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Hemagglutinating Virus of Japan Envelope Vectors as High-Performance Vehicles for Delivery of Small RNAs

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Abstract

Hemagglutinating virus of Japan envelope (HVJ-E) vectors are particulate forms of the Sendai virus, and are characterized by maintained cell membrane fusion activities and completely inactivated genomes. HVJ-E vectors can be safely used as a non-viral transfection tools for laboratory research without the need for special protocols or equipment. HVJ-E particles are loaded with molecules such as DNA, proteins, antisense oligonucleotides, or small RNAs, to form HVJ-E vectors that carry these molecules into target cells by virtue of their membrane fusion activity. Interference by small RNAs such as small interfering RNA (siRNA) and microRNA (miRNA) is now established as an important biological strategy for gene silencing, and is becoming an essential method for analyzing biological processes. Various delivery reagents are currently available globally; however, delivery to non-adherent immune cells, particularly primary immune cells, remains extremely difficult. The simple and effective delivery capabilities of HVJ-E vectors overcome the above obstacle. Here we describe examples and demonstrate the utility and potential applications of HVJ-E vectors as high-performance vehicles for delivery of small RNAs.

Keywords: HVJ envelope vecto; Sendai virus; Cell membrane fusion; Non-viral vector, small RNA delivery; siRNA; miRNA

Introduction

RNA interference (RNAi) is a well-known endogenous mechanism for gene regulation, and was originally identified in *Caenorhabditis elegans* by Fire et al. in 1998 [1]. The RNAi pathway is found in many eukaryotes including animals and is initiated by the enzyme Dicer, which cleaves long double-stranded RNA (dsRNA) molecules into short double-stranded molecules containing approximately 20 nucleotides. These are known as small interfering RNAs (siRNAs). After internalization, siRNAs are incorporated into RNA-induced silencing complexes (RISC), in which the catalytic component of the RISC complex Argonaute-2 unwinds the siRNAs. The two strands of the siRNAs are referred to as passenger and guide strands; the passenger strand gets cleaved and the activated guide strand moves out and cleaves complimentary mRNA [2]. Powerful and specific gene silencing by siRNAs is becoming an essential strategy for analyzing biological processes, and has numerous applications in drug discovery, diagnosis, and medical therapy.

Numerous *in vitro* and *in vivo* siRNA delivery strategies have been developed to date [3-9], and their common fundamental aim is to efficiently localize siRNAs to functional sites of target cells. Generally, viral vectors are more efficient for gene delivery and expression than non-viral ones; however, viral vectors pose greater risks than non-viral vectors. Thus, viral vectors are generally not used for drug delivery, and instead non-viral vectors are used for delivering anticancer agents, synthetic oligonucleotides, antibodies, and small RNA molecules such as siRNA and microRNA (miRNA).

In the last 10 years, tremendous efforts have been made to develop therapeutic applications of gene silencing using non-viral vectors. Cationic lipids such as dioleoyl phosphatidylethanolamine (DOPE) form lipocomplexes with negatively charged siRNAs. These pH-sensitive liposomes escape from endosomes by fusing with endosomal membranes, and they are routinely used for the delivery of siRNA or plasmid DNA into mammalian cells *in vitro*. However, surface interactions of cationic liposomes with tumor cells produce an electrostatic binding-site barrier effect, inhibiting further association of delivery systems with tumor spheroids [10]. In addition, although cationic liposomes efficiently take up siRNA, limited success has been achieved with these systems in *in vivo* gene silencing, probably due to their intracellular stability, which results in failure to release enclosed siRNA. Also, their effectiveness is limited by their toxicity [7].

Polymeric nanoparticles are solid, biodegradable, colloidal systems that have been widely investigated as drug or gene carriers [11], and have been classified as either natural or synthetic polymers. Natural polymers for siRNA delivery include cyclodextrin, chitosan, and atelocollagen [12]. Synthetic polymers such as polyethylenimine (PEI), poly(dl-lactide-*co*-glycolide), and dendrimers have been extensively investigated [13]. PEI is commonly used for drug delivery because it can disrupt endosomal membranes through the proton–sponge effect [14]. Moreover, it has high transfection efficiency and has been widely studied for siRNA delivery [12-14]. PEI forms small and compact structures that spontaneously assemble polyplexes with negatively charged siRNA through a simple and rapid polycation process [12]. The resulting PEI–siRNA complexes protect siRNA from degradation by nucleases, resulting in prolonged half-lives. In addition, complete encapsulation of siRNA prevents off-target effects such as immune activation by toll-like receptor-dependent mechanisms [15]. However, the significant toxicity associated with PEI complexes has limited

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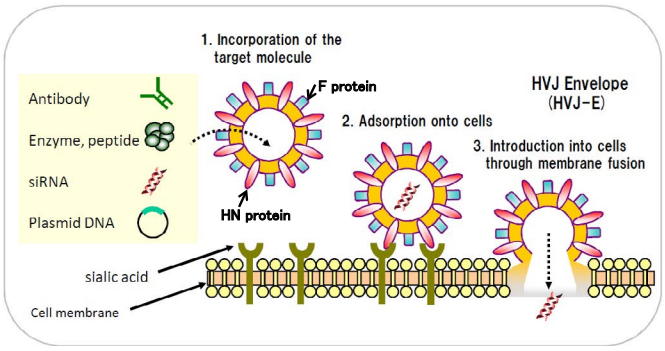
their assessment in clinical trials [7,16]. Molecular mechanisms of PEI cytotoxicity include membrane damage and activation of mitochondrial apoptotic programs following PEI-induced channel formation in outer mitochondrial membranes [7,17].

To overcome the limitations of each type of vector system, virosomes equipped with chimeric viral and non-viral vector apparatuses have been developed [18]. Initially, viral liposomes with fusogenic envelopes were constructed from hemagglutinating virus of Japan (HVJ) (Sendai virus) to compensate the limitations of one vector system with the advantages of another. This approach not only enables efficient drug delivery and gene expression but also reduces cytotoxicity of various vector components. Although virosomes have disadvantages, such as immunogenicity and instability in circulation, they have unique characteristics that may make them suitable for cancer therapy.

In this system, loaded DNA liposomes were fused with UV-inactivated HVJ to form fusogenic liposomes hybrids that had higher levels of trapped DNA than reconstituted virosomes, leading to direct transmission of their cargo to the cytoplasm by bypassing endocytotic uptake [19,20]. However, fusion of liposomes with inactivated HVJ requires a complicated procedure, and fusogenic abilities are reduced (approximately 2% relative to native HVJ) due to dilution of HVJ glycoproteins [21]. To address these issues, HVJ envelope (HVJ-E) vectors have been developed, in which the cargo, such as DNA, is incorporated directly into intact, though inactivated, HVJ (reviewed by Kaneda et al. [21-23]). HVJ-E vectors are unique non-viral vectors that carry viral membrane components with cell membrane fusion activities [24]. HVJ-E vectors have been shown to have superior performance in transferring genes into tissues of animals as well as into various cultured cell lines [25-31]. The vector kit employs delivery principles that are distinctively different from those of cationic non-viral vectors, which are aggressively marketed, and it enables effective intracellular delivery of negatively charged plasmid DNAs, low molecular weight nucleic acids (siRNA, miRNA, antisense/decoy oligonucleotides), and biologically active substances such as peptide proteins (including antibodies) with various electric charges (isoelectric points) [32-36]. These delivery principles (Figure 1), features, procedures, and application examples have been outlined in our previous publications [21,23,27,28,32], and the vector kit is commercially available in both Japan [37] and USA [38].

