Viral vector mediated insulin gene expression in liver of diabetic rats

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Viral vector mediated insulin gene expression in liver of diabetic rats

Directed by Professor Hyun Chul Lee

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This certifies that the Doctoral Dissertation of

Seonock Woo is approved.

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Christine Seonock Woo

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ABSTRACT

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A lot of therapeutic approaches to cure diabetes have been tried but still remains unsatisfied. The present treatments for diabetes involve frequent monitoring of blood glucose and life-long insulin injection or alternate combination with diabetic medicine. This research purposed to express furin-cleavage human proinsulin in liver of diabetic animal using adenovirus and adeno-associated virus (AAV) and to provide constant insulin level for basal requirement in diabetes. Adenovirus and adeno-associated virus expressing furin-cleavage human proinsulin (AAV-FhPI) under CMV promoter were administered into portal vein of

streptozotocin (STZ)-induced diabetic rats. These resulted in the decrease of blood glucose levels within 24 hr after adenovirus injection and within 3 day after AAV injection respectively. In adenovirus-FhPI treated rats, serum insulin levels were increased and glucose tolerance was significantly improved although these effects lasted for less than 3 weeks. For a long-term insulin gene expression, AAV-FhPI was administered in STZ-induced diabetic rats and the treated rats exhibited the normalized blood glucose levels in fasting state over 3 months. The ability of glucose clearance approximated to non-diabetic control and the hemoglobin A1c concentrations were decreased in AAV-FhPI treated rats. Immunohistochemical studies of pancreas in adenovirus-FhPI treated rats and AAV-FhPI treated rats revealed the pancreatic β-cells were mostly destroyed while adenovirus-FhPI and AAV-FhPI transduced hepatocytes produced human insulin. In conclusion, viral vector-mediated furin-cleavage proinsulin gene expression using the constitutive promoter is feasible gene therapy to supply basal insulin, lessening the frequency of insulin injection. And portal vein injection is suitable to enhance the exogenous gene delivery into liver. Furthermore, the therapeutic potential of this approach would be realized through the study on regulating insulin secretion, for example, regulating promoter-based gene expression with glucose/insulin responsible elements.

Key words: gene therapy, diabetes, furin, insulin, liver, hyperglycemia

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I. INTRODUCTION

Diabetes mellitus is divided into two types, type 1 diabetes mellitus and type 2 diabetes mellitus.[1] Type 1 diabetes mellitus is caused by autoimmune destruction of pancreatic β-cells, leading to severe insulin deficiency.[2] Patients are treated with multiple daily insulin injections based on the number and content of meals eaten and the sensitivity of each patient to insulin.[3-5] Type 2 diabetes mellitus, which hyperglycemia is caused primarily by a resistance to insulin, also known as adult-onset diabetes, is complex disease characterized by end-organ unresponsiveness to the effects of insulin,[6-7] as well as by β-cell dysfunction and eventual β-cell

failure.[8] In these patients, blood insulin level may even be higher than normal and blood glucose is usually controlled by diet and antihyperglycemic, pharmacological agents.[9] However, type 2 diabetes eventually results in β-cell dysfunction and a drastic reduction in insulin production.[10-11]

Both forms share the characteristics of peripheral organ damage caused by the toxic effects of hyperglycemia.[12] The long-term hyperglycemia causes the clinical problems, including renal failure, retinopathy, neuropathy and heart disease.[8,9,11] Although intensive exogenous insulin injection to closely mimic the physiological control of glucose, can delay or prevent the onset of chronic complications, sometimes multiple daily administrations or continuous subcutaneous infusion of insulin causes hypoglycemia.[13-16] Even with strict management, normoglycemia is not easily achieved in diabetics despite improvements in insulin preparation and delivery. Therefore, there have been considerable interests in the transplantation of whole pancreas/islets [5,12,17] or transplanting genetically engineered cells capable of producing insulin [18-25], or using gene therapy to supply insulin.[26-28]

Delivery of insulin by gene therapy represents an attractive alternative to current insulin treatments. Gene therapy for diabetes needs target organs and an effective gene delivery system.[29-34] The recent studies have chosen muscle, pancreas and liver as target organs for insulin gene expression. Since liver plays a

critical role between glucose production and glucose utilization,[35-36] liver is selected as a major target. Several studies have reported that hepatic insulin production showed to prevent lethal problems and development of long-term complications associated with diabetes.[37-39] Also proinsulin gene should be modified for processing by non-endocrine cells,[40-42] because the maturation process of proinsulin needs the action of two endopeptidases proprotein convertase (PC1 and 2 or PC1 and 3) [43-44] , which are specifically expressed in the β-cells and some neuroendocrine cells.[45-46] The processing can be accomplished by adding furin-cleavage sites at 60th ends of C-peptide in non-endocrine cells.[47-50]

Another important factor of gene therapy is an efficiency of therapeutic gene delivery. There have been some therapeutic trials using non-viral vector systems but viral vector systems have been noticed for an effective gene delivery.

Unlike other viral vectors as gene delivery vehicles, potential application and impact of adeno-associated virus have been expanded rapidly.[51] Long-term expression of transgene and no immune responses to transduced cells have been suggested distinctive therapeutic expectations of adeno-associated virus using gene therapy. High-titer recombinant adeno-associated virus production is a main hurdle but recent advances in virus production techniques have brought to a settlement.

Concerning that gene therapeutic approaches have been concentrated on type 1 diabetes mainly, this research focused on solving the insulin deficiency in both

types of diabetes. As constant physiological level of insulin is needed in both types of diabetes, this study purposed, i) to transfer the therapeutic genetic material to specific target organ using constitutive strong promoter in a safe manner, ii) a constitutive gene expression for providing a relatively constant background level of insulin to supply the basal requirement of insulin, iii) a long-term insulin production achieving adequate control of fasting hyperglycemia. This research evaluates that constitutive furin-cleavage proinsulin gene expression using adeno-associated viral vector is feasible gene therapy to supply basal insulin avoiding complex regimens.

II. MATERIALS AND METHODS

1. Plasmids construction

Human preproinsulin cDNA (Dr. M. Walker's laboratory, Weizmann Institute, Israel) was modified by overlap extension PCR to generate the furin recognition motif at the B-chain/C-peptide and the C-peptide/A-chain junctions. Preproinsulin with cleavable furin sites, was generated using the site-directed mutagenesis Quickchange kit (Stratagene, La Jolla, CA, USA). This modified furin-cleavage human preproinsulin cDNA was inserted into pBluescript SK (+) vector. The fragment between BamH I and Xba I was inserted into pShuttle-CMV vector. pAdeno-CMV/FhPI was selected in clones after homologous recombination with pAdEasy, which included adenoviral DNA, followed by adenovirus production system procedure (Figure 1). And the fragment between BamH I and Xba I was inserted into pAAV-CMV/MCS vector followed by adeno-associated virus production system procedure (Figure 2).

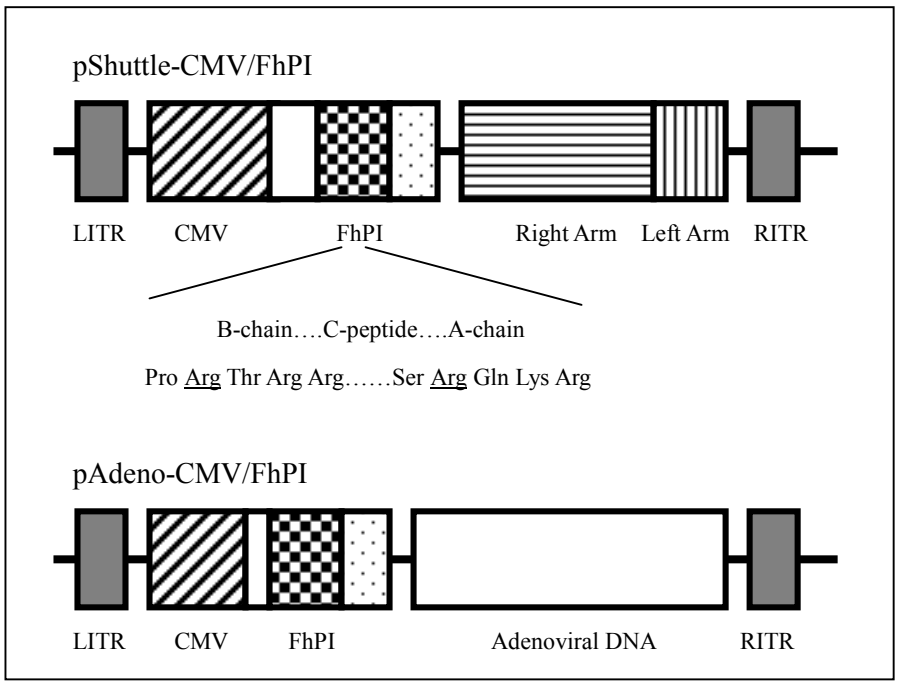

Figure 1. Construction of adenoviral vector for expression of furin-cleavage human proinsulin. The modified furin-cleavage human proinsulin cDNA (mosaic bar) and insulin signal peptide (white bar) were inserted into pShuttle-CMV vector. The vector pShuttle-CMV contained a multiple cloning site sandwiched between the CMV promoter (hatched bar) and the SV40 polyadenylation signal (dotted bar) and the regions indicated as arms are stretches of sequence homology with AdEasy-1 where the homologous recombination occurs. The RITR and LITR regions are short inverted terminal repeats (left and right), which have a role in replication of the viral DNA. Recombinant pAdeno-CMV/FhPI was completed after homologous recombination in bacteria followed by adenovirus production system procedure.

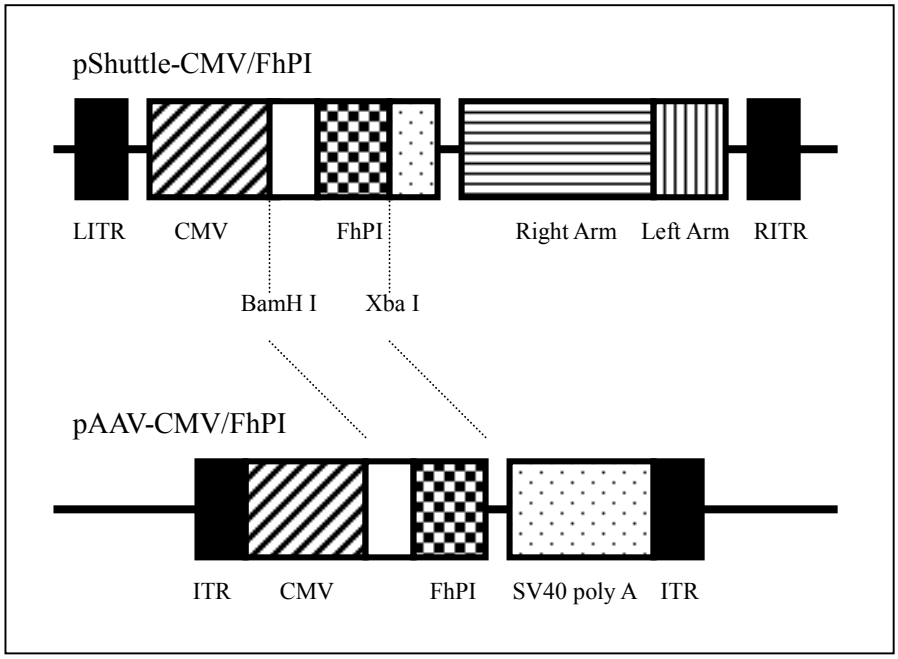

Figure 2. Construction of adeno-associated viral vector for expression of furin-cleavage human proinsulin. The first step in the production of recombinant AAV particles in the AAV-Helper-Free system was cloning the furin-cleavage human proinsulin into the plasmid pAAV-MCS. The vector provided the CMV promoter (hatched bar) and other elements for high-level gene expression in mammalian cells. The furin-cleavage human proinsulin gene (mosaic bar) was cloned into pAAV-MCS at the multiple cloning sites using BamH I/Xba I fragment from pShuttle-CMV/FhPI. White bar, insulin signal peptide; dotted bar, SV40 poly A.

