

Aberrant Expression of Rest4 Gene in Low-Functioning Pancreatic Beta Cell Line

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ABSTRACT

Repressor element-1 silencing transcription factor (Rest) is not expressed in pancreatic beta cells and neuronal cells. However, Rest4, a truncated form of Rest, is expressed in high passaged MIN6 (HP-MIN6) cells, a pancreatic beta cell line that lost glucose-responsive insulin secretion. Rest4 is also expressed in injured MIN6 cells and isolated islets. Herein, the forced expression of dominant negative form of Rest in HP-MIN6 cells was subjected to microarray analysis of gene expression to investigate the role of Rest4 gene in MIN6 cells. Furthermore, the forced expression of Rest4 gene in MIN6 cells was subjected to microarray analysis of gene expression to investigate the function of Rest4 in normal insulin-producing cells. The results showed that Rest4 inhibits cell proliferation and DNA and RNA metabolism and stimulates secretory mechanisms and nervous system gene expression. These findings suggest that Rest4 may act defensively against cellular injury in pancreatic beta cells.

Introduction

Pancreatic beta cells and neuronal cells share similar functions. Repressor element-1 silencing transcription factor (Rest) is important for the terminal differentiation of pancreatic beta cells and neuronal cells through the regulation of neuronal and pancreatic endocrine gene [1–5]. Generally, the Rest gene is not expressed in mature pancreatic beta cells and beta cell line [6] and is classified as a disallowed gene in pancreatic beta cells [7].

Recent studies revealed that the Rest gene is highly expressed in adult neurons and that dysregulation of the Rest gene is involved in the pathogenesis of Alzheimer's disease (AD), Huntington's disease, and epilepsy [8–15]. Thus, the Rest gene may be involved in the dysregulation of pancreatic beta cells. However, no studies have investigated Rest gene expression in pancreatic beta cells.

MIN6 cells are a transformed β -cell line obtained from mouse insulinoma; they retain glucose-stimulated insulin secretion (GSIS) and are widely used as an in vitro model for insulin secretion [16]. However, the GSIS capacity of MIN6 cells is lost in long-term culture. Recently, my colleagues and I developed a MIN6 subclone, designated as MIN6 clone 4, which retains GSIS even after long-term culture [17]. We compared the gene expression profiles of parental MIN6 cells and MIN6 clone 4 cells after short-term culture (Pr-LP and C4-LP, respectively) and long-term culture (Pr-HP and C4-HP, respectively) to identify the genes involved in the maintenance of GSIS capacity [17].

Rest was one of the differentially expressed genes among Pr-LP, Pr-HP, C4-LP, and C4-HP cells. It was only expressed in high passaged MIN6 (HP-MIN6) cells, which are known to have reduced

insulin secretion in response to glucose. Therefore, in the present study, the expression of Rest gene in pancreatic beta cell line was further investigated.

Materials and Methods

Culture of MIN6 cells and mouse islets

MIN6 cells that were established in a previous study [16] were maintained in Dulbecco's Modified Eagle's Medium containing 25 mM glucose, 13 % heat-inactivated fetal bovine serum, 0.1 mM 2-mercaptoethanol, 100 units/ml penicillin, and 0.05 mg/ml streptomycin in humidified 5 % CO₂ at 37 °C. MIN6 clone 4 cells are a subclone isolated from low-passage-number parental MIN6 cells through the limiting dilution method. They were maintained in the same culture conditions as the parental cells and retained good GSIS even after 6 months of continuous culture. Cells passaged 17–20 and 35–40 times were used as low-passage-number parental MIN6 cells (Pr-LP) and high-passage-number MIN6 cells (Pr-HP), respectively. Seventeen to 20 passages were also used for the low-passage-number MIN6 clone 4 cells (C4-LP), and 40 to 50 passages were used for the high-passage ones (C4-HP) [17]. In the following sections, the parental MIN6 cells and their subclone were treated identically.

