Development of novel cell lines of diabetic dysfunction model fit for cell-based screening tests of medicinal materials

Mikako Saito · Aya Hayakawa ·
Nobuya Inagaki · Hideaki Matsuoka

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Abstract *Pdx-1* and *Irs-1*, genes highly associated with diabetes onset, were knocked down in mouse embryonic stem (ES) cells in order to develop cell line models for diabetes. ES cells with different gene knockdown levels were induced to differentiate to the stage of insulin production. Among the cell lines that differentiated, we identified two in which the levels of expression of both genes were 20–40 % of that of control cells. These cell lines showed appreciable deficiencies in three characteristic malfunctions associated with diabetes, namely, insulin production, insulin reception signaling, and glucose-stimulated insulin secretion. These dysfunctions were consistent with results reported elsewhere from in vivo and in vitro studies. Both cell lines did not show any abnormal morphology such as size, shape, color, and surface roughness. No abnormal expression profiles for 17 genes relevant to diabetes were observed. Therefore, these cell lines fulfilled the criteria for a validated cell model for diabetes. The model cell lines developed here are promising biomaterials for cell-

based screening tests of new medicines that may be effective in treating diabetes.

Keywords Diabetic dysfunction model cell · Cell-based screening test · Mouse embryonic stem cell · Double knockdown · Validation

Introduction

There is an urgent need for viable cell-based screening methods to test new drugs with potential for use in the management and treatment of diabetes. Such cell-based methods are required to be responsive to the effects of candidate drugs across the range of dysfunctions associated with diabetes. The principal effects of the disease in vivo are deficiencies in insulin production, insulin reception, and glucose-stimulated insulin secretion (GSIS). GSIS is a combination of glucose reception and insulin secretion functions. A number of studies have examined the genetic basis of diabetes and identified specific genes for insulin production (Melloul et al. 2001), insulin reception (Kubota et al. 2008; Guo et al. 2009; Dobrzyn et al. 2010), and GSIS (Pal 2009; Bennett et al. 2010). It has been speculated that the genes for these various roles are functionally linked. From the standpoint of screening candidate drugs, it is important for model cell lines to be sensitive to the effect of candidate drugs on any of these dysfunctions.

M. Saito (✉) · A. Hayakawa · H. Matsuoka
Department of Biotechnology and Life Science, Tokyo
University of Agriculture and Technology, 2-24-16,
Naka-cho, Koganei, Tokyo 184-8588, Japan
e-mail: mikako@cc.tuat.ac.jp

N. Inagaki
Department of Diabetes and Clinical Nutrition, Graduate
School of Medicine, Kyoto University, Sakyo-ku,
Kyoto 606-8507, Japan

Our strategy is to selectively knock down expression of genes that are involved in the dysfunctions described above. It is known that a number of genes contribute to the risk of diabetes, and several cases of double gene knockout, such as *Pdx-1* and *Irs-1* (Kulkarni et al. 2004), *Pdx-1* and *HNF-1 α* (Shih et al. 2002), *Irs-1* and *Gk* (Terauchi et al. 1997), *Kir6.2* and *Irs-2*, *Gk* and *Shp*, have been reported to be suspected to cause diabetes in mice.

In order to achieve a quantitative regulation of multiple genes, we adopted RNAi-based knockdown methodologies (Santoyo et al. 2005; Sliva and Schnierle 2010; Li and Mahato 2011). We first sought to identify RNAi sequences that were effective at knocking down the genes listed above. RNAi sequences against every gene except *Gk* were developed (unpublished data). Two of the genes, *Pdx-1* and *Irs-1*, were then selected for the development of double knockdown cell lines.

Pdx-1 has a well-understood and central role in insulin production by directly activating insulin production genes such as *Ins1* and *Ins2* (Melloul et al. 2001). It has been speculated that *Pdx-1* can regulate the size of the β -cell mass and also the insulin production activity of each β -cell, so as to maintain insulin levels within the required range (McKinnon and Docherty 2001; Melloul et al. 2002; Gannon et al. 2008). The available data all point to *Pdx-1* having a role as a general stabilizer of the insulin production level. When the insulin production level goes up to such an abnormally high level as after loss of *Irs-1*, it is possible that *Pdx-1* might act to reduce insulin production to the normal level.

Irs-1 is involved in insulin reception deficiency (insulin resistance). Insulin resistance is observed not only in the cells of peripheral organs, such as hepatocytes and skeletal muscle cells (Wada et al. 2009; Franchini et al. 2010; Ketsawatsomkron et al. 2010), but also in pancreatic β -cells (Terauchi et al. 2007). Typically in β -cells, insulin resistance is caused by an insufficiency of *Irs-1*, which encodes a protein that is a substrate of tyrosine kinase. *Irs-1* constitutes the intracellular part of the insulin receptor and plays a role in signal transduction from insulin reception to several downstream pathways (Guo et al. 2009). In these pathways, *Irs-2* is of particular interest. *Irs-2* expression is activated in compensation for the loss of *Irs-1* function (Kubota et al. 2008; Dobrzyn et al. 2010; González-Rodríguez et al. 2010; Yang

et al. 2010). Following its activation, *Irs-2* stimulates expression of further downstream genes associated with insulin production. This activation cascade often leads to an abnormal increase in β -cell mass and islet hyperplasia. Such an abnormal response is thought to be facilitated by *Pdx-1* (Kulkarni et al. 2004).