2. Adenovirus preparation

AdEasy™ Adenoviral Vector System (Stratagene, La Jolla, CA, USA) was used for adenovirus production.[52-55] Purified recombinant pAdeno-CMV/FhPI was digested with *Pac* I to expose its inverted terminal repeats (ITR), and then used to transfect HEK293 cells. Transfections were carried out at 37°C using the calcium phosphate transfection system (Life Technologies, Minden, NV, USA) according to the manufacturer's specifications. In brief, 24 hr prior to the transfection HEK293 cells were plated at 70-80 % confluence on 10 cm culture plate. Ten microgram of *Pac* I digested pAdeno-CMV/FhPI were mixed with 1 ml of 0.3 M $CaCl_2$. The 1 ml of 2X HBS (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.10) solution was added in DNA and $CaCl_2$ mixture gently. The DNA/ $CaCl_2$/HBS suspension was applied to the plate of cells in drop-wise fashion. The culture plate was returned to the 37°C incubator for 6 hr and then growth media was changed. The morphological changes of cells were monitored and then cells were harvested (Figure 3). After 7-10 days, cells were harvested by centrifugation at 3000 rpm for 10 min, washed in PBS twice and then resuspended in PBS. Recombinant adenovirus was released by freezing and thawing four times. The crude lysate was clarified by centrifugation at 12,000 rpm for 10 min. To isolate an adenovirus clone including transgene, plaque isolation was performed with primary

viral solution. A solution of 5 % SeaPlaque agarose (BioWhittaker Molecular Applications, Verviers, Belgium) in sterile PBS was prepared and autoclaved, and stored in 10 ml aliquots at 4°C in 50 ml sterile conical tubes. Prior to use, agarose was melted by placing the tube in a beaker of boiling water. Once melted, the agarose was cool down to 45°C. And 30 ml of growth medium was added previously and equilibrated to 37°C and mixed. This made the final agarose concentration 1.25 %. After complete removal the growth medium from the wells that were infected with recombinant adenovirus, the agarose overlay was received. Three milliliter of agarose/growth medium was gently pipetted and mixed very gently along the side of the well and allowed to completely cover the bottom of the well. The plate was incubated at 37°C. Plaques, having the appearance of small white spots, were visible to the naked eye within 12 − 21 days. Under the sterile conditions, plaques were core-out well and isolated with agarose. It was transferred to 250 ul of growth medium in a sterile microcentrifuge tube with a sterile pasteur pipette. Virus was eluted for 24 hr at 37°C. Then viral DNA was extracted and transgene was confirmed by PCR with specific primers. To amplify the adenovirus, infection of HEK293 cells were achieved simply by adding viral solution to adherent cells in tissue culture dishes. Cultures of HEK293 cells were prepared 70 −80 % confluent and the diluted primary virus stock was added in the cultured cells. Cells were incubated at 37°C during the infection reactions for 2 hr, preferably on a

rocking platform to disperse the solution evenly. After two hours, additional growth medium was supplied. After 3 day of incubation, cells were harvested in a minimal volume of PBS. The cell lysates were prepared by four rounds of freezing and thawing, and then fractionated by sonication for 5 min to break down cell membranes. The obtained lysates were then loaded onto a CsCl step gradient of 1.2 g/ml and 1.4 g/ml. Adenovirus particles were then purified by CsCl gradient centrifugation in a SW41Ti rotor (Sorvall, Newton, CT, USA) at 70,000 rpm for 6 hr. The clear white region in the tube was gathered inserting 5 ml syringe with 21 G needle. Viral solution was dialyzed against phosphate buffered saline (PBS) at 4°C overnight or was desalted by Sephadex PD-10 column (Amercham Bioscience, Uppsala, Sweden) and stored at –80°C after mixing with one volume of in 2X adenovirus storage buffer. To determine titer, the number of plaques was counted after agarose overlay. The counts were averaged from duplicate wells and multiplied that number by the dilution factor to estimate pfu/ml.

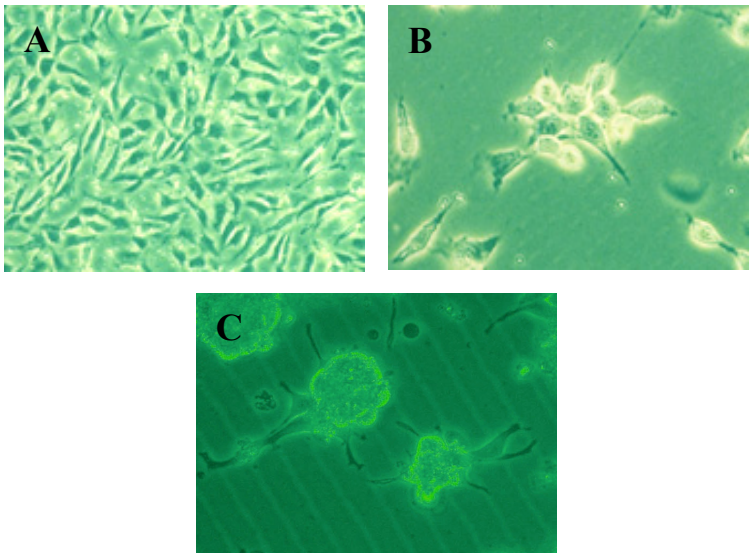

Figure 3. Adenovirus and adeno-associated virus production monitoring. Morphological changes were observed post-transfection with pAdeno-CMV/FhPI and pAAV-CMV/FhPI under the microscope. Cells were in the form of rounded, refractile and swollen. A, Normal HEK293 cells (x 100); B, HEK293 cells at 7 day post-adenoviral vector transfection (x 400); C, HEK293 cells at 3 day post-3 plasmids cotransfection for adeno-associated virus production (x 400).

3. Adeno-associated virus preparation

AAV Helper-Free System (Stratagene, La Jolla, CA, USA) was used for adeno-associated virus production. HEK293 cells were maintained in DMEM supplemented with 5 % fetal bovine serum and incubated in humidified environment with 5 % CO_2 at 37°C. Briefly, helper plasmid, vector plasmid and RC plasmid were cotransfected into HEK293 cells at a ratio of 1:1:1 by calcium phosphate precipitation. For each 15 cm tissue culture dish, a total of 75 ug DNA was used. The transfected cells were then maintained in DMEM containing 2 % fetal bovine serum. At 3 day post-transfection, the cells were harvested monitoring the morphological changes and virus was purified by CsCl gradient (Figure 3).

The harvested cell pellet after transfection was resuspended in PBS. After four freezing - thaw cycles in liquid nitrogen bath and 37°C water bath, cell lysates were fractionated by sonication for 5 min to break down cell membranes (40 burst, level 2). The obtained lysates were then loaded onto a CsCl step gradient. Recombinant AAV particles were then purified by CsCl gradient centrifugation in a SW41Ti rotor at 60,000 rpm for 16 hr. Twelve to sixteen fractions of ten drops each were collected by inserting a 21 G needle. To select the fractions including AAV, PCR method was used. The selected six fractions were dialyzed against phosphate buffered saline at 4°C overnight and stored at -80°C. Recombinant AAV titer was

determined by competitive PCR method with the viral DNA. Quantification of adeno-associated virus was determined by the DNA concentration out of competitive PCR analysis. To convert DNA into copy number the following equations were used; a) (number of base pairs) (average weight of single stranded DNA) / Avogadro's number = grams / viral DNA molecules, b) (grams/ul) / (grams/ viral DNA molecules) = Viral DNA molecules / ul.

4. Animal experiments

Male Sprague-Dawley rats (300 g) were obtained from Daehan BioLink (Eumsung, Chungbuk, Korea). Animals were housed in hanging wire cages in temperature-controlled rooms (22°C) with a 12 hr light-dark cycle and fed Purina chow (Ralston-Purina, St. Louis, MO, USA) and given deioned water *ad libitum*. Diabetes was induced in the rats by injection of streptozotocin 70 mg/kg in 0.05 M citrate buffer (pH 4.5) intraveneously. Induction of diabetes was confirmed by measuring fasting blood glucose levels. General operation for portal vein injection was performed after anesthesia with ketamine hydrochloride (10 mg/kg) for administration of adenovirus-FhPI or AAV-FhPI. Ventral hair was shaved and skin was washed with povidone-iodine solution and wiped with sterile water. A midline abdominal incision was made and 1 ml of adenovirus/adeno-associated virus was

injected into the hepatic portal vein of animals.

5. Measurements of blood glucose and hemoglobin A1c

Blood glucose level was measured by the tail vein blood being placed on glucose reagent strips (Johnson & Johnson, Milpitas, CA, USA) and read in a glucometer (Johnson & Johnson, Milpitas, CA, USA). Hemoglobin A1c was measured by Roche Cobas Integra HbA1c cassette with whole blood (Roche, Basel, Swiss).

6. Intraperitoneal/oral glucose tolerance test

Prior to intraperitoneal/oral glucose tolerance test, food was withdrawn for 6 hr and then 2 g/kg glucose was loaded intraperitoneally/orally. Blood glucose was measured at 30, 60, 90 and 180 min.

7. Analysis of tissue mRNA expression.

A. RNA extraction: 0.5 g of rat tissues were mixed with 1 ml of TRIzol reagent (GIBCO BRL, Tulsa, OK, USA) by pipetting and homogenized in glass homogenizer. After incubation on ice for 5 min, 0.2 ml of chloroform/1ml TRIzol

reagent was added and mixed well with vigorous shaking for 15 sec. Centrifugation, 13,000 rpm for 20 min at 4°C proceeded after 2 min incubation on ice. The colorless upper aqueous phase was transferred to a fresh tube and mixed with 0.5 ml of isopropyl alcohol. Centrifugation, 13,000 rpm for 20 min at 4°C proceeded after 10 min incubation on ice. After removal of supernatant, 1 ml of 75 % EtOH was added and centrifuged 12,000 rpm, 5 min, 4°C. RNA pellet was air-dried for 10 min and dissolved in DEPC-treated distilled water.

B. RT-PCR: Total RNA was extracted and stored at -80°C until analysis. For the first strand cDNA synthesis, reverse transcription system (Promega, Madison, WI, USA) was used. For the detection of exogenous vector-derived FhPI transcript, the forward primer within the coding sequence and the reverse primer within the vector-specific region were used for PCR reaction. All PCR products were first confirmed by direct sequencing analysis. Primers used in RT-PCR reaction are as follows.

