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## Establishment of an erythroid cell line from primary CD36<sup>+</sup> erythroid progenitor cells

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### Abstract

**Objective.**—Most continuous cell lines with erythroid characteristics are derived from patients with myelogenous leukemia or erythroleukemia. Among them, a few cell lines have been reported to be positive for CD36. We tried to establish a continuous erythroid cell line from the primary CD36<sup>+</sup> erythroid progenitor cells (EPCs) by the lentivirus-mediated gene transduction system.

**Materials and Methods.**—A lentiviral vector carrying *SV40T*, *hTERT*, or the human papillomavirus type 16 (HPV16) *E6* and *E7* (*E6/E7*) viral oncogenes, was introduced into CD36<sup>+</sup> EPCs, singularly or combined. Transformed cells were characterized in terms of histology, phenotype, karyotype, and gene expression profile.

**Results.**—The lentiviral vector carrying *HPV16 E6/E7* genes successfully transformed CD36<sup>+</sup> EPCs, creating a continuous cell line, CD36E. Immunophenotype analysis revealed that the CD36E cells had characteristics of erythroid progenitors, among which about 27% of the cell population produced hemoglobin. Colony-forming cell assay demonstrated that the CD36E cells were capable of forming erythroid colonies. Using cytokines or chemical agents, attempts were made to induce differentiation of the CD36E cells but were ineffective, indicating the irreversible erythroid lineage commitment of the cells. The gene expression profile of the CD36E cells displayed a marked difference from that of the CD36<sup>+</sup> EPCs.

**Conclusions.**—The continuous CD36E cell line is an erythroid progenitor cell line possessing the ability to produce hemoglobin. The CD36E cell line would be an excellent tool for applied research involving erythroid lineage cells and comparative studies with primary CD36<sup>+</sup> EPCs.

Studies involving erythroid progenitor cells have been limited to primary cells or cell lines established from leukemia. Recent advances in differentiating and generating large numbers of specific lineages of hematopoietic cells from CD34<sup>+</sup> hematopoietic stem cells (HSCs) have provided valuable tools and extensive uses. Differentiating cells from CD34<sup>+</sup> HSCs toward the erythroid lineage increasingly express the CD36 transmembrane protein, a member of the scavenger receptor family, on the surface [1]. However, as erythroid

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progenitors evolve into more mature and differentiated cells, the CD36 expression decreases [2,3]. Therefore, the CD36 expression is defined as a determinant of erythroid progenitors [3–5]. In addition to erythroid progenitors, the CD36 expression is also detected on the surface of different hematopoietic cells, including platelets and megakaryocytes [5], and various other cell types, such as endothelial cells, mast cells, smooth muscle cells, and dendritic cells.

There are various cell lines that display erythroid characteristics, such as K562 [6], KU812Ep6 [7], JK-1 [8], KMOE [9], TF1 [10], HEL [11], B1647 [12], MB-02 [13], M-07 [14], and UT7/erythropoietin (EPO) [15]. These cell lines are derived from patients with leukemia, some of which produce varying amounts of hemoglobin innately or under induction.

We previously developed a culture system (a modification of Freyssinier et al. [16] and Giarratana et al. [17]) that generated a large and relatively pure population of erythroid progenitor cells (EPCs) from peripheral blood–derived CD34<sup>+</sup> HSCs [3]. In this study, we sought to generate an EPC line from primary CD36<sup>+</sup> EPCs by a lentivirus-mediated gene transduction and successfully established a CD36E cell line using the human papillomavirus type 16 (HPV16) *E6* and *E7* (*E6/E7*) genes.

## Materials and methods

### Cell culturing, cell proliferation monitoring, and cell sorting

CD34<sup>+</sup> cells were purified from granulocyte colony-stimulating factor–mobilized peripheral blood stem cells using the Isolex 300i Magnetic Cell Selection System (Baxter Healthcare Corporation, Deerfield, IL, USA) [3]. CD34<sup>+</sup> cells (10<sup>4</sup> cells/mL) were initiated in the serum-free expansion medium composed of  $\alpha$ -minimum essential medium (Mediatech, Manassas, VA, USA) with 100 ng/mL recombinant human (rhu) stem cell factor (SCF) (Stem Cell Technologies, Vancouver, British Columbia, Canada), 5 ng/mL rhu interleukin-3 (IL-3; R&D Systems, Minneapolis, MN, USA), 3 IU/mL rhuEPO (Amgen, Thousand Oaks, CA, USA), and other factors described previously. UT7/EPO-S1 and K562 cells were cultured as described previously [18]. Cell proliferation was monitored in triplicate using the Nexcelom Cellometer Auto T4 (Isogen Life Science, De Meern, Netherlands). Single cell sorting into 96-well plates was performed using the MoFlo Cytometer (Beckman Coulter, Fort Collins, CO, USA).

### Induction of CD36E cells with various cytokines and chemical agents

CD36E cells were grown in the expansion medium, but with different combinations or concentrations of cytokines (EPO, SCF, IL-3, and thrombopoietin). Further, CD36E cells were cultured with hemin, dimethyl sulfoxide (DMSO), or phorbol-12- myristate-13-acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) at the indicated concentrations.

### Generation of lentiviral vector carrying SV40T, hTERT, or HPV16 E6/E7 genes

A lentiviral vector plasmid carrying *SV40T* alone, *hTERT* alone, or *SV40T* plus *hTERT* was developed by CDI Technologies (Madison, WI, USA). To generate a lentiviral vector

plasmid with *HPV16 E6/E7* genes, a *HPV16 E6/E7*-coding region was amplified by polymerase chain reaction (PCR) from a p16HH plasmid (kind gift from John Schiller) [19] using primers modified from Akimov et al. [20], followed by insertion into BamHI and EcoRI sites of a pCDF1 plasmid, creating pCDF1-E6/E7.

Pseudotyped lentiviral vectors were created using the Lentiviral Packaging System (CDI Technologies), according to manufacturer's instruction.

CD36<sup>+</sup> EPCs (10<sup>6</sup> cells) were infected with *SV40T* alone, *hTERT* alone, *HPV16 E6/E7* alone, *SV40T* plus *hTERT* (in the same vector), *SV40T* plus *hTERT* (in separate vectors), or *HPV16 E6/E7* plus *hTERT* (in separate vectors) on 3 consecutive days. Half of the culture was subjected to puromycin selection at a concentration of 1 µg/mL for 2 weeks, as the lentiviruses had a puromycin-resistant gene.

### Histology, immunophenotyping, spectral karyotyping, and chromosome banding

Cells ( $2 \times 10^4$  to  $5 \times 10^4$ ) were stained with Wright-Giemsa using the MidasIII Automatic Slide Stainer (EMD; Gibbstown, NJ, USA) or with 1% benzidine (Sigma-Aldrich), followed by microscopy.

Approximately 10<sup>5</sup> cells were subjected to flow cytometry analysis using the Beckman Coulter Cytomics FC500 System. Fluorescein isothiocyanate-conjugated antibodies were purchased through BD Biosciences (Franklin Lakes, NJ, USA): CD2, CD3, CD19, CD33, CD36, CD41a, and CD61. R-phycoerythrin-conjugated antibodies were obtained as follows: CD34, CD49e, CD44, CD10, CD71, CD110, and CD235a (glycophorin A) from BD Biosciences; CD133 from Miltenyi Biotec (Auburn, CA, USA); CD235 phycoerythrin/Cy5 from BioLegend (San Diego, CA, USA). Unconjugated anti-globoside antibody (Metreya, Pleasant Gap, PA, USA) was used with secondary antibody, fluorescein isothiocyanate goat anti-rabbit IgG antibody (Invitrogen).

