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ARTICLE

Erythropoietic differentiation of a human embryonic stem cell line harbouring the sickle cell anaemia mutation

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Abstract Herein is reported efficient erythropoietic differentiation of a human embryonic stem cell (ESC) line derived from a pre-implantation genetic diagnosis (PGD)-screened embryo that harbours the homozygous sickle cell disease (SCD) haemoglobinopathy mutation. This human ESC line possesses typical pluripotency characteristics and forms multilineage teratomas *in vivo*. SCD-human ESC efficiently differentiated to the haematopoietic lineage under serum-free and stromal co-culture conditions and gave rise to robust primitive and definitive erythrocytes. Expression of embryonic, fetal and adult sickle globin genes in SCD PGD-derived human ESC-derived erythrocytes was confirmed by quantitative real-time PCR, intracytoplasmic fluorescence-activated cell sorting and in-situ immunostaining of PGD-derived human ESC teratoma sections. These data introduce important methodologies and paradigms for using patient-specific human ESC to generate normal and haemoglobinopathic erythroid progenitors for biomedical research.

KEYWORDS: haematopoiesis, human embryonic stem cells, preimplantation genetic diagnosis, red blood cells, sickle cell anaemia

Introduction

Preimplantation genetic diagnosis (PGD) is routinely used in assisted reproduction to screen inherited genetic disorders in gametes or cleavage-stage embryos during IVF cycles to avoid births with severe genetic disorders. More than 100 testable genetic conditions, including severe haematological disorders such as beta-thalassaemia, Fanconi anaemia and sickle cell disease (SCD), can be PCR-screened using the polar body of an unfertilized oocyte or from a microbiopsied blastomere. The derivation of human embryonic stem cell (ESC) lines from preimplantation embryos with

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genetic disorders has been extensively reported, and these PGD-derived human ESC are untested, yet potentially valuable, tools for investigating cellular and molecular events of human embryogenesis in diseased states (Ben-Yosef et al., 2008; Eiges et al., 2007; Kuliev et al., 2001; Strelchenko et al., 2004; Verlinsky et al., 2005, 2006).

A more recent approach for creating human pluripotent stem cells (PSC) that harbour genetic disorders is via the generation of induced PSC using defined transgenic pluripotency factors. For example, a great deal of interest has recently been invested in modelling the developmental pathology of haematological disorders using disease-affected induced PSC and in treating haematological disorders with genetically corrected haematopoietic stem cells that are derived from autologous induced PSC (Hanna et al., 2007; Park et al., 2008a). Sickle cell haemoglobinopathy, a classic inherited monogenic disorder resulting from the substitution of glutamate to valine at position 6 of the beta-haemoglobin chain, is an important candidate for such stem cell-based therapies. A proof of principle for induced PSC-based cellular/genetic therapy was recently demonstrated in a murine model of sickle cell anaemia (Hanna et al., 2007). However, human PSC lines generated by nascent induced PSC technology may face several caveats in their differentiation capacity that may limit their use in disease modelling, such as incomplete reprogramming and viral integration effects. Thus, disease-affected, bona-fide human ESC derived from IVF-derived PGD-selected preimplantation embryos can serve as gold standards for preclinical validation of induced PSC-based therapies.

This study significantly advances several of these concepts by reporting the characterization and haematopoietic differentiation of a novel PGD-derived human ESC line harbouring the homozygous mutation for SCD haemoglobinopathy. More importantly, these data demonstrate the utility and feasibility of using patient-specific human ESC for generating erythroid progenitors for haematological disease modelling and therapeutics.

