Expert Opinion

Applications of Hemagglutinating Virus of Japan in therapeutic delivery systems

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Background: Efficient and minimally invasive vector systems appear to be the most appropriate for both gene therapy and drug delivery. Numerous viral and non-viral vectors have been developed. Each vector has its own advantages and limitations. *Objective*: New vectors have been required for overcoming the limitations of both viral vectors and non-viral vectors. The idea is to compensate the limitations of one vector system with the advantages of another. This can enable efficient drug delivery and gene expression, while reducing the cytotoxicity of the various vector components. *Methods*: The Hemagglutinating Virus of Japan (HVJ; Sendai virus) envelope vector was developed using fusion-competent inactivated HVJ particle. Briefly, the viral genome was destroyed by UV-irradiation, and the inactivated viral particles were mixed with plasmid DNA, proteins or siRNA in the presence of mild detergent. After centrifugation, those molecules were incorporated into the viral envelope. *Conclusion*: The HVJ-E vector can efficiently deliver therapeutic molecules such as genes, siRNA, decoy oligonucleotides, proteins, and anti-cancer drugs to various tissues *in vivo*. It is also available for high throughput screening of therapeutic genes. A number of anti-cancer effects of HVJ-E have been identified, specifically activation of both T cell immunity and non-T cell immunity against cancers. Furthermore, a tissue-targeting HVJ-E vector has been constructed using a unique approach for virus engineering and by conjugation with biocompatible polymers. Therefore, the HVJ-E vector is expected to enable effective cancer therapy through the delivery of molecular therapy and through its immunotherapeutic effects.

Keywords: drug delivery, fusion, gene therapy, Hemagglutinating Virus of Japan, immunotherapy

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1. Introduction

Efficient and minimally invasive vector systems appear to be the most appropriate for both gene therapy and drug delivery. Numerous viral and non-viral (synthetic) methods for gene transfer and drug delivery have been developed [1-5]. Each viral and synthetic system has its own set of advantages and limitations.

In general, viral methods are more efficient than non-viral methods for gene delivery to cells. The introduction of therapeutic molecules into the cytoplasm poses a barrier to non-viral delivery systems [6-8]. The nuclear import of therapeutic DNA is another challenge that needs to be overcome in order to deliver effective gene therapy [8]. Researchers of non-viral vectors have developed various techniques to solve these problems. For example, to escape from the endosome, pH-sensitive liposomes with dioleoylphosphatidylethanolamine [9] or DMRIE-C (a 1:1 mixture of *N*-[1-(2,3-dimyristyloxy)propyl]-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide and cholesterol) [10] are able to fuse with the endosomal

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membrane. Polyethylenimine has been used for drug delivery because it can disrupt the endosomal membrane by a proton-sponge effect [11]. However, viral vectors readily deliver genes to the cytoplasm due to components that can fuse with the cell membrane or disrupt endosomes [12,13]. To enhance gene expression following the nuclear import of DNA using non-viral vectors, some recommend the conjugation of a nuclear localizing signal peptide derived from SV40, and others use a non-classical nuclear localizing signal of heterogeneous nuclear ribonucleoprotein [14-16]. A recent paper suggests that the nuclear migration of plasmid DNA might be sequence-dependent [17] because sequence-specific binding with transcription factors facilitates the nuclear migration of exogenous DNA [18]. Although the nuclear import of therapeutic DNA is not feasible using retrovirus vectors, it is easily achieved with adenovirus and lentivirus vectors [19]. Thus, some viral vectors are naturally equipped with the functional apparatus required for efficient gene delivery to cells. To impart this capability to non-viral vectors, extensive modifications are required. Although this approach for vector development is attractive from the standpoint that a non-viral vector can be used, the requirement for extensive modifications of the vector system often prohibits mass production of clinical grade vectors.

On the other hand, viral vectors do not permit the delivery of agents such as proteins, synthetic oligonucleotides and small drug molecules. Non-viral vectors have been widely used for the delivery of these types of therapeutic agents [20]. Furthermore, safety is a concern with viral vectors due to the concomitant introduction of genetic elements from parent viruses, as well as leaky expression of viral genes, immunogenicity and changes in the host genome structure, whereas non-viral vectors are less toxic and less immunogenic [5,6].

To overcome the limitations of each vector system, chimeric viral and non-viral vectors have been developed. The idea is to compensate the limitations of one vector system with the advantages of another. This can enable efficient drug delivery and gene expression, while reducing the cytotoxicity of the various vector components.

In these chimeric vector systems, viral fusion activity has been used [8]. To enhance the gene transfer efficiency of one receptor-mediated gene delivery system, a fusion peptide derived from influenza virus hemagglutinin was combined with a poly L-lysine–DNA complex [21]. Fusion-competent viral liposomes with envelopes derived from the Hemagglutinating Virus of Japan (HVJ; Sendai virus) were also constructed [22,23].

HVJ was the first virus isolated in Japan in the early 1950s. It is a mouse parainfluenza virus belonging to the paramixoviridae genus. It is 150 – 600 nm in diameter, and contains negative strand RNA (15,383 bases) inside its viral envelope (**Figure 1A**). Two glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) protein, are present on the viral envelope [24]. The first step of infection involves the binding of HN to its acetylated sialic acid receptor. HN has neuraminidase activity and is thought to ligate carbohydrate chains on the viral envelope (**Figure 1B**). Following this, the hydrophobic region of F protein, which is thought to function as a fusion peptide, invades into the lipid bilayer through its association with lipid molecules, such as cholesterol. F protein is first produced as inactive F0 protein when the virus is excreted from cells, after which it is cleaved to F1 and F2 by protease (**Figure 1C**), such as tryptase Clara in rat bronchial epithelium [25] and factor X in chorioallantoic fluid [26]. Experimentally, mild protease treatment such as trypsin (0.05%) is required to obtain fusion-competent virus [24]. The fusion of HVJ occurs at a neutral pH [24], as with herpes virus and human immunodeficiency virus, whereas fusion occurs only at acidic pH for a number of other viruses, such as influenza virus [27], vesicular stomatitis virus [28] and Semliki forest virus [29]. Therefore, endocytotic uptake is not necessary for the fusion of HVJ. Following fusion-mediated gene transfer, therapeutic molecules are rescued from degradation before reaching the cytoplasm. The viral nucleocapsid containing the RNA genome is directly introduced into the cytoplasm. At the time of membrane fusion, depolymerization of the actin filament is transiently facilitated, which is important for the nuclear targeting of exogenous DNA as described below [30]. After introduction of the viral genome, abundant viral proteins are produced in infected cells, which are toxic to most cells. Moreover, viral proteins, especially nucleocapsid protein, are highly immunogenic [31]. Therefore, the viral genome was destroyed by UV irradiation [24] and only the viral envelope used. Although there are several strains of HVJ, VR-105 parainfluenza1 Sendai/52 – one of the commonly used Sendai virus strains – was used after inactivation by UV irradiation, as the fusion activity of this strain is more pronounced than other strains of HVJ, with minimal toxicity, both in cultured cells and in animals.

