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Simian fetal brain progenitor cells for studying viral neuropathogenesis

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The pathogenesis of neurologic dysfunctions caused by human immunodeficiency virus type 1 (HIV-1) infection is not yet well understood. Simian immunodeficiency virus (SIV) infection of macaques is an important animal model for HIV-1 infection. This is the first report to characterize brain progenitor cells (BPCs) isolated from embryonic brain of cynomolgus monkeys (*Macaca fascicularis*) by neurosphere assay and utilize BPC-derived cell culture for studying SIV infection. The self-renewal and multilineage differentiation properties of BPCs are convenient for planning viral infection experiments. The BPC-derived culture does not contain macrophage/microglial cells, fibroblasts, or endothelial cells. Thus, this culture is appropriate for studying direct relation between SIV infection and neuronal and glial cells. First, the authors characterized undifferentiated and differentiated simian BPCs by immunocytochemistry, flow cytometry analysis, real-time polymerase chain reaction (PCR), and reverse transcriptase (RT)-PCR. The BPCs induced to differentiate by the addition of 1% fetal bovine serum (FBS) were composed of heterogeneous cells expressing nestin, glial fibrillary acidic protein (GFAP), and/or tubulin beta III isoform (Tuj). None of them expressed the monocyte/macrophage/microglial marker. mRNA expression of CD4, CXCR4, CCR5, GPR1, STRL33, and APJ in both undifferentiated and differentiated BPCs were shown by RT-PCR method, suggesting that SIV would infect and replicate in this culture system. Then, it was confirmed that the neurotropic SIV strain, SIV17/E-Fr, replicated productively in BPC-derived cells. The SIV/17E-FrΔnefGFP was inoculated to identify the infected cells and immunocytochemistry analysis revealed that green fluorescent protein (GFP)-expressing cells were mostly GFAP positive and coexpressed with SIV p27 antigen. Thus, BPC-derived cell culture system is applicable for studying SIV infection in glial and neuronal cells. *Journal of NeuroVirology* (2007) **13,** 11–22.

Keywords: encephalitis; macaque; neurosphere assay; SIV

Introduction

Human immunodeficiency virus type 1 (HIV-1) induces encephalitis, dementia, or a milder form of neurologic dysfunction called minor cognitive/motor disorder (MCMD) in the era of highly active antiretroviral therapy (HAART) (Gonzalez-Scarano and Martin-Garcia, 2005). The pathogenesis of HIV encephalitis (HIVE) is not yet well understood. There is a consensus that the principle central nervous system (CNS) cell types productively infected by HIV

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are perivasucular macrophages and microglial cells and that the neurological abnormalities are caused by some inflammatory cytokines, chemokines, and viral antigens released from these cells (Fischer-Smith *et al*, 2001; Kim *et al*, 2003). On the other hand, there is no consensus that cells of neural origin (neurons, astrocytes, oligodendrocytes) support a productive infection of HIV-1. With more sensitive detection techniques, such as *in situ* polymerase chain reaction (PCR) (Bagasra *et al*, 1996; Nuovo *et al*, 1994) and microdissection-PCR (Fassunke *et al*, 2004; Torres-Munoz *et al*, 2001), HIV RNA and HIV DNA were detected also in neurons or astrocytes, though so far productive infection in these cells has not yet been reported. It was reported that HIV infections in astrocytes were supposedly restricted to early gene products and were not productive both *in vivo* and *in vitro* infection (Gorry *et al*, 1999; Messam and Major, 2000; Tornatore *et al*, 1994). The contribution of this restricted infection in astrocytes to neuropathogenesis remains unknown. simian immunodeficiency virus (SIV) infection of macaques is an important animal model for studying the neuropathogenesis of HIV infection (Gonzalez *et al*, 2000; Kestler *et al*, 1990; Zink *et al*, 1998). As for SIV infection, neurotropic strains of SIV had been reported by the John Hopkins university laboratory (Flaherty *et al*, 1997; Mankowski *et al*, 1997; Sharma *et al*, 1992; Zink *et al*, 1997). The molecular clone of infectious SIV, SIV/17E-Fr, were shown to replicate productively in primary rhesus macaque astrocytes (Overholser *et al*, 2003, 2005). Primary fetal astrocyte cultures have been generally used for investigating the mechanism of SIV infection in CNS, though it was difficult to establish a culture consisting of more than 99% astrocytic marker–positive cells and the cultures often had the risk of being contaminated with microglial cells, in which SIV might replicate. In addition, they have limited in the number of experiments that can be performed on them. On the other hand, primary neural stem cells (NSCs) and brain progenitor cells (BPCs) isolated from embryonic brains by neurosphere assay have the capacity to proliferate and to differentiate into precursor cells expressing neuronal or glial phenotypes (Reynolds *et al*, 1992; Reynolds and Weiss, 1992). The differentiated BPC cultures do not contain any monocyte/macrophage, microglial and endothelial phenotypes and it is possible to analyze SIV infection in neuronal or glial cells directly. Recently, NSCs and BPCs have been used for studying mouse cytomegalovirus (CMV) infection (Kosugi *et al*, 2000), HIV-1 infection (Lawrence *et al*, 2004), and JC virus (JCV) infection (Messam *et al*, 2003). Thus we planned to use these cultures for studying SIV infection. There have been extensive studies on rodent or human BPCs (Davis and Temple, 1994; Roy *et al*, 2000; Uchida *et al*, 2000; Westerlund *et al*, 2003), but relatively fewer reports exist on nonhuman primates, and most studies were about progenitor cells derived from embryonic stem (ES) cells (Asano *et al*, 2002; Calhoun *et al*, 2003; Vrana *et al*, 2003).

In this study, we characterized NSCs/BPCs isolated from embryonic brain of cynomolgus monkeys (*Macaca fascicularis*) by neurosphere assay. Then, we confirmed the utility of BPC-derived cell culture system for studying SIV infection in glial and neuronal cells.

Result

Isolation and differentiation of multipotential simian progenitor cells

The cells isolated from simian fetal brain were cultured in serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Only growth factor–responsive cells proliferated as floating clusters termed neurospheres within a few days (Figure 1A). Under these neurosphere-generating conditions, both NSCs and progenitor cells survived. We treated these cells together as BPCs. They easily adhered to the plastic and began to extend processes, so the flasks were gently knocked every day to prevent attachment. The spheres were dissociated by trypsinization twice a week. A single dissociated cell is 10 to 15 μm in diameter (Figure 1B). The primary neurosphere-derived BPCs proliferated again to form secondary spheres. *In vitro* differentiation was induced by culturing the dissociated cells with medium containing 1% fetal bovine serum (FBS) without growth factors. Cells with apparent morphological changes appeared at 1 day post induction of differentiation (dpid). At 14 dpid, these cells consisted of small cells with neurite-like processes and elongated cells with a polygonal appearance (Figure 1C).

