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Reprogramming of bone marrow derived mesenchymal stromal cells to human induced pluripotent stem cells from pediatric patients with hematological diseases using a commercial mRNA kit

Sfougataki Irene[a,f,*], Grafakos Ioannis[a], Varela Ioanna[a], Mitrakos Anastasios[b], Karagiannidou Angeliki[a], Tzannoudaki Marianna[c], Poulou Myrto[b], Mertzanian Anny[a,b], Roubelakis G. Maria[d], Stefanaki Kalliope[e], Traeger-Synodinos Joanne[b], Kanavakis Emmanuel[b,f], Kitra Vasiliki[a], Tzetis Maria[b], Goussetis Evgenios[a]

[a] Stem Cell Transplant Unit, Aghia Sophia Children's Hospital, Athens 11527, Greece
[b] Department of Medical Genetics, Medical School, National and Kapodistrian University of Athens, 11527, Greece
[c] Department of Immunology and Histocompatibility, Aghia Sophia Children's Hospital, Athens 11527, Greece
[d] Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece
[e] Department of Histopathology, Aghia Sophia Children's Hospital, Athens 11527, Greece
[f] Research Institute for the Study of Genetic and Malignant Disorders in Childhood, Aghia Sophia Children's Hospital, Athens 11527, Greece

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ABSTRACT

The potential use of patient-specific induced pluripotent stem cells (hiPSCs) in the study and treatment of hematological diseases requires the setup of efficient and safe protocols for hiPSC generation. We aimed to adopt a reprogramming method for large-scale production of integration-free patient-specific hiPSC-lines in our stem cell processing laboratory, which supports a pediatric hematopoietic stem cell transplant unit located at a tertiary care children's hospital. We describe our 5-year experience in generation of hiPSC-lines from human bone marrow-derived mesenchymal stromal cells (BM-MSCs) using synthetic mRNAs encoding reprogramming factors. We generated hiPSC-lines from pediatric patients with β-Thalassemia, Sickle Cell Anemia, Blackfan-Diamond Anemia, Severe Aplastic Anemia, DOCK8 Immunodeficiency and 1 healthy control. After optimization of the reprogramming procedure, average reprogramming efficiency of BM-MSCs was 0.29% (range 0.25–0.4). The complete reprogramming process lasted 14–16 days. Three to five hiPSC-colonies per sample were selected, expanded to 5 culture passages and then frozen. The whole procedure took an average time of 1.8 months (range 1.6–2.2). The hiPSC-lines expressed embryonic stem cell markers and exhibited pluripotency. This mRNA reprogramming method can be applicable in a hematopoietic stem cell culture lab setting and would be useful for the clinical translation of patient-specific hiPSCs.

1. Introduction

The successful long term reconstitution of the human lymphohematopoietic system by infusing allogeneic or autologous hematopoietic stem cells (HSCs) into patients with hematological diseases is the only clinical proof of principle for stem cell-based therapies. Since the first description of allogeneic hematopoietic stem cell transplantation (HSCT) 50 years ago [1], the use of HLA-matched allogeneic HSCT to treat many genetic blood diseases has become a clinical standard. Moreover, during the last 5 years there have been several studies using related haploidentical donors, with encouraging results [2]. However, the lack of suitable HLA-matched donors and the potential immunological post-transplant complications remain significant clinical barriers limiting the successful application of allogeneic HSCT [3,4]. During the last two decades efficient gene therapy protocols have been developed for many genetic diseases [5–7], which are curable only by allogeneic HSCT. In most gene therapy strategies, patient's bone marrow or peripheral blood stem cells are collected, *ex vivo* genetically

Abbreviations: HSCs, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; BM, bone marrow; MSCs, mesenchymal stromal cells; hiPSCs, human induced pluripotent stem cells; hiPSCLs, human induced pluripotent stem cell lines; hESC, human embryonic stem cells; array-CGH, array comparative genomic hybridization analysis; EBs, embryoid bodies

* Corresponding author at: Stem Cell Transplant Unit, Aghia Sophia Children's Hospital, Levadias 8, 11527 Athens, Greece.
E-mail address: irene_sf_@hotmail.com (I. Sfougataki).

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corrected and then intravenously infused. While there has been much progress in the development of HSC gene therapy protocols and several early-phase clinical reports have been recently published [8–10], more efficient methods in cell processing and genetic cell correction of HSCs are needed before gene therapy becomes clinical standard for genetic diseases. One limiting factor is that the cell processing manipulations needed for stem cell enrichment and genetic modification may cause significant cell losses. Hence, higher numbers of HSCs are required than those that could usually be obtained from bone marrow or peripheral blood. *Ex vivo* expansion of HSCs using growth factors or several small molecules leads to a proliferation of progenitor cells rather than expansion of stem cells with repopulating ability [11]. In this regard, the development of human induced pluripotent stem cell (hiPSCs) technologies [12] offers a new gene/cell therapy approach by generating an unlimited number of hiPSCs from the individual patient, correcting the genetic defect at the stage of pluripotent cell, and then differentiating the corrected hiPSCs into transplantable HSCs. Indeed, over the last decade, disease and patient-specific hiPSCs combined with genome editing have been successfully used in preclinical studies for disease modeling and cell therapies [13–15]. However, current protocols for differentiating hiPSCs into HSCs are insufficient in obtaining fully functional stem cells making, thus, the clinical translation of hiPSCs based gene/cell therapy for inherited hematological diseases premature [16].

