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Table 1

Luciferase activity at 24 h after transfection of *HGF* gene via the cisterna magna

	RLU/mg tissue
Cerebral cortex	151.2 ± 224.7 (n=4)
Medulla	225.3 ± 88.2 (n=4)
Cerebellum	112.3 ± 61.4 (n=4)
Cochlea	146.6 ± 44.7 (n=8)

After intrathecal injection of the luciferase gene via the cisterna magna, luciferase activity was measured from tissues dissected from the cerebral cortex, medulla, cerebellum, and cochlear. Transgene expression was not detected in other organs including the liver, lung, and spleen.

Fig. 1

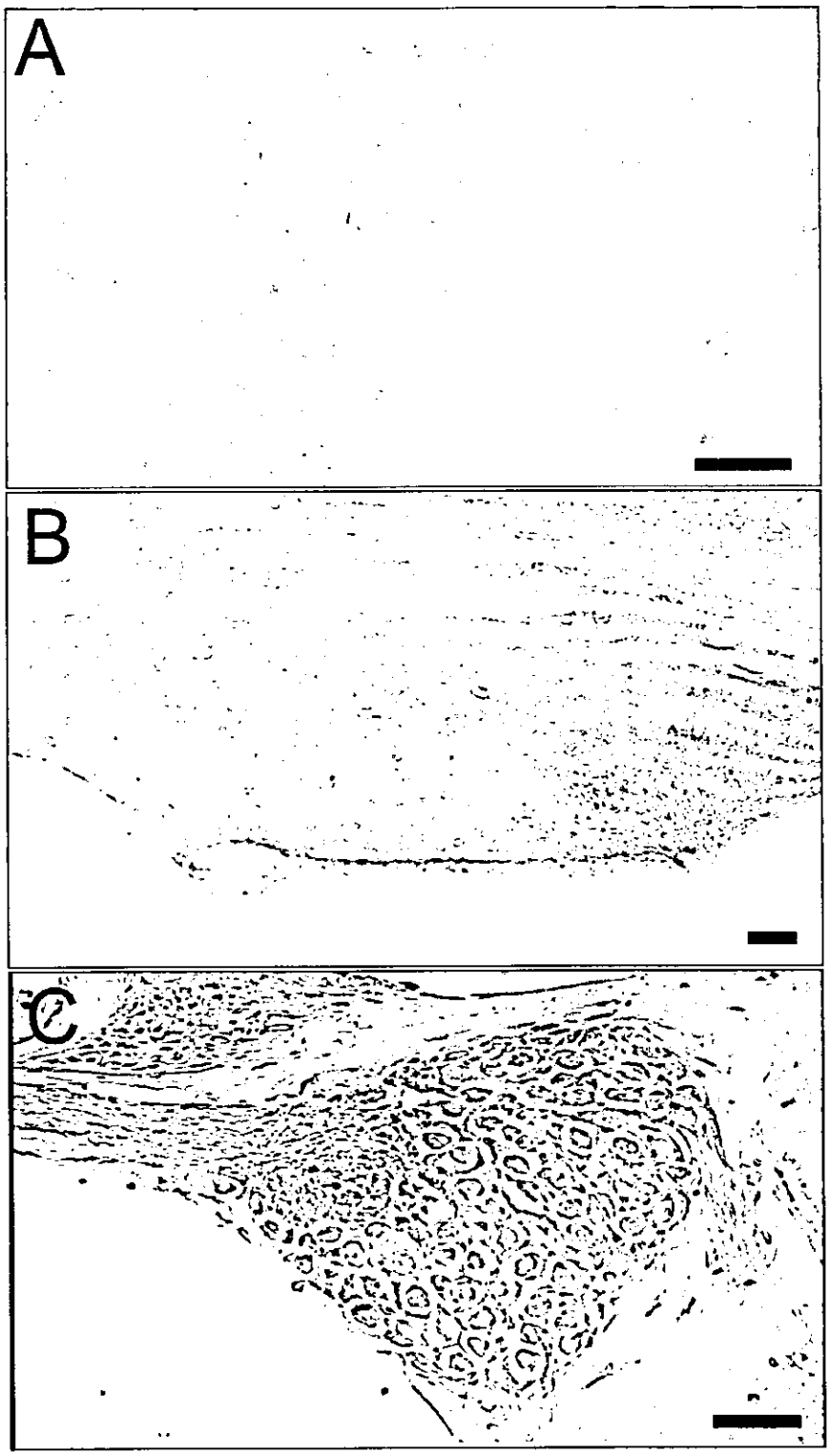

Figure 1. Localization of *lacZ* expression in the brain and cochlea. *lac*Z expression in the medulla (*A*), cochlear nucleus (*B*), and SGCs (*C*) of normal rats was detected by X-gal staining on day 7 after intrathecal injection of HVJ-E containing the *E. coli* β-galactosidase gene *lac*Z. Scale bar: 100μm.

Fig. 2

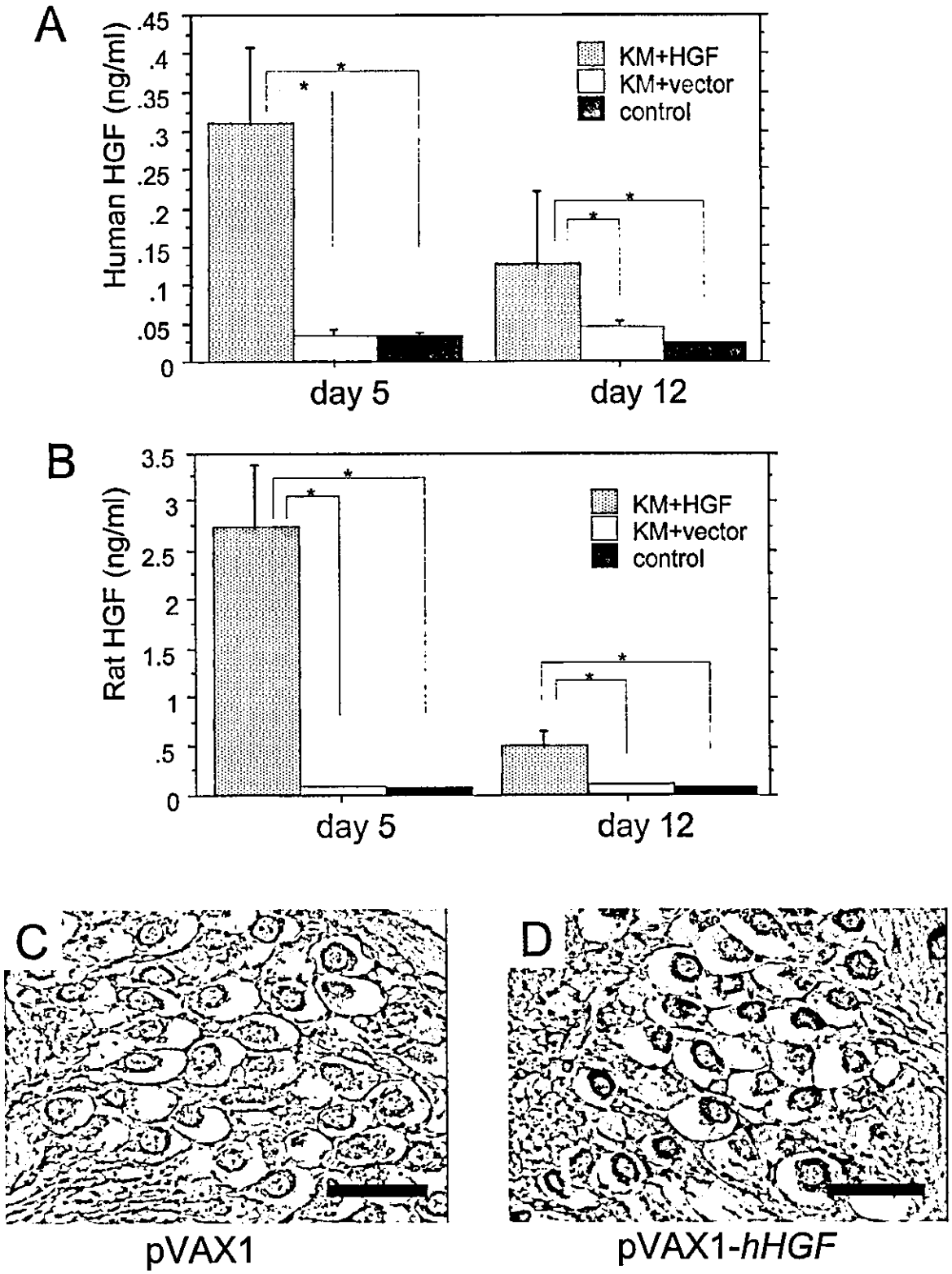

Figure 2. Expression levels of exogenous and endogenous HGF in CSF and SGCs. Exogenous human HGF (*A*) and endogenous rat HGF (*B*) in CSF from the KM + HGF, KM + vector, and control groups were measured on days 5 and 12 after transfection with the human HGF transgene (n=4 for each). SGCs from the mid-turn of cochleae treated with KM + vector (*C*) or KM + HGF (*D*) were immunostained with anti-human HGF antibody. *$P < 0.01$. Scale bar: 50 μm; n=4 for each group.

