Role of Erythropoietin Receptor Signaling in Parvovirus B19 Replication in Human Erythroid Progenitor Cells

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Parvovirus B19 (B19V) infection is highly restricted to human erythroid progenitor cells. Although previous studies have led to the theory that the basis of this tropism is receptor expression, this has been questioned by more recent observation. In the study reported here, we have investigated the basis of this tropism, and a potential role of erythropoietin (Epo) signaling, in erythroid progenitor cells (EPCs) expanded ex vivo from CD34+ hematopoietic cells in the absence of Epo (CD36+Epo− EPCs). We show, first, that CD36+Epo− EPCs do not support B19V replication, in spite of B19V entry, but Epo exposure either prior to infection or after virus entry enabled active B19V replication. Second, when Janus kinase 2 (Jak2) phosphorylation was inhibited using the inhibitor AG490, phosphorylation of the Epo receptor (EpoR) was also inhibited, and B19V replication in ex vivo-expanded erythroid progenitor cells exposed to Epo (CD36+Epo+ EPCs) was abolished. Third, expression of constitutively active EpoR in CD36+EpoR EPCs led to efficient B19V replication. Finally, B19V replication in CD36+Epo+ EPCs required Epo, and the replication response was dose dependent. Our findings demonstrate that EpoR signaling is absolutely required for B19V replication in ex vivo-expanded erythroid progenitor cells after initial virus entry and at least partly accounts for the remarkable tropism of B19V infection for human erythroid progenitors.

Parvovirus B19 (B19V) is pathogenic to humans. It replicates autonomously and belongs to the genus Erythroivirus in the family Parvoviridae (14). Clinical manifestations of B19V infection vary among different health conditions. The most common manifestation is erythema infectiosum. However, B19V infection often results in bone marrow failure under the following conditions (9, 10, 62). In patients with increased destruction of erythrocytes and a high turnover of erythrocytes (e.g., sickle cell disease patients), acute B19V infection can cause transient aplastic crisis. In immunocompromised patients, persistent B19V infection may develop manifestations as pure red-cell aplasia, a chronic anemia. Moreover, B19V fetal infection can cause severe anemia in the fetus, resulting in nonimmune hydrops feticus and fetal death (1, 2, 16, 47, 57).

Erythropoiesis is the process whereby a fraction of primitive multipotent hematopoietic stem cells (CD34+) commit to the erythroid lineage, forming burst-forming units-erythroid (BFU-E: earlier erythroid progenitor) cells, CFU-erythroid (CFU-E: later erythroid progenitor) cells, normoblasts, erythrophasts, reticulocytes, and ultimately, mature erythrocytes. B19V infection shows a remarkable tropism for BFU-E and CFU-E progenitors in human bone marrow and fetal livers. Notably, both cell types express the cell surface marker CD36 (30, 39, 50, 60). The clinical manifestations of B19V infection seen in both aplastic crisis and pure red-cell aplasia are direct outcomes of cell death of the erythroid progenitors that are targets of B19V replication, and this cell death is due to direct cytotoxicity of the virus infection (9, 13). Progressive host cell apoptosis has been observed during B19V infection of erythroid progenitor cells (29, 49, 60), and this is likely induced during infection of the abundantly expressed 11-kDa nonstructural protein of the virus (12). Apoptosis of erythroid progenitor cells is also characteristic of B19V-induced hydrops feticus (60).

Polyadenylation at the proximal site [(pA)p], which is located in the center of the B19V genome, precludes the inclusion of the capsid-encoding open reading frame (ORF) in transcripts under some conditions (38, 61). We have recently shown that replication of the B19V genome enhances readthrough of the (pA)p and, thereafter, the polyadenylation of B19V transcripts at the distal site. Therefore, replication of the B19V genome facilitates the production of a sufficient number of full-length transcripts encoding the viral capsid proteins to achieve productive infection (21). The remarkable tropism of B19V for human erythroid progenitors was initially believed to be due to cell type-specific expression of the blood group P antigen (globoside), which serves as the cellular receptor for B19V (8). However, recent findings revealed an additional requirement for the coreceptors integrin α5β1 (55) and KU80 (32); i.e., erythrocyte P antigen is necessary, but not sufficient, for B19V binding and entry into the cell (54).

More about the B19V tropism has been learned from the fact that in addition to the native target cells for B19V infection in human bone marrow and fetal livers, a few cell lines (the megakaryoblastoid cell line UT7/Epo-S1 [31] and the erythroid leukemic cell line KU812Ep6 [28]) support B19V rep-
lication, albeit at a limited efficiency (7, 56). Recently, \textit{ex vivo}-expanded CD36$^+$ erythroid progenitor cells (CD36$^+$ EPCs) have proven to be highly permissive to B19V infection and to support active B19V replication (\approx 100-fold increase in the B19V genome copy number) (19, 21, 43, 49, 56). All B19V-semipermisive cell lines and primary CD36$^+$ EPCs require erythropoietin (Epo) to sustain proliferation, suggesting that Epo is required for B19V infection. Epo has been confirmed to be essential for the susceptibility of human bone marrow cells to B19V infection (52), and based on this, it was concluded that the B19V target cells are of the erythroid lineage, including BFU-E progenitors through erythroblasts, with the susceptibility to B19V infection increasing with maturity (52). Thus, the main role of Epo in B19V permissiveness was thought to be differentiation of bone marrow hematopoietic stem cells (HSCs) into the stage of erythroid progenitors.

