ventral prostate-1 (RVP-1) possesses a sequence and function similar to those of CPE-R [46]. Morita et al. revealed CPE-R and RVP-1 to be claudin-4 and claudin-3, respectively [37]. CPE is cytotoxic to claudin-3- and -4-positive cells, but this effect is lost if its N-terminus is removed. Treatment of cells with the C-terminal fragment of CPE, corresponding to amino acids 184–319 (C-CPE184) reduces transepithelial electrical resistance, which is a typical indicator of the barrier function of TJs. TJ barrier integrity recovers after the removal of C-CPE184. Treatment of cells with C-CPE184 decreases the level of claudin-4 protein [47]. Claudin-4 levels recover after the removal of C-CPE184. C-CPE184 treatment does not affect claudin-1 protein levels. Claudin contains the clathrin-sorting signal, and endocytosis of claudin occurs during the remodeling of TJ strands [48,49]. C-CPE184-bound claudin-3/-4 may be taken up by endocytosis; this is followed by degradation of claudins. Two possible mechanisms were proposed to explain C-CPE184-induced modulation of the TJ-barrier [47]. C-CPE184 may bind directly to claudin-3/-4 within TJ strands and then evoke depolymerization of TJ strands. Alternatively, C-CPE184 may bind to claudin-3/-4 in the non-junctional area, and this binding to claudin may suppress the polymerization of TJ strands. Furthermore, C-CPE184 increases the paracellular permeability of 4- and 10-kDa dextran in epithelial cell sheets. Inhibition of the claudin-based TJ-barrier by using claudin binders would provide a new means to modulate the TJ barrier and improve the bioavailability of drugs to target organs.

3.2.3. Claudin-targeted drug delivery

The efficacy of claudin binders as a novel strategy for mucosal absorption of drugs was demonstrated 6 years after the idea was first proposed (Table 2). C-CPE184 dose-dependently enhances intestinal absorption of 4-kDa dextran [50]. The absorption-enhancing activity is more than 400 times that of capric acid, an enhancer used clinically. A C-CPE184 mutant lacking the claudin-3/-4-binding domain does not exhibit absorption-enhancing activity. C-CPE184 enhances intestinal absorption of 4-, 10-, and 20-kDa dextran but not 40-kDa dextran. There is no mucosal injury in the intestines of C-CPE184-treated animals. Therefore, the use of a claudin-3/-4 binder is a novel method to enhance mucosal absorption. CPE also binds to claudin-6, -7, -8 and -14, and CPE interacts with the second extracellular loop of claudins [51]. Electrostatic interaction could be involved in the interaction between CPE and the second extracellular loop of claudins [52]. Claudin-7 and -8 are also expressed in the intestine, but any interaction between C-CPE184 and claudin-7 and/or -8 in the intestine remains unclear [53].

Recent progress in genomic and proteomic technologies has yielded some new biologics, including peptides, proteins, and nucleic acids, as pharmaceutical candidates. Most of these compounds are hydrophilic molecules that are poorly absorbed by the mucosa. Although injection is a compelling route for the administration of biologics, a transmucosal delivery system would be an ideal route because it is noninvasive and therefore would enable easy, pain-free administration by patients. C-CPE184 enhances nasal, but not jejunal and pulmonary, absorption of a peptide drug [54]. The solubility of C-CPE184 is <0.3 mg/ml. C-CPE194 is a derivative of C-CPE184 with a deletion of 10 N-terminal amino acids. C-CPE194 has greater solubility (10 mg/ml) than C-CPE184. C-CPE194 is also a claudin-3/-4 binder that enhances jejunal and pulmonary absorption of a peptide drug [54]. These findings support the idea, proposed by Sonoda et al. in 1999, that claudin binders may improve drug bioavailability. Modulation of the claudin barrier could be useful for development of non-invasive administration of biologics, most of which must be injected in patients.

3.2.4. Development of claudin binders

A series of studies using C-CPEs have revealed the possibility of developing claudin-targeted drug absorption [50,54]. The claudin family has at least 27 members. Several claudins are expressed in the duodenum, jejunum, ileum, and colon, and the expression profiles of claudins differ throughout the segments of the intestinal tract (see review in [53]). Development of claudin binders with broad and narrow specificity for claudins will be needed for the development of oral drug delivery systems based on modulation of the claudin barrier. However, claudins have four transmembrane domains, are hydrophobic, and have small extracellular loop domains. Therefore, preparation of recombinant proteins and antibodies to these extracellular domains is difficult. Only claudin-4 protein has been purified [55], and the development of specific claudin binders has been delayed. Claudin binders are classified into three categories: C-CPE derivatives, antibodies, and claudin peptides.