Various small RNA delivery reagents are currently available globally. However, delivery to non-adherent immune cells, particularly to primary immune cells, remains extremely difficult [39]. In this context, a promising feature of HVJ-E vectors is that they offer a simple and effective delivery system, even to these cells. Numerous *in vitro* and *in vivo* examples of HVJ-E-mediated delivery of small RNAs have been reported since 2003 [40]. Indeed, HVJ-E vectors have been widely used to deliver a number of different molecules to various adherent and non-adherent cells *in vitro* (Table 1 and 2) and to various target organs and tissues *in vivo* (Table 3). In this study, we describe examples of *in vitro* and *in vivo* delivery of small RNAs, and their utility and potential applications as transfection tools.



1. siRNA is incorporated into HVJ-E .
2. The HN proteins on HVJ-E bind receptor moieties of sialic acid on the membrane of the target cells.
3. The molecules (siRNAs) included in HVJ-E vectors are directly delivered into the target cells via the membrane fusion induced by the F proteins.

Figure 1: Delivery of molecules (siRNAs) into cells using HVJ-E vectors.

Delivery to Transfection-resistant Non-adherent Immune Cells

Delivery of siRNAs that target Eg5 and Cdc2 (Cyclin-Dependent Kinase 1; CDK1) has been performed in various non-adherent immune cells, including Jurkat, U-937, THP-1, HL-60, Raji, and K-562 cells, for which currently available lipofection reagents are ineffective. The kinesin-like motor protein Eg5 is essential for the formation of spindle microtubules during cell division, and impaired function of this protein leads to cell cycle arrest and apoptosis. Utilizing this phenomenon, the effects of siRNA delivery were assessed quantitatively using a viable cell count method (WST-8 assay). Cdc2 is known to form a complex with cyclin B and regulates the M phase of the cell cycle. Hence, knockdown of Cdc2 mRNA was determined using real-time PCR to assess the use of the vector kit (Table 1). These experiments showed extremely efficient intracellular delivery and high knockdown effects in various non-adherent immune cells. In contrast, although lipofection products allowed effective delivery to adherent HeLa S3 cells, with few exceptions, stable delivery in non-adherent immune cells was not achieved.

Numerous other examples of HVJ-E vector-mediated delivery of small RNAs have been reported *in vitro* (Table 2) and *in vivo* (Table 3) since 2003 [40]. Here we describe some examples of delivery to transfection-resisitant cells such as non-adherent immune cells.

Although autoantibodies to SS-A/Ro52 (Ro52) are frequently associated with autoimmune diseases such as Sjogren's syndrome, the physiological function of Ro52 is poorly understood. In 2003, Ishii et al. used HVJ-E vectors to deliver siRNA duplexes against SS-A/Ro52 (Ro52) into Jurkat T cells overexpressing Ro52, and produced the first evidence that Ro52 is involved in CD28-mediated production of interleukin (IL)-2 [41].

To address the significance of Brap2 expression in cytoplasmic p21 localization, Asada et al. used HVJ-E vectors to deliver Brap2 siRNA into U97/CB6-p21 cells [45].

Another study demonstrated essential regulation of total white blood cell numbers by the protein Bim in Bim-deficient mice. To examine whether the upregulation of Bim contributes to imatinib-induced apoptosis, K562 cells were transfected with Bim siRNA using HVJ-E vectors, resulting in effective rescue from imatinib-induced apoptosis. This result suggests that Bim plays an important apoptotic role in early hematopoietic progenitors [46].

Fichtner-Feigl et al. transfected J774 mouse macrophage-like cells with IL-13Rα2 siRNA using HVJ-E vectors, and showed blockade of TGF-β1 and collagen production in bleomycin-induced lung fibrosis [51].

To examine the effect of cathepsin G knockdown on the

Cell line[*1]	Target of siRNA[*2]	Evaluation method[*3]	HVJ-E[*4]		R Reagent[*5]		X Reagent[*6]	
			Silencing efficiency (%)	Viable cells (%)	Silencing efficiency (%)	Viable cells (%)	Silencing efficiency (%)	Viable cells (%)
(Suspension)								
Jurkat	Eg5	WST-8	81	84	31	91	6	80
U-937	Eg5	WST-8	93	83	4	100	6	100
THP-1	Eg5	WST-8	94	100	45	100	3	100
HL-60	Eg5	WST-8	63	89	0	100	4	97
Raji	Cdc2	RT-PCR	73	100	12	94	30	100
K-562	Cdc2	RT-PCR	85	98	81	100	52	100
(Adherent)								
HeLa S3	Eg5	WST-8	80	100	76	100	53	77

[*1]Jurkat: Human Acute T cell Leukemia; U-937: Human Histiocytic Lymphoma; THP-1: Human Acute Monocytic Leukemia; HL-60: Human Acute Promyelocytic Leukemia; Raji: Human Burkitt's Lymphoma; K-562: Human Chronic Myelogenous Leukemia; HeLa S3: Human Adenocarcinoma
[*2]Eg5: Silencer KIF11(Eg5) siRNA(Ambion, Code No.AM4639); Cdc2: Very High Potency Hs_CDC2 siRNA (QIAGEN, Cat. No. 1027273); Negative control #1 siRNA (Ambion, Code No.4611G)
[*3]WST-8: Cell Count Reagent SF (Nacalai, Code No.07553-44); RT-PCR: TaqMan Gene Expression Master mix (ABI, Cat No.4369016)
[*4]HVJ-E-mediated delivery was done using a kit (GenomONE-Si™)
[*5]Lipofectamine® RNAiMAX Transfection Reagent (Lifetechnologies, No.13778-075)
[*6]X-tremeGENE siRNA Transfection Reagent (Roche Applied Science, No.4476093)

Table 1: Delivery of siRNA to non-adherent immune cell strains (Comparison with lipofection reagents).