The current understanding of the principal pathway for GSIS is as follows: a blood glucose increase is first detected by pancreatic β -cells. The β -cells, which produce and store insulin, respond to the glucose increase by secreting insulin into the blood via ATP-dependent K^+ channel closure, Ca^{2+} influx, the fusion of insulin stored vesicles with the cell membrane, and the final step of insulin secretion. Blood insulin is then transferred to and detected by peripheral cells such as hepatocytes and skeletal muscle cells. These cells remove glucose from the blood and lower the blood glucose levels. The reduction in blood glucose is detected by the β -cells and, finally, insulin secretion slows down. This pathway therefore constitutes an in vivo feedback regulation cycle. The principal genes involved in this cycle are *Gk*, a glucose sensor, and *Kir6.2*, a subunit of an ATP-dependent K^+ channel. Since *Gk* is known to be partially regulated by *Pdx-1* (Watada et al. 1996), the dysfunction of GSIS is thought to be caused by *Pdx-1* deficiency.

In light of the available information, we speculated that a double knockdown of *Pdx-1* and *Irs-1* would provide a model cell system for the principal dysfunctions associated with diabetes. The goal of this study was to establish ES cell lines in which expression of both genes was knocked down to levels that were not too low to inhibit their differentiation into β -like insulin producing cells but not so high that dysfunctions associated with diabetes were not manifested.

Methods

Mouse ES cell culture and transfection

A feeder-free mouse ES cell line (EB3) was obtained from H. Niwa (RIKEN, Kobe, Japan) and cultured in ES cell medium (ESM) in gelatin-coated dishes. ESM is composed of Glasgow minimum essential medium, 10 % fetal bovine serum, $1 \times$ non-essential amino acids, 200 mM L-glutamine, 1 mM sodium pyruvate, 10^{-4} M 2-mercaptoethanol and 1,000 U/ml leukemia inhibitory factor (LIF). For transfection, 1×10^7 ES cells in a

0.4 cm cuvette were electroporated with 50 µg of linearized plasmid DNA at 800 V and 3 µF using a Bio-Rad gene pulser. Selection was carried out using 100–200 µg/ml hygromycin or 100–300 µg/ml G418 (Sigma-Aldrich, Saint Louis, MO) for 7–10 days.

Preparation of shRNA expression vector

At first we developed ES cell lines in which the expression of *Pdx-1* or *Irs-1* was visualized by means of a fluorescent reporter protein, EGFP. Since this check should be performed in ES cells, undifferentiated state, we used a promoter such as CAG applicable to the constant expression of those genes in ES cells. In the transfection vector, the CAG promoter was followed by the sequences of *Pdx-1* (or *Irs-1*), *IRES*, and *EGFP* in tandem. In these cells, equimolar Pdx-1 (or Irs-1) and EGFP proteins are thought to be generated. These cell lines were designated as *Pdx-1:EGFP* and *Irs-1:EGFP*.

Several candidate RNAi sequences against *Pdx-1* or *Irs-1* were designed using siSearch (Chalk et al. 2004) and applied to *Pdx-1:EGFP* or *Irs-1:EGFP*. The effectiveness of the RNAi sequences was evaluated by monitoring EGFP fluorescence intensities and the optimum siRNA sequences against *Pdx-1* and *Irs-1* were determined. Based on these siRNA sequences, the optimum shRNAs against *Pdx-1* (shRNA_{Pdx-1}) and *Irs-1* (shRNA_{Irs-1}) were designed (Fig. 1a–c). Vectors expressing shRNA_{Pdx-1} or shRNA_{Irs-1} were prepared by inserting the appropriate hair-pin type sequences into pSilencer3.1-H1-hygro vector or pSilencer3.1-H1-neo vector (Ambion, Austin, TX, USA) (Fig. 1d). The shRNA_{Pdx-1} expression vector, pshRNA_{Pdx-1}-hygro, was introduced into *Pdx-1:EGFP*. The shRNA_{Irs-1} expression vector, pshRNA_{Irs-1}-neo(21mer) or pshRNA_{Irs-1}-neo(29mer), was introduced into *Irs-1:EGFP*. 21mer and 29mer indicate the chain lengths of the active form of the siRNA produced in the cells. Preliminary experiments suggested that activity of siRNA_{Irs-1} was greatest at these chain lengths. Therefore, we used these 2 different vectors for shRNA_{Irs-1}. Consequently the fluorescent intensity decreased markedly in comparison with that of the control cell line. These indicated that shRNA_{Pdx-1} and shRNA_{Irs-1} expression vectors actually caused the decrease of Pdx-1 and Irs-1 proteins.

