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# Evolution of drug delivery system from viewpoint of controlled intracellular trafficking and selective tissue targeting toward future nanomedicine

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ABSTRACT

Due to the rapid changes that have occurred in the field of drug discovery and the recent developments in the early 21st century, the role of drug delivery systems (DDS) has become increasingly more important. For the past 20 years, our laboratory has been developing gene delivery systems based on lipid-based delivery systems. One of our efforts has been directed toward developing a multifunctional envelope-type nano device (MEND) by modifying the particle surface with octaarginine, which resulted in a remarkably enhanced cellular uptake and improved intracellular trafficking of plasmid DNA (pDNA). When we moved to *in vivo* applications, however, we were faced with the PEG-dilemma and we shifted our strategy to the incorporation of ionizable cationic lipids into our system. This resulted in some dramatic improvements over our original design and this can be attributed to the development of a new lipid library. We have also developed a mitochondrial targeting system based on a membrane fusion mechanism using a MITO-Porter, which can deliver nucleic acids/pDNA into the matrix of mitochondria. After the appearance of antibody medicines, Opdivo, an immune checkpoint inhibitor, has established cancer immunology as the 4th strategy in cancer therapy. Our DDS technologies can also be applied to this new field of cancer therapy to cure cancer by controlling our immune mechanisms. The latest studies are summarized in this review article.

## 1. Introduction

We initiated our gene delivery research project in 2000 at Hokkaido University by focusing on the delivery of plasmid DNA (pDNA). By introducing cell penetrating peptides, especially octaarginine (R8) into a system, cellular uptake as well as endosomal escape was increased efficiently [1] and a multifunctional envelope-type nano device (MEND) modified with R8 induced transfection activities as high as that for the adenovirus in tumor cell lines [2]. To apply the R8-MEND for *in vivo* gene delivery, we shielded the positive charge of R8 with polyethyleneglycol (PEG), which improved the *in vivo* pharmacokinetics of the R8-MEND, resulting in an enhanced tumor delivery as well as an extended circulation time, however, the transfection activities of the R8-MEND were greatly decreased in tumor tissue, causing us to face the PEG-dilemma [3]. To clarify this problem, we proposed a new strategy for selectively cleaving PEG from the MEND in tumor tissue, and this resulted in an enhanced transfection activity of PPD-MEND [4].

Due to a Nobel Prize for RNA interference that was awarded in 2006, gene delivery research has shifted from pDNA to short interfering RNA (siRNA), since siRNA medicine can be classified not as gene therapy in which the regulatory process requires much less time compared to that for gene therapy. We also shifted our focus from pDNA delivery to siRNA delivery and we improved the MEND by introducing GALA to overcome the PEG-dilemma, which succeeded to enhancing gene silencing in tumor tissue [5]. In 2010, a breakthrough technology appeared when it was found that the use of a pH-sensitive (ionizable) cationic lipid, a cationic lipid as DLin-KC2-DMA, resulted in an enhanced *in vivo* gene silencing efficiency of a marker gene in the liver by two orders of magnitudes (50% effective dose ($ED_{50}$): 0.02 mg siRNA/kg from 2 mg siRNA/kg) from the original compound DLin-DMA [6]. Based on our analysis, the endosomal escape efficiency of the R8/GALA-MEND, which has the highest efficiency of the particles developed in our laboratory, was estimated to be 80% and the $ED_{50}$ for the R8/GALA-MEND was similar to that of DLin-DMA. Under such circumstances, it was difficult for us to compete with the efficiency of a pH-sensitive cationic system. We then shifted our strategy from the R8/GALA-MEND to a pH-sensitive cationic lipid and succeeded in designing YSK05, YSK12, YSK 13, and related compounds and these new lipids were found to enhance gene silencing in liver much more efficiently than the R8/GALA-MEND and succeeded in curing hepatitis C/B infected chimeric mouse model [7,8]. The latest research is updated in this paper.

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We also developed a MITO-Porter to deliver encapsulated compounds to mitochondria *via* a membrane fusion mechanism. R8 played an important role in enhancing cellular uptake as well as in mitochondrial targeting. We then examined the targeting of a small molecular drug (propidium iodide), a peptide (mastoparan), a protein (DNase), to mitochondria however it was difficult to prove that we successfully delivered the compounds into the matrix of mitochondria [9]. We then applied an antisense oligo nucleotide to knock down gene expression in mitochondria, because silencing can only be detected when an oligo nucleotide is delivered to the mitochondrial matrix [10]. At that time, there was no reporter gene available for mitochondria gene delivery, but we have many reporter genes for the nucleus such as luciferase, green fluorescent protein (GFP), *etc.* We were waiting for the development of a reporter gene for mitochondria, but it did not appear. Therefore, we decided to develop it by ourselves. In this article, we discussed mitochondrial gene therapy from a view point of mitochondrial RNA therapy. In addition, recent progress in mitochondrial delivery technology for translational studies are also explained.

In 2014, Opdivo was approved in Japan for use as an antibody drug for the treatment of melanomas and was subsequently extended to lung cancer, renal cell carcinoma, Hodgkin's lymphoma, *etc.* Professor Tasuku Honjo was awarded a Nobel Prize in 2018, along with James Patrick Allison for his work on the mechanism of immune checkpoint inhibition. The Nobel Prize as well as the clinical success of cancer immunotherapy by immune checkpoint inhibitors established cancer immunotherapy as the 4th strategy. We are also in the process of developing nano devices for cancer immunotherapy, since a DNA vaccine was the first goal of our gene delivery research because we thought it may be the easiest way to reach the goal for gene therapy. We initially focused on delivering pDNA to dendritic cells (DC). However, DC are completely different from tumor cell lines and we thought that the nuclear membrane might be a key step in developing DNA vaccines, since tumor cell lines frequently divide while DC do not. We developed a T-MEND [11] to overcome this barrier and succeeded in developing a KALA-MEND to transfect DC, antigen presentation, cytotoxic T-cell induction and antitumor activity under *ex vivo* conditions [12]. We attempted to find a way to apply the KALA-MEND *in vivo*, however, the KALA-MEND cannot exert its activity under *in vivo* conditions. Therefore, we learned how difficult it is to develop a DNA vaccine and, since then, have developed another strategy for contributing to cancer immunotherapy using our delivery technologies for *in vivo* applications. Some aspects of our recent progress are described in this article.

## 2. Development of lipid nanoparticles for RNA delivery

### 2.1. Delivery technology in RNA therapeutics

Disease phenotypes of various diseases including genetic diseases, infectious diseases, and cancer, may be derived from abnormal genetic status including upregulation, downregulation, mutation and the deletion of the responsible genes [13–16]. These therapeutic targets are sometimes resistant to treatment by small molecule drugs [17]. Therefore, RNAs including short RNAs, messenger RNAs, and RNAs are required for gene editing (*e.g.* clustered regularly interspaced palindromic repeat/Cas), represent an alternative approach for the treatment of these refractory diseases. Despite their great potential, the unique characterization of the RNAs, which includes ease of enzymatic degradation in biological fluids, poor pharmacokinetics and biodistribution, severely hinders their utility *in vivo*. Therefore, the development of sophisticated technologies which stabilize and facilitate the delivery of RNAs is an integral issue for the translation of RNAs to clinical use.

siRNA, a double stranded RNA with a 20–25 nt length, was discovered by Tsuchl and colleagues in 2001 [18], and can suppress the expression of a specific gene through RNA interference (RNAi), which was initially discovered by Fire and Mello in *C. elegans* in 1998 [19]. Many efforts have been made to apply siRNAs for therapeutics to date [20–29]. In 2018, only 20 years from the discovery of RNAi, Patisiran (ONPATTRO™), a treatment for familial amyloid polyneuropathy (FAP) caused by mutations in the transthyretin (TTR) gene, was approved by the U.S. Food and Drug Administration as a first-ever RNAi medicine [30]. Patisiran is also a first-ever approved lipid nanoparticle (LNP) containing therapeutic RNA. Patisiran rapidly accumulates in liver tissue and is taken up in hepatocytes, which mainly produce TTR proteins, by endocytosis upon intravenous administration. Upon endocytosis, ionizable amino lipids contained in the Patisiran formulation develop a cationic charge in response to the acidified interior of endosomes and the siRNAs are released to the cytosol through membrane fusion between Patisiran and endosomal membranes following electrostatic interaction.

