

BRIEF COMMUNICATION

Establishment and characterization of immortalized erythroid progenitor cell lines derived from a common cell source

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Immortalized erythroid progenitor cell lines, which exhibit potential for enucleated red blood cell (RBC) production, are expected to serve as an in vitro source of RBCs. These erythroid progenitor cell lines have previously been established from a variety of sources; however, large numbers of cell lines have not been established, characterized, and compared from a common cell source. In the present study, 37 cell lines were established from human bone marrow cells from a single donor. The time required for the establishment of each cell line varied greatly from 46 to 246 days. Of these lines, five were selected and their characteristics were analyzed. The cell lines established at the earliest time point showed better results in terms of both karyotype and differentiation potential than those established the latest. Moreover, obvious differences were noted even when cell lines were established at the earliest time point from the same source. These results suggest that it is important to select the best cell lines from ones established at the earliest time point for generating cell lines with low genomic abnormality and high differentiation ability. We have successfully generated an adult type of cell line with 50% cells carrying a normal karyotype and with 25% enucleation efficiency. These findings could be valuable in the development of an optimal method for establishing cell lines. © 2018 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

Recently, attempts have been made to produce large quantities of red blood cells (RBCs) in vitro using cell culture techniques [1–5]. Although hematopoietic stem/progenitor cells or pluripotent stem cells have been considered primary candidates for in vitro RBC production because they possess excellent characteristics for this technology [6–11], we have successfully established immortalized erythroid progenitor cell lines (imERYPCs) that can differentiate into enucleated

RBCs [12]. The main advantage of these cell lines is that they can proliferate indefinitely, can produce enucleated RBCs rapidly, and can be handled with ease. Trakarnsanga et al. successfully established a cell line that can produce up to 30% enucleated RBCs (reticulocytes) [13], suggesting that imERYPCs could be an important tool for in vitro RBC production. Moreover, imERYPCs are considered to be useful research tools with which to study the regulation of globin gene expression [14–18]. The imERYPCs have previously been established from various sources [12,13,19,20]; however, there have been no reports regarding the establishment of large numbers of cell lines from a common cell source. Therefore, it is unclear whether differences exist among these cells in terms of their properties and how large these differences might be.

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We attempted to derive large numbers of cell lines from a common source with the aim of addressing these issues. These findings can shed light on the optimal method for establishing imERYPCs.

Methods

The methods used in this study are described in the supplementary materials (online only, available at www.exphem.org).

Results and discussion

Establishment of imERYPCs derived from a common cell source

To establish large quantities of imERYPCs from a common cell source, HPV E6/E7-transduced cells were divided into groups of 20 cells in a 96-well plate during the early culture phase (16th day after the start of culture) (Figure 1A). Subsequently, 43.2% (166/384) of all wells reached 70% confluence and stable proliferation was observed by the 60th day in most wells (Figures 1B and 1C). However, some wells took longer to reach 70% confluence (up to 120 days for induction). There were large variations in the time required for cells to reach the cryopreservation stage (three confluent 24-well plate wells); this duration ranged from 46 to 246 days (Supplementary Table E1, online only, available at www.exphem.org). This raises the possibility that, when using traditional, nondivided establishment methods, high-proliferative clones dominate and low-proliferative clones are eliminated. Therefore, it is important to oligo-clone cells in the early phase to reduce their distinctive features, which have previously been difficult to analyze. We successfully established 37 lines from a common cell source and assigned these cell lines a BMDEP-1 establishment number.

Karyotype analysis

To evaluate chromosomal abnormalities of the established cell lines, a total of five cell lines were subjected to karyotype analysis: BMDEP-1-01 (BM-1-01) and BMDEP-03 (BM-1-03), which were established at the earliest time point; BMDEP-1-35 (BM-1-35) and BMDEP-37 (BM-1-37), which were established the latest; and BMDEP-1-22 (BM-1-22), which was established in an intermediate period. Several chromosomal abnormalities were observed in all cell lines, consistent with a previous report [21], whereas the karyotype abnormality tended to be more severe as the cell lines were established later (Figure 1D and Supplementary Figure E1, online only, available at www.exphem.org). However, the common chromosomal abnormalities were not found in these cell lines. Among them, BM-1-01 cells showed relatively lesser abnormalities. G-banding revealed that the modal chromosome number

was 46 or 47 and 86% (43/50) and 50% of the analyzed cells (10/20) had normal karyotypes (46, XY). These data indicate that E6/E7 gene expression is only sufficient to immortalize cells without drastic chromosomal changes. Nevertheless, the time required for the establishment of each cell line varied greatly. It was speculated that the expression level or integration site of the E6/E7 gene and some genomic deficiencies such as point mutations or short deletions were all synergistically involved in immortalizing the cells, rather than frequent chromosomal changes.