3.2.5. C-CPE derivatives

C-CPE184 is the first claudin binder and the only one discovered before 2005. C-CPE184 is a toxin fragment, and overcoming its antigenicity is critical for its clinical application [56]. Two approaches have been utilized to develop a claudin binder by using C-CPE184. One approach is the development of a binder by using C-CPE184 as a prototype. A deletion analysis revealed that the C-terminal segment, corresponding to 30 or 16 amino acids, is essential for the interaction of C-CPE184 with claudin-4 [57,58]. Synthetic peptides, corresponding to the 30 amino acids, bind to claudin-4 and modulate the TJ barrier in human intestinal cell lines [59]. Deletion of a portion of the N-terminal segment of C-CPE184 improves its solubility, and the improved C-CPE enhances mucosal absorption of a bioactive peptide drug [54]. Therefore, the deletion approach is one method of developing claudin binders, but there are no reports of a synthetic short peptide that can enhance mucosal absorption. Random mutagenesis of C-CPE184 is another approach. A site-directed mutagenesis analysis identified the functional amino acids of C-CPE184, and a C-CPE mutant library was formed by randomly mutating functional residues into other amino acid residues [60,61]. Recombinant claudin proteins are needed for the selection of claudin binders. It is generally difficult to obtain recombinant membrane proteins, including claudins, with an intact structure, and a protocol has been established only for recombinant claudin-4 [55]. Functional membrane proteins are heterogeneously expressed on the budded baculovirus, and interactions between membrane proteins can be detected by using receptor- and ligand-displaying budded baculoviruses [62–64]. Very recent studies suggest that claudins on budded baculovirus possess the native form and the claudin-displaying baculovirus functions as a screening system for claudin binders [60]. Indeed, claudin binders with broad specificity for claudins were isolated from the C-CPE mutant library by using the baculoviral display system, and the binders enhance mucosal absorption (our unpublished data). Chemical claudin binders are ideal for clinical application in terms of costs and antigenicity. Structural information about claudins and C-CPEs can provide the theoretical basis for claudin binders, but the three-dimensional structure of claudins has never been identified. An X-ray diffraction analysis revealed that C-CPE194 contains nine beta-sheets and one alpha helix and that the loop between beta-sheets 8 and 9 is a binding site of C-CPE194 to claudin-3 and -4 [65]. CPE interacts with the second loop of claudin-3 [51,66]. A series of analyses of the interaction between CPE and claudins indicated that the negatively charged cleft of C-CPE surrounded by Tyr306, Tyr310, Tyr312, and Leu315 might interact with the positively charged CPE-sensitive region [52]. Future determination of complex interactions between claudins and C-CPEs will facilitate the development of chemical claudin binders.

3.2.6. Antibodies and claudin peptides

The first antibodies to the extracellular domains of claudins were prepared by immunizing chickens with synthetic peptides corresponding to the extracellular domains [67]. Some of the polyclonal antibodies bound to claudin-3 and -4. Single-chain Fv fragments that bound to claudin-3 were isolated through selection from a human recombinant

antibody library containing $>10^9$ possible antibody combinations by using synthetic peptides corresponding to the extracellular domain of claudin-3 [68]. Immunization of immune-deficient mice or mice with claudin-4-expressing cells results in the production of monoclonal antibodies that bind claudin-4 or both claudin-3 and -4 [69–71]. Genetic immunization of mice with claudin-1-expressing DNA vectors led to the successful production of anti-claudin-1 monoclonal antibodies [72]. These findings did not include any data on modulation of the TJ barrier by antibodies. Therefore, progress has been made in the development of anti-claudin antibodies that recognize the extracellular domain.

Claudins have two extracellular loop domains. The intercellular interaction of the extracellular domains is thought to regulate paracellular permeability [73,74]. There may be homophilic interaction between claudins. Claudins have a *cis* interaction within the plane of the membrane to form dimers, followed by *trans* interactions between claudins in adjacent cells and additional *cis* interactions to assemble claudin oligomers into intramembrane TJ strands [75,76]. These findings suggest that peptides mimicking the extracellular loop domains of claudins interfere with the interactions of claudins, leading to modulation of the TJ barrier. A specific claudin-1 extracellular loop peptide mimetic corresponding to a 53- to 80-amino acid domain reversibly decreases the TJ barrier integrity and increases paracellular permeability to solutes in epithelial cells through its interaction with claudin-1 and -3 and occludin [77]. The peptide mimetic also enhances mucosal absorption in vivo [77].

3.2.7. Progression in development of absorption enhancers mediated by claudins

Some claudin binders truly enhance mucosal absorption of solutes through the paracellular route in the epithelium. What is the difference between first- and second-generation absorption enhancers? Can second-generation absorption enhancers overcome the problems associated with first-generation absorption enhancers? What progress has there been in the development of absorption enhancers mediated by claudins?