Characterization of BPC phenotypes by immunocytochemistry

Immunocytochemistry was performed on BPCs before and after induction of differentiation using various cell type–specific phenotypic markers for progenitor cells, astrocytes, neurons, oligodendrocytes, and microglia. Nestin is an intermediate filament protein and is expressed in the developing CNS (Lendahl *et al*, 1990). The prospective phenotypic marker of BPCs in the neurosphere cultures is unknown, though nestin is used as a retrospective phenotypic marker of stem cells and progenitor cells (Messam *et al*, 2000, 2002).

Before induction of differentiation, almost all BPCs expressed nestin (Figure 1D) and the astrocytic marker glial fibrillary acidic protein (GFAP) (Figure 1E, H). Some BPCs expressed the neuronal marker beta-III-tubulin (Tuj) (Figure 1G). Double-immunofluorescence staining showed colocalization of nestin and GFAP (Figure 1F). Unexpectedly some cells coexpressed GFAP and Tuj (Figure 1I).

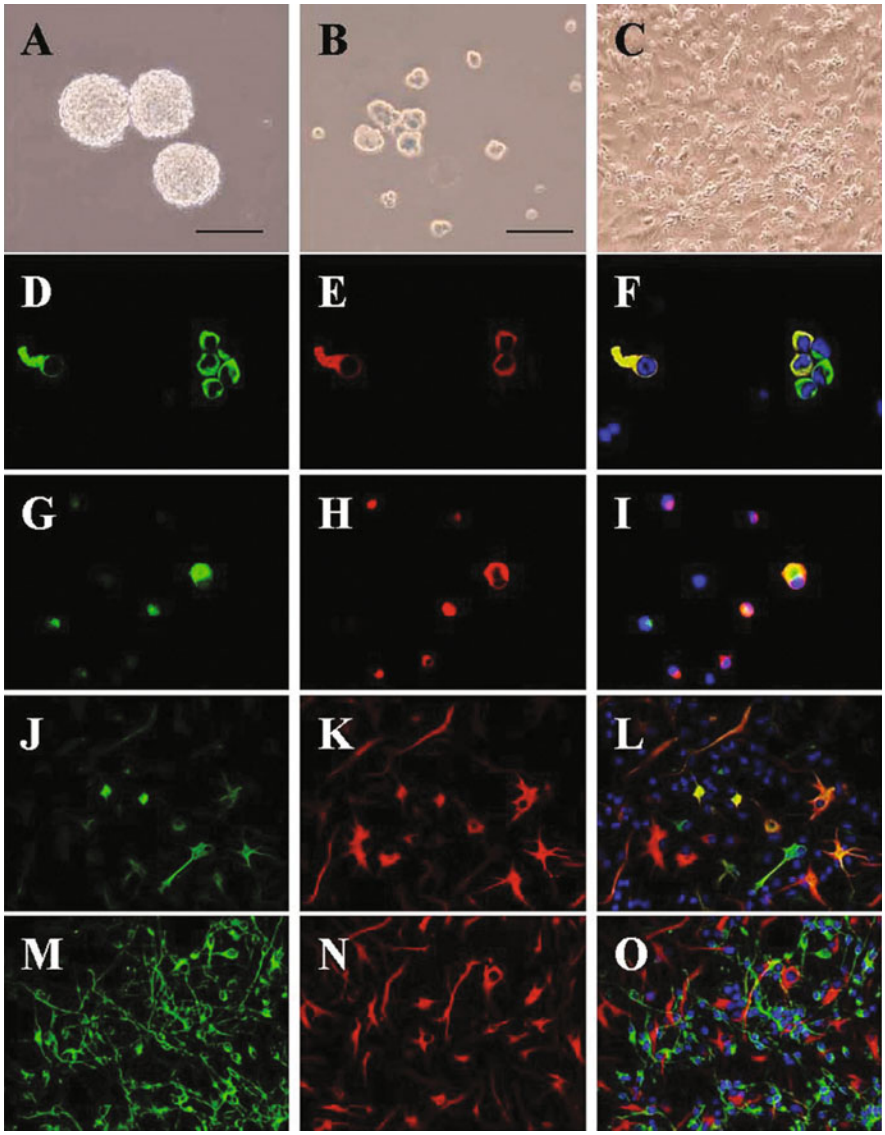

Figure 1 (**A–C**) Phase-contrast photographs of fetal macaque brain progenitor cells (BPCs). (**A**) The BPCs form neurospheres in the presence of EGF and bFGF. (**B**) A single BPC and dividing BPCs. (**C**) The BPCs 14 days post induction of differentiation (dpid). These BPC-derived cells consist of small cells and elongated cells. (**D–O**) Immunofluorescence staining of BPCs before (**D–I**) and after (**J–O**) induction of differentiation. Double-immunofluorescence stainings show GFAP (**E, H, K, N**) immunoreactivity *(red)* and nestin (**D, J**) or Tuj (**G, M**) immunoreactivity (*green*) colocalization in some cells (**F, I, L, O,** *yellow*). Nucleic acids are counterstained with topro-3 *(blue)*. Cells were imaged using an LSM 410 inverted laser-scanning microscope. (**A, B**) Scale bar, 50 μm. Original magnifications: (**A**, **B**, **J–O**) $\times$200, (**C**) $\times$100, (**D–I**) $\times$630.

At 14 dpid, the cells still expressed nestin (Figure 1J) and some of them expressed also GFAP (Figure 1L). Most cells expressed GFAP or Tuj (Figure 1M, N). The expression of another neuronal marker, mitogen-activated protein (MAP)-2, was also detected (data not shown). Morphologically, the elongated polygonal cells were positive for GFAP, and the small cells with neurite-like processes were positive for Tuj. The cells coexpressing GFAP and Tuj were hardly observed at this time point (Figure 1O). We failed to detect cells expressing the oligodendrocyte marker, O4. The BPCs, neither undifferentiated nor differentiated, expressed the monocyte/macrophage/microglial marker (HAM56) (data not shown).