Analyses of differentiation potentials of imERYPCs derived from a common cell source

We induced the differentiation of five cell lines. The growth of BM-1-01, BM-1-03, and BM-1-22 cells remained healthy throughout induction of differentiation, whereas BM-1-35 and BM-1-37 cells showed gradual cell death approximately 4 days after the induction of differentiation (dd4). The resultant BM-1-35 and BM-1-37 cells at dd10 showed low viabilities (Supplementary Figure E2, online only, available at www.exphem.org) and hemoglobin synthesis (data not shown). Therefore, BM-1-01, BM-1-03, and BM-1-22 cells were used for further analyses.

Flow cytometric analysis revealed that BM-1-01, BM-1-03, and BM-1-22 cells exhibited highly similar expression patterns of surface markers (Supplementary Figure E3, online only, available at www.exphem.org). These expression patterns demonstrated that the established BM-1-01, BM-1-03, and BM-1-22 cells underwent a similar course of differentiation, which was similar to the differentiation of normal erythroid cells.

Next, enucleated RBC production efficiency was analyzed. Although immature erythroblasts represented a large proportion of each of these cell lines before differentiation, condensation of nuclei and morphological changes occurred with the induction of differentiation; by day 10 after induction, the majority of cells had differentiated into orthochromatic erythroblasts and some enucleated RBCs were observed (Figure 2A and Supplementary Figure E4, online only, available at www.exphem.org). The enucleation efficiency was then calculated as 25.2% of its maximum with BM-1-01 cells, 6.7% with BM-1-03 cells, and 17.3% with BM-1-22 cells (Figure 2B). Furthermore, as the dd10 culture product from BM-1-22 cells was processed through the leucocyte reduction filter, nucleated cells, expelled nuclei, and dead cells were removed, demonstrating the feasibility of obtaining highly pure enucleated RBCs (Figure 2C).

The results of imERYPC differentiation implied that there are differences with respect to enucleation efficiency even when cell lines are established from the same source. Further analysis is needed to observe the

