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A novel intranuclear RNA vector system for long-term stem cell modification

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Abstract

Genetically modified stem and progenitor cells have emerged as a promising regenerative platform in the treatment of genetic and degenerative disorders, highlighted by their successful therapeutic use in inherent immunodeficiencies. However, biosafety concerns over insertional mutagenesis resulting from integrating recombinant viral vectors have overshadowed the widespread clinical applications of genetically modified stem cells. Here, we report an RNA-based episomal vector system, amenable for long-term transgene expression in stem cells. Specifically, we used a unique intranuclear RNA virus, Borna disease virus (BDV), as the gene transfer vehicle, capable of persistent infections in various cell types. BDV-based vectors allowed for long-term transgene expression in mesenchymal stem cells (MSCs) without affecting cellular morphology, cell surface CD105 expression, or the adipogenicity of MSCs. Similarly, replication-defective BDV vectors achieved long-term transduction of human induced pluripotent stem cells (iPSCs), while maintaining the ability to differentiate into three embryonic germ layers. Thus, the BDV-based vectors offer a genomic modification-free, episomal RNA delivery system for sustained stem cell transduction.

Keywords

BDV vector; iPSCs; pluripotent stem cells; mesenchymal stem cells; RNA virus

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INTRODUCTION

Genetically modified stem and progenitor cells can provide powerful tools to treat genetic and degenerative disorders. To date, integrating retro- or lenti-viral vectors have been extensively used for sustained genetic modification. For instance, in X-linked severe combined immunodeficiency (X-SCID) gene therapy trials, patients with X-SCID received hematopoietic stem cells transduced with retroviral vectors to express the common gamma chain from IL2RG[1–3]. Although these trials demonstrated successful therapy for X-SCID[3–6], a subset of the treated patients subsequently developed leukemia due to the activation of host oncogenes by integrated retroviral sequences, i.e., insertional mutagenesis[1, 7], illustrating a biosafety concern of integrating vectors. Various non-integrating DNA transfer platforms, such as adenoviral or adeno-associated viral vectors, are currently available. However, these vectors typically achieve transient transgene expression and, in general, cannot support long-term genetic modification in rapidly proliferating cells[8].

Borna disease virus (BDV) is a non-segmented, negative-strand RNA virus with a broad host range[9]. BDV causes neuronal disorders in horses, sheep, cats, and cattle[9, 10]. In humans, BDV infection has been linked to various neurological disorders, such as major depressive disorder, bipolar disorder, and schizophrenia. However, a recent multi-center study, with standardized methods for clinical assessment and blinded to serological and molecular analysis of 396 subjects (198 matched controls), found no BDV sequences in any samples. The study did report a 2% immuno-reactivity to BDV (8 of 396); however, there was no link found between BDV sero-positivity and neurological disorders[11]. This observation, among others, strongly suggests no relationship between BDV and the pathogenesis of human psychiatric disorders. Although a novel variegated squirrel bornavirus (VSBV) was recently identified and linked to fatal human encephalitis cases in Germany, VSBV is different from well-characterized BDV strains[12].

BDV has unique biological properties as an RNA virus, such as intranuclear replication and transcription[13, 14], along with employing splicing, in order to express overlapping open reading frames[15–17]. Our recent study has revealed that the BDV ribonucleoprotein interacts directly with the host chromosome using core histones as a docking platform throughout the cell cycle, facilitating persistent intranuclear BDV infection[18]. Additionally, BDV replication is not lytic and extremely slow, supporting persistent infection in the nervous system[19] or peripheral blood mononuclear and bone marrow cells[20]. These biological characteristics are ideal for a non-integrating vector system capable of sustained transgene expression. Staeheli and colleagues have established a prototypic BDV-based gene transfer vector system, which encodes an expression cassette for green fluorescent protein (GFP) at a site near the 5’ end of the BDV genome[21]. Recently, we have developed both replication-competent and replication-defective BDV vector systems, allowing transgene expression from an intercistronic non-coding region[22]. Like wild-type BDV, BDV-based vectors also replicate intranuclearly, evident by intranuclear viral speckle of transcripts (vSPOTs)[18, 22], facilitating sustained transgene expression in cultured cells as well as in neurons[21–23].

In this study, we employed replication-competent BDV and M and G proteins-deleted replication-defective BDV vectors in order to genetically modify two stem cell types;

mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs). Our results demonstrate long-term transgene expression in MSCs and iPSCs without impairing their differentiation potential.