In this study, we show that Epo, specifically the Epo/Epo receptor (EpoR)/Janus kinase 2 (Jak2) pathway, plays a direct role in supporting replication of the B19V genome. We prepared CD36$^+$ EPCs by \textit{ex vivo} expanding CD34$^+$ HSCs in the absence of Epo (Epo$^-$) or presence of Epo (Epo$^+$). CD36$^+$ EPCs expanded in StemCell medium (no Epo) (Table 1), namely, CD36$^+$/Epo$^-$ EPCs, were not permissive to B19V infection, although virus did enter the cells. However, when Epo was added either prior to or after virus infection, CD36$^+$/Epo$^+$ EPCs became permissive to B19V replication. Moreover, inhibition of EpoR signaling, by applying either the Jak2-specific inhibitor AG490 or a Jak2-specific short hairpin RNA (shRNA), reduced B19V replication in CD36$^+$/Epo$^+$ EPCs (Table 1). Expression of constitutively active EpoR in CD36$^+$/Epo$^-$ EPCs led to an increase in B19V replication, bringing it to a level comparable to that observed in CD36$^+$/Epo$^+$ EPCs. Thus, our study brings to light a novel indispensable role of the Epo/EpoR/Jak2 pathway in B19V replication, which partially accounts for the fact that B19V propagates only in cells that require Epo for their proliferation.

**MATERIALS AND METHODS**

**Generation of CD36$^+$ EPCs.** CD34$^+$ HSCs were purchased from the National Disease Research Interchange (NDRI), Philadelphia, PA.

For the generation of CD36$^+$/Epo$^+$ EPCs, CD34$^+$ HSCs were \textit{ex vivo} expanded in Wong medium (Table 1) upon arrival of the cells (day 0) and stored in liquid nitrogen on day 4, as described previously (21, 56). Once the day 4 stock cells were thawed, they were cultured in Wong medium for an additional 4 days before being used in experiments.

StemCell medium was prepared from StemSpan serum-free expansion medium (SFEM; StemCell Technologies Inc., Vancouver, British Columbia, Canada) and a combination of cytokines (Table 1). CD34$^+$ HSCs were \textit{ex vivo} expanded in StemCell medium from day 0 continuously until day 8. These cells were used only for experiments described in the legend to Fig. 1.

For the generation of CD36$^+$/Epo$^-$ EPCs, 3 \times 10^6 CD34$^+$ HSCs were cultured in StemCell medium. At day 6 or 8, approximately 2 \times 10^3 to 3 \times 10^3 expanded cells were spun down at 300 \times g for 10 min, resuspended in 1 ml of autoMACS rinsing buffer (Miltenyi Biotec, Auburn, CA), and incubated with mouse anti-human CD36 antibody (10\(^6\) cells/\mu l; BD Biosciences) for 15 min at 4°C. Then cells were spun down at 2,250 rpm for 2 min and washed three times with the autoMACS rinsing buffer. After incubation with anti-mouse IgM-coated magnetic microbeads (Miltenyi Biotec) for 15 min at 4°C, cells were loaded onto a MiniMACS column, and the labeled cells were eluted by following the manufacturer's instructions (catalog no. PT3132-1; Clontech).

**Viral vector and production of retroviral and lentiviral vectors.** (i) Retroviral vectors expressing EpoR/ Jak2. Plasmid pMSCV-EpoR(J29C)-IRES-GFP was constructed by inserting the EpoR(J29C) gene (20) into the BamHI-XbaI-digested pMSCV-MCS-IRES-GFP-WPRE vector. Retrovirus (Retro-EpoR and Retro-green fluorescent protein [GFP]) were produced by transfecting pMSCV-EpoR(J29C)-IRES-GFP and pMSCV-IRES-GFP with pCMV-SVG in GP293 cells (Clontech). Concentration of retroviral vectors was carried out by following the manufacturer's instructions (catalog no. PT3132-1; Clontech).

(ii) Lentiviral vectors expressing shRNAs. We obtained the pLKO.1 cloning vector and the pLKO-Scramble-shRNA vector from Addgene Inc. (Cambridge, MA). The puromycin resistance gene in the pLKO vectors was replaced with the GFP ORF from pC1GFP (Clontech), using the BamHI/KpnI sites, generating pLKO-GFP and pLKO-GFP-Scramble-shRNA, respectively. The validated Jak2 shRNA1 sequence (TRC0000003818; Sigma) (GenBank accession no. NM_004972) was cloned into pLKO-GFP using the AgeI and EcoRI sites, generating plKO-GFP-Jak2-shRNA. Lentivirus was generated and concentrated by following Addgene's instructions (http://www.addgene.org/plko/).