Fig. 3

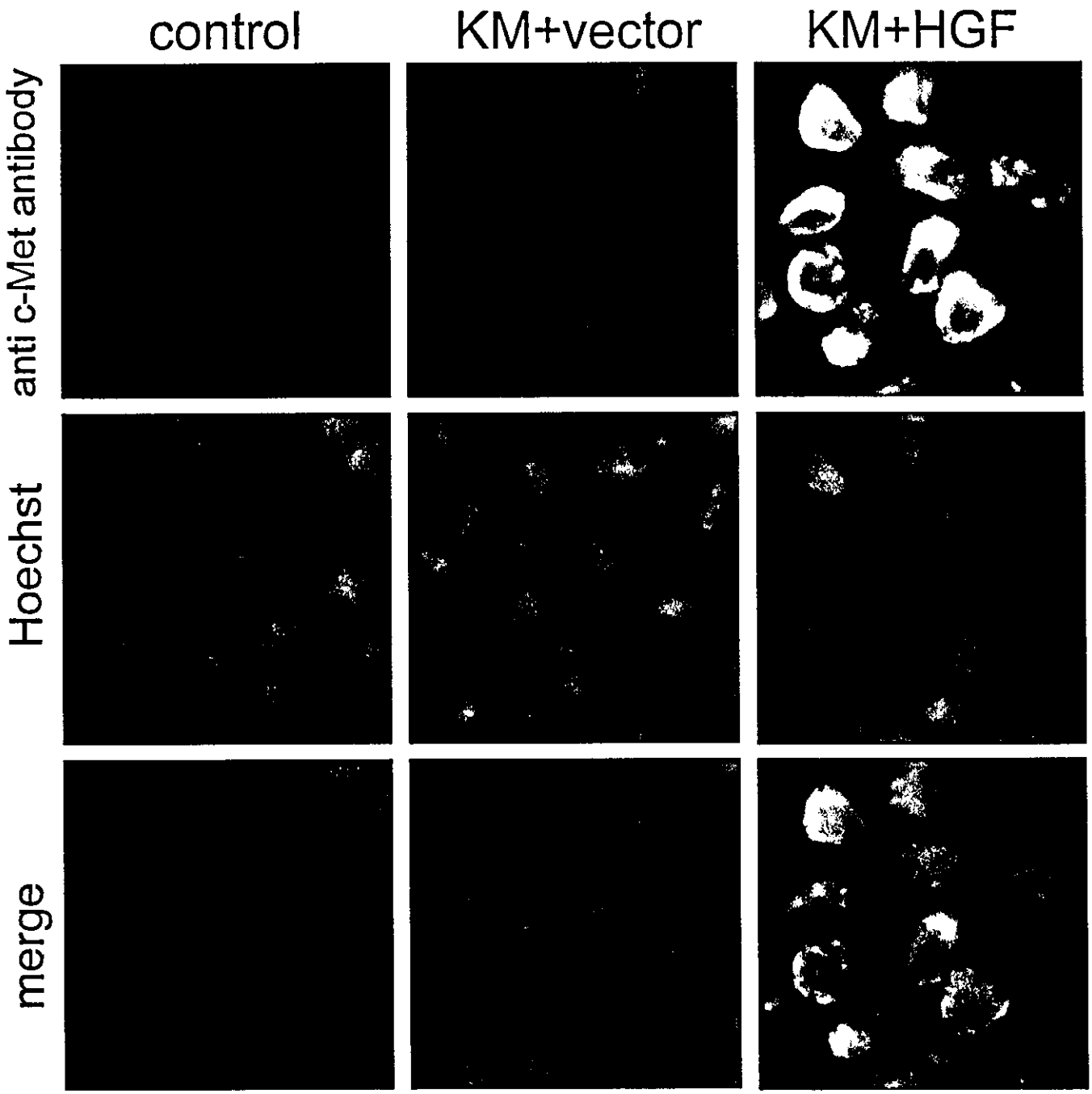

Figure 3. Enhancement of c-Met expression in SGCs by *HGF* gene transfer. Immunohistochemistry of SGCs from the intact rats, KM + vector group, and KM + HGF group was performed. Samples were stained with anti-c-Met antibody (upper) and counterstained with Hoechst 33342 (middle). Merged images are also presented (bottom).

Fig. 4

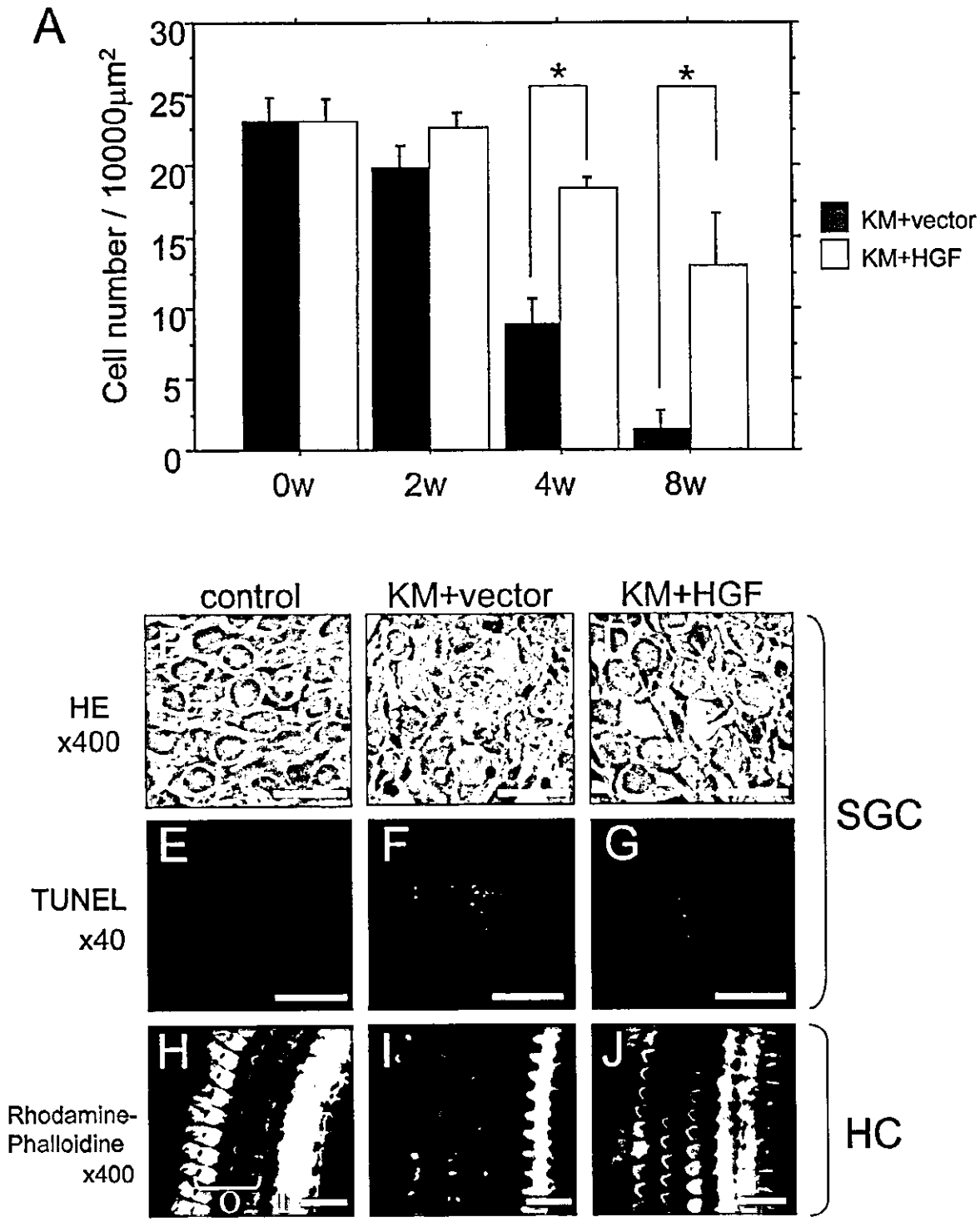

Figure 4. Protective effect of the HGF transgene on SGC and HC treated with kanamycin. Numbers of hematoxylin-positive cells of SGC of the rats treated with KM + vector or KM + HGF are counted at various time points (***A***; n=6 for each group). Mid-modiolar 10 μm cryosections from rats without treatment (control; ***B***), treated with kanamycin and HVJ-E containing control vector (***C***), or HVJ-E containing the human HGF gene (***D***) were stained with hematoxylin on week 4. TUNEL staining of the contiguous sections of SGCs from the same rats as described above is shown in ***E***, ***F***, and ***G***. Fluorescent image of HC of the rats in the control, KM + vector group, and KM + HGF group is shown in ***H***, ***I***, and ***J***. O: outer hair cell; I: inner hair cell. Scale bar: 50 μm in ***B-D*** and ***H-J***, 500 μm in ***E-G***. *$P < 0.01$.