Based on these properties, inactivated HVJ is often used as a drug delivery vector. A more direct and practical approach involves the conversion of this fusigenic virion to a carrier of exogenous genes. To simplify and enhance the effectiveness of this gene delivery system, technology has been developed that enables the incorporation of plasmid DNA into the inactivated HVJ particle itself without liposomes [32]. HVJ was first inactivated with β-propiolactone (0.0075% – 0.001%) or by UV irradiation (99 – 198 mjoule/cm^2; **Figure 2A**), and then purified by ion-exchange column chromatography and gel filtration. The diameter of the HVJ envelope (HVJ-E) was 220 nm and the zeta potential was ~ -5 mV. The amount and molar ratios of F and HN fusion proteins within the HVJ-E vector were similar to that of native HVJ [32]. Therefore, the fusion activity of the HVJ-E vector was as robust as wild-type HVJ.

Exogenous plasmid DNA was incorporated into inactivated HVJ by treatment with mild detergent and

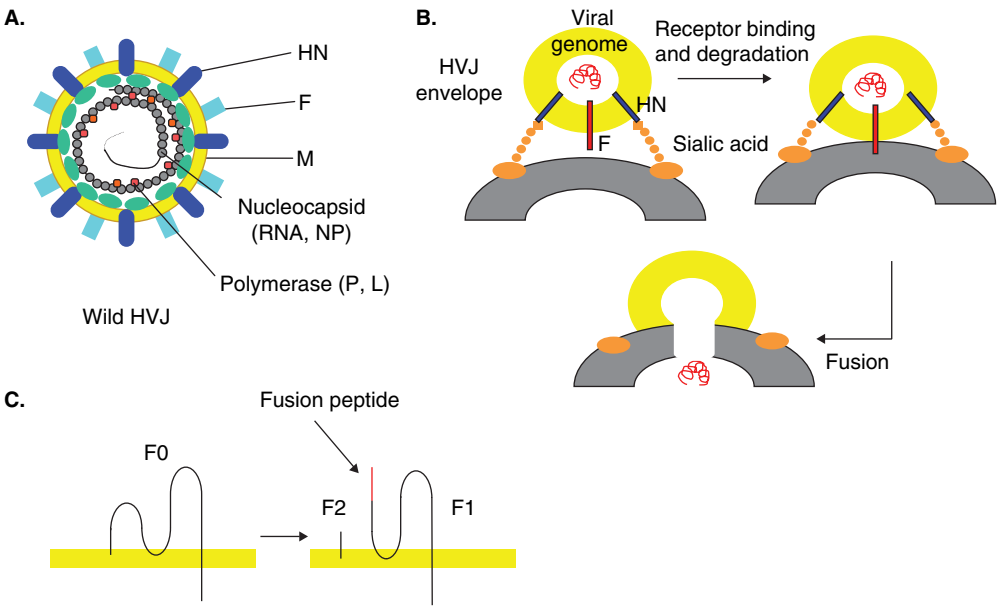

Figure 1. A. The structure of wild-type HVJ. A nucleocapsid containing ~ 15 kb of the viral RNA genome and nucleocapsid protein (NP), as well as polymerase P and polymerase L, is contained inside wild-type HVJ. F and HN on the envelope are associated with M protein beneath the envelope. **B.** The initial step of HVJ infection. HN protein first binds to acetylated sialic acid on the cell surface, after which it degrades the receptor with its neuraminidase. Then, a hydrophobic peptide of F protein associates with lipid molecules in the lipid bilayer and membrane fusion occurs between the HVJ envelope and the cell membrane. **C.** Cleavage of F protein of HVJ. F0 is an inactive fusion protein of HVJ produced by infected cells and then processed into F1 and F2 by endogenous proteases or trypsin treatment. F1 contains a fusion-competent hydrophobic peptide at its amino terminus.
F: Fusion; HN: Hemagglutinin-neuraminidase; HVJ: Hemagglutinating Virus of Japan; M: Membrane.

centrifugation (10,000 g, 5 – 10 min; **Figure 2A**). Numerous detergents, such as Triton X-100, Nonidet P-40 and deoxycholate, were available for preparation of the HVJ-E vector [32]. In the absence of detergent treatment, DNA does not become incorporated into the viral particle. The DNA-trapping efficiency of the HVJ-E vector using this method was ~ 15 – 20%. Electron microscopy confirmed that DNA was incorporated into all of the inactivated HVJ particles. The largest molecule of DNA tested was a 14 kb DNA plasmid, with a trapping efficiency of ~ 18%.

Electron microscopy confirmed that fusion between the HVJ-E vector and the cell membrane occurred within 3 – 5 s after attachment of the plasmid-containing HVJ-E vector to the cell surface. Cy3-pDNA reached the nucleus, specifically the nucleolus, within 30 min of fusion-mediated delivery via the HVJ-E vector [30], and DNA was retained in the cytoplasm during the entire period of observation following delivery by cationic liposomes. Luciferase gene expression by HVJ-E was ~ 10-times higher than that by cationic liposomes. HVJ-E treatment transiently depolymerized actin filaments, and nucleolar entry of microinjected DNA was accelerated following treatment with either empty HVJ-E or cytochalacin D – an inhibitor of actin polymerization – prior to microinjection. These results suggest that plasmid DNA can be rapidly transported from the cytoplasm to the nucleolus when actin filaments are depolymerized. Thus, the HVJ-E vector can accelerate the transport of DNA to the nucleolus by actin depolymerization.

The vector is commercially available both in Japan and the USA. The vector kit has been used for numerous applications.

