functions, and there is no DNA phase during its life cycle, so that the possible transformation of cells due to the integration of vector materials into the cellular genome is not a concern [1]. These properties make SeV vectors very promising for applications to gene therapy (cytoplasmic gene therapy) and vaccination via the expression of therapeutic genes and antigens [2,3]. In particular, a clinical trial protocol for therapeutic induction of angiogenesis, which is for the treatment of critical limb ischemia using an SeV vector carrying the fibroblast growth factor-2 gene [4,5], has been approved by the Ministry of Health, Labour and Welfare (MHLW) of Japan and will begin soon. In addition, cancer treatments including treatments for brain tumors [6] and many types of aggressive tumors [7], and a variety of vaccination protocols such as for the human immunodeficiency virus [8,9], using SeV vectors are planned.

The viral envelope comprises a lipid bilayer derived from the host plasma membrane and two inserted viral glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) proteins. Beneath the envelope is a lining consisting of the matrix or membrane (M) protein. The F and HN proteins are primarily required during the entry of SeV into cells. The F protein is involved in virus penetration, hemolysis and cell fusion [10]. The HN protein mediates the viral attachment to cells by interacting with cell-surface sialic acid containing receptor(s). The HN protein is known to be one of the major targets of the host humoral immune responses against SeV infection, and to induce NK and cytotoxic T lymphocyte responses [11,12]. The M protein promotes vesiculation of the membrane and the release of particles into the extracellular medium without the aid of other viral proteins [13,14]. We previously succeeded in the recovery of high titers of F-gene-deleted (SeV/ΔF) [15], M-gene-deleted (SeV/ΔM) [16], HN-gene-deleted (SeV/ΔHN), and both M- and F-genes-deleted (SeV/ΔMΔF) [17] SeV vectors in addition to other types of SeV vectors [18] by using packaging cell lines that express the respective proteins encoded by the deleted gene(s). All the vectors showed efficient infectivity and transgene expression in various types of cell lines and primary cells *in vitro*. Deletion of the F gene made the SeV vector non-transmissible, deletion of the M gene worked well to render the vector incapable of directing the formation of particles in infected cells, and deletion of the HN gene was expected to reduce the host immune response against the SeV vector. The single-gene-deleted SeV vectors were also rescued and propagated by another group [19], but multiple-genes-deleted SeV vectors have not been propagated at high titer. For the wide-range application of SeV vectors, such as for the treatment of chronic diseases, virus-gene-derived protein expression should be reduced as much as possible. High-level transgene expression from the SeV vector results from the high-level transcription associated with the genome replication that is directed by nucleoprotein (NP), phospho (P) and large (L) proteins [20]. To keep the high-level expression of SeV vectors, the NP, P and L genes should not be removed. Therefore, the most advanced SeV vector, which reduces the viral genome as much as possible, is the M-, F- and HN-genes-deleted SeV vector (SeV/ΔMΔFΔHN) at present. However, the recovery of SeV/ΔMΔFΔHN has hitherto been very difficult because the efficient complementation of all three proteins (M, F and HN) needed to form the virion particle was essential for its propagation.

In this study, we succeeded in the establishment of a packaging cell line that expresses all three envelope proteins, M, F and HN, by using a Cre/*loxP* induction system. Using this cell line, we succeeded in producing the SeV vector deleted for the M, F and HN genes at a titer of more than 10^8 cell infectious units (CIU)/ml. SeV/ΔMΔFΔHN possesses only the NP, P and L genes in its genome. This vector showed efficient transduction capability and reduced cytopathic effect. Importantly, the immune reaction against SeV was also reduced when SeV/ΔMΔFΔHN was used *in vivo* and *ex vivo*.

Materials and methods

Cells and viruses

Monkey kidney cell lines, LLC-MK_2 and CV-1, were maintained in monolayer cultures in minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum and penicillin-streptomycin in the presence of 5% CO_2. The molecular clone of SeV Z strain with attenuated virulence was used as the starting material for genome modifications in this study. The F, M or both M/F gene(s)-deleted SeV vectors were prepared by using LLC-MK_2 cells stably transformed with the F gene (LLC-MK2/F7 [15], M gene (LLC-MK2/F7/M62 [16]), or both M and F genes (LLC-MK2/F7/M#33 [17]). The SeV/ΔMΔFΔHN was prepared in a new packaging cell line (LLC-MK2/F7/M#33/A/HN7; this report). An adenovirus vector, AxCANCre [21], expressing Cre recombinase, was used for the induction of respective protein(s) encoded by these deleted gene(s).

