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ARTICLE *in* MOLECULAR PHARMACEUTICS · NOVEMBER 2008

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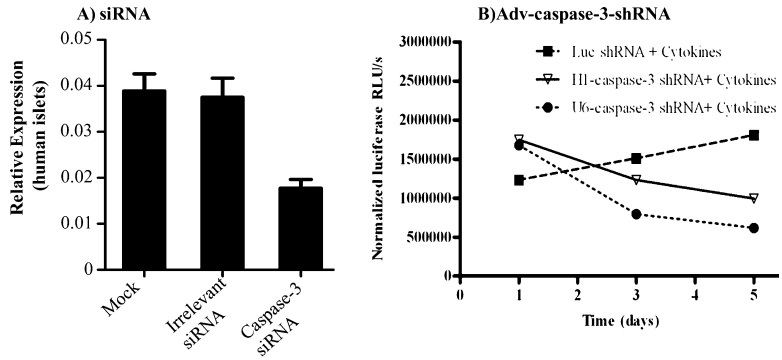
Mol. Pharmaceutics, **2008**, 5 (6), 1093-1102• DOI: 10.1021/mp800093f • Publication Date (Web): 02 October 2008



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Caspase-3 Gene Silencing for Inhibiting Apoptosis in Insulinoma Cells and Human Islets

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Received July 16, 2008; Revised Manuscript Received September 18, 2008; Accepted September 19, 2008

Abstract: Although islet transplantation has great potential to treat type I diabetes, most islet grafts do not function due to the host immune rejection, nonspecific inflammatory response and poor revascularization. Since caspase-3 plays a crucial role in apoptosis of transplanted islet cells, we used chemically synthesized small interfering RNAs (siRNAs) to silence caspase-3 in insulinoma (INS-1E) cells and human islets, and then determined whether caspase-3 gene silencing can prevent these cells from cytokine-induced apoptosis. Transfection of INS-1E cells and islets with siRNAs reduced caspase-3 transcripts by 50−67% and 50%, respectively. Additionally, apoptosis in transfected insulinoma cells was markedly inhibited. Since gene silencing did not last beyond two days, we converted potent siRNA into shRNA and constructed replication deficient adenoviral (Adv) vectors encoding these shRNAs driven by a U6 or H1 promoter. Compared to chemically synthesized siRNA, Adv-caspase-3-shRNA efficiently transduced islets, showed relatively higher and prolonged levels of gene silencing beyond five days, with higher gene silencing with a U6 promoter, and protected islets from cytokine-induced apoptosis. Finally, return to normoglycemia was achieved at 1 day post-transplantation of Adv-caspase-3-shRNA transduced islets under the kidney capsules of streptozotocin induced nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice and maintained beyond two weeks. Blood glucose levels returned to ≥325 mg/dL upon removal of the islet graft-bearing kidney at 32 days after transplantation, confirming that transplanted islets were functional.

Keywords: Caspase-3; gene silencing; apoptosis; insulinoma cells; human islets

Introduction

Human islet transplantation following the Edmonton protocol has the potential to replace pancreatic endocrine function in type 1 diabetic patients. Successful islet transplantation offers the advantages of attaining normal or near normal blood glucose levels without predisposing the patient to the risks of severe hypoglycemia associated with intensive insulin therapy.[1] Although significant progress has been made in this treatment modality, most recipients return to insulin therapy because up to 70% of transplanted islets undergo apoptosis within the first week post-transplantation.[2−9]

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10.1021/mp800093f CCC: $40.75
Published on Web 10/02/2008

Therefore, success of islet transplantation greatly depends on minimizing apoptotic cell death of islet grafts during this period.

Programmed cell death is a cascade of events leading to upregulation of caspases resulting in apoptosis through intrinsic and extrinsic pathways. Caspase-3 is a converging point of these apoptotic pathways,[10] and its peptide inhibitors have been shown to prevent islet apoptosis and improve graft function in a dose dependent manner.[4,5] Montolio et al.[11] demonstrated that incubation of islets with 500 μM caspase-3 inhibitor, *N*-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD.fmk) reduced islet apoptosis and lowered blood glucose level compared to the control and 200 μM z-VAD.fmk-treated groups when these islets were transplanted into streptozotocin-induced diabetic C57BL/6 mice. Shapiro and associates[2] also demonstrated significant improvement in islet function in renal subcapsular transplantation, where 90% of z-VAD.fmk-treated mice became euglycemic versus 27% of the control animals. The benefit of z-VAD.fmk therapy was further demonstrated after intraportal transplantation, where 75% of zVAD.fmk-treated animals established euglycemia with only 500 islets, and all of the controls remained severely diabetic. A short course of z-VAD.fmk therapy could prevent metabolic dysfunction of islet grafts over time. In addition, short-term zVAD-FMK treatment significantly reduced post-transplant apoptosis in islet grafts and resulted in preservation of graft insulin reserve over time. Furthermore, Liadis et al. demonstrated that caspase-3 knockout (Casp3−/−) mice were protected from developing diabetes and their islets were resistant to apoptosis following streptozotocin administration.[12] Contrary to these findings, Aikin et al.[13] reported that incubation of human islets with z-VAD.fmk for 72 h resulted in increased necrosis and decreased cell viability in both human and canine islets. This discrepancy may be due to prolonged incubation of islets with z-VAD.fmk at high doses, resulting in toxic side effects of this caspase inhibitor to the islets.[4]

Previous studies indicated that infiltrating T cells in islets is also attributed to the dysfunction of islet grafts by secreting proinflammatory cytokine and inducing immune response. Furthermore, lymphocyte infiltration of islets was completely absent in Casp3−/− mice and caspase-3 is required for activation and proliferation of T cells,[12] suggesting that down-regulation of caspase-3 can deactivate T-cell mediated immune response.

Since caspase-3 plays a critical role in apoptosis of islet graft, in the present study we investigated the effect of caspase-3 gene silencing on apoptosis of insulinoma cells and human islets using chemically synthesized siRNAs against caspase-3. However, an islet is a cluster of 200−1000 cells, and introduction of exogenous nucleic acids into islets is known to be difficult.[14] Bain et al. also demonstrated efficient gene silencing after transduction of adenoviral (Adv) vector encoding shRNA in pancreatic islets.[15] Therefore, we constructed replication deficient Adv vectors encoding caspase-3 shRNA driven by different polymerase III promoter (H1 and U6) and evaluated their silencing efficiency in human islets. Finally, we determined whether ex vivo caspase-3 gene silencing improves the outcome of islet transplantation in nonobese diabetic severe combined immunodeficient (NOD-SCID) mice.

