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Efficient production of platelets from mouse embryonic stem cells by enforced expression of Gata2 in late hemogenic endothelial cells

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ABSTRACT

Platelets are essential for blood circulation and coagulation. Previous study indicated that overexpression of Gata2 in differentiated mouse embryonic stem cells (ESCs) resulted in robust induction of megakaryocytes (Mks). To evaluate platelet production capacity of the Gata2-induced ESC-derived Mks, we generated i*Gata2*-ESC line carrying the doxycycline-inducible *Gata2* expression cassette. When doxycycline was added to day 5 hemogenic endothelial cells in the *in vitro* differentiation culture of i*Gata2*-ESCs, c-Kit$^-$Tie2$^-$CD41$^+$ Mks were predominantly generated. These i*Gata2*-ESC-derived Mks efficiently produced CD41$^+$CD42b$^+$CD61$^+$ platelets and adhered to fibrinogen-coated glass coverslips in response to thrombin stimulation. Transmission electron microscopy analysis demonstrated that the i*Gata2*-ESC-derived platelets were discoid-shaped with α-granules and an open canalicular system, but were larger than peripheral blood platelets in size. These results demonstrated that an enforced expression of Gata2 in late HECs of differentiated ESCs efficiently promotes megakaryopoiesis followed by platelet production. This study provides valuable information for *ex vivo* platelet production from human pluripotent stem cells in future.

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1. Introduction

Platelets are essential blood components for coagulation and maintenance of vascular functions. Because the shelf life of these cells is short, new technologies for platelet generation from human-induced pluripotent stem cells (iPSCs) are currently under exploration [1]. Previously, Nakamura et al. utilized human iPSC-derived megakaryocyte (Mk) progenitor cell lines to generate large numbers of platelets [2], and Feng et al. developed an efficient serum-/feeder cell-free method for large-scale production of platelets from human iPSCs [3]. More recently, Noh et al. reported a new methodology for obtaining scalable amounts of platelets from mouse embryonic stem cells (ESCs) by controlling *Gata1* gene expression levels in Mk progenitors [4]. Although these studies clearly advanced the fields of platelet generation and engineering, such protocols require long culture times before platelets can be harvested from ESCs/iPSCs.

Blood cells are generated by two waves of developmental processes during mammalian embryogenesis, i.e., primitive hematopoiesis and definitive hematopoiesis [5]. Platelets are mainly produced during definitive hematopoiesis by way of Mk

Abbreviations: ESC, embryonic stem cell; Mk, megakaryocyte; iPSC, induced pluripotent stem cell; HSC, hematopoietic stem cell; HEC, hemogenic endothelial cell; AGM, aorta-gonad-mesonephros; dpc, postcoitum; EHT, endothelial-to-hematopoietic transition; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PS, penicillin-streptomycin; LIF, leukemia inhibitory factor; α-MEM, α-Minimum Essential Medium; Dox, doxycycline; RT-PCR, reverse transcription PCR; ECL, enhanced chemiluminescence; FACS, Fluorescence-activated cell sorting; PBS, phosphate-buffered saline; APC, allophycocyanin; FITC, fluorescein isothiocyanate; TPO, thrombopoietin; CFU, colony forming unit; PGE_1, prostaglandin E_1; TEM, transmission electron microscopy; BSA, bovine serum albumin; SE, standard error.

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progenitors derived from hematopoietic stem cells (HSCs). HSCs are themselves generated from hemogenic endothelial cells (HECs) present in aorta-gonad-mesonephros (AGM) regions of the day 10.5 postcoitum (dpc) mouse embryos via endothelial-to-hematopoietic transition (EHT) [6]. Runx1 is absolutely essential for the initiation of EHT and HSC induction [7]. In addition, Gata2 and Notch signaling participate in EHT at specific sites in the developing mouse embryo [8–10]. Erythroid-myeloid progenitors are also directly produced from HECs in the AGM regions and yolk sacs [11]. Primitive and definitive Mk types were previously reported in the yolk sacs of the mouse embryos between 8.5 and 9.5 dpc before the emergence of HSCs [12–14]. However, in the early embryo, whether Mks are directly generated from HECs or they are differentiated from Mk progenitors remains to be determined.

Many steps in primitive and definitive hematopoiesis can be recapitulated by *in vitro* differentiation culture of mouse ESCs. HECs are induced from ESCs on day 3–5 of differentiation, and can then be isolated as c-Kit$^+$Tie2$^+$CD41$^-$ cells [15]. Subsequently, pre-HSCs are generated from HECs by EHT and begin to express CD41 [16]. Conditional expression of Gata2 in differentiated mouse ESCs reportedly enhances hemangioblast generation [17]. In addition, it was previously reported that enforced expression of Gata2 in differentiated mouse ESCs resulted in the induction of Mk and erythroid progenitors [18]. In this study, we re-examined the previous observations in more precise manners and revealed that Gata2 overexpression in HECs on day 5 in the *in vitro* differentiation of mouse ESCs leads to the production of c-Kit$^-$Tie2$^-$CD41$^+$ Mks with the capacity to generate platelets. We present evidence for well-developed ultrastructure and functionality of these Gata2-induced ESC-derived platelets.