Ref. NO	Cells	Origin	Target genes
[40]	MIN6	Mouse pancreatic βcell	GPR40
[41]	Jurkat	Human acute T cell leukemia	SS-A/Ro52
[42]	C2C12 Mouse	myoblast(differentiated)	Bin1
[43]	primary monocyte	Human monocyte	Caveolin-1
[44]	primary cardiac myocyte	Rat cardiac myocyte	phospholamban
[45]	U937 Human leukemic	monocyte cell	Brap2
[46]	K562	Human chronic myelogenous leukemia	Bim
[47]	HUVEC	Human umbilical vein endothelial cell	TSAd
[48]	CMK6G3 Monkey ES cell	Monkey ES cell (stably expresses EGFP)	EGFP
[49]	primary monocyte	Human monocyte	Tollip, IRAK-
[50]	HuH-6, HuH-7, HepG2	Human hepatoblastoma cell Human hepatocecellular carcinoma cell	β-catenin
[51]	J774	Mouse macrophage cell	IL-13 Receptor α2
[52]	MIN6	Mouse pancreatic β cell	GPR40
[53]	primary T cell	Human peripheral blood	Human CARMA1
[54]	Primary calvarial osteoblasts	Mouse calvarial osteoblasts	OPG
[55]	primary mast cell	Mouse bone marrow-derived	GATA-1,GATA-2
[56]	INS-1E, NIH-3T3	Rat β cell, Mouse embryonic fibroblast	Sox6
[57]	primary macrophage	C3H mouse peritoneal resident	Mcl-1
[58]	primary granulosa cell	Mouse granulosa cell	Snap25
[59]	primary granulosa cell	Rat granulosa cell	TACE/ADAM17
[60]	BASMC	Bovine aortic smooth muscle cell	Bovine TE(tropoelastin)
[61]	U937	Human leukemic monocyte cell	Human cathepsin G
[62]	A549, H1299,TE13, PCNA-1, MIAPaCa-2, Du-145, ME-180	Human carcinoma (lung, esophageal, pancreas, prostate, cervical)	Ku80
[63]	HMVEC-dLyNeo(LEC)	Human neonatal dermal lymphatic microvascular endothelial cell	TLR4
[64]	U251MG, D54MG	Human glioma(p53 mutated at codon 273) Human glioma(p53 wild type)	Survivin, p53
[65]	granulosa cell	Mouse granulosa cell	TLR2, TLR4
[66]	granulosa cell	Mouse granulosa cell	Mkp3
[67]	COLO201	Human colon cancer	FKBP51
[68]	alveolar type II epithelial cells	Mouse alveolar type II epithelial cells	Nedd4, Nedd4-2
[69]	primary alveolar type2 cell	mouse lung alveolar type2 cell	LPCAT1
[70]	Primary neonatal cardiac ventricular myocyte	Rat primary neonatal cardiac ventricular myocyte	TFAM,TFB2M
[71]	U937	Human leukemic monocyte cell	VEGFA*[1]
[72]	Mouse suprachiasmatic nucleus slice cultures	Mouse suprachiasmatic nucleus	Atf4
[73]	Human alveolar epithelial type Ilike (ATI-like) cells	Human alveolar epithelial	Nrf2
[74]	U937	Human leukemic monocyte cell	PP2ACα
[39]	U937	Human leukemic monocyte cell	Mk2
[75]	Human alveolar epithelial type Ilike (ATI-like) cells	Human alveolar epithelial	Nrf2
[76]	U937	Human leukemic monocyte cell	PP2ACα
[77]	Artery smooth muscle cells	Porcin e	ROCK2, MLCK, ZIPL, RORα
[78]	primary cardiomyocytes	Neonatal rat	HMGB1

[*1]miRNA

Table 2: siRNA transfection studies using the HVJ-E (*in vitro*).

Ref. No	Organ/Tissue	Host	Target genes	Delivery route	Dose
[62]	transplanted tumor (H1299)	BALB/C nude mouse	Ku80	intratumor injection	day0(1shot) or day 2, 4(2 shots)
[79]	transplanted tumor (HeLa)	SCID mouse	Rad51	intratumor injection	35 μg, Day 0, 2, 4
[85]	transplanted tumor (B16-F10)	Mouse	Rad51	intratumor injection	2.5 nmol, day 6,8,10
[89]	transplanted tumor (U-118MG)	Mouse	Eg5	intratumor injection	200 pmol, day7,10,13
[94]	transplanted tumor (colon26)	Mouse	bcl-2	intratumor injection	40 μg/1shot
[97]	transplanted tumor(A549)	Mouse	Nrf2	intratumor injection	day 1,2,3
[80]	submandibular gland	Rat	rCLCA(Ca2+-dependent clchannels), CFTR(cystic fibrosis transmembrane conductance regulator)	retrograde ductal injection	2 nmol, day 0
[83]	submandibular gland	Rat	CFTR (cystic fibrosis transmembrane conductance regulator)	retrograde ductal injection	2 nmol day 0
[81]	colon	Mouse	IL-13 Receptorα2	intrarectal injection	
[82]	colon	Mouse	IRF4	intrarectal injection	100 μg, day-2,1-,0,1
[87]	colon	Mouse	Toll like receptor(TLR)-2	intrarectal injection	1 mg
[86]	spinal cord	Mouse	GlyT1, GlyT2, GlyRα3	spinal cord injection	0.45 pmol
[88]	spinal cord	Mouse	GlyR α3	spinal cord injection	0.45 pmol
[100]	spinal cord	Mouse	Phospholipase C-related but catalytical inactive protein (PRIP)	spinal cord injection	0.45 pmol
[92]	brain	Rat	Ca(v)3.2	intrathecal(i.t) injection	9 μg/1shot
[98]	brain	Mouse	CD45	intracerebroventricular (ICV) injection	5 μg
[99]	brain	Mouse	GlyRα3 siRNA	intrathecal injection	
[96]	dorsal horn	Rat	NK1 receptor	intrathecal injection	45 pmol/1shot, day0
[51]	lung	Mouse	IL-13 Receptorα2	Intratracheal injection	100 μg, day0,2,4,6,8,10, 12, 14, 16, 18, 20, 22
[84]	inguinal and popliteal LN cells (enrich in lymphoblasts)	SJL/J mouse (*ex vivo*)	orphan nuclear receptor NR4A2	adoptive transfer experimental autoimmune encephalomyelitis (EAE) model in irradiated mice/ intraperitoneal injection of NR4A2 siRNA-treated LN cells	
[90]	spleen	Mouse	Histamine H4 Receptors	tail vein	50 μg
[91]	stomach	Mouse	Stat1	intraperitoneal injection	100 μg
[93]	uterus	Mouse	RCAS1	uterine cavity	
[95]	dorsal skin	Mouse	Int6	subcutaneous injection	40 μg

Table 3: siRNA transfection studies using the HVJ-E (*in vivo*).

degradation of 4-hydroxy-2-nonenal (HNE)-modified glyceraldehyde-3-phosphate (GAPDH), U937 cells were transfected with a cathepsin G-specific siRNA (CTSG siRNA) using HVJ-E vectors. The resulting specific knockdown of cathepsin G successfully demonstrated its role in the degradation of HNE-modified GAPDH [61].

Jafarifar et al. transfected miRNAs into U937 cells using HVJ-E vectors, and demonstrated roles of their targets in hypoxia-induced expression of vascular endothelial growth factor-A (VEGFA). These experiments demonstrated that miR-297 and -299 were endogenous negative regulators of VEGFA expression in human monocytic cells, and elucidated a new mechanism of VEGFA regulation by hypoxia [71].

Using HVJ-E vectors, Kobayashi et al. transfected siRNA against the serine/threonine phosphatase protein phosphatase 2A (PP2A) into U937 cells to investigate the role of PP2A on corticosteroid sensitivity in severe asthma. Knockdown of PP2A significantly blocked the inhibitory effects of dexamethasone on TNFα-induced IL-8 release in U937 cells [74,76].

Tietz and Berghoff failed to create an efficient knockdown of MAPK-activated kinase 2 (MK2) in U937 cells, despite transfecting siRNA and shRNA using various delivery reagents and methods [39]. Subsequently, with the use of HVJ-E vectors, these investigators successfully transfected siRNA into U937 cells and showed >90% reduction in MK2 expression. Moreover, analyses of cell viability showed 85% survival 48 h after transfection. These data lead to the conclusion that knockdown of MK2 using HVJ-E vectors is a simple and rapid method for transfection without the use of harsh conditions [39].