Materials and Methods

siRNAs. Chemically synthesized, double-stranded siRNAs targeting rat caspase-3 (NM_012922) and human caspase-3 (NM_004346, NM_032991) as well as a control siRNA were purchased from Ambion (Austin, TX). The most potent siRNA sequences for human caspase-3 and a nontargeting siRNA sequences were converted into shRNA.

Cell and Islet Culture and Transfection. Rat insulinoma (INS-1E) cells, a gift from Professor Claes B. Wolheim (University Medical Center, Geneva, Switzerland), were cultured as described by De Paula et al.[16] INS-1E cells were seeded at a density of 6×10^5 cells per 24-well plate. For human islet culture, human islets were sent from one of the Islet Cell Resource Centers (ICR) through ICR Services for Basic Science Application. On arrival at our facility, islets were cultured in 24-well plates (about 1000 islets per well) in CMRL 1066 medium (Cellgro, Manassas, VA) supplemented with 10% FBS and 100 μg/mL penicillin-streptomycin. Caspase-3 siRNAs and irrelevant siRNA were transfected into INS-1E cells and human islets after complex formation with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at doses of 150 nM and 400 nM, respectively. For some experiments, the transfected cells or human islets were added with the fresh medium containing cytokine cocktail composed of IL-1β (25ng/mL), TNF-α (5ng/mL) and IFN-γ (25ng/mL) (R&D System, Minneapolis, MN) and incubated for the indicated times.

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Real-Time RT-PCR. At the indicated time post-transfection/transduction, INS-1E cells or human islets were collected for determining caspase-3 gene silencing by real-time RT-PCR. Briefly, total RNA was isolated from the transfected and control INS-1E cells or islets with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. One microgram of RNA was added to a reverse transcript transcription reaction containing MultiScribe Reverse Transcriptase Reagent and random hexamers (Applied Biosystems, Foster City, CA) by incubation at 25 °C for 10 min, followed by reverse transcription at 48 °C for 30 min and enzyme inactivation at 95 °C for 5 min. PCR was performed with 1 μL of cDNA in a final volume of 10 μL containing 5 μL of SYBR Green master mixture (Roche, Basel, Switzerland) and 20 pmol of sense and antisense primers of caspase-3 (rat sense 5′-CAT GAC CCG TCC CTT GAA-3′ and antisense 5′-CCG ACT TCC TGT ATG CTT ACT CTA-3′; human sense 5′-CAG TGT TCT CCA TGG ATA CCT TTA TT-3′ and antisense 5′-CTG GTT TTC GGT GGG TGT-3′). To normalize the samples for RNA amount, a PCR reaction was also performed with primers of ribosomal proteins (rat s5 sense 5′-GCT TGC TCC CTA CGA TGA GA-3′ and antisense 5′-ACC CCG GAG GTA CAG GTG-3′; human s19 sense 5′- GAC TGA GAA GCC CGG TTT G-3′ and antisense 5′-CTT GAT GTC CGG GGT CTC T-3′). Real-time PCR was carried out in a Light Cycler 480 (Roche, Basel, Switzerland) using the following thermal cycling profile: 95 °C for 5 min, followed by 40 cycles of amplification (95 °C for 10 s, 58 °C for 30 s, 72 °C for 10 s). All samples were run in triplicate.

Caspase Activity. Since caspase-7 is the downstream of caspase-3 and plays an important role in apoptosis, we used Glo 3/7 assay kit (Promega, Madison, WI) to determine caspase activities in INS-1E cells and human islets. This assay kit provides a proilluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, which is cleaved to release aminoluciferin.[17] Briefly, INS-1E cells treated with siRNA were washed by PBS, and we added 80 μL of fresh medium without FBS to each well in 24-well plates; then we added 80 μL of Caspase-Glo 3/7 reagent to each well and gently mixed the content of the wells using a plate shaker at 400 rpm for 30 s. The plates were incubated at room temperature for 90 min to allow the reaction. Finally, 100 μL of reaction solution was measured using a luminometer (Berthold, Bad Wildbad, Germany). Protein concentration of each reaction solution was determined using the bicinchoninic acid (BCA) protein assay kit and Compat-Able protein assay preparation reagent set (Pierce, Rockford, IL) for normalizing luciferase. After transduction with Adv-caspase-3-shRNA and incubation at the indicated time, islets were first washed with PBS, and then spun at 200*g* for 5 min. The supernatant was removed. Two hundred microliters of hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Pefablock, and protease inhibitors) was added to each pellet and lysed using a homogenizer and subsequently centrifuged (15 min, 13,000 rpm, 4 °C). The protein concentration of supernatant was adjusted to 100 μg/mL with the extraction buffer. An equal volume of reagents was added to a tube and incubated at room temperature for 1 h. The luminescence of each sample was measured in a luminometer (Berthold, Bad Wildbad, Germany).

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling Assay (TUNEL). INS-1E cells after transfection with caspase-3 siRNAs and irrelevant siRNA were incubated with the cytokine cocktail for additional 12 h. β-Cell apoptosis was detected by DeadEnd Colorimetric TUNEL system (Promega, Madison, WI) according to the manufacture's manual.

