

Development of Defective and Persistent Sendai Virus Vector A UNIQUE GENE DELIVERY/EXPRESSION SYSTEM IDEAL FOR CELL REPROGRAMMING^{*†}

Received for publication, September 9, 2010, and in revised form, December 5, 2010. Published, JBC Papers in Press, December 7, 2010, DOI 10.1074/jbc.M110.183780

Ken Nishimura,^{a,b} Masayuki Sano,^a Manami Ohtaka,^{a,c} Birei Furuta,^d Yoko Umemura,^a Yoshiro Nakajima,^a Yuzuru Ikehara,^e Toshihiro Kobayashi,^{f,g} Hiroaki Segawa,^{h1} Satoko Takayasu,^{a,c} Hideyuki Sato,^{f,g} Kaori Motomura,^{a,c} Eriko Uchida,ⁱ Toshie Kanayasu-Toyoda,^d Makoto Asashima,^{a,j} Hiromitsu Nakauchi,^{f,g} Teruhide Yamaguchi,^d and Mahito Nakanishi^{a2}

From the ^aResearch Center for Stem Cell Engineering, ^eResearch Center for Medical Glycoscience, and ^hOrgan Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Central 4, Tsukuba, Ibaraki 305-8562, ^bPRESTO, Japan Science and Technology Agency, 4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, ^cJapan Biological Informatics Consortium, TIME24 Building, 2-4-32 Aomi, Koto-ku, Tokyo 135-8073, ^dDivision of Biological Chemistry and Biologicals and ⁱDivision of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1 Kami-Yoga, Setagaya-ku, Tokyo 158-8501, ^fDivision of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, the Institute of Medical Science, the University of Tokyo, and the ^gNakauchi Stem Cell and Organ Regeneration Project, ERATO, Japan Science and Technology Agency, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, and the ^jDepartment of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

The ectopic expression of transcription factors can reprogram differentiated tissue cells into induced pluripotent stem cells. However, this is a slow and inefficient process, depending on the simultaneous delivery of multiple genes encoding essential reprogramming factors and on their sustained expression in target cells. Moreover, once cell reprogramming is accomplished, these exogenous reprogramming factors should be replaced with their endogenous counterparts for establishing autoregulated pluripotency. Complete and designed removal of the exogenous genes from the reprogrammed cells would be an ideal option for satisfying this latter requisite as well as for minimizing the risk of malignant cell transformation. However, no single gene delivery/expression system has ever been equipped with these contradictory characteristics. Here we report the development of a novel replication-defective and persistent Sendai virus (SeVdp) vector based on a non-cytopathic variant virus, which fulfills all of these requirements for cell reprogramming. The SeVdp vector could accommodate up to four exogenous genes, deliver them efficiently into various mammalian cells (including primary tissue cells and human hematopoietic stem cells) and express them stably in the cytoplasm at a prefixed balance. Furthermore, interfering with viral transcription/replication using siRNA could erase the genomic RNA of SeVdp vector from the target cells quickly and thoroughly. A SeVdp vector installed with *Oct4/Sox2/Klf4/c-Myc* could reprogram mouse primary fibroblasts quite efficiently; ~1% of the cells were reprogrammed to Nanog-positive induced pluripotent stem cells without chromosomal gene integration. Thus, this SeVdp vector has poten-

tial as a tool for advanced cell reprogramming and for stem cell research.

The generation of induced pluripotent stem (iPS)³ cells by reprogramming tissue cells with defined factors opened the door for realizing the medical application of patient-derived engineered stem cells (1). iPS cells were established originally by the ectopic expression of multiple transcription factors (e.g. Oct3/4, Sox2, Klf4, and c-Myc) using a retroviral vector (1). Since then, researchers have established iPS cells by several different approaches (and by their combination), including gene transfer, protein transduction, and treatment with chemical compounds (2). However, because of superior reproductibility and efficacy, ectopic expression of reprogramming factors by gene transfer is still the primary method of choice.

Various lines of evidence indicate that efficient cell reprogramming requires the sustained and simultaneous expression of several (usually 4) exogenous factors for at least 10–20 days (3). On the other hand, after reprogramming has been completed, these exogenous factors should be replaced promptly with their endogenous counterparts if the cells are to acquire autoregulated pluripotency (3). For this reason, retroviral and lentiviral vectors have been used preferentially; chromosomal insertion of the vector genome allows for stable gene expression, whereas epigenetic modification of the viral promoter shuts off the vector-mediated gene expression after cell reprogramming has been accomplished. Nevertheless, cell reprogramming with these insertional vectors has a crucial disadvantage in that silencing and reactivation of the inte-

* This work was supported in part by a grant from the New Energy and Industrial Technology Development Organization of Japan, by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and by JST PRESTO Program.

† The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2 and Figs. S1–S4.

¹ Present address: Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical Co. Ltd., 1-135, Komakado, Gotemba, Shizuoka, 412-8513, Japan.

² To whom correspondence should be addressed. Tel.: 81-29-861-3040; Fax: 81-29-861-2798; E-mail: mahito-nakanishi@aist.go.jp.

³ The abbreviations used are: iPS, induced pluripotent stem; ES, embryonic stem; SeV, Sendai virus; SeVdp, defective and persistent Sendai virus; Bs, blasticidin S; Zeo, zeocin; Zeo, phleomycin-binding protein gene; Bsr, blasticidin S deaminase gene; Hyg, hygromycin B phosphotransferase gene; EGFP, enhanced green fluorescent protein; KO, Kusabira Orange; KR, Keima Red; MEF, mouse embryonic fibroblast; NP, nucleocapsid protein; CFU, colony-forming unit.

grated reprogramming genes is often unmanageable, which might affect the differentiation potency of iPS cells and the safety of the iPS-derived cells. Thus, investigators have focused on generating iPS cells carrying no exogenous genetic materials either by repetitive transient gene expression (4, 5), by passive elimination of stable episomal DNA (6), or by recombinase-mediated excision of integrated genes from the chromosome (Refs. 7 and 8; for review, see Ref. 9). However, all of these approaches are not only inefficient but also laborious in practice, and development of a simpler gene delivery/expression system suitable for cell reprogramming is needed.

Sendai virus (SeV) is a nonsegmented negative-strand RNA virus belonging to the *Paramyxoviridae* (10). As SeV can infect various animal cells with an exceptionally broad host range and is not pathogenic to humans, various applications have been explored for SeV as a recombinant viral vector capable of transient but strong gene expression (11). We have demonstrated the potential of SeV as a tool for stable gene expression through an analysis of the Cl.151 strain (12). This unique variant was originally isolated as a mutant capable of persistent infection at a nonpermissive temperature (38 °C) (13). We cloned the entire genome of SeV Cl.151 and determined that more than two genetic elements were responsible independently for the establishment of stable persistent infections (12). We also demonstrated that SeV Cl.151 installed with a single exogenous gene could express it stably without chromosomal insertion (12). As this characteristic is advantageous for cell reprogramming, we planned to optimize this gene delivery/expression system through a more extensive analysis of SeV-mediated stable gene expression.

Here we describe the replication-defective and persistent Sendai virus (SeVdp) vector, a novel gene transfer/expression system based on SeV Cl.151, with the following characteristics, 1) efficient, harmless, and simultaneous delivery of up to four exogenous genes installed on a single vector, 2) stable and reproducible expression of installed genes at a pre-fixed balance without chromosomal integration, and 3) quick and complete erasure of the vector genome by interfering with viral RNA-dependent RNA polymerase using siRNA. We also demonstrated that an SeVdp vector installed with *Oct4/Sox2/Klf4/c-Myc* could reprogram mouse primary fibroblasts efficiently. These characteristics should make SeVdp a universal tool for stem cell research, especially for advanced cell reprogramming.

EXPERIMENTAL PROCEDURES

Reconstitution of SeVdp Vector by Reverse Genetics—All recombinant DNA experiments were performed according to our institutional guidelines and under the permission of the institutional recombinant DNA experiment committee of the National Institute of Advanced Industrial Science and Technology and of the National Institutes of Health Sciences. Replication-competent SeV was reconstituted as described (12). Full-length SeVdp vector genomic cDNA for SeV (Cl.151 strain and Nagoya strain) and for installed genes was constructed on the lambda Dash II vector as described in [supplemental Fig. S1](#). In brief, the *M*, *F*, and *HN* genes were replaced with exogenous genes cloned between *KasI* and *MluI* restric-

tion sites (for *M*), between *BglIII* sites (for *F*), and between *NheI* and *SphI* sites (for *HN*). Additional extra genes were inserted into an *NheI/NotI* site created between the *P/C/V* and *M* genes. cDNAs encoding blasticidin S deaminase (*Bsr*), phleomycin-binding protein (*Zeo*), enhanced green fluorescent protein (*EGFP*), *Cypridina noctiluca* luciferase (*CLuc*), humanized Kusabira Orange (*KO*), and human gp91phox (*CYBB*) were amplified by polymerase chain reaction using pCX4-*bsr* (14), pUT58 (15), pEGFP-1 (Takara Bio, Otsu, Japan), pCLm (ATTO, Tokyo, Japan), pHKO1-MN1 (Medical & Biological Laboratories, Nagoya, Japan), and gp91phox-pCI-neo as templates, respectively. cDNA encoding humanized Keima Red (*KR*) was synthesized by GenScript (Piscataway, NJ), according to a published peptide sequence (16).

The reconstructed cDNA plasmids (2 μ g) and the expression vector plasmids for SeV nucleocapsid protein (NP), P/C, and L proteins (1 μ g each) and pSRD-HN-Fmut (17) (2 μ g) were transfected into BHK/T7/151M(SE) cells using Lipofectamine LTX Plus reagent (Invitrogen). BHK/T7/151M(SE) cells were established by expressing humanized T7 RNA polymerase and the M protein of the SeV Cl.151 strain stably in BHK-21 cells and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The SeVdp vector was reconstituted in cells from positive-strand antigenome RNA transcribed from this cDNA and the SeV NP, P/C, L, Fmut, HN, and M (Cl.151) proteins. The vector-packaging cells harboring the SeVdp vector were established by selecting with antibiotics (blasticidin S at 10 μ g/ml, zeocin at 500 μ g/ml, or hygromycin B at 200 μ g/ml) except for SeVdp(*c-Myc/Klf4/Oct4/Sox2*). The SeVdp vector was rescued by transient expression of the SeV *Fmut*, *HN*, and *M* (Cl.151) genes (driven by the SR α promoter derived from pcDL-SR α) (18) in the packaging cells as described above and recovered into the culture supernatant after incubation at 32 °C for 4 days. *Fmut*, a modified F gene for expressing the protease-susceptible SeV F protein, was generated as described (17). The supernatant was filtered through 0.45- μ m cellulose acetate filters and stored in small aliquots at -80 °C. Titers of SeVdp vectors were determined by examining LLCMK₂ cells infected with a diluted SeVdp vector suspension using indirect immunofluorescence microscopy with an anti-NP rabbit polyclonal antibody.

Cell Culture, Fluorescence Microscopy, and Flow Cytometry—Long term stability of gene expression mediated by the SeVdp vectors was examined in LLCMK₂ cells and in human primary fibroblasts (TIG3) cultured in Eagle's minimum essential medium supplemented with 10% FCS. For examining stability under antibiotic selection, the cells were incubated in the presence of blasticidin S (Bs) (5 μ g/ml), hygromycin B (200 μ g/ml), or a mixture of blasticidin S (5 μ g/ml) and zeocin (Zeo) (100 μ g/ml). For examining stability without selection, the cells were preselected with antibiotics and then cultured without selection. The presence of SeVdp was determined by detecting SeV NP antigen with indirect immunofluorescence microscopy, counterstained with DAPI. EGFP, KO, and KR were detected by fluorescence microscopy (Zeiss, Oberkochen, Germany) using specific filters customized for these proteins. Flow cytometry was performed using a

Novel Sendai Virus Vector Ideal for Cell Reprogramming

FACSCalibur (BD Biosciences; see Figs. 2B and 3, E and F) and with FISHMAN R (On-chip Biotechnologies, Tokyo, Japan; Fig. 3, G and H) according to the standard procedures provided by the manufacturers.

Gene Delivery to Human Hematopoietic Stem Cells—All experiments using human resources were performed according to National Institute of Advanced Industrial Science and Technology and National Institutes of Health Sciences guidelines. OP9 cells (19) (provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan) were cultured with minimal essential medium- α containing 20% FCS and 4 mM l-glutamine. Human umbilical cord blood was collected after a normal pregnancy and delivery after obtaining informed consent from the mothers. Human mononuclear cells were isolated from the cord blood using Lymphoprep (Axis-Shield, Oslo, Norway) according to the protocol provided by the manufacturer. CD133/1(+) cells were prepared from mononuclear cells using CD133 microbead kits and an AutoMACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) with a purity greater than 90%, confirmed by flow cytometry using a FACSCalibur.