Most work on pluripotent stem cells is taking place in established sophisticated core facilities, which are part of large research institute centers. We applied a synthetic mRNA kit for reprogramming bone marrow derived mesenchymal stromal cells (BM-MSCs) in our hematopoietic stem cell processing lab with the aim to generate integration-free hiPSC lines (hiPSCLs) from pediatric hematological diseases. Herein, we communicate our experience in setting up a clinically relevant reprogramming method in a clinically oriented laboratory and the successful generation of hiPSC-lines from patients with various pediatric hematological diseases.

2. Materials and methods

2.1. Patients

The research protocol was approved by the Ethical Committee of the Aghia Sophia Children's Hospital (Athens, Greece). Bone marrow (BM) samples were harvested from hematological pediatric patients with β-Thalassemia, Sickle Cell Anemia, Diamond-Blackfan Anemia, Aplastic Anemia and Combined Immunodeficiency due to DOCK8 defect. BM was harvested from the posterior iliac crest for autologous backup BM grafts and samples of 2–15 ml were used for the derivation of mesenchymal stromal cells (MSCs), after written informed consent from the parents. Furthermore, BM sample from a healthy bone marrow transplant donor was harvested.

2.2. Cell culture

MSCs were derived from the BM samples according to standard protocols [17]. BJ human foreskin fibroblasts were purchased from Stemgent (Cambridge, MA, USA). Bone marrow MSCs (BM-MSCs) and BJ were cultured in Dulbecco's Modified Eagle Medium (DMEM; GlutaMAX, Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Stemcell Technologies, Vancouver, BC, Canada) with medium replacement every 3–4 days.

2.3. Reprogramming of BM-MSCs with synthetic modified mRNA

The initial experiments, regarding β-Thalassemia and normal samples were performed with the commercial kit of Stemgent (Cambridge, MA, USA), [18] containing synthetic modified mRNAs encoding five transcription factors OCT4, KLF4, SOX2, c-MYC and LIN28 and eGFP mRNA (ratio 3;1;1;1;1;1). The procedure was performed on irradiated NuFF feeder cells (GlobalStem, Gaithersburg, MD, USA), according to the manufacturer's recommendations.

In the next experiments, regarding the other hematological diseases, we used StemMACS mRNA Reprogramming kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), that additionally contained mRNA encoding for Nanog transcription factor (ratio was 3;1;1;1;1;1;1 of OCT4;KLF4;SOX2;C-MYC;LIN28;NANOG;eGFP) and was also suitable for reprogramming without feeder cells. The detailed protocol is described in Appendix. 3-7 × 10^4 MSCs, were seeded in 1 well of 12-well plate coated with Matrigel hESC-qualified Matrix (BD Bioscience, Franklin Lakes, NJ, USA) in NuFF-conditioned StemMACS Repro-Brew XF Medium (Miltenyi). After 1–2 days, cells were prepared for transfection, by incubation with 200 ng/ml B18R interferon inhibitor and daily transfections were performed, following an mRNA dose ramping, as recommended by the manufacturer. On the first days of transfection, daily mRNA dose was 500 ng/well. We increased mRNA dose to 750 ng/well on day 4–6, depending on the condition of the cells and the observed toxicity. Fluorescence of eGFP protein allowed for monitoring of the mRNA delivery of the cells. BJ fibroblasts were simultaneously transfected, as positive control. After colony emergence, transfections were stopped and cells were allowed for 1–3 days for further growth. When in adequate size, hiPSCs colonies were examined for TRA-1-60 expression with anti-TRA-1-60 live staining (Miltenyi Biotec), then were isolated and reseeded individually in matrigel-coated wells, in mTeSR1 medium (Stemcell). hiPSCs were passaged using Dispase (Stemcell). hiPSCs were tested for their genetic stability, pluripotency and for their patient-specific origin. After passage 5, the obtained hiPSC lines (hiPSCLs) were stored in liquid nitrogen. HUES-9 and HUES-7 embryonic stem cell (hESC) lines, kindly provided by Prof. D.A. Melton, Harvard University [19], were initially used in our laboratory for obtaining experience in pluripotent stem cell cultures and during our work in hiPSCs, they served as cells of reference for comparative assessment of our hiPSC results.

2.4. Flow cytometry

OCT4 and SSEA-4 primary antibodies (Stemcell Technologies) were used for the characterization of hiPSCs by flow cytometry. Staining with unconjugated mouse isotypic controls was performed in parallel. Fluorescein isothiocyantate (FITC)-conjugated goat anti-mouse immunoglobulin M (IgM) was used as a secondary antibody. For intracellular OCT4 staining, IntraPrep Permeabilization Reagents (Beckman Coulter Inc., Nyon, Switzerland) were used. Samples were analyzed using FC500 instrument (Beckman Coulter Inc., Miami. USA).