One problem with first-generation absorption enhancers is the influx of solutes other than drugs across epithelial cell sheets after the TJ seals are opened. The enhancers widen the paracellular space by inhibiting TJ seals, leading to the movement of solutes. Solutes with molecular masses of 600–800 Da were found to cross the epidermal and blood–brain barriers in claudin-1- and -5-deficient mice, respectively [40,42]. C-CPE184 enhances the epithelial permeability and mucosal absorption of dextran in a molecular size-dependent manner [47,50]. Claudin-based TJ strands also function in paracellular

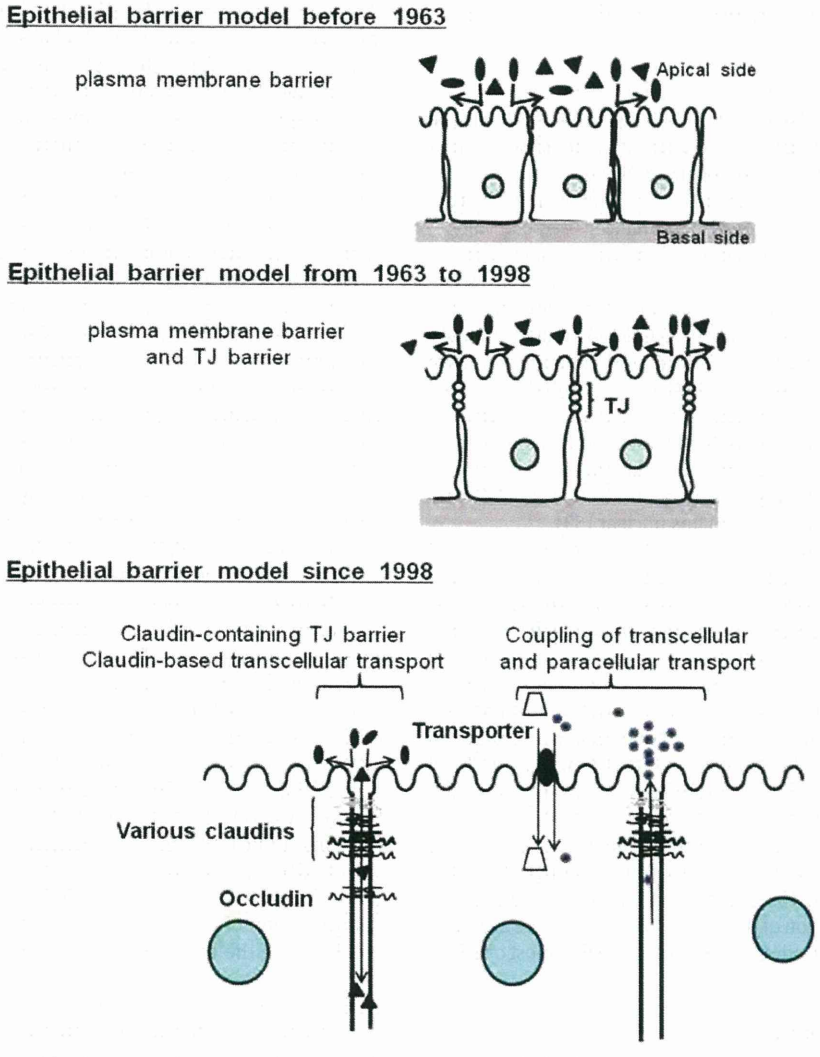

Fig. 1. Progression in paracellular transport research through understanding of the biology of the epithelial barrier. The plasma membrane was originally considered to be the primary barrier in epithelial cell sheets. TJ strands were subsequently identified as the intercellular sealing components [4,5]. Claudins were later found to be key structural and functional components of TJ seals. They function as intercellular seals and are also involved in the intercellular transport of ions. Claudins are coupled to both transcellular and paracellular transport. Claudin-based paracellular transport can be charge and size selective [35].

ion transport. Claudin-16 is required for paracellular magnesium resorption in the kidney [38]. Claudin-2 forms a leaky, sodium ion-selective paracellular channel in the kidney [78]. Paracellular influx of cations or anions is regulated by the expression of different claudins [79]. Claudins represent a family consisting at least 27 proteins, and TJ strands contain several types of claudins in a tissue-specific manner. The combination and mixing ratios of claudins are important for the tightness of TJ strands [80]. Incorporation of claudin-4 and -8 into TJ strands requires a paracellular anion channel in the kidney [81]. Interactions between claudin-16 and -19 are required for their assembly into TJ strands and renal reabsorption of magnesium [82]. Therefore, claudins function as a biological barrier that prevents the influx of solutes and pathogens, and they also function in the paracellular transport of solutes across TJ strands. Charge- and molecular size-dependent aqueous pores within claudin-based TJ strands for small molecules such as inorganic ions have been proposed [35]. Modulation of claudin-based aqueous pores suitable for a drug using claudin binders might allow the specified drug to pass through the mucosal epithelium only through paracellular routes. Future combinations of structural biological approaches involving claudin and the development of claudin binders will lead to a breakthrough in the development of a paracellular drug transport system that overcomes the drawbacks of first-generation absorption enhancers.

4. Future direction of research in transmucosal absorption

Hegel proposed the concept of "interpenetration of opposites" in his dialectic; this means that opposites will develop to interpenetrate each other. The final section will discuss the coupling of transcellular and paracellular drug delivery systems.