The purified CD133/1(+) cells were infected with SeVdp(*Bsr*/ ΔF /KO) at a multiplicity of infection of 4 at 37 °C for 2 h. For examining the efficiency of gene delivery, the infected cells were cultured for 10 days in Iscove's modified Dulbecco's medium supplemented with 20% FCS, 8 mM L-glutamine, 50 μ M 2-mercaptoethanol, 50 ng/ml human stem cell factor, 5 ng/ml interleukin-6 (IL-6), 5 ng/ml IL-3, 25 ng/ml flt-3 ligand (all from PeproTech, Rocky Hill, NJ), and 50 ng/ml human thrombopoietin (Kirin, Tokyo, Japan), and the fraction of KO-positive cells was determined by flow cytometry (Fig. 2B). For long term culture-initiating cell assays (20), 20–200 SeVdp-infected cells were seeded on 1.25×10^4 γ -ray-irradiated OP9 cells in 96-well plates in Iscove's modified Dulbecco's medium with 12.5% FCS, 12.5% horse serum, 8 mM L-glutamine, 50 μ M 2-mercaptoethanol, and 1 μ M hydrocortisone for 5 weeks; half of the medium was exchanged every week. Then whole cells in a well were recovered and cultured in 300 μ l of semisolid colony-forming cell assay medium (MethoCult GF+ H4433, StemCell Technologies, Vancouver, Canada) in 48-well plates. After culturing for 2 weeks, the numbers of KO-positive colonies were determined.

RNA Interference Analysis—The sequences of siRNAs against SeV NP, P, and L mRNAs (designed and synthesized by iGENE Therapeutics, Tokyo, Japan) used in this study are listed in supplemental Table S1. For examining the effect of siRNAs on the infection of replication-competent SeV vectors (supplemental Fig. S4A), 2×10^4 HeLa cells were seeded in 48-well plates with DMEM containing 10% FCS on day 0. On day 1, the cells were treated with siRNAs (100 nM) mixed with Lipofectamine 2000 (Invitrogen) for 6 h, then infected with SeV Cl.151(*EGFP*) at a multiplicity of infection of 100. The medium was replaced on day 2, and the cells were examined using fluorescence microscopy on day 4. For examining the effect of siRNAs on the removal of SeVdp vector from BHK-21 cells expressing the T7 RNA polymerase and F pro-

tein of the SeV Nagoya strain constitutively (BHK/T7/NaF cells) (supplemental Fig. S4B), 1×10^4 cells harboring SeVdp(*M/EGFP/Bsr*) were seeded in 48-well plates on day 0. On day 1 the cells were treated with siRNA (100 nM) mixed with Lipofectamine 2000 as described above. On day 5, the cells were examined using fluorescence microscopy. In both of these experiments, siRNA against firefly luciferase (21) was used as a control.

For examining the effect of siRNA on the removal of SeVdp vector using luciferase activity as a quantitative index (Fig. 4), 1.5×10^5 (Fig. 4B) or 3×10^4 (Fig. 4C) HeLa cells harboring SeVdp(*KO/Hyg/EGFP/Luc2CP*) were seeded with siRNAs (40 nM) mixed with Lipofectamine RNAiMAX (Invitrogen) as described above on day 0 in a 6-well plate (Fig. 4B) or in a 24-well plate (Fig. 4C), respectively. siRNA against *Renilla reniformis* luciferase (22) was used as a control. The cells were passaged with fresh siRNA on days 3 and 7, and the culture medium was replaced on the next day at each point. Firefly luciferase activity in the cell extract was determined on the indicated day using a luciferase assay system (Promega, Madison, WI). Specific luciferase activity was determined by normalizing against the amount of protein, determined using a Bradford protein assay kit (Bio-Rad). The cell lysates prepared on days 3, 7, and 12 were also analyzed by Western blotting using affinity-purified anti-SeV L protein rabbit antibody (2 μ g/ml). For certifying complete removal of the SeVdp genome, the cells harboring SeVdp(*KO/Hyg/EGFP/Luc2CP*) and treated with siRNA as described above were cultured in the absence of siRNA for 4 weeks. Then, 1×10^4 of the cells were seeded in a 6-well plate and cultured in the presence of hygromycin B (100 μ g/ml) for 10 days. The surviving cells were fixed, then stained with 0.01% crystal violet.

Biochemical Assays—SDS-PAGE and protein blotting were performed as described (23) using SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific, Waltham, MA). For filter trap assays to detect the NP antigen (Table 1), culture supernatants of the cells harboring SeVdp vectors were passed through 0.45- μ m cellulose acetate filters, trapped onto supported nitrocellulose membranes (0.2 μ m, Bio-Rad) by vacuum filtration, and probed with an anti-SeV NP monoclonal mouse antibody.

Cell Reprogramming—Isolation and culture of mouse embryonic fibroblasts (MEFs) from a Nanog/GFP knock-in mouse (provided by the Riken BioResource Center) (MEF/Nanog-GFP), reprogramming with retroviral vectors and culture of mouse iPS cells were performed as described previously (24). Retroviral vectors installed separately with *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* (RvMX4) were prepared as described (1) using template DNA obtained from Addgene (Cambridge, MA). For reprogramming with SeVdp vectors installed with *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*, 1.25×10^5 of MEF/Nanog-GFP-expressing cells were infected with SeVdp vectors at 32 °C for 14 h. Then 1.0×10^3 of infected cells were seeded onto the feeder cells in 6-well plates and cultured as indicated in the legend to Fig. 5. The numbers of iPS colonies expressing GFP were determined using fluorescent microscopy. At 10 days after SeVdp infection, GFP-positive clones were isolated and treated with siRNA L527 as described above.

Characterization of iPS Cells—Semi-quantitative RT-PCR Assays were performed using GoTaq qPCR Master Mix (Promega) and the primer sets listed in supplemental Table S2. Template cDNA was synthesized with random primers using SuperScript III reverse transcriptase (Invitrogen) from 2 μ g of total cellular RNA isolated using ISOGEN (Nippon Gene, Tokyo, Japan). Bisulfite sequencing analysis was performed using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) with primer sets listed in supplemental Table S2. The PCR DNA fragments were cloned into pCR2.1 vector (Invitrogen), and sequenced by TAKARA BIO INC. (Shiga, Japan). Telomerase activity was determined using the Quantitative Telomerase Detection kit (Allied Biotech, Vallejo, CA). Teratoma formation was performed by subcutaneous injection of 1×10^6 SeVdp-iPS cells (clone #13) into SCID mice. Tumors recovered at necropsy after 6 weeks were processed for fixing and paraffin wax embedding, sectioned (4 μ m), and stained with hematoxylin and eosin. Histological findings were evaluated using a DM3000 microscope (Leica, Wetzlar, Germany). Chimera animals were generated by microinjection of iPS cells into eight-cell or morula stage embryos. The embryos were collected in Medium 2 (Millipore, Billerica, MA) from oviduct and uterus of ICR female mice 2.5 days post-coitum. These embryos were transferred into potassium simplex optimized medium with amino acids (KSOM-AA, Millipore) and cultured for 1–2 h. iPS cells were trypsinized and suspended in iPS cell culture medium. A piezo-driven micro-manipulator (Prime Tech, Tokyo, Japan) was used to drill zona pellucida under the microscope, and 10–15 iPS cells were introduced into the subzonal space of individual 8-cell or morula-stage embryos. After injection, embryos underwent follow-up culture in KSOM-AA for 24 h (until blastomere stage) and then were transferred into the uteri of pseudopregnant recipient ICR female mice.

RESULTS

Basic Design of the SeVdp Vector—The SeV genome consists of six independent cistrons (NP, P/C/V, M, F, HN, and L), encoding eight proteins (10). Each cistron is preceded by a gene-start signal (3'-UCCCNNUUUC) and is followed by a gene-end signal (3'-AUUCUUUUU), which are the only essential *cis*-elements for transcription (25). This simple structure of each cistron makes it easier to design a defective viral vector by gene replacement. The NP, P, and L genes of SeV encode a major NP and two subunits of RNA-dependent RNA polymerase (P and L), respectively. All of these are indispensable for viral transcription and replication (10). We revealed previously that the L gene of the SeV Cl.151 strain with four missense mutations (V981I, S1088A, C1207S, and V1618L) contributes to long term persistence by providing the mechanism to escape from interferon β (IFN β) induction (12). Among these mutations, V1618L is most critical; SeV with a mutant L protein (V1618L) is defective in IFN β induction as with the Cl.151 strain.⁴ We also found that uncapped read-through transcripts synthesized in an early stage of infection with wild-type SeV were only barely detectable in cells in-

⁴ K. Nishimura and M. Nakanishi, unpublished information.

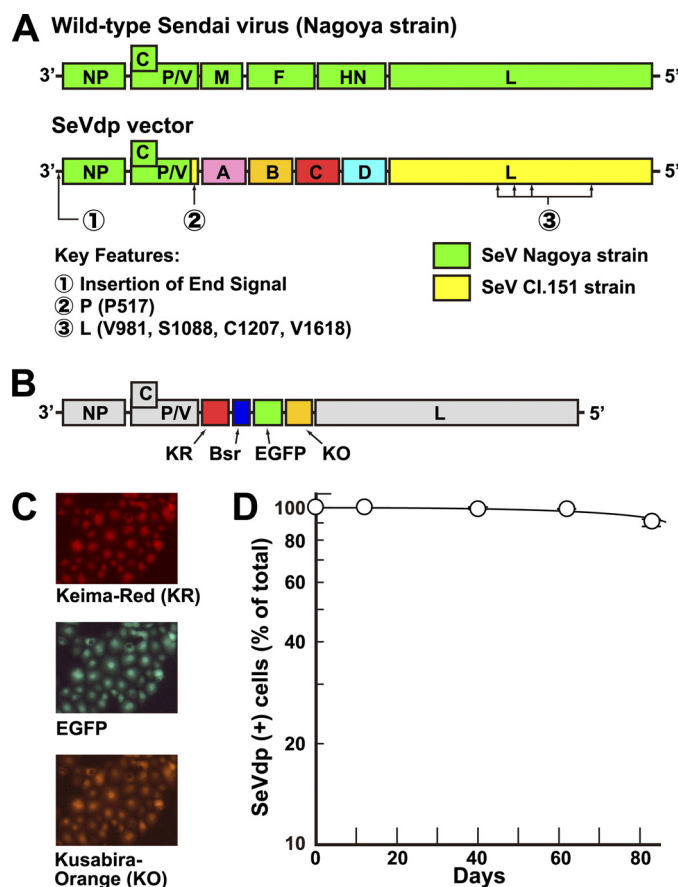


FIGURE 1. Design and characteristics of the SeVdp vectors. A, genome structure of the Sendai virus and an SeVdp vector is shown. Exogenous genes installed on the SeVdp vector are indicated as A–D. B, genome structure of SeVdp(KR/Bsr/EGFP/KO) is shown. C, expression of the fluorescent marker genes installed on the SeVdp vector is shown. LLCMK₂ cells were infected with SeVdp(KR/Bsr/EGFP/KO) at a multiplicity of infection of 0.1, selected with blasticidin S (5 μ g/ml), and examined by fluorescence microscopy with dye-specific filters. D, stability of gene expression induced by the SeVdp vector is shown. LLCMK₂ cells were infected with SeVdp(KR/Bsr/EGFP/KO) and selected with blasticidin S as described in C. The cells were then cultured for the indicated period in the absence of blasticidin S. The ratio of SeV NP antigen-positive cells in the total cells was determined by fluorescence microscopy, as described under “Experimental Procedures.”

fectured with SeV/L (V1618L).⁴ We hypothesized that the defect in IFN β induction might be correlated with the altered transcription of uncapped read-through RNA by the mutant SeV RNA polymerase.

