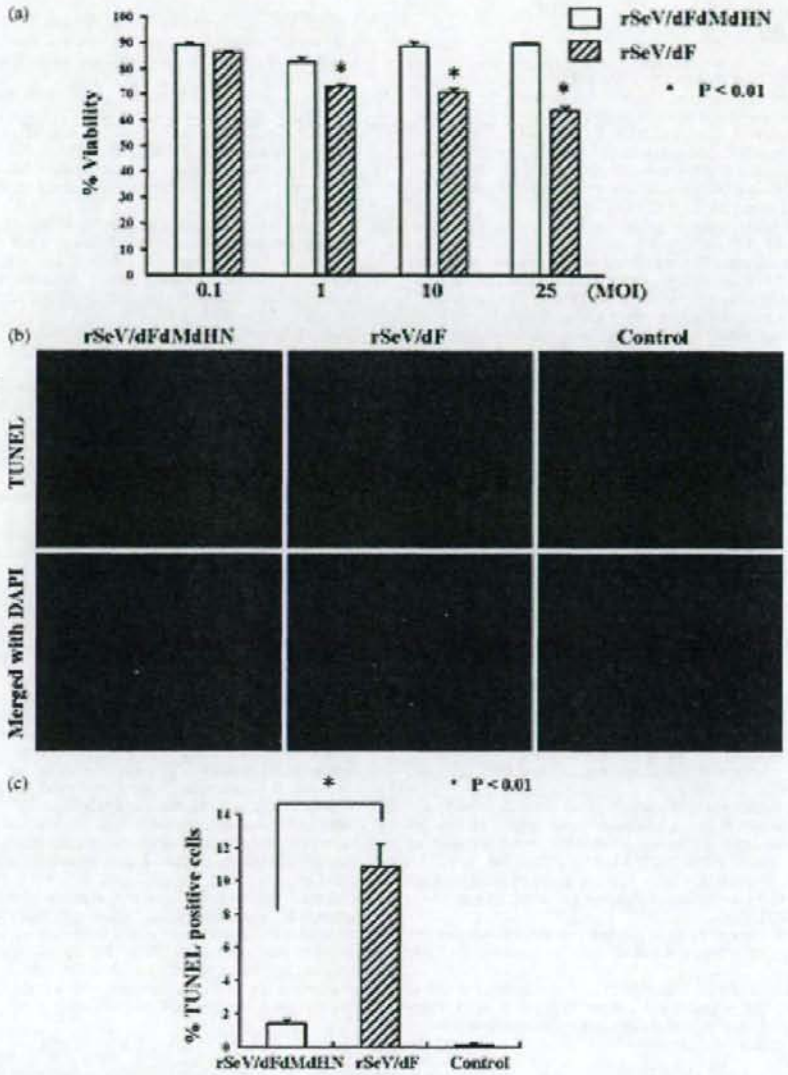

Figure 7. Quantitative analysis of rSeV infection-dependent cytotoxicity in vitro. (a) *In vitro* cytotoxicity of rSeVs to ARPE-19. After 48 h of culturing in the presence of rSeV/dF-EGFP or rSeV/dFdMdHN-EGFP at a multiplicity of infection (MOI) of 0.1, 1, 10 and 25, cell viability was assessed. Note the reduction of SeV-induced cytotoxicity in rSeV/dFdMdHN-infected cells ($n = 4$ each; $^*p < 0.01$). (b, c) TUNEL staining (b) and quantitative analysis of the TUNEL-positive apoptotic nuclei in ARPE-19 cells infected with each rSeV at a MOI of 10. Note the reduction of SeV-induced apoptosis in rSeV/dFdMdHN-infected cells ($n = 5$ each; $^*p < 0.01$)

Acknowledgements

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Supplementary Material

The supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1099-498X/suppmat/.

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New cancer therapy using genetically-engineered oncolytic Sendai virus vector

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1. ABSTRACT

We have developed a new type of Sendai virus-(SeV) based gene transfer vectors for cancer therapy. The matrix gene-, indispensable for particle formation, deficient and fusion gene-, essential for cell-fusion and deciding viral tropism, redesigned SeV vector loses vector particle formation from transduced cells and gains cell-to-cell spreading in protease-dependent, namely controllable, manner. For the selective delivery to malignant tumor cells expressing matrix metalloproteinases (MMPs) or urokinase-type plasminogen activator (uPA), we introduced MMP-cleavage (PLGMTS) or uPA-cleavage (SGRS) sequences, respectively, immediately prior to the cleavage site for activation of fusion protein with remaining essential sequences for cell-fusion. The MMP-targeted SeV vector demonstrated syncytia formation in MMP expressing HT1080 cell line in vitro, and growth inhibition of HT1080 subcutaneous xenografts in vivo. The uPA-targeted one showed the same effects in uPA expressing PC-3 cell line. Severe apoptosis occurred in fused-cells. Thus, the vector selectively spreads to tumor cells in tumor-protease dependent manner and demonstrates the antitumor effects in solid tumors, indicating the value of selective targeting and killing of tumors by recombinant SeV technology.

2. SENDAI VIRUS VECTORS FOR GENE THERAPY

The cytoplasmic RNA vector would be promising for use in gene therapy and gene vaccines to large population of patients because of its important genotoxicity-free nature. Sendai virus (SeV), a murine parainfluenza virus belongs to a family *Paramyxoviridae*, infects and multiplies its genome copy in most mammalian cells. Its replication is strictly in cytoplasm and independent of nuclear functions of host cells (1) (Figure 1), so SeV-based vectors do not need to be concerned about the transformation of cells by integration of vector materials into the host chromosomes (2,3). These properties of the vector enable us to propose the new concepts, CYTOPLASMIC GENE THERAPY (4-8) and CYTOPLASMIC VACCINATION (9-11) with ribonucleoprotein (RNP)-based treatment.

In order to establish such SeV vector system, we have taken the strategy to generate the gene(s)-deleted or modified SeV vectors. Thus, we have succeeded in the recovery in high titers of fusion (F) gene-deleted (SeV/ΔF) (12), matrix (M) gene-deleted (SeV/ΔM) (13), hemagglutinin-neuraminidase (HN) gene-deleted (SeV/ΔHN), both M and F genes-deleted (SeV/ΔMΔF)

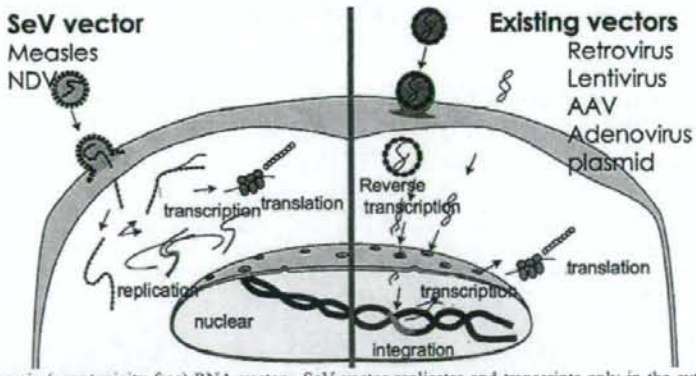

Figure 1. Cytoplasmic (genotoxicity free) RNA vector. SeV vector replicates and transcripts only in the cytoplasm, does not have DNA phase and never interacts with chromosomes. Therefore, this type vector is completely free from genotoxicity. For the broad range of use for gene therapy, such free from genotoxicity is very important. In contrast, existing vectors such as retrovirus and lentivirus are integrated into the chromosome to express the transgene. Even in the cases of adeno-associated virus, Adenovirus, and plasmid, some parts of them are known to integrate into the chromosome.

