Short Communication

Generation of Human Induced Pluripotent Stem Cells Using Genome Integrating or Non-integrating Methods

(hiPSCs / lentivirus / Sendai virus / episomal reprogramming)

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Abstract. Preclinical studies have demonstrated the promising potential of human induced pluripotent stem cells (hiPSCs) for clinical application. To fulfil this goal, efficient and safe methods to generate them must be established. Various reprogramming techniques were presented during seven years of hiPSCs research. Genome non-integrating and completely xeno-free protocols from the first biopsy to stable hiPSC clones are highly preferable in terms of future clinical application. In this short communication, we summarize the reprogramming experiments performed in our laboratories. We successfully generated hiPSCs using STEMCCA lentivirus, Sendai virus or episomal vectors. Human neonatal fibroblasts and CD34$^+$ blood progenitors were used as cell sources and were maintained either on mouse embryonic feeder cells or in feeder-free conditions. The reprogramming efficiency was comparable for all three methods and both cell types, while the best results were obtained in feeder-free conditions.

Introduction

Pluripotent stem cells are characterized by their self-renewal ability and potential to differentiate into three embryonic germ layers (ectoderm, endoderm and mesoderm), and further to progenitor and adult cells of various tissues. Human embryonic stem cells (hESCs) are derived from the blastocyst, while human induced pluripotent stem cells (hiPSCs) are genetically reprogrammed adult cells. Both cell types are being intensively studied in the laboratories all around the world because of their tremendous potential for therapeutic use and disease modelling (reviewed in Simara et al., 2013). Clinical applications of hiPSCs would offer an unlimited supply of patient-derived transplantable cells, reduced need for immunosuppression, as well as the chance to edit disease-causing genetic mutations. Although to date only a few hESC-based therapeutic approaches entered clinical trials (Strauss, 2010; Baker, 2011; Schwartz et al., 2012), it is expected that hiPSCs will follow in the near future.

Improvements of the methods for hiPSC generation are sought to ensure efficacy and safety. The first induced pluripotent stem cells (iPSCs) were derived from murine fibroblasts using retroviruses as vectors for the transcription factors Oct3/4, Sox2, Klf4 and c-Myc (OSKM; Takahashi and Yamanaka, 2006), and the same delivering system was successfully used for the first hiPSCs (Takahashi et al., 2007). James Thomson's group chose lentivirus to deliver Oct3/4, Sox2, Lin28 and Nanog reprogramming factors (Yu et al., 2007). Subsequently, the lentiviral reprogramming system was improved by introducing a "stem cell cassette" (STEMCCA) containing all of the OSKM transcription factors in a single vector (Sommer et al., 2009). However, despite an effort to reduce the number of genome integration sites, both retroviruses and lentiviruses inherently modify the host genome (Hacein-Bey-Abina et al., 2003), which substantially increases the risk of tumorigenicity. Therefore, DNA non-integrating approaches seem to be more optimal for future clinical application.

Fusaki et al. (2009) presented a Sendai virus-based vector system for reprogramming somatic cells feasible for such applications. Sendai virus eliminates the risk of viral DNA integration into the host genome because it replicates only in the cytoplasm of infected cells. The number of virus-associated transgenes decreases during

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Abbreviations: EBNA-1 – Eppstein-Barr nuclear antigen-1, hiPSC – human induced pluripotent stem cell, hESC – human embryonic stem cell, iPSC – induced pluripotent stem cell, MEF – mouse embryonic fibroblast, OSKM – Oct3/4, Sox2, Klf4 and c-Myc, STEMCCA – stem cell cassette.

cell division and any remaining virus-infected cells can be removed based on HN antigen surface expression.

Episomal reprogramming offers completely viral-free hiPSC generation. Six reprogramming factors (Oct3/4, Sox2, Klf4, Lin28, Nanog and c-Myc) were delivered on episomal vectors with an oriP/EBNA-1 (Eppstein-Barr nuclear antigen-1) backbone in a previously published study (Yu et al., 2009). The plasmids do not require viral packaging for transfection and transgene-free hiPSCs can be isolated merely through subcloning.