Characterization of BPC phenotypes by flow cytometry

We examined the expression levels of cellular markers in BPCs before and after induction of differentiation by flow cytometry. The FSC/SSC (forward scatter/side scatter) profiles showed that the BPCs were composed of heterogeneous cell populations

Table 1 Percentage of analyzed cells expressing nestin, GFAP, and Tuj by flow cytometric analysis

	Percentage of analyzed cells expressing each phenotypic marker (%)								
Gate*	Nestin	GFAP	Tuj	N+/G+	N+/G−	N−/G+	T+/G+	T+/G−	T−/G+
R1	96.1	98.2	89.5	75.8	18.8	3.6	68.5	16.2	12.2
R2	91.9	99.0	59.4	91.4	4.0	4.1	61.3	2.5	35.1
R3	89.8	93.6	29.2	81.3	1.9	13.4	14.9	1.0	80.3
R4	18.9	54.4	54.4	11.1	8.9	55.7	29.3	27.8	18.5
R5	41.9	91.6	93.1	19.3	6.4	25.2	58.0	31.5	5.3
R6	34.3	87.7	73.9	22.6	1.8	44.4	46.1	14.9	31.2
R7	36.3	96.8	41.1	30.6	0.7	60.1	29.3	3.0	65.5
R8	30.5	99.2	44.3	8.9	0.4	84.6	13.7	0.7	83.9

*Gates defined in Figure 2. R1 to R3 are gates of BPCs before induction of differentiation. R4 to R8 are gates of BPC-derived cells 28 days post induction of differentiation.
N: nestin; G: GFAP; T: Tuj.

(Figure 2). According to these profiles, we set up three gates (R1, R2, R3) for the undifferentiated BPCs (Figure 2A) and five gates (R4, R5, R6, R7, R8) for the differentiated BPCs (Figure 2B). The expression levels of cellular markers in the cells of each gate were examined and summarized in Table 1.

Firstly, the expression level of nestin, GFAP, and Tuj in BPCs of each gate were examined with single-color flow cytometry by using specific antibodies as described previously in Materials and Methods. Before induction of differentiation, nestin was expressed in 96.1%, 91.9%, 89.8%, and GFAP was expressed in 98.2%, 99.0%, 93.6% of cells in fraction R1, R2, R3, respectively. Tuj was expressed in 89.5% in cells of a smaller size (R1) and 29.2% in cells of a larger size (R3). After induction of differentiation, at 28 dpid, the percentage of nestin-positive cells decreased to 32.3% from 92.6% on average. GFAP was expressed in 99.2% in larger cells (R8) and Tuj was expressed in 93.1% of smaller cells (R5).

Secondly, coexpression of GFAP and nestin, GFAP, and Tuj was examined by two-color flow cytometry. Before induction, the percentages of nestin(+)/GFAP(+) double-positive cells were 75.8%, 91.4%, and 81.3% and for Tuj(+)/GFAP(+) double-positive cells were 68.5%, 61.3%, and 14.9% of cells in fraction R1, R2, R3, respectively. These percentages of double positive cells decreased at 28 dpid. The percentage of nestin(+)/GFAP(−) cells was higher in smaller cells (18.8%, R1) and decreased to 8.9% (R4) at 28 dpid. The percentage of nestin(−)/GFAP(+) cells was higher in larger cells (13.4%, R3) and increased to 84.6% (R8) at 28 dpid. The percentage of Tuj(+)/GFAP(−) cells was higher in smaller cells (16.2%, R1) and increased to 31.5% (R5) at 28 dpid. In summary, double-positive cells and nestin-positive cells decreased and GFAP- or Tuj-positive cells increased after induction of differentiation. GFAP-positive cells were mainly larger cells (R8), and Tuj-positive cells were mainly smaller cells (R4, R5).

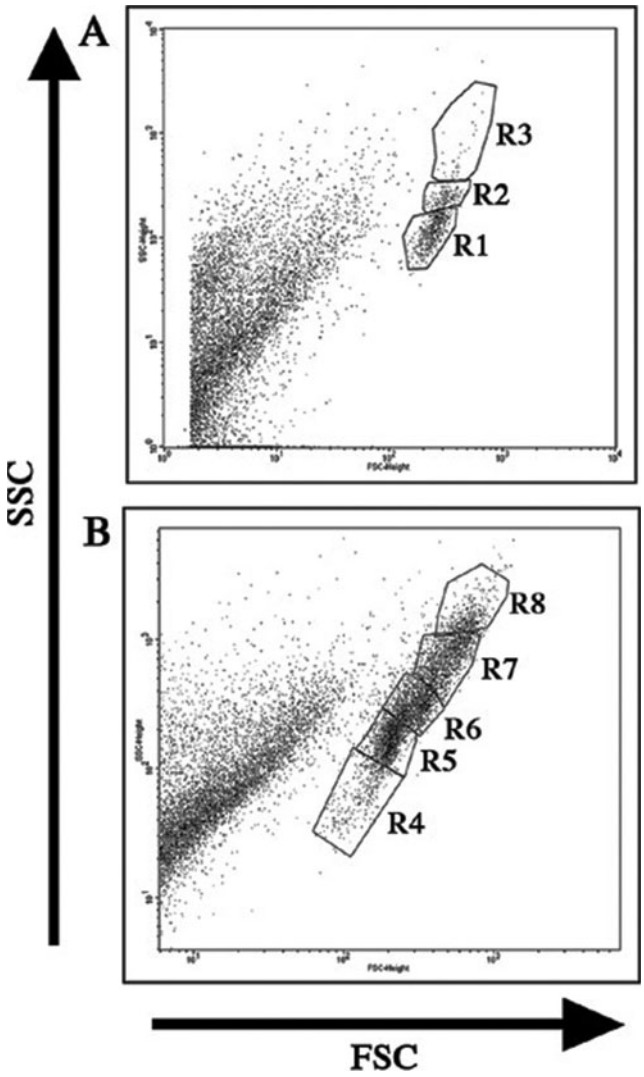

Figure 2 Flow cytometric analysis of BPCs before and after induction of differentiation. FSC/SSC (forward scatter/side scatter) profiles show the heterogeneity of the cells. Conventionally, three gates were set (R1, R2, and R3) for the undifferentiated BPCs (**A**) and five gates (R4, R5, R6, R7, and R8) for the differentiated BPC-derived cells (**B**). The expression levels of cellular markers in the cells of each gate were examined and summarized in Table 2.

