A Dissertation

for the Degree of Master of Science

Efficient gene transfection to liver cells by a multifunctional polylactitol-based gene transporter

폴리락티톨 기반 간세포 표적 다기능성 유전자 전달체의 개발

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치의과학과 분자유전학전공

김영동

폴리락티톨 기반 간세포 표적
다기능성 유전자 전달체의 개발

지도교수 Rohidas B. Arote

이 논문을 김영동 석사학위논문으로 제출함

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서울대학교 대학원

치의과학과 분자유전학전공

김 영 동

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위 원 장 우 경 미 (인)

부위원장 (인)

위 원 조 종 수 (인)

Abstract

Young-Dong Kim
School of dentistry, molecular genetics
The Graduate School
Seoul National University

In recent years, the introduction of non-viral gene transfer systems for treatment of inherited and acquired liver diseases has attracted a lot of attention. To mediate liver-directed gene delivery, the strategy of liver cell targeting and intracellular control of gene trafficking for designing an ideal non-viral gene delivery system are a crucial and great challenge. In order to meet this needs, a new multifunctional gene carrier, polylactitol-based gene transporter (PLT) was prepared by crosslinking low molecular weight polyethylenimine (LMW PEI) with lactitol diacrylate (LDA) composed of D-galactose and D-sorbitol provides synergistic effects to increase cellular uptake, to get liver cell targeting, and to have rapid release of gene from endosome, because hyperosmotic property of polysorbitol part selectively stimulates caveolae-mediated endocytosis, polygalactose part provides liver cell targeting ability and PEI part assists rapid endosomal escape of gene due to its proton sponge effect. With these unique multifunctions, PLT/DNA nanocomplexes showed low cytotoxicity, high transfection efficiency, liver cell targeting in vitro and in vivo, and selective transition of cellular uptake pathway into the caveolae-mediated endocytosis avoiding lysosomal degradation. Taken together, PLT was confirmed as a safe and efficient vector, which will highlight a potential

candidate for targeted gene therapy in the hepatic diseases.

keywords : Gene delivery, Lactitol, PEI, Liver cell targeting, Caveolae-mediated endocytosis

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List of Abbreviations

PEI: polyethylenimine

ASGPR: asialoglycoprotein receptors

GLUT2: glucose transporter-2

PLT: poly(lactitol-co-PEI)

rAAV: recombinant adeno-associated virus

DOPE: dioleoylphosphatidyl-ethanolamine

DOTAP: N-1(-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniumethyl - sulphate

DOTMA: N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride

CME: clathrin-mediated endocytosis

CvME: caveolae-mediated endocytosis

NCME: non-caveolin-mediated endocytosis

CPP: cell-penetrating peptides

PMT: polymannitol-co-PEI

Polyplexes: polymer/gene complexes

EGF: epidermal growth factor

BPEI: branched PEI

DMSO: dimethylsulfoxide

DMF: dimethylformamide

CH: chloropromazine

GE: genistein

M- β -CD: methyl-beta-cyclodextrin

WO: wortmannin

Cy5.5: Cyanine5.5

RPMI: Roswell Park Memorial Institute

DMEM: Dulbecco's modified Eagle's medium

FBS: fetal bovine serum

LDA: lactitol diacrylate

GPC: gel permeation chromatography column

EF-TEM: energy-filtering transmission electron microscopy

DDW: double distilled water

TAE: tris–acetate

SDS: sodium dodecyl sulfate

A549 cells: lung adenocarcinoma epithelial cells, human

HepG2 cells: hepatocellular carcinoma, human

293T cells: embryonic kidney cells, human

RLU: relative light units

Bafilomycin A1: an inhibitor of vacuolar-type H^+-ATPase

SDA: sorbitol diacrylate

LMW/HMW: low molecular weight/high molecular weight

EPR effect: enhanced permeability and retention effect

I. Introduction

Gene therapy has emerged as a future medicine to treat a wide range of the human diseases such as various types of cancers and inherited genetic disorders. The liver diseases have been of high interest in the field of gene therapy because the liver plays a wide variety of roles such as metabolism, detoxification, and synthetic activity in the body [1], and numerous inherited liver disorders impair those critical functions [2]. Gene therapy is able to provide treatment of the ultimate underlying cause of liver originated inherited diseases via directly correcting defective gene or supplanting the functional genes.

In this regard, tremendous efforts have been made toward developing effective liver-directed gene delivery system focused mainly on viral vectors which promise great transfection efficiency [3, 4], however, several crucial limitations related to safety concerns have led to the pursuit of nanotechnology-based non-viral vectors as alternatives [5-9]. Among the non-viral vectors, polymeric nanocarriers are currently attracted by their excellent properties over viral vectors including low immunogenicity, tunable size, no limitation of length of DNA, ease of preparation, scale-up, and surface functionalization. Nevertheless, relatively low transfection efficacy and lack of site-specific delivery of the polymeric carriers still remain great challenge for utilizing their advantages in liver gene therapy.

To overcome those problems, numerous strategies based on modification of mechanistic modules of gene carriers have been investigated. Firstly, to confer

targeting specificity to liver, galactose moiety as a targeting ligand has been widely introduced into polymeric gene carriers including polyethylenimine (PEI) [7], chitosan [10], polyspermine [11], and polyphosphoramidate [12] for specific interaction with asialoglycoprotein receptors (ASGPR) [6] or glucose transporter-2 (GLUT2) [13, 14] which are abundantly expressed in hepatocytes. Secondly, to enhance the transfection efficiency, strategy of avoidance of lysosomal degradation [15] has been investigated as a critical factor in intracellular delivery of gene. Also, a "proton sponge effect" which provides rapid endosomal escape before fusion with lysosome was commonly confirmed by the PEI as a golden standard of polycationic non-viral vector. However, high cytotoxicity of the generally used PEI is still one of major barriers to clinical use of PEI although the cytotoxicity of the PEI depends on the MW and structure of the PEI. Our group has previously developed a variety of polyol-based gene transporters composed of degradable PEI part and osmolyte part including polyglycerol [16, 17], polysorbitol [18-20], polymannitol [21, 22], and polyxylitol [23, 24] as a strategy of controlling intracellular trafficking. We demonstrated that hyperosmotic properties of polyol-based transporters are able to stimulate caveolae-mediated endocytosis pathway offering an effective internalization of nucleic acids via non-digestive routes [25] showing enhanced transfection in vitro and in vivo.

Although much efforts has been devoted in improvement of efficacy of liver-targeted gene delivery, non-viral vectors still face multiple challenges owing to single functionality requiring multifunctionality of polymeric carrier. In general, the multifunctionality is constituted by multistage chemical conjugations of

functional moieties. However, such modifications are multistep processes not straightforward and complicated due to technical difficulties and moreover, each functional part can lose the activity during multistage chemical conjugations of the functional parts.

To address the issues associated with synthetic complexity of multifunctional carriers in liver-directed gene delivery system, herein, we employed a novel multifunctional gene carrier by simple chemical processes, poly(lactitol-co-PEI) (PLT) using a lactitol composed of D-galactose and D-sorbitol as a multifunctional material because galactose provides liver-targeting specificity and sorbitol has osmotic activity. The hyperosmolarity of polysorbitol part in PLT could mechanically stimulate cells inducing hyperosmotic stress [26], which may shifts the mode of endocytosis pathways biased toward caveolae-mediated endocytic pathway enhancing gene delivery efficiency. Also, polygalactose part in PLT could act as molecular Trojan horse providing a liver-directed gene deliver via specific binding to ASGPR. Furthermore, PEI part in PLT could assist rapid endosomal escape of gene due to its proton sponge effect.

Altogether, based on above mentioned approaches, we hypothesized that PLT has great potentials as a multifunctional carrier for liver gene therapy: 1) liver cell targeting specificity by polygalactose part, 2) selective stimulation of caveolae-mediated endocytosis by polysorbitol part, and 3) induction of rapid endosomal escape by PEI part. In this study, synergistic effects of multi-functional PLT on overcoming multiple biological barriers were examined in vitro and in vivo.

II. Review of Literature

1. Introduction of gene therapy

1) General overview of gene therapy

The therapeutic delivery of genes (DNA, RNA, or synthetic nucleic acids) into a patient's cells to treat disease is described as ‘Gene therapy’. As shown in Fig. 1, there are common forms of gene therapy. Delivered genes can be expressed as proteins, correct genetic mutations, and interfere or control with the expression of proteins [27]. Therefore, gene therapy has great potential for treating both genetic and infectious diseases by various forms of gene therapy.

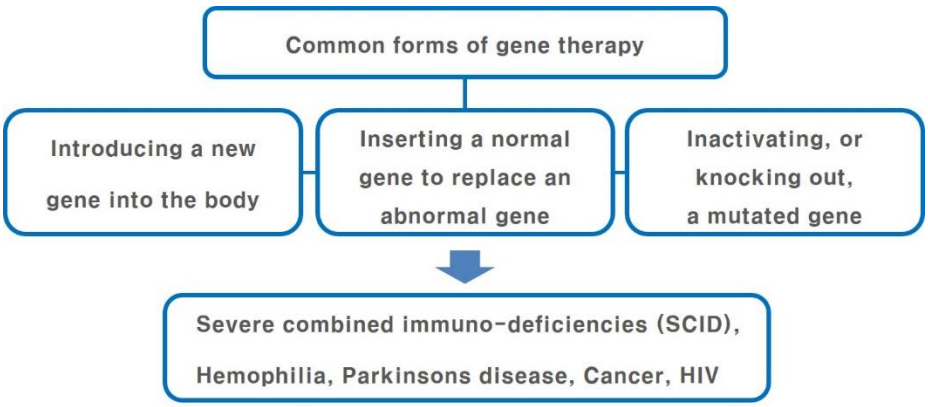

Figure 1. Common forms of gene therapy.

In gene therapy, the nucleic acid molecule has large size and the negative charge, their delivery is typically mediated by carriers or vectors, which is the most common form of gene delivery system. For an ideal gene delivery, vector should be safe, have target specificity, and can be categorized into viral and non-viral vectors according to their origin of building blocks.

Viral vectors such as recombinant adeno-associated virus (rAAV) vectors have emerged as highly promising for use in gene transfer for a variety of reasons, including high transfection efficiency, lack of pathogenicity and wide host range [28]. However, several crucial limitations related to safety concerns such as immune responses and insertional mutagenesis risk have led to the pursuit of nanotechnology-based non-viral vectors as alternatives.

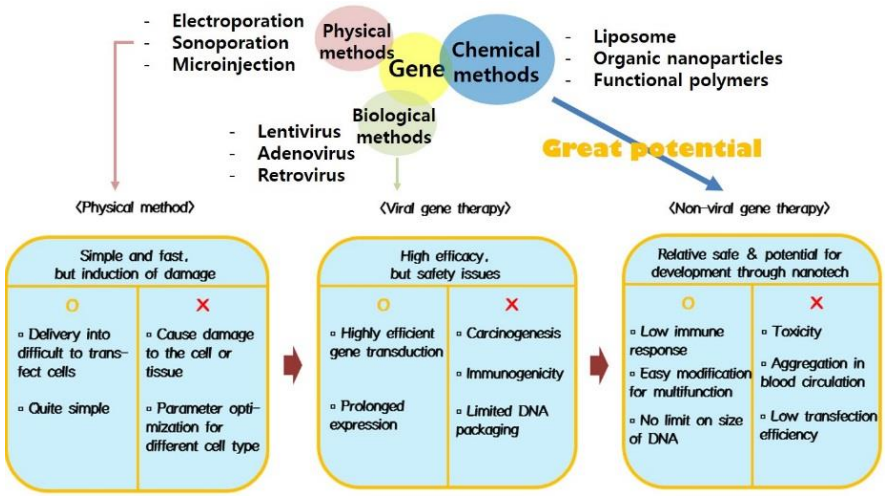

Figure 2. Prospect of gene therapy by non-viral vectors.