RESULTS

Infection of cells from different species with recombinant BDV vectors

We first assessed the infectivity of our replication-defective BDV vector with deletion in the G protein, rBDV ΔMGP/M-GFP, on various cell lines at a multiplicity of infection (MOI) of 0.05 (determined in Vero cells). BDV vectors infected all cell lines tested, with some variation in transduction efficiency. One week post infection, GFP-positive cell populations were analyzed by flow cytometry (Fig. 1A). Human neuronal U251 cells and canine renal D17 cells were particularly susceptible to the rBDV ΔMGP/M-GFP vector. Similar results were observed with our replication-competent recombinant BDV, rBDV P/M-GFP (not shown). As reported previously, rBDV ΔMGP/M-GFP vector-infected cells remained GFP-positive 2 months post infection, indicating sustained transduction of various cell types (Fig. 1B). Similarly, GFP-positive cells were found in cells infected by replication-competent rBDV P/M-GFP virus, although expanded GFP signals were found only in select cell lines, such as 293T, U251, Vero, D17, DK and CRFK cells (Fig. 1B). Intriguingly, when cell-free BDV stocks were prepared from these cell lines, as described in the Materials and Methods, only Vero and DK cells showed over 10^5 infectious units (IU)/mL of BDV titers, while less than 10^3 IU/mL of cell-free BDV were produced from 293T, U251, D17 and CRFK cells. Thus, although various cell types from a range of species are permissive to persistent BDV infection, select cell lines appear to support efficient BDV production.

BDV vectors achieve long-term transgene expression in mesenchymal stem cells without affecting their adipogenic potential

Adipose-derived MSCs were first analyzed for their expression of major MSC markers. Flow cytometry analysis verified high levels of expression of CD44, CD90 and CD105 (Fig. 2A). When MSCs were infected with cell-free recombinant BDV vector, rBDV P/M-GFP, at a multiplicity of infection (MOI) of 0.1, approximately 10% of cells became GFP-positive one week after infection. The GFP-positive cell population had increased to >80% positive at 6 weeks post infection (p.i.) (Fig. 2B). Importantly, BDV vector infection showed no notable effect on the growth or morphology of infected MSCs, with surface CD105 expression unchanged in BDV-infected GFP-positive cells (Fig. 2C).

To assess the influence of BDV vector infection on the differentiation capacity of MSCs, we cultured MSCs infected with rBDV P/M-GFP for 6 weeks in adipogenesis differentiation medium. One week after differentiation, formation of small lipid droplets were evident in infected and uninfected MSCs. Two weeks of adipogenic differentiation resulted in the generation of adipocytes with numerous large lipid droplets, confirmed by Oil Red O staining (Fig. 2D). Importantly, BDV-infected cells remained GFP positive throughout the differentiation, and BDV infection did not impair the adipogenic potential of MSCs (Fig. 2D).

BDV vectors efficiently transduce human iPSCs but not mouse iPSCs

Next, we assessed the permissivity of mouse and human iPSCs to BDV vector transduction. For this, we used our replication-defective recombinant BDV vector with deletion in the G protein (rBDV ΔMGP/M-GFP). To achieve more efficient BDV vector transduction we cocultured rBDV ΔMGP/M-GFP vector-producing cells with iPSCs. Specifically, dissociated mouse and human iPSCs were seeded on a cell-cycle-arrested BDV vector producer cell line, pretreated by Mitomycin C. Co-cultivation of mouse iPSCs with BDV producer cells did not lead to the generation of GFP-positive iPSCs, even after prolonged co-cultivation and passaging, suggesting that mouse iPSCs are resistant to BDV vector transduction (Fig. 3A). In contrast, GFP-positive human iPSCs appeared as early as 4 days post co-cultivation (Fig. 3A). Through serial passaging, rBDV ΔMGP/M-GFP-infected human iPSCs, with stable GFP expression, were successfully expanded (Fig. 3B). Flow cytometry analysis revealed GFP-positive iPSC population at 2 and 6 weeks post co-cultivation as 29.6% and 21.2%, respectively. Similarly, human iPSCs were permissive to a replication-competent GFP-expressing BDV vector (Fig. 3B, right panels). Four weeks after infection with cell-free rBDV P/M-GFP at MOI of 0.1, 36.6% of human iPSCs were GFP positive. BDV vector infection did not affect the growth or the morphology of infected cells, and rBDV ΔMGP/M-GFP-infected human iPSCs showed comparable expression levels of pluripotency-associated factors, including OCT4, SOX2, SSEA4 and TRA-1–60 (Fig. 3C).

BDV vector-infected human iPSCs maintain pluripotency

To assess the influence of BDV vector infection on the pluripotency of iPSCs, uninfected and BDV vector-infected cells were allowed to form embryoid bodies (Fig. 4A). Further spontaneous differentiation in vitro resulted in the generation of cells from all three germ layers, including βIII-tubulin positive neuronal cells (ectoderm), FOXA2-positive endoderm, CD31/PECAM1-positive endothelial cells (mesoderm), and cardiac troponin-positive cardiomyocyte (mesoderm), with no notable difference between control and BDV-infected human iPSCs (Fig. 4B). To assess the long-term BDV infection on the differentiation propensity of iPSCs, BDV-infected human iPSCs were further cultured for 4 months, and the percent GFP-positive cell populations were determined by flow cytometry (Fig. 4C). Nearly 100% of iPSCs became GFP positive, indicating long-term persistent infection of BDV virus in human iPSCs (Fig. 4C, lower panels). Upon guided differentiation into definitive endoderm cells, the BDV-GFP virus-infected cells showed over 100-fold induction of definitive endoderm markers (FOXA2 and SOX17 transcripts), comparable to those of control iPSCs (Fig. 4D). These observations demonstrated the utility of BDV vectors for sustained iPSC modification without affecting the pluripotency.