The above example strongly suggests that controlling both the biodistribution and endosomal escape processes are integral parts for the efficient delivery of therapeutic RNA, maximizing their therapeutic potential, and facilitating the translation of RNAs to clinical use. In this section, we describe the mechanism of hepatic accumulation of the LNPs and the impact of the chemical structure of an ionizable amino lipid on endosomal escape.

### 2.2. Controlling biodistribution: in vivo hepatic delivery of RNAs

The liver is the primary target of RNA-loaded LNPs because it is estimated that genetic disorders in hepatocytes, parenchymal cells in liver tissue are the cause of thousands of human diseases [31]. The unique structural and functional features in liver tissues enable LNPs to efficiently enter parenchymal cells from the blood stream. The structural features include the presence of small pores, referred to as fenestrae, in liver sinusoidal endothelial cells (LSECs) and the absence of basement membranes between hepatocytes and the LSECs. Because it is known that the diameter of the fenestrae are approximately 107 nm and 141 nm in human and mouse, respectively [32], controlling size of LNPs to 100 nm or less in diameter is important in terms of delivering RNAs to hepatocytes [7,33–35]. The functional feature is the presence of the apolipoprotein E/low-density lipoprotein receptor (ApoE/LDLR) mediated the endogenous cellular uptake pathway in hepatocytes. Upon the intravenous administration of LNPs, ApoE proteins are adsorbed on the surface of the LNPs and are recognized by LDLRs, which are expressed at high levels on hepatocytes, resulting in the endocytosis of ApoE-bound LNPs. ApoE proteins are typically found in endogenous lipoproteins, including very low-density lipoproteins (VLDLs), high density lipoproteins, (HDLs), and chylomicrons (CMs), and are involved in promoting the cellular uptake of CMs and VLDLs by hepatocytes to manage lipid metabolism in the body [36,37]. Yan et al. first demonstrated the involvement of ApoE proteins in the elimination of liposomes from the blood stream by hepatocytes in mice [38]. They showed a 3.6-fold faster blood clearance and over a 20-fold higher accumulation in hepatocytes of neutral liposomes in wild-type mice compared to ApoE-deficient mice, which was not observed for negatively-charged liposomes. In a subsequent study, Akinc et al. clearly demonstrated that nearly-neutral LNPs composed of an ionizable amino lipid with an acid dissociation constant (pKa) of approximately 6.7, referred to as ionizable LNPs (iLNPs), are taken up by hepatocytes through the ApoE-LDLR-mediated endogenous cellular uptake pathway [39]. On the other hand, cationic LNPs (cLNPs) composed of relatively cationic lipid-like materials (lipidoids) were taken up by hepatocytes and this process was not dependent on ApoE proteins. Considering the above collective information, electrostatically near-neutral LNPs that are composed of phosphatidylcholine derivatives and/or ionizable amino lipids with relatively lower pKa values have a tendency to adsorb ApoE proteins. On the other hand, it has been reported that ionizable amino lipids composed of linear saturated scaffolds or a constrained adamantane scaffold have the ability to avoid accumulation in hepatocytes, indicating that the ApoE-mediated pathway is lacking [40,41]. Further investigations will be needed to develop a precise understanding of the

relationships between chemical structure requirements and ApoE-mediated accumulation to hepatocytes. Akinc et al. also showed that decoration of *N*-acetyl-D-garactosamine (GalNAc) clusters on the surface of the siRNA-loaded iLNPs restored gene silencing in hepatocytes in ApoE-deficient mice or LDLR knockout mice. It is known that GalNAc and galactose are recognized by asialoglycoprotein receptors (ASGPRs), which are expressed in hepatocytes [42]. Due to following impressive features of ASGPRs, decorating GalNAc on nanoparticles is an alternative strategy for targeting hepatocytes [43–45]: 1) a specific and high (~500,000 molecules per individual cell) expression in hepatocytes, 2) a rapid (~15 min) recycling time. It has also been reported that decorating GalNAc on siRNA-loaded LNPs contributed to improving hepatocyte-specificity and reduced cationic lipid-associated hepatic toxicity which is caused by the off-target accumulation of the LNPs to LSECs and activation of the same cell type followed by neutrophil-mediated inflammation [46].

Many researchers have demonstrated that both ApoE-LDLR (endogenous) and GalNAc-ASGPR (exogenous) pathway are useful for efficiently delivering RNAs to hepatocytes so far. However, little was known regarding the kinetics of the delivery process including blood clearance, hepatic accumulation and cellular uptake in hepatocytes between the endogenous and exogenous targeting mechanisms. We recently investigated this using siRNA-loaded LNPs, the surfaces of which were modified differently so as to modify the mechanism for hepatocyte targeting (Fig. 1) [47]. The blood half-life of unmodified (bare) LNPs that target hepatocytes through the endogenous mechanism was only 1.8 min, which was much (approx. 18-fold) faster than that of GalNAc/Shielded LNPs (modified with both GalNac and PEG) that target hepatocytes though the exogenous mechanism. The bare LNPs accumulated rapidly in liver tissue within 10 min upon intravenous administration, were located in or near LSECs for approximately 10 min, and were then endocytosed 30 min after the administration. Electron microscopic observations revealed that most of the LNPs were located in the space of Disse even at early time point (3 min after administration). Both rapid blood clearance and the accumulation of the bare LNPs which were absent in ApoE-deficient mice, were restored by the co-administration of recombinant ApoE protein. On the other hand, the GalNAc/Shielded LNPs gradually accumulated in liver tissue and were endocytosed by hepatocytes in wild-type mice. The ApoE is known to bind to heparan sulfate proteoglycans (HSPGs) as well as LDLR through the arginine-rich receptor-binding domain [48]. Treatment with heparin, which can compete with HSPGs, even 10 min after administration of the bare LNPs significantly suppressed both the accumulation and gene silencing of the LNPs in hepatocytes, suggesting that HSPGs in the liver are involved in the rapid accumulation of the bare LNPs. The relatively slow uptake of the bare or GalNAc/Shielded LNPs could be explained by assuming that receptors are mainly distributed on microvilli but not on coated pits and therefore a certain time would be required for the particles to migrate to coated pits for internalization [49,50].