Plasmid construction

For the construction of genomic cDNA of M-, F- and HN-genes-deleted SeV carrying the green fluorescent protein (GFP) gene, LitmusSalINheIhfrag-ΔMΔFGFP that was used to generate the cDNA of SeV/ΔMΔF-GFP (pSeV/ΔMΔF-GFP) containing the GFP and HN genes in the *Sal I/Nhe* I site in LITMUS38 (NEB) [17] was utilised. Inverse polymerase chain reaction (PCR) was conducted with primer pairs of 5′-GAGGTCGCGCGTTAATTAAGCTTTCACCTCAAACAAGC-ACAGATCATGG-3′ and 5′-GCATGTTTCCCAAGGGGAGA-GTTAATTAACCAAGCACTCACAAGGGAC-3′ to introduce the *Pac* I site just behind the P gene. The PCR product was digested with *Pac* I and *Dpn* I and then self-ligated; thus both GFP and HN genes were deleted from LitmusSalINheIhfrag-ΔMΔFGFP and generated

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LitmusSalINheIhfrag-ΔMΔFΔHN-Pac I. To insert the GFP gene with end and start signals (EIS) between the P and L genes, PCR was conducted with primer pairs of 5′-CTGCGATCGCGCCCCAAGCAGACACCACCT-3′ and 5′-TACGCGATCGCTGATAATGGTCGTGATCAT-3′ on pSeV18+/ΔF-GFP [15] as a template. The amplified GFP fragment was digested with *Sgf* I and inserted into the *Pac* I site of LitmusSalINheIhfrag-ΔMΔFΔHN-PacI to generate LitmusSalINheIhfrag-ΔMΔFΔHN-GFP. The 5.9 kb *Sal* I- and *Nhe* I-digested fragment containing the GFP gene was substituted for the corresponding fragment of pSeV18+/ΔMΔF-GFP to generate pSeV/ΔMΔFΔHN-GFP. To introduce the mutations of persistent infection into the P and L genes, site-directed mutagenesis was conducted using a QuickChange site-directed mutagenesis kit (Stratagene) with the primer pairs 5′-ctcaaacgcatcacgtctcTtTccctccaaagagaagc-3′ (sense) and 5′-gcttctctttggagggAaAgagacgtgatgcgtttgag-3′ (antisense) for L511F in the P gene, and 5′-gttctatcttcctgacTC-tatagacctggacacgcttac-3′ (sense) and 5′-gtaagcgtgtccaggt-ctataGAgtcaggaagatagaac-3′ (antisense) for N1197S and 5′-ctacctattgagcccettagttgacGaAgataaagataggcta-3′ (sense) and 5′-tagcctatctttatcTtCgtcaactaaggggctcaatagg-tag-3′ (antisense) for K1795E in the L gene used on pSeV/ΔMΔFΔHN-GFP as a template. Thus, full-length genomic cDNA of pSeV/PLmutΔMΔFΔHN-GFP was generated and its structure was confirmed by sequencing. For the plasmid expressing the HN protein under the control of the *Cre/loxP* induction system [22] a PCR-generated 1.8 kb fragment containing the HN gene from SeV cDNA was inserted into the *Swa* I site of pCAL-NdLw [22] to generate pCALNdLw/HN, in which the HN gene was located after the drug-resistant gene sandwiched between *loxP* sequences. Hence, the expression of the HN protein in pCALNdLw/HN-introduced cells, LLC-MK2/F7/M#33/A/HN7, was induced after expressing Cre recombinase from AxCANCre.