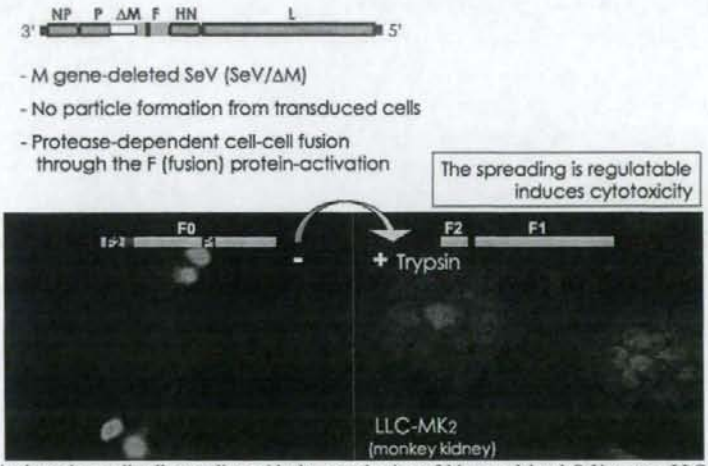

Figure 2. Trypsin-dependent cell-cell spreading with the transduction of M gene-deleted SeV vector. LLC-MK_2 cells were transduced with SeV/ΔM-GFP at an MOI of 0.01. Expression of GFP protein was detected 2 days after the transduction under fluorescence microscopy.

(14), all of the envelop-related genes-deleted (SeV/ΔMΔFΔHN) (15) SeV vectors by using the packaging cell lines which express respective proteins of those deleted gene(s). Among them, SeV/ΔM vector was selected in generating an oncolytic SeV vector targeting a solid tumor tissue.

3. CELL-CELL SPREADING OF M GENE-DELETED SeV VECTOR

M protein plays central role in virus assembly and budding. Therefore, deletion of the M gene from SeV almost completely abolished virus maturation into infectious particles from transduced cells and instead caused cell-to-cell vector spreading via membrane fusion and formed large syncytia (Figure 2)(13). The addition of trypsin to the culture medium was indispensable for the spreading in this case. That is, the significant matter for this vector is that the spreading is regulatable by the presence of selected F protein-activating proteases.

4. SeV VECTOR TROPISM : POSSIBLE CONVERSION TO ECM DEGRADATION ENZYME-DEPENDENT SPREADING

The initiation of infection with SeV involves virus attachment to the cell surface via HN protein and the

subsequent envelope-plasma membrane fusion mediated by F protein, resulting in entry of the viral genome into the cytoplasm. The F protein is synthesized as a biologically inactive precursor Fo and is converted to the active F1 (and F2) by posttranslational proteolytic processing. SeV displays a narrow spectrum of tissue tropism, its multiplication being restricted to the respiratory tract of mice, because the expression of the virus activating proteases, tryptase Clara in this case, are highly specific for particular tissues, such as respiratory tracts (16,17). These facts brought the assumption that the tropism of SeV could be converted by modifying the tryptic cleavage site of F0.

Invasive metastatic, that is malignant, tumors are known to express high levels of matrix metalloprotease (MMP) and plasminogen activators (uPA, tPA) (18,19). Since extracellular matrix (ECM) around tumor cells serves as a barrier and blocks tumor cell migration for infiltration and metastasis, tumors are considered to express ECM-degrading enzymes. We also confirmed the both types of proteases are over-expressed in many kinds of tumor cell lines such as prostate, esophagus and breast cancers. Therefore, we selected these proteases for the targeting, and the cleavage sequence of F protein of SeV/ΔM was changed to that susceptible to MMP or uPA.

5. ALTERATION OF F PROTEIN TO RENDER IT SUSCEPTIBLE TO MMP OR uPA

The sequence encoding the activation site was redesigned to acquire MMP- or uPA-dependent cleavage. There have been many reports of the sequences of cleavable substrates of MMP, and it is possible to apply them. However, the N-terminal region of F1 of paramyxovirus F protein is important for fusion activity and amino acid mutation in the region may lose its fusion activity (20). Therefore, we left the sequence of the N-terminal region of F1 unmodified. Although the N-terminal was not modified, insertion of the common cleavable substrate of MMP consisting of six residues adds three residues to the N-terminal of F1 upon cleavage by MMP (Figure 3), which possibly affects the fusion activity of F1, although F protein is cleaved by MMP. Accordingly, to design an F protein that is activated by MMP-dependent cleavage, it is necessary to consider the following two points: MMP substrate specificity and retention of fusion activity of F protein after cleavage.

We selected the substrate sequence MMP-subII (PLG-MTS) that was modified from the original substrate sequence of MMP2 and 9, PLGMWS (21), according to the consensus sequence in MMP9, Pro-X-X-Hy-(Ser/Thr), which has been clarified by phage display (22). The newly designed sequence, PLG-MTS, fulfills the two requirements, MMP substrate specificity and retention of fusion activity of F protein after cleavage. The uPA-subII (SGR-S) was also designed for the sequence that is susceptible to uPA. Thus, the two types of F gene-modified M gene-deleted SeV vectors, SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subI)ΔM-GFP, were constructed and recovered using M protein-expressing packaging cell line (13). For the propagation of SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subI)ΔM-GFP, collagenase type IV and trypsin were added to culture medium, respectively. Both vectors were successfully recovered in high titers (up to 10^8 cell infectious unit (CIU)/ml) without any concentration procedure (23).

6. CONFIRMATION OF ALTERATION OF TROPISM OF F-MODIFIED SeV/ΔM

Alteration of tropism, infection causing protease-dependent cell fusion, was confirmed using tumor cells. As shown in Figure 3, several ten-fold or more spread of SeV/F(MMP-subII)ΔM-GFP transduction was observed only in HT1080 cells (human sarcoma) that highly express MMPs[24] and not in the SW620 cells (human colon carcinoma) expressing MMPs at very low level. Spread of cell fusion type transduction of SeV/F(uPA-subI)ΔM-GFP was observed only in PC-3 cells (human prostate cancer) that highly express uPA and not in the LNCaP cells (human prostate cancer) expressing uPA at very low level (data not shown). Similar transduction experiments in other tumor cells were performed, and SeV/F(MMP-subII)ΔM-GFP caused syncytium formation in high MMP-expressing lines, U87MG, A172, and U251, and SeV/F(uPA-subI)ΔM-GFP caused syncytium formation in uPA-expressing LS174 cell line (data not shown).

7. ANTITUMOR EFFECT OF F-MODIFIED SeV/ΔM IN TUMOR-BEARING NUDE MICE

HT1080 tumor-bearing nude mice were prepared and the effect of F-modified SeV/ΔM was evaluated *in vivo*. To confirm spreading of the modified SeV vector, 5 $x10^7$ CIU of the vector was injected once into the subcutaneously transplanted HT1080 tumor. Two days later, the skin was externally irradiated and observed by fluorescence microscopy. When the parent vectors, wild type SeV carrying GFP gene (SeV-GFP) and SeV/ΔM-GFP, were administered, fluorescence was detected only around the administration site. In contrast, when SeV/(MMP-subII)ΔM-GFP was administered, GFP was spread over the tumor (Figure 4). In the magnified figure on the right, fluorescence was detected in each cell in animals treated with SeV-GFP and SeV/ΔM-GFP, while the cell boundaries were not clear in animals treated with SeV/F(MMP-subII)ΔM-GFP, strongly suggesting cell fusion.

Next, the size of the tumor was measured every other day. When saline and the parent vectors, SeV-GFP and SeV/ΔM-GFP, were administered, tumors grew rapidly. In contrast, when SeV/F(MMP-subII)ΔM-GFP was administered, tumor cell growth was markedly inhibited (Figure 4). As a part of elucidation of the tumor cell growth inhibition mechanism, TUNNEL staining was performed. Since each vector contained the GFP gene, staining with anti-GFP antibody was also performed to identify transduced cells. When SeV/ΔM-GFP was administered, transduction was localized to the administration site and there were only a few TUNNEL-positive cells. When SeV/F(MMP-subII)ΔM-GFP was