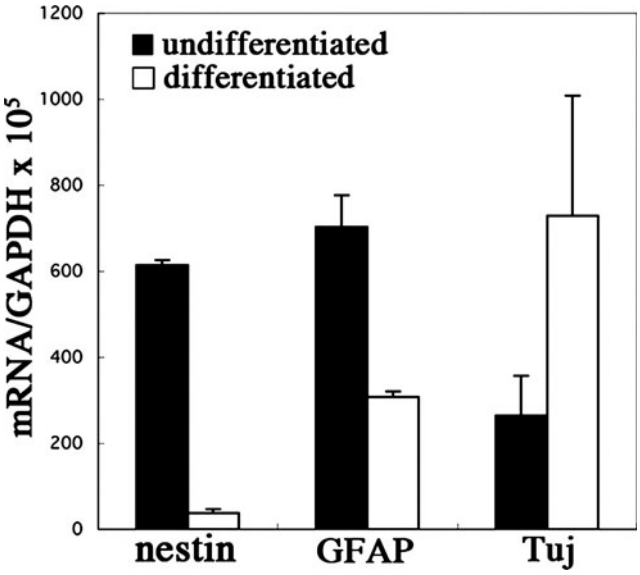

Figure 3 mRNA expression levels of nestin, GFAP and Tuj in undifferentiated BPCs and differentiated BPCs (14 dpid) were analyzed by real-time PCR. In undifferentiated BPCs *(black bars)*, mRNA expression levels of nestin and GFAP were higher than that of Tuj, while in differentiated BPCs (14 dpid) *(white bars)*, mRNA expression levels of nestin and GFAP decreased significantly, and that of Tuj increased. This study was performed three times with similar results.

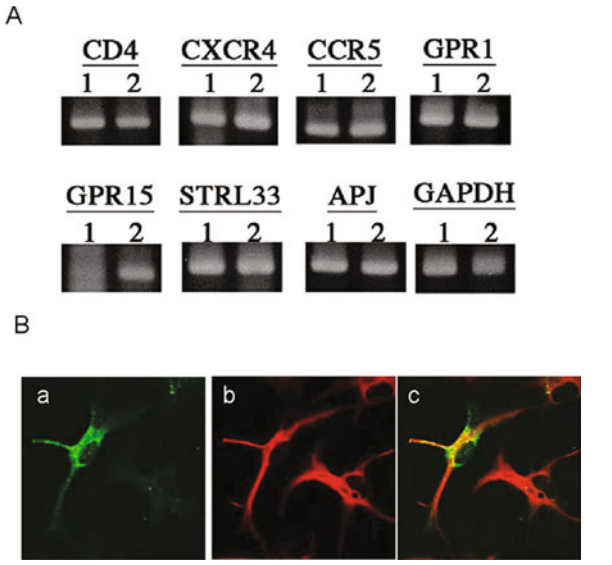

Figure 4 (**A**) mRNA expression of CD4, CXCR4, CCR5, GPR1, GPR15, STRL33, and APJ, examined by RT-PCR analysis with 1 μg of total RNA isolated from BPCs before and after induction of differentiation. Amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide. GAPDH served as an internal control for the standardization of each product. CD4 and the examined chemokine receptors except GPR15 mRNAs were all expressed in undifferentiated and differentiated BPCs. These results were representative for at least three independent experiments and individual samples were analyzed three times by RT-PCR. Lane 1: undifferentiated BPCs. Lane 2: differentiated BPCs. (**B**) Double-immunofluorescence stainings show CCR5 (**a**) immunoreactivity (*green*) and GFAP (**b**) immunoreactivity (*green*) colocalization (**c**, *yellow*) in the differentiated BPCs. Original magnifications: (**a. b, c**) ×400.

mRNA expression levels of Nestin, GFAP, and Tuj in BPCs before and after differentiation

To confirm the results of immunofluorescence analysis further, we examined mRNA expression levels of nestin, GFAP, and Tuj in BPCs cultures before and after induction of differentiation by real-time PCR (Figure 3). The mRNA expression levels of nestin in BPCs decreased significantly after induction of differentiation. GFAP mRNA was expressed at high levels before induction and the levels decreased at 14 dpid. mRNA expression levels of Tuj increased after induction.

Expression of CD4 and chemokine receptors on simian BPCs

In the CNS, chemokines and their receptors are involved in the migration, differentiation, and activation of some cells and in the proliferation of glial and neuronal cells. Reportedly, many members of the CXCR family and the CCR family are expressed in the brain (Ji *et al*, 2004; Klein *et al*, 1999). It is known that the entry of SIV into cells involves a series of interactions with CD4 and coreceptors and also that several SIV strains are capable of using CCR5 as a primary receptor to infect CD4-negative cells (Edinger *et al*, 1997, 1999; Overholser *et al*, 2005). The receptor or the coreceptor can be one of a variety of seven transmembrane G-protein-coupled chemokine receptors. We examined the mRNA expression of CD4 and several chemokine receptors that are candidates for coreceptors of SIV; CXCR4, CCR5, GPR1, GPR15, STRL33, and APJ (Choe *et al*, 2000; Edinger *et al*, 1998; Westmoreland *et al*, 2002) in BPCs before and after induction of differentiation. Reverse transcriptase (RT)-PCR analysis confirmed the expression of CD4 and the examined chemokine receptors except GPR15 in both undifferentiated and differentiated BPCs. The expression of GPR15 was turned to be positive after induction of differentiation (Figure 4A). As for CD4 and CCR5, immunocytochemistry was performed on the differentiated BPCs. As shown in Figure 4B, some of the GFAP-positive cells coexpressed with CCR5 (Figure 4B), though we failed to detect CD4-positive cells (data not shown).

SIV infection in differentiated simian BPC-derived cell culture

We examined whether the differentiated simian BPC cultures supported SIV infection and the infection was productive or restricted. When the dissociated BPCs were plated on poly-O-coated 6-well plate with medium containing 1% FBS (MHM1%FBS) for induction of differentiation, they were inoculated with SIVmac239 and SIV/17E-Fr. The clone SIV17EΔnefGFP was also inoculated to identify infected cells. As shown in Figure 5A, the neurotropic SIV/17E-Fr replicated most efficiently, but the parental SIVmac239 replicated hardly in the