Based on these preliminary experiments, we finally prepared optimum knockdown cell lines by introducing the shRNA_{Pdx-1} expression vector or the shRNA_{Irs-1} expression vector into EB3 cells by electroporation.

In parallel with these cell lines, we prepared 2 kinds of controls: negative control cell lines and non-vector control cells. The negative control cell lines NC-1, NC-2, and NC-3 were established by introducing a pSilencer3.1-H1-hygro, a pSilencer3.1-H1-neo vector, and both vectors into EB3 cells, respectively. The non-vector control cells EB3 cells that had not been transfected with a vector.

Evaluation of the effects of shRNA expression vectors against *Pdx-1* and *Irs-1*

Since *Pdx-1* is not expressed in ES cells, the effect of shRNA_{Pdx-1} on intrinsic expression of *Pdx-1* was evaluated at stage 4 of the differentiation process (defined below). The effect of shRNA_{Irs-1} on the expression of *Irs-1* was evaluated at differentiation stages 2 and 4.

In vitro differentiation

ES cells were induced to differentiate into insulin producing cells as previously described (Moritoh et al. 2003). Briefly, the procedure has 4 stages: stage 1, 2–3 days culture in ESM for multiplication; stage 2, suspension culture in ESM for 4–5 days to obtain embryoid bodies; stage 3, selection of nestin-positive cells; stage 4, induction of insulin producing cells. Selection at stage 3 was performed by suspending embryoid bodies in DMEM (Gibco/BRL) containing 10 % fetal bovine serum (FBS) and culturing for 2 days. The medium was then replaced by DMEM/F-12 (Gibco/BRL) and 50× ITSFn (Gibco/BRL) and the culture continued for 4–7 days. The cells were dislodged from culture dishes by treatment with 100 µl of 0.1 % trypsin/EDTA and then suspended in 1 ml ESM. The cells were collected by centrifugation (1,000 rpm, 3 min) and washed twice with 3 ml phosphate buffer saline (PBS). At stage 4, the cells were suspended in differentiation inducing medium (DIM), centrifuged, then re-suspended in fresh DIM. DIM is DMEM/F12 (Gibco/BRL) containing 50 × N2 (Gibco/BRL), 10 ng/ml human keratinocyte growth factor (PeproTech), 25 ng/ml basic fibroblast growth factor (R&D Systems), 20 ng/ml epidermal growth factor (Sigma), 50 × B27 supplement (Invitrogen), and 10 ng/ml nicotinamide. They were transferred to gelatin-coated dishes at a concentration of 0.5 – 2.0 × 10⁵ cells/cm². The culture was continued for 6–8 days, and the culture medium was refreshed every 24 h.

Fig. 1 shRNA expression vectors. **a** shRNA_{Pdx-1} expression cassette (21mer). BH, SS, LP, AS, RP, and HD designate BamHI site, Sense strand, Loop, Antisense strand, RNA Polymerase III terminator, HindIII site, respectively. **b** shRNA_{Irs-1} expression cassette (21mer). **c** shRNA_{Irs-1} expression cassette (29mer). **d** Construct of pSilencer3.1-H1-hygro (or -neo) vector

RNA isolation, RT-PCR and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was prepared with ISOGEN reagent (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. Oligo(dT)-primed cDNA was prepared from 1 µg of total RNA using SuperScript reverse transcriptase (Clontech-Takara Bio, Otsu, Japan). The primers for RT-PCR were designed using the information described in Moritoh et al. (2003) or mouse sequences reported elsewhere. The primer sequences used here are listed in Table 1. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

qRT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The amount of target RNA was normalized against *Gapdh* mRNA. The following primer pair sequences were used:

Pdx-1; forward 5'-GAA-ATC-CAC-CAA-AGC-TCA-CG-3' and

reverse 5'-CGG-GTT-CCG-CTG-TGT-AAG-3';
Irs-1; forward 5'-CCA-TGC-CTT-CTT-CTA-GA-T-GCT-C-3' and
 reverse 5'-AGC-CAT-GGC-CAC-TGG-TAC-T-3';
Irs-2; forward 5'-TCC-TCT-GCC-TCT-CTA-GA-T-GAA-TAC-AC-3' and
 reverse 5'-AAG-GGT-TGT-AGG-CCA-CTT-TG-G-3';
Ins1; forward 5'-GCC-CTT-AGT-GAC-CAG-CT-A-TAA-TCA-G-3' and
 reverse 5'-GGG-TAG-GAA-GTG-CAC-CAA-CA-G-3';
Ins2; forward 5'-GAG-CCC-TAA-GTG-ATC-CG-C-TAC-A-3' and
 reverse 5'-AGG-AAG-CGC-ATC-CAC-AGG-3';
Gk; forward 5'-TCC-CTG-TAA-GGC-ACG-AAG-ACA-3' and
 reverse 5'-GCC-ACC-ACA-TCC-ATC-TCA-AG-G-3';
Gapdh; forward 5'-TGT-GTC-CGT-CGT-GGA-TCT-GA-3' and
 reverse 5'-CCT-GCT-TCA-CCA-CCT-TCT-TGA-3'.