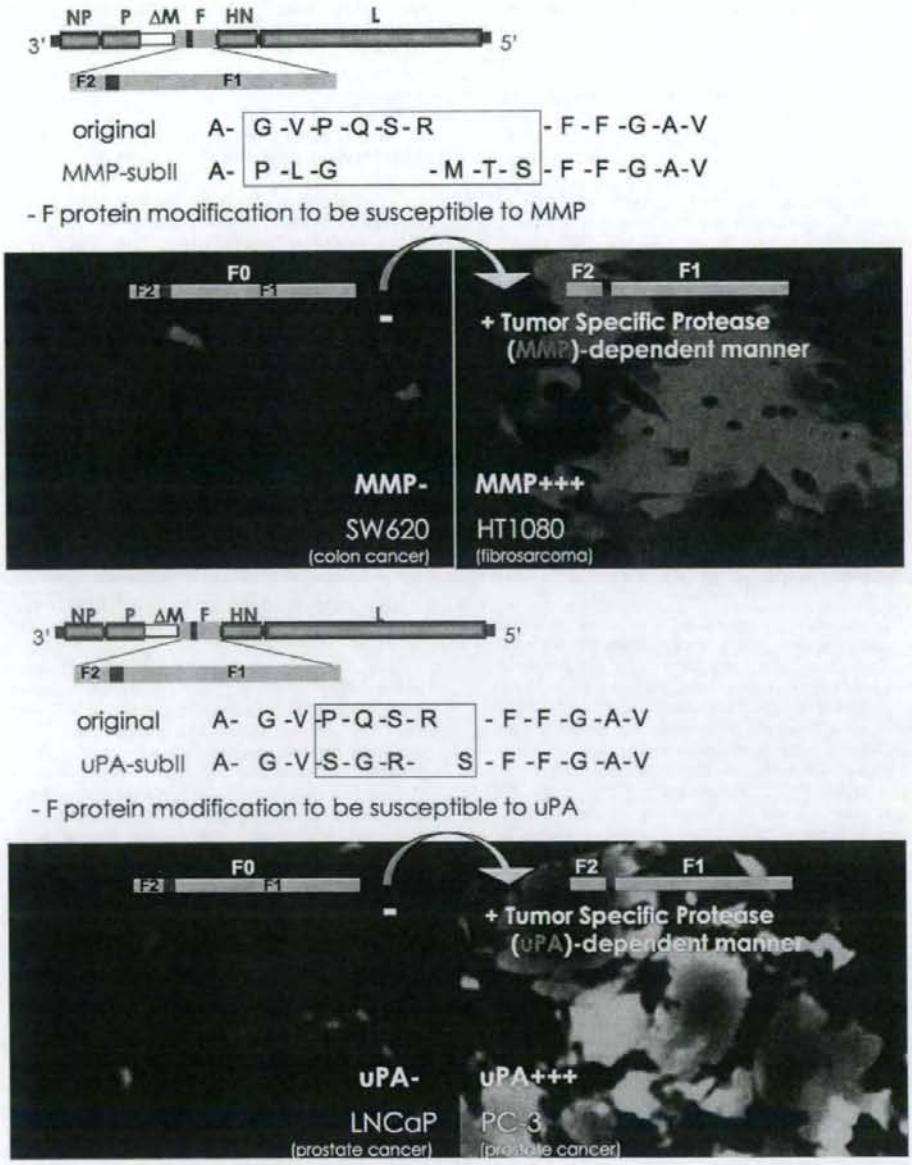

Figure 3. Cell-cell spreading of F-modified M gene-deleted SeV vector dependent on endogenous protease from tumor cells. MMP-expressing HT1080 and SW620 expressing low levels of MMP and uPA-expressing PC-3 and LNCaP expressing low levels of uPA were transduced with SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subII)ΔM-GFP, respectively, at an MOI of 0.02. Those cells were cultured for four days with the culture medium containing 1% fetal bovine serum, and cell fusion was observed.

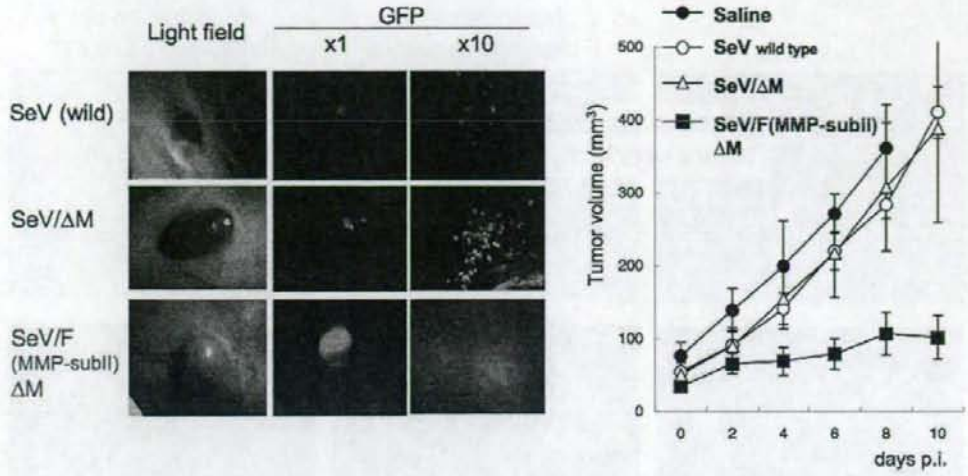

Figure 4. Antitumor effect of F-modified SeV/ΔM *in vivo*. Cell-cell spreading of F-modified SeV/ΔM. 5 x 10^7 CIU of viral vectors, SeV-GFP, SeV/ΔM-GFP, and SeV/F(MMP-subII)ΔM-GFP, were injected once directly into the subcutaneously transplanted HT1080 tumors. Two days later, GFP expression was observed from outside the body under fluorescence microscopy. F-modified SeV/ΔM inhibits the tumor growth. After the vector injection, tumor length, width and height were measured with time. Tumor volume was calculated with the formula (Volume = p/6 x abc :(length(a), width(b), height (c)) and expressed by an average (n=7). Statistical differences were observed between the group administered SeV/F(MMP-subII)ΔM-GFP and all other groups ($P < 0.05$, Student's t-test) at 8 and 10 days after injection.

administered, GFP protein flowed out from the fused cell region during preparation of sections and little GFP-positive cells were observed, but spread of GFP was observed in the tip outside there, and TUNNEL staining was positive over the cell fusion area (23), confirming that apoptosis was induced in a wide cell-cell spreading area after infection with SeV/F(MMP-subII)ΔM-GFP. Thus, the concept of the vector design of "F-modified SeV/ΔM" was proved.

8. FUSOGENIC ACTIVITY IMPROVEMENT OD F-MODIFIED SeV/ΔM VECTOR

Both SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subI)ΔM-GFP showed extensive spreading in the tumor and led to significant inhibition of the tumor growth in the mice (23). However, as we observed tumor re-growth in some cases even after the vector treatment, we try to increase the fusogenic and tumor-killing activity by further modifications of F protein. One modification is the partial deletion of the cytoplasmic domain of F protein (F-truncated), and the other one is the genetic fusion of the F and HN (F/HN fusion). In the latter design, F and HN genes were fused with a linker sequence. Thus generated SeV vectors, F-truncated and F/HN fusion vectors, showed drastically increased fusogenic activity (enhanced cell-fusion) even in the tumor cells that express in low level of tumor-specific proteases (data not shown). That is, both F-truncated and F/HN fusion vectors showed quite increased cell-fusion. Typical example is shown in Figure 5. The first modification of F cleavage sequence enables the tumor-specific spreading, and the second modification of the cytoplasmic domain efficiently increased the cell-killing activity of the vector. Such characteristic change was achieved only by the genetic modifications of SeV vector.

Another method to potentiate F-modified SeV/ΔM is expectative by integrating therapeutic gene(s) such as immune stimulating or suicide genes. Our results showed that the tropism-modified cell-cell spreading of F-modified SeV/ΔM exhibit a strong antitumor effect on tumor cells without carrying a therapeutic gene, and thus, the vectors may be promising for tumor therapy. Loading an effective therapeutic gene(s) on the vectors will be effective to further increase the therapeutic effect, and we are improving and evaluating the vectors in this direction.

9. CONCLUSION : A NOVEL ONCOLYTIC VECTOR BASED ON AN ENGINEERED VECTOR TROPISM

We generated a new type of oncolytic SeV/ΔM vector that is activated by MMPs or uPA but no longer by trypsin like proteases (23). The virus vector spreads selectively in MMP- or uPA-expressing tumor cells and those engrafted to mice, leading to rapid death of these cells and strong inhibition of their growth (Figure 6). Malignant tumor-specific cell fusion leading to efficient cell death was produced by transducing a novel genetically-engineered oncolytic Sendai virus vector.