In addition, we identified a missense mutation of the P gene (P517H) that is also essential for establishing long term persistency (Fig. 1A, supplemental Fig. S2A). The P protein makes a complex with the NP and L proteins, and the C terminus of P protein (amino acids, 479–568) has been assigned as a binding region for the NP (26). However, the precise role of this alteration in long term persistency remains to be determined. On the other hand, the 3'-distal region (nucleotides 1–2870) of the genome of SeV Cl.151 consists of the whole NP gene and part of the P/C/V genes but does not contribute to viral persistency (12) (Fig. 1A, supplemental Fig. S2A). Rather, we found that replacement of this 3'-distal region with that of the wild-type Nagoya strain significantly improved the recovery of the recombinant SeV from full-length genomic cDNA (supplemental Fig. S2B). We also inserted a

Novel Sendai Virus Vector Ideal for Cell Reprogramming

gene-end signal just upstream of the gene-start signal of the *NP* gene (Fig. 1A, supplemental Fig. S2C). This modification further stabilized SeV-mediated gene expression through more stringent control of IFN β induction by forced termination of uncapped read-through transcripts.⁴

We then planned to expand the capacity of the SeV Cl.151-based vector by deleting all the viral genes dispensable for stable gene expression. We have revealed previously that mutations within the central region (nucleotides 2871–9594) of the SeV Cl.151 genome contributed to viral persistence independently from the altered *L* gene (12). The region consists of *M*, *F*, and *HN* genes, which encode a matrix protein underlining the viral envelope (*M*) and envelope glycoproteins (*F* and *HN*), respectively. These structural genes are essential for production of infectious virions but are dispensable for viral transcription/replication, as SeV vectors with all of the *M*, *F*, and *HN* genes deleted could be generated successfully (27). However, as these defective vectors could not support stable gene expression, the role of these structural genes in long term persistency remains obscure.

We found previously that cells infected with SeV Cl.151 expressed large quantities of *F* and *HN* proteins on their surface (28). This observation suggested that the accumulation of the structural gene products (proteins and/or mRNAs) might interfere with the lytic infection cycle by a negative feedback mechanism, as proposed previously for the *M* protein (29). To examine this premise directly, we characterized recombinant SeVs with a dysfunction in each of the *M*, *F*, or *HN* genes either by deletion or by nonsense mutation. A selective marker gene (*Bsr*) conferring resistance to blasticidin S was used for estimating persistency rapidly (supplemental Fig. S3A). We found that all of these single-gene defective viruses established stable *Bs*-resistant colonies (supplemental Fig. S3A). Moreover, any two of these structural genes could be replaced with exogenous genes without affecting the persistent phenotype (supplemental Fig. S3A). To examine the role(s) of the *M*, *F*, and *HN* proteins further, we coexpressed these proteins directly from the cloned cDNAs and found that those derived from wild-type SeV strains induced much stronger cytopathic effects than did those derived from the SeV Cl.151 strain (supplemental Fig. S3B). From these results, we conclude that all of the *M*, *F*, and *HN* genes of SeV Cl.151 are dispensable and can be replaced with exogenous genes without disturbing viral persistency. We also succeeded in expanding the capacity of the vector by inserting an extra gene cassette between the *P/C/V* genes and the *M* gene without affecting viral persistency (Fig. 1A).

Dysfunction of structural genes is also essential for preventing self-replication of the vector. As the vectors used for cell reprogramming are installed with tumorigenic genes, such as *c-Myc* and *LIN28*, avoiding the production of secondary infectious particles from the gene-transferred cells is important not only to observe the regulation of recombinant DNA experiments but also to guarantee the safety of the vector in any therapeutic application. In the case of SeV, cultured cells infected with SeV variants defective in single structural genes produced significant amounts of virus-like particles (30, 31), suggesting that a dysfunction in single genes is insufficient for

TABLE 1

Determination of infectious virions and the NP protein in the culture supernatant of the cells harboring SeV Cl.151-based vectors

All the vectors were installed with the *Bsr* gene encoding blasticidine S deaminase. Aliquots of 10⁶ of LLCMK₃ cells harboring each SeVdp vector were seeded in 90-mm wells with 8 ml of medium. After culturing for 3 days, culture supernatant was recovered and filtered through 0.45- μ m cellulose acetate membranes. NP protein was determined by blotting 0.04–20 μ l of the supernatant on nitrocellulose membranes as described under “Experimental Procedures.” The supernatant was also incubated with 10⁶ uninfected LLCMK₃ cells for 14 h and then cultured in the presence of *Bs* (10 μ g/ml) for 7 days. The numbers of cell colonies resistant to *Bs* were determined by staining with crystal violet.

Structural genes			NP protein	Number of <i>Bs</i> ^r colonies
			ng/day/10 ⁵ cells	
<i>M</i> ^a	<i>F</i> ^a	<i>HN</i> ^a	8.75	> 10 ⁶
– ^b	<i>F</i>	<i>HN</i>	5.36	366
<i>M</i>	– ^b	<i>HN</i>	7.62	2
<i>M</i>	<i>F</i>	– ^b	72.05	10
<i>M</i>	– ^b	– ^b	2.51 ^c	0
– ^b	<i>F</i>	– ^b	2.76 ^c	0
– ^b	– ^b	<i>HN</i>	2.61 ^c	0
– ^b	– ^b	– ^b	2.56 ^c	0

^a Replication-competent vector.

^b Corresponding genes were deleted or replaced with exogenous genes.

^c Background caused by spontaneous cell lysis.

the complete blockage of self-replication. To reexamine this phenomenon, we determined the numbers of infectious particles and the amount of NP antigen in the culture supernatant of cells infected with various SeV Cl.151-derived defective viruses carrying the *Bsr* gene (Table 1). We found that cells infected with SeVs bearing a defect in one of the *F*, *HN*, or *M* genes produced significant amounts of NP antigen as well as infectious particles capable of transmitting *Bs* resistance to naïve cells (Table 1). This phenomenon was not observed when the viruses carried defects in at least two of the structural genes (Table 1). Therefore, we conclude that all three structural genes should be eliminated for maximizing the safety of the SeVdp vector through abolishing self-replication and for maximizing the vector capacity for installing exogenous genes.

In summary, we have designed the basic genome structure of the SeVdp vector. This consists of three separate genetic elements (Fig. 1A) as follows. 1) The 3'-terminal structure comprises the *NP* and *P/C/V* genes derived from the Nagoya strain with an alteration for supporting stable gene expression. 2) Internal gene cassettes capable of installing up to four exogenous genes, created by deletion/replacement/insertion of *M*, *F*, and *HN* genes. 3) The *L* gene and the 5'-terminal structure derived from the Cl.151 strain with four missense mutations necessary for stable gene expression and for escaping from IFN β induction.

Characterization of SeVdp Vector-mediated Gene Expression—We then prepared the SeVdp vectors installed with four exogenous genes and characterized vector-mediated gene expression. We first constructed SeVdp(*KR/Bsr/EGFP/KO*) installed with *Bsr* and three marker genes encoding *KR*, *EGFP*, and *KO* (Fig. 1B). All the cells infected with this vector expressed the three marker genes stably after selection with *Bs* (Fig. 1C). Furthermore, even in the absence of selection, 98.2% of cells retained the vectors for 62 days (Fig. 1D). Stability of gene expression induced by SeVdp vectors was solely dependent on the vector backbone described above and was not affected either by the installed genes or by the characteris-

TABLE 2
Stability of gene expression induced by SeVdp vectors

Cells harboring the SeVdp vectors were cultured in the presence of Bs (10 $\mu\text{g}/\text{ml}$) to certify that 100% of the cells were SeVdp (+). On day 0 the cells were set up in the medium either with Bs (Bs(+)) or without Bs (Bs(-)). Expression of SeV NP antigen was recorded periodically, and the day on which 100% ($T_{100\%}$), 95% ($T_{95\%}$), or 80% ($T_{80\%}$) of the cells expressed NP is indicated. The gene cassette no. corresponds to those shown in Fig. 1A. *Bsr*, blasticidin S deaminase; *KO*, Kusabira Orange; *EGFP*, enhanced green fluorescent protein; *CLuc*, *Cypridina noctiluca* luciferase; *CYBB*, gp91 phox; *aGal*, human α -galactosidase; *KR*, Keima Red; ND, not determined because of the limited lifespan of the cells.

Gene cassette no.				Bs (-)		Bs (+)
A	B	C	D	$T_{95\%}$	$T_{80\%}$	$T_{100\%}$
<i>Days</i>						
- ^a	<i>Bsr</i>	- ^a	<i>KO</i>	80 ^b	205 ^b	>180 ^b
- ^a	<i>Bsr</i>	- ^a	<i>KO</i>	>80 ^c	ND ^c	ND ^c
- ^a	<i>Bsr</i>	<i>EGFP</i>	<i>CLuc</i>	65 ^b	105 ^b	>180 ^b
- ^a	<i>Bsr</i>	<i>EGFP</i>	<i>CYBB</i>	65 ^b	170 ^b	>180 ^b
- ^a	<i>Bsr</i>	<i>EGFP</i>	α -Gal	60 ^b	112 ^b	>180 ^b
<i>KR</i>	<i>Bsr</i>	<i>EGFP</i>	<i>KO</i>	70 ^b	195 ^b	>180 ^b

^a No exogenous gene was installed.

^b Determined in LLCMK₂ cells.

^c Determined in normal human fibroblasts.

tics of host cells (Table 2). Under selection with antibiotics, nearly 100% of cells could retain the expression of all the marker genes for at least 6 months (Table 2). Reflecting the characteristics of its parental virus, the SeVdp vector could deliver and express the installed genes stably in various host cells, including cell lines derived from the mouse (NIH3T3), hamster (CHO, BHK-21), monkey (LLCMK₂, CV-1, COS-7), and human (HeLa, U937) as well as human and mouse primary fibroblasts (12). Thus, we proved that the SeVdp vectors had preserved the same characteristics of the parental SeV Cl.151 to establish stable persistent infection after it had been modified with four exogenous genes.

We then examined the feasibility of using the SeVdp vectors in stem cell research, focusing on their biological inertness. Most gene delivery/expression systems using either recombinant viruses or physical/chemical means often trigger cellular defense systems against pathogenic microbes (32). The stimulated cells secrete various cytokines, which affect the proliferation, differentiation, and survival of stem cells. Wild-type SeV and conventional SeV vectors based on the wild-type Z strain powerfully induced the production of IFN β , IFN γ , TNF α , IL-1 β , IL-6, IL-8, and many other cytokines (33–35), resulting in the apoptotic death of target cells. On the other hand, we revealed previously that SeV Cl.151 has a defect in inducing these inflammatory cytokines (12), suggesting strongly that the SeV Cl.151-based vector is biologically inert. To verify this under more stringent experimental conditions, we examined the effect of SeVdp-mediated gene transfer/expression on human hematopoietic stem cells (HSCs) by long term culture-initiating cell assays (20).

We isolated a CD133 (+) HSC-enriched fraction from human cord blood, infected it with the SeVdp vector bearing the *KO* gene (SeVdp(*Bsr*/ Δ F/*KO*)) on day 0, and cultured it further in standard conditions. More than 90% of the cells in the HSC-enriched fraction were susceptible to the SeVdp vector under this infection protocol and sustained strong *KO* expression on days 3 (Fig. 2A) and 10 (Fig. 2B). Seven weeks after culturing on OP9 stromal cells, all kinds of myeloid lineage colonies derived from human HSCs, including colony-form-

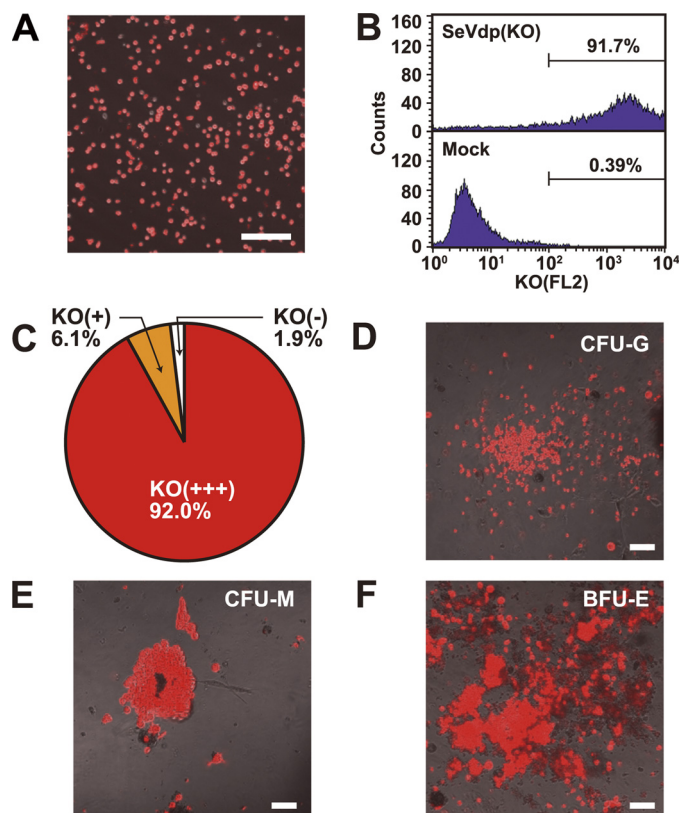


FIGURE 2. Expression of *KO* in human hematopoietic stem cells and in their descendant cells. A and B, expression of *KO* in CD133 (+) cord blood cells is shown. CD133 (+) cells were purified with magnetic beads conjugated with anti-CD133 antibody (Miltenyi Biotec). The cells were infected with SeVdp(*Bsr*/ Δ F/*KO*) at a multiplicity of infection of 4 at 37 °C for 2 h. The cells were then cultured for 3 days (A) and 10 days (B) and examined using fluorescence and phase-contrast microscopy (A) and with flow cytometry using a FACSCalibur (BD Biosciences) (B), respectively. C–F, expression of *KO* in descendant colonies differentiated *in vitro* is shown. Cells infected with SeVdp(*Bsr*/ Δ F/*KO*) as described above were cultured on OP9 cells in a 96-well plate for 5 weeks for lineage commitment. The cells in each well were then harvested, cultured in semisolid medium for 2 weeks, and examined for the expression of *KO* using fluorescence microscopy. C, the ratio of *KO*-positive colonies; 2931 differentiated colonies were examined. *KO* (+++), colonies expressing *KO* strongly; *KO* (+), colonies expressing *KO* weakly or heterogeneously; *KO* (-), colonies with no detectable *KO* expression. D–F, fluorescence and phase-contrast micrographs of typical colonies representative of each lineage. D, *CFU-G*, *CFU*-granulocytes. E, *CFU-M*, *CFU*-macrophages. F, *BFU-E*, burst-forming unit-erythroid cells. Scale bar, 100 μm .

ing unit (*CFU*)-granulocytes (*CFU-G*), *CFU*-macrophages (*CFU-M*), *CFU*-granulocyte-macrophage, and burst-forming unit-erythroid (*BFU-E*) cells were readily detectable (Fig. 2D–F). Most importantly, 92% of these colonies still expressed *KO* very strongly on the seventh week (Fig. 2C), indicating that the SeVdp vector can deliver the gene quite efficiently into HSCs and is inert enough to sustain gene expression without affecting the differentiation of multipotent HSCs.

