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Efficient production of erythroid, megakaryocytic and myeloid cells, using single cell-derived iPSC colony differentiation

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ABSTRACT

Hematopoietic differentiation of human induced pluripotent stem cells (iPSCs) provide opportunities not only for fundamental research and disease modelling/drug testing but also for large-scale production of blood effector cells for future clinical application. Although there are multiple ways to differentiate human iPSCs towards hematopoietic lineages, there is a need to develop reproducible and robust protocols. Here we introduce an efficient way to produce three major blood cell types using a standardized differentiation protocol that starts with a single hematopoietic initiation step. This system is feeder-free, avoids EB-formation, starts with a hematopoietic initiation step based on a novel single cell-derived iPSC colony differentiation and produces multipotential progenitors within 8–10 days. Followed by lineage-specific growth factor supplementation these cells can be matured into well characterized erythroid, megakaryocytic and myeloid cells with high-purity, without transcription factor overexpression or any kind of pre-purification step. This standardized differentiation system provides a simple platform to produce specific blood cells in a reproducible manner for hematopoietic development studies, disease modelling, drug testing and the potential for future therapeutic applications.

1. Introduction

The discovery that human somatic cells can be reprogrammed into a pluripotent stem cell state, has created a novel approach for developmental studies, drug screening/discovery, disease modelling, and regenerative medicine (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wu and Hochedlinger, 2011). Recent advances in hematopoietic differentiation of human induced pluripotent stem cells (iPSCs) opened opportunities not only for fundamental research and disease modelling/drug testing but also for large-scale manufacture of blood products with the expectancy of clinical application (Giarratana et al., 2011; Ross and Akimov, 2014). However, this potential is influenced by the I) variations at (*epi*)genetic level between iPSC lines or between clones, II) incomplete understanding of differentiation cues, III) lack of standard (GMP-grade) protocols, IV) low cellular yield and V) low purity, often requires pre-purification. These also make direct comparisons between the final results obtained in the different laboratories difficult. Thus, there is a need of improvement and standardization of iPSCs differentiation to hematopoietic cells in order to obtain a reproducible method which yields cells enough for downstream applications. In contrast to mouse embryonic stem cells (ESCs)/iPSCs, human iPSC are sensitive for single cell-passaging due to detachment-induced apoptosis (Chen et al., 2010; Ohgushi et al., 2010; Amit and Itskovitz-Eldor, 2002). Therefore, clump-passaging is still the preferred method for human ESC or iPSC maintenance (Hartung et al., 2010; Reubinoff et al., 2000). Besides its labour intensiveness, clump-passaging technically leads to heterogeneity in clump size, causing potential reproducibility issues. Dissociation-induced apoptosis of iPSCs is mediated by the Rho-associated protein kinases (ROCK) pathway (Chen et al., 2010; Ohgushi et al., 2010; Watanabe et al., 2007). The use of ROCK inhibitors during single cell-dissociation significantly increases cell survival, colony formation and decreases cell death without affecting pluripotency, thereby enabling single cell-passaging (Watanabe et al., 2007; Kurosawa, 2012; Kajabadi et al., 2015; Zhang et al., 2016).

Differentiation of iPSCs towards the hematopoietic lineage can be achieved by 2D- or 3D methods. Forced or spontaneous clustering of

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iPSCs into a spherical 3D structures (embryoid body (EB)) recapitulates in some aspects the complex assembly during embryogenesis (Amit and Itskovitz-Eldor, 2002; Doetschman et al., 1985; Ng et al., 2005), as evidenced by the formation of the three germ layers (Amit and Itskovitz-Eldor, 2002; Itskovitz-Eldor et al., 2000). Differentiation can be directed towards cell types of interest by controlling the EB size, or by using extracellular matrix and/or growth factors. With the development of forced aggregation, EB size can be better controlled but spontaneous agglomeration of multiple EBs is still an unregulated factor (Ng et al., 2008). Release of progenitors from the EBs involves either attaching EBs to a matrix coated surface or applying physical shear stress sometimes combined with the use of proteases (Olivier et al., 2016; Ko et al., 2014; Fujita et al., 2016). These are harsh treatments, affecting viability and the ability to further culture these cells. As an alternative to the 3D approach, iPSCs can be differentiated in 2D monolayers on a feeder cell layer or on feeder-free matrix coated dishes (Salvagiotto et al., 2011; Niwa et al., 2011; Feng et al., 2014; Smith et al., 2013; Choi et al., 2009a, 2009b; Weisel et al., 2006). The 2D method enables the collection of hematopoietic cells without the use of proteases or physical shear stress, increasing the yield of viable cells (Salvagiotto et al., 2011; Niwa et al., 2011; Feng et al., 2014). Currently, 2D protocols have been initiated using iPSC clumps of variable size, hindering reproducibility and robustness. We hypothesized that tight control of iPSC differentiation starting conditions will increase the reproducibility of hematopoietic specification and differentiation.

In the present study a novel, feeder-free, single iPSC-derived, monolayer differentiation system was designed to culture 3 major myeloid blood cell types starting from a single hematopoietic initiation step. The derived multi-potential hematopoietic progenitors could further mature into erythroid, megakaryocytic and myeloid cells with high purity. This protocol provides a reproducible platform not only for hematopoietic developmental research but for disease modelling/drug testing and it has the potential for large-scale production of specific blood cell types for future clinical application.