DISCUSSION

Integrating retro- and lenti-viral vectors have been extensively used for stem cell gene therapy trials. Recent trials using self-inactivating (SIN) lentiviral vectors have demonstrated promising tumor-free therapy[30–32]. Nevertheless, the biosafety concern over vector-mediated insertional mutagenesis still remains a major challenge for stable stem cell modification, as SIN lentiviral vectors have been shown to induce leukemia in a mouse model of X-SCID[33, 34]. In this context, recent advances in site-directed gene editing technologies,

including Zinc finger nucleases[35], TALE nuclease[36] and CRISPR systems[37], are notable, as they allow genetic modification without random integration. At present, however, these site-directed gene editing technologies are relatively inefficient and still entertain risks of off-target cleavage, presenting a formidable barrier in any immediate clinical applications. Additionally, screening stem and progenitor cells with correct gene edits typically requires expansion and screening of single cell clones, which is not feasible for many stem and progenitor cell types. It should be noted that prolonged ex-vivo culturing and expansion of stem and progenitor cells often leads to impaired differentiation and decreased repopulation efficiency[38]. Here, we demonstrated that BDV vector system facilitates sustained transgene expression in MSCs and iPSCs without affecting their proliferation or differentiation capacity. Importantly, the BDV vector system has biological properties highly attractive for stem cell modification: (i) BDV achieves persistent intranuclear infection through close association with host chromosomes throughout the cell cycle[18], (ii) BDV persistently infects neural stem/progenitor cells[39], (iii) BDV G protein plays a critical role in the release of infectious BDV particles allowing for the generation of replication- and particle-release-free BDV vectors through the G protein deletion[22], and (iv) BDV vectors show long-term transgene delivery in vivo without notable toxicity[22]. Thus, the BDV vector system offers a promising non-integrating RNA-based platform for sustained stem cell modification.

Currently, a major limitation of the BDV vector system is the difficulty in obtaining high infectious titer vector stocks. After purification, BDV vector titers typically reach 10^5 to 10^6 infectious units per ml (IU/ml). The titers are comparable to those of unconcentrated gammaretroviral vectors, but not comparable to VSV-G-pseudotyped lentiviral vectors, which can reach 10^{10} IU/ml after concentration by ultracentrifugation. Like low titer retroviral vectors, introduction of an antibiotic resistant gene into BDV vectors would allow selection of transduced cells. Since additional genes would increase the vector genome size, further studies are required to define the packaging capacity of BDV. BDV has an 8.9 kb genome, which is relatively short for an RNA virus. Our preliminary data has demonstrated successful generation of a BDV vector carrying the 1.6 kb luciferase gene. Accordingly, the BDV vector should be able to accommodate at least a 10.5 kb genome. Removal of non-essential viral genes, such as the G and M protein genes, will further increase the packaging capacity of BDV vectors.

Another limitation is the possible influence of persistent BDV infection on cellular functions. For instance, BDV-P protein has been shown to interact with multifunctional protein, HMGB1, and inhibits its function in cultured neural cells[40]. The BDV-P interaction with HMGB1 also blocks the tumor suppressor p53, or p53-mediated activation of the major senescence-inducing factor p21[41]. Although BDV infection induces no or low levels of interferon, due to post-transcriptional removal of the RIG-I-activating RNA 5’ triphosphate group[42] and BDV-X protein interfering with the type 1 IFN signaling[43], long-term, persistent BDV vector infection may also affect normal cellular functions through activation of interferon-stimulated genes. Thus, further studies are necessary to elucidate the influence of sustained BDV infection on stem cell fates or functionality of stem cell-derived cell products.

A potential issue is the BDV integration into the host genome. This concern is based on our recent reports on identification of endogenized bornavirus-related elements from ancient bornaviruses in mammalian genomes[44, 45]. However, this concern is not specific to BDV vectors. We have shown that integrated bornavirus-related sequences have poly-A tails and target site duplications, suggesting that ancient bornavirus-integration events were mediated by non-specific mRNA reverse transcription and integrations by LINE-1 (long interspersed nuclear element-1), a non-LTR retrotransposon[45, 46]. In theory, LINE-1-meidated integration can occur for any RNA viruses and cellular transcripts. Indeed, similar fossilized RNA viral elements from ebolavirus/marburgvirus and tamana bat virus, and various poly-A tailed cellular transcripts-like DNA sequences, have been found in vertebrate genomes[46–48]. Thus, bornaviral integration process is not particularly efficient, likely because the process requires both reverse transcription and integration by non-specific interaction with LINE-1 components[48, 49]. Recently, we have demonstrated that the integration of BDV or rBDV vector sequences is rare. Even when over 90% of cells persistently infected by BDV for 8 weeks, we found 0 and 0~0.007 copies/cell of BDV-specific DNA sequences in 293T and human oligodendroglioma OL cells, respectively[49]. Clearly, this is in sharp contrast to other integrating vectors, which are based on retro- or lenti-viruses.

