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Generation of human induced pluripotent stem cells from a Bombay individual: Moving towards "universal-donor" red blood cells

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ABSTRACT

Bombay phenotype is one of the rare phenotypes in the ABO blood group system that fails to express ABH antigens on red blood cells. Nonsense or missense mutations in fucosyltransfrase1 (FUT1) and fucosyltransfrase2 (FUT2) genes are known to create this phenotype. This blood group is compatible with all other blood groups as a donor, as it does not express the H antigen on the red blood cells. In this study, we describe the establishment of human induced pluripotent stem cells (iPSCs) from the dermal fibroblasts of a Bombay blood-type individual by the ectopic expression of established transcription factors Klf4, Oct4, Sox2, and c-Myc. Sequence analyses of fibroblasts and iPSCs revealed a nonsense mutation 826C to T (276 Gln to Ter) in the FUT1 gene and a missense mutation 739G to A (247 Gly to Ser) in the FUT2 gene in the Bombay phenotype under study. The established iPSCs resemble human embryonic stem cells in morphology, passaging, surface and pluripotency markers, normal karyotype, gene expression, DNA methylation of critical pluripotency genes, and *in-vitro* differentiation. The directed differentiation of the iPSCs into hematopoietic lineage cells displayed increased expression of the hematopoietic lineage markers such as CD34, CD133, RUNX1, KDR, α-globulin, and γ-globulin. Such specific stem cells provide an unprecedented opportunity to produce a universal blood group donor, *in-vitro*, thus enabling cellular replacement therapies, once the safety issue is resolved.

Introduction

Blood supply shortages could be potentially life-threatening in patients with massive blood loss. Despite approximately 81 million units of whole blood and 20 million liters of plasma being donated annually [1], shortages are still prevalent. Blood supply constraints are vital concerns particularly in developing countries. While 61% of the global blood supply has been donated by developed countries between 2001 and 2002; only 39% was donated in developing countries that support 82% of the world's population [1]. Unfortunately, it is also well recognized that there is no safety guaranteed on the donor blood products, in developed countries [2].

To overcome this concern, several sources of progenitors to generate large-scale transfusable red blood cells (RBCs) including cord blood, bone marrow, and peripheral blood [3–5] have been investigated. Recently, Lu et al. [25] successfully generated oxygen-carrying erythroid cells from hESCs highly suited to scale-up, thus promoting hESCs as a potential donorless RBC transfusion source. Regardless of the many remaining biological challenges, it appears likely that human embryonic stem cells (hESCs) and related technologies will surpass cord blood and other donated material as a source of starting material, eliminating supply constraints [2] on many counts.

Alternatively, generating human induced pluripotent stem cells (hiPSCs) by reprogramming the somatic cells into an embryonic-like state would enable RBC production. In 2006, Takahashi and Yamanaka successfully reprogrammed adult mouse fibroblasts into iPSCs through the ectopic expression of selected transcription factor groups *viz.*: Oct4, Sox2, Klf4, and c-Myc [6]. Later, the generation of human iPSCs [7,8] would provide an invaluable contribution to the

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field of regenerative medicine. Several studies demonstrated that iPSCs were certainly very similar to embryonic stem cells (ESCs) when investigated across a rigorous set of assays [9–11].

The hiPSCs generated from the somatic cells of Bombay blood-type individuals would allow for ABO and RhD production of compatible and pathogen-free "universal-donor" RBCs. The Bombay phenotype is characterized by the total absence of ABH antigens of the ABO blood group system, both on red blood cells and in secretions because of the lack of the *H* gene (*FUT1*)- and *Secretor* gene (*FUT2*)-encoded α (1,2) fucosyltransferase activities. Para-Bombay is characterized by the absence or very weak expression of the H antigen on RBCs, and by the presence of ABH substances in secretions due to the absence of the *H* gene (*FUT2*)-encoded α (1,2) fucosyltransferase activity and the presence of *Secretor* gene (*FUT2*)-encoded 1,2 fucosyltransferase activity [12–14]. The *FUT1* and *FUT2* genes on chromosome 19, share approximately 70% DNA sequence similarity. A wide variety of point mutations and deletions inactivate these two genes in the Bombay and para-Bombay individuals [12,15–18].