Routes for passing through the mucosal epithelium are classified as transcellular and paracellular [83–87]. Early research in this process focused on the paracellular routes, leading to the development of first-generation absorption enhancers. Transcellular uptake of drugs is mediated by simple diffusion and receptor- and transporter-dependent transcellular routes. Many different drug transporters are expressed in various tissues, including intestinal and hepatic epithelial cells and brain capillary endothelial cells [88–91]. Some of these transporters are involved in intestinal absorption and tissue distribution of drugs and can be used to determine the pharmacokinetic characteristics of various drugs. Although a number of receptors and transporters are available for drug delivery, each drug needs to be modified for recognition by these receptors and transporters. These findings indicate that the safety of transcellular transport may be superior to that of paracellular transport, because nonspecific influx of solutes other than drugs does not occur through carrier-recognized transport. Therefore, transcellular transport is thought to be the ideal intestinal absorption system. Paracellular and transcellular drug delivery systems will be coupled, on the basis of Hegel's "interpenetration of opposites," and the coupling will result in progression in the development of mucosal absorption enhancers.

Coupling of transcellular and paracellular transport can be categorized as production of the driving forces for solutes moving through either route and regulation of cellular signaling that controls permeability to solutes [92]. Intestinal absorption of nutrients, including sugars and amino acids, is coupled with Na^+ absorption [93]. Claudin-based TJ functions as a charge-selective channel in the paracellular route [74,79,94]. Claudin-15 is responsible for transepithelial permeability to extracellular monovalent cations, especially Na^+. Claudin-15-deficient mice exhibit low luminal Na^+ levels and low glucose absorption in the intestine, indicating that paracellular transport of Na^+ through claudin-15-based TJ strands may be coupled to the transcellular transport of glucose through a glucose transporter [95]. Modulation of the claudin barrier may be a novel mode of action of mucosal absorption enhancers that can modulate both the paracellular and transcellular transport of drugs.

In summary, strategies for intestinal absorption of drugs through the paracellular route have made dramatic progress because of progress in our understanding of the cell biology of the epithelial barrier. Two paradigm shifts have occurred in the consideration of paracellular route-mediated epithelial absorption of drugs (Fig. 1). The first paradigm shift was from modulation of the plasma membrane to the opening of TJ seals, leading to the development of absorption enhancers with lower cytotoxicity. The second paradigm shift was from the opening of TJ seals to the modulation of TJ components, leading to the development of molecular-based absorption enhancers. Moreover, the second shift revealed the possibility of enhancing intestinal absorption of drugs by regulating the nonspecific influx of substances other than drugs. A subsequent third paradigm shift may occur: coupling of paracellular and transcellular transport. The third shift suggests that future absorption enhancers will regulate both the transcellular and paracellular transport of drugs. Orally administered drugs are ideal pharmaceutical agents because they are less expensive and facilitate high patient compliance. The TJ-based strategy for mucosal absorption will lead to progress in the development of strategies for oral absorption of drugs.

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Short communication

Suppression of hepatitis C virus replicon by adenovirus vector-mediated expression of tough decoy RNA against miR-122a

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ABSTRACT

Recent studies have demonstrated that the liver-specific microRNA (miRNA) miR-122a plays an important role in the replication of hepatitis C virus (HCV). Antisense nucleotides against miR-122a, including locked nucleic acid (LNA), have shown promising results for suppression of HCV replication; however, a liver-specific delivery system of antisense nucleotides has not been fully developed. In this study, an adenovirus (Ad) vector that expresses tough decoy (TuD)-RNA against miR-122a (TuD-122a) was developed to suppress the HCV replication in the liver hepatocytes. Ad vectors have been well established to exhibit a marked hepatotropism following systemic administration. An in vitro reporter gene expression assay demonstrated that Ad vector-mediated expression of TuD-122a efficiently blocked the miR-122a in Huh-7 cells. Furthermore, transduction with the Ad vector expressing TuD-122a in HCV replicon-expressing cells resulted in significant reduction in the HCV replicon levels. These results indicate that Ad vector-mediated expression of TuD-122a would be a promising tool for treatment of HCV infection.

Hepatitis C virus (HCV) is a hepatotropic human virus belonging to a member of the family *Flaviviridae* and possessing a 9.6-kb positive-sense RNA genome. HCV infection causes chronic hepatic inflammation and fibrosis, leading to hepatocellular carcinoma (Hoofnagle, 2002). Currently, 170 million people worldwide are infected with HCV, and suffering from or at risk for the diseases described above. In order to suppress the replication of HCV, PEGylated interferon alpha and ribavirin, which is a nucleotide analogue, have been used as standard-of-care therapy; however, the therapeutic efficiency has been limited, in spite of relatively severe side effects, including fever and malaise (Chisari, 2005; Feld and Hoofnagle, 2005). Another therapeutic strategy should be developed to efficiently suppress the HCV infection and HCV-caused diseases.