With the aim of preparing the vector for use in clinical trials, a clinical grade HVJ-E vector is being produced by a venture company. Until now, the virus has only been produced in chick eggs [24]; however, egg-derived HVJ has limitations preventing its use in clinical trials. It was difficult to produce large amounts of virus in cultured cells. However, recently, the technology to produce large amounts of HVJ in human cells using animal product-free medium has been developed [33]. Using this new system, human cell-derived HVJ can be obtained at an efficiency exceeding 10^{10} particles/ml of culture medium. A pilot plant for the commercial production of clinical-grade HVJ-E vector has recently been established. Thus, a human cell-derived HVJ-E vector is now ready for clinical use.

2. Therapeutic gene expression *in vivo*

As described above, it is expected that the efficient and rapid transfer of exogenous DNA into the cytoplasm and then the nucleus can be achieved using the HVJ-E vector system. Therefore, the HVJ-E vector is hoped to permit gene therapy for the treatment of intractable human

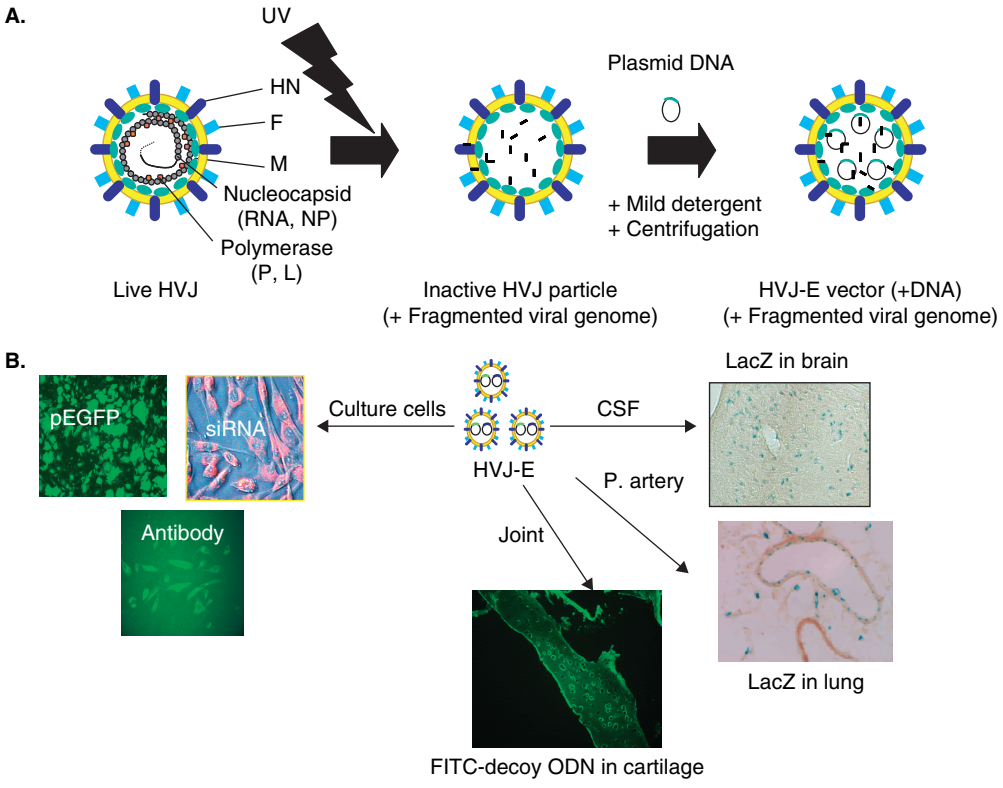

Figure 2. A. Method of construction of the HVJ-E vector. Live HVJ was inactivated with UV irradiation. The inactivated particle containing the fragmented viral genome was then mixed with plasmid DNA in the presence of mild detergent. Following this, the mixture was centrifuged to precipitate the viral particle in order to form the HVJ-E vector. Plasmid DNA was incorporated into the HVJ-E particle with ~ 15 – 20% efficiency. **B.** The delivery of various molecules to both cultured cells and animal tissue using the HVJ-E vector. siRNA, antibody and plasmid DNA were delivered to cultured cells with > 80% efficiency. *LacZ* gene expression in the brain and lung, as well as the delivery of decoy ODNs to cartilage was achieved using the HVJ-E vector.

CSF: Cerebrospinal fluid; HVJ-E: Hemagglutinating Virus of Japan envelope; NP: Nucleocapsid protein; ODN: Oligonucleotide; P. artery: Pulmonary artery.

diseases. The utility of HVJ-E vector in gene therapy has been demonstrated in various animal models. Gene delivery to various brain regions using the HVJ-E vector was first examined to test the potential applicability of HVJ-E to the treatment of diseases of the central nervous system. Direct injection of vectors into the brain has resulted in gene delivery [34], but carries a theoretical risk of brain damage. In an attempt to achieve gene delivery with minimal toxicity, the HVJ-E vector was injected into cerebrospinal fluid. When HVJ-E-vector containing *LacZ* or luciferase genes were intrathecally injected into the cerebrospinal fluid of rats, gene expression was detected in the brain stem, cerebellum, cerebral cortex (**Figure 2B**), medulla and inner ear tissue, including spiral ganglion cells (SGCs) of the cochlea [35,36]. Luciferase activity was not found in the lung, spleen or liver of luciferase-injected rats, nor in any of the organs tested from rats injected with empty HVJ-E vector. These results suggest that the HVJ-E vector might be used to deliver gene therapy for the treatment of hearing loss due to inner ear damage caused by kanamycin, or brain infarction due to occlusion of the middle cerebral artery in rats [35].

The potential of hepatocyte growth factor (HGF) as a therapeutic gene was examined. HGF is a secretory protein, which is known to function in an autocrine/paracrine manner in epithelial cells [37] and the nervous system [38,39]. When HVJ-E vector containing the human *HGF* gene (HVJ-E/HGF) was intrathecally injected into rats, human HGF expression was immunohistochemically detected in > 70% of SGCs of the inner ear. Within cerebrospinal fluid, the expression of both human and rat HGF was detected 12 days after transfection in rats.

It was then examined whether HGF might prevent the loss of hair cells and SGCs induced by kanamycin insult [35]. Kanamycin produces a significant loss of SGCs and hair cells due to apoptosis. However, the intrathecal administration of HVJ-E/HGF prior to kanamycin insult reduced the loss of SCGs and hair cells within the cochlea by ~ sixfold, compared with rats administered empty HVJ-E (13.3 ± 3.2 cells/10,000 μm^2 versus 2.2 ± 1.8 cells/10,000 μm^2, $p < 0.05$). The investigators confirmed that the intrathecal injection of HVJ-E vector alone did not damage SGCs.