For efficient and reproducible cell reprogramming, it is also important to express the various reprogramming genes at a fixed balance in each target cell (36–39). The SeVdp vector installed with four exogenous genes could deliver these genes simultaneously, so is theoretically superior to those systems delivering the genes separately. To clarify this issue further, we prepared an SeVdp vector installed with *KO* and *EGFP* together (SeVdp(*KO*/*Hyg*/*EGFP*/*Luc2CP*)) (Fig. 3A) and two

Novel Sendai Virus Vector Ideal for Cell Reprogramming

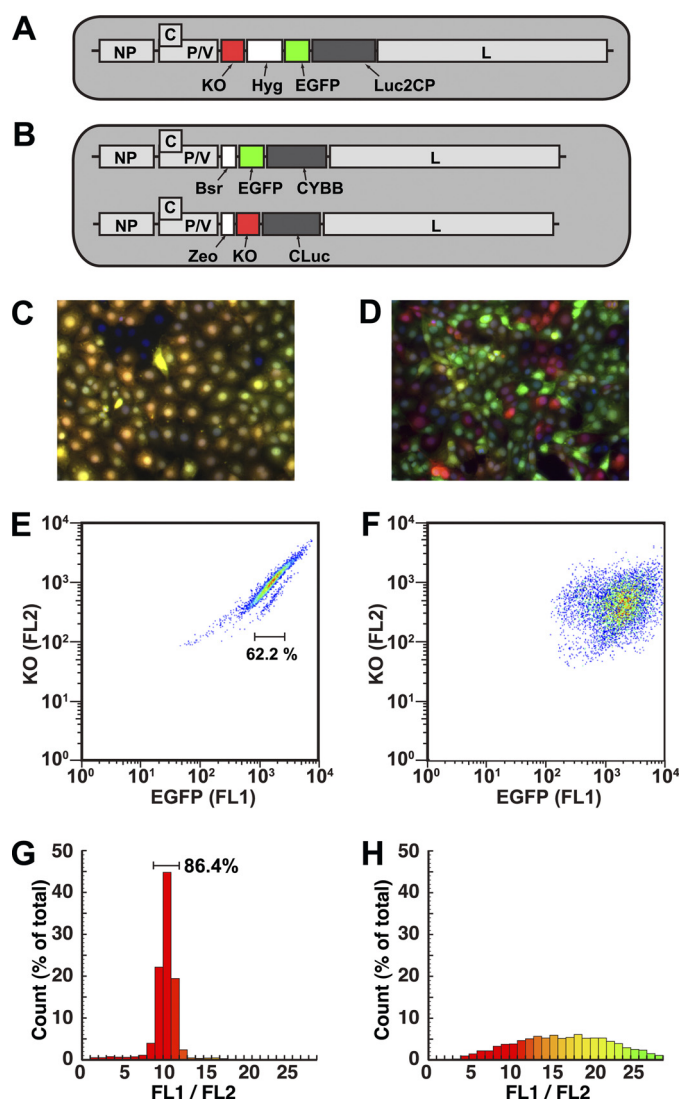


FIGURE 3. Compatibility of two independent SeVdp vectors in a single cell. *A*, genome structure of SeVdp(KO/Hyg/EGFP/Luc2CP) is shown. *B*, genome structure of SeVdp(Bsr/EGFP/CYBB) and SeVdp(Zeo/KO/CLuc), coexisting in a single cell is shown. *C* and *D*, fluorescence and phase-contrast micrographs of the cells described in schema (*C*) and in schema *B* (*D*) are shown. LLCMK₂ cells were infected with the SeVdp vectors as described in Fig. 1C and selected with hygromycin B (200 μ g/ml) (*C*) or with zeocin (100 μ g/ml) and blasticidin S (5 μ g/ml) (*D*). Fluorescence images of KO and EGFP were obtained separately with specific filter sets, converted to artificial color (green for EGFP and red for KO), and merged using iVision software (BioVision Technologies, Exton, PA). *E–H*, quantitative analysis of EGFP and KO expression by flow cytometry is shown. The cells shown in *C* (*E* and *G*) or in *D* (*F* and *H*) were harvested as single-cell suspensions with trypsin, and the fluorescent signals were analyzed using a FACSCalibur (BD Biosciences) for quantifying the signals of EGFP (FL1, 515–545 nm) and KO (FL2, 564–606 nm) after compensation (*E* and *F*) and analyzed with FISHMAN R (On-tip Biotechnologies) for determining the ratio of the signals of EGFP and KO in each cell as a histogram (*G* and *H*).

others installed with *KO* and *EGFP* separately on different SeVdp vectors (SeVdp(Bsr/EGFP/CYBB) and SeVdp(Zeo/KO/CLuc)) (Fig. 3*B*). We then characterized the expression levels of *KO* and *EGFP* induced by a single infection with SeVdp(KO/Hyg/EGFP/Luc2CP) and by coinfection with SeVdp(Bsr/EGFP/CYBB) and SeVdp(Zeo/KO/CLuc) (Fig. 3). When the cells were infected solely with SeVdp(KO/Hyg/EGFP/Luc2CP) (Fig. 3*A*), they expressed both *KO* (red) and *EGFP* (green) at a constant balance, shown by a uniform yellow

low color in merged microscopy images (Fig. 3*C*). In contrast, the cells coinfecting with SeVdp(Bsr/EGFP/CYBB) and SeVdp(Zeo/KO/CLuc) (Fig. 3*B*) expressed *KO* and *EGFP* at a significantly different balance even after selection under Zeo plus Bs conditions (Fig. 3*D*). We then examined these cells quantitatively by flow cytometry (Fig. 3, *E–H*). When coinfecting with SeVdp(Bsr/EGFP/CYBB) and SeVdp(Zeo/KO/CLuc), nearly 100% of the infected cells expressed both *KO* and *EGFP* after antibiotic selection (Fig. 3*F*), but the balance of expression varied widely (Fig. 3, *F* and *H*). In contrast, 86.4% of the cells infected with SeVdp(KO/Hyg/EGFP/Luc2CP) expressed *KO* and *EGFP* at a fixed balance (Fig. 3, *E* and *G*) and at a constant level (62.2% of the cells expressed *EGFP* and *KO* within a 3-fold range) (Fig. 3*E*). From these results, we conclude that only the SeVdp vector installed with all the genes required to be expressed from a single genome can express these genes reproducibly at a fixed balance, thus providing a significant advantage for cell reprogramming.

Elimination of SeVdp Vector with siRNA—The last hurdle for efficient cell reprogramming is to establish a method for eliminating the vector genome from those cells harboring it stably. Although the viral family *Paramyxoviridae* includes major human pathogens (e.g. measles virus and respiratory syncytial virus), there is no specific small-molecule antiviral drug available. Instead, siRNAs against viral genes have been investigated with the aim of interfering with viral replication (40). However, the effects of siRNAs on stable persistent infections such as the SeVdp system have not been established. Therefore, we examined the effect of knocking down the viral replication machinery on the stability of the SeVdp genome using specific siRNAs. We designed siRNAs against each of the *NP*, *P*, and *L* genes (Fig. 4*A*) and examined their effects on the infection of a replication-competent SeV Cl.151 installed with the *EGFP* gene (SeV Cl.151(EGFP)). When the cells were treated with these siRNAs just before infection, the replication of SeV Cl.151(EGFP) was blocked almost completely (supplemental Fig. S4*A*). However, the effects of these siRNAs on the cells already harboring an SeVdp vector stably were quite different; siRNA against the *L* gene was most effective, and that against the *NP* gene showed almost no effect (supplemental Fig. S4*B*). This phenomenon might simply reflect the relative abundance of the target gene products; *NP* mRNA is about 34 times more abundant than *L* mRNA (41). Otherwise, suppression of a catalytic subunit of RNA polymerase (*L* protein) might interfere with the replication of the SeVdp genome more profoundly.

We then examined the time course with which an SeVdp vector would be eliminated by siRNA against the *L* gene (Fig. 4). To monitor elimination quantitatively, we used a cell line harboring the SeVdp vector installed with a destabilized firefly luciferase gene (SeVdp(KO/Hyg/EGFP/Luc2CP)) and determined luciferase activity as a faithful marker of gene expression from the SeVdp vector. As shown in Fig. 4*B*, the siRNA blocked expression of the *L* protein quite efficiently after day 3. In parallel with this suppression, the SeVdp was eliminated at a half-life of 17.5 h after a short time lag; the luciferase activity fell below the detection limit after day 8 (Fig. 4*C*). This elimination was irreversible; when the cells