Construction of Adenoviral Vectors Encoding shRNAs and Transduction. Adv-caspase-3-shRNAs were generated using the Knockout RNAi system (Clontech, Mountain View, CA). One shRNA specific for human caspase-3 using different promoters (U6 and H1), named as H1-caspase-3 shRNA and U6-caspase-3 shRNA (H1-caspase-3 shRNA sense 5′ GCG GAT CCG GGG TAC TTT AAG ACA TAC TTT CAA GAG AAG TAT GTC TTA AAG TAC CCC TTT TTT AAG CTT CG 3′, H1-caspase-3 shRNA antisense 5′CGA AGC TTA AAA AAG GGG TAC TTT AAG ACA TAC TTC TCT TGA AAG TAT GTC TTA AAG TAC CCC GGA TCC GC 3′; U6-caspase-3 shRNA sense 5′ GCG GAT CCG GGG TAC TTT AAG ACA TAC T TTC AAG AGA AGT ATG TCT TAA AGT ACC CCT TTT TTG AAT TCG C3′, U6- caspase-3 shRNA antisense 5′ GCG AAT TCA AAA AAG GGG TAC TTT AAG ACA TAC TTC TCT TGA AAG TAT GTC TTA AAG TAC CCC GGA TCC GC 3′) were synthesized by IDT. These oligonucleotide pairs were annealed and inserted into *Bam*HI/*Hin*dIII sites of pRNAT-h1.1/Shuttle (GenScript, Piscataway, NJ) for H1-caspase-3 shRNA and *Bam*HI/*Eco*RI sites of pSIREN-shuttle (Clontech, Mountain View, CA) for U6-caspase-3 shRNA. Then, the recombinant shuttle plasmids were digested by I-*Ceu*I and PI-*Sce*I to dissect the shRNA expression cassette, and the excised shRNA cassettes were cloned into the pAdenovirus viral DNA (Clontech, Mountain View, CA). Recombinant plasmids were selected with antibiotic-resistant LB plate, and cloned regions were further verified by restriction analysis and DNA sequencing. The positive recombinant adenoviral DNAs were digested with *Pac*I to expose the inverted terminal repeat location. The linearized adenoviral DNAs with shRNA expression cassette were transfected into AD293 cells (Stratagene, La Jolla, CA) for producing adenoviral particles. Infectious titer of each viral lysate was determined by Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA) according to the manufacture's manual. About 600−1000 human islets were cultured in a well of 24-well plates in 1 mL of CMRL 1066 medium supplemented with 10% FBS and 100 μg/mL penicillin-streptomycin. Then islets were transduced with Adv-caspase-3-shRNA for 18 h at a dose of 2.8×10^4 pfu/islet, then

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Table 1. Small Interfering RNA (siRNA) Sequences against Human and Rat Caspase-3

names	sequences	targeting region	RefSeq no.
human	sense 5′ GGGUACUUUAAGACAUACUtt3′	1203−1221	NM_004346
siRNA	antisense 5′ AGUAUGUCUUAAAGUACCCtc3′	1036−1054	NM_032991
rat s1	sense 5′GCAGUUACAAAAUGGAUUAtt3′	160−178	NM_012922
siRNA	antisense 5′UAAUCCAUUUUGUAACUGCtg3′		
rat s2	sense 5′CCUUACUCGUGAAGAAAUUtt3′	326−344	NM_012922
siRNA	antisense 5′AAUUUCUUCACGAGUAAGGtc3′		
rat s3	sense 5′GGAGUAAUUUUUGGAACGAtt3′	429−447	NM_012922
siRNA	antisense 5′UCGUUCCAAAAAUUACUCCtt3′		

washed with phosphate-buffered saline (PBS), and had fresh medium added with or without the cytokine cocktail, and then were collected at the indicated days for determining caspase-3 gene silencing effect.

Western Blotting. At day 2 post-transfection of INS-1E cells and human islets with siRNA or at days 1, 3 and 5 post-transduction of human islets with Adv-shRNA, protein extracts were prepared by washing cells with PBS and lysing in RIPA buffer containing protease inhibitor (Roche, Basel, Switzerland). Human islets were homogenized and lysates were spun at 15000*g* for 15 min to remove insoluble material. Protein concentrations were determined by BCA protein assay. Proteins were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) (Bio-Rad, Hercules, CA) and transferred to a PVDP membrane (Bio-Rad, Hercules, CA), blocked with TBST buffer composed of 50 mM Tris (pH 7.6), 150 mM NaCl and 0.05% Tween supplemented with 5% fat-free milk at 4 °C for 1 h. After a brief rinse, the membrane was incubated overnight at 4 °C in TBST containing 0.5% milk with rabbit antihuman caspase-3 (Abcam, Cambridge, MA) (1:1000). Rabbit anti-human β-actin polyclonal antibody (Abcam, Cambridge, MA) (1:5000) was used for detecting an internal control protein. Then, blots was washed four times using TBST for 1 h at room temperature. Immunodetection was performed using a horseradish peroxidase (HRP) CHEMI Blot Starter Kit (Biorad, Hercules, CA) according to the manufacture's instruction.

Insulin Release Assay. In vitro islet function was determined by static incubation. Following transduction with Adv-shRNA or transfection with Lipofectamine 2000/siRNA complexes, islets were washed and incubated in 2.5 mM glucose containing media followed by sequential incubation in low (2.5 mM) and high (22 mM) glucose media at 37 °C for 1 h each. Supernatants were collected after incubation and insulin concentration was determined by ELISA (Alpco Diagnostics, Salem, NH). Ratio of the amount of insulin released at 22 mM glucose to that at 2.5 mM represents the stimulation index.

Islet Transplantation Studies. To induce diabetes, STZ (40 mg/kg) was administered to NOD-SCID mice by intraperitoneal injection for 5 consecutive days. We considered nonfasting blood glucose level less than 200 mg/dL as normoglycemia, and that above 325 mg/dL on two consecutive measurements as hyperglycemia. Before transplantation, human islets were transduced with Adv-caspase-3-shRNA at 1000 MOI for 18 h and washed with PBS. About 1200 infected islets and uninfected islets were transplanted into diabetic mice under the left kidney capsule. The nonfasting glucose levels were measured every day for the first 8 days post-transplantation and on alternating days thereafter using a Touch Ultra Glucometer (LifeScan, Inc.). In some animals, the graft-bearing kidneys were removed to confirm the function of islet grafts by the return to blood glucose levels to $\geq$325 mg/dL for two consecutive days.

Results

Caspase-3 Gene Silencing after Transfection with siRNA. To mimic the apoptotic dysfunction of islets after transplantation, INS-1E cells or human islets in most experiments were incubated with inflammatory cytokines consisting of IL-1β, TNF-α and IFN-γ. Upon incubation with the cytokine cocktail, caspase-3/7 activity has been significantly increased as determined by Caspase-Glo 3/7 Assay (Supplementary Figure 1 in the Supporting Information). Three different siRNA duplexes, named as s1, s2 and s3, were designed to target different regions of rat caspase-3 (NM_012922): start sites of 160−178, 326−344 and 429−447 (Table 1). As shown in Figure 1a, all three different siRNAs could trigger the suppression of caspase-3 at the transcription level as determined by real time RT-PCR. These siRNA duplexes showed varying degrees of caspase-3 gene silencing efficiency ranging from 53% to 72%. However, the irrelevant siRNA treatment did not significantly alter the levels of caspase-3 transcript. Additionally, the silencing effect was much better at 12 h of incubation with the cytokines than those at 24 h of incubation (Figure 1a). As siRNAs are presumed to act directly on the transcript of a target gene, we further examined RNAi effect on caspase-3 protein level using the polyclonal antibody to detect the precursor caspase-3 by Western blotting at 2 days after transfection. As shown in Figure 1b, transfection of s1 and s2 siRNA in INS-1E cell resulted in the reduction of precursor caspase-3 as compared to irrelevant siRNA.

