performed according to the method described previously [16]. LLC-MK$_2$ cells (1×10^6) in 6-well plates were infected at an MOI of 3 with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP or SeV/ΔMΔFΔHN-GFP and incubated in serum-free MEM at 37 °C. Three days after transduction, the cells were recovered and solubilized in a sample buffer (Biolabs) for SDS-PAGE. Proteins separated by SDS-PAGE were transferred to Immobilon-PVDF membranes (Millipore). The membranes were probed with a rabbit polyclonal anti-M antibody [16] or mouse monoclonal anti-F, γ236 [23], and anti-HN, HN-2 [24], antibodies. Secondary antibodies were goat anti-rabbit IgG (Santa Cruz Biotechnology) or goat anti-mouse IgG+IgM (Bioscience) conjugated with horseradish peroxidase. The protein bands were detected by chemiluminescence using ECL Western blotting detection reagents (Amersham Biosciences) following the manufacturer's protocol.

Quantitative analysis of cytotoxicity

Confluent CV-1 cells grown in 96-well plates were infected at an MOI of 0.1, 0.3, 1, 3, 10 or 30 with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP or SeV/PLmutΔMΔFΔHN-GFP and incubated at 37 °C in serum-free MEM. The culture supernatants were collected 3 days after transduction and assayed with a cytotoxicity detection kit (Roche) that measures lactase dehydrogenase (LDH) activity released from damaged cells [25].

SEAP assay

Confluent LLC-MK$_2$ cells grown in 96-well plates were infected at an MOI of 3 with SeV^{18+}SEAP/ΔF-GFP, SeV^{18+}SEAP/ΔMΔF-GFP or SeV^{18+}SEAP/PLmutΔMΔF-ΔHN-GFP and incubated in serum-free MEM at 37 °C. The culture supernatants were collected every 24 h and assayed for SEAP activities using an SEAP reporter assay kit (Toyobo) with an LAS 1000 image analyser (Fuji Film). Means were calculated from three replicate samples.

In vivo study of M-, F- and HN-genes-deleted SeV vector

Twelve BALB/cA mice (Charles River), 7 weeks old weighing 20–25 g, were used. In half of the mice, submandibular lymph nodes (SMLN) were removed before injection. Mice were anaesthetised with diethyl ether, and 5 µl of SeV/ΔF-GFP (5×10^6 GFP-CIU/head) or SeV/PLmutΔMΔFΔHN-GFP (5×10^6 GFP-CIU/head) was injected into the ear auricle intradermally (n = 3). GFP expression was assessed by examining the ear auricle under a fluorescence stereomicroscope. The surface of the auricle was photographed and the GFP intensity was quantified with NIH image software. Sera of injected mice were collected at weekly intervals and stored at -80 °C

until the measurement of neutralizing and total anti-SeV antibody levels.

Immunohistochemical staining

The auricle was frozen and sliced into 10 µm thick sections with a cryotome (Coldtome CM-502; Sakura Seiki). The frozen sections were pretreated with 0.3% H$_2$O$_2$ in methanol. Migrated macrophages and pan-T cells were detected using a rabbit polyclonal anti-CD11b (Santa Cruz) and anti-CD3 (Santa Cruz) antibodies, respectively, followed by using a Histofine SAB-PO(R) kit (Nichirei). Immunopositive cells were visualised with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

Ex vivo transduction using the M-, F- and HN-genes-deleted SeV vector

MC57G cells, an isogenic cell line of C57BL/6, were inoculated with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP at an MOI of 3. Two days later, the cells were transplanted subcutaneously in the back of C57BL/6 mice. Sera of transplanted mice were collected at weekly intervals.

Quantitation of neutralizing and total anti-SeV antibodies

To quantify neutralizing antibody against SeV, serial 1:5 dilutions of sera previously inactivated at 56 °C for 30 min were mixed with wild-type SeV carrying the GFP gene (SeV^{18+}GFP (wild)) at 37 °C for 1 h. These virus-serum mixtures were inoculated to LLC-MK$_2$ cells in 96-well plates and cultured in serum-free MEM at 37 °C for 3 days. The remaining infectivity of SeV^{18+}GFP (wild) was estimated by measuring the fluorescence of GFP with a fluorescence microplate reader (Cytofluor II; Biosearch/Millipore). Results are expressed as percent inhibition by setting the values from the cells infected with SeV^{18+}GFP (wild) alone as 0% and those from the uninfected cells as 100%. Total anti-SeV antibody level in the sera was subsequently measured by a test kit for HVJ (Denka Seiken) according to the manufacturer's protocol. In this test, the quantities of anti-SeV antibody were measured as relative ones represented by OD450 because the absolute quantity of anti-SeV antibody could not be determined. Those were determined using 1:300 dilutions of the sera and measured simultaneously.

Characterization of serum antibodies by Western blotting

The mixture of NP and HN viral proteins which was prepared from infected cells and whole virion proteins were separated by SDS-PAGE and transferred

to Immobilon-PVDF membranes (Millipore) as described before. Sera collected from transplanted mice were used as the first antibody. Secondary antibodies were goat anti-mouse IgG+IgM conjugated with horseradish peroxidase.

Results and discussion

Establishment of M-, F- and HN-expressing packaging cell line

For the recovery of SeV/ΔMΔFΔHN virion particles from cDNA, the missing M, F and HN genes must be complemented *in trans*. To establish such an M-, F- and HN-expressing packaging cell line, a Cre/loxP induction system [22] was employed as has been reported [15,16]. Moreover, we previously established an M/F-expressing packaging cell line (LLC-MK$_2$/F7/M#33), in which M- and F-genes-deleted SeV (SeV/ΔMΔF) was successfully recovered [17]. Therefore, it was thought that the introduction of the HN gene into LLC-MK$_2$/F7/M#33 would make this cell line capable of supporting the recovery of SeV/ΔMΔFΔHN. Early-passage LLC-MK$_2$/F7/M#33 cells were transfected with pCALNdLw/HN. After incubation at 37 °C for 2 weeks, clones were selected and expanded. The HN protein expression was examined by the Western blot analysis of the cellular proteins after infection with the Cre-encoding adenovirus vector, AxCANCre [21], at a MOI of 5. The clones expressing the HN protein were then subjected to two rounds of subcloning, resulting in the final selection of one cell line, LLC-MK$_2$/F7/M#33/A/HN7.

Construction of a vector with M, F and HN gene deletions

SeV genomic cDNA carrying the GFP gene in place of the M, F and HN genes (SeV/ΔMΔFΔHN-GFP) was constructed (Figure 1A). The GFP gene in SeV cDNA allows us to confirm easily the successful recovery of the SeV/ΔMΔFΔHN vector. Using the established cell line and the plasmid-based reverse genetics technology [26], we propagated SeV/ΔMΔFΔHN-GFP, leading to a titer of 1×10^8 CIU/ml in the culture supernatant. During the reconstitution of SeV/ΔMΔFΔHN-GFP, some degree of cytotoxicity toward the packaging cell lines, which had not been observed with previous SeV vector reconstitutions, was observed (data not shown). The cause of this cytotoxicity is still obscure, but one possibility is that the L protein might be expressed in relative excess and drive the transcription and replication of the vector beyond the capacity of the packaging cells because of the shorter genome size of SeV/ΔMΔFΔHN-GFP. Based on this hypothesis, we prepared the SeV/PLmutΔMΔFΔHN-GFP vector, which has amino acid substitutions in P (L511F) and L (N1197S, K1795E). These substitutions had been identified in SeV strains capable of persistent infection *in vitro* with slightly reduced transcription and

replication [27]. As expected, SeV/PLmutΔMΔFΔHN-GFP was propagated without obvious cytotoxicity during reconstitution, leading to a titer of 5×10^8 CIU/ml in the culture supernatant.