Forward 5'-CGG GAT CCA TGG CCC TGT GGA TGC GCC TCC-3'

Reverse 5'-CCA CTA GTT ACA ATA GTT CTC CAG CTG GTA G-3'

C. Northern blotting

(A) RNA electrophoresis and membrane transfer: 0.6 g of agarose was

melted in 38 ml of distilled water. 12 ml of 10X MOPS solution and 10.8 ml of formaldehyde were added after cooling and then poured on gel caster. Twenty microgram of RNA were mixed with 2 ul of 10X MOPS, 3.4 ul of formaldehyde and 10 ul of formamide. Sample was denatured at 70°C for 5 min and loaded with 2 ul of loading buffer and 1 ul of 0.4 mg/ml Ethidiumbromide solution. Electrophoresis was performed under 70 - 80 Volt for 2 hr. After three times washing gel in DEPC water, the gel was rewashed three times in 10X SSC and laid top-side down and followed the general procedure.

(B) Probe: 10 ng of *Bam*H I/*Xba* I fragment of pAAV-CMV/FhPI was used to make probe. Random labeling kit (Roche, Basel, Switzerland) and 5 μl of [a-^{32}P]dCTP were used for probe labeling.

(C) Hybridization: The fixed membrane by UV-cross linking was pre-hybridized in 10 ml of rapid hybridization buffer (Amersham Biosicence, St. Gail, UK) at 65˚C for 15 min and hybridized at 65˚C for 2 hr. Then membrane was washed with 2X SSC, 0.1 % SDS at room temperature for 10 min twice, 1X SSC, 0.1 % SDS at 65˚C for 15 min, 0.1X SSC, 0.1 % SDS at 65˚C for 10 min twice and wrapped and exposed overnight.

8. Insulin immunohistochemical staining

Liver and pancreas were removed from adenovirus-FhPI and AAV-FhPI injected rats. Fixation, embedding and sectioning were followed by the general procedure. For insulin immunohistochemical staining, immunohistochemistry kit AEC (Innogenex, San Ramon, CA, USA) was used and all procedures were followed by manufacturer's instructions. Briefly, guinea pig anti-insulin (DAKO, Glostrup, Denmark) was used as primary antibody, and pepsin (Biogenex, San Ramon, CA, USA) was used for antigen removal. The endogenous peroxidase was inactivated by 3 % hydrogen peroxide. Prior to antibody labeling, 10 % goat serum was applied to the samples to prevent non-specific antibody labeling. The slides were incubated with the primary antibody [guinea pig insulin antibody (1: 100)] for 1 hr at 37°C. After washing in PBS including 0.1 % Tween 20 the slides were allowed to react with the secondary antibody [anti-rabbit immunoglobulin (1:500)], (DAKO, Glostrup, Denmark) for 5 min at 37°C. This was followed by sequential labeling with biotinylated anti-rabbit immunoglobulin and streptavidin peroxidase conjugate. Coloration was produced with substrate solution containing hydrogen peroxidase and 3-amino-9-ethylcarbazole. For the negative control, primary antibody was omitted by replacing the antibody with PBS. Normal rat pancreas was immunohistochemically stained as the positive control. After immunostaining

the nuclei were counterstained with Mayer's hematoxylin (Sigma Aldrich, St. Louis, MO, USA).

9. Statistical analysis

Data were shown as means ± S.D. For group differences, the Student's *t*-test was used. A *P*-value less than 0.05 was considered significant.

III. RESULTS

1. Human insulin secretion from vectors-transduced cells

The transduction efficiency of FhPI carrying vectors in HEK293 cells was evaluated exploiting the vector-derived expression of the GFP marker. After a single exposure to the vectors, more than 90 % of HEK293 cells were induced to express the GFP molecule (data not shown). The release of human insulin from cells transduced with the pShuttle-CMV/FhPI and pAAV-CMV/FhPI was measured in culture media (Figure 4). The transfection experiments were performed in triplicate separately and insulin levels in media were measured at 2 day after transfection. The pShuttle-CMV/EGFP and pAAV-CMV/EGFP vectors were used for control transfection. The pShuttle-CMV/FhPI and pAAV-CMV/FhPI transduced HEK293 cells released insulin in amounts ranging from 208 to 224 uIU/ml and 81 to 86 uIU/ml respectively. On the other hand, insulin was undetectable in the culture media of both pShuttle-CMV/EGFP and pAAV-CMV/EGFP vectors transduced cells. Since all transfection experiments were performed in serum free conditions, this result suggested the detected insulin was derived from pShuttle-CMV/FhPI or pAAV-CMV/FhPI vector-transduced cells.

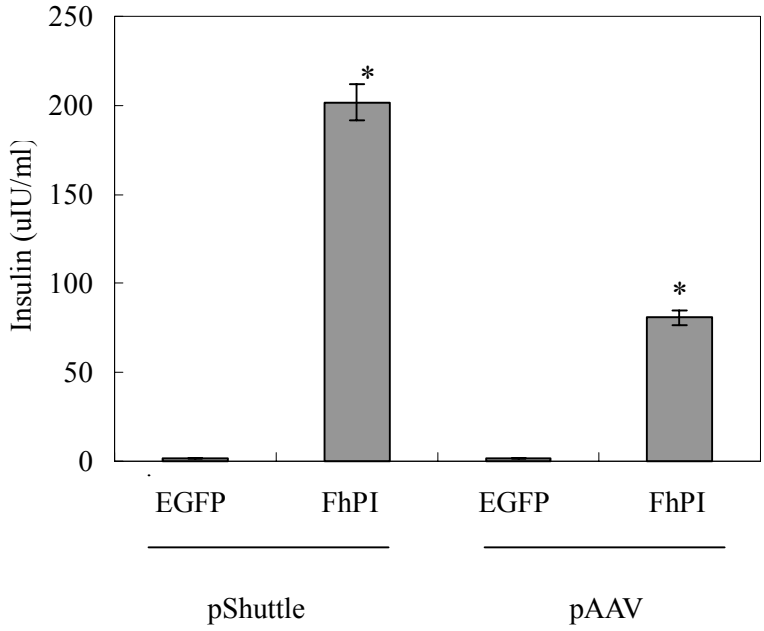

Figure 4. Human insulin release from pShuttle-CMV/FhPI and pAAV-CMV/FhPI transduced HEK293 cells. HEK293 cells were transfected with pShuttle-CMV/FhPI and pAAV-CMV/FhPI. Human insulin levels were increased in culture media of the cells transduced with pShuttle-CMV/FhPI and pAAV-CMV/FhPI vectors but insulin was undetectable in media of the cells transduced with pShuttle-CMV/EGFP and pAAV-CMV/EGFP. Values are expressed as means $\pm$ S.D. of triplicate experiments. * $P < 0.05$ vs. EGFP controls.

2. Human insulin release from the cells transduced with recombinant adenovirus

To determine whether adenovirus-FhPI effectively transduced hepatocyte-derived cells, the content of immunoreactive insulin in culture media of adenovirus-FhPI-infected Huh7 cells was measured (Figure 5). Huh7 cells were exposed by adenovirus-FhPI at a multiplicity of infection (MOI) of 50 for 2 hr and then media was changed. Insulin contents in growth media were measured at 48 hr post-infection. For a negative control, Huh7 cells were infected by adenovirus-empty. At 48 hr post-infection, the immunoreactive insulin level in serum-free media of adenovirus-FhPI exposed cells was 161 $\pm$ 10.7 uIU/ml, whereas in control (adenovirus-empty exposed cells), the levels of immunoreactive insulin were below 1.5 $\pm$ 0.25 uIU/ml. Immunoreactive insulin in culture media of adenovirus-FhPI exposed cells was derived from recombinant adenovirus infection because the experiments were performed under serum-free condition and also that of adenovirus-empty control indicated that insulin was produced specifically in transduced cells by infection of adenovirus-FhPI.

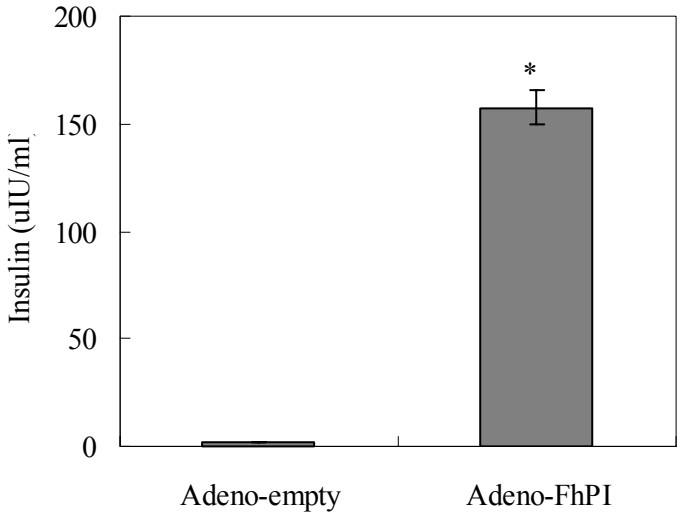

Figure 5. Human insulin release from Huh7 cells by infection of adenovirus-FhPI. Huh7 cells were exposed to adenovirus-empty (open bar) or adenovirus-FhPI (closed bar) and then insulin levels in media were measured at 48 hr post-infection. Values are expressed as means $\pm$ S.D. of triplicate experiments. * $P < 0.05$ vs. adenovirus-empty control.

3. Reversal of hyperglycemia in STZ-induced diabetic rats by adenovirus-FhPI treatment

The ability of adenovirus-expressed furin-cleavage human proinsulin to control hyperglycemia was examined in animal model of streptozotocin-induced diabetes. Four groups of streptozotocin-induced, severely diabetic rats (6 hr-fasting blood glucose > 500 mg/dl) were administered with adenovirus-FhPI in different doses and a group of STZ-induced diabetic rats was administered with adenovirus-empty into portal vein (Figure 6). Group A (n=4) administered with 1 x 10^{12} particles of adenovirus-empty, which did not show any change of blood glucose level (above 500 mg/dl) and insulin level (1.5 to 2.5 uIU/ml). A second group (B, n=4) administered with 5 x 10^{12} particles of adenovirus-FhPI showed drastic decrease in the blood glucose level from 511 mg/dl to hypoglycemic level (below 20 mg/dl) in 24 hr and died within 2 day. A third group (C, n=4) received 2.5 x 10^{12} particles of adenovirus-FhPI, which released a large amount of human insulin (136.91 uIU/ml) at 24 hr post-administration. The blood glucose level was progressively decreased from 451 mg/dl to 53 mg/dl in average. However, the animals were hypoglycemic by day 5 (43 mg/dl < BG <51 mg/dl) and reported slightly increased blood glucose levels (171 mg/dl < BG < 379 mg/dl) by day 10 and became hyperglycemic until sacrificed at day 18. Group D (n=4), which was

administered with 1.5 x 10^{12} particles of adenovirus-FhPI, also showed marked decline of blood glucose level to around 60 mg/dl until 3 days and gradual increase to approximately 300 mg/dl at 10 days after treatment. The dose of 6 x 10^{11} particles of adenovirus-FhPI, slightly decreased the blood glucose level but maintained hyperglycemic levels (from 372 mg/dl to 454 mg/dl) for 11 day and then the severe hyperglycemic blood glucose levels (BG > 600 mg/dl) by day 18. Furin-cleavage human proinsulin producing adenovirus treatment resulted in significant reduction of blood glucose in proportion to injected doses of adenovirus-FhPI compared with persistently elevated blood glucose levels in adenovirus-empty treated diabetic animals. Basal insulin production by adenovirus-FhPI treatment further reduced the blood glucose levels of diabetic animals to euglycemic range. However, in adenovirus-FhPI treated diabetic rats, the daily blood glucose levels in fed state were observed with a high degree of variation between animals.