2.5. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Rneasy Plus Mini-kit (Qiagen GmbH, Hilden, Germany) was used for total RNA extraction from hiPSCs. RNA was then reversely transcribed into cDNA using Superscript II Reverse Transcriptase kit (Invitrogen). cDNA was used in polymerase chain reaction (PCR) with specific primer sequences for *OCT4, REX1, SOX2, NANOG,* to identify the presence of the pluripotency-associated markers in the extracts. GAPDH gene was used as an internal control. The primer sequences used for each gene were the following. *OCT4:* forward 5′-CTT GCT GCA GAA GTG GGT GGA GGA-3′, reverse 5′-CTG CAG TGT GGG TTT CGG GCA-3′; *SOX2:* forward 5′-ATG CAC CGC TAC GAC GTG A-3′, reverse 5′-CTT TTG CAC CCC TCC CAT TT-3′; *REX1:* forward 5′-TGA AAG CCC ACA TCC TAA CG-3′, reverse 5′-CAA GCT ATC CTC CTG CTT TGG-3′; *NANOG:* forward 5′-CCA AAG GCA AAC AAC CCA CT-3′, reverse 5′-TGA ATT GTT CCA GGT CTG GTT G-3′; *GAPDH:* forward 5′-GCT CAG ACA CCA TGG GGA AGG T-3′, reverse 5′-GTG GTG CAG GAG GCA TTG CTG A-3′.

2.6. In vitro and in vivo assays of pluripotency

hiPSCs were allowed to form embryoid bodies (EBs) by culture in ultra-low attachment plates in Knockout DMEM supplemented with 20% Knockout serum replacement, 1 × MEM NEAA, 1 mM L-Glutamine and 0.1 mM β-mercaptoethanol (All from Invitrogen). For *in vivo* pluripotency assay, hiPSCs were collected from 25cm^2 flasks, diluted in a mixture of Matrigel and DMEM/F12 (Stemcell) (1:1 ratio) and injected subcutaneously into NOD/SCID mice (n = 3). Mice were maintained at the Animal Facility of the Biomedical Research Foundation of the Academy of Athens (BRFAA), according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). Mice were euthanized 8–10 weeks after the injection. Tumors were excised, fixed in parafolmaldehyde 4% and embedded in paraffin. Hematoxylin and eosin (H&E) staining of sections was used to examine the morphological characteristics of the derived tissues.

2.7. Comparative genomic hybridization analysis (array-CGH)

Total genomic DNA was extracted with Qiagen Blood Mini-kit. The extracted DNA was processed as previously described [20] and hybridized to Agilent SurePrint G3 Human 4x180k CGH + SNP microarrays, composed of 110,712 [comparative genomic hybridization (CGH) + 59,647 [single-nucleotide polymorphism (SNP)] 60-mer oligonucleotide probes with 25.3-kb overall median probe spacing (5-kb in ISCA regions). Agilent Feature Extraction Image Analysis Software (v. 10.7.3) was used to extract data from raw microarray image files. Data visualization and analysis was performed using Agilent Cyto Genomics (v. 2.7) software, as previously described [20].

2.8. In vitro hematopoietic differentiation

In order to investigate the hematopoietic differentiation potential of the hiPSCs that were derived with the current method, 3 hiPSCLs were cultured in 6-well plates, in hematopoietic differentiation medium, consisting of STEMDiff APEL Medium (StemCell Technologies) supplemented with cytokines, according to the manufacturer's instructions. After 13 days of culture, cells were collected, analyzed with FACs for the expression of CD34 hematopoietic marker, and 1 × 10^5 cells were transferred in methylcellulose (MethoCult H4230, StemCell Technologies), with the addition of 5 U/ml EPO, 100 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml GM-CSF and 50 ng/ml G-CSF (R&D Systems, Minneapolis, MN, USA) for hematopoietic colony formation. After 13 days, colony formation was examined under the microscope.