To investigate siRNA effect on caspase-3 in human islets, one validated siRNA duplex (Table 1) was transfected in human islets. As shown in Figure 1c, treatment of human islets with caspase-3 siRNA led to 60% reduction in caspase-3 at the transcript level. We also observed that the

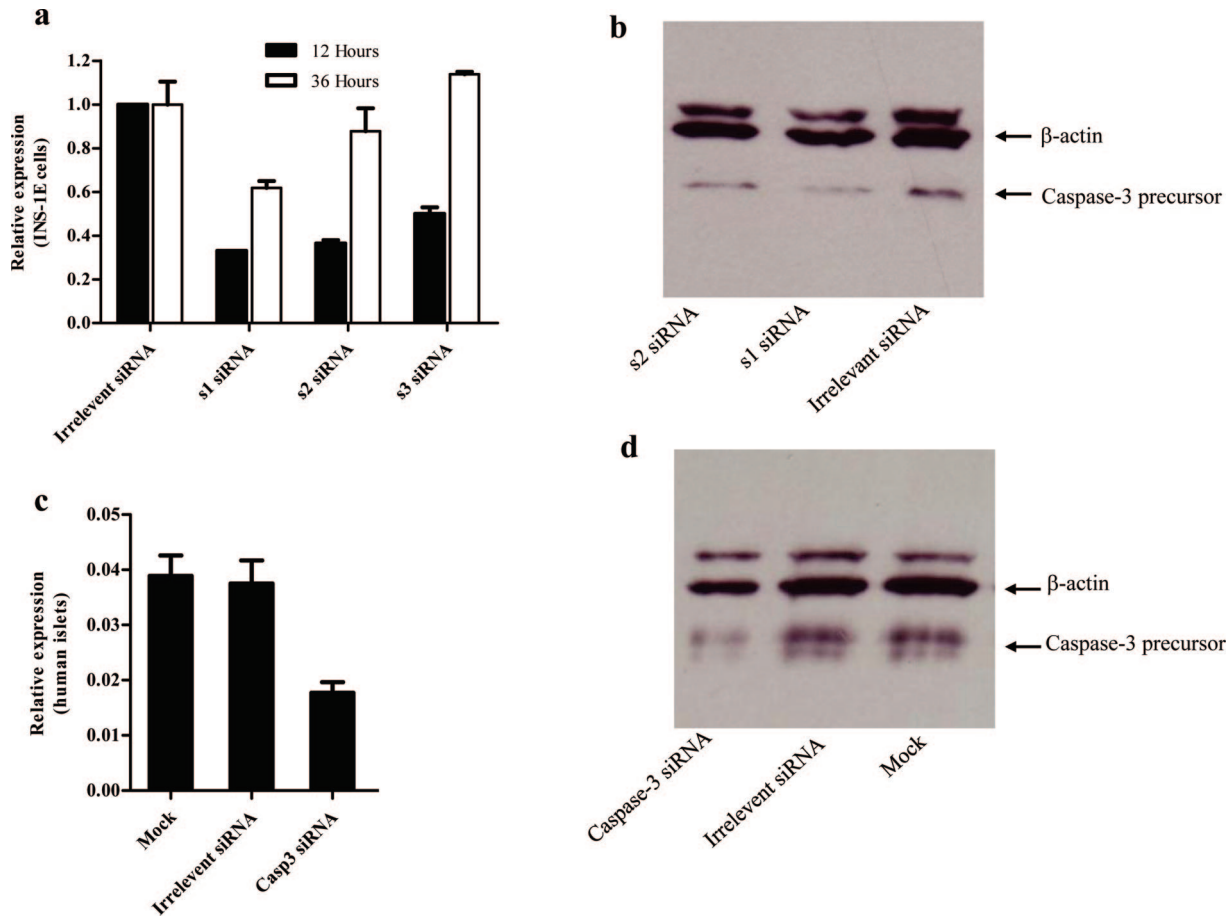

Figure 1. Effect of siRNA transfection on caspase-3 in INS-1E cells and human islets. (a) Caspase-3 gene silencing in INS-1E cells at the transcription level. INS-1E cells were transfected with siRNA/Lipofectamine 2000 complexes and incubated for additional 12 and 36 h with the cytokine cocktail. The total RNA was isolated from the transfected INS-1E cells, and the mRNA levels of caspase-3 were measured by real-time RT-PCR with normalization by the rat ribosomal protein s5. Error bar means the standard deviation based on the triplicates. (b) Western blot analysis at 2 days post-transfection for determining precursor caspase-3 protein expression after transfection of INS-1E cells with siRNA/Lipofectamine 2000 complexes. (c) Caspase-3 gene silencing at the transcript level in human islets. Following transfection with siRNA/Lipofectamine 2000 complexes, about 1000 islets were incubated for additional 24 h with the cytokine cocktail. Total RNA was isolated from the islet pellets for determining caspase-3 gene silencing by real-time RT-PCR with normalization by the human ribosomal protein s19. Error bar means the standard deviation based on the triplicate. (d) Western blot analysis for determining precursor caspase-3 protein expression at two days after transfection of islets with caspase-3 siRNA/Lipofectamine 2000 complexes.

expression of precursor caspase-3 at the protein level was significantly suppressed in human islets at 2 days after transfection with siRNA by Western blotting (Figure 1d).

Effect of Caspase-3 Gene Silencing on Apoptosis. Since caspase-3 is the converging point of apoptotic pathways, we determined whether siRNA targeting caspase-3 results in the reduction of caspase activity and consequent apoptosis. As shown in Figure 2, all three different siRNAs decreased caspase-3/7 activity by 45−38% in INS-1E cells. Among them, s1 siRNA showed the highest reduction in caspase activity. These observations were consistent with the results derived from real-time RT-PCR. Since apoptosis leads to nuclear DNA breakdown, we also examined the DNA fragments that resulted from apoptosis using the TUNEL assay. As shown in Figure 2b, nuclear DNA breakdown in situ was significantly decreased in the INS-1E cells with all three caspase-3 siRNAs treatment compared to that with irrelevant siRNA treatment. The result is in good agreement with the reduction in caspase-3/7 activity. To determine siRNA silencing effect on caspase-3/7 activity at different time points, s1 siRNA was transfected into INS-1E cells. As shown in Figure 2c, caspase-3/7 activity dramatically decreased at 24 h post-transfection, maintained this effect up to 48 h, then slowly increased, and was recovered completely after 96 h.

