Ph.D. Dissertation of Science

# Protective effects of renal fibrosis by renal-specific chitobionic acid- conjugated polysorbitol gene transporter carrying hepatocyte growth factor

HGF 유전자가 도입된 신장 특이적 키토산 접합
폴리소르비톨계 전달체를 이용한
신장 섬유화의 방어효과

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# ABSTRACT

# Protective effects of renal fibrosis by renal-specific chitobionic acid-conjugated polysorbitol gene transporter carrying hepatocyte growth factor

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Chronic kidney disease (CKD) refers to kidney disease that results in structural or functional damage to the kidneys due to a variety of causes, resulting in irreversible loss of nephron and renal function. The incidence and mortality rate of CKD is increasing worldwide, due to the

absence of treatment methods and the existence of various causes of disease. Renal fibrosis is a chronic and progressive kidney disorder that is associated with many kidney diseases. Recovery from acute kidney injury involves the regeneration of the tissue, in which the damaged tissue is replaced by new cells of the same lineage. Abnormal or incomplete recovery lead to renal fibrosis, which is a cause of chronic, progressive renal failure. Considerable research has focused on the development of efficient and specific methods to treat renal diseases. Gene therapy using gene carriers has the advantages of high transfer efficiency, low toxicity, and application to various diseases. In addition, non-viral gene carriers also have potential for gene therapy, due to their ease of modification and biocompatibility. This study was demonstrated that the protective effects of kidney - specific chitonic acid-conjugated polysorbitol gene transporter (CBA-PSGT) carrying the gene for hepatocyte growth factor (HGF) as an anti fibrotic factor, against renal fibrosis progression.

First, CBA, a kidney specific ligand, was synthesized, and conjugated it to a PSGT carrier. CBA can effectively target vimentin to the kidney. Vimentin is an intermediate filament protein that is found in vascular endothelial cells, vascular smooth muscle cells and fibroblasts. Therefore, vimentin, which is expressed on the surface of kidney cells or kidney tissues, can be used as a target for gene delivery. Moreover, vimentin is expressed in the renal interstitial region, as well as in normal and abnormal renal tissues. In particular, vimentin can be considered as a

marker of renal tubular disorder, owing to its high expression in those disorders, including renal atrophy, inflammation, and fibrosis. The kidney-specific gene transporter CBA-PSGT as a gene carrier was synthesized and evaluated. CBA-PSGT was confirmed a successful gene transporter with high stability, due to DNA complexation, minimal toxicity, and high gene expression efficiency with the gene. In addition, CBA-PSGT was effectively protected from digestion by DNase.

Second, unilateral ureteral obstruction (UUO) animal models were constructed for the evaluation of the protective effects of CBA-PSGT/HGF against renal fibrosis. In the UUO model, the expression of HGF was increased by treatment with CBA-PSGT/HGF. The UUO model causes renal tissue damage, mainly resulting in atrophy of the interstitial tissue of the tubule and fibrosis. In addition, infiltration of inflammatory cells into the tubulointerstitial tissue causes inflammatory cytokine release in the UUO model. The CBA-PSGT/HGF delivery group was detected less damage to the kidney tissue by histological analysis. BUN and creatinine levels were used as kidney damage markers, and the expression levels of inflammatory cytokines, interleukin-6 (IL-6) and IL-1$\beta$ were deceased in the group that treated of CBA-PSGT/HGF. Renal fibrosis lead to the formation and accumulation of collagen in the renal tissues. Therefore, collagen formation and accumulation were reduced by gene therapy by histological analysis and assessment of the levels of collagen. In addition, the expression level of collagen-related proteins, such as ICAM-1,

TIMP-1 and α-SMA, which highly expressed in the setting of renal injury, as well as metalloproteinase (MMPs), which are enzymes that inhibits the formation of extraellular matrix (ECM), were decreased in the CBA-PSGT/HGF complex group.

In summary, a CBA-conjugated gene transporter was successfully synthesized as kidney specific ligand, which targets the vimentin that is highly expressed in the setting of renal disease and damage. It is characterized that the kidney-specific gene transporter. CBA-PSGT was stable due to DNA complexation and it was protected from degradation by DNase; the construct also had low toxicity and high delivery efficiency. In addition, The study was demonstrated that CBA-PSGT carrying HGF, an anti-fibrotic factor, protected against the progression of renal fibrosis in a UUO model. CBA-PSGT should be further investigated, to explore its potential to alleviate UUO and kidney-related disease using high affinity kidney targeting.

**Keyword** : Chitonic-acid, HGF, Gene therapy, Kidney fibrosis

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# INTRODUCTION

## *1. Gene delivery system*

Gene and drug delivery systems, which use a variety of facilitate the uptake of genes that have been selected to target the cells or tissues, have great therapeutic potentials for various diseases (Kim *et al.*, 2011; Langer *et al.*, 1998). Optimization of a gene delivery system requires complete understanding of the interactions between the target cells or tissues and delivery system. Cell-targeting gene delivery, the most o a specific compartment or tissue, which is the most commonly used mechanism in endocytosis gene therapy, particularly in cellular uptake of non-vial gene delivery system (Langer *et al.*, 2007). Gene therapy, defined as a procedure used to treat a disorder or improve the health of a patient by genetically modifying the patient`s cells (Stachan *et al.*,1990), is considered an alternative for enzyme or protein replacement therapy. Despite various methods or types of gene therapy, the identification of mutant genes are responsible for the cause of the diseases. The therapeutic gene is loaded in a vehicle, called the vector. The function of the vector is to deliver the therapeutic gene to the target cells or organ. The most critical step in successful gene therapy is the selection of the vector (Ramamoorth *et al.*,2015). The optimal vector and delivery system depends on the target cell and its characteristics, as well as the required duration of expression of the gene of interest and the size of the genetic

material to be incorporated in the vector (Somia *et al.*, 2000), which may fall into the category of a viral, non-viral, or engineered vector.

## 1.1. Viral vector gene delivery system

Viral-based gene delivery systems consist of viruses that are modified to be replication-deficient, which can deliver genes to target sites to facilitate expression (Liao *et al.*,2009). The most commonly used viral vectors are based on retroviruses (Yee *et al.*, 2001), lentiviruses, herpes simplex viruses (HSVs), adenoviruses (Jooss *et al.*, 2003), and adeno-associated viruses (AAVs) (Jooss *et al.*, 2003; Oligino *et al.*, 2000).

Retro viral vectors are the most frequently used for gene delivery as they can transfect dividing cells by passing through the nuclear pores of mitotic cells, unlike other viral vectors, including adenoviral and lentiviral vectors (Bushman *et al.*, 2007). In addition, retroviral vectors have the ability to integrate linearly into the host cell genome, which is useful for *ex vivo* delivery therapy (Fischer *et al.*, 2005). They have been applied for familial hyperlipidemia gene therapy and tumor vaccination. However, retroviral vectors have limitations, including low efficiency *in vivo*, immunogenicity, the inability to transduce non-dividing cells, and the risk of off-target insertion. Thus, they can cause oncogene activation or

tumor-suppressor gene inactivation (Fischer *et al.*, 2005).

Lentiviruses, a subclass of retroviruses, have recently been used as gene delivery vectors because of their ability to naturally integrate with non-dividing cells, unlike other retroviruses. Lentiviruses can deliver 8 kb of sequence; they have been extensively used for *ex vivo* gene transfer to the central nervous system, with fewer instances of immune responses and unwanted side effects. The advantages of lentiviral gene delivery systems are high efficiency of infection; long-term, stable expression of a transgene; low immunogenicity; and their ability to accommodate larger transgenes (Federici *et al.*, 2009).

HSV vectors are among the most recent viral candidates for gene delivery. They can deliver up to 150 kb of transgenic DNA and, due to their neurotropic features, they have potential for gene delivery to the nervous system (Liu *et al.*, 2008), as well as to cancer cells (Goss *et al.*, 2002; Goins *et al.*, 2009).

Adenoviral vectors have been isolated from more than 100 different species. Adenovirus serotypes are commonly used in gene therapy, particular types 2 and 5. Adenoviral vectors can be utilized for transfer into both dividing and non-dividing cells; due to their low host specificity, they can be used for gene delivery into a large range of tissues (Thrasher *et al.*, 2006). Adenoviral vectors can deliver large-scale DNA particles, up

to 38 kb; larger particles do not integrate into the host genome, so the resulting gene expression is brief. Although the risk of serious disease following clinical application is minimal, gene therapy using adenoviral vectors has caused severe side effects and even death (Reid *et al.*, 2002; Raper *et al.*, 2003).

The advantage of using viral vectors is their natural ability to enter cells and express their own proteins. Viral characteristics allow for high titer production, high expression levels, and the ability to infect both actively dividing and quiescent cell types (Phillips *et al.*, 2005; Worgall *et al.*, 2005). Viruses have a high capacity for the physical condensation of DNA within the viral capsid (Ledley *et al.*, 1996). IIn addition, the carrier system features a viral capsid that protects it from enzymatic degradation. This highly evolved and specialized capacity for DNA condensation ensures efficient gene delivery from the cell surface to the nucleus, and subsequent expression of the encoded protein. DNA condensation enhances binding to the cell surface, penetration into the cell, escape from intracellular compartments, and transport of the DNA into the nucleus. Therefore, viral gene delivery systems are considered the most efficient methods of DNA delivery (Mansouri *et al.*, 2004). However, the use of these system in gene therapy could be limited by various factors. First, there are safety issues with using viral vectors, such as toxicity, immune activation, and inflammatory responses (Yee *et al.*, 2001). Second, random insertion of DNA into host cell chromosomes may lead to insertional

oncogenesis or mutagenesis. In addition, recombination with other viruses can produce new, competent viruses (Mansouri *et al.*, 2004). Third, the use of viral vectors for gene therapy is limited by the fact that only small sequences of DNA can be inserted in the viral genome; thus, large-scale alterations may be difficult to achieve. Finally, viral gene delivery systems are typically restricted to targeting specific cell types and are associated with high production costs (Luo *et al.*, 2000). The limitations of viral vectors, especially the safety concerns, have led to the evaluation of alternative approaches and the development of non-viral systems.

## 1.2. Non-viral vector gene delivery system

Non-viral vector gene delivery systems have been developed as an alternative to viral-based systems. They are classified into physical and chemical methods, and they include delivery by gene gun, electroporation, particle bombardment, ultrasound utilization, magnetofection, or cationic liposomes and polymers. Although these systems have lower gene transduction efficiencies than viral systems, they are superior in terms of cost effectiveness, availability, and, most importantly, lower rates of immune activation. In addition, these systems have no limitations with regard to the size of the transgenic DNA constructs, unlike viral delivery systems (Nayerossadat *et al.*, 2012).

Physical methods of non-viral gene delivery have been applied for *in*

*vitro* and *in vivo* gene delivery. They are based on transiently penetrating the cell membrane by mechanical, electrical, ultrasonic, hydrodynamic, or laser-based means, in order to facilitate the targeting of cells or tissues. Naked DNA is susceptible to degradation by DNase and cannot efficiently overcome the cellular barriers for delivery to the cell nucleus, due to its large size and negative surface charge. Naked DNA alone is able to directly deliver a gene (219 kb) to the skin, thymus, cardiac muscle, or skeletal muscle, following intravenous, mucosal, or intramuscular administration (Blomberg *et al.*, 2002; Bragonzi *et al.*, 1999). Although injection of naked DNA is safe and simple, it has low gene delivery efficiency and is only appropriate for some applications, such as DNA vaccination.

The use of a gene gun for DNA particle bombardment is an ideal alternative to the injection of naked DNA. Gold and tungsten coated with plasmid DNA are typically used for gene delivery. This strategy is a modification of a technique that was developed for generating transgenic plants, but it is now used in animals, both *in vitro* and *in vivo*, as well (Knapp *et al.*, 2004; Herweijer *et al.*, 2003).

Electroporation is used to enhance cell membrane permeability by applying an electrical field. Cell membrane breakdown leads to pore formation, which allows the transgene to pass into the cell. This method has been used *in vivo* for many type of tissues, including skin, muscle, and

lung (Dean *et al.*, 2003), as well as for tumor treatment (Heller *et al.*, 2005). On the other hand, the high voltage applied to the tissue can damage the organ and affect genomic DNA stability (Young *et al.*, 2005; McMahon *et al.*, 2004). Ultrasound utilization has also been used to enhance cell membrane permeability for the delivery of DNA particles, in a safe, highly flexible manner. However, the greatest limitation of the system is its low efficiency, particularly *in vivo.*

Chemical systems are more commonly used than physical systems; they typically involve nanomeric complexes, including cationic liposomes (micelles) or cationic polymers. These systems are the 2 major types of non-viral gene delivery vectors currently under investigation. Both cationic liposomes (micelles) and cationic polymers interact electrostatically with negatively charged DNA to form complexes, called lipoplexes or polyplexes, respectively. The most important advantages of these non-viral systems are their ease of use and reliability. In addition, they have good bio-safety with low immunotoxicity, and offer diversity in modification. Although these 2 non-viral delivery systems have several advantages over viral vectors, their success has been limited (Goss *et al.*, 2002). They have relatively low transfection efficiencies, in comparison with viral gene delivery systems.

A number of barriers must be overcome in order to increase the effectiveness of non-viral vectors. Transfer across the plasma membrane

is the most critical barrier to effective DNA transfection. In order for naked nucleic acids to cross the cell membrane, a physical method or carrier must be used to facilitate cellular uptake, which includes 4 steps (Al-Dosari *et al.*, 2009): (1) nonspecific interaction between cationic particles and the cell surface, (2) endocytosis into intracellular vesicles (endosomes), (3) compaction and release of the DNA particle from the endosome, and (4) translocation of the DNA particle into the nucleus, via membrane receptors, and transgenic expression (Mair *et al.*, 2009). When lipoplex or polyplex particles associate with the cell surface, they enter the cell by endocytosis. Many studies have reported receptor-mediated delivery via interactions between carbohydrates and cell surface lectins, including interactions between galactose and glycoprotein receptors in hepatocytes (Hashida *et al.*, 1998; Nie *et al.*, 2011; Wang *et al.*, 2008; Oh *et al.*, 2016).

Cationic lipids have been used for the delivery of encapsulated drugs, as well as the delivery of vectors for gene therapy (Shi *et al.*, 2002). Interactions between anionic nucleic acids and the surfaces of cationic liposomes result in the formation of multilamellar structures. Cationic liposomes have several advantageous characteristics, including the capability to incorporate hydrophilic and hydrophobic drugs, low toxicity, and targeted delivery of bioactive compounds to the site of action (Hofland *et al.*, 2002; Mastrobattista *et al.*, 2002). However, they also have several disadvantages, that the reticuloendothelial system and the inability to

achieve sustained drug delivery over a prolonged period of time, owing to their rapid degradation. To overcome these drawbacks, the systems magnificate the surface of liposomes with hydrophilic polymers, for example, polyethylene glycol (PEG) (Son *et al.*, 2000), integration of the pre-encapsulated drug-loaded liposomes within depot polymer-based systems (Thomsen *et al.*, 2015).

Like cationic lipids, cationic polymers, such as poly-L-lysine (PLL), polyethylenimine (PEI), and polyamidoamine dendrimers, interact with the negatively charged DNA phosphate groups. These carriers are generally accepted due to their ability to efficiently condense DNA and interact with cells; they are more stable than the systems that use cationic lipids. These carriers form complexes with DNA, which protect it against nuclease-mediated degradation (Rasouloanboroujeni *et al.*, 2017).

PLL was the first cationic lipid, followed by PEI. PLL is still the most widely investigated cationic polymer. However, PEI is the most important cationic polymer; it is one of the most positively charged, dense polymers. PEI has high transfection activity *in vitro* and moderate activity *in vivo*. Its linear forms have lower toxicity and higher efficiency than its branched forms (McKenzie *et al.*, 1999). Given that PEI has the greatest number of primary, secondary, and tertiary amino groups, it also has a strong buffering capacity at almost any pH. In constant, Some limitations of PEI have application *in vivo* delivery. However, there are a number of

some advantages and disadvantages. The notable factors affecting its *in vivo* application are toxicity and transfection efficiency.

## 1.3. Chitosan conjugated gene delivery system

Among non-viral vectors, chitosan is considered a good gene delivery candidate. It is known as a biocompatible, biodegradable system with low toxicity and high cationic potential (Safari *et al.*, 2012). Chitosan, which belongs to a family of linear binary polysaccharides, is comprised of $\beta$ (1-4) linked 2-amimo-2-deoxy-b-D-glucose (GlcN; D-unit) and N-acetylated analogue (GlcNAc;A-unit). It provides a biocompatible alternative to cationic polymers, and is widely used as a promising non-viral gene delivery system (Akbuga *et al.*, 2016; Thanou *et al.*, 2002). Nucleic acids can be complexed with chitosan by simple complexation or entrapped in chitosan nanoparticles via ionic gelation. Chitosan-conjugated DNA nanoparticles have the disadvantages of low aqueous solubility and low transfection efficiency (Mansouri *et al.*, 2004; Thanou *et al.*, 2002; Corsi *et al.*, 2003; Mansouri *et al.*, 2004). However, recently, several chitosan derivatives have been developed to enhance transfection efficiency and solubility at neutral pH. Chitosan-conjugated gene carriers have also been modified for high transfection efficiency, and can be successfully administered by parenteral, oral, intranasal, pulmonary, and dermal routes (Akbuga *et al.*, 2016).

Previous studies were demonstrated that vimentin can be targeted with high affinity by CBA, prepared by dimerization of N-acetylglucosamine (GlcNAc), when conjugated with cationic polymers; we found that CBA-conjugated polymers showed higher transfection efficiency in vimetin-expression cells (the human embryonic kidney 293T cells and epithelial HeLa cells) than their unconjugated counterparts (Kim *et al.*, 2011; Singh 2014). Mechanistically, Kim *et al.*, also showed the proof-of-concept that these CBAconjugated polymers had no effect when surface expression of vimentin was knocked down by RNA interference (Kim *et al.*, 2011). This remarkable targeting affinity of CBA for vimentin motivated us to combine CBA with recently developed gene transpoter (PSGT); PSGT has shown remarkable efficacy for the delivery a variety of genetic materials, such as DNA (Kim *et al.*, 2014; Luu *et al.*, 2012; Nguyen *et al.*, 2014; Islam *et al.*, 2011), siRNA (Islam *et al.*, 2012; Islam *et al.*, 2014A; Islam 2014B; Cho *et al.*, 2015) and microRNA (Muthiah *et al.*, 2014; Muthiah *et al.*, 2015), and exhibited remarkable potential for the treatment of diseases, such as lung carcinoma, breast cancer and restenosis.

## *2. Kidney disease*

### 2.1. Kidney injury animal model

Chronic kidney disease (CKD), a leading cause of kidney failure, is still a major health problem, which causes millions of deaths worldwide. In the United States alone, 1 in 3 adults are at high risk of kidney diseases, and 82,000 people die from kidney failure each year. The current treatments have poor efficacy in preventing the progression of the disease; in addition, there is no treatment to permanently reverse the damage of kidney failure, except kidney transplantation, which is expensive and reliant on donor matching and availability (Grande *et al.*, 2015). Recently, considerable researches have been conducted recently to find out the key regulators (such as HIPK2 (Jin *et al.*, 2012), epithelial to mesenchymal transition (Lovisa *et al.*, 2015), defective fatty acid oxidation in renal epithelial cells (Kang *et al.*, 2015), mitochondrial abnormality (Zhang *et al.*, 2015), fibroblast and myofibroblast (Palumbo-Zerr *et al.*, 2015; Strutz *et al.*, 2006)) as well as identify the underlying mechanisms causing CKD leading to kidney failure; it must be a priority to develop a clinically relevant treatment strategy to prevent kidney disease, one of the most devastating human health problems.