Materials and methods

Derivation, culture, characterization and genotyping of a pluripotent PGD-derived human ESC line affected with the homozygous SCD mutation

Human ESC line SC233 (NIH human ESC registry; RG-233) was established by Reproductive Genetics Institute (Chicago, IL, USA) via original techniques from donated morula-stage embryos (Ley et al., 1983; Verlinsky et al., 2006). Patients undergoing PCR-based PGD selection to avoid the homozygous SCD mutation gave institutionally approved informed consent for the IVF process, the PGD selection process, as well as for the derivation of human ESC from surplus, disease-affected embryos. All human ESC lines used in these studies were evaluated and approved by the JHUSM Embryonic Stem Cell Research Oversight (ESCRO) Committee to assure derivation a informed consent guidelines conformed with those recommended by the National Academy of Sciences for research involving human ESC. Line SC233, as well as control H1 (WA01) and H9 (WA09) lines obtained from WiCell, were cultured in standard conditions. Line SC233 was obtained and expanded at low passage (about p6).

Karyotyping was performed using high resolution G-banding (JHUSM Cytogenetics Core). In the study laboratory, line SC233 displays classic human ESC morphology in typical culture conditions and has a high level of expression for pluripotency markers (e.g., alkaline phosphatase, SSEA3, SSEA4, TRA-1—60, TRA-1—81 and OCT4 (ES Cell Marker Kit; Chemicon). The SCD beta-6 mutation in line SC233 was detected by *Dde*I (New England Biolabs) restriction digest of PCR-amplified genomic DNA. The SCD AT transversion point mutation was further confirmed by direct DNA sequencing of the PCR-amplified beta-globin locus. Genomic DNA from SC233 and control H1 human ESC was isolated using DNeasy reagents (Qiagen). Diluted genomic DNA was PCR-amplified using primers specific to exon I of the beta-globin locus with the following primers: forward HBB: 5′-AGC CAG TGC CAG AAG AGC-3′; reverse HBB: 5′-AGG GGA AAG AAA ACA TCA AGG GTC-3′. A specific 688-bp PCR product was purified using Qiagen spin columns, digested with *Dde*I and analysed on 4% agarose gels, or subcloned (TOPO XL/TA Cloning kit; Invitrogen), and subsequently sequenced directly.

Teratoma formation and immunostains

Teratomas were prepared in immunodeficient mice, as previously described (Park et al., 2008b). Immunohistochemical staining using anti-human beta-globin (Santa Cruz), anti-human CD34 and murine Ter119 (Becton Dickinson) antibodies was performed using standard protocols.

Erythroid differentiation of human ESC

Human embryoid bodies (EB) from normal (H1, WA01; H9, WA09) and SC233 ESC were differentiated as described before (Zambidis et al., 2005, 2008) but with minor modifications. ESC cultures with minimal differentiation were grown to about 60—80% confluence on gelatinised plates and irradiated primary mouse embryo fibroblasts. Twenty-four hours prior to EB formation, serum-free human ESC medium was switched to an adaptation medium (Zambidis et al., 2005, 2008) consisting of serum-free expansion medium (SFEM; StemCell Technologies) supplemented 15% fetal bovine serum (FBS; StemCell Technologies), 50 mg/ml ascorbic acid (Sigma), 1% enhancement media supplement EX-CYTE (Millipore), 0.5% insulin/transferrin/selenium-X supplement (Invitrogen), 3.5% PFHM-II (Invitrogen) and 100 U/100 g penicillin-streptomycin (Invitrogen). ESC were harvested 24 h later with 1 mg/ml collagenase IV (Invitrogen) and cultured for an additional 2—3 days in 6-well ultra low-attachment plates (Corning) for EB formation. One well of ESC was transferred to 1-well ultra low-attachment plates for EB formation in 2.5—3 ml/well of methylcellulose medium (SF H4236; StemCell Technologies) supplemented with 15% FBS (StemCell Technologies), 50 μg/ml ascorbic acid, 0.5% enhancement media supplement EX-CYTE, 3.5% PFHM-II and 5—10 mmol/l Rock inhibitor (Y-27632; Calbiochem). Formed EB were collected 2—3 days later, washed with phosphate-buffered saline and replated into ultra low-attachment 6-well plates (1 well of human EB to 1—2 wells) in SFEM which consisted of all the ingredients