Gene structure of the recovered SeV vector

The vector gene structure was confirmed by RT-PCR. The DNA fragment of SeV/ΔMΔFΔHN-GFP from the 5′-terminus of the P gene to the 3′-terminus of the L gene containing the GFP gene was amplified from the vector genome, and the amplified fragment was compared with the corresponding fragments amplified
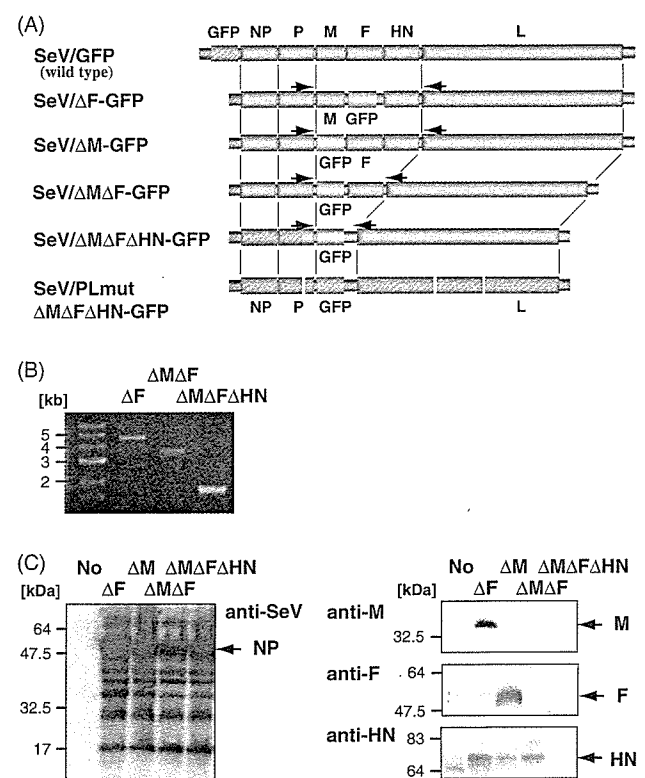

Figure 1. Construction of M-, F- and HN-genes-deleted SeV vector carrying the GFP gene, and confirmation of its structure. (A) The structures of recombinant SeV genomes. The open reading frame of the GFP gene was inserted with the SeV end and start signals (EIS) in the respective positions of the deleted gene(s). The positions of the primers for RT-PCR are shown by arrows. (B) Viral genome structure was confirmed by RT-PCR. The DNA fragment of SeV/ΔMΔFΔHN-GFP (ΔMΔFΔHN) from the 5′-terminal of the P gene to the 3′-terminal of the L gene (containing the GFP gene) was amplified from the vector genome, and the fragment was compared to the corresponding fragments amplified from the vector genomes of SeV/ΔF-GFP (ΔF) and SeV/ΔMΔF-GFP (ΔMΔF). (C) Viral proteins were detected by Western blot analysis. LLC-MK$_2$ cells were infected with SeV^{18+}GFP (wild), SeV/ΔF-GFP (ΔF), SeV/ΔM-GFP (ΔM), SeV/ΔMΔF-GFP (ΔMΔF) or SeV/ΔMΔFΔHN-GFP (ΔMΔFΔHN) at an MOI of 3. The viral proteins in the cells 2 days after transduction were detected by Western blotting using anti-M, anti-F, anti-HN and anti-SeV (which mainly detects NP protein) antibodies after transferring the proteins to a PVDF membrane

from the vector genomes of SeV/ΔF-GFP and SeV/ΔMΔF-GFP. Amplification of 1719-bp, 3576-bp and 4773-bp DNAs for SeV/ΔMΔFΔHN-GFP, SeV/ΔMΔF-GFP and SeV/ΔF-GFP, respectively, was expected based on the genome structures. The results of RT-PCR clearly showed that SeV/ΔMΔFΔHN-GFP had the M, F and HN genes deleted from its genome (Figure 1B). In the case of SeV/PLmutΔMΔFΔHN-GFP, the deletion of the M, F and HN genes was examined by RT-PCR as in the case of SeV/ΔMΔFΔHN-GFP, and newly introduced mutations in the P and L parts were also confirmed by determining the sequences of the DNA fragment amplified from the cDNA (data not shown). The absence of the M, F and HN genes was also confirmed by Western blot analysis of the protein expression of LLC-MK$_2$ cells infected at an MOI of 3 with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP or SeV/ΔMΔFΔHN-GFP. In these analyses, anti-M, anti-F, anti-HN or anti-SeV (which mainly detects the NP protein) antibodies were used. The NP protein, but not M, F or HN proteins, was detected in the cells transduced with SeV/ΔMΔFΔHN-GFP (Figure 1C). Similarly, neither the M nor the F protein was observed in cells transduced with SeV/ΔMΔF-GFP. These results clearly indicated the absence of the envelope genes in these vectors.

Cytopathic effect of SeV is efficiently diminished by M, F and HN gene deletion

Infection with SeV vectors causes a cytopathic effect (CPE) in some types of cells. Therefore, it was important to characterise the newly recovered SeV/ΔMΔFΔHN-GFP and SeV/PLmutΔMΔFΔHN-GFP in terms of CPE. The CPE was investigated in CV-1 cells, which are known to be very sensitive to SeV infection-dependent cytotoxicity [17]. CV-1 cells plated in 96-well plates were transduced with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP and SeV/PLmutΔMΔFΔHN-GFP, respectively, and incubated in serum-free MEM for 3 days. Then, their CPE was quantitatively measured by using Decker's method [25]. The CPE of SeV/ΔMΔFΔHN-GFP was approximately equal to that of SeV/ΔMΔF-GFP. However, that of SeV/PLmutΔMΔFΔHN-GFP was greatly reduced as compared to that of all other types of SeV vectors (Figure 2A). Morphological damage to the cells infected with vectors at an MOI of 20 was also examined under a fluorescence microscope. CV-1 cells transduced with SeV/PLmutΔMΔFΔHN-GFP showed little CPE (Figure 2B). Thus, the combination of the deletion of all envelope-related genes and the amino acid substitutions in P and L was found to be quite effective in reducing the CPE of SeV *in vitro*. In addition to membrane fusion and apoptosis, rapid transcription and replication increase SeV-induced cytotoxicity. The substitutions in P and L that induce slightly reduced transcription and replication [27] brought about additional reduction of CPE.
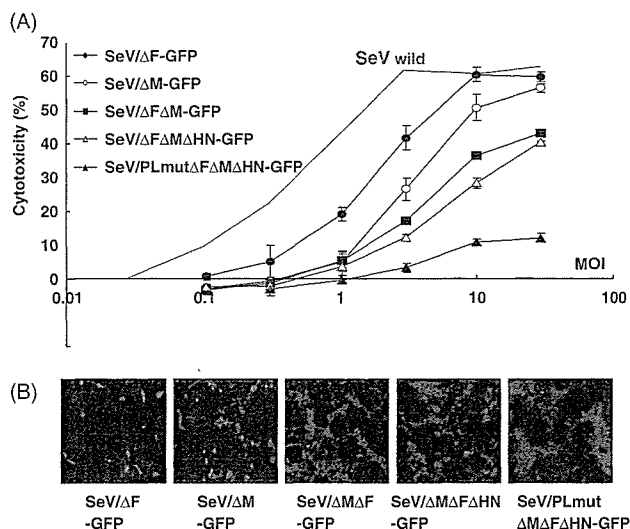
(A)

