Ex vivo generated CD36+ erythroid progenitors are highly permissive to human parvovirus B19 replication

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Abstract

The pathogenic parvovirus B19 (B19V) has extreme tropism for human erythroid progenitor cells. In vitro, only a few erythroid leukemic cell lines (JK-1 and KU812Ep6) or megakaryoblastoid cell lines (UT7/Epo and UT7/Epo-S1) with erythroid characteristics support B19V replication, but these cells are only semi-permissive. Using recent advances in generating large numbers of human erythroid progenitor cells (EPCs) ex vivo from hematopoietic stem cells (HSCs), we produced a pure population of CD36$^+$ EPCs expanded and differentiated from CD34$^+$ HSCs, and assessed the CD36$^+$ EPCs for their permissiveness to B19V infection. Over more than three weeks, cells grown in serum-free medium expanded >800,000-fold, and 87-96% of the CD36$^+$ EPCs were positive for globoside, the cellular receptor for B19V. Immunofluorescence (IF) staining showed ~77% of the CD36$^+$ EPCs positive for B19V infection, compared to ~9% of UT7/Epo-S1 cells. Viral DNA detected by real-time PCR increased by >3 logs in CD36$^+$ EPCs, as compared to 1 log in UT7/Epo-S1 cells. Due to the extensive permissivity of CD36$^+$ EPCs, we significantly improved the sensitivity of detection of infectious B19V by real time RT-PCR and IF staining to 100- and 1000-fold, respectively, greater than that of UT7/Epo-S1 cell-based methods. This is the first description of an ex vivo method to produce large numbers of EPCs which are highly permissive to B19V infection and replication, offering a cellular system that mimics in vivo infection with this pathogenic human virus.
INTRODUCTION

Parvovirus B19 causes fifth disease in children, transient aplastic crisis in individuals with high red cell turnover, and pure red cell aplasia in immunosuppressed patients (40). B19V has a small (22 nm), nonenveloped, icosahedral capsid which packages a single-stranded DNA genome of approximately 5,600 nucleotides. Transcription of the B19 viral genome is controlled by a single promoter (p6) located at map unit 6. The p6 promoter regulates the synthesis of all nine viral transcripts (1, 7, 29). There is a single non-spliced transcript for the nonstructural protein (NS), and eight other transcripts generated by a combination of different splicing events that encode the two capsid proteins (VP1 and VP2) and two smaller proteins of unknown functions (5, 11, 21).

B19V is highly tropic for human erythroid progenitor cells (EPCs); restriction of infection to these cells is at least in part due to their selective expression of globoside (P antigen), the receptor for B19V (2). The targets of in vitro infection of parvovirus B19 are erythroid progenitors of the bone marrow (16). While stem cells are not infected, the most permissive target cells for B19V infection and replication are erythroid progenitors, blast forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E), and erythroblasts (32). Preincubation of BFU-Es and CFU-Es derived from bone marrow with viremic human serum abrogates erythroid colony formation but does not affect the myeloid lineage (16).

Replication of B19V in continuous cell lines is also restricted. Only a few permissive cell lines have been described, including erythroleukemic cell lines such as JK-1 (31, 33) and KU812Ep6 (13), and megakaryoblastoid cell lines, such as MB-02 (18), UT7/Epo (25), and UT7/Epo-S1, a subclone of UT7/Epo.
(15). Recently, a comparative study of various cell lines that are permissive to B19V infection demonstrated that the UT7/Epo-S1 cells provided the greatest sensitivity to B19V replication and expression (38), but even these cells were only semi-permissive, with limited viral production.

Erythroid progenitors can be obtained from bone marrow and cultured in vitro for use as permissive systems for B19V replication (22, 23, 27). Erythroid progenitors that can be infected are also present in peripheral blood (24), umbilical cord blood (28) and in fetal liver (3, 39). By immunohistochemistry, permissive cells in fetal liver express erythroid markers, specifically CD36 (6, 20), and glycophorins A (GPA) and C (14). Although CFU-Es generated from CD34+ cells from peripheral blood have been used for B19V infectivity assays (30), these cells have not been assessed for their ability to generate large quantities of virus.