Institute of Cancer Research mice were sacrificed, and their pancreatic islets were isolated according to a previously described method [18, 19]. The isolated islets were cultivated in RPMI medium with 11 mM glucose, 10 % fetal bovine serum, and antibiotics. After 20 hours cultivation with or without trichostatin A (TSA), the islets were washed with phosphate buffered saline and transferred to microcentrifuge tubes (10 islets per tube).

MIN6 cells or isolated islets were exposed to 1 μM thapsigargin [an endoplasmic reticulum (ER) stress inducer], 0.5 mM streptozotocin (STZ, which is toxic to pancreatic beta cells), or 0.01 or 0.1 μM TSA (a histone deacetylase inhibitor) for 24 hours. The concentration of TSA was selected to avoid nonspecific cytotoxic effect of TSA. Therefore, a concentration of TSA corresponding to 70 % survival of MIN6 cells after 24 hours incubation was selected [20].

Adenoviral vectors and infection

MIN6 cells were infected with adenoviral vector expressing Rest4 (Adv-CAG-Rest4) or dominant negative form of Rest (Adv-CAG-dnREST) using a standard technique [21]. Briefly, MIN6 cells were dissociated by trypsin-EDTA; suspended in growth medium; and infected with Adv-CAG-Rest4, Adv-CAG-dnREST, or a control Adv expressing enhanced green fluorescent protein (Adv-CAG-EGFP) at a multiplicity of infection of 20. The cells were then plated in a culture dish and incubated for 4 days. Fluorescence microscopy showed that Adv-CAG-EGFP infected more than 98 % of MIN6 cells under these conditions. The dbREST construct was based on the report of Chen et al. [5]. The Rest4 construct was derived from Pr-HP MIN6 cells.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured cells or pancreatic isolated islets via the acid guanidinium-phenol-chloroform (AGPC) method. cDNA was prepared from total RNA using a ReverTra Ace-α kit (Toy-

obo, Tokyo, Japan) with oligo dT primers in accordance with the manufacturer's instructions. PCR was performed with Taq DNA polymerase (Promega, Madison, WI) within the log phase of the reaction (25–30 cycles).

The following primers were used to detect Rest genes: Rest-F1 (exon 5) primer: 5'-tgtgaccgctgtggatacaa-3', Rest-R1 (exon 6): 5'-cagttgaactgccgtgggtt-3', REST4-F1 (exon N): 5'-cagagtgatgatctagatggg-3', and Rest-R2 (exon 6): 5'-attaagcaggtcagcctctc-3'. The combination of Rest-F1 and Rest-R1 resulted in the following PCR products: original Rest, 520 bp; REST4, 536 bp. REST4-F1 and Rest-R2 primers produced REST4 product (353 bp), whereas no product was obtained in the original REST.

cDNA microarray analysis

MIN6-C4 cells were infected with Adv-CAG-Rest4 or Adv-CAG-EGFP as a control, and MIN6-HP cells were infected with Adv-CAG-dnREST or Adv-CAG-EGFP as a control. Four days later, total RNA was extracted from these cells by using the AGPC method. The quality of the purified total RNA was examined with an Agilent 2100 Bioanalyzer. About 500 ng of verified RNA was utilized to obtain cyanine 3 (Cy3)- or Cy5-labeled cRNA using a low RNA input fluorescent linear amplification kit (Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's instructions. After the cRNA was purified using the RNeasy Mini Spin Columns (Qiagen, Hilden, Germany), 0.75 μg each of Cy3- and Cy5-labeled cRNA was combined, fragmented, and hybridized to a high-density oligonucleotide microarray (Agilent). The image was quantified by Agilent Feature Extraction Software.