(B)

Figure 2. Analysis of SeV infection-dependent cytotoxicity. (A) Quantitative measurement using SeV infection-sensitive cells. CV-1 cells were infected with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP or SeV/PLmutΔMΔF-ΔHN-GFP at an indicated MOI. Cytotoxicity was determined by the quantity of LDH released from damaged cells. The assay was carried out 3 days after infection using the supernatants of cultures in serum-free medium. The percentage of cytotoxicity (%) was calculated using the low control (0%) from the supernatant of uninfected cells and the high control (100%) from the supernatant of cell lysates after treatment with 2% Triton X-100. Cytopathic effect of a wild-type SeV (SeV^{18+}GFP (wild)) was also examined and shown in the thin line. (B) Morphology of CV-1 cells infected with respective SeV vectors at an MOI of 20 on day 3 after infection

Quantitative analysis of foreign gene expression of M-, F- and HN-genes-deleted SeV

To quantify the expression level of a foreign gene(s) carried in SeV/ΔMΔFΔHN, the gene for the secreted form of human placental alkaline phosphatase (SEAP), which is an easily detectable marker of protein production, was inserted upstream of the open reading frame of NP. The thus-generated SeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP vector was used for the SEAP assay. LLC-MK$_2$ cells were infected at an MOI of 3 with SeV^{18+}SEAP/ΔF-GFP, SeV^{18+}SEAP/ΔMΔF-GFP [17] or SeV^{18+}SEAP/PLmutΔ-MΔFΔHN-GFP and the culture supernatants were collected every 24 h. The expression level of SEAP in the culture supernatant of SeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP was rather low as compared to that of SeV^{18+}SEAP/ΔF-GFP, but was similar to that of SeV^{18+}SEAP/ΔMΔF-GFP (Figure 3). This supported the notion that the M protein might participate not only in viral assembly and budding, but also in the control of transgene transcription [28].

Prolonged transgene expression of M-, F- and HN-genes-deleted SeV *in vivo*

We next examined the transduction efficiency of SeV/PLmutΔMΔFΔHN-GFP *in vivo*. SeV/PLmutΔMΔF-
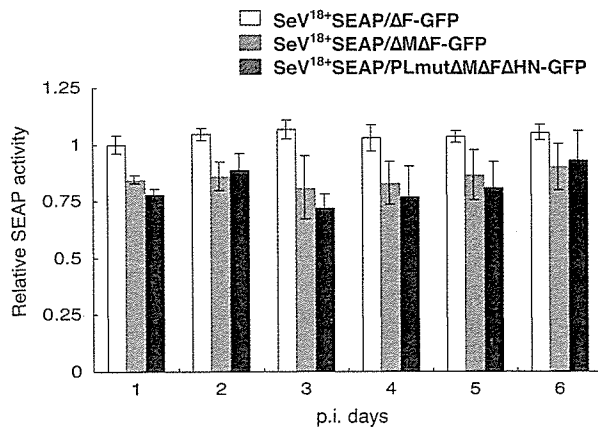
Figure 3. Comparison of expression performance of SeV vectors carrying the SEAP gene. The culture medium of $LLC-MK_2$ cells was collected every 24 h after infection with $SeV^{18+}SEAP/\Delta F$-GFP, $SeV^{18+}SEAP/\Delta M\Delta F$-GFP or $SeV^{18+}SEAP/\Delta M\Delta F\Delta HN$-GFP at an MOI of 3. SEAP activity was determined as a relative activity by setting the value from the medium of uninfected cells as zero (0) and that from the medium of $SeV^{18+}SEAP/\Delta F$-GFP-infected cells (MOI = 3, 1 day post-infection.) as one (1). Average of three experiments; bar: SD
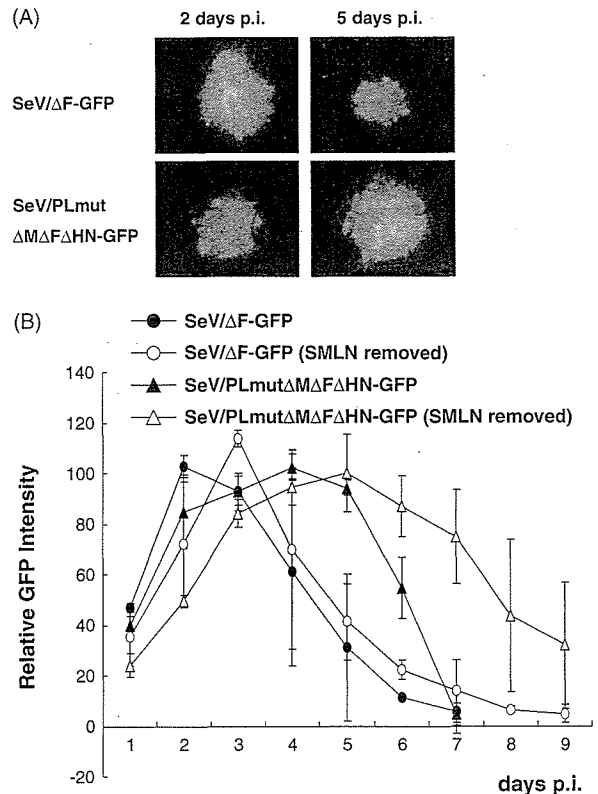

Figure 4. Gene transfer by M-, F- and HN-genes-deleted SeV vector in vivo. (A) Gene transfer to the ear auricle. SeV/ΔF-GFP or SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP ($5 \times 10^6$ GFP-CIU/head) was administered to the ear auricle of BALB/cA mice with or without submandibular lymph nodes (SMLN) by a single intradermal injection (n = 3). GFP expression was detected by a fluorescent stereomicroscope from the skin surface of the ear auricle under a fixed condition. (B) Quantitative analysis of GFP fluorescence. The expression of GFP was calculated by multiplying its fluorescence intensity and its area by NIH image software and expressed as relative ones