Furthermore, when HVJ-E/HGF was administered 2 weeks after kanamycin insult, the number of SGCs recovered to nearly half the normal level. Based on the observed recovery of SGCs, and probably hair cells, intrathecal injection of HVJ-E/HGF likely resulted in significant recovery of hearing function in rats following kanamycin insult. These results suggest that *HGF* gene delivery into cerebrospinal fluid using the HVJ-E vector has potential for the prevention and treatment of hearing loss [35].

Next, the therapeutic effect of HGF on brain injury was investigated using a rat model of permanent middle cerebral artery occlusion [40]. Gene transfer into the brain was performed by injection of the human *HGF* gene using the HVJ-E vector into cerebrospinal fluid via the cisterna magna. Over expression of the *HGF* gene significantly decreased the area of infarcted brain, compared with rats transfected with control vector, after 24 h of ischemia. A consistent significant reduction in neurological deficit was observed in rats transfected with the *HGF* gene 24 h after the ischemic event. The stimulation of angiogenesis was also observed in rats transfected with the *HGF* gene, compared with controls. Importantly, no cerebral edema or destruction of the blood–brain barrier was observed in rats transfected with the *HGF* gene. Thus, the reduction of brain injury by HGF suggests its potential for use in the treatment of cerebrovascular disease.

3. Therapeutic gene silencing *in vivo*

The HVJ-E vector is also available for drug delivery, in addition to gene delivery (**Figure 2B**). Viral vectors cannot be used to deliver proteins, synthetic oligonucleotides or small drug molecules. Thus, gene silencing through the delivery of short interfering RNA (siRNA) or decoy oligonucleotides (ODNs) *in vivo* using the HVJ-E vector was investigated.

siRNA is a very attractive tool for specific inhibition of gene expression by recruiting endogenous RNase to a specific mRNA template [41,42]. A major problem of the siRNA strategy is an effective delivery system. Fusion-mediated direct delivery to the cytoplasm by HVJ-E appears to be an ideal delivery tool for siRNA. In fact, luciferase gene expression has been shown to be lowered to an undetectable level by the delivery of luciferase-specific siRNA using HVJ-E in osteogenic progenitor cells transfected with luciferase gene, and no inhibition was observed in cationic liposomes. Given the potent ability of siRNA to knock down gene expression, significant attention has focused on the use of siRNA for cancer treatment. However, it is difficult to inhibit tumor growth with siRNA alone, especially *in vivo*, as it is impossible to deliver siRNA into all cancer cells within a tumor mass. A more practical use of siRNA in cancer therapy will be the enhancement of the anti-cancer effects of chemotherapy or radiotherapy. *Cis*-diamminedichloroplatinum (II) (CDDP) – one of the most widely used anti-cancer drugs – inhibits cellular growth by inducing breaks in double-stranded DNA [43,44]. However, cells can use their DNA repair machinery to respond to DNA damage, lending resistance to anti-cancer drugs in human cancer cell lines. Rad51 plays a major role in homologous recombination repair machinery, which repairs double-stranded DNA breaks generated by CDDP [45]. Indeed, over expression of the human *Rad51* gene in HeLa cells renders HeLa cells resistant to CDDP. When *Rad51* siRNA was delivered into HeLa cells using HVJ-E, *Rad51* expression was completely suppressed. Consequently, the colony number of HeLa cells incubated with 0.02 μg/ml CDDP was < 10% of that observed for HeLa cells incubated without CDDP, presumably due to the transfer of *Rad51* siRNA [46]. Similar increases in CDDP sensitivity following the introduction of *Rad51* siRNA have been observed in a number of other human cancer cell lines, such as mammary carcinoma, pancreatic cancer, lung cancer and prostate cancer. By combining CDDP with the delivery of *Rad51* siRNA using the HVJ-E vector, a significant reduction in the growth of HeLa tumors was observed. *Rad51* siRNA also enhanced the anti-cancer effect of another chemotherapeutic drug, bleomycin (BLM).

Decoy double stranded synthetic ODNs encoding transcription factor binding sequences, have been widely used to inhibit gene expression by trapping transcription factors [47]. The difficulty of using ODNs is delivery, especially *in vivo*. Binding of endogenous nuclear factor-kappa B (NF-κB) to the promoter regions of several genes involved in inflammation, cell survival and cell adhesion, is inhibited by NF-κB decoy oligonucleotides. NF-κB decoy oligonucleotides have been shown to reduce myocardial reperfusion injury by inhibiting the expression of cytokines such as IL-6 and IL-8, and adhesion molecules in aortic endothelial cells [48], as well as to prevent atopic dermatitis [49]. Research suggests that idiopathic inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis are caused by inflammation due to inappropriate and/or excessive responses to antigens present in the normal bacterial microflora [50]. In both human and murine models of IBDs, inflammation appears to depend, at least in part, on the activation and nuclear translocation of NF-κB family members [51]. Thus, NF-κB decoy ODNs may have therapeutic potential in the treatment of IBDs. The local (intrarectal) and systemic (intraperitoneal) administration of NF-κB decoy ODNs using HVJ-E vectors prevented and ameliorated trinitrobenzene sulfonic acid-induced colitis (a Crohn's disease model), as well as oxazolone-induced colitis (an ulcerative colitis model) [52]. Furthermore, when given late in the course of a model of chronic trinitrobenzene sulfonic acid-induced colitis, treatment prevented the development of fibrosis. Thus, combining a NF-κB decoy ODN with the HVJ-E vector effectively treated both forms of IBD and prevented some of the complications of these illnesses in the models examined.

Similarly, AP-1 is a therapeutic target in the treatment of tissue fibrosis [53]. IL-13 produces fibrosis in a number of chronic infectious and autoimmune diseases. It stimulates transforming

growth factor-β1 in macrophages through induction of IL-13 receptor α_2 and activation of an AP-1 variant containing c-jun and Fra-2. Therefore, AP-1 decoy ODNs, as well as IL-13 receptor α_2 siRNA, are effective treatments of tissue fibrosis when delivered using the HVJ-E vector.