Among several host factors involved in HCV infection, the abundant liver-specific microRNA (miRNA), miR-122a has been demonstrated to be crucial for efficient replication and/or translation of the HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007). The HCV genome has two closely spaced miR-122a-binding sites in the 5′-untranslated region (UTR), which contains overlapping *cis*-acting signals involved in translation and RNA synthesis (Jopling et al., 2005). Although the mechanism of the miR-122a-mediated enhancement of HCV replication is controversial (Henke et al., 2008; Jopling et al., 2005; Machlin et al., 2011; Roberts et al., 2011; Wilson et al., 2011), antisense oligonucleotides complementary to miR-122a, including locked nucleic acid (LNA) oligonucleotides, have been shown to significantly inhibit miR-122a and reduce the HCV genome, and thereby to exhibit superior therapeutic effects (Henke et al., 2008; Jopling et al., 2005; Krutzfeldt et al., 2005; Lanford et al., 2010). Intravenous administration of LNA oligonucleotides against miR-122a into HCV-infected chimpanzees resulted in the long-lasting suppression of HCV viremia without viral resistance or severe side effects (Lanford et al., 2010). In addition, the 5′-UTR of the HCV genome is composed of highly conserved structural domains, suggesting that a mutant lacking the miR-122a-binding sites in the genome is unlikely to appear. These results indicate that miR-122a is a promising target for the treatment of HCV-related diseases; however, LNA oligonucleotides accumulate in the kidney immediately after intravenous administration and are excreted into the urine (Fluiter et al., 2003).

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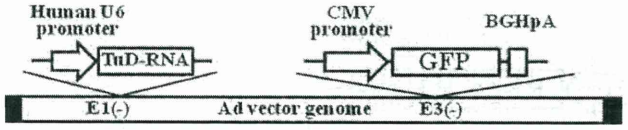

Fig. 1. Structure of Ad vectors used in this study. The human U6 promoter-driven TuD-RNA expression cassette was inserted into the E1-deleted region of the Ad vector genome. The CMV promoter-driven GFP expression cassette was inserted into the E3-deleted region of the Ad vector genome.

Systems which efficiently deliver or express anti-miR-122a drugs in the liver are necessary to efficiently treat HCV-related diseases.

Recently, tough decoy (TuD)-RNAs against miRNAs, which efficiently and specifically inhibit miRNAs, were developed by Haraguchi et al. (2009). TuD-RNAs are composed of two miRNA-binding sequence (MBS) regions and two stem structures with 3-nucleotide linkers. The MBS in the TuD-RNA is considered to tightly bind to miRNAs, leading to the inhibition of miRNAs. The inhibition activity of the TuD-RNA against miRNAs is higher than that of LNA oligonucleotides and miRNA sponges (Haraguchi et al., 2009). Another advantage of the TuD-RNA is that it can be expressed by viral and non-viral vectors. miRNAs can be persistently suppressed by lentivirus vector- and retrovirus vector-mediated expression of the TuD-RNA. Furthermore, liver-specific expression of the TuD-RNA is thought to be achievable by an adenovirus (Ad) vector and adeno-associated virus vector, because these vectors can express transgenes in a liver-specific manner after systemic administration. These properties of the TuD-RNA are highly promising for inhibition of miR-122a in the liver and suppression of HCV replication.

In the present study, we developed an Ad vector expressing the TuD-RNA against miR-122a (TuD-122a) to efficiently inhibit miR-122a and to suppress the HCV replication. Transduction with an Ad vector expressing TuD-122a efficiently inhibited miR-122a in vitro. In HCV replicon-expressing cells, HCV replicon levels were significantly reduced by Ad vector-mediated TuD-122a expression.

First, in order to examine the transduction efficiencies of the Ad vectors constructed in this study in the HCV replicon-expressing cells, Huh-7.5.1 1bFeo cells, which is a genotype 1b HCV replicon cell line (Yokota et al., 2003), were transduced with an Ad vector expressing TuD-122a (Ad-TuD-122a) or the control TuD-RNA (Ad-TuD-NC). Ad-TuD-122a and Ad-TuD-NC were prepared as described in Supplemental materials and methods. Structure of Ad vectors used in this study is shown in Fig. 1. The ratio of particles-to-biological titer was between 6 and 9 for each Ad vector used in this study. Both Ad-TuD-122a and Ad-TuD-NC carry the TuD-RNA expression cassette and the green fluorescence protein (GFP) expression cassette in the E1-deleted and E3-deleted region, respectively (Fig. 1). Both Ad-TuD-NC and Ad-TuD-122a efficiently transduced Huh-7.5.1 1bFeo cells (Fig. 2). More than 80% of the cells were found to be GFP-positive following transduction with Ad-TuD-122a and Ad-TuD-NC, respectively, at a multiplicity of infection (MOI) of 100. The averages of GFP-positive cells following transduction with Ad-TuD-NC were slightly higher than those with Ad-TuD-122a; however, statistically significant differences were not found for either group. Apparent cellular toxicity was not found following transduction with Ad-TuD-122a or Ad-TuD-NC (data not shown). These results indicate that Ad-TuD-122a and Ad-TuD-NC efficiently transduce Huh-7.5.1 1bFeo cells.