Using recent technical advances to generate large numbers of EPCs from HSCs (8, 9), we developed a modified cell culture system which allows differentiation and expansion of CD34+ HSCs into erythroid progenitors presenting the surface antigen CD36. These CD36+EPCs expressed the B19V cellular receptor, globoside, on the cell surface and were highly permissive to B19V infection. The cell culture system developed in this study will be useful to generate infectious B19V and to study the pathogenicity of the virus, including mechanisms of viral replication, permissivity, and host-virus interaction.
MATERIALS AND METHODS

Cells. Human CD34+ cells were isolated from G-CSF-mobilized peripheral blood stems cells (PBSC) from normal donors who provided informed consent according to protocols approved by NCI IRB using the Isolex 300i Magnetic Cell Selection System (Baxter Healthcare Corporation, Deerfield, IL). UT7/Epo-S1 cells (15, 25, 26) were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Mediatech, Herndon, VA), enriched with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), penicillin, streptomycin, L-glutamine (Invitrogen, Carlsbad, CA) and 2 international units/ml recombinant human (rhu) erythropoietin (EPO; Amgen, Thousand Oaks, CA) at 37°C with 5% CO2.

Generation of CD36+ erythroid progenitor cells. CD34+ cells were cultured in serum free expansion medium modified from a protocol previously developed by Freyssinier et al. (8). Briefly, ~10^4 cells/ml were initiated in expansion medium containing a 1:5 dilution of BIT 9500 (Stem Cell Technologies, Vancouver, British Columbia, Canada) in alpha minimum essential medium (AMEM; Mediatech), obtaining a final concentration of 10 mg/ml of bovine serum albumin, 10 µg/ml of rhu insulin, 200 µg/ml of iron-saturated human transferrin, and supplemented with 900 ng/ml of ferrous sulfate (Sigma, St. Louis, MO), 90 ng/ml of ferric nitrate (Sigma), 10 nM hydrocortisone (Sigma), 100 ng/ml of rhu stem cell factor (SCF; Stem Cell Technologies), 5 ng/ml of rhu Interleukin-3 (IL-3; R&D Systems, Minneapolis, MN), and 3 international units/ml of rhu EPO. After 4 days of culture in the expansion medium, one volume of cell culture was expanded into four volumes with fresh medium, and
thereafter maintained at < 2 ×10^6 cells/ml. Otherwise, the cultured cells were cryopreserved in 10% dimethylsulfoxide and stored in liquid nitrogen.

Differentiating cells were characterized by the presence of cell surface antigens from days 0 to 10, and then on days 15 and 20 in culture using flow cytometry.

**Flow cytometry.** Approximately 5 ×10^5 cells were used for flow cytometry analysis. Cells were labelled with unconjugated or FITC-conjugated antibodies. Antibodies to CD34 (BD Biosciences, Franklin Lakes, NJ), CD36 (BD Biosciences), GPA (BD Biosciences), and CD71 (BD Biosciences) were used for phenotyping. Antibodies against globoside (Metlraya, Pleasant Gap, PA), CD49e (BD Biosciences), and KU80 (Calbiochem, San Diego, CA) were used for detection of the B19V cellular receptors (17, 37). Flow cytometry was performed using the Beckman Coulter Cytomics FC 500 Flow Cytometry System (Beckman Coulter, Fullerton, CA).

**Virus and infection.** Viremic plasma containing high titer B19V (2 ×10^{12} genome equivalent/ml, sample V1) was obtained from a normal blood donor and provided to our laboratory by Aris Lazo at V.I. Technologies (Watertown, MA). This stock was determined to have 1 infectious unit/2 ×10^4 genome equivalents which was determined by endpoint serial dilutions (38).

The infection was carried out in a 96-well plate with 10 μl of cell suspension (2 ×10^4 cells) and 10 μl of the defined concentration of B19V. The cells were incubated for 2 h at 4°C and then expanded with 80 μl of expansion medium. The samples were collected at various time points post-inoculation for subsequent analyses. The infection was scaled up as necessary.
Real-time RT-PCR and real-time PCR. As described previously (38), real-time RT-PCR for B19V NS transcripts and real-time PCR for B19 viral DNA were carried out using the Quantitect Probe Multiplex PCR Kit (Qiagen, Valencia, CA) and the Quantitect Probe PCR Kit (Qiagen), respectively. For RT-PCR, RNA was extracted from the cells using the TurboCapture Kit (Qiagen) and converted to cDNA using random primers with M-MLV reverse transcriptase (Invitrogen). One set of primers was used for real-time RT-PCR: the NS primers, B19-NS-F (5’-GTTTTATGGGCCGCGCCAAGTA-3’) and B19-NS-R (5’-ATCCCAGACCACCAAGCTTTT-3’); B19-NS-Probe (5’-6-FAM-CCATTGCTAAAAGTGTTCCA-BHQ1-3’). Real-time RT-PCR reaction was performed using 5 µl of the resulting cDNA which was amplified as a multiplex with β-actin as an internal control.