To investigate the mechanisms of disease and effects of interventions, a variety of animal kidney disease models have been

developed, including spontaneous, genetic, and induced models. The animal kidney disease models provide a fundamental understanding of kidney biology, physiology, and pathophysiology, and aid in the development of therapies. Among the models, the animal CKD model provides the opportunity to investigate kidney disease-specific mechanisms and molecular pathogenesis, as well as assess potential therapies (Lim *et al.*, 2014). However, many of these models do not accurately reflect human disease.

Various kidney injury models have been developed, in different species and to model specific diseases. An optimal experimental study design must include appropriate choices of model, time course, and readouts (Lim *et al.*, 2014). Ischemia commonly causes acute kidney injury (AKI) in the human population, which is associated with an increased risk of developing subsequent CKD (Bellomo *et al.*, 2012; Bucaloiu *et al.*, 2012).

Diabetes nephropathy (DN) models, which address the single largest cause of CKD, can be artificially induced, spontaneous, or genetically engineered (Lim *et al.*, 2014; Becker *et al.*, 2012). Type I diabetes has been most commonly modelled by injection of streptozotocin (STZ). Animals in STZ-induced models develop a modest degree of proteinuria and kidney toxicity, as indicated by elevated serum levels of creatinine and BUN. Patients with type II diabetes often have DN. Type II diabetes is

commonly modelled by single-gene mutations of the leptin receptor, as in the db/db mouse (Soler *et al.*, 2012). These models are characterized by obesity, hyperglycemia, hyperinsulinemia, and mild to moderate hypertension. These abnormalities precede the development of albuminuria and glomerular injury (Stevenson *et al.*, 1999).

The rodent unilateral ureteral obstruction (UUO) model generates progressive renal fibrosis. Although complete ureteral obstruction is not a common cause of human renal diseases, this animal model for renal fibrosis aids in building an understanding of the disorder under investigation. The UUO model is useful to examine the mechanisms driving tubulointerstitial fibrosis *in vivo* (Chevalier *et al.*, 2009; Lim *et al.*, 2014; Yang *et al.*, 2010). In the UUO model, the obstruction is created surgically, and can be experimentally manipulated with respect to timing, severity, and duration; reversal of the obstruction permits the study of recovery. Renal fibrosis is regarded as the final common pathway for most forms of progressive renal disease, as most renal diseases lead to renal fibrosis (Yang *et al.*, 2010). Since then, the UUO model has been refined to elucidate the pathogenesis of obstructive nephropathy, as well as the mechanisms responsible for progressive renal fibrosis (Sharma *et al.*, 1993). Within 24 hours, animals in the UUO model experience reduced renal blood flow and a lower rate of glomerular filtration. Accordingly, hydronephrosis, interstitial inflammatory infiltration, and tubular cell death, attributable to apoptosis and necrosis, follow within several days.

Progressive fibrosis and tubular cell death appear to be closely associated (Docherty *et al.*, 2006). Therefore, these are useful in the study of the pathogenesis of renal fibrosis. Lesions, which develop more slowly, may be better suited to the study of therapeutic interventions (Chen *et al.*, 2007; Windt *et al.*, 2006).

The surgical procedure for inducing UUO is relatively straightforward. Ligation of the ureter is the most frequently used technique. A piece of Silastic tubing can be folded perpendicularly across the ureter to create an obstruction, or the tubing can be slit and fitted around the ureter longitudinally to form a sleeve that creates a partial obstruction (Chevalier *et al.*, 2009). The obstructed kidney reaches end stage in approximately 10-14 weeks (Chevaliar *et al.*, 2009; Chevaliar *et al.*, 2006).

## 2.2. Mechanism of kidney fibrosis

Renal fibrosis is considered a final, common outcome when the kidney faces chronic, sustained injury. In response to injurious stimuli, affected tissues initially undergo a cascade of events in an attempt to repair, heal, and recover from the damage. Renal fibrosis can be quantitated by histological analysis of renal collagen distribution in tissue sections stained for type I, III, and IV collagen fibrils (Wada *et al.*, 2002). One study found that expression of urinary-specific matrix metalloproteinases (MMPs)

correlated with the deposition of collagen type I and III in a renal fibrosis model (Papasotiriou *et al.*, 2015). Several major pathways are associated with the development of renal interstitial fibrosis following UUO in rats, including interstitial infiltration by macrophages (Xie *et al.*, 2004), tubular cell death by apoptosis, and interstitial fibrosis (Wu *et al.*, 2010). Classically activated macrophages can generate tumor necrosis factor-$\alpha$ (TNF-$\alpha$), which regulates pro-apoptotic signaling and renal tubular cell apoptosis following UUO (Yeh *et al.*, 2011). Wound-healing processes are also activated, including the production and secretion of chemokines and cytokines, recruitment of inflammatory cells to the injured sites, activation of fibroblasts to produce extracellular matrix (ECM), and the regeneration of damaged tissue through cell proliferation and differentiation. However, upon exposure to chronic injury, the tissue overproduces ECM, ultimately causing fibrotic lesions and tissue scarring (Klahr *et al.*, 2002; Genovese *et al.*, 2014). Numerous factors are known to regulate the fibrogenic process after tissue injury, including pro-fibrotic (transforming growth factor-$\beta$ or TGF-$\beta$, HIF, plasminogen activator inhibitor-1 or PAI-1, platelet-derived growth factor, vascular endothelial growth factor or VEGF) and anti-fibrotic (hepatocyte growth factor or HGF, and bone morphogenetic protein 7 or BMP7) factors (Kellenberger *et al.*, 2015; Ognoon *et al.*, 2013; Wong *et al.*, 2014; Persson *et al.*, 2017). These factors have a variety of functions depending on the duration and type of injury (Kellenberger *et al.*, 2015).

A reduction in renal oxygenation occurs in CKD, along with a combination of pathophysiological and morphologic changes that correlate with CKD [117]. A reduction in renal oxygenation in CKD induces the oxygen-sensitive HIF-1, which is primarily expressed in the tubular segments, peritubular endothelial cells, fibroblasts, and glomerular cells (Persson *et al.*, 2017). Reduction renal oxygenation in CKD is the induction of the oxygen-sensitive HIF-1 , is primarily expressed in the tubular segments, in peritubular endothelial cell and fibroblasts as well as in glomerular cells (Persson *et al.*, 2017; Haase *et al.*, 2006). HIF activation during hypoxia may contribute to renal fibrosis by direct transcriptional regulation of target genes that control ECM turnover, and by interaction with the pro-fibrotic factor TGF-$\beta$1 (Haase *et al.*, 2006; Higgins *et al.*, 2008).

Hepatocyte growth factor (HGF) promotes hepatocyte proliferation and liver regeneration (Lin *et al.*, 2002; Liu *et al.*, 2004). Several reports demonstrate that HGF regulates such diverse cellular processes as cell survival, proliferation, migration, and differentiation, via binding to the *c*-mesenchymal epithelial transition factor (*c*-met) receptor(Higgins *et al.*, 2006; Imamura *et al.*, 2017). HGF binding triggers the activation of the c-met receptor through tyrosine autophosphorylation. The kidney abundantly expresses HGF and its receptor (Higgins *et al.*, 2008). Furthermore, HGF is a potent anti-fibrotic factor that prevents progression of chronic renal fibrosis by inhibiting TGF-$\beta$ activation and

epithelialmesenchymal transition (Kellenberger *et al.*, 2015; Mizno *et al.*, 2008).

Some studies have demonstrated that administration of exogenous HGF attenuates renal interstitial fibrosis in animal models of obstructive nephropathy induced by UUO (Gao *et al.*, 2002; Yang *et al.*, 2002A). Administration of HGF not only inhibits the onset and progression of CKD but also exhibits therapeutic efficacy even when tissue injury is already established. HGF has emerged as an intrinsic factor that prevents kidney tissues from the development of fibrotic lesions after chronic injury [123]. The reciprocal balance of TGF− and HGF plays a critical role in determining if the injured tissues undergo recovery or fibrogenesis. Exogenous or endogenous HGF expression would likely provide an effective means for the treatment of a wide range of chronic fibrotic disorders (Liu *et al.*, 2004; Yang *et al.*, 2002A; Matsumoto *et al.*, 2014; Sakai *et al.*, 2015).

Many types of kidney injury induce inflammation as a protective response. Conversely, inflammation promotes progressive renal fibrosis, which can occur in renal disease, and kidney inflammation leads to the production and release of pro−fibrotic cytokines and growth factors (Meng *et al.*, 2014; Guiteras *et al.*, 2016). Macrophages have been implicated in glomerular and interstitial fibrosis, which can cause the progression of CKD (Meng *et al.*, 2014). In addition, the balance between type 1 and 2

macrophages is associated with renal injury and influences CKD progression (Guiteras *et al.*, 2016).

## 2.3. Gene delivery system in kidney disease

For the past several decades, gene therapy has been considered one of the most promising strategies for the treatment of inherited or acquired diseases, and several clinical trials of gene therapy products have indicated a promising future for the translation of this field into tangible bedside applications. In this context, a targeted gene therapy specific to the kidneys could be an ideal strategy to improve the therapeutic efficacy of a desired gene, as wall as an approach to identify an effective method to treat kidney-related diseases.

Applications of gene therapy for the kidney face some unique challenges due to the structure-function relationship of the nephron (Van der Wouden *et al.*, 2004). The kidney is a well-organized and differentiated organ with specialized compartments, consisting of tubules, vasculature, glomeruli, and interstitium, all of which represent hurdles for the gene delivery system; gene carriers encounter these anatomic barriers before reaching their specific transfection sites (Chevaliar *et al.*, 2004). The nephron consists of many unique, highly specialized, quiescent differentiated cell types.

A vector system must be able to gain access via the ureter, and possess the ability to manipulate an organ *ex vivo* at the time of transplantation (Imai *et al.*, 2001; Ojeda *et al.*, 2011; Gong *et al.*, 2003). To achieve therapeutic effects in the kidney and reduce side effects, including transfection of non-target cells in the kidney, the selection of an appropriate delivery vector is critical. New therapeutic techniques in renal gene therapy typically use non-viral, viral, and cellular vectors (van der Wouden *et al.*, 2004; Imai *et al.*, 2001).

The selection of the route of administration is important for improving transfection efficiency. The different methods of administrations allow the targeting of different cell types in the kidney. There are 5 routes of administration, including (1) renal arterial injection, (2) direct parenchymal injection, (3) renal venous injection, (4) retrograde delivery via the ureter, and (5) subcapsular injection.

In general, viral vectors are more efficient for gene therapy than non-viral vector systems. The viral gene vectors used in renal gene therapy are typically adenoviral, because adenoviruses can transfect a wide range of cells, including dividing and non-dividing cells. However, adenoviral transfection is transient, and gene expression must be enhanced by prolonged incubation or perfusion of the kidney. Other types of viruses, such as adeno-associated viruses (AAV), retroviruses, and lentiviruses, are also used for renal gene therapy, and can provide long-term gene

expression compared with that induced by adenoviruses.

A non-viral vector system that has been applied to renal gene therapy is the injection of naked plasmid DNA. Direct injection of naked plasmid DNA into the renal parenchyma was not employed in renal tissues. In general, transfection of naked plasmid DNA results in a low transfection efficiency; thus, several techniques have been attempted to enhance its efficiency, including physical and chemical transfection methods. The physical methods include electroporation and microbubbles with ultrasound. After injection of the plasmid into the renal artery, electroporation of the kidney leads to a high efficiency of transfection; in addition, the use of microbubbles and ultrasound is an efficient technique for the delivery of genes to glomerular cells, endothelial cells, and fibroblast.

Although non-viral vector systems using liposomes are generally nontoxic and non-immunogenic, they have the limitation of low transfection efficiency, Therefore, a technique has been developed to enhance their transfection efficiency. Hemagglutinating virus of Japan (HVJ) liposomes were developed into glycoproteins used to enhance liposome-mediated gene transfer. The liposomes comprise a parainfluenza virus that expresses 2 glycoproteins on its surface, which cooperate to achieve viruscell fusion. HVJ liposomes are more efficient vectors than normal liposomes, but they lead to only transient expression, and HVJ liposomes are immunogenic. Injection of HVJ liposomes via the ureter or renal vein to target interstitial

fibroblasts seems to be the best renal gene therapy (Wu *et al.*, 2005).

New gene therapy methods are usually developed using reporter genes, such as reporter genes (Wu *et al.*, 2005), green fluorescent protein (GFP) (Picconi *et al.*, 2014), and vimentin (Deng *et al.*, 2015). They kidney has some endogenous ndogenouss some activity and displays high autofluorescence. For the quantification of transfection efficiency, luciferase would be the optimal reporter gene.

## *3. Experimental purpose*

In the present study, UUO models were prepared, which a widely used animal model to study kidney disease (Cavagliri *et al.*, 2015). This study investigated that a strategy to treat the UUO rat model using CBA-conjugated, PSGT-mediated delivery of HGF, using kidney-specific targeting. HGF as a potential genetic factor was used to ameliorate UUO in rats because several previous gene therapy studies showed that HGF blocks the progression of chronic obstructive nephropathy (Dai *et al.*, 2004; Gao *et al.*, 2002; Yang *et al.*, 2002A). CBA-PSGT mediated high affinity kidney targeting was observed and led to the expression of HGF specifically in the kidneys. As expected, CBA-PSGT dramatically alleviated urethral obstruction in the UUO rat model; This study investigated the underlying molecular mechanism of this ameliorative effect.

# MATERIALS AND METHODS

## Materials

Branched PEI (molecular weight: 12 and 25kDa) and 3-(4, 5-dimethyl thioazol-2-yl)-2, 5-diphenyl tetrazolium bromide reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorbitol diacrylate (SDA) (MW, 290.27 Da) was purchased from Monomer-Polymer & Dajac Labs (Trevose, PA, USA). Chitobiose was kindly provided by Yaizu Suisankagaku Industry Co. Ltd. (Shizuoka, Japan). HGF plasmid was cultivated in *Escherichia coli* and purified with a plasmid DNA purification kit (Cosmo Genetech, Seoul, Korea).

## Synthesis and characterization of the PSGT, CBA and CBA-PSGT

As we previously described (Islam *et al.*, 2011; Islam *et al.*, 2012; Islam *et al.*, 2014A), the PSGT was synthesized by reaction between SDA and PEI (12 kDa) using a Michael addition reaction and characterized by $^{1}H$ nuclear magnetic resonance(NMR) (Avance$^{TM}$ 600, Bruker, Mannheim, Germany) and gel permeation chromatography (GPC) to confirm the success of the reaction. For GPC measurement, a column coupled with multi angle laser light scattering (GPC-MALLS) and a Sodex OHpack

SB-803HQ (Phenomenox, Torrance, CA, USA) were used to measure the actual MW of the polymer. The column temperature was 25° Cata flow rate of 0.5㎖/min and as mobile phase,0.5 M ammonium acetate was used.

To synthesize chitobionic acid (CBA), first a water and methanol mixture (5mL) was used to dissolve chitobiose (0.5g). Then iodine (1.425g) dissolved in methanol (20mL) was added drop by drop into chitobiose solution and mixed for 1 h at 40℃. The color of the solution became dark brown after adding iodine solution to CBA. The solution was mixed with 4% (weight percent) potassium-hydroxide solution(in methanol) until the color of iodine solution disappeared and stirred for 2h at 40℃. After cooling, the sample was recrystallized in ethe r and ether was removed carefully to get precipitated sample. The recrystallized precipitated sample was then dissolved in distilled water and chitobionic acid [$(GlcNAc)_2$-COOH] was obtained by separating the synthesized product using an amberlite IR-120 column and repeated this separating process 2 to 3 times to obtain more purified product. The purified CBA solution was then lyophilized and stored at -70℃ until use later.

To synthesize CBA-PSGT, CBA was conjugated with PSGT using DCC/NHS chemistry as coupling agents where the mole ratios of all reactants in the reaction are 1: 2: 2: 5 for CBA: DCC: NHS: PSGT, respectively. Methanol was used as the dissolving solvent for all the reactants. Briefly, CBA was dissolved in methanol at 2 mM concentration.

The DCC and NHS were added separately (each at 4 mM concentration) and stirred the reaction at room temperature for overnight (16 h) at inert condition. The PSGT (10mM concentration in methanol) was then added and kept the reaction at RT (and inert condition) for additional 24 h. The sample was then dialyzed (MWCO: 3500 Da) in distilled water at 4℃ for 24h. Any unwanted precipitation (because of the formation of dicyclohexyl urea in the reaction) was removed by centrifugation and supernatant was collected, lyophilized and CBA-PSGT was stored at -70℃ until use later. To characterize the successful synthesis of CBA and conjugation of CBA to PSGT, $^{1}$HNMR was performed in $D_2O$ and the spectra of PSGT, CBA and CBA-PSGT were analyzed.

## Characterization of the CBA-PSGT/DNA complexes

The ability of the CBA-PSGT to complex DNA was evaluated by gel retardation procedure according to various N/P (phosphate groups of siRNA/amine groups of the polymer) ratios (from 0 to 10). PGL3 was used as a plasmid DNA control. Briefly, the CBA-PSGT/PGL3 (0.1㎍) complexes at N/P ratios 0.1, 0.5, 1, 3, 5, and 10 were prepared by incubating the complexes for 30 min at RT. The final volume was 12㎕ for each sample, including the loading dye mixture (Biosesang, Seoul, Korea). The samples were loaded onto an agarose gel (1 %) and run at 100 V with Tris/borate/EDTA (TBE) buffer for 40 min. Finally, an ultraviolet illumination was used to observe the gel. The protection and release of

PGL3 was also investigated by gel electrophoresis assay. Briefly, the CBA-PSGT/PGL3 complexes (N/P 10) were prepared by incubating the complexes for 30 min at RT and further incubated at 37° C for 30 min in DNase I. Then, 4㎕ EDTA was added and further incubated for 30 min at RT to inactivate DNase. Finally, 5㎕ of sodium dodecyl sulfate (SDS) (1% in distilled water) was added and incubated at RT for additional 2 h. Electrophoresis was performed for 1 h on a 1 % agarose gel at 50 V with TBE running buffer. To check particle morphology, the CBA-PSGT/PGL3 complexes (N/P 10) were observed by TEM (LIBRA 120, Carl Zeiss, Oberkochen, Germany). Particle size and surface charge of the CBA-PSGT/PGL3 complexes were analyzed using a DLS (DLS 8000, Otsuka Electronics, Osaka, Japan), as we reported earlier (Islam *et al.*, 2011; Islam *et al.*, 2012; Islam *et al.*, 2014A).