Vector Delivery to Primary Immune Cells

Delivery of miRNA and siRNA to primary immune cells, to which it is considered to be more difficult to deliver, was examined using HVJ-E vectors.

Delivery of miRNA to activated T cells

Primary mouse T cells were isolated from splenocytes of 7–12-week-old female BALB/c mice using the Pan T Cell Isolation Kit II (Miltenyi Biotec), and were co-stimulated with phorbol 12-myristate 13-acetate (PMA, 5 nM) and ionomycin (1 μg/ml). After 24 h, HVJ-E vectors were

used to deliver an miRNA mimic (Dharmacon RNAi Technologies) targeted at the housekeeping gene cyclophilin B (CyB) in primary T cells. After 48 h, real-time PCR showed high knockdown efficacy with extremely low miRNA doses (1 nM or less; Figure 2A). Concurrent Western blot analysis corroborated this potent inhibitory effect at the protein level (Figure 2B).

Delivery of miRNA to activated B cells

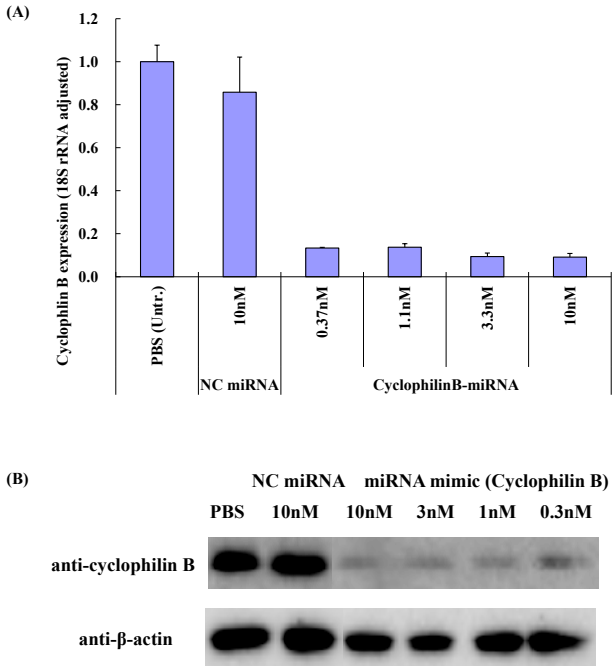
Primary mouse B cells were isolated from splenocytes of 7–12-week-old female BALB/c or C57BL/6 mice using the Pan B Cell Isolation Kit (Miltenyi Biotec), and were co-stimulated with anti CD40 antibody (1 μg/ml) and IL-4 (34.5 ng/ml). After 24 h, delivery of an miRNA mimic (Dharmacon RNAi Technologies) targeted at CyB was performed using HVJ-E vectors. Subsequent Western blot analysis at 48 h after transfection indicated a high inhibitory effect at the protein level (Figure 3A). Moreover, after stimulation of primary B cells with anti-CD40 antibody and lipopolysaccharide (1 μg/ml) a specific knockdown effect was observed (Figure 3B).

Delivery of siRNA to activated T cells

Delivery of CyB siRNA into primary T cells co-stimulated with PMA and ionomycin was performed using the same test system as described above, and mRNA levels were evaluated using real-time PCR. Again, mRNA knockdown was observed in a dose-dependent manner at 50 nM or less (Figure 4).

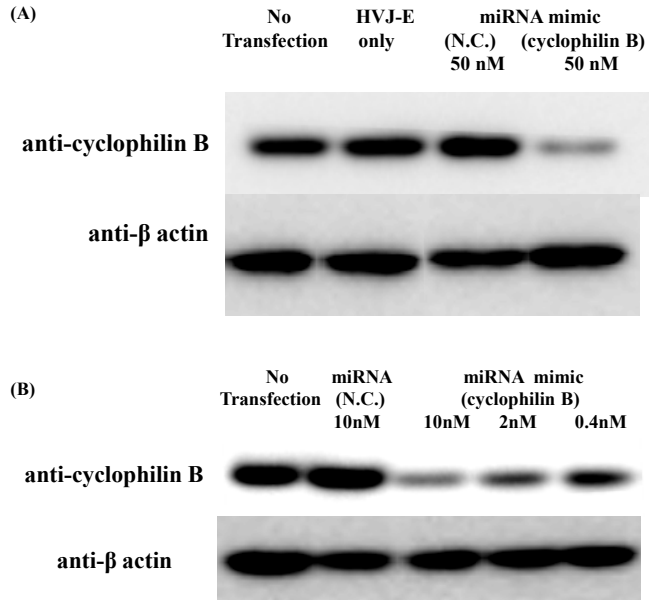
Delivery of siRNA to naive T cells

The effects of ZAP70 siRNA in mouse naïve thymocytes were evaluated using Western blot analysis. ZAP70 is a Syk family protein tyrosine kinase that forms a complex with T-cell antigen receptors and plays an important role in transducing downstream signals. Of 3 delivered ZAP70 siRNAs, 2 exerted inhibitory effects at the protein level (Figure 5).



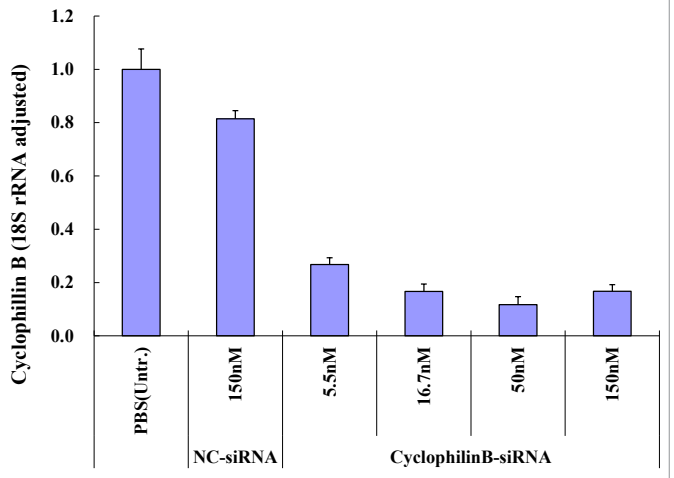
T cells isolated from murine splenocytes using the T Cell isolation kit were co-stimulated with PMA (5nM) and ionomycin (1 μg/ml). After 24 hrs, miRNA mimic targeted at cyclophilin B was transfected into the activated primary T cells using HVJ-E vectors. Real-time PCR evaluation (A) and western blot analysis (B) were performed at 48 hrs after the transfection

Figure 2: Delivery of miRNA into activated T cells using HVJ-E vectors.



B cells isolated from murine splenocytes using the B cell isolation kit were co-stimulated with CD40 antibody/IL-4 or CD40 antibody/LPS. After 24hrs, miRNA mimic targeted at cyclophilin B was transfected into the activated primary B cells using HVJ-E vectors. Western blot analysis in the B cells activated by CD40 antibody/IL-4 (A) or CD40 antibody/LPS (B) was performed at 48hrs after the transfection

Figure 3: Delivery of miRNA into activated B cells using HVJ-E vectors.