For real-time PCR, DNA was extracted using the QIAmp DNA Mini Kit (Qiagen) and 5 µl of the resulting DNA was used for analysis. One set of capsid primers was used for real-time PCR: B19-Cap-F (5’-TACCTGTCTGGATTGCAAAGC-3’) and B19-Cap-R (5’-GATGGGTTTTCTAGGGGATTATC-3’); B19-Cap-Probe (5’-6-FAM-ATG GTG GGA AAG TGA TGA TGA ATT TGC TA-BHQ1-3’).

All reactions were performed using the Chromo4 Real-Time Detector (Bio-Rad, Hercules, CA). The reaction started with an activation of the polymerase at 95°C for 15 min, followed by 45 cycles of 15 s (or 1 min for the multiplex) at 94°C and 1 min at 60°C. The quantitation of each amplicon was
performed by interpolation with the respective standard curve to each target (NS, capsid, or β-actin) constructed with serial dilutions of the corresponding plasmid.

**Plasmids and transfection.** Plasmid pB19-M20 containing the full-length B19V genome (41) was constructed in our laboratory and the plasmid pEGFP-F was purchased from BD Biosciences.

The plasmid pEGFP-F was used to optimize conditions for transfecting CD36+ EPCs with the Amaxa Nucleofection System (Amaxa, Gaithersburg, MD). After transfection, cells were examined daily for expression of EGFP by UV microscopy and flow cytometry analysis. Conditions that gave the maximum number of cells expressing EGFP with minimum cytotoxicity were chosen.

UT7/Epo-S1 cells were transfected with the same plasmid DNA, following the condition previously optimized (Nucleofector Reagent R and Program T-20) (41). After transfection, the cells were harvested at various time points post-transfection and used for DNA, RNA, and immunofluorescence studies. For infection studies, cells were harvested at 72 h post-transfection and cell lysates were prepared by three cycles of freeze–thaw. After centrifugation at 10,000 \( \times \) g for 10 min, the clarified supernatant was treated with RNase (a final concentration of 1 U/\( \mu \)l; Roche) and collected for further infections.

**Immunofluorescence for B19V capsid proteins.** Cells were harvested and cytocentrifuged at 1,500 rpm for 8 min in a cytospin funnel (Shandon Cytospin, Thermo-Fisher Scientific, Waltham, MA). Cells were fixed in a mixture of acetone and methanol (1:1) at –20°C for 5 min, washed twice in phosphate-buffered saline (PBS), and then incubated with monoclonal antibody 521-5D
(1:500 dilution) in PBS with 10% FBS for 1 h at 37°C. For IF staining, FITC-labeled goat anti-mouse IgG (BD Biosciences) was used as secondary antibody and counterstained with Evans Blue. Slides were subsequently observed by UV microscopy.
RESULTS

Generating large numbers of erythroid progenitor cells. Human

CD34+ cells isolated from G-CSF-mobilized PBSC were cultured in serum-free medium with a cytokine mixture containing rhu SCF, rhu IL-3, and rhu EPO. The cell culture system resulted in a robust cell population expansion, increasing to a plateau of about 800,000-fold by day 19. Because cells amplified exponentially, aliquots of cells were cryopreserved on day 4 and revived later. Although thawed cells grew slightly slower than did the original culture, the mean cell expansion reached over 700,000-fold, indicating that freezing cells on day 4 of culture did not significantly alter cell proliferation.