Real-time PCR analysis

The microarray data were validated by real-time PCR for several selected genes. Real-time PCR was performed on an ABI 7300 or StepOnePlus Real-Time PCR System using the SYBR Green PCR Reagents detection system (Takara, Shiga, Japan). PCR was performed with an initial step of 10 seconds at 95 °C followed by 40 cycles of 5 seconds at 95 °C and 31 seconds at 60 °C. The expression levels of targeted genes were normalized to that of Rpl32. The following primers were used to detect REST genes: REST-F2 primer: 5'-acatcgcgactcattcag-3' and NRSF-R2: 5'-attaagcaggtcagcctctc-3'. The following primers were used to detect Rpl32 genes: Rpl32-F primer: 5'-caatgtctctctaagaaccgaaa-3' and Rpl32-R: 5'-cctggcgttgaggattgg-3'.

Rest4-related gene enrichment analysis

Further functional enrichment analysis of target genes of Rest4 in pancreatic beta cells was performed using Metascape [22]. Two sets of microarray analyses were performed to investigate the function of Rest4: one involving MIN6-C4 cells (forced expression of Rest4 against forced expression of EGFP) and another involving MIN6-HP cells (forced expression of dominant negative Rest against forced expression of EGFP). The enrichment of differentially expressed genes was performed by Metascape (<https://metascape.org/>) [22]. Genes that showed more than two-fold difference in expression level were subjected to gene enrichment analysis.

Results

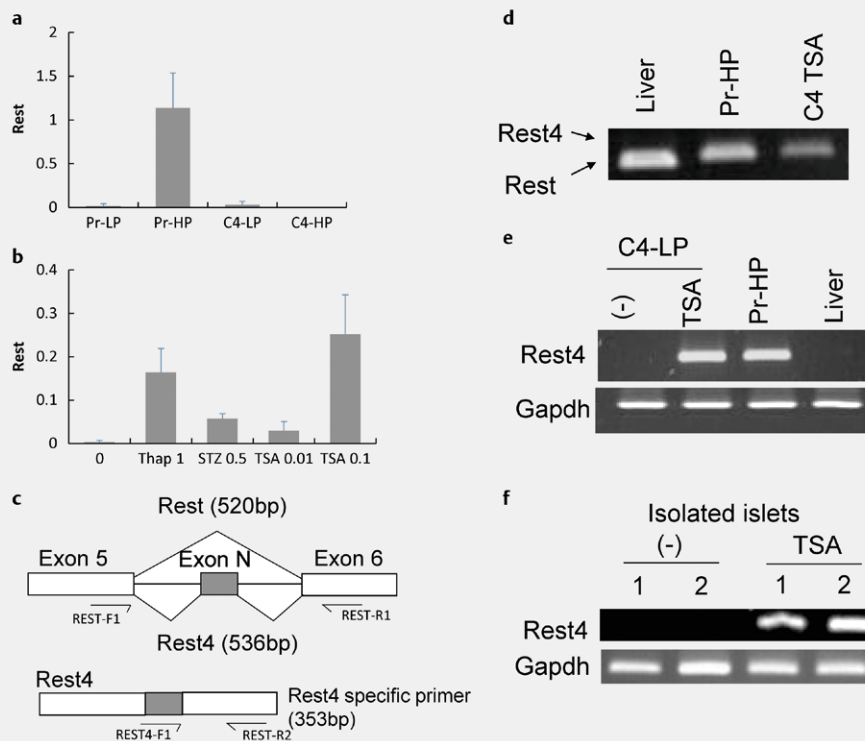
The expression of the Rest gene was extremely low in the well-regulated MIN6 cells (Pr-LP, C4-LP, and C4-HP), which is consistent with a previous study on a pancreatic beta cell line [6] (► Fig. 1a). However, it was high in the dysregulated cells (Pr-HP) (► Fig. 1a). Interestingly, the PCR product of Pr-HP was relatively shorter than that of the original one (► Fig. 1c, d, PCR product of liver was 520 base pairs, whereas those of Pr-HP and C4 treated with TSA were 536 base pairs). Sequence analysis of the PCR product of Pr-HP revealed that the Rest gene of Pr-HP was Rest4, an alternative splicing variant of the Rest gene. RT-PCR analysis using a specific primer for Rest4 confirmed that Rest4 was expressed in Pr-HP cells (► Fig. 1c, e). Quantitative PCR analysis using a specific primer to detect the Rest gene with and without exon N also showed that the Rest gene without neuron-specific exon N was detected at a low level in Pr-HP cells (data not shown), showing that Rest4 is expressed in MIN6 cells, but not full-length Rest.