Islet Function after Caspase-3 Gene Silencing. The transfection process and caspase-3 gene silencing should not affect islet function. Therefore, we measured insulin secretion in response to glucose challenge after transfection with siRNA or transduction with Adv-U6-caspase-3-shRNA. The stimulation index (SI) was determined as a ratio of insulin secreted at higher (22 mM) glucose to that at lower (2.5 mM)

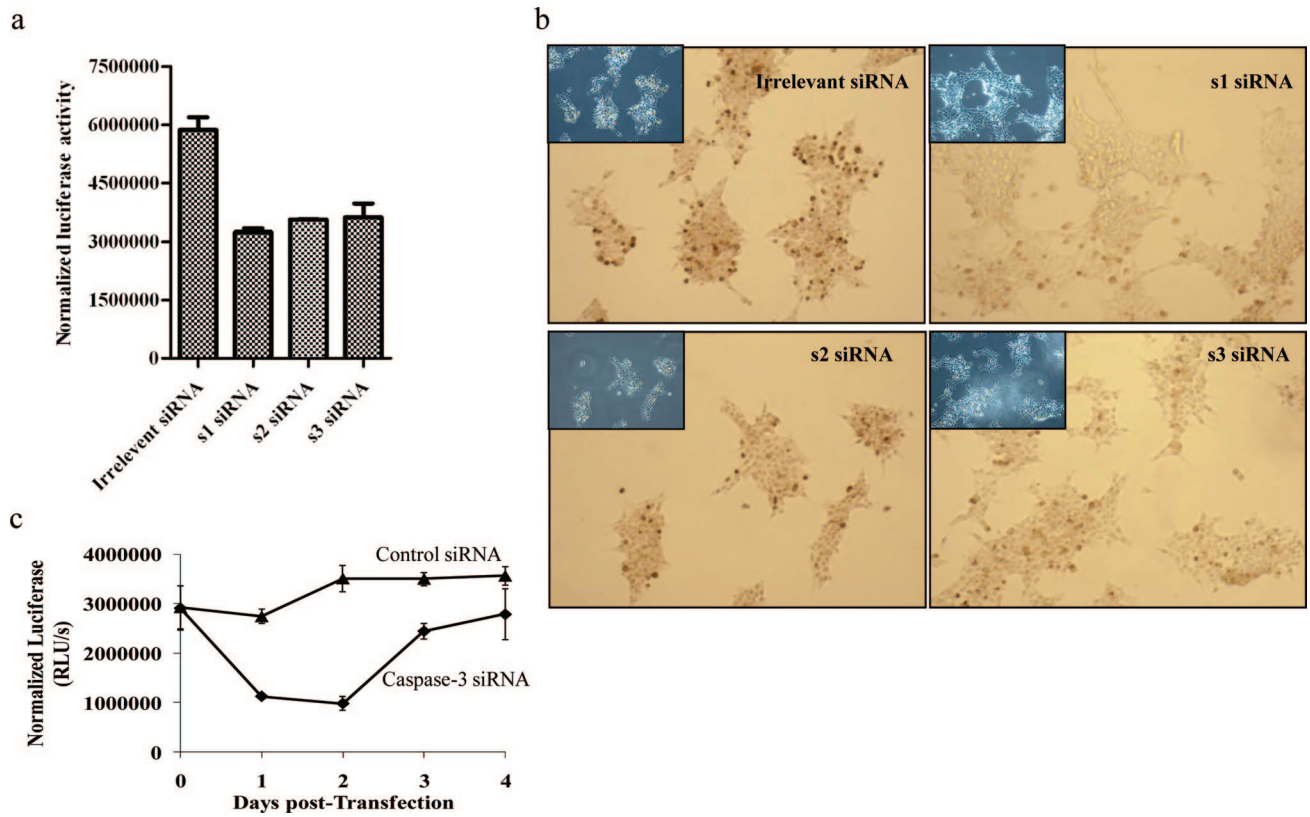

Figure 2. Effect of caspase-3 siRNAs silencing on caspase-3/7 activity and apoptosis in INS-1E cells. (a) siRNA silencing effect on caspase 3/7 activity. After transfection of INS-1E cells with siRNA/Lipofectamine 2000 complexes, the cells were incubated with the cytokine cocktail for additional 16 h. Then caspase 3/7 activity was determined by Caspase-Glo 3/7 Assay. Error bar means the standard deviation based on the triplicates. (b) Caspase-3 gene silencing inhibits apoptosis induced by cytokine cocktail in INS-1E cells as determined by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay. Brown staining represents the cells undergoing apoptosis. (c) Time course of s1 siRNA silencing effect on caspase-3/7 activity. Chemically synthesized s1 siRNA and control siRNA were transfected into INS-1E cells at a dose of 150 nM. Then the cells were collected at the indicated days for determining caspase-3/7 activity using Caspase-Glo 3/7 Assay. Error bar means the standard deviation based on the triplicates.

glucose concentration after siRNA transfection. There was no significant alteration in SI between the untreated human islets and caspase-3 siRNA transfected human islets (data not shown). Similar results were also obtained with the islets transduced with for Adv-U6-caspase-3-shRNA 1000 or 1500 MOI (data not shown). This result suggests that our process of siRNA transfection or Adv-shRNA transduction, and caspase-3 gene silencing in human islets did not affect islet function.

Construction and Transduction of Adenovirus Encoding shRNAs into Human Islets. Since an islet is a cluster of 200−1000 cells, introduction of exogenous nucleic acids into islets is known to be difficult and the duration of siRNA mediated silencing lasted only for two days, we constructed Adv-caspase-3-shRNA driven by a U6 or H1 promoter (Figure 3a) to improve the level and duration of caspase-3 gene silencing. Transduction efficiency was first determined using Adv-GFP. As shown in Figure 3b, there was strong fluorescence signal throughout the islet after transduction.

To determine caspase-3 gene silencing effect, human islets were transduced with Adv-H1-caspase-3-shRNA without incubation with inflammatory cytokines. Caspase-3 gene silencing at the transcription level was determined at days 1, 3 and 5 post-transduction. As shown in Figure 3c, transduction of human islets with Adv-caspase-3-shRNA led to significant reduction in caspase-3 mRNA ranging from 46% to 68% at days 3 and 5 as compared with that at day 1, but transduction with Ad-Luc shRNA did not significantly alter caspase-3 mRNA. We also determined caspase-3/7 activity by Caspase-Glo 3/7 assay after transduction with Adv-caspase-3-shRNA or Adv-control-shRNA. As shown in Figure 3d, transduction of Adv-H1-caspase-3 shRNA markedly decreased caspase-3/7 activity at days 3 and 5.