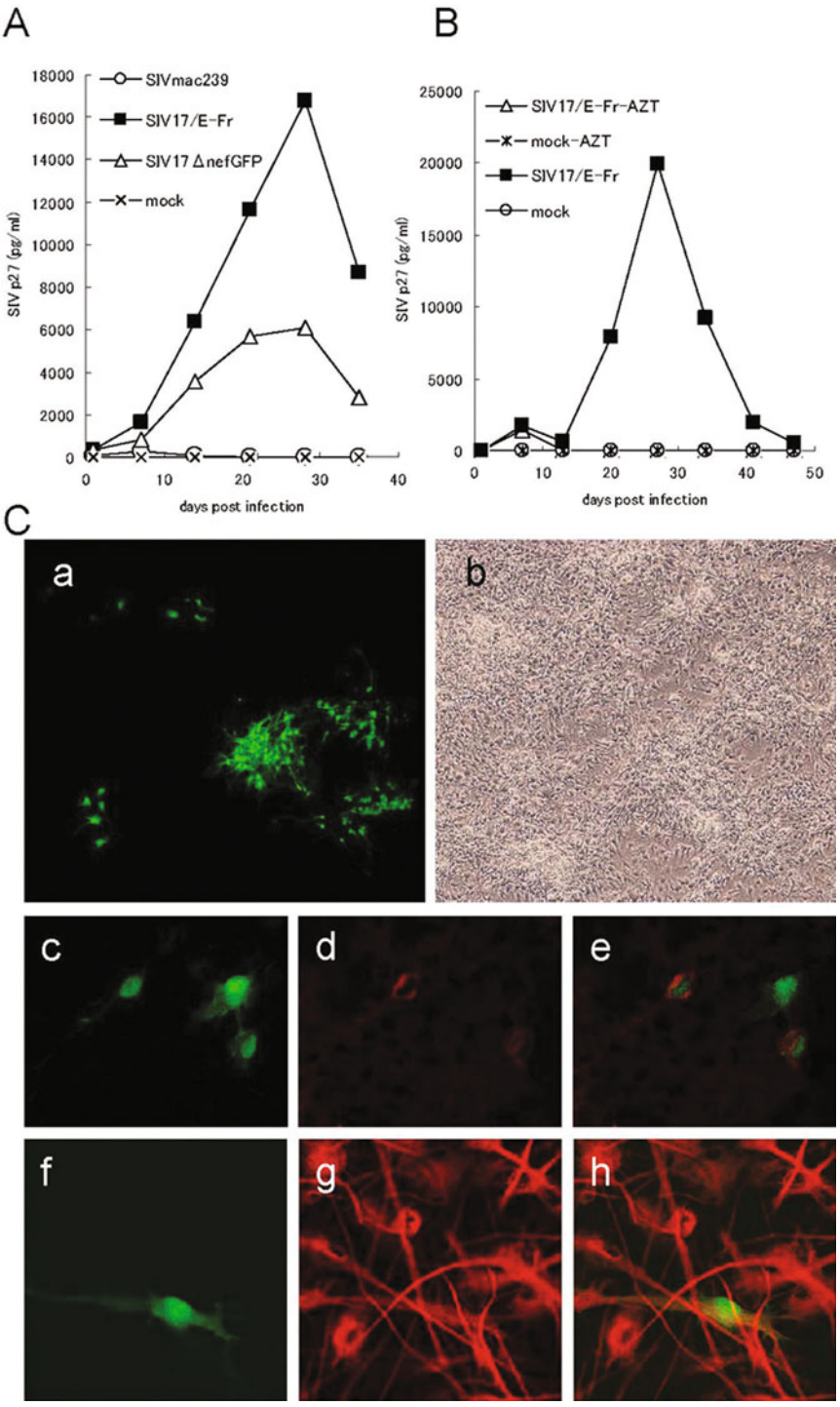

Figure 5 SIV infection of differentiated BPCs. The dissociated BPCs were plated on poly O-coated plates and were incubated with each SIV stock (100 ng of SIV p27) for 16 h in MHM1%FBS. After infection, the cells were washed 3 times extensively and fresh MHM1%FBS was added and the culture supernatants were sampled at various days post infection (dpi) and assayed for SIV p27. (**A**) SIV/17E-Fr replicates most efficiently, though the parental SIVmac239 replicates at very low levels. The SIV17EΔnef GFP also replicates efficiently, more than SIVmac 239 but less than SIV/17E-Fr. Results shown are representative of several independent experiments with BPC cultures from several macaques. (**B**) SIV p27 production depended on reverse transcription (RT). To examine whether the peak of the SIV p27 value depended on the RT step, 10 mM AZT (RT inhibitor) was added to the media every 3 days after overnight infection. SIV17/E-Fr replicates hardly in the presence of AZT in media. (**C**) (**a**) The expression of GFP in the cells infected with SIV17EΔnef GFP were observed under a fluorescent microscope (14 dpi). (**b**) Phase-contrast photographs of (**a**). (**c, d, e**) A cell infected with SIV17EΔnef GFP expressing GFP (**c,** *green*) and immunostained with mouse monoclonal anti-SIVp27 antibody using Alexa568-conjugated secondary antibody (d, *red*). Marged image (**e,** *yellow*). (**f, g, h**) A cell infected with SIV17EΔnef GFP (**f,** *green*) identified as GFAP-positive by immunostaining with rabbit polyclonal anti-GFAP antibody using Alexa568-conjugated secondary antibody (**g,** *red*). Marged image (**h,** *yellow*). Original magnifications: (**a, b**) ×100, (**c–h**) ×400.

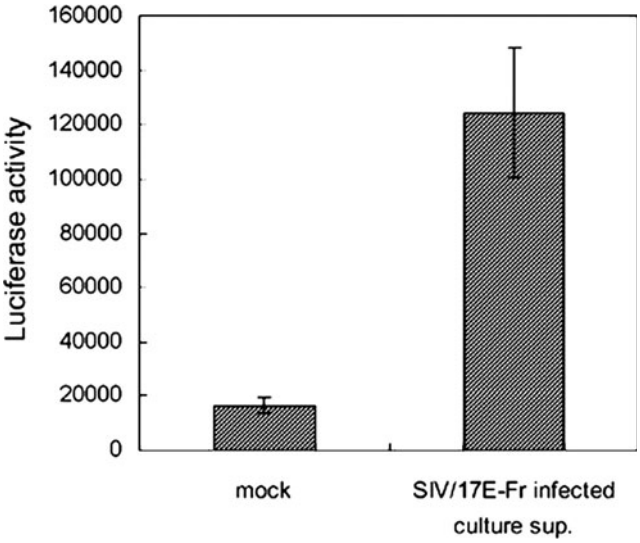

Figure 6 Luciferase activity in LuSIV cells at 72 h post infection with the supernatant of BPC-derived cell culture infected with SIV/17E-Fr and mock. The fold induction of luciferase activity over background was 7.57 ± 0.64.

differentiated BPC cultures. The SIV17EΔnefGFP also replicated more efficiently than SIVmac 239 but less than SIV/17E-Fr. The experiments were performed twice independently and the same results were obtained (Figure 5A).

To investigate the infectivity of SIV released from the SIV/17E-Fr–infected BPC-derived cell culture, the culture supernatant was assayed by the LuSIV system (Roos *et al*, 2000; Overholser *et al*, 2003). The culture supernatants were collected from the other infection experiment on 36 dpi. Though the assayed supernatant contained only 1.86 ng of SIV p27, the fold induction of luciferase activity over background was 7.57 ± 0.64 (Figure 6).