Metaphase spreads were prepared from exponentially growing CD36E cells. Spectral karyotyping was carried out by fluorescence in situ hybridization with chromosome-specific painting probes labeled with different fluorochromes. Chromosome Giemsa-banding (G-banding) was performed to display a unique banding pattern of each chromosome. These analyses were conducted by the Comparative Molecular Cytogenetics Core Facility (<http://web.ncifcrf.gov/research/cytogenetics/>).

### Colony-forming cell assay

CD36E cells ( $5 \times 10^4$ ) were subjected to colony-forming cell assay using two different methylcellulose-based media (Stem Cell Technologies) according to manufacturer's instructions: MethoCult GF<sup>+</sup> (Catalog #H4435) complete methylcellulose medium supports hematopoietic colony formation of erythroid, granulocyte, macrophage, and multipotential progenitors; MethoCult (catalog #H4230) methylcellulose medium with a cocktail used for the expansion medium (supplemented MethoCult) supports erythroid colony formation. On day 12, colony cultures were stained with Wright-Giemsa or benzidine.

## PCR amplification and Southern blotting

PCR amplification and Southern blotting were performed to examine whether the *E6/E7* genes were integrated in CD36E genomic DNA. PCR was carried out using 100 ng DNA and the same PCR primers used for the *E6/E7* gene amplification (described here), according to manufacturer's instruction, followed by detection on a 1% agarose gel.

After digestion with BamHI or EcoRI, genomic DNA (20 µg) was subjected to Southern blot analysis using a <sup>32</sup>P-labeled probe and then analyzed by autoradiography. The probe was prepared by excising a BamHI–EcoRI fragment from the pCDF1-E6/E7 plasmid and labeled with <sup>32</sup>P using the Prime-It Random Primer Labeling kit (Stratagene/Agilent Technologies, Santa Clara, CA, USA).

## Real-time reverse transcriptase (RT)-PCR

A custom PCR array targeting hematopoietic genes and a modified-cataloged cytokine array (to include *EPO*, *EPOR*, and *CSF* genes) were created by SABiosciences (Frederick, MD, USA). Total RNA extracted using the Qiagen microRNA Extraction kit was converted to complementary DNA and subjected to real-time RT-PCR following manufacturer's instructions. Data were analyzed by the  $C_t$  method using the SABiosciences Data Analysis Web Portal (<http://www.SABiosciences.com/pcrarraydataanalysis.php>), and genes with fivefold or greater differences were extracted. Fold changes represent ratios of gene expression levels between CD36E cells and parental CD36<sup>+</sup> EPCs.

## Immunoblotting

Whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–12%) and immunoblot analysis was performed using primary and then secondary antibodies. Signals were detected by chemiluminescent detection method. The following antibodies were used: hemoglobin- $\alpha$  (H-80, sc-21005), hemoglobin- $\beta$  (37-8, sc-21757), hemoglobin- $\gamma$  (51-7, sc-21756), and glyceraldehyde phosphate dehydrogenase (sc-47724) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); HRP-rabbit anti-mouse IgG antibody (61-6520) from Invitrogen.

## Results

### Immortalization of CD36<sup>+</sup> EPCs using the HPV16 E6/E7 lentiviral vector

CD34<sup>+</sup>-selected cells cultured for 8 days (typically >95% CD36<sup>+</sup> cells) were transduced by infection on 3 consecutive days with the pseudotyped lentiviral vectors. At 2 weeks post transduction, half of the culture was subjected to puromycin selection. As the puromycin-treated cells exhibited significant growth retardation, possibly due to its cytotoxic effect on CD36<sup>+</sup> EPCs, these cell cultures were terminated.

Nontransduced CD36<sup>+</sup> EPCs generally proliferated for ~1 month, and survived up to 1.5 months. Compared with nontransduced cells, the lifespan of CD36<sup>+</sup> EPCs was not significantly extended by infection with the lentiviral vector carrying *SV40T* alone, *hTERT* alone, or *SV40T* plus *hTERT* (both in the same lentiviral vector), or by coinfection with lentiviral vectors containing *SV40T* and *hTERT* (as a separate vector) (data not shown). In

contrast, cells infected with the *HPV16 E6/E7* lentiviral vector or co-infected with the *HPV16 E6/E7* and the *hTERT* lentiviral vectors continued to proliferate. Using these two kinds of cells, attempts were made to generate single cell clones by single cell deposition into individual wells of 96-well plates by cell sorting with or without CD36<sup>+</sup> EPC feeder cells, but failed to produce viable clones. As a next trial, 10 or 20 cells were seeded into individual wells of ten 96-well plates and allowed to incubate until colonies appeared. After 2 to 4 weeks, 53 of these cultures were expanded into 24-well plates and evaluated for their proliferation capabilities and appearance of hemoglobin. Clone 35 derived from the *HPV16 E6/E7* alone transduced cells displayed the greatest proliferative capability with the strongest appearance of hemoglobin. Therefore, the clone 35 termed CD36E was selected to use in further experiments. As shown in Figure 1A, a doubling time of the CD36E cells in the expansion medium ranged from 3 to 4 days, depending on the cell density. *HPV16 E6/E7* gene integration into CD36E genomes was confirmed by PCR amplification with specific primers (Fig. 1B) and by Southern blotting (Fig. 1C). Attempts were again made to isolate a single cell clone from CD36E cells, but were unsuccessful.

### CD36E cells display characteristic features of EPCs

CD36E cells were stained with Wright-Giemsa and compared with CD34<sup>+</sup> HSCs and parental CD36<sup>+</sup> EPCs (Fig. 2A). CD34<sup>+</sup> HSCs were round and uniform in size and shape. By incubation in the expansion medium, CD34<sup>+</sup> HSCs were forced to differentiate toward CD36<sup>+</sup> EPCs, resulting in a gradual increase of their sizes. On day 8, more than half of CD36<sup>+</sup> EPCs developed microvilli on the surface. Compared with parental CD36<sup>+</sup> EPCs, the CD36E cells were larger with round nuclei, were less homogenous in size and shape, and more than half had microvilli on the surface. The CD36E cells grew in clusters and some were semi-adherent in liquid culture, but easily disassociated.

Hemoglobin production of CD36E cells was examined by benzidine staining and compared with that of parental CD36<sup>+</sup> EPCs using peripheral blood cells as a positive control (Fig. 2B). A higher ratio of hemoglobin-positive cells was observed in the CD36E cells (~27%) compared with the parental CD36<sup>+</sup> EPCs (~2%). CD36E cell pellet was redder in color than those of K562 and UT7/EPO-S1, indicating the highest hemoglobin production among the three cell lines (Fig. 2C). Immunoblot analysis was performed to identify  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globin chains. When compared with the parental CD36<sup>+</sup> EPCs, the CD36E cells produced a higher level of  $\gamma$ -chain, whereas an  $\alpha$ - or  $\beta$ -chain level was similar or lower, respectively (Fig. 2D).