Non-viral vectors have been successfully proposed as the potential vector to address safety concerns of viral vector (Fig. 2). Also, it is typically easier to synthesize and has abilities to deliver larger genetic payloads [29]. Although relatively low transfection efficiency of non-viral vectors is pointed out as drawback, it is improved by the rapid progress of nanotechnology, which gives versatilities for overcoming various cellular barriers and enhancing gene transfer such as hydrophobicity/hydrophilicity, safety, serum stability, lack of immunogenicity, target specificity. In order to control and modify these advantages, various materials-based non-vectors were developed and being

researched such as liposome, polycationic polymers, inorganic nanoparticles, and hybrid form of two or more materials.

2) Non-viral gene delivery vectors

Non-viral gene delivery vectors can be categorized in two major types: cationic lipids and cationic polymers, which are formed by self-assembling process: at physiological pH, positive charges of vectors interact with polyanionic nucleic acids and spontaneously lead to the formation of nanoscaled complexes named lipoplexes and polyplexes for lipids and polymers, respectively [30].

2-1) Lipid-based non-viral vectors

In non-viral gene delivery, cationic liposomes are one of the most widely and successfully used lipid-based vectors for gene transfer, since their introduction as gene delivery systems in 1987 [31].

Cationic liposomes are able to interact spontaneously with negatively charged genes and are formed by the self-assembly of dissolved lipid molecules. Hydrophilic head group and hydrophobic tail of lipid molecule form the bilayer and energetically favorable circular shape. Their usual form includes a neutral lipid such as DOPE (dioleoylphosphatidyl-ethanolamine) into the formulation, which assist membrane fusion. Various cationic lipids have been developed to effectively deliver genes. However, maybe the best potent types are DOTAP

(N-1(-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniumethyl sulphate) and DOTMA (N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride). These are commercially available lipids, which are widely used in gene delivery fields such as human clinical gene therapy trials as well as in cell transfection applications for biological research [32].

However, despite their many advantages such as ease of production/preparation, biodegradability, protection of nucleic acids from nuclease degradation and renal clearance, and promotion of cellular uptake, acceptable toxicity profile, cationic liposomes contribute to >8% of the vectors used in clinical trial and the efficiency of these vectors is not high enough to provide significant therapeutic effects [33].

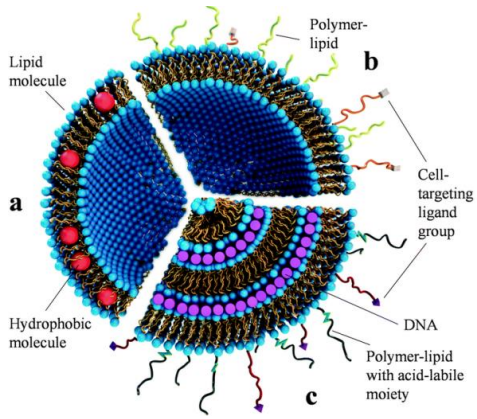

Figure 3. The evolution and developments for functional liposome. (a) A unilamellar liposome consisting of a self-assembly of amphiphilic lipid molecules for trapping hydrophobic molecules. (b) A "stealth" liposome for avoid immune cells. (c) A Cationic liposome–DNA complex consisting of an onion-like multilamellar structure with DNA (purple rods) sandwiched between cationic membranes. [34]

To enhance their efficacy and draw potentials, a variety of developments for functional liposome (Fig. 3) are ongoing by using various strategies such as modifying the chemical features of the lipids and controlling their biological behaviors via fusion with other materials, which made immune-, pH sensitive-, long circulating-, and fusogenic-liposome. These efforts have been contributed for developing efficient lipid delivery vectors.

2-2) Polymer-based non-viral vectors

Synthetic cationic polymeric vector specifically designed by comprising a variety of chemistries for gene delivery, which has nucleic acid binding moieties including amines, as well as other positively charged groups and linear, branched and dendritic structures. Because of the flexibility of polymer chemistries, they have great potential for human gene therapy [29].

However, poor gene-transfer efficiency of synthetic polymeric vector has limited their clinical application, because usually they don’t have efficient modes to overcome many extra- and intra-cellular gene delivery obstacles without functionalities. To get over barriers related with delivery efficacy, since the first demonstration of polycation-mediated gene transfection in 1987 [35], various formulations of cationic polymers have been synthesized and developed as non-viral vectors for effective gene delivery.

2-2-1) PEI

Among them, one of the most effective and widely studied polymeric gene delivery systems is the PEI (Polyethylenimine), which has high concentration of positively charged nitrogen atoms. It can interact with negatively charged nucleic acid such as DNA and siRNA resulting in the formation of polyplex. Also, PEI can enhance transfection efficiency by overcome intracellular barriers, including the escape from lysosomes, nuclear localization and DNA unloading [36]. However, the exact mechanisms about intracellular transport remain largely unexplained, only the most widely accepted hypothesis is 'proton sponge effect' [37]. This hypothesis suggests that the PEI becomes more protonated at a low pH in endosomes, and the proton buffering capacity of protonated amino groups of PEI triggers an influx of Cl- ions with protons leading to a water influx and finally the swelling and rupturing of the endosomes (Fig. 4). Ion-pair formation or proton-sponge effect can induce proper releasing of genes from the carrier by preventing the endo-lysosomal entrapment.

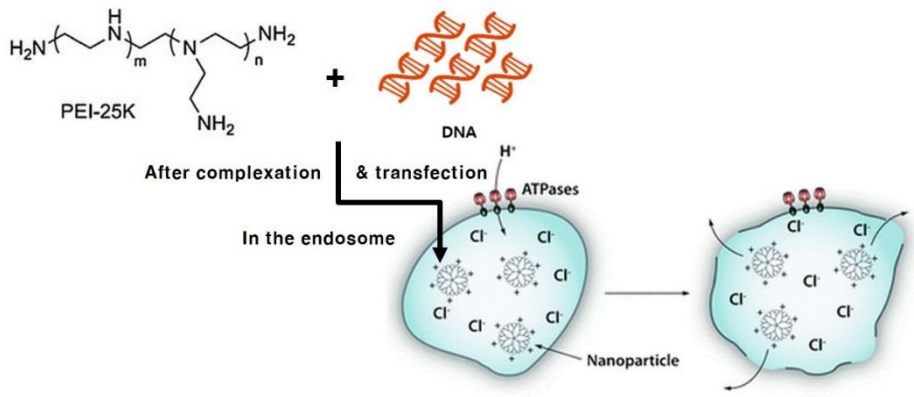

Figure 4. Mechanism of proton sponge effect. [modified from [38]]

Although high concentration of PEI's cation enhance transfection efficiency by forming stable polyplexes, its non-cleavable structure and excess of positive surface charges induces greater cytotoxicity. Therefore, various strategies for reducing cytotoxicity such as use of low molecular weight PEI, introduction of biodegradable linkages, cross-linking with anionic polymers, and further modifications have been investigated and have improved gene delivery efficacy.

2-2-2) Osmotic active polyol-based gene transporter

Transfection efficiency is largely affected to cellular uptake pathways which can determine the intracellular fates and cytosolic delivery of the carried gene by directing them toward the digestive or non-digestive route [39]. Although endocytic and intracellular pathways remain largely unanswered, endocytosis pathway are largely affected by nanocomplexes of physical, chemical, biological and geometrical cues [40].

In this regards, our group has previously developed a variety of polyol-based gene transporters composed of degradable PEI part and osmolyte part including polyglycerol [16, 17], polysorbitol [18-20], polymannitol [21, 22], and polyxylitol [23, 24] as a strategy of controlling intracellular trafficking.

In our previous studies, osmotic active molecules (polyols) have two crucial role for effective gene delivery. Firstly, abundant hydroxyl groups in polyol backbone form strong intermolecular hydrogen bonding between polymers with hydroxyl groups and nucleic acid, and can shield the surface charge of the polyplexes. Therefore, our polyplexes have nearly neutral surface charge,

which are significantly lower than those of PEI/DNA complexes. It can contribute to reduce cytotoxicity, because high cytotoxicity of PEI is associated with its high positive charges which lead electrostatic interaction with the negatively charged components of the cell membrane and cause membrane disruption and damage [29].

Another important role is that hyperosmotic properties of polyol-based transporters are able to stimulate caveolae-mediated endocytosis pathway by their hyperosmolality. Osmotic cellular stress offering an effective internalization of nucleic acids via non-digestive routes [25] showing enhanced transfection in vitro and in vivo. Even though there are some necessity of further modifications, osmotically active polyol-based gene transporter must be the most potent candidate as polymer-based non-viral vector.

2. Challenges for liver-directed non-viral gene therapy

In the field of gene therapy, the liver diseases are one of the most challengeable major target for adopting gene therapy, because the liver plays a wide variety of roles such as metabolism, detoxification, and synthetic activity in the body [1], and numerous inherited liver disorders impair those critical functions [2].

However, many liver diseases lack satisfactory treatment, and there are proper alternative therapeutic options. In these regards, gene therapy is great option for treating a variety of liver disease including inherited metabolic defects, chronic viral hepatitis, liver cirrhosis and primary and metastatic liver

cancer. Also, introduction of new gene via gene transfer to the liver can be used as a factory of specific secreted proteins that do not affect the liver itself [41].

Tremendous efforts have been made toward developing effective liver-directed gene delivery system focused mainly on viral vectors which promise great transfection efficiency [3, 4], however, several crucial limitations related to safety concerns have led to the pursuit of nanotechnology-based non-viral vectors as alternatives [5-9]. As discussed in section (2-2-2), among the non-viral vectors, polymeric nanocarriers are currently attracted by their excellent properties over viral vectors including low immunogenicity, tunable size, no limitation of length of DNA, ease of preparation, scale-up, and surface functionalization. Nevertheless, synthetic non-viral vectors are usually unsatisfactory because they deficient one or several of the necessary function such as site-specific delivery. It still remains great challenge for utilizing their advantages in liver gene therapy. In this section, extra- and intracellular barriers in liver-directed non-viral gene delivery and strategies using nanotechnology will be covered (Fig. 5).

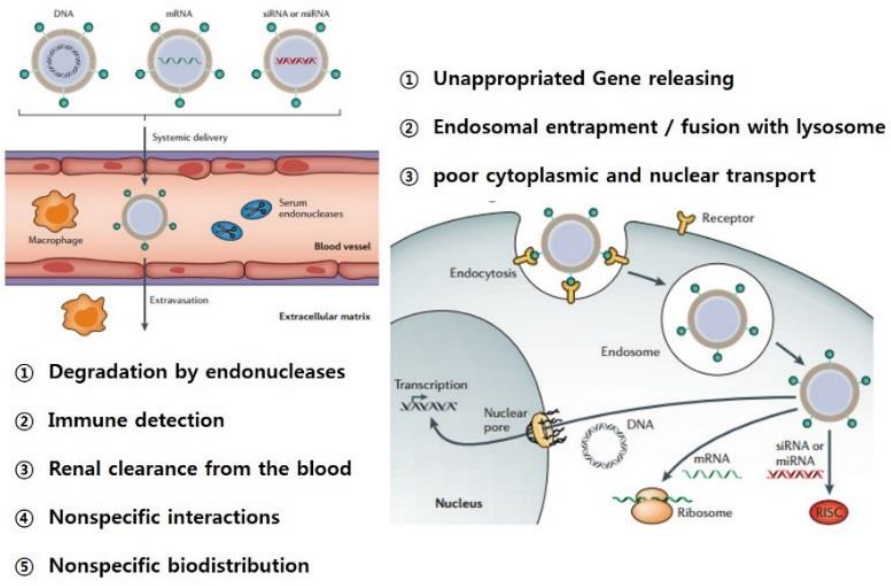

Figure 5. Delivery mechanisms and Barriers for in vivo gene therapy [42].