## Cell line and culture conditions

The human embryonic kidney 293T cells and the human adeno carcinomatous type II alveolar epithelial A549 cells were obtained from American Type Culture Collection, and maintained in Dulbecco' s Modified Egles`s Medium (DMEM) at 37° C in humidified air with 5% $CO_2$, supplemented with heat-inactivated fetal bovine serum(10%)(FBS, Hyclone, Logan, UT, USA) and 1% penicillin/ streptomycin (complete culture medium). Cells were subcultured every 2-3days when subconfluent. Unless otherwisestated, cells were plated at $1x10^5$㎖/well in

24-well culture plates during experimental procedures and invitro treatments were done when the cells are in 80% confluent.

## *In vitro* toxicity and transfection assay

Evaluation of *in vitro* toxicity of polyplexes was tested with the MTT assay. Briefly, $1x10^5$cells/mL/well in 24-well culture plates were exposed to the polyplexes(1 mg pGL3) at various NP ratios (at 2.5, 5, 10 and 20) for 72 h in serum-free medium. The metabolically active (living) cells were spectrophotometrically measured as reduction of the tetrazolium MTT dye to insoluble formazan produced by cellular enzymes, as previously described (Islam *et al.*, 2011; Islam *et al.*, 2012; Islam *et al.*, 2014A).

To evaluate transfection efficiency, cells (10 x $10^4$cells/mL/well in24-wells culture plates) were grown to approximately 80% confluence before treatment with fresh serum free media containing polyplexes(1mg pGL3)at various N/P ratios (at 5,10 and 20) and incubated for 4-6h at 37℃ under the standard incubation condition. The cells were incubated for an additional 48h by replacing serum free media with fresh serum containing media. The luciferase assay was carried out as previously described (Islam *et al.*, 2011).

## *In vivo* transfection and luciferase expression in various organs of wild-type mice

Balb/c mice were obtained from the Human Cancer Consortium, National Cancer Institute Breeding Colony (Frederick, MD, USA) and maintained in a laboratory animal facility with temperature and relative humidity maintained at 23±2° C and 40±100% under a 12 h light/dark cycle. Mice (n=3) received PBS, naked pGL3, PSGT/pGL3 and CBA-PSGT/pGL3 polyplexes (30㎍ of pGL3/mouse) intravenously via tail-vein injection. Mice were sacrificed after 72 h and whole organs (lung, heart, liver, spleen and kidney) were collected immediately for extraction of total RNA using TRizol solution (Invitrogen, USA). Reverse transcription of total RNA to cDNA was performed using AccuPower CycleScript RT PreMix (Bioneer, South Korea). Luciferase and GAPDH mRNAs were quantified by quantitative real-time PCR (qRT-PCR) analysis using TOPreal qPCR Premix (Enzynomics, South Korea) on iCycler Real-Time Detection System (BioRad, USA) using following primers; Table 1. Finally, luciferase mRNA expression level was normalized with GAPDH mRNA expression.

## Preparation of UUO rat model and administration of HGF gene

Five weeks old male Sprague Dawley (SD) rats were purchased from Nara Biotech. Inc. (Seoul, Korea) and kept in a laboratory animal facility

with a constant temperature of 23±2℃ and relative humidity of 40±10% under a regular 12h light/dark cycle. The rats underwent surgery to induce UUO. Briefly, under pentobarbital anesthesia, the left ureter was exposed through a lateral incision and ligated with 4-0 silk (Ethicon). After surgery; the rats were kept in the laboratory animal facility for 14 days to induce UUO. The sham (healthy control) rats were un-operated, thus the ureters were not ligated. The rats were randomly divided into the following five groups: sham (healthy control rat) and four other UUO groups (UUO control, naked HGF, PSGT/HGF and CBA-PSGT/HGF complexes). PSGT/HGF and CBA-PSGT/HGF complexes were prepared at N/P ratio of 10 as explained earlier (50㎍ HGF gene/rat) and injected intravenously at days 3, 7 and 10 after UUO surgery before sacrificing the rat on day 14. All the experimental procedures in this study were performed using processes approved by the animal care and use committee of Seoul National University (SNU-131205-2).

## Pathological assays

The kidney tissues were fixed in 4% paraformaldehyde and embedded in paraffin for histological evaluation. Paraffin-embedded renal samples were sectioned, prepared at a thickness of 5㎛ and placed on charged glass slides (Fisher Scientific, Fairlawn, NJ, USA). The samples were scanned for histopathological examination, Sirius red staining for evaluating the accumulation of collagen, and immunohistochemistry. The slides were

stained with hematoxylin and eosin (H&E) for histopathological analysis. The accumulation of collagen was evaluated via Sirius red staining according to manufacturer' s instructions (Sigma, Germany). The degree of tubular injury was judged by H&E staining. The tissue injury score was estimated based on random observation of three fields per each examined kidney and the magnitude of tubular injury was scored into five levels on the basic of the percentage of affected tubular in a high-power filed under light microscope. The tissue injury score was recorded according to this estimation: 0, none; 1, <25%; 2, 23-50%;3, 50-75%, 4> 75% (Imai *et al.*, 2001). The blood urea nitrogen (BUN) (Arbor Assays) and creatinine (Cayman Chemical) levels in serum were measured according to the manufacturer instructions.

## Western blot analysis

Kidney tissues were homogenized and protein concentrations were measured by a Pierce BCA Protein Assay kit (Thermo, Rockford, IL, USA). An equal amount of protein (25$\mu$g) was loaded onto a SDS-containing gel and transferred to nitrocellulose membrane using Neon transfection kit (Invitrogen, Camarillo, CA, USA). The membranes were blocked and incubated with their corresponding primary antibodies at 4°C. Immunoblotting was performed with primary antibodies of HGF (Abcam Inc., Cambridge, MA, USA), ICAM-1, TIMP-1 (Santa Cruz, CA, USA), and $\alpha$-SMA (AbFrontier, Seoul, Korea). After washing with TTBS, the

membranes were incubated with secondary antibody of GAPDH (AbFrontier, Seoul, Korea). The bends of interest were obtained with Ez-Capture MG image analyzer (ATTO, Tokyo, Japan).

## Quantitative analysis of PCR products

Total RNA was extracted by Quick Gene RNA cultured cell kit (Kurabo, Japan) following the manufacturer`s protocol and used for cDNA synthesis with a reverse transcription kit by GeNet Bio. The values of gene expression were normalized against GAPDH. The sequences of the primers that were used for the qPCR were as follows Table 2.

## Statistical analysis

Quantification of Western blot analyses was performed with the Multi-Gauge ver. 2.02 program (Fujifilm, Tokyo, Japan). Quantitative data are given as mean ±SD. The results were analyzed by an independent samples *t*-test (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered as significant, whereas $P<0.01$ and $P<0.001$ were considered as highly significant, respectively.

Table 1. List of primers using luciferase assay

| | Primer | |
|---|---|---|
| Luciferase | F | 5`-CTCACTGAGACTACATCAGC-3' |
| | R | 5`-TCCAGATCCACAACCTTCGC-3' |
| GAPDH | F | 5`-TTGATGGCAACAATCTCCAC-3 |
| | R | 5`-CGTCCCGTAGACAAAATGGT-3' |

Table 2. List of primers using real time-PCR

| Gene | Primer | |
|---|---|---|
| HGF | F | 5`-TGATCCAAACATCCGAGTTG-3' |
| | R | 5`-CCATTGCCACGATAACAATCT-3' |
| type I collagen | F | 5`- ATCCTGCCGATGTCGCTA-3' |
| | R | 5`- CCACAAGCGTGCTGTAGGT-3' |
| typr II collagen | F | 5`- CTGGTCCTGTTGGTCCATCT-3' |
| | R | 5`- ACCTTTGTCACCTCGTGGAC-3' |
| GAPDH | F | 5`-TTCAACGGCACAGTCAAG-3' |
| | R | 5`-TACTCAGCACCAGCATCA-3' |

# RESULTS

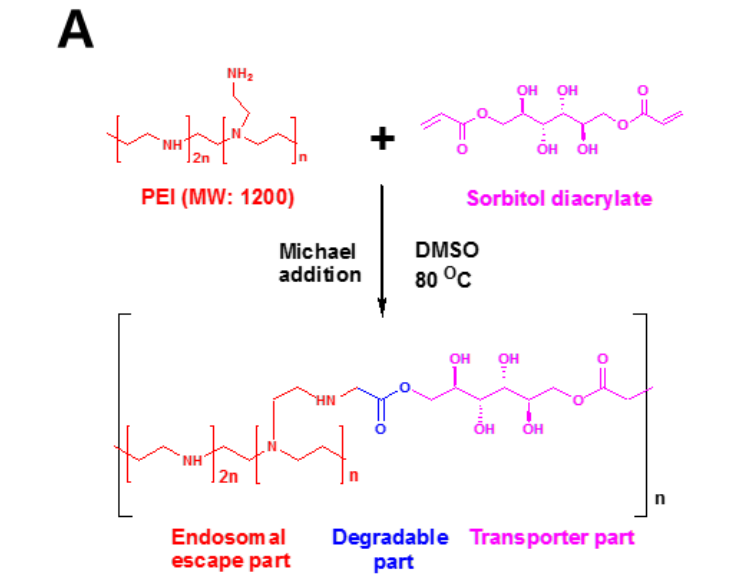
## Characterization of PGST, CBA, and CBA-PSGT

Michael-type addition reaction was employed to synthesize PSGT prepared from the reaction between SDA and low molecular weight PEI as the researchers reported previously (**Fig. 1A**) (Islam *et al.*, 2014A). A transporter for DNA motivated me to apply PSGT for kidney-specific therapy by tuning the chemistry of this novel biomaterial with synthesized according to the previous report (Kim *et al.*, 2011; Singh *et al.*, 2014) (**Fig. 1B**). Conjugated with PSGT using DCC/NHS chemistry synthesize CBA-PSGT gene carrier.

In the reaction, the DCC and NHS activated the carboxylic acid groups of CBA and promoted their reaction with amine groups of PSGT, forming amide linkages to synthesize CBA-PSGT (**Fig. 2**). The PSGT, CBA and CBA-PSGT were found completely water soluble due to their hydrophilic property. $^{1}H$ NMR was performed in $D_2O$ to characterize these compounds. The $^{1}H$ NMR spectrum of CBA-PSGT (after reaction) was compared with the individual spectrum of PSGT and CBA (before reaction) in order to validate their successful conjugation.

In this regard, **Figure 3,** shows that CBA has its major proton peaks at ppm value around 2.0, whereas no peaks appeared at this range for PSGT (before reaction), however, CBA-PSGT has those clear peaks at

that region, comfirming that CBA was successfully conjugated to PSGT and it was estimated by $^{1}H$ NMR that about 2.41 mol-% of CBA was remained attached in CBA-PSGT product.



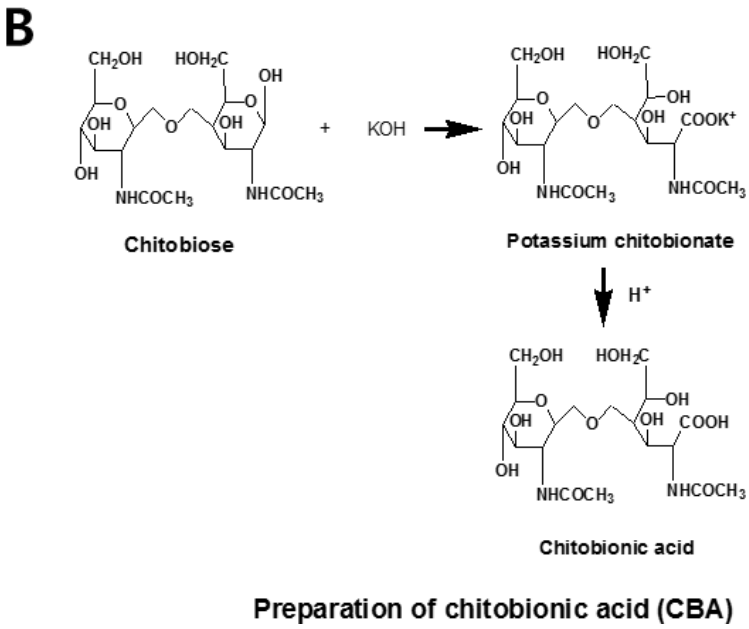




Figure 1. Synthesis and physicochemical characterization of CBA-PSGT. (A) PSGT was synthesized by Michael addition reaction between SDA and PEI (12kDa) in DMSO at 80℃. (B) synthesis of chitobionic acid (CBA).

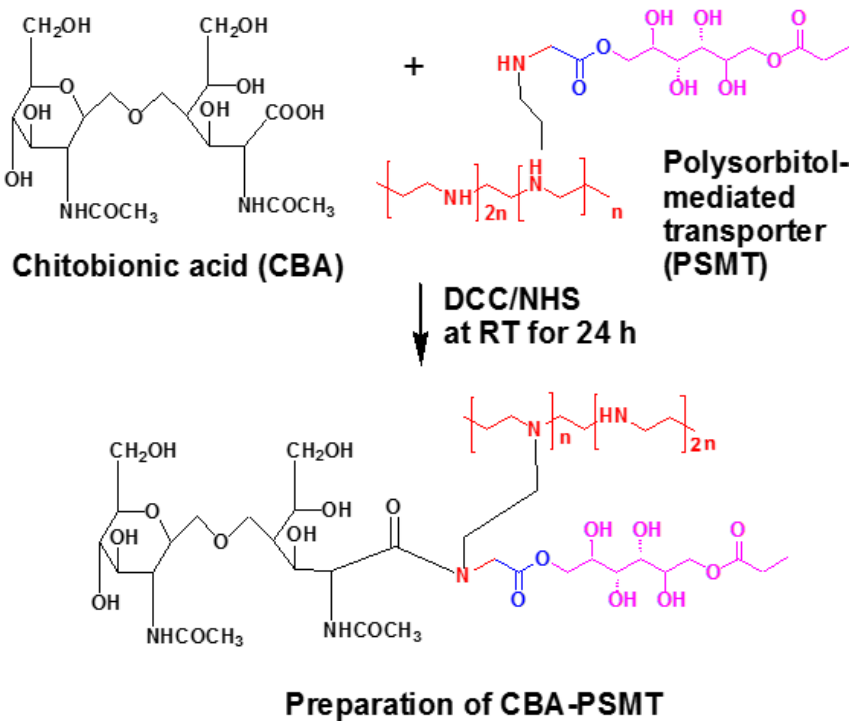



Figure 2. Synthesis and physicochemical characterization of CBA-PSGT. (C)synthesis of CBA-PSGT. CBA was conjugated with PSGT using DCC/NHS chemistry as coupling agents where the mole ratios of all reactants in the reaction are 1:2:2:5 for CBA:DCC:NHS:PSGT, respectively. Materials and methods section explain the entire synthesis scheme in detail.

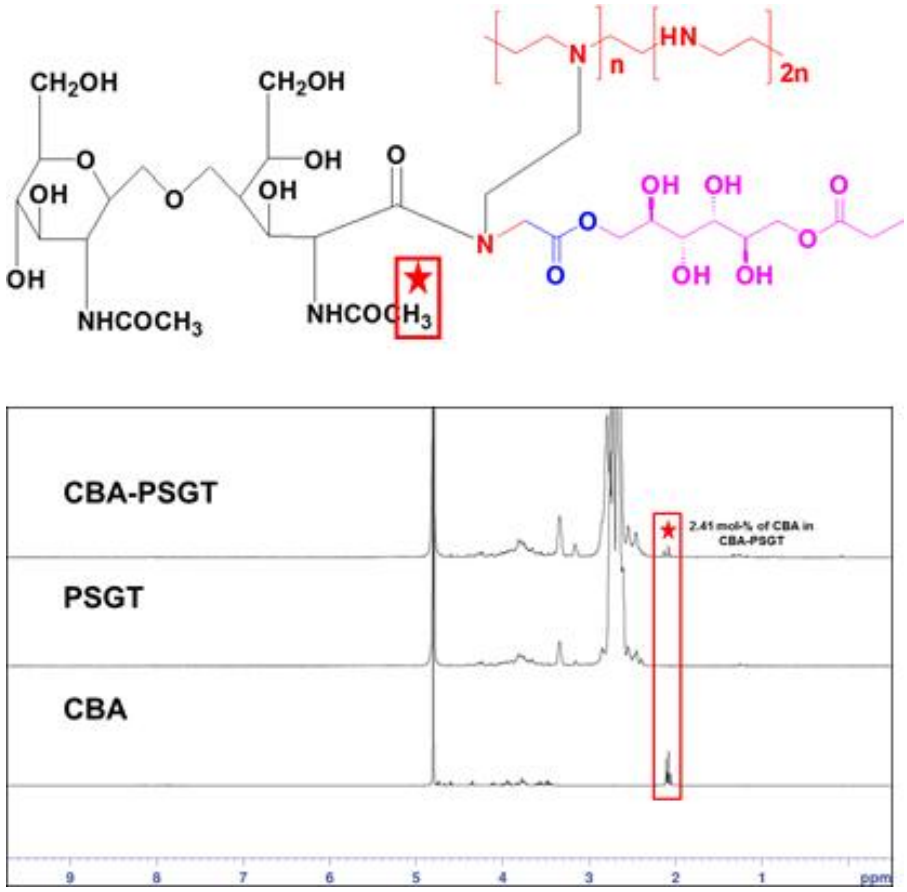



Figure 3. Representative $^{1}H$ NMR spectra of CBA, PSGT, and CBA-PSGT. CBA-PSGT is showing a successful conjugated of CBA to PSGT and mod-% of CBA in the conjugate.

## CBA-PSGT stably complexes with DNA and protects DNA from enzymatic degradation

I ultimate goal of this work was to prepare a kidney-targeting carrier system which can effectively deliver hepatocyte growth factor (HGF) in a form of plasmid DNA. Thus, in order to determine the DNA complexation ability of CBA-PSGT, gel retardation assay was performed at various N/P ratios ranging from 0.1 to 10. PGL3 was used as a model DNA. It was found that CBA-PSGT was remarkably effective to condense DNA at N/P ratio as low as 0.5 (**Fig. 4**), suggesting an excellent DNA condensation ability by the synthesized targeted polymer. The CBA-PSGT is effective in condensation of DNA. To check whether CBA-PSGT can protect the complexed DNA from degradative enzymes and facilitate releases, gel electrophoresis was performed in the presence or absence of DNase I enzyme. **Figure. 5** shows that the naked DNA was degraded eventually in the presence of DNase. Whereas CBA-PSGT completely protected DNA from the enzyme, suggesting that CBA-PSGT is very efficient to protect it from enzymatic degradation and facilitate the release of the complexed DNA.

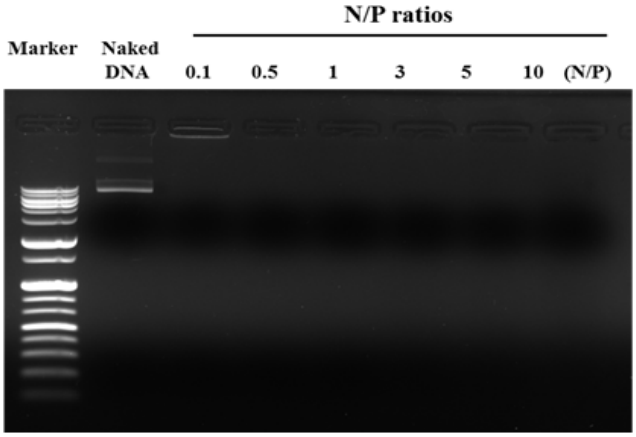



**Figure 4. The stability of DNA complexation of CBA-PSGT** Agarose gel electrophoresis of CBA-PSGT/DNA complexes at various N/P ratios of 0.1-10. One microgram of PGL3was used as DNA to complex with CBA-PSGT.