Due to the lack of ABH antigens, a Bombay blood-type person is a universal blood donor and can donate blood to types A, B, AB, and O Bombay persons; however, they can receive blood safely only from other Bombay donors. Therefore, the Bombay blood type is just a step closer to becoming a universal donor comparable with the O blood type.

Further, the Bombay phenotype and para-Bombay phenotype are relatively rare. In India, where H deficiency was first discovered, the frequency of both phenotypes combined is 1 in 10,000, though slightly more common in Taiwan, affecting 1 in 8000 people [19]. Given that this is such a rare blood type, it is unlikely that any blood bank would have blood available for a Bombay blood-type individual requiring an urgent blood transfusion.

In this study, we present hiPSCs generated from the dermal fibroblasts (HDF) of a Bombay blood-type individual by ectopic expression of the defined transcription factors Oct4, Sox2, Klf4, and c-Myc. The established iPSCs displayed many similarities to the hESCs showing an increased expression of hematopoietic lineage markers, during differentiation into hematopoietic lineage cells.

Materials and methods

Determination of Bombay genotyping. Genomic DNA was extracted from the dermal fibroblast of a Bombay phenotype using a QIAamp DNA mini kit (QIAGEN). The *FUT1* and *FUT2* gene sequences were determined using the PCR product. The primer sequences were: F: 5′acctttaacttcctctttccc3′ and R: 5′tggatacgggca cccatttg3′ for the FUT1 gene and F: 5′aagtagaagcacacacacac3′ and R: 5′agatttctgttacttgcagcc3′ for the *FUT2* gene. Sequencing was performed with a 3130 Genetic Analyzer (ABI) sequencer.

Dermal fibroblast culture of Bombay individual. Under the human research subject and stem cell protocols approved by the Institutional Review.

Board of Royan Institute, a healthy 32-year-old Iranian Bombay female was recruited to donate skin biopsies. Written informed consent was obtained from the donor participant as well as permission to use the sample in reprogramming studies and hiPSC production. The Bombay phenotype was detected based on serological analysis (data not shown). Dermal fibroblasts were isolated and cultured as previously described [20].

Generation of iPSCs from Bombay fibroblasts. Bombay-iPSCs (B-iPSCs) were established by transduction of viral vectors containing Oct4, Sox2, c-Myc and Klf4, as described earlier [20].

The transduced cells were passaged on serum and feeder layer free-culture medium (FFM) or purely hESC medium supplemented with 100 ng/ml basic fibroblast growth factor (bFGF). The hESC medium contained DMEM/F12 medium (Gibco, 21331-020) supplemented with 20% knock-out serum replacement (KOSR, Gibco, 10828-028), 2 mM L-glutamine (Gibco, 25030-024), 0.1 mM β-mercaptoethanol (Sigma; M7522), 1% nonessential amino acids (Gibco, 11140-035), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, 15070-063), and insulin–transferrin–selenite (ITS, Gibco, 41400-045). About 2 weeks later, a few colonies showing hESC morphology (Figs. 2A and B) were identified. Three lines were selected from these for initial characterization. For passaging, the hiPSCs were washed once with PBS (Gibco, 14287-072) and then incubated with DMEM/F12 containing 1:1 collagenase IV (0.5 mg/ml, Gibco, 17104-019), dispase (1 mg/ml, Gibco, 17105-041) at 37 °C, for 5–7 min. When colonies on the dish periphery began to dissociate from the base, the enzyme was removed and the colonies were washed with PBS. Cells were gently pipetted out.

Karyotype and gene expression analyses. Karyotype analysis was performed according to Mollamohammadi et al. [21]. Using the Nucleospin RNAII kit (MN) the total RNA was isolated and treated with DNaseI, RNase Free Kit (Fermentas) to remove genomic DNA contamination. Two micrograms of total RNA was used for the reverse transcription reaction with the RevertAid First Strand cDNA synthesis kit (Fermentas) and oligo dT primers, following manufacturer's instructions. Quantitative PCR reactions were set up in three biological replications with the Power SYBR Green Master Mix (Applied Biosystems) and analyzed with the 7500 real-time PCR system (Applied Biosystems). Expression values were normalized to the average expression of the housekeeping gene GAPDH. The primer sequences are presented in Supplementary Table 1.