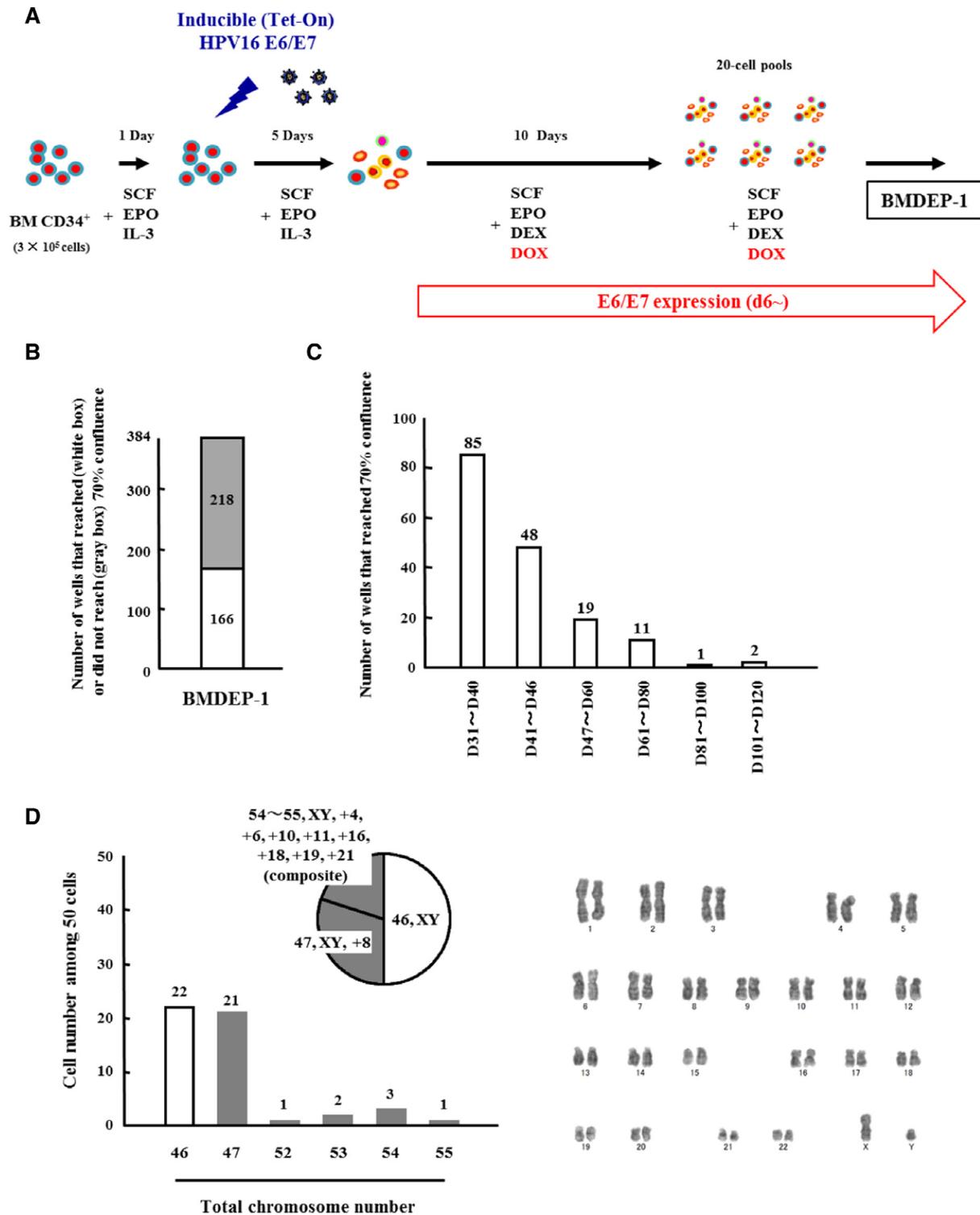


Figure 1. Establishment of erythroid progenitor cell lines derived from a common cell source. **(A)** Outline of the establishment of erythroid progenitor cells. After overnight culture of bone marrow CD34⁺ cells derived from a single donor, the human papillomavirus (HPV) E6/E7 gene was transduced using a tetracycline-inducible lentivirus vector. On the sixth day, doxycycline (DOX) was added to the culture medium to induce E6/E7 gene expression. On the 16th day, cells were subdivided into smaller units and transferred to 96-well plates at 20 cells per well for indefinite culture. Cell lines established after DOX treatment were termed BMDEP-1. **(B)** From four 96-well plates, the number of proliferative cultures that could be serially passaged and the number of nonproliferative cultures were determined. **(C)** Number of wells that could be serially passaged at each time point. **(D)** Karyotype analysis of BM-1-01 cells. The distribution of total chromosome number (bar graph) and structural descriptions (pie chart) are shown (left). The normal G-banding pattern of BM-1-01 cells is also shown (right).