To compare the silencing effect of Adv-caspase-3-shRNAs driven by different promoters (H1 and U6), human islets were transduced with Adv-H1-caspase-3 shRNA, Adv-U6-caspase-3 shRNA, and Adv-Luc-shRNA at a dose of 2.8×10^4 pfu/islet followed by incubation with the cytokine cocktail. The infected islets were collected at days 1, 3 and 5 post-transduction for determining caspase-3/7 activity. As shown in Figure 3e, transduction with Adv-H1-caspase-3-shRNA and Adv-U6-caspase-3-shRNA resulted in significant reduc-

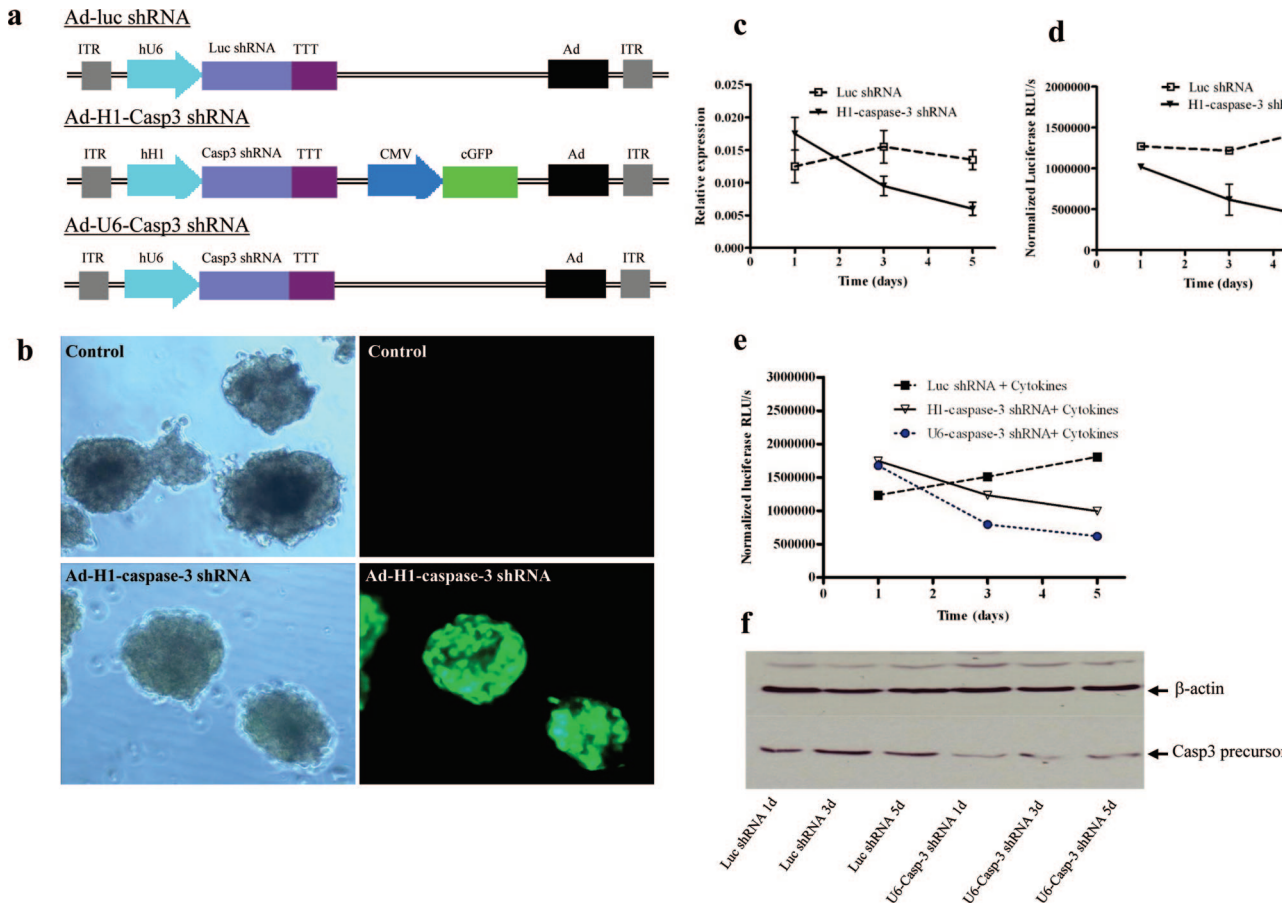

Figure 3. Schematic representation of recombinant adenoviruses and caspase-3 gene silencing after transduction with recombinant adenoviruses encoding shRNAs. (a) Schematic representation of recombinant adenoviruses encoding shRNAs. Ad-Luc shRNA was set as control; Adv-H1-caspase-3-shRNA encoding caspase-3 shRNA under a H1 promoter; Ad-U6-caspase-3 shRNA encoding caspase-3 shRNA under a U6 promoter. (b) The transduction efficiency of Adv-shRNA in human islets. At day 3 post-transduction with Adv-H1-caspase-3-shRNA, islets were observed under a fluorescent microscope. The uninfected islets were set as control. (c) Adv-H1-caspase-3-shRNA silencing effect on caspase-3 in human islets at the transcription level. Islets were then incubated with the fresh medium without cytokines, then were collected at the indicated days for determining caspase-3 gene silencing at the transcription level. Error bar means the standard deviation based on the triplicates. (d) Adv-H1-caspase-3-shRNA silencing effect on caspase 3/7 activity in human islets. Upon transduction, islets were incubated in the fresh medium without cytokines, then were collected at the indicated days for determining caspase 3/7 activity. Error bar means the standard deviation based on the triplicates. (e) Comparison of different promoter driven shRNA silencing effects on caspase activity induced by cytokine cocktail. After transduction with Adv-U6-caspase 3 shRNA, Adv-H1-caspase-3-shRNA and Ad-Luc shRNA, the infected and control islets were incubated with the fresh medium containing the cytokine cocktail, then were collected at the indicated days for determining caspase activity. Results are represented as the average of two independent treatments. (f) Evaluation of Adv-U6-caspase-3 shRNA silencing effect by Western blotting. After transduction with Adv-U6-caspase-3 shRNA and Ad-Luc-shRNA, islets were incubated with the fresh medium containing the cytokine cocktail, then were collected at the indicated days for Western blotting analysis.

tion in caspase-3/7 activity. However, U6 promoter (Adv-U6-shRNA) was more efficient than H1 promoter (Adv-H1-shRNA) (Figure 3e). Additionally, Western blot was carried out to corroborate Adv-U6-caspase-3-shRNA silencing effect in human islets. As shown in Figure 3f, transduction of Adv-U6-caspase-shRNA also led to significant reduction in precursor caspase-3.