Alkaline phosphatase and immunofluorescence staining. The hiPSCs were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min, and blocked in 10% goat serum in PBS for 1 h. Incubation of cells with primary antibody was done for 1 h at 37 °C, then washed and incubated with FITC-conjugated secondary antibodies, anti-mouse IgM (1:100, Sigma, F9259), anti-rat IgM (1:200, eBioscience, 11-0990), and anti-mouse IgG (1:200, Sigma, F9006) as appropriate, for 1 h at 37 °C.

The following primary antibodies, anti TRA-1-60 (1:100, Chemicon MAB4360), TRA-1-81 (1:100, Chemicon MAB4381), Oct4 (1:100, Santa Cruz Biotechnology, SC-5279), and SSEA-4 (1:100, Chemicon, MAB4304) for undifferentiated determination; anti-neuron specific tubulin-III (1:250, Sigma, T5293), and microtubule-associated protein (MAP2; 1:200, Sigma; M1406) for neural cell detection; anti-α-actinin (1:800, Sigma, A7811) and Troponin I (1:250, Chemicon, MAB169) for mesodermal differentiation, and anti α-feto protein (AFP; 1:200, DAKO) for endodermal differentiation were used. Nuclei were counterstained with DAPI (Sigma, D8417) or propidium iodide (PI, Sigma, P4170) and the cells were analyzed under a fluorescent microscope (Olympus, Japan).

Alkaline phosphatase staining was conducted based on the manufacturer's recommendations (Sigma, 86R).

Bisulfite sequencing. The human Oct4 and Nanog gene promoter regions were amplified with PCR, as described earlier [20]. PCR products were subcloned into the InsTAclone PCR Cloning kit (Fermentas). Ten clones of each sample were verified by sequencing with the M13 universal primer and analyzed using the BIQ Analyzer software [22].

In-vitro differentiation. To demonstrate if the established clones were pluripotent an assay was conducted to ascertain their ability to differentiate into lineages representative of the three embryonic germ layers, by embryoid body (EB) formation and spontaneous differentiation [21]. The hiPSC1 were differentiated into a neural lineage in a defined adherent culture using retinoic acid, noggin and bFGF [23]. Further, the hiPSC1 were induced into endodermal lineages by Activin A and hepatocyte growth factor (HGF) [24].

Hematopoietic lineage differentiation was performed following the Lu et al., protocol [25].

Results and discussion

Verification of Bombay phenotype

The *Fut1* and *Fut2* alleles of the Bombay blood-type individual and B-hiPSCs were sequenced to identify the differences in the sequences. As shown in Fig. 1, a nonsense mutation 826C → T(276Gln → Ter) in *Fut1* and a missense mutation 739G → A (247Gly → Ser) in the *Fut2* genes were detected in a Bombay individual, which confirmed the Bombay genotype based on the earlier reports [12,14]. All the established B-hiPSCs maintained the *Fut1* and *Fut2*-related mutations.

Generation of B-hiPSCs in serum and feeder-free conditions

Reprogramming the human fibroblasts was performed following the earlier-described method under serum-free and feeder-free conditions [20]. After approximately 14 days following infection, some colonies that emerged in the infected fibroblast cultures were observed to adopt a tightly packed morphology with refractive edges and three-dimensional growth, and were highly reminiscent of the hESC colonies (Fig. 2A). These compact colony cells expressed high nucleus to cytoplasm ratios besides prominent nucleoli (Figs. 2B and C). Twenty-eight days after transduction, 14 hESC-like colonies were retrieved and manually expanded by mechanical disaggregation, without enzymes. This stage was termed passage one. The medium was changed every other day, until finally eight propagating and proliferating B-hiPSCs were obtained. Colonies B-hiPSC1, B-hiPSC11, and B-hiPSC12 were selected for further analyses.

Characterization of established B-hiPSCs

All three B-hiPSC lines revealed compact colonies with high nucleus to cytoplasm ratios. Also, the clones exhibited strong ALP activity and expressed several hESC-associated antigens (Oct4, SSEA-4, Tra-1-60, and Tra-1-81) (Fig. 2). Additionally, all putative B-hiPSC lines also maintained a normal karyotype (Fig. 2). The colonies were permitted to passage for at least 6 months by weekly passaging with a split ratio of 1:3 to 1:6. Colonies were cultured in hESC medium (with KOSR and bFGF) without feeders and were propagated using enzymatic protocols.