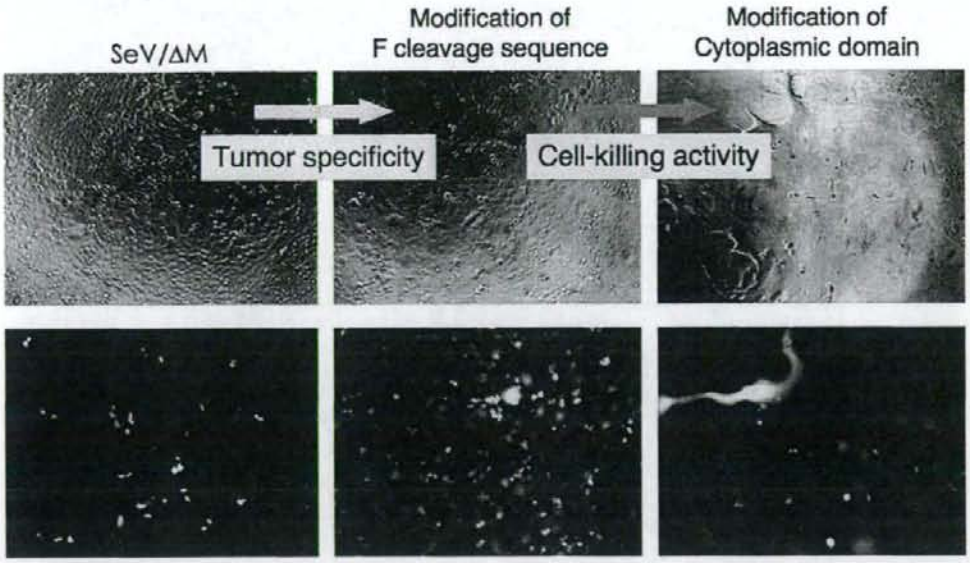

Figure 5. Typical example of synergistic effect of F-modification. The first modification of F cleavage sequence enables the tumor-specific spreading, and the second modification of the cytoplasmic domain efficiently increased the cell-killing activity of the vector.

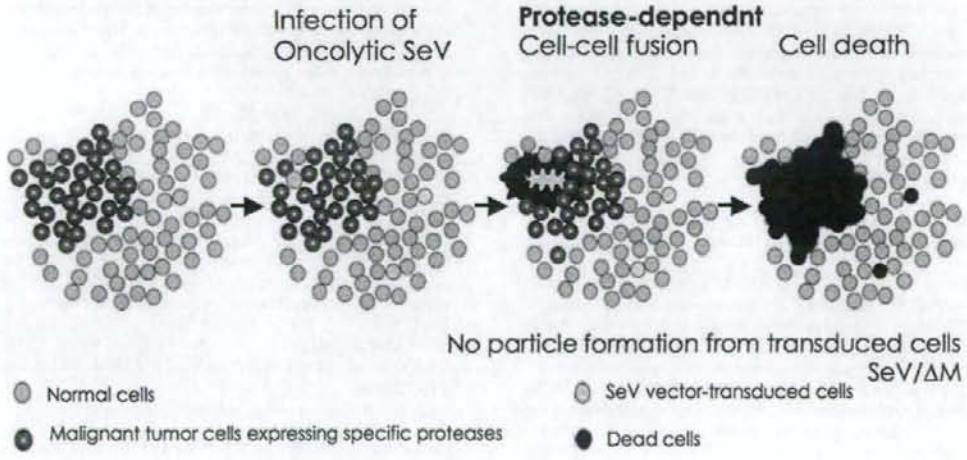

Figure 6. Oncolytic strategy using F-modified M gene-deleted SeV vector. The many types of malignant tumor cells are known to express the tumor-specific proteases such as MMP and uPA for their malignancy. F-modified SeV/ΔM transduces both tumor and normal cells, and spread only in the tumor cells through the tumor-specific protease-dependent manner and with cell-cell spreading, and lyses the malignant tumor cells. One of the important characteristics of this strategy is that there is no virion formation from transduced cells because SeV/ΔM is used, indicating shedding problem will be minimum.

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Abbreviations: Sev: Sendai virus; SeV/ΔM: matrix (M) gene-deleted SeV vector; MMPs: matrix metalloproteinases; uPA: urokinase-type plasminogen activator; CIU: cell infectious unit

Key Words: Sendai virus, oncolytic virotherapy, MMP, uPA, Review

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In vivo repopulation of cytoplasmically gene transferred hematopoietic cells by temperature-sensitive mutant of recombinant Sendai viral vector ☆

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Abstract

Recent clinical studies revealed 'proof of concept' of gene therapy targeting hematopoietic stem cells (HSCs) to treat hematopoietic disorders. However, vector integration-related adverse events of retroviral vectors have slowed progress in this field. As an initial step to overcoming this hurdle, we examined the potential of an improved cytoplasmic RNA vector, temperature-sensitive mutant non-transmissible recombinant Sendai virus (ts-rSeV/dF), for gene transfer to murine HSCs and progenitors. Both conventional vector and ts-rSeV/dF-GFP showed efficient gene transfer to T-lymphocyte-depleted syngeneic bone marrow cells (BMCs) (>85%), but only BMCs treated with ts-rSeV/dF-GFP but not with conventional vector efficiently repopulated in the recipient mice, associated with multilineage differentiation *in vitro* and *in vivo*. To our knowledge, this is the first demonstration of the *in vivo* reconstruction of hematopoietic series by cytoplasmically gene transferred BMCs, that warrants further investigation to realize this strategy in clinical settings.

Keywords: Sendai viral vector; Hematopoietic stem cells; Cytoplasmic gene therapy; Severe immunodeficiency syndrome; Bone marrow transplantation

Gene therapy targeting hematopoietic cells is a promising strategy for treating inherited hematopoietic disorders. Hematopoietic stem cells (HSCs) in particular are an ideal target, because they can reconstitute the entire hematopoietic system in a recipient during a lifetime. Recent clinical success in clinical gene therapy for X-linked SCID using a retroviral vector expressing the common γ-chain revealed the 'proof of concept' of the strategy and has given encouragement to physicians, patients, and scientists, but a serious adverse event, T-cell leukemia, which is related to the insertion of a pro-viral genome into the host chromosome

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mediated by retroviral vector, is an obstacle to developing the gene therapy strategies in this field [1–4].

We recently developed a novel viral vector for efficient gene transfer, recombinant Sendai virus (rSeV), and demonstrated highly efficient gene transfer to various organs *in vivo*, including airway epithelial cells, vascular tissue, skeletal muscle, and joint synovium [5–8] by using replication-competent additive-type rSeV; these findings are also consistent with a clinically available fusion gene-deleted non-transmissible rSeV (rSeV/dF) [9]. Since rSeVs uses a cytoplasmic transcription system, it can mediate gene transfer to a cytoplasmic location without the DNA phase [10] and therefore theoretically avoid a vector integration-related adverse event. In addition, there are technical advantages in the use of rSeVs for gene transfer to HSCs. First, vector particles can be easily concentrated to high titers, unlike retroviral vectors. Second, and most importantly, the modalities of target cell processing and viral transduction are technically non-demanding and feasible in clinical situations that require transduction into large numbers of target cells. Using replication-competent additive-type rSeV, we previously demonstrated a highly efficient gene transfer to HSCs derived from human cord blood preserving their functions to differentiate into the entire hematopoiesis series *in vitro* [11]; however, repopulation of HSCs has been unsuccessful when these cells were treated with additive-type rSeV as well as rSeV/dF (unpublished data). Therefore, we have again turned our focus to improving the rSeV system in view of reduced immunogenicity and cytotoxicity.

Recently, we newly developed temperature-sensitive mutant non-transmissible rSeV ($rSeV/dFP^{ts}M^{ts}HN^{ts}L^{ts}$, abbreviated as ts-rSeV/dF) [12,13], as an improved version of rSeV/dF. This new vector loses the expression of envelope-related genes M and HN at 37 °C [12,13], resulting in dramatically reduced cytotoxicity as well as innate immune responses in the murine lung [14].

In this study, therefore, we examined the potential of SeV as a cytoplasmic transcription system for gene transfer to HSCs and hematopoietic progenitors.