**Virus and infection.** We obtained B19V viremic plasma samples, P20 (21) and P32, from ViraCor Laboratories (Lee’s Summit, MO) and quantified the number of B19V genomic copies (gc) for each (10\(^{12}\) gc/ml, as previously described (21). Infection was performed at a multiplicity of infection (MOI) of 1 fluorescent focus-forming unit (FFU) per cell (approximately 5,000 gc/cell). Except for the experiments illustrated in Fig. 1, in which plasma sample P20 was used, all other experiments were carried out using P32 for the sake of consistency. B19V infection of CD36$^+$ EPCs expanded in both samples was carried out at culture day 8.

For lentiviral and retroviral transduction, concentrated lentiviral retrovirus was added to CD36$^+$ EPCs at day 6 of culture at an MOI of 4 FFU/cell. B19V infection was carried out at 48 h posttransduction (p.t.).

**Viral entry and replication quantification assays.** We performed a viral entry assay as previously described (3). Briefly, cells were infected with B19V at an MOI of 1. After 2 h of incubation with the virus, the cells were washed with AMEM (alpha modification of Eagle’s medium; Mediatech, Manassas, VA), and spun down at 2,200 rpm for 3 min. The cell pellet was then resuspended at 0.5 million cells per 100 \(\mu\)l of trypsin-EDTA (0.25% trypsin in 20 mM EDTA buffer) for 5 min at 37°C. Total DNA was extracted using the blood DNA minikit (Qiagen), according to the manufacturer’s instructions. The number of B19V genome copies in the extracted DNA was then assayed by quantitative real-time PCR (qPCR) as described previously (22). The replicated viral DNA was extracted as described above and quantified by qPCR at 48 h postinfection (p.i.), except in the case of experiments carried out at 24 h p.i. (see Fig. 5).

**Southern blot analysis.** At 48 h p.i., cells were harvested for Hirt DNA extraction, and Hirt DNA samples were analyzed by Southern blotting as described previously (21, 22). Blots were exposed to a GE phosphor imaging screen and quantified using a phosphor imager (Storm 856) and ImageQuant TL software, version 2005 (GE Healthcare).

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**TABLE 1. Media used for CD36$^+$ EPC expansion**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Serum-free base</th>
<th>SCF</th>
<th>IL-6</th>
<th>IL-3</th>
<th>Flt-3-L</th>
<th>TPO</th>
<th>Epo</th>
</tr>
</thead>
<tbody>
<tr>
<td>StemCell</td>
<td>SFEM</td>
<td>100 ng/ml</td>
<td>20 ng/ml</td>
<td>20 ng/ml</td>
<td>50 ng/ml</td>
<td>None</td>
<td>3 U/ml</td>
</tr>
<tr>
<td>Wong</td>
<td>SFEM plus BIT</td>
<td>100 ng/ml</td>
<td>None</td>
<td>20 ng/ml</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

\(^a\) Serum-free expansion medium (SFEM) and BIT 9500 (BIT) were obtained from StemCell Technologies, Inc. (Vancouver, British Columbia, Canada). With the exception of Epo, which was obtained from Amgen, all cytokines were purchased from Invitrogen. SCF, stem cell factor; IL-3, interleukin-3; Flt-3-L, Flt-3 ligand; TPO, thrombopoietin. In addition to the components listed, Wong medium also contains 900 ng/ml of Fe$^{3+}$, 90 ng/ml of Fe$^{2+}$, and 1 \(\mu\)M hydrocortisone, as described previously (21, 56).
Reverse transcription (RT) and RT-qPCR. We extracted mRNA using a TurboCapture mRNA kit (Qiagen) by following the manufacturer’s instructions. We then performed reverse transcription directly in the TurboCapture tubes, using random hexamers (Promega) and Moloney murine leukemia virus (MMLV) RT (Invitrogen). A multiplex RT-qPCR was performed to detect B19V VP2-encoding mRNA and the B19V mRNAs spliced from the D1 donor site to the A1-2 acceptor site (D1/A1-2-spliced mRNA) (21), with /H9252-actin mRNA serving as an internal control, as previously reported (21, 22).

Immunofluorescence. Infected cells were cytocentrifuged at 1,500 rpm for 5 min and fixed in a mixture of acetone and methanol (1:1 dilution) at 20°C for 15 min. The staining was performed as previously described using anti-B19V capsid (clone 521-5D) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (22).

Western blot analysis. Cell lysates were prepared at 48 h posttreatment and used for Western blot analysis as previously described (46).

Cell viability assay. We examined cell viability using the CellTiter-Glo kit (Promega), which determines the number of viable cells in culture based on quantification of the ATP presence in cells by following the manufacturer’s instructions.