4. Protein delivery enabling therapy and bioimaging *in vivo*

To demonstrate the efficiency of delivery of proteins using HVJ-E vector *in vivo*, fluorescent bovine serum albumin (BSA) was delivered into nasal mucosa without damaging the mucosa using the HVJ-E vector delivery system in a previous study [54]. Ten microliter drops of 3000 hemagglutinating unit of HVJ-E vector containing BSA–Alexa Fluor® (Invitrogen) 488 conjugate (HVJ-E/Alexa488-BSA) were dropped into the nasal cavities of mice using a micropipette, after which all mice inhaled the suspension. After 24 h, stereoscopic examination revealed fluorescence in the nasal mucosa of all mice treated with HVJ-E/Alexa488-BSA, but not in mice treated with Alexa488-BSA alone. Microscopic analysis of coronal nasal sections confirmed that Alexa488-BSA was effectively incorporated into the nasal epithelium of mice treated with HVJ-E/Alexa488-BSA, but not Alexa488-BSA alone. None of the treatments induced significant pathological changes in the nasal mucosa. Using this delivery method, ovalbumin (OVA)-induced allergic rhinitis was successfully treated in OVA-sensitized Balb/c mice. Following intranasal treatment with HVJ-E vector containing OVA (HVJ-E/OVA), Th2 cytokines, such as IL-4 and -5, were reduced and Th1 cytokines, such as IFN-γ, were elevated in splenocytes. Alteration of this Th1/Th2 balance by intranasal administration of HVJ-E/OVA dramatically suppressed the production of serum OVA-specific IgE, which is normally increased > 40-fold in the mouse model of allergic rhinitis compared with control mice.

Apart from its therapeutic use, protein delivery by HVJ-E is also available for bioimaging [55]. Microglia are supposed to reduce levels of amyloid-β through phagocytosis. In the present study, microglial cells were labeled with super-magnetic iron particles (Resovist®; Bayer Schering) using the HVJ-E vector and transplanted into the lateral ventricles of rat brains. The labeled microglial cells were then detected by magnetic resonance imaging. When human amyloid-β42 was injected into the rat hippocampus, labeled microglia accumulated in amyloid-β plaques and amyloid-β42 was significantly reduced in the rats transplanted with microglia. Thus, the transplantation of exogenous microglia might be useful in the treatment of Alzheimer's disease.

5. High throughput functional screening to identify therapeutic genes

In designing molecular therapy for the treatment of human diseases, the identification of target molecules is critically important, in addition to vector development. For this purpose, high throughput screening of cDNA libraries to evaluate specific functions is necessary. The HVJ-E vector has some distinct advantages for use in high throughput screening. Approximately 100 copies of plasmid DNA can be incorporated into one HVJ-E particle when 200 μg of 7 kb plasmid is mixed with 3×10^{10} particles of inactivated HVJ. Furthermore, the HVJ-E vector enables two different plasmids to be delivered to the same cell, as confirmed by concurrent LacZ and green fluorescence protein (GFP) expression in transfected cells [33]. Other benefits of the HVJ-E vector include rapid preparation of HVJ-E vector containing the cDNA library (~ 30 min) and easy cloning of candidate genes by transformation of *Escherichia coli* (12 – 16 h; **Figure 3**). In order to demonstrate the ease of high throughput screening using the HVJ-E vector, several novel angiogenic genes were identified using the HVJ-E vector [56]. A human heart cDNA library was randomly infused into the HVJ-E vector for transfection into human aortic endothelial cells (HAECs), which were subsequently seeded into 96-well plates. HAEC proliferation was then measured using a [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] (MTS) assay, and DNA was extracted from the cell population demonstrating the greatest degree of proliferation, after which the DNA was directly transfected into bacteria. After screening, 1588 independent clones were isolated and sent for sequencing to eliminate empty and repetitive plasmids. Seven hundred and eighty one clones were selected and further characterized to identify clones that exhibited multiple hits (291 clones). Each of the 291 clones was transfected into HAECs and evaluated by MTS assay. Six genes showed more potent effects on endothelial cell viability than vascular endothelial growth factor, and were further evaluated using a *c-fos* promoter assay. Three of these genes had more potent effects than vascular endothelial growth factor in this assay. Thus, it is possible to isolate genes of interest in 1 month using this screening system. This system is much more convenient and effective for gene screening than viral vector systems [57,58], because neither genetic engineering for vector development nor polymerase chain reaction (PCR) amplification of cDNA to isolate candidate genes is necessary (**Figure 3**).

Next, a novel gene capable of inhibiting the growth of both aortic and lymphatic endothelial cells was identified from the cDNA library of a mouse lung cancer line (LL/2) using the HVJ-E vector system [59]. cDNA encoding mouse cold shock domain protein A (CSDA) was obtained after two cycles of screening of the library. Over expression of CSDA significantly inhibited cell proliferation and c-*fos* promoter activity in aortic, venous and lymphatic endothelial cells. CSDA is thought to be a DNA binding protein that binds to the hypoxia response element. CSDA might directly bind to the serum response element sequence, resulting in inhibition of serum response element, which may result in

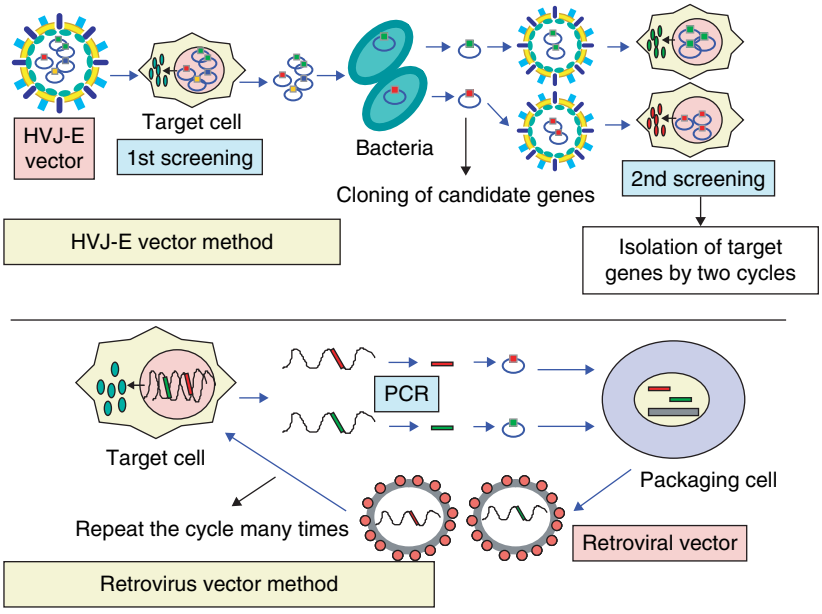

Figure 3. High throughput functional screening of therapeutic genes using the HVJ-E vector (upper panel) and retrovirus vector (lower panel). Using the HVJ-E vector, candidate genes were isolated by two-cycle screening. Plasmid DNA was purified from cells demonstrating a desired function after the first round of screening. Cloning of candidate genes was achieved by transferring their DNA to bacteria (*Escherichia coli*). The function of each cloned DNA molecule was then examined in the second screening cycle. Neither PCR nor genetic engineering for vector construction was needed when using a retrovirus vector for screening.
HVJ-E: Hemagglutinating Virus of Japan envelope.