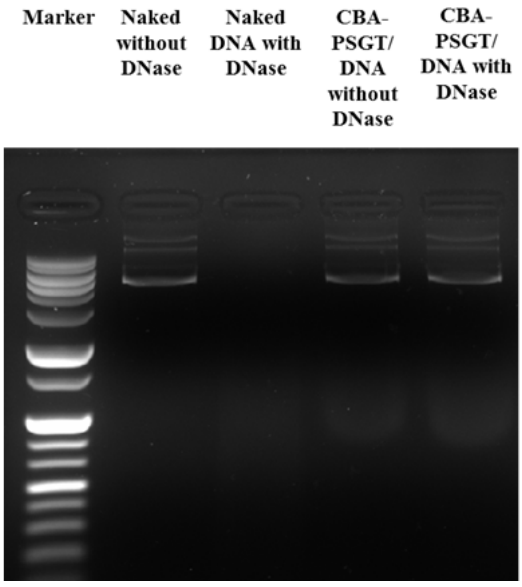



**Figure 5. CBA-PSGT and DNA complexes gene successfully protect from DNA degradation enzyme.** Naked PGL3 and CBA-PSGT/PGL3 complexes (N/P 10) were incubated with or without DNase I for 30min at 37℃ with gentle shaking. Sodium dodecyl sulfate (1%) was used to release the PLG3 from the complexes.

## Physicochemical characterizations of CBA-PSGT

The physicochemical characterizations of the polyplexes are the prerequisite factors to determine their efficacy because the therapeutic effectivity largely depends on the particle size, surface charge and shapes. The particle sizes were ranged from 110.6, 114.9 and 129.3 nm for N/P ratios of 2.5, 5 and 10, respectively measured by DLS. (**Fig. 6A**). The surface charge of the polyplexes was near neutral as expected (about 4-5mV) because of the presence of many hydroxyl groups in CBA-PSGT which hindered the surface charge of the particles (Ialam *et al.*, 2011; Islam *et al.*, 2012; Islam *et al.*, 2014A). TEM images showed that CBA-PSGT/DNA complexes are nanometer-scaled size, uniformly distributed and compact in their shape as shown in **Figure 6B,** which supports the DLS analysis.

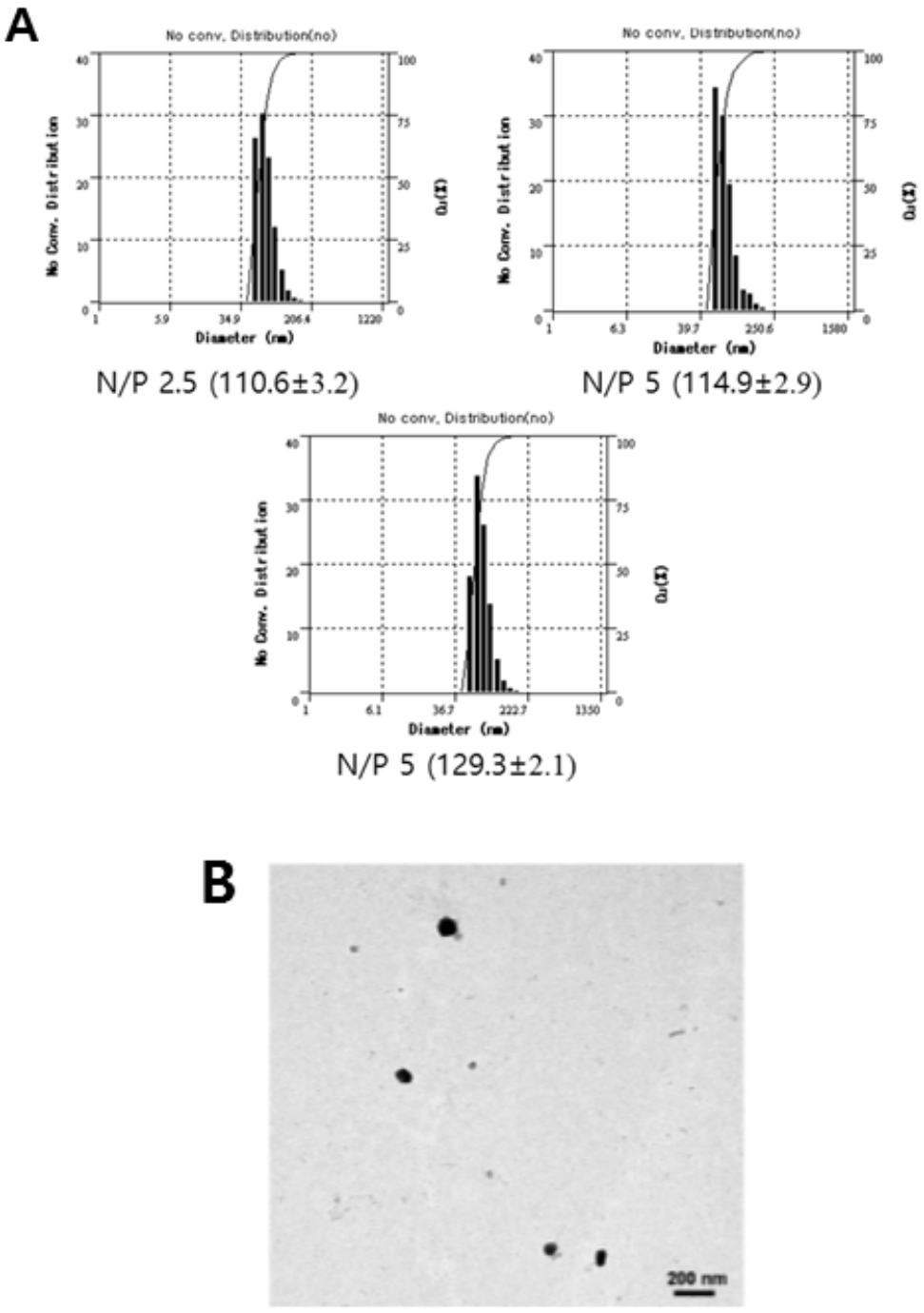



**Figure 6. Physicochemical characterizations of CBA-PSGT.** (A) Particle size were measured at various N/P ratios of 2.5, 5, and 10 (n=3; mean± SD). (B) Transmission electron microscopic (TEM) image of CBA-PSGT/PLG3 complexes (N/P 10;bar denotes 200nm).

## CBA-PSGT/HGF complexes exhibit minimal toxicity

Toxicity is one of the major concerns to develop an effective polymeric gene delivery system having negligible side effects. To test *in vitro* toxicity of CBA-PSGT, 293T and A549 cells were investigated by incubating them with the polyplexes. PSGT/DNA and PEI 25kDa/DNA complexes were also checked and compared with CBA-PSGT/DNA complexes. The results from **Figure 7** showed that both PSGT and CBA-PSGT complexes exhibited similar cell viability percentages at various N/P ratios from 2.5 to 20, whereas the toxicity of PEI 25kDa polyplexes was increased with the increase of N/P ratio in both the cell lines. At N/P ratio of 2.5, cell viabilities of PEI 25 kDa polyplexes were about 85 % in both 293T and A549 cells, however cell viability drastically dropped to near 40 % and 50 %, respectively in 293T (**Fig. 7A**) and A549 cells (**Fig. 7B**) as the N/P ratio increased to 20. One the other hand, at N/P ratios from 2.5 to 10, both PSGT and CBA-PSGT exhibited near or over 90 to 95 % of viable cells in both the cell lines. At N/P ratio of 20, cell viability reduced slightly, however still maintained near or over 80 % cell viability, suggesting that the modification of PSGT with CBA did not affect on cytotoxicity of CBA-PSGT polymer.

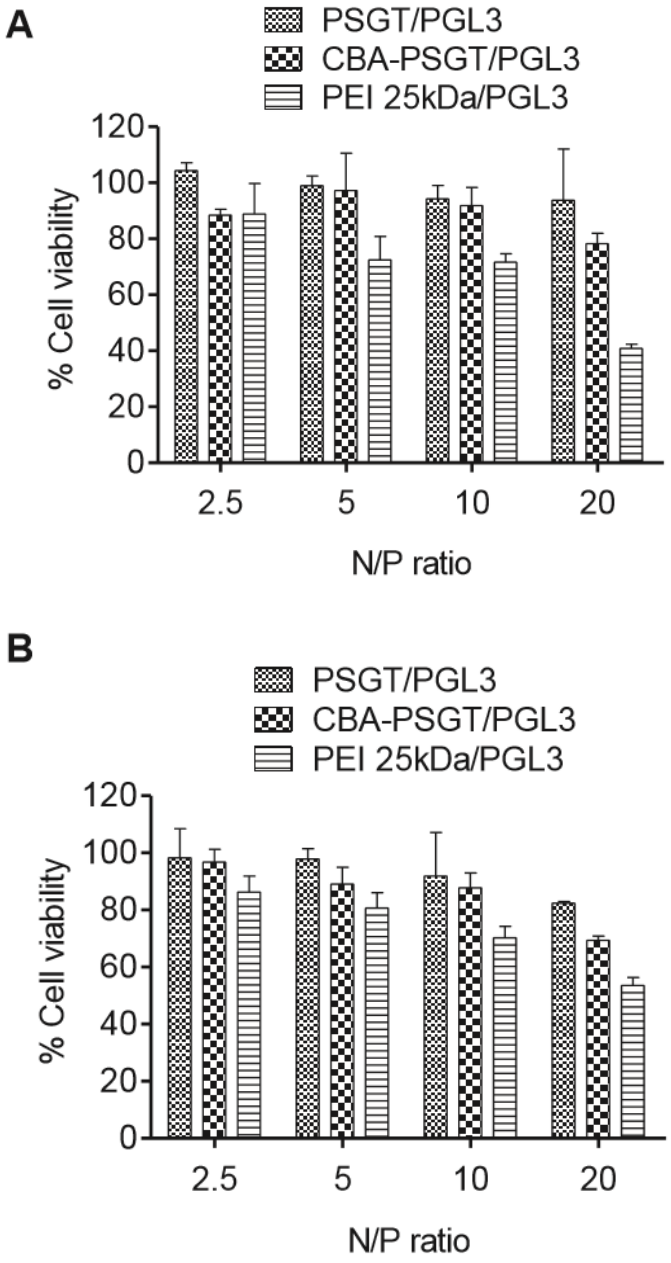



Figure 7. Cytotoxicity at various N/P ratios (from 2.5 to 20) of CBA-PSGT/PGL3 and PEI 25kDa/PLG3 complexes in (A) 293T (n=3, mean± SD) and (B) A549 cells (n=3, mean± SD).

## CBA-PSGT/HGF complexes greater transfection efficiency in embryonic kidney cells *in vitro*

To investigate the differences in transfection activity among CBA-PSGT, PSGT and PEI 25kDa, an embryonic kidney carcinoma cell line (293T) was used and compared the transfection with A549 cells, which is a lung cancer cell line to depict the fact that CBA-PSGT, due to have high-specificity, could be able to provide an increase transfection activity in kidney cells compared to other types and its unmodified form. PGL3 was used as a DNA at various N/P ratios with the polymers and luciferase assay was performed to estimate the luciferase relative light unites (RUL) per mg of protein. The results in **Figure 8A** showed that the transfection efficiencies of CBA-PSGT at various N/P ratios were significantly higher in 293T cells compared with that of unmodified PSGT and also better than PEI 25kDa as well as Lipofectamine 2000 used as controls. On the other hand, there were no differences in transfection efficiencies between PSGT and CBA-PSGT in A549 cells (**Fig. 8B**); rather the transfection of PSGT at N/P ratio of 20 was higher than CBA-PSGT, suggesting that CBA-PSGT was no better than PSGT to transfect A549 cells but highly effective in 293T cells. The differences in transfection efficiencies of PSGT and CBA-PSGT between 293T and A549 cells imply that CBA-PSGT had a more effective interaction with 293T cells presumably because of its targeting CBA moiety with the cells having vimentin receptors which A549 cells do not possess, suggesting that CBA-PSGT may be useful as kidney targeting gene carrier.

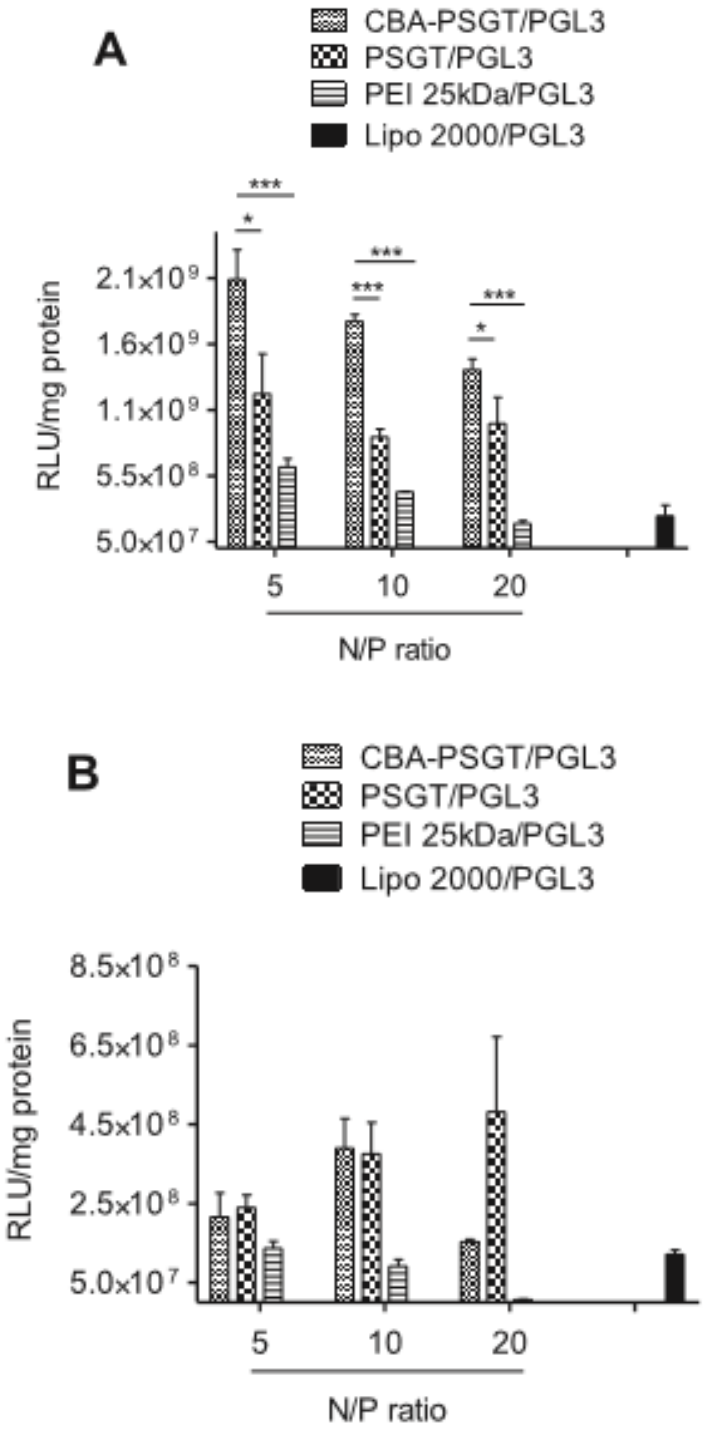



Figure 8. Transfection efficiency of CBA-PSGT/PGL3, PSGT/PGL3 and PEI 25kDa/PGL3 complexes at various N/P ratio in (A) 293T and (B) A549 cell lines.

## *In vivo* kidney-specific targeting in wild-type mice by CBA-PSGT/HGF complexes

To elucidate better understanding on *in vivo* kidney targeting ability of CBA-PSGT system, mice were intravenously injected with CBA-PSGT/PLG3 polyplexes and luciferase mRNA expression was evaluated in kidney as well as in various other organs including lung, heart, liver, and spleen compared to that of PSGT/PGL3 (**Fig. 9**). PBS and naked PGL3 were injected as negative controls. Luciferase mRNA expression in organs were normalized with that of the house-keeping GAPDH mRNA expression and it was found that the CBA-PSGT was remarkably effective to transfect kidneys since it showed high luciferase mRNA expression which was more than two-fold higher in kidneys compared to the expression in spleen and about 10-fold higher than that in lung, heart, and liver. The negative controls showed minimal luciferase expression in all organs. As expected, it was found that the luciferase activity of CBA-PSGT polyplexes in kidney was more than 3-fold higher($P<0.001$) than that of unmodified PSGT polyplexes, rendering that CBA-PSGT is highly effective to target kidneys *in vivo* and holds a promising potential to use as therapeutic gene delivery system against kidney-related disorder.

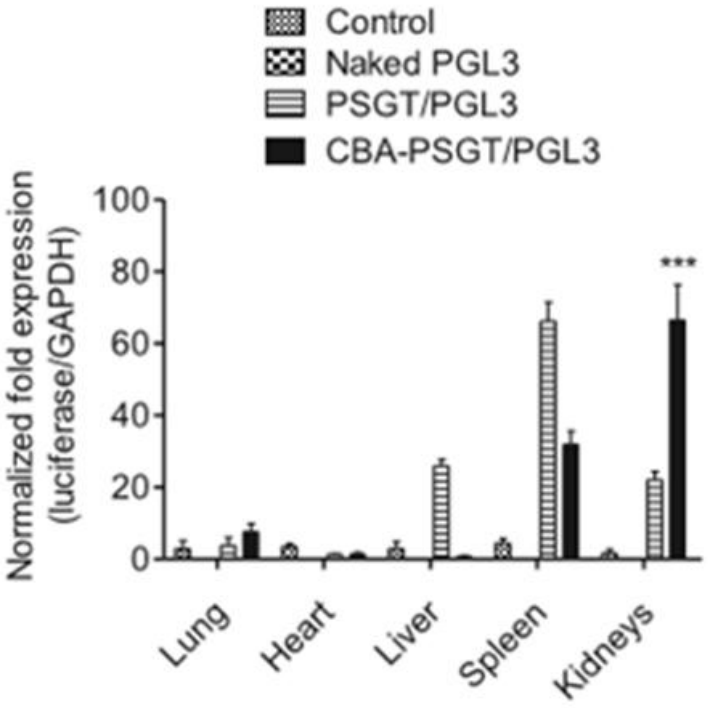



Figure 9. *In vivo* kidney-specific targeting in wild-type mice by CBA-PSGT/HGF complexes *in vivo* kidney targeting efficacy of CBA-PSGT system.

