Differential Gene Expression in GPR40-Overexpressing Pancreatic β-cells Treated with Linoleic Acid

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“G protein-coupled receptor 40” (GPR40), a receptor for long-chain fatty acids, mediates the stimulation of glucose-induced insulin secretion. We examined the profiles of differential gene expression in GPR40-activated cells treated with linoleic acid, and finally predicted the integral pathways of the cellular mechanism of GPR40-mediated insulinotropic effects. After constructing a GPR40-overexpressing stable cell line (RIN-40) from the rat pancreatic β-cell line RIN-5f, we determined the gene expression profiles of RIN-5f and RIN-40. In total, 1004 genes, the expression of which was altered at least twofold, were selected in RIN-5f versus RIN-40. Moreover, the differential genetic profiles were investigated in RIN-40 cells treated with 30 μM linoleic acid, which resulted in selection of 93 genes in RIN-40 versus RIN-40 treated with linoleic acid. Based on the Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG, http://www.genome.jp/kegg/), sets of genes induced differentially by treatment with linoleic acid in RIN-40 cells were found to be related to mitogen-activated protein (MAP) kinase- and neuroactive ligand-receptor interaction pathways. A gene ontology (GO) study revealed that more than 30% of the genes were associated with signal transduction and cell proliferation. Thus, this study elucidated a gene expression pattern relevant to the signal pathways that are regulated by GPR40 activation during the acute period. Together, these findings increase our mechanistic understanding of endogenous molecules associated with GPR40 function, and provide information useful for identification of a target for the management of type 2 diabetes mellitus.

Key Words: G protein-coupled receptor 40, Linoleic acid, Insulin secretion, Pancreatic β-cell, Type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM, OMIM 125853) is a complex disease of various etiologies. A major characteristic of the pathological condition in T2DM is impairment of insulin secretion [1]. This deficit in insulin secretion involves the function of pancreatic β-cells and is aggravated by loss of β-cells and inappropriate signaling pathway activity [2]. Impaired insulin secretion leads to elevated blood glucose levels and multiple complications, including cardiovascular disease, nephropathy, retinopathy, lipid disorders, neuropathy, and ketonuria. Thus, regulation of insulin release is considered a target for intervention in T2DM [3,4].

“G protein-coupled receptor 40” (GPR40) is a potential therapeutic target in T2DM and has been suggested to play an important role in regulating glucose-stimulated insulin secretion by pancreatic β-cells [5,6]. GPR40 is activated by free fatty acids (FFAs), such as linoleic acid, and mediates the majority of the effects of FFAs on β-cells [7]. Experiments using GPR40 KO mice (GPR40 -/-) showed that loss of GPR40 resulted in impaired insulin secretory responses to fatty acids, suggesting a possible role for the receptor in insulin secretion [8]. In another study, human GPR40 transgenic mice under the control of an insulin promoter were developed and displayed improved oral glucose tolerance, as well as enhanced glucose- and fatty acid-stimulated insulin secretion, compared with wild-type mice [9]. Thus, these observations suggest that GPR40 is linked to glucose-stimulated insulin secretion in pancreatic β-cells.

Whether a GPR40 agonist or antagonist would be better

ABBREVIATIONS: GPR40, G protein-coupled receptor 40; KEGG, Kyoto Encyclopedia of Genes and Genomes Pathway; MAP, mitogen-activated protein; PLC, phospholipase C; ERK1/2, extracellular signal-regulated kinase 1/2; SEA, singular enrichment analysis; Arrdc3, arrestin domain containing 3; Egr1, early growth response 1; PKC, protein kinase C.
for alleviating T2DM remains controversial. Whereas acute stimulation of GPR40 by FFAs has beneficial effects of increasing insulin secretion, chronic exposure to FFAs might cause lipotoxicity, such as β-cell dysfunction and death [10]. The efforts to discover non-fatty acid agonists for GPR40 began only recently [8,11-14]; however, an orally bioavailable GPR40 agonist, TAK-675, has been discovered and entered phase II clinical testing for T2DM in Japan [15]. Thus, the activation of GPR40 might increase glucose-stimulated insulin secretion in the management of T2DM.