Effect of Caspase-3 Gene Silencing on Islet Transplantation. Since Adv-caspase-3-shRNA significantly suppressed caspase-3/7 activity, we next tested the effect of caspase-3 gene silencing on the outcome of islet transplantation after infusing Adv-caspase-3-shRNA transduced islets under the left kidney capsules of these diabetic mice. As shown in Figure 4a, although the blood glucose levels in all recipient mice were significantly decreased upon transplantation, the use of Adv-U6-caspase-3-shRNA transduced islets resulted not only in a decrease in blood glucose below 200 mg/dL at the early period of post-transplantation but also in a prompt return to normoglycemia in all recipient mice (Figure 4a and 4b). The animal reverted to diabetes upon removal of the islet graft-bearing kidney at 32 days after transplant (Figure 4a).

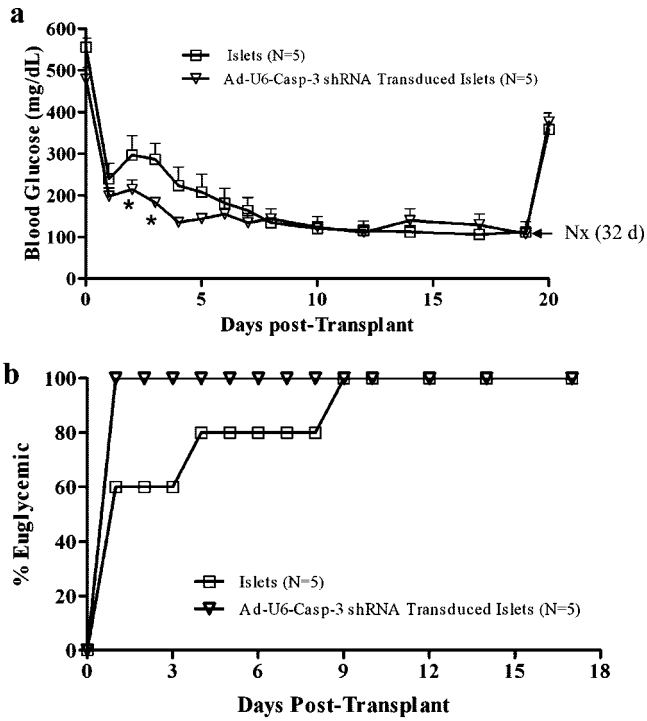

Figure 4. Ad-U6-caspase-3 shRNA transduction improves the outcome of islet transplantation in STZ-induced diabetic mice. (a) STZ-treated diabetic mice received subcapsular transplant of uninfected and Adv-U6-caspase-3-shRNA transduced (MOI = 1000) islets on day 0 and nephrectomy (Nx) on day 32. Data is mean ± S.E., * means $P < 0.05$ (uninfected islets vs Adv-U6-Caspase-3-shRNA-transduced islets) by ANOVA analysis. (b) Blood glucose levels in transplant recipients, expressed as percentage of animals normalized. The day of normalization was considered the second of consecutive days where blood glucose was ≤200 mg/dL.

Discussion

Human islet transplantation has great potential to provide type 1 diabetic patients with sustained and improved glycemic control and a period of insulin independence. However, up to 70% of the transplanted islets often get destroyed in the early days post-transplantation due to apoptosis.[18,19] Several factors are known to attribute to the dysfunction of islets after transplantation. First, prolonged hypoxia due to poor revascularization is one of the key factors attributed to islet dysfunction. Second, proinflammatory cytokines that are secreted by infiltrating T cells also further contributes to islet demise. Given the limited supply of donor pancreatic islets and the prevalence of type 1 diabetes, considerable efforts have been made to prevent the loss of islet mass in the post-transplantation period.[7,20−23] For example, overexpression of molecules known to enhance revascularization, such as vascular endothelial growth factor, had been attempted in our laboratory.[24] In contrast to the overexpression of therapeutic genes, ex vivo silencing of harmful genes also offers an alternative approach to improve islet viability after transplantation.

Most islet grafts undergo apoptosis, leading to islet dysfunction and cell death. We recently demonstrated significant decrease in apoptosis of insulinoma cells and human islets by iNOS gene silencing.[25] Programmed cell death is a cascade of events leading to the upregulation of procaspase-9 → caspase-9 → procaspase-3 → caspase-3 → apoptosis. Caspase-8 and caspase-9 are the upstream caspases involved in the extrinsic and intrinsic pathways, respectively. Hypoxia and oxidative stress are involved in apoptosis via intrinsic mitochondrial pathway.[10] Similarly, cytokines and/or elevated glucose level upregulate Fas, which is known to activate caspase-8 through the extrinsic apoptotic pathway.[26] Caspase-3 is a converging point of apoptosis for intrinsic and extrinsic pathways, and its upregulation leads to the generation of caspase-6 and caspase-7, which in turn degrade a number of intracellular protein substrates. This leads to the classical morphological changes associated with apoptosis, including chromatin condensation, nuclear degeneration, and cellular dehydration.

Hypoxia, oxidative damage, inflammatory cytokines and other factors induce apoptosis via increased caspase activity in the islets. Since caspase-3 is the "converging point" of apoptosis, in this study we used chemically synthesized siRNA to silence caspase-3 in INS-1E cells and human islets to determine whether caspase-3 gene silencing can block apoptosis induced by inflammatory cytokines. We observed all three chemically synthesized siRNAs significantly suppressed caspase-3 at both transcript and protein levels at 12 h post-transfection. There is not 100% homogeneity in rat caspase-3 mRNA (GenBank Accession NM_012922) and human caspase-3 mRNA (GenBank Accession NM_004346). Therefore, we designed siRNA against rat caspase-3 to determine whether caspase-3 gene expression can be inhibited after transfection of INS-1E rat insulinoma cell lines with chemically synthesized siRNA and whether caspase-3 silencing can protect β-cells from apoptosis. We then tested siRNA against human caspase-3 in human islets and then converted this siRNA into shRNA and cloned into replication

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deficient adenoviral (Adv) vectors. Since our Adv vector encoding shRNA is designed to target human caspase-3, we could not evaluate this Adv vector in rat INS-1E cells. To test Adv-caspase-3-shRNA in INS-1E cell lines, we need to convert the potent siRNA sequence against rat caspase-3 into shRNA and then into Adv-shRNA. Since generation of Adv-shRNA is very time-consuming and our purpose to use INS-1E was simply to confirm the usefulness of caspase-3 gene silencing in rat insulinoma cell lines before moving to human islets, we did not design and construct Adv-shRNA against rat caspase-3.