In CBIA laboratories we established three protocols for hiPSC generation: STEMCCA lentivirus, Sendai virus, and episomal reprogramming. We compared the various reprogramming approaches, source cell types and culture conditions in terms of reprogramming efficacy.

Material and Methods

We reprogrammed two cell types in our experiments: neonatal human dermal fibroblasts (Life Technologies, Carlsbad, CA) and $CD34^+$ hematopoietic progenitors. $CD34^+$ cells were isolated from 3 ml of peripheral blood (sample kindly provided by the Department of Internal Medicine – Haematology and Oncology, Masaryk University and University Hospital Brno). Briefly, mononuclear cells were isolated using Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St. Louis, MO) and subsequently enriched for $CD34^+$ cells using magnetic-activated cell sorting (MACS) with a CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Before reprogramming, fibroblasts were expanded in DMEM medium (Life Rechnologies), supplemented with 20 % foetal bovine serum, 2 mM L-glutamine and 100 μM non-essential amino acids (all from Life Technologies). $CD34^+$ cells were expanded in StemPro®-34 medium (Life Technologies) supplemented with growth factors (100 ng/ml SCF; 50 ng/ml IL-3; 25 ng/ml GM-CSF; all factors were purchased from Peprotech, Rocky Hill, NJ).

Both cell types were reprogrammed using (1) lentivirus containing OSKM transcription factors (Human STEMCCA Constitutive Polycistronic Lentivirus Reprogramming Kit; Millipore, Darmstadt, Germany), (2) Sendai virus containing OSKM transcription factors (CytoTune™-iPS Reprogramming Kit; Life Technologies) and (3) episomal vectors containing OSKM and Lin28 transcription factors (Epi5™ Episomal iPSC Reprogramming Kit; Life Technologies) according to the manufacturer's instructions. For STEMCCA lentivirus and Sendai virus we used multiplicity of infection (MOI) = 6. Episomal transfection required electroporation of the cells with 1650 V for 10 ms three times for 10^5 cells in the Neon electroporator (Life Technologies). Reprogrammed cells were then seeded either on irradiated mouse embryonic fibroblast feeder cells (MEFs; $3x10^5$ per well of a 6-well plate) or on the Geltrex® matrix (Life Technologies). After reprogramming, cells on MEFs were cultured in DMEM/F12 (1 : 1), supplemented with 20% knock-out serum replacement, 2 mM L-glutamine, 100 μM non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol and 10 ng/ml bFGF (all from Life Technologies). Cells on Geltrex® were grown in Essential 8™ medium (Life Technologies). Medium was changed daily. Approximately 10 days after reprogramming colonies started to appear. Between days 17–28, the colonies were manually picked with a needle and transferred onto a fresh plate.

Cells were stained immunohistochemically for markers of pluripotency: Oct-3/4 (Santa Cruz Biotechnology, Dallas, TX), Nanog (Cell Signaling, Danvers, MA), Sox2 and SSEA4 (both from R&D Systems, Minneapolis, MN). Briefly, cells on plates were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton-X (both from Sigma-Aldrich). Cells were incubated with primary antibodies overnight at 4 °C, followed by 2 h incubation with secondary antibody conjugated with Alexa 488 (Cell Signalling). Nuclei were stained by Hoechst dye (BisBenzemide H33258; 1 μg/ml; Sigma-Aldrich). Fluorescent signal was detected in an inverted microscope Olympus IX71 (Olympus, Hamburg, Germany).

Results and Discussion

We successfully derived hiPSCs from both neonatal human dermal fibroblasts and $CD34^+$ hematopoietic progenitors using three methods: STEMCCA lentivirus, Sendai virus, and episomal reprogramming (the only exception was the STEMCCA lentivirus reprogramming, which was only performed with fibroblasts).

After application of reprogramming factors, the transformed cells were allowed to grow either on a MEF feeder cell layer or on basement membrane matrix – Geltrex®. The overall reprogramming efficacy in our experiments was between 5 to 50 colonies per 10^5 transfected cells in a 6-well plate (Table 1). Based on the morphology, we picked 5 to 13 clones for further expansion. We observed better reprogramming results while using feeder-free conditions. hiPSC generation efficacy may be compromised by unknown components released into the medium and increased exhaustion of nutrients by MEFs. Moreover, there is a high batch-to-batch variability amongst MEFs, and feeders or feeder-conditioned medium could potentially transmit pathogens. Therefore, an animal origin-free extracellular matrix in combination with precisely defined xeno-free medium seems to be more appropriate and confirms previously published data (Draper et al., 2004; Elefanty and Stanley, 2010; Chen et al., 2011).