### 2.3. Controlling endosomal escape: impact of the chemical structure of ionizable cationic lipid

The endosomal escape process is now recognized as the rate-limiting step for the cytosolic delivery of RNAs [51]. LNPs that contain ionizable amino lipids are known be processed through membrane fusion following electrostatic interaction with the negatively charged endosomal membranes in acidified endosomes. In this step, phase transition of endosomal membranes from a lamellar (L) to an inverted hexagonal ($H_{II}$) phase is required for successful membrane fusion [52]. Heyes et al. reported that the presence of *cis*-unsaturated bond(s) in hydrophobic scaffolds of ionizable cationic lipids are important for facilitating membrane fusion and for inducing the cytosolic delivery of siRNAs [53]. The unsaturated bond can allow complexes between ionizable cationic lipids and anionic lipids to adopt a cone-shape, which can disrupt the bilayer structure and thus facilitate membrane fusion. The subsequent rational design of linker structures between hydrophilic head groups and hydrophobic scaffolds of ionizable amino lipid successfully maximized their fusogenic activity and the cytosolic delivery of siRNA [6,54]. The attachment of two hydrophobic scaffolds from a single carbon atom is currently adopted as a linker of the ionizable amino lipids. Jayaraman et al. screened ~100 types of ionizable amino lipids and found DLin-MC3-DMA (MC3) to be the most potent lipid with an $ED_{50}$ of 0.005 mg/kg in a mouse factor 7 (F7) model, which was approximately a 100-fold higher efficiency compared to a benchmark lipid, DLin-DMA (Fig. 2A) [21]. The MC3 is used in the Patisiran formulation. While the efficiency of cytosolic delivery of siRNAs was

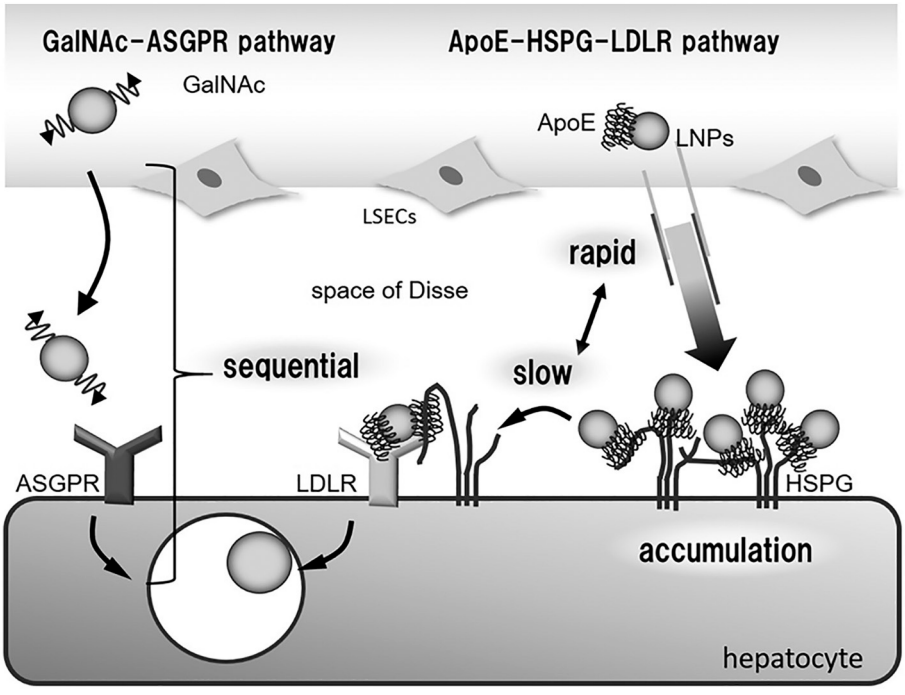


**Fig. 1.** Schematic illustration of the hepatic uptake of the LNPs *via* the ApoE-HSPG-LDLR pathway and the GalNAc-ASGPR pathway.
Regarding the former pathway, the blood clearance rate is rapid due to ApoE-mediated binding to hepatic HSPGs, resulting in accumulation in the space of Disse. After this accumulation, the LNPs are taken up by hepatocytes. Regarding the latter pathway, the LNPs are gradually cleared from the blood circulation and are taken up by hepatocytes in a sequential manner. ApoE; apolipoprotein E, ASGPR; asialoglycoprotein receptor, GalNAc; *N*-acetyl-D-galactosamine, HSPG; heparan sulfate proteoglycan, LDLR; low density lipoprotein receptor, LNP; lipid nanoparticle, LSEC; liver sinusoidal endothelial cell. This figure is reprinted from [47] with permission from Elsevier.

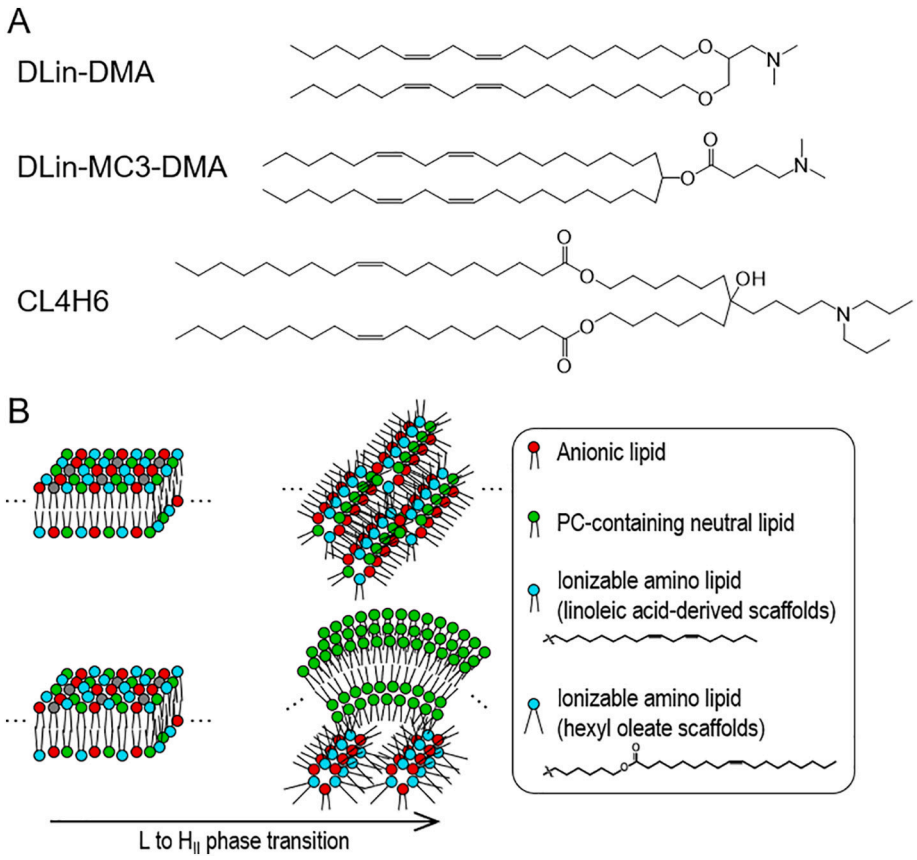


**Fig. 2.** Importance of the hydrophobic scaffold structure of ionizable amino lipids.
(A) Chemical structure of ionizable amino lipids, DLin-DMA, DLin-MC3-DMA, and CL4H6. (B) Differences in lipid polymorphism during lamellar (L) to inverted hexagonal ($H_{II}$) phase transition between ionizable amino lipids with different hydrophobic scaffold structures. This figure is reprinted from [58] with permission from Elsevier.

greatly improved, the efficiency was estimated to be only approximately 2–3% based on various analyses using electron microscopy and fluorescent microscopy [55,56].