Materials and methods

Animals. Female 7-week-old C57BL/6 mice of Charles River grade were obtained from KBT Orientals Co., Ltd. (Tosu, Saga, Japan) and kept under specific pathogen-free and humane conditions. The GFP-TG mice (C57BL/6-TgN(act-EGFP) OsbC14-Y01-FM131) [15] were a gift from Prof. Okabe, Osaka University, Osaka, Japan. These mice were used for all experiments except the CTL assay. All animal experiments were carried out according to the protocols approved by the Institutional Committee for Animal Experiments and by the Institutional Committee for Recombinant DNA and Infectious Pathogen Experiments, Kyushu University. The experiments were carried out in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government.

rSeV vectors. rSeV/dF-GFP and ts-rSeV/dF-GFP were constructed as previously described [9,12,13]. A series of F-deficient SeV vectors were prepared by using recombinant LLC-MK_2cells carrying the F gene (LLC-MK_2/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used for the induction of F protein in LLC-MK_2/F7 cells (referred to as LLC-MK_2/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave UV irradiation, and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units [9].

Gene transfer to BM cells and BMT. BMCs from donor mice were flushed under sterile conditions with RPMI 1640 with 10% fetal calf serum from the medullary cavities of tibiae and femurs using a 23-gauge needle. Red blood cells were depleted using Lysing buffer (0.38% NH_4Cl, Tris-HCl, pH 7.65), and mature T-cells were depleted with anti-Thy1.2 monoclonal antibody (Sigma, St. Louis) plus selected rabbit complement (Cedalene, Ontario, Canada). Gene transfer was carried out by simply adding MOI = 10 of vector solution to the media at 37 °C for 1 h, and 2×10^5 (C57BL/6) BMCs were given by tail vein injection. For bone marrow ablation, the recipient mice were treated by whole-body γ-irradiation at lethal dose (10 Gy) 4–5 h before BMT. For blood cell count, PBCs were obtained via the tail vein, diluted, and counted by an automatic cytometer (CelltacaαMEK-6158 NIHON KOHDEN, Tokyo, Japan).

Flow cytometry analyses. At appropriate time points, PBCs or tissue samples were obtained and subjected to flow cytometry analysis. For immune cell subset analysis, cells from each organ were stained with CD3-APC/DX5-PE (for pan-T-cells), CD3-APC/CD4-PE (CD4/T-cells), CD3-APC/CD8-PE (CD8/T-cells), CD11b-PE/CD11c-APC (monocyte/macrophages), and B220-APC/IgM-PE (B-cells) (all antibodies were from BD Pharmingen, CA) by FACSCalibur (Becton Dickinson, CA). For *in vitro* analysis of each progenitor population, whole BMCs, 48 h after ts-rSeV/dF-GFP exposure, were stained with biotin-conjugated anti-lineage (lineage panel; BD pharmingen), anti-Sca-1-APC, anti-c-kit-PE antibodies and streptavidin for 30 min. Propidium iodide-positive dead cells were excluded. Data were evaluated using Cellquest™ (Becton Dickinson) or Flowjo (Tree Star Inc., CA) software.

Enrichment of HSCs and colony assay. Enrichment of HSC via FACS Aria (Becton Dickinson) was previously described [16,17]. Briefly, mononuclear cells were obtained from BMCs supplemented with Lympholyte-M (Cedalene), and $lineage^+$ cells were removed with sheep anti-rat IgG conjugated magnetic beads (Dynal, A.S.), and with anti-c-kit-APC antibody and anti-APC microbeads (Dynal, A.S.). The remaining cells were stained with biotin-conjugated anti-lineage-cocktail (BD Pharmingen), streptavidin-perCP-Cy5, anti-c-Kit-APC, and anti-Sca-1-PE, and were sorted in duplicate. For the colony assays, an enriched HSC population was cultured in MethoCult™ Media (M3434; StemCell Technologies, Vancouver, BC, Canada), and exposed to ts-rSeV/dF-GFP for 1 h. After washing, 1000 cells were cultured in 1 ml Methocult media per dish (35 mm) for 10–12 days. CFU-Mix colonies were observed and analysed by BZ-8000 and BZ-Analyzer (KYENECE, Osaka, Japan).

Statistical analysis. All data are expressed as the mean ± SD, and were analyzed by one-way ANOVA with Fisher's adjustment, with the exception of the data on animal survival. Survival was plotted using Kaplan–Meier curves, and statistical relevance was determined using a log-rank comparison. $P < 0.05$ was considered significant.

Results

Efficient engraftment and repopulation of cytoplasmically gene transferred murine bone marrow cells by ts-rSeV/dF in C57BL/6 syngeneic recipient mice

We first evaluated the gene transfer efficiency of recombinant ts-rSeV/dF-GFP compared to that with rSeV/dF-GFP mediated gene transfer to murine (7-week-old female C57BL/6) BMCs depleted with erythrocyte and mature T-cells. We here used whole BMCs instead of an enriched stem cell fraction to avoid the loss of experimental animals

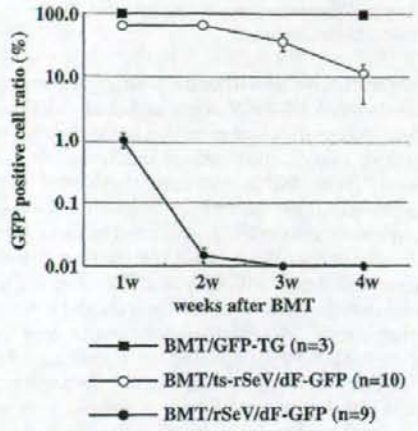
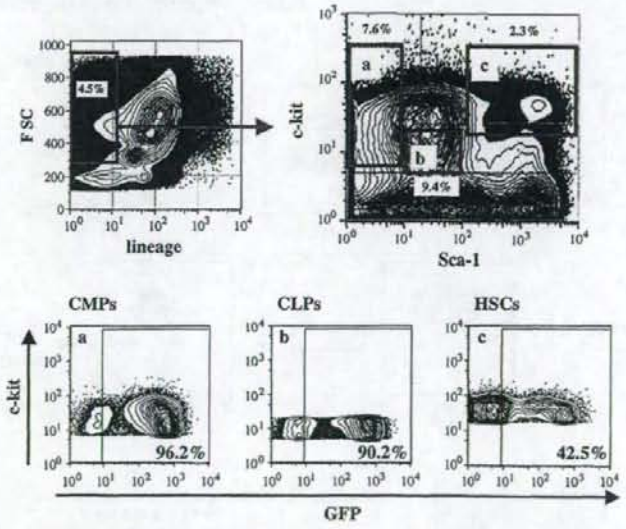


Fig. 1. Time course of GFP gene transferred cell ratio in peripheral blood cells (PBCs) after bone marrow transplantation (BMT). After bone marrow transplantation of 2×10^7 BMCs treated with ts-rSeV/dF-GFP (BMT/ts-rSeV/dF-GFP, $n = 10$) or conventional rSeV/dF-GFP (BMT/rSeV/dF-GFP, $n = 9$), the PBCs were collected via the tail vein of C57BL/6 recipient mice at each time point, and GFP expression was determined by FACS analysis. BMCs from GFP-transgenic mice ($n = 3$) were used as a positive control.

from unsuccessful reconstruction after lethal irradiation. BMCs were isolated from medullary cavities of tibiae and femurs. Erythrocytes were depleted in lysing buffer (0.38% NH_4Cl in Tris–HCl, pH 7.65) and mature T-lymphocytes were removed by anti-Thy1.2 antibody (mouse ascitis IgM monoclonal antibody) with rabbit complement; the removal of more than 99.9% of T-cells was constantly done and checked by FACS analysis. These BMCs were incubated with vectors at a multiplicity of infections 10 (MOI = 10) for only 1 h at 37 °C, and GFP expression was determined 48 h later, as described previously [15]. Both vectors constantly showed high gene transfer efficiency (>85%) to murine BMCs in repeated experiments (data not shown; similar data are shown in Fig. 2A).

Next, we engrafted these BMCs (2×10^7 cells/200 μl/head, 1 h after virus exposure) to lethally irradiated (γ-X ray 10 Gy) female C57BL/6 mice via the tail vein, and then the GFP expression in the cells from peripheral blood (PBCs) was monitored every week. BMCs from β-actin promoter-driven GFP transgenic mice (C57BL/6-TgN(act-EGFP)OsbC14-Y01-FM131) [15] were also used as a positive control of successful bone marrow transplantation (BMT).