As Pr-HP cells are considered as a model of dysregulated pancreatic beta cells, the induction of Rest gene expression was relevant to the malfunctional status of pancreatic beta cells. Thus, the effect of toxic agents for pancreatic beta cells on Rest4 gene expression was evaluated. STZ induces oxidative stress in pancreatic beta cells, thapsigargin induces ER stress, and TSA impairs the function of pan-

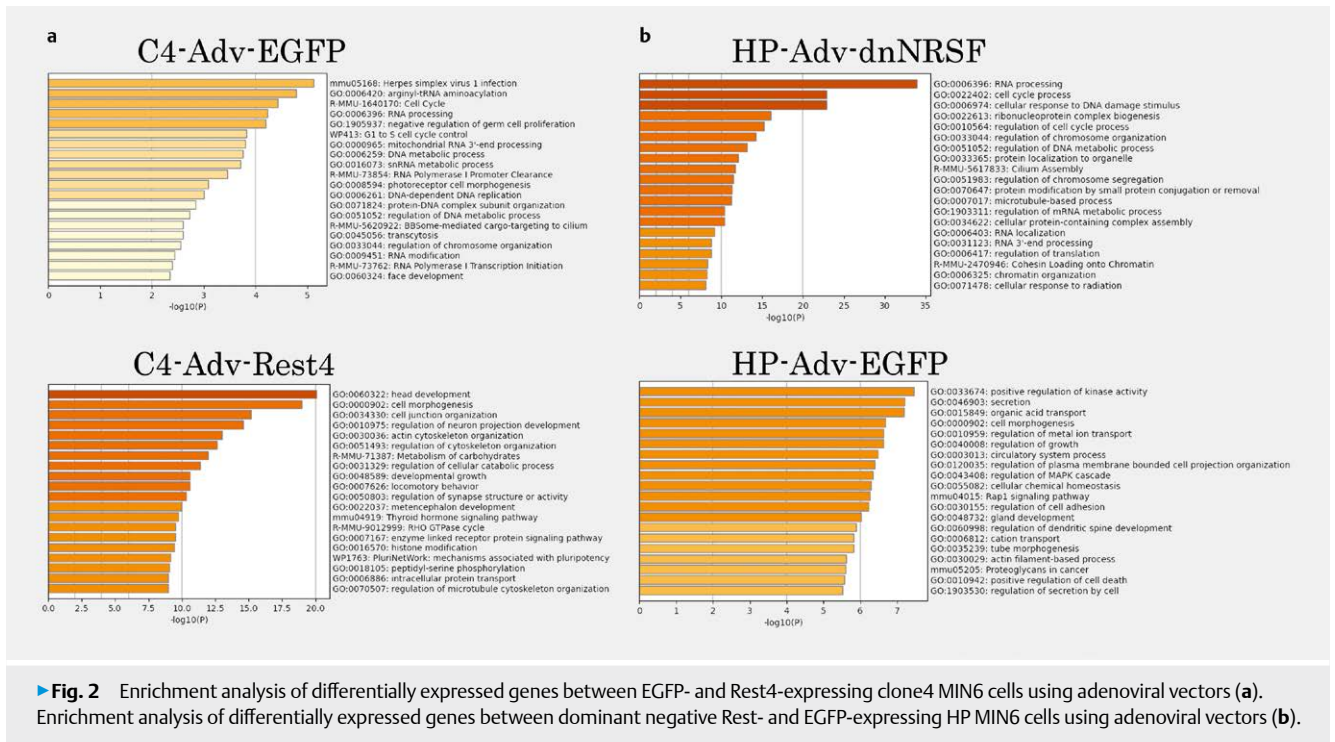
creatic beta cell line [20]. In the present study, STZ, thapsigargin, and TSA increased Rest gene expression in MIN6 cells. TSA also increased Rest4 gene expression in mouse islets (► Fig. 1f).