Fig. 5

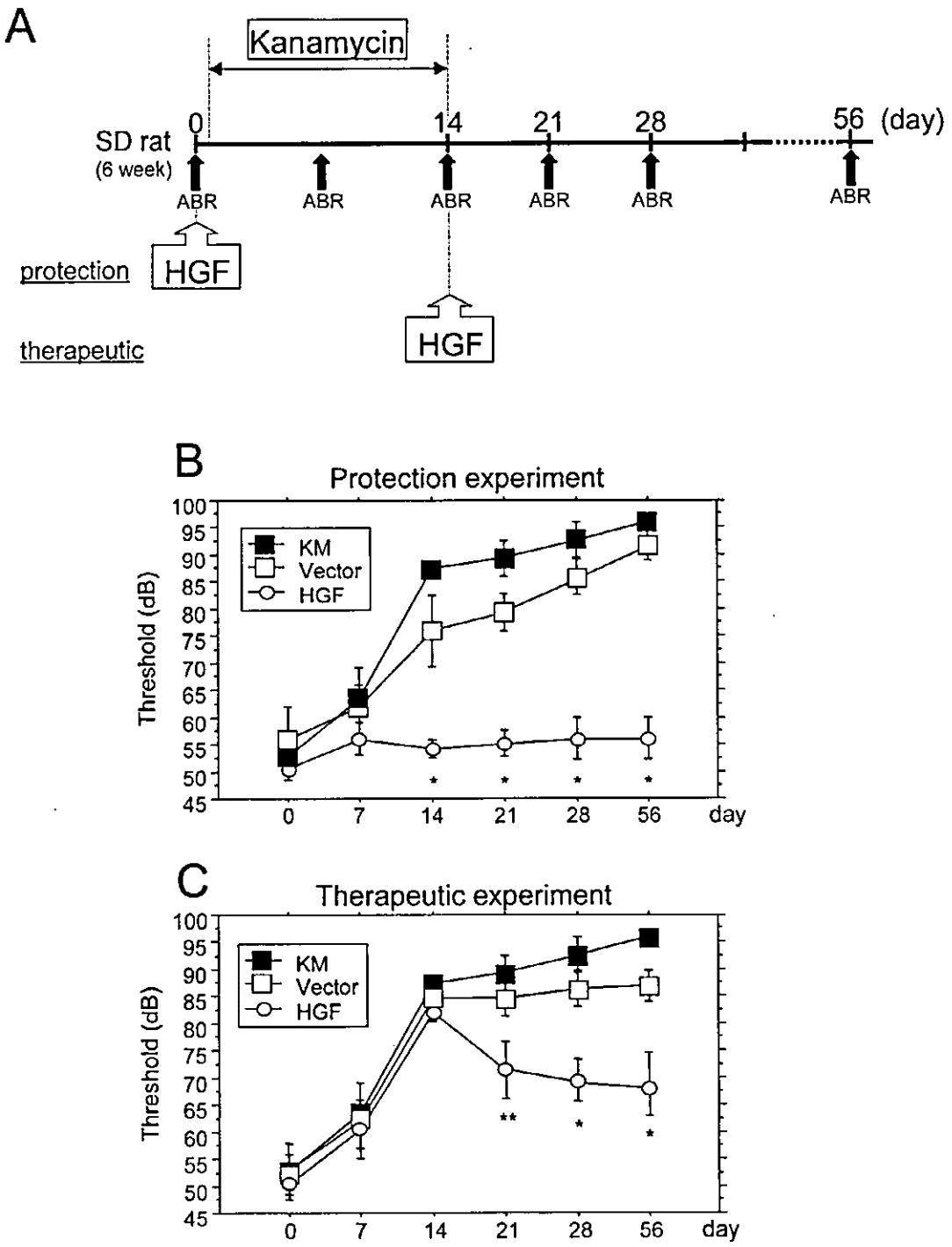

Figure 5. Hearing function of rats treated with KM, KM + vector, or KM + HGF was evaluated by the auditory threshold using ABR. Time course of the experiment was illustrated in *A*. In the protection experiment (*B*), rats treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) immediately before the kanamycin insult underwent evaluation of the auditory threshold on days 0, 7, 14, 21, 28, and 56. In the therapeutic experiment (*C*), rats were treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) 14 days after the kanamycin insult and the auditory threshold was measured at each time point. KM means the auditory threshold of rats treated only with kanamycin. Six rats were used in each group. Means ± SD of each value are indicated. ■: KM group; □: KM + vector group; ○: KM + HGF group. $*P < 0.01$; $**P < 0.05$.

The HVJ-Envelope as an Innovative Vector System for Cardiovascular Disease

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Abstract: Recently promising results of gene therapy clinical trials have been reported for treatment of peripheral vascular and cardiovascular diseases using various angiogenic growth factors and other therapeutic genes. Viral vector and non-viral vector systems were employed in preclinical studies and clinical trials. Adenoviral vector and naked plasmid have been used most in the clinical studies. HVJ (hemagglutinating virus of Japan or Sendai virus)-liposome vector, a hybrid non-viral vector system with fusion of inactivated HVJ virus particle and liposome, has developed and demonstrated high transfection efficiency in preclinical studies of many different disease models, including a wide range of cardiovascular disease models. However, some limitations exist in the HVJ-liposome technology, especially in the scalability of its production. Recently an innovative vector technology, HVJ envelope (HVJ-E) has been developed as a non-viral vector, consisting of HVJ envelope without its viral genome, which is eliminated by a combination of inactivation and purification steps. HVJ-E is able to enclose various molecule entities, including DNA, oligonucleotides, proteins, as single or multiple therapeutic remedies. The therapeutic molecule-included HVJ-E vector can transfect various cell types in animals and humans with high efficiency. In this review, vector technology for cardiovascular disease and the biology of HVJ-E vector technology is discussed.

INTRODUCTION

Gene therapy, as an approach to treat diseases, uses vectors carrying therapeutic gene or genes. In the cardiovascular area, naked plasmid DNA and adenoviral vectors have been used most for gene therapy of ischemic heart disease (IHD) and lower extremity ischemia (LEI) with angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hypoxia inducible factor 1 (HIF-1) and hepatocyte growth factor (HGF). Adenoviral vectors demonstrated relative high transduction efficiency in skeletal muscle and myocardium compared to that of naked plasmid. However, the replication-deficient adenoviral vector system has its deficiencies for gene therapy applications, such as size limitation, viral toxicity and immunogenicity. The adenoviral vectors have been employed in a significant number of clinical trials with extensive safety considerations. In contrast, it has been considered safer for naked plasmid DNA as the vector carrying VEGF, FGF or HGF to treat IHD or LEI in the clinical trials. However, naked plasmid DNA is generally unstable while it is taken up by endocytosis. The *in-vivo* transfection efficiency of naked plasmid DNA also needs to be improved. Most non-viral vectors are much less efficient in delivery of genes into cells *in-vivo* as compared to recombinant viral vectors. In most cases the introduced DNA with non-viral vectors is taken up by endocytosis mechanism of the host cells and gets into lysosomes, resulting in rapid degradation. Therefore, there has been a demand to develop an improved non-viral vector technology, which can deliver genes efficiently and perform high efficacy with high safety in humans. Upon such a demand, HVJ (Hemagglutinating Virus of Japan)-liposome vectors were developed and then a further improved vector system called HVJ-envelope (HVJ-E) technology was innovated (Kaneda *et al.*, 2002) in order to overcome the deficiencies of both viral and other current non-viral vector systems.

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CURRENT GENE THERAPY VECTORS FOR CARDIOVASCULAR DISEASE

Since gene therapy emerged as a new approach to the treatment of cardiovascular disease in the late 1980s and early 1990s (Swain 1989; Nabel *et al.*, 1991), some promising results from gene therapy clinical trials of cardiovascular diseases have been reported recently, which are summarized in (Table **1**).

Diseases and Target Genes

A majority of the reported clinical trials, 15 clinical trials out of the 19 clinical trials listed in (Table **1**), focused on therapeutic angiogenesis for IHD or LEI caused by coronary artery disease (CAD) or peripheral artery disease (PAD). The early Phase I and Phase I/II clinical trials, using VEGF165 (Losordo *et al.*, 1998; Vale *et al.*, 2000; Huwer *et al.* 2001; Lathi *et al.* 2001; Sarkar *et al.*, 2001; Freedman *et al.*, 2002), VEGF121 (Rosengart *et al.*, 1999; Rajagopalan *et al.* 2001; Rajagopalan *et al.*, 2002), VEGF167 (Huwer *et al.*, 2001),

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Table 1. Clinical Trials of Gene Therapy for Cardiovascular Diseases

Disease Indication	Target Gene	Vector	Delivery	Clinical Trial	References
Angina (CMI)	VEGF165	Plasmid DNA	Intra-myocardial injection, invasive surgery.	Phase I (5 patients, OL); Phase I (13 patients, OL); Phase I (30 patients, OL); Phase I (7 patients, OL).	Losordo *et al.*, 1998; Vale *et al.*, 2000; Lathi *et al.*, 2001; Sarkar *et al.*, 2001.
PAD	VEGF165	Plasmid DNA	Intra-muscular injection	Phase I (34 patients).	Freedman *et al.*, 2002.
Angina (CAD)	VEGF121	Recombinant adenovirus	Intra-myocardial injection, invasive surgery (with CABG for Phase IA)	Phase II (71 patients, DB, R); Phase IA/IB (15/6 patients, OL).	Stewart 2002; Rosengart *et al.*, 1999.
IC or RP (PAD)	VEGF121	Recombinant adenovirus	Intra-muscular injection	Phase I (6 patients, OL).	Rajagopalan *et al.*, 2001; Rajagopalan et al, 2002.
Angina (CMI)	VEGF-2	Plasmid DNA	Intra-myocardial injection by catheter.	Phase I (6 patients, SB); Phase I/II (19 patients, DB, R).	Vale *et al.*, 2001; Losordo *et al.*, 2002.
Angina (CAD)	VEGF165, VEGF167	Plasmid DNA	Intra-myocardial injection, invasive surgery.	Phase I (24 patients, OL).	Huwer *et al.*, 2001.
LLI	VEGF	Recombinant adenovirus or plasmid/liposome	Catheter intra-arterial infusion after PTA	Phase II (54 patients, DB, R).	Makinen *et al.*, 2002.
Angina (CAD)	FGF-4	Recombinant adenovirus	Single intra-coronary injection.	Phase I/II (79 patients, DB, R).	Grines *et al.*, 2002.
RP or TN (PAD)	FGF-1	Plasmid DNA	Intra-muscular injection	Phase I (51 patients, OL).	Comerota *et al.*, 2002.
IC (PAD)	FGF-2	Plasmid DNA	Intra-arterial infusion	Phase II/III (190 patients, DB, R).	Lederman *et al.*, 2002.
Restenosis	Anti-c-myc	Single strand ODN	Intra-coronary local delivery after coronary stent implantation	Phase I/II (85 patients, DB, R).	Kutryk *et al.*, 2002.
Vein graft failure of PABG or CABG	E2F-decoy	Double strand ODN	*Ex-vivo* treatment of vein grafts prior to CABG	Phase I/II (41 patients, DB, R); Phase II (200 patients, DB, R).	Mann *et al.*, 1999; Terashima *et al.*, 2002.
Homozygous familial hypercholesterilemia	LDLR	Recombinant retrovirus	*Ex-vivo* primary hepatocyte transduction and implantation back to liver.	Phase I (5 patients, OL).	Raper *et al.*, 1997.