We recently developed an original library of ionizable amino lipids and identified the most potent lipid for the hepatic delivery of siRNAs, referred to as CL4H6 [41]. The CL4H6 lipid contains two very long hexyl oleate chains (C24 + O1) (Fig. 2A). The CL4H6-LNPs exhibited an $ED_{50}$ of 0.0025 mg/kg in a mouse F7 model. We found that 4.2% of the siRNA was loaded in the RNA-induced silencing complex (RISC) at 24 h after intravenous administration, suggesting that endosomal escape was better compared to MC3. The CL4H6 also exhibited a better tolerability compared to MC3-LNPs due to the fact that the CL4H6 is biodegradable. It is known that phosphocholine (PC)-containing neutral lipids, including phosphatidylcholines and sphingomyelins (SMs), are cylindrical-shaped and therefore stabilize bilayers and strongly inhibit membrane fusion [52,57]. We revealed that the long hexyl oleate scaffolds can overcome the inhibitory effect of PC-containing neutral lipids on the fusogenicity of the LNPs [58]. Detailed analyses of lipid polymorphism by small-angle X-ray scattering (SAXS) and $^{31}P$ nuclear magnetic resonance (NMR) spectroscopy revealed that the CL4H6 lipids have tendency to segregate PC-containing neutral lipids when they form an $H_{II}$ phase after electrostatic binding to anionic lipids, while it was not observed in their counterparts with conventional linoleic acid-derived hydrophobic scaffolds (C18 length) (Fig. 2B). PC-containing neutral lipids in the endosomal membranes mix with ion complexes of ionizable cationic lipids and anionic lipids by diffusion and can then inhibit membrane fusion (L to $H_{II}$ phase transition). However, the immiscible properties of CL4H6 with PC-containing neutral lipids when it forms an $H_{II}$ phase allows it to escape from the inhibitory effect and contribute to an enhanced endosomal escape.

## 3. Challenge for mitochondrial RNA therapy using a mitochondrial DDS

The functional breakdown of mitochondria can affect various functions and cause a variety of diseases. It has been reported that mutations/deletions in mitochondrial DNA (mtDNA) cause to the onset of some of these diseases [59,60]. Therefore, research focusing on mitochondrial gene therapy is expected to lead to the development of innovative medicines. This section focuses on our research efforts on “mitochondrial drug delivery system (DDS) for mitochondrial RNA therapy”.

### *3.1. Significance of an innovative therapy to target the mitochondrial genome and essential mitochondrial delivery based on DDS technology*

Mitochondria contain their unique genome, mtDNA, and plays an important role to sustaining a variety of essential functions. Many physicians and researchers have reported that mutations/deletions in mtDNA are responsible for the onset of many mitochondria dysfunction diseases [61,62]. In the case of humans, mtDNA encodes for 13 kinds of mRNAs, 22 kinds of tRNA, and 2 kinds of rRNA, and all of these mRNA are translated into essential proteins constituting the mitochondrial respiratory chain complex. Therefore, if the mitochondrial genome is damaged, the mitochondrial respiratory chain complex would not function, thus disrupting energy production, and the mitochondrial function of cells and organs would eventually be impaired, leading to the development of diseases [63–65].

Moreover, it has been reported that the causes of neurodegenerative diseases, diabetes, and cancer are implicated with the accumulation of mtDNA mutations [66–68]. As described above, mitochondrial genomic abnormalities (the accumulation of mtDNA mutations, *etc.*) would be associated with the onset of various types of diseases. However, the mainstream of current therapy for diseases with mitochondrial

dysfunctions is symptomatic therapy with the goal of providing temporary support for mitochondrial function by continuous administration.

Thus, an innovative therapy for treating the mitochondrial genome has been attracting considerable attention as a fundamental therapeutic alternative to conventional therapeutic methods. To achieve such an innovative therapy, mitochondrial DDS to access the mitochondrial genome is highly required. Conventional mitochondrial DDS such as a mitochondrial targeting signal peptide would not result in the mitochondrial transfection of nucleic acids and genes [9,69], which are candidate molecules for gene therapy, thus, making it difficult to exert a therapeutic effect. We focused on the fact that mitochondria are continuously repeating fusion and fission in living cells and this permits bio macromolecules (nucleic acids, mtDNA, proteins and etc) to be shared between mitochondria, and we proposed the concept of a MITO-Porter, a liposome to achieve mitochondrial delivery of various types of cargoes *via* a membrane fusion. We successfully identified highly fusible mitochondrial fusogenic lipid compositions for this purpose [9,70–72].

A MITO-Porter, a particle in which the surface is modified with the membrane-permeable peptide (R8) is efficiently taken up by cells. Such carriers would bind to mitochondria, and then fuse with the mitochondrial membrane to deliver cargoes to mitochondria (Fig. 3) [9,70,71]. This strategy for the mitochondrial delivery of cargoes *via* mitochondrial membrane fusion is not limited by the physical properties or size of the delivery molecule. Intracellular observations showed that the MITO-Porter could deliver various molecules including chemicals, proteins and supermolecules to the mitochondria of living cells [70,71,73–75]. We successfully packaged nucleic acids into a MITO-Porter [76].

Research directed toward the development of nanomedicines based on MITO-Porter technology includes fields such as mitochondrial gene therapy [77–79], cancer therapy [80–84], ischemic diseases therapy [85,86] and cell therapy [87]. Our research outcomes regarding validating a mitochondrial gene therapeutic strategy are summarized in the following section, with a particular focus on mitochondrial RNA therapy.

### 3.2. Road to gene therapy for mitochondrial related diseases

Mitochondrial genomic abnormalities such as the accumulation of mtDNA mutations would be predicted to cause the onset of various types of diseases. Mitochondrial genetic material including mtDNA, mitochondrial RNA (mRNA, tRNA, rRNA) is concentrated inside the mitochondrial matrix, that is tightly enclosed by double mitochondrial membranes. Delivery of therapeutic cargoes into the mitochondrial matrix is needed to successfully achieve gene therapy targeting mitochondria. Our previous findings showed that the MITO-Porter achieved mitochondrial matrix delivery [88].

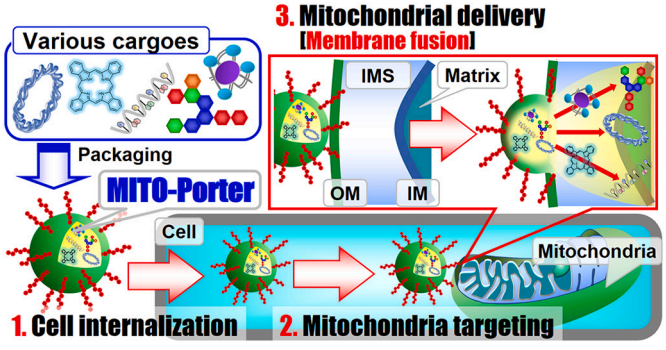


**Fig. 3.** Mitochondrial drug delivery by MITO-Porter.
The MITO-Porter is internalized into the cells, and targeted to mitochondria. Finally, the MITO-Porter delivers its cargoes to mitochondria *via* membrane fusion. IM, inner membrane; IMS, intermembrane space; outer membrane.

It is also noteworthy that therapeutic macromolecules such as nucleic acids can be efficiently packaged in the carrier. Controlling mitochondrial function is necessary for sufficient amounts of cargoes to be delivered to mitochondria. To date, we succeeded in efficiently packaging nanoparticles of therapeutic nucleic acids with polycations in the MITO-Porter. These cargoes include circular DNA [89,90], an antisense RNA oligonucleotide (ASO) [91,92], tRNA [93] and mRNA [94,95]. In the following, we summarize our efforts in mitochondrial gene therapy based on the MITO-Porter technology.

#### 3.2.1. Validation of mitochondrial knockdown by mitochondrial delivery of ASO

With the goal of achieving gene therapy targeting mitochondria, we have mainly focused on research based on the use of a MITO-Porter system, including “Functional molecule delivery aimed at mtDNA [96,97]“, “Construction of mitochondrial gene expression DNA [89,98,99]“and “Nucleic acid therapy targeting mitochondria [10] [91,93,95]”. As our first trial to validate the nucleic acid delivery targeting mitochondria, we attempted mitochondrial RNA knockdown. In this study, we targeted an endogenous mitochondrial mRNA that encodes the cytochrome *c* oxidase subunit II (COX2) protein. The COX2 protein functions to maintain the membrane potential of mitochondria. It was presumed that the knockdown of the mRNA [COX 2] would decrease the levels of expression of the target proteins, and therefore reduce the mitochondrial membrane potential (Fig. 4A) [10][91].