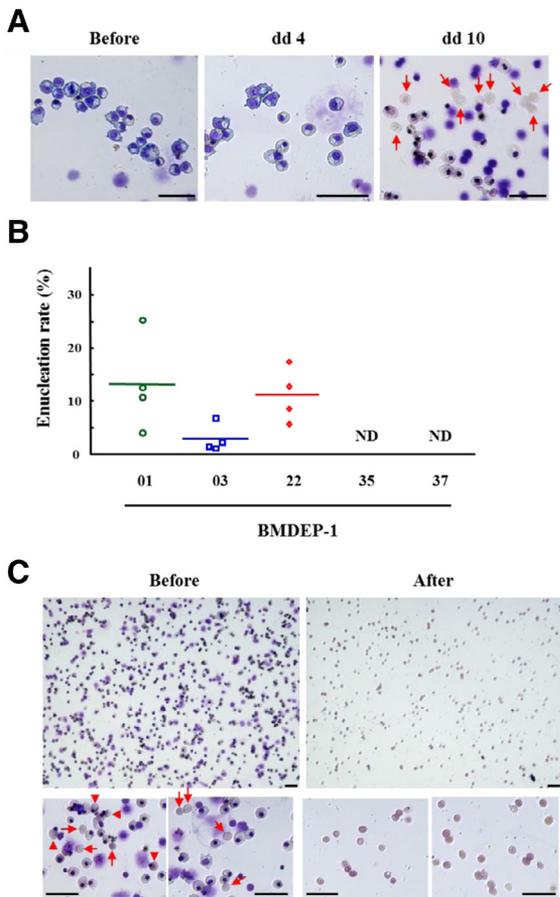


Figure 2. Analysis of enucleation efficiency using BMDEP-1 cell lines. (A) Typical morphological changes in BMDEP-1-01 cells before differentiation (before), at dd4, and at dd10. Arrows indicate enucleated RBCs. Scale bar indicates 50 μ m. (B) Enucleation efficiency at dd10. ND=not determined because of low cell viability. (C) Purification of enucleated RBCs. Cytopsin images before and after application of the leukocyte-reduction filter are shown. Example images at low magnification (top) and high magnification (bottom) are shown. Arrows indicate enucleated RBCs. Arrowheads indicate cells in the process of enucleation. Scale bar indicates 50 μ m.

maximum differences in enucleation efficiency for each cell line established from a common source; however, to establish good lines with a high differentiation ability, it might be important to subdivide them as early as possible and to select the best cells. The cell lines established the latest should be omitted to obtain better cell lines.

Hemoglobin analyses

Finally, hemoglobin analyses were conducted (Figure 3). We visually observed that the color of pellets from BM-1-01, BM-1-03, and BM-1-22 cells before differentiation was light or pale red, with a mixture of red and white (Figure 3A, data not shown). On the 10th day of differentiation (dd10), the pellets uniformly exhibited a

deep red color (Figure 3A, data not shown). To analyze these results in detail, we measured heme content using a spectrophotometer. The extracts from BM-1-01 and BM-1-03 cells prior to differentiation showed lower absorbance values at approximately 414 nm, which was thought to be attributed to heme (Figure 3B). In contrast, BM-1-01 and BM-1-03 dd10 samples showed a large increase in peak values, which were on an average of 13.1-fold and 15.4-fold greater, respectively, compared with those for samples tested before differentiation (Figure 3B). Unfortunately, we could not measure the heme content of BM-1-22 cells because more than half of the hemoglobin apparently rested in the cell pellet using the same method. However, the pellet of BM-1-22 cells exhibited the same deep red color as that seen for BM-1-01 or BM-1-03 cells. This revealed a large increase in hemoglobin quantity after differentiation in all tested cell lines.

For analyzing the hemoglobin β -chain of BM-1-01, BM-1-03, and BM-1-22 cells, a high-resolution multiple reaction monitoring assay on a quadrupole time-of-flight mass spectrometer was performed. To the best of our knowledge, little has been reported on human cell lines predominantly expressing β -globin, except for the HUDEP-2 [12] and BEL-A cell [13] lines recently established by our group and our collaborators, respectively. Although the imERYPCs that can produce adult hemoglobin are useful research tools with which to study the regulation of globin gene expression [14–18], their abnormal karyotype with multiple chromosome numbers often makes these experiments challenging. Therefore, establishing additional adult types of cell lines, possibly with a near-normal karyotype, would be highly valuable in the field.

In BM-1-01, BM-1-03, and BM-1-22 cells, β -globin predominated with an average proportion of 96.2%, 95.3%, and 97.0%, respectively, whereas the proportion of γ -globin (total of γ 1 and γ 2) was 2.5%, 2.6%, and 1.0%, respectively (Figure 3C). Therefore, it was revealed that the BMDEP lines established at this time contained a large proportion of adult-type globin. Karyotype abnormalities in BM-1-01 cells were notably milder than those of HUDEP-2 cells (Figure 1 and Supplementary Figure E5, online only, available at www.exphem.org), which have been widely used in recent hemoglobin studies. This indicates that BM-1-01 cells are more valuable for hemoglobin studies and are also preferable for the preparation of blood products.

In summary, imERYPCs show some differences in their karyotype and enucleation efficiency even when cell lines are established from the same source. To obtain good cell lines, it is recommended to select the best cell lines from ones established at the earliest time point. Here, we have successfully generated an adult type of cell line with 50% of cells carrying a normal