1) Challenges for overcoming extracellular barriers

Extracellular barriers include obstacles for transporting genes from the test tube to the membrane of a target cells: nanoparticle's physio-chemical properties (gene packaging and particle size), in vivo barriers (stability in the blood circulation and target specificity). In this section, extracellular barriers related with liver-directed non-viral gene delivery will be covered.

1-1) Gene packaging for protection

For in vivo systemic gene delivery to the liver, one of the challenge is the gene packaging for protection from degradation of the therapeutic gene in extracellular region. The half-life of plasmid DNA was estimated to be ten

minutes following intravenous injection in mice [43]. In this regard, non-viral vector must be protect from endonucleases and prolong circulation time. For instance, our group's gene protection & releasing assay results using polyol-based gene transporters shown superior gene packaging at N/P ratio 1 by electrostatic complexation and protection from degradation by nucleolytic enzymes. However, in issues related to complexation, strong binding or/and efficient DNA condensation do not indicate directly gene-delivery efficiency, because rigid binding may prevents transcription [29]. In another view, poly-plexes are spherical and nearly monodisperse. It is strongly encouraged feature of non-viral vector because neutral polyplexes in physiological salt concentrations quickly form large aggregates, which hinder effective gene-delivery and can be toxic due to embolism in lung capillaries. Also, abundant hydroxyl groups in polyol backbone shield remaining surface positive charge, which protect either colloidal instability or interaction with blood components (such as serum proteins and erythrocytes), and reduce rapid clearance by circulating macrophages [42].

1-2) Liver cell specificities

For specific accumulation in target tissue of interest, target-cell specificity is the major challenge for in vivo gene therapy. Especially, in cancer gene therapy, target tissue and cell-specific gene delivery is the most important because main purpose is to kill the cells. Although polymers usually lack the targeting abilities, flexible modification of targeting moieties can provide specificities.

In this regard, non-viral polymeric vector is modified using ligand for targeting many membrane-bound receptor proteins because receptor-ligand interaction initiates receptor-mediated endocytosis.

In case of liver, to confer targeting specificity to liver cells, galactose moiety as a targeting ligand has been widely introduced into polymeric gene carriers including polyethylenimine (PEI) [7], chitosan [10], polyspermine [11], and polyphosphoramidate [12] for specific interaction with asialoglycoprotein receptors (ASGPR) [6] or glucose transporter-2 (GLUT2) [13, 14] which are abundantly expressed in hepatocytes. The asialoglycoprotein receptor (ASGPR) is primarily expressed on hepatocytes and minimally on extra-hepatic cells, and it has high affinity for carbohydrates specifically galactose, N-acetylgalactosamine and glucose [44]. Interaction of ASGPR with ligands of gene carrier initiates internalization of polyplexes by clathrin-mediated endocytosis. This liver targeting strategies are widely explored for liver-directed gene delivery and minimize concerns of toxicity by reducing non-specific accumulation in normal tissues.

2) Challenges for overcoming intracellular barriers

After reach target tissues by extravasation from the bloodstream, gene delivery vectors encounter various intracellular obstacles such as endosomal entrapment, unappropriated gene releasing from vector, and poor cytoplasmic and nuclear transport, which related to considerably low transfection efficiency of non-viral vector. In this section, major challenges for overcoming

extracellular barriers of liver-directed non-viral gene delivery will be covered.

2-1) Endo-lysosomal escape

Generally, the polyplexes are enter into the cells by endocytic vesicles. After that, they may go into the late endosomes through maturation process via the endocytosis pathways, then ATPase proton-pump enzyme in the vesicle membrane rapidly act and promote acidify to pH 5–6. Subsequently, further trafficking to the lysosome and acidification (~4.5) of internal space of vesicles leads to degradation of genes with degradative enzymes.

Therefore, to protect from lysosomal gene degradation, endo-lysosomal escape is one of the most challenge for developing efficient non-viral vector. There are three major strategies. Firstly, carrier can be designed to bypass endo-lysosomes by selective stimulation into the special endocytosis pathway. Caveolae-mediated endocytosis (CvME) is able to evade from endo-lysosomes. This will be discussed later. As discussed in section 2-2-1, second strategy is use of functional polymers such as PEI. Its unique property, 'proton sponge effect', induce rapid endo-lysosomal escape. Abundant amine groups in PEI that can be protonated and consume influxed-protons in vesicles. It increase concentration of endosomal chloride anion, which leads influx of water. Finally, endosome was disrupted by osmotic pressure, which is called osmotic swelling [45]. This specific mode of action of PEI has been widely corporate in design of polymeric non-viral vectors. Final strategy is utilizing modification of cell-penetrating peptides (CPPs) with polymeric vectors. Most of the CPPs usually

has a high density of amino acids, and it can interact with anionic surfaces of the plasma membrane and enhances cellular entry. Also, several synthetic amphipathic peptides such as (GALA, KALA, JTS-1, and ppTG20) are used to enhance endosomal escape. Generally short sequences of only 20 amino acids induce membrane destabilization, and their an a -helical structure at endosomal pH leading to hydrophobic and hydrophilic faces that can form pore at endosomal membrane and leas to disruption [46].

2-2) Selective stimulation of CvME

As referred to earlier, CvME is one of endocytosis pathway that can bypass the endo-lysosomes in Fig. 6. In this regard, selective stimulation of CvME by functional molecules in non-viral vector can improve transfection efficiency. For instance, in our group, Park. et al. designed new non-viral vector, PMT (polymannitol-co-PEI), which shifted the uptake route into CvME by using hyperosmotic activity of PMT. Results strongly indicate that PMT/DNA complexes efficiently avoid lysosomal degradation via osmotic activity of polymannitol activated Src-kinase and stimulated caveolae-mediated endocytosis [21]. In detail, proposed possible mechanism is that generated osmotic gradient osmolytes by draw water from the intracellular space to extracellular region, which can cause cell shrinkage. Subsequently, phosphorylation of caveolin-1 is mediated by Src-kinase, and it is required for caveolae budding and induction of CvME.

Other factors for stimulating CvME are particle size and ligand modification.

Firstly, there is repot that small particle size is more favorable for uptake mechanisms. Boussif, O. et al. [47] compared uptake efficiency via CvME by using PEI polyplexes (20, 40, and 100 nm) of three particle sizes in endothelial cells. In their results shown 20 and 40 nm nanoparticles have 5−10 times higher uptake efficiency levels than 100-nm particles, indicating that small particle size is more efficient to facilitate CvME. In addition, CvME can be mediated through some specific ligand−receptor binding. Activation of some receptors such as insulin receptor [48], epidermal growth factor (EGF) receptor [49] mediated CvME. Furthermore, interaction of caveolae or caveolin with specific ligands like folic acid [50] can trigger the rapid internalization of caveolae [51]. These improved understanding of factors for stimulating CvME may facilitate the design of efficient non-viral gene transfer systems.

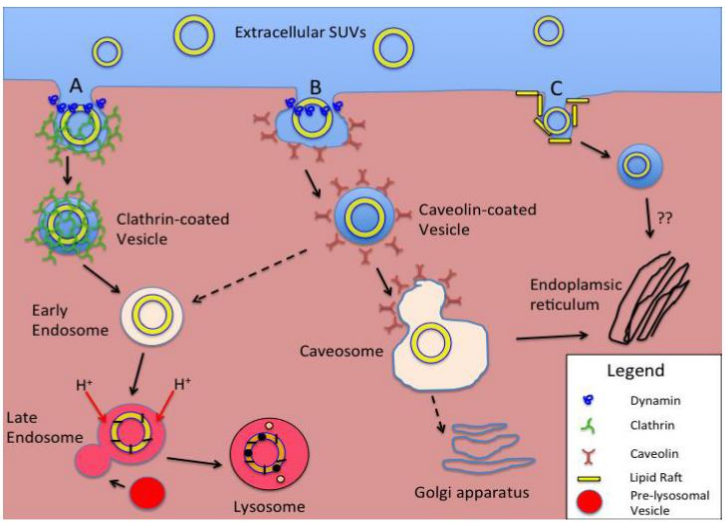

Figure 6. Schematic illustration of intracellular processing of nanocarriers. (A) Clathrin-mediated endocytosis (CME), (B) Caveolin-mediated endocytosis (CvME), (C) Non-clathrin- or non-caveolin-mediated endocytosis (NCME). [52]

III. Materials and methods

1. Materials

Branched PEI (BPEI) (Mn: 1200 Da and 25 kDa), dimethylsulfoxide (DMSO), dimethylformamide (DMF), D-lactitol monohydrate, pyridine, bafilomycin A1, chloropromazine (CH), genistein (GE), methyl-beta-cyclodextrin (M-β-CD), wortmannin (WO), tetrazolium reagent (MTT reagent), D(+)-galactose were purchased from Sigma (St. Louis, Mo, USA). Luciferase reporter gene for in vitro transfection study and pGL3-Control vector with SV-40 promoter, and enhancer encoding firefly (Photonus pyralis) luciferase and PureYield plasmid maxiprep system for plasmid purification, and RQ1 RNase-Free DNase were purchased from Promega (Madison, WI, USA). The concentration of purified DNA was determined at 260 nm UV absorbance. Cy5.5-labeled scrambled DNA was obtained from Bioneer Inc. (Daejeon, Korea). Roswell Park Memorial Institute (RPMI)-1640 culture medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from HyClone (Logan, Utah).

2. Synthesis of lactitol diacrylate and polylactitol-based gene transporter (PLT)

Lactitol diacrylate (LDA) was prepared by reaction between lactitol and acryloyl chloride (molar ratio 1:2). Briefly, 1 g of D-lactitol monohydrate was dehydrated for 24 h using vaccum oven, and anhydrous lactitol was dissolved

in 30 ml DMF in the presence of 10 ml pyridine solution. After stirring for 30 min, acryloyl chloride dissolved in 7.5 ml DMF was added drop wise. The reaction was done for 24 h at 4°C. For precipitation of LDA, diethyl ether as a poor solvent was used, and dried overnight at room temperature. After preparation of LDA, PLT was synthesized with lactitol diacrylate and LMW BPEI (MW: 1200 Da) by Michael addition reaction. LDA (Mw: 442 Da) dissolved in anhydrous DMSO was added into the LMW BPEI dissolved in DMSO solution at a molar ratio of 1:1, and the reactant was stirred at 80°C for 18 h with N_2 bubbling. After reaction, the reaction mixture was dialyzed using dialysis membrane (MW cut off 3500) at 4°C for 24 h. The dialyzed polymer solution was lyophilized for 3 days.

3. Characterization of PLT

PLT was characterized using ^{1}H high resolution NMR Spectrometer (AVANCE 600, Bruker - 600 mHz, Germany) to estimate the composition of PEI in PLT. Molecular weight of PLT was measured using a gel permeation chromatography column (GPC) with TSKgel G5000PWxl-CP+TSKgel G3000PWxl-CP. The column temperature was kept at 45°C with a flow rate of 1.0 ml/min and 0.1 M $NaNO_3$ was used as the mobile phase.