## Establishment of UUO model for identification of CBA-PSGT transporter efficiency

To investigate the targeting specificity of CBA-PSGT/HGF complexes to renal lesion, the UUO model in rats were developed by operating and tieing the left ureter of rats The left ureter was operated via a lateral incision and ligated (**Fig. 10A**). The obstructed kidneys displayed activation of fibrosis on day 7 after surgery and caused damage of kidney both medulla and cortex tissues. Following surgery to induce UUO, HGF gene was delivered (as naked HGF, or as complexed with PSGT or as CBA-PSGT) at days 3, 7, and 10 via intravenous injection. HGF gene was applied because it is widely used in kidney gene therapy studies for the hope to treat chronic renal disease such as kidney fibrosis (van der Wouden *et al.*, 2004; Imai *et al.*, 2001; Ojeda *et al.*, 2011). **Figure 10B** showed that the right nonobstructed kidney or left obstructed kidney was analyzed 14 days after the operation.

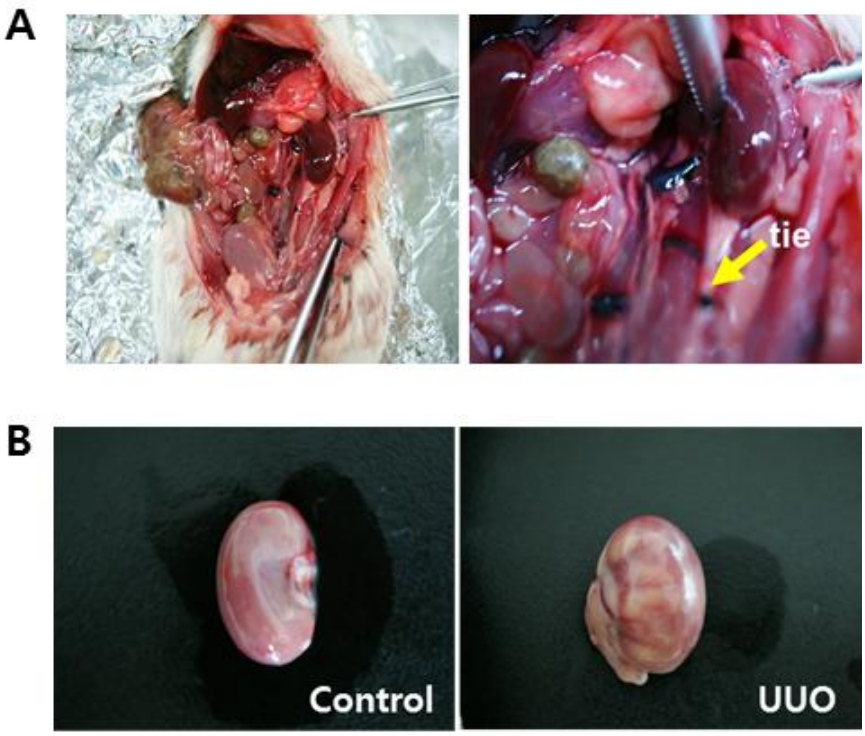



**Figure 10. Establishment of UUO model for identification of CBA-PSGT transporter efficiency** (A) Operation in kidney and tie of left ureter, (B) the kidney after establishing of UUO model.

## Targeted HGF delivery to renal lesion of UUO rat by CBA-PSGT/HGF complexes

The untreated UUO rat model was used as a negative control. I found that the CBA-PSGT/HGF complexes successfully targeted kidneys, determined through the expression of HGF gene. The results showed a significantly increased expression of HGF in CBA-PSGT/HGF group since the DAB signals in CBA-PSGT/HGF was mush higher compared to that in control, naked HGF, and PSGT/HGF group in UUO model rats showed in immunohistochemistry analysis (**Fig 11A**). Futhermore, the mRNA level of HGF was significantly elevated for CBA-PSGT/HGF complexes compared to all other groups (**Fig. 11B**). The protein level of HGF gene for CBA-PSGT/HGF group was highly expressed significantly more than that of naked HGF and PSGT/HGF as well as the control group determined by Western blot assay (**Fig. 11C**) and it`s represented quantitative analysis. These results clearly represent that the CBA-PSGT/HGF complexes can successfully target kidneys to deliver and express therapeutic gene.

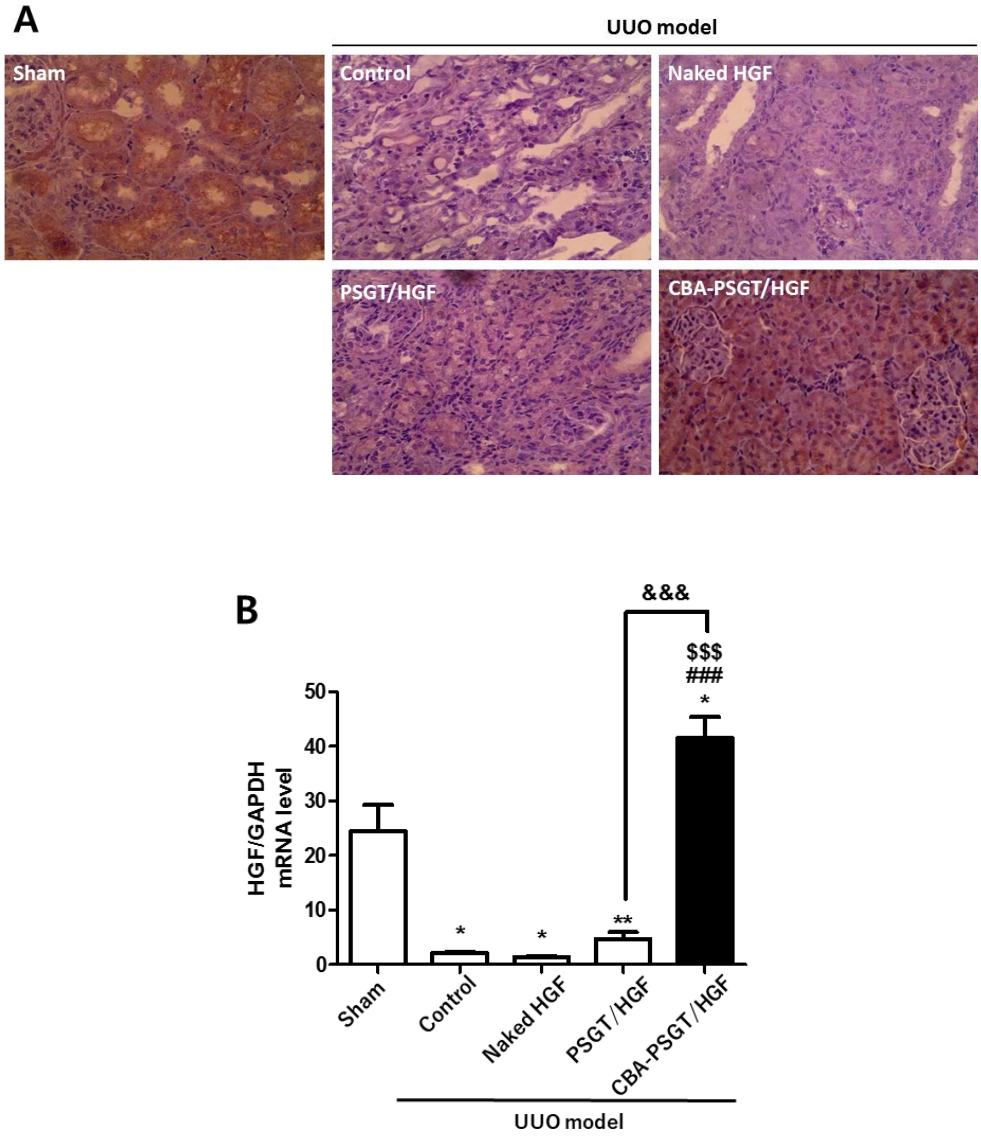
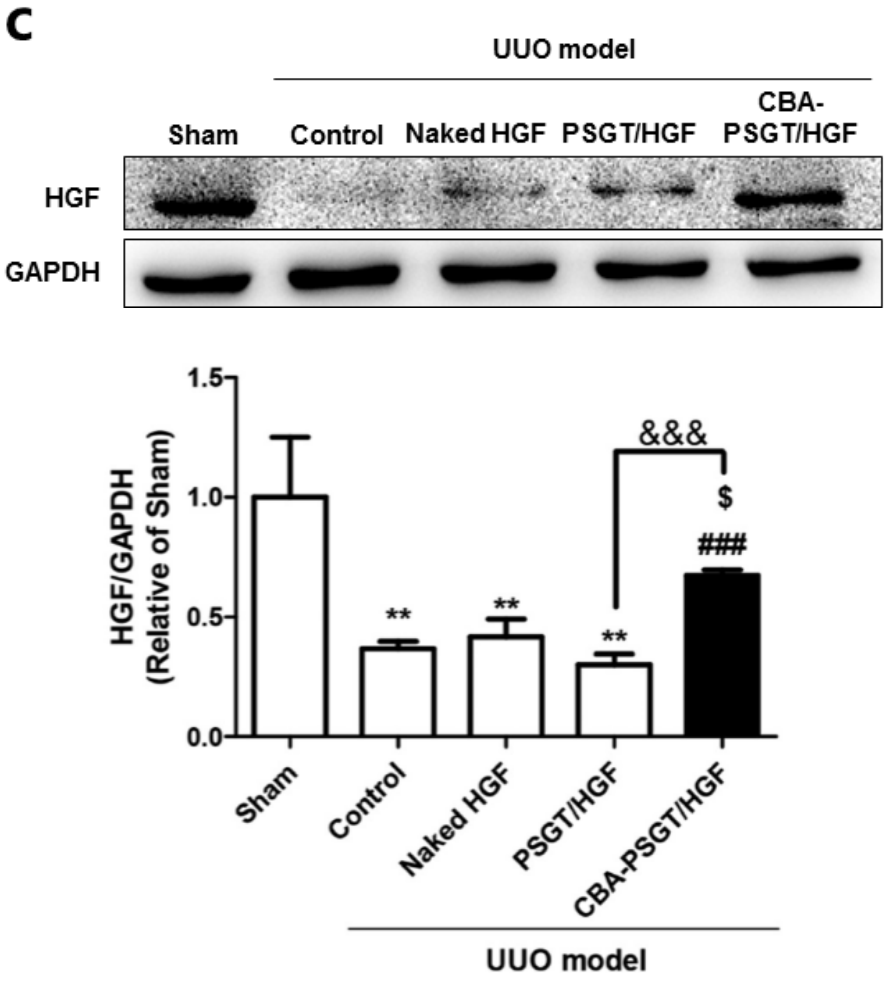



**Figure 11. The efficiency of CBA-PSGT/HGF complexes to target kidneys was demonstrated by specific HGF expression in kidney tissues.** The expression of HGF in UUO model rats were examined and confirmed by (A) immunohistochemistry (magnification, x40). Total kidney lysate were also used to evaluate the delivery efficacy of CBA-PSGT/HGF complexes to the kidneys of UUO model rats. (B) The mRNA levels of semi-quantitative RT-PCR analysis. (n=3; mean±SD; $*P<0.05$ and $**P<0.01$ compared to sham; $^{\$\$\$}P<0.001$ compared to control, $^{\#\#\#}P<0.001$ compared to naked HGF; $^{\&\&\&}P<0.001$ compared to PSGT/HGF).(Continued next page)



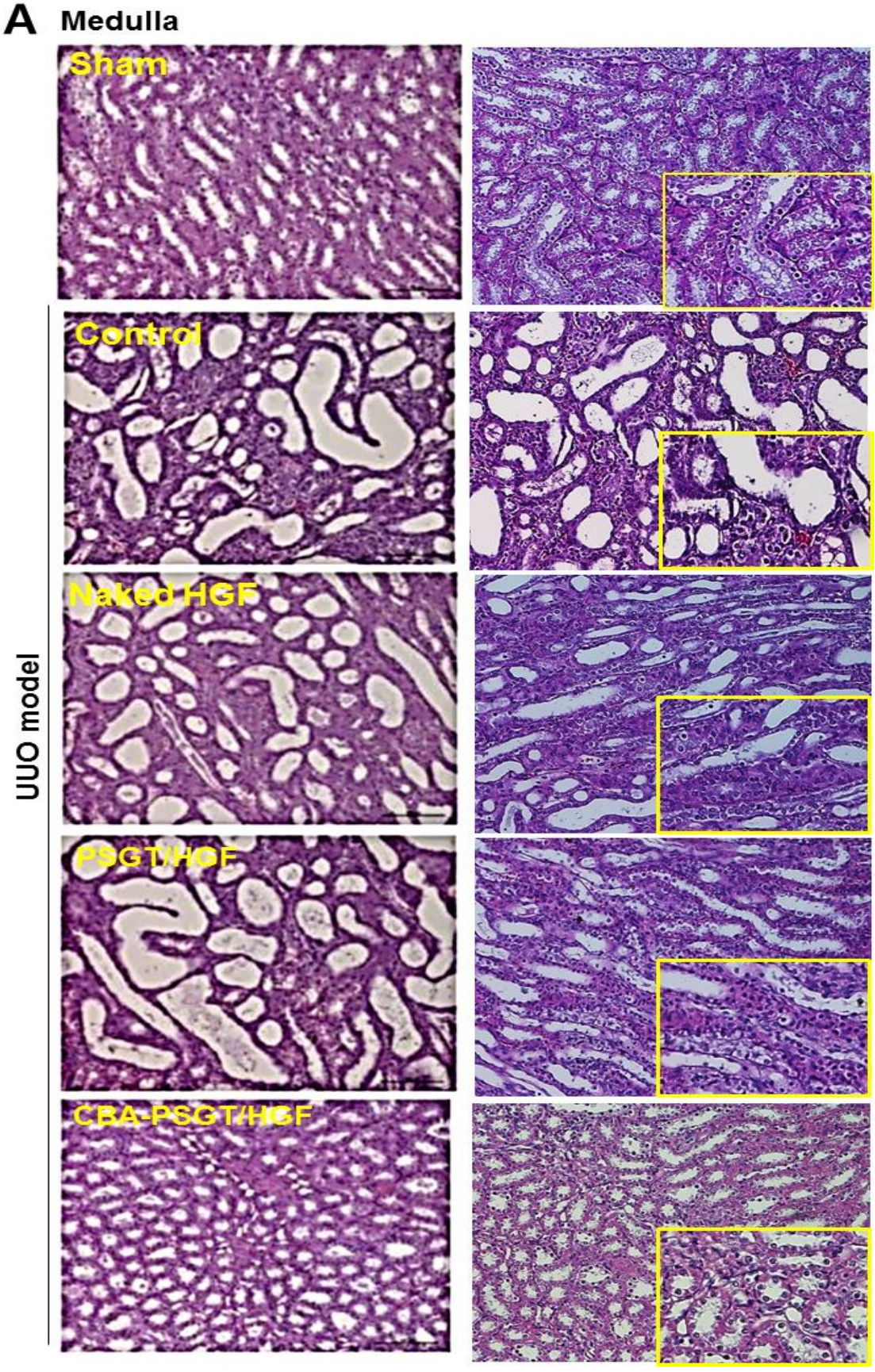

(Continued) **Figure 11. The efficiency of CBA-PSGT/HGF complexes to target kidneys was demonstrated by specific HGF expression in kidney tissues.** The expression of HGF in UUO model rats were examined and confirmed by (C) Western blot assay. GAPDH represents a house-keeping control gene expression. (n=3; mean±SD; $^{**}P<0.01$ compared to sham; $^{\$}P<0.05$ compared to control, $^{\#\#\#}P<0.001$ compared to naked HGF; $^{\&\&\&}P<0.001$ compared to PSGT/HGF).

## CBA-PSGT/HGF complexes remarkably reduced renal pathogenesis

To check the prevent effects of CBA-PSGT/HGF complex on the progression of renal morphologic lesions and interstitial fibrosis, the UUO kidneys were examined histopathological analysis. The untreated, naked HGF, and PSGT/HGF- delivered UUO kidneys were used as controls. **Figure 12** showed that the tubules in both medulla (**Fig. 12A**) and cortex (**Fig. 12B**) region. The UUO model groups including control, naked HGF, and PSGT/HGF developed severe renal lesions at 14 days after UUO, which observed numerous urinary casts. In UUO model groups, pathological changes in the renal tissue were observed renal tubular epithelium damage as well as a urinary cast in medulla region and glomerular enlargement, increased mesangial matrix, and nodular glomerulosclerosis in cortex region (Wu *et al.*, 2010). On the other hand, CBA-PSGT/HGF reversed these morphologic injuries including tubular damage (in both cortex and medulla region) and also showed minimal urinary casts similar to the sham control. In particular, UUO models groups including control, naked HGF, and PSGT/HGF were obviously observed interstitial fibrosis with interstitial accumulation of mononuclear leukocytes. In addition, histological evaluation showed that the development of nodular glomerulosclerosis in cortical area.

The results suggest that CBA-PSGT ameliorated the tubular damage by targeted delivery of HGF to UUO kidney region. In addition, it also identified by a quantitative scoring of tubular injury in H&E staining (**Fig. 12C**). The index score of tubular damage on CBA-PSGT/HGF group was

significantly reduced similar for sham control group. By contrast, there were significantly effects on the induction of the tubular damage score by UUO groups including control, naked HGF, and PSGT/HGF. Although the UUO model slightly improved the morphologic injury in both cortex and medulla region, CBA-PSGT/HGF resulted in a remarkable inhibition of morphologic damages seen in this model.


A
Medulla
Sham
Control
Naked HGF
PSGT/HGF
CBA-PSGT/HGF
UUO model

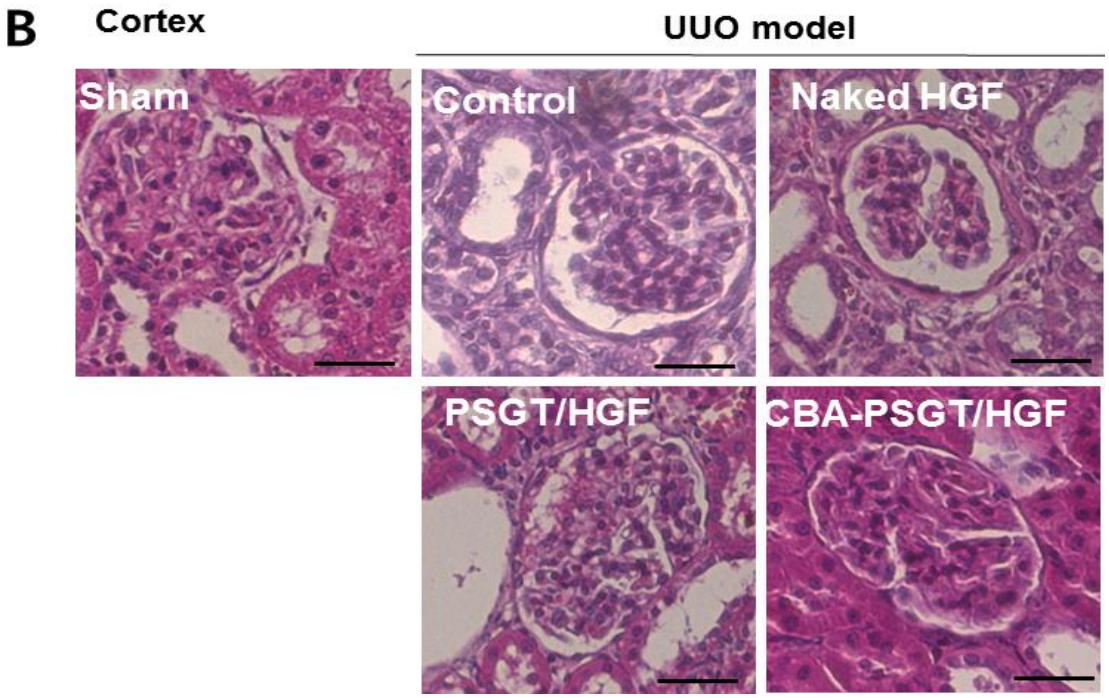
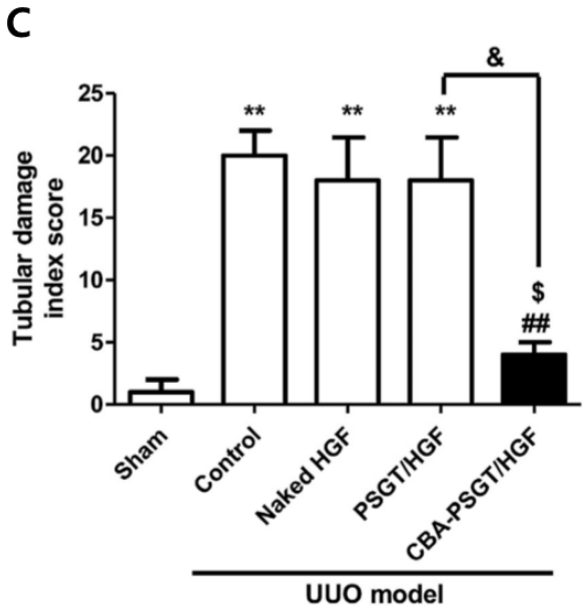



Figure 12. The effect of CBA-PSGT/HGF complexes on pathological change in kidney tissues (both medulla and cortex) of UUO model (A) A medulla and (B)cortex renal morphology in UUO model through H&E staining (magnification, 20X). (Continued next-page)




(Continued) **Figure 12. The effect of CBA-PSGT/HGF complexes on urinary casts formation and collagen accumulation in kidney tissues of UUO model** (C) Scale of renal tubular injuries. The significance was analyzed using paired *t*-test (n=3; mean±SD; $^{**}P<$ 0.01 compared to Sham, $^{\#\#}P<$ 0.01 compared to control, $^{\$}P<$ 0.01 compared to Naked HGF, $^{\&}P<0.05$ compared to PSGT/HGF).