To examine whether the peak of SIV p27 value in the culture supernatant infected with SIV17/E-Fr depended on the reverse transcription step or not, 10 mM of AZT (reverse transcriptase inhibitor) was added to the medium every 3 days after overnight infection. As shown in Figure 5B, SIV17/E-Fr replicated hardly in the presence of AZT in the medium. As for SIV17EΔnefGFP infection, GFP expression was observed under fluorescence microscopy every day post infection. GFP-positive cells appeared at 4–6 days post infection and variable shapes of GFP-positive cells were observed at 14 days post infection (Figure 5C, a). The number of GFP-positive cells increased in parallel with the SIV p27 antigen titer in the supernatant (Figure 5C, a, b). The GFP-positive cells were confirmed to express SIV p27 gag antigen by immunostaining (Figure 5C, c to e). We examined the phenotype of the GFP-positive cells by immunostaining with antibodies for GFAP and Tuj. Most of these cells were positive for GFAP (Figure 5C, f to h). These results suggested that the simian fetal BPC-derived cell culture can be used for studying neuropathogenesis of SIV in the CNS.

Discussion

In the present study, we attempted to establish an *in vitro* culture system for investigating neuropathogenesis of SIV encephalitis using primary BPCs isolated from embryonic brains of Cynomolgus macaque (*Macaca fascicularis*) by neurosphere assay. The neurosphere assay has been developed primarily as a valuable tool for isolating NSCs (Reynolds and Weiss, 1992, 1996). However, recently, the NSCs isolated by neurosphere assay were found to be mostly non-stem BPCs (Maric and Barker, 2004; Suslov *et al*, 2002) and the assay expanded both stem and non-stem progenitor cell population (Reynolds and Rietze, 2005). Thus, we treated all the cells isolated by the neurosphere assay as BPCs in this report.

Primary BPC-derived cell culture has an advantage over conventional primary brain cell culture. The self-renewal and multilineage differentiation properties of BPCs are very convenient for planning viral infection experiments. It is possible to increase cells with mitogens and to prepare cell cultures by inducing differentiation by adding appropriate reagents to the culture medium according to each purpose (Caldwell *et al*, 2001). Therefore, there are no limitations for repeated experiments and the valuable simian embryonic brain can be used effectively. It is generally difficult to completely exclude any monocyte/macrophage/microglial cells, fibroblasts, and endothelial cells from conventional primary brain cell culture, though BPC-derived culture never contain such cells.

We induced differentiation of simian BPCs by culturing the cells with the medium (Dulbecco's modified Eagle's medium [DMEM]/F-12 plus hormone mix) supplemented with 1% fetal bovine serum (FBS). We characterized both undifferentiated and differentiated BPCs by examining the expression of nestin, GFAP, and Tuj in BPCs by immunocytochemistry, flow cytometry, or quantitative RT-PCR and the results obtained with these different methods were in agreement. The BPCs revealed heterogeneity both before and after induction of differentiation as shown in Figure 1 and 2. Before induction, more than 90% of cells expressed nestin or GFAP and some cells expressed Tuj. There were even nestin(+)/GFAP(+) cells and Tuj(+)/GFAP(+) cells. The mRNA expression level of GFAP was as high as the expression level of nestin before induction and the levels decreased at 14 dpi. This was because GFAP-positive cells did not always differentiate into astrocytes after induction. Several reports supported the fact that astrocytes, which are defined as GFAP-positive cells, globally possess NSC attributes and form neurospheres that give rise to both neurons and glias (Doetsch, 2003; Doetsch *et al*, 1999;

Laywell *et al*, 2000; Seri *et al*, 2001). After induction of differentiation, the number of nestin-positive cells decreased, which is shown in Figure 3. The decrease in mRNA expression level of GFAP suggested that some GFAP-positive cells changed to GFAP-negative cells after induction of differentiation. In total, the number of Tuj(+)/GFAP(+) cells decreased and nestin(−)/GFAP(+) or Tuj(+)/GFAP(−) cells increased. No detectable expression of monocyte/macrophage/microglia markers (data not shown) was confirmed.

The final aim of this study was to establish an *in vitro* culture system consisting of only neuronal and glial cells to investigate the direct relation between SIV infection and these cells. SIV infects and replicates much more efficiently in macrophages and microglial cells than in neuronal and glial cells. If the culture contains macrophages or microglial cells, it is difficult to determine whether neural damage is due to a direct effects of SIV infection or due to secondary effects of infected macrophages or microglial cells. SIV17/E-Fr is a neurotropic SIV strain that replicates productively in astrocytes (Flaherty *et al*, 1997). SIV17/E-Fr replicated also in the BPC-derived cells (Figure 5), though the value of SIV p27 gag protein in the culture supernatant was not so high. This was because the culture consisted of various cell types in neuronal and glial lineage and only a few of them were permissive to SIV infection. This heterogeneity of the culture changed and depended on the day post induction of differentiation (dpid). Thus, we did the infection experiments also at 1, 3, 7, and 14 dpid. The results of each experiment were similar among them, if the BPCs were derived from the same simian fetus. We should investigate in detail the differences of the direction of differentiation between SIV-infected BPCs and mock-infected BPCs.

To identify the permissive cells, we inoculated SIV17EΔnefGFP and observed the GFP-expressing cells under a fluorescent microscopy after infection. The SIV17EΔnefGFP also replicated efficiently in BPC-derived cells but less than SIV/17E-Fr. SIV17EΔnefGFP might require the Nef protein for efficient and optimal replication (Gorry *et al*, 1998; Overholser *et al*, 2003). The number of GFP-positive cells was less than expected. It was confirmed that GFP-expressing cells (Figure 5C, c) expressed SIV p27 gag antigen (Figure 5C, d, e) and almost all GFP-expressing cells were GFAP positive (Figure 5C, f to h) and Tuj negative (data not shown). These suggested that SIV/17E-Fr and SIV17EΔnefGFP infected astrocytes in the BPC-derived cell culture. It was reported that SIV/17E-Fr used CCR5 for entry and replication in the primary rhesus macaque astrocytes (Overholser *et al*, 2003). In our study, the coreceptor utilization was not clearly determined, though we confirmed some GFAP-positive cells co-expressed with CCR5 in the BPC-derived cell culture (Figure 4B). We also determined whether recombinant human RANTES, one of the ligands of CCR5, could block the entry of SIV/17E-Fr into the BPC-derived cell. The peak of the value of SIV p27 decresed to 21% in the supernatant of culture treated with 100 ng/ml of RANTES (Peprotech, NJ, USA) compared with that in the absence of RANTES (data not shown). Thus, RANTES partially inhibited SIV entry and replication in the BPC-derived cell culture and CCR5 was utilized by SIV/17E-Fr. The GFP-expressing cells should be characterized further in detail and should be cloned, if possible.