In relation to low BDV vector titers, we also noticed that BDV vector infection of certain cell lines was strongly inhibited at higher vector doses. For instance, when U251 and NP2 cell lines were infected by GFP-expressing BDV at MOI 0.1 and 0.5 (standardized in Vero cells), the GFP-positive cell populations were 39.1 and 5.5% for U251 and 4.1 and 2.0% for NP2 cells, respectively. Similar findings have been reported with lenti-viral vectors pseudotyped with amphotropic murine leukemia virus (MLV-A) Env, largely due to high levels of dissociated particle-free MLV-A Env protein in the vector preps competing with infectious vector particles[50]. VSV-G-pseudotyped vectors do not show this phenotype because of the stability of VSV-G and its ubiquitous receptor, facilitating efficient vector infection. It is plausible that BDV vector preps contain high levels of particle-free BDV G protein, or defective BDV vector particles, which antagonize the interaction between infectious BDV vector particles and functional entry receptors on target cells. It is also conceivable that BDV uses an entry receptor which is not abundant at least in U251 and NP2 cells. The use of a gel-filtration column to remove excessive particle-free BDV G protein during vector purification may improve the problematic, low vector infectivity at high vector doses. Alternatively, incorporation of a heterologous viral glycoprotein, such as a modified VSV-G[22], for pseudotyped BDV vectors, would allow production of higher titer vectors with improved infectivity and stability.

For validating the safety of gene transfer vectors, as well as the feasibility of gene therapy strategies, it is critical to have a relevant small animal model. Ideally, BDV vectors would transduce both human and preclinical animal models equally well. Unfortunately, we found that BDV vectors failed to transduce mouse iPSCs. Since no BDV-infected cell was found after several attempts, it is conceivable that mouse iPSCs do not express a functional BDV receptor. It is also notable that mice and mouse-derived cells are generally more resistant to BDV infection[51]. Previous studies have established replicating mouse-adapted BDV strains through serial passaging of BDV in rat brains[51, 52], resulting in two amino acid substitutions, R66K and L116R in the P and L proteins[52]. Notably, Ackermann et al. developed a GFP-

carrying, mouse-adapted BDV, facilitating viral dissemination in the mouse nervous system[23]. We are currently in the process of generating a replication-defective BDV vector system with R66K and L116R mutations for an improved BDV vector mouse-system.

In conclusion, our data demonstrate sustained transgene expression in human MSCs and iPSCs by BDV vectors. Our preliminary study also showed human CD34+ cells are permissive to BDV infection (Supplementary Fig. S1). Although further improvements in increased vector titers are necessary, the BDV-based vectors offer a unique episomal RNA delivery platform for sustained stem cell modification without genomic modification.

Materials and Methods

All studies were approved by Mayo Clinic Institutional Biosafety Committee.

Cells

Human U251, 293T, and PANC1 cells, rhesus monkey FrhK4 cells, African Green Monkey Vero and CV1 cells, Dog D17 and DK cells, mouse Min6 cells, feline CRFK cells, bovine MDBK cells, and rat NRK cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics as described previously[24]. Mouse iPSCs were generated from murine fibroblasts by lentiviral transduction of four reprogramming factors[25] and cultured without feeder cells on Matrigel-coated plates (BD Bioscience) in ESGRO-2i medium (Millipore) supplemented with 45% Dulbecco's modified Eagle's medium (DMEM), 5% FCS, 25 U/ml penicillin, and 25 μg/ml streptomycin. Transgene-free iPSCs were reported previously[26], which were cultured in Pluriton medium (Stemgent, Cambridge, MA) supplemented with 25% mTeSR1 medium (Stemcell Technology) and 50 U/ml penicillin and 50 μg/ml streptomycin. Adipose-derived MSCs were derived and cultured as described previously[27].

BDV vectors

A replication-competent, recombinant BDV vector harboring a GFP gene in the intercistronic region between the P and M genes (rBDV P/M-GFP), and a replication-defective BDV vector with deletion in the BDV M and G genes (rBDV ΔMGP/M-GFP) were reported previously[22]. Briefly, the replication-competent recombinant BDV vector was rescued in 293T cells transfected with BDV vector plasmid, pCA-N, pCXN2-P, and pCA-L[28], or with additional pBDV-G for the ΔMGP/M vector[22], followed by co-culturing of the cells with Vero or Vero-BG cells[22]. rBDV P/M-GFP and rBDV ΔMGP/M-GFP vectors were expanded in Vero cells and BDV M and G protein-expressing Vero cells, respectively. For preparation of cell-free BDV vectors, 100% confluent BDV producer cells in 6 × 10 cm dishes were harvested and resuspended in 1.2 ml of DMEM medium supplemented with FCS, sonicated by BioRuptor (UCD-300) at power 5 (high) for 30 seconds × 4 times, and centrifuged at 1,200g at 4 °C for 25 min. Viral supernatants were then stored at −80 °C. Viral titers were then determined by flow cytometry analysis of GFP-positive cell populations in Vero cells infected with serially diluted BDV vector stocks.