Surface phenotype of CD36E cells was characterized by flow cytometry using various monoclonal antibodies against the surface antigens of human hematopoietic cells (Table 1). The CD36E cells and parental CD36<sup>+</sup> EPCs were clearly not HSCs, as indicated by the loss of CD34 on their cell surface. The number of cells positive for CD33 (a myeloid cell marker) was much lower in the CD36E cells than the CD36<sup>+</sup> EPCs. None of the following lymphoid surface antigens were detected in the CD36E cells or CD36<sup>+</sup> EPCs: CD2 (T cells), CD3 (T cells), and CD19 (B cells). The CD36, CD44, and CD71 surface antigens marking erythroid lineage cells remained positive in the majority of the CD36E cells, which was similar to the parental CD36<sup>+</sup> EPCs. As for other erythroid lineage markers, the CD36E

cells positive for CD235a (glycophorin A) were considerably increased (96%) compared with the CD36<sup>+</sup> EPCs (64%), whereas globoside-positive CD36E cells were decreased to 81%, compared with the CD36<sup>+</sup> EPCs (99%). When the CD36E cells were evaluated for surface antigens for megakaryocytes, in this case CD41a, CD61, and CD110, cell populations with these antigens were lower than those of the CD36<sup>+</sup> EPCs: CD41a and CD61, <1%; CD110, ~20%.

### **Karyotype analysis of CD36E cells**

CD36E cells were evaluated for chromosomal abnormalities by spectral karyotyping and G-banding at 52 weeks (Fig. 3). Analysis of 10 cells revealed that the CD36E cells showed some chromosomal instability. The majority of abnormalities seemed to result from translocations with losses and gains of chromosomal materials, such as T(1;3), translocation involving chromosomes 1 and 3 and Is(6;10), insertion of a fragment from chromosomes 6 into 10. Chromosomes 1, 2, 5, 6, 7, 8, 17, and 21 were trisomy, while chromosome 19 was monosomy. The chromosome number of the CD36E cells ranged between 43 and 53, with a modal number of 53. Although the CD36E cells were originated from the 20-cell sorted culture, the cells were highly clonal as determined by karyotyping, implicating that a progressively expanded clone eventually took over the culture or that the initial culture was comprised of clonal cells when expanded.

### **Erythroid colony-forming ability of CD36E cells**

The colony-forming cell assay was performed to examine a functional potentiality of CD36E cells to proliferate and differentiate into colonies in semi-solid media in response to cytokine stimulation. CD36E cells and CD36<sup>+</sup> EPCs were cultured in two kinds of methylcellulose-based media and evaluated on days 7, 10, and 12: the MethoCult GF<sup>+</sup> complete methylcellulose medium for hematopoietic colony formation of erythroid, granulocyte, macrophage, and multipotential progenitors; the supplemented MethoCult methylcellulose medium for erythroid colony formation. On day 7, erythroid colonies were observed in cultures of the CD36E cells and the CD36<sup>+</sup> EPCs in either the MethoCult GF<sup>+</sup> or the supplemented MethoCult, although colonies were generally too small to count (Fig. 4A). On day 10, colonies were increased in size and allowed to continue to day 12. By day 12, all cultures of the CD36E cells and CD36<sup>+</sup> EPCs in the MethoCult GF<sup>+</sup> or the supplemented MethoCult had <20 erythroid colonies/well, with no significant difference in the number of colonies in each culture (Fig. 4A). However, the CD36<sup>+</sup> EPCs in the MethoCult GF<sup>+</sup> appeared more granular and were decreased in size. Also not observed in cultures of the CD36E cells or CD36<sup>+</sup> EPCs were colonies of granulocytes, macrophages, or eosinophils that can be cultured in the MethoCult GF<sup>+</sup>.

To examine the morphology and hemoglobin production of colony-forming cells, all 12-day colonies in individual wells cultured in the MethoCult GF<sup>+</sup> or the supplemented MethoCult were stained with Wright-Giemsa or benzidine (Fig. 4B). Wright-Giemsa staining showed that colonies derived from CD36<sup>+</sup> EPCs in either culture medium contained differentiated erythroid cells in addition to undifferentiated cells. Small erythroid cells were also observed in specimens from the CD36E-derived colonies in either culture medium, while a relatively high number of undifferentiated CD36E cells (large in size) were also seen. To check

hemoglobin production of the cells, benzidine staining was performed, confirming that colonies from the CD36E cells and CD36<sup>+</sup> EPCs contained hemoglobin-positive cells. Collectively, the CD36E cells were capable of erythroid colony formation in semi-solid culture with EPO, but not other types of hematopoietic colonies. This was highly conceivable because the parental CD36<sup>+</sup> EPCs were erythroid progenitors.

### CD36E cells are growth factor–dependent

Effects of cytokines on the survival and proliferation of CD36E cells were assessed by culture in various conditions where cytokines, alone or in combination, were excluded from the original expansion medium (3 IU/mL EPO + 100 ng/mL SCF + 5 ng/mL IL-3). In addition, an effect of thrombopoietin (100 ng/mL) was also examined. CD36E cells proliferated continuously in the presence of EPO and SCF with or without IL-3, indicating that IL-3 was not required for growth (Fig. 5A and Supplementary Table E1; online only, available at [www.exphem.org](http://www.exphem.org)). CD36E cells cultured in any other conditions showed decreased proliferation and fewer viable cells, indicating both EPO and SCF were indispensable for CD36E growth. Thrombopoietin (100 ng/mL) appeared to have no enhancing effect on the cells. We next examined whether increased concentrations of EPO and/or SCF improved CD36E cell proliferation (Fig. 5B and Supplementary Table E2; online only, available at [www.exphem.org](http://www.exphem.org)). Higher concentrations (three- and nine-fold) of EPO plus SCF inhibited cell proliferation. Removal of a specific cytokine or higher cytokine concentrations appeared to have no stimulating effects on cell differentiation.

To examine whether hemin, DMSO, or PMA induced CD36E differentiation, cells were cultured with each of these reagents at two different concentrations as described in previous studies [21–26]. The treated cells were subjected to measurement of cell proliferation, microscopic analysis, and immunoblotting (Supplementary Figure E1 and Supplementary Table E3; online only, available at [www.exphem.org](http://www.exphem.org)). From these results, it appears that the three reagents had no inducible effects on CD36E differentiation.

### Gene expression profiling of CD36E cells using real-time RT-PCR arrays

We carried out real-time RT-PCR array analysis to investigate a gene expression profile of CD36E cells compared with that of parental CD36<sup>+</sup> EPCs. The CD36E cells expressed a number of genes with greater differences from those in the CD36<sup>+</sup> EPCs (Fig. 6 and Table 2). The following genes were expressed with > 50-fold differences: IRF4 (1,450-fold), GALNAC4S-6ST (520-fold), FAM3B (138-fold), MAL (100-fold), and MLF1 (63-fold) were upregulated; PF4 (869-fold), PTPRC (100-fold), KITLG (82-fold), and LRMP (54-fold) were downregulated. Among these, IRF4, GALNAC4S-6ST, MAL, PTPRC, and LRMP are lymphoid-relating factors. As for lymphocyte-relating factors with < 50-fold differences, BLNK and CD86 molecules were considerably upregulated, while LEF1, TLR3, TLR4, and IRF8 were highly downregulated. Among erythroid-relating factors, ERAF expression was fairly decreased (13-fold) in the CD36E cells compared with the CD36<sup>+</sup> EPCs. Within the interleukin group, expression levels of several factors, including IL-1A, IL-4, IL-6, IL-10, IL-11, IL-12A, IL-13, and IL-15, were highly elevated.