Insertion of the SEAP gene

To quantify the expression of a foreign gene carried in the SeV genome, the gene for the secreted form of human placental alkaline phosphatase (SEAP) was inserted upstream of the open reading frame of the NP gene. The SEAP gene with the EIS element [17] was introduced into the *Not* I site located in the non-coding sequence between the start (S) signal and the translation initiation codon (ATG) of the NP gene of pSeV/PLmutΔMΔFΔHN-GFP to generate pSeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP.

Cloning and analysis of packaging cell lines

LLC-MK$_2$/F7/M#33 cells were transfected with pCAL-NdLw/HN using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instruction. Two weeks after transfection, viable clones of single cell origin in 96-well plates were expanded in 12-well plates, and, when they reached a nearly confluent state, they were infected with AxCANCre at an multiplicity of infection (MOI) of 5 [21,22]. After culturing for 2 days at 32 °C, the cells were recovered and subjected to semiquantitative Western blotting with anti-HN antibody according to a method described previously [16].

M-, F- and HN-genes-deleted SeV vector recovery from cDNA

Preparation of cell lysate containing RNPs and primary virions of SeV/ΔMΔFΔHN-GFP was carried out according to the method described previously [16] with minor modification. Briefly, approximately 1×10^7 LLC-MK$_2$ cells seeded in Ø10-cm dish were transfected with pSeV/ΔMΔFΔHN-GFP and pCAG-plasmids each carrying the NP, P, M, F, HN or L gene (Ban *et al.*, personal communication). The cells were cultured in MEM containing trypsin (7.5 µg/ml). Twenty-four hours after transfection, the cells were overlaid with LLC-MK2/F7/M#33/HN7 cells after induction of M, F and HN proteins by AxCANCre infection at an MOI of 5 and cultured for another 48 h. The cells were harvested and lysed by repeating a freeze/thaw cycle three times in Opti-MEM (Invitrogen). The cell lysate was infected into new LLC-MK2/F7/M#33/HN7 cells after AxCANCre infection. After that, these cells were cultured at 32 °C in MEM containing trypsin for 10 to 20 days. When spread of GFP expression to neighbour cells was seen by fluorescence microscopy, it was considered that viral vectors were recovered in the culture supernatants. Those viral vectors were further amplified by several rounds of propagation. Titers were determined by the proportion of GFP-expressing cells (GFP-CIU) per milliliter [15]. The culture supernatant of the fourth passage was collected and stored at −80 °C, after adding bovine serum albumin (BSA) solution to a final concentration of 1% (w/v), until usage in all the experiments described below.

RT-PCR

Total viral RNA from SeV/ΔMΔFΔHN-GFP was extracted from the supernatant containing viral particles using a QIAamp viral RNA minikit (Qiagen). Reverse-transcription (RT)-PCR was performed in a one-step process using the Superscript RT-PCR system (Invitrogen). RT-PCR amplication was performed with random hexamers and the primer pair 5′-AGAGAACAAGACTAAGGCTACC-3′ (forward primer specific for the P gene) and 5′-TATTCAACCAAAGATCCT-GGAACCC-3′ (reverse primer specific for the L gene) probed.

Detection of viral proteins by Western blotting

Analysis of viral proteins by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was

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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_2O_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

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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

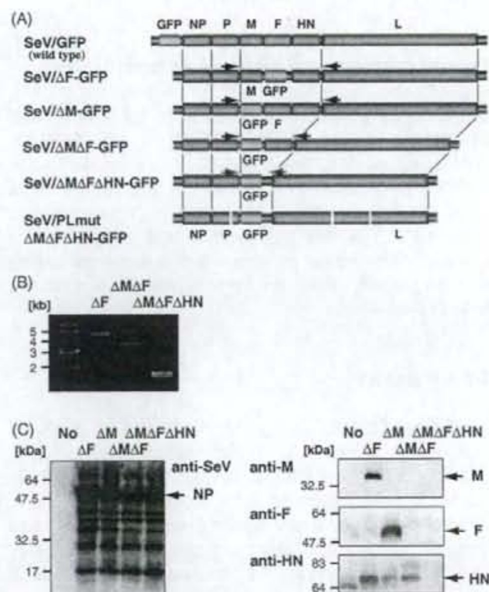

Figure 1. Construction of M-, F- and HN-genes-deleted SeV vector carrying the GFP gene, and confirmation of its structure. (A) The structures of recombinant SeV genomes. The open reading frame of the GFP gene was inserted with the SeV end and start signals (EIS) in the respective positions of the deleted 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

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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.