Differentiation of MSCs into adipocytes

Semi-confluent MSCs were cultured for 2 weeks in StemPro Adipogenesis differentiation kit (Life Technologies). Generation of adipocytes with lipid droplets was verified by Oil Red O staining. Specifically, differentiated cells were fixed by formalin, followed by fixation of Isopropanol (60%) treatment for 5 min and incubation with Oil Red O solution for 10 min. After washing, cells were incubated with Hematoxylin for 1 min to counter-stain nuclei, as per manufacturer's directions.

Spontaneous differentiation

iPSCs were dissociated by Versene (Life Technologies) for 30 min and cultured in the human iPSC medium on low adhesion plates, allowing formation of embryoid bodies (EBs). EBs were cultured as suspension for 4 days in the iPSC medium, and then cultured in DMEM medium supplemented with 20% FBS for one week. EBs were then cultured on Matrigel-coated Lab-Tek Chamber slides in DMEM with 20% FBS for one week.

Guided differentiation of iPSCs into definitive endoderm

iPSCs cultured on Matrigel-coated plates were treated with 100 ng/ml Activin A (Peprotech) and 25 ng/ml Wnt3a (R&D Systems) in advanced RPMI (Invitrogen) for 1 day, followed by treatment with 100 ng/ml Activin A in advanced RPMI medium supplemented with 0.2% fetal bovine serum for 2 days. Total RNA was isolated with Trizol reagent (Invitrogen), used for real-time reverse transcription-PCR analysis as reported previously[29]. For SOX17-specific primers (5'-ACGCCGAGTTGAGCAAGA-3' and 5'-TCTGCCTCCTCCACGAAG-3', with Roche Universal Probe #61) and FOXA2-specific primers (5'-CGTTCCGGGTCTGAACTG-3' and 5'-ACCGCTCCCAGCATACTTT-3' with Roche Universal Probe #68) were used to detect SOX17 and FOXA2 transcripts as definitive endoderm markers.

Immunostaining

For immunostaining, cells were cultured in matrigel-coated Lab-Tek Chamber slides (Nunclone), fixed in 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 and blocked in PBS supplemented with 5% FBS. Cells were stained by specific antibodies; SSEA-4 and TRA-1–60 (Millipore #SCR001), OCT4 (Cell Signaling Technology #2750), SOX2 (Cell Signaling Technology #2748), FOXA2 (Millipore #07-633), β-III tubulin (Abcam # 41489), CD31 (Santa Cruz Biotechnology # SC1506), cardiac troponin T (Abcam #ab8295).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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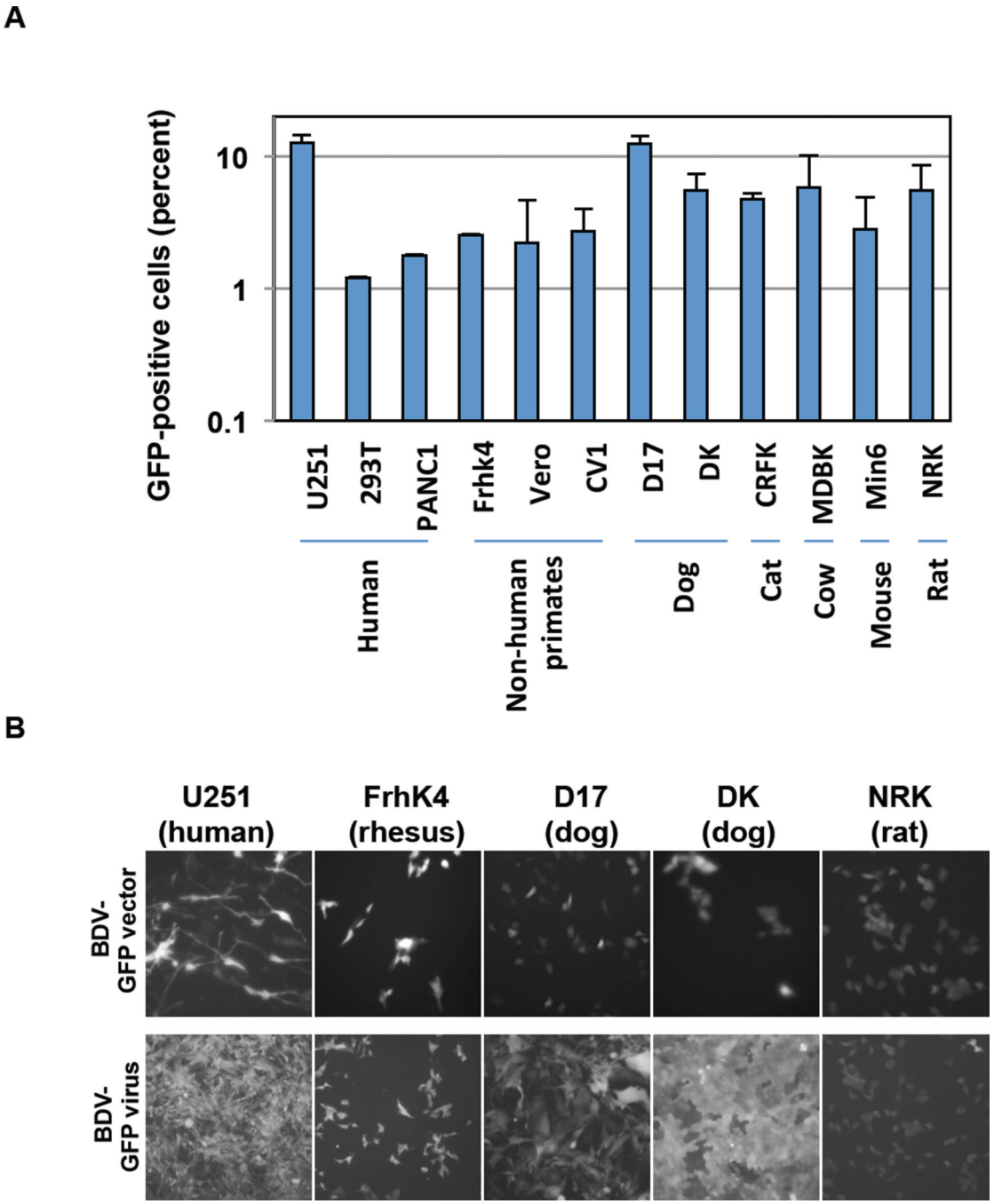