The hiPSC clones established by all three methods demonstrated hESC-like morphology (Fig. 1, left column). In order to confirm the pluripotent state of the hiPSCs, random colonies of the particular clone were passaged on a 12-well plate and stained with antibodies against pluripotent markers Oct-3/4, Sox2, Nanog and

Table 1. Summary of the reprogramming experiments. Two cell types were reprogrammed using three methods. Cells were grown either on MEF feeder cells or on Geltrex®. STEMCCA lentiviral reprogramming was performed only in fibroblasts and grown on MEF feeder cells. Only colonies with proper hESC-like morphology were counted.

Source cell type	Reprogramming method	Surface support	Input cell number	Number of colonies	Passage number
Fibroblasts	STEMCCA lentivirus	MEF	10^5	11	29
		Geltrex	-	-	-
	Sendai virus	MEF	10^5	7	5
		Geltrex	10^5	21	5
	Episomal vectors	MEF	10^5	10	5
		Geltrex	10^5	27	5
$CD34^+$ cells	STEMCCA lentivirus	MEF	-	-	-
		Geltrex	-	-	-
	Sendai virus	MEF	10^5	5	5
		Geltrex	10^5	16	5
	Episomal vectors	MEF	10^5	14	5
		Geltrex	10^5	50	5

SSEA4 (Adewumi et al., 2007). The majority of the colonies were positive for all four markers (Fig. 1, right column), which implies their pluripotent state. We are able to grow and maintain all our hiPSC lines in an undifferentiated state over multiple passages (Table 1).

For our experiments we chose three methods representing distinct approaches to hiPSC generation: DNA integrating virus, DNA non-integrating virus and viral-free episomal vectors. The first approach, lentiviral transfection, requires integration into the host DNA. Integrating viruses (retrovirus and lentivirus) are considered a more reliable and efficient method to deliver reprogramming factors and were used in the first successful hiPSC generation (Takahashi et al., 2007; Yu et al., 2007). However, the exogenous viral DNA integration may induce insertional mutagenesis (Hacein-Bey-Abina et al., 2003). Although the viral transgenes may be removed from the cells after transformation (Sommer et al., 2010), this method is not very suitable for clinical applications. The second approach, Sendai virus reprogramming, represents a significant improvement in terms of safety. A negative-strand RNA virus does not go through DNA phase and replicates only in the cytoplasm, and is therefore very suitable as a vector for human gene therapy (Yonemitsu et al., 2000; Masaki et al., 2001; Tokusumi et al., 2002; Ferrari et al., 2007). OSKM reprogramming factors in Sendai virus vectors ensure fast expression of the transgenes at a high level, and have been shown to be sufficient to establish transduction (Fusaki et al., 2009). As a result, reprogrammed hiPSCs possess a genetically intact genome. The third approach used in our experiments involves highly effective episomal reprogramming (Yu et al., 2009). With this system we were able to generate and maintain hiPSCs completely viral-free and xeno-free throughout all the process, with comparable or higher reprogramming efficacy than in the previous two methods. The replication of oriP/EBNA-1 DNA vectors is extrachromosomal and applicable to many human cell types. There is no need for removing the remaining vectors because around