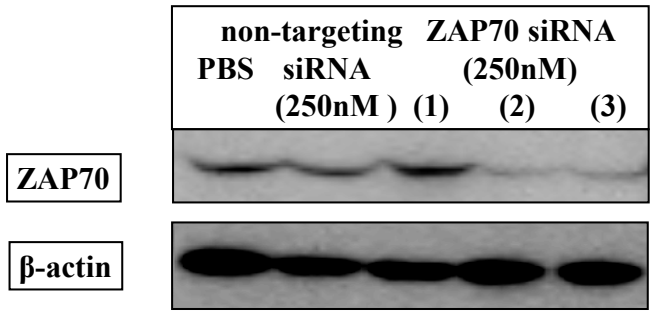


T cells isolated from murine splenocytes using the T Cell isolation kit were co-stimulated with PMA (5nM) and ionomycin (1 μg/ml). After 24 hrs, siRNA targeted at cyclophilin B was transfected into the activated primary T cells using the HVJ-E. Real-time PCR evaluation was performed at 48 hrs after the transfection

Figure 4: Delivery of siRNA into activated T cells using HVJ-E vectors.

Other important studies in primary immune cells

Using HVJ-E vectors, Ohnuma et al. transferred caveolin-1



Three kinds of siRNA sequences (1-3) selected as being specific to ZAP70 were transfected into naïve thymocytes using HVJ-E vectors. Western blot analysis was performed at 48hrs after the transfection.
Sequence of siRNA (1): GAAGCUGAAGGCGGAUGGACUAAUU
(2): CACGCUUAGCGUCAUCGACAGACAA
(3): GCCCUGGGUGCUGACGACAGCUAUU

Figure 5: Delivery of siRNA into naive T cells using HVJ-E vectors.

siRNAs into human peripheral monocytes to study the role of CD26–caveolin-1 interactions in tetanus toxoid (TT)-loaded monocytes. Their results suggested that the CD26-caveolin-1 interaction plays an important role in the up-regulation of CD86 in TT-loaded monocytes, and indicated subsequent engagement with CD28 on T cells [43]. They also identified Toll-interacting protein- and IL-1 receptor-associated serine/threonine kinase 1 (IRAK-1) as caveolin-1-interacting proteins in monocytes. Subsequent transfection of siRNAs against each target molecule into human peripheral monocytes demonstrated their function in signaling pathways of antigen-presenting cells [49]. Moreover, transfection of siRNAs into freshly isolated human T cells from healthy adult peripheral blood mononuclear cells demonstrated the role of CARD-containing MAGUK protein 1 on CD26-mediated T-cell co-stimulation. This study provided novel insights into the regulation of T-cell co-stimulation via CD26 [53].

Using HVJ-E vectors, Masuda et al. transfected GATA-1 and GATA-2 siRNAs into bone marrow-derived mast cells (BMMCs) that were isolated from femoral BM cells of BALB/c mice, and examined the role of GATA transcriptional factors in differentiated MCs. Delivery of these siRNAs into BMMCs resulted in markedly decreased levels of GATA-1 and GATA-2 proteins. Subsequently, they showed that repression of GATA activity leads to impaired cell survival and IgE-induced degranulation and cytokine production, indicating that GATA plays a critical role in the activation of MCs [55].

Delivery of siRNA using HVJ-E Vectors *in vivo*

Many *in vivo* examples of HVJ-E vector-mediated siRNA delivery have been reported since 2005 (Table 3).

Ito et al. [79] used HVJ-E vectors to deliver siRNAs against *Rad51*, which mediates homologous recombination in dsDNA break repair, and showed enhanced sensitivity to cisplatin (CDDP) *in vitro* and in *vivo*. 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.

In another notable study, 3 days after bleomycin challenge, Fichtner-Feigl et al. [81] administered HVJ-E-encapsulated IL-13Rα2-specific siRNAs by intra-rectal instillation (0.1 mg/instillation) into mice every second day for 24 days. On days 13 and 24, expression of IL-13Rα2 in lung tissue was greatly diminished, and TGF-β1 secretion and collagen formation were also significantly decreased. These data suggest that IL-13Rα2 signaling during prolonged inflammation is an important therapeutic target for prevention of TGF-β1-mediated fibrosis [51]. Additional studies of chronic 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis showed that fibrosis was dependent on the development of IL-13 responses that induced TGF-β1 via a novel cell surface-expressed IL-13 receptor [81].

Ishibashi et al. injected rCLCA siRNA (2 nmol) into submandibular glands of rats in a retrograde manner using HVJ-E vectors. After 48 hrs, significantly higher Cl^- levels and suppression of Cl^- channel proteins were observed on siRNA-injected sides. This report provided the first *in vivo* evidence for the physiological significance of rCLCA and CFTR in transepithelial Cl^- transport in ductal systems of rat submandibular glands [80]. They also, using HVJ-E vector-mediated siRNA delivery, revealed a significant involvement of apical P2Y2 receptor-regulated CFTR activity in rat submandibular gland [83].

Morita et al. [86-88], Kitayama et al. [100] used HVJ-E vectors to deliver siRNAs against Glycine transporter (GlyT) 1, GlyT2, glycine receptorα3 subunit (GlyRα3), protein phospholipase C-related but catalytically inactive protein (PRIP)-1 and PRIP-2 by injection into the subarachnoid space between the L_5 and L_6 vertebrate of mice. A peak of gene suppression occurred at 2-3 days post-injection, and this recovered to original levels approximately 8 days after injection. The results suggested that suppressed expression of PRIPs induced an elevated expression of K^+-Cl^--cotransporter-2 in the spinal cord, resulting in inhibition of nociception and amelioration of neuropathic pain in PRIP-1 and PRIP-2 double knockout mice.

Conclusions and Future Outlook

With the advent of next-generation sequencers, identification and detection of small RNAs undetectable using conventional methods have now become possible, and these small RNAs are being sequenced in various tissues from a variety of organisms. Although small RNAs that function in RNA silencing are roughly classified into 3 groups (siRNA, miRNA, and piRNA), novel small RNAs are expected to be discovered in the future. Hence, targeted delivery of small RNAs is essential for analyzing biological functions and screening their potential therapeutic targets. In this study, we showed that delivery of small RNAs using HVJ-E vectors resulted in high-performance knockdown in various cell types, including transfection-resistant cells such as non-adherent immune and primary immune cells. Numerous *in vivo* studies corroborate the utility of HVJ-E vectors as high-performance vehicles for delivery of small RNAs. HVJ-E vectors are expected to contribute profoundly to biological research, and promise applications in drug discovery and diagnosis as quick functional assessment tools for small RNAs.

HVJ-E vector itself induces anti-tumor immunity, including T cell-mediated and non-T cell-mediated immunity through multiple pathways (activation of natural killer cells, and dendritic cells, suppression of regulatory T cells through interleukin-6 secretion from dendritic cells). These activities may be desirable in the treatment of cancer, although these phenomena may adversely affect outcomes in other applications of HVJ-E, including siRNA delivery. Interestingly, 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 vectors. Using two different detergents at the incorporation step, it is possible to construct HVJ-E vectors with or without anti-tumor immunity [28].