growth inhibition of endothelial cells. In an LL/2-inoculated mouse model, tumor growth was significantly inhibited in a mCSDA-injected group. The expression of blood and lymphatic endothelial cell markers was significantly reduced in mCSDA-injected mice. Thus, CSDA may be useful in cancer gene therapy in the future.

6. HVJ envelope as a novel anti-cancer reagent

Recently, it has been discovered that HVJ-E vector itself induces anti-tumor immunity, including T cell-mediated and non-T cell-mediated immunity through multiple pathways.

HVJ-E vector injected into murine colon carcinoma tumors of CT 26 cells growing in syngeneic Balb/c mice eradicated 60 – 80% of the tumors and obviously inhibited the growth of the remainder, and adenovirus vector was not effective for inhibiting tumor growth [60]. Induction of adaptive anti-tumor immune responses played an obvious role in tumor eradication, as the effect was abrogated in severe combined immunodeficient mice. Murine and human dendritic cells (DCs) underwent dose-dependent maturation upon exposure to HVJ-E vector *in vitro*. Cytokine profiles secreted by DCs after HVJ-E vector stimulation showed induction of IL-6 release comparable to that elicited by live HVJ. Real-time reverse transcription (RT)-PCR and immunohistochemistry revealed marked infiltration of DCs, $CD4^+$ and $CD8^+$ T cells into tumors by HVJ-E vector. In addition, CT26-specific cytotoxic T lymphocytes were induced with evidence of enhanced $CD8^+$ T cell activation in a $CD4^+CD25^-$ T cell-dependent manner. On the other hand, conditioned medium from DCs stimulated by HVJ-E vector rescued $CD4^+CD25^-$ effector T cell proliferation from $Foxp3^+CD4^+CD25^+$ regulatory T cell-mediated suppression, and IL-6 presumably played a dominant role in this phenomenon. Furthermore, a significant increase in IL-6-producing dendritic cells was observed in tumor beds and draining lymph nodes after injection of HVJ-E vector into tumor masses. When regulatory T cells and effector T cells were isolated from draining lymph nodes after HVJ-E vector treatment, proliferation of effector T cells was refractory to inhibition of regulatory T cells in mixed culture. This is the first report to demonstrate that HVJ-E vector alone can eradicate tumors by enhancing T cell immunity against cancers and inhibiting regulatory T cell-mediated suppression (**Figure 4**).

Along with T cell immunity, non-T cell immunity was also induced by HVJ-E vector treatment [61]. Microarray analysis revealed that direct injection of HVJ-E vector induces CXCL10 expression in established Renca tumors. CXCL10 was secreted by dendritic cells within the tumors after HVJ-E vector injection. Quantitative real-time RT-PCR and immunohistochemistry revealed infiltration of HVJ-E-injected tumors with $CXCR3^+$ cells, predominantly natural killer (NK) cells. Moreover, HVJ-E vector injection

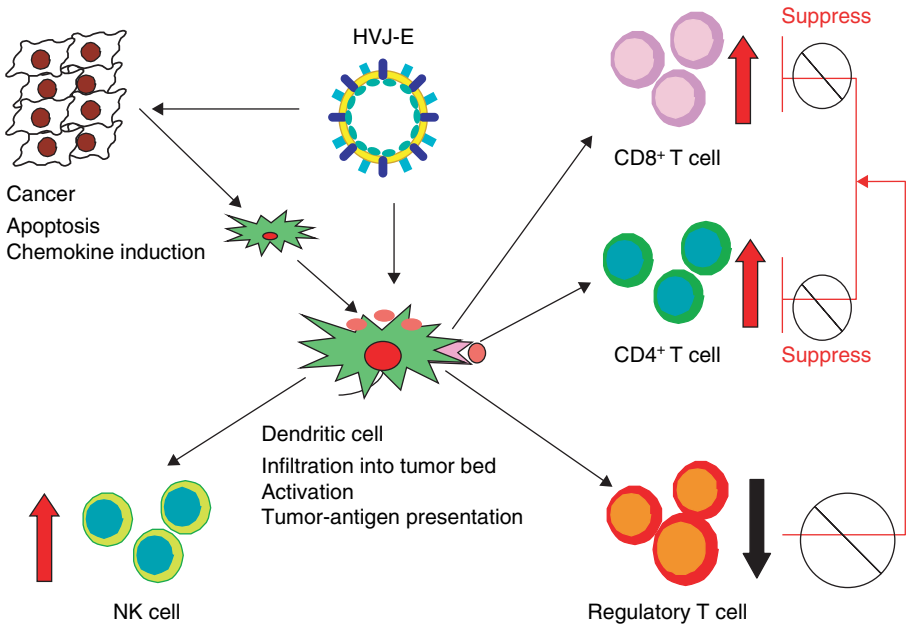

Figure 4. The multiple anticancer effects of the HVJ-E vector. When injected into tumors, HVJ-E vector stimulated the secretion of chemokines from tumor cells to facilitate the migration of DCs to the tumor bed. Following this, HVJ-E vector enhanced DC maturation and the production of various cytokines and chemokines from DCs. Due to these cytokines and chemokines, $CD4^+$ and $CD8^+$ T cells, as well as NK cells, migrated to the tumor bed and were activated to kill tumors. Furthermore, IL-6 was produced from DCs by HVJ-E vector, which suppressed the action of regulatory T cells, resulting in continuous activation of effector T cells.
DC: Dendritic cell; HVJ-E: Hemagglutinating Virus of Japan envelope; NK: Natural killer.

caused systemic activation of NK cells and enhanced their cytotoxity against tumor cells (**Figure 4**). In an *in vivo* experiment, ~ 50% of tumors were eradicated by HVJ-E injection, and the activity of HVJ-E vector against Renca tumors was largely abolished by NK cell depletion using anti-asialo GM1 antibody. However, during HVJ-E vector treatment of Renca tumors, cytotoxic T lymphocytes against Renca also increased. A time-course analysis of the production of immune cells in tumors after HVJ-E vector treatment demonstrated that the non-T cell immune reaction mediated by NK cells occurred in the early phase of treatment (i.e., within 24 h), after which T-cell immunity became more evident.