Table 1 Primers used in RT-PCR and the sizes of their respective PCR products

Category	Gene	Forward primer	Reverse primer	Size of product (bp)
a. Pancreatic cell markers	<i>Ins2</i>	CCCTGCTGGCCCTGCTCTT	AGGTCTGAAGGTCACCTGCT	211
	<i>SS</i>	TCGCTGCTGCCTGAGGACCT	GCCAAGAAGTACTTGGCCAGTTC	232
	<i>PP</i>	ACTAGCTCAGCACACAGGAT	AGACAAGAGAGGCTGCAAGT	364
	<i>Glucagon</i>	ACTCACAGGGCACATTACCC	CCAGTTGATGAAGTCCCTGG	353
	<i>Amylase</i>	CAGGCAATCCTGCAGGAACAA	CACTTGCGGATAACTGTGCCA	484
b. Transcription factors	<i>Ngn3</i>	TGGCACTCAGCAAACAGCGA	ACCCAGAGCCAGACAGGTCT	444
	<i>Pax6</i>	CAGTCACAGCGGAGTGAATC	CGCTTCAGCTGAAGTCGCAT	658
	<i>Pax4</i>	AAATGGCGCAGGCAAGAGAA	ATGAGGAGGAAGCCACAGGA	280
	<i>Nkx2.2</i>	AACCGTGCCACGCGTCAAA	AGGGCCTAAGGCCTCCAGTCT	220
	<i>Nkx6.1</i>	TCTTCTGGCCCGGGGTGATG	AGCCGCGTGCTTCTTCCTCCA	283
	<i>Isl-1</i>	AGATATGGGAGACATGGGCGAT	ACACAGCGGAAACACTCGATG	327
	<i>Beta2</i>	CTTGGCCAAGAACTACATCTGG	GGAGTAGGGATGCACCGGGAA	222
	<i>HNF-1α</i>	CATCCCTGTTCTTAGGAC	GAGTCGCCTCATTTCCAG	492
	<i>HNF-1β</i>	ATGGCTCCCCTCACCATCAG	AGGTGTTGAGGCTCTGTGCAA	360
	c. GSIS relevant genes	<i>Glut2</i>	CGGTGGGACTTGTGCTGCTGG	CTCTGAAGACGCCAGGAATTCCAT
<i>Kir6.2</i>		GGCTCCTAGTGACCTGCACCA	CCACAGCCACACTGCGCTTGCG	317
<i>Pc2</i>		AGAGATTCCATTGTGTGGGA	CAAAATGGACTTGGTGCCCA	215
Control	<i>Gapdh</i>	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452

Immunostaining of insulin producing cells

After stage 4, the culture medium was removed and the cells attached to the bottom of the dish were washed with PBS. The cells were fixed by addition of 2 ml of 4 % paraformaldehyde for 45 min. The fixed cells were washed three times with PBS and then treated with 100 % ethanol to permeabilize the cell membrane. The cells were washed three times with a glycerol/PBS solution and with 2 % gelatin for 15 min. Finally, the cells were washed three times with a glycerol/PBS solution and placed in a PBS solution containing 0.1 % bovine serum albumin (BSA) for 5 min. After removal of the BSA/PBS solution, guinea pig anti-mouse insulin antibody and rat anti-mouse C-peptide antibody were added to the dish. The cells were incubated with the antibodies at 37 °C for 40 min. The reaction solution was then removed and the cells were washed six times with BSA/PBS solution. The cells were stained with Alexa-568-conjugated goat anti-guinea pig antibody and Alexa-488-conjugated goat anti-rat antibody at 37 °C for 40 min. The cells were then washed six times with BSA/PBS solution.

Preparation of double knockdown ES cells

Pdx-1 knockdown cell lines were cultured and induced to differentiate to stage 4; *Pdx-1* expression levels were then assayed in the differentiated cells. The cell line with the lowest level of *Pdx-1* expression was selected as the recipient of the shRNA_{Irs-1} expression vector. Similarly, the *Irs-1* knockdown cell line with lowest level of *Irs-1* expression was selected from each of the two groups using pshRNA_{Irs-1}-neo(21mer) or pshRNA_{Irs-1}-neo(29mer). Thus, two cell lines were selected as recipients for the shRNA_{Pdx-1} expression vector.

Measurement of GSIS

Stage 4 cell clusters were washed four times with DMEM containing 3.3 mM glucose, 10 mM HEPES, and no insulin. After incubation at 37 °C for 30 min in the same medium, the cell clusters were washed five times with the same medium to eliminate any residual insulin contamination from the original culture medium. Then Krebs–Ringer solution containing 0, 3.3, or 33 mM glucose was applied to the cell clusters.

After incubation at 37 °C for 1 h, the insulin concentration in the reaction solution was measured using an Ultrasensitive Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden).