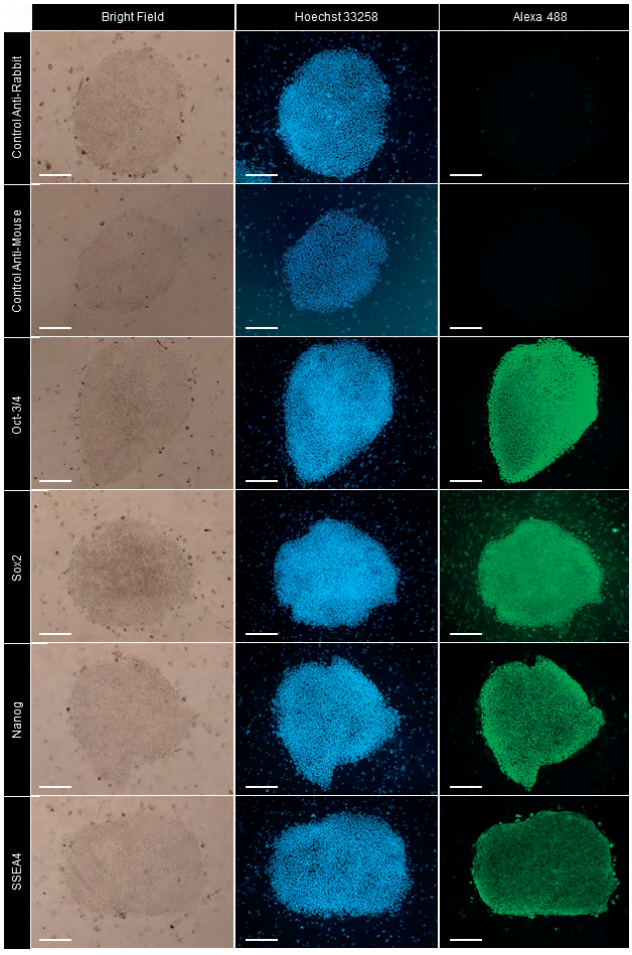

Fig. 1. Immunohistochemistry of pluripotency markers in hiPSCs derived from $CD34^+$ cells reprogrammed with Sendai virus. Pluripotency markers Oct-3/4, Sox2, Nanog and SSEA4 are highly expressed in hiPSCs in passage 4. Primary antibodies are mouse anti-human, except for Nanog, which is rabbit anti-human. Oct-3/4, Sox2 and Nanog are intracellular markers, SSEA4 is a surface antigen. No signal was detected in control samples stained only with secondary antibody anti-mouse or anti-rabbit conjugated with Alexa 488 fluorescent dye (upper right corner). Scale bar, 100 µm.

5 % are lost per cell cycle due to defects in plasmid synthesis and partitioning (Nanbo et al., 2007). For fibroblast-derived hiPSCs, Yu et al. (2011) assessed 14 passages to completely lose episomal vectors. The reprogramming efficiency in the first successful reprogramming of human fibroblasts with oriP/EBNA-1 vectors was low (~3 to 6 colonies/10^6 input cells) (Yu et al., 2009). Interestingly, it was found that the reprogramming induces apoptosis via activation of the p53 pathway, and therefore knock-down of this pathway was shown to improve the efficacy of reprogramming (Hong et al., 2009; Kawamura et al., 2009). Moreover, adding EBNA-1 vectors helps to transiently increase the expression of reprogramming plasmids (Middleton and Sugden, 1994; Kitamura et al., 2006). Therefore, five reprogramming factors (Oct3/4, Sox2, Klf4, Lin28, and c-Myc) were used in combination with genes for EBNA-1 and for a dominant negative mutation of p53.

Genome non-integrating reprogramming and animal origin-free (human or recombinant origin only) conditions in the process of hiPSC generation, expansion and subsequent differentiation into specialized cell types is essential for future transition of this technology into the clinic. All three methods established in our laboratories prove the high efficiency of hiPSC generation and are applicable to various human cell types. From the tested protocols, the Sendai virus or episomal vector reprogramming in combination with feeder-free culturing best fulfils the high criteria for possible future clinical application.

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Short Communication

Haematopoietic Developmental Potential of Human Pluripotent Stem Cell Lines

(human pluripotent stem cells / haematopoietic development / embryoid bodies / $CD34^+$ cells)

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Abstract. The generation of haematopoietic progenitors from human pluripotent stem cells (hPSCs) presents great promise for cell-replacement therapies. However, current protocols for haematopoietic differentiation of hPSCs suffer from low efficiency and functional defects in the derived cells. The technology is also limited by variable ability of hPSC lines to generate blood cells *in vitro*. To address this issue, methodologies for haematopoietic differentiation in feeder-free conditions were applied to available human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) lines in this study. It was found that these cell lines did not generate haematopoietic progenitors to such an extent as did H1 and H9 hESC lines that were used for this purpose in the vast majority of relevant studies. These results suggest that for clinical application of blood cells derived from hPSCs, possibly from autologous hiPSCs, it is necessary to overcome the variability in the haematopoietic developmental potential of individual hPSC lines.