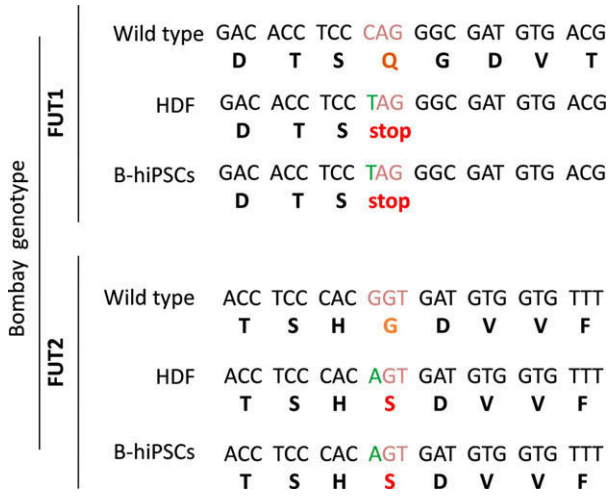

Fig. 1. Sequence differences between wild-type alleles and H-deficient alleles for the study individual. A nonsense mutation 826C → T (276Gln → Ter) in the *FUT1* gene and a missense mutation 739G → A (247 Gly → Ser) in the *FUT2* gene produced the Bombay phenotype. Both the nucleotide sequence and predicted amino acid sequence are shown for each sequence difference.

The qRT-PCR analysis, using primers specific for retroviral transcripts, indicated that the B-hiPSC clones suppressed the expression of the retroviral transgenes excepting Klf4 (Fig. 3A–D). Although there was no expression of the Oct4 and Nanog stem cell marker genes in the HDFs, their expression levels in the B-hiPSCs were comparable to those in hESCs (Fig. 3E), suggesting that the B-hiPSCs are efficiently reprogrammed and are not dependent on continuous transgene expression for self-renewal.

Bisulfite genomic sequencing analyses were also performed to investigate the methylation status of the cytosine guanine dinucleotides (CpG) in the promoter regions of the two prominent pluripotent-associated genes, Oct4 and Nanog. The results revealed that the promoter region of these two genes was highly unmethylated, whereas the CpG dinucleotides of the regions were highly methylated in the parental HDFs (Fig. 3F). These findings indicated that the above mentioned promoter regions were active in B-hiPSCs, and somatic cell reprogramming was accompanied by demethylation of critical pluripotency gene promoters [26,27].

Embryoid body-mediated differentiation of B-hiPSCs

To demonstrate the pluripotency of established B-hiPSC clones, we examined their differentiation into lineages representative of the three embryonic germ layers by EB formation. After 12 days of growth in suspension for in hESC medium in the absence of bFGF, the EBs were replated under adherent conditions, for 8 days. The expression analyses of the lineage specific markers for ectoderm (Sox1, Pax6, and Nestin), endoderm (α-FP, FoxA2, Sox17, Alb, and Pdx1), and mesoderm (MEF2C, Brachyury, TNNT2, and PPAR) revealed the absence of these markers in the undifferentiated hESCs and hiPSCs. They were strongly induced only after differentiation to EBs (Fig. 4A). These results demonstrated that B-hiPSC EBs shared an ability similar to the hESC to up-regulate different lineage markers. Only pluripotent cells are known to possess the ability to up-regulate all three embryonic germ layer markers, thus providing strong evidence for the pluripotency of B-hiPSC clones.

Some EBs manifested spontaneous beating which demonstrated the formation of contractile cardiomyocytes (Supplementary video). We also examined if lineage-directed differentiation of B-hiPSCs could be induced by the methods reported for hESCs. On inducting neural differentiation in adherent conditions by retinoic acid (RA), bFGF, and noggin, the rosette and neural-tube-like structure formation and neural generation dramatically increased (Fig. 4B). Immunostaining revealed that the differentiated cells strongly expressed two neural markers, β-tubulin-III (Fig. 4C) and MAP2 (Fig. 4D). The beating cells expressed α-actinin (Fig. 4E) and Troponin I (Fig. 4F). Further, treatment of the cells by Activin A strongly induced an endodermal marker, Sox17 (Fig. 4G).

Directed differentiation of B-hiPSCs to hematopoietic stem cells

The differentiation of B-hiPSCs to hematopoietic stem cells (HSCs) was also examined using BMP4, and VEGF induction of B-hiPSCs in forming EBs and later, to HSCs. RT-PCR analyses showed that the hematopoietic lineage markers like CD34, Runx1, CD133, KDR and even genes related to final stage red blood cells such as α-globulin and γ-globulin were highly expressed in these cells, indicating a strong commitment toward this lineage (Fig. 4H).