Flow cytometry revealed that the expanding population of cells increasingly expressed CD36 and progressively lost CD34 on the cell surface (Fig. 1). A population shift towards the erythroid lineage was evident by day 8 as 90% of the cells were CD36+. Cells also increasingly expressed GPA, from 0% on day 0 to 64% on day 8. Cells also expressed the B19V receptor, globoside, from 5% on day 0 to 87% on day 8, and then 96% on day 15. Cells continuously expressed CD71 (transferrin receptor) and α5β1 integrin (CD49e, a reported B19V co-receptor) over the entire period of proliferation and differentiation (Table 1). However, expression of Ku80 (a reported B19V co-receptor) (17) remained undetectable or at an extremely low level. Subsequent experiments were conducted using predominantly day-8 cells, as the cell population at this stage was comprised of relatively pure erythroid progenitors (90% CD36+) and
amplified to a reasonable scale (~100-fold); therefore, day-8 cells were designated as CD36+ EPCs in the following work.

**CD36+ erythroid progenitor cells are highly permissive to B19V infection.**

In order to investigate the susceptibility of the EPCs at different maturation stages to B19V infection, cells cultured in the expansion medium were harvested daily from days 0 to 10, and then on days 15 and 20. Each cell harvest was inoculated with $5 \times 10^2$ infectious units/cell of B19V and constant volumes of cell cultures were collected at 0 h and 24 h post-inoculation. B19V NS transcript production, detected by real-time RT-PCR, served as a marker of infection. When comparing 0 h with 24 h post-inoculation, an approximate 2-log increase of NS transcripts was first detected in cells harvested on day 3, and similar increases (2- to 4-log) were also observed in the cell harvests from days 4 to 10, as well as on days 15 and 20 (Fig. 2A). Differentiating cells thus appeared to become permissive to B19V infection by day 3 and maintain the permissiveness up to day 20 in the expansion medium as described above.

Cell harvests from days 4 to 10, and then on days 15 and 20 were inoculated with $5 \times 10^2$ infectious units/cell ($10^3$ genome equivalents/cell) of B19V and constant volumes of cell cultures were collected at 0 h and 72 h post-inoculation. Viral DNA replication was analyzed by real-time PCR targeting the capsid protein gene. By comparing 0 h with 72 h post-inoculation, a gradual increase in viral DNA yields was observed in the cell harvests on days 4, 5, and 6 (Fig. 2B). Viral DNA was maintained at similar levels, ~100-fold increase, in the cell harvests on days 7 and 8, and then declined over the course of next four
samplings, suggesting that the differentiating cells cultured in the expansion medium from days 6 to 8 were optimal for viral DNA replication.

The permissiveness of the differentiating cells was also examined by IF staining using a monoclonal antibody specific for viral capsid protein. Over the course of the first six samplings, cell harvests on days 0, 4, 5, 6, 7, and 8, there was a progressive increase in positive cells, from 0% to 77%, while positive cells decreased from ~70% to 28% in the cell harvests on days 9, 10, 15, and 20. (Fig. 2C). Taken together, our results indicated that the optimal time for B19V infection of differentiating cells was from days 7 to 9.

To determine if B19V infection affected cell proliferation, CD36+EPCs were exposed to serial dilutions of B19V or mock-infected and the cells were enumerated daily. Over a period of 4 days, a total cell number increased 15-fold in the mock-infected cells, but only 12-, 8-, 3.5-, and 0-fold increases were found in the cells inoculated with $5 \times 10^{-4}$, $5 \times 10^{-3}$, $5 \times 10^{-2}$, and $5 \times 10^{-1}$ of infectious units/cell, respectively, indicating that B19V infection suppressed proliferation of CD36+EPCs in a dose-dependent manner.