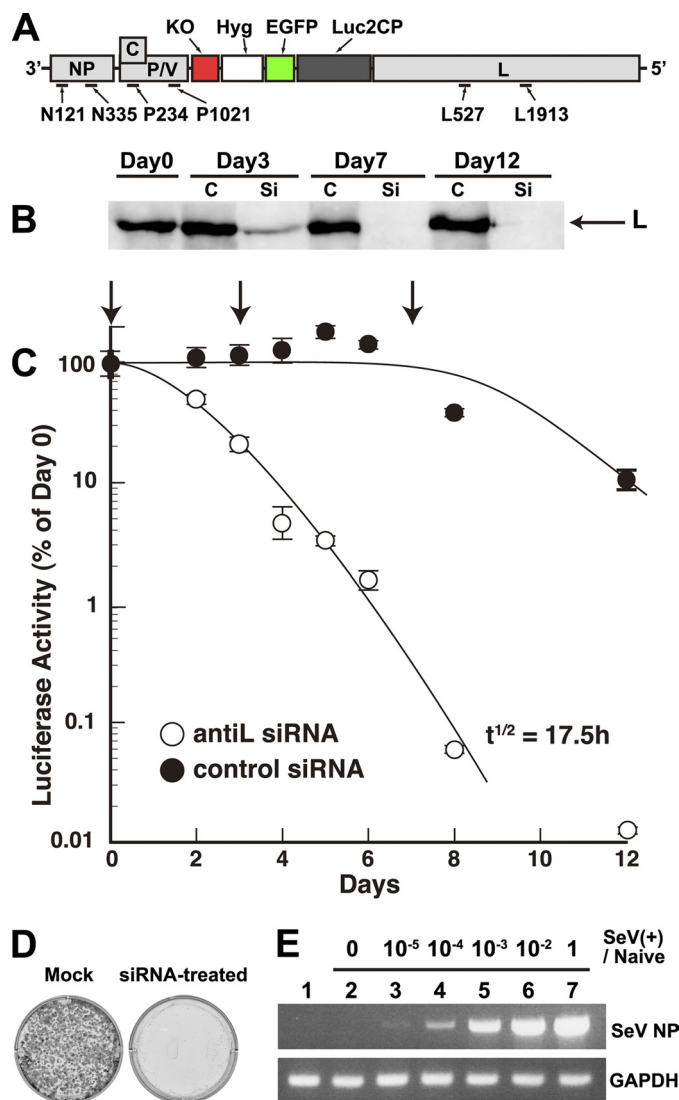


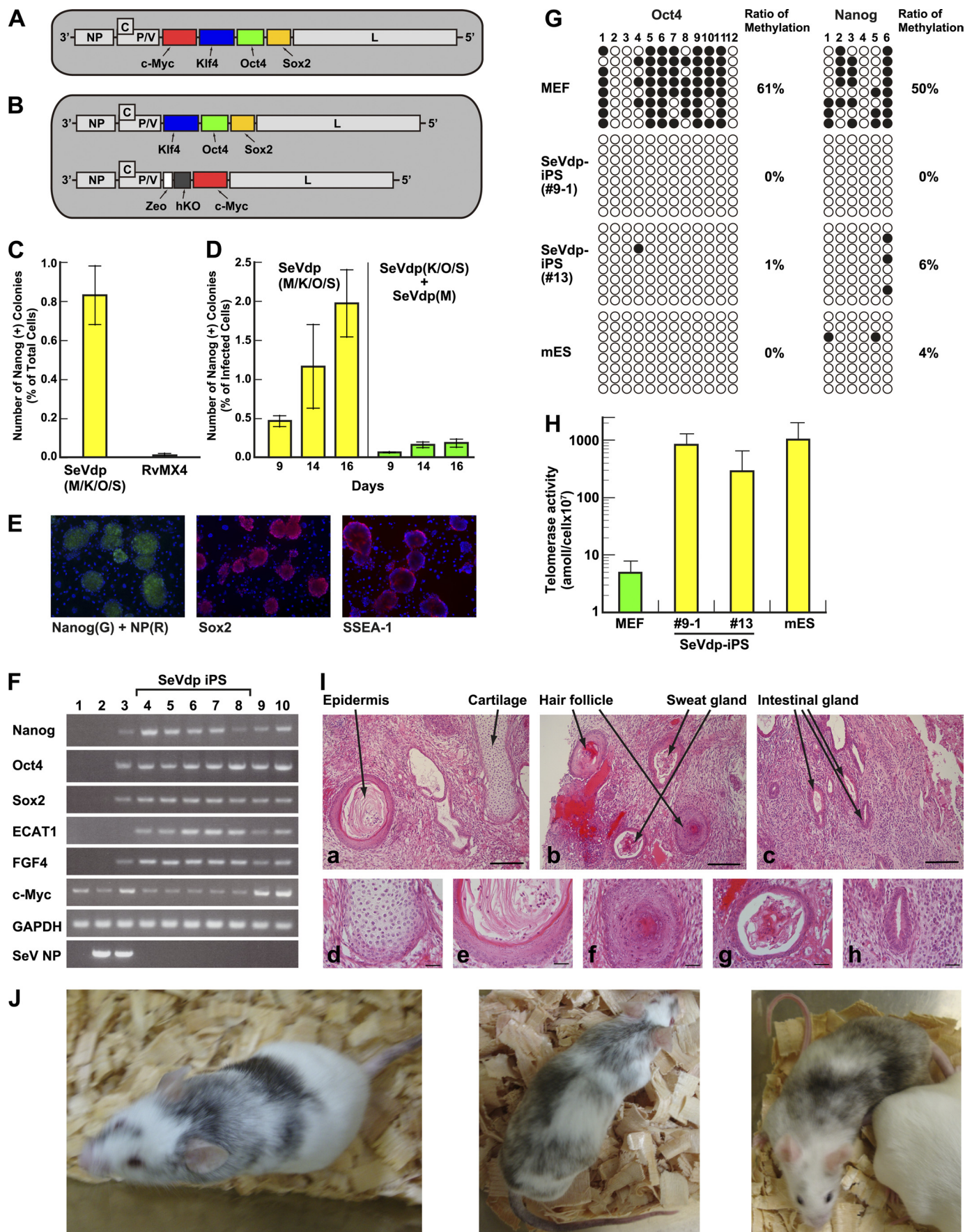
FIGURE 4. Elimination of SeVdp vectors from the cells with specific siRNAs. *A*, the genome structure of SeVdp(KO/Hyg/EGFP/Luc2CP) and target sites of siRNAs is shown. *B*, quantitative analysis of L protein by Western blotting is shown. HeLa cells carrying SeVdp(KO/Hyg/EGFP/Luc2CP) were cultured in the absence of hygromycin B and treated with siL527 (Si) or with control siRNA (C) complexed with Lipofectamine RNAiMAX (Invitrogen) on days 0, 3, and 7. The cells were harvested periodically as indicated, and 50- μg aliquots of cell extracts were separated on SDS-PAGE. The amount of L protein was determined by Western blotting probed with an anti-SeV L protein rabbit polyclonal antibody. *C*, quantitative analysis of SeVdp-mediated gene expression is shown. The HeLa cells carrying SeVdp(KO/Hyg/EGFP/Luc2CP) were treated with siRNA as described in *B*. The cells were harvested periodically as indicated, and the specific firefly luciferase activity was determined as described under "Experimental Procedures." Open circles, treated with siL527; closed circles, treated with control siRNA; vertical arrows, the day of siRNA treatment. *D*, detection of the cells carrying the SeVdp vector after treatment with siL527 is shown. HeLa cells carrying SeVdp(KO/Hyg/EGFP/Luc2CP) and treated with siL527 for 8 days as described in *C* were further cultured for 4 weeks in the absence of the siRNA and of hygromycin B. Aliquots of 1×10^4 cells were then seeded into 6-well plates with medium containing hygromycin B (100 $\mu\text{g}/\text{ml}$) and cultured for 10 days. The cells were fixed then stained with 0.01% crystal violet. *E*, detection of SeVdp by semiquantitative RT-PCR is shown. cDNAs were prepared by using 2- μg aliquots of total cellular RNAs as indicated, and the cDNA corresponding to 10^4 cells was analyzed by RT-PCR to determine SeV NP mRNA as described under "Experimental Procedures." Lane 1, HeLa cells carrying SeVdp(KO/Hyg/EGFP/Luc2CP) and treated with siL527 as described in *D*; lanes 2–7, naïve HeLa cells containing SeVdp(+) cells at the ratio indicated.

harboring SeVdp(KO/Hyg/EGFP/Luc2CP) had been treated with the siRNA for 8 days and were further cultured for 4 weeks in the absence of the siRNA, all the cells became susceptible to hygromycin B, indicating that the SeVdp genome was no longer present in the cells (Fig. 4D). We further confirmed the complete erasure of the SeVdp genome by sensitive RT-PCR analysis; we could not detect any SeV genome under conditions capable of detecting a single SeV(+) cell among 10^5 naïve cells (Fig. 4E). From these data, we conclude that siRNA against the L gene is an effective tool for erasing the SeVdp genome thoroughly from the cells.

Generation of Mouse iPS Cells with SeVdp Vectors Installed with Reprogramming Genes—We then constructed an SeVdp vector installed with four reprogramming genes (SeVdp(*c-Myc/Klf4/Oct4/Sox2*)) (Fig. 5A) and examined its potential to reprogram mouse fibroblasts. First, we compared the efficiency of reprogramming by an infection of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) with that produced by the coinfection of ecotropic retrovirus vectors installed with the same genes separately (RvMX4), which is a current standard approach for iPS generation (24) (Fig. 5C). For assessing the expression of Nanog, a well known marker of fully reprogrammed iPS cells, we used embryonic fibroblasts derived from a Nanog-GFP knock-in mouse (MEF/Nanog-GFP) (24) and monitored the expression of GFP. We found that SeVdp(*c-Myc/Klf4/Oct4/Sox2*) and pMX4 reprogrammed 0.83 and 0.01% of MEF cells to Nanog (+) iPS cell-like colonies, respectively, on day 14 after vector infection (Fig. 5C). As the efficiency of emergence of Nanog-GFP (+) colonies from MEF with retrovirus vectors was consistent with that reported previously (24), we concluded that the SeVdp vector could reprogram MEF about 100 times more efficiently than standard procedures using retrovirus vectors. We also prepared SeVdp vectors installed with *Klf4/Oct4/Sox2* genes (SeVdp(*Klf4/Oct4/Sox2*)) and with a *c-Myc* gene (SeVdp(*Zeo/hKO/c-Myc*)) (Fig. 5B) separately. We found that the coinfection with these two SeVdp vectors reprogrammed MEF/Nanog-EGFP much less efficiently than did the single infection with SeVdp(*c-Myc/Klf4/Oct4/Sox2*) (Fig. 5D). These results clearly proved our assumption that installing all the necessary genes on a single vector was critical for maximizing the potential of the vector to reprogram cells.

After treating these Nanog (+) cells with siRNA L527, we obtained SeV antigen-free iPS cells with typical characteristics (Fig. 5). First, they expressed ES/iPS cell markers detectable by fluorescence microscopy (*Nanog*, *Sox2*, and *SSEA-1*) (Fig. 5E) as well as by RT-PCR (*Nanog*, *Sox2*, *Oct4*, *c-Myc*, *ECAT1*, and *FGF4*) (Fig. 5F). The primer sets used in this RT-PCR assay detected the expression of mouse genes but not that of human genes installed on the SeVdp vector. Some of the endogenous iPS marker genes (*Nanog*, *Oct4*, and *Sox2*) were detectable as early as on day 5 after gene transfer (Fig. 5F, lane 3), suggesting rapid cell reprogramming by the SeVdp vector. Second, the promoter regions of the *Oct4* and *Nanog* genes were epigenetically remodeled similar to ES cells (Fig. 5G). Third, telomerase activity was increased by 50–200-fold to the same level as in ES cells (Fig. 5H). Fourth, they differentiated into derivatives of all three germ layers in teratomas (Fig.

Novel Sendai Virus Vector Ideal for Cell Reprogramming



5f). Finally, we can generate chimera animals by injecting the SeVdp-iPS cells into embryos (Fig. 5j).

DISCUSSION

Discovery of the methods for achieving cell reprogramming with ectopic expression of defined factors has had a great impact on modern bioscience. However, to establish this technology for practical applications, we still have to overcome several difficult hurdles, such as the dramatic improvement of reprogramming efficiency and reproducible generation of fully differentiation-competent iPS cells without allowing the expression of residual exogenous genes on the chromosomes. Among these issues, chromosomal integration of reprogramming genes is the most critical factor affecting the characteristics of iPS cells (9). Incomplete suppression of exogenous reprogramming genes might affect the pluripotency of iPS cells. Furthermore, even if the integrated gene cassette is suppressed by epigenetic modification, differentiation of mouse iPS cells has frequently reactivated the integrated genes and induced cancers in iPS cell-derived offspring, as some of the reprogramming genes have intrinsic tumorigenic activity (24). Therefore, methodologies for generating fully pluripotent human iPS cells carrying no remnant of exogenous genes have been investigated but with limited success (9). As far as we know, the SeVdp vector described here is the only system that fulfills all the requirements to accomplish this goal with high efficiency and without laborious procedures.

Efficient gene delivery and expression are the primary factors affecting iPS cell generation, and this SeVdp vector has a great advantage over other delivery systems. As a typical recombinant viral vector, it can deliver genes much more efficiently than a nonviral system. In addition, its exceptionally broad host range should be quite beneficial; these viruses can infect almost all cell types from avian to human (42). Among others, the efficiency of the SeVdp vector to induce stable gene expression in human HSCs (~80%) is remarkably higher than that of retroviral vectors under multiple infection cycles with the aid of recombinant fibronectin fragments (~30%)

(43). These characteristics have emerged partly because the SeVdp recognizes ubiquitous sialic acid as the primary receptor. Furthermore, the SeVdp has its own RNA-dependent RNA polymerase and requires only ubiquitous cytosolic proteins for transcription/replication (44). Thus, the SeVdp vector induces active transcription just after the nucleocapsid is delivered into the cytoplasm.

The enablement of stable gene expression without chromosomal integration is the most remarkable characteristic of these SeVdp vectors. DNA-based vectors (including retro/lentiviral vectors) can accomplish stable gene expression either by chromosomal integration or by episomal replication, depending on the cellular replication machinery. However, these characteristics make it difficult to remove the stabilized DNA from the cells; this depends either on a complex excision process using DNA recombinase or on passive elimination in the absence of selection. On the other hand, recombinant RNA viral vectors (except for retro/lentiviral vectors) cannot achieve stable gene expression, partly because they trigger cellular defense systems and induce apoptotic death in the host cells. The SeVdp vector is the only RNA-based platform inert enough to allow stable gene expression in sensitive HSCs, thanks to its unique gene mutations/alterations for escaping the host defense system. Furthermore, as the stability of SeVdp genomic RNA depends on the activity of viral RNA polymerase, interference of the polymerase with siRNA can be used to eliminate the genome from infected cells, as shown here.