Criteria for validation of model cell lines

In order to use the developed cell lines as a model system for cell-based tests, it is important to guarantee that the cell lines are thoroughly validated. Here, we used the following criteria for validation:

- (1) The expression levels of *Pdx-1* and *Irs-1* should be sufficient to allow differentiation to stage 4, i.e., to cells capable of producing insulin. The model system was conceived as using cells at this stage, as it was believed that it would not be feasible to simulate more serious dysfunctions that might block differentiation or cause cell death during differentiation.
- (2) By contrast to criterion (1), the levels of *Pdx-1* and *Irs-1* expression need to be as low as possible. They should certainly be lower than 50 %, i.e., the heterozygous knockout level, to be able to simulate appreciable dysfunctions in insulin production activity, insulin reception activity, or GSIS activity.
- (3) The cells should not show any unexpected or abnormal phenotypes, such as unusual expression profiles of diabetes-relevant genes, as a consequence of the *Pdx-1* and *Irs-1* double knockdown. In the present study, we examined pancreatic cell markers (5 genes), transcription factors (9 genes), and GSIS related genes (3 genes) that are relevant to diabetes.

Results

Pdx-1 and/or *Irs-1* knockdown ES cell lines

Ten ES cell colonies carrying the pshRNA_{*Pdx-1*}-hygro (21mer) vector were obtained using the hygromycin selection protocol. The levels of *Pdx-1* expression were not determined in these candidate *Pdx-1* knockdown cell lines as the gene was either not expressed or only expressed at a low level during stages 1–3. Six of the cell lines were selected for differentiation to stage 4, i.e., to the insulin production stage. All 6 cell lines,

designated as P-1 to P-6, successfully differentiated to stage 4 and their *Pdx-1* expression levels were analyzed by qRT-PCR. The P-1 cell line showed the lowest expression level (32 %) compared to non-vector control cells (Fig. 2a).

Irs-1 knockdown cell lines were established using the same protocol as above except that G418 was used instead of hygromycin. We obtained 5 colonies with pshRNA_{*Irs-1*}-neo(21mer) and 7 colonies with pshRNA_{*Irs-1*}-neo(29mer). The cell lines were named I₂₁-1 to I₂₁-5 and I₂₉-1 to I₂₉-7, respectively. Examination of *Irs-1* expression showed that the I₂₁-1 and I₂₉-1 cell lines had the lowest expression levels, 19 and 51 %, respectively, compared to non-vector control cells (Fig. 2b).

Double knockdown cell lines were constructed by introducing the *Irs-1* knockdown vector into *Pdx-1* knockdown cells, or vice versa. Three recipient cell lines were used, namely P-1, I₂₁-1, and I₂₉-1. In total, 24 cell lines were established: (1) pshRNA_{*Irs-1*}-neo(21mer) was introduced into P-1 cells to generate 4 lines (P/I₂₁-1 to P/I₂₁-4); (2) pshRNA_{*Pdx-1*}-hygro was introduced into I₂₁-1 cells to generate 11 lines (I₂₁/P-1 to I₂₁/P-11); (3) pshRNA_{*Pdx-1*}-hygro was introduced into I₂₉-1 cells to generate 9 lines (I₂₉/P-1 to I₂₉/P-9). The integration of both vectors in each cell line was confirmed by genomic PCR. Each cell line was cultured to form embryoid bodies (stage 2) and *Irs-1* expression levels were measured. Two or three cell lines with the lowest levels of *Irs-1* expression were selected from each of the three groups. In total, 8 cell lines were finally selected and allowed to differentiate to stage 4. All 8 cell lines successfully differentiated to stage 4. The *Pdx-1* and *Irs-1* expression levels of each cell line were measured by qRT-PCR. *Pdx-1* expression levels ranged from 34 to 77 %, whereas *Irs-1* expression levels were in the range 32–46 % (Fig. 2c). The I₂₁/P-1 line showed low levels of *Pdx-1* and *Irs-1* expression: 34 % for both compared to the non-vector control.

Classification of the cell lines according to gene expression levels (Table 2) indicated that two lines, I₂₁/P-1 and P/I₂₁-1, were the best candidates for testing of the validation criteria outlined above.

Deficiency of insulin producing activity

Reduction in the *Pdx-1* expression level to 32 % caused a reduction in expression of the principal genes

Fig. 2 Knockdown cell lines with different levels of *Pdx-1* and *Irs-1* expression. Gene expression level in knockdown cell lines and negative control cell lines (NC) is expressed as relative intensity (%) compared to non-vector control cells. Error bar indicates mean $\pm$ SEM for $n = 3$. **a** *Pdx-1* single knockdown cell lines. **b** *Irs-1* single knockdown cell lines. **c** *Pdx-1* and *Irs-1* double knockdown cell lines. More details on the cell lines used here are provided in the legend to Table 2

for insulin production, *Ins1* and *Ins2*, to 19 % (Fig. 3a). In contrast, their expression levels increased to 120 % when *Irs-1* expression level decreased. In double knockdown cells, *Ins1* and *Ins2* expression levels were predominantly dependent on the level of *Pdx-1* expression (Fig. 3b). Therefore, *Ins1* and *Ins2* appear to be less sensitive to variation in expression of *Irs-1* than of *Pdx-1*. The double knockdown cell lines thus satisfy the requirement of an appreciable deficiency in insulin production.