Introduction

Haematopoietic differentiation of hPSCs provides a unique model of human haematopoietic stem cell (HSC) development and it is also of great therapeutic interest. hPSC-derived haematopoietic progenitors are considered to be an alternative source of HSCs for cell-replacement therapies.

Non-genetic strategies to derive HSCs from hPSCs are based on the ability to mimic hematopoietic niches *in vitro*. hPSCs are differentiated on feeder cells supporting haematopoiesis (Vodyanik et al., 2005; Ledran et al., 2008) or in the form of embryoid bodies (EBs) in the presence of growth factors promoting mesoderm, haemangioblast and HSC development (Chadwick et al., 2003; Zhang et al., 2008; Chicha et al., 2011; Kennedy et al., 2013; Ferrell et al., 2014). For clinical applications, protocols with fully defined, serum- and stroma-free conditions are considered.

Being widely used as a marker of HSCs, $CD34^+$ cells derived from hPSCs were compared to their *in vivo* $CD34^+$ counterparts. Whereas morphological properties and CFU ability were shown to be similar in both population, proper molecular characteristics, generation of definitive haematopoiesis, and the ability to engraft recipient bone marrow remain to be confirmed for hPSC-derived $CD34^+$ cells (Chadwick et al., 2003; Lu et al., 2004; Tian et al., 2004; Vodyanik et al., 2005; Zambidis et al., 2005; Tian et al., 2006; Ledran et al., 2008; Martin et al., 2008; Timmermans et al., 2009; Chicha et al., 2011; Dravid et al., 2011; Melichar et al., 2011; Niwa et al., 2011).

Taking the above information in consideration and despite the various methodological approaches, haematopoietic differentiation of hPSCs remains relatively ineffective and the derived HSCs possess functional defects. Therefore, several issues need to be elucidated and improved before clinical application of this process may begin. One of them is the issue of original hPSCs entering the differentiation, considering both hESCs and autologous and allogeneic iPSCs. The reason is that there are marked differences in the ability to differentiate into cells of various germ layers and into haematopoietic progenitors among hESC lines (Chang et al., 2008; Osafune et al., 2008; Melichar et al., 2011). Furthermore, this heterogeneity was found not only in various hESC lines, but also in individual cell lines. According to the specific surface markers, hESC sub-populations with different potential to generate haematopoietic progeni-

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Abbreviations: EBs – embryoid bodies, hESCs – human embryonic stem cells, hiPSCs – human induced pluripotent stem cells, hPSCs – human pluripotent stem cells, HSCs – haematopoietic stem cells, MEFs – mouse embryonic fibroblasts.

tors were defined and this potential was correlated with the chromatin landscape of mesoderm-associated genes (Hong et al., 2011).

In this study, we applied protocols described in the literature for haematopoietic differentiation of hPSCs to available hESC and hiPSC lines with the aim to determine their haematopoietic developmental potential.

Material and Methods

The hESC lines CCTL-12 and CCTL-14 and hiPSC lines STENF and IPSCF were used in this study. hiPSC lines were reprogrammed from human fibroblasts using Human STEMCCA Constitutive Polycistronic (OSKM) Lentivirus Reprogramming Kit (Millipore, Darmstadt, Germany) according to manufacturer's instructions. Cells were maintained undifferentiated as colonies on irradiated mouse embryonic fibroblasts (MEFs) in hESC medium and/or adapted to single-cell passage with TrypLE Select (Life Technologies, Carlsbad, CA) in feeder-free conditions with MEF-conditioned medium. Cell lines CCTL-12, CCTL-14 and IPSCF were kindly provided by the laboratory of Petr Dvořák (Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic).