3. Results

3.1. Setting up the mRNA reprogramming method

In our existing hematopoietic stem cell culture lab we set out to expand our translational research interests in the field of pluripotent stem cell biology. In order to accomplish this, one staff member, after completing a training course in hESC culture, set up the maintenance of the well-characterized hESC lines HUES-9, HUES-7 as the "gold standard" for all subsequent hiPSCs work. After considering the advantages and disadvantages of different integration-free reprogramming methods available at that time (2011), we chose the method of synthetic mRNA reprogramming, because it was commercially available as ready to use and was highly efficient in reprogramming of human fibroblasts. Due to the accessibility of our laboratory to obtaining BM samples, we aimed to set up the method on BM-MSCs from hematological pediatric patients, to generate disease-specific hiPSCLs. All patients had an autologous back-up BM harvest prior to allogeneic HSCT as per policy of our HSCT-unit. The adopted mRNA method using commercial kit, was standardized through a 5 year experience, in which, its application on BM-MSCs was successfully established for reprogramming. BM-MSCs were daily transfected with the synthetic mRNAs, until colonies emerged. For the first attempts of reprogramming, (normal and the β-thalassemia samples), the kit containing mRNAs for 5 transcription factors and requiring a feeder layer, was used. In these experiments, 18 daily consecutive daily transfections were required till the formation of hiPSC colonies with the typical morphology. The procedure yielded 5 hiPSC colonies per 10^4 target cells, with a reprogramming efficiency of 0.05% [18]. For the next reprogramming experiments, several optimizations took place, leading to a significant increase in the reprogramming efficiency. The initial reprogramming kit was replaced with a kit that additionally contained Nanog mRNA and was optimized for feeder-free reprogramming. Adjustment of BM-MSC seeding densities, according to the growth rates of each line, typically starting from 3 to 7 × 10^4 MSCs per well of 12-well plate, helped to ameliorate the toxicity events that initially occurred in the cells. The increase of the mRNA transfection dose, depending on the observed toxicity, helped to maintain cells in a healthy condition during the reprogramming process. Combination of coating with matrigel and feeding with NuFF-Conditioned Medium, proved to be a suitable culture formula. The efficiency of the standardized experiments reached an average of 0.29% (0.25–0.4%) yielding to the emergence of 25–40 hiPSC colonies per 10^4 MSCs (Table 1). The efficiency for BJ fibroblasts reached to 3.7%. First hiPSC colonies were visible after 9–11 days and transfections stopped after 14–16 days (Fig.1A). With daily refeeding, hiPSC colonies grew rapidly in size and were ready to be picked after 1–3 days. The reprogramming process, up to manual picking of colonies, took approximately 18–20 days. The selection of hiPSC colonies was performed according to morphological criteria (Fig.1B) and after live staining with anti-TRA-1-60 antibody, which confirmed the pluripotency of the emerged colonies (Fig.1B). From each reprogramming experiment, 3–5 hiPSC colonies of typical hESC-like morphology and adequate size were selected and replated individually so as to obtain different clones from each parental MSC line. hiPSCs expanded rapidly for 5 passages in a time interval of 1.1–1.6 months, till hiPSCLs were established in *in vitro* culture conditions (Table 1).

3.2. Characterization of disease specific hiPSCLs

3.2.1. Evaluation of pluripotency

Examination with RT-PCR confirmed that hiPSCLs from all patients, expressed pluripotency genes, *OCT4* and *NANOG, SOX2* and *REX1*. Representative results of the RT-PCR of the hiPSCLs, in parallel with

Table 1
Generation and establishment of the patient-specific hiPSCLs using the synthetic mRNA method.

Molecular disorder	Colonies/10^4 MSCs	Efficiency (%)	Days of transfection (d)	Time required for reprogramming and expansion till passage 5 (d)	Whole procedure time interval beginning from BM harvesting (d)
b-Thalassemia	5	0.05	18	66	81
Sickle Cell Anemia	26	0.26	16	56	71
DOCK8 Immunodeficiency	40	0.4	14	48	62
Diamond-Blackfan Anemia	25	0.25	14	51	66
Aplastic Anemia	26	0.26	15	53	67