The ASO was transfected into mitochondria of cells using the MITO-Porter, and the antisense effect was then evaluated by the quantification of mRNA [COX 2] levels using quantitative reverse transcription (RT)-PCR. The findings indicated that the mRNA [COX 2] expression levels were depressed by the mitochondrial transfection of ASO compared with a control oligonucleotide (Fig. 4B) and the knockdown effect was maintained for periods of up to 48 h after the transfection [91]. While no knockdown effect was observed when transfection by Lipofectamine, a commercially available transfection reagent, was used.

The membrane potential of mitochondria was also evaluated using the JC-1 dye. Using JC-1, when the membrane potential was decreased, a green fluorescence is observed in the cytoplasm, red colored mitochondria are observed in the case where cells possess normal mitochondria with a membrane potential (Fig. 4C (a)) [91]. In cells where the mitochondrial transfection of ASO was performed, a green fluorescence was observed in the cytoplasm (Fig. 4C (b)). These findings suggest that the direct mitochondrial transfection of ASO could control mitochondrial functions *via* mitochondrial knock down.

#### 3.2.2. Validation of mitochondrial RNA therapeutic strategy targeting mitochondrial disease cells

The next topic is the validation of the mitochondrial RNA therapeutic strategy targeting diseased cells. This study was initiated as an independent clinical research “Study for establishment of a drug treatment for a mitochondrial disease” and was a collaborative effort between three facilities, namely, the Faculty of Pharmaceutical Sciences in Hokkaido University, Hokkaido University Hospital and Sapporo City General Hospital. As the first validation, we used G625A cells obtained from a patient with a mitochondrial disease, who possesses a large amount of mtDNA with a G625A point mutation located in the region coding for $tRNA^{Phe}$ (heteroplasmic mutation) [101]. The mutation rate of the mtDNA is 80%, the mitochondrial complex III activity is reduced, and the mitochondrial membrane potential/ATP production ability is reduced.

As shown in Fig. 5A, we carried out the mitochondrial transfection of wild-type mitochondrial $tRNA^{Phe}$ (WT-tRNA) into G625A cells that carried the mutant $tRNA^{Phe}$ (MT-tRNA) using the MITO-Porter system, in an attempt to reduce the ratio of the MT-tRNA content in mitochondria. The quantification of the content ratio indicated that a decrease in the content ratio of MT-tRNA in mitochondria was observed when the mitochondrial transfection of WT-tRNA was performed,

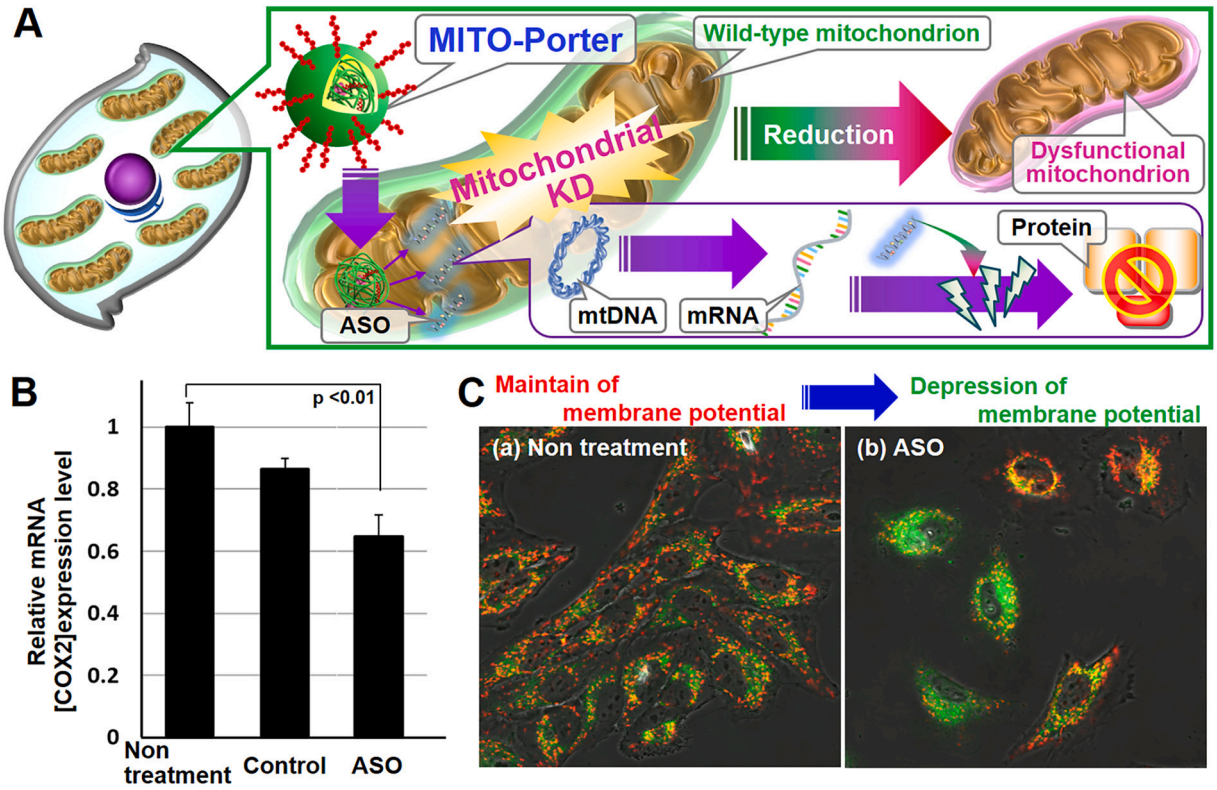


**Fig. 4.** Validation of mitochondrial knockdown using a MITO-Porter system.
(A) Conceptual image of mitochondrial knockdown. After reaching the mitochondria, a nanoparticle of ASO is imported into the mitochondrial matrix. The binding of the ASO to the target mRNA would be the degradation or the inhibition of the translation process. Finally, the expression levels of target mitochondrial protein would be decreased, followed by a reduction in mitochondrial function. (B) Evaluation of mitochondrial mRNA expression levels after mitochondrial transfection of ASO. The knockdown effect of the mitochondrial mRNA (COX2) was estimated by quantitative RT-PCR. Bars indicate the mean with SEM (n = 3–7). (C) Fluorescent image of mitochondrial membrane potential. After the mitochondrial transfection of ASO by MITO-Porter, the cells were stained with JC-1 [fluorescent dye to detect mitochondrial membrane potential] ((a) Non-treatment, (b) ASO). These figures [91] are reproduced with permission from Elsevier.