Figure 1. Infection of cells from different species with recombinant BDV vectors

A. Various cell lines were infected with the replication-defective rBDV ΔMGP/M-GFP vector at MOI of 0.05. One week post infection, GFP-positive cell populations were determined by flow cytometry analysis. Data are shown as averages of two independent experiments with error bars representing standard error of the mean. **B.** GFP expression was monitored in cells infected with replication-defective and –competent BDV vectors 2 months post infection.

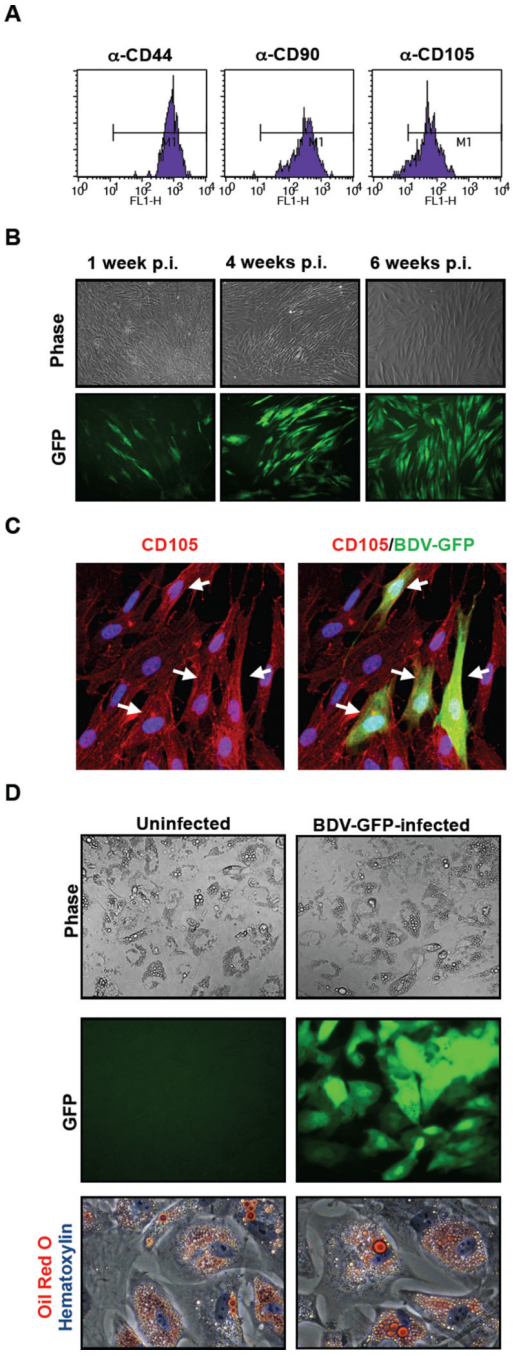

Figure 2. BDV vectors achieve long-term transgene expression in mesenchymal stem cells without affecting their adipogenic potential

A. Flow cytometry analysis of adipose-derived MSCs demonstrated expression of major MSC markers CD44, CD90 and CD105. **B.** MSCs were infected with a GFP-expressing BDV vector at MOI 0.1, and monitored for 6 weeks. The BDV vector achieved long-term GFP expression in MSCs without showing notable toxicity. **C.** Cell surface CD105 expression on MSCs was assessed by immuno-staining at 4 weeks after BDV infection. Nuclei were counter-stained by DAPI. BDV-infected cells are indicated by arrows. **D.** BDV-

infected and uninfected MSCs at 6 weeks post infection were differentiated into adipocytes through guided differentiation in adipogenesis differentiation medium for two weeks (top panels). Sustained transgene (GFP) expression was monitored by UV microscope (middle panels). Successful adipogenesis was verified by Oil Red O staining (lipid droplets, red; nuclei, blue).