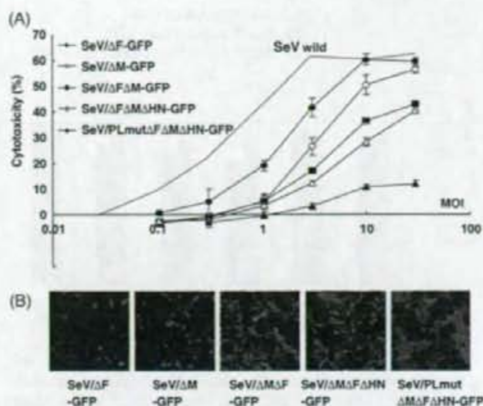

Figure 2. Analysis of SeV infection-dependent cytotoxicity. (A) Quantitative measurement using SeV infection-sensitive cells. CV-1 cells were infected with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP or SeV/PLmutΔMΔF-ΔHN-GFP at an indicated MOI. Cytotoxicity was determined by the quantity of 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-

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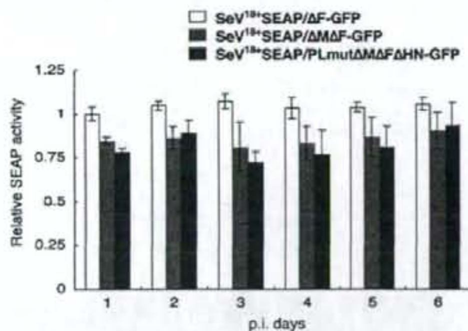

Figure 3. Comparison of expression performance of SeV vectors carrying the SEAP gene. The culture medium of LLC-MK_2 cells was collected every 24 h after infection with SeV^{18+}SEAP/ΔF-GFP, SeV^{18+}SEAP/ΔMΔF-GFP or SeV^{18+}SEAP/ΔMΔFΔHN-GFP at an MOI of 3. SEAP activity was determined as a relative activity by setting the value from the medium of uninfected cells as zero (0) and that from the medium of SeV^{18+}SEAP/ΔF-GFP-infected cells (MOI = 3, 1 day post-infection.) as one (1). Average of three experiments; bar: SD

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/PLmutΔMΔFΔHN-GFP (Figure 4A). However, the peak of expression was prolonged in the case of SeV/PLmutΔMΔFΔHN-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/PLmutΔMΔFΔHN-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/PLmutΔMΔFΔHN-GFP administration compared with that of SeV/ΔF-GFP (Figures 5A and 5B). These results clearly indicate that the immunoreaction against SeV/PLmutΔMΔFΔHN-GFP is weak and delayed compared to that against SeV/ΔF-GFP. Efficient transduction and prolonged expression of SeV/PLmutΔMΔFΔHN 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/PLmutΔMΔFΔHN-GFP on the induction of anti-SeV antibodies and

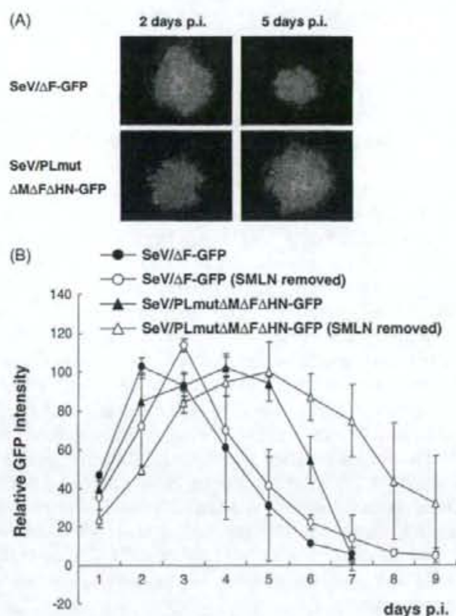

Figure 4. Gene transfer by M-, F- and HN-genes-deleted SeV vector *in vivo*. (A) Gene transfer to the ear auricle. SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP (5×10^6 GFP-CIU/head) was administered to the ear auricle of BALB/cA mice with or without submandibular lymph nodes (SMLN) by a single intradermal injection (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