## Discussion

Some reports have demonstrated that stable expression of *hTERT*, a catalytic subunit of telomerase, is sufficient to extend the lifespans of various human cells [27,28]. However, other reports have described that several types of cells, including corneal keratinocytes [29] and mammary epithelial cells [30], require additional genetic hits to achieve immortality, such as viral oncogenes or dominant negative-acting transgenes either alone or in combination with *hTERT* expression. Three viral oncogenes, *SV40T*, *HPV16 E6*, and *HPV16 E7*, are well-documented for cellular immortalization and transformation properties and widely used to establish cell lines [31,32]. *SV40T* binds to and inactivates Rb and p53 tumor suppressor proteins. Similarly, *HPV16 E6* is involved in targeted degradation of p53 and thereby facilitates cell proliferation. *HPV16 E7* binds to Rb by displacing the E2F transcription factor, resulting in the activation of E2F and subsequently cell-cycle progression.

In this work, we successfully generated immortalized cells from the CD36<sup>+</sup> EPCs by introducing *HPV16 E6/E7* alone or *HPV16 E6/E7* plus *hTERT*, but failed to immortalize the cells by transducing with *SV40T* alone, *hTERT* alone, or *SV40T* plus *hTERT*. Similarly, a previous work that used CD34<sup>+</sup> stem/progenitor cells from human cord blood (CB) has reported the immortalization and transformation of the cells by transduction with lentiviral vectors containing *HPV16 E6/E7* alone or in concert with *hTERT*, but not with *hTERT* alone [20]. Considering this work in addition to ours, *hTERT* transduction alone appears to be insufficient for the immortalization of hematopoietic stem/progenitor cells, and additional elements, such as the viral *HPV16 E6/E7* oncoproteins, are required for successful transformation.

Zhang et al. [33] serially examined cytogenetic events of *HPV16 E6/E7*-immortalized esophageal epithelial cells; there was a progressive increase of chromosomal aberrations during the early stage of crisis; in the later stage, the karyotypes were more convergent with an increased karyotypic complexity. It is generally suggested that most of the chromosomal rearrangements are randomly generated during the early stage of the crisis (a state characterized by genomic instability, cell death, and the eventual emergence of rare replicatively immortal variants) [34–37], and some chromosomal aberrations may accelerate cellular proliferation, whereby cells that acquired these aberrations can overcome the crisis. The serial steps may contribute to transforming the CD36<sup>+</sup> EPCs cells to establish the CD36E cells. In fact, the CD36E cells possessed various chromosomal abnormalities and aneuploidy, although they were still dependent on EPO and retained other features of erythroid progenitors. As for other hematopoietic cell lines, various cell lines derived from leukemic patients also display chromosomal aberrations with aneuploidy and a number of translocations; for example, the modal chromosome numbers are 54 in the TF-1 erythroleukemic cells [10]; 45 in the MB-02 megakaryoblastic cells [13]; and 92 in the UT7 megakaryocytic cells, a parental cell to the UT7/EPO cells [15]. In the *HPV16 E6/E7- or HPV16 E6/E7* plus *hTERT*-transduced cell lines derived from human CB CD34<sup>+</sup> cells, numerous chromosomal alterations are also observed [20], but with much less than those seen in the CD36E cells. Because the adult CD36<sup>+</sup> EPCs are more mature and differentiated compared with the CB CD34<sup>+</sup> cells, it is highly possible that the CB CD34<sup>+</sup> cells may have



great plasticity intracellularly, allowing immortalization without radical changes of chromosomal structure and number. In addition to the hematopoietic cell lines, chromosomal abnormalities are consistently observed in other cell types transformed with the *HPV16 E6/E7* genes [38], which display progressively increased chromosomal aberrations during the early stage of cellular crisis. Among the *E6/E7*-transduced cells, the nonrandom occurrence of chromosome-20 gain or chromosome-20q amplification has been frequently reported in immortalized epithelial lineage cells [33,39]. Similarly, trisomy of chromosome 20 has been described in one of the cell lines derived from the *HPV16 E6/E plus hTERT*-transduced CB CD34<sup>+</sup> cells [20]. However, this phenomenon was not seen in the CD36E cells.

One of the remarkable characteristics of the CD36E cells was the ability to produce hemoglobin with ~27% of benzidine-positive cells, a higher percentage than the parental CD36<sup>+</sup> EPCs. Compared with the CD36E cells, other cell lines had a lower ratio of benzidine-positive cells in their normal culture conditions. The JK-1 cell line derived from a chronic myelogenous leukemia patient is shown to have the highest number of benzidine-positive cells (~20%) [8]. The UT7/EPO cells, which are committed more to erythroid lineage than the UT-7 cells, have 4% benzidine-positive cells [15]. K562 [40], KMOE [9], and HEL [41] have <1% to 5% of benzidine-positive cells in normal culture condition.

There are various hematopoietic cell lines with erythroid features derived from leukemic patients, most of which have immunoprofiles typical of immature erythroid cells. Most cell lines, including K562, KMOE, and HEL, grow autonomously. Other cell lines are constitutively cytokine-dependent; mainly on granulocyte macrophage colony-stimulating factor or IL-3 like UT7, TF-1, and MB-02; strictly on EPO like UT7/EPO. Compared with these cell lines, the CD36E cells appear distinctive due to the strict dependency on both EPO and SCF for viability and proliferation. However, higher concentrations (three- and nine-fold) of EPO plus SCF inhibited cell growth, probably due to selection or adaptation of the cells during culture at the original cytokine concentrations.

EPO is uniquely associated with erythroid lineage differentiation. As reported previously, EPO plays a role as a differentiation inducer to promote erythropoiesis for some hematopoietic cell lines, such as UT7, TF-1 [42], M-TAT [43], MB-02 [13], and NS-Meg [44]. In the case of the CD36E cells, there were no apparent signs of differentiation induction with EPO, even at a ninefold higher concentration, alone or in combination with SCF. As the CD36E cells evolved from the culture supplemented with EPO, SCF, and IL-3, it is highly plausible that the main actions of EPO for the cells are to promote proliferation and to maintain viability, but not to induce differentiation.

Hemin, DMSO, and PMA are chemicals known to induce differentiation of various hematopoietic cell lines. CD36E cells treated with hemin, PMA, or DMSO did not exhibit the desired result of differentiation as reported with some erythroleukemic cell lines, such as K562 [22,25], HEL [25] UT7 [15], U937 [24], and HL-60 [24]. DMSO rather negatively affected cell viability.

In cell lines derived from patients with hematological malignancies, acquired elevation of hemoglobin (Hb) F has been reported with or without induction by measuring HbF or  $\gamma$ -globin chain expression: for example, HbF is a major hemoglobin subtype in UT7/EPO [15] and JK-1 [8]; HbF is induced in K562 [45]. In the CD36E cells, enhanced  $\gamma$ -chain expression concomitant with reduced  $\beta$ -chain expression was observed, whereas  $\alpha$ - and  $\beta$ -chains were predominant in the CD36<sup>+</sup> EPCs with only a trace amount of  $\gamma$ -chain. The results indicate that switching of HbA to HbF occurred during the establishment of the CD36E cells from the CD36<sup>+</sup> EPCs. Previous studies have reported that an increased HbF production was observed in hemin-treated cells, such as primary erythroid progenitors [45] and K562 cells [45,46]. However, treatment of the CD36E cells with hemin or PMA had no clear effect on HbF production when evaluated by  $\gamma$ -chain expression.