CMI indicates chronic myocardial ischemia; PAD, peripheral artery disease; CAD, coronary artery disease; IC, intermittent claudication; RP, rest pain; TN, tissue necrosis; LLM, lower-limb ischemia; PABG, peripheral artery bypass grafting; CABG, coronary artery bypass grafting; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; LDLR, low density lipoprotein receptor; ODN, oligodeoxynucleotide; PTA, percutaneous transluminal angioplasty; OL, open labeled; DB, double blind; SB, single blind; R, randomized.

VEGF-2 (Vale *et al.*, 2001; Losordo *et al.*, 2002), FGF-1 (Comerota *et al.*, 2002), FGF-2 (Lederman *et al.*, 2002) and FGF-4 (Grines *et al.*, 2002), demonstrated general safety in the therapeutic genes and the delivery procedures, and also promising indication in clinical efficacy. Two recent reports on double-blind randomized Phase II clinical trials, using VEGF genes to treat CAD patients (Stewart *et al.*, 2002; Makinen *et al.*, 2002), demonstrated statistical significant efficacy of the therapeutic angiogenesis gene therapy that warrants Phase III pivotal clinical trial.

Coronary restenosis, a vasoproliferative disease, was treated with antisense single-stranded oligodeoxynucleotides (ODN) anti-c-myc, targeting the cell cycle regulator c-myc, in a double blind and randomized phase I/II clinical trial (Kutryk *et al.*, 2002). Vein grafts were treated with intra-operative *ex-vivo* transfection of double-stranded ODN decoy for the DNA-binding site of E2F, a transcription factor necessary for the expression of genes that are involved in proliferation of smooth muscle cells, in a double blind and randomized phase I/II (Mann *et al.*, 1999) clinical trail for

peripheral artery bypass grafting and a phase II (Terashima *et al.*, 2002) clinical trial for coronary artery bypass grafting. The *ex-vivo* transfection of vein grafts with E2F ODN decoy for the artery bypass grafting was safe, feasible, and effective in ODN transfection of the vein grafts with potential therapeutic benefits on reduction of bypass-graft failure. Homozygous familial hypercholesterolemia was treated with low density lipoprotein receptor (LDLR) gene in a phase I clinical trial (Raper *et al.*, 1997).

There have also been many cardiovascular diseases under preclinical and clinical studies, demonstrating the potential of novel gene therapy remedies with different target genes. In the field of therapeutic angiogenesis, hypoxia inducible factor 1-α (HIF-1α) has entered phase I clinical trials for CAD and PAD patients (Rasmussen *et al.*, 2002). Hepatocyte growth factor (HGF) has also demonstrated angiogenic efficacy in preclinical studies and entered phase I clinical trials for PAD patients (Morishita 2002). The genes of nitric oxide synthases (iNOS and eNOS) (Chen *et al.*, 2002 & references therein), tissue factor pathway inhibitor (Yin *et al.*, 2002), anti-monocyte chemoattractant protein-1 (Usui *et al.*, 2002) and C-type natriuretic peptide (Ohno *et al.*, 2002) have been tested in preclinical studies to prevent restenosis after coronary intervention (Rutanen *et al.*, 2002 & references therein). The genes of anti-monocyte chemoattractant protein-1 (Inoue *et al.*, 2002), heme oxygenase-1 (Juan *et al.*, 2001) and dominant-negative Rho-kinase (Morishige *et al.*, 2001) have been tested in various animal models for the treatment of hypercholesterolemia and arteriosclerosis (Kawashiri and Rader, 2000 & references therein). The genes of prostacyclin synthase (Suhara *et al.*, 2002), antisense angiotensin II type I receptor (Pachori *et al.*, 2002), antisense angiotensinogen (Makino *et al.*, 1998; Wang *et al.*, 2001), antisense β1-adrenergic receptor (Zhang *et al.*, 2000) and eNOS (Lin *et al.*, 1997; Champion *et al.*, 1999) have been tested for the treatment of hypertension. The genes of HGF (Miyagawa *et al.*, 2002), antisense phospholamban (Eizena *et al.*, 2000; del Monte *et al.*, 2002), and sarcoplasmic reticulum Ca^{2+}-ATPase (del Monte *et al.*, 2001) have been tested for the treatment of heart failure. Moreover, expression of KCNE3 gene, encoding a regulatory subunit of pore-forming potassium channel, in the left ventricular cavity of a guinea pig model shortened the QT interval of electrocardiogram, demonstrating the potential for treatment of cardiac arrhythmias and sudden cardiac death (Mazhari *et al.*, 2002; Zhao *et al.*, 2002). Over-expression of a G1 cell cycle regulator gene, cdk inhibitor p16INK4a, demonstrated the suppression of left ventricular hypertrophy in a rat model (Nozato *et al.*, 2002).

In addition to the target gene, the delivery method and the vector system are vital for the success of cardiovascular gene therapy.

Delivery Method

Most of the reported clinical trials, 16 clinical trials out of the 19 clinical trials, employed various *in-vivo* local delivery methods, such as intra-myocardial direct injection with invasive surgery (7 clinical trials) (Losordo *et al.*, 1998; Rosengart *et al.* 1999; Vale *et al.*, 2000; Lathi *et al.*, 2001; Sarkar *et al.*, 2001; Huwer *et al.*, 2001; Stewart *et al.*, 2002), intra-myocardial injection with catheter (2 clinical trials) (Vale *et al.*, 2001; Losordo *et al.*, 2002), local direct intra-muscular injection (3 clinical trials) (Rajagopalan *et al.*, 2001; Rajagopalan *et al.*, 2002; Freedman *et al.*, 2002; Comerota *et al.*, 2002), local intra-coronary delivery (2 clinical trials) (Grines *et al.*, 2002; Kutryk *et al.*, 2002), local intra-arterial infusion (2 clinical trials) (Lederman *et al.*, 2002; Makinen *et al.*, 2002). *Ex-vivo* delivery methods were used in 3 reported clinical trials. In the clinical trials of PREVENT (Mann *et al.*, 1999) and PREVENT II (Terashima *et al.*, 2002) for the treatment of vein graft failure, the ODN E2F-decoy was delivered to the vein grafts by *ex-vivo* pressure-mediated transfection prior to grafting of the CABG surgery. The LDLR gene was delivered to the autologous hepatocyte culture by *ex-vivo* transduction before implantation back to patients' liver in the phase I clinical trial to treat homozygous familial hypercholesterolemia (Raper *et al.*, 1997).

Although *ex-vivo* was the choice of delivery method for many early gene therapy clinical trials, it became less favorable for the later gene therapy clinical trials because most cardiovascular diseases need to be treated *in-vivo* and also because of the cost of individualized *ex-vivo* process and the difficulties in scaling-up the *ex-vivo* process for commercial manufacturing. In some cases, such as the *ex-vivo* transfection of vein graft immediately prior to CABG surgery (Mann *et al.*, 1999; Terashima *et al.*, 2002), it can be attractive and efficacious.

Because of toxicity and safety concerns, none of the clinical trials in (Table 1) used the *in-vivo* systemic delivery. However, in most cases effective local delivery requires specific procedures and delivery devices, such as invasive surgeries, catheters, imaging instruments, etc., which may cause additional complications of adverse incidents and are more costly. Development of targeting vector technology can make *in-vivo* systemic delivery safer, more effective and economically sound. At that time *in-vivo* systemic delivery may become a more attractive choice for cardiovascular gene therapy.

Vector System

The naked plasmid DNA or ODN was the most frequently used vector system in the reported gene therapy clinical trials on cardiovascular disease and the adenovirus was the choice of viral vector system. As listed in (Table 1), naked plasmid DNA or ODN was used by 13 clinical trials, replication-deficient recombinant adenovirus was used by 5 clinical trials, only one clinical trial used liposome and one clinical trial used replication-deficient recombinant retrovirus.

In preclinical studies, adeno-asociated virus (AAV) has been tested as the vector system to deliver therapeutic genes in a mouse ischemic heart model (Su *et al.*, 2002) and in a rat hind limb ischemia model (Shimpo *et al.*, 2002). It was also demonstrated that a lentivirus vector can successfully deliver genes into adult cardiac myocytes *in-vitro* and *in-vivo* (Martin *et al.*, 2002). In addition to the non-viral vector technologies, such as liposomes and cationic polymers, some physical treatments, such as *in-vivo* electroporation (Nakano *et al.*, 2001) and endovascular therapeutic ultrasound

(Amabile *et al.*, 2001), have demonstrated the enhancement of plasmid DNA delivery efficiency into tibialis anterior muscles and femoral arteries in animal models.