ΔHN-GFP or SeV/ΔF-GFP was administered intradermally to the ear auricles of BALB/cA mice (5×10^6 CIU/head). We can directly observe the fluorescence from expressed GFP over a period of time through the skin surface of the ear auricle without sacrificing animals. Efficient transduction from both vectors was confirmed and the level of GFP expression was approximately equal between SeV/ΔF-GFP and SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP (Figure 4A). However, the peak of expression was prolonged in the case of SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP (Figure 4B). In an additional study, SeV vectors were administered after the removal of submandibular lymph nodes in order to avoid direct transduction to them. In this case, GFP expression derived from SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP was significantly prolonged (Figure 4B). In fact, both anti-CD11b (macrophage marker) and anti-CD3 (Pan-T cell marker) staining of the frozen sections of the inoculated auricles showed the delayed migration of macrophages and T cells to the site of SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP administration compared with that of SeV/ΔF-GFP (Figures 5A and 5B). These results clearly indicate that the immunoreaction against SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP is weak and delayed compared to that against SeV/ΔF-GFP. Efficient transduction and prolonged expression of SeV/PLmut$\Delta M\Delta F\Delta HN$ were also observed in the cases of transduction to the airway epithelial cells and neuronal cells in the brain (data not shown).

Humoral immune reaction of M-, F- and HN-genes-deleted SeV in vivo

We next examined the effect of SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP on the induction of anti-SeV antibodies and

neutralizing antibodies. SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP or SeV/$\Delta F$-GFP was administered to BALB/cA mice, and sera of mice were collected weekly for 3 weeks. Phosphate-buffered saline (PBS)-injected mice were used as controls. The level of serum neutralizing antibodies in the mice administered SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP was reduced to about half of that in the mice administered SeV/ΔF-GFP (Figure 5C). On the other hand, the overall quantity of anti-SeV antibodies was only slightly reduced by using the new SeV vector. The envelope proteins of SeV/PLmut$\Delta M\Delta F\Delta HN$-GFP particles supplied by the packaging cells may contribute to the induction of both overall anti-SeV antibodies and a certain amount of neutralizing antibodies against the vector.
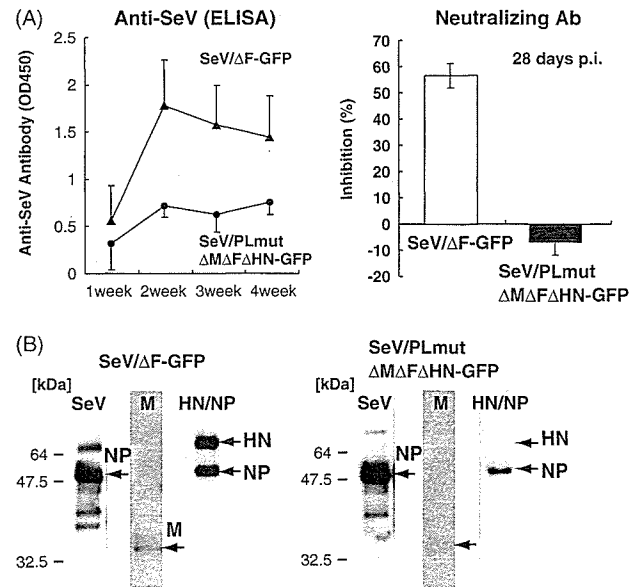
No neutralizing antibody production after M-, F- and HN-genes-deleted SeV transduction ex vivo

We then examined whether the envelope gene deletions reduce the induction of humoral immunoreaction

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(A)

(B)

(C)
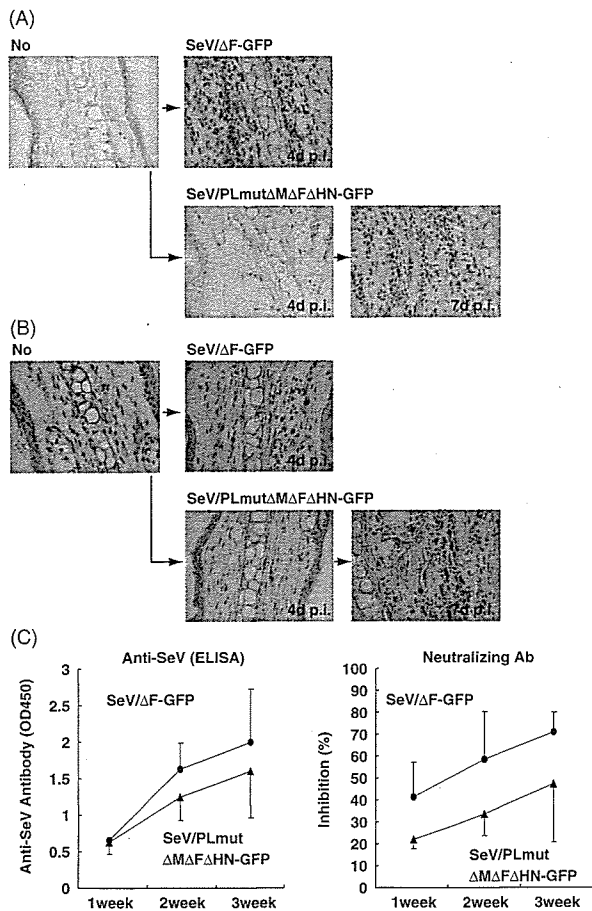
Figure 5. Immune reaction to SeV vectors introduced by *in vivo* transduction. Immunohistochemical analysis of the migrations of (A) macrophages and (B) T lymphocytes. The frozen sections of ear auricles of BALB/cA mice intradermally administered with SeV/ΔF or SeV/PLmutΔMΔFΔHN. (C) Sera from mice administered with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP were assayed for overall anti-SeV antibodies as well as neutralizing antibodies directed against SeV. Neutralizing antibody level is shown as a percent inhibition of SeV[18+]GFP (wild) infectivity

Figure 6. Immune reaction to SeV vectors following transplantation of SeV-infected cells. (A) Serum levels of anti-SeV antibody (left) following transplantation of isogenic cells (1×10^6 cells/head) infected with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP at an MOI of 3 were examined. Neutralizing antibody level was measured in the sera on days 28 after transplantation (right). (B) Serum was analysed by Western blotting. The purified M protein, the mixture of NP and HN viral proteins that were prepared from infected cells, and whole virion proteins were loaded and transferred to a membrane. Western blotting was carried out using sera collected from mice transplanted with isogenic cells infected with SeV vectors as a primary antibody

in the case of *ex vivo* transduction. Isogenic cells (MC57G; 1×10^6 cells) transduced by SeV/PLmutΔMΔFΔHN-GFP or SeV/ΔF-GFP were transplanted subcutaneously in the backs of C57BL/6 mice. Sera were collected at weekly intervals, and the levels of anti-SeV and SeV-neutralizing antibodies were measured. Figure 6A shows that, in comparison with the overall quantity of anti-SeV antibody produced in the case of transduction with SeV/ΔF-GFP, the production of anti-SeV antibody was greatly reduced when SeV/PLmutΔMΔFΔHN-GFP was used. Furthermore, neutralizing antibody was undetectable in mice transplanted with cells transduced by SeV/PLmutΔMΔFΔHN-GFP even at 28 days after the transplantation. The serum antibodies were characterised by Western blot analysis. Anti-HN and anti-M antibodies were not detected in the serum from the mice transplanted with SeV/PLmutΔMΔFΔHN-GFP-transduced cells (Figure 6B). However, anti-NP antibody

was detected in mice transplanted with either SeV/ΔF-GFP- or SeV/PLmutΔMΔFΔHN-GFP-transduced cells. As the HN protein is known to be one of the major targets for neutralizing antibodies against SeV [29], the abolishment of neutralizing antibody in the case of SeV/PLmutΔMΔFΔHN-GFP would be caused by the absence of anti-HN antibody in the serum. Also, we previously showed that deletion of both the M and F genes from the vector genome made the SeV vector non-transmissible and caused a lack of the formation of particles that might have enhanced the immunogenicity [12]. These results clearly indicate that the deletion of all three envelope-related genes (M, F and HN) from the SeV genome significantly reduced the humoral immune reaction against the SeV vector, especially in *ex vivo* transduction.