Three different techniques were used to generate EBs from hPSCs: collagenase IV-disrupted colonies cultured in suspension, single cells cultured in suspension, and spin EBs formed in 96-well plates from single-cell suspension (Sheridan et al., 2012). Briefly, cell colonies were disrupted to small aggregates using collagenase IV (1 mg/ml, 30 minutes, 37 °C) and resuspended in differentiation medium at a ratio 1 : 1. TrypLE-adapted cells were dissociated into single-cell suspension by TrypLE Select (2 min, room temperature) and cultured in suspension at a density of 500,000 cells per ml in 6-well plates or as spin EBs in 96-well plates (3,000 cells pelleted in 100 µl at 400 *g* for 4 min). Low-attachment tissue culture dishes were used during the differentiation processes to prevent EB attachment.

EBs were differentiated in BPEL medium (Ng et al., 2008) or StemPro-34 medium (Life Technologies) supplemented with the following factors: protocol a) day 0–8: 40 ng/ml SCF, 20 ng/ml VEGF, and 20 ng/ml BMP-4 (Ferrell et al., 2014); protocol b) day 0–1: 0,5 ng/ml BMP-4, day 1–4: 10 ng/ml BMP-4, 5 ng/ml bFGF, day 4–8: 100 ng/ml SCF, 100 ng/ml VEGF, 5 ng/ml bFGF, 100 ng/ml FLT3-L, 40 ng/ml TPO (Chicha et al., 2011); protocol c) day 0–1: 10 ng/ml BMP-4, day 1–2: 10 ng/ml BMP-4, 5 ng/ml bFGF, day 2–4: 10 ng/ml BMP-4, 5 ng/ml bFGF, 0,3 ng/ml activin A, day 4–7: 5 ng/ml bFGF, 15 ng/ml VEGF, 150 ng/ml DKK-1, 10 ng/ml IL-6, 5 ng/ml IL-11, 25 ng/ml IGF-1 (Kennedy et al., 2013) (all factors were purchased from Peprotech, Rocky Hill, NJ).

After eight days of differentiation, EBs were dissociated with trypsin/EDTA (0.05%, Life Technologies). Cell suspensions were analysed via flow cytometry for the presence of haematopoietic progenitors after staining with CD34-, VEGFR2-, CD45-, CD31-specific antibodies (Miltenyi Biotec, Boston, MA). Data were collected in a BD FACS Canto II device (BD Biosciences, San Jose, CA) and analysed with the FACSDiva (BD Biosciences) and Flowing software (Turku Centre for Biotechnology, University of Turku, Finland).

Results and Discussion

Haematopoietic developmental potential of hPSC lines was determined after the application of differentiation protocols based on EB formation in the presence of growth factors.

The ability to form EBs differed among differently propagated hPSCs. TrypLE-adapted cells were subjected to the spin EB method. While BPEL medium was characterized by generation of uniform EBs (Fig. 1A), this result was not observed in StemPro-34 medium (Fig. 1B). This difference was given by polyvinylalcohol included in the BPEL medium, which improved the initial formation of EBs (Ng et al., 2008). In suspension culture, TrypLE-adapted cells also formed EBs, which were heterogeneous in size due to spontaneous aggregation of hPSCs (Fig. 1C). Non-adapted hPSCs grown in colonies on MEFs did not survive the generation of EBs, when protocols including the single-cell suspension step were applied (Fig. 1E, F). On the contrary, using collagenase IV disruption of colonies into cell clumps, the EBs were formed. Nevertheless, the process was ineffective with considerable cell loss and the EB population was characterized by great heterogeneity. EBs differed in both size and shape, and the EBs with cystic structure (Fig. 1D), indicating efficient hPSC differentiation (Kim et al., 2011), were in a minority.

Considering the above analysis, it became clear that for efficient and reproducible haematopoietic differentiation of hPSCs, it is necessary to consider the generation of EBs itself. TrypLE-adapted cells were shown to efficiently form a population of uniform EBs, but no cystic structure was observed. In future studies we will, therefore, focus on: a) optimization of hPSC transfer into differentiation cultures; b) comparison of haematopoietic differentiation efficiency among differentially propagated hPSCs to determine whether the cell selection during TrypLE adaptation affects the process of differentiation.