Two sets of microarray analyses were performed to investigate the function of Rest4: one involving MIN6-C4 cells (forced expression of Rest4 against forced expression of EGFP) (► Fig. 2a) and another involving MIN6-HP cells (forced expression of dominant negative Rest against forced expression of EGFP) (► Fig. 2b). Compared with MIN6-HP cells, which expressed Rest4, the dominant negative form of Rest was introduced in the zinc-finger domain. This fragment is necessary and sufficient for DNA binding and lacks active domains, outside of the DNA binding region [5]. The enrichment of differentially expressed genes was performed by Metascape. The 1498 genes whose gene expression levels were more than doubled by Rest4 gene transfer and the 840 genes whose gene expression levels were more than doubled by EGFP gene transfer were analyzed. The 1697 genes whose gene expression levels were more than doubled by dnRest gene transfer and the 1073 genes whose gene expression levels were more than doubled by EGFP gene transfer were analyzed.

Enrichment analysis was performed using Metascape to evaluate gene expression in HP cells transfected with Adv-dnREST and Adv-EGFP. The results showed that Rest4 gene expression was sup-



► **Fig. 1** Quantitative RT-PCR analysis of Rest (a) in Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells. Values are means \pm SD. Quantitative RT-PCR analysis of Rest4 (b) in MIN6 cells cultured with 1 μ M thapsigargin (Thap1), 0.5 mM STZ (0.5), and 0.01 or 0.1 μ M TSA for 24 h. Values are means \pm SD. Structure of the mouse Rest gene transcript and its alternative splicing variant, Rest4 (c). Exons are shown in white box, additional exons are shown in gray box, and introns are shown in lines. The primers of RT-PCR are indicated by the right and left arrows. RT-PCR profiling of Rest and Rest4 in liver cells (control), Pr-HP MIN6 cells, and C4 MIN6 cells cultured with 0.1 μ M TSA for 24 h (d). Rest4 gene expression using Rest4 gene-specific primers in C4 MIN6 cells cultured with 0.1 μ M TSA for 24 h, Pr-HP MIN6 cells, or liver cells (control) (e). Rest4 gene expression using Rest4 gene-specific primers in isolated mouse islets cultured with or without 0.1 μ M TSA for 24 h (f).



pressed when gene expression was increased by Adv-dnREST. As gene groups, genes related to the cell cycle, DNA, RNA, and nucleic acid metabolism were found to be upregulated by Adv-dnREST. When gene expression was increased by Adv-EGFP, the Rest4 gene promoted the expression of genes related to hormone secretion, intracellular trafficking, and intracellular signal transduction. In EGFP and C4 cells, the expression of genes increased with Adv-EGFP transfection; that is, the Rest4 gene suppressed the expression of genes related to the cell cycle, DNA, RNA, and nucleic acid metabolism. These results were similar to those observed in HP cells. In addition, the expression of genes related to nervous system formation and cell adhesion was increased by Adv-Rest4 (i. e., genes whose expression was promoted by the Rest4 gene).

Discussion

The prolonged culture of MIN6 cells promotes the expression of Rest, especially its truncated form, Rest4. The Rest4 gene was expressed only in HP-MIN6 cells, not in LP-MIN6 or C4 cells. HP-MIN6 cells are known to have reduced insulin secretion in response to glucose. The Rest4 gene was also expressed in injured MIN6 and islet cells.

Rest is expressed in developmental [23–25] and injured neuronal cells [8, 26, 27]. Rest4 is reported to counteract the repression of Rest on target genes in a dominant negative manner, modulating the function of neuronal cells [28]. Thus, the Rest4 gene may regulate the function of pancreatic beta cells, especially when they are injured. To date, no study has reported on Rest4 gene expression in pancreatic beta cells. As injured beta cell lines express the Rest4 gene, functional analysis of Rest4 can be used to explore

the novel mechanism of pancreatic beta cells. Therefore, GeneChip analysis was performed to investigate the function of the Rest4 gene in pancreatic beta cells.