Next, in order to examine the inhibitory effects of TuD-122a expressed by the Ad vector on miR-122a, a reporter gene assay using the miR-122a complementary sequence-encoded plasmid, psiCheck-122aT, was performed in Huh-7 cells. Huh-7 cells endogenously express a high level of miR-122a (Suzuki et al., 2008). Huh-7 cells were transduced with the Ad vectors at MOIs of 25 and 100 for 1.5 h. After a 24-h incubation, the cells were transfected with

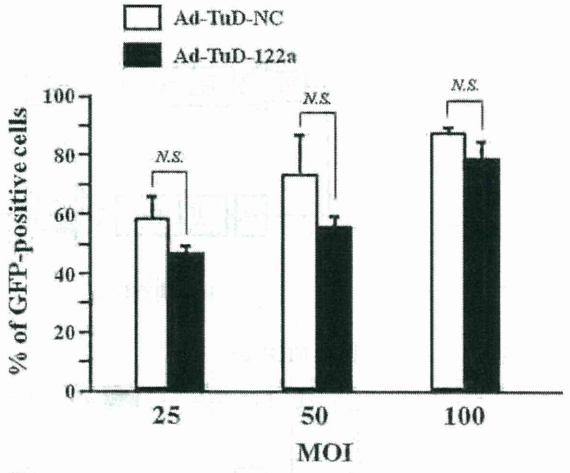

Fig. 2. Transduction efficiencies of Ad-TuD-122a and Ad-TuD-NC in Huh-7.5.1 1bFeo cells. The cells were transduced with Ad-TuD-122a or Ad-TuD-NC at multiplicities of infection (MOIs) of 25, 50, and 100 for 1.5 h. At 48 h after transduction, GFP expression was evaluated by flow cytometry. The data are expressed as the means ± S.D. ($n = 3$). The percentage of GFP-positive cells in the mock-transduced group was less than 0.2%. N.S.: not significant.

psiCheck-2 or psiCheck-122aT. The renilla and firefly luciferase expression was evaluated 48 h after transfection with the plasmid DNA. psiCheck-122aT, plasmid DNA containing the two copies of miR-122a complementary sequences in the 3′-UTR of the renilla luciferase gene, was constructed by ligation of *NotI*/*XhoI*-digested psiCheck-2 (Promega, Madison, WI) with the oligonucleotides 122aT-F and 122aT-R. The sequences of the oligonucleotides 122aT-F and 122aT-R are described in the Supplemental information. In mock-transduced cells, the relative renilla luciferase expression level by psiCheck-122aT was about 5-fold lower than that by the control plasmid psiCheck-2, which does not possess miR-122a target sequences, due to the endogenous expression of miR-122a in Huh-7 cells (Fig. 3). The renilla luciferase expression profiles following transfection with psiCheck-122aT were similar in the mock-transduced cells and Ad-TuD-NC-transduced cells, indicating that expression of the control TuD-RNA does not inhibit the miR-122a. Ad-TuD-122a did not alter the renilla luciferase expression level by psiCheck-2; on the other hand, psiCheck-122aT-mediated renilla luciferase expression was significantly restored by Ad-TuD-122a. The cells transduced with Ad-TuD-122a exhibited 2.8-fold and 3.5-fold higher renilla luciferase expression at MOIs of 25 and 100, respectively, than the mock-transduced cells following transfection with psiCheck-122aT. These results indicate that miR-122a is efficiently inhibited by Ad-TuD-122a. We also performed quantitative RT-PCR analysis for miR-122a following transduction with Ad-TuD-122a and Ad-TuD-NC in Huh-7 cells. No significant differences in the miR-122a expression levels were found in the cells transduced with Ad-TuD-122a and the cells transduced with Ad-TuD-NC (data not shown), probably because TuD-RNA does not induce degradation of miRNA, although TuD-RNA tightly binds to the target miRNA (Haraguchi et al., 2009).

Next, in order to examine whether TuD-122a-mediated inhibition of miR-122a suppresses the HCV replicon, Huh-7.5.1 1bFeo cells were transduced with Ad-TuD-122a and Ad-TuD-NC at the indicated MOIs. Huh-7.5.1 1bFeo cells express an mRNA consisting of the HCV 5′-UTR and the upstream part of the core region, connected in-frame with the firefly luciferase gene, which allows the simple evaluation of the HCV replicon levels by measuring the firefly luciferase activity in the cells (Yokota et al., 2003). Huh-7.5.1 1bFeo cells were transduced with the Ad vectors at MOIs of 25, 50, and 100 for 1.5 h. After a total 48-h incubation,