Flow cytometry analysis. For cell surface staining, 10⁶ cells were incubated with the first antibody at a 1:100 dilution in a volume of 100 μl of phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) for 30 min at room temperature. After being washed twice with PBS-FCS, the cells were incubated with FITC-conjugated secondary antibody at a dilution of 1:100 for 30 min at room temperature. After being washed, the cells were fixed in 1% paraformaldehyde.

For intracellular staining, annexin V/propidium iodide (PI) and DAPI (4’,6-diamidino-2-phenylindole) staining were performed essentially as described previously (11).

RESULTS

CD36⁺ EPCs differentiated from CD34⁺ HSCs in the absence of Epo are not permissive to B19V infection. To distinguish between potential roles for Epo in differentiating human HSCs and in supporting B19V replication, we generated erythroid progenitor cells that possess the erythroid progenitor marker CD36 (17, 37). This was accomplished by stimulating the differentiation of human bone marrow derived-HSCs (CD34⁺) in two expansion media, Wong medium (with Epo) and StemCell medium (without Epo), which contain different combinations of cytokines, as described in Table 1.

At day 8 in culture, cells grown in the two media were analyzed by flow cytometry with antibodies to a panel of ery-
thyroid markers (CD36, glycophorin A [GPA], EpoR, and CD71), the B19V receptor (Globoside) (8) and its coreceptors (CD49e and KU80), (32, 55), and the CD41 megakaryoblastic marker (53), as well as CD34 of the HSC lineage. Of the cells that were ex vivo expanded in Wong medium and StemCell medium, 99.4% and 41.5% expressed CD36, respectively (Fig. 1A). Cells expanded in StemCell medium retained a considerable level of CD34 (23.3%), and a low level of CD41 (7.4%) (Fig. 1A), indicating that they had differentiated toward an erythroid progenitor fate. CD71, the transferrin receptor, was expressed at 96.8% and 16.7% on cells expanded in Wong and StemCell media, respectively (Fig. 1A). Expression of GPA was significantly enhanced (97.2%) on the CD36/H11001 EPCs expanded in Wong medium, whereas only 30.0% on the cells expanded in StemCell medium (Fig. 1A). Cells expanded in both media expressed high levels of globoside, CD49e, the primary receptor and coreceptor for B19V infection (8, 32, 55), and EpoR (Fig. 1A). Interestingly, KU80, which has been proposed to serve as a coreceptor for B19V (32), was expressed on approximately 65.3% of the cells expanded in StemCell medium but on only 4.6% of the cells expanded in Wong medium (Fig. 1A).

We then infected the two types of CD36+ EPCs with B19V in their respective media for 2 days. B19V infectivity was then examined by immunofluorescence staining with an anti-B19V capsid antibody (Fig. 1B). Unexpectedly, CD36+ EPCs expanded in StemCell medium (no Epo) were not permissive to B19V infection, as shown by a lack of anti-capsid staining. Conversely, in CD36+ EPCs expanded in Wong medium (with Epo at 3 units/ml), more than 80% of the cells were positive for capsid detection, indicating that infection was effective (56). This result suggests that the committed erythroid progenitor cells cultured in the absence of Epo are not permissive to B19V infection and that Epo not only is required for the differentiation of erythroid progenitor cells but also may be necessary for B19V infection of erythroid progenitor cells.

Epo-dependent B19V infection of CD36+/Epo− EPCs leads to replication of the viral genome. We next purified CD36+ EPCs from the cells expanded in StemCell medium. The purified cells, referred as CD36+/Epo+ EPCs, were analyzed by flow cytometry and uniformly found to express markers of erythroid progenitors (i.e., CD36 at 87.5%, GPA at 88.2%, and EpoR at 84.5%) and high levels of the B19V receptor (globoside at 87.9%) and its coreceptors (CD49e at 39.4% and KU80 at 85.7%). Interestingly, most of these purified cells (82.5%) expressed both CD34 and CD36 (Fig. 2B, 0 h), indicating that they represented BFU-E cells or cells in transition from the BFU-E to the CFU-E stage (37). StemSpan SFEM (the base medium used here) supplemented with cytokines has been widely used for the culture and expansion of human HSCs. To ensure that CD34+ HSC expansion toward CD36+ EPCs was optimal and to guarantee that the purified CD36+ EPCs entered S phase to complete cell division, even in the absence of Epo, we used the following cytokine combination: stem cell factor (SCF), Flt-3, interleukin-3 (IL-3), IL-6, and thrombopoietin (TPO) (technical bulletin no. 29954;
As shown in Fig. 2C, most of the purified CD36+/Epo− EPCs (approximately 70%) survived through day 8 of culture in the absence of Epo, and these surviving cells entered S phase at a rate of 17%. This result indicates that the combination of cytokines in the StemCell medium enabled the survival of the CD36+/Epo− EPCs in the absence of Epo and that it is suitable for parvovirus B19V infection experiments. Similar to unpurified cells expanded in StemCell medium (Fig. 1B), purified CD36+/Epo− EPCs were not susceptible to B19V infection, as revealed by a lack of detectable capsids and B19V mRNAs (Fig. 3A to C).