Consistent with the parental CD36<sup>+</sup> EPCs, lymphoid-specific markers, CD2, CD3, CD10, and CD19, were undetectable on the surface of the CD36E cell. Nevertheless, when measured by RT-PCR array analysis, mRNA expression levels of some lymphoid-relating factors, such as IRF4, GALNAC4S-6ST, and MAL, were extremely elevated compared with those of the parental CD36<sup>+</sup> EPCs, although mRNA levels of other lymphoid factors such as PTPRC, LRMP, and LEF1 were markedly decreased. Of note, IRF4 expression was dramatically activated in the CD36E cells (> 1,000-fold). IRF4, which is primarily expressed in B cells and activated T cells, is an important regulator of B-cell development and inducible by antigen stimulation. Overexpression of IRF4 may facilitate the CD36E proliferation because IRF4 was reported as a master regulator of the malignancy-specific gene expressions in multiple myelomas in which IRF4 constitutes a positive autoregulatory loop with MYC [47]. The MAL (CD88 adaptor-like) protein expression level was also significantly elevated (100-fold) in the CD36E cells, similar to T- and B-lineage malignant cells [48,49] in which overexpression of MAL have been described. MAL is expressed highly in thymocytes (the CD4<sup>+</sup> cells > CD8<sup>+</sup> T cells) and acts to recruit MyD88 to TLR4, resulting in activation of the signaling pathway. As the *TLR4* expression was fairly downregulated (12-fold) in the CD36E cells, MAL may act in a different manner in the CD36E cells. It is unclear whether the skewed expressions of lymphoid lineage markers in the CD36E cells were due to the *HPV16 E6/E7* expression or they were randomly acquired by the CD36E cells over the course of chromosomal rearrangements. Previously, Gubin et al. has reported the gene expression profile of normal human erythroid cells sorted with anti-CD71 antibody using a method involving suppression subtractive hybridization [50]. Further, two groups have demonstrated gene expression profiling of a growth factor-dependent erythroblast cell line derived from fetal liver of the p53-deficient mouse using the Atlas complementary DNA arrays or custom-made hematopoietic microarrays [51,52]. Here we examined a gene expression profile of the CD36E cells using the custom real-time PCR array targeting 92 hematopoietic genes and compared it with that of the parental CD36<sup>+</sup> EPCs. Thus, due to the differences of cell origins and experimental conditions between their experiments and ours, we were unable to directly compare gene expression profiles of the CD36E cells with those of others. However, as shown in Table 1, surface proteins, CD36, CD71, and glycophorin A (CD235a) were detected in CD36E cells, consistent with data described by Gubin et al.

To the best of our knowledge, the CD36E cells are the first cell line established from primary CD36<sup>+</sup> EPCs. The cell line would create promising opportunities for various studies using EPCs. We would like to freely distribute the cell line upon request, or better yet, deposit the cells into a public repository, such as ATCC.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

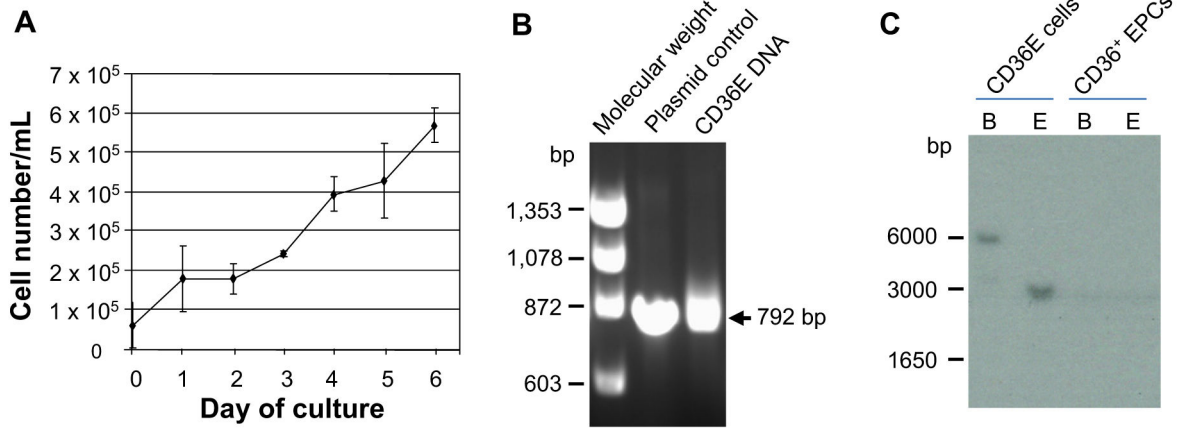
This work was supported by the National Institutes of Health (NIH) Intramural Research Program (Bethesda, MD, USA). We thank Andre Larochelle, Hematology Branch, National Heart, Lung and Blood Institute (NHLBI), NIH for his helpful discussion. We also thank the following people and facilities for their technical support: Leigh Samsel and Phil McCoy at the NHLBI Flow Cytometry Core Facility, NIH; Sandra Burkett at National Cancer Institute Frederick SKY facility, NIH (Frederick, MD, USA); Bey-Dih Chang and Thomas Primiano at CDI Technologies, Madison, WI, USA; and Rick Dreyfuss, Medical Arts Photography Branch, NIH (Bethesda, MD, USA).

