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β Cell cytoprotection using lentiviral vector-based iNOS-specific shRNA delivery

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Abstract

Cytokine-induced β cell pathophysiology is characterised by the induction of iNOS expression. Inhibition of iNOS expression protects β cells from cytokine-mediated destruction. The development of vector-based shRNA strategies capable of stably suppressing iNOS expression may provide a novel platform to protect β cells from cytokine toxicity. In this report the utility of lentiviral shRNA vectors to silence iNOS expression was evaluated with respect to insulinoma cell viability, the induction of iNOS expression and the accumulation of nitrite in a cytokine-induced β cell toxicity model. Here, we report for the first time on the use of lentiviral vector-based shRNA delivery to efficiently suppress the IL-1β-mediated induction of iNOS expression, the accumulation of nitrite and provide significant protection against the cytotoxic effects of IL-1β exposure. Moreover, non-specific knockdown of endogenous β cell nNOS did not occur.

Keywords: shRNA; Lentiviral vector; Rat insulinoma cells; Cytokines; IL-1β; iNOS

Pancreatic islet β cells display an increased susceptibility to cytokine-mediated damage, in particular, IL-1β [1]. Among the hallmarks of β cell pathophysiology following IL-1β exposure are the induction of iNOS and the accumulation of nitrite [2]. The pivotal nature of iNOS expression in this process is highlighted by the fact that both gene-based and chemical inhibition of iNOS induction in insulin-producing cells results in significant protection from IL-1β damage [3–5].

RNA interference (RNAi) entails the phenomenon whereby double stranded RNA (dsRNA) inhibits eukaryotic gene expression in a potent and specific manner by targeting the complementary messenger RNA (mRNA) for degradation [6]. RNAi harbours potential as a therapeutic tool to silence deleterious gene expression. We hypothesized that RNAi may be employed to suppress the deleterious induction of iNOS following IL-1β exposure.

The utility of RNAi-mediated suppression of cytokine-induced iNOS activation is provided by a recent study wherein siRNAs specific for iNOS were delivered to insulinoma cells to suppress iNOS expression and significantly preserve viability following cytokine exposure [7]. Although successful, the clinical applicability of this strategy is questionable giving the slow-dividing nature and poor transfection efficiencies of islet β cells. Moreover, long-term stable iNOS suppression will be required in the humans to preserve β cell viability following inflammatory mediators (i.e., IL-1β and iNOS) exposure. Vector-based small hairpin RNA (shRNA) expression systems provide a novel platform for the unlimited delivery of shRNAs to target cells in a stable long-term manner compared to synthetic siRNA delivery. Consequently, a number of groups have developed vector-based systems to facilitate stable and efficient expression of siRNA and shRNA in mammalian cells [8,9]. Recently, lentiviral vector systems which infect both dividing and non-dividing cells, including pancreatic β cells have been engineered that express shRNAs [10,11].

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In this report we demonstrate for the first time, the successful exploitation of vector-based RNAi for efficient and long-term knockdown of deleterious iNOS gene expression in a β cell line. Based on these data, we suggest that lentiviral vector-based shRNA expression systems may harbour therapeutic potential for the preservation of β cell viability following transplantation and prolongation of islet allograft longevity following proinflammatory cytokine exposure.

Research design and methods

Materials. RIN-r cell stocks were a gift from Prof. Jørn Nerup of the Steno Diabetes Centre, Gentofte, Denmark. Tissue culture plastics were supplied by Nunc and Sarstedt. RPMI 1640 culture media and supplements were supplied by Cambrex. Primary antibodies against iNOS, nNOS, and GAPDH were supplied by Transduction Laboratories and Amersham, respectively. Secondary antibodies conjugated to horseradish peroxidase (HRP), were supplied by Amersham. Recombinant human IL-1β was obtained from PromoCell, Heidelberg, Germany. All other reagents were obtained from Sigma unless otherwise stated.