As shown in Fig. 1, all recipients receiving BMCs from GFP transgenic mice (GFP-TG) demonstrated nearly



	CMPs	CLPs	HSCs	whole BMCs
GFP-positive ratio (%)	97.5 ± 1.3	77.3 ± 13.0	63.9 ± 21.4	91.7 ± 5.0

(n=3)

Fig. 2. *In vitro* gene transfer efficiencies of ts-rSeV/dF-GFP to populations of hematopoietic stem cells (HSC) and common myeloid and lymphoid progenitors (CMPs and CLPs). A typical result of the FACS analyses is given as panels, and the summary of triplicate experiments is demonstrated in the bottom table. Each enriched population was gated by $Lin^-/c\text{-}Kit^+/Sca\text{-}1^-$ for CMPs, $Lin^-/c\text{-}Kit^{low}/Sca\text{-}1^{low}$ for CLPs, and $Lin^-/c\text{-}Kit^{high}/Sca\text{-}1^{high}$ for HSCs. Data are expressed as mean ± SD.

100% GFP-positive cells in PBCs, indicating successful engraftment of, and almost total replacement by, donor cells under this experimental condition. In contrast, PBCs from mice of BMT/rSeV/dF-GFP showed very low repopulation of GFP-positive cells (~1%) at 1 week, and were rapidly eliminated during the experimental course, a representative result of our previous experiments (unpublished data). Importantly, recipient mice with BMT/ts-rSeV/dF-GFP exhibited a relatively high repopulation of PBCs (50–70%) 1 week after BMT; the ratio gradually declined to ~10% at 4 weeks.

To our knowledge, this is the first demonstration of the efficient repopulation of donor cells that were transferred by cytoplasmic RNA vector.

Efficient ts-rSeV/dF-mediated gene transfer to HSCs and progenitor populations

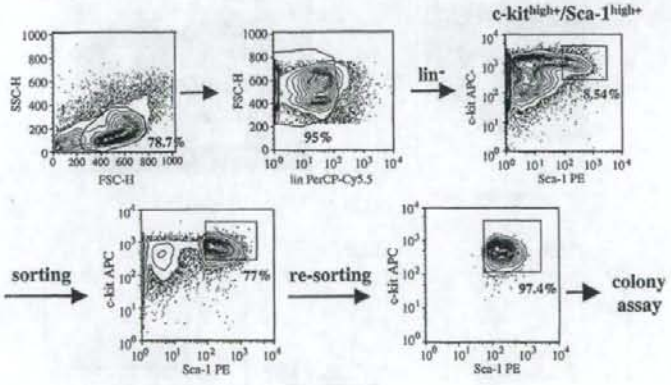
We examined the gene transfer efficiencies of ts-rSeV/dF-GFP to common myeloid progenitors (CMPs: enriched in Lin$^-$Sca-1$^-$c-Kit$^+$) [16], common lymphoid progenitors (CLPs: enriched in Lin$^-$Sca-1^{low}c-Kit^{low}) [17], and HSCs (enriched in Lin$^-$Sca-1^{high}c-Kit^{high}) by flow-cytometric analysis (Fig. 2). Constant high gene transfer, over 90%, was seen in T-cell-depleted BMCs by ts-rSeV/dF-GFP, and relatively high gene transfer also occurred in these populations (mean = 97.5% of CMPs, 77.3% of CLPs, and 63.9% in HSCs, $n = 3$).

Multilineage differentiation of cytoplasmically gene transferred HSCs in vitro

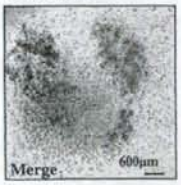
Subsequently, we investigated whether gene transferred HSCs by ts-rSeV/dF-GFP might sufficiently differentiate to various types of colonies by a colony-forming assay. As shown in Fig. 3, 1000 cells of highly enriched HSCs were sorted from BMCs twice and transfected with the GFP gene, and then sparsely cultured for 12 days [16]. Colony-forming units (CFU) were then assessed by fluorescence microscope. We counted the number of multilineage mixed colonies (CFU-Mix) as CFU from single cells with pluripotency. The result in Fig. 3 shows, a reduction in the number of CFU-Mix was not seen in cells treated with ts-rSeV/dF-GFP, suggesting no significant effect of gene transduction to cell differentiation. In addition, the ratio of GFP-positive colonies was almost comparable to gene transfer efficiency to HSCs, suggesting no significant effect on cell growth by ts-rSeV/dF-GFP, at least *in vitro*.

In vivo repopulation and multilineage differentiation of cytoplasmically gene transferred BMCs

Using six animals 5 weeks after BMT with BMC treated ts-rSeV/dF-GFP, we next examined the distribution, repopulation and differentiation of GFP-positive cells in typical lymphoid organs (thymus, spleen, bone marrow)



CFU-MIX



	HSCs (1,000 cells)	
	naïve	ts-rSeV/dF-GFP
number of CFU-MIX	65.3 ± 10.0	88.0 ± 11.1
GFP positive (%)	0.0	63.6 ± 8.6

Fig. 3. *In vitro* colony-formation assay. Cells were sorted twice (FACS panels) in order to enrich HSC fraction, a typical photomicrograph of GFP-positive CFU-Mix (bottom left), suggesting multilineage differentiation, and the table summary of the quantitative data (bottom right) are given. Data are expressed as mean ± SD.

as well as PBCs by flow cytometry. As shown in Fig. 4A, these six mice showed a representatively high GFP-positive ratio (55.9 ± 5.9%, $n = 6$) in PBCs, and the thymus, spleen, and bone marrow of these animals also exhibited a mean = 47.7%, 79.3% and 17.1% of GFP-positive cells, respectively, confirming the repopulation of transplanted cells in each lymphoid organ. A simultaneous experiment using the BMT/SeV/dF-GFP of three animals did not show any GFP-positive cells at all 5 weeks after BMT (data not shown).

Subsequently, we investigated the GFP-positive ratio of each cell type in the spleen and bone marrow by FACS analyses. Cells from each organ were determined by antibodies specific for the following surface-markers: NK cells = $CD3^-/DX5^+$, NKT cells = $CD3^+/DX5^+$, pan-T-cells = $CD3^+/DX5^-$, CD4/T-cells = $CD3^+/CD4^+$, CD8/T-cells = $CD3^+/CD8^+$, dendritic cells (DCs) = $CD11b^+/CD11c^+$, monocytes/macrophages (Mφ) = $CD11b^+/CD11c^-$, B-cells = $B220^+/IgM^+$. As shown in Fig. 4B, even though BMCs were used after complete depletion of T-cells, all these subsets contained GFP-positive cells at various ratios including T-cell lineages, suggesting that these GFP-positive cells were from HSCs and progenitors. Interestingly, DCs and Mφs, both from CMPs [16], included a relatively large number of GFP-positive cells in both the spleen and bone marrow, probably reflecting the high gene transfer to CMPs (Fig. 2).

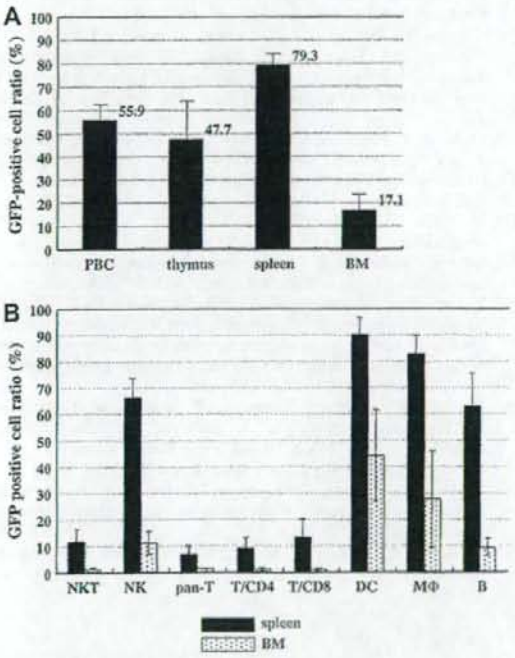


Fig. 4. *In vivo* repopulation and multilineage differentiation of BMCs transfected by ts-rSeV/dF-GFP after BMT. Data are expressed as mean ± SD. (A) Repopulation efficiency of ts-rSeV/dF-GFP treated BMCs in typical lymphoid organs at 4 weeks after BMT *in vivo*. (B) FACS analysis of ration of GFP expression in various cellular subsets in spleen and bone marrow of recipient mice at 5 weeks after BMT. Each cell subset was determined as follows: NK = $CD3^-/DX5^+$, NKT = $CD3^+/DX5^+$, pan-T-lymphocytes = $CD3^+/DX5^-$, CD4/T-cells = $CD3^+/CD4^+$, CD8/T-cells = $CD3^+/CD8^+$, DCs = $CD11b^+/CD11c^+$, Mφ = $CD11b^+/CD11c^-$, and B-lymphocytes = $B220^+/IgM^+$.