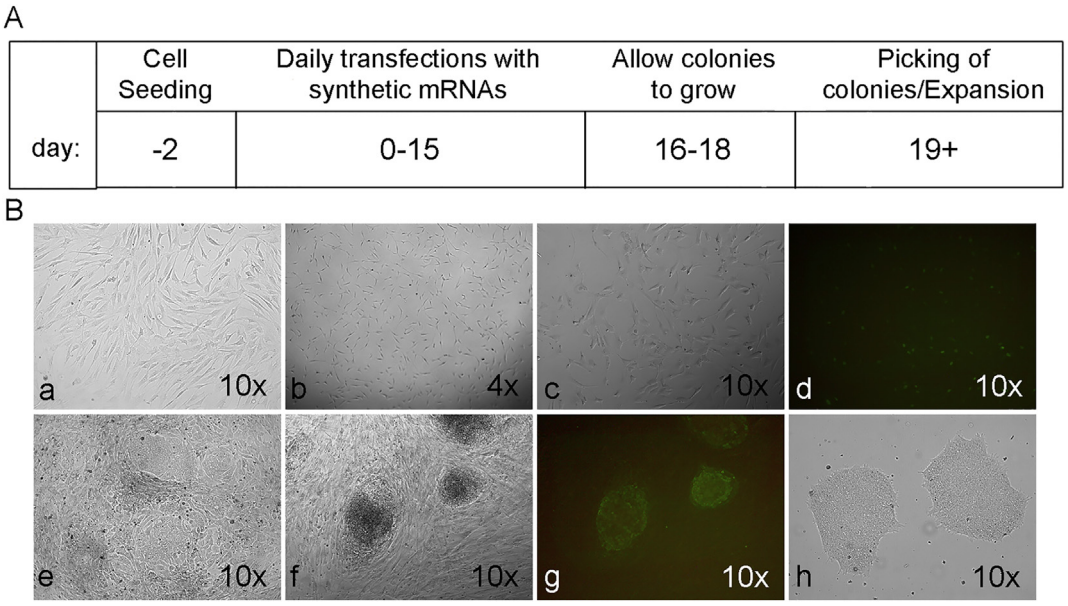

Fig. 1. Generation of hiPSCLs from BM-MSCs. (A) Timeline of the mRNA reprogramming procedure. (B) Morphology of cells during the stages of the reprogramming process. a) Target BM-MSCs in standard culture medium (Magnification 10×). b) Target BM-MSCs seeded in appropriate density (7×10^4 cells) on matrigel, on the first day of transfections (day 0) (4×). c) BM-MSCs at day 1, 19 h post-transfection (10×) and d) fluorescence of nGFP-expressing cells (10×). e) Transition of mesenchymal to epithelial morphology of cells on day 11 (10×). f) Fully reprogrammed colonies with typical hESC-like morphology on day 17 (10×) and g) immunofluorescence of pluripotent stem cells expressing Tra-1-60 surface antigen in the same colonies (10×). h) hiPSCs colonies after 2 passages of culture on matrigel in mTeSR1 medium (10×).

hESC-line HUES-9, are shown in Fig.2A. In addition, hiPSCs were tested with flow cytometry, which showed that the majority of cells were positive for OCT4 and SSEA-4 markers (Fig.2B). Functional pluripotency of hiPSCLs was examined *in vitro* under culture in low-attachment plates, where EB formation was observed during the first 3–5 days (Fig.2C). *In vivo* injection of hiPSCLs in NOD/SCID mice led to teratoma formation. After 2–3 months, tumors were visible at the injection sites. Staining with H&E revealed the presence of tissues that originate from all three germ layers (Fig.2D).

3.2.2. Genetic stability

In order to investigate whether the reprogramming process causes genetic instability to the cells, array-CGH was performed, from DNA extracted from the initial expansion of the clones. hiPSCLs had a normal karyotype after reprogramming. Most lines exhibited a few mainly common microduplications and microdeletions characterized as normal Copy Number Variations (CNVs) (Supplementary Table S1). These CNVs did not occur in regions containing oncogenes, tumor suppressor genes or other genes related to cell proliferation, with the exception of one Diamond-Blackfan line, which acquired a gain of 0.6 Mb in 2p24.3 containing *MYCNUT, MYCNOS* and *MYCN* genes and a duplication of 1.7 Mb in 7q31.32, previously reported [21]. The karyotype of the parental MSC lines was normal, with no chromosomal aberrations.

3.2.3. Patient-specific origin

To confirm the parental origin of the hiPSCLs, in the cases of patients with hereditary disorders with known mutations (β-Thalassemia, Sickle cell anemia, DOCK8 Immunodeficiency) (Table 2), the hiPSCLs were examined with Sanger sequencing, in parallel with the parental BM-MSCs, for the detection of the specific molecular defects. In the case of the Aplastic Anemia patient, (idiopathic disorder) and the Diamond-Blackfan patient (no known gene mutation associated with the disease has been found, after whole exome sequencing), testing that the hiPSCLs were derived from the parental cells was performed using common STRs. All hiPSCLs were proven to have been derived from their parental MSCs.

3.3. In vitro hematopoietic differentiation of the hiPSCLs

In order to investigate whether the hiPSCs which are derived with the current protocol are capable of hematopoietic differentiation *in vitro*, appropriate hematopoietic differentiation cultures took place in thawed hiPSCs. After 13 days of culture, the formation of round cells, emerging from the adherent cells, was observed (Fig.3B). The derived cells, as indicated by FACs, contained 5–10% CD34+ cells. After culture in methylcellulose, an average of approximately 8 BFU-E colonies per 1×10^5 cells had developed (Fig.3C).

4. Discussion

hiPSCs technology is a valuable tool for the production of HSCs that can be used both in the study of the pathogenesis of hematological diseases and the development of gene and cellular therapy clinically relevant protocols. The traditional methods of reprogramming, using retroviral vectors, involve the random integration of the transgenes into the genome and insertional mutagenesis, which may lead to gene dysregulations or tumorigenic potential of cells. During the last years, research has focused on the development of integration-free methods for the derivation of hiPSCs with potential use in regenerative medicine. So far, reprogramming approaches using recombinant proteins have been proven to be extremely inefficient, making the derivation of hiPSCs a very difficult procedure [22]. Episomal vectors and Sendai virus vectors (SeV) are currently used in the majority of studies that aim to produce integration-free hiPSCs. Episomal vector reprogramming has been successful in peripheral blood and cord blood cells with efficiencies varying from low to high (up to 0.1%) [23–27], although with low efficiencies for fibroblasts [28]. SeV has been reported to have low to very high efficiencies (0.01–1%) [29–32]. For these methods, screening of the derived hiPSCs is essential in order to confirm that the vectors have been lost from the cell populations. Typically, transgenes persist for approximately 10 passages [30,33–37]. The mRNA method, developed during the last years and applied mainly in fibroblasts [38–44], presents a very promising approach, completely free of integration. Synthetic modified mRNA method has been reported in previous studies