4. Observation of morphology of complexes and measurement of particle size of complexes.

The morphology of the PLT/DNA (pGL3) nanocomplexes was observed at N/P ratio 10 by energy-filtering transmission electron microscopy (EF-TEM) (LIBRA 120, Carl Zeiss, Germany). A drop of the nanocomplexes in double distilled water (DDW) was put on a copper grid. After drying grid, morphology was observed by EF-TEM. The particle size and zeta-potential of PLT/DNA and PEI/DNA nanoparticles were assessed using a Zeta-potential & Particle size Analyzer (ELSZ-1000, OTSUKA ELETRONICS, Japan). The nanocomplexes were prepared at various N/P ratios (5, 10, 20 and 30) with 40 μg pGL3 for each in 2 ml of total volume, and each batch was analyzed in triplicate.

5. Gel retardation assay

Gel retardation assay was performed by a common gel electrophoretic technique. After complexation between pGL3 plasmid of 0.1 μg and PLT at various N/P ratios (1, 5, 10, 20, and 30), they were incubated at room temperature for 30 min. Subsequently, 2 μl of loading dye was added in the prepared each complex solution, complexes were loaded in total solution of 12 μl for electrophoretic separation on 1% agarose gel with tris–acetate (TAE) running buffer and electrophoresed at 100 V for 30 min.

6. Measurement of osmolarity

To measure osmolarity, 50 μl of each sample (PLT, PEI 1.2 kDa and PEI 25 kDa in DW with various concentrations) was transferred in cryoscopic tube and osmolarity was measured using cryoscopic osmometer (Osmomat 010, Gallay Medical & Scientific, Australia).

7. DNA protection and release assay

DNA protection in a DNase1 environment and release of DNA from complexes were investigated by a common gel electrophoretic technique. PLT/DNA (pGL3) complexes and naked DNA (0.2 μg) were separately incubated for 30min at room temperature. RQ1 RNase-Free DNase (0.2 μl) and RQ1 RNase-Free DNase 10X Reaction Buffer (0.2 μl) were added to each sample, and they were incubated for 30 min at 37°C. After incubation, RQ1 DNase Stop Solution (1 μl) was added to each sample and incubated for 10 min at 65°C for DNase 1 inactivation. Then, sodium dodecyl sulfate (SDS) was added to each sample and incubated for 5 h at room temperature. Subsequently, DNA protection and release of DNA were examined by 1% agarose gel electrophoresis.

8. Cell lines and cell culture

A549 cells (lung adenocarcinoma epithelial cells, human) and HepG2 cells (hepatocellular carcinoma, human) were cultured in Roswell Park Memorial

Institute medium (RPMI 1640). 293T cells (embryonic kidney cells, human) were cultured in Dulbecco's modified Eagle's medium (DMEM). Culture media contained 10% heat-inactivated FBS with 1% penicillin/streptomycin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

9. In vitro cell cytotoxicity study

In vitro cell cytotoxicity studies in three different cell lines (A549, HepG2, and 293T) were conducted using MTT assay. Cells were seeded at density of 5 x 10^4 cells/ml and incubated 18-20 h to obtain 80-90% confluence in 24-well plates. PLT or PEI complexed with pGL3 DNA was incubated for 30 min at room temperature with various N/P ratios in the 500 μl serum free media, and complexes were treated in each well. After 24 h incubation, MTT reagent was treated to the each well and incubated for an additional 4 h for formation of MTT formazan. Subsequently, medium was aspirated carefully, DMSO (100 μl) was treated to dissolve MTT formazan, and optical density was measured at 540 nm using a microplate reader (VERSAmax, Molecular Devices, USA).

10. In vitro transfection efficiency

In vitro transfection efficiency of three different cell lines (A549, HepG2, and 293T) was measured by luciferase assay. PEI 25K and Lipofectamine 2000

were used as positive controls against PLT. Cells were seeded at density of 10 x 10^4 cells/ml and incubated for 18-20 h to obtain 80-90% confluence in 24-well plates. Serum-free media containing PLT/DNA complexes were prepared at various N/P ratios. PLT/pGL3 complexes were treated to each well and incubated for 4 h. After 4 h, old medium was aspirated carefully, the complete media (10% FBS) was added, and plate was incubated for additional 24 h. After aspiration of all the media, 100 µl of passive lysis (1X) buffer was treated to each well. Luciferase assay was performed according to the manufacturer's instruction (luciferase assay system protocol-Promega). Luciferase activity was measured using luminometer (Infinite® 200 PRO, TECAN, Switzerland) to measure relative light units (RLU), which were normalized via measured total proteins by a BCA protein assay. To investigate serum stability of PLT/DNA complexes, in vitro cell transfection efficiency in the presence of different serum concentrations (0, 10, 20 and 30%) was performed in HepG2 cells at an N/P ratio of 20. All experiment was performed in triplicate and luciferase activity was measured as described above.

11. Competition assay

The competition assay was performed to confirm the in vitro liver cell targeting efficacy of PLT/DNA complexes at an N/P ratio 20. HepG2 cells were seeded at density of 10 x 10^4 cells/ml and incubated for 18-20 h to obtain 80-90% confluence in 24-well plate. Before transfection of PLT/DNA complexes,

galactose solution as a competitor was prepared by dissolving in serum-free media with various concentrations (0 mM, 10 mM, 20 mM, and 30 mM) and treated to each well. After incubation for 30 min, the transfection of PLT/DNA complexes was performed and luciferase activity was measured by the same method as described in section 10.

12. Effect of cellular uptake inhibitors on transfection efficiency

To identify endocytosis pathway of PLT/DNA complexes, HepG2 cells were seeded at density of 10×10^4 cells/ml and incubated for 18-20 h to obtain 80-90% confluence in 24-well plates. Each cellular uptake inhibitors (CH, M-β-CD, GE and WO) were prepared by dissolving in DMSO. Subsequently, these solutions were mixed with serum free media at various concentrations and treated to the cells. After incubation for 1 h at 37°C in a humidified atmosphere of 5% CO_2, PLT/DNA and PEI/DNA complexes at N/P 20 were added and incubated without eliminating inhibitors for 4 h. Effects of cellular uptake inhibitors were investigated by measuring luciferase activity in triplicate.

13. Effect of bafilomycin A1 on transfection efficiency

HepG2 cells were seeded at density of 10×10^4 cells/ml and incubated for 18-20 h to obtain 80-90% confluence in 24-well plates. Before transfection with

PLT/DNA and PEI/DNA complexes at N/P 20, bafilomycin A1 (200 nM, 250 μl/well) as an inhibitor of vacuolar-type H^+-ATPase (V-ATPase) was treated to the cells for 15 min. Luciferase activity was measured as described earlier.

14. Imaging of biodistribution and liver cell targeting efficacy

To detect the biodistribution and liver cell targeting efficacy of PLT, Cy5.5-labeled DNA/PLT complexes were intravenously injected in the mice. Six-week-old C57BL/6 (4 mice/group) were used following the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University, Korea) and were kept in the laboratory animal facility with temperature and relative humidity at 23 ± 2 ℃ and 50 ± 20%, respectively, under 12 h light/dark cycle. All experimental protocols were reviewed and approved by the Animal Care and Use Committee at Seoul National University (SNU-140730-1). Cy5.5-labeled DNA (10 μg) was complexed with PLT and PEI at an N/P ratio of 10 in serum-free media. After 4h post-injection, the mice were sacrificed, the various tissues, including liver, spleen, kidney, heart, and lung were dissected, and washed with PBS. Subsequently, their ex vivo fluorescence images were obtained by Optix-MX3-FX (Advanced Research Technologies, Montreal, Canada), and images were analyzed using Optix-MX3 ART Optiview Software (version 3.02.00).

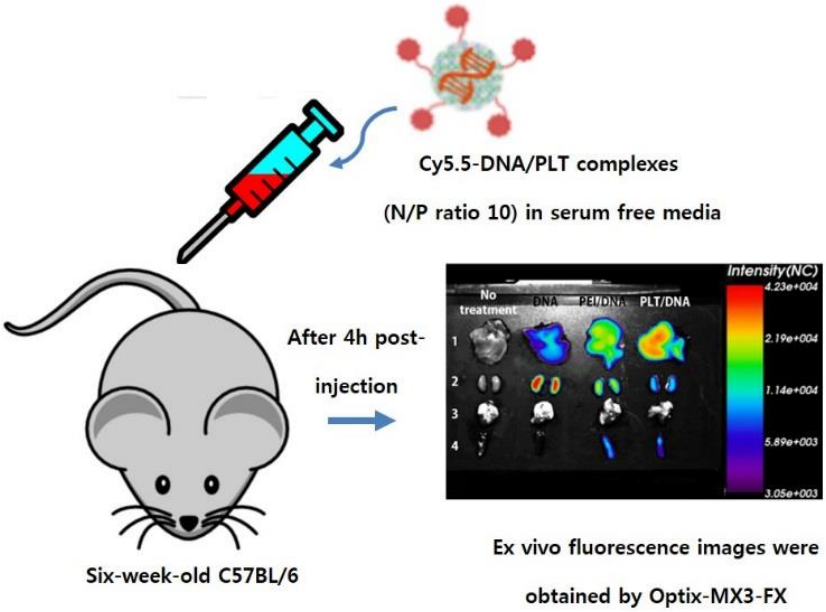

Figure 7. Schematic illustration of ex vivo imaging.

IV. Results and Discussion

1. Synthesis and characterization of PLT

Lactitol diacrylate (LDA) was prepared by reaction of lactitol and acryloyl chloride under molar ratio of 1:2 in the presence of pyridine at 4℃ for 24 h as shown in Fig. 8. Diacrylation of lactitol can preferably occur at 1 and 6 positions of hydroxyl groups of D-sorbitol backbone in lactitol because hydroxyl groups of 1° alcohols are more reactive than those of 2° or 3° alcohols. The synthesis of LDA was confirmed by assessing ^{1}H-NMR of the peaks of vinyl groups in primary alcohol position of D-sorbitol backbone in lactitol (δ: 5.3-6.5) (Fig. 9). Also, the peaks of vinyl groups of lactitol appeared in similar fashion to that of sorbitol diacrylate (SDA; commercial product of Sigma) in our group's previous report [18], indicating that diacrylation of lactitol selectively occurred at 1° alcohol positions of D-sorbitol backbone in lactitol. After preparation of LDA, PLT was synthesized by crosslinking of LMW BPEI (MW: 1.2 kDa) with LDA as a crosslinking agent via the Michael addition reaction. The reaction scheme of the PLT synthesis, and functional structures are described in Fig. 8, which is classified by their functional parts. The composition of polylactitol in PLT was estimated to 49.09 mol% by analyzing ^{1}H-NMR. Also, the final molecular weight (Mw) of PLT measured by GPC was 9,871 Da as shown in Table 1.

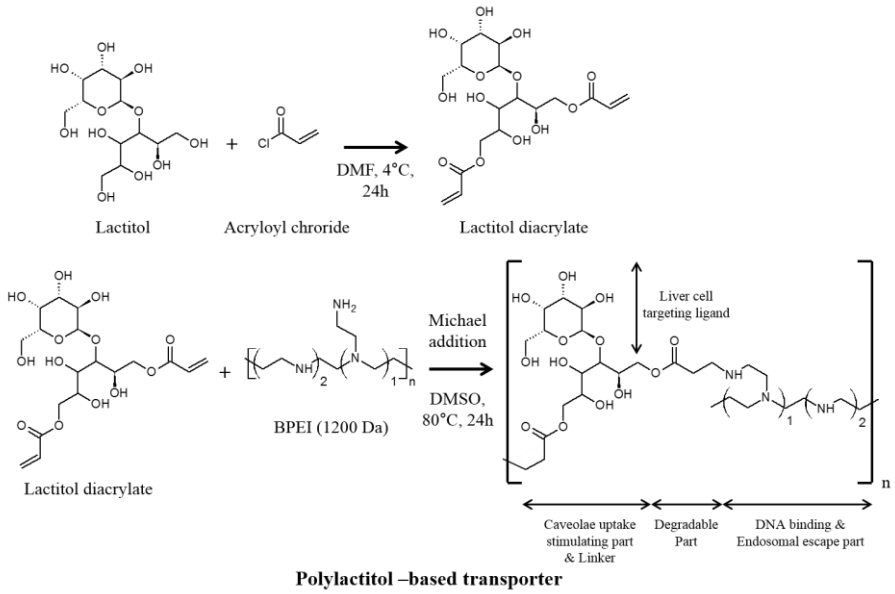

Figure 8. Schematic illustration of PLT synthesis.