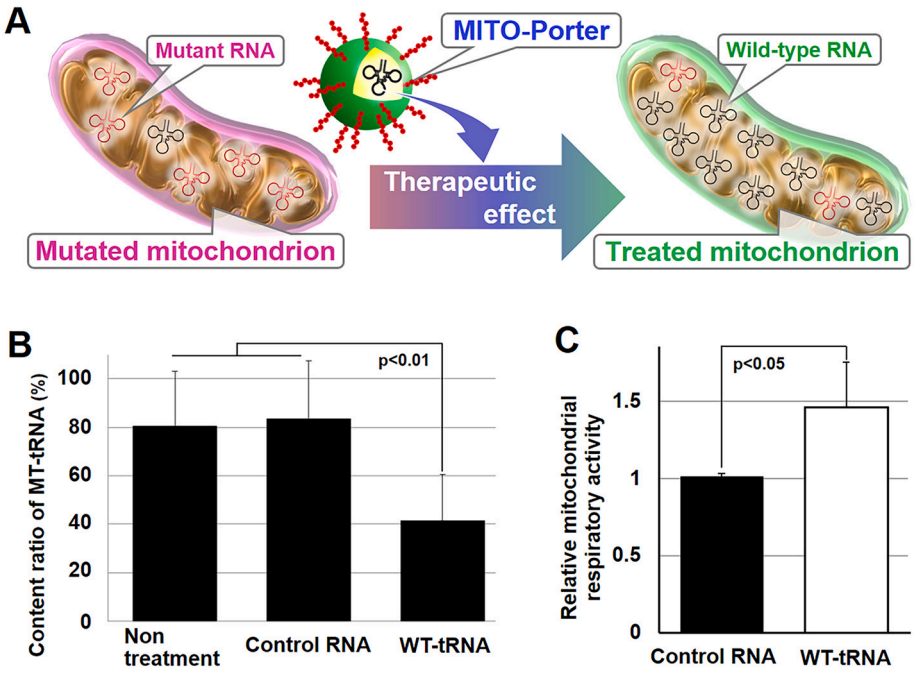


**Fig. 5.** Attempts of mitochondrial RNA therapy targeting disease cells.
(A) Schematic image of mitochondrial RNA therapy targeting mutated mitochondria is shown. Mitochondrial transfection of WT-tRNA in G625A fibroblasts is achieved *via* a MITO-Porter system in order to decrease the content ratio of MT-tRNA in mitochondria. (B) Evaluation of the content ratio of MT-tRNA in mitochondria after transfection with WT-tRNA or negative control RNA. The content ratio was quantitatively estimated. Bars indicate the mean with SEM (n = 3–7). (C) Evaluation of mitochondrial function. Mitochondrial respiratory activities of G625A fibroblasts were estimated by measuring the mitochondrial oxygen consumption rate after the mitochondrial transfection of WT-tRNA or negative control RNA. Data are represented as the mean ± S.E. (n = 3–5). These figures [93] are reproduced.

whereas the transfection of the control sequence had no effect on the content ratio (Fig. 5B). Furthermore, mitochondrial function (mitochondrial respiratory activity) after the mitochondria transfection of the therapeutic RNA was significantly improved compared to that of control RNA (Fig. 5C) [93].

As the next challenge, we verified a gene therapy strategy that targets a disease cell carrying mutant mRNAs. In this validation, we used $LS^{ND3}$ cells, fibroblasts of a Leigh encephalopathy patient, who possesses a large amount of mtDNA with a T10158C point mutation which is located in the region coding for in the NADH dehydrogenase subunit 3 (ND3) protein. This protein plays an important role in mitochondrial respiration. Through a series of studies, we succeeded in delivering the therapeutic mRNA (ND3) to mitochondria in the of $LS^{ND3}$ cells and reducing the content ratio of mutant mRNA (ND3) from 80% to 10%. We also confirmed that the rate of mitochondrial oxygen consumption (mitochondrial function) increased with increasing ratio of mutant mRNA (ND3) [95]. These findings suggest that the mitochondrial RNA delivery therapeutic strategy using the MITO-Porter is a potentially useful innovative therapeutic method for the treatment of mitochondrial diseases.

### 3.3. Perspective of mitochondrial nano medicine based on mitochondrial DDS technology

In this section, we focus on our research dealing with RNA therapy targeting mitochondria. In the near future, we plan to initiate a research project for “Drug discovery targeting mitochondria”. As part of this activity, we will participate in the drug discovery project “7 SEAS PROJECT”, the aim of which is to conduct research and development for “mitochondrial diseases”, and to promote research aimed at contributing to medical care, the economy, and society in general. Based on the MITO-Porter system, BioVenture, LUCA Science Co., Ltd. was established on December 25 of 2018, and the goal of this effort is to promote research on the development of mitochondrial nano medicine, as scientific advisors. In addition, Hokkaido University's Industry Creation Laboratories (Laboratory for Biological Drug Development base on DSS Technology) was opened in April 2020 and this has further accelerated the research and development of “nano-medicine”.

## 4. Development of a lipid-based nano DDS of use in cancer immunotherapy

Immunotherapy is now well established in the field of cancer therapy based on the success of immune checkpoint inhibitors (ICIs). However, the clinical benefits of ICIs are limited in minor portion of cancer patient. To overcome this problem, combination therapies of ICIs with other types of therapies have been investigated. In this situation, the demand for nano DDS technology has been increasing.

### 4.1. Role of nano DDS in cancer immunotherapy

Our immune system protects us from cancer invasion, which is represented by a series of spatiotemporal events, namely the Cancer-Immunity Cycle [102]. Cancer cell derived antigens (neoantigens) are recognized by DCs, followed by DC maturation and antigen presentation. The mature DCs then move to the lymph nodes (LNs) and prime T cells. Finally, the activated T cells infiltrate the tumor tissues and eliminate the cancer cells. The ICIs such as the programmed cell death 1 (PD-1) and the programmed cell death ligand 1 (PD-L1) antibodies block the immune suppression of activated T cells by cancer cells. That is, PD-1/PD-L1 antibodies improve the latter steps after T cell activation. Therefore, therapies that can effectively induce T cell activation should be suitable candidates for such combination therapies.

The priming of T cells by mature DCs is essential for inducing effective T cell activation. Antigens and adjuvants are often employed to enhance this process. Antigens are supplied in various ways such as proteins, peptides, mRNA and DNA [103]. In any case, since they are easily degraded, when used naked, they would not be expected to be effective. Various types of adjuvants are currently available, but agonists of innate immune receptors such as toll-like receptors (TLRs) have been used most recently [104]. Most of the agonists are also unstable in the body or are insoluble, because they are nucleic acids or lipid components. Furthermore, the drawbacks associated with the use of antigens and adjuvants significantly reduce the efficiency of their delivery to target cells and tissues. Therefore, the use of a nano DDS technology should be a promising strategy for their efficient delivery [105]. We have been developing lipid-based nano DDS such as liposomes and LNPs for delivering antigens and adjuvants [76,105,106].

### 4.2. Modification of R8 peptide on nano DDS for efficient delivery of antigens and adjuvants

To induce effective antitumor immunity, antigen presentation on major histocompatibility complex class I (MHC-I) by antigen presenting cells (APCs) such as DCs is necessary for the induction of cytotoxic T lymphocytes (CTLs) [107]. CTLs are differentiated from naïve $CD8^+$ T cells by MHC-I antigen presentation and can eliminate cancer cells *via* their ability to recognize tumor antigen peptides on MHC-I in cancer cells. Delivering antigens to the cytosol in APCs is essential for MHC-I antigen presentation, namely cross-presentation. The use of a combination of the R8 peptide, a cell-penetrating peptide, and fusogenic liposomes would allow efficient endosomal escape to occur, resulting in the cytosolic delivery of antigens [108]. An ovalbumin (OVA) loaded R8 peptide modified liposome (R8-Lip/OVA) was found to induce MHC-I specific antigen presentation in mouse DCs. Interestingly, the enhancement in the C-terminal trimming of the antigen peptide by the R8-Lip contributes to the efficient MHC-I antigen presentation [109]. Furthermore, the *co*-encapsulation of polyinosinic-polycytidylic acid (polyI:C), a TLR3 and melanoma differentiation-associated gene 5 (Mda5) agonist, into the R8-Lip/OVA drastically enhanced the OVA-specific CTL response and antitumor activity against E.G7-OVA tumor [110]. The effect of polyI:C appeared to be maximized by its incorporation into the inner aqueous phase of the R8-Lip for the recognition by TLR3 in endosomes and Mda5 in the cytosol. The fact indicates that the suitable position of an adjuvant in a nano DDS regarding recognition by innate immune receptors is an important issue for maximizing the effect.