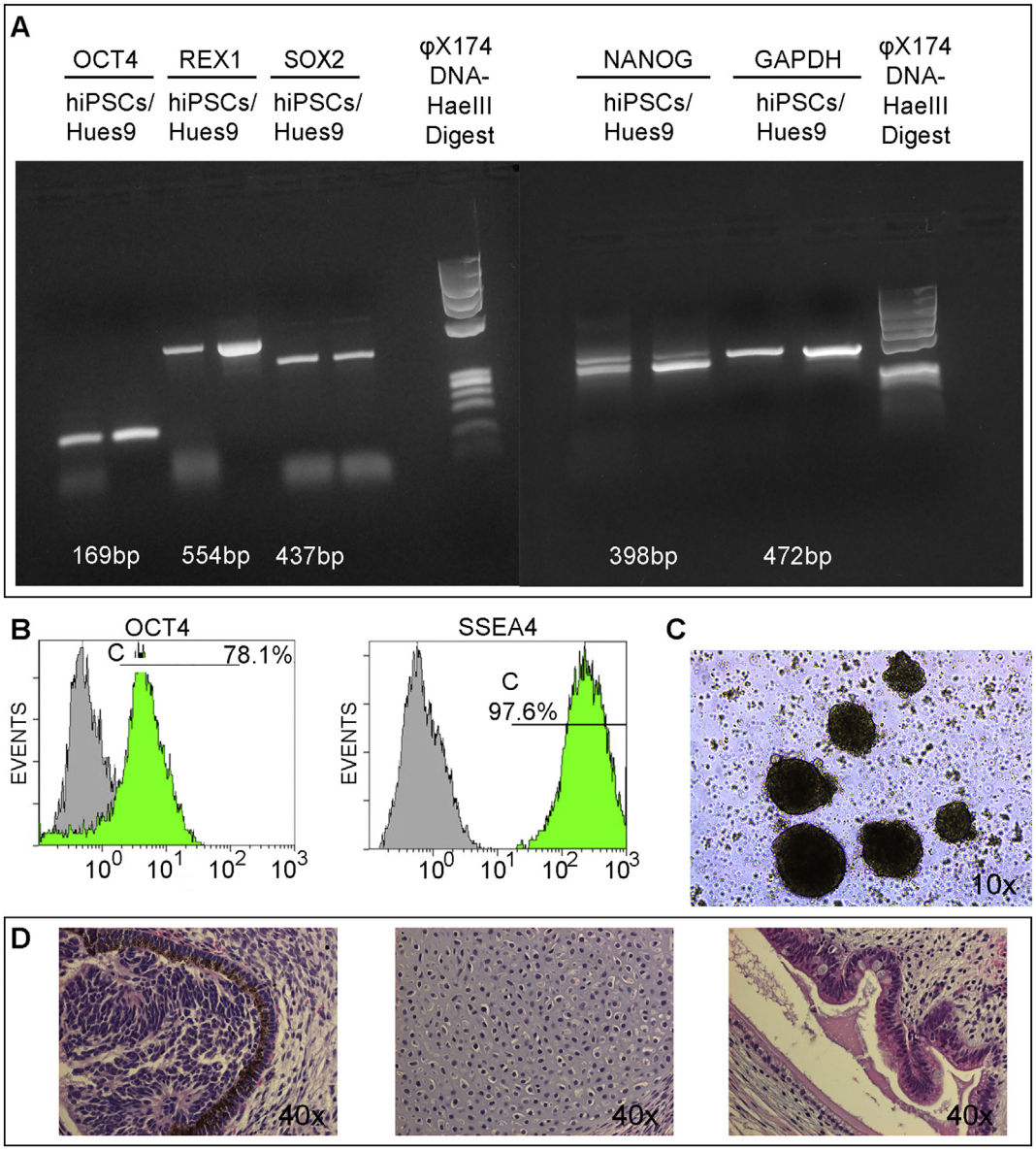

Fig. 2. Confirmation of pluripotency of the patient-specific hiPSCLs. (A) RT-PCR results showing that, similarly to hESCs, hiPSCLs express pluripotency associated genes *OCT4, SOX2, NANOG, REX1*. Expression of the internal control *GAPDH* was also evident. (B) Flow cytometry results, showing the expression of master regulator OCT4 and surface antigen SSEA-4. (C) Spontaneous embryoid body formation after 5 days of culture in ultra-low attachment plates (Magnification 10×). (D) H&E staining of the formed teratomas, showing the presence of tissues (from left to right) originating from ectoderm (neuroepithelium/ pigmented epithelium), mesoderm (cartilage) and endoderm (gut-like epithelial tissue) (Magnification 40×). All pictures display results of one representative experiment.

to exhibit high efficiencies in fibroblasts of up to 4.4% [38] and can also be very efficient in endothelial cells of different origins [43]. The major disadvantage of the mRNA method is that it is more time-consuming than the other methods, requiring daily transfections usually for approximately 2 weeks, due to the limited lifespan of the mRNA in the cell.