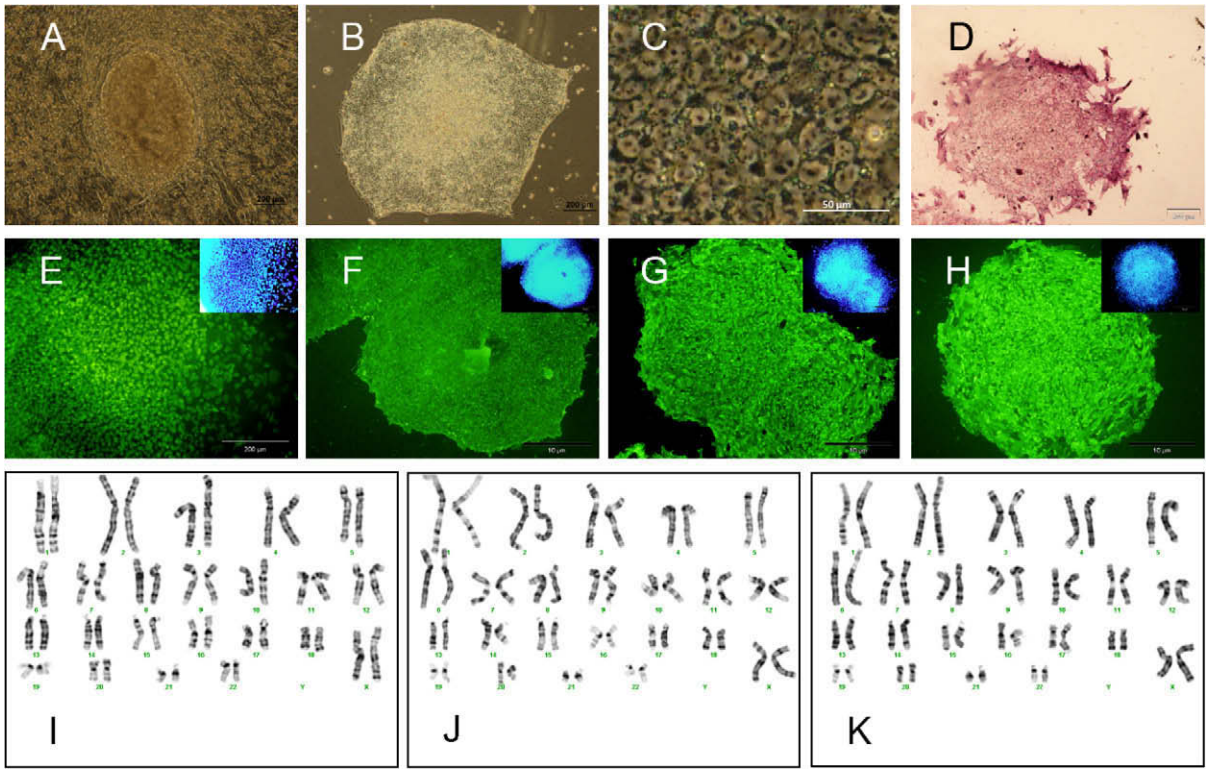

Fig. 2. Characteristics of established B-hiPSCs. (A) Phase contrast microscopy of established B-hiPSC colony at day 20 after infection under feeder- and serum-free conditions. (B) Established colony on feeder-free matrix, (C) Higher magnification of B-hiPSCs, (D) Alkaline phosphatase staining, (E) Expression of pluripotency marker Oct4, (F) SSEA-4, (G) Tra1-60, (H) Tra1-81. (I), (J), and (K) represent normal karyotypes for B-hiPSC1, B-hiPSC11, and B-hiPSC12, respectively.