In conclusion, we have successfully recovered and propagated a high titer of more than 10^8 CIU/ml of a new SeV vector (SeV/ΔMΔFΔHN), in which all envelope-related genes were deleted from the vector genome using a newly established packaging cell line. Amino acid substitutions in P and L proteins that induce a slower rate of transcription caused additional attenuation of this vector (SeV/PLmutΔMΔFΔHN). This new vector showed efficient transduction capability both *in vitro* and *in vivo*. Furthermore, *in vivo* as well as *ex vivo* experiments showed a significant reduction of its immunogenicity compared with that of SeV/ΔF. That is, the deletion of

gene(s) from the SeV genome and additional introduction of amino acid substitutions into P and L is a very effective way to reduce both the immunogenic and cytopathic reactions induced by the SeV vector. These modifications are expected to improve the safety and broaden the range of clinical applications of SeV vectors such as for vaccine treatments [8,9] and cell-based treatment for hematopoietic damage including repeated dosing. The new vector, SeV/PLmutΔMΔFΔHN, is one of the most advanced genotoxicity-free cytoplasmic RNA virus vectors. This vector will also become a possible source for further improvement of SeV vector systems by introducing the additional mutations on the NP gene to modify the immune response against the vector and for alternative vector candidates such as virus-like particles (VLPs) and self-replicating ribonucleoprotein (RNP) complexes [3].

Acknowledgements

References

1. Lamb RA, Kolakofsky D. *Paramyxoviridae*: the viruses and their replication. In *Fields Virology*, Fields BN, Knipe DM, Howley PM (eds). Lippincott-Raven: Philadelphia, 1996; 1177–1204.
2. Bitzer M, Armeanu S, Lauer UM, *et al*. Sendai virus vectors as an emerging negative-strand RNA viral vector system. *J Gene Med* 2003; 5: 543–553.
3. Griesenbach U, Inoue M, Hasegawa M, *et al*. Sendai virus for gene therapy and vaccination. *Curr Opin Mol Ther* 2005; 7: 346–352.
4. Masaki I, Yonemitsu Y, Yamashita A, *et al*. Angiogenic gene therapy for experimental critical limb ischemia: acceleration of limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2. *Circ Res* 2002; 90: 966–973.
5. Shoji T, Yonemitsu Y, Komori K, *et al*. Intramuscular gene transfer of FGF-2 attenuates endothelial dysfunction and inhibits intimal hyperplasia of vein grafts in poor-runoff limbs of rabbit. *Am J Physiol Heart Circ Physiol* 2003; 285: H173–182.
6. Iwadate Y, Inoue M, Saegusa T, *et al*. Recombinant Sendai virus vector induces complete remission of established brain tumors through efficient interleukin-2 gene transfer in vaccinated rats. *Clin Cancer Res* 2005; 11: 3821–3827.
7. Kinoh H, Inoue M, Washizawa K, *et al*. Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases. *Gene Ther* 2004; 11: 1137–1145.
8. Matano T, Kobayashi M, Igarashi H, *et al*. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. *J Exp Med* 2004; 199: 1709–1718.
9. Takeda A, Igarashi H, Nakamura H, *et al*. Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. *J Virol* 2003; 77: 9710–9715.
10. Morrison T, McQuain C, McGinnes L. Complementation between avirulent Newcastle disease virus and a fusion protein gene expressed from a retrovirus vector: requirements for membrane fusion. *J Virol* 1991; 65: 813–822.
11. Arnon TI, Lev M, Katz G, *et al*. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* 2001; 31: 2680–2689.
12. Guertin DP, Fan DP. Stimulation of cytolytic T cells by isolated viral peptides and HN protein coupled to agarose beads. *Nature* 1980; 283: 308–311.
13. Justice PA, Sun W, Li Y, *et al*. Membrane vesiculation function and exocytosis of wild-type and mutant matrix proteins of vesicular stomatitis virus. *J Virol* 1995; 69: 3156–3160.
14. Mebatsion T, Konig M, Conzelmann KK. Budding of rabies virus particles in the absence of the spike glycoprotein. *Cell* 1996; 84: 941–951.
15. Li H-O, Zhu YF, Asakawa M, *et al*. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J Virol* 2000; 74: 6564–6569.
16. Inoue M, Tokusumi Y, Ban H, *et al*. A new Sendai virus vector deficient in the matrix gene does not form virus particles and shows extensive cell-to-cell spreading. *J Virol* 2003; 77: 6419–6429.
17. Inoue M, Tokusumi Y, Ban H, *et al*. Recombinant Sendai virus vectors deleted in both the matrix and the fusion genes: efficient gene transfer with preferable properties. *J Gene Med* 2004; 6: 1069–1081.
18. Inoue M, Tokusumi Y, Ban H, *et al*. Nontransmissible virus-like particle formation by F-deficient Sendai virus is temperature sensitive and reduced by mutations in M and HN proteins. *J Virol* 2003; 77: 3238–3246.
19. Bernloehr C, Bossow S, Ungerechts G, *et al*. Efficient propagation of single gene deleted recombinant Sendai virus vectors. *Virus Res* 2004; 99: 193–197.
20. Hamaguchi M, Yoshida T, Nishikawa K, *et al*. Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* 1983; 128: 105–117.
21. Kanegae Y, Takamori K, Sato Y, *et al*. Efficient gene activation system on mammalian cell chromosomes using recombinant adenovirus producing Cre recombinase. *Gene* 1996; 181: 207–212.
22. Arai T, Matsumoto K, Saitoh K, *et al*. A new system for stringent, high-titer vesicular stomatitis virus G protein-pseudotyped retrovirus vector induction by introduction of Cre recombinase into stable prepackaging cell lines. *J Virol* 1998; 72: 1115–1121.
23. Segawa H, Kato M, Yamashita T, *et al*. The roles of individual cysteine residues of Sendai virus fusion protein in intracellular transport. *J Biochem (Tokyo)* 1998; 123: 1064–1072.
24. Miura N, Uchida T, Okada Y. HVJ (Sendai virus)-induced envelope fusion and cell fusion are blocked by monoclonal anti-HN protein antibody that does not inhibit hemagglutination activity of HVJ. *Exp Cell Res* 1982; 141: 409–420.
25. Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 1988; 115: 61–69.
26. Kato A, Sakai Y, Shioda T, *et al*. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* 1996; 1: 569–579.
27. Bossow S, Neubert WJ. A set of four mutations in the P and L genes derived from persistent Sendai virus variants attenuates viral replication. *Negative Strand Viruses* 2000; 157.
28. Ogino T, Iwama M, Ohsawa Y, *et al*. Interaction of cellular tubulin with Sendai virus M protein regulates transcription of viral genome. *Biochem Biophys Res Commun* 2003; 311: 283–293.
29. Tozawa H, Komatsu H, Ohkata K, *et al*. Neutralizing activity of the antibodies against two kinds of envelope glycoproteins of Sendai virus. *Arch Virol* 1986; 91: 145–161.