The prominent concerns in regards to the gene therapy vectors in clinical use are always the issue of safety, especially for the viral vector systems. That may be the reason for the majority of reported clinical trials to choose naked DNA or ODN as the vector system. The potential of generation of replication competent virus (e.g. replication competent retrovirus, replication competent adenovirus) during *in-vitro* packaging or *in-vivo* application, the potential of insertional mutagenesis and germline mutations by integrating viral vectors, such as retrovirus, AAV and lentivirus, the potential of acute and chronic toxicities of the viral components carried by the viral vectors, and the potential of adverse effects due to over expression or unspecific expression of transgenes in non-targeted tissues or organs are a few of the top concerns on the list of safety issues. On the other hand, efficiency of the gene delivery is the major challenge for naked DNA-based vector technology. Although many promising non-viral vectors and gene delivery-enhancing technologies, such as liposomes, *in-vivo* electroporation and ultrasound, have been developed; most of them are still in early preclinical studies except liposomes, which have been used in some early clinical trials. The emergence of many technical hurdles and safety-toxicity issues with clinical use of the non-viral vectors and gene delivery-enhancing technologies is largely responsible for slowing the development of these approaches.

An ideal vector system should combine the gene delivery efficiency of a viral vector and the safety profile of the naked DNA. The HVJ-liposome vector and HVJ-E non-viral vector are candidates of such ideal vector systems as described in the rest of this review.

HVJ-LIPOSOME VECTOR

Hemagglutinating virus of Japan (HVJ) or Sendai virus is a member of the murine paramyxovirus family, containing a single-stranded RNA virus genome with an envelope. The HVJ-envelope contains two glycoproteins, HN (hemagglutinating neuraminidase) and F (fusion protein) proteins, which possess hemagglutinating and fusion activity respectively (Fig. 1). These HVJ-envelope proteins are

- **Murine paramyxovirus discovered in Japan (1950s)**
- **Cell fusion activity (monoclonal Antibody, chromosome mapping)**
- **Viral genome : single-strand RNA (minus strand)**
- **Envelope proteins : two glycoproteins (F, HN)**
- **Diameter : 150-600nm (average 300nm)**

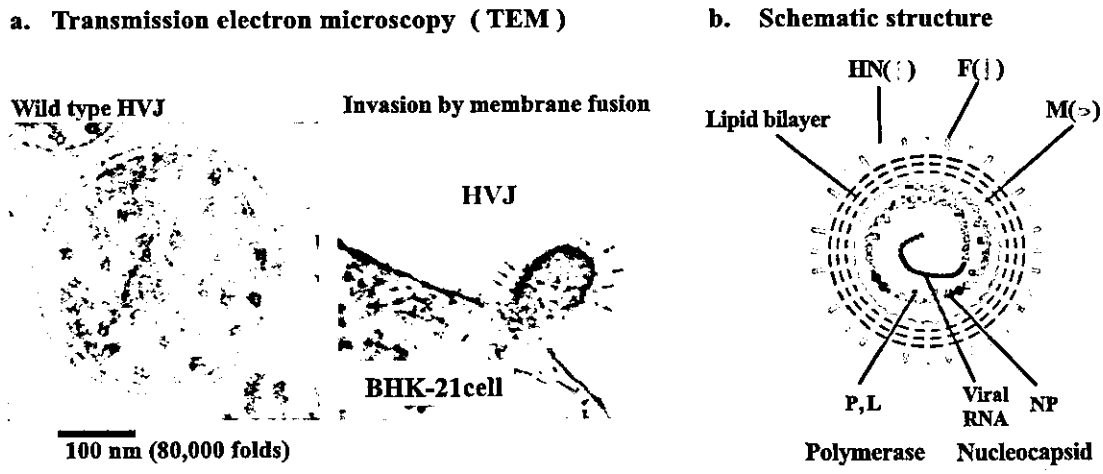

Fig. (1). Structure of hemagglutinating virus of Japan (HVJ)

a. Transmission electron microscopic observation of HVJ.

HVJ was discovered at Sendai Japan in 1952 as a pathogen for rodent animals, so it also called "Sendai virus" (box). HVJ is belongs to paramyxovirus group and its structure resembles influenza virus (upper panel). The envelope portion is a lipid bilayer derived from host cell membrane and dense materials inside envelope are nucleocapsid, which contains minus strand RNA genome and nucleocapsid protein (left panel). The average size of viral particles is 300 nm (left panel). HVJ is nonpathogenic for human, though it is able to infect human cells. The major character of HVJ is fusogenic activity (right panel). The spike proteins (F and HN) of viral envelope are indicated by arrow. Hybridoma cells producing monoclonal antibody is originally prepared using this activity by Dr. Köller and Dr. Milshtein in Cambridge University in 1970s. And this activity was also used for preparation of chimeric cells that were essential for chromosome mapping.

b. Schematic structure of HVJ

The viral particle of HVJ consists of three component, envelope, nucleocapsid and polymerase. Viral envelope is a lipid bilayer containing two glycoproteins: fusion (F) and hemagglutinating neuraminidase (HN) proteins. Nucleocapsid portion contains viral genome and nucleocapsid protein (NP). The virus particle contains two kinds of polymerases (P and L) and a matrix protein (M). The envelope portion of HVJ is used for the preparation of an HVJ-envelope vector. F and HN proteins are involved in the membrane fusion activity.

involved in cell fusion. HVJ virus is an enveloped large particle ranging from 300-600 nm in diameter. The viral particle is negatively charged and attaches to sialic acid (the HVJ receptor), fuses with cell membrane, and releases its genome into cytoplasm directly, rather than via the endocytosis.

HVJ-liposome gene transfer technology was developed in late 1980s (e.g. Kaneda *et al.*, 1987) and early 1990s (e.g. Tomita *et al.*, 1993; Morishita *et al.*, 1993) to introduce nucleic acid, ODN, and protein with high efficiently. The molecules included in HVJ-liposomes are delivered directly into various types of mammalian cells by means of the virus-cell fusigenic character of HVJ (Fig. 2) (Dzau *et al.* 1996). The first generation of HVJ-liposome was constructed by a combination of inactivated viral particles and multi- or uni-lamellar cationic liposomes to produce a non-viral gene transfer system. The HVJ-liposomes can deliver nucleic acids (e.g. Hirano *et al.*, 1998) or ODN (e.g. Morishita *et al.*, 1994) more efficiently than other non-viral vectors (e.g. liposomes). Moreover, the ODN delivered by HVJ-liposome were accumulated in the nucleus rapidly and persisted up to 2 weeks, whereas liposome-mediated delivery of ODN did not result in nuclear accumulation and rapidly decayed within a few days (Morishita *et al.* 1994), demonstrating the advantage of fusigenic gene delivery over endocytotic gene delivery. With modification of liposome composition from cationic to anionic, the second generation HVJ-AVE (artificial viral envelope) liposome showed a 5- to 10-fold higher gene expression in liver and muscle than the first generation HVJ-liposome vector. In addition, the high level of gene expression in muscle delivered by HVJ-AVE persisted as long as 30 days (Saeki *et al.* 1997). Delivered by HVJ-AVE liposome, the Fas-ligand protected the liver transplantation in rats from graft rejection for 20 day (Li *et al.*, 1998) similar to the protection achieved by adenovirus-delivered Fas-ligand (Okuyama *et al.*, 1998), implying the delivery efficiency of HVJ-AVE liposomes in liver was comparable to that of adenoviral vector. A more recent development of the HVJ-liposome technology was the reconstituted HVJ-fusion liposomes (Suzuki *et al.*, 2000b), which reconstituted purified fusion proteins from the HVJ-envelope into liposomes and demonstrated the gene delivery efficiency comparable to the HVJ-liposomes both *in-vitro* and *in-vivo*.

The HVJ-liposome system has exhibited therapeutic potential in various animal models for different disease indications such as liver cirrhosis (Ueki *et al.*, 1999), arthritis (Tomita *et al.*, 1999), transplantation rejection (Li *et al.* 1998) and cancer (Zhou *et al.*, 1999). More extensively HVJ-liposome technology has been tested as the vehicle for delivery of genes and ODNs in a variety of cardiovascular diseases, including vein graft failure (Suzuki *et al.*, 1997a; Matsumoto *et al.*, 1998; Mann *et al.*, 1995; Suzuki *et al.*, 2000a), restenosis (Morishita *et al.*, 1993; Morishita *et al.*; 1994, Morishita *et al.*, 1995; Yonemitsu *et al.*, 1996; Yonemitsu *et al.*, 1997; Morishita *et al.*, 1998; Aoki *et al.*, 1999; Morishita *et al.*, 2000), hypertension (Tomita *et al.*, 1993; Tomita *et al.*, 1995; Nakamura *et al.*, 1999),