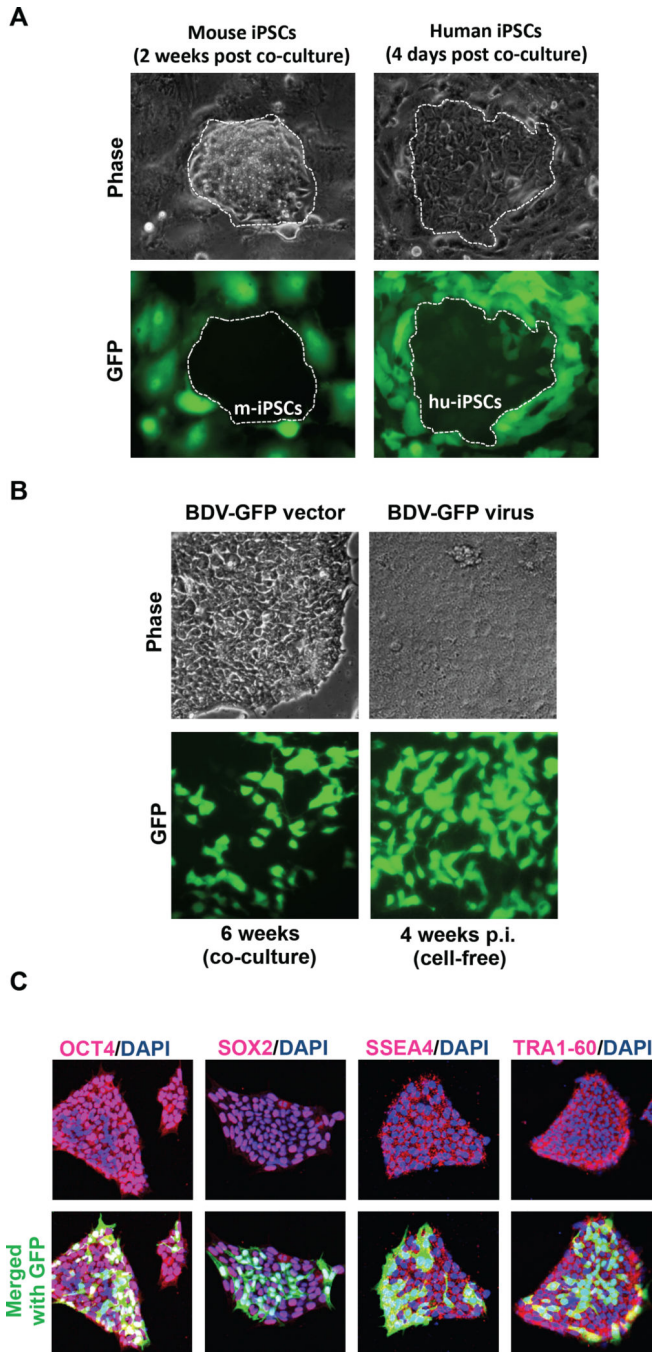

Figure 3. BDV vectors efficiently transduce human iPSCs

A. A replication-defective, GFP-expressing BDV vector (rBDV ΔMGP/M-GFP)-producing cells were cell cycle-arrested by Mitomycin C, followed by co-cultivation with dissociated mouse and human iPSCs (m-iPSCs and hu-iPSCs, respectively). Transgene GFP expression in iPSC colonies was monitored. Borders of mouse and human iPSC colonies are indicated by broken white lines. **B.** Human iPSCs infected by replication-defective BDV vector (rBDV ΔMGP/M-GFP), were expanded and monitored for transgene expression at 4 and 6 weeks post co-cultivation (after 8 and 12 passages). Human iPSCs infected by cell-free replication-

competent BDV vector (rBDV P/M-GFP) were also monitored 4 weeks after infection at MOI 0.1. **C.** Human iPSC colonies were characterized through immunocytochemistry analysis using a panel of pluripotency markers. Nuclei were counter-stained by DAPI (blue). Expression of pluripotency-associated factors, OCT4, SOX2, SSEA4 and TRA-1–60 (red), were monitored in human iPSCs infected by rBDV ΔMGP/M-GFP. BDV vector-infected cells (green) showed comparable expression of pluripotency-associated factors.