CD1 molecules present lipid antigens that are different from MHC molecules, leading to T cell activation and the activation of natural killer T (NKT) cells [111]. Glycolipids derived from tuberculosis are well-known lipid antigens that are presented on CD1 molecules, but the insolubility of these molecules in aqueous media has hampered research progress in this area and their application to tuberculosis vaccines. To overcome this problem, we incorporated glycolipids such as glucose monomycolate (GMM) and glycerol monomycolate (GroMM) into the lipid membrane of the R8-Lip, resulting in the water-dispersion and efficient uptake by APCs [112,113]. We demonstrated that GMM and GroMM are potent target antigens for T cell responses against tuberculosis by using GMM and GroMM loaded R8-Lips, and the GMM and GroMM loaded R8-Lips were found to be useful as tuberculosis vaccines in rhesus macaques and guinea pigs [112–115]. On the other hand, alpha-galactosylceramide (αGC) is a lipid antigen that is presented on CD1d molecules and is expected to act as an adjuvant for NKT cell activation [116]. We also incorporated αGC into the lipid membrane of the R8-Lip [117]. The αGC-loaded R8-Lip drastically enhanced αGC presentation on CD1d in APCs, the activation of NKT cells and exerted antitumor effect in a B16-F10 melanoma lung metastasis mouse model, compared with solution types of αGC. These findings suggest that the R8-Lip represents a potent lipid-based nano DDS for delivering lipid antigens.

Since mycobacterium bovis Bacille Calmette-Guerin (BCG) contains various antigens and adjuvants, BCG is a very successful

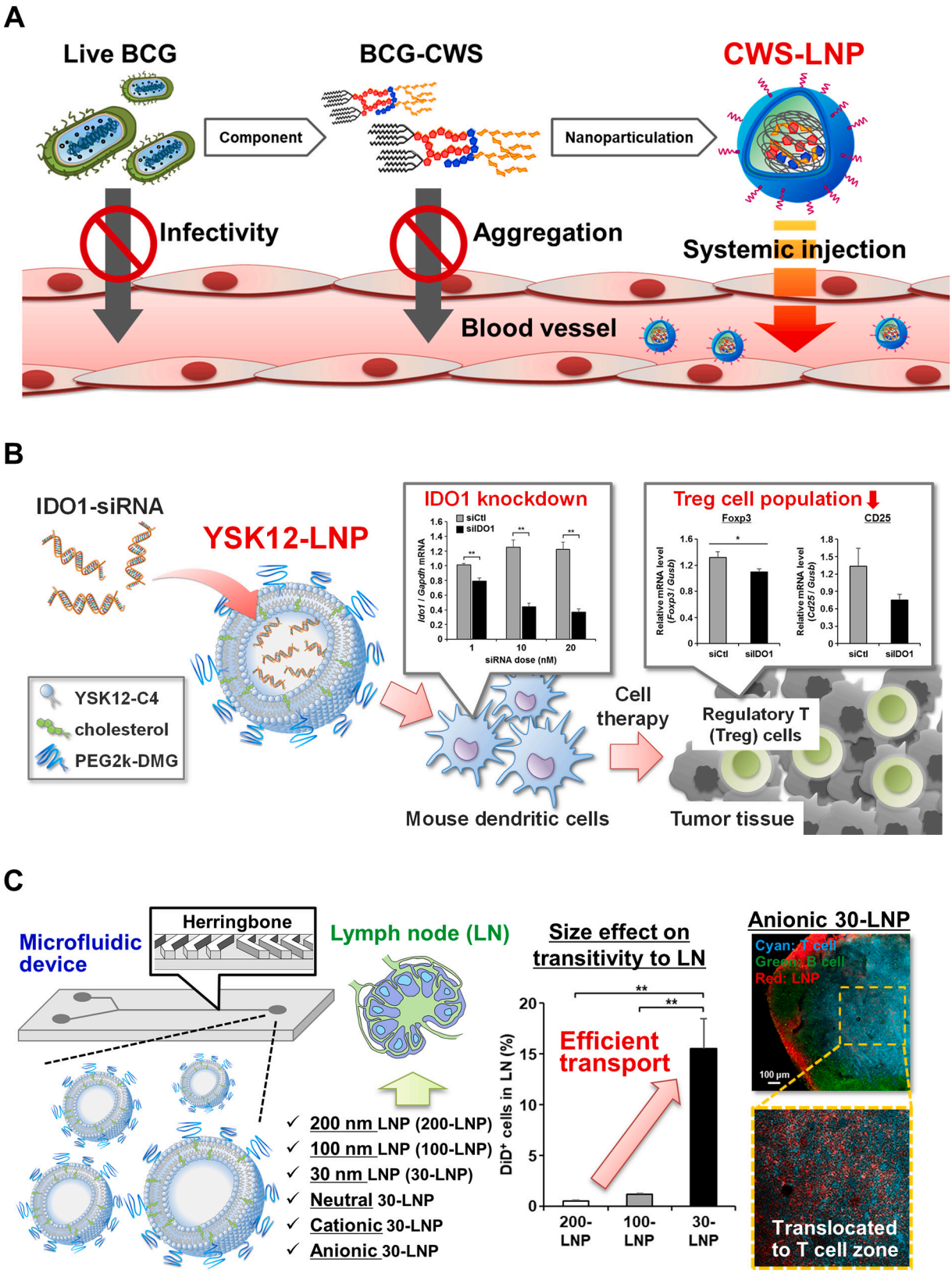


**Fig. 6.** Development of an LNP system toward cancer immunotherapy.
(A) Application of CWS-LNP to the systemic cancer adjuvant. Live BCG and BCG-CWS have been limited to the systemic cancer adjuvant duo to the infectivity and the aggregation, respectively. However, the CWS-LNP effectively functions as a systemic cancer adjuvant, because it is non-infectious and can be dispersed in water. Reprinted with permission from [124]. Copyright 2018 American Chemical Society. (B) IDO1-silenced DC therapy using YSK12-LNP. The administration of IDO1-silenced DC by YSK12-LNP induces the reduction of Treg cell population in tumor. (C) LN targeting using LNP prepared by microfluidic device. Microfluidic device produces LNPs having different sizes and charges. The 30 nm LNP is efficiently taken up by LN cells. Adding an anionic charge to the 30 nm LNP achieves the infiltration to T cell zone in the LN. Reprinted with permission from [148]. Copyright 2020 American Chemical Society.

immunotherapeutic drug. In addition to its use as a tuberculosis vaccine, BCG is intravesically infused in non-muscle invasive bladder cancer patients. The therapeutic rate is more than 70% and is significantly higher than that of ICIs [118,119]. However, patients suffer from a high frequency of serious side effects due to the use of the live mycobacterium [120]. In this situation, the BCG cell wall skeleton (BCG-CWS) is potent candidate for use instead of BCG, because it is the main immune active component in the BCG drug [121]. Clinical applications of BCG-CWS have, however, been limited due to the huge molecular size and the insolubility of the cell wall material in both aqueous solutions and organic solvents. To overcome the unfavorable properties of BCG-CWS, we encapsulated BCG-CWS into an R8-modified LNP (CWS-LNP) by the liposome evaporated *via* emulsified lipid (LEEL) method [122]. The CWS-LNP is a nano-sized particle formulation and is a highly homogenous dispersion in water. Treatment with the CWS-LNP induces strong antitumor responses in MBT-2 (mouse bladder cancer) tumor mouse model, a naturally developed balder cancer rat model, and human immune cells. A study using an MBT-2 tumor mouse model indicates that the antitumor immune responses against bladder cancer by the CWS-LNP is initiated by the internalization of the CWS-LNP into bladder cancer cells, but not APCs [123]. This mechanism appears to be similar to that for BCG-mediated antitumor immunity. Thus, we applied the CWS-LNP to a systemic cancer adjuvant, because, unlike BCG, the CWS-LNP is non-infectious (Fig. 6A). The intravenous administration of CWS-LNP enhanced the induction of OVA-specific CTL and the growth suppression of E.G7-OVA tumor [124]. On the other hand, a biochemical analysis in blood after the intravenous administration of CWS-LNP and an evaluation of weight change during the immunization of CWS-LNP suggest that the CWS-LNP caused no side effects. Furthermore, an investigation of the distribution of the CWS-LNP in the spleen after the intravenous administration revealed that the CWS-LNP is mainly taken up by B cells and a part of CWS-LNP is taken up by DCs. Interestingly, the internalization of CWS-LNP by DCs, but not B cells may contribute to the effective induction of CTL [125]. Collectively, the CWS-LNP can be expected to be applied not only as an immunotherapeutic drug against bladder cancer, but also as a systemically administered cancer adjuvant.