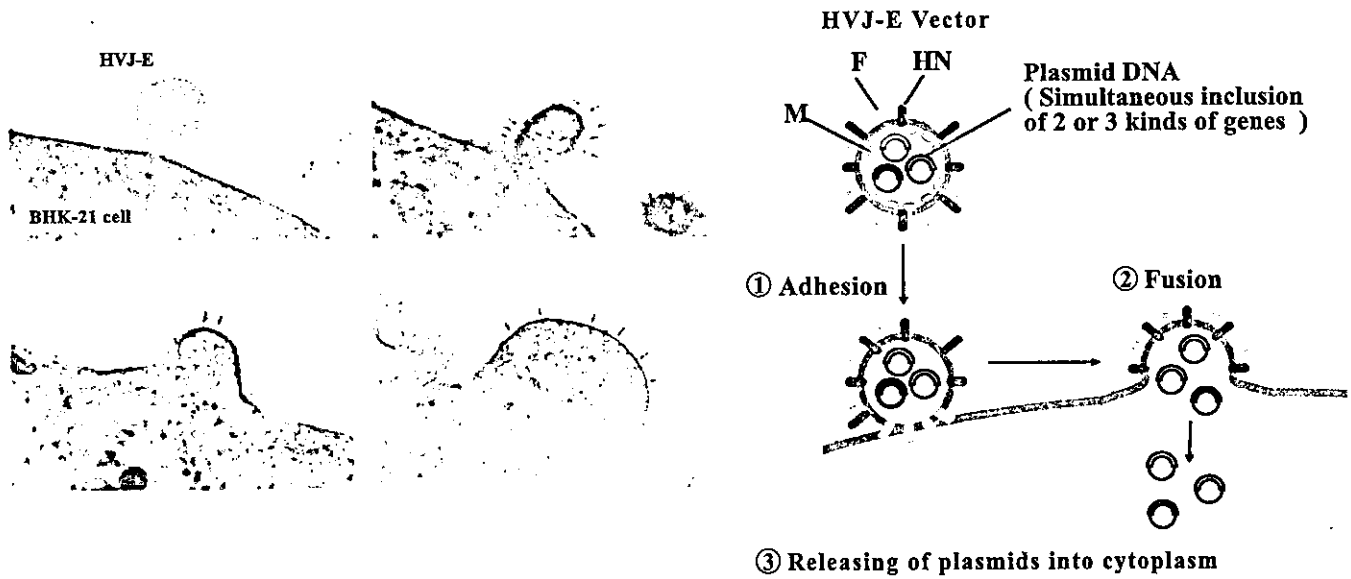

Fig. (2). Mechanism of gene transfer mediated by HVJ-E vector

a. Transmission electron microscopic observation of membrane fusion between HVJ and target cell (BHK-21).
The fusogenic activity of HVJ is utilized for the delivery of incorporated materials. Similar to wild type virus, HVJ envelope vector attaches to the cell surface and directly injects the incorporated materials into target cell cytoplasm (BHK-21). The reaction occurs within a few seconds.

b. Simultaneous gene transfer by HVJ-E vector
Direct injection of incorporated materials by membrane fusion permits the simultaneous gene transfer to identical target cells. Immediately after the attachment of HVJ-E particle containing two or three kinds of plasmid vector to the target cells, membrane fusion between vector and target cell occurs and plasmid DNAs are released into the cytoplasm of target cells.

myocardial protection (Suzuki *et al.*, 1997b; Sawa *et al.*, 1997; Sawa *et al.*, 1998; Suzuki *et al.*, 1999) and therapeutic angiogenesis (Aoki *et al.*, 2000).

Remarkably, the HVJ-liposome vectors could be administered repeatedly into rat liver without decreasing the level of gene expression, implying low immunogenicity and low pathogenicity (Hirano *et al.*, 1998). A safety study with repetitive intramuscular administration and single intravenous injection into cynomologus monkeys demonstrated the safety, feasibility, and therapeutic potential of the HVJ-AVE liposome vector for humans (Tsuboniwa *et al.*, 2001).

HVJ ENVELOPE (HVJ-E) VECTOR TECHNOLOGY

In the course of developing a vector technology for *in-vivo* gene delivery with high efficiency and low toxicity, which are critical to the success of therapeutic goals, HVJ-liposome hybrid vector has been utilized successfully in many preclinical studies as mentioned above. However, compared to wild type HVJ viruses, the HVJ-liposome has lower fusion activity probably due to the dilution of HVJ-envelope proteins by hybridizing with liposomes. In addition, there are substantial technical hurdles for the development of a scalable process to produce large quantity of the HVJ-liposomes in supporting a real clinical application.

The HVJ-E vector technology has been developed to overcome these hurdles (Kaneda *et al.*, 2002). In contrast to a recombinant HVJ viral vector (e.g. Yonemitsu *et al.*, 2000), the HVJ-E is a non-viral vector system that consists of an envelope derived from wild type HVJ virus by inactivation and purification processes (Fig. 3). Without the viral genome in the HVJ-E vector, there are no replication and viral gene expression in the cells transfected with the HVJ-E vector, whereas the recombinant HVJ viral vector replicates and expresses viral genes after its infection of cells as illustrated in (Fig. 4). A comparison of the characteristics between recombinant HVJ and HVJ-E vectors is listed in (Table 2). Virus replication and viral gene expression of the recombinant HVJ vector cause serious toxicity concerns and high immunogenicity, which make it less desirable for repeated administration of the recombinant HVJ vector. In contrast, when plasmid DNA carrying luciferase gene was delivered by HVJ-E in the mice, which had been immunized twice with HVJ-E vector, the luciferase expression in the immunized mice was as high as in the naïve mice, which were first time injected with luciferase-included HVJ-E (data not shown). It indicates that repeated administration is possible for the HVJ-E vector to deliver therapeutic genes.

Fusion between HVJ-E vector envelope and cell membrane, as shown in the transmission electron microscopy pictures of Fig. 2 (data not published), occurs within only 3-5 seconds immediately after the attachment of

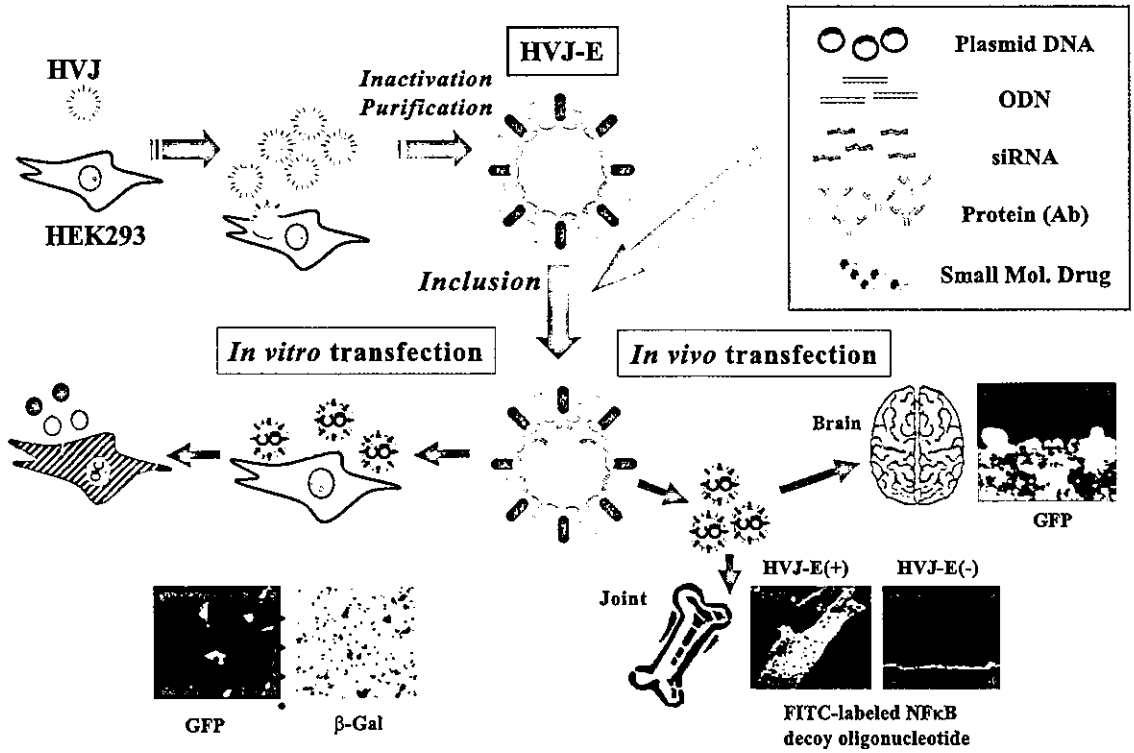

Fig. (3). Creation of HVJ-E vector with ability to transfect *in vitro* and *in vivo*.

HVJ particles are produced by human HEK293 cells. Empty HVJ envelop (HVJ-E) vector particles are prepared by inactivation of viral genome with chemical agent and removal of viral genome by purification. Various biomolecules, including plasmid DNA, oligonucleotides, protein and antibody, are incorporated into empty particles and used for transfection of many kinds of cells and organs. The left corner shows the BHK21 cells co-transfected with HVJ-E included GFP and β-Gal plasmid DNA, where both GFP and β-Gal expressed in the same cells. The right corner shows two *in vivo* HVJ-E transfection experiments: GFP expression in rat brain through carotid artery injection of EVJ-E included GFP plasmid; FITC-labeled NF-κB decoy double-stranded oligonucleotides penetrated into cartilage cells when included by HVJ-E. The major advantages of HVJ-E vector are summarized in the bottom text box.