Cell cultures generating EBs were subjected to haematopoietic differentiation according to protocols found in the literature (Chicha et al., 2011; Kennedy et al., 2013; Ferrell et al., 2014). They were based on the differentiation of EBs in feeder-free conditions supplemented with growth factors promoting the stepwise development, proliferation and survival of mesodermal cells, haemangioblasts, and $CD34^+$ cells (Chadwick et al., 2003; Ng et al., 2005; Kennedy et al., 2007; Pick et al., 2007). Various combinations of the following parameters were tested to optimize the differentiation efficiency: original cells (hiPSCs or hESCs propagated as colonies on MEFs or adapted to TrypLE), EB formation

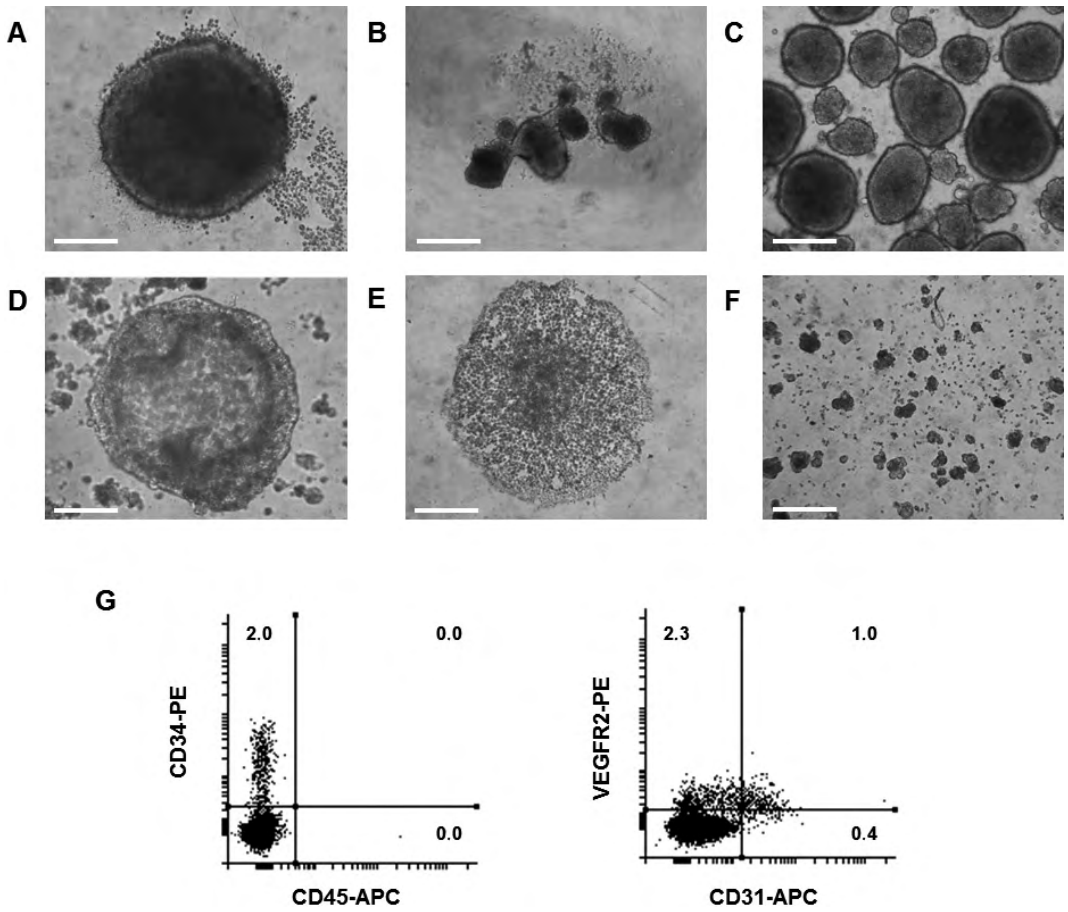

Fig. 1. Haematopoietic differentiation of hPSC in the form of EBs. The morphology of EBs generated from differently propagated hPSC lines after eight days of differentiation is shown (**A-F**). TrypLE-adapted cells (**A-C**) formed single EBs in BPEL medium (**A**) in contrast to non-uniform EBs in StemPro-34 medium (**B**) during the spin EB method (3,000 of hPSCs per well). Suspension cultures of TrypLE-adapted cells were characterized by EB population heterogeneous in size (**C**). Generation of EBs from hPSCs grown in colonies on MEFs was very poor with protocols including the single-cell suspension step, meaning both the spin EB method (**E**) and suspension culture (**F**). EBs with cystic structures were found in suspension cultures of collagenase IV-disrupted hPSC colonies (**D**). TrypLE-adapted CCTL-14 cells (**A-C**) and STENF cells grown in colonies on MEFs (**D-F**) were used to form EBs in BPEL medium (**A, E**) and StemPro-34 medium (**B-D, F**). Scale bar, 100 µm. Phenotypic analyses of dissociated EBs after eight days of haematopoietic differentiation (**G**). Representative data from flow cytometry analysis of differentiating CCTL-14 line using the spin EB method, in StemPro-34 medium with growth factors included according to the protocol b) (see Material and Methods) are shown.

method (spin EB or suspension culture of single cells or collagenase IV-disrupted colonies), differentiation medium (BPEL or StemPro-34), and cytokine cocktail (see protocol a)-c) in Material and Methods). After eight days of differentiation, dissociated EBs were analysed by flow cytometry. Cells characterized by markers for haemangioblasts and haematopoietic progenitors, VEGFR2, CD31, and CD34, were detected; $CD45^+$ cells were not present in EBs at this stage of differentiation (Fig. 1G). The yield of $CD34^+$ cells ranged between 2 and 7 %, regardless of the combination of parameters mentioned above. Such haematopoietic differentiation can be considered as relatively low efficiency. The representation of $CD34^+$ cells was almost an order of magnitude lower than in the studies from which the used differentiation protocols were adapted and where it reached up to 20 to 30 % (Chicha et al., 2011; Kennedy et al., 2013; Ferrell et al., 2014).