Cell culture and cytokine treatment. RIN-r cells were cultured at 37 °C in RPMI 1640 supplemented with 10% (v/v) FBS, 20 mM Hepes, 2 mM L-glutamine, 24 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2. Cells were passaged every 3–4 days by tryspinisation. Cells were allowed to recover for 24 h after plating before exposure to cytokines. Cells were exposed to IL-1β alone at 100 U/ml. HEK 293FT cells and HeLa cells were cultured in T-75 cell culture plates in DMEM supplemented with 10% (v/v) heat-inactivated FBS (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2. Cytokine incubation periods varied depending on the experiments. Media samples for nitrite analysis were taken at 0, 24, 48, 72, 120 h, and 7 days post IL-1β exposure. Viability was measured after 0, 24, 48, 72, and 120 h of IL-1β exposure. For vector-based shRNA experiments, viability was also monitored after both 7 and 14 days of IL-1β exposure. To assess the impact of RNAi on iNOS and nNOS expression RIN-r whole cell lysates were extracted at 6, 72 h, and 7 days post IL-1β exposure.

Cell viability. Cell viability was assayed using a Cell Proliferation Kit 1 (MTT assay) (Roche Diagnostics, Mannheim, Germany). RIN-r cells were seeded in 48-well microtitre plates at a density of 2.5×10^4 cells per well in a humidified atmosphere of 5% CO_2, 37 °C. Twenty-four hours after plating the cells were transfected as appropriate. After 48 (transient transfection experiments) or 96 h (lentiviral transduction experiments), cytokine incubations commenced. 0.5 mg/ml MTT labelling reagent was then added to each well and incubated at 37 °C for 4 h before addition of solubilization solution. After allowing to stand overnight at 37 °C the A_{550nm} was recorded using a microtitre plate reader (SpectraMax, Molecular Devices). MTT assay was also used to monitor vector-associated toxicity.

Griess assay. Nitrite accumulation was monitored as a marker for the activation of iNOS in RIN-r exposed to IL-1β. Briefly, RIN-r cells (2.5×10^4 cells/well)) were plated in 48-well microtitre plates and transfected or transduced as appropriate 24 h later. For plasmid transfection and lentiviral transduction experiments cells were allowed to recover for 48 and 96 h, respectively, before cytokine incubations commenced. 50 μl samples of media were taken at appropriate time-points and incubated with Griess reagent (1:1) (0.1% naphthyl ethylenediamine and 1% sulphanilamide in 0.1 mol/l HCl, 1:1 vol/vol) for 15 min in the dark at 25 °C. Cells were harvested, lysed and assayed for protein using the BCA protein assay. The A_{540nm} was then measured and the sample nitrite concentrations were normalized to total protein (expressed in nM of accumulated nitrite per mg total protein).

Western blotting. Western blotting was used to monitor alterations in iNOS and nNOS protein expression. The house-keeping protein GAPDH served as a loading control. RIN-r cells were plated at 3×10^5 cells/well in 6-well plates. 24 h later cells were transfection or transduction as appropriate and allowed to recover for 48 and 96 h, respectively, before exposure to IL-1β as described. Cells were harvested by centrifugation at 5000 rpm for 5 min. Total protein was extracted from cell pellets following suspension in whole cell lysis buffer (5 mM Tris–HCl, 0.1 mM EDTA, 0.1% 10% (v/v) SDS, 1% IGEPAL, 10% (v/v) protease inhibitor cocktail, pH 7.5) on ice for 20 min. Supernatant was collected and total protein concentration determined by the BCA protein assay. 15 μg of protein were loaded on 4% stacking/10% separating SDS–PAGE. Resolved proteins were transferred to a 0.2 mm nitrocellulose membrane on a semidry electrophoretic transfer system (Bio-Rad) before blots were placed in blocking buffer (5% non-fat milk in phosphate buffer saline/0.05% Tween 20) overnight at 4 °C. Membranes were incubated with the primary antibodies (i.e., either anti-iNOS (1:5000), anti-nNOS (1:500), or anti-GAPDH (1:10,000)) diluted in blocking buffer overnight at 4 °C. After washing, the membranes were incubated with the appropriate secondary antibodies conjugated to HRP for 1 h at room temperature. Protein bands were detected using enhanced chemiluminescence (ECL) (Amersham Life Sciences) and autoradiography.