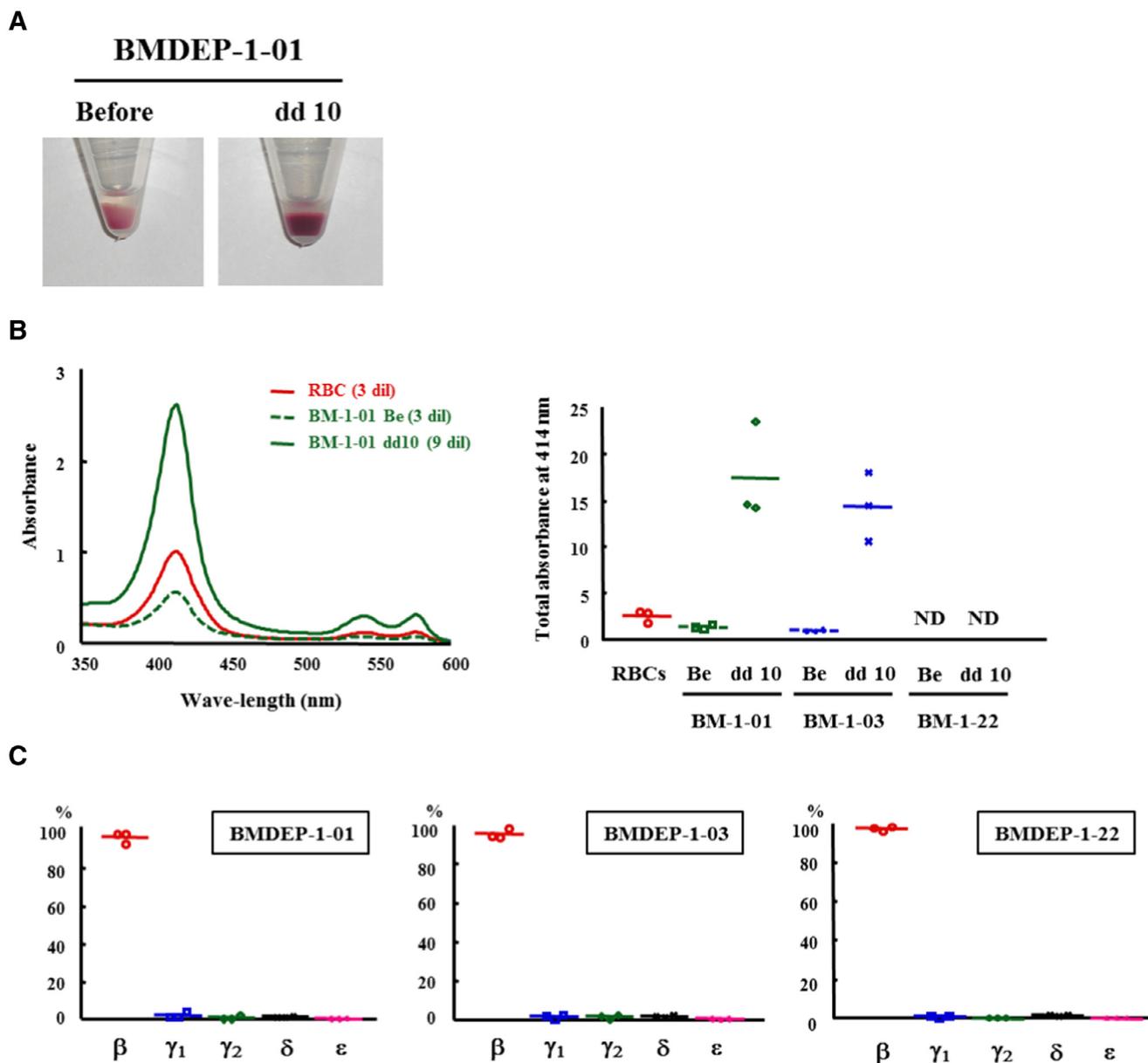


Figure 3. Hemoglobin analysis of BMDEP-1 cell lines. (A) Images of cell pellets before differentiation and at dd10. (B) Typical patterns of the absorbance spectra for cell extracts using an ultraviolet and visible spectrophotometer are shown (left). Peripheral blood and cell extracts before the induction of differentiation were diluted threefold, whereas the cell extract after differentiation was diluted ninefold for measurement. Be=before induction of differentiation. The total absorbance values for each sample at 414 nm are shown on the right. ND=not determined because of low recovery of hemoglobin from the cells. (C) Relative proportion of β -chain protein of each globin based on a high-resolution, multiple reaction monitoring assay.

karyotype and with 25% enucleation efficiency. These results could be valuable in the development of an optimal method for establishing cell lines with low abnormality and high differentiation ability.

Conflict of interest disclosure

The authors declare no competing financial interests.

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