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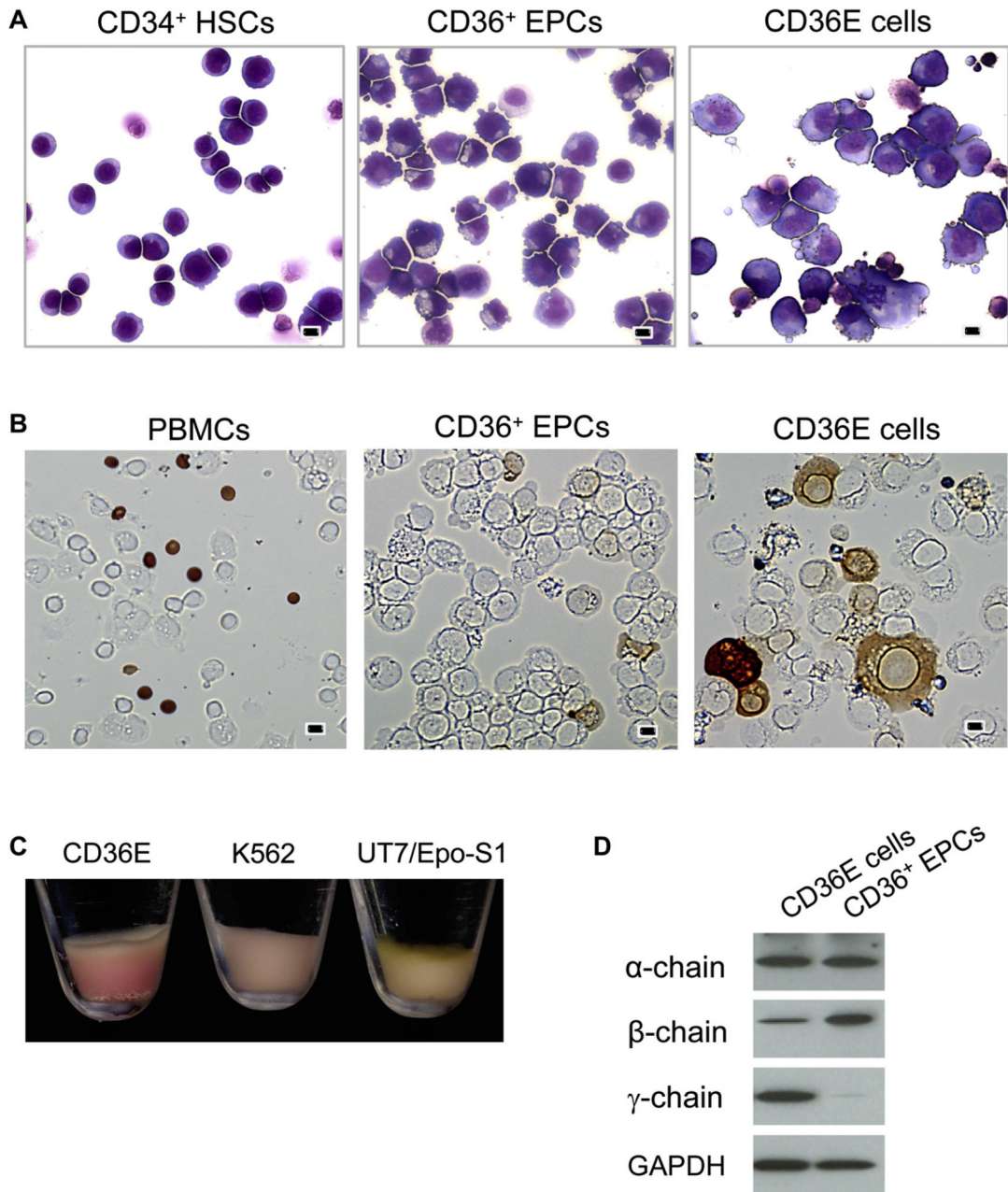
**Figure 1.** Growth curve of CD36E cells and detection of *HPV16 E6/E7*-gene integration in the genomes. **(A)** CD36E cells were cultured in the serum-free expansion medium. Cell number and viability were assessed by the Trypan blue-dye exclusion. Each value is the mean  $\pm$  SD of three experiments. **(B)** Genomic DNA isolated from CD36E cells or CD36<sup>+</sup> EPCs was PCR-amplified to examine the integration of *HPV16 E6/E7* genes into the genomes using the adequate primer set. An arrow represents a position of the amplified fragment from the *HPV16 E6/E7* genes. DNA size markers (bp) are denoted on the left. **(C)** Genomic DNA isolated from CD36E cells or CD36<sup>+</sup> EPCs was digested with BamHI or EcoRI, and subjected to Southern blot analysis. Numbers on the left indicate molecular weight marker positions.

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**Figure 2.**

Comparison of morphological features and hemoglobin production. CD36E cells were cytocentrifuged on glass slides for staining. **(A)** After Wright-Giemsa staining, morphological features of CD36E cells were observed by microscopy at 200× magnification, and compared with those of CD34<sup>+</sup> HSCs and CD36<sup>+</sup> EPCs. Scale bars: 10 μm. **(B)** Hemoglobin production in CD36E cells, peripheral blood mononuclear cells (PBMCs), and CD36<sup>+</sup> EPCs was examined by benzidine staining followed by microscopy. Scale bars: 10 μm. **(C)** Shown are pictures of cell pellets prepared from CD36E, K562, and UT7/EPO-S1 (a subclone of UT7/EPO). **(D)** Whole cell lysates obtained from CD36E cells and CD36<sup>+</sup>

EPCs were analyzed by immunoblot using antibodies against  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globin chains, and glyceraldehyde phosphate dehydrogenase (an internal control).