These findings, therefore, strongly suggested that BMCs transfected with ts-rSeV/dF-GFP were capable of differentiating the whole hematopoietic series *in vivo*.

Discussion

Physicians and scientists in the gene therapy community have been greatly encouraged by the dramatic outcome of clinical studies to treat X-linked SCID conducted in France [1]. However, this trial has also raised safety concerns about vector integration to the host chromosomes.

Using vectors that express the transgene in cellular cytoplasm is one possible way of addressing safety concerns; in the present study, therefore, we attempted to seek the potential of a cytoplasmic RNA vector, rSeV, as a new modality for gene therapy to treat hematopoietic disorders. To the best of our knowledge, this is the first study to make use of a newly developed version of the cytoplasmic RNA vector, ts-rSeV/dF, to show the high gene transfer efficiency to hematopoietic stem/progenitor populations and their successful repopulation associated with differentiation to multiple lineages of hematopoietic series.

Apparently, an advance seen in this study is that the repopulation of cytoplasmically gene transferred BMCs could be done by using an improved design of rSeV/dF, a temperature-sensitive mutant ts-rSeV/dF, but not of conventional rSeV/dF (and prototype additive rSeV, unpublished data). However, there is no definitive explanation as to why ts-rSeV/dF realized an efficient repopulation. As previously demonstrated, ts-rSeV/dF shows a reduced cytotoxic effect on some types of cells, but this is not a likely explanation for its ability to realize BMC repopulation because our *in vitro* study using murine HSCs, which is shown in Fig. 3, along with a previous study using human HSCs [11], could not demonstrate the apparent cytotoxicity and the disturbance of their differentiation caused by any type of rSeV vector. It is therefore possible that the reduced expression of HN protein due to the insertion of temperature-sensitive mutations enabled BMCs to repopulate in the recipient.

The findings obtained in this study suggested the possible application of 'cytoplasmic gene therapy' for hematopoietic disorders; however, our preliminary study assessing long-term engraftment also revealed the potential limitations of the current construct *in vivo* in view of its clinical application. In other words, more than 40% of recipient mice showing efficient hematopoietic gene transfer were dead in later phase due to severe pancytopenia (Yoshida K and Yonemitsu Y, unpublished data), therefore, more studies and biological information are thus

needed to realize cytoplasmic gene therapy for hematopietic diseases.

In summary, we here demonstrated the successful repopulation and reconstruction of hematopoietic series by cytoplasmically gene transferred BMCs using newly developed ts-rSeV/dF. This study is, to the best of our knowledge, the first report reconstituting whole hematopoietic series using a bone marrow gene transferred by cytoplasmic transcription, but the transient repopulation is an issue that must be resolved. Since 'cytoplasmic gene therapy' is an attractive and challenging strategy for physicians and scientists in this field, further studies are needed to overcome the current problems.

Competing interest statement

Dr. Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

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Neurospheres From Human Adipose Tissue Transplanted Into Cultured Mouse Embryos can Contribute to Craniofacial Morphogenesis: A Preliminary Report

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Adipose-derived stromal cells (ASCs) are one of the most promising stem cell populations that differentiate into the mesodermal as well as neural lineages in vitro. In this study, we examined the neural differentiating potential of human ASCs by a neurosphere culture method. Neurospheres derived from human ASCs expressed *Nestin* and *Musashi-1* genes, which are marker genes for neural stem cells. When these cells were labeled with *green fluorescent protein* gene transfection by Sendai virus vector and transplanted into the head region of mouse embryos using a whole embryo culture system, these cells were incorporated into the craniofacial development. Some transplanted cells appeared to migrate along the second branchial arches, implicating some similarity to the cranial neural crest cells. Although preliminary, our results support an idea that ASC-derived neurospheres have properties of neural progenitors in vitro and in vivo.

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Adipose-derived stromal cells (ASCs) were originally reported as a subtype of the mesenchymal stem cells (MSCs) isolated from liposuction aspirates differentiating into the mesodermal tissues such as bone, cartilage, and adipose tissue.[1] Characterization of ASCs has recently been studied world wide by many groups, including ours.[2–4] ASCs are now regarded as one of the most promising adult stem cells for regenerative medicine because they can be harvested safely by liposuction, and a good yield can be anticipated.

Advances in stem cell research have resulted in a novel concept of cellular plasticity of differentiation beyond the boundary of germ layers. MSCs and ASCs can differentiate into neuronal (and thus ectodermal) derivatives, although these cells are primarily mesodermal.[5,6] Recent reports further indicate that stem cells with neural characteristics can be isolated from the mesodermal tissues such as the dermis and the heart.[7–9] In these cases, the cells were harvested by a neurosphere method, which was originally developed as a culture method of isolating spheres of neural stem cells from the embryonic and adult brain.[10–12] However, it is to be elucidated whether this method is also applicable for obtaining neural stem cells from the adipose tissue or ASCs.

In this study, the neurospheres expressing neural stem cell marker genes were obtained from human ASCs. We also transplanted these cells into mouse embryos cultured in vitro to examine whether these cells behave similar to neuronal cells in vivo.

Fig 1 Neurosphere formation of adipose-derived stromal cells cultured in neurosphere medium for 5 days (A), 6 days (B), and 7 days (C) (magnification ×200).

Materials and Methods

Isolation of Human ASCs and Neurosphere Cell Culture

ASCs were isolated from the human liposuction aspirates as reported previously.[3] The suctioned fat was digested with 0.075% collagenase in phosphate-buffered saline (PBS) for 30 minutes on a shaker at 37°C. Mature adipocytes and connective tissues were eliminated by centrifugation. Blood cells were also eliminated by treating with erythrocyte lysis buffer, and resultant ASC pellets were obtained. Alternatively, ASCs could be isolated from the fluid portions of liposuction aspirates by treating with erythrocyte lysis buffer and density gradient centrifugation with Ficoll (GE Healthcare Bio-sciences, Piscataway, NJ).

Neurosphere culture was performed as described previously with slight modification.[12] Freshly isolated ASCs were plated at a density of 2×10^7 cells in 10 cm uncoated dishes and cultured in the neurosphere culture medium at 37°C in an atmosphere of 5% CO_2 in humid air. The neurosphere medium was a Dulbecco's Modified Eagle's Medium/F12 (1:1)-based medium supplemented with human recombinant epidermal growth factor (EGF, 20 ng/mL, PeproTech, Rocky Hill, NJ), human recombinant basic fibroblast growth factor (FGF, 20 ng/mL, Kaken Pharmaceutical, Tokyo, Japan), 2% B27 supplement (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Half of the medium was replaced with a fresh medium on the fourth to fifth day, and the passaging was performed on the eighth day.

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Total mRNA was extracted using RNeasy-mini kit (Qiagen, Hilden, Germany) from the neurosphere cells derived from passage one ASCs, which were precultured in the normal medium containing M199 medium and 10% fetal bovine serum (FBS). The preculturing was necessary for reducing the contamination of blood cells. Control mRNA was also extracted from the passage one undifferentiated ASCs cultured in M199 plus 10% FBS.