ES細胞のゲノム操作
Genetic manipulation of ES cells

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ヒト ES 細胞の樹立と利用

　1998 年アメリカのグループがヒト ES 細胞株の樹立を発表した[1]. ES 細胞は，三胚葉いずれの細胞にも分化しうる多分化能をもつうえ，この多分化能を維持した状態で無限増殖が可能である. この驚くべき性質をもつヒト ES 細胞をさまざまな疾患や障害の治療に用いようと研究者が注目するのは当然の結果である. ヒト ES 細胞から特定の細胞への分化については，神経細胞，血液細胞，心筋細胞などへの分化の成功例がすでに報告されている[2-4].

　ES 細胞の作製と利用にあたっては，かねてから 2 つの問題が指摘されていた. ひとつは，作製時の初期胚破壊にともなう倫理的な問題である. これに対しては，初期胚を破壊しない ES 細胞作製技術など，倫理的問題の少ない方法が工夫されている[5]. ふたつめの問題は，HLA 不一致にともなう移植後の免疫拒絶の問題である. これに対して韓国のグループが体細胞核移植技術を利用した「自家」ES 細胞作製に成功したと Science 誌に報告して脚光を浴びたが，データは捏造であることが発覚し，自家 ES 細胞作製に関しては振り出しに戻った. 一方，とりあえず 150 個のヒト ES 細胞株を樹立すれば実用レベルのバンキング事業が可能だとする報告があって[6]，免疫拒絶問題の克服のために ES 細胞のバンキングも今後期待される.

ES 細胞の遺伝子操作法

　ヒト ES 細胞へ効率よく安定に遺伝子を導入する方法は，ES 細胞の分化制御やカスタム化のために今後重要性を増す技術である. 従来 ES 細胞への遺伝子導入というと遺伝子改変マウスの作製用途が多かった. この場合は，電気穿孔法やリポフェクション法といった，いわゆるトランスフェクションが一般的である.

しかし，この方法で霊長類 ES 細胞への安定な遺伝子導入効率は 10⁵分の 1 程度である．

一方，ES 細胞の分化制御やカスタム化(テーラーメード)といった，移植・再生医療への応用を考えると，大量の ES 細胞にもっと効率よく遺伝子を導入する必要が出てくる．そのような場合はウイルスベクターを利用することが多い．もっとも，遺伝子改変マウス作りにも，後述のとおりウイルスベクター(特にレンチウイルスベクター)が用いられることがある[7,8]．そもそもウイルスは宿主細胞に効率よく感染すべく「進化」を遂げてきたので，その性質を利用するわけである．よく使われるウイルスベクターには，アデノウイルス，アデノ随伴ウイルス，レトロウイルス，レンチウイルス，センダイウイルス由来のものがある．それぞれ独特の特徴をもつので，用途に応じた使い分けが重要である．

レンチウイルスベクターとレトロウイルスベクター

ヒト ES 細胞はその使用にあたってさまざまな制約があるため，筆者らは同じ霊長類のサル(カニクイザル)の ES 細胞を用いて研究を行っている[9]．アデノウイルスベクターやアデノ随伴ウイルスベクターを用いた遺伝子導入法では，サル ES 細胞への導入効率はたかだか 20 ％で，しかも導入遺伝子の発現が一過性であり安定した遺伝子発現は望めない．これらのベクターは宿主ゲノムに組み込まれないので，感染後，細胞が分裂するたびにベクターが希釈されるからである．

一方，宿主ゲノムに組み込まれ，細胞分裂後も安定した遺伝子発現が得られるレンチウイルスベクターを使うと，霊長類 ES 細胞へきわめて効率よく(>50 ％)遺伝子導入できることがわかった[10]．従来のレトロウイルスベクターはどうかというと，残念ながらサル ES 細胞に対する遺伝子導入効率はあまり高くない(<20 ％)．霊長類 ES 細胞への遺伝子導入にはレンチウイルスベクターが断然優れている．

しかも，レトロウイルスベクターを使って ES 細胞に導入した遺伝子は，発生や分化の途上でサイレンシングを受け，その発現は次第に抑制されてしまう．そのためレトロウイルスベクターを用いる ES 細胞への遺伝子導入法では，導入遺伝子がきちんと発現するトランスジェニックマウスは得られないことが知られていた．一方，マウス ES 細胞にレンチウイルスベクターで遺伝子を導入して，それを胚盤胞に戻しキメラマウスを作ると，生まれたマウスで導入遺伝子の発現が認められたという[7,8]．

レンチウイルスベクターとレトロウイルスベクターで ES 細胞に導入した際の遺伝子発現のこの顕著な違い(サイレンシングの有無)は，これらのウイルスの生活環に関係すると思われる．レトロウイルスは，その伝播を，生殖細胞を通じた垂直感染に依存している．したがって，宿主ゲノムに組み込まれたレトロウイルスの遺伝子発現を抑えなければ，ウイルスはその宿主個体内に広がるのみならず，生殖を通じて後代まで伝わることになる．これを避けるために，哺乳動物はゲノムに組み込まれたレトロウイルスをメチル化などによってサイレンシングする機構を備えるようになったと思われる．一方，レンチウイルスは，その内因性ウイルスが哺乳類でみつかっていないことからわかるとおり，その伝播は，生殖細胞を介さないような水平または垂直感染に依存している．したがって，ゲノムに組み込まれたレンチウイルスをサイレンシングする機構を進化させる必要が生じなかったのであろう．