## CBA-PSGT/HGF complexes reduce renal injury and inflammatory response in UUO rat model

To evaluation of renal injury, the renal injury was evaluated by measuring the level of blood urea nitrogen (BUN) and creatinine in serum of UUO rat model using enzymatic method after treatment with the targeted CBA-PSGT/HGF system. The BUN in serum significantly increased in UUO models including control, naked HGF, and PSGT/HGF. The delivery with CBA-PSGT/HGF complexes provided a greater reduction of BUN than other UUO models, but similar sham control group. Likewise, CBA-PSGT/HGF complexes was significantly affected by reduction of creatinine levels in serum (**Fig. 13A** and **13B**). The inflammatory response was further examined by analyzing the expression pro-inflammatory cytokiens such as IL-6 and IL-1$\beta$, which could be secreted by immune response and injured tubule cells (Cui *et al.*, 2013) were significantly down-regulated in CBA-PSGT/HGF complexes similar for sham (**Fig. 14A** and **14B**). These results suggest that the delivery with CBA-PSGT/HGF complexes may lead to alleviate renal injury and decrease inflammation levels compared to other UUO models.

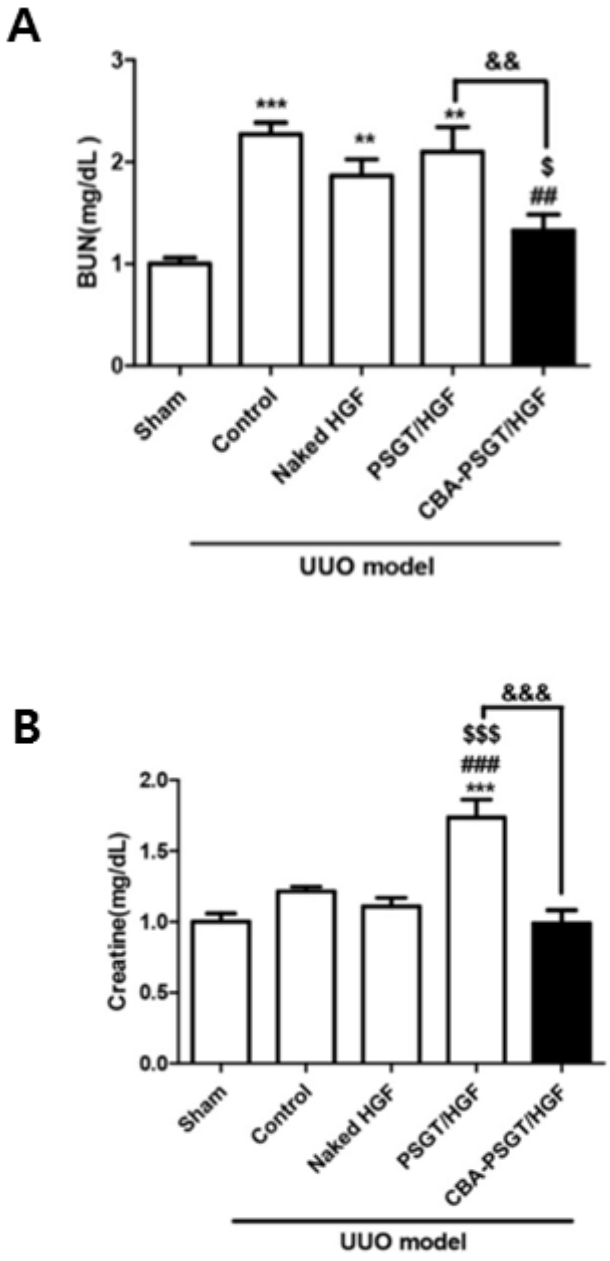



**Figure 13. The effects of CBA-PSGT/HGF complexes on renal injury** on (A) blood urea nitrogen (BUN) and (B) creatinine levels. The significance was analyzed using One-Way ANOVA (n=3; mean±SD; $^{**}P<0.01$ and $^{***}P<0.01$ compared to sham; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to control group; $^{\$}P<0.05$ and $^{\$\$\$}P<0.001$ compared to Naked HGF; $^{\&\&}P<0.01$ and $^{\&\&\&}P<0.001$ compared to PSGT/HGF).

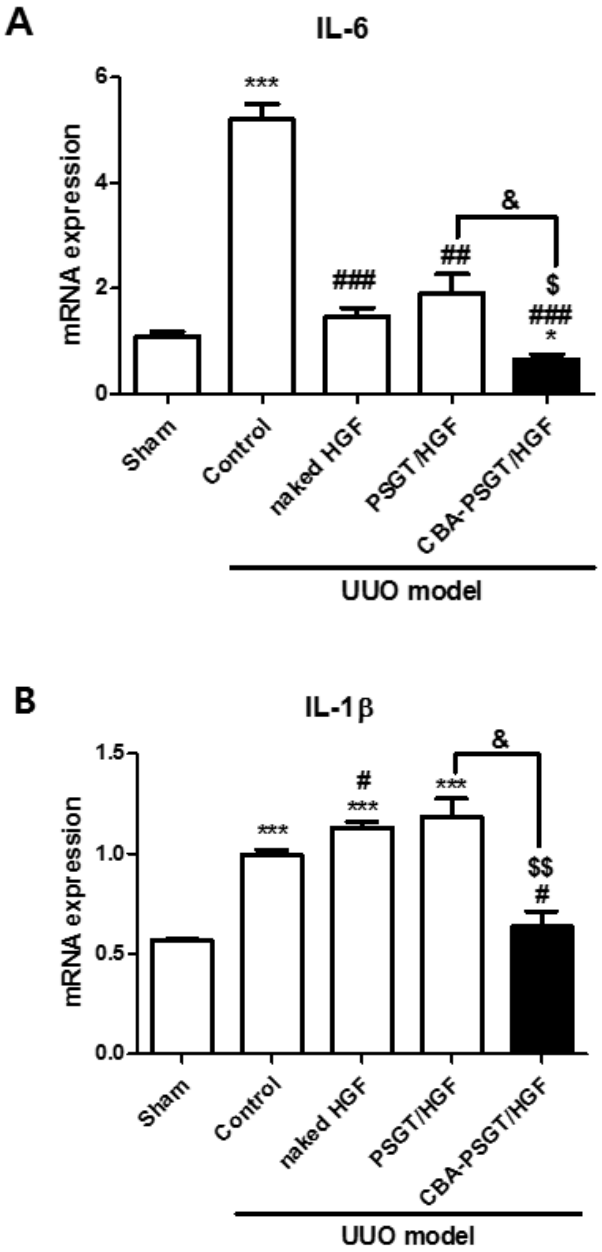



**Figure 14. The effects of CBA-PSGT/HGF complexes on renal inflammation with UUO model** on (A) IL-6 and (B) IL-1$\beta$ The significance was analyzed using One-Way ANOVA (n=3; mean±SD; $^{*}P<0.05$ and $^{***}P<0.01$ compared to sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to control group; $^{\$}P<0.05$ and $^{\$\$}P<0.01$ compared to Naked HGF; $^{\&}P<0.05$ compared to PSGT/HGF).

## CBA-PSGT/HGF complexes remarkably reduced accumulation and deposition of collagens

Collagens are the main components of the extracellular matrix (ECM) and strongly associated with the progressive renal fibrosis and injury (Chevalier *et al.*, 2009), since the processing of the pathogenesis of renal fibrosis starts by excessive accumulation and deposition of ECM. The accumulation and deposition of collagen I and II was examined in UUO model by qRT-PCR. **Figure. 15A** and **15B** showed collagen type I and II mRNA expression in renal tissues, respectively and it was found that the high affinity targeting of HGF by CBA-PSGT eliminated collagen accumulation specifically and remarkably compared to that of all other groups. UUO significantly increased kidney collagen contents, the induction of collagen deposition was observed in UUO control, naked HGF and PSGT/HGF unlike the targeted CBA-PSGT/HGF group which is similar to sham control. CBA-PSGT/HGF inhibited collagen I and II expression, resulted in marked suppression in the kidney. Furthermore, to evaluate collagen deposition in renal tissues, the Sirius red staining was performed in the renal of UUO model indicating by red color. Compared with CBA-PSGT/HGF, the UUO model groups including control, naked HGF, and PSGT/HGF showed much more collagen deposition and more prominent fibrosis (**Fig 16A**) which was nearly similar to the healthy sham control mice and also determined the levels of total renal collagen sections were quantified by image analysis software using Image J as shown in **Figure. 16B**.

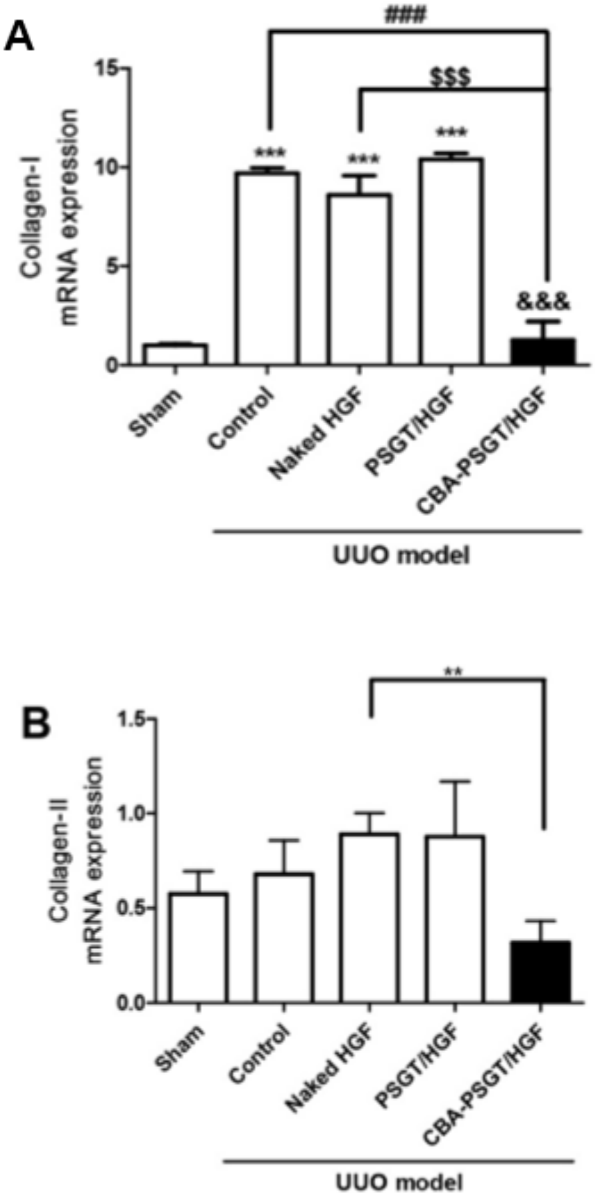
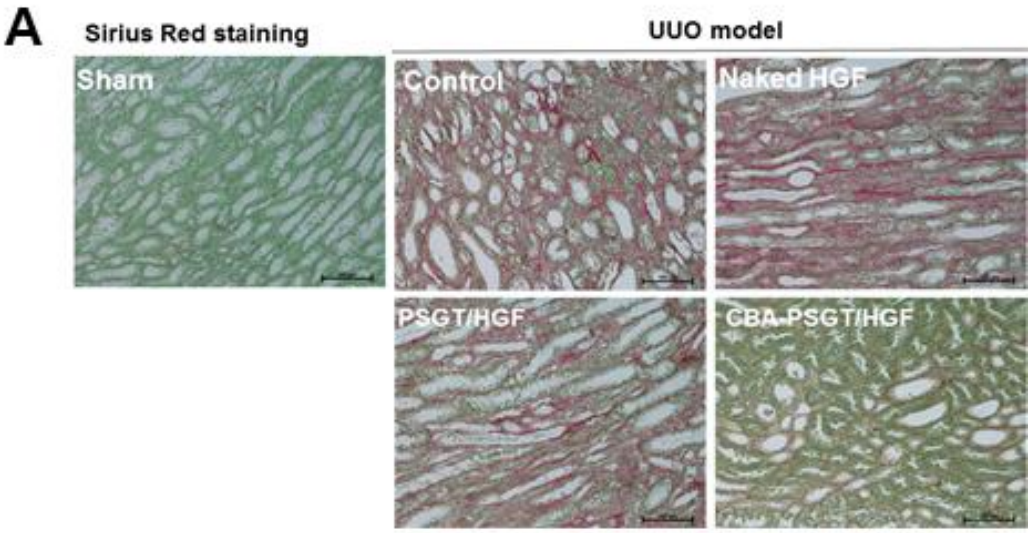



**Figure 15. The effect of CBA-PSGT/HGF in collagen expression in kidneys of UUO model** The effect of CBA-PSGT/HGF complexes on (A) Type I and (B) II collagen mRNA levels. The significance was analyzed using *t*-test (n=3; mean±SD; $^{**}P<0.01$ and $^{***}P<0.01$ compared to sham; $^{\#\#\#}P<0.001$ compared to control group; $^{\$\$\$}P<0.001$ compared to Naked HGF; $^{\&\&\&}P<0.001$ compared to PSGT/HGF).



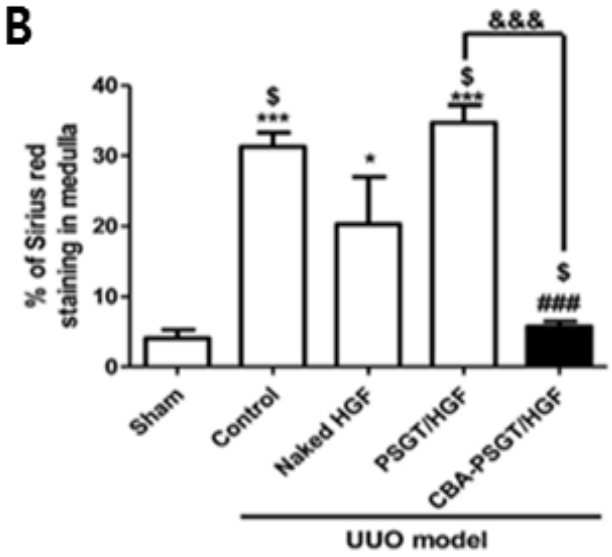




**Figure 16. The effect of CBA-PSGT/HGF complexes on collagen accumulation in kidney tissues of UUO model** (A) Collagen accumulation was confirmed by Sirius red staining (magnification, 20X), and (B) quantification of the percentage of collagen accumulation with Sirius red staining in medulla by densitometer analysis. The significance was analyzed using paired *t*-test (n=3; mean±SD; $^{*}P<$ 0.05 and $^{***}P<$ 0.001 compared to Sham, $^{\#\#\#}P<$ 0.001 compared to control, $^{\$}P<$ 0.01 compared to Naked HGF, and $^{\&\&\&}P<$0.001 compared to PSGT/HGF).

## CBA-PSGT/HGF complexes remarkably reduced collagen-related renal fibrotic factors in UUO model

To checked expression of collagen-related proteins, the levels of ICAM-1 and TIMP-1, collagen-related proteins were confirmed in renal tissues of UUO model treated by the experimental groups using immunoblotting assay as shown in **Figure 17A** and **17B**, respectively. The results showed that both ICAM-1 and TIMP-1 expression were markedly down-regulated by HGF delivery using CBA-PSGT, but suppressed in UUO control, naked HGF and PSGT/HGF, respectively. The ICMA-1 expression as also critical factor involved in the recruitment of leukocyte to injured renal tissues. As shown in **Figure 12** and **14**, the inflammatory response was enhanced on renal injury tissue in UUO model including control, naked HGF, and PSGT/HGF. In addition, although PSGT/HGF had an effect to slightly reduce TIMP-1 level, TIMP-1 expression level increased in UUO model with control, naked HGF and PSGT/HGF, which was not superior compared to that of CBA-PSGT/HGF. These results suggests that CBA-PSGT has a high targeting efficacy to delivered HGF to renal tissues and down-regulates the fibrotic related factors.