BM-MSCs, which were used in this study as target cells for reprogramming, are easily expandable cells. They have low costs of culture and similar characteristics with fibroblasts, such as adherence to the surface and rapid proliferation, which enable daily transfections of mRNAs. A previous study has reported reprogramming of MSCs, derived from adipose tissue of a middle-aged person, with non-modified mRNAs, with an efficiency of 0.05% [45]. In the current study, feeder-free, modified mRNA method was highly efficient in multiple pediatric BM-MSCs, reaching up to 0.4%, which is enough to obtain more than 100 colonies per well. In our hands, optimal cell input of pediatric BM-

Table 2
Patient-specific genotypes of the derived hiPSCLs from the cases of hereditary disease with diagnosed mutation.

Patient with specific molecular disorder	Affected Gene	Genotype
b-Thalassemia	*HBB*	c.[92 + 6T > C];[-137C > T]
Sickle cell anemia	*HBB*	c.[20A > T];[20A > T]
DOCK8 immunodeficiency	*DOCK8*	c.[4408_4411delAACT];[4408_4411delAACT]

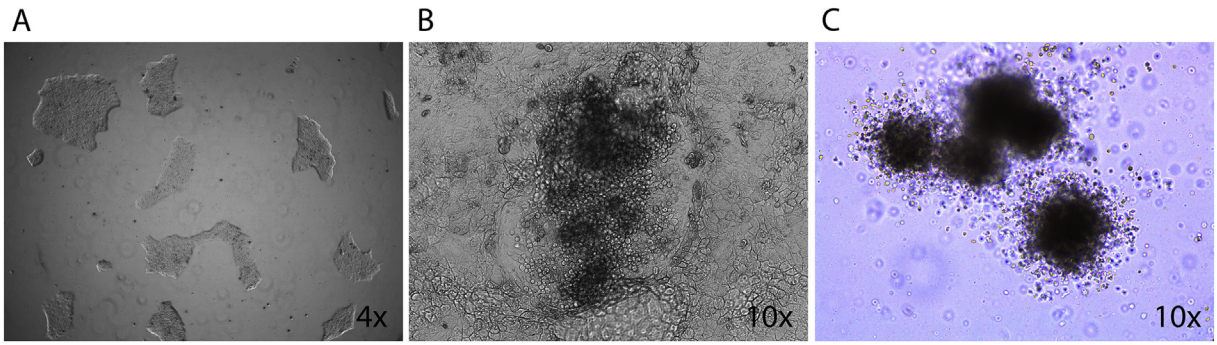

Fig. 3. *In vitro* hematopoietic differentiation. (A) Undifferentiated hiPSCs on the first day of the differentiation experiment (Magnification 4×). (B) Differentiated colony consisting of round cells emerging from the adherent cells (Magnification 10×). (C) BFU-E colony formed after 13 days of culture of the differentiated cells in methylcellulose (10×). (Pictures of a representative experiment).

MSCs for reprogramming is about 3-7 × 10^4 cells per well, which can be easily obtained from as low as 2 ml of BM. After establishment and optimization of the protocol on MSC target cells, we succeeded in reprogramming for all samples. The current work represents an improvement upon our initial attempts for mRNA reprogramming [18]. The method was further adapted using feeder-free conditions, an additional mRNA encoding for NANOG pluripotency factor and an mRNA dose ramp that allowed toxicity reduction and better survival of the target cells. The above allowed a significant increase of the reprogramming efficiency, as well as the establishment of feeder-free hiPSC lines. The optimized protocol is more reliable and ready to use for systematic hiPSC derivation in a daily routine laboratory. All reprogramming experiments were conducted on frozen-thawed BM-MSCs, which underscores that the method does not necessarily require a fresh sample and the experiment commencement can be suitably arranged.

Our experience has shown that large-scale derivation of hiPSCLs from hematological pediatric patients with feeder-free and integration-free mRNA reprogramming is feasible. The protocol used is reproducible and reliable for obtaining integration-free patient-specific lines, appropriate for usage both for research and in potential clinical applications in the future. The method can be easily applied in a HSCT unit laboratory for reprogramming of BM-MSC samples and can be easily performed by anyone with technical expertise on BM-MSC and hESC cultures. It is feasible for one trained staff member to apply the protocol for the production of hiPSCLs, alongside the daily routine work. Further optimizations of the protocols may reduce hands-on time especially regarding daily mRNA transfections. Rohani et al., managed to produce higher stability mRNAs, leading to successful reprogramming of fibroblasts with a transfection frequency of 48 h [46]. Another study used a combination of modified mRNAs and microRNAs and managed successful reprogramming with three transfections [44]. Importantly, reprogramming with only a single transfection of fibroblasts has been accomplished, using synthetic self-replicating RNA [47], offering the option of integration-free hiPSCs generation with minimal hands-on time needed and more convenient application in a daily routine laboratory. Moreover, it would be ideal for a clinical hematology laboratory to be able to produce integration-free hiPSCLs, beginning from a small amount of peripheral blood as a sample source. For this reason, application of the mRNA method on blood cells, as has been accomplished for other methods (SeV and episomal vectors) [23,30], is one of our goals, on which we are currently working.