Lentiviral vectors encoding shRNAs of interest. shRNA-expressing vectors were generated using the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen). Two vectors were prepared, one expressing shRNA specific for rat iNOS (top strand: 5′-CACCGGATGACCCTAAGAGT CACTTGAGAAAGTGACTCTTAGGGTCATCC-3′; bottom strand: 5′-AAAAGGATGACCCTAAGAGTCACTTTCTCAAGTGACTCTTA GGGTCATCC-3′) and the other expressing shRNA specific for the unrelated control gene, lamin A/C (as supplied by the manufacturers) according to the manufacturer's guidelines. Virus-containing supernatants were harvested 48 h post-transfection, isolated by centrifugation at 3000 rpm for 5 min at 4 °C and concentrated by centrifugation at 22,500 rpm for 2 h before resuspension in basal RPMI medium. Virus was then aliquoted and stored at −80 °C. Lentiviral vector preparations were titred at 1.5×10^7 transduction units per ml (TU/ml) and 1.7×10^7 TU/ml for the vectors expressing the lamin A/C-specific shRNA and iNOS2-specific shRNA, respectively. The optimal MOI for RIN-r cell gene transfer was determined using a 2nd generation lentiviral vector encoding the GFP marker gene. Briefly, cells were transduced over a range of MOIs (0, 0.1, 1, 10, 50, 100, and 150) and the number of GFP-positive cells quantified by flow cytometry 96 h after transduction.

Transduction of RIN-r cells and analysis. RIN-r cells were plated in 6-well plates at 3×10^5 cells/well (Western blots) or in 48-well plates at 2.5×10^4 cells/well (Griess and MTT studies) and allowed to attach for 24 h before transduction at MOI 100 (predetermined to be sufficient to achieve a transduction efficiency of $83.9 \pm 1.35\%$) at 37 °C overnight whereupon the virus containing media was removed and replaced with complete culture medium. 72 h later, cytokine incubations commenced and cells were harvested as appropriate and assayed for inhibition of iNOS activation as appropriate.

Statistical analysis. The experimental data are expressed as means ± SD. Treatment groups were compared by means of two-tailed Student's t test, assuming unequal variances; significance was established at $p < 0.05$ unless otherwise stated.

Results

Optimising lentiviral vector-mediated gene transfer to RIN-r cells

The gene transfer efficiency of a 2nd generation lentiviral vector expressing the GFP marker gene was evaluated by flow cytometry 96 h after transduction. Briefly, the efficiency of gene transfer increased with increasing MOIs. Percentage gene transfer efficiencies were observed to be 0.7 ± 0.18, 3.93 ± 0.25, 10.58 ± 0.75, 26.26 ± 0.76, 56.48 ± 2.15, 86.17 ± 1.24, 86.91 ± 1.31 at MOIs of 0,

0.1, 1, 10, 50, 100, and 150. The optimal MOI was determined to be 100. At MOIs beyond this, no appreciable increase in transduction efficiency was observed.

Expression profile of lipofectamine 2000 and lentiviral vector-mediated gene delivery to RIN-r cells

To compare the efficiency, duration and associated toxicity of transgene expression conferred in RIN-r cells using either lipofectamine 2000 (reverse transfection) or lentiviral gene delivery, gene expression was monitored at days 1, 4, 7, 14, and 21 days. Lipofected RIN-r cells displayed an onset of GFP transgene expression at 24 h post transfection with expression levels of GFP declining steadily to near non-detectable levels at 14 and 21 days post transfection (Table 1A). In contrast, lentiviral vector-transduced RIN-r cells exhibited prolonged transgene expression together with an overall higher level of gene transfer efficiency. Transgene expression was increased as determined by flow cytometric analysis in lentiviral vector-transduced cells versus lipofected cells 36.44 ± 6.28-fold and 58.12 ± 3.03-fold, respectively. Moreover, the duration of transgene expression was markedly prolonged, when compared to lipofection, using the lentiviral gene delivery system with $70.88 \pm 2.85\%$ +GFP-expressing cells at 21 days post transduction (Table 1A) versus $1.25 \pm 0.26\%$ of lipofected RIN-r cells expressing GFP at the same time-point. These results highlight the superior gene expression profiles achievable using lentiviral vectors versus lipofection.

Notably, however, viability studies conducted in parallel experiments revealed that both lipofection and lentiviral-mediated gene delivery exerted a mild but significant impact ($p < 0.05$) on viability relative to unmodified control cells (Table 1B). For the purposes of these experiments non-transfected/non-transduced RIN-r cells were taken to be 100% viable at the respective time-points. Briefly, it was observed that both lipofection and transduction were associated with significant decreases in viability at all time-points ($p < 0.05$). The cytotoxic effect of both lipofection and transduction were exacerbated at the later time-points. No significant cytotoxic differences between lipofection and lentiviral transduction were observed at any of the time-points examined.