Expressions of undifferentiated neural stem cell marker genes *Nestin* and *Musashi-1*[13] and adipogenic differentiation marker *Leptin* were analyzed by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) using an ABI PRISM 7700

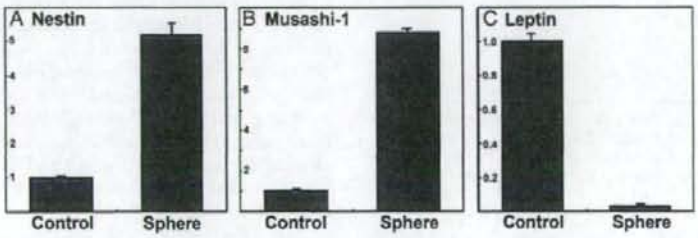

Fig 2 Quantitative real-time reverse-transcription polymerase chain reaction analysis of gene expressions of neural stem cell marker *Nestin* (A), *Musashi-1* (B), and adipogenic differentiation marker *Leptin* (C). Control = undifferentiated adipose-derived stromal cells; Sphere = neurospheres. Assays were performed in triplicate, and standard errors are indicated by error bars.

(Applied Biosystems, Foster City, CA), as reported previously. Gene expression of the target sequence was normalized to that of the housekeeping gene *β-actin*. Transcript level in the control (undifferentiated ASC) group was arbitrarily expressed as 1. TaqMan chemistry and assay by design primers and probe sets were used for human *Nestin*, *Musashi-1*, *Leptin*, and *β-actin*. All the primers and probe sets were purchased from Applied Biosystems.

Mouse Whole Embryo Culture and Transplantation of Neurosphere-Like Cells

Neurospheres derived from human ASCs were transfected with *green fluorescent protein* (*GFP*) gene using the Sendai virus vector (DNAVEC Corp. Tsukuba, Japan), as reported previously.[14,15] The original vector SeV/ΔF lacks the F gene encoding fusion protein necessary for penetration of ribonucleoprotein complex into infected cells, and is thus nontransmissible and nonpathogenic.[14] The modified SeV/ΔF vector has additional mutations to reduce its cytotoxicity,[15] and we used the modified vector in the present study. Neurospheres were incubated for 1 hour in the medium with the modified SeV/ΔF carrying the *GFP* gene at a multiplicity of infection of 250 and rinsed with PBS.

Mouse whole embryo culture was performed as reported previously.[16–19] Nine mouse embryos at embryonic day (E) 8 were dissected out without damaging yolk sacs, and the GFP-transfected neurosphere cells were transplanted using micropipettes into the head region of the embryos. The embryos were cultured for approximately 40 hours, and presence or absence of the GFP-positive transplanted cells was investigated under a fluorescent dissecting microscope. All experimental procedures were performed at the University of Tokyo under approval of the ethical committee.

Results

We first cultured human ASCs in the neurosphere culture medium containing EGF and basic FGF without serum. On the third day of culture of freshly prepared ASCs, the floating ASCs started to form small masses (data not shown). The neurosphere-like cellular aggregates were clearly observed on the fifth day (Fig 1A). The number and the size of the spheres became increasingly larger within the next 2 days (Fig 1, B and C). The passaging was performed on the eighth day when the spheres were dissociated and resuspended in the new medium. The spheroids were newly formed after culturing again for several days (data not shown), suggesting self renewal of the neurosphere cells.

To characterize the neurosphere cells, we next examined expressions of neural stem cell marker *Nestin* and *Musashi-1* genes and adipocyte marker *Leptin* by quantitative real-time RT-PCR. Expressions of *Nestin* and *Musashi-1* genes were remarkably upregulated in the neurosphere cells compared with the control ASCs without culturing in the neurosphere medium (Fig 2, A and B), suggesting characteristics of neural progenitor. Conversely, *Leptin* expression

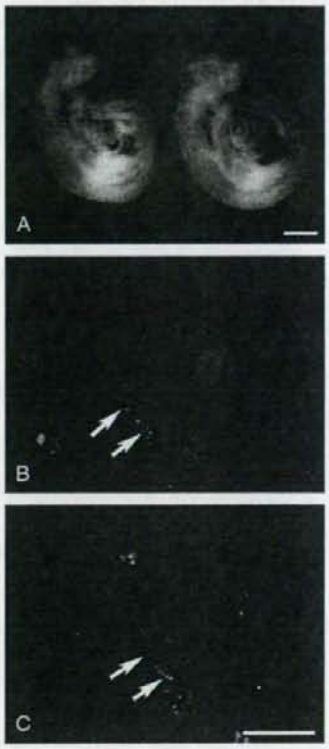

Fig 3 Neural crest-like migrations of green fluorescent protein (GFP)-transfected, adipose-derived stromal cell-derived neurospheres grafted into mouse embryo cultured in vitro. (A) Appearances of mouse embryos cultured for 40 hours from embryonic day 8. (B and C) Fluorescent views of embryos. GFP-positive neurosphere cells were arranged in a row (arrows), suggesting their migration along second branchial arch. Bars = 500 μm.

was dramatically reduced in the neurospheres (Fig 2C), indicating loss of adipogenic potential.

To investigate functions of the neurosphere cells in vivo, we labeled these cells by the modified Sendai virus vector carrying the *GFP* gene and transplanted them into the head region of the E8 mouse embryos. After the embryos were cultured for approximately 40 hours in vitro, the transplanted GFP-positive cells were clearly observed and appeared viable in only two embryos of the nine cultured embryos. The GFP-positive cells were incorporated into the craniofacial region as well as the heart and the trunk in these two embryos (Fig 3). Notably, the transplanted cells were arranged in a row along the second branchial arch (arrows in Fig 3, B and C) in a quite similar pattern to the neural crest cells migrating within the second branchial arch. Although not confirmatory, this result suggests a intriguing possibility that neurosphere cells derived from ASCs have neural crest-like properties.

Discussion

ASCs are probably one of the most well-known stem cells among plastic surgeons. ASCs were originally reported by Zuk et al[1] from the clinical samples of liposuction aspirates. According to their broad spectrum of differentiation potential, ASCs have been used in a number of preclinical animal studies of in vivo regeneration of a various tissues such as bone,[20,21] cartilage,[22] vessels,[4,23,24] soft tissue,[4] bone marrow,[25] and so on. Even a clinical case was reported, in which a calvarial defect was repaired by ASCs combined with scaffold.[26] Several groups reported neural differentiation of ASCs in vitro,[5,6,27] and Kang et al[28] reported functional recovery of the rat model with cerebral infarction after ASC transplantation in vivo.

The neurosphere method was originally reported by Reynolds et al[10,11] and is one of the most frequently used methods for isolating neural stem cells from the embryo or from the adult central nervous systems. However, this method has not yet been applied for obtaining neural stem cells from adipose tissue or the ASC population. In this preliminary study, we obtained neurospheres from the ASCs in human liposuction aspirates. Proliferation of these cells was quite rapid, possibly faster than other neurospheres from various tissue origins such as the dermis and the heart,[7–9] suggesting advantages of ASCs as a origin of neuronal progenitors for regenerative medicine. These neurosphere cells expressed *Nestin* and *Musashi-1*, marker genes for neural stem cells, probably reflecting their tendency of differentiating into neuronal progenitors. This view is further supported by inhibition of their expression of *Leptin*, a marker for adipogenic differentiation and maturation.

Do the ASC-derived neurosphere cells behave as neuronal progenitors in vivo? Our attempt of grafting these cells into the cultured mouse embryo revealed that some of the cells migrate along the second branchial arch and contribute to craniofacial morphogenesis. Their migratory pattern is quite similar to that of cranial neural crest cells, as we reported previously.[16] The neural crest cells are an embryonic cellular population characterized by extensive migration and a unique repertoire of differentiation.[29] The neural crest cells are often regarded as stem or progenitor cells for peripheral neurons and Schwann cells, and the craniofacial skeletal mesenchyme is also neural-crest derived.[17,19,29,30] Recent studies indicate that the neural crest stem cells can be harvested from the seemingly "mesodermal" tissues of adult animals, such as the dermis,[7] the hair follicular dermal papilla,[8] or the heart,[9] by means of the neurosphere method, implicating that it is also the case in the adipose tissue. Because our data are preliminary and we have a small sample size, further studies such as those with detailed expression analysis of neural/neural crest marker genes and large-scale in vivo grafting are necessary to confirm this interesting idea.

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