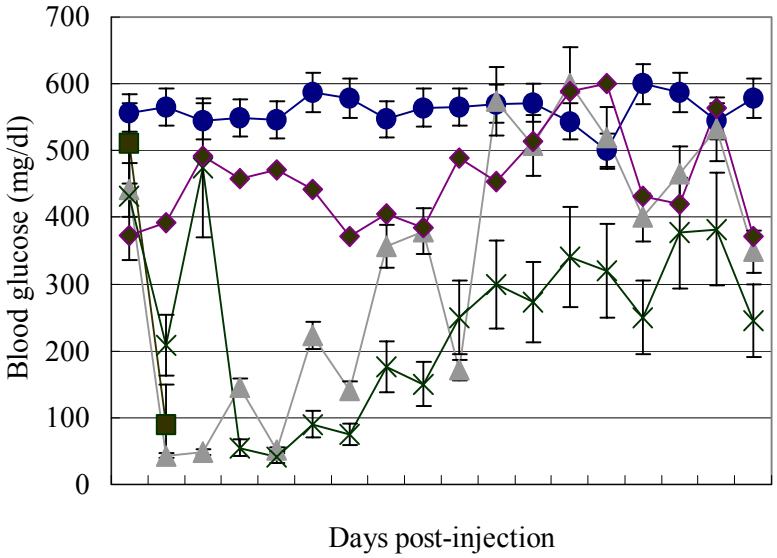

Figure 6. Blood glucose levels in adenovirus-FhPI treated rats. Various doses of adenovirus-FhPI, 5 x 10^{12} adenoviral particles (■, n=4), 2.5 x 10^{12} adenoviral particles (▲, n=4), 1.5 x 10^{12} adenoviral particles (x, n=4), and 6 x 10^{11} adenoviral particles (◆, n=4) were administered into portal vein of STZ-induced diabetic rats. 1 x 10^{12} particles of adenovirus-empty (●, n=4) was control injected. The blood glucose levels of adenovirus-FhPI injected rats were significantly reduced in a dose-dependent manner over 2 weeks. Values are expressed as the means $\pm$ S.D.

4. Improved glucose tolerance in adenovirus-FhPI-treated animals

To study the efficacy of blood glucose disposal, an oral glucose tolerance test (OGTT) was performed at 8 day post-adenovirus injection. Withdrawal of food for 6 hr preceded the glucose (2 g/kg) loading. As shown in Figure 7, adenovirus-FhPI -treatment significantly improved the glucose tolerance and their elevated blood glucose levels after the glucose loading returned to the pre-loading levels within 2.5 hr and their profile approximated that of non-diabetic controls. In contrast, STZ-induced diabetic controls showed persistent hyperglycemic levels during whole oral glucose tolerance test. To observe the serum insulin levels during blood glucose disposal, the blood was collected from tail vein 30 min interval during the glucose tolerance test (Figure 8). At the beginning of the test, serum insulin level of adenovirus treated groups was 2-3 fold higher than normal control (17.5 $\pm$ 3 uIU/ml vs. 7.0 $\pm$ 0.5 uIU/ml). Challenged glucose was reflected in the increasing serum insulin level in normal control until 60 min (from 7.0 $\pm$ 0.5 uIU/ml to 24.1 $\pm$ 1.5 uIU/ml) but inversely in the reduction of serum insulin levels in 2.5 x 10^{12} particles (from 20.5 $\pm$ 9.5 uIU/ml to 9.4 $\pm$ 3.8 uIU/ml) and 1.5 x 10^{12} particles treated rats (from 14.5 $\pm$ 1.8 uIU/ml to 10.5 $\pm$ 0.9 uIU/ml). During the proceeding 60 min, elevated insulin levels in non-diabetic rats were decreased following glucose sensing regulatory system in the body and returned to pre-challenging level

(from 24.1 $\pm$ 1.5 uIU/ml to 10.4 $\pm$ 2.3 uIU/ml) but in adenovirus treated groups, irrelevant insulin level was maintained by constitutive insulin secretion through adenovirus treatment. In contrast, serum insulin levels of 6×10^{11} particles injected rat were not significantly changed for those 3 hr (2.6 uIU/ml – 2.4 uIU/ml) and STZ-diabetic rats exhibited constant low insulin levels during whole test hour (1.5 uIU/ml – 1.5 uIU/ml) and insulin was barely detectable.

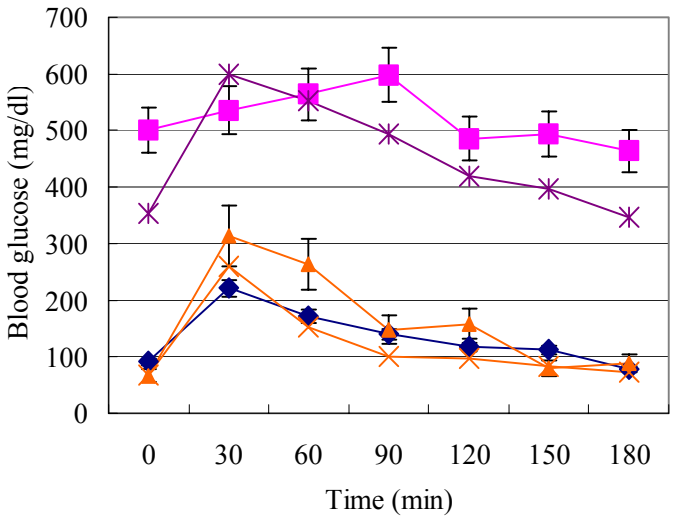

Figure 7. Blood glucose profiles in a glucose tolerance test of adenovirus-FhPI treated rats. At 8 day post adenovirus-FhPI treatment with doses of 2.5 x 10^{12} particles (▲, n=4), 1.5 x 10^{12} particles (x, n=4) and 6 x 10^{11} particles (✱, n=4), rats were fasted for 6 hr and then administered glucose (2 g/kg) orally. Normal rats (◆, n=4) and streptozotocin-induced diabetic rats (■, n=4) were used as controls. The blood glucose levels were determined at the indicated time intervals. Values are expressed as the means ± S.D.

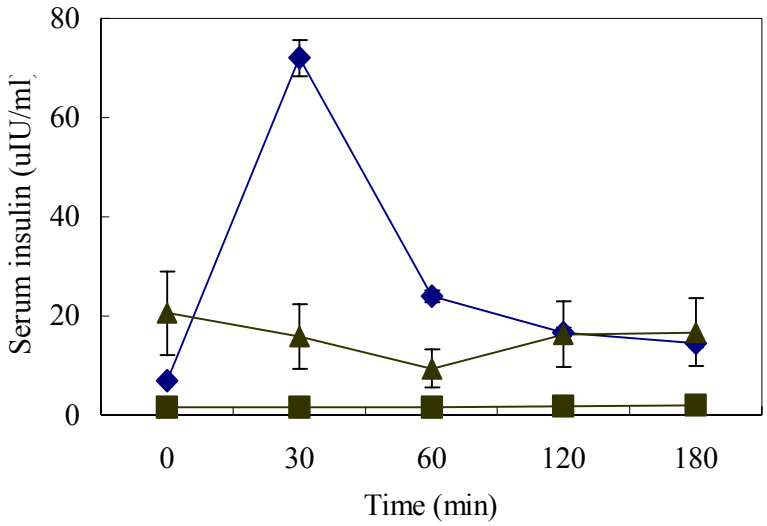

Figure 8. Serum insulin levels in a glucose tolerance test of adenovirus-FhPI treated rats. Blood was collected from tail vein at indicated time interval during the 3 hr test and serum insulin levels were measured in normal control group (◆, n=4), STZ-diabetic group (■, n=4), 2.5 x 10^{12} particles treated group (▲, n=4). Insulin was barely detectable in STZ-diabetic group. Values are expressed as the means ± S.D.

5. Transient insulin gene expression in adenovirus-FhPI treated animals

The effects of the ectopic insulin were monitored over a short period (up to 3 weeks). As shown in Figure 6, the hypoglycemic effect induced by adenovirus-FhPI treatment lasted for 8 – 18 days with the variation between the injected doses and the blood glucose levels were reversed to the hyperglycemic degree. To examine insulin gene expression by adenovirus treatment with advancing days, serum insulin levels of 2.5 x 10^{12} particles treated rats and 1.9 x 10^{12} particles treated rats were measured at day 1, 7, 14, 21 post-treatment. Serum insulin level of adenovirus-empty injected rat was compared as a control. Ectopic insulin gene expression by adenovirus treatment peaked in day 1 - 7 with a variation between injected particles (from 2 uIU/ml to 136.5 $\pm$ 10.5 uIU/ml in 2.5 x 10^{12} particles treated rats and from 2 uIU/ml to 55.2 $\pm$ 4.5 uIU/ml in 1.9 x 10^{12} particles treated rats) and progressively insulin levels were reduced to 33 $\pm$ 4.8 uIU/ml, 23 $\pm$ 3.4 uIU/ml at day 7 and 16.7 $\pm$ 2.0 uIU/ml, 10.5 $\pm$ 2.5 uIU/ml at day 14 respectively (Figure 9).

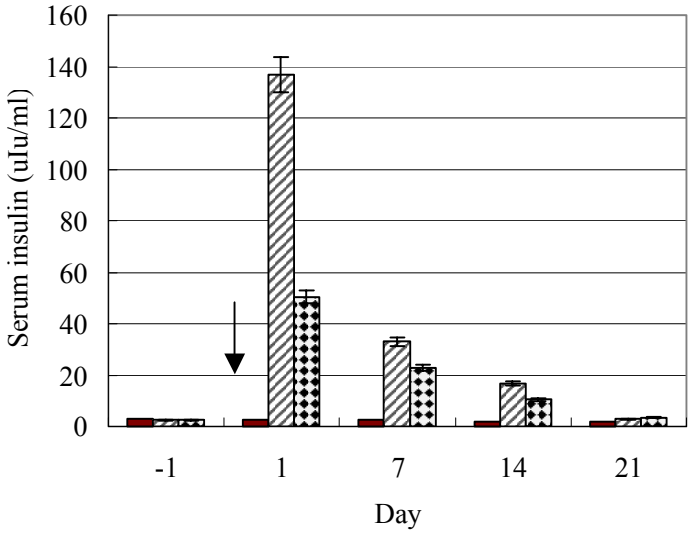

Figure 9. The effect of adenovirus-FhPI treatment on serum insulin levels. Serum insulin was significantly increased in adenovirus-FhPI treated rats at 1 day post injection comparing with Adeno-empty treated rats ($p < 0.001$, 2.5×10^{12} particles treated vs. adeno-empty treated; $p < 0.05$, 1.9×10^{12} particles treated vs. adeno-empty treated). Arrow indicates the time point that adenovirus was administered. Values are expressed as the means $\pm$ S.D. Closed bar, adeno-empty; hatched bar, 2.5×10^{12} particles treated rats; dotted bar, 1.9×10^{12} particles treated rats.

