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

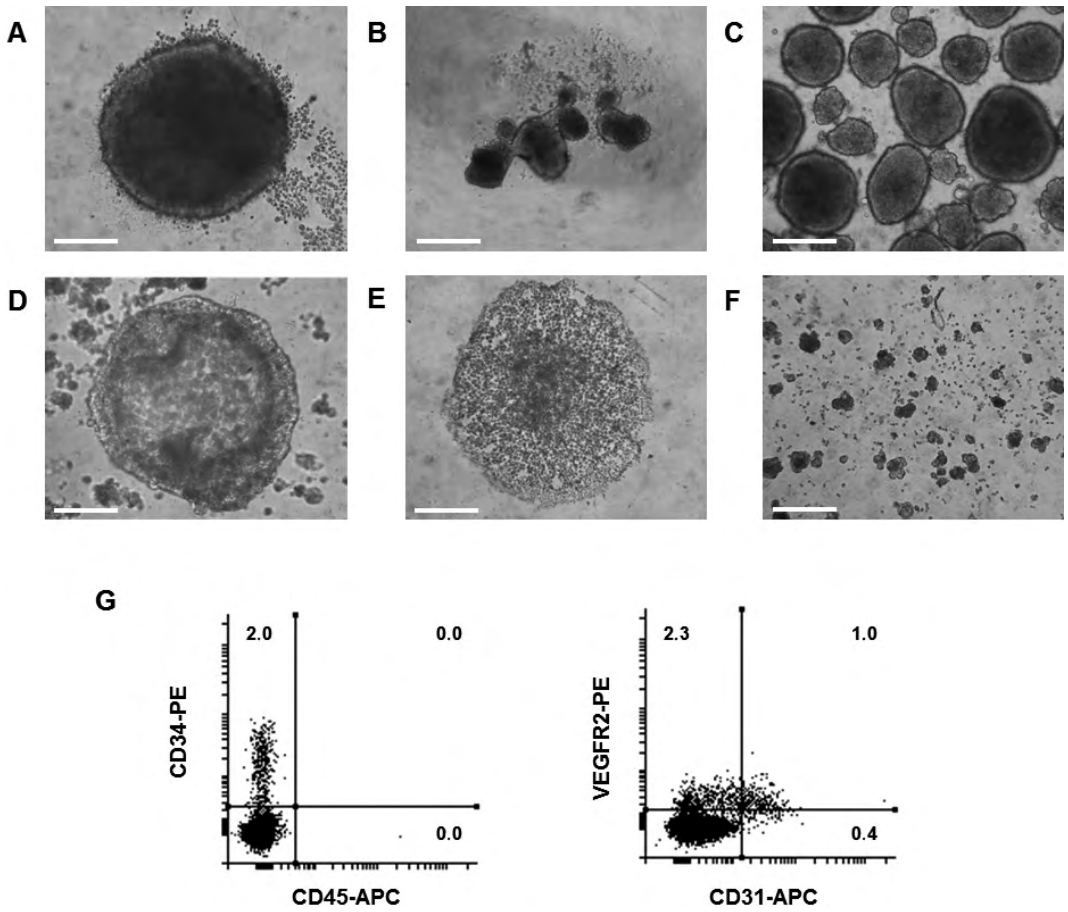

Fig. 1. Haematopoietic differentiation of hPSC in the form of EBs. The morphology of EBs generated from differently propagated hPSC lines after eight days of differentiation is shown (**A-F**). TrypLE-adapted cells (**A-C**) formed single EBs in BPEL medium (**A**) in contrast to non-uniform EBs in StemPro-34 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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