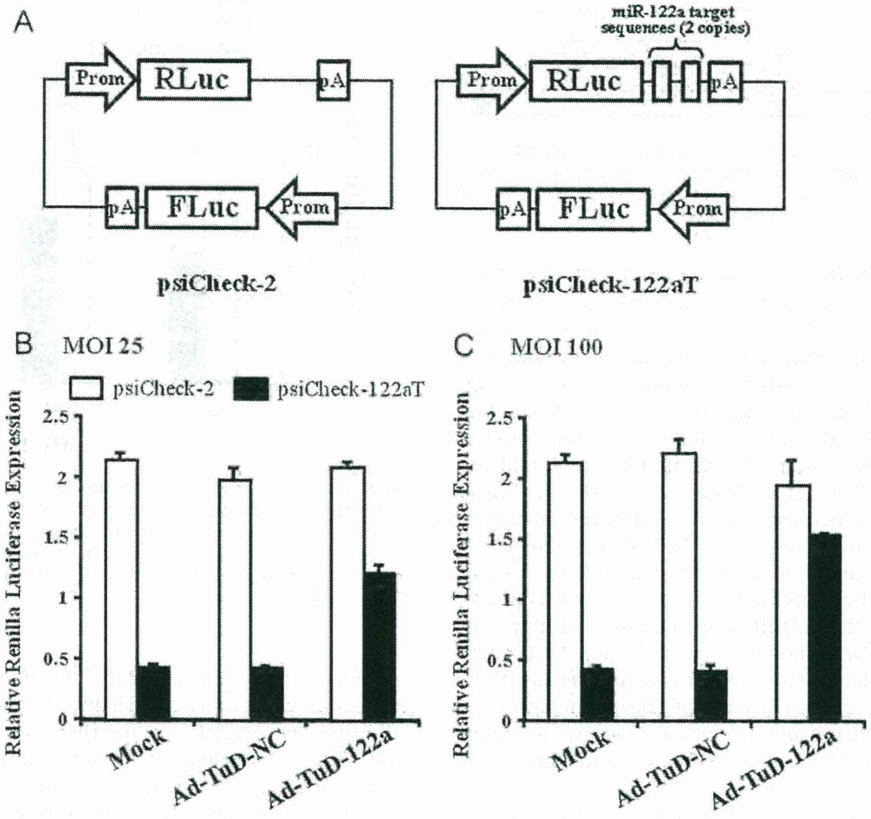

Fig. 3. Inhibition of miR-122a by Ad vector-meditated TuD-122a expression. (A) Structure of the reporter gene-expressing plasmids psiCheck-2 and psiCheck-122aT. (B and C) Relative renilla luciferase expression levels following transduction with Ad-TuD-NC or Ad-TuD-122a at MOIs of 25 (B) and 100 (C). The data are expressed as the means ± S.D. ($n = 4$)..

firefly luciferase expression levels were determined. Ad-TuD-122a significantly reduced the firefly luciferase expression levels in a dose-dependent manner (Fig. 4a). The firefly luciferase expression level was reduced to 29% of that in the cells transduced with Ad-TuD-NC at MOI of 100 by transduction with Ad-TuD-122a at MOI of 100. In contrast, no significant changes in the firefly luciferase expression were found by transduction with Ad-TuD-NC.

To examine whether inhibition of miR-122a by Ad vector-mediated TuD-122a expression leads to a reduction in HCV replicon RNA levels, strand-specific real-time RT-PCR analysis was performed to determine the HCV replicon RNA levels. Briefly, Huh-7.5.1 1bFeo cells were transduced with the Ad vectors as described above, and the total RNA was isolated 48 h after transduction. Real-time RT-PCR analysis for the HCV positive-strand RNA genome was performed as follows. Briefly, 2 μg of total RNA was reverse-transcribed to cDNA using the primer specific for the HCV positive-strand genome (RC21; 5′-ctc ccg ggg cac tcg caa gc-3′). Real-time RT-PCR was performed using the primers (RC21 and RC1; 5′-gtc tag cca tgg cgt tag ta-3′) and SYBR Premix Ex Taq II (Takara Bio Inc., Kyoto, Japan). Similarly to the results for the firefly luciferase expression in Fig. 4A, HCV replicon RNA levels were significantly reduced by Ad-TuD-122a (Fig. 4B). There was an approximately 2.2-fold decline in the HCV replicon RNA level in the cells transduced with Ad-TuD-122a at an MOI of 100, compared with the HCV replicon RNA level in the cells transduced with Ad-TuD-NC at an MOI of 100. Ad-TuD-NC did not apparently decrease the HCV replicon RNA levels. These results indicate that the inhibition of miR-122a by Ad vector-mediated TuD-122a expression efficiently suppresses the replication of the HCV replicon.

The present study demonstrates that Ad vector-mediated TuD-122a expression significantly inhibits the function of miR-122a and replication of the HCV replicon. Replication of the HCV genome is promoted by the direct interaction between miR-122a and the complementary sequences in the 5′-UTR of the HCV genome (Henke et al., 2008; Jangra et al., 2010), indicating that sequestration of miR-122a leads to suppression of the HCV replication. In order to suppress the HCV replicon by inhibiting miR-122a, TuD-RNA was selected as an inhibitor of miRNA in this study, because TuD-RNA potently inhibits miRNA by strongly binding to miRNA (Haraguchi et al., 2009). In addition, TuD-RNA can be expressed by conventional gene delivery vectors, including virus vectors. One drawback of TuD-RNA is that TuD-RNA does not discriminate miRNA members that belong to the same miRNA family (Haraguchi et al., 2009); however, miR-122a does not constitute a family of miRNA, suggesting that TuD-122a would not inhibit other miRNAs.