Comparison of permissiveness and sensitivity of CD36+ erythroid progenitor cells and UT7/Epo-S1 cells. Among the few cell lines which are currently used for B19V cell culture, UT7/Epo-S1 cells are believed to be the most susceptible. A comparative evaluation of B19 viral DNA replication in CD36+EPCs and UT7/Epo-S1 cells was performed by real-time PCR. Cells were inoculated with $5 \times 10^{-2}$ infectious units/cell ($10^{3}$ genome equivalents/cell) and harvested at 0 h, 24 h, 48 h, and 72 h post-inoculation and analyzed by real time
PCR. DNA production was not apparent between 0 h and 24 h post-inoculation, but a gradual increase in DNA production was observed in both cell types thereafter. The period when viral DNA was not being produced indicated a time lag between inoculation and entry of viral DNA into the cell, especially the nucleus. At 48 h post-inoculation, an increase in DNA production of >1.6 logs was observed in the CD36$^+$ EPCs as compared to input levels whereas UT7/Epo-S1 cells increase only by ~0.6 logs. More significantly, at 72 h post-inoculation, CD36$^+$ EPCs generated a 3.5-log increase of viral DNA over the input, whereas only a 1-log increase was seen in UT7/Epo-S1 cells (Fig. 3A). Permissiveness to B19V infection was also evaluated by B19V protein production which was determined by IF staining using a monoclonal antibody specific for viral capsid protein. After inoculation with the same amount of B19V (5 infectious units/cell), ~78% of the CD36$^+$ EPCs was positive at 24 h and the cell population noticeably decreased at 72 h whereas <0.1% UT7/Epo-S1 cells were positive at 24 h and ~9% positive at 72 h (Fig. 3B). Taken together, our results indicated that the permissiveness of CD36$^+$ EPCs to B19V infection was much higher than that of UT7/Epo-S1 cells.

In order to compare the sensitivity of the methods to detect B19V infection based on CD36$^+$ EPCs and UT7/Epo-S1 cells (38), serial dilutions of viremic plasma containing defined infectious units were used to infect CD36$^+$ EPCs and UT7/Epo-S1 cells. Sensitivity to infection was defined by the endpoint of detection of NS transcript production using real-time RT-PCR and capsid protein production using IF staining (Table 2). Between 0 h and 72 h post-inoculation, a
significant increase of NS transcripts was consistently detected by real-time RT-PCR at a viral input of $5 \times 10^{-5}$ infectious units/cell in CD36$^+$ EPCs, compared to $5 \times 10^{-3}$ infectious units/cell in UT7/Epo-S1 cells (Table 2). IF staining for capsid protein production detected infected cells at $5 \times 10^{-5}$ infectious units/cell in CD36$^+$ EPCs, and $5 \times 10^{-2}$ infectious units/cell in UT7/Epo-S1 cells (Table 2). Therefore, by using CD36$^+$ EPCs, the detection limits were significantly increased by at least 100-fold by real-time RT-PCR and 1000-fold by IF staining.

**Transfection and infectious clone analysis.** We previously showed that transfection of the B19V infectious clone (pB19-M20) into UT7/Epo-S1 cells led to production of infectious virus. In order to determine if CD36$^+$ EPCs could similarly produce infectious particles, we first optimized conditions for transfecting plasmid DNA into CD36$^+$ EPCs using the plasmid pEGFP-F. The highest transfection efficiency (~70%) with minimum cytotoxicity (~20%) was achieved with Reagent V and program T-19 (Amaxa Nucleofection System) using 3 µg of plasmid DNA and $2 \times 10^6$ CD36$^+$ EPCs. At 72 h post-transfection, approximately 15% of the transfected CD36$^+$ EPCs were positive for B19V capsid proteins detected by IF staining (data not shown). Subsequent infection assays were performed by inoculating the supernatant prepared from the cell lysates of transfected cells into uninfected CD36$^+$ EPCs, followed by detection of increasing viral NS transcripts production by real-time RT-PCR which served as a marker for successful viral infection. At 72 h post-inoculation, a 1.6-log increase in NS transcripts was detected in samples derived from CD36$^+$ EPCs and a 0.4-log increase from UT7/Epo-S1 cells (Fig. 4), indicating that transfection of
CD36⁺ EPCs with the B19V infectious clone produced infectious viral particles and yields of viral progeny were higher than with UT7/Epo-S1 cells.
DISCUSSION

Since the discovery that parvovirus B19 inhibited erythroid colony formation in bone marrow cultures and targeted erythroid progenitor cells (16), numerous approaches have been undertaken to culture the virus in vitro. Culture methods using primary cells and cell lines have not been efficient for propagation of B19V. Although UT7/Epo-S1 cells have been shown to be the most susceptible, they are not highly permissive to B19V infection (38). In using unselected primary cells for B19V infection, such as bone marrow and hematopoietic cells from fetal liver, only a subset of these cells are permissive for B19V infection and to date, none can generate virus in practically useful quantities. Similarly, CFU-Es generated from CD34^+ cells derived from peripheral blood have been employed in B19V infectivity assays (30) but have not been assessed for their ability to generate large quantities of virus. The unavailability of an in vitro culture system to productively grow the virus not only limits the source of infectious virus to sera or plasma of viremic donors but retards study of the pathogenicity of B19V. Based on recent advances in obtaining relatively pure population of EPCs (6, 8, 9, 19, 20), we sought to determine if these culture conditions could provide cells that would support productive replication of B19V. In this study, we show that CD36^+EPCs were highly permissive to B19V infection and able to efficiently produce infectious viral particles after infection with viremic sera or plasma, or transfection with an infectious clone.