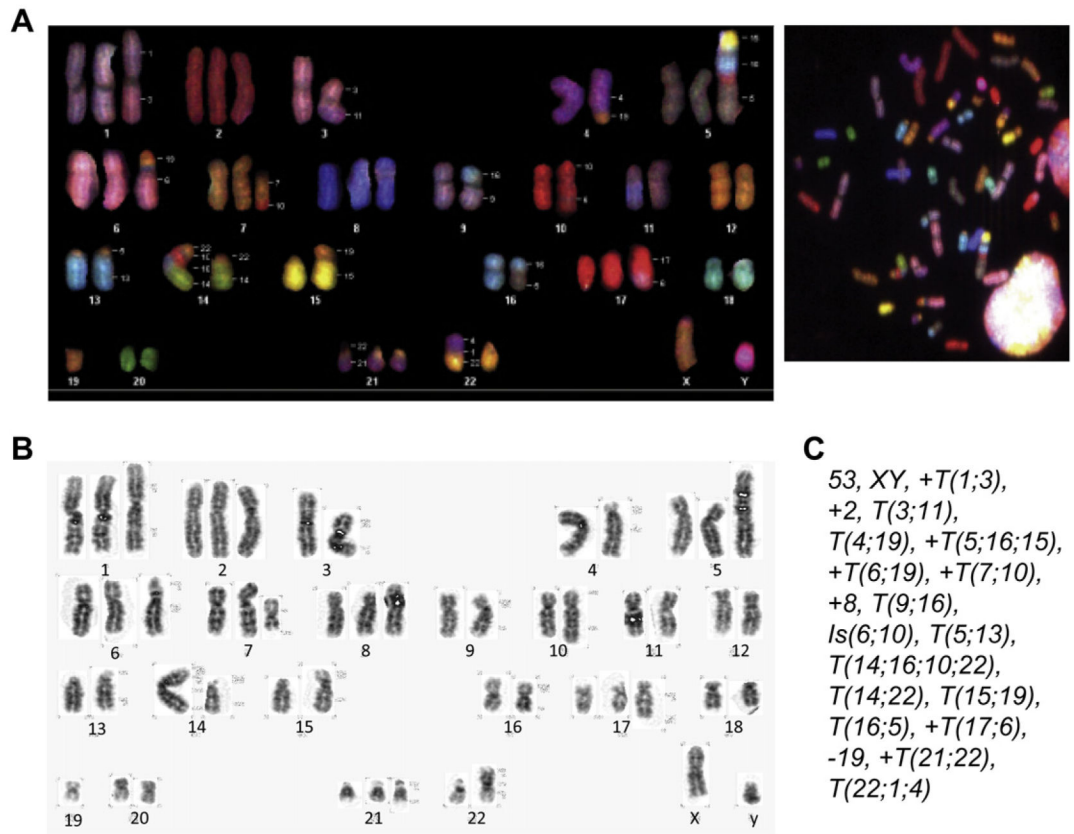
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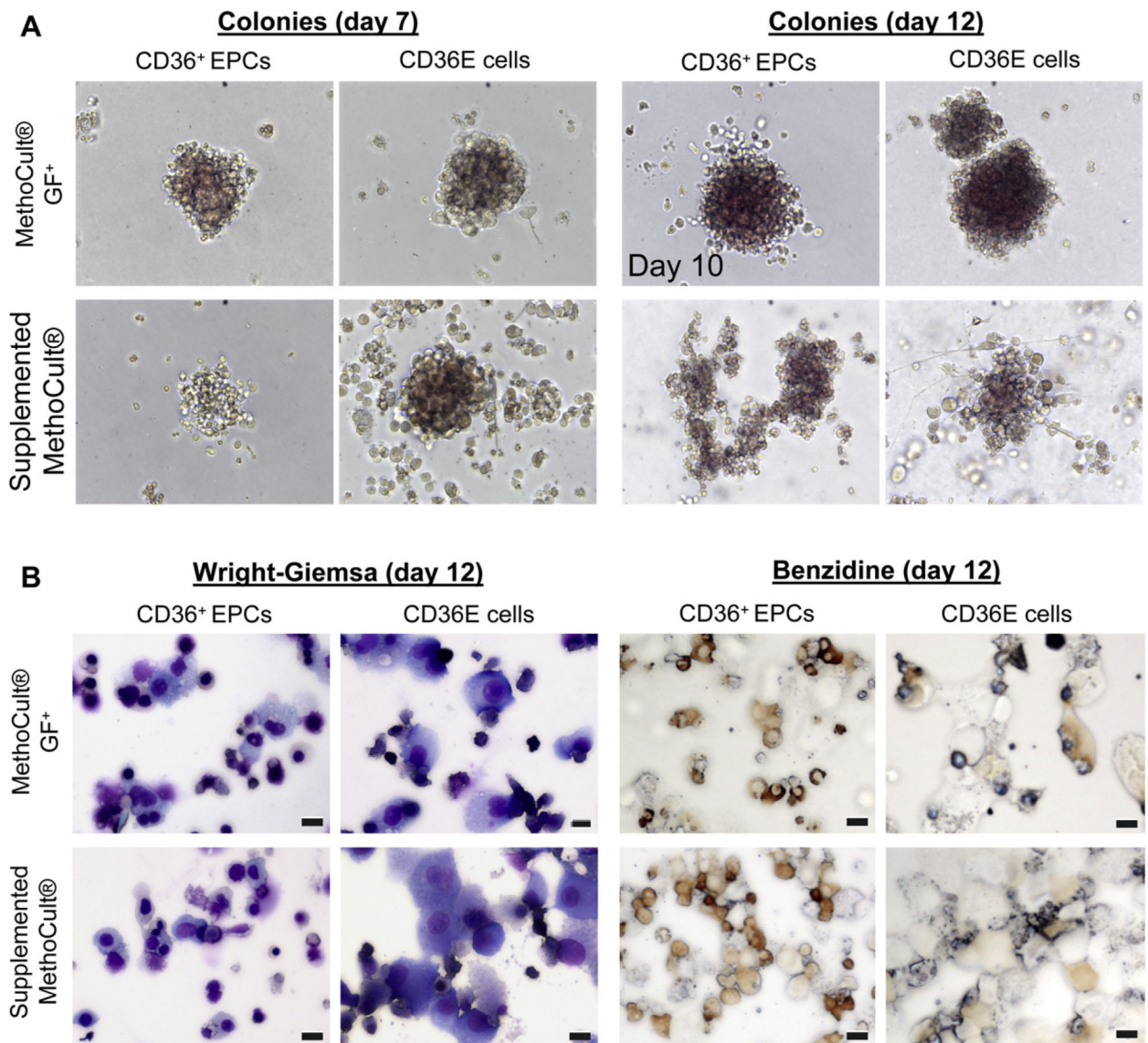
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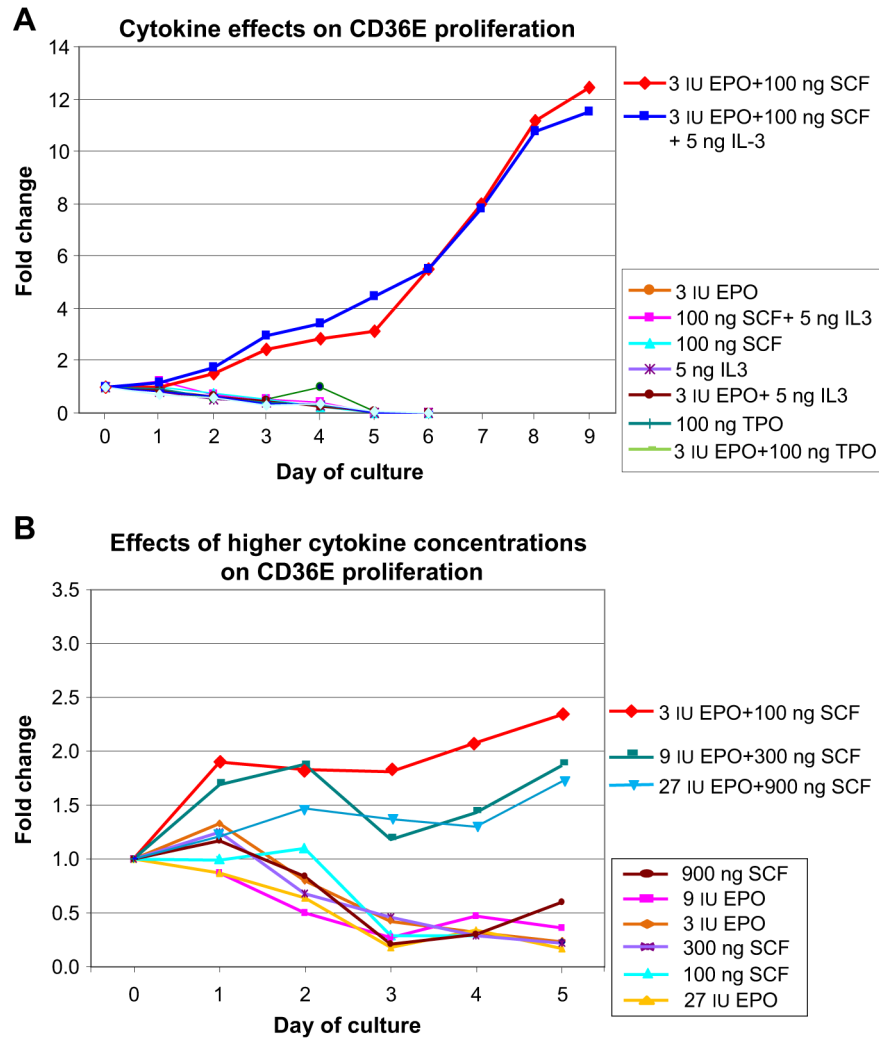
**Figure 3.**

Chromosome analyses of CD36E cells by spectral karyotyping and G-banding. Metaphase spreads were prepared from exponentially growing CD36E cells. Chromosomal analyses were conducted using 10 metaphase spreads and one representative is shown. (A) Spectral karyotyping was performed by fluorescence in situ hybridization with chromosome-specific painting probes. Structural chromosomal abnormalities are designated on the left side of each chromosome. (B) G-banding was carried out to generate dark and light banding patterns unique to individual chromosomes. (C) Detailed numerical and structural descriptions of the CD36E karyotype.