Similar anti-tumor effects of HVJ-E vector were observed during combined treatment with an anti-cancer reagent in an orthotopic tumor model [62]. An orthotopic tumor model was established by intravesical administration of mouse bladder carcinoma MB49 cells. Combined intravesical instillation of HVJ-E vector and doxorubicin hydrochloride (DXR) resulted in a significantly greater numbers of tumor-free mice (11/21), compared with mice treated with DXR alone (3/19, $p < 0.05$). Median survival was > 60 days for intravesical instillation of HVJ-E vector and DXR, compared with 29 days for DXR instillation alone ($p < 0.05$). Following combination therapy, the surviving mice did not develop bladder tumors after intravesical re-instillation of MB49 cells.

The production of cytokines and chemokines in dendritic cells by HVJ-E vector was abolished by the treatment of HVJ-E with Triton-X100, but maintained by Tween 80-treated HVJ-E (unpublished data by Y Kaneda.). Both detergents were available for the incorporation of therapeutic molecules into HVJ-E vector. For cancer treatment, both immunological activities and drug delivery potency of HVJ-E are necessary, but, for the therapy of diseases other than cancers, induction of cytokine and chemokine may be harmful. Thus, using two different detergents at the incorporation step, it is possible to construct HVJ-E vector with or without anti-tumor immunity.

7. Conclusion

The HVJ-E vector is a sort of hybrid between a viral and non-viral vector, in which most of the limitations of both vector systems cancel each other out in the HVJ-E vector system. An inherent problem with non-viral vectors is the difficulty in achieving gene delivery *in vivo*. The HVJ-E vector enables the efficient *in vivo* delivery of therapeutic molecules, such as virus vectors, due to fusion-mediated direct delivery into the cytoplasm and rapid transport of DNA into the nucleus. From a safety standpoint, there is concern about using viral vectors in human trials [63,64] due to the possibilities of insertional mutagenesis and potential production of replication-competent viruses. As the viral genome is eliminated in the HVJ-E vector, replication does not occur and viral genes are not expressed in cells

transfected with the HVJ-E vector. With the HVJ-E vector, transgene expression does not depend on viral genome structure, but is based on plasmid DNA [32]. Although viral protein components remain, *de novo* synthesis of viral proteins is not detected in the cells treated with HVJ-E vector. Neither insertional mutagenesis nor viral replication occurs with the HVJ-E vector. However, caution is advised regarding the use of the HVJ-E vector in clinical trials, as it has not yet been examined in humans. In Japan, recombinant Sendai virus vector containing the fibroblast growth factor-2 gene [65] has been injected into human muscle to treat arteriosclerosis obliterance. So far, severe side effects related to the vector have not occurred.

Therefore, the HVJ-E vector may be superior to presently available vectors for the treatment of intractable human diseases.

8. Expert opinion

Approximately two thirds of human gene therapy clinical trials have involved cancer treatments. It is very difficult to achieve successful results with cancer gene therapy, as gene therapy is generally permitted only to the patients at the terminal stage under the informed consent and gene therapy technology is not yet sufficient for cancer eradication. As a result, the therapeutic efficacy of gene therapy appears limited at this point in time with little progress made since the early stages of its development. A common challenge of cancer therapy is the prevention of metastasis and cancer recurrence. As a result, a number of researchers have focused on tumor immunotherapy in recent times. Cancer induces immunological tolerance of the host immune system by several mechanisms [66-70]. Therefore, successful cancer therapy must have both tumor killing activity and induce anti-tumor immunity. The HVJ-E vector has a number of unique characteristics enabling the delivery of molecular therapy and immunotherapy. HVJ-E has been used for drug delivery *in vitro* and *in vivo* [32,33], and the vector itself enhances anti-tumor immunity [60-62]. Enhancement of general immunity by a number of vectors, such as liposomes [71], and naked DNA with the CpG motif [72], has been described. However, in the case of HVJ-E treatment, tumor-specific immunity is generated. Following HVJ-E treatment, tumor cells underwent apoptosis due to the induction of type I interferon. However, the tumor-killing activity of HVJ-E itself is insufficient for the eradication of large tumor masses (i.e., > 5 mm in diameter for tumors composed of CT26 cells) and tumors with rapid growth, such as MB49 tumors [62]. In such cases, the HVJ-E vector can be used to deliver therapeutic molecules that further enhance its anti-tumor effects eradicate tumors.

For example, an HVJ-E vector containing an anti-cancer drug, BLM, was injected into a tumor mass (> 5 mm in diameter) derived from CT26 in mice [73]. Three intra-tumoral injections of BLM-containing HVJ-E (HVJ-E/BLM) with single intraperitoneal administration of CDDP eradicated CT26 tumors with > 75% efficiency. When tumor cells were intradermally injected into the flank opposite the initial tumor site 10 days after the first inoculation of tumor cells, tumors on both sides disappeared in the majority of mice treated with combination HVJ-E/BLM and CDDP therapy. The surviving mice were then re-challenged with CT26 cells 8 months after eradication of the initial tumor. Tumors did not grow following re-challenge in any of the mice treated with combination therapy. Cytotoxic T lymphocytes specific for CT26 were generated in the surviving mice. This result suggests that the HVJ-E vector itself can induce anti-tumor immune responses within tumor masses, as well as assisting in delivering anti-tumor drugs.

Thus, HVJ-E is an ideal vector system for cancer therapy because it can deliver molecular therapy and induce immunotherapy.