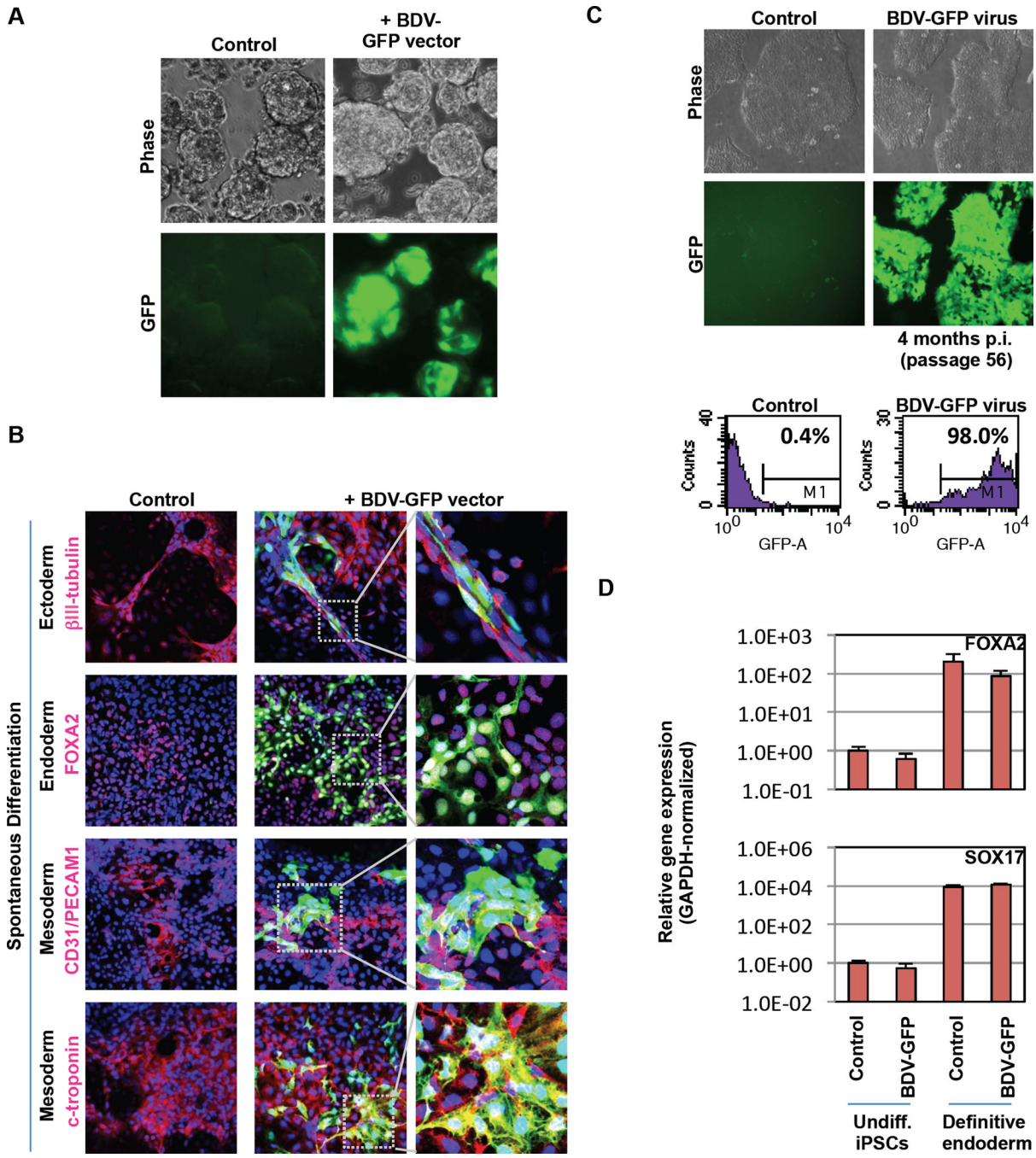

Figure 4. BDV vector-infected human iPSCs maintain pluripotency

A. BDV vector-infected and uninfected human iPSCs were cultured in suspension. Both infected and uninfected cells formed embryoid bodies. **B.** Pluripotency of control and BDV vector-infected iPSCs were verified through spontaneous differentiation *in vitro*. iPSC progeny cells were analyzed via immunocytochemistry for lineage markers for three germ layers (ectoderm, βIII-tubulin; endoderm, FOXA2; mesoderm, CD31 and c-troponin). Nuclei were counter-stained by DAPI. **C.** Replication-competent BDV-GFP virus-infected iPSCs were cultured for 4 months and the percent GFP-positive cell populations were

determined by flow cytometry. **D.** Control iPSCs and replication-competent BDV-GFP virus-infected iPSCs from **C** were subjected to the guided differentiation into definitive endoderm cells. Three days after differentiation, definitive endoderm markers FOXA2 and SOX17 transcript levels were compared by real-time RT-PCR. Data are shown as averages of two independent experiments with error bars representing standard error of the mean.