Deficiency of insulin reception signaling function

Deficiency in insulin reception signaling function occurred as a consequence of the knockdown of *Irs-1*. Therefore, cell lines with low expression levels of *Irs-1* (20–40 % compared to the non-vector control) fulfill the criterion of an appreciable deficiency in insulin reception signaling function.

Loss of *Irs-1* activity is also known to be compensated by *Irs-2* activation. In the present case, expression of *Irs-2* was markedly increased by the decrease in *Irs-1* expression level (Fig. 4). In double knockdown cell lines, however, *Irs-2* expression decreased. This

decrease depended upon the level of *Pdx-1* expression rather than that of *Irs-1*. Therefore, the requirement of an appreciable deficiency in insulin reception signaling function is satisfied in the double knockdown cells.

Deficiency of GSIS activity

The level of *Gk* expression decreased when the *Pdx-1* expression level decreased (Fig. 5a). There was no difference between *Gk* expression profiles in single or double knockdown cells, indicating that *Gk* expression was insensitive to changes in *Irs-1* expression.

GSIS activity was measured and, under conditions of no glucose, insulin secretion was observed in all the cell lines. In the absence of glucose, 2.4–2.8 ng/mg protein was secreted; in the presence of 3.3 mM glucose, the level rose to 4.3–5.3 ng/mg protein, and at 33 mM glucose, to 5.0–7.2 ng/mg protein. We designated these levels of insulin secretion at 0, 3.3, and 33 mM glucose as I_0 , I_L , and I_H , respectively, and constructed a GSIS indicator using the formula: $(I_H - I_0)/(I_L - I_0)$. As shown in Fig. 5b, the GSIS indicator decreased as *Pdx-1* expression level decreased. Therefore, the double knockdown cell lines fulfill

Table 2 *Pdx-1* and *Irs-1* expression levels in the single and double knockdown cell lines generated in the present study

<i>Pdx-1</i> expression level					
20–40 %	40–60 %	60–80 %	>80 %	>95 %	
(a)					
<u>P-1</u>	<u>P-2</u>	P-3 <u>P-4</u> P-5	P-6	<u>NC-1</u>	
<i>Irs-1</i> expression level					
20–40 %	40–60 %				>95 %
(b)					
<u>I₂₁-1</u>	<u>I₂₉-1</u>				<u>NC-2</u>
<i>Pdx-1</i> expression level					
20–40 %	40–60 %	60–80 %	>80 %	>95 %	
(c)					
<i>Irs-1</i> expression level					
20–40 %	<u>I₂₁/P-1</u> <u>P/I₂₁-1</u>				
40–60 %	<u>I₂₉/P-1</u>	<u>P/I₂₁-2</u>	<u>I₂₁/P-3</u> <u>I₂₁/P-2</u>	I ₂₉ /P-2 I ₂₉ /P-3	
>95 %					NC-3

(a) *Pdx-1* knockdown cell lines and their negative control cell line (NC-1), (b) *Irs-1* knockdown cell lines and their negative control cell line (NC-2), (c) *Pdx-1* and *Irs-1* double knockdown cell lines and their negative control cell line (NC-3). The cell lines used in the experiments illustrated in Figs. 3, 4 and 5 are underlined

Fig. 3 Influence of *Pdx-1* or *Irs-1* expression level on *Ins1* (open circle) and *Ins2* (closed circle) expression levels in single (a) or double knockdown (WKD) cells (b). a The solid lines indicate *Pdx-1* expression profiles obtained with the cell lines underlined in Table 2a, while the broken lines indicate *Irs-1* expression profiles obtained with cell lines underlined in

Table 2b. b *Ins1* (solid lines) and *Ins2* (broken lines) expression profiles in WKD cell lines were obtained with cell lines underlined in Table 2c, respectively; these profiles were determined by the least-squares method. Relative gene expression level and error bars are as in Fig. 2

the requirement of an appreciable deficiency of GSIS activity.