neutralizing antibodies. SeV/PLmutΔMΔFΔHN-GFP or SeV/ΔF-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/PLmutΔMΔFΔHN-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/PLmutΔMΔFΔHN-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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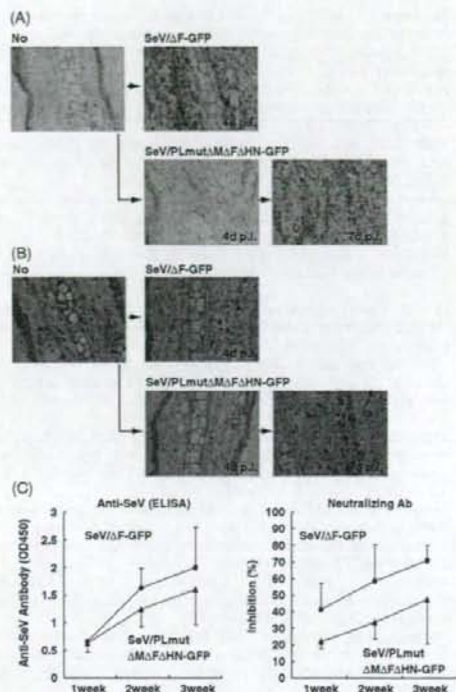

Figure 5. Immune reaction to SeV vectors introduced by *in vivo* transduction. Immunohistochemical analysis of the migrations of (A) macrophages and (B) T lymphocytes. The frozen sections of ear auricles of BALB/cA mice intradermally administered with SeV/ΔF or SeV/PLmutΔMΔFΔHN. (C) Sera from mice administered 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

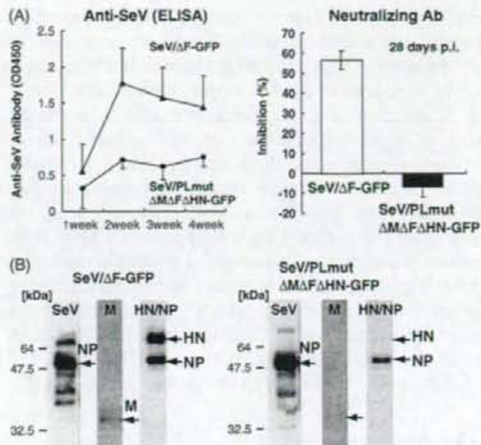

Figure 6. Immune reaction to SeV vectors following transplantation of SeV-infected cells. (A) Serum levels of anti-SeV antibody (left) following transplantation of isogenic cells (1×10^6 cells/head) infected with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP at an MOI of 3 were examined. Neutralizing antibody level 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

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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].

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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10^5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

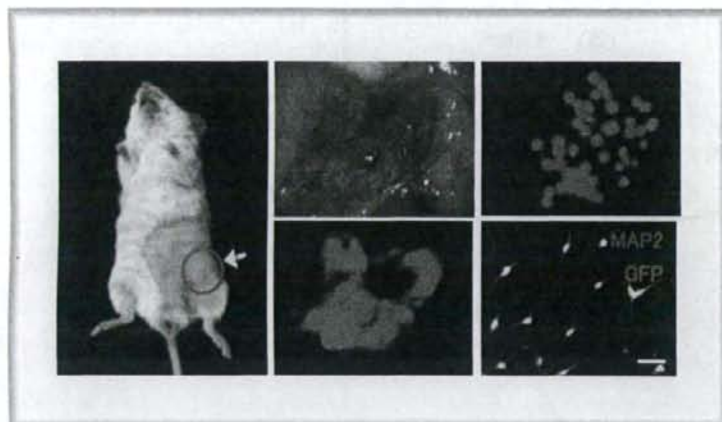

図1 センダイウイルス(SeV)ベクターを用いたES細胞への遺伝子導入
SeVベクターを用いてサルES細胞へGFP遺伝子を導入後，その細胞を免疫不全マウスへ移植した．移植部位にテラトーマを形成し(左)，そのテラトーマはGFPを発現していた(中)．また，SeVベクターによってGFP遺伝子を導入したサルES細胞を，好中球(右上)や神経細胞(右下)に分化させても，GFPの発現は衰えなかった．(文献11より引用)