The gene cluster of the cell cycle was enriched in HP-Adv-dnREST and C4-Adv-Rest4. This phenomenon suggested that dnREST suppressed the intrinsic expression of Rest4 in HP cells. Thus, Rest4 expression was suppressed by dnREST expression. Rest4 is a truncated form of Rest that suppresses the activity of Rest in other cells in neurons. However, in the present study, dnREST expression suppressed Rest4-activated and cell-cycle-related genes.

Rest has been shown to promote tumorigenesis in neurons. However, it has been reported to suppress tumorigenesis in non-neuronal cells [29–31]. In the present study, Rest4 showed an inhibitory function on cell proliferation in a pancreatic beta cell line that resembles neurons in terms of Rest gene expression. This fact is also considered interesting in examining the behavior of Rest against tumors.

The inhibition of cell proliferation also plays an important role in the physiological aging system and is thought to inhibit tumorigenesis. In fact, Rest genes have also been shown to play an important role in maintaining normal aging in neurons [11, 32–34].

These findings also suggest that the expression of the Rest4 gene by injured MIN6 cells may be a mechanism to protect them from injury. Interestingly, the suppression of the Rest4 gene by dnRest gene transfer to HP-MIN6 cells revealed a group of genes related to secretion and secretory gland development. In other words, the Rest4 gene seems to positively regulate gene expression in the secretory system in HP-MIN6 cells, where the insulin secretory mechanism is malfunctioning, suggesting that the Rest4 gene may also act defensively in injured insulin-secreting cells.

When the Rest4 gene was forced to express in MIN6 cells with normal insulin secretion mechanism, no secretion-related genes were induced. However, nervous system genes were induced, suggesting that the Rest4 gene is involved in de-repression of a group of nervous system genes by Rest genes in neurons. Pancreatic beta cells are known to have some expression of nervous system genes [6]. Thus, it is possible that the nervous system genes were induced by the de-repression mechanism of the Rest4 gene observed in the nerve cells.

Rest4 also suppressed DNA synthesis, RNA synthesis, RNA processing, and cell proliferation in HP cells. This shows that Rest4 contributes to cell stabilization. A similar trend was observed in normal C4 cells, suggesting that Rest4 plays a role in cell stabilization. The mechanism of stabilizing cellular functions and preventing over-responses to stimuli is observed during cellular senescence [33]. Thus, it is possible that Rest4 maintains mechanisms to maintain cellular functions, including suppression of tumorigenesis.

Rest plays a regulatory role in neuronal homeostasis and hyper-responsiveness through the repression of various neuronal gene clusters [8, 10, 35]. In other words, Rest may be cytoprotective in some situations. Rest is known to be expressed in aging brain and neurodegenerative diseases, suggesting the protective action of Rest in mature neurons [33, 34]. In AD, Rest acts as an antioxidant stressor, plays a cytoprotective role against amyloid accumulation, and promotes longevity by suppressing cellular hyperreactivity [11]. Thus, it is possible that Rest acts on neurons in an injurious or protective manner, depending on the situation. Since pancreatic beta cells are similar to neurons and the expression of Rest is lower than that of neurons, it is possible that Rest plays some role in pancreatic beta cells.

In summary, this study demonstrates for the first time that Rest4, an alternative splicing gene of Rest, is expressed in a pancreatic β -cell line. The Rest gene is expressed during pancreatic β -cell damage. Moreover, gene cluster analysis revealed that the Rest gene is involved in suppressing cell proliferation and promoting the expression of secretion-related genes in pancreatic β -cells, suggesting that the Rest gene is involved in the maintenance of pancreatic β -cell function. Further analysis of the Rest gene is needed to elucidate the mechanism of pancreatic β -cell injury and the pathogenesis of diabetes mellitus.

Conflict of Interest

The authors declare that they have no conflict of interest.

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