To examine whether Epo is required for B19V infection, we pretreated purified CD36+/Epo− EPCs with Epo (3 units/ml) for 2 h or overnight (approximately 16 h) prior to B19V infection. Interestingly, cells treated with Epo for either lengths of time became susceptible to B19V infection to some extent (Fig. 3A to C). To pretreated purified CD36+/Epo− EPCs with Epo (3 units/ml) for 2 h or overnight (approximately 16 h) prior to B19V infection. Interestingly, cells treated with Epo for either lengths of time became susceptible to B19V infection to some extent (Fig. 3A to C). Following overnight Epo treatment, approximately 10% of the purified CD36+/Epo− EPCs expressed the B19V capsid, as well as B19V VP2-encoding mRNA (0.7% relative to the β-actin mRNA) and D1/A1-2-spliced mRNA (0.4% relative to the β-actin mRNA). These results were consistent with findings obtained from virus entry and DNA replication assays, which revealed that the virus genome copy (gc) number did not increase in purified CD36+/Epo− EPCs in the absence of Epo, whereas the gc number increased by 2.5- and 13-fold in cells treated with Epo for 2 h and overnight, respectively (Fig. 3D). Control CD36+/Epo+ EPCs supported an approximately 93-fold increase of the entered B19V genome. Interestingly, viral entry was most dramatic in CD36+/Epo− EPCs, with a 17-fold increase compared to that in the untreated CD36+/Epo− EPCs and an approximately 5- to 8-fold increase compared to that in cells treated with Epo for 2 h or overnight (Fig. 3D). The cell surface marker profile for CD36+/Epo− EPCs treated with Epo for 2 h and overnight was slightly shifted toward the profile for CD36+/Epo− EPCs, especially with respect to levels of the CD34, CD41, and CD71 markers (Fig. 2A and B). These results indicate that Epo treatment facilitates differentiation of BFU-E progenitors to CFU-E progenitors, that the latter loses both the CD34 and CD41 markers (37), and that Epo treatment enhances B19V virus entry into infected cells.

Taken together, these results demonstrate that CD36+/Epo− EPCs do not support replication of the B19V genome, although B19V entered these cells; Epo treatment led to an increase of B19V replication. The notion that Epo/EpoR signaling is likely essential for B19V replication is further supported by the fact that an increase in levels of phosphorylated Jak2 (pJak2) and phosphorylated EpoR (pEpoR) (markers of Epo treatment) (25) was observed in cells treated with Epo for 2 h and overnight, relative to the levels in cells not treated with Epo (Fig. 4). Thus, our results suggest that the Epo/EpoR signaling is likely essential for B19V replication.

Epo stimulation in CD36+/Epo− EPCs after virus entry is sufficient to stimulate B19V replication. We next confirmed...
the role of Epo in supporting B19V replication by performing a B19V entry assay prior to Epo stimulation. B19V replication was assayed at 24 h.p.i. to minimize reinfection by progeny virus. Under these conditions, the numbers of viral genomes in the cells in different test groups were exactly the same at the time of Epo treatment. Again, CD36/Epo− EPCs were used as controls. Whereas no replication was observed at 24 h p.i. in the absence of Epo stimulation, replication in cells treated with Epo for 2, 8, and 24 h led to 2.2-, 3.9-, and 8.3-fold increases, respectively, in the viral genome copy numbers (Fig. 5A). Replication in the control CD36/Epo− EPCs expanded in Wong medium were used as controls. (A) For each treatment group, phosphorylation of EpoR and Jak2 prior to B19V infection was assessed by flow cytometry immediately before infection. Primary antibodies used were anti-pEpoR (Tyr 456) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-pJak2 (Tyr 1007) (GenScript, Piscataway, NJ), and anti-B19V NS1 (12). (B) Quantification of data shown in panel A, with average mean fluorescence intensities (MFI) indicated by the bars and the background fluorescence indicated by the reference line. bkg, secondary antibody only.

**FIG. 4.** An Epo pulse triggers EpoR signaling in CD36/Epo− EPCs. CD36/Epo− EPCs were pulsed with Epo (3 U/ml) for different lengths of time prior to B19V infection. Analysis was performed immediately after treatment and prior to B19V infection. CD36/Epo− EPCs expanded in Wong medium were used as controls. (A) For each treatment group, phosphorylation of EpoR and Jak2 prior to B19V infection was assessed by flow cytometry immediately before infection. Primary antibodies used were anti-pEpoR (Tyr 456) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-pJak2 (Tyr 1007) (GenScript, Piscataway, NJ), and anti-B19V NS1 (12). (B) Quantification of data shown in panel A, with average mean fluorescence intensities (MFI) indicated by the bars and the background fluorescence indicated by the reference line. bkg, secondary antibody only.