Cell cluster sizes and insulin producing patterns

These parameters were analyzed to detect a symptom of any malfunction that should occur in cells either during differentiation or at the final stage of insulin

producing cells. Three cell lines, P-1, I₂₁-1, and I₂₁/P-1, were selected on the basis of their low *Pdx-1* or *Irs-1* expression levels (Table 2) and their properties were compared with those of non-vector control cells. The cell clusters formed by the P-1 and I₂₁/P-1 lines, which have low *Pdx-1* expression levels, ranged from 85 to 130 μ m and were slightly smaller than those of the non-vector control, which ranged from

Fig. 4 Effect of *Irs-1* expression level on *Irs-2* expression level in *Irs-1* knockdown cell lines obtained with cell lines underlined in Table 2b, and the effect of *Pdx-1* expression level on *Irs-2* expression level in double knockdown (WKD) cells obtained with cell lines underlined in Table 2c. Relative gene expression level and error bars are as in Fig. 2

115 to 170 μm (Fig. 6). By contrast, the clusters in the I_{21} -1 line, which has a low *Irs-1* expression level, seemed to contain larger clusters which ranged from

Fig. 5 Influence of *Pdx-1* expression level on GSIS related responses. **a** *Gk* expression profiles in *Pdx-1* knockdown (SKD) cells obtained with cell lines underlined in Table 2a (open circle), and in double knockdown (WKD) cells obtained with

115 to 200 μm . The results of this analysis were consistent with those reported previously that the knockdown deficiency of *Irs-1* expression is associated with an increase in β -cell mass.

Insulin producing activities in the 3 cell lines were analyzed by immunochemical staining. In P-1, which has a relatively low level of *Pdx-1* expression (Table 2), insulin producing activity was lower than in non-vector control cells (Fig. 7). By contrast, insulin production was similar to that of the non-vector control cells in I_{21} -1, which has a relatively low *Irs-1* expression level. In the double knockdown cells (I_{21} /P-1), the insulin producing pattern was similar to that of *Pdx-1* deficient cells (P-1).

Therefore, the double knockdown cell lines developed here satisfy the requirements that they should not display abnormal phenotypes nor have unusual patterns of insulin producing activity as a consequence of *Pdx-1* and *Irs-1* knockdown.

Expression profiles of cell type markers

The expression of pancreatic cell markers was investigated in the P-1, I_{21} -1, and I_{21} /P-1 lines. Five genes were selected, namely *Ins2*, *SS*, *PP*, *Glucagon*, and *Amylase*, which respectively act as diagnostic markers for δ -cells, PP-cells, α -cells, and exocrine cells. The influence of *Pdx-1* and/or *Irs-1* knockdown on the expression of these genes is summarized in Table 3a. Expression of all 5 genes was reduced by *Pdx-1*

cell lines underlined in Table 2c (closed circle). **b** GSIS indicator (see text for details) determined using the cell lines underlined in Table 2c (closed circle). Relative gene expression level and error bars are as in Fig. 2

Fig. 6 Phase contrast images of cell clusters in cultures of the P-1, I₂₁-1, and I₂₁/P-1 cell lines, and non-vector control (NVC) at stage 4 of differentiation. Bar indicates 300 μm

knockdown, but was apparently unaffected by knockdown of *Irs-1*. Therefore, the increase in cell cluster sizes in I₂₁-1 (Fig. 6) was unlikely to be a result of an increase in β -cell mass but rather of all cell types. In the double knockdown cell line, I₂₁/P-1, expression of the five genes was similar to that in the *Pdx-1* single knockdown cell line, P-1. This indicates that suppression of expression of these genes was predominantly a consequence of *Pdx-1* knockdown.

Expression profiles of transcription factors

We examined expression of a range of transcription factors that are markers of different cell types from endoderm to pancreatic cells. In the following description, the name of the transcription factor is given in parentheses after the name of the cell type in which it is normally expressed. Endoderm cells (*Hnf-1 α*) differentiate into pancreatic progenitor cells (*Isl-1*), which give rise to exocrine progenitor and endocrine progenitor cells (*Ngn3*). Exocrine progenitor cells differentiate into exocrine cells (*Amylase*). Endocrine progenitor cells have two differentiation pathways: one towards α -cell progenitor cells (*Pax6*),

the other towards the common progenitor (*Beta2*, *Nkx2.2*, *Pax4*, *Nkx6.1*) of β -cells (*Pax6*, *Isl-1*, *Beta2*), δ -cells, and PP-cells.

In the P-1 line, with knockdown of *Pdx-1*, the expression of *Ngn3* and *Nkx2.2* decreased markedly (Table 3b). This suggests that differentiation from pancreatic progenitor into endocrine progenitor and further into pancreas β -, δ -, and PP-cells was dramatically suppressed. This suppression of differentiation might explain the reduced size of clusters in P-1 (Fig. 6). By contrast, reduction in *Irs-1* expression in the I₂₁-1 line did not affect expression of these transcription factors. In the double knockdown cells, I₂₁/P-1, the expression profiles of these factors was similar to those in *Pdx-1* single knockdown cells. Therefore, the expression of the transcription factors was predominantly regulated by *Pdx-1*.

Expression profiles of GSIS relevant genes

As mentioned above, *Gk* is regulated by *Pdx-1* expression level. Here, the influence of *Pdx-1* and *Irs-1* knockdown on other genes associated with GSIS was investigated. As shown in Table 3c, *Kir6.2* and *Pc2* expression was reduced dramatically when *Pdx-1*

Fig. 7 Immunofluorescence stained cell clusters in cultures of the P-1, I₂₁-1, and I₂₁/P-1 cell lines and non-vector control (NVC). *Left column*: Alexa-568 staining of insulin production

expression level decreased as in P-1 cell line but not when the *Irs-1* expression level decreased as in I₂₁-1 cell line.