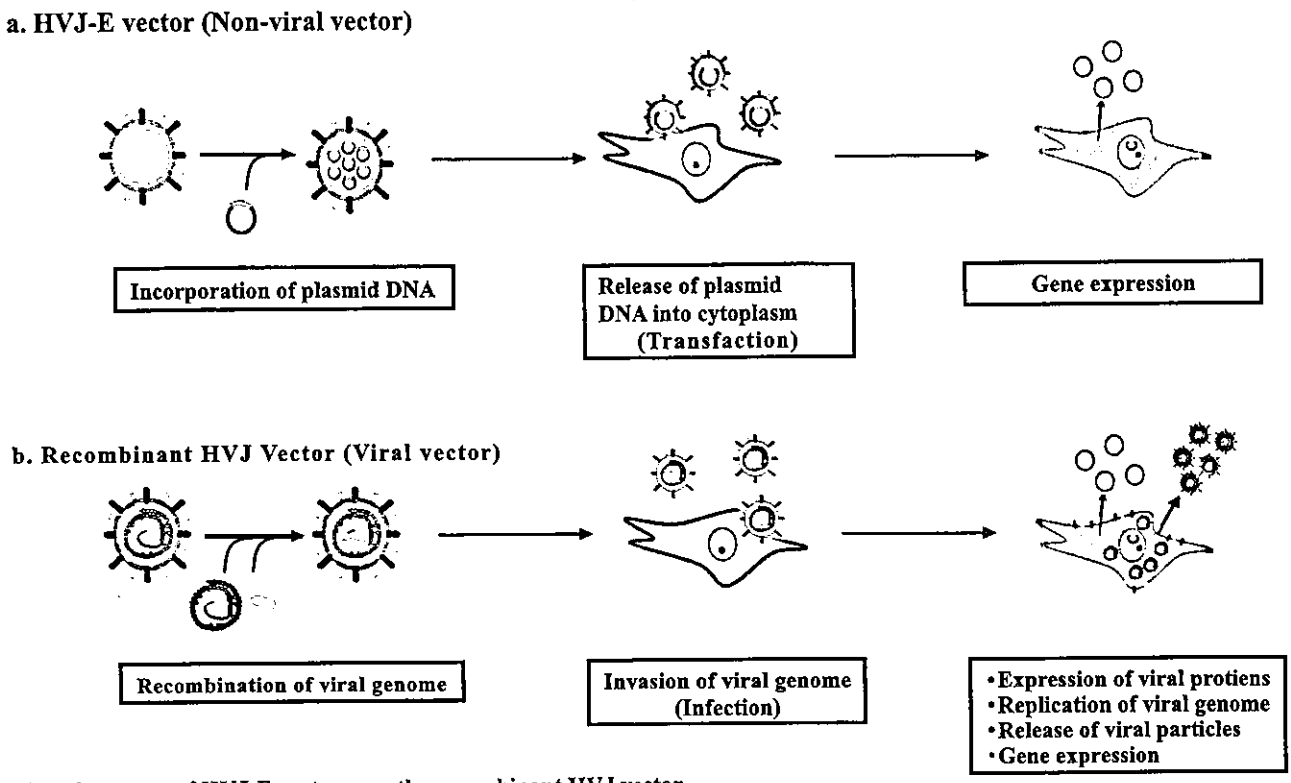

Fig. (4). Advantages of HVJ-E vector over the recombinant HVJ vector.
HVJ-E vector system has a lot of advantages over another type of vector using HVJ, the recombinant HVJ vector system. The recombinant HVJ vector system causes viral replication and production of viral proteins in target cells (lower panel). One viral protein, NP protein, is highly antigenic and strongly induces immune reaction *in vivo*. Therefore, the repeated injection of vector is difficult in case of recombinant HVJ vector system. So the major drawback of recombinant HVJ vector system is biosafety. In contrast, HVJ-E induces only the expression of transgene (upper panel) and can be used for the repetitive administration.

Table 2. Characteristics of HVJ-E and Recombinant HVJ Vectors

	HVJ-E	Recombinant HVJ
Replication of viral genome	No	Yes
Production of viral proteins	No	Yes
Release of virus particles	No	Yes
Toxicity	Low	Moderate
Immunogenicity	Low	High
Suitability for repeated administrations	Yes	Possible

the plasmid-containing HVJ-E vector to a cell surface. The plasmid was directly released into cytoplasm through the cell-HVJ-E fusion hole, but not through endocytosis. The plasmid is transported in cytoplasm, not taken into lysosomes. Thus the plasmid is not degraded by lysosomal enzymes, resulting in higher and more efficient gene expression in the host cells. Advantages of the HVJ-E vector technology are (1) rapid incorporation of therapeutic molecules into an envelope, eliminating recombinant DNA construction steps; (2) no viral replication and viral gene expression, eliminating the major safety concerns for viral vectors; (3) ability to include single therapeutic molecule entity as well as a mixture of different types of therapeutic molecular entities for combination therapies. Figure 5 shows that the HVJ-E vectors, containing GFP plasmid DNA, NF-κB decoy ODN, immunoglobulin G, and BSA respectively, introduced each molecule into cells at high efficiency (data not published).

HVJ-E vector can efficiently transfect various types of human and mammalian cells, such as BHK-21, SAS, HEK 293, HuH-7, K-562, as well as human aortic endothelial primary cells and rat aortic primary cells (Table 3, Kaneda *et al.*, 2002, and data not published). In animal studies, HVJ-E vectors deliver genes effectively in organs such as liver, brain, skin, uterus, tumor masses, lung and eye of animals including mouse, rat, rabbit and monkey (data not shown). The pictures in Fig. 3 (data not published) demonstrate high GFP expression in rat brain by administration of the HVJ-E via carotid artery and high transfection of a decoy FITC-labeled ODN into a rat cartridge tissue by intra joint administration of the HVJ-E. These indicate the powerful penetration activity of HVJ-E vectors.

In comparison to HVJ-liposome and liposome of lipofectin, HVJ-E shares many favorable characteristics with HVJ-liposome, such as high level of transgene expression and low cytotoxicity, whereas liposome exhibits much higher cytotoxicity. Nevertheless, HVJ-E vector possesses higher fusion activity reflected in more rapid transfection time and requires much simpler preparation process reflected

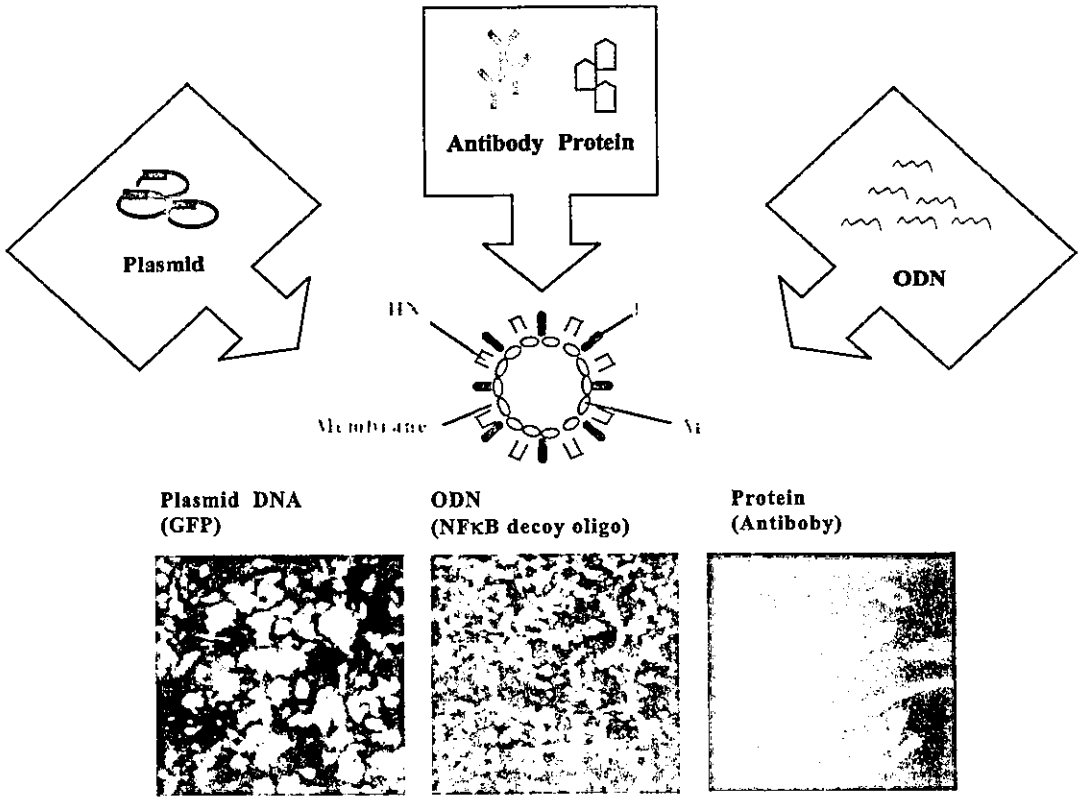

Fig. (5). HVJ-E vector as delivery system for various biomacromolecules
HVJ-E vector has a capability for delivering biomolecules and synthetic molecules with high molecular weight. Various kinds of biomolecules including plasmid DNA, antibody, enzyme, other proteins and oligonucleotide can be incorporated into the empty particles of HVH-E vector (upper panel). Lower pictures of fluorescence microscope demonstrate the transfection results of GFP expression vector (left), FITC-labeled oligonucleotides (center) and FITC-labeled antibody (right) delivered by HVJ-E vector. As shown in the pictures, over 90% of the target cells were transfected by HVJ-E vector.