2. Materials and methods

2.1. In vitro differentiation of ESCs

A cDNA encoding mouse Gata2 or green fluorescent protein (GFP) was inserted into the *hprt* locus of A2Lox.cre mouse ESCs by homologous recombination to generate the inducible *Gata2* ESC and *GFP* ESC lines (i*Gata2*-ESCs and i*GFP*-ESCs), as previously described [19]. The i*Gata2*-ESCs and control i*GFP*-ESCs were maintained on a feeder layer of mitomycin-C (Sigma, St. Louis, MO)-treated mouse embryonic fibroblasts in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) containing 2% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA), 15% KnockOut Serum Replacement (Thermo Fisher Scientific), 0.5% penicillin-streptomycin (PS; Sigma), 50 μM β-mercaptoethanol (Sigma), and 10^3 U/ml leukemia inhibitory factor (LIF; Thermo Fisher Scientific).

In vitro differentiation of i*Gata2*-ESCs and i*GFP*-ESCs was carried out as previously described [20]. Briefly, ESCs (10^4 cells per well in a six-well plate) were cultured on a feeder layer of mouse OP9 stromal cells in α-Minimum Essential Medium (α-MEM; Thermo Fisher Scientific) supplemented with 20% FBS (JRH Biosciences, Lenexa, KS) and 0.5% PS for 3 or 5 days in the absence of LIF. Doxycycline (Dox, 1 μg/ml; Sigma) was then added to the culture medium to induce transgene expression.

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNAs were prepared from ESC-differentiated cells by using TRIzol Reagent (Thermo Fisher Scientific). RNA (1 μg) isolated from each sample was reverse transcribed by using PrimeScript Master Mix and random primers (Takara, Otsu, Japan). A portion of the cDNA (1/50) was used for PCR reactions with an annealing temperature of 56 °C, recombinant Taq DNA polymerase (Takara), and the primer sets listed in Supplementary Table S1.

2.3. Western blot analysis

Protein extracts from ESCs (10^6 cells) were fractionated by electrophoresis in a 10–20% SuperSep Precast Polyacrylamide Gel (Wako, Osaka, Japan) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The blot was incubated with primary rabbit anti-Gata2 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK), and subsequently subjected to enhanced chemiluminescence (ECL) detection of immunoreactive proteins by using an ECL Plus Kit (GE Healthcare) and an ImageQuant LAS-4000 Biomolecular Imager (Fujifilm, Tokyo, Japan).

2.4. Fluorescence-activated cell sorting (FACS)

Differentiated ESCs were dissociated by digestion with 0.25% trypsin-EDTA in phosphate-buffered saline (PBS; Sigma) for 5 min at 37 °C, and stained with allophycocyanin (APC)-conjugated anti-c-Kit antibody (clone 2B8; BioLegend, San Diego, CA), phycoerythrin-conjugated anti-Tie2 antibody (clone TEK4; BD Biosciences, San Jose, CA), and fluorescein isothiocyanate (FITC)-conjugated anti-CD41 antibody (MWReg30; Biolegend). Next, c-Kit$^+$Tie2$^+$CD41$^-$ cells were isolated via FACS on a FACSAriaIII System (BD Biosciences) and subcultured with mouse OP9 cells in α-MEM medium supplemented with 20% FBS, 0.5% PS, and 10 ng/ml mouse thrombopoietin (TPO; Kirin Brewery, Takasaki, Japan) for 4–12 days. FACS data were analyzed by using FlowJo Software (Tomy Digital, Tokyo, Japan).

2.5. In vitro hematopoietic progenitor cell assay

ESC-derived hematopoietic cells were harvested by repeated pipetting of trypsinized OP9 cell-containing cocultures. A colony forming unit (CFU)-Mk assay was performed by using MegaCult®-C Medium and double-chamber slides (STEMCELL Technologies). Cells were fixed onto glass microscope slides with Cytospin 4 (Thermo Shandon, Pittsburgh, PA), and then stained with Diff-Quick (Sysmex, Kobe, Japan) or acetylcholinesterase staining solution (0.1 M phosphate buffer, pH 6.0, 0.05% acetylthiocholine iodide, 0.1 M sodium citrate, 30 mM copper sulfate, and 5 mM potassium ferricyanide).

2.6. Mouse care and platelet preparation

Adult C57BL/6 mice were purchased from Nihon SLC (Hamamatsu, Japan). All mice were maintained under a 12-h light/dark cycle in a pathogen-free animal facility. Experimental procedures involving mice were pre-approved by the Ethics Committee of the Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan).

Platelets were prepared as previously described [21]. Briefly, blood was taken from 12 to 16- week-old male C57BL/6 mice and suspended in a solution of 111 mM dextrose, 71 mM citric acid, and 85 mM sodium citrate containing 1 μM prostaglandin E_1 (PGE_1; Calbiochem, San Diego, CA) and apyrase (1 U/ml; Sigma). White and red blood cells were pelleted by centrifugation at 150 × *g* for 10 min at 30 °C, and the supernatant was collected and centrifuged at 900 × *g* for 10 min at 30 °C to collect platelets. For i*Gata2*-ESC experiments, PGE_1 was added one day before harvesting platelets. Pelleted platelets were resuspended in Tyrode-Hepes buffer (5 mM glucose, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 138 mM $NaCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes-HCl, pH 7.4), fixed in 2% paraformaldehyde in Tyrode-Hepes buffer for 60 min at room temperature, and stained with FITC-conjugated anti-CD41 antibody together with PE-conjugated mouse anti-

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