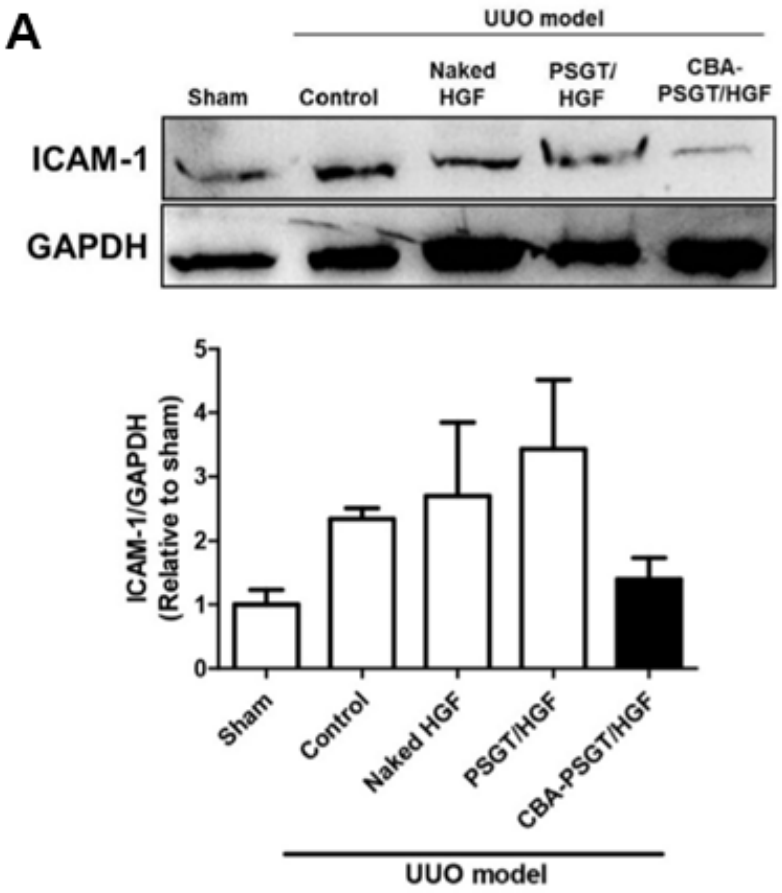
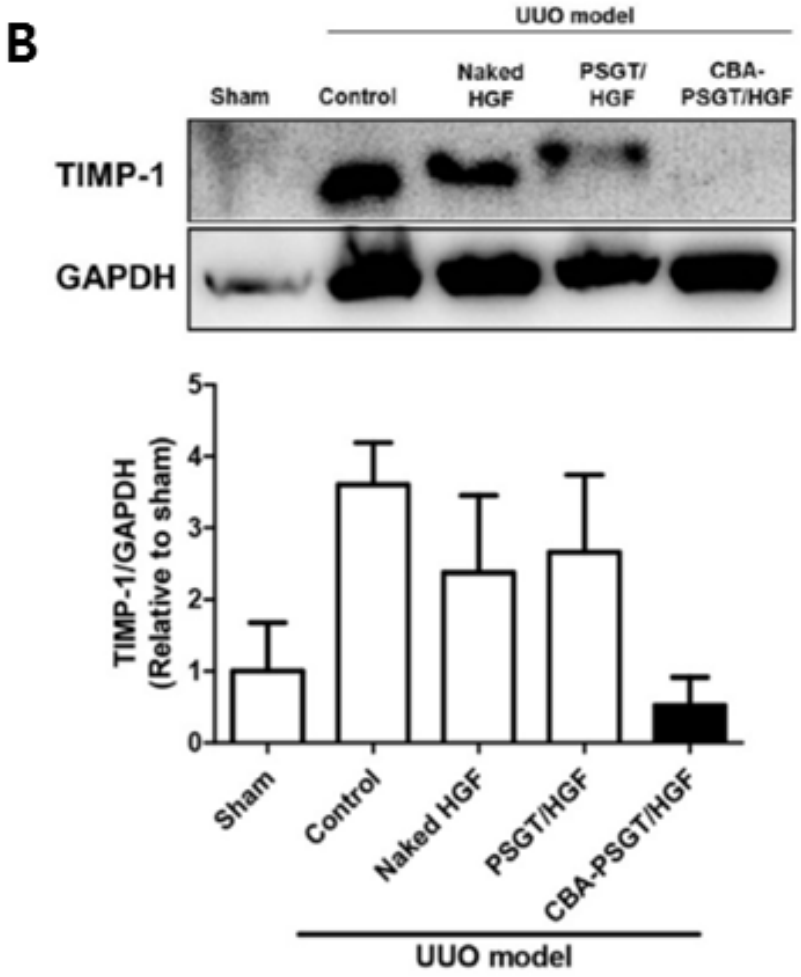



**Figure 17. The effect of CBA-PSGT/HGF in collagen-related protein expression in kidneys of UUO model** The expression of collagen-related proteins; (A) ICAM-1 (Continued next page)




(Continued) **Figure 17. The effect of CBA-PSGT/HGF in collagen-related protein expression in kidneys of UUO model rats.** The expression of collagen-related proteins; (B) TIMP-1 in kidney tissues of UUO models and sham rats. The significance was analyzed using *t*-test (n=3; mean±SD; $^{**}P<0.01$ and $^{***}P<0.01$ compared to sham; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to control group; $^{\$}P<0.05$ and $^{\$\$\$}P<0.001$ compared to Naked HGF; $^{\&\&}P<0.01$ and $^{\&\&\&}P<0.001$ compared to PSGT/HGF).

## CBA-PSGT/HGF complexes significantly down-regulate α-SMA expression in UUO model

**Figure 18**, demonstrated that the effects of CBA-PSGT/HGF in obstructed kidneys among five different treatment groups by immunohistochemistry (IHC), immunofluorescence (IF) assays, mRNA level, and protein expression level of α-SMA. α-SMA is well known as a key protein that causes progression of renal fibrosis (Zhou *et al.*, 2013). The CBA-PSGT/HGF complexes remarkably reduced α-SMA expression and retained a similar level to sham control compared to that of UUO control, neked HGF and non-targeted PSGT/HGF system by IHC and IF assays (**Fig. 18A** and **B**, respectively). In addition, the mRNA and protein expression of α-SMA were also significantly inhibited by CBA-PSGT/HGF compared to that of all other groups (**Fig. 18C** and **18D**), futher supports the results of IHC and IF assays. These results strongly demonstrate that CBA-PSGT/HGF delivery system could provide a potential and effective therapy for renal dysfunction *in vivo.* The overall mechanism of kidney-targeted HGF-delivered CBA-PSGT system to prevent renal fibrosis is schematically represented in **Figure 19**.

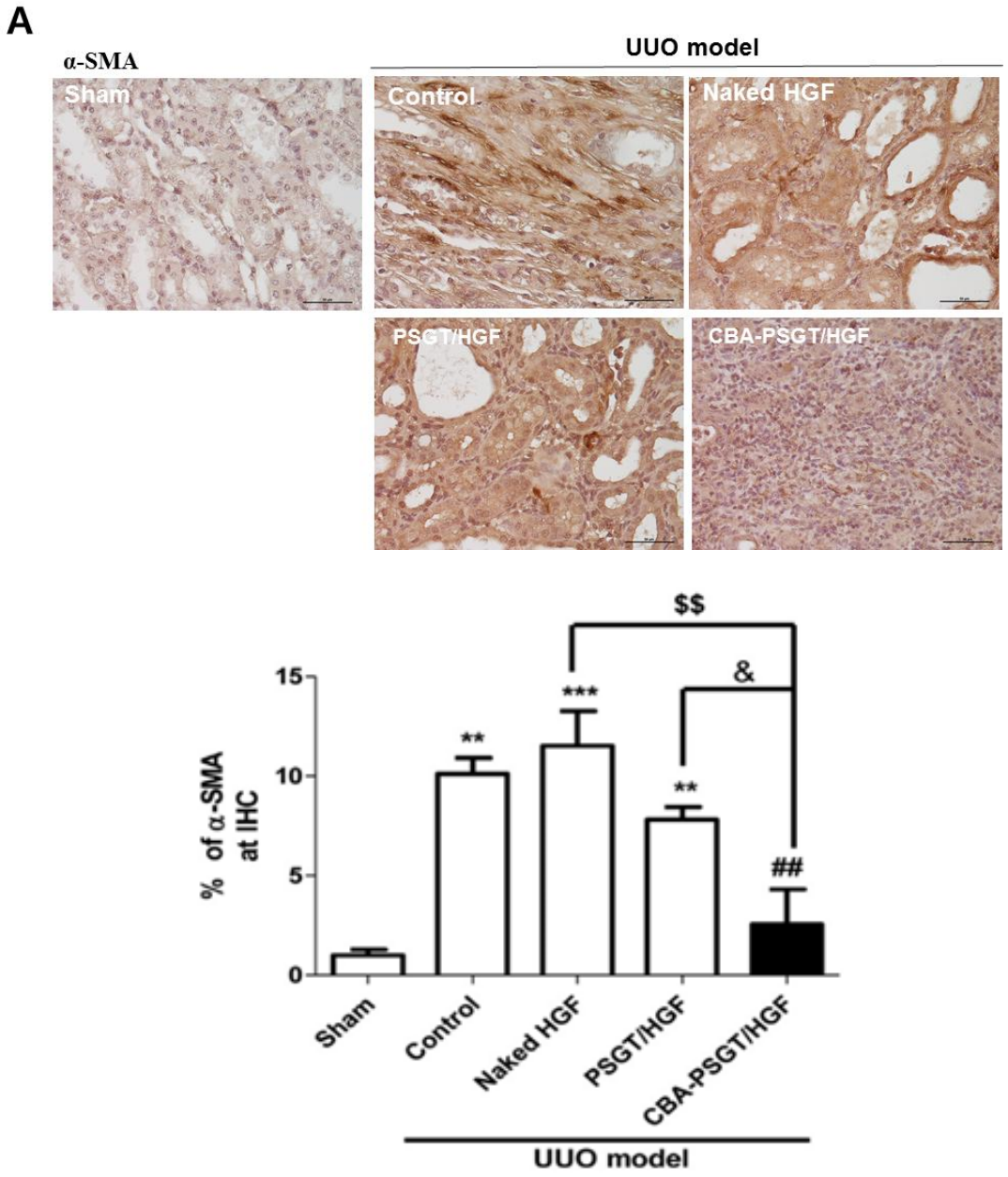
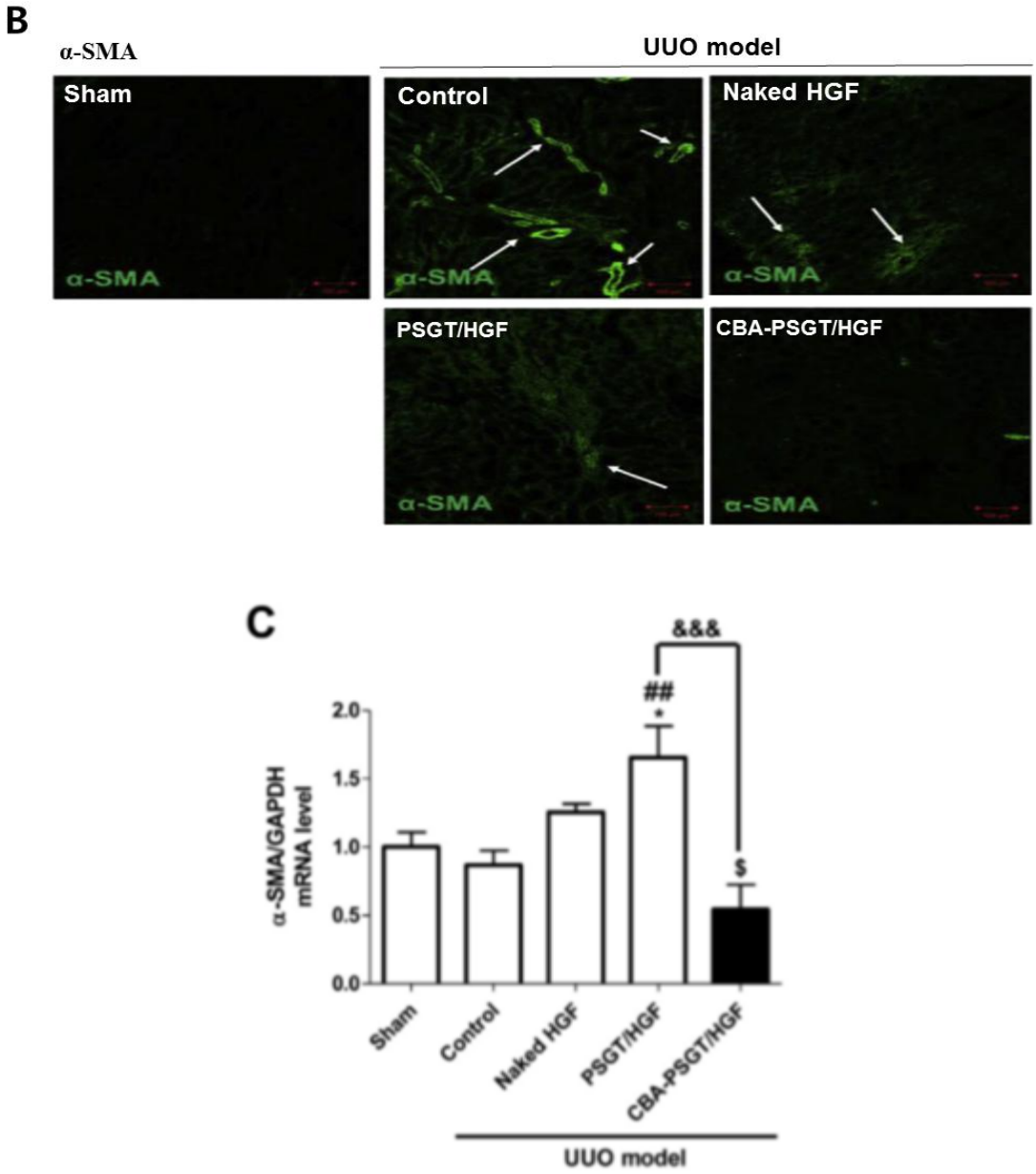
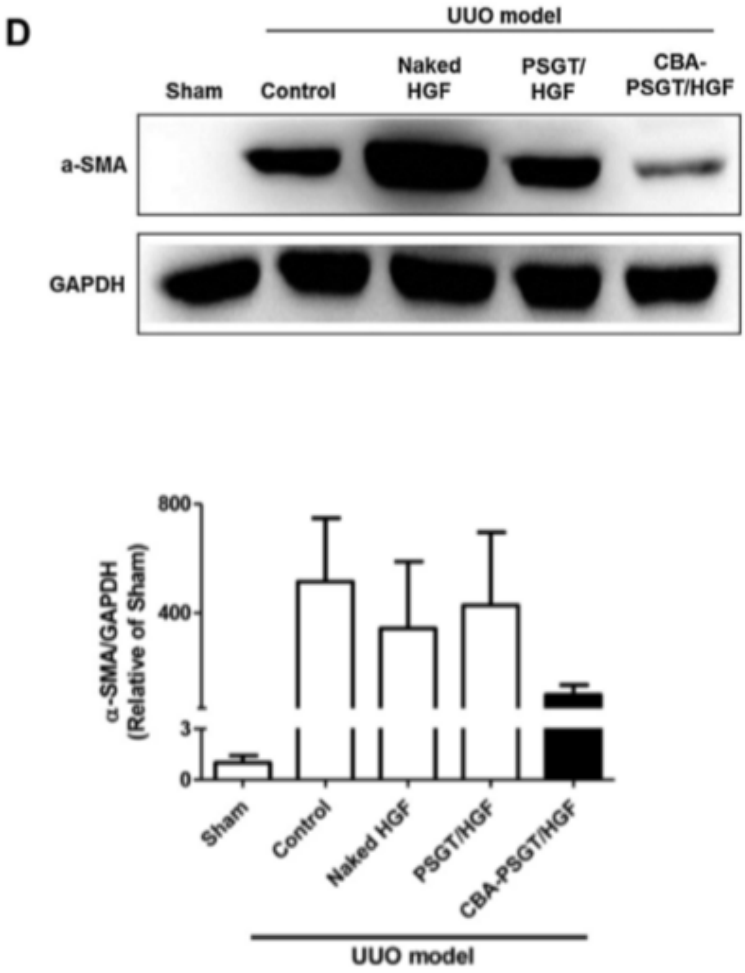



Figure 18. Delivery of HGF by CBA-PSGT/HGF complexes suppresses $\alpha$-SMA expression in UUO model (A) Immunohistochemistry of $\alpha$-SMA expression in kidney tissues of CBA-PSGT/HGF complexes delivered rats (magnification, 20X). and Quantification of the percentage of $\alpha$-SMA expression with immunohistochemistry by densitometer analysis. (Continued next page)




(Continued) **Figure 18. Delivery of HGF by CBA-PSGT/HGF complexes suppresses α-SMA expression in UUO model** (B) immunofluorescence of α-SMA expression (C) Quantification of α-SMA mRNA levels in kidney tissues of CBA-PSGT/HGF complexes delivered animal model (Continued next page)




(Continued) **Figure 18. Delivery of HGF by CBA-PSGT/HGF complexes suppresses α-SMA expression in UUO model** (D) Western blot of α-SMA expression (GAPDH represents a house-keeping control gene expression). The significance was analyzed using One-Way ANOVA (n=3; mean±SD; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to sham; $^{\#\#}P<0.01$ compared to control; $^{\$}P<0.05$ and $^{\$\$}P<0.01$ compared to naked HGF; $^{\&}P<0.05$ and $^{\&\&\&}P<0.001$ compared to PSGT/HGF).

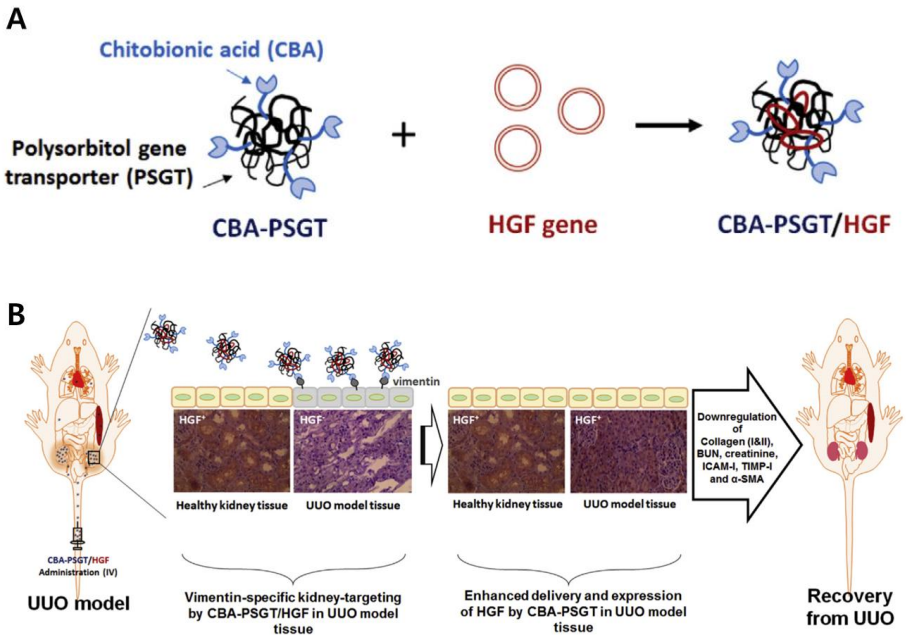



**Figure 19. A schematic representation on the overall mechanism of kidney-targeted HGF-delivered CBA-PSGT system to prevent renal fibrosis in UUO model** (A) The schematic illustration on the successful synthesis of CBA-PSGT and stable complexation of CBA-PSGT with HGF gene. (B) The mechanism of CBA-PSGT/HGF to deliver HGF and prevent renal fibrosis in UUO model rats.

# DISCUSSION

In light of targeted gene therapy to alleviate kidney dysfunction, CBA-PSGT/HGF complexes exhibited remarkable efficacy. Renal fibrosis is considered as the final common progressive renal diseases, and involves glomerular sclerosis or interstitial fibrosis. Studies have been conducted to develop kidney target gene transporter to treat CKD. This study clearly demonstrate that delivery of HGF gene with CBA-PSGT protects renal fibrosis, following to (1) mechanism of kidney specific targeting gene transporter, (2) UUO model with features of renal fibrosis, and (3) mechanism of protect of renal fibrosis.