6. RT-PCR analysis with liver-tissue derived mRNA

Human insulin mRNA was detected by reverse transcriptase (RT)-PCR in liver of rat which was administered with 5 x 10^{11} adenovirus-FhPI particles and of which the blood glucose level was significantly lowered post-treatment. And the liver RNA was extracted from 5 x 10^{11} adenovirus-empty particles injected rat, non-diabetic rat, STZ-diabetic rat for the controls. The observed comparable signal for an invariant β-actin indicated that an approximately equal amount of starting cellular material was used from each tissue sample in PCR. Representative profiles of insulin signal in the indicated tissue of normal control, STZ-diabetic control, one adenovirus-empty treated rat and one adenovirus-FhPI treated rat were shown in Figure 10. The results of PCR amplification clearly showed that presence of human insulin mRNA was in liver tissue of adenovirus-FhPI injected rat. The amplified PCR product was used for sequence analysis and furin-cleavage human proinsulin gene was confirmed by nucleotides sequence analysis.

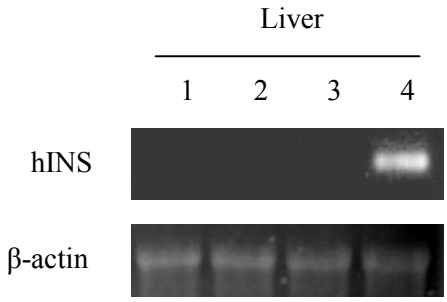

Figure 10. Detection of human insulin mRNA in liver of adenovirus-FhPI treated rat. Liver RNA was prepared from normal control rat, STZ-diabetic control rat, adenovirus-empty injected rat, and adenovirus-FhPI injected diabetic rat and RT-PCR was performed with sequence specific primers. Targeted PCR product was produced in adenovirus-FhPI injected rat specifically and no product was produced in other control groups. Lane 1, normal rat; lane 2, STZ-diabetic rat; lane 3, adenovirus-empty injected rat; lane 4, adenovirus-FhPI injected rat.

7. Exogenous proinsulin gene expression in diabetic rats

Intraportal administration of recombinant adenovirus resulted in an efficient transgene expression, specifically in liver. At 5 day post-adenovirus treatment, northern blotting with RNA extracted from various tissues of 5 x 10^{11} adenovirus-FhPI injected rat was performed comparing with RNA from adenovirus-GFP injected rat (Figure 11). Quantitative RNA expression indexed to β-actin. To examine exogenous proinsulin gene might express in other tissues, total RNA extracted from stomach, heart, kidney, spleen, muscle, fat, brain and testis were compared in northern blot analysis. Specific region of proinsulin gene was used for the probe and liver RNA of adenovirus-GFP injected rat was used for standard negative. As a result of northern blotting, exogenous proinsulin gene was expressed specifically in liver of recombinant adenovirus injected rat and it was not detected in other tissues.

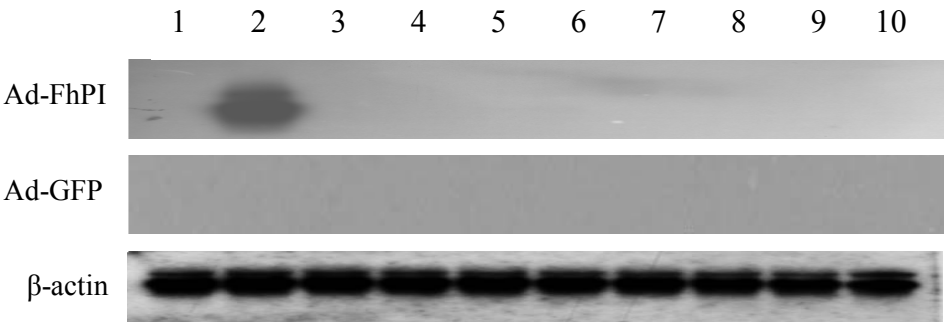

Figure 11. Northern blot analysis in adenovirus-FhPI injected rats. A representative profiles of insulin signal in the indicated tissues of insulin gene–treated diabetic rat and GFP gene–treated rat were shown. Exogenous proinsulin gene was detected in liver of adenovirus-FhPI injected rat and it was not detected in other tissues. Lane 1, pancreas; lane 2, liver; lane 3, stomach; lane 4, heart; lane 5, kidney; lane 6, spleen; lane 7, muscle; lane 8, fat; lane 9, brain; lane 10, testis.

8. Insulin immunostaining in adenovirus-FhPI treated animals

Immunohistochemistry of liver and pancreas proceeded in 2.5 x 10^{12} particles of adenovirus-FhPI administered rats and normal control rats. Immunohistochemical studies revealed that in response to STZ treatment, pancreatic β-cells were mostly destroyed in adenovirus-FhPI treated rats (that remained normoglycemic). Pancreatic insulin staining in normal control rat was indexed to compare. The islets in pancreas of normal rat were big round-shaped contrasting the islets of adenovirus-FhPI treated rat shown irregular and fragmented (Figure 12A, 12B). The countable number of islets in pancreas of normal rats was 19 $\pm$ 2 islets/100 mm^2 compared with 3 $\pm$ 1.5 islets/100mm^2 in adenovirus-FhPI treated rats (data not shown). By contrast, hepatic insulin staining of adenovirus-FhPI treated rat resulted in strongly positive comparing that normal rat liver showed completely negative (Figure 12C). Exogenous insulin produced by adenovirus-FhPI injection was detected on whole liver tissue (Figure 12D) and insulin positive staining regions were mainly located in cytoplasm and around the nuclear membrane of hepatocytes (Figure 12E). Comparing with normal rat liver tissue, immunohistochemical data proved that the immunostained insulin in liver was produced by adenovirus-FhPI injection and blood glucose level was lowered by hepatic insulin production in adenovirus-FhPI treated rats.

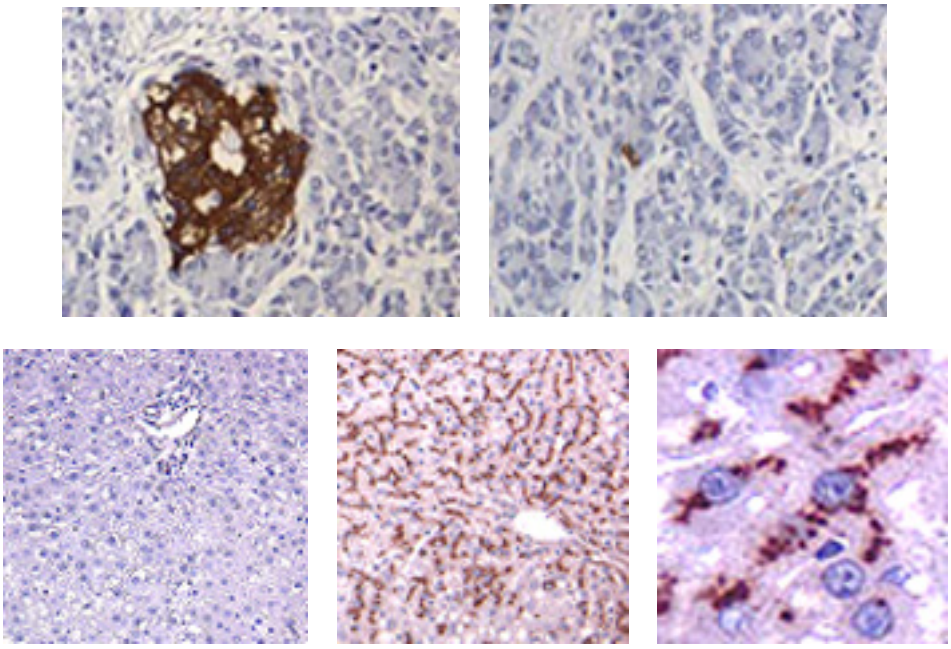

Figure 12. Immunohistochemistry of pancreas and liver in adenovirus-FhPI treated rats. Immunostaining results showed that exogenous insulin was produced in liver strongly by adenovirus-FhPI injection (2.5 x 10^{12} particles) in STZ-induced diabetic rat. Comparing with normal pancreas, most of the islets were destroyed in adenovirus-FhPI treated diabetic rat. Positively immunostained-regions were widely scattered in liver and the produced insulin was located in cytoplasm and around the nuclear membrane in hepatocytes. Normal liver tissue was completely negative to insulin antibody. A, normal rat pancreas (x 400); B, pancreas of adenovirus-FhPI injected diabetic rat (x 400); C, normal rat liver (x 200); D, liver of adenovirus-FhPI injected rat (x 200); E, magnification (x 1000).

9. Production of adeno-associate virus including furin-cleavage human proinsulin gene (AAV-FhPI) and insulin release by AAV-FhPI infection *in vitro*

To examine that purified AAV-FhPI expressed human insulin gene *in vitro*, 9 x 10^{10} particles/ml of AAV-FhPI was infected in 7 x 10^{6} Huh7 cells and insulin levels in media were measured at 48 hr post-infection. As a standard control, 9 x 10^{10} particles/ml of AAV-GFP was used for the infection of 7 x 10^{6} Huh7 cells and insulin levels in media of AAV-GFP infected Huh7 cells were measured and compared with AAV-FhPI infected cells. Insulin levels were 378 $\pm$ 9 uIU/ml in AAV-FhPI infected cells but insulin was not detected in media of AAV-GFP infected cells (insulin levels < 1.5 uIU/ml) (Figure 13).

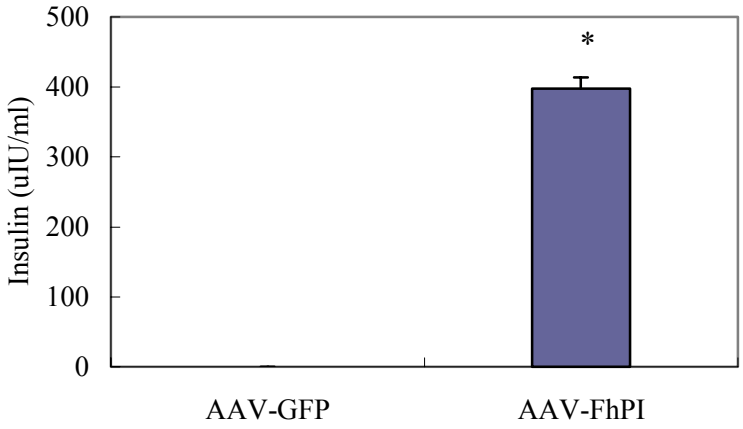

Figure 13. Insulin secretion of AAV-FhPI infected Huh7 cells. AAV-FhPI was exposed in Huh7 cells and insulin levels in growth media were measured at 48 hr post-infection. As a standard control, insulin level in media of AAV-GFP infected Huh7 cells was compared. Insulin level was 378 $\pm$ 9 uIU/ml in AAV-FhPI infected cells but insulin was not detected in media of AAV-GFP infected cells (* < 1.5 uIU/ml). Values are expressed as means $\pm$ S.D. of three separate experiments. $P <$ 0.05 vs. AAV-GFP control.