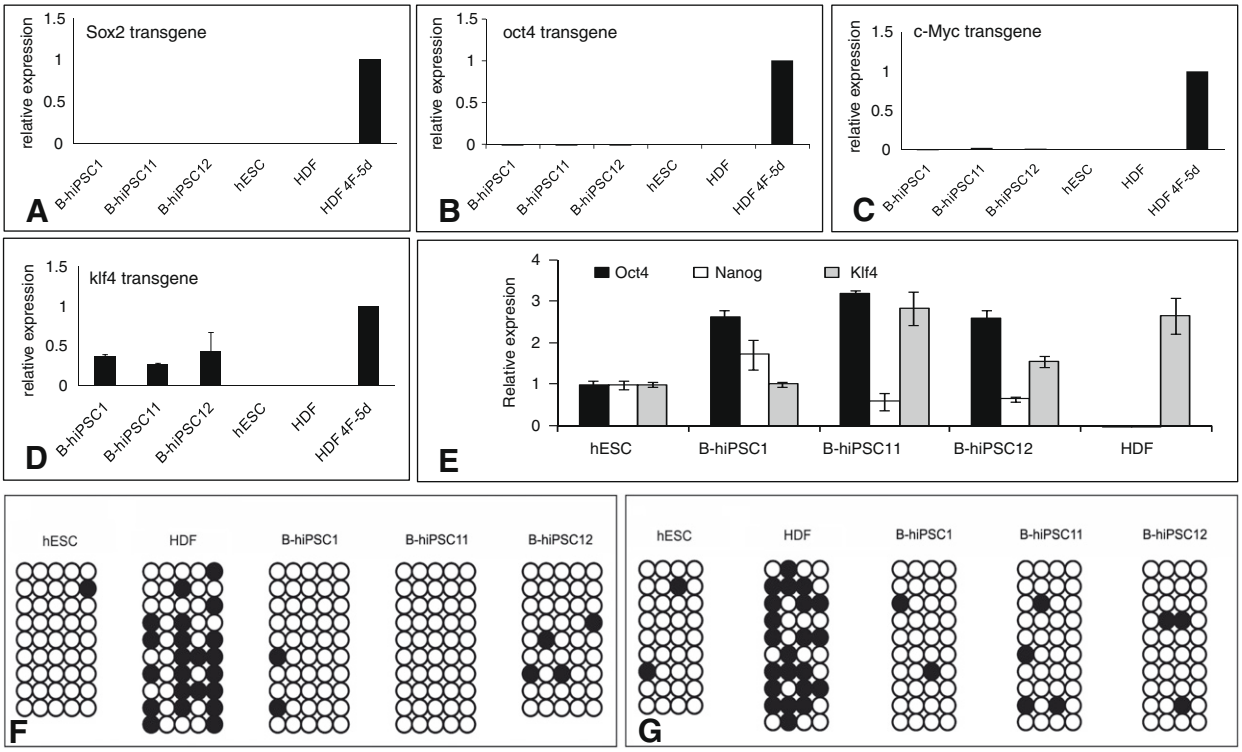

Fig. 3. Expression of hESC-marker genes in B-hiPSCs. (A–D) Expression levels of transgenes Oct4 (A), Sox2 (B), Klf4 (C), and c-Myc (D) assessed by qRT-PCR. The values from the infected HDF fibroblasts (isolated 5 days post-infection, HDF 5 days) were set to one and transcript levels were normalized to GAPDH. Uninfected HDF were used as negative controls. The error bar indicates standard deviation (SD). (E) The expression levels of endogenous factors were quantified by qRT-PCR and plotted relative to GAPDH expression. Bisulfite genomic sequencing of the Oct4 (F) and Nanog promoter regions. (G) Open and closed circles indicate unmethylated and methylated CpGs.