In order to treat metastatic tumor foci, targeted drug delivery following systemic vector administration is desired. Tissue-targeting HVJ-E vectors have been constructed using two different approaches. One approach uses conjugation with biocompatible polymers [74]. When combined with cationized gelatin (CG), CG-HVJ-E vector demonstrated a high level of luciferase gene expression, primarily within tumor deposits [75]. Forty-eight hours after introducing colon cancer cells into the peritoneum of experimental mice, CG-HVJ-E vector with or without BLM was injected into the abdominal cavity. Following six injections of BLM-incorporated CG-HVJ-E vector, complete responses were observed in 40% of the mice examined. All of the mice that received either empty CG-HVJ-E vector or BLM alone died within 40 days of having the cancer cells introduced into their peritoneum. When the mice with complete responses were re-challenged with colon cancer cells from the same cell line, no tumors developed. Thus, CG-HVJ-E vector may suppress peritoneal dissemination of cancer in mice.

The second approach involves modification of fusion proteins with targeting molecules by genetic engineering (**Figure 5**) [76]. Chimeric genes were constructed using various deletion mutants of viral F protein and GFP. One chimera containing a signal peptide, a transmembrane domain, and the cytoplasmic tail of F protein was transported to the cell surface and incorporated into new viruses released from HVJ-infected LLCMK2 cells. To enable tissue targeting, GFP within the above construct was replaced with a single chain antibody (scFv) against mouse desmoglein 3 (mDsg3), a desmosomal cadherin found within basal layer keratinocytes in skin. Chimeric HVJ containing scFv and deleted F was observed to bind to mDsg3-coated plates with significantly greater efficiency than wild-type HVJ. When chimeric HVJ was injected into skin blisters in a mouse model of epidermolysis bullosa, in which there is defective expression of type VII collagen securing the epidermis to the underlying dermis, viral F protein expression was detected in most of the basal keratinocytes. Furthermore, the chimeric HVJ-E

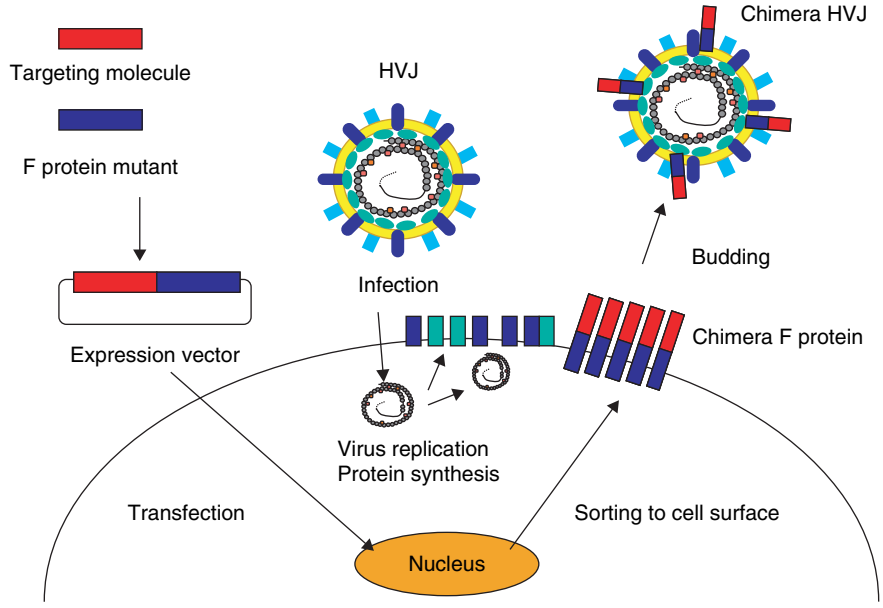

Figure 5. The method of construction for a chimeric HVJ containing a targeting molecule. First, a chimeric gene was constructed using viral F protein and a targeting molecule. After this, the chimeric gene was transfected into cultured cells to form stable transformants. After the chimeric molecules became inserted into the plasma membranes of transformed cells, the targeting molecule was incorporated into the viral envelope during virus budding following infection of the transformants with wild-type HVJ.
F: Fusion; HVJ: Hemagglutinating Virus of Japan.

vector introduced a type VII collagen expression plasmid more efficiently than wild-type HVJ into basal keratinocytes in mouse skin deficient in type VII collagen, resulting in efficient amelioration of the genetic defect. Thus, the production of chimeric HVJ enabled tissue-targeting of the HVJ-E vector and successfully targeted epidermal keratinocytes both *in vitro* and *in vivo*.

However, HVJ has HN protein in addition to F protein on its viral membrane. HN binds to its receptor, acetylated sialic acid, on cell membranes. This protein is also involved in the agglutination of red blood cells. To increase the targeting specificity and decrease hemagglutination, HN-depleted HVJ is desired. For this purpose, HN-depleted HVJ have been developed using HN-specific siRNA. By combining the construction of chimera containing the F protein and a targeting molecule with the use of HN-specific siRNA to produce HN-depleted HVJ [77], tumor-targeting HVJ-E vector without HN was developed [78]. This unique approach has not been used in viral and non-viral vector systems so far. The tissue-targeting HVJ-E vector will greatly extend the capabilities of this vector system in the future.

Thus, the efficacy of HVJ-E for cancer treatment in animal models has been evaluated, and the preclinical safety test is being performed. Repeated injection of HVJ-E may cause an immune response. Preliminary toxicity tests revealed that faint erythema was seen in mouse skin. More precise safety testing is will be performed. As hemolysis occurrs by the systemic injection of HVJ-E, direct intratumoral injection of the vector will be performed in clinical trials. Therefore, the leakage of HVJ-E into blood will be examined when the vector is injected into a tumor mass in mice. Vector production system has been established and clinical-grade vector is being produced. Based on the information on the HVJ-E vector system, clinical applications of the vector are being planned. First, a Phase I study to evaluate the safety and anti-tumor immune reactions of empty HVJ-E will commence in 2009 with melanoma patients in stage IV. This is a dose-escalation study by repeated injection of empty HVJ-E to skin lesions of melanoma patients. If the safety of the vector is confirmed and some immune reactions against cancers are recognized in the Phase I study, gene therapy using HVJ-E containing a therapeutic gene will be performed in cancer patients including melanoma, bladder carcinoma and malignant glioma by 2011. This step-by-step strategy is necessary because no clinical trials have been performed by repeated administration of HVJ-E in cancer patients, although a safety evaluation has been done by the single intranasal injection of live HVJ to healthy human adults [79]. The final goal is the systemic injection of tumor-targeting HN-depleted HVJ-E incorporating therapeutic molecules to various refractory cancers.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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