10. Reversed hyperglycemia in AAV-FhPI treated rats

The hypoglycemic effect of AAV-FhPI treatment in STZ-diabetic rats was examined by intraportal administration. AAV-FhPI and AAV-GFP were administered in diabetic rats and blood glucose levels and serum insulin levels were determined. Prior to adeno-associated virus treatment, food was withdrawn for 6 hr and animals over 500 mg/dl of fasting blood glucose level were selected for the experiments. Various doses of AAV-FhPI and AAV-GFP were injected into portal vein of diabetic rats and their hypoglycemic effects were investigated. At 3 day post-AAV-FhPI treatment, blood glucose levels of AAV-FhPI treated rats in fed state turned to 84 mg/dl - 368 mg/dl, and 20 mg/dl - 121 mg/dl in 6 hr fasting state contrasting AAV-GFP injected rats consistently showed hyperglycemic levels (486 $\pm$ 8.2 mg/dl < BG < 543 $\pm$ 4.5 mg/dl) in both states. The fasting blood glucose levels were traced for 3 months to observe the long-term insulin gene expression by adeno-associated virus treatment (Figure 14). Also serum insulin levels were measured 10 days interval after single injection. Serum insulin levels were 71 uIU/ml, 40 uIU/ml, 18 uIU/ml in average from 2×10^{12}, 1×10^{12}, 5×10^{11} particles of AAV-FhPI injected rats respectively contrasting that insulin levels were noted below 1.5 uIU/ml in AAV-GFP injected rats (Figure 15).

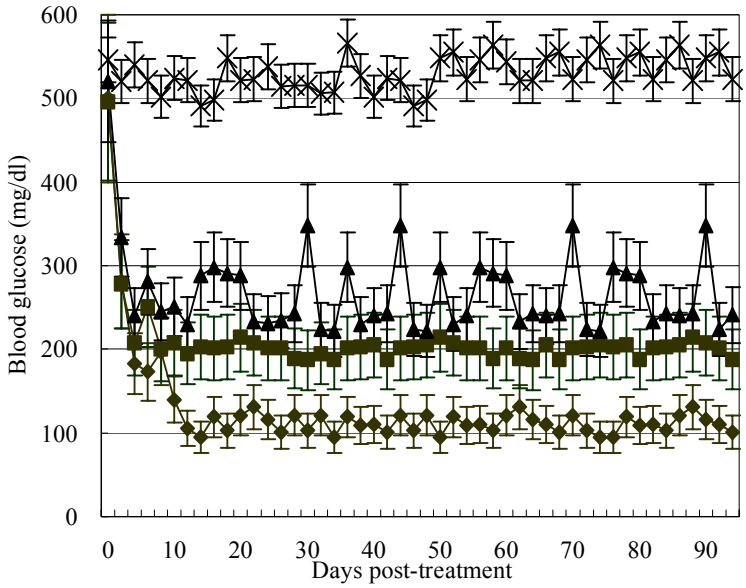

Figure 14. Blood glucose levels in AAV-FhPI treated rats. Various doses of recombinant AAV-FhPI, 2 x 10^{12} (◆, n=4), 1 x 10^{12} (■, n=4), and 5 x 10^{11} particles (▲, n=4) were injected into portal vein of STZ-induced diabetic rats and blood glucose levels were measured in fasting state. The fasting blood glucose levels of AAV-GFP treated group (x, n=4) were reported and compared with AAV-FhPI treated group. Especially the blood glucose levels of 2 x 10^{12} turned to euglycemic range at 3 day post-AAV injection and maintained over 3 months. Values are expressed as the means $\pm$ S.D.

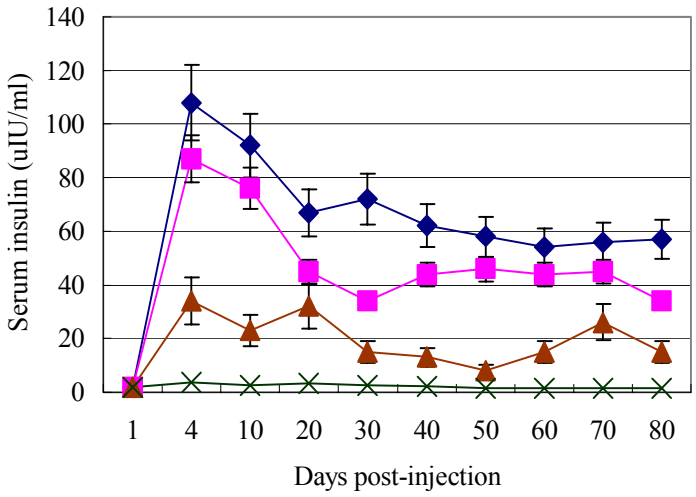

Figure 15. Long-term insulin secretion by AAV-FhPI treatment. Various doses of recombinant AAV-FhPI particles (♦, 2 x 10^{12}; ■, 1 x 10^{12}; ▲, 5 x 10^{11}) and 1 x 10^{12} particles of AAV-GFP were administered in diabetic rats. Serum insulin levels were measured 10 days interval after single injection of AAV-FhPI and AAV-GFP. Basal level of insulin was significantly increased in dose-dependent manner by AAV-FhPI treatment comparing with AAV-GFP treatment ($P < 0.05$ vs. AAV-GFP control).

11. Improved glucose tolerance by AAV-FhPI treatment

The ability of glucose clearance was completely improved like as normal animals exhibited (Figure 16). To study the efficacy of blood glucose disposal, intraperitoneal glucose tolerance test (IPGTT) was performed at 3 day post-adeno associated virus injection. Withdrawal of food for 6 hr from non-diabetic rats (n=4), AAV-GFP injected rats (n=4), and different doses of AAV-FhPI treated rats proceeded prior to intraperitoneal glucose (2 g/kg) loading. 2 x 10^{12} particles and 1 x 10^{12} particles of AAV-FhPI treated rats showed the normalized glucose tolerance comparing that AAV-GFP injected rats showed invariable hyperglycemic profiles. Their elevated blood glucose levels after the glucose loading returned to the pre-loading levels within 2 hr and their profile approximated that of non-diabetic controls in 2 x 10^{12} particles and 1 x 10^{12} particles of AAV-FhPI treated rats. In contrast, AAV-GFP injected diabetic controls showed over 450 mg/dl of blood glucose levels during the whole glucose tolerance test.

To observe serum insulin levels during the blood glucose disposal, blood was collected from tail vein every 30 min during the glucose tolerance test in normal rats, STZ-diabetic rats, AAV-GFP treated rats and 2 x 10^{12} particles of AAV-FhPI treated rats (Figure 17). Challenged glucose was reflected in the increasing serum insulin levels in normal rats until 30 min (from 11.82 $\pm$ 4.5 uIU/ml to 74 $\pm$ 1.5

uIU/ml) but inversely in the slight reduction of serum insulin levels in AAV-FhPI treated group (from 42.52 $\pm$ 3 uIU/ml to 32.58 $\pm$ 3.8 uIU/ml). For the proceeding 60 min, the elevated insulin levels in normal rats were decreased following glucose sensing regulatory system in the body and returned to pre-challenging level (from 24. $\pm$ 1.5 uIU/ml to 14.39 $\pm$ 2.3 uIU/ml) but in AAV-FhPI treated group, constitutive insulin production by adeno-associated virus was maintained irrelevantly to the glucose disposal in absence of external glucose input. In contrast, serum insulin levels in AAV-GFP treated rats were not significantly changed for those 2 hr (2.0 uIU/ml – 2.5 uIU/ml) and also STZ-diabetic rats exhibited constant low insulin levels during whole test (1.5 uIU/ml – 1.5 uIU/ml) and serum insulin was barely detectable in both groups.

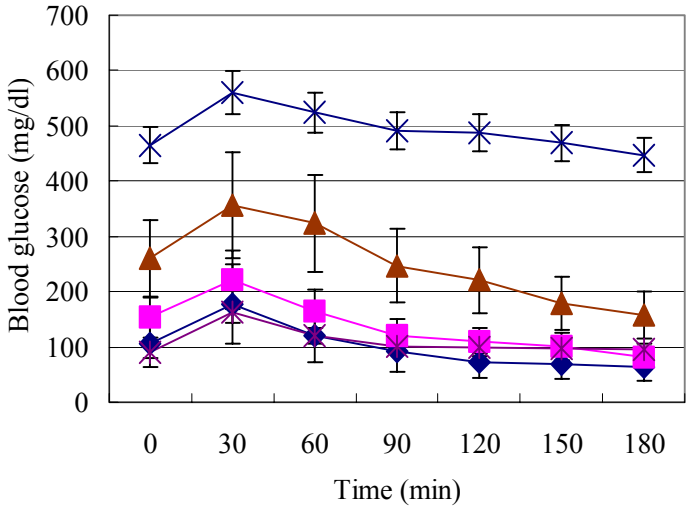

Figure 16. Blood glucose profiles in a intraperitoneal glucose tolerance test of AAV-FhPI injected rat. Glucose was loaded intraperitoneally in non-diabetic rats (∗, n=4), AAV-GFP injected rats (x, n=4), and three different doses of AAV-FhPI treated rats (♦, 2 x 10^{12}; ■, 1 x 10^{12}; ▲, 5 x 10^{11}, n=4 in each group). The blood glucose levels were determined at the indicated time intervals. Values are expressed as the means ± S.D.

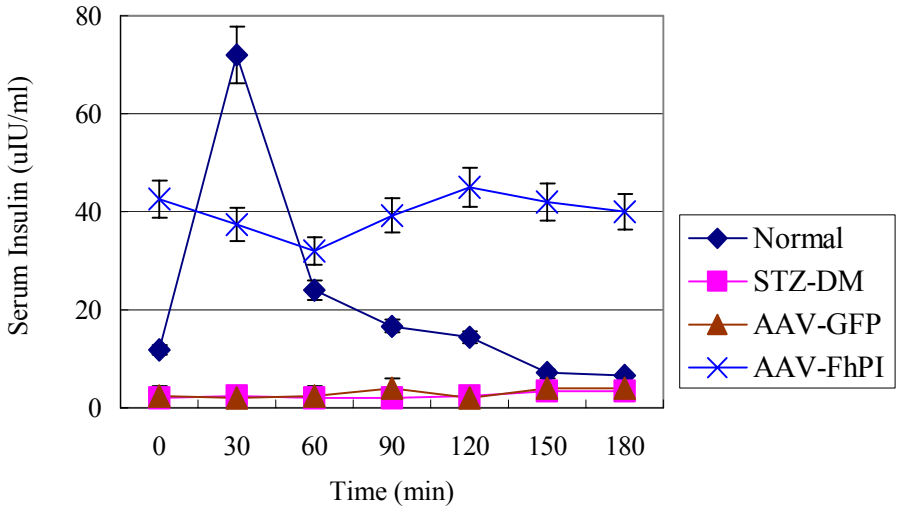

Figure 17. Serum insulin levels in a intraperitoneal glucose tolerance test of AAV-FhPI injected rat. Serum insulin levels were measured in normal (◆), STZ-diabetic (■), AAV-GFP treated (▲) and AAV-FhPI treated (x) rats during the IPGTT. Insulin levels in AAV-FhPI treated rat were invariably higher that those of STZ-diabetic and AAV-GFP groups during whole test hour. Values are expressed as the means ± S.D.