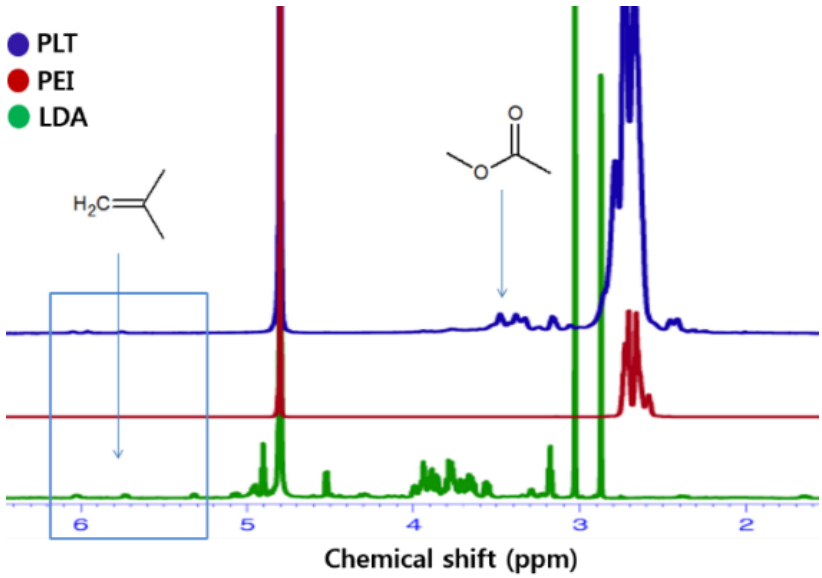

Figure 9. ^{1}H NMR spectra of PLT, PEI, and LDA.

Table 1. Molecular weight of PLT measured using GPC.

Sample	Mn (Da)	Mw (Da)	Polydispersity
PLT	4574	9871	2.158229

Mn (number average mol weight) and Mw (weight average mol weight)

2. Physicochemical characteristics of PLT/DNA complexes

The particle size and surface charge of PLT/DNA complexes were measured using a Zeta-potential & Particle size Analyzer. The overall sizes of PLT/DNA complexes were ranged from 62.1 to 81.1 nm (Table 2). The particle sizes of PLT/DNA complexes at N/P ratio of 10 monitored by TEM were similar with those measured by a Zeta-potential & Particle size Analyzer, and complexes have a single uniform population of size and shape (Fig. 10). The results suggested the potential for use of PLT as gene carrier for intracellular delivery because particle sizes of PLT/DNA are in a range of optimal particles size for EPR effect [53].

Table 2. Size and zeta potential of PLT/DNA and PEI/DNA complexes.

N/P ratio	Size (nm)		Zeta potential (mV)	
	PLT/DNA	PEI/DNA	PLT/DNA	PEI/DNA
5	78.5 ± 1.4	67.1 ± 2.6	8.0 ± 0.5	25.9 ± 2.0
10	62.1 ± 4.0	62.0 ± 3.3	7.8 ± 0.1	30.4 ± 0.3
20	78.9 ± 1.7	48.0 ± 3.1	7.6 ± 0.2	41.7 ± 0.7
30	81.1 ± 1.6	26.4 ± 1.1	6.1 ± 0.1	44.6 ± 1.4

The zeta-potential of PLT/DNA complexes ranged from +6.1 to +8.0 mV (Table 2), which is nearly neutral and significantly lower than those of PEI/DNA complexes due to negative charges of the hydroxyl groups of polylactitol, which neutralize positive charges of PEI. High cytotoxicity of PEI is in connection with the its high positive charge which leads electrostatic interaction with the negatively charged components of the cell membrane and causes membrane disruption and damage [54].

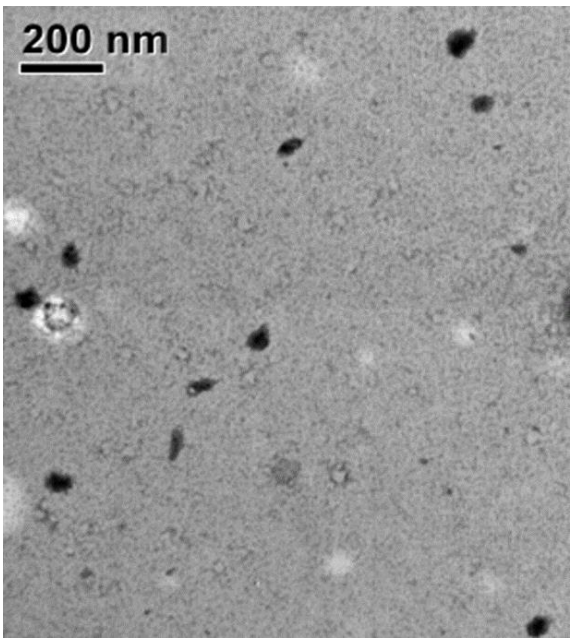

Figure 10. Transmission electron microscope (TEM) image of PLT/DNA complexes (N/P 10).

Gel retardation assay was performed to evaluate electrostatic interaction and condensation between cationic PLT and negatively charged DNA. From the

results of gel retardation assay, PLT/DNA complexes did not show any DNA bands at N/P ratio 1, indicating perfect complexation at N/P ratio 1 (Fig. 11. A). Also, DNA protection assay was conducted for investigating protection from nuclease degradation. DNase 1-exposed naked DNA was fully digested, whereas DNA complexed with PLT was not degraded by DNase 1 (Fig. 11. B) and DNA was released without degradation, indicating that PLT effectively protected from nuclease degradation and released DNA.

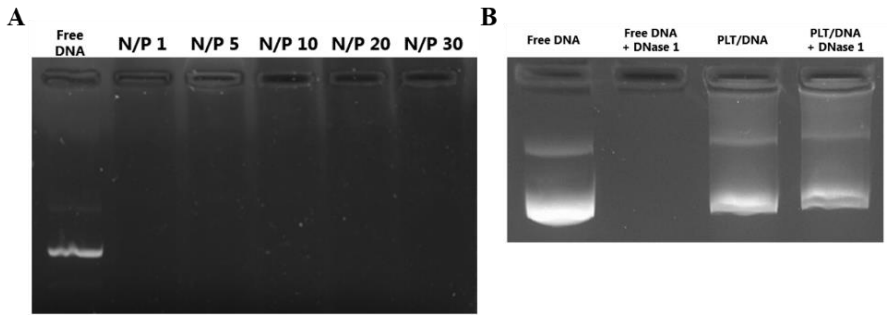

Figure 11. Gel retardation and DNA protection assay. (A) gel electrophoresis of PLT/DNA complexes at various N/P ratios. (B) DNA was released from by PLT/DNA complexes at N/P ratio 5 by SDS.

In our previous report, polyol-based gene transporter had high osmolality, which is critical for contributing increased cellular uptake of enhancement of transfection efficiency via selective caveolae-mediated pathway. In this regard, osmolarity of PLT was measured and compared with BPEI 1.2 kDa and BPEI 25 kDa at various concentrations using a cryoscopic osmometer (Fig. 12). The osmolarity of PLT almost was exponentially increased along with an increase

of concentration in contrast to the nearly linear increase of BPEI 1.2 kDa and BPEI 25 kDa. The osmolarity of PLT is 2.5- and 6.5-fold higher than BPEI 1.2 kDa at concentration 50 and 250 mg/ml, respectively, indication of hyperosmolarity of PLT.

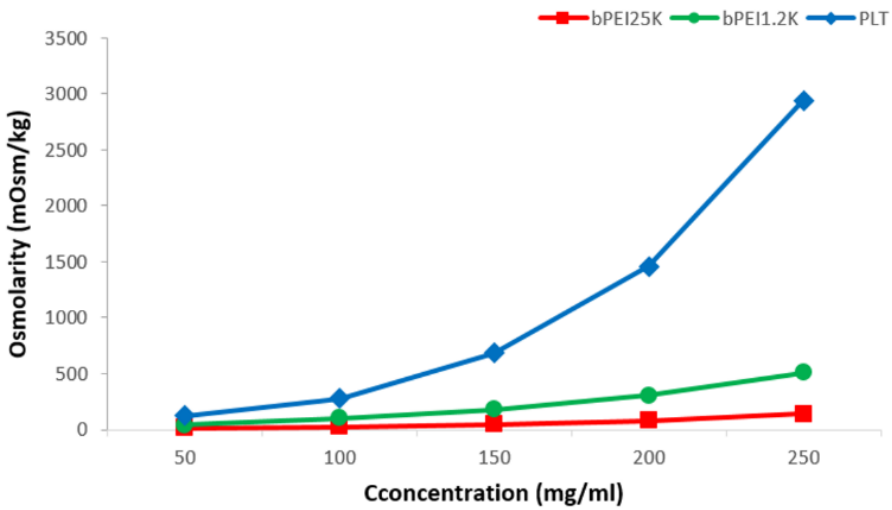

Figure 12. Osmolarity of PLT, PEI 1.2 kDa, and PEI 25 kDa measured by osmometer.

3. In vitro cytotoxicity of PLT/DNA complexes

The safety of gene delivery vector is the most important factor in a routine clinical application, because the toxicity is still an obstacle to the application of non-viral vectors for gene therapy [55]. In this regard, in vitro cytotoxicity study was performed to evaluate our carrier's safety at A549, 293T, and HepG2 cell lines using an MTT assay method. High cytotoxicity of high MW PEI hinders effective gene delivery as well as medical applications due to lack of degradability, molecular weight-dependent number of primary amine groups

[56], and type of PEI structure. To minimize cytotoxicity of PLT, we used less cytotoxic LMW BPEI instead of HMW PEI and crosslinked LMW BPEI using LDA as a crosslinking agent through degradable and hydrolysable ester linkages which facilitates the degradation of PLT to the low molecular weight non-toxic building blocks by hydrolysis in physiological conditions. LMW BPEI has low cytotoxicity compared to their high MW counterparts [57], and negative charge of hydroxyl groups of polylactitol part contribute to neutralize remaining positive charges of PEI part (Table 2), which may bring a synergistic effect on the reduction of cytotoxicity and enhancement transfection efficacy. From the results of cell viability, the PLT/DNA complexes revealed significantly low cytotoxicity in three different cell lines (A549, 293T, and HepG2) than PEI 25 kDa, particularly at high N/P ratios (Fig. 13), which supports the above mentioned our hypothesis. Interestingly, PLT/DNA complexes were remarkably less cytotoxic to target cells (HepG2 cell) than A549 and 293T cells. In addition, differences between cytotoxicity of PLT and PEI was dramatically increased with the increase of N/P ratios in three different cell lines.