センダイウイルスベクター

センダイウイルス(SeV)ベクターは，ヒトへの病原性がなく，DNA を介さない細胞質型 RNA ベクターである．したがって，レトロウイルスベクターで問題になる挿入変異や相同組換えによる変異ウイルス産生といった心配がない．SeV ベクターを用いるとサル ES 細胞に対して効率よく外来遺伝子を導入できる[11](図1)．1 回のみの感染で 2 日後には約 60 ％の細胞が GFP 蛍光を発した．これはレンチウイルスベクター
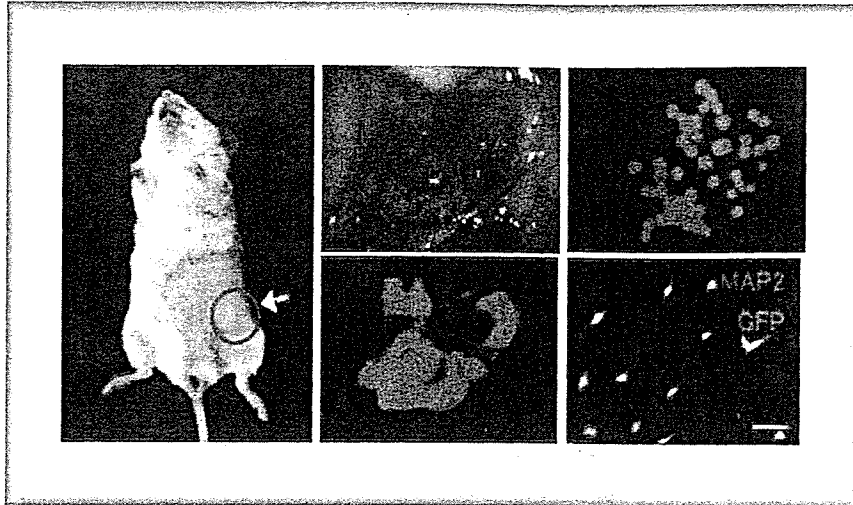
図1　センダイウイルス(SeV)ベクター
　　を用いた ES 細胞への遺伝子導入
SeV ベクターを用いてサル ES 細胞へ GFP 遺
伝子を導入後，その細胞を免疫不全マウスへ
移植した．移植部位にテラトーマを形成し
(左)，そのテラトーマは GFP を発現していた
(中)．また，SeV ベクターによって GFP 遺伝
子を導入したサル ES 細胞を，好中球(右上)
や神経細胞(右下)に分化させても，GFP の発
現は衰えなかった．　　　(文献 11 より引用)

による遺伝子導入効率に匹敵する．SeV ベクターは
それ自身が自己複製能をもつので，細胞分裂によって
ベクターが希釈されることなく，GFP の発現は感染
後数カ月以上にわたって安定していた．しかも，SeV
ベクターは ES 細胞の三胚葉分化能を損わず，成熟細
胞に分化後も導入遺伝子の発現は衰えることがなかっ
た．さらに，SeV ベクターで導入した遺伝子の発現
を抗ウイルス剤(リバビリンなど)の添加によって調節
するという，全く新しい遺伝子発現調節法も検討され
ており，今後の展開が期待されるベクターである．

　以上，筆者らは，サル ES 細胞への効率的な遺伝子
導入法として，レンチウイルスベクターと SeV ベク
ターが優れていることを明らかにした．いずれも
RNA ウイルスベクターである．レンチウイルスベク
ターは宿主ゲノムへ組み込まれることによって安定な
遺伝子発現が得られる．一方，SeV ベクターは自ら
がもつ増殖能によって安定な遺伝子発現が得られる．
遺伝子導入の目的や用途に応じて，2 つのベクターを
使い分けることができよう．

ES 細胞遺伝子治療モデル

　1990 年いわゆる「アニッサ事件」が起った．白血
病の少女アニッサを助けるためには骨髄移植しかなか
った．しかし適合ドナーがみつからないなか，両親は
もう 1 人子供を作る決心をした．その子供の骨髄をア
ニッサに移植しようと．HLA が適合する 4 分の 1 の
可能性に両親は賭けた．1990 年妹マリッサが誕生し
た．幸い HLA が適合し，翌年妹マリッサからアニッ
サへの移植手術が行われ無事成功した．現在，姉妹と
も健在である．しかし「手段」として子どもを作った
ことに対する強い批判が起こった．

　その 10 年後，新たな事例が起った(「2000 年版アニ
ッサ事件」)．ファンコニー貧血の少女モリーにはやは
り適合ドナーがみつからなかった．両親はもう 1 人子
供を作る決心をする．ただし今回の方法は体外受精だ
った．作られた受精卵は全部で 15 個．受精卵のなか
から，その病気にかかっておらず組織が適合するもの
を選んで母親の子宮に戻した．その結果，弟のアダム
が生まれる．当然のことだが HLA は適合した．2000
年 10 月移植手術は成功，モリーは元気になって退院
した．今回は子どもを作る際に行った「遺伝子の選
別」に対して強い批判が起こった．

　これらは実話である．さて，X 年後，アニッサはど
のような治療を受けることになるか予想してみよう(図
2)．白血病に冒されたアニッサを助けるには組織が適
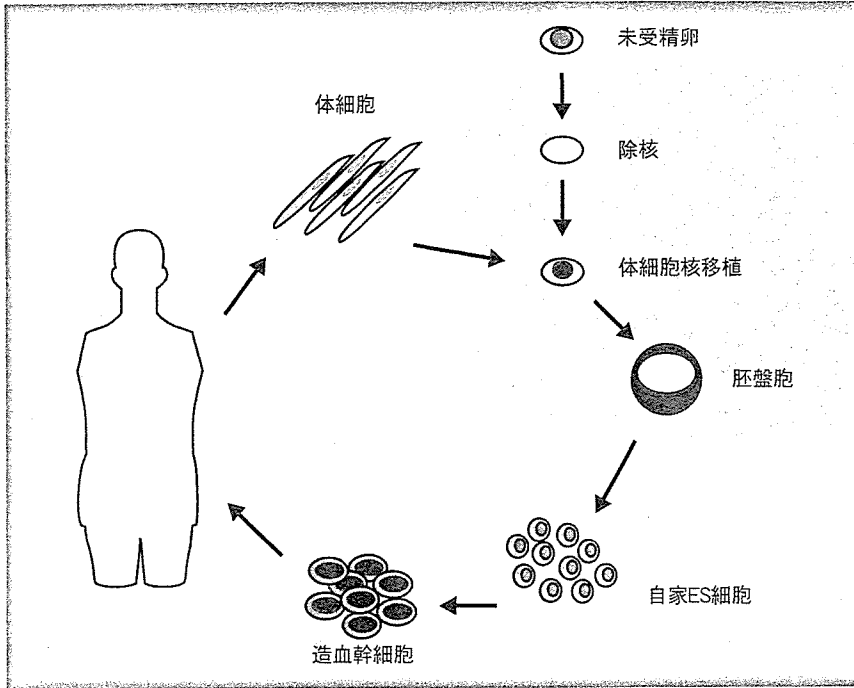合する骨髄移植しかないが，適合ドナーはやはりみつ
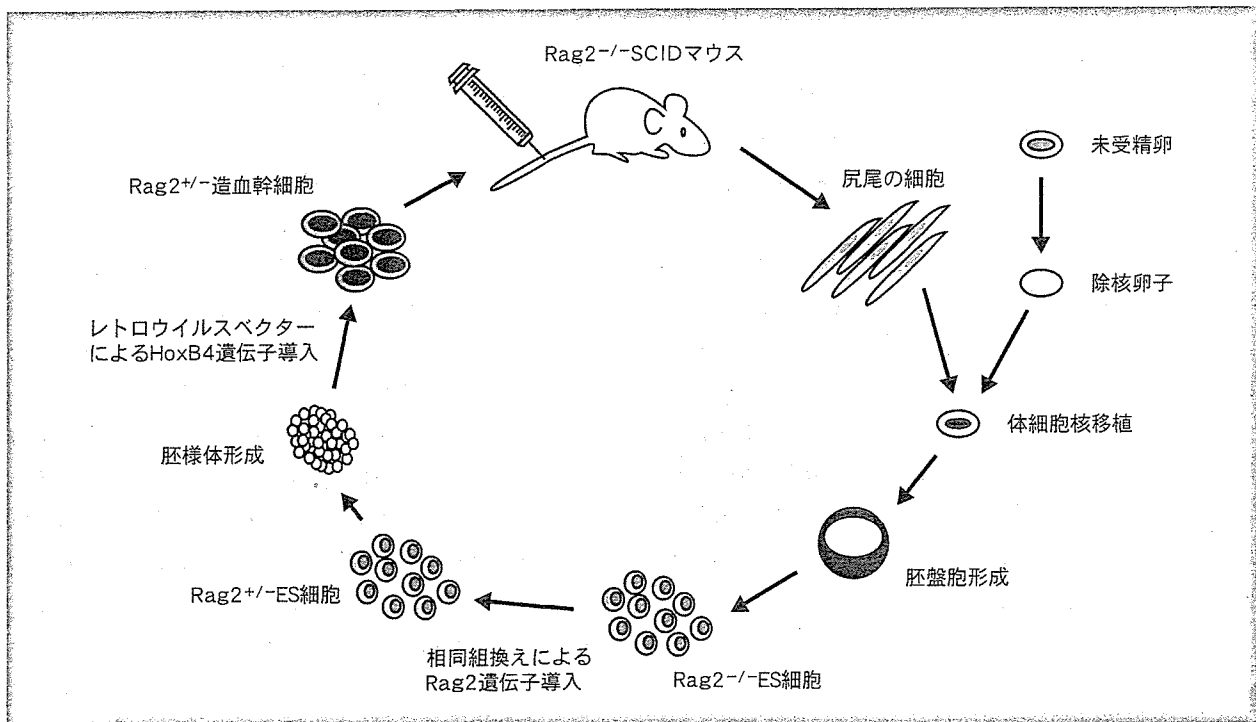
図2 近未来の白血病治療
自家 ES 細胞を利用する新しい遺伝子治療が可能になるかもしれない.