Lentiviral vector-based shRNA delivery to RIN-r cells exposed to IL-1β

Following generation and transduction of RIN-r cells with either lamin (control) or iNOS2 shRNA expressing lentiviral vectors, RIN-r cells were exposed to IL-1β over a range of time-points. Briefly, nitrite accumulation, cellular viability and the induction of iNOS were all examined. In order to evaluate the potential of this vector system to confer long-term protection from the deleterious effects of IL-1β-induced iNOS expression, a 7 day time-point of IL-1β exposure was also investigated. Using an MOI of 100 of the lenti.shRNA-iNOS2 vector, the accumulation of nitrite was suppressed significantly versus controls at all time-points examined (Table 2A). That is, nitrite levels were suppressed 2.02 ± 0.23-fold, 1.81 ± 0.52-fold, 2.64 ± 0.18-fold, 2.84 ± 0.38-fold, and 2.82 ± 0.39-fold at 24, 48, 72, 120 h, and 7 days, respectively. It was notable too, that significant suppression persisted even at the latest time-point examined, i.e., 7 days. Concomitantly, transduction conferred significant protection upon the RIN-r cells exposed to IL-1β over all time-points examined. The percentage viability of each of the respective groups is depicted in Table 2B.

Western blot data are consistent with the nitrite accumulation data. The induction of iNOS expression in appropriately transduced RIN-r cells was monitored over 3 time-points of exposure to IL-1β. Relative to the mock-transduced and lamin shRNA-transduced controls, RIN-r cells expressing the iNOS2 shRNA displayed markedly reduced levels of iNOS protein expression after 6 and 72 h, and 7 days of IL-1β exposure (Fig. 1).

Table 1
Comparative analysis of the gene transfer profile of lipofection versus lentiviral vector transduction

	Day 1	Day 4	Day 7	Day 14	Day 21
A					
Control	1.24 ± 0.06	2.01 ± 0.38	0.97 ± 013	1.12 ± 0.3	1.08 ± 0.33
Plasmid	46.95 ± 1.85	34.9 ± 1.75	4.05 ± 1.16	3.54 ± 0.63	1.25 ± 0.26
Lentiviral vector	4.19 ± 0.8	81.83 ± 1.35	76.33 ± 2.13	77.16 ± 1.77	70.88 ± 2.85
B					
Control	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Plasmid	96.32 ± 1.38[a]	94.39 ± 1.96[a]	90.8 ± 1.69[a]	82.83 ± 1.16[a]	81.03 ± 3.37[a]
Lentiviral vector	95.19 ± 1.53[a]	92.99 ± 0.99[a]	92.07 ± 2.92[a]	83.07 ± 2.31[a]	77.26 ± 2.03[a]

(A) Percentage transduced (GFP +'ve) cells in control, lipofected and lentiviral vector transduced RIN-r cell populations at days 1, 4, 7, 14, and 21 as determined by flow cytometry. (B) Cytotoxicity of RIN-r cells lipofected via lipofectamine 2000 reverse transfection and lentiviral vector-mediated transduction. Non-transfected/non-transduced RIN-r cells were normalized to 100% viability. Experiments were performed at $n = 3$ in triplicate. Data are expressed as means ± SD. While lipofection and transduction resulted in significant levels of cytotoxicity that were exacerbated over time no significant differences between the treatments were observed.

[a] Significance was established at $p < 0.05$.

Table 2
Impact of lentiviral-mediated shRNA transfer to RIN-r on iNOS activation and associated toxicity

	0 h	24 h	48 h	72 h	120 h	7 Day
A						
Lamin shRNA	6.82 ± 1.66	52.65 ± 5.98	133.02 ± 19.52	183.45 ± 16.28	230.9 ± 10.02	344.2 ± 21.2
iNOS2 shRNA	6.48 ± 1.48	21.84 ± 1.17[a]	57.5 ± 6.15[a]	67.17 ± 7.18[b]	103.45 ± 10.95[b]	159.96 ± 18.29[b]
Mock shRNA	6.73 ± 1.56	49.23 ± 4.9	138.81 ± 20.85	185.19 ± 22.02	258.67 ± 17.34	360.48 ± 19.47
	24 h	48 h	72 h	120 h	7 Day	14 Day
B						
Nonexposed	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Lamin shRNA	98.54 ± 1.08	83.35 ± 1.13	77.67 ± 1.43	45.05 ± 1.41	18.3 ± 1.06	4.52 ± 0.97
iNOS2 shRNA	102.98 ± 0.65[a]	101.75 ± 1.23[b]	96.38 ± 0.97[b]	92.43 ± 1.09[b]	75.63 ± 2.1[b]	63.06 ± 1.26[b]
Mock	99.76 ± 1.51	85.23 ± 1.27	80.98 ± 0.53	50.84 ± 1.08	30.3 ± 1.05	11.63 ± 1.29