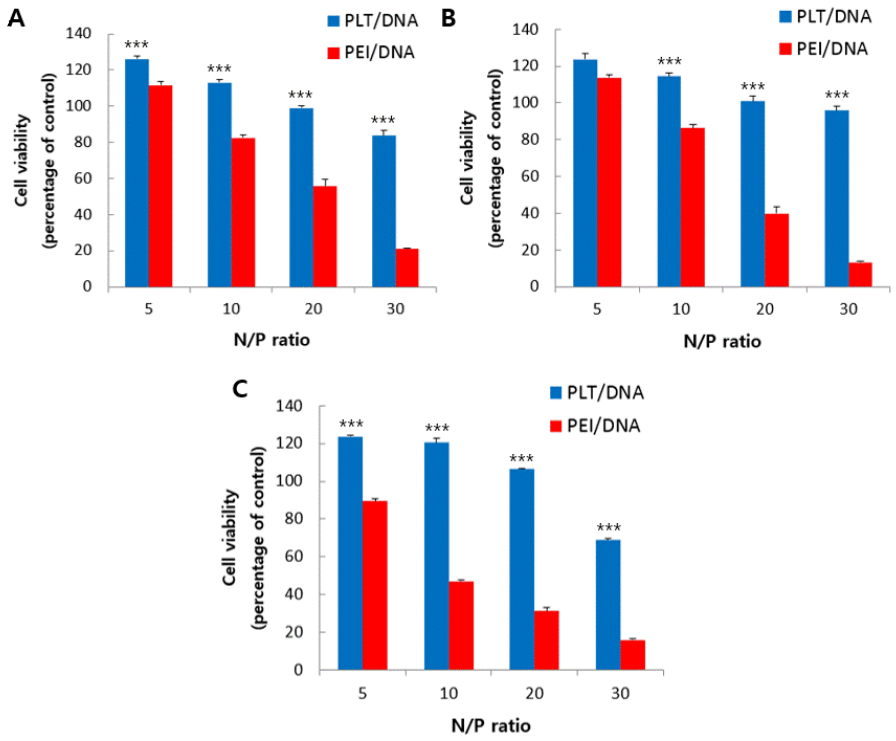

Fig. 13. Cytotoxicity of PLT/DNA and PEI/DNA complexes was measured by MTT assay method in (A) A549 cells, (B) HepG2 cells, and (C) 293T cells. (n = 3, error bar represents standard deviation, *p < 0.05, **p < 0.05, ***p < 0.01, one-way ANOVA compared to that of PEI/DNA complexes).

4. In vitro transfection efficiency of PLT/DNA complexes

Most of non-viral gene delivery vectors suffer from insufficient transfection efficiency compared to viral vectors. Therefore, we designed a new polymeric carrier to improve the transfection efficiency by the synergistic effect of three functionalities of PLT as shown in Fig. 8: polygalactose (liver cell targeting ability), polysorbitol (caveolae-mediated endocytosis pathway), and PEI (proton sponge effect). To confirm in vitro transfection ability of PLT, we performed luciferase assay in three different cell lines using PLT, PEI 25K, and

Lipofectamine 2000 at various N/P ratios. As shown in Fig. 14 (A-C), the transfection efficiency of PLT/DNA complexes was remarkably high in all three cell lines despite of numerous hydroxyl groups of PLT, particularly at the high N/P ratios of 20 and 30 compared to PEI 25K. At N/P ratio of 30, PLT/DNA complexes showed approximately 12 and 4-folds higher luciferase expression levels than that of PEI 25K in A549, and 293T cells, respectively. Also, PLT/DNA complexes showed approximately 13-fold higher luciferase expression levels than PEI 25K at the high N/P ratio of 30 in HepG2 cells, indicating receptor specificity and increasing transfection efficiency via ASGPR. Furthermore, in the HepG2 and 293T cells, transfection efficiency of PLT was higher than Lipofectamine 2000 at high N/P ratios. Although high transfection efficiency of PLT/DNA complexes was found optimum, most synthetic vectors were unstable, and their positive charge can interact with negatively charged proteins or enzymes in the presence of serum, which leads to toxicity of nanoparticles [58]. In this regard, to identify the serum effect on transfection efficiency of PLT/DNA complexes, we performed luciferase assay in various serum percentages (0, 10, 20, and 30) in HepG2 cells at an N/P ratio of 20 using PLT/DNA and PEI/DNA complexes. As shown in Fig. 14D, luciferase expression level of PLT/DNA complexes was not significantly affected by the presence of serum while that of PEI/DNA complexes was significantly decreased by increase of concentration of serum. This phenomenon is due to both the shielding of positive charges by hydroxyl groups of PLT and the hydration of polyol. The polysorbitol and polygalactose parts in our transporter system contain numerous hydroxyl groups, which has shielding

effect [18, 20] from the cationic charges of the nanocomplexes and simultaneously may encourage hydration of the nanocomplexes by producing a repulsive hydration shell with water molecules, hence reducing intermolecular interactions. The interaction between water and polyol molecules such as polysorbitol for hydrogen bonding with surrounding waters was suggested by using molecular dynamics computer simulations [59]. Consequently, shieling effect and hydration of PLT might prevent interaction of serum protein and the losing transfection function of PLT.

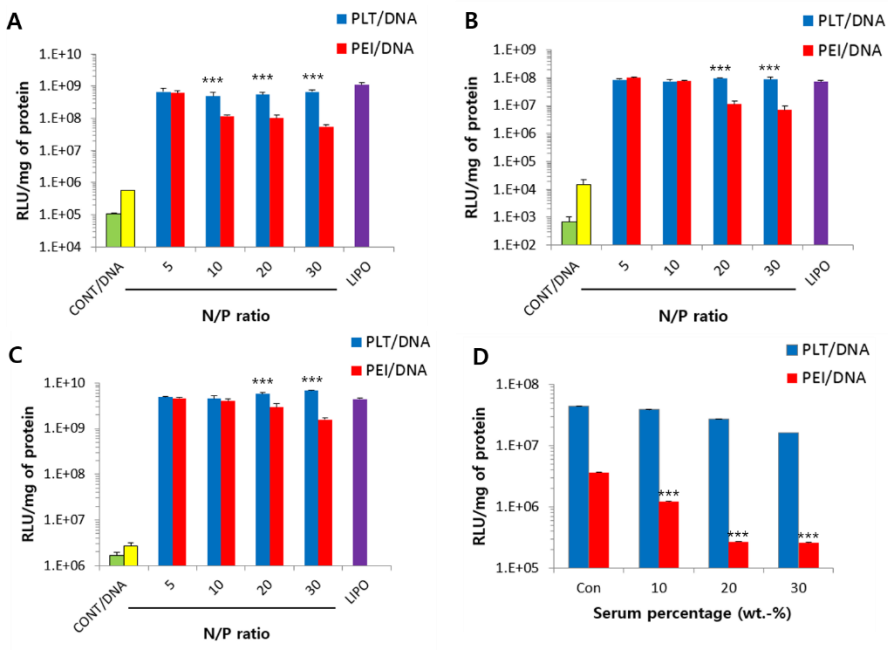

Fig. 14. In vitro transfection efficiency of PLT/DNA complexes. The PLT, PEI and Lipofectamine 2000 were complexed with pGL3 plasmids at various N/P ratios and transfected in (A) A549 cells, (B) HepG2 cells, and (C) 293T cells without serum. (D) The PLT and PEI were complexed with pGL3 plasmids at N/P ratio 20 and transfected to HepG2 cells in the presence of various

concentration of serum. Chemo luminescence was measured 24 h after transfection and normalized with the amount of protein. (n = 3, error bar represents standard deviation; *p < 0.1, **p < 0.05, ***p < 0.01, one-way ANOVA compared to that of PEI/DNA complexes (A-C) and control (D)).

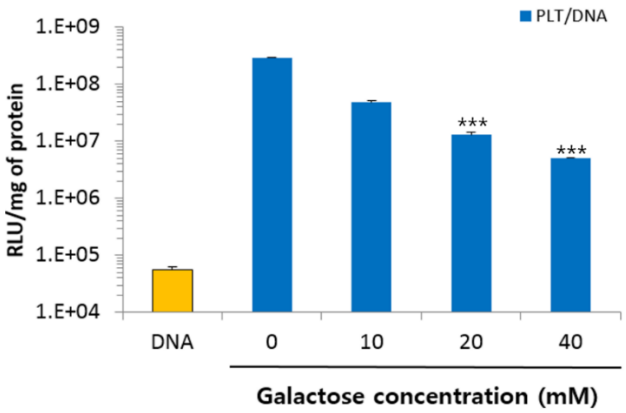

Fig. 15. Competition assay of PLT/pGL3-control complexes on HepG2 cells prepared at an N/P ratio of 20 by adding free galactose (0, 10, 20 and 40 mM) as a competitor. Luminescence was measured 24 h after transfection and normalized with the amount of protein. (n = 3, error bar represents standard deviation; *p < 0.1, **p < 0.05, ***p < 0.01, one-way ANOVA compared to the PLT/DNA complexes at free galactose (0mM)).

In addition, we performed competition assay to investigate the above mentioned liver cell specificity via interaction between polygalactose of PLT and receptors expressed on liver cells including ASGPR. The PLT/DNA complexes were prepared at an N/P ratio of 20 and were transfected to HepG2 cells in the presence of various concentrations of free galactose (0, 10, 20, and 40 mM) as a competitor for polygalactose part in PLT as shown in Fig. 15. The level of luciferase expression of PLT/DNA complexes were reduced by 6, 22,

and 57 times compared to that of control group by pre-treatment of galactose at concentrations of 10, 20, and 40 mM respectively. It may be regarded that specific recognition of galactose on the PLT/DNA complexes by ASGPR induces the receptor-mediated endocytosis in liver cells. In that respect, decrease of transfection efficiency of PLT/DNA complexes in excess of free galactose is caused by saturation of binding sites (ASGPR) of targeting moieties of PLT. Collectively, these results revealed that the polygalactose part of PLT could contribute to the recognition and binding to ASGPRs expressed on the surface of the HepG2 cells, and thereby enhanced transfection efficiency through receptor-ligand interaction. Overall results strongly suggested that our PLT/DNA nanocomplexes are a highly efficient gene transporter system by high transfection efficacy, good serum stability, and liver cell targeting ability.

5. Mechanism of gene delivery by PLT

5.1. Endocytosis pathway of PLT/DNA complexes

It became evident that transfection efficiency is largely attributed to uptake pathways which can determine the intracellular fates and cytosolic delivery of the carried gene by directing them toward the digestive or non-digestive route [39]. Although endocytic and intracellular pathways remain largely unanswered, endocytosis pathway are affected by nanocomplexes of physical, chemical, biological and geometrical cues [40].

In our previous studies, polyol-based gene transporters using polyglycerol [16, 17], polysorbitol [18-20], polymannitol [21, 22], and polyxylitol [23, 24] showed remarkably high transfection efficacy via selective caveolae-mediated endocytic pathway due to their unique osmolality. We hypothesized that polylactitol as a kind of polyol enhances the transfection efficiency by shifting the mode of endocytosis into the caveolae-mediated pathway. To investigate the endocytosis pathway of the PLT/DNA complexes, three types of cellular uptake pathways were blocked by pre-treatment of metabolic inhibitors: chlorpromazine (CH) as an inhibitor of clathrin-mediated endocytosis (CME), methyl-beta-cyclodextrin (M-β-CD) and genistein (GE) as inhibitors of caveolae-mediated endocytosis (CvME), and wortmannin (WO) as an inhibitor of fluid-phase endocytosis are four different inhibitors of cellular uptake, and the variations of transfection efficiency in both PLT/DNA and PEI/DNA complexes were checked. Before using inhibitors, we performed the cell viability test to optimize the concentration of the inhibitors which may induce cytotoxicity at high concentrations (data not shown).