Table 3. Transfection of Various Cells by HVJ-E

Cell type	Species	Source of cells	Transfection efficiency
Adherent cells			
HeLa	Human	Epitheoid carcinoma	+
293	Human	Primary embryonic kidney	+++
SAS	Human	Tongue squamous carcinoma	+++
HuH-7	Human	Hepatoma	+++
BHK-21	Hamster	Kidney	+++
Blood cells			
K-562	Human	Chronic myelogenous leukemia	++
CCRF-CEM	Human	Acute lymphoblastic leukemia	–
NALM-6	Human	T cell leukemia	+
Primary cells			
HAEC	Human	Aortic endothelial cells	++
RAC	Rat	Aortic cells	++

in the much shorter preparation time (Table 4, data not published).

Figure 6 illustrates a process for HVJ-E production. The HVJ-E is produced by cell culture followed by downstream processes, including inactivation, purification and inclusion of therapeutic molecules into the envelope particles. Wild type HVJ is produced in a suspension culture of cloned 293 cells in serum free medium in a bioreactor. The viral particles were collected and inactivated by the treatment with beta-propiolactone and then purified by column chromatography. The purified HVJ-E particles were treated with a mild detergent and then mixed with the molecules of interests for inclusion. The included HVJ-E vectors are further purified with a buffer exchange into final formulation

Table 4. Characteristics of Transfection Mediated by HVJ-E, HVJ Liposome, and Liposome (Lipofectin) (*In Vivo* and *In Vitro*)

	HVJ liposome	HVJ-E	Liposome (Lipofectin)
Gene expression level	+ _++	++ _+++	+ _+++
Homogeneity of gene expression	+++	+++	+
Cell Toxicity	-	-	++++
Time necessary for gene expression	16 hrs	16 hrs	48 hrs
Time necessary for transfection	2 hrs	5 min	4 _ 24 hrs
Capability of multiple gene transfection	+++	+++	+
Sample preparation time	4 hrs	15 min	40 min

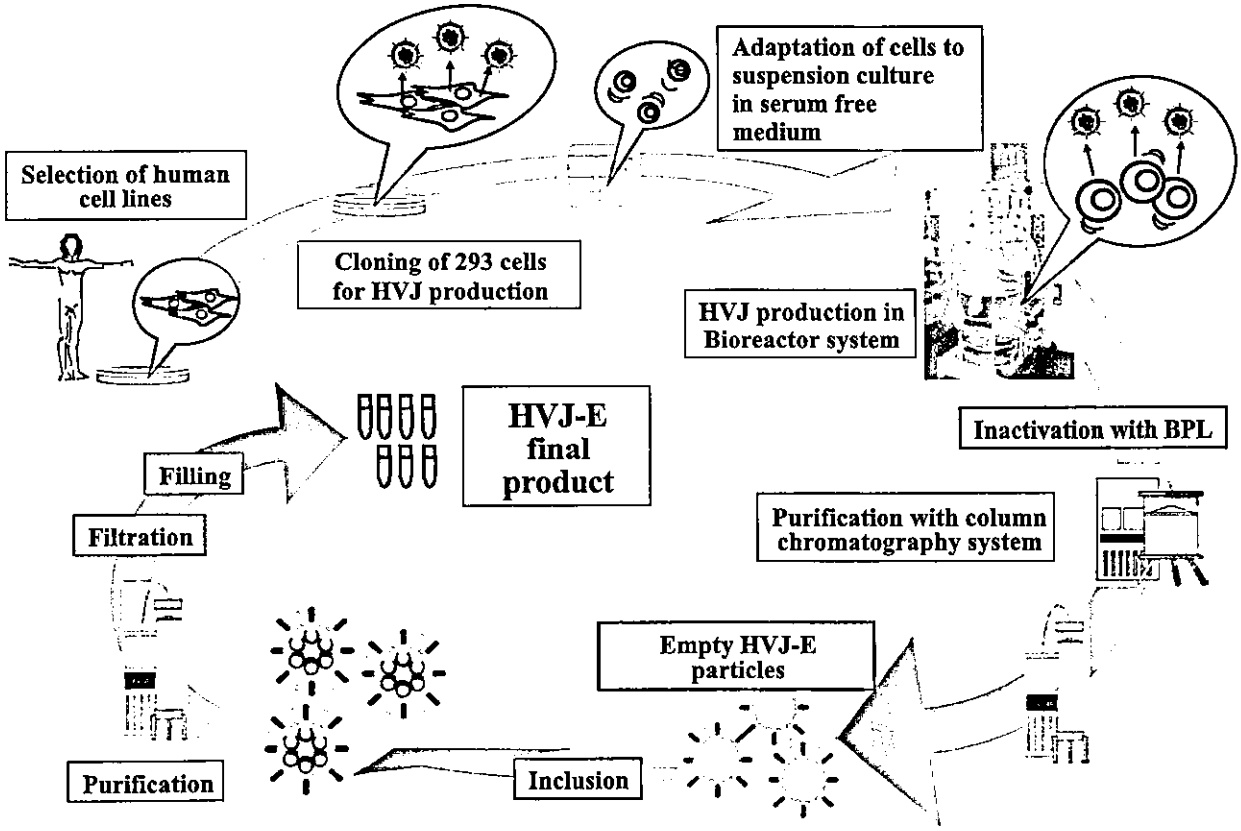

Fig. (6). Process development and manufacturing of HVJ-E vector for clinical application.
A GMP production process of HVJ-E vectors has been developed for clinical use such as treatment of cardiovascular diseases. After the screening of human cell lines suitable for GMP production, cloning of parental 293 cells was conducted. Cloned 293 cells have been adapted to serum-free/animal protein-free medium in suspension culture and used for HVJ production in stirred tank bioreactor. This automated bioreactor system is able to scale up to 100L or larger. After inactivation of the HVJ viral genome, the HVJ-E is purified by multiple steps of filtration and column chromatography to remove viral genome, viral proteins, host cell-derived proteins and host cell nucleic acids. After inclusion to incorporate various biomolecules, the biomolecule-included HVJ-E is further purified for removal of unincorporated materials, formulated, sterile-filtrated and subjected to final filling as HVJ-E final product.

buffer for either immediate application or storage. This is a scalable process that can meet future demands of large quantity HVJ-E production to supply real clinical applications.

With the versatility in inclusion of a wide range of different molecules and high transfection efficiency into a variety of cells and tissues both *in-vitro* and *in-vivo*, the HVJ-E vector technology not only can deliver various therapeutic molecular entities, such as therapeutic genes, ODNs or proteins, but can play an important role in functional genomics and proteomics, as well as in high throughput drug screening for the discovery of new target

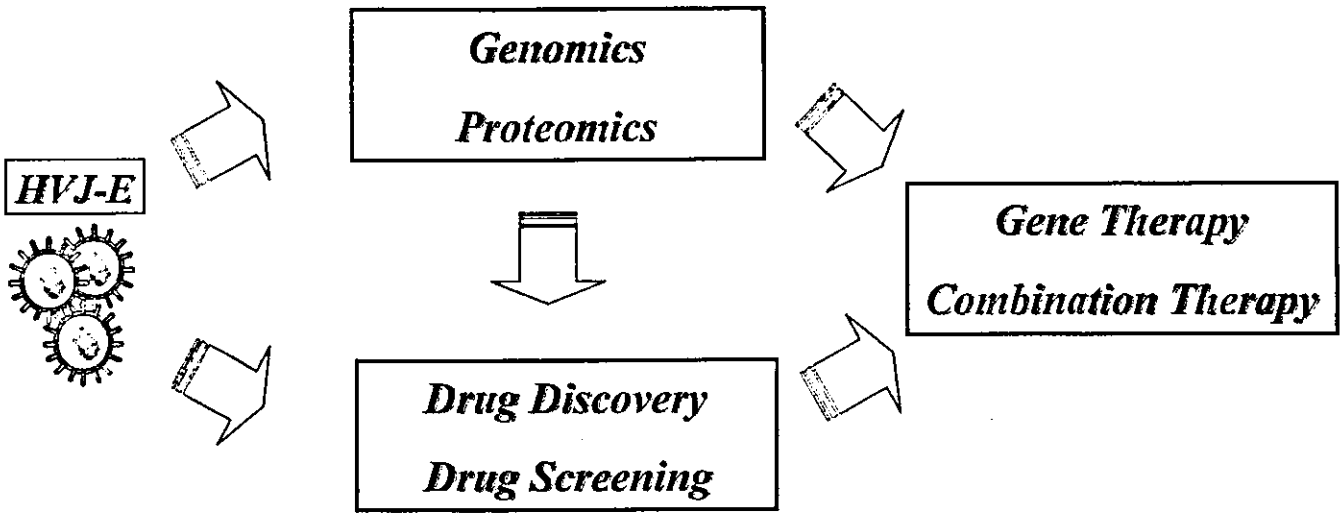

Fig. (7). Application of HVJ-E non-viral vector technology

HVJ-E non-viral vector system is useful tool for two fields, basic science and drug development. For genomics and proteomics analyses, cell array system (or vector array system) using HVJ-E vector in solid phase is under development (upper box). Drug delivery system (DDS) using HVJ-E vector is also developed in parallel (lower box). HVJ-E non-viral vector will become a tool for drug discovery and drug screening, since it could be used for both *in vivo* and *in vitro* delivery of various kinds of molecules including conventional drugs.

genes and new drugs (Fig. 7). As an emerging novel delivery system with no precedent case of clinical applications, systemic safety and toxicology studies are required for the clinical use of HVJ-E. Nevertheless, delivery by HVJ-E possibly allows repeated administration of therapeutic genes or therapeutic molecules and results in more persistent gene expression in comparison to other gene delivery technologies, the HVJ-E vector technology has the potential being not only safer but also more efficacious for the treatment of cardiovascular disease, as well as many other clinical applications.

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