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

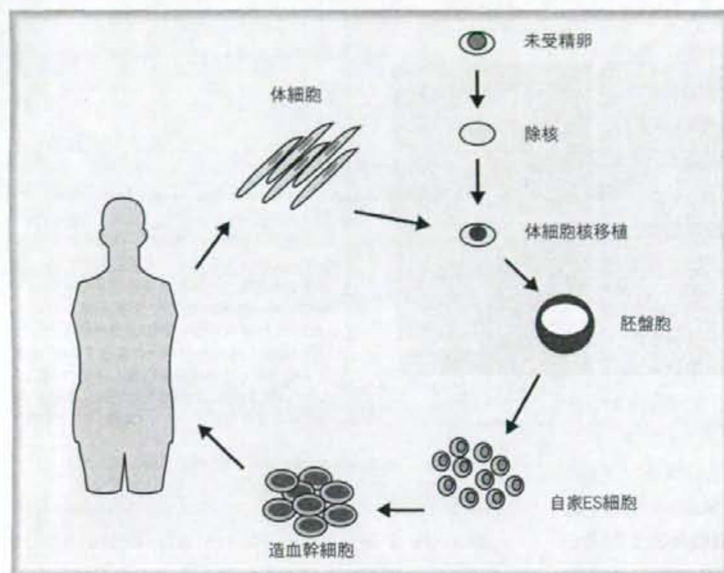

図2　近未来の白血病治療
自家 ES 細胞を利用する新しい遺伝子治療が可能になるかもしれない．

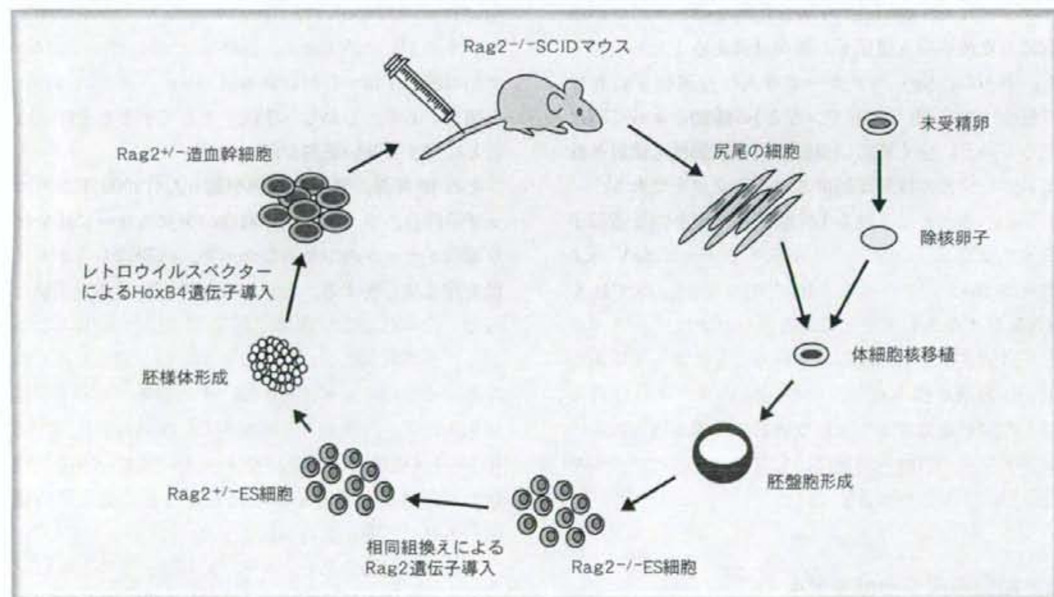

図3　SCID マウスに対する ES 細胞遺伝子治療
SCID マウスでは，その体細胞核を未受精卵に移植することによって自家 ES 細胞を作り出し，その細胞を遺伝子修復したうえで，造血幹細胞に分化させることに成功した．（文献 12 より引用）

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．

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