(A) Nitrite accumulation (nM/mg total protein) in RIN-r cells exposed to 100 U/ml IL-1β over 0, 24, 48, 72, and 120 h, and 7 days. RIN-r cells transduced with lenti.shRNA-iNOS2 displayed significantly reduced levels of nitrite following cytokine treatment. (B) Percentage viabilities of RIN-r cells appropriately transduced with shRNA expressing lentiviral vectors or controls and exposed to 100 U/ml of IL-1β for either 24, 48, 72, and 120 h and 7 and 14 days. Relative to mock transduced and lamin.shRNA-transduced controls, transduction with the lenti.shRNA-iNOS2 vector preserved viability significantly over all time-points examined. Data are expressed as means ± SD. All experiments were performed at $n = 3$ in triplicate.

[a] Significance established at $p < 0.05$.

[b] Significance established at $p < 0.005$.

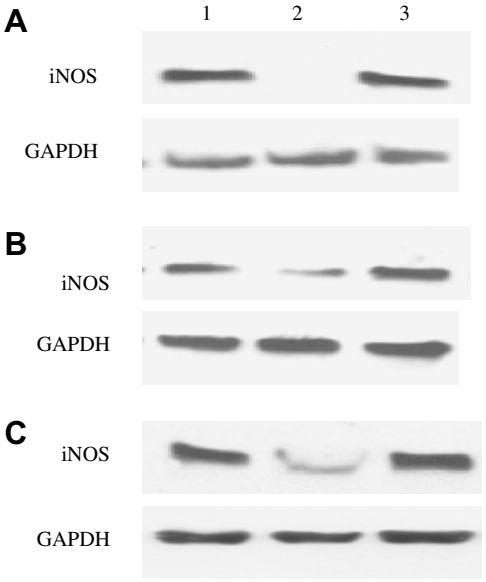

Fig. 1. Western blot for the induction of iNOS after 6 (A), 72 h (B), and 7 days (C) of IL-1β. Lane 1, lamin shRNA-expressing control RIN-r cell lysates; lane 2: lenti.shRNA-iNOS2-expressing RIN-r cell lysates; lane 3: mock transduced RIN-r cell lysates. Blots are representative images from experiments performed in duplicate at $n = 2$. GAPDH was also used as a loading control for each of the three time-points examined.

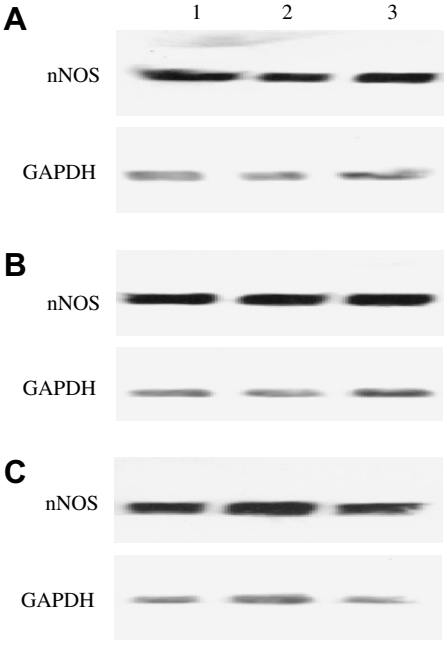

Fig. 2. nNOS expression remains unchanged as determined by Western blotting in RIN-r cells transduced with lenti.shRNA-iNOS2. nNOS expression was monitored after 6 (A), 72 h (B), and 7 days (C) of IL-1β exposure. Lane 1, RIN-r cells transduced with lentiviral vector expressing the lamin A/C-specific shRNA; lane 2 RIN-r cells transduced with the lenti.shRNA-iNOS2 vector; lane 3, Mock-transduced RIN-r cells. Blots are representative images of experiments performed in duplicate. GAPDH was also used as a loading control for each of the three time-points examined.