As described above, an Ad vector is suitable for liver-specific expression of TuD-RNA due to the strong hepatotropism. Previous studies demonstrated that Ad vectors expressing short-hairpin RNA (shRNA) or antisense RNA against the HCV genome successfully exhibited the suppressive effects on HCV infection in vivo (Gonzalez-Carmona et al., 2011; Sakamoto et al., 2008). Another advantage of using an Ad vector for treatment of HCV-related diseases is that in vivo administration of an Ad vector induces type I interferon (IFN) production via innate immune responses (Huarte et al., 2006; Zhu et al., 2007). Our group previously demonstrated that VA-RNA, which is a small non-coding RNA expressed from a replication-incompetent Ad vector as well as wild-type Ad, stimulates type I IFN production in an IFN-β promoter stimulator-1 (IPS-1)-dependent manner (Yamaguchi et al., 2010). Ad vector-induced type I IFN would contribute to suppression of HCV infection. The anti-HCV activity of Ad-TuD-122a can also be up-regulated by insertion of an expression cassette of an

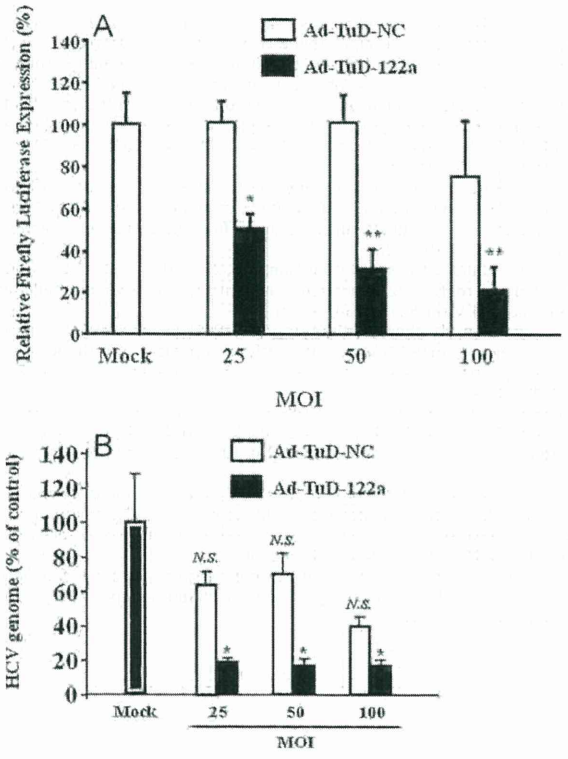

Fig. 4. Suppression of the HCV replicon by Ad vector-mediated TuD-122a expression. (A) Firefly luciferase expression levels and (B) HCV replicon RNA levels in Huh-7.5.1 1bFeo cells following transduction with the Ad vectors. All the data are shown as the means ± S.D. ($n = 3$). N.S.: not significant. $^{*}P < 0.05$, $^{**}P < 0.005$ between mock-transduced cells and cells transduced with Ad-TuD-122a.

anti-HCV gene, including type I IFN genes and short-hairpin RNA (shRNA) or antisense RNA against the HCV genome, into the Ad vector genome. Our group has developed various types of Ad vectors in which two or three transgene expression cassettes can be inserted into a single Ad vector genome (Mizuguchi et al., 2001, 2005, 2003).

Previous studies have demonstrated that lipid droplets, which are lipid-storage intracellular organelles, are crucial for the production of infectious HCV particles (Hinson and Cresswell, 2009; Miyanari et al., 2007). Miyanari et al. demonstrated that HCV capsid proteins recruit the non-structural proteins and the replication complex to the lipid droplet-associated membrane (Miyanari et al., 2007). miR-122a is an important factor that regulates cholesterol and fatty-acid metabolism in the hepatocytes (Esau et al., 2006; Iliopoulos et al., 2010). Intravenous administration of the antisense oligonucleotide against miR-122a resulted in a reduction in the plasma levels of cholesterol and triglycerides (Esau et al., 2006; Lanford et al., 2010). In addition to the enhancement of accumulation and translation of the HCV genome, miR-122a might up-regulate HCV infection by regulating lipid metabolism in the hepatocytes.

Almost similar levels of reduction in the HCV replicon RNA copy numbers were found for Ad-TuD-122a at MOIs of 25, 50, and 100, although there was dose-dependent reduction in the firefly luciferase expression following transduction with Ad-TuD-122a. It remains unclear why dose-dependent reduction in the HCV replicon RNA copy numbers was not found, however, miR-122a plays a crucial role in the enhancement of both translation and stability of HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007; Shimakami et al., 2012). Stability of HCV genome might be more susceptible to inhibition of miR-122a than translation. The averages of HCV replicon RNA levels were also reduced following transduction with Ad-TuD-NC, although statistically significant differences were not found, compared with the mock-transduced cells. Replication-incompetent Ad vectors express non-coding small RNA (VA-RNA), which forms RNA-induced silencing complex (RISC) with argonaute 2 (Ago2) (Xu et al., 2007). Ago2 is an important factor for miRNA processing (Diederichs and Haber, 2007). Processing of miR-122a might be slightly disturbed by Ad vector-expressed VA-RNA, leading to the reduction in the HCV replicon RNA levels.

In summary, we efficiently suppressed the HCV replicon levels by Ad vector-mediated expression of TuD-122a, which blocks the function of miR-122a. This study indicates that Ad vector-mediated expression of TuD-122a in liver hepatocytes would offer an alternative approach for the treatment of HCV infection.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2012.02.003.

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