However, HVJ-E vectors have HN protein in addition to F protein on their envelope membrane. The HN protein binds to its receptor (acetylated sialic acid) on the cell membrane. This protein is also involved in the agglutination of red blood cells. Namely, systemic administration of HVJ-E vector results in functional inactivation with hemagglutination in the blood. Therefore, it is advisable to select a route of administration involving less exposure to blood (direct injection to organs or tissue is recommended) or to perform perfusion of the animal prior to administration. To overcome the limitations of HVJ-E vector, HN-depleted HVJ was developed using HN-specific siRNA. LLCMK2 cells were transformed with HN-specific siRNA. The HVJ progeny produced from these cells lost HN but retained F protein. No hemagglutination was detected by the HN-depleted HVJ. Finally, by combining the construction of chimera containing the F protein and transferrin with the use of HN-specific siRNA, tumor-targeting HVJ-E vector without HN was developed [101]. Final goal is the systemic injection of tumor-targeting HN-depleted HVJ-E incorporating therapeutic molecules to various refractory cancers [28].

A clinical grade HVJ-E vector is currently being produced for use in clinical trials. Thus far, the virus has only been produced in chick eggs [24], however, egg-derived HVJ is difficult to use in clinical trials. It is also difficult to produce large amount of the virus in cultured cells. However, we recently succeeded in producing large amount of HVJ in human cells using animal product-free medium. Now we can produce more than 10^{10} particles/ml of culture medium of human cell-derived HVJ. A pilot plant to commercially produce clinical grade HVJ-E vector has already been established. Thus, a human cell-derived HVJ-E vector is now ready for clinical use [26,28].

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References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, et al. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806-811.
2. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, et al. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-498.
3. Juliano R, Alam MR, Dixit V, Kang H (2008) Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Res 36: 4158-4171.
4. Dominska M, Dykxhoorn DM (2010) Breaking down the barriers: siRNA delivery and endosome escape. J Cell Sci 123: 1183-1189.
5. Kanasty RL, Whitehead KA, Vegas AJ, Anderson DG (2012) Action and reaction: the biological response to siRNA and its delivery vehicles. Mol Ther 20: 513-524.
6. Sahay G, Querbes W, Alabi C, Eltoukhy A, Sarkar S, et al. (2013) Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. Nat Biotechnol 31: 653-658.
7. Lee JM, Yoon TJ, Cho YS (2013) Recent developments in nanoparticle-based siRNA delivery for cancer therapy. Biomed Res Int 2013: 782041.
8. van den Boorn JG, Schlee M, Coch C, Hartmann G (2011) SiRNA delivery with exosome nanoparticles. Nat Biotechnol 29: 325-326.
9. Lund PE, Hunt RC, Gottesman MM, Kimchi-Sarfaty C (2013) Pseudovirions as vehicles for the Delivery of siRNA. Pharm Res 27: 325-326.
10. Kostarelos K, Emfietzoglou D, Papakostas A, Yang WH, Ballangrud A, et al. (2004) Binding and interstitial penetration of liposomes within avascular tumor spheroids. Int J Cancer 112: 713-721.
11. Egusquiaguirre SP, Igartua M, Hernández RM, Pedraz JL (2012) Nanoparticle delivery systems for cancer therapy: advances in clinical and preclinical research. Clin Transl Oncol 14: 83-93.
12. Wang Y, Li Z, Han Y, Liang LH, Ji A (2010) Nanoparticle-based delivery system for application of siRNA in vivo. Curr Drug Metab 11: 182-196.
13. Yuan X, Naguib S, Wu Z (2011) Recent advances of siRNA delivery by nanoparticles. Expert Opin Drug Deliv 8: 521-536.
14. Wu ZW, Chien CT, Liu CY, Yan JY, Lin SY (2012) Recent progress in copolymer-mediated siRNA delivery. J Drug Target 20: 551-560.
15. Merkel OM, Beyerle A, Beckmann BM, Zheng M, Hartmann RK, et al. (2011) Polymer-related off-target effects in non-viral siRNA delivery. Biomaterials 32: 2388-2398.
16. Alameh M, Dejesus D, Jean M, Darras V, Thibault M, et al. (2012) Low molecular weight chitosan nanoparticulate system at low N:P ratio for nontoxic polynucleotide delivery. Int J Nanomedicine 7: 1399-1414.
17. Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, et al. (2005) A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Mol Ther 11: 990-995.
18. Kaneda Y (2005) Biological barriers to gene therapy. Polymeric Gene Delivery, CRC Press, Florida: 29-42.
19. Dzau VJ, Mann MJ, Morishita R, Kaneda Y (1996) Fusigenic viral liposome for gene therapy in cardiovascular diseases. Proc Natl Acad Sci U S A 93: 11421-11425.
20. Nakamura N, Hart DA, Frank CB, Marchuk LL, Shrive NG, et al. (2001) Efficient transfer of intact oligonucleotides into the nucleus of ligament scar fibroblasts by HVJ-cationic liposomes is correlated with effective antisense gene inhibition. J Biochem 129: 755-759.
21. Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, et al. (2002) Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. Mol Ther 6: 219-226.
22. Kaneda Y (2003) New vector innovation for drug delivery: development of fusigenic non-viral particles. Curr Drug Targets 4: 599-602.
23. Kaneda Y (2012) Virosome: a novel vector to enable multi-modal strategies for cancer therapy. Adv Drug Deliv Rev 64: 730-738.
24. Okada Y (1993) Sendai virus-induced cell fusion. Methods Enzymol 221: 18-41.
25. Kotani H, Nakajima T, Lai S, Morishita R, Kaneda Y (2004) The HVJ-envelope as an innovative vector system for cardiovascular disease. Curr Gene Ther 4: 183-194.
26. Kaneda Y, Yamamoto S, Nakajima T (2005) Development of HVJ envelope vector and its application to gene therapy. Adv Genet 53: 307–332.
27. Kaneda Y, Tabata Y (2006) Non-viral vectors for cancer therapy. Cancer Sci 97: 348-354.
28. Kaneda Y (2008) Applications of Hemagglutinating Virus of Japan in therapeutic delivery systems. Expert Opin Drug Deliv 5: 221-233.
29. Kitani A, Fichtner-Feigl S (2010) Efficient in vivo delivery of plasmids and oligonucleotides using hemagglutinating virus of Japan envelope (HVJ-E) vector in immunological disease models. Curr Protoc Immunol Chapter 10: Unit 10.
30. Zhang Q, Li Y, Shi Y, Zhang Y (2008) HVJ envelope vector, a versatile delivery system: its development, application, and perspectives. Biochem Biophys Res Commun 373: 345-349.
31. Lund PE, Hunt RC, Gottesman MM, Kimchi-Sarfaty C (2010) Pseudovirions as vehicles for the delivery of siRNA. Pharm Res 27: 400-420.
32. Kato M (2002) High-performance transfection kit HVJ Envelope vector kit (GenomONETM). Bio Clinica 17: 614-619.
33. Kato F, Yuki S, Kondo Y, Kato M (2003) HVJ envelope vector kit (GenomONE): Application as a biological function analysis tool by direct intracellular delivery of peptides or proteins. Medical Science Digest 29: 122-125.
34. Kondo Y, Miyata K, Kato F (2010) Efficient delivery of antibody into living cells using hemagglutinating virus of Japan (HVJ) envelope. Curr Protoc Immunol Chapter 2: Unit 2.
35. Kondo Y, Miyata K, Kato F (2010) Use of the hemagglutinating virus of Japan

(HVJ) envelope as a versatile delivery system for nucleic acids and proteins to leukocytes in vitro. Curr Protoc Immunol Chapter 10: Unit 10.