The number of GFP-expressing cells increased in parallel with the value of SIV p27 in the culture supernatant. Both the number of GFP-expressing cells and the value of SIV p27 were reduced by adding AZT to the culture medium (Figure 5B). This suggested that the production of SIV p27 and the expression of GFP depended on the step of reverse transcription. Thus, the value of SIV p27 in the supernatant around 28 dpi were supposed to be derived from newly produced SIV/17E-Fr. Adding this, the results of LuSIV assay showed the infectivity of SIV released from the SIV/17E-Fr-infected BPC-derived cell culture (Figure 6). In summary, BPC-derived cells supported productive infections of SIV17/E-Fr and SIV17EΔnefGFP, though the amount of viral products was low.

We showed that simian BPC-derived cell culture supported a productive infection of SIV. This culture system is easy to establish and maintain and, if necessary, it is possible to coculture with microglial cells or monocytes/macrophages (data not shown). It is applicable to investigate viral neuropathogenesis other than SIV.

Materials and methods

Cell culture

BPCs were isolated from the fetal brain of Cynomolgus macaque (*Macaca fascicularis*) at 8 to 11 weeks of gestation in accordance with institutional ethical guidelines. Fetal brains were removed, cut into 1-mm coronal sections and triturated gently with a pipette in phosphate-buffer saline (PBS). After mechanical dissociation, cells were washed in PBS, centrifuged at 600 rpm for 5 min, and resuspended in Neural Progenitor Cell Medium (NPBM; Clonetics, San Diego, USA) including epidermal growth factor (EGF) (20 ng/ml; Progen, Heidelberg, Germany) and basic fibroblast growth factor (bFGF) (20 ng/ml; Progen) as described by Reynolds and Weiss (Reynolds *et al*, 1992; Reynolds and Weiss, 1992). The cell suspension was cultivated in uncoated 75-cm^2 cell cultureflasks (Asahi Techno Glass, Tokyo, Japan) at a density of 1.0×10^6/ml at 37°C with 5% CO_2. The cells formed spheres within approximately one week. Every 5 to 7 days, the spheres were dissociated with 0.125% trypsin-EDTA (Invitrogen, CA, USA) for 10 min at 37°C. Following rinsing with 1 mg/ml trypsin inhibitor (Roche Diagnostics, Boehringer Mannheim,

Germany), single cells were centrifuged at 1000 rpm for 5 min and resuspended in fresh medium. After more than five passages of growth factor selection, the cells were used for the experiments in the present study.

For the induction of differentiation, the dissociated cells were plated on 6-well plates precoated with 30 μg/ml of poly-L-ornithine hydrobromide (poly O; Sigma-Aldrich, St. Louis, MO, USA), at a density of 3 $\times$ 10^5 cells per well. The differentiated BPC culture medium (MHM1%FBS) was composed of DMEM/F-12 (Invitrogen) supplemented with a defined hormone and salt mixture composed of insulin, transferrin, progesterone, putrescine, and sodium selenite (all from Sigma) supplemented with 1% fetal bovine serum (FBS), amphotericin B (1.25 μg/ml), penicillin (50 μg/ml), streptomycin (50 μg/ml), and neomycin (100 μg/ml) (all from Invitrogen) and lacking growth factors. The medium was changed every 7 days.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature, rinsed in PBS, permeabilized with 0.1% Triton-X (Wako Chemicals, Osaka, Japan)/PBS for 10 min at room temperature, and blocked with normal goat serum for 30 min at room temperature. Cells were then incubated at 4°C overnight with mouse monoclonal antibodies against human nestin (1:10; R&D Systems, MN, USA), tubulin beta III isoform (Tuj) (1:200; Chemicon), MAP-2 (1:200; Chemicon), oligodendrocyte marker O4 (O4) (1:50; Chemicon), human macrophage (HAM56, 1:50; DakoCytomation, Kyoto, Japan), CD4 clone 1F6 (1:10; NICHIREI Bio. Osaka, Japan), CCR5 clone 3A9 (1:100; BD PharMingen, CA, USA), and SIV p27 gag protein (1:100) or a rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (1:1; DakoCytomation). After 3 washes in PBS, cells were incubated in either Alexa 488–conjugated goat anti-mouse immunoglobulin G (IgG) (1:200; Molecular Probe, Eugene, OR), Alexa 568–conjugated anti-mouse IgG (1:200; Molecular Probes), Alexa 568–conjugated anti-rabbit IgG (1:200; Molecular Probes), or fluorescein-conjugated anti-mouse IgM (Vector laboratories, Burlingame, CA) for 1 h at 37°C. Nucleic acids were counterstained with topro-3 (1:1000; Molecular Probes). Fluorescence was analyzed with an LSM 410 inverted laser-scanning microscope (Carl-Zeiss, Jena, Germany).

Flow cytometry
For flow cytometry, 0.125% trypsin-treated undifferentiated and differentiated BPCs were fixed with 4% PFA/PBS at 4°C for 30 min. After 3 washes in PBS and an incubation with 5% goat serum/PBS for 30 min on ice, cells were stained with the following primary antibodies: mouse monoclonal antibodies against human nestin (1:10; R&D), Tuj (1:500; Chemicon), or a rabbit polyclonal antibody against GFAP (1:1; DakoCytomation), for 30 min on ice at 10^6 cells/ml. The mouse IgG_{2b} isotype control (1:10; Cymbus Biothechnology) and N-universal negative control (1:1; Dako Cytomation) were used as negative controls for mouse monoclonal antibodies and the rabbit polyclonal antibody, respectively. After 3 washes in PBS, phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin specific polyclonal antibody (1:200; BD Biosciences Pharmingen, San Diego, CA, USA) or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin-pecific polyclonal antibody (1:200; BD Bioscience Pharmingen) were added for secondary labeling. The stained cells were washed with PBS, and resuspended in 0.1% bovine serum albumin (BSA)/PBS for flow cytometry using FACS caliber (BD, NJ, USA).

RT-PCR and real-time PCR analysis
Total RNA was extracted from BPCs using Quick gene RNA cultured cell kit S and Quick gene-800 (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. The concentration of total RNA was estimated by measuring the optical density. The extracted RNA samples were subjected to reverse transcription (RT) using oligo$(dT)_{15}$ and the Omniscript RT kit (Qiagen, CA, USA) according to the manufacturer's protocol. PCR was performed in 20 μl of reaction mixture containing cDNA derived from 1 μg of RNA, each primer pair (Table 2) and the TAKARA Premix (Takara Biotechnology, Tokyo, Japan) or Hi-fidelity premix kit (Roche). The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for 35 cycles (CD4, CXCR4, GPR1, STRL33, APJ, GAPDH), or denaturation at 94°C for 30 s annealing at 65°C for 30 s, extension at 72°C for 1 min for 35 cycles (CCR5, GPR15). As a negative control, the cDNA template was substituted for an equal volume of water. Amplification was performed in an i-Cycler BIO RAD (Bio-Rad Laboratories, Tokyo, Japan). The amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control in RT-PCR analyses for the standardization of each amplified product.