Coinfection of conventional F-defective SeV vectors installed with *Oct4*, *Sox2*, *Klf4*, and *c-Myc* separately has been reported to generate iPS cells (45, 46). Although these reports demonstrated the potential of SeV vectors in cell reprogramming, our data have clearly demonstrated that this SeVdp vector provides a superior alternative to this approach. Among other factors, the cytopathic nature of the conventional SeV vectors based on wild-type SeV, including simple F-defective SeV vectors (30, 47), limits their utility in cell re-

FIGURE 5. Reprogramming of MEFs with SeVdp vectors installed with reprogramming genes. *A*, the genome structure of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) is shown. *B*, shown is genome structure of SeVdp(*Klf4/Oct4/Sox2*) and SeVdp(*Zeo/hKO/c-Myc*), coexisting in a single cell, is shown. *C* and *D*, shown is the efficiency to reprogram MEF/Nanog-GFP. MEF/Nanog-GFP cells (1.25×10^5) were infected with SeVdp vectors, and retroviral vectors were installed with *c-Myc/Klf4/Oct4/Sox2* as described under "Experimental Procedures." Then 1.0×10^3 of infected cells were seeded onto the feeder cells in 6-well plates and cultured for 14 days (*C*) or for the indicated days (*D*). The number of iPS colonies expressing GFP was determined under fluorescent microscopy. Reprogramming efficiency was indicated as the ratio of the number of EGFP-positive colonies to that of MEF/Nanog-GFP seeded in the well (*C*) or to that of infected MEF/Nanog-GFP seeded in the well (*D*). *C*, shown is a comparison of reprogramming efficiency with the SeVdp(*c-Myc/Klf4/Oct4/Sox2*) vector and with retroviral vectors. SeVdp(*M/K/O/S*), SeVdp(*c-Myc/Klf4/Oct4/Sox2*); RvMX4, coinfection of ecotropic retroviral vectors installed with *c-Myc*, *Klf4*, *Oct4*, and *Sox2* separately. *D*, shown is a comparison of reprogramming efficiency by a single infection of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) (SeVdp(*M/K/O/S*)) and by coinfections of SeVdp(*Klf4/Oct4/Sox2*) and SeVdp(*Zeo/hKO/c-Myc*) (SeVdp(*K/O/S*) + SeVdp(*M*)). *E*, characterization of mouse iPS cells generated with SeVdp(*c-Myc/Klf4/Oct4/Sox2*) is shown. The ES-like colonies emerging from MEF/Nanog-GFP cell lines were fixed, incubated with specific primary antibodies against SeV NP antigen (*left*), Sox2 (*middle*), and SSEA-1 (*right*), then stained with secondary antibodies conjugated with Alexa 555. The cells were then counterstained with DAPI and examined by fluorescence microscopy as described under "Experimental Procedures." Nanog (*left*), expression of GFP driven by the Nanog promoter. *F*, gene expression analysis with semiquantitative RT-PCR is shown. Aliquots (2 μ g) of total RNA prepared from the cells indicated were analyzed as described under "Experimental Procedures." Lane 1, MEF; lane 2, MEF infected with control vector (SeVdp(*Bsr/ Δ F/KO*)); lane 3, MEF infected with SeVdp(*M/K/O/S*) on day 5 infection; lane 4, SeVdp-iPS cell clone #2-1; lane 5, SeVdp-iPS cell clone #9-1; lane 6, SeVdp-iPS cell clone #13; lane 7, SeVdp-iPS cell clone #16; lane 8, SeVdp-iPS cell clone #21; lane 9, mouse iPS cell generated with retroviral vectors (RvMX4); lane 10, mouse ES cell (clone D3). *ECAT1*, ES cell-associated transcript 1; *FGF4*, fibroblast growth factor 4. *G*, methylation analysis of Oct4 and Nanog promoters is shown. Methylation profile of CpG in genomic DNA was analyzed by bisulfite sequence analysis as described under "Experimental Procedures." Open circles, unmethylated cytosine; closed circles, methylated cytosine. The ratio of methylated cytosine is indicated as a percentage of total cytosine residues analyzed. *H*, a telomerase assay is shown. Telomerase activity in total cell extract prepared from 1×10^3 cells was analyzed as described under "Experimental Procedures" and is indicated as the amount of (dT-TAGGG)_n synthesized. *I*, histology of teratomas derived from SeVdp-iPS cells is shown. Teratoma formation was studied at 6 weeks after the subcutaneous injection of 1×10^6 SeVdp-iPS cells from clone #13 into SCID mice. *a-c*, low magnification; scale bar = 100 μ m. *d-h*, high magnification observation; scale bar = 20 μ m. *d*, cartilage; *e*, epidermis; *f*, hair follicle; *g*, sweat gland; *h*, intestinal gland. *J*, adult chimeras derived from SeVdp-iPS cells (clone #13) are shown. Dark hair indicates donor contribution.

Novel Sendai Virus Vector Ideal for Cell Reprogramming

programming. As revealed in our previous work (12) and in the present article, the cytopathogenicity of SeV depends on multiple factors including escaping from cytokine induction and acute membrane dysfunction, and it is not possible to evade this by simple deletion of all the structural genes (*M*, *F*, and *HN*) (27). Furthermore, the use of a single-gene defective SeV vector raises safety and regulatory concerns, as the potential of the SeV vector to self-replicate was diminished but was not completely abolished by a single gene defect, as shown in Table 1. This issue was neither investigated nor addressed in previous reports describing single gene defective vectors (30, 47) but has been resolved here for the first time.

Cell reprogramming depends on the simultaneous delivery of multiple genes, on their balanced expression, and on their prompt suppression/removal. These factors affect both the efficiency of iPS cell generation and the quality of the iPS cells (36, 39). Nevertheless, this issue has not been investigated in detail, partly because most of the current viral vectors can accommodate only one or a few extra genes. The capacity of SeVdp vectors to install four reprogramming genes on a single vector was critical both for expressing these genes at a pre-fixed balance (Fig. 3) and for highly efficient reprogramming (Fig. 5); coinfection of two independent SeVdp vectors failed to accomplish either of these goals (Figs. 3 and 5). This latter phenomenon might be caused by homologous viral interference, which has been observed after coinfections of two independent paramyxoviruses including SeV (48). Otherwise, this phenomenon could reflect that the SeVdp genome is a multicopy replicon with about 40,000 copies per cell (12). In general, it is difficult to manage to produce two (or more) independent multicopy replicons under equal balance when they share the same replication machinery. Active and rapid erasure of SeVdp vectors with specific siRNAs (Fig. 4) is also advantageous to the generation of homogeneous and vector-free iPS cells compared with the passive and unmanageable vector removal that depends on the sequential passages of the cells (45, 46).

Synthetic modified mRNA encoding reprogramming factors has been reported to generate iPS cells highly efficiently (49). This nonviral approach and our SeVdp vector have their own advantages and disadvantages, but the escape from the cellular antiviral defense system is a critical characteristic common to these advanced reprogramming systems. The former has the advantage that the combination of exogenous reprogramming genes and the balance or duration of their expression can be adjusted flexibly. The nonviral approach has another advantage, as the use of recombinant viruses is regulated strictly in general. On the other hand, it is highly dependent on the gene delivery system, as it requires repetitive transfection for 16 days. Therefore, it might not be applicable to cells that are difficult to transfect, such as primary peripheral blood cells. In contrast, adjustment of reprogramming genes and of their expression is not so easy using SeVdp vectors and needs the construction of different vectors for tuning these features. However, once established, the SeVdp vector can always induce steady expression of the installed genes at a fixed balance, and this feature allows highly reproducible and uniform cell reprogramming. Furthermore, pro-

longed gene expression by a single infection procedure and the wider host range available are advantageous for practical applications, such as reprogramming of blood cells. Combining the results obtained with these two approaches depending on specific research purposes will enable us to create a more advanced system for cell reprogramming.

The field of cell reprogramming is expanding very rapidly, and the tools for safer and efficient reprogramming have become increasingly important both for basic research and for development of medical applications. We are presently investigating the utility of the SeVdp vectors in the genomic reprogramming of human cells.

Acknowledgments—We thank Dr. Hideharu Taira and Dr. Yutaka Takebe for providing pSRD-HN-Fmut and pcDL-SR α , respectively, and Dr. Akinori Masago for preparing anti-SeV L antibody and Naeko Sakai for excellent technical assistance.

REFERENCES

1. Takahashi, K., and Yamanaka, S. (2006) *Cell* **126**, 663–676
2. Müller, L. U., Daley, G. Q., and Williams, D. A. (2009) *Mol. Ther.* **17**, 947–953
3. Jaenisch, R., and Young, R. (2008) *Cell* **132**, 567–582
4. Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008) *Science* **322**, 949–953
5. Jia, F., Wilson, K. D., Sun, N., Gupta, D. M., Huang, M., Li, Z., Panetta, N. J., Chen, Z. Y., Robbins, R. C., Kay, M. A., Longaker, M. T., and Wu, J. C. (2010) *Nat. Methods* **7**, 197–199
6. Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I. I., and Thomson, J. A. (2009) *Science* **324**, 797–801
7. Kaji, K., Norrby, K., Paca, A., Mileikovskiy, M., Mohseni, P., and Woltjen, K. (2009) *Nature* **458**, 771–775
8. Woltjen, K., Michael, I. P., Mohseni, P., Desai, R., Mileikovskiy, M., Hämläinen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H. K., and Nagy, A. (2009) *Nature* **458**, 766–770
9. O'Malley, J., Woltjen, K., and Kaji, K. (2009) *Curr. Opin. Biotechnol.* **20**, 516–521
10. Lamb, R. A., and Kolakofsky, D. (2001) in *Fundamental Virology* (Knipe, D. M., and Howley, P. M., eds.) 4th Ed., pp. 689–724, Lippincott Williams & Wilkins, Philadelphia
11. Griesenbach, U., Inoue, M., Hasegawa, M., and Alton, E. W. (2005) *Curr. Opin. Mol. Ther.* **7**, 346–352
12. Nishimura, K., Segawa, H., Goto, T., Morishita, M., Masago, A., Takahashi, H., Ohmiya, Y., Sakaguchi, T., Asada, M., Imamura, T., Shimotono, K., Takayama, K., Yoshida, T., and Nakanishi, M. (2007) *J. Biol. Chem.* **282**, 27383–27391
13. Yoshida, T., Nagai, Y., Maeno, K., Iinuma, M., Hamaguchi, M., Matsumoto, T., Nagayoshi, S., and Hoshino, M. (1979) *Virology* **92**, 139–154
14. Akagi, T., Sasai, K., and Hanafusa, H. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13567–13572
15. Drocourt, D., Calmels, T., Reynes, J. P., Baron, M., and Tiraby, G. (1990) *Nucleic. Acids Res.* **18**, 4009
16. Kogure, T., Karasawa, S., Araki, T., Saito, K., Kinjo, M., and Miyawaki, A. (2006) *Nat. Biotechnol.* **24**, 577–581
17. Taira, H., Sato, T., Segawa, H., Chiba, M., Katsumata, T., and Iwasaki, K. (1995) *Arch. Virol.* **140**, 187–194
18. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **8**, 466–472
19. Nakano, T., Kodama, H., and Honjo, T. (1994) *Science* **265**, 1098–1101
20. Croisille, L., Auffray, I., Katz, A., Izac, B., Vainchenker, W., and Coulombel, L. (1994) *Blood* **84**, 4116–4124
21. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* **411**, 494–498
22. Sano, M., Sierant, M., Miyagishi, M., Nakanishi, M., Takagi, Y., and