Our main objective was to determine whether caspase-3 can be efficiently silenced in human islets using RNAi approaches. We demonstrated that transfection of INS-1E cells and islets with siRNAs reduced caspase-3 transcripts by 50−67% and 50%, respectively (Figure 1a and 1c). Gene silencing did not last beyond two days and was not detectable after 4 days post-transfection of human islets with siRNA (Figure 2c). Because an islet is a cluster of 200−1000 cells, we applied high dose of siRNA (400nM) for transfection to increase caspase-3 gene silencing. Caspase-3 mRNA was reduced by 50%, and the precursor of caspase-3 protein was also significantly suppressed as determined by Western blotting at 2 days after transfection (Figure 1c and 1d). However, after 5 days post-transfection caspase-3 gene silencing effect on caspase activity was also undetectable in human islets (data not shown).

We have previously demonstrated efficient transfection and transient gene silencing in β-cells,[16] but in human islets were relatively low.[25] Moreover, the high dose of siRNA required for gene silencing in human islets can preferentially cause the off-target effect.[27] Since the apoptosis begins during islet isolation process, peaks 2−3 days post-transplantation, and continues for approximately 7−14 days until the remains of the graft has stabilized and become vascularized, duration of caspase-3 gene silencing is important. To overcome these obstacles by increasing the transfection efficiency and prolonging the duration of caspase-3 gene silencing, we converted potent siRNA into shRNA and constructed replication deficient Adv vectors encoding these shRNAs driven by a U6 or H1 promoter (Figure 3a). In this study, three strategies had been applied to minimize innate and adaptive immune response caused by adenovirus backbone. First, we applied relatively low-dose adenovirus to transduce islets for transplantation that had been tested in our laboratory. Second, we washed islets with PBS after transduction to remove free adenovirus prior to transplantation. Finally, previous studies indicated that infiltrating T cells in islets, which is one of the factors attributed to the dysfunction of graft islets by secreting proinflammatory cytokine and inducing immune response. Transduction of adenovirus encoding shRNA in human islets can also result in T-cells caspase-3 gene silencing. Liadis et al. demonstrated that caspase-3 knockout (Casp3−/−) mice protected from developing diabetes and their islets were resistant to apoptosis following streptozotocin administration. Furthermore, lymphocyte infiltration of islets was completely absent in Casp3−/− mice and caspase-3 is required for activation and proliferation of T cells,[12] suggesting that downregulation of caspase-3 can deactivate T-cell mediated immune response. Consequently, capase-3 gene silencing in human islets as well as infiltrated T-cells can to some extent inhibit T-cell mediated immune response for adenovirus backbone.

Our results demonstrated that transduction of Adv-caspase-3-shRNA driven by either a human H1 promoter or U6 promoter can significantly inhibit caspase-3/7 activity despite the incubation of human islets with the cytokine cocktail (Figure 3e). Moreover, U6 promoter showed better caspase-3 gene silencing. This observation is consistent with the results published by Makinen et al. who demonstrated that U6 promoter was more efficient than H1 promoter both in vitro and in vivo.[28]

Bain et al. also determined the gene silencing efficiency of an Adv vector encoding shRNA in pancreatic islets.[15] These authors constructed an Adv vector encoding shRNA driven by a CMV promoter targeting glucose transporter-2 (GLUT2) gene. Transduction of islets at a dose of 1.9×10^6 pfu/islet resulted in a ~95% reduction in GLUT2 expression.[15] In our study, human islets were transduced at a dose of 2.8×10^4 pfu/islet for 18 h and then treated with the cytokine cocktail up to 5 days. Under these conditions the majority of islets (~80%) did not alter their morphologies as observed under microscopy. To further increase caspase-3 gene silencing, we also infected islets at a dose of 1.4×10^5 pfu/islet. However, most islets underwent morphological changes and there was no increase in caspase-3 gene silencing (data not shown). In our study, we did not observe any changes in morphology when islets were infected with Adv-caspase-3-shRNA.

Although the blood glucose levels in all recipient mice were significantly decreased upon transplantation, the use of Adv-U6-caspase-3-shRNA transduced islets at 1000 MOI resulted not only in a decrease in blood glucose levels below 200 mg/dL at the early period of post-transplantation of 1200 islets but also in a prompt return to normoglycemia in all recipient mice. In contrast, blood glucose level below 200 mg/dL was achieved in only 60% at day 1, 80% at 4 and 100% at day 8 of mice transplanted with untreated islets (Figure 4a). Islet graft bearing kidneys were removed at day 32 post-transplantation to confirm the function of islet grafts. As expected, blood glucose levels returned to ≥325 mg/dL (Figure 4a), confirming that transplanted islets were functional. Our results are in good agreement with the work of Shapiro and associates who incubated islets with a peptide caspase inhibitor for

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2 h prior to transplantation and also administered this caspase inhibitor intraperitoneally for 5 consecutive days postransplantation.[2] These results indicate that caspase-3 gene silencing is likely to reduce the islet mass required to achieve normoglycemia. Since the performance of islet grafts greatly depends not only on the number of islets but also on their quality, we cannot directly correlate our results with the work of Nakano et al.,[4] who demonstrated that all of six mice transplanted with 1000 islets after pretreatment with 100 μmol/l z-VAD.fmk became normoglycemic by days 9 ± 2 post-transplantation, while only 3 of 8 mice transplanted with the control islets achieved normoglycemia by 17.7 ± 6.4 days post-transplantation. We plan to determine the effect of MOI and islet number in achieving normoglycemia and apoptosis after transplantation of Adv-caspase-3-shRNA transduced NOD-SCID mice. We will report these results in a subsequent publication.

In conclusion, these results demonstrated that caspase-3 gene silencing could be a possible approach to reduce islet apoptosis post-transplantation and help to improve the outcome of islet transplantation.

Acknowledgment. The study was supported by the NIH (1R01DK69968).

Supporting Information Available: Supplementary Figure 1 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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