**FIG. 5.** An Epo pulse is critical for replication of the B19V genome in CD36/Epo− EPCs following virus entry. We recovered purified CD36/Epo− EPCs from StemCell medium for 1 h and then infected cells with B19V. One-fifth of the cells were used to quantify viral entry, and the other four-fifths were evenly divided into four groups treated with Epo for 0, 2, 8, and 24 h, respectively. Analysis was performed at 24 h.p.i. CD36/Epo− EPCs expanded in Wong medium were used as controls. (A) B19V entry and replication were quantified by qPCR and are presented as the average numbers of B19V genome copies per cell (gc/cell). (B and C) The B19V VP2-encoding (B) and D1/A1-2-spliced (C) mRNAs were quantified by RT-qPCR and are presented as the average numbers of mRNA copies per β-actin mRNA (internal control). Standard deviations are shown in the panels and were calculated from at least three independent experiments. UD, undetectable.

B19V replication in CD36/Epo− EPCs requires Jak2 phosphorylation. Epo ligation triggers EpoR dimerization and, in turn, phosphorylates EpoR-associated Jak2 (25). Phosphorylated Jak2 then phosphorylates tyrosine residues in the cytoplasmic tail of EpoR. We investigated whether this pJak2-mediated Epo/EpoR signaling facilitates B19V replication using AG490, a Jak2-specific inhibitor (27). The inhibitory effect of AG490, at 2 and 5 μM, was confirmed by Western blotting for pJak2 and pEpoR (Fig. 6A) and was found to be dose dependent. In the sample treated with 5 μM AG490, pJak2 and pEpoR were decreased to approximately 20% and 30%, respectively, of the level in the dimethyl sulfoxide (DMSO) controls. The cytotoxicity of AG490 in CD36/Epo− EPCs was monitored using the CellTiter-Glo kit obtained from Promega (Fig. 6B). No significant reduction of cell viability was observed at either concentration. Furthermore, AG490-treated cells did not show a significant level of cell death by annexin V/PI staining or that of cell cycle arrest by DAPI staining compared to those of their DMSO-treated counterparts (Fig. 6C). However, even at 2 μM AG490, B19V infection was significantly inhibited. B19V replication was reduced by 20-fold (Fig. 7A, compare lanes 3 and 2), and expression of both the B19V VP2-encoding and D1/A1-2-spliced mRNAs was reduced (Fig. 7B). Strikingly, when AG490 at 5 μM was used, both replication of the B19V DNA and expression of the VP2-encoding and D1/A1-2-spliced mRNAs were undetectable (Fig. 7A, lane 4, and Fig. 7B and C), indicating that B19V infection of CD36/Epo− EPCs is sensitive to AG490 treatment. Notably, AG490 treatment did not affect B19V entry to the cells (Fig. 7C).

To confirm the specificity of the requirement for Jak2 signaling in B19V replication, we tested the effects of Jak2 knock-
down using a lentivirus expressing a Jak2-specific shRNA (Lenti-GFP-Jak2-shRNA) and a control lentivirus expressing scrambled shRNA (Lenti-GFP-Scramble-shRNA). CD36/Epo EPCs were pretreated with these lentiviruses 2 days prior to B19V infection. At 2 days p.i., GFP-expressing cells were gated and analyzed by flow cytometry using anti-Jak2 and anti-B19V NS1 antibodies. Consistent with the decrease in Jak2 levels, in GFP-Jak2-shRNA-expressing cells (with approximately 50% knockdown), the level of B19V NS1 detection was decreased by approximately 50%, relative to that in GFP-Scramble-shRNA-expressing cells (Fig. 8A). However, GFP-expressing cells did not show a significant level of cell death or cell cycle arrest compared with that of the GFP-negative cells (data not shown). Replication of the B19V genome, assessed based on levels of B19V VP2-encoding and D1/A1-2-spliced mRNAs, decreased by approximately 3-fold in Lenti-GFP-Jak2-shRNA-transduced cells (Fig. 8B and C), confirming the importance of Jak2 in B19V infection of CD36/Epo EPCs. Overall, these results indicate that the Epo/EpoR/Jak2 signaling pathway is crucial to B19V infection of CD36/Epo EPCs.

CD36/Epo EPCs are permissive to B19V infection when EpoR is constitutively activated. To further corroborate the role of the Epo/EpoR/Jak2 signaling pathway during B19V infection, we generated a retroviral vector that expresses constitutively active EpoR (20). Purified CD36/Epo EPCs were transduced with Retro-EpoR or Retro-GFP (as a control) 48 h prior to B19V infection. At 48 h p.i., GFP-expressing cells (approximately 50% of the total cell population) were gated and analyzed by flow cytometry, following intracellular staining with anti-pEpoR, anti-pJak2, and anti-B19V NS1 antibodies. Retro-EpoR-transduced cells expressed constitutively activated EpoR, as shown by an increase in the level of pEpoR (presumably phosphorylated by detected active pJak2), compared to that in Retro-GFP-transduced cells (Fig. 9A). The expression of constitutively active EpoR caused cells of the erythroid lineage to take on an expression profile typical of CD36/Epo EPCs, including a decrease in the level of CD34 and an increase in the levels of GPA and CD71 (Fig. 9B).