Assessing the off-target silencing effects of iNOS-specific vector-based shRNA on nNOS expression

Given the pivotal role of constitutive nNOS expression in regulating insulin secretion from the pancreatic β cell, we evaluated whether or not iNOS-directed gene silencing would adversely impact nNOS expression. RIN-r whole cell lysates were analysed for the presence and quantity of nNOS expression following transduction with either vector control, mock vector or the lenti.shRNA-iNOS2 vector. Our results clearly indicate that despite the reported sequence homology between iNOS and nNOS, no detectable off-target effects on nNOS expression were observed at any of the three time-points of IL-1β exposure (i.e., 6, 72 h, and 7 days) examined (Fig. 2). These data highlight the specificity of the vector-based shRNA expression system under study.

Discussion

Given that iNOS represents a pivotal mediator of cytokine-induced β cell pathophysiology [3], we hypothesized that silencing deleterious iNOS expression using RNAi represents a promising therapeutic strategy for the preservation of β cell integrity following cytokine exposure. To date one report has demonstrated the utility of RNAi-mediated iNOS knockdown in insulin-producing cells [7]. While the aforementioned report provided useful information regarding proof of feasibility of RNAi strategies to silence iNOS expression in a β cell line, the study had several limitations. First, siRNA transfection strategy employed, provided only short term, low level protection over a relatively short period of exposure to cytokines. Second, the clinical applicability of siRNA transfection by non-viral means to confer iNOS knockdown in primary β cells is questionable given the reported poor transfection efficiencies of this cell type (largely attributable to their relatively slow proliferation rate and their centralized location within the islet as a whole) [12,13]. The development of more efficient vector-based shRNA strategies designed to mediate gene knockdown would benefit this field of research. In this study, we used lentiviral vector-mediated gene transfer of shRNA specific to iNOS and evaluated cytoprotection against IL-1β.

Preliminary gene transfer studies confirmed the superior gene transfer profile of lentiviral vectors compared to plasmid vectors with respect to duration and efficiency. We suggest that the observed trend towards decreased gene expression in the later time-points examined may be attributable, in part at least, to the long-term cytotoxic effects of the GFP transgene on the RIN-r cells and perhaps also due to a degree of instability of lentiviral vector transgene integration as has been reported elsewhere [14,15]. However, prolonged exposure to the control lentiviral vector too conferred a comparable cytotoxic impact. The observed toxicity was thus likely a function of the relatively high MOIs required to confer high transduction efficiencies in RIN-r cells. In this respect, primary β cells are markedly more permissive to lentiviral vector-mediated gene delivery [10].

Consequently, we engineered the iNOS2 shRNA construct into a second generation VSV-g pseudotyped lentiviral vector. Lentiviral vector-based shRNA delivery provided long-term knockdown of iNOS expression following prolonged exposure to IL-1β. The degree of knockdown conferred was such that iNOS levels were below the levels of detection at 6 h by Western blot in the lenti.shRNA-iNOS2 transduced cells, and although detectable thereafter, they were significantly reduced at all time-points examined relative to controls. Concomitantly, nitrite levels were significantly reduced at all time-points examined. The degree of suppression afforded is particularly important given the rapid transcription rate of iNOS expression following cytokine exposure. Previous data from our group has shown that the p65 subunit of the cytosolic NF-κB complex translocates to the nucleus <45 min after cytokine exposure whereupon it binds to upstream elements in the iNOS gene to induce its transcription [3]. Notably, the suppression of iNOS expression was sufficient to confer prolonged significant preservation of RIN-r cell viability.

Given the reports of the off-target gene silencing effects of shRNAs [16,17] and the fact that pancreatic β cells express nNOS which shares >50% sequence homology with iNOS to facilitate controlled insulin secretion [18]. Given these facts we investigated whether or not our RNAi strategy would induce off-target gene silencing effects on nNOS expression. At all time-points examined, nNOS expression remained unchanged relative to GAPDH loading controls highlighting the utility of vector-based RNAi approaches to silence gene expression in a highly efficient and specific manner.

This investigation provides novel evidence for lentiviral vector-based RNAi-mediated long-term and efficient knockdown of iNOS expression in a β cell line exposed to the proinflammatory cytokine, IL-1β. Moreover, the data herein provides proof of principle that vector-based shRNA expression strategies are sufficient to silence the deleterious expression of iNOS in insulin-producing cells for prolonged periods. Strategies such as that outlined in this study, which aim to modulate the accumulation of cytotoxic levels of NO, may be useful in preserving β cell viability in the face of proinflammatory cytokine exposure. Similarly, such strategies may hold therapeutic utility in disease models where NO toxicity represents a key determinant of cell fate.

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