2. Material and methods

2.1. Chemicals

The chemicals were purchased from Sigma-Aldrich (Munich, Germany), the culture reagents from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and all growth factors from Peprotech (Pittsburgh, USA) unless otherwise specified.

2.2. Statement

Informed consent was given in accordance with the Declaration of Helsinki and Dutch national and Sanquin internal ethic boards.

2.3. iPSC lines

Four iPSC lines were used in this study. MML-6838-Cl2: megakaryoblast origin, hOKSM lenti-virus vector (Passage number (p)23, p24, p34, p36, p38, p39, p44); MEL1171.CL7: erythroblast origin, hOKSM lenti-virus vector (p35); SANi003-B: erythroblast origin, hOKSML episomal plasmid (transgene-free) (p22), SANi003-A: erythroblast origin, hOKSML episomal plasmid (transgene-free) (p22) (Hansen et al., 2017; Varga et al., 2017). All used lines are registered at the human pluripotent stem cell registry (https://hpscreg.eu).

2.4. iPSC single cell-maintenance

All cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2. The iPSCs were cultured feeder-free on matrigel (BD Biosciences) in essential 8 medium (E8). Passaging occurred every 5–7 days using TrypLE Select according to manufacturer's instruction, with a seeding density of 1-2 × 10^4 cells/6 cm dish in E8 + Revitacell supplement. If necessary, differentiated parts were removed by scraping. Cells were counted on a Casy cell counter and analyser (Roche).

2.5. iPSC colony differentiation towards hematopoietic lineages

In order to reach the required colony number (5–15 colonies/6 cm dish) the cells were harvested as described above. The iPSCs were single cell-seeded at lower density, 250–400 cells/6 cm dish on matrigel (BD Biosciences) coated dishes. Single cell-derived colonies were grown for 8–9 days prior to differentiation. If necessary, surplus colonies were removed by scraping. At day 0 of differentiation, cells were washed 1× with DPBS then hematopoietic differentiation was initiated by adding Stemline II supplemented with 1% Pen/strep, 50 ng/ml bFGF, 40 ng/ml VEGF, 20 ng/ml BMP4 and 100× insulin-transferrin-selenium (ITS). At day 6, colonies were washed 1× with DPBS then culture media was added containing lineage specific growth factor cocktails (described below) and re-freshed every 2–3 days.

2.6. Specification towards erythrocytes

After initiation of differentiation for 6 days medium was changed to Cell-Quin (home-made media, published by others with minor modifications) 100× ITS, 5 U/ml EPO, 50 ng/ml hSCF, 10 ng/ml TPO, 50 ng/ml IL-6, 50 ng/ml IL-3, 1% Pen/strep (Migliaccio et al., 2010). Between day 9–15 of iPSC differentiation (day 9, 12, 15) suspension cell were harvested and analysed. Day 12 suspension cells were collected and further expanded for 9 days (day 12 + 9) in erythroblast (EBL)-specific media (Cell-Quin supplemented with: 1% Pen/strep, 2 U/ml EPO, 100 ng/ml hSCF, 10^{-6} M Dexamethasone) (Heideveld et al., 2015; van den Akker et al., 2010). Expanded iPSC-EBLs were further maturated by adding terminal differentiation mix Cell-Quin suppl. with: 1% Pen/strep, 10 U/ml EPO, 5 U/ml heparin, 5% pooled human plasma. 1 × 10^7 cells were collected and analysed for hemoglobin (Hb) isoform expression by high-performance cation-exchange liquid chromatography (HPLC) on Waters Alliance 2690 equipment and the column was purchased from PolyLC (van Zwieten et al., 2014).

2.7. Specification towards megakaryocytes

After 6 days of differentiation, medium was changed to Cell-Quin with 10 ng/ml VEGF, 20 ng/ml BMP4, 1 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml TPO, 50 ng/ml hSCF, 100× ITS, 1% Pen/strep and 10 ng/ml IL-1β. Suspension cells were harvested between day 11–15 of iPSC differentiation and terminally differentiated in Cell-Quin with 1% Pen/strep, 10 ng/ml IL-1β and 50 ng/ml TPO for 6 days (day 11 + 6). For megakaryocyte colony assays (CFU-Mk) day 11 of iPSC differentiation, suspension cells were expanded for 6 days in Cell-Quin supplemented with 10 ng/ml IL-6, 50 ng/ml hSCF, 50 ng/ml TPO and 10 ng/ml IL-1β. The expanded cells were seeded into the Mk-CFU assay chamber slide (Stem cell technologies) at 5000 cells/well. Mk-CFU were fixed and stained according to manufactures instructions after 10 days of culture. For $CD34^+$ mobilized peripheral blood (MPB) differentiation towards the megakaryocytic linage, cells were differentiated in Cell-Quin with 50 ng/ml hSCF, 1 ng/ml IL-3, 20 ng/ml IL-6, 50 ng/ml TPO for 4 days followed by 10 days differentiation in Cell-Quin supplemented with 50 ng/ml TPO and 10 ng/ml IL-1β. Isolation of $CD34^+$ MPB was performed as described before (Heideveld et al., 2015).

2.8. Specification towards myeloid cells

From day 6 of differentiation, cells were cultured in Stemline II with 1% Pen/strep, 10 ng/ml VEGF, 20 ng/ml BMP4, 1 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml TPO, 50 ng/ml hSCF, 100× ITS and 50 ng/ml FLT-3. Suspension cells were collected on day 12 of iPSC differentiation and