- Sutou, S. (2008) *Nucleic. Acids Res.* **36**, 5812–5821
23. Okabe, J., Eguchi, A., Wadhwa, R., Rakwal, R., Tsukinoki, R., Hayakawa, T., and Nakanishi, M. (2004) *Hum. Mol. Genet.* **13**, 285–293
 24. Okita, K., Ichisaka, T., and Yamanaka, S. (2007) *Nature* **448**, 313–317
 25. Plattet, P., Strahle, L., le Mercier, P., Hausmann, S., Garcin, D., and Kolokofsky, D. (2007) *Virology* **362**, 411–420
 26. Horikami, S. M., Smallwood, S., and Moyer, S. A. (1996) *Virology* **222**, 383–390
 27. Yoshizaki, M., Hironaka, T., Iwasaki, H., Ban, H., Tokusumi, Y., Iida, A., Nagai, Y., Hasegawa, M., and Inoue, M. (2006) *J. Gene Med.* **8**, 1151–1159
 28. Eguchi, A., Kondoh, T., Kosaka, H., Suzuki, T., Momota, H., Masago, A., Yoshida, T., Taira, H., Ishii-Watabe, A., Okabe, J., Hu, J., Miura, N., Ueda, S., Suzuki, Y., Taki, T., Hayakawa, T., and Nakanishi, M. (2000) *J. Biol. Chem.* **275**, 17549–17555
 29. Ogino, T., Iwama, M., Ohsawa, Y., and Mizumoto, K. (2003) *Biochem. Biophys. Res. Commun.* **311**, 283–293
 30. Inoue, M., Tokusumi, Y., Ban, H., Kanaya, T., Tokusumi, T., Nagai, Y., Iida, A., and Hasegawa, M. (2003) *J. Virol.* **77**, 3238–3246
 31. Sugahara, F., Uchiyama, T., Watanabe, H., Shimazu, Y., Kuwayama, M., Fujii, Y., Kiyotani, K., Adachi, A., Kohno, N., Yoshida, T., and Sakaguchi, T. (2004) *Virology* **325**, 1–10
 32. Takeuchi, O., and Akira, S. (2010) *Cell* **140**, 805–820
 33. Hua, J., Liao, M. J., and Rashidbaigi, A. (1996) *J. Leukoc. Biol.* **60**, 125–128
 34. Murakami, Y., Ikeda, Y., Yonemitsu, Y., Tanaka, S., Kondo, H., Okano, S., Kohno, R., Miyazaki, M., Inoue, M., Hasegawa, M., Ishibashi, T., and Sueishi, K. (2008) *J. Gene Med.* **10**, 165–176
 35. Zidovec, S., and Mazuran, R. (1999) *Cytokine* **11**, 140–143
 36. Papapetrou, E. P., Tomishima, M. J., Chambers, S. M., Mica, Y., Reed, E., Menon, J., Tabar, V., Mo, Q., Studer, L., and Sadelain, M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 12759–12764
 37. Carey, B. W., Markoulaki, S., Hanna, J., Saha, K., Gao, Q., Mitalipova, M., and Jaenisch, R. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 157–162
 38. Gonzalez, F., Barragan Monasterio, M., Tiscornia, G., Montserrat Puelido, N., Vassena, R., Batlle Morera, L., Rodriguez Piza, I., and Izpisua Belmonte, J. C. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 8918–8922
 39. Chin, M. H., Pellegrini, M., Plath, K., and Lowry, W. E. (2010) *Cell Stem Cell* **7**, 263–269
 40. Haasnoot, J., Westerhout, E. M., and Berkhout, B. (2007) *Nat. Biotechnol.* **25**, 1435–1443
 41. Homann, H. E., Hofschneider, P. H., and Neubert, W. J. (1990) *Virology* **177**, 131–140
 42. Nakanishi, M., Mizuguchi, H., Ashihara, K., Senda, T., Akuta, T., Okabe, J., Nagoshi, E., Masago, A., Eguchi, A., Suzuki, Y., Inokuchi, H., Watabe, A., Ueda, S., Hayakawa, T., and Mayumi, T. (1998) *J. Control Release* **54**, 61–68
 43. Hanenberg, H., Hashino, K., Konishi, H., Hock, R. A., Kato, I., and Williams, D. A. (1997) *Hum. Gene Ther.* **8**, 2193–2206
 44. Ogino, T., Kobayashi, M., Iwama, M., and Mizumoto, K. (2005) *J. Biol. Chem.* **280**, 4429–4435
 45. Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2009) *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **85**, 348–362
 46. Seki, T., Yuasa, S., Oda, M., Egashira, T., Yae, K., Kusumoto, D., Nakata, H., Tohyama, S., Hashimoto, H., Kodaira, M., Okada, Y., Seimiya, H., Fusaki, N., Hasegawa, M., and Fukuda, K. (2010) *Cell Stem Cell* **7**, 11–14
 47. Li, H. O., Zhu, Y. F., Asakawa, M., Kuma, H., Hirata, T., Ueda, Y., Lee, Y. S., Fukumura, M., Iida, A., Kato, A., Nagai, Y., and Hasegawa, M. (2000) *J. Virol.* **74**, 6564–6569
 48. Shimazu, Y., Takao, S. I., Irie, T., Kiyotani, K., Yoshida, T., and Sakaguchi, T. (2008) *Virology* **372**, 64–71
 49. Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y. H., Li, H., Lau, F., Ebina, W., Mandal, P. K., Smith, Z. D., Meissner, A., Daley, G. Q., Brack, A. S., Collins, J. J., Cowan, C., Schlaeger, T. M., and Rossi, D. J. (2010) *Cell Stem Cell* **7**, 618–630

Supplemental Figure Legends

Fig. S1. Construction of SeVdp vector genomic cDNA. The source materials for the vector backbone were genomic cDNAs of the Nagoya strain (corresponding to nucleotides, nt 1–2870) and of the Cl.151 strain (corresponding to nt 2871–15384) (12). *M*, *F* and *HN* genes were replaced with exogenous genes cloned between *KasI* and *MluI* restriction sites (for *M*), between *BglIII* sites (for *F*) and between *NheI* and *SphI* sites (for *HN*). Additional extra genes were inserted into *NheI* and *NotI* sites created between the *P/C/V* and *M* genes.

Fig. S2. Structure of the 3' region of the SeVdp genome. *A.* Effect of the 3' region on the persistent phenotype of the virus. Replication-competent chimeric SeVs with genomic RNA derived from the SeV Cl.151 strain (yellow) and from the SeV Nagoya strain (green) were prepared as described (12). LLCMK₂ cells were infected with these chimeric SeVs at a multiplicity of infection (MOI) of 5 on day 0 and examined by phase-contrast microscopy on day 1 (*Middle*). The infected cells were further cultured for 2 weeks, and long-term persistency was determined using stable expression of the nucleocapsid (NP) protein as an index. *B.* Effect of the 3' region on the efficiency of reconstitution of the virus from cDNA. Genomic cDNA of the chimeric SeV was constructed as described (12). SeVs carrying the *Bsr* gene were reconstituted from SeV proteins expressed from plasmid vectors and from the positive-strand antigenome RNA transcribed from the genomic cDNAs in BHK/T7/151M(SE) cells. The efficiency of reconstitution was estimated from the numbers of blasticidin S (Bs)-resistant cell colonies appearing in the culture. *C.* Sequences of the 3'-terminal region of the SeVdp vector RNA genome. Nucleotides 43–52 of the wild-type SeV (AUCUGUCCUA) were replaced with a transcription termination signal (AAUUCUUUUU).

Fig. S3. Roles of structural genes (*M*, *F* and *HN*) on the persistent phenotype of SeV vectors. *A.* Effects of deletions and nonsense mutations in structural genes on the persistent phenotype of SeV Cl.151-based vectors. Persistency was determined using Bs resistance as an index. Nx indicates the genes with nonsense mutations. *B.* Cytotoxicity induced by transient expression of SeV structural proteins. *M*, *F* and *HN* genes derived from the SeV Z, Nagoya and Cl.151 strains were cloned into the expression plasmid as described (8) and were expressed transiently in BHK-21 cells by transfection. Syncytium formation was examined using phase-contrast microscopy.

Fig. S4. Effects of SeV-specific siRNAs on SeV vector-mediated gene expression. *A.* Effect of pretreatment of cells with SeV-specific siRNAs. (*Upper*) Genome structure of replication-competent SeV Cl.151-based vector carrying the *EGFP* gene (1) and target sites of siRNAs. N121 and N335 were siRNAs targeted to NP mRNA, P234 and P1021 were siRNAs targeted to P mRNA, and L527 and L1913 were siRNAs targeted to L mRNA. (*Middle*) Design of the experiment. HeLa cells were treated with the given siRNA (100 nM) complexed with Lipofectamine 2000 (Life Technologies) for 6 h just before infection with SeV Cl.151(*EGFP*) (notated as rCl.151-*EGFP* in (12)) at 37 °C. The cells were cultured for 3 days and were examined by fluorescence microscopy to detect EGFP. (*Bottom*) Image of the cells treated with the indicated siRNAs. Key: si Control, siRNA against firefly luciferase (21). *B.* Effect of treatment of cells carrying an SeVdp vector with SeV-specific siRNAs. (*Upper*) Genome structure of SeVdp(*M/EGFP/Bsr*) and target sites of the siRNAs. (*Middle*) Design of the experiment. BHK/T7/NaF cells carrying SeVdp(*M/EGFP/Bsr*) were treated with the siRNAs and were examined by fluorescence microscopy to detect EGFP 4 days after the treatment. (*Bottom*) Image of the cells treated with the indicated siRNAs. Key: si Control, siRNA against firefly luciferase (21).

Name of siRNA	Sequence
siN121	5'— GGCCCAAGUGUGACUGAUGAG—3' 3'—UACCGGGUUCACACUGACUAC —5'
siN335	5'— GGACGAAGACAGACGGAUUAG—3' 3'—UACCUGCUUCUGUCUGCCUAA —5'
siP234	5'— GACCCAAGAUAAUCGAUCAAG—3' 3'—UACUGGGUUCUAUUAGCUAGU —5'
siP1021	5'— GCUCAAGAAUUCGAGUCAUAG—3' 3'—UACGAGUUCUUAAGCUCAGUA —5'
siL527	5'— GGUUCAGCAUCAAUAUGAAG—3' 3'—UACCAAGUCGUAGUUUAUACU —5'
siL1913	5'— GGUCCAGACAUGAAUCAAAG—3' 3'—UACCAGGUCUGUACUUAAGUU —5'
Control siRNA #1 (Fig. S3)	5'— CUUACGCUGAGUACUUCGATT—3' 3'—TTGAAUGCGACUCAUGAAGCU —5'
Control siRNA #2 (Fig. 4)	5'— CAAUAGUUCACGCUGAAAGUG—3' 3'—UCGUUAUCAAGUGCGACUUUC— 5'

Table S1.

Nucleotide sequences of siRNAs used in this study.

Application	Sequence (5' to 3')	Length (nt)
RT-PCR <i>Nanog</i>	ACTAGCTAGCAGAATATATGAAAACATTTAACATTTCTCA	40
	GGTCCACGCGTTTTAATTCGGGTATATTTGA	32
RT-PCR <i>Oct4</i>	AATTGGCGCCAGCCACCATGGCCAAGTTGACCAGTGCCGT	40
	GGTCCACGCGTTTCAGTCCTGCTCCTCGGCCACGAAGTG	40
RT-PCR <i>Sox2</i>	ACGAAGATCTCCGGTCGCCACCATGGTGAG	30
	ACGAAGATCTTTACTTGTACAGCTCGTCCA	30
RT-PCR <i>ECAT1</i>	ACTAGCTAGCGGGGACCATGGTGAGCGTGA	30
	GGTCCGCATGCTTCAGCAGTGGGCCACGGCGT	32
RT-PCR <i>FGF4</i>	ACTAGCTAGCCCTTATGAAGACCTTAATTCTTGC	34
	GGTCCGCATGCTCTATTTGCATTCATCTGGTACT	34
RT-PCR <i>c-Myc</i>	ACTAGCTAGCTGCCACCATGGGGAAGTGGGCTGTGAATGA	40
	GGTCCACCGGTGTTAGAAGTTTTCTTGTGTA	32
RT-PCR <i>GAPDH</i>	TATCTAGACCTTATGCAGCTGAGGAACCCAG	31
	CCGCATGCATTAAGTAAGTCTTTAATGACATC	34
RT-PCR <i>SeV NP</i>	GAAAGAAATTTACCGCTAGCGCGGCCGCATGCTAACACGGCGCAATG	48
	CATTGCGCCGTGTTAGCATGCGGCCGCGCTAGCGGTGAAATTTCTTTC	48
Bisulfite sequencing <i>Oct4</i> promoter	AATTGGCGCCAGCCACCATGGCCAAGTTGACCAGTGCCGT	40
	GGTCCACGCGTTTCAGTCCTGCTCCTCGGCCACGAAGTG	40
Bisulfite sequencing <i>Nanog</i> promoter	ACGAAGATCTCCGGTCGCCACCATGGTGAG	30
	ACGAAGATCTTTACTTGTACAGCTCGTCCA	30

Table S2.

Nucleotide sequences of PCR primers used in this study.