12. RT-PCR analysis with liver-tissue derived mRNA in AAV-FhPI treated rats

At 8 day post-treatment, total liver RNA was prepared from rat which was administered with 2 x 10^{12} particles of AAV-FhPI and of which the blood glucose levels was significantly lowered post-treatment. And liver RNA was extracted from rat that was administered with 2 x 10^{12} particles of AAV-GFP, non-diabetic rat, and STZ-diabetic rat for the controls. The observed comparable signal for an invariant β-actin indicated that an approximately equal amount of starting cellular material was used from each tissue sample in PCR. Representative profile of insulin signal in indicated tissues of normal control, STZ-diabetic control, one AAV-GFP treated diabetic and one AAV-FhPI treated was shown (Figure 18A). The results of PCR amplification clearly showed that presence of human insulin mRNA was specifically in liver tissue of AAV-FhPI injected rats. Also the insulin mRNA expression in 3.2 x 10^{12}, 2 x 10^{12}, 1 x 10^{12} and 5 x 10^{11} particles of AAV-FhPI injected rats showed a dose-dependent results (Figure 18B). The amplified PCR product was used for sequence analysis and furin-cleavage human proinsulin gene was confirmed by nucleotides sequence analysis.

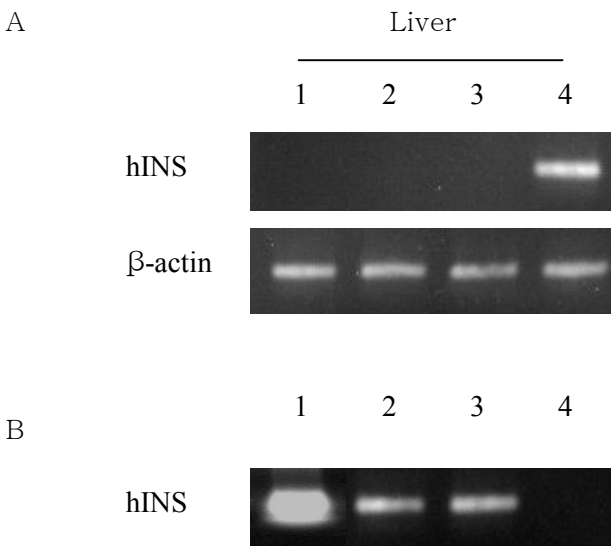

Figure 18. Detection of human insulin mRNA in AAV-FhPI treated rats. Liver RNA was extracted from normal rat, STZ-diabetic rat, AAV-GFP injected rat, and AAV-FhPI injected rat. RT-PCR was performed with sequence specific primer. PCR amplification was produced with cDNA derived from mRNA of AAV-FhPI injected rat and no product was exhibited in other control groups. A. lane 1, normal rat; lane 2, STZ-diabetic rat; lane 3, AAV-GFP injected rat; lane 4, AAV-FhPI injected rat. B. Lane 1, 3.2 x 10^{12}; lane 2, 2 x 10^{12}; lane 3, 1 x 10^{12}; lane 5 x 10^{11} particles of AAV-FhPI injected rat.

13. Northern blot analysis in AAV-FhPI treated rats

At 14 day post-AAV treatment, total RNA was extracted from various tissues of 2 x 10^{12} particles of AAV-FhPI injected rat and northern blotting was performed and compared with AAV-GFP injected control rat (Figure 19). The observed quantitative RNA of an invariant 28S indicated that an approximately equal amount of starting cellular material was used from each tissue sample in northern blot analysis. Total RNA extracted from stomach, heart, kidney, spleen, muscle, fat, brain and testis in both AAV-FhPI and AAV-GFP injected rat were used in analysis to examine whether exogenous proinsulin gene expressed in other tissues. Specific region of proinsulin gene was used for probe and was used for the standard negative. It was found that exogenous proinsulin gene was expressed specifically in liver of AAV-FhPI injected rat but was not detectable in other tissues or any tissue of AAV-GFP injected rat.

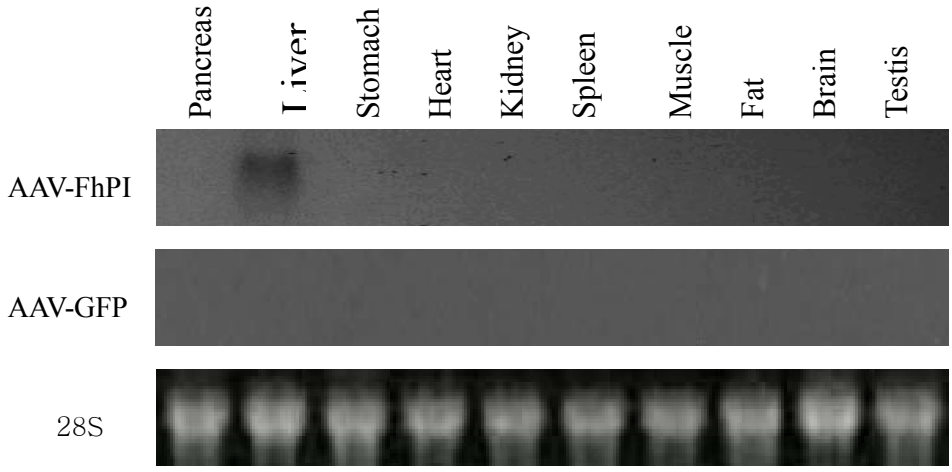

Figure 19. Northern blot analysis in AAV-FhPI injected rat. RNA was extracted from each tissue of 2 x 10^{12} AAV-FhPI treated rat and northern blotting was performed and compared with AAV-GFP treated rat. Exogenous proinsulin gene was expressed specifically in liver of AAV-FhPI treated rat and was not detected in other tissues or any tissue of AAV-GFP treated rat.

14. Insulin immunohistochemical staining in AAV-FhPI treated rats

Immunohistochemistry was performed in liver and pancreas tissues of 2 x 10^{12} particles of AAV-FhPI administered rats and compared with non-diabetic control rats (Figure 20). Immunohistochemical studies revealed that in response to STZ treatment, pancreatic β-cells were mostly destroyed in AAV-FhPI treated rat (that remained normoglycemic) and diabetic rat (that remained hyperglycemic). Pancreatic insulin staining in normal control rat was indexed to compare. The countable number of islets in normal pancreas was 19 $\pm$ 2 islets/100mm^2 compared with 4 $\pm$ 1 islets/100mm^2 in AAV-FhPI treated rats and diabetic control rats (data not shown). By contrast, hepatic insulin staining in AAV-FhPI treated rat showed the positive result comparing that insulin immunostaining in normal rat liver showed completely negative. Exogenous insulin produced by adeno-associated virus injection was detected in whole/parts of liver tissue and also it was observed that insulin positive-stained regions were mainly distributed around veins. Positive-stained regions were harbored in cytoplasm and around nuclear membrane of hepatocytes. Comparing with normal rat liver tissue, immunohistochemical data of AAV-FhPI treated rats proved that positive-stained insulin was produced by transduced hepatocytes by AAV-FhPI. And hepatic insulin secretion lowered blood glucose levels in AAV-FhPI treated rats because immunohistochemical data of

pancreas noted that most of the islets were destroyed by streptozotocin and very few islets were poorly stained against insulin antibody.

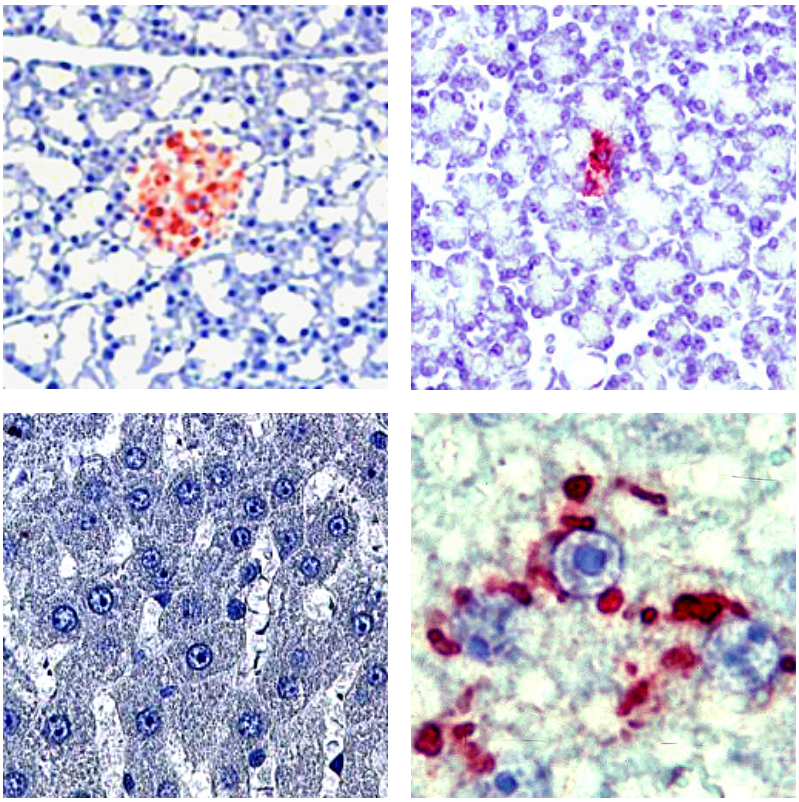

Figure 20. Insulin immunostaining in AAV-FhPI treated rat. Most of pancreatic islets in AAV-FhPI treated rats were destroyed and the size was decreased comparing with normal islets. Insulin was detected specifically in liver of AAV-FhPI treated rat. A, normal pancreas (x 200); B, pancreas of AAV-FhPI treated rat (x 200); C, normal liver (x 200); D, liver of AAV-FhPI treated rat (x 1000).

15. Reduced hemoglobin A1c levels in AAV-FhPI treated rats

To verify the basal insulin produced by furin-cleavage human proinsulin gene expression influenced the qualified fasting blood glucose management after AAV-FhPI treatment, blood hemoglobin A1c levels were measured in non-diabetic group (n=4), diabetic group (n=4), AAV-GFP injected group (n=4), 1 x 10^{12} AAV-FhPI treated group (n=4), and 2 x 10^{12} AAV-FhPI treated group (n=4) at 90 day post-treatment. HbA1c levels were 3 %, 8.0 %, 7.9 %, 4.9 % and 3.9 % in non-diabetic, diabetic, AAV-GFP injected, 1 x 10^{12} AAV-FhPI treated and 2 x 10^{12} AAV-FhPI treated groups respectively. As shown in Figure 21, HbA1c levels were elevated in diabetic group and AAV-GFP injected group comparing with non-diabetic group. However, single treatment of AAV-FhPI significantly reduced the blood HbA1c concentrations covering a 3-month period in contrast to AAV-GFP injection and 2 x 10^{12} particles treatment further reduced the blood HbA1c concentration to near-normal level.