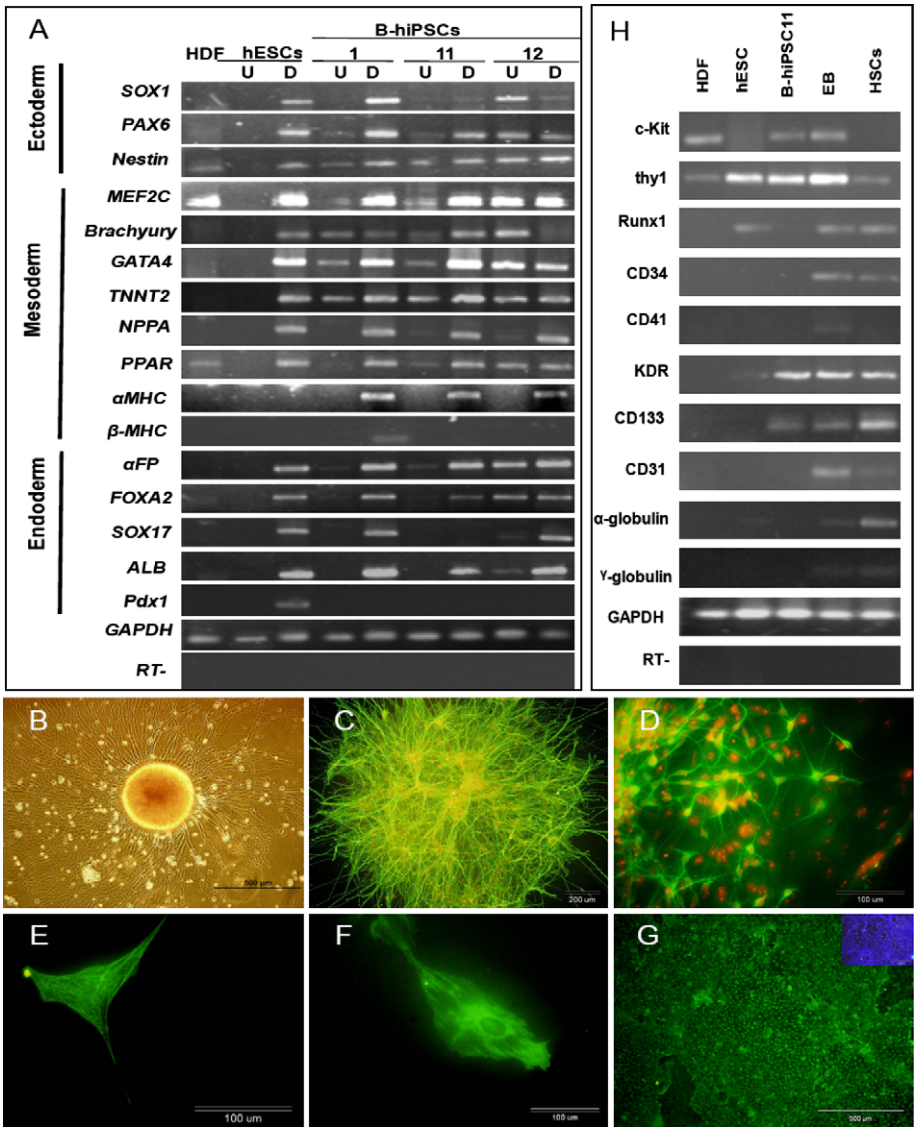

Fig. 4. Differentiation of B-hiPSCs. (A) RT-PCR analyses of various differentiation markers for the three germ layers by EB-mediated differentiation "D" in comparison with undifferentiated state "U". Directed differentiation of B-hiPSC1 into neural cells in the presence of Noggin, RA, and bFGF (B–D). Phase contrast photomicrographs of neural cells on laminin/poly-L-ornithine for 14 days. (B) Immunocytofluorescence of the cells shown in (C) with Tubulin III (green) and (D) MAP2 (green) antibodies. Nuclei were stained with propidium iodide (red). Beating cardiomyocytes by EB-mediated differentiation expressed α-actinin (E) and Troponin I (F). AFP positive cells (green) after treatment of B-hiPC11 with Activin A and HGF (G). DAPI (blue)-stained nuclei. (E) Directed differentiation of B-hiPSC11 to hematopoietic lineage and expression of these lineage-related genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Concluding remarks

We produced B-hiPSCs from the HDF of a Bombay phenotype individual using Yamanaka factors [7]. The established B-hiPSCs displayed great similarity to hESCs in view of the expression of pluripotency surface markers and pluripotency genes, demethylation of the promoter region of the pluripotency genes such as Oct4 and Nanog, and the spontaneous and direct differentiation potency. The established cells suppressed the retroviruses, thus representing their independence from the transgenes to maintain pluripotency. Thus spontaneous and directed differentiation resulted in good commitment toward the mesoderm, heart and hematopoietic lineages appropriate for RBC production. The hiPSC generation from the Bombay phenotype can lead to the production of universal donor RBCs for all recipients and provide unique resources for the study of hemangiogenesis. The production of virus-free hiPSCs using the newly developed methods such as Cre-excisable viruses [28], oriP/EBNA1-based episomal expression system [29], with small molecules [30–34] and protein transduction [35] could result in safer hiPSCs for application in regenerative medicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.058.

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