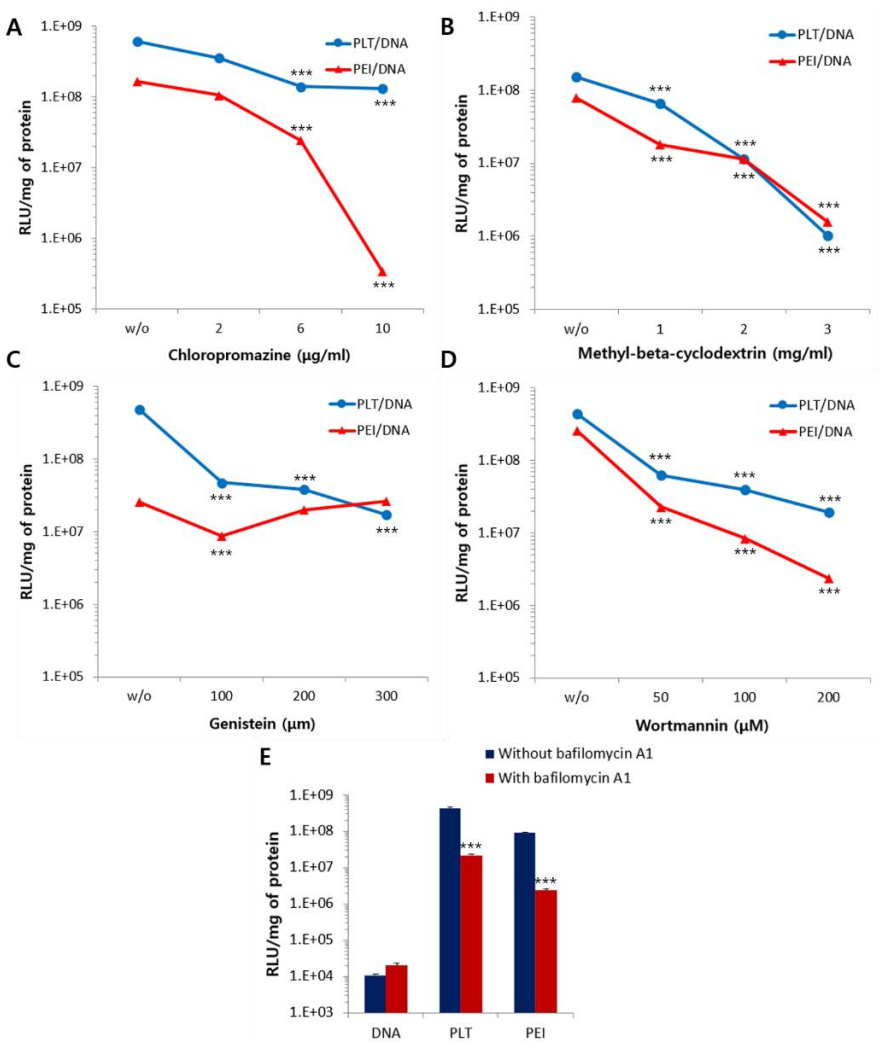

Fig. 16. Mechanistic investigation for high transfection of PLT: effect of endocytosis inhibitors and bafilomycin A1 on transfection efficiency of PLT/DNA complexes and PEI/DNA complexes in HepG2 cells. After pre-incubation of cells with various concentration of (A) chloropromazine, (B) methyl-beta-cyclodextrin, (C) genistein, and (D) wortmannin. PLT/pGL3 and PEI/pGL3 complexes (N/P 20) were transfected and luciferase assay was conducted. Cells were pre-incubated with (E) 200 nM of bafilomycin A1. PLT/pGL3 and PEI/pGL3 complexes (N/P 20) were transfected and luciferase expression was checked. (n = 3, error bar represents standard deviation; *p < 0.1, **p < 0.05, ***p < 0.01, one-way ANOVA compared to that of control without inhibitor).

As shown in Fig. 16, levels of luciferase expression of PLT/DNA and PEI/DNA complexes were changed according to the various concentrations of cellular uptake inhibitors. In the M-β-CD and GE-treated HepG2 cells, PLT/DNA complexes showed 147-fold and 28-fold lower transfection efficiency than untreated cells at concentrations of 3 mg/mL and 300 μM, respectively (Fig. 16. B and C), which strongly suggests that gene transfer is largely dependent on CvME by hyperosmotic PLT having 49-mol% of polylactitol part. In contrast, as shown in Fig. 16. A and D, the transfection efficiency of the PLT/DNA complexes was a little decreased by treatments of CH and WO compared to that of PEI/DNA complexes, indicating that PLT-mediated transfection is not dependent on the clathrin or fluid-phase endocytic pathway than PEI. Hyperosmolality of polylactitol (Fig. 12) can be operated as a mechanical stress to the cells [60], and stimulate CvME with transient down-regulation of CME [61], suggesting that hyperosmotic polylactitol part in PLT can shift the mode of endocytosis into CvME. On the other hand, gene transfer of PEI/DNA complexes was largely affected by treatment of CH, M-β-CD, and WO although less affected by GE, indicating PEI-mediated transfection is dependent on random endocytic pathway.

As described in section 4, it was demonstrated that internalization of PLT/DNA complexes are initiated by receptor-ligand interaction in HepG2 cells and reasonably assumed that galactose on PLT/DNA complexes would interact with galactose binding receptors-ASGPR expressed on HepG2 cells. Because

ligand binding ASGPR is localized in detergent resistant lipid rafts [62], the PLT/DNA complexes recognized by ASGPR could be internalized into CvME, which would be promoted by hyperosmotic property of polylactitol as shown in Fig. 17.

Consequently, the results of endocytosis pathway consistently indicate that internalization of PLT/DNA complexes is dependent on both receptor- and caveolae-mediated cellular uptake pathways. It is a strong advantage for developing more effective gene carriers by protecting the lysosomal degradation fate of internalization, because CvME can effectively avoid the endo-lysosomal pathway [63]. As it is known, some pathogens such as viruses and bacteria avoid the lysosomal digestion by using caveolae as their portal of entry escape delivery [64]. Thus, only the DNA in polyplexes that enters the cell by CvME can evade the lysosomal fate and effectively transfer gene [65].

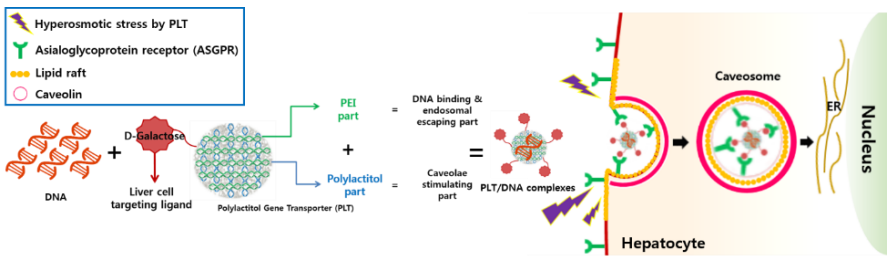

Fig. 17. Schematic illustration of PLT-mediated transfection.

5.2. Proton sponge effect of PEI part in PLT

One of the most effective and widely studied polymeric gene delivery systems is the PEI [66], which has the ability to combine both DNA and siRNA.

It has a relatively high transfection efficiency due to its intrinsic 'proton sponge effect' [67]. This hypothesis suggests that the PEI becomes more protonated at a low pH in endosomes, and the proton buffering capacity of non-protonated amino group of PEI triggers an influx of Cl^- ions with protons leading to a water influx and finally the swelling and rupturing of the endosomes [39]. Ion-pair formation or proton-sponge effect can induce proper releasing of genes from the carrier by preventing the endo-lysosomal entrapment [68].

Therefore, we assumed that high transfection efficiency of our carrier was partially related to the proton sponge effect of PEI part in PLT. To observe the proton sponge effect of PLT, we transfected PLT/DNA complexes at an N/P ratio of 20 in the HepG2 cells after pre-treatment of 200 nM of bafilomycin A1: a specific vacuolar type H^+ ATPase inhibitor. As shown in Fig.16.E, transfection efficiency of PLT/DNA and PEI/DNA complexes were significantly decreased by 20 and 40-folds respectively compared to that of control group by treatment of bafilomycin A1. Apparently, PLT was less affected by blocking H^+ ATPase compared to PEI. Although the high transfection efficiency of PLT cannot be fully understood using the concept of 'proton sponge effect', our results reveal that the proton sponge effect of PEI part in PLT contributes to PLT-mediated high transfection as a synergistic effect.

6. Imaging of biodistribution and liver cell targeting efficacy

To clarify the potential of PLT for in vivo application as a gene delivery carrier, we intravenously administered Cy5.5-labeled DNA/PLT and Cy5.5-

labeled DNA/PEI complexes to tumor-free mice. We used an ex vivo imaging system to effectively visualize the fluorescence intensity of Cy5.5-DNA/PLT complexes and prevent optical impedance of the fluorescent signals by soft tissues [69]. Although ex vivo imaging is an invasive method, it is more precise compared to non-invasive whole-animal imaging and provides better understanding of mechanistic accumulation of polyplexes. After 4 h post-injection, naked Cy5.5-DNA showed highest fluorescence intensity in the kidney and less accumulation in the liver (Fig. 18. A), because oligonucleotides preferably accumulated in the liver and kidney by renal excretion as well as non-specific RES uptake [70]. In contrast, Cy5.5-DNA/PLT complexes showed higher fluorescence intensity in the liver than other treatment groups, and less accumulated in both kidneys and the spleen compared with the naked DNA and PEI/DNA complexes. The reason for this phenomenon is related with liver cell targeting specificity of polygalactose part in PLT. To quantify in vivo liver cell targeting efficacy of PLT/DNA complexes, only the liver was separated from organs and imaged again to prevent interference with strong fluorescence signals from other organs, and the mean fluorescence intensity was measured and analyzed using the ART OptiView system (Fig. 18. B). The Cy5.5-DNA/PLT complexes presented 27-fold and 1.5-fold higher fluorescence signal in the liver than naked DNA and PEI/DNA, respectively (Fig. 18. C), consistent with the results of the in vitro targeting efficiency. The results of ex vivo imaging strongly indicates that genes were effectively delivered to the target site by liver cell targeting ability of PLT system, which can be possibly applied for the gene therapy for treating liver disease.

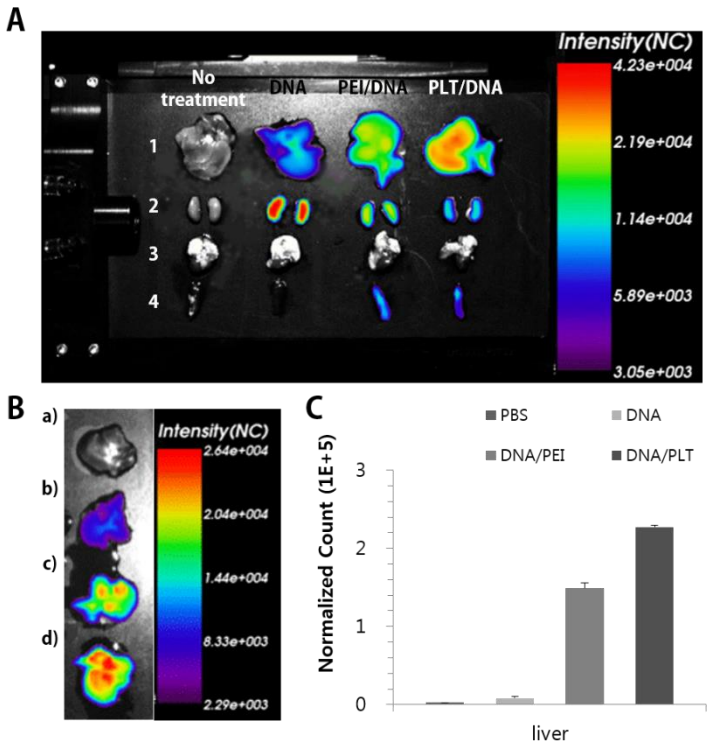

Fig. 18. Biodistribution and liver cell targeting efficacy of PLT/DNA complexes in tumor-free mice. (A) Optical image of bio-distribution [PBS (no treatment, control), DNA-Cy5.5, DNA-Cy5.5/PEI, and DNA-Cy5.5/PLT]. Various DNA formulations were intravenously injected in tumor-free mice at a dose of 15 μg DNA/mouse. 1: liver, 2: kidney, 3: lung/heart, and 4: spleen. (B) Fluorescence scanning of liver was performed for confirming liver cell targeting efficacy of PLT/DNA complexes without other organs fluorescent interferences. a: PBS (no treatment, control), b: DNA-Cy5.5, c: DNA-Cy5.5/PEI, and d: DNA-Cy5.5/PLT. (C) To investigate liver cell targeting efficacy of PLT/DNA complexes, the mean fluorescence intensity of image (B) was quantified and analyzed using the ART OptiView 3.0 software package. Graphed data represent the mean + s.d., and NC = normalized counts.