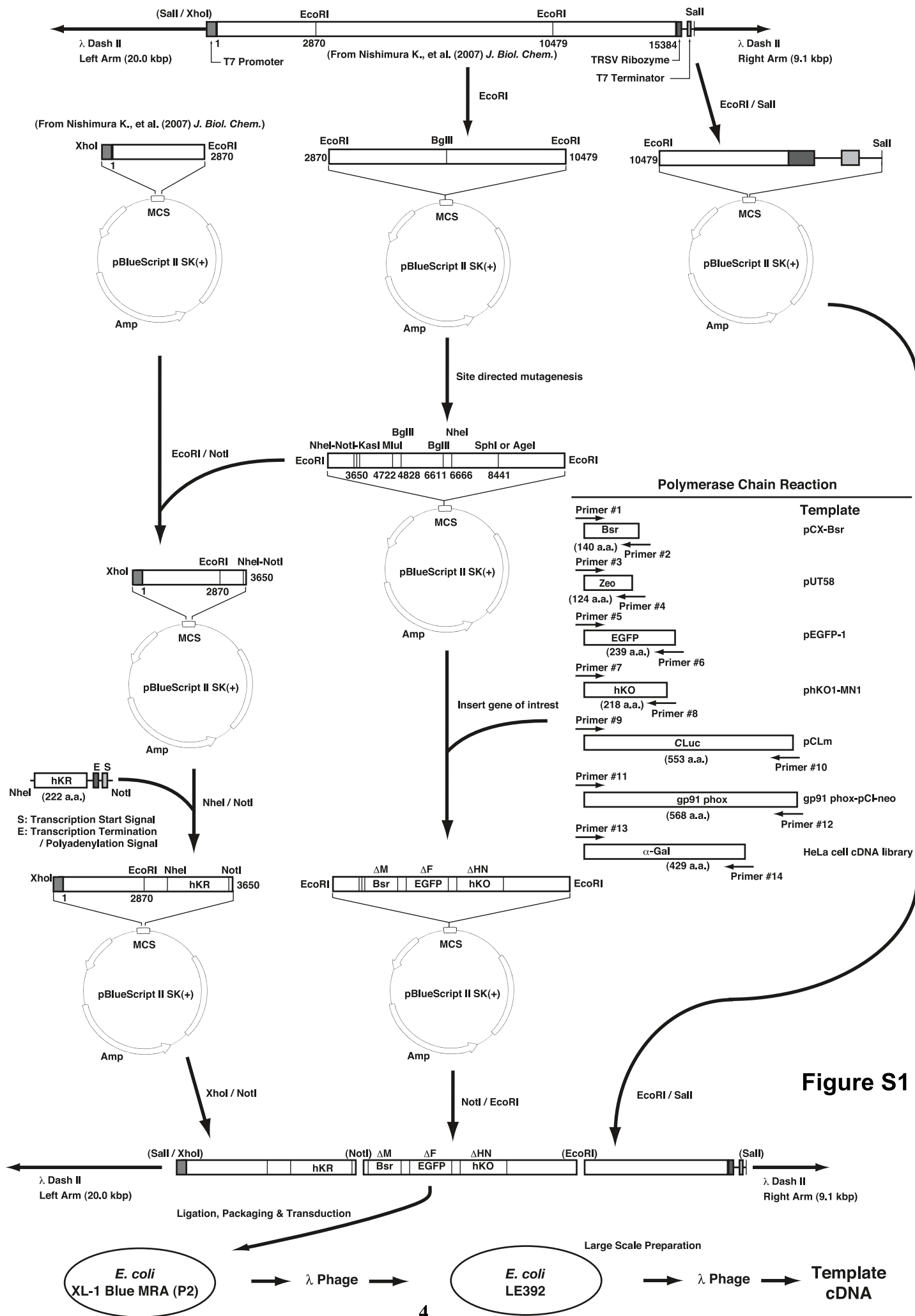


Figure S1

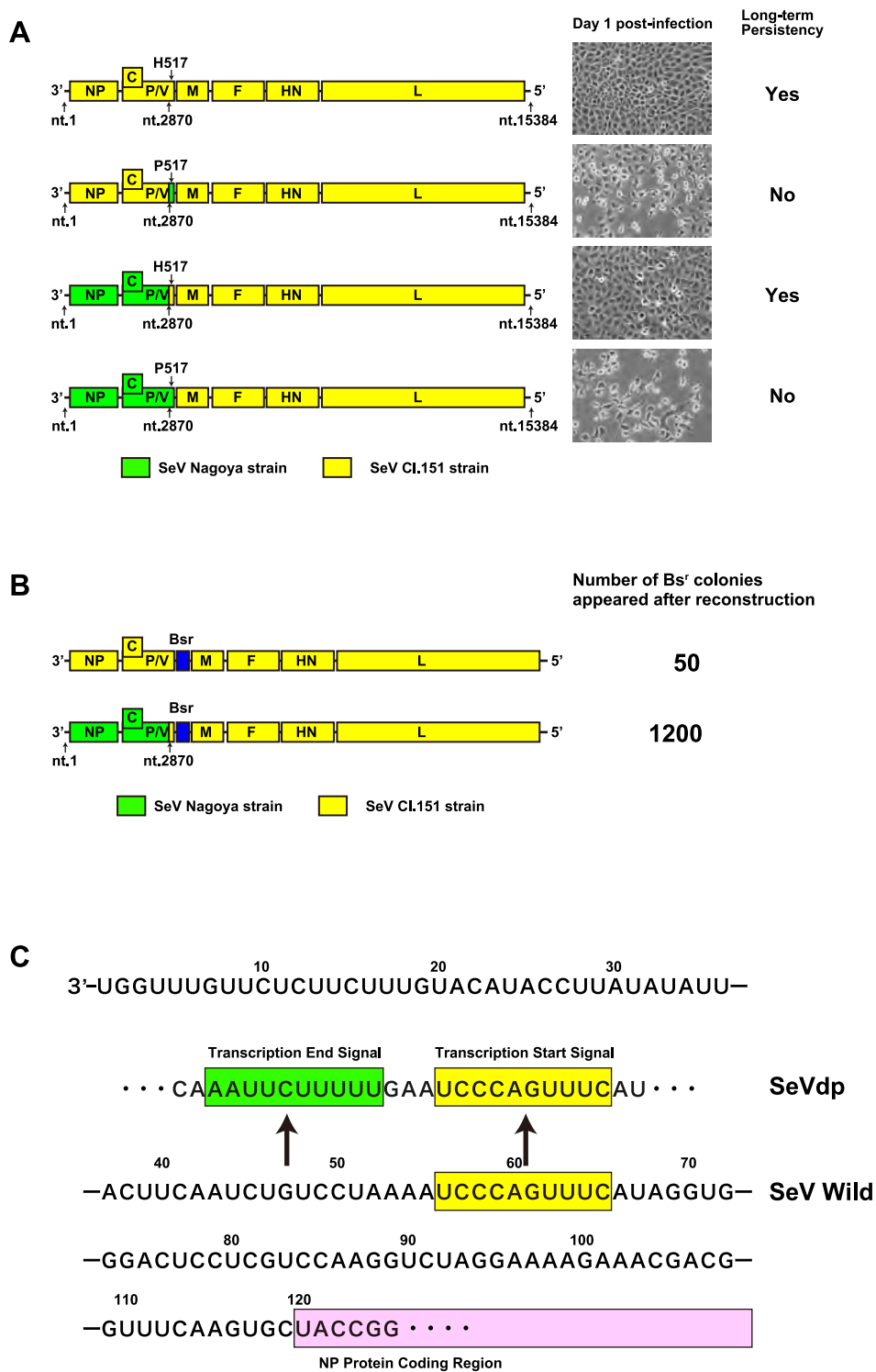


Figure S2

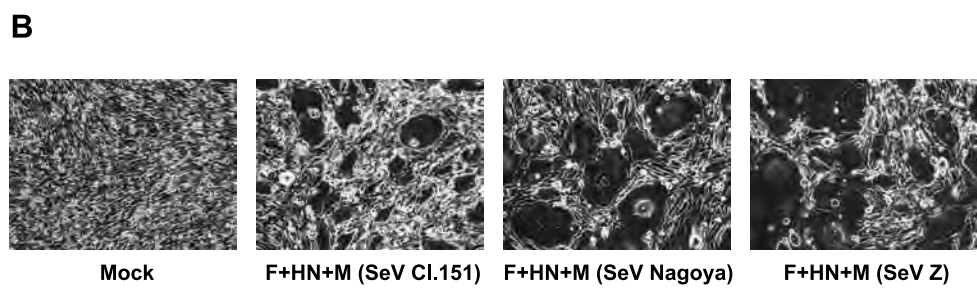
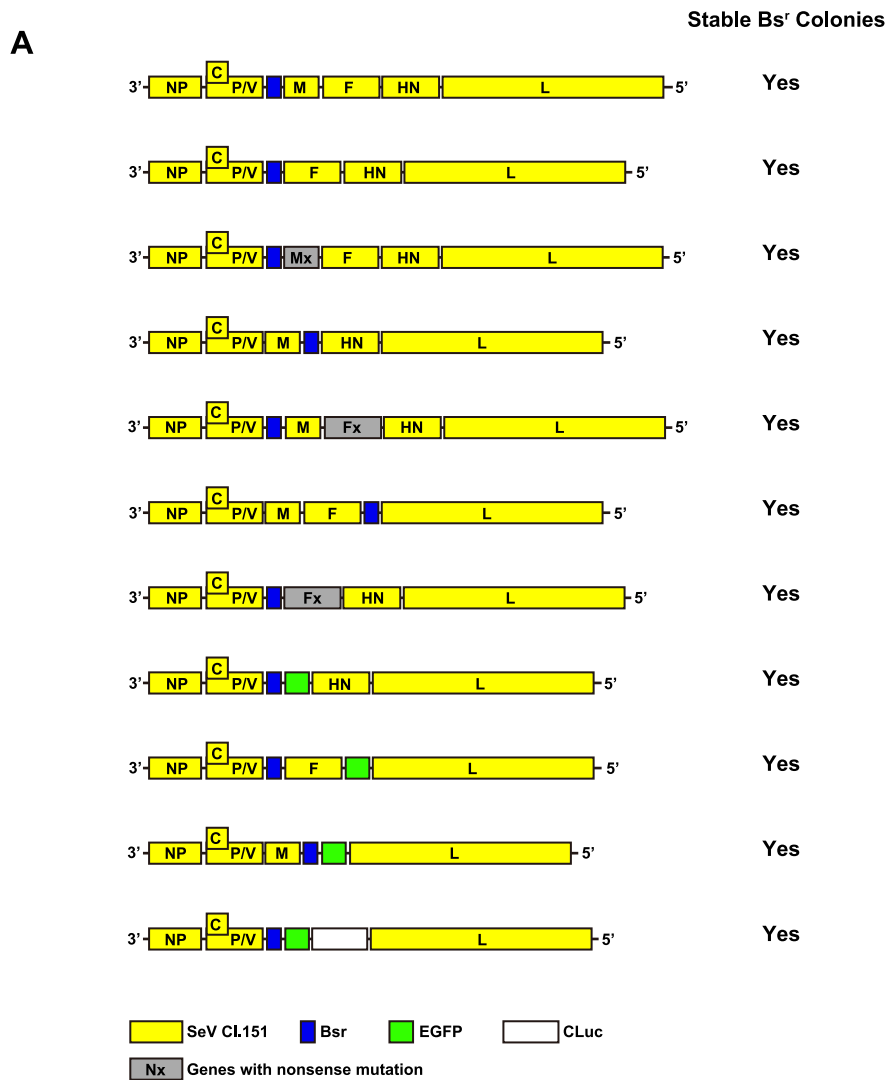
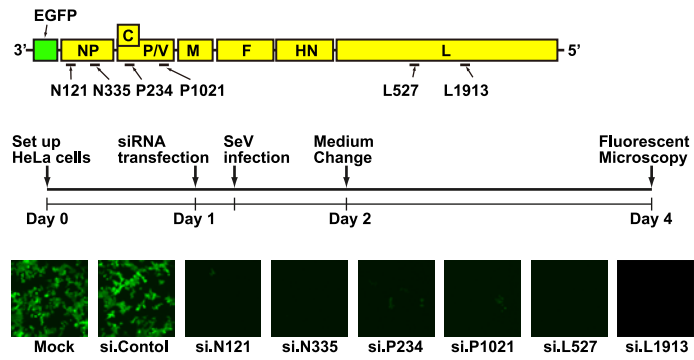
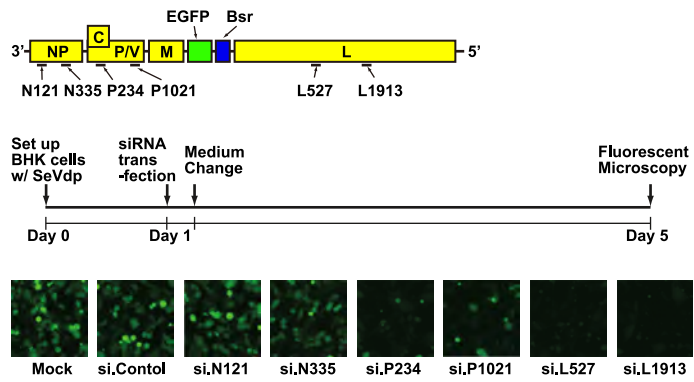


Figure S3

A**B****Figure S4**