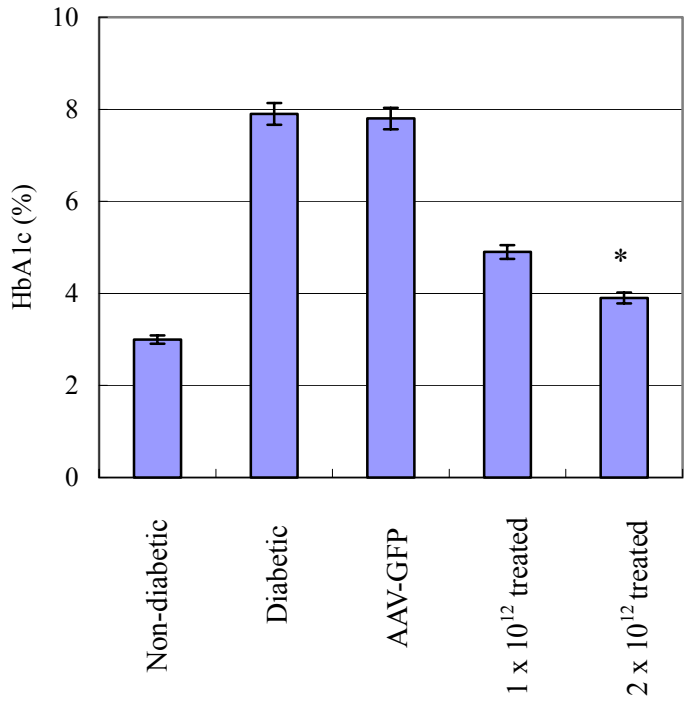

Figure 21. Improved HbA1c levels in AAV-FhPI treated rats. HbA1c levels were decreased in AAV-FhPI treated groups in dose-dependent manner and 2 x 10^{12} particles treated group showed the significantly ameliorated blood glucose management comparing with diabetic and AAV-GFP controls. Values are expressed as the means $\pm$ S.D. * $p < 0.05$ vs. diabetic and AAV-GFP treated.

IV. DISCUSSION

Therapeutic gene construct has been generated with the CMV promoter containing modified human proinsulin properly processed by the endogenous protease furin. This gene construct expressed insulin using adenoviral and adeno-associated viral vectors. The biological active insulin normalized fasting blood glucose level and improved glucose tolerance in STZ-induced diabetic rats. Especially the data of adeno-associated virus treatment demonstrated that modified human proinsulin gene expression resulted in a long-term normoglycemia in fasting state and a marked increase in plasma levels of insulin. The significantly ameliorated blood glucose profiles conferred profound physiological benefits in diabetes reflecting glucose tolerance improvement and the decreased blood HbA1c concentrations.

The lowering of blood glucose in diabetic rats subjected to gene therapy was caused by human insulin produced in liver and was not the consequence of remaining pancreatic cells resulting from an incomplete destruction. Immunohistochemical data confirmed that pancreatic islets were destroyed by streptozotocin in adenovirus and adeno-associated virus treated animals showing reduction of countable number and size of islets in insulin gene treated animals (Figure 12, 20).

The intraperitoneally administered glucose led to clear improvement in glucose tolerance in adenovirus-FhPI and AAV-FhPI treated rats (Figure 7, 16). Those data were related to the significantly increased insulin levels in adenovirus-FhPI and AAV-FhPI treated animals, indicating that constitutive insulin gene therapy elevated basal insulin secretion (Figure 8, 17). These results were consistent with the data reported glucose intolerance and impaired insulin response in diabetic state.[56-57] Both adenovirus-FhPI treated and AAV-FhPI treated groups showed a substantial rise in glucose levels in response to glucose load, resulting in normal glucose tolerance curves. Especially high and constant serum insulin levels during intraperitoneal glucose tolerance test in AAV-FhPI treated animals proved the increased basal insulin level by constitutive insulin gene therapy significantly ameliorated blood glucose profiles physically related with glucose tolerance improvement.

In both type 1 and a part of type 2 diabetes, insulin deficiency is accompanied by hepatic gluconeogenesis [58] and the elevated glucagons secretion results in severe hyperglycemia [59-61] and it requires at least twice-daily insulin injections.[62-63] Fasting hyperglycemia fundamentally caused by shortage of basal insulin plays a key role for developing long-term complications in diabetes.[64-65] In present study, furin-cleavage human proinsulin gene was delivered and expressed in non-β cells to supply insufficient basal insulin. Non-β cells are being considered

for the expression of insulin due to concerns about autoimmune destruction of islets Langherhans. Especially liver is considered as an ectopic organ for insulin gene expression among the non-β cells because it is a major target organ of insulin action and plays an important role in glucose homeostasis.[66] In this study, hepatic gene expression increased circulating plasma levels of insulin and lowered fasting blood glucose. And those data demonstrated that the secretion of basal insulin not only recovered fasting blood glucose levels to normal or near-normal but also decreased glycosylated hemoglobin concentration reflecting controlled blood glucose (Figure 14, 21).

Gene therapy using furin-cleavage proinsulin by various vectors and target organs has been previously tried. Viral and non-viral vectors have been used for insulin gene delivery in the different tissues including muscle, pituitary, submandibular gland and exocrine pancreas and in the different ways including subcutaneous, intravenous, intramuscular and intraportal injection.[67-70] However, the results from adeno-associated virus mediated constitutive gene expression in liver and the normalized glucose tolerance have not been obtained. Also portal vein injection helped the exogenous gene to be expressed in liver specifically. Those data were consistent with the notion that cells in liver were transduced by adeno-associated virus and the transduced hepatocytes induced a long lasting and comprehensive human proinsulin gene expression. Despite post-prandial demand

of insulin after meals was still inadequate in constitutive gene therapy, relatively abundant ectopic human proinsulin gene expression in liver was significantly effective in preventing fasting hyperglycemia in type 1 diabetic animal model in this research.

Recombinant adenoviral vector is useful tool for gene delivery. It has been characterized, relatively easy to produce and efficiently expressed transgenes. However, transgene expression was transient and immune responses with host are main difficulties. Adeno-associated virus seems to be an optimal tool used for gene delivery *in-vivo* for the purpose of inducing a long-term exogenous gene expression. Importantly, present study demonstrated that simplified procedure for the adeno-associated virus production was contributed in acquisition of high tittered adeno-associated virus and high level of infectivity. Primary viral solution after harvesting and centrifugation stored relatively high activity and limited loss without exposing to high concentration of salt. At this point immune incompatibility still remains in subjects between the amount of variability and cellular immune responses. The detailed immune responses and inflammatory reactions derived from primary recombinant adeno-associated virus are needed to trace the sustained gene expression at the same time survival rates. Additional studies are needed to fully align the tight index between produced amount of insulin and administrated adeno-associated viral dosage and possibly additional

proinsulin processing by liver furin is to be statistically calculated.

A number of studies have addressed to derive glucose-sensitive insulin production by driving insulin gene expression with a variety of glucose-dependent promoter elements.[71-73] However, achieving glucose-dependent insulin release continues to limit clinical application owing to the narrow therapeutic window of insulin gene expression for the treatment of diabetes. The dual system of glucose-stimulated as well as self-limited insulin production with a set of basal insulin providing gene therapy is to be helpful to develop the controlling diabetes. Furthermore, the therapeutic potential of this approach would be realized through the study on regulating insulin secretion, for example, regulating promoter-based gene expression with glucose/insulin responsible elements.

V. CONCLUSION

This study showed that adeno-associated virus mediated furin-cleavage human proinsulin gene therapy in liver can ameliorate fasting hyperglycemia in streptozotocin-induced diabetic rodent. Gene therapy using various routes and methods of furin-cleavable insulin gene delivery has been previously explored but, using adeno-associated viral vector, results comparable to this study have not been reported. Long-term constitutive secretion of insulin by intraportal vein injection of recombinant adeno-associated virus in liver provides a potential approach to gene therapy for diabetes mellitus. And also this study demonstrates *in vivo* basal level insulin secretion leads to fasting euglycemia, an improved glucose tolerance, and decreased blood HbA1c concentration over time. Thereby it supports the feasibility of constitutive gene therapy for treatment of type 1 diabetes mellitus and facilitates the development of novel strategies for gene therapy of type 2 diabetes. In further studies, a mechanism for regulating insulin secretion will be necessary to realize the therapeutic potential of this approach.

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ABSTRACT (IN KOREAN)

바이러스 벡터를 이용한 당뇨백서 간에서의 인슐린 유전자 발현

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당뇨병을 치료하기 위하여 여러 가지 방법들이 시도되어 오고있으나 만족스럽지 못한 실정이다. 현재로서는 식후의 혈당을 조절하기 위한 방법과 기저치의 인슐린을 공급하는 방법을 적절하게 조합하여 시행되고 있다. 이 연구는 기저치의 인슐린을 제공하기 위하여 실행하고 있는 하루 두세 번 정도의 인슐린 주사법을 대신하여 유전자 요법으로 일정한 양의 인슐린을 공급하는 것을 목적으로 하였다. 구조적인 프로모터하에 퓨린에 의해 잘려지는 휴먼 프로인슐린을 아데노 바이러스와 아데노 수반 바이러스 벡터를 이용하여 스트렙토조토신에 의해 유도된 당뇨백서의 간문맥에 주입하고 혈당변화와 인슐린의 발현을 측정하였다. 아데노 바이러스를 이용한 유전자요법으로 혈액내의 인슐린농도를 증

가시켜 당뇨백서에서 공복시의 고혈당을 개선하였고 아데노 수반 바이러스를 이용한 유전자 요법에 의해 3 개월이상 고혈당 상태에 있던 당뇨백서의 공복혈당이 정상으로 변화되었으며 당 분해능력과 당화 헤모글로빈 농도가 현저히 개선되었다. 유전자 요법이 실행된 백서에서 공복시의 혈중 인슐린의 농도 또한 일정하게 증가되어 기록되었다.

치료된 백서의 간에서 추출한 mRNA를 이용한 역전사 효소 중합효소 연쇄반응 (RT-PCR) 과 연쇄반응의 생산물의 뉴클레오타이드 분석을 통해 전달된 휴먼 프로인슐린 유전자의 발현을 확인하였다. 유전자 치료된 당뇨백서의 간조직과 췌장조직에서 면역조직염색법을 통하여 스트렙토조토신으로 췌도가 대부분 파괴되었음을 확인하였고 간에서 생성된 인슐린에 의하여 혈당강하의 효과가 나타났음을 보여주었다. 치료된 백서의 여러 가지 조직에서 추출한 RNA를 가지고 노던 블러팅을 실행한 결과 주입된 유전자의 발현이 간 조직에서 이루어졌음을 보여주었다. 이상의 결과로 구조적인 프로모터를 이용한 유전자 요법의 실행은 식후 혈당의 변화를 조절하기에는 제한적이었으나 일정한 양의 인슐린을 발현하고 공급함으로써 공복 상태의 고혈당을 개선하고 혈중 인슐린의 양을 증가시키는 것에 성공적인 결과를 보여주었다. 또한 아데노 수반 바이러스 벡터를 이용한 유전자 치료법은 한번의 주입으로 오랜 기간동안 외부 인슐린 유전자를 발현함으로써 안정적이고 비교적 안전한 유전자 치료법임을 시사하였다.

핵심되는 말 : 유전자 치료, 당뇨병, 퓨린, 인슐린, 간, 고혈당