V. Conclusion

Polylactitol-based gene transporter as a multifunctional nanocarrier for liver cell targeting through cellular regulation was designed and successfully demonstrated to be an advanced multifunctional gene delivery system. The PLT reveals several excellent features: (1) reduction of synthesis step provides technical ease for design and preparation of nanocarrier by lactitol composed of sorbitol and galactose, (2) a single uniform population and ideal size for employing in biological applications, (3) significantly low cytotoxicity by adopting low MW PEI and hydrolysable ester linkages, and (4) an unprecedented good transfection efficiency by the synergistic effect of each functional backbone's unique properties: polygalactose part has superior liver cell targeting ability in vitro, as well as in vivo and facilitates receptors-mediated endocytosis. The PEI part induces a rapid endosomal escape of genes and prevents gene degradation within the late endosome and lysosome due to its proton sponge effect. The hyperosmotic activity of polylactitol can selectively stimulate the caveolae-mediated endocytosis. Our study demonstrated that targeted and controlled gene delivery with a polylactitol-based gene transporter provides a new insight and concept for designing a multifunctional gene nanocarrier and a solution regarding several barriers in therapeutic applications of liver-directed non-viral gene therapy.

VI. Literature Cited

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VII. Publications

1. Young-Dong Kim, Tae-Eun Park, Singh Bijay, Sushila Maharjan, Yun-Jaie Choi, Pill-Hoon Choung, Rohidas B.Arote, Chong-Su Cho. Nanoparticle-mediated delivery of siRNA for effective lung cancer therapy (2015), Nanomedicine.

2. Yong-Dong Kim, Tae-Eun Park, Bijay Singh, Sushila Maharjan, Kye-Soo Cho, Kyoung-Pyo Park, Yun-Jaie Choi, Rohidas B. Arote, Chong-Su Cho. Image-guided nanoparticle based siRNA delivery for cancer therapy (2015), Current Pharmaceutical Design, Accepted.

3. Young-Dong Kim, Prasad Pofali, Tae-Eun Park, Prajakta Dandekar, Ratnesh Jain, Yun-Jaie Choi, Rohidas Arote, Chong-Su Cho. Gene therapy for bone tissue engineering (2015), Tissue Engineering and Regenerative Medicine, Submitted.

4. Young-Dong Kim, Tae-Eun Park, Bijay Singh, Kye-Soo Cho, Jaiprakash N. Sangshetti, Yun-Jaie Choi, Rohidas B. Arote, Chong-Su Cho. Efficient gene transfection to liver cells by a multifunctional polylactitol-based gene transporter via cellular regulation (2015), Acta Biomaterialia, manuscript under preparation.

Summary in Korean

질병의 원인이 되는 유전자 및 단백질을 직접적으로 조절하는 유전자 치료법은 효율적인 치료 방법이 없는 질병들의 새로운 돌파구로 인식되고 있다. 특히, 간은 우리 몸의 대사의 중심이며, 다양한 단백질을 합성하는 vital organ이다. 때문에 유전자 치료 적용을 위한 주요 타깃으로 다양한 유전자 전달 시스템을 이용한 연구가 이루어지고 있다. 그러나 간세포에서 과발현되는 몇몇의 유전자 발현 조절을 제외하고는 간세포 암종을 표적하는 간 특이적 유전자 치료 연구가 많이 진행되어있지 않고, 각종 간질병에 대한 정확한 기전과 진행 과정에 대한 잘 밝혀지지 않고 있다.

간세포를 표적하는 유전자 치료를 위해서는 유전자를 체내로 안전하고 정확하게 전달할 수 있는 전달체가 필요하다. 유전자 치료를 위한 전달체는 질병의 특성에 따른 장단점을 고려하여 선택되며, 전달체를 이루는 물질의 기원에 따라 크게 바이러스와 비바이러스 벡터로 구분된다. 앞서 언급한대로 전달체를 이용한 유전자 치료의 주요 쟁점은 독성과 면역 문제, 그리고 타깃세포에 특이적으로 표적전달이 어렵다는 점이다. 많은 연구들이 바이러스 벡터를 사용하고 있으나 여러 차례 주사하기에는 면역으로 인한 염증 반응과 숙주 세포 염색체내로 무작위로 삽입된다는 문제가 있다. 반면 비바이러스 벡터는 목적에 맞게 표면을 유연하게 변화시킬 수 있어 표적에 효과적으로 전달이 가능하고 면역 독성의 문제가 훨씬 적어 여러 차례 투여 가능하므로 지속적인 치료가 가능 한 것이 장점이다. 그러나 비바이러스 벡터의 유전자 전달 효율이 바이러스 벡터에 비해 상대적으로 낮기 때문에, 본 연구에서는 효과적인 유전자 전달을 위해 세포 외부와 내부에서의 장벽들을 극복한 간세포를 타깃하는 다기능성 유전자

전달체를 구축하였다.

새로운 천연물 유래의 물질을 이용한 다기능성 고분자 유전자 전달체 [poly(lactitol-co-PEI)]는 기존의 비바이러스성 유전자 전달체에 비해 우수한 생체 안전성을 갖고, 높은 수준의 유전자 전달 효율을 보였다. 한가지 물질을 이용함으로서 표적 전달과 삼투 특수성을 동시에 이용할 수 있는 천연물인 젖당 유래 물질 락티톨 (lactitol)을 이용하여 유전자 전달체를 고안하였다. 유전자 전달체로 잘 알려진 저분자량의 폴리에틸렌이민 (PEI : polyethyleneimine)에 삼투 물질인 lactitol diacrylate (LDA)를 공중합체 시켜 합성함으로써 ① PEI의 효과로 인해 유전자가 엔도좀을 빠르게 탈출하여 세포질로 방출 되도록 유도하고 (proton sponge effect), ② D-sorbitol의 우수한 삼투활성으로 세포 내부의 특이적인 라이소좀 분해 회피 기전 (카비올레)을 선택적으로 이용하며, ③ Lactitol의 D-galactose기를 이용하여 간세포 표적성의 효과를 동시에 얻을 수 있다. Asialoglycoprotein receptor (ASGPR)를 표적하여 in vitro, in vivo에서 우수한 간세포 특이성을 가졌으며, 3) 폴리락티톨의 다수의 -OH기가 세포에 삼투 스트레스를 유발함으로서 카비올레 세포내 이입을 촉진시킴과 동시에 PEI의 proton sponge effect가 나노입자를 세포질로 빠르게 탈출하도록 유도시켜 뛰어난 유전자 전달 효율을 보였다.

개발된 유전자 전달체는 간 질환 치료를 목표로 한 다양한 치료용 유전자를 간세포에 표적 전달함으로서, 간과 관련된 질병에 확대 적용할 수 있고 유전자 발현을 조절함으로서 근본적이고 효과적인 치료를 유도함과 동시에 치료 연구에 상승효과를 가져올 것이다.

국문초록

유망한 신약개발 분야인 유전자 치료는 질병의 원인이 되는 유전자 및 단백질을 직접적으로 억제하여 효율적인 치료 방법이 없는 난치성 질환이나 유전 질환 등의 새로운 돌파구가 되고 있다. 특히, 우리 몸의 주요 장기로서 각종 대사 작용과 해독 작용을 담당하는 간과 관련된 질병들은 치료법이 제한적이기 때문에 유전자 치료의 주요 타깃이 되고 있다. 간 질병에 유전자 치료를 도입함으로써 근원적 치료가 가능하며, 기능의 저하로 인한 관련된 여러 임상적 문제까지도 해결할 수 있다는 가능성을 갖고 있다. 그러나 간세포에서 과발현되는 몇몇의 유전자 발현 조절을 제외하고는 간세포를 표적하는 유전자 치료 연구가 많이 진행되어있지 않다.

본 연구의 목적은 간세포 외부와 내부에서의 문제점을 극복한 효율적인 다기능성 유전자 전달체를 구축하는 것에 있다. 따라서, 새로운 천연물 유래의 당알코올 (lactitol)을 이용한 다기능성 고분자 유전자 전달체 [poly(lactitol-co-PEI)]를 개발하였으며, 기존의 비바이러스성 유전자 전달체에 비해 우수한 안전성을 갖고 유전자 전달 효율이 높으며, 한가지 물질을 이용함으로서 표적 전달과 삼투 특수성을 동시에 이용할 수 있다는 장점이 있다. 유전자 전달체로 잘 알려진 저분자량의 폴리에틸렌이민 (PEI: polyethyleneimine)에 삼투 물질인 lactitol diacrylate (LDA)를 공중합체 시켜 합성함으로써 ① PEI의 효과로 인해 유전자가 엔도좀을 빠르게 탈출하여 세포질로 방출되도록 유도하고 (proton sponge effect), ② D-sorbitol의 우수한 삼투활성으로 세포 내부의 특이적인 라이소좀 분해 회피 기전 (카비올레)을 선택적으로 이용하며, ③ Lactitol의 D-galactose기를 이용하여 간세포 표적성의 효과를 동시에 얻을 수 있다.

본 연구에서 개발된 [poly(lactitol-co-PEI)]의 간세포를 표적하는

비바이러스성 다기능성 유전자 전달체로서 효율을 검증하였다. 1) 저분자량의 PEI와 에스터 결합을 도입함에 따라 독성이 낮고, 2) 폴리락티톨의 갈락토오스 분자 골격을 통해 세포 외부의 asialoglycoprotein receptor (ASGPR)를 표적하여 in vitro, in vivo에서 우수한 간세포 특이성을 가졌으며, 3) 폴리락티톨의 다수의 -OH기가 세포에 삼투 스트레스를 유발함으로서 카비올레 세포내 이입을 촉진시킴과 동시에 PEI의 proton sponge effect가 나노입자를 세포질로 빠르게 탈출하도록 유도시켜 뛰어난 유전자 전달 효율을 보였다.

개발된 유전자 전달체는 간 질환 치료를 목표로 한 다양한 유전자 (DNA, siRNA)를 간세포에 표적 전달함으로서, 간과 관련된 질병에 확대 적용할 수 있고 유전자 발현을 조절함으로서 근본적이고 효과적인 치료를 유도함과 동시에 치료 연구에 상승효과를 가져올 것이다.

주요어 : 유전자 전달, 락티톨, 폴리에틸렌이민, 간세포 표적, 카비올레 세포내 이입

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