Human CD34^+ HSCs are able to proliferate and differentiate into fully mature red blood cells ex vivo (8, 9). These experimental systems were designed
to mimic the marrow microenvironment by the supplementation of cytokines in both liquid culture and coculture on stromal cells. In our system, CD34+ cells were cultured serum-free in the presence of growth factors (SCF, IL3, and EPO), specifically directing erythroid lineage differentiation (CFU-E or BFU-E). Within about 8 days, the majority of CD34+ HSCs had differentiated into CD36+ EPCs in the serum-free medium, and cell numbers were amplified about 100-fold. The presence of EPO in culture was important to promote the erythroid lineage expansion, as erythroblasts show greatest sensitivity to EPO (4). IL-3 acts by maintaining or arresting the cells in a blastoid state and preventing complete differentiation, and glucocorticoids such as hydrocortisone enhance proliferation of EPCs (34). Hematopoietic precursors can be identified by their cell-surface markers (14, 35). CD36 is typically found on erythroid and megakaryocytic progenitor cell, but appears earlier on the cells in erythroid lineage and has been defined as a marker for EPCs (6, 20). During maturation to erythroblasts, cells also express CD71 (12) and GPA (12). Analysis of the cell surface antigens of the CD34+ cells selected from PBSCs indicated an absence of CD36 and GPA. After 1 day cultured in expansion medium, cells began to present CD36 on their cell surface and by day 8, a pure population of CD36+/GPA+/CD34− EPCs was generated.

We have previously indentified that the necessary cellular receptor for B19V infection is globoside (P antigen) (2) and others have reported co-receptors to be α5β1 integrin (37) and/or autoantigen Ku80 (17). The permissiveness to B19V infection of the cells relies primarily on viral entry. In the present study, we
showed that an increasing cell population of CD36/GPA/globoside cells coincided with the susceptibility of the cells to B19V infection, confirming the importance of globoside in mediating viral infection. However, α5β1 integrin-expressing cells declined from 99% on day 0 to 59% on day 8, when infectivity had increased from 0% to 77%. In addition, the expression of Ku80 remained undetectable or presented in an extremely low level during differentiation from CD34+ HSCs to CD36+EPCs. Therefore, the roles of α5β1 integrin and Ku80 in B19V viral infection remain to be further investigated.

The CD36+EPCs continued to proliferate after 8 days but after this point the cells became less homogeneous, evident by a decline of CD36+ cells, from 90% on day 8 to 72% on day 15, and then to 55% on day 20. Although the population of globoside-expressing cells peaked at 96% on day 15 and was maintained at 84% on day 20, the susceptibility of the cells to B19V infection decreased from 77% on day 8 to 50% on day 15 and then 28% on day 20. These results agree with previous studies (36), suggesting that globoside is necessary but not sufficient for B19V entry and the existence of putative co-receptor(s) for efficient entry of B19V into human hematopoietic cells. In addition to viral receptor(s), cellular factors for optimal transcriptional activation of the B19V promoter or involved in transcript maturation may also contribute to the restriction of permissiveness (10).
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REFERENCES


FIGURE LEGENDS

FIG. 1. Flow cytometry of cell surface-antigen expression of differentiating cells. CD34+ cells isolated from G-CSF-mobilized PBSC were cultured in serum-free expansion medium supplemented with growth factors. The cells were collected daily from days 0 to 10, and then on days 15 and 20, followed by flow cytometry analysis using specific antibodies against CD34, CD36, and globoside. Grey, isotype controls; black, stained cells. Numbers above gates show percentages of positive cells in the gate.