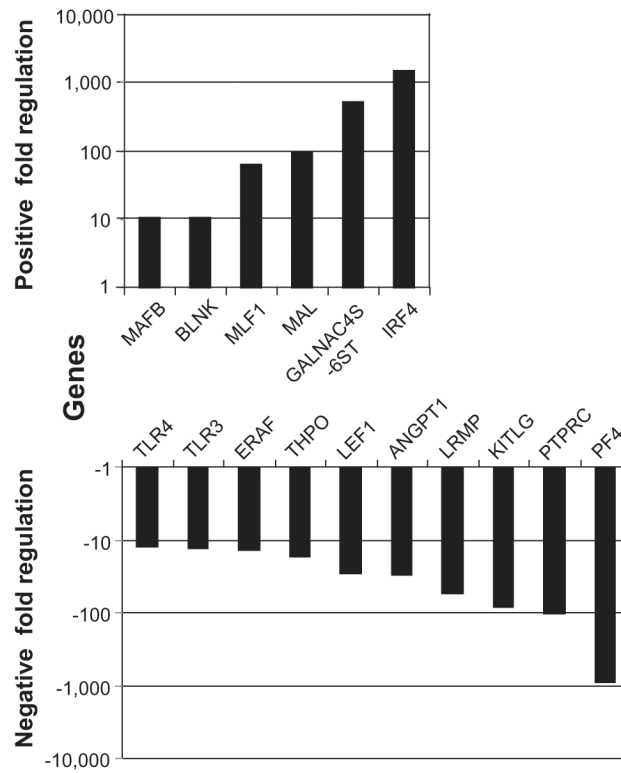


**Figure 4.**

Colony-forming cell assay of CD36E cells. CD36E cells were cultured in two different methylcellulose-based media: the MethoCult GF<sup>+</sup> for various kinds of hematopoietic colony formation; the supplemented MethoCult for erythroid colony formation. (A) Using a light microscope at 200× magnification, colonies were evaluated on days 7 and 12, but colonies from CD36<sup>+</sup> EPCs cultured in the MethoCult GF<sup>+</sup> were evaluated on day 10, instead of day 12, due to overgrowth. (B) On day 12, individual colony cultures were harvested, cytocentrifuged onto glass slides, and stained with Wright-Giemsa or benzidine followed by microscopy. Scale bars: 10 μm.



**Figure 5.** Effects of cytokines on CD36E proliferation. **(A)** CD36E cells were cultured under various conditions in which cytokines alone or in combination were excluded from the original serum-free expansion medium or replaced by 100 ng/mL thrombopoietin. These cultures were compared with cells grown in the standard cytokine cocktail (3 IU/mL EPO + 100 ng/mL SCF + 5 ng/mL IL-3). Cell proliferation was measured at indicated time points in triplicate by Trypan blue staining. Indicated cytokine amounts, 3 IU, 100 ng, and 5 ng, are per mL. **(B)** CD36E cells were grown in serum-free expansion medium supplemented with higher concentrations (three- or nine-fold) of EPO and/or SCF alone or in combination. Because IL-3 had no effect on CD36E proliferation, the cytokine was kept at a constant 5 ng/mL in all conditions. The cultures were compared with the standard cytokine concentrations of 3 IU/mL EPO and 100 ng/mL SCF. Cell proliferation was monitored on various days in triplicate culture conditions.



**Figure 6.**

Gene expression profiling of CD36E cells using the real-time RT-PCR arrays. Total RNA was isolated from CD36E cells using the Qiagen microRNA Extraction kit and converted to complementary DNA, followed by real-time RT-PCR assay using the Custom PCR array targeting hematopoietic genes and the modified-cataloged cytokine array. Fold changes represent ratios of gene expression levels with fivefold or greater differences between the CD36E cells and the parental CD36<sup>+</sup> EPCs.

Table 1.

Flow cytometry analysis of cellular surface antigens

Antigen	Description	CD34 <sup>+</sup> (day 0)	CD36 <sup>+</sup> (day 8)	CD36E	UT7/EPO-S1
CD2	T-cell surface antigen T11, E-rosette receptor	<1	0	0	0
CD3	T-cell antigen receptor complex	<1	0	0	0
CD10	Common acute lymphocytic leukemia antigen	0	0	6.1	0
CD19	B-lymphocyte surface antigen B4	<1	0	0	0
CD33	Sialic acid-binding Ig-like lectin 3	46.2	58.6	30.0	91.3
CD34	Hematopoietic progenitor cell antigen	96.6	1.0	0	0
CD36	Collagen type I recepto, thrombospondin receptor	11.1	97.9	99.5	99.0
CD41a	Alpha IIb integrin chain		2.9	<1	
CD44	Extracellular matrix receptor III	98.9	98.0	98.5	99.0
CD61	Integrin $\beta$ 3-subunit		2.4	<1	
CD71	Transferrin receptor	61.3	97.0	99.0	96.5
CD110	Thrombopoietin receptor		58.0	19.3	
CD235a	Glycophorin A	<1	63.9	96.0	26.6
Globoside	Glycosphingolipid with N-acetylgalactosaminea	4.7	99.1	81.2	57.6

Values represent percentages of cells.

**Table 2.**

Up- and downregulation of selected genes analyzed by realtime RT-PCR

Gene symbol	Fold change <sup>a</sup>	Description
IRF4	1,449.5	Interferon regulatory factor 4
GALNAC4S-6ST	520.2	B-cell RAG associated protein
FAM3B	137.5	Family with sequence similarity 3, member B
MAL	99.6	Mal, T-cell differentiation protein
MLF1	63.1	Myeloid leukemia factor 1
GDF2	38.2	Growth differentiation factor 2
TGFB2	28.8	Transforming growth factor, $\beta$ 2
BLNK	10.7	B-cell linker
MAFB	10.6	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
IL12A	10.6	Interleukin 12A
IL15	9.2	Interleukin 15
IL4	9.1	Interleukin 4
HDAC9	9.0	Histone deacetylase 9
IL6	8.1	Interleukin 6 (interferon, $\beta$ 2)
CD86	8.1	CD86 molecule
IL13	7.4	Interleukin 13
IL1A	7.0	Interleukin 1, $\alpha$
IL10	6.9	Interleukin 10
TNF	6.4	Tumor necrosis factor (TNF superfamily, member 2)
GDF9	5.7	Growth differentiation factor 9
IL11	5.7	Interleukin 11
PDGFA	5.6	Platelet-derived growth factor $\alpha$ polypeptide
BMP1	5.5	Bone morphogenetic protein 1
CD34	-5.7	CD34 molecule
LMO2	-6.0	LIM domain only 2 (rhombotin-like 1)
ETV6	-7.9	Ets variant gene 6 (TEL oncogene)
PECAM1	-8.2	Platelet/endothelial cell adhesion molecule (CD31 antigen)
IRF8	-8.6	Interferon regulatory factor 8
MAP4K1	-9.8	Mitogen-activated protein kinase kinase kinase kinase 1
TLR4	-12.3	Toll-like receptor 4
TLR3	-12.9	Toll-like receptor 3
ERAF	-13.2	Erythroid associated factor
THPO	-16.7	Thrombopoietin
LEF1	-28.1	Lymphoid enhancer-binding factor 1
ANGPT1	-29.8	Angiopoietin 1
LRMP	-53.7	Lymphoid-restricted membrane protein
KITLG	-81.8	KIT ligand
PTPRC	-99.5	Protein tyrosine phosphatase, receptor type, C
PF4	-868.8	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)

<sup>a</sup>More than fivefold expression changes.

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