36. Kato F, Kondo Y, Yagi T, Fujieda T, Miyata K (2012) Transfection of small RNA (siRNA/miRNA) into suspension-cultured immune cells using GenomONE-Si (HVJ-E vector kit). Bio Clinica 27: 880-883.

37. http://www.iskweb.co.jp/hvj-e.

38. www.cosmobiousa.com.

39. Tietz SM, Berghoff M (2012) Gene silencing of MK2 in hard-to-transfect human U937 cells. J Biomol Tech 23: 47-50.

40. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, et al. (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422: 173-176.

41. Ishii T, Ohnuma K, Murakami A, Takasawa N, Yamochi T, et al. (2003) SS-A/Ro52, an autoantigen involved in CD28-mediated IL-2 production. J Immunol 170: 3653-3661.

42. Kojima C, Hashimoto A, Yabuta I, Hirose M, Hashimoto S, et al. (2004) Regulation of Bin1 SH3 domain binding by phosphoinositides. EMBO J 23: 4413-4422.

43. Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Yoshikawa N, et al. (2004) CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. Proc Natl Acad Sci U S A 101: 14186-14191.

44. Watanabe A, Arai M, Yamazaki M, Koitabashi N, Wuytack F, et al. (2004) Phospholamban ablation by RNA interference increases Ca2+ uptake into rat cardiac myocyte sarcoplasmic reticulum. J Mol Cell Cardiol 37: 691-698.

45. Asada M, Ohmi K, Delia D, Enosawa S, Suzuki S, et al. (2004) Brap2 functions as a cytoplasmic retention protein for p21 during monocyte differentiation. Mol Cell Biol 24: 8236-8243.

46. Kuribara R, Honda H, Matsui H, Shinjyo T, Inukai T, et al. (2004) Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. Mol Cell Biol 24: 6172-6183.

47. Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, et al. (2005) VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J 24: 2342-2353.

48. Takada T, Nemoto K, Yamashita A, Kato M, Kondo Y, et al. (2005) Efficient gene silencing and cell differentiation using siRNA in mouse and monkey ES cells. Biochem Biophys Res Commun 331: 1039-1044.

49. Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Iwata S, et al. (2005) CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells. Mol Cell Biol 25: 7743-7757.

50. Sangkhathat S, Kusafuka T, Miao J, Yoneda A, Nara K, et al. (2006) In vitro RNA interference against beta-catenin inhibits the proliferation of pediatric hepatic tumors. Int J Oncol 28: 715-722.

51. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A (2006) IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med 12: 99-106.

52. Feng DD, Luo Z, Roh SG, Hernandez M, Tawadros N, et al. (2006) Reduction in voltage-gated K+ currents in primary cultured rat pancreatic beta-cells by linoleic acids. Endocrinology 147: 674-682.

53. Ohnuma K, Uchiyama M, Yamochi T, Nishibashi K, Hosono O, et al. (2007) Caveolin-1 triggers T-cell activation via CD26 in association with CARMA1. J Biol Chem 282: 10117-10131.

54. Nakamichi Y, Udagawa N, Kobayashi Y, Nakamura M, Yamamoto Y, et al. (2007) Osteoprotegerin reduces the serum level of receptor activator of NF-kappaB ligand derived from osteoblasts. J Immunol 178: 192-200.

55. Masuda A, Hashimoto K, Yokoi T, Doi T, Kodama T, et al. (2007) Essential role of GATA transcriptional factors in the activation of mast cells. J Immunol 178: 360-368.

56. Iguchi H, Urashima Y, Inagaki Y, Ikeda Y, Okamura M, et al. (2007) SOX6 suppresses cyclin D1 promoter activity by interacting with beta-catenin and histone deacetylase 1, and its down-regulation induces pancreatic beta-cell proliferation. J Biol Chem 282: 19052-19061.

57. Kubota Y, Kinoshita K, Suetomi K, Fujimori A, Takahashi S (2007) Mcl-1 depletion in apoptosis elicited by ionizing radiation in peritoneal resident macrophages of C3H mice. J Immunol 178: 2923-2931.

58. Shimada M, Yanai Y, Okazaki T, Yamashita Y, Sriraman V, et al. (2007) Synaptosomal-associated protein 25 gene expression is hormonally regulated during ovulation and is involved in cytokine/chemokine exocytosis from granulosa cells. Mol Endocrinol 21: 2487-2502.

59. Yamashita Y, Kawashima I, Yanai Y, Nishibori M, Richards JS, et al. (2007) Hormone-induced expression of tumor necrosis factor alpha-converting enzyme/A disintegrin and metalloprotease-17 impacts porcine cumulus cell oocyte complex expansion and meiotic maturation via ligand activation of the epidermal growth factor receptor. Endocrinology 148: 6164-6175.

60. Saito E, Wachi H, Sato F, Sugitani H, Seyama Y (2007) Treatment with vitamin k(2) combined with bisphosphonates synergistically inhibits calcification in cultured smooth muscle cells. J Atheroscler Thromb 14: 317-324.

61. Tsuchiya Y, Okuno Y, Hishinuma K, Ezaki A, Okada G, et al. (2007) 4-Hydroxy-2-nonenal-modified glyceraldehyde-3-phosphate dehydrogenase is degraded by cathepsin G. Free Radic Biol Med 43: 1604-1615.

62. Nimura Y, Kawata T, Uzawa K, Okamura J, Liu C, et al. (2007) Silencing Ku80 using small interfering RNA enhanced radiation sensitivity in vitro and in vivo. Int J Oncol 30: 1477-1484.

63. Sawa Y, Ueki T, Hata M, Iwasawa K, Tsuruga E, et al. (2008) LPS-induced IL-6, IL-8, VCAM-1, and ICAM-1 expression in human lymphatic endothelium. J Histochem Cytochem 56: 97-109.

64. Saito T, Hama S, Izumi H, Yamasaki F, Kajiwara Y, et al. (2008) Centrosome amplification induced by survivin suppression enhances both chromosome instability and radiosensitivity in glioma cells. Br J Cancer 98: 345-355.

65. Shimada M, Yanai Y, Okazaki T, Noma N, Kawashima I, et al. (2008) Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. Development 135: 2001-2011.

66. Fan HY, Shimada M, Liu Z, Cahill N, Noma N, et al. (2008) Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. Development 135: 2127-2137.

67. Mukaide H, Adachi Y, Taketani S, Iwasaki M, Koike-Kiriyama N, et al. (2008) FKBP51 expressed by both normal epithelial cells and adenocarcinoma of colon suppresses proliferation of colorectal adenocarcinoma. Cancer Invest 26: 385-390.

68. Conkright JJ, Apsley KS, Martin EP, Ridsdale R, Rice WR, et al. (2010) Nedd4-2-mediated ubiquitination facilitates processing of surfactant protein-C. Am J Respir Cell Mol Biol 42: 181-189.

69. Bridges JP, Ikegami M, Brilli LL, Chen X, Mason RJ, et al. (2010) LPCAT1 regulates surfactant phospholipid synthesis and is required for transitioning to air breathing in mice. J Clin Invest 120: 1736-1748.