### 4.3. Application of nucleic acids loaded LNPs to cancer immunotherapy

Nucleic acids are a satisfactory modality that functions as antigens, adjuvants and controllers at the gene level. DNA and cyclic dinucleotides (CDNs) are recognized by cyclic GMP–AMP synthase (cGAS), the stimulator of interferon genes (STING) pathway, and also function as adjuvants [126]. Stimulation of the cGAS-STING pathway induces the production of type I interferons (IFNs) and proinflammatory cytokines. We previously demonstrated the potential of DNA or CDN loaded LNPs in cancer immunotherapy [127–129]. Since the cGAS-STING pathway is operative in the cytosol, the agonists must be delivered to the cytosol. We encapsulated a cyclic di-GMP (*c*-di-GMP), a type of CDNs, into the YSK05-LNP for enhancing the cytosolic delivery of *c*-di-GMP [127]. The *c*-di-GMP-loaded YSK05-LNP (cdGMP/YSK05-LNP) induced higher type I IFN production than commercially available transfection reagents in a macrophage cell line. In addition, the subcutaneous administration of the cdGMP/YSK05-LNP with OVA induced an OVA-specific CTL response and a strong inhibition of E.G7-OVA tumor growth. On the other hand, a cdGMP/YSK05-LNP treatment strongly activated NK cells, resulting in the therapeutic effect in a B16-F10 melanoma lung metastasis [128]. B16-F10 cells are nearly lacking in MHC-I molecules. These findings suggest that the cdGMP/YSK05-LNP could show antitumor activity against not only immunogenic tumors, but also tumors that escape from CTL killing. In addition to cancer immunotherapy, the strong production of systemic type I IFNs by the cdGMP/YSK05-LNP may show therapeutic effects against virus pneumonia such as COVID-19 pneumonia, because inflammatory type 2 conventional DCs that optimally prime T cell responses in the lung are generated by type I IFNs [130].

The regulation of gene expression in immune cells by siRNA appears to be a useful method for controlling complicated cancer immune responses. However, it is well known that it is difficult to introduce siRNA to immune cells using non-viral vectors. We previously developed siRNA-loaded LNPs that target various immune cells [131–136]. An LNP composed of YSK12-C4, a cationic lipid with fusogenic capability, efficiently introduced siRNA to mouse DCs and to human immune cell lines (Jurkat, THP-1, KG-1 and NK-92) compared with commercially available siRNA transfection reagents [133,134,136]. Moreover, mouse DCs that contain immune suppressive genes such as the suppressor of cytokine signaling 1 (SOCS1) and indoleamine 2,3-dioxygenase 1 (IDO1) that were silenced by the YSK12-LNP resulted in a significantly strengthened antitumor effect in DC-based therapy [133,136]. In particular, IDO1 is intimately associated with the formation of immune suppressive tumor microenvironments, leading to a poor prognosis of cancer patients [137,138]. Because the production of IDO1 results in the inhibition of effector T cells, the induction of regulatory T (Treg) cells and the activation of myeloid-derived suppressor cells (MDSCs) [139]. Thus, IDO1 is a potent target for a combination therapy with ICIs. In our study, the treatment of IDO1-silenced DCs by the YSK12-LNP appeared to be accompanied by a decrease in the Treg cell population in tumor tissue (Fig. 6B) [136]. We conclude that the YSK12-LNP can be potent nano DDS for siRNA delivery to immune cells.

### 4.4. LN targeting by small-sized LNPs produced by microfluidic devices

Given the Cancer-Immunity Cycle, LNs are central organs for initiating T cell responses against tumors [102]. Thus, LNs are attractive delivery targets for cancer immunotherapy. For direct delivery *via* the lymphatic system by using nano DDS, particles size is a critical factor and the suitable size appears to be in the range from 20 nm to 50 nm [140–142]. An important technology in the success of the Patisiran preparation is microfluidic mixing. Microfluidic devices are used for this purpose, since they can continuously mix a lipid solution and a drug solution, resulting in manufacturing on an industrial scale [143–145]. Moreover, the continuous rapid mixing with microfluidic devices can generate LNPs with sizes below 30 nm [143,146,147]. It therefore appears that LNP technology will be increasingly applied to the field of LN targeting.

We recently reported on the effect of LNP properties prepared by a microfluidic device on LN delivery (Fig. 6C) [148]. To our knowledge, only a few such studies have been reported [149,150]. A comparative analysis between 30 nm, 100 nm and 200 nm neutral LNPs clearly demonstrated that the 30 nm LNP are drastically captured by LN cells and CD8α DCs in the LNs compared with other LNPs. Furthermore, charge property of 30 nm LNPs greatly influences the transport capability to the LNs and the distribution in the LNs. The anionic 30 nm LNP showed a superior transport capability to LNs compared with the cationic and neutral 30 nm LNPs. Interestingly, the anionic 30 nm LNP was distributed to the inner parenchyma, namely the cortex and paracortex and was captured by B cells and T cells. Although the mechanism for the transport of anionic 30 nm LNPs may be associated with lymphatic endothelial cells in a subcapsular sinus [151–153], further investigations will clearly be needed. Our collective findings indicate that even for 30 nm LNPs, charge has a significant effect on their transport to and distribution within the LN.

Our results indicate that the small-sized LNPs produced by microfluidic devices should be potent delivery systems for targeting LNs. We expect that developing new cancer immunotherapy using the LNP technology will now accelerate. Because tumor antigens flow to tumor draining LNs, tumor draining LNs are promising targets for the effective induction of antitumor immunity [154]. Furthermore, spontaneous immune responses against immunogenic tumors can be initiated in the tumor microenvironments and the draining LNs [155]. Consequently, using such small-sized LNPs for delivering DNA and mRNA coding

antigens or adjuvants is expected to become standard procedure for the induction of effective antitumor immunity in the future.

## 5. Perspectives

During 20 years of gene delivery research, we have learned how exciting it is to develop original delivery systems by interacting with many collaborators as well as competitors. We have sometimes discovered unexpected mechanisms and succeeded in developing one of the most efficient delivery systems reported so far. However, we have faced numerous difficulties when we moved into translational studies with Venture Companies, Pharmaceutical Companies, *etc.* LUCA Science is one of the most committed Venture Company who plan to take these breakthrough technologies into clinical practice. Lilac Pharma is another venture company originated from Hokkaido University and is now focusing on microfluidic technology for scaling up the production of lipid nanoparticles. We are currently creating networking relationships with companies who are interested in our technologies and in developing Nanomedicines. We hope to see a new era of Nanomedicine in the near future keeping in mind the words of Thomas Edison “Many of life's failures are people who did not realize how close they were to success when they gave up”.

## Declaration of competing interest

The authors (Y.S. and T.N.) have no conflicts of interest to declare. The authors (Y.Y. and H.H.) have a conflict of interest to disclose with this study as follows: This study was financially supported by LUCA Science Inc. (Tokyo, Japan) for Research and Patent licensing fees.

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