(1) *Mechanism of kidney specific targeting gene transporter* : Gene transporters should be suited precisely on stability of DNA condensation, protection from degradation enzyme, low toxicity, and high efficiency with gene targeting to targeted region. CBA-PSGT was demonstrated excellent DNA condensation and protection from enzyme-mediated degradation together with suitable physicochemical properties (**Fig. 4 and 5**) and minimal toxicity which favored them to apply as a carrier for HGF to specifically target and treat kidney dysfunction (Nakamura *et al.*, 2010). Moreover, CBA-PSGT showed the highest kidney specific-delivery efficiency at both *in vitro* and *in vivo*.

Previous study demonstrated that vimentin and desmin show GlcNAc-binding lectin-like activity on the surface of cells and possess strong avidity to GlcNAc ligands (Kobayashi *et al.*, 2009; Ise *et al.*, 2010). GlcNAc-conjugated agent can be targeted to vimentin- and desmin-expressing cells and tissues (Kobayashi *et al.*, 2010). Vimentin is a type III intermediate filament protein found in cells of mesenchymal origin, vascular endothelial cells, vascular smooth muscle cells, and fibroblasts (Herrmann *et al.*, 2000). In addition, Singh *et al.* was demonstrated that vimentin are recently known to bind N-acetylglucosamine (GlcNAc) residue, therefore, the cell surfaces of vimentin-expression cells could be targeted by using the GlcNAc residue as a specific ligand for receptor-mediated gene delivery (Singh *et al.*, 2014). Vimentin is expressed on the surface of kidney cells or tisssue, in particular, glomerular or renal interstitial tissues in normal and/or abnormal kidney tissues (Wu *et al.*, 2010). Targeting vimentin receptors can be a successful strategy for efficient and specific delivery of genes to the kidney because vimentin, which is a type of intermediated filament protein, is expressed in developing and adult human kidney (Holthfer *et al.*, 1984). Several reports have also described the distribution of vimentin in both normal and carcinoma kidney tissues (Skinnider *et al.*, 2005; Gonlusen *et al.*, 2001; Beham *et al.*, 1992). Moreover, Scanziani *et al.* reported that vimentin not only expresses in human kidneys (normal or damaged) tissues, but also expresses in bovine kidneys with nephritis at interstitial regions (Scanziani *et al.*, 1993). In normal kidneys, vimentin can be found

in arterioles, interstitial fibroblasts, glomeruli, but not in tubules. Interestingly, however, vimentin expression is abundant after tubular injury (atrophy, inflammation and/or fibrosis) in experimental models such as urethral obstruction (Klahr *et al.*, 2001), kidney aging (Nakatsuji *et al.*, 1998), ischemic injury (Villanueva *et al.*, 2006; Matos *et al.*, 2007), proteinuria (Kikuchi *et al.*, 2000), nephropathy by adriamycin (Shu *et al.*, 2002), suggesting that overexpression of vimentin can be considered as a marker for tubular injury (Vansthertem *et al.*, 2010; Stefanovic *et al.*, 1996). Moreover, it has been demonstrated that, after kidney transplantation, vimentin expression can be assessed as an indicator of renal dysfunction (Matos *et al.*, 2007). These evidences strongly suggest that vimentin can be a potent receptor used to target the kidneys, especially the damaged or obstructive kidney tissues. In addition, vimentin can be effectively targeted with high affinity kidney targeting by chitobionic acid are prepared. Therefore, it should be possible to use surface-expressed vimentin in cells or tissues as targeting sites for gene delivery (Kim *et al.*, 2012). Kim *et al.* identified that vimentin can be effectively targeted with high affinity by chitobionic acid (CBA), prepared by dimer of *N*-acetylglucosamine (GlcNAc) as a specific ligand to the vimentin-expressing cells, when conjugated with cationic polymers, because we found that the CBA-conjugated polymer showed higher transfection efficacy in vimentin expressing cells (the human embryonic kidney 293T cells and epithelial HeLa cells) compared to their un-conjugated counterparts (Kim *et al.*, 2011; Singh *et al.*, 2014).

Mechanistically we also showed the proof-of-concept that these CBA-conjugated polymers had no effect when the surface-vimentin was knocked-down by RNA interference (Kim *et al.*, 2011). This study determined that gene transporter as a kidney targeting ligand was successfully synthesized, CBA-PSGT (**Fig. 1 and 2**). The evidence that CBA-PSGT was obtained CBA prepared by dimer of N-acetylglucosamine(GlcNAc) and conjugated with the novel gene carrier PSGT was investigated.

(2) *UUO model with features of renal fibrosis* : The model of UUO induces progressive renal fibrosis that is independent of hypertension and immune diseases. In addition, UUO model also present several typical features of obstructive nephropathy such as tubular injury, interstitial macrophages infiltration, and proliferation of renal interstitial fibroblast and their transformation into myofibroblasts (Fu *et al.*, 2006). To determine an effective delivery of HGF gene with CBA-PSGT specifically to kidney showed incredible improvement of UUO symptoms in model rats, UUO models were prepared, and confirmed recurrent the healthy condition of kidney tissues (**Fig. 10**). **Figure 12.** was showed severe tubular dilatation and atrophy, and widened interstitial space with a greater number of interstitial cells, and infiltrating leukocytes in obstructed kidney with the exception of CBA-PSGT/HGF complexes group.

(3) *Mechanism of protect of renal fibrosis* : To repair and regenerate tubular function after acute renal disease, HGF is a key protein (Gao *et al.*, 2002; Yang *et al.*, 2002A; Gong *et al.*, 2003; Staunton *et al.*, 1988) which is not only considered as an endogenous anti-fibrotic factor, but also as therapeutic element that blocks renal tissue fibrosis and repair dysfunction (Zuo *et al.*, 2009). In addition, HGF prevents accumulation of collagen in renal fibrosis (Zuo *et al.*, 2009; Kobayashi *et al.*, 2009). Some studies were demonstrated HGF gene or its protein prevents the development and progression of renal lesions and dysfuction (Cui *et al.*, 2013; Yang *et al.*, 2002B). Many factors are known to regulate the fibrogenic process after tissue injury, in which transforming growth factor (TGF)-$\beta$ is believed to the principal one that plays a central role. HGF is an intrinsic antifibrotic factor that directly antagonizes the profibrotic actions of TGF-$\beta$ (Liu *et al.* 2004). The data showed that CBA-PSGT delivered HGF gene reduced renal injury, decreased the levels of BUN, creatinine in serum, and significantly lowered the levels of collagen and markedly down-regulated collagen-related proteins such as ICAM-1, TIMP-1, and $\alpha$-SMA levels which are primarily responsible for obstructive kidney function. It was previously reported that the level of BUN was significantly increased with longer duration of UUO (Tapmeier *et al.*, 2008). In another study, June et al. described that both BUN and creatinine were significantly elevated in UUO rat model compared to those control aniamls (Li *et al.*, 2015), suggesting that BUN and creatinine are also good indicators to evaluate renal injury in blood serum of UUO rat

model. Proinflammatory cytokines such as IL-6 and IL-1$\beta$ (**Fig. 14**) could be secreted by inflammatory response and injured tubule cells. ICAM-1 (**Fig. 17A**) is also a critical factor involved recruitment of leukocytes to injured tissues. Ureteral obstruction was observed inflammatory cells in many forms of chronic kidney diseases, and persistent inflammatory response. It contribute to the development of tubulointerstitial fibrosis with function impairment. HGF induces the degradation of ECM as well as blocks the synthesis of ECM by activating of protease such as matrix metalloproteinase (MMPs) (Ojeda *et al.*, 2011; Gong *et al.*, 2003). In addition, HGF also influences the balance of MMPs secretion and their activators. To maintain ECM deposition, the TIMP-1 play a key role which inhibits the activation of MMPs (Fu *et al.*, 2006; Staunton *et al.*, 1988; Mizno *et al.*, 2000). Therefore, ECM degradation is catalyzed by MMPs, which is prevented by the expression of TIMPs (Zhang *et al.*, 2006; Sharma *et al.*, 1995). In addition, ICAM-1, which is regulated by MMPs in renal injury, also upregulates in several renal diseases and relates to the progression of renal lesion (Staunton *et al.*, 1988; Zhang *et al.*, 2006). In my data, HGF delivered with CBA-PSGT group express less ICAM-1 and TIMP-1 than other UUO groups including control, naked HGF, and PSGT/HGF. Because imbalance of MMPs and TIMP-1 and ICAM-1, as several inflammatory mediators, is substractes of MMPs, resulting to TIMP-1 affect ICAM-1 through MMPs and subsequently promote renal fibrosis (Zhang *et al.*, 2006). TIMP-1 could enhance renal fibrosis, which was contribute to promoting inflammation

though upregulation of ICAM-1. α-SMA was the major source of excessive ECM deposition and the prognostic indicator of renal disease (Zuo *et al.*, 2009), manifesting a marked activation of the metrix-producing myofibroblast cell (Yang *et al.* 2002B).

Taken together, these results demonstrated that CBA-PSGT/HGF complexes were highly effective to deliver HGF specifically to the kidney tissues which showed enhanced recovery of the renal dysfunction, impressively by negative regulation of important fibrotic factors such as collagen accumulation, ICAM-1, TIMP-1, α-SMA, BUN, and creatinine level. In conclusion, it is suggested that CBA-PSGT, might be a novel and effective therapeutic to kidney diseases as well as fibrosis.

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# LIST OF ABBREVIATION

| | |
|---|---|
| AAV | Adeno-associated virus |
| AKI | Acute kidney injury |
| α-SMA | alpha-Smooth muscle actin |
| BMP7 | Bone morphogentic protein |
| BUN | Blood urea nitrogen |
| CBA | Chitobionic acid |
| CKD | Chronic kidney disease |
| DN | Diabetes nephropathy |
| ECM | Extracellular matrix |
| GFP | Green fluorescence protein |
| GlcNAc | *N*-acetylglucosamine |
| GPC | Gel permeation chromatography |
| H&E | Hematoxylin and eosin |
| HGF | Hepatocyte growth factor |
| HIF | Hypoxia-inducible factor |
| HSV | Herpes simplex virus |
| HVJ | Hemagglutinating virus of Japan |
| ICAM-1 | Intercellular Adhesion Molecule 1 |

| | |
|---|---|
| IL-6 | Interleukin 6 |
| I.M. | Intramuscular |
| I.V. | Intravenous |
| MMP | Matrix metalloproteinase |
| NMR | Nuclear magnetic resonance |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PEG | Poyethylene glycol |
| PEI | Polyethylenimine |
| PLL | Poly-L-lysine |
| PSGT | Polysorbitol gene transporter |
| SD | Sprague Dawley |
| STZ | Streptozotocin |
| TEM | Transmission electron microscopic |
| TGF-$\beta$ | Transforming growth factor-$\beta$ |
| TIMP-1 | Tissue inhibitor of metalloproteinases-1 |
| TNF-$\alpha$ | Tumor necrosis factor-$\alpha$ |
| UUO | Unilateral urethral obstruction |
| VEGF | Vascular endothelial growth factor |

# 국문요약문

종양생물학 협동과정 전공
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만성신장질환(Chronic kidney disease)은 다양한 원인에 의해서 신장의 구조 또는 기능적 손상이 일어나 신원(nephron)의 감소와 신기능의 소실이 비가역적으로 발생하는 신장질환을 말한다. 만성신장질환은 전 세계적으로 다양한 발병 원인으로 인한 치료법의 부재에 따른 발병률과 사망률이 증가하는 추세이다. 그 중 신장 섬유증은 만성적이고 점진적인 신장장애의 원인으로 여러 질병으로 존재한다. 급성 신장 손상으로 인한 비정상적이고 불완전한 복구는 조직의 재생단계를 포함하고, 이 단계에서 손상된 세포들은 동일한 계열의 세포들에 의해 교체된다. 섬유증성 단계는 결합 조직이 정상적인 실질조직을 교체하고, 복구는 손상된 부위가 지속되지 않거나 섬유성 병소가 점진적인 기관장애로 발전한다. 이로써, 신장 섬유증은 만성적 진행성 신장장애의 원인이다. 이러한 질병을 치료하기 위해 효율적이고, 정확한 신장질환 치료 방법의 개발을 위해, 많은 시도들이 행해져 왔다. 그 중, 유전자 전달체를 이용한 유전자 치료방법은 높은 전달효율과 낮은 독성의 이점을 지녀 다양한 질환 치료에 적용할 수 있다. 또한, 비-바이러스성 유전자 전달체는 쉽게 변형이 가능하고, 생체 적합한 성질을 지녀 유전자 전달체 치료방법으로 각광받고 있다.

이번 연구에서는, 항섬유증 인자인 HGF(hepatocyte growth factor)를 도입한 신장 특이적 키토산접합 폴리소르비톨계 (CBA-PSGT) 유전자 전달체를 이용하여 신장 섬유증 진행의 방어효과를 확인하였다. 먼저, 신장 특이적 리간드로서 키토산(Chitonic acid)를 폴리소르비톨계 (PSGT) 전달체에 접합하여 합성하였다. 키토산은 비멘틴(Vimentin)을 표적화하여 신장으로 효과적으로 전달 할 수 있다. 더욱이 비멘틴은 정상 신장조직 및 비정상신장조직 뿐만 아니라, 신장의 간질부분에서 많이 발현된다. 특히나, 비멘틴은 신장위축, 염증, 섬유증을 포함한 신세뇨관의 장애에서 그 발현량이 과다하게 증가함에 따라 이 유전자는 신세뇨관 장애의 마커로서 고려될 수 있다. 그러므로 신장세포 또는 신장조직의 표면에 발현하는 비멘틴은 유전자 전달체의 표적으로서 사용된다. 신장 특이적 유전자 전달체(CBA-PSGT)를 합성하여, 유전자 전달체로서의 효율을 평가하였다. 유전자 전달체는 전달하고자하는 유전자와의 높은 결합유무, 낮은 독성, 높은 효율성으로 목표하고자 하는 표적에 정확히 작용해야한다. 위 조건에 충족하기 위해 먼저 합성한 CBA-PSGT와 DNA와의 결합성을 확인하였다. 각 N/P ratio에 따라 DNA와 CBA-PSGT의 결합력을 확인한 결과, N/P ratio 0.5 이하에서 가장 효과적으로 나타났다. CBA-PSGT와 콤플렉스를 이룬 DNA는 생체 내에서 많은 효소들의 공격으로부터 보호되어야 한다. 위 실험에서 CBA-PSGT/DNA 콤플렉스가 DNase로부터 효과적으로 보호됨을 확인하였다. CBA-PSGT의 세포 내 독성을 확인하기 위해 신장암세포(293T)와 폐암세포(A549)에서 세포독성실험을 진행하였다. 그 결과 두 세포에서 CBA-PSGT 전달 그룹에서 90-95%의 세포 생장률을 보여 생체 내에서 CBA-PSGT가 낮은 독성을 보임을 확인하였다. CBA-PSGT 유전자 전달체의 높은 신장 타겟 효율성을 확인하기 위해, 293T세포와 A549세포에서의 전달효율을 확인한 결과, 신장암세포에서 폐암세포에 비하여 CBA-PSGT 전달효율이 더 높은 것으로 확인되었다. 마찬가지로 동물 조직에서 각 장기별 CBA-PSGT 전

달 효율을 확인한 결과 폐, 심장, 간, 비장에 비해 CBA-PSGT 전달이 신장 특이적으로 나타남을 확인하였다. 따라서, 합성한 CBA-PSGT는 DNA와의 높은 결합력, 낮은독성 및 신장으로 높은 전달 효율을 가지는 유전자전달체이다.

두 번째로, 항섬유증 인자인 HGF가 도입된 CBA-PSGT 전달체를 이용한 신장 섬유증 방어효과를 평가하기 위한 동물모델로서 일축성 요관 폐색(unilaternal ureteral obstruction;UUO) 동물모델을 제작하였다. UUO 동물모델에서 HGF가 도입된 CBA-PSGT를 전달한 결과 CBA-PSGT/HGF 콤플렉스 그룹에서 유의적으로 HGF의 발현량이 증가함을 확인하였다. UUO 동물모델은 일반적으로 신장 조직 손상을 일으키는 질환으로서 주로 세뇨관의 간질조직의 위축과 간질의 섬유화를 초래한다. 또한, 이로 인한 세뇨관 간질조직 내 염증세포의 침투로 인하여 염증성 사이토카인이 증가하게 된다. 신장의 조직학적변화를 확인한 결과 항섬유증인자인 HGF가 도입된 CBA-PSGT 전달그룹에서 신장 조직손상이 덜 일어남을 확인하였다. 신장 조직의 손상은 지표 중 하나인 혈액 내 BUN과 크레아틴(Creatinine)의 농도와 염증성 사이토카인인 IL-6와 IL-1$\beta$의 발현량이 CBA-PSGT/HGF 콤플렉스 전달체 그룹에서 유의적으로 감소하였다. 또한, 신장의 섬유화가 진행되면 조직 내 콜라겐 형성이 증가하게 된다. 콜라겐 I과 II의 발현량 및 신장의 콜라겐축적의 조직학적 성상을 확인한 결과에서 CBA-PSGT/HGF 콤플렉스 전달체 그룹에서 콜라겐 형성 및 축적이 억제됨을 확인하였다. 마찬가지로, 신장 섬유화가 진행되면 콜라겐 형성 및 축적이 이루어지면서 관련 단백질의 발현량이 증가하게 된다. 본 실험에서 콜라겐 형성과 관련된 단백질인 ICAM-1 과 TIMP-1의 발현량을 확인하였다. ICAM-1과 TIMP-1은 각각 신장 질환 조직에서 높은 발현량을 보이고, 세포외 기질(Extracellular matrix;ECM) 형성을 막는 효소인 MMP (matrix metalloproteinase) 의 활성을 조절한다. CBA-PSGT/HGF 콤플렉스를 전달시킨 결과 콜라겐 형성 및 축적 관련 단백질인 ICAM-1과 TIMP-1의 발현량이 감소함

을 확인하였다. 더나아가, 섬유증 진행과 가장 밀접한 연관있는 단백질 중 하나인 $\alpha$-SMA의 발현량 역시 CBA-PSGT/HGF 콤플렉스 전달 그룹에서 억제됨을 확인하였다.

이 연구들의 결과를 요약하면, 신장 특이적 리간드로서 키토산을 접합시킨 유전자 전달체를 합성하는 데에 성공하였고, 이는 신장질환 및 조직손상에 많이 발현되는 비멘틴을 표적화하여 신장 특이적 유전자 전달체의 특징을 지니고 있다. 합성에 성공한 CBA-PSGT 전달체는 유전자 전달체로서 가지는 DNA 와의 결합력과 효소로부터의 방어력, 낮은 독성 및 특정 표적으로의 높은 전달 효율성을 모두 지니고 있다. 또한, 신장 섬유증을 완화시키는 항섬유증인자인 HGF를 도입하여 신장 섬유증을 유발시킨 동물모델에서 섬유증 진행이 방어됨을 보여주었다. 따라서 이러한 결과들은 CBA-PSGT가 신장질환 치료를 위한 유전자 전달체로서 높은 가치를 지니고 있다.

**주요어** : 키토산, HGF, 신장섬유증, 유전자치료

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