Our results clearly indicate that various hPSCs differ in the haematopoietic development potential. Cell lines of hESCs and hiPSCs used in our study did not generate more than 7 % of $CD34^+$ cells, whereas in other studies, the same or similar differentiation protocols yielded up to 30 % of haematopoietic precursors (Chadwick et al., 2003; Ng et al., 2005; Zhang et al., 2008; Chicha et al., 2011; Kennedy et al., 2013; Ferrell et al., 2014). In the vast majority of these studies H1 and H9 hESC lines were used for differentiation, which suggests their increased potential to develop into the cells of blood lineage. These H1 and H9 haematopoietic predispositions were demonstrated by their direct comparison with other hESC lines (Chang et al., 2008; Melichar et al., 2011). The reasons for variable haematopoietic development potential of hPSCs remain to be fully defined. The epigenetic status of hPSCs was suggested to be one of them based on the fact that there is great epigenetic variety

between hPSC lines (Brunner et al., 2009). This hypothesis was supported by Hong et al., who found the differentiation potential of hPSCs to be encoded by histone modifications, and the ability to generate haematopoietic progenitors was correlated to epigenetic marks on mesoderm-associated genes (Hong et al., 2011).

The clinical application of hPSC-derived HSCs requires improvement of the differentiation protocols to become more efficient and generate HSCs functionally equivalent to their *in vivo* $CD34^+$ counterparts. Based on our results and other studies, it is clear that one of the issues to be considered is the ability of original hPSCs to differentiate into haematopoietic cell lineage. It will be important to identify factors determining the haematopoietic differentiation potential. A relatively easy and cheap methodology, e.g. hPSC co-cultivation with stromal cells supporting haematopoiesis, could be used to distinguish between cells with and without haematopoietic potential. Finding molecular differences between these two cell types could help to reveal the basis for haematopoietic differentiation potential and to define the putative markers for evaluation whether hPSCs in use are suitable for differentiation or not, possibly for selection of cells with haematopoietic potential from mixed cell population. Finally, the ability to transfer hPSCs with haematopoietic potential to differentiation cultures with fully defined, serum- and stroma-free conditions will be the prerequisite for clinical applications of hPSC-derived HSCs.

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