Consistent with the observed change in the differentiation stage, the Retro-EpoR-transduced cells showed a significant increase in B19V NS1 expression (approximately 40% of the
cells) versus 1.1% of Retro-GFP-transduced cells (Fig. 9A). Levels of B19V mRNAs and replication of the B19V genome were also found to be significantly high in Retro-EpoR-transduced cells but not detectable in the Retro-GFP-transduced control cells (Fig. 9C and D). Consistent with the observations documented in Fig. 3D, B19V entry was increased by approximately 6.2-fold in Retro-EpoR-transduced cells compared to that in Retro-GFP-transduced cells; however, the B19V genome in these cells replicated by approximately 86-fold (Fig. 9C), suggesting that expression of constitutively active EpoR facilitates B19V entry into the cells and, more importantly, accommodates replication of these B19V genomes. These results are direct evidence that expression of constitutively active EpoR renders B19V-nonpermissive CD36+/Epo− EPCs susceptible to B19V infection, confirming the role of the Epo/EpoR/Jak2 signaling pathway in B19V infection of erythroid progenitor cells.

**B19V permissiveness of CD36+/Epo+ EPCs is sensitive to the concentration of Epo.** We next sought to confirm that Epo plays a role in B19V infection of CD36+/Epo+ EPCs expanded in Wong medium. To this end, we cultured cells from day 4 stocks in Wong medium with a range of concentrations of Epo. These cells were infected at day 8 and analyzed at 48 h.p.i. We found that as the Epo concentration rose from 0.1 to 0.5 and 2 U/ml, the percentage of NS1-expressing cells also increased (from 12% to 30% and 50%, respectively), as determined by flow cytometry using anti-NS1 antibody (Fig. 10A). No significant difference in NS1 expression between the groups treated with Epo at 2 and 10 U/ml was observed. Consequently, with this observation, the phosphorylation of both EpoR and Jak2 increased proportionally in the groups exposed to Epo at levels from 0.1 to 0.5 and 2 U/ml but was similar in the groups treated with 2 and 10 U/ml Epo (Fig. 10A). In addition, we observed an increase of B19V infection in these groups, as determined based on the levels of the B19V VP2-encoding and D1/A1-2-spliced mRNAs and the levels of B19V DNA replication (Fig. 10B and C). The levels of B19V entry into cells, in contrast, were found to be similar across all treatment groups (Fig. 10C), as well as those of cell survival and entered S-phase entry (Fig. 10D). Collectively, these experiments reveal that the level of B19V infection in CD36+/Epo+ EPCs is dependent on the Epo concentration, confirming that Epo plays an essential role in B19V replication.

**DISCUSSION**

In this study, we provide strong evidence that the Epo/EpoR/Jak2 signaling pathway is important for B19V replication in erythroid progenitor cells. Although a previous study had shown that Epo is essential for the differentiation of human hematopoietic stem cells into a population susceptible to B19V (52), the role of Epo in B19V infection was thought to be solely directing this differentiation specifically to BFU-E progenitors, CFU-E progenitors, and erythroblasts.

**Effects of Epo on differentiation of erythroid progenitor cells.** Erythropoiesis is a regulated process whereby hematopoietic progenitor cells give rise to committed erythroid progenitor cells, differentiate, and proliferate into mature red blood cells. This process involves an Epo-independent early phase, commitment of pluripotent hematopoietic stem cells to the erythroid lineage, and an Epo-dependent late phase during which these precursors mature into terminally differentiated, circulating enucleated erythrocytes. The commitment of hematopoietic stem cells to an erythroid progenitor (BFU-E and CFU-E) fate is independent of Epo expression (36, 40, 58, 59) and can be triggered by stem cell factor (SCF) (15, 33, 40, 59), interleukin-6 (IL-6) (51), and IL-3 (40). Epo acts late in erythropoiesis, is erythroid lineage specific, and supports the proliferation and maturation of committed erythroid progenitors (24, 36). Interestingly, Epo-independent erythrocyte production has also been reported (51).

We demonstrate that key markers of the erythroid progenitor fate, which are expressed on cells expanded in Wong medium (CD36+/Epo− EPCs), define these cells as CFU-E-type progenitors on day 8, and their counterparts expanded in StemCell medium (CD36+/Epo− EPCs) are defined as BFU-E-type progenitors, potentially at the verge of transitioning from the BFU-E to the CFU-E stage.