Real-time PCR was performed with the Quantitect probe PCR kit (Qiagen) on the ABI PRISM 7900HT sequence detector (Applied Biosystems, CA, USA). The sequence of primers and probes of nestin, GFAP, Tuj, and GAPDH were as described in previous reports (Fassunke *et al*, 2004; Li *et al*, 2005) or designed using the software Primer Express v2.0 (ABI) (Table 2). Three parallel PCRs for each sample were performed and data are shown in columns as each sample/monkey GAPDH by mean $\pm$ SD.

Viruses
SIVmac239 is a pathogenic lymphocyte-tropic molecular clone that infects macaque monkeys effectively and induces simian acquired

Table 2 Primers and probes

Genes	*Sequence*	*Product (bp)*	*Accession no./reference*
CD4	F: 5′-GTGGCACCTGGACATGC-3′ R: 5′-GGTCCCCACACTTCACA-3′	462	Mukhtar *et al*, 2002
CXCR4	F:5′-ATATACACTTCAGATAACTACACC-3′ R: 5′-CATAGGAAGTTCCCAAAGTACC-3′	311	Weatmoreland *et al*, 2002
CCR5	F: 5′-TCTCTGACCTGCTTTTCCTTCTTA -3′ R: 5′-TGCAGGTGTAATGAAGACCTTCTC-3′	314	Accession no. U77672
GPR1	F: 5′-CATGGAAGATTTGGAGGAAAC-3′ R: 5′-AGAGTTCTTGAGGGTTCGATGCCG-3′	462	Edinger *et al*, 1998
GPR15	F: 5′-CTGGTTTATCTCCTGCCTGCTGGG-3′ R: 5′-GGCAGCCAGGAGACAAGAAAGGC-3′	293	Edinger *et al*, 1998
STRL33	F: 5′-CCAGGAGGAGCATCAAGACTTCC-3′ R: 5′-AGGTCATCCTGTTGGCTTGCTGG -3′	393	Edinger *et al*, 1998
APJ	F: 5′-TACACAGACTCCAAATCCTCG-3′ R: 5′-TGCACCTTGGTGGTGTTCTCC-3′	481	Mukhtar *et al*, 2002
GAPDH	F: 5′-ATTCCATGGCACCGTCAAGGCT-3′ R: 5′-TCAGGTCCACCACTGACACGTT-3′	572	Accession no. J04038
Nestin	F: 5′-GCCCTGACCACTCCAGTTTA-3′ R: 5′-GGAGTCCTGGATTTCCTTCC-3′ P: 5′-TGGAGAATCCGGTGGGTCTAGAGTGTTCA-3′	201	Li *et al*, 2005
GFAP	F: 5′-AGAACCGGATCACCATTCCC-3′ R: 5′-TCTTGAGGTGGCCTTCTGACA-3′ P: 5′-TGCAGATCCGAGAAACCAGCCTGG-3′	96	Fussunke *et al*, 2004 Accession no. AY650314
Tuj	F: 5′-CATGGACAGTGTCCGCTCAG-3′ R: 5′-CAGGCAGTCGCAGTTTTCAC-3′ P: 5′-TGGATTCGGTCCTGGATGTGGTGC-3′	175	Li *et al*, 2005
GAPDH	F: 5′-AAGCTTGTCATCAATGGAAATCC-3′ R: 5′-CATCGCCCCACTTGATTTTG-3′ P: 5′-ACCATCTTCCAGGAGCGAGATCCC-3′	73	Accession no. J04038

F: forward primer; R: reverse primer; P: probe.

immunodeficiency syndrome (AIDS) (Kestler *et al*, 1990; Regier and Desrosiers, 1990). SIV17/E-Fr, which is a molecularly cloned macrophage-tropic and neurovirulent virus with the backbone of the clone SIVmac239, was kindly donated by Dr. M. Christine Zink (John Hopkins University School of medicine) (Flaherty *et al*, 1997). SIV17EΔnef GFP was newly constructed. A fragment of CMV-EGFP was amplified from pEGFP-C1 (BD Biosciences Clontech, Mountain View, CA, USA) by PCR using primers tagged with the *Bsp*HI site (5′-gggtcatgatag taatcaattacggggtc-3′, 5′-gggtcatgactagtacagctcgtccat gc-3′). The purified PCR product was inserted in the *Nco*I site of the nef region of pSIV17/E-Fr. Virus stocks were prepared by transfecting infectious viral plasmid DNA (pSIVmac239, pSIV17/E-Fr, and pSIV17EΔnef GFP) into 293T cells with Fugene 6 transfection reagent (Roche). Two days after transfection, supernatants were filtered through 0.45-μm filters (PALL, MI, USA) and subjected to RNase-free DNase I digestion (2 U/ml, Roche) for 30 min at 37°C in the presence of 0.01 M of $MgCl_2$. The amount of virus was standardized by the assay for p27 gag antigen with the SIV core antigen assay kit (Coulter, FL, USA).

SIV infection of differentiated stem cell cultures

The dissociated BPCs were plated on 6-well plates at a density of 3×10^5 cells/well and were incubated for 16 h in medium supplemented with 1% FBS (MHM1%FBS) and SIV stocks containing 100 ng of p27. After infection, the cells were washed 3 times with MHM extensively and fresh MHM-1%FBS was added. For 3′-azido-3′-deoxythymidine (AZT) treatment, 10 mM of AZT was also added to the media every 3 days after overnight infection. The culture medium was exchanged once a week and the culture supernatants were collected for measuring SIV p27 gag antigen with the SIV core antigen assay kit (Coulter). The expression of GFP in the cells infected with SIV17EΔnefGFP was observed under a fluorescent microscope (Olympus, Tokyo, Japan) after infection.

LuSIV assay

The infectivity of the supernatant of SIV/17E-Fr or mock-infected culture was assayed with the LuSIV system (Roos *et al*, 2000; Overholser *et al*, 2003). The LuSIV cells were maintained in RPMI supplemented with 10% FBS and 300 μg/ml of hygromycin B (Wako, Tokyo, Japan). The LuSIV cells (2×10^5 cells) were incubated in triplicate with the supernatant of SIV/17E-Fr or mock-infected culture. The value of SIV p27 in the culture supernatant collected on 36 dpi was 12380 pg/ml and 150 μl (1.86 ng of SIV p27) of the supernatant was used for LuSIV assay. After 72 h incubation, LuSIV cells were washed and assayed for the presence of luciferase activity with Luciferase Assay system (Promega). The luciferase activity was measured with Centro LB960 (Berthold). The fold induction of luciferase activity over background was calculated.

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