FIG. 2. Quantitative evaluation of susceptibility of differentiating cells to B19 infection. (A) Quantitative analysis of NS transcripts by real-time RT-PCR. Cells cultured in expansion medium were harvested daily from days 0 to 10, and then on days 15 and 20. Each cell harvest was inoculated with $5 \times 10^{-2}$ infectious units/cell of B19V. mRNA was extracted from the cells at 24 h post-inoculation and quantitated by real-time RT-PCR. The abundance of NS transcripts is represented as a fold change at any time point versus time point 0. (B) Quantitative analysis of B19 viral DNA replication in differentiating cells. Cells were harvested daily from days 4 to 10, and then on days 15 and 20. Each cell harvest was inoculated with $5 \times 10^{-2}$ infectious units/cell of B19V. Total DNA was extracted from the cells at 72 h post-inoculation and viral DNA was quantitated by real-time PCR. Increase of viral DNA is represented as a fold change between 0 h and 72 h post-inoculation. (C) Immunofluorescence of B19 capsid protein in infected cells. Cells cultured were harvested daily from days 0 to 10, and then on
days 15 and 20 and inoculated with 5 infectious units/cell of B19V. Cells were collected at 24 h post-inoculation and cytocentrifuged onto glass slides. B19 capsid proteins were detected by IF staining. Magnification, × 750. All results shown are mean values obtained from three independent experiments.

FIG. 3. Comparison of susceptibility of CD36\(^+\) EPCs and UT7/Epo-S1 cells to B19V infection. (A) Quantitative comparison of B19V DNA replication in CD36\(^+\) EPCs and UT7/Epo-S1 cells. Cells were inoculated with 5 \(\times\) \(10^2\) of infectious units/cell of virus. Total DNA was extracted from the cells at 0 h, 24 h, 48 h, and 72 h post-inoculation. The abundance of viral DNA was measured by real-time PCR. Quantitations are given as genome equivalents/µl of DNA extraction. (B) Immunofluorescence of B19 capsid protein in infected cells. The cells were inoculated with 5 infectious units/cell of virus and collected at 0 h, 24 h, and 72 h post-inoculation. B19 capsid proteins were detected by IF staining. Magnification, × 750. Results shown are mean values of three independent experiments.

FIG. 4. Quantitation of B19V NS transcripts in CD36\(^+\) EPCs and UT7/Epo-S1 cells transfected with infectious clones or inoculated with the cell lysates derived from transfected cells. At 72 h post-transfection, cells were lysed by freeze-thaw and clarified supernatants were inoculated into uninfected cells. mRNAs were extracted from the cells at 72 h post-transfection, and 0 h and 72 h post-inoculation and measured by real-time RT-PCR. Quantitations are given as copy
numbers of NS transcripts/µl of cDNA reaction mixture. Results shown are mean values from three independent experiments.
Figure 1, Wong et al.
Figure 2, Wong et al.
Figure 4, Wong et. al.

The graph shows the number of viral copies (in millions) at different time points post-transfection and post-inoculation for CD36+ EPC and UT7/Epo-S1 cells. The y-axis represents the number of viral copies, and the x-axis shows the cell types. The data points indicate a significant increase in viral copies at 72 hr post-transfection compared to 0 hr post-inoculation and 72 hr post-inoculation, with CD36+ EPC cells showing higher viral copy numbers than UT7/Epo-S1 cells.
<table>
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<th>Surface antigen</th>
<th>Day-0 cells</th>
<th>Day-8 cells</th>
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ND, not done.
<sup>a</sup> Transferrin receptor = CD71
<sup>b</sup> GPA (Glycophorin A) = CD235a
<sup>c</sup> α5 integrin = CD49e, exists as a heterodimer with β1 integrin.
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<th>Dilution</th>
<th>Real-time RT-PCR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IF (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD36&lt;sup&gt;e&lt;/sup&gt; EPC</td>
<td>UT7/Epo-S1</td>
</tr>
<tr>
<td>IU/cell</td>
<td>0 hpi</td>
<td>72 hpi</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>29</td>
<td>1,754,750</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>4</td>
<td>41,152,500</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>UD</td>
<td>2,306,250</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>UD</td>
<td>11,301</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>UD</td>
<td>1,293</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

IU, infectious units; ND, not done; UD, undetected; hpi, hours post-inoculation.

<sup>a</sup> Mean value of capsid transcripts/μl of cDNA reaction in two independent experiments.

<sup>b</sup> Mean value of percentage of positive cells in three independent experiments.

<sup>c</sup> Results from separate experiments to determine the endpoint in which cells were permissive for infection.