Detection of genomic abnormalities remains a matter of concern. In this study, the derived hiPSCLs, compared to their parental MSCs, maintained a stable karyotype, with the exception of a duplication detected in a Diamond-Blackfan specific line containing *MYCNUT, MYCNOS,* and *MYCN* genes. It is known that chromosomal aberrations such as aneuploidies and CNVs are a phenomenon widely observed in hiPSCs [21,48–50]. This raises concerns of whether the aberrations may give oncogenic potential to the derived cells. High resolution strategies, such as array-CGH containing SNPs, are crucial to monitor the genomic stability of hiPSCLs.

Currently, disease-specific hiPSCs are used for the modeling of various hematological diseases, for drug screening and for the development of therapeutic approaches for the treatment of these disorders [27,51–57]. Furthermore, recently developed gene-targeting techniques, such as CRISPR/Cas9 technology are a promising tool for application in hiPSCs with the aim of autologous transplantation of the corrected hematopoietic derivatives in monogenic disorders [53,56,58]. The hiPSCLs generated during this study, are currently available for research involving the study of the relevant disorders. Hematopoietic differentiation of hiPSCLs has recently been our aim and initial attempts have led to a yield of 10% production of hematopoietic CD34+ stem cells [18]. However, the efficiencies of current protocols are not sufficient so as to produce large amounts of functional HSCs. A better understanding of the mechanisms involved in hematopoietic differentiation during embryonic development will lead to the establishment of more robust protocols for large-scale production of hiPSC-derived HSCs and will facilitate their use for the study and treatment of hematological diseases. Furthermore, extensive *in vivo* studies in animals will elucidate if the derived populations are true hematopoietic stem cells with potential of long-term hematopoietic reconstitution.

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Declaration of interest

The authors declare that there are no conflicts of interest.

Author contributions

SI designed and performed experiments and wrote the paper. GI performed experiments and wrote the paper. VI designed and performed experiments. MA performed analyses. KA designed and performed experiments. TM performed analyses and interpreted data. PM performed analyses. MA performed experiments. RGM performed experiments. SK interpreted data. TSJ interpreted data. KE, KV supervised the experiments. TM, GE designed and supervised the experiments, interpreted the data. All authors have read and approved the final manuscript.

Appendix A. Detailed protocol of reprogramming with synthetic mRNAs

For feeder-free reprogramming of BM-MSCs, StemMACS mRNA Reprogramming kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), that contains synthetic modified mRNAs encoding transcription factors OCT4;KLF4;SOX2;C-MYC;LIN28;NANOG;eGFP (ratio 3;1;1;1;1;1;1) is used. 2 days prior to seeding for reprogramming, BM-

MSC culture medium is switched to reprogramming medium StemMACS Repro-Brew XF (Miltenyi), previously conditioned in Newborn Human Foreskin Fibroblasts (NuFF), with addition of 5 ng/ml bFGF. BM-MSCs of passage 1 or 2, are collected from flasks using trypsin 0.05% (Gibco, BRL) with 4 min incubation at 37 °C. Depending on the growth rates of each BM-MSC line, $3 - 7 \times 10^4$ cells are seeded in 1 well of 12-well plate coated with Matrigel hESC-qualified Matrix (BD Bioscience, Franklin Lakes, NJ, USA) in NuFF-conditioned StemMACS Repro-Brew XF Medium (Miltenyi). After 1–2 days, cells are prepared for transfection, by incubation with 200 ng/ml B18R interferon inhibitor in fresh equilibrated reprogramming medium, for at least 3 h. First transfection is then performed beginning with 500 ng/ml total mRNA per well, followed by incubation for 4 h. Afterwards, medium is changed with fresh, equilibrated reprogramming medium, containing B18R. Daily transfections are performed similarly, following an mRNA dose ramping. On the first 5–7 days of transfection, daily mRNA dose is 500 ng/well. We increase mRNA dose to 750 ng/well on day 4–6, depending on the observed toxicity and the proliferation capability of cells. If observed toxicity remains high and proliferation of cells remains too slow, low dose of mRNA should be prolonged for more days. On the first 3 days fluorescence microscopy can be used to observe eGFP protein expression in cells, which represents mRNA delivery from cells. Mesenchymal to epithelial transition is observed after approximately 9–11 days of tranfection. After 15–16 days, colonies with the typical hESC morphology are formed and transfections can be stopped. Cells are allowed for 1–3 days for further growth, with daily change of the reprogramming medium, without addition of transfection reagent. When in adequate size, hiPSCs colonies are examined for pluripotency with anti-TRA-1-60 live staining (Miltenyi Biotec). Then, pluripotent colonies with adequate size are isolated and reseeded individually in matrigel-coated wells, in mTeSR1 medium (Stemcell). Colony isolation is performed manually, under the microscope, by separating the desired colony from the neighbouring cells with a sterile needle and transferring with the pipette into the new well. During the first 1–2 passages, when hiPSC colonies are still few in numbers, they should be passaged manually, as described. Thereafter, they can be passaged enzymatically using Dispase (Stemcell), with incubation at 37 °C for 1 min. After 5 passages, cells are collected using Dispase, frozen in mFreSR medium (StemCell) with slow programmable freezing and stored in liquid nitrogen.

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