In a similar fashion to that found for the pancreatic cell markers and transcription factors, the expression profiles of GSIS related genes in the double knockdown I₂₁/P-1 cell line were similar to those in the *Pdx-1* knockdown cell line.

Center column: Alexa-488 staining of C-peptide production. *Right column*: merged image. *Bar* indicates 100 μ m

Discussion

In this study, we used RNAi knockdown methodologies to develop knockdown cell lines, rather than the knockout strategies adopted by previous studies on cell lines and mice. At first we would like to discuss the stability of transgenes during long-term culture: the ES cell culture for no longer than 1 month followed

Table 3 Summary of the effects of *Pdx-1* and/or *Irs-1* on the expression of pancreatic marker genes

Category	Gene	NVC	P-1	I ₂₁ -1	I ₂₁ /P-1
a. Pancreatic cell marker	<i>Ins2</i>	++	↓↓	→	↓↓
	<i>SS</i>	+++	↓	→	↓↓
	<i>PP</i>	+++	↓	→	↓
	<i>Glucagon</i>	+	↓↓	→	↓↓
	<i>Amylase</i>	+	↓	→	↓
b. Transcription factors	<i>Ngn3</i>	++	↓↓	→	↓↓
	<i>Pax6</i>	+++	→	→	↓
	<i>Pax4</i>	+++	→	→	↓↓
	<i>Nkx2.2</i>	++	↓↓	→	↓↓
	<i>Nkx6.1</i>	++	↓	↓	↓
	<i>Isl-1</i>	++	↓	→	→
	<i>Beta2</i>	+	→	→	↓
	<i>HNF-1α</i>	+	↓	→	→
	<i>HNF-1β</i>	++	↓	→	↓↓
	<i>Glut2</i>	+++	→	→	↓
	<i>Kir6.2</i>	+	↓↓	→	↓
c. GSIS relevant genes	<i>Pc2</i>	+++	↓↓	→	↓↓
	<i>Gapdh</i>	++	→	→	→

Gene expression intensities were judged after ethidium bromide staining: (+++) intense, (++) less intense, (+) weak

Gene expression intensity changes in comparison to non-vector control cells (NVC): (↓↓) marked decrease, (↓) decrease, (→) no change

by the differentiation culture for ca. 3 weeks. We initiated differentiation culture at different times during the ES cell culture and then measured the expression intensities of *Pdx-1* and *Irs-1* at stage 4. We confirmed that the knockdown levels of both genes were reproducible within the standard error of means for the repetition of 3 times indicated in Fig. 2. The results showed that the transgenes were sufficiently stable enough for the quantitative experiments at stage 4.

Next we discuss the validation criteria. Here, two cell lines, I₂₁/P-1 and P/I₂₁-1, were found to meet the necessary validation criteria in order for them to be used as a model cell system. Their characteristics can be summarized as follows:

- (i) A decrease in the *Pdx-1* expression level reduced *Ins1* and *Ins2* expression levels (*Pdx-1* in Fig. 3a). This is consistent with previous reports that conclude *Pdx-1* is a regulator of *Ins1* and *Ins2* (Melloul et al. 2001).
- (ii) A decrease in the level of expression of *Irs-1* increased *Irs-2* expression (*Irs-1* knockdown cell lines in Fig. 4). This is thought to be

compensation for the loss of *Irs-1* function described e.g. in Kubota et al. (2008) and Dobrzyn et al. (2010).

- (iii) A decrease in the level of expression of *Pdx-1* reduced *Irs-2* expression, and this effect surpassed the reverse effect described above in (ii) as demonstrated in the double knockdown cells (double knockdown cell lines in Fig. 4). This observation is a novel finding but does not contradict previous reports Kulkarni et al. (2004) that insufficiency of *Pdx-1* limits compensatory islet hyperplasia.
- (iv) A decrease in the level of expression of *Pdx-1* reduced *Gk* expression (Fig. 5a). This is consistent with previous reports by Chalk et al. (2004).

The characteristics of the knockdown cell lines listed above, together with other results obtained in this study, can be explained by the model gene expression regulation scheme illustrated in Fig. 8.

We have outlined the criteria that candidate cell lines must satisfy before they can be considered as a validated cell model system. Two cell lines, I₂₁/P-1

Fig. 8 Schematic summary of the gene expression regulation pathways consistent with the results of this study. Arrowed line indicates positive regulation. Line with closed circle indicates negative regulation. The numbers (i)–(iv) refer to the points described in the “Discussion”. “Tab. 3” refers to the results summarized in Table 3

and PI_{21-1} , produced by a double knockdown of *Pdx-1* and *Irs-1*, were shown to satisfy these criteria. The successful generation of these cell lines indicates the feasibility of applying the RNAi method and validation scheme to other combinations of diabetes-related genes. Therefore, the results of this study will be of general significance for ES cell engineering.

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