**Effects of Epo on B19V infection of erythroid progenitor cells, implication for mechanism of virus entry, and a major role in virus genome replication.** Our observation that CD36+/Epo− EPCs expanded in Wong medium allow B19V to enter cells at a particularly high efficiency level (approximately 17
times higher than that of the CD36/Epo− EPCs) was surprising. This could potentially be due to the observed increase in expression of the B19V coreceptor CD49e (55) or a consequence of the activities of as-yet-unidentified coreceptors. However, it is unlikely a consequence of KU80 binding as previously proposed (32), given that fewer than 5% of CD36/Epo− EPCs expressed KU80, yet more than 80% of these cells were infected by B19V (Fig. 1) (56). In addition, although a significantly high percentage of CD36/Epo− cells expressed KU80 (approximately 86%), the level of B19V entry into these cells was much lower than that into CD36/Epo+ EPCs (Fig. 2D). All of these results question KU80 as a coreceptor for B19V infection. Clearly, the mechanism by which B19V enters cells warrants further investigation.

On day 8 of culture, CD36+EPCs expanded in StemCell and Wong media were at a similar stage along the erythropoiesis pathway, and B19V entered both types of CD36+EPCs, albeit at different levels. A major difference in the absence of Epo stimulation, however, was the failure of B19V genomes present in CD36/Epo− EPCs to replicate. In light of our previous finding that expression of adenosivirus genes enables the B19V genome to replicate in B19V-nonpermissive 293 cells (22), we hypothesize that the current results reflect a requirement for a unique nuclear microenvironment for B19V replication. We propose that such an environment is created in erythroid progenitor cells by the Epo/EpoR/Jak2 signaling pathway.

Key role of the Epo/EpoR/Jak2 pathway in B19V infection of erythroid progenitor cells. Jak2 plays a pivotal role in Epo signal transduction (34, 42). This kinase associates with EpoR at a membrane-proximal region of the cytoplasmic domain, where its binding to EpoR is thought to induce a conformational alteration of the EpoR cytoplasmic domain. This structural change in turn leads to the juxtaposition of Jak2 molecules in a manner conducive to transphosphorylation within the activation loop and, thus, to Jak2 activation. Upon activation, Jak2 phosphorylates 8 tyrosine residues in the EpoR cytoplasmic domain and autophosphorylates a number of additional sites (25). In addition, phosphorylated EpoR recruits and mediates the activation of SH2-binding factors (including STATs, Grb2, and phosphatidylinositol 3-kinase [PI3K]) that signal through the Jak2/STAT5, Ras/MAPK, and PI3K/AKT pathways. Activation of these pathways synergistically prevents apoptosis of committed erythroid progenitors, allowing them to undergo a predetermined program of terminal proliferation and erythroid differentiation. We have shown here that inhib-
bition of either Jak2 activity or EpoR phosphorylation abolishes B19V replication in infected erythroid progenitor cells. Together with the results obtained using the Jak2-specific shRNA, these results lead us to believe that Epo/EpoR/Jak2 signaling must be involved in B19V replication and provide a strong support for further investigation into the mechanisms underlying B19V replication in erythroid progenitors.

Previous studies have shown that expression of the constitutively active EpoR \([\text{the EpoR(R129C) mutant}]\) can activate Jak2 in the absence of Epo binding (26). Our demonstration that Jak2 phosphorylation upon expression of this activated receptor in CD36 \(^-\)/Epo \(^-\) EPCs (Fig. 9A), and an accompanying increase in B19V infection, could be exacerbated by exposing cells to Epo at various concentrations (Fig. 10A) argues that the Epo/EpoR/Jak2 signaling pathway is critical to B19V infection. These conclusions are further supported by the fact that treating CD36 \(^-\)/Epo \(^-\) EPCs with Epo led to activation of B19V replication and to corresponding increases in the phosphorylation of both Jak2 and EpoR (Fig. 4). Collectively, our results strongly support the notion that signal transduction in response to EpoR activation involves the initial phosphorylation of Jak2, subsequent EpoR phosphorylation, and ultimately, B19V genome replication.

Interestingly, previous studies have indicated that that B19V can infect immortalized human microvascular endothelial cells and the human hepatocyte cell line HepG2 (18, 45), which might play a role in B19V infection-caused myocarditis (4, 6) and fulminant hepatitis (5, 23, 41, 48), respectively. EpoR is detectable on endothelial cells (35) and on hepatocytes (44), which may explain the B19V permissiveness of these cells under certain conditions.

In conclusion, our study provides evidence that Epo/EpoR/Jak2 signaling is essential for B19V replication and that EpoR activation either by Epo ligation or expression of constitutively active EpoR makes previously nonpermissive cells susceptible to B19V infection, activating replication of the B19V genome. It also indicates that Jak2-mediated phosphorylation of EpoR is necessary for this activation. This is a previously unappreciated role of Epo/EpoR/Jak2 signaling in supporting B19V infection and accounts for the unique tropism of B19V infection for human erythroid progenitors. In addition, our demonstration that AG490 abolishes B19V replication provides a potential starting point for the development of anti-B19V drugs.

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