70. Watanabe A, Arai M, Koitabashi N, Niwano K, Ohyama Y, et al. (2011) Mitochondrial transcription factors TFAM and TFB2M regulate Serca2 gene transcription. Cardiovasc Res 90: 57-67.

71. Jafarifar F, Yao P, Eswarappa SM, Fox PL (2011) Repression of VEGFA by CA-rich element-binding microRNAs is modulated by hnRNP L. EMBO J 30: 1324-1334.

72. Koyanagi S, Hamdan AM, Horiguchi M, Kusunose N, Okamoto A, et al. (2011) cAMP-response element (CRE)-mediated transcription by activating transcription factor-4 (ATF4) is essential for circadian expression of the Period2 gene. J Biol Chem 286: 32416-32423.

73. Kosmider B, Messier EM, Chu HW, Mason RJ (2011) Human alveolar epithelial cell injury induced by cigarette smoke. PLoS One 6: e26059.

74. Kobayashi Y, Mercado N, Barnes PJ, Ito K (2011) Defects of protein phosphatase 2A causes corticosteroid insensitivity in severe asthma. PLoS One 6: e27627.

75. Kosmider B, Messier EM, Janssen WJ, Nahreini P, Wang J, et al. (2012) Nrf2 protects human alveolar epithelial cells against injury induced by influenza A virus. Respir Res 13: 43.

76. Kobayashi Y, Mercado N, Miller-Larsson A, Barnes PJ, Ito K (2012) Increased corticosteroid sensitivity by a long acting ß2 agonist formoterol via ß2 adrenoceptor independent protein phosphatase 2A activation. Pulm Pharmacol Ther 25: 201-207.

77. Saito T, Hirano M, Ide T, Ichiki T, Koibuchi N, et al. (2013) Pivotal role of Rho-associated kinase 2 in generating the intrinsic circadian rhythm of vascular contractility. Circulation 127: 104-114.

78. Funayama A, Shishido T, Netsu S, Narumi T, Kadowaki S, et al. (2013) Cardiac nuclear high mobility group box 1 prevents the development of cardiac hypertrophy and heart failure. Cardiovasc Res 99: 657-664.

79. Ito M, Yamamoto S, Nimura K, Hiraoka K, Tamai K, et al. (2005) Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin. J Gene Med 7: 1044-1052.

80. Ishibashi K, Yamazaki J, Okamura K, Teng Y, Kitamura K, et al. (2006) Roles of CLCA and CFTR in electrolyte re-absorption from rat saliva. J Dent Res 85: 1101-1105.

81. Fichtner-Feigl S, Fuss IJ, Young CA, Watanabe T, Geissler EK, et al. (2007) Induction of IL-13 triggers TGF-beta1-dependent tissue fibrosis in chronic 2,4,6-trinitrobenzene sulfonic acid colitis. J Immunol 178: 5859-5870.

82. Watanabe T, Asano N, Murray PJ, Ozato K, Tailor P, et al. (2008) Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. J Clin Invest 118: 545-559.

83. Ishibashi K, Okamura K, Yamazaki J (2008) Involvement of apical P2Y2 receptor-regulated CFTR activity in muscarinic stimulation of Cl(-) reabsorption in rat submandibular gland. Am J Physiol Regul Integr Comp Physiol 294: R1729-1736.

84. Doi Y, Oki S, Ozawa T, Hohjoh H, Miyake S, et al. (2008) Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. Proc Natl Acad Sci U S A 105: 8381-8386.

85. Kiyohara E, Tamai K, Katayama I, Kaneda Y (2012) The combination of chemotherapy with HVJ-E containing Rad51 siRNA elicited diverse anti-tumor effects and synergistically suppressed melanoma. Gene Ther 19: 734-741.

86. Morita K, Motoyama N, Kitayama T, Morioka N, Kifune K, et al. (2008) Spinal antiallodynia action of glycine transporter inhibitors in neuropathic pain models in mice. J Pharmacol Exp Ther 326: 633-645.

87. Boirivant M, Amendola A, Butera A, Sanchez M, Xu L, et al. (2008) A transient breach in the epithelial barrier leads to regulatory T-cell generation and resistance to experimental colitis. Gastroenterology 135: 1612-1623.

88. Morita K, Kitayama T, Morioka N, Dohi T (2008) Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice. Pain 138: 525-536.

89. Matsuda M, Yamamoto T, Matsumura A, Kaneda Y (2009) Highly efficient eradication of intracranial glioblastoma using Eg5 siRNA combined with HVJ envelope. Gene Ther 16: 1465-1476.

90. Matsuda N, Teramae H, Futatsugi M, Takano K, Yamamoto S, et al. (2010) Up-regulation of histamine H4 receptors contributes to splenic apoptosis in septic mice: counteraction of the antiapoptotic action of nuclear factor-kappaB. J Pharmacol Exp Ther 332: 730-737.

91. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, et al. (2010) NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J Clin Invest 120: 1645-1662.

92. Takahashi T, Aoki Y, Okubo K, Maeda Y, Sekiguchi F, et al. (2010) Upregulation of Ca(v)3.2 T-type calcium channels targeted by endogenous hydrogen sulfide contributes to maintenance of neuropathic pain. Pain 150: 183-191.

93. Tskitishvili E, Nakamura H, Kinugasa-Taniguchi Y, Kanagawa T, Shimoya K, et al. (2010) The effect of tumor-associated protein RCAS1 gene silencing on blood pressure and urinary protein excretion in pregnant mouse: a pilot study. Am J Obstet Gynecol 203: 364.

94. Fujioka T, Matsunaga N, Okazaki H, Koyanagi S, Ohdo S (2010) Hypoxia-response plasmid vector producing bcl-2 shRNA enhances the apoptotic cell death of mouse rectum carcinoma. J Pharmacol Sci 113: 353-361.

95. Chen L, Endler A, Uchida K, Horiguchi S, Morizane Y, et al. (2010) Int6/eIF3e silencing promotes functional blood vessel outgrowth and enhances wound healing by upregulating hypoxia-induced factor 2alpha expression. Circulation 122: 910-919.

96. Naono-Nakayama R, Sunakawa N, Ikeda T, Nishimori T (2011) Knockdown of the tachykinin neurokinin 1 receptor by intrathecal administration of small interfering RNA in rats. Eur J Pharmacol 670: 448-457.

97. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, et al. (2012) Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. Cancer Cell 22: 66-79.

98. Jin J, Lam L, Sadic E, Fernandez F, Tan J, et al. (2012) HIV-1 Tat-induced microglial activation and neuronal damage is inhibited via CD45 modulation: A potential new treatment target for HAND. Am J Transl Res 4: 302-315.

99. Motoyama N, Morita K, Kitayama T, Shiraishi S, Uezono Y, et al. (2013) Pain-releasing action of Platelet-activating factor (PAF) antagonists in neuropathic pain animal models and the mechanisms of action. Eur J Pain 17: 1156-1167.

100. Kitayama T, Morita K, Sultana R, Kikushige N, Mgita K, et al. (2013) Phospholipase C-related but catalytically inactive protein modulates pain behavior in a neuropathic pain model in mice. Mol Pain 9: 23.

101. Shimbo T, Kawachi M, Saga K, Fujita H, Yamazaki T, et al. (2007) Development of a transferrin receptor-targeting HVJ-E vector. Biochem Biophys Res Commun 364: 423-428.

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