図3 SCID マウスに対する ES 細胞遺伝子治療

SCID マウスでは, その体細胞核を未受精卵に移植することによって自家 ES 細胞を作り出し, その細胞を遺伝子修復したうえで, 造血幹細胞に分化させることに成功した.
(文献 12 より引用)

からなかった．しかし，両親はもはやもう1人子ども を作る必要はない．ES細胞遺伝子治療が可能になっ たからである．まずアニッサの皮膚細胞を取り，その 核を提供された卵子に移植する(体細胞核移植)．そこ からできる胚盤胞からES細胞を取り出す．遺伝子操 作によってES細胞を造血幹細胞に分化させ，それを 移植する．自分の細胞からできた造血幹細胞だから完 全に適合する．そしてアニッサは元気になって退院す る．

これらの技術が本当に実用化されるかはわからない． ただ最近の科学の進歩によって，この話はにわかに現 実味を帯びてきたのは事実である．2002年4月，マ ウスの系で，Rag2欠損型複合型免疫不全症($Rag2^{-/-}$ SCID)に対して，ES細胞遺伝子治療の試行例がCell 誌に報告された(図3)[12]．遺伝子治療と再生医学が融 合したこのハイブリット治療は，新しい時代の幕開け を思わせる見事な実験であった．前述の方法はマウス ではすでに実現したのである．ES細胞の遺伝子操作 技術は，こうした新時代の治療法へつながっていくと 思われる．

References
1) Thomson JA et al：*Science* 282：1145-1147, 1998
2) Reubinoff BE et al：*Nat Biotechnol* 19：1134-1140, 2001
3) Kaufman DS et al：*Proc Natl Acad Sci USA* 98：10716-10721, 2001
4) Kehat I et al：*J Clin Invest* 108：407-414, 2001
5) Chung Y et al：*Nature* 439：216-219, 2006
6) Taylor CJ et al：*Lancet* 366：2019-2025, 2005
7) Pfeifer A et al：*Proc Natl Acad Sci USA* 99：2140-2145, 2002
8) Lois C et al：*Science* 295：868-872, 2002
9) Suemori H et al：*Dev Dyn* 222：273-279, 2001
10) Asano T et al：*Mol Ther* 6：162-168, 2002
11) Sasaki K et al：*Gene Ther* 12：203-210, 2005
12) Rideout WM 3 rd et al：*Cell* 109：17-27, 2002

Eradication of the Sendai virus vector genome in primate embryonic stem cells by Knockout Serum Replacement (KSR)

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Background. Efficient and regulated transgene expression in primate embryonic stem (ES) cells is highly desirable for future applications of the cells. We have previously shown that Sendai virus (SeV) vector can introduce foreign genes efficiently and stably into cynomolgus monkey ES cells without spoiling pluripotency of the cells. For most of clinical applications, however, the transient expression of genes of interest would be suitable and the removal of the SeV genome from the infected cells would be required before transplantation of the cells into human subjects in such cases. To this end, we have already shown that SeV-mediated transgene expression levels can be reduced by the addition of an anti-virus drug, ribavirin, to the culture medium. Ribavirin, however, cannot completely eradicate the SeV genome in the infected ES cells and shows cytopathic effects at high concentrations. In the present study, we examined the effect of the Knockout Serum Replacement (KSR) against the SeV vector replication in cynomolgus ES cells. **Methods and Results**. Cynomolgus ES cells were infected with the SeV vector (F-defective) carrying the green fluorescent protein (GFP) gene. We plucked fluorescent ES-cell colonies under a fluorescent microscope after infection and propagated them in the regular FBS-supplemented medium (GFP-positive cells > 90%). We cultured GFP-positive ES cells in the 20% KSR-supplemented medium in the presence of 1.5% or 15% FBS. In the former condition (20% KSR/1.5% FBS), the GFP expression dramatically decreased to zero and the SeV genome was not detectable even as assessed by PCR at 77 days after the exposure of KSR, whereas the GFP expression in the latter condition (20% KSR/15% FBS) remained unchanged. On the other hand, the GFP expression was still reversible when GFP-positive ES cells dropped to 12% in the KSR-supplemented medium: When we returned the medium to the regular FBS-supplemented one at the time, the GFP expression was restored. The KSR inhibitory effect on the SeV-mediated GFP expression was not observed in the rhesus monkey kidney cell line LLC-MK2, a standard control cell line for SeV infection. **Discussion**. Although KSR was originally developed to maintain ES cells in an undifferentiated state, the present results show that the exposure of ES cells to KSR-supplemented media eradicates the SeV genome and spoils the SeV vector-mediated transgene expression in the cells. This inhibitory effect can be cancelled by the addition of 15% FBS. Thus, KSR-supplemented media would be useful for the eradication of the SeV genome and complete reduction of its transgene expression in primate ES cells.