GPR40 can be coupled to Gαq with a subsequent increase in cytosolic Ca2+ concentrations [6,16] by activating phospholipase C (PLC) or the Ltype Ca2+ channel [17-19]. Since the involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) in the GPR40-mediated signaling pathway was suggested [6,20], a recent report demonstrated that FFA-mediated ERK1/2 activation was through c-Raf (RAF, a proto-oncogene serine/threonine-protein kinase) and MEK1/2 (ERK kinase) via Rac1 [21]. Although attention was focused on changes in intracellular Ca2+,” the possible involvement of ERK1/2 in GPR40-mediated insulin secretion remains unclear.

In this regard, to better understand the molecular mechanisms in the intracellular response following stimulation of GPR40, a stably GPR40-overexpressing pancreatic β-cell line ("RIN-40") was established from a rat immortalized pancreatic β-cell line ("RIN-5f"), and the differential expression of genetic profiles was examined in RIN-5f versus RIN-40 cells, and in RIN-40 versus linoleic-acid-treated, active RIN-40 cells. Based on microarray data, the involvement of ERK1/2 in GPR40-mediated insulin secretion was confirmed.

METHODS

Reagents

Linoleic acid as a GPR40 agonist (C18H32O2; mol. wt. 280.45; purity 99%) and G418 (an aminoglycoside antibiotic, similar in structure to gentamicin B1) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All reagents were of analytical grade or complied with the standards for cell culture. The rat immortalized pancreatic β-cell line RIN-5f (CRL-2058) was purchased from ATCC and maintained in RPMI 1640 medium containing 2.05 mM L-glutamine, 17.86 mM sodium bicarbonate, 25 mM glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% heat-inactivated FBS. Cells were incubated in a humidified atmosphere at 37°C with 5% CO2.

Vector constructs

For construction of the pCMV6-Neo-GPR40 expression plasmid, the vector pEZ-M02-GPR40 was purchased from GeneCopoeia (MD, USA) and inserted into the pCMV6-Neo vector (ORIGENE, MD, USA). E. coli DH-5α cells harboring the selected plasmid DNA were cultured in LB broth media. Plasmid DNA was extracted using a plasmid mini kit (Qiagen, Hilden, Germany). The extracted plasmid DNA was treated with the XmnI and XhoI restriction enzymes. The digested GPR40 gene was separated in a 1.0% agarose gel, eluted using a MEGA-spin Agarose Gel Extraction Kit (Intron, Seongnam, Korea), and ligated into the pCMV6-Neo vector plasmid. The pCMV6-Neo-GPR40 was verified by XmnIXhoI double digestion and sequencing (Bioneer, Daejeon, Korea). The confirmed vector was prepared using a QIAfilter Plasmid Midi Kit (Hilden, Germany) and used to transfect the cells.

Establishment of a stably GPR40-overexpressing pancreatic β-cell line

To construct a stable cell line for human GPR40 (NM_005303), RIN-5F cells were transfected with pCMV6-Neo-GPR40 in the transfection reagent Nucleofector Solution V using an Amaxa Nucleofector II device (Amaxa Biosystems, Cologne, Germany). The transfected cells were then selected and maintained in culture medium containing 300 μg/ml G418; this stably GPR40-overexpressing cell line was designated “RIN-40.” RIN-40 cells were maintained in G418 selective medium and confirmed by assaying GPR40 mRNA and protein levels.

Measurement of intracellular Ca2+

The cells were plated in 96-well plates (2×105 per well) for 2 days (to 70 ~ 80% confluence). On the day of the experiment, the cell culture medium was aspirated and the plate was washed twice with Krebs-Ringer bicarbonate HEPES (KRBB) buffer. Cells were rested at 37°C for 30 min in KRBB containing 25 mM glucose. Compounds were dissolved in DMSO and added to cells for 2 min. After the reaction, cells were fixed with 10% formalin for 1 h. The fixed cells were treated with 2 μM Fura-2AM in 1 mM EGTA buffer for 30 min. Intracellular Ca2+ was measured using a total internal reflection fluorescence (TIRF) microscope.

Measurement of insulin secretion

RIN-5F and RIN-40 cells were seeded in 24-well plates at a density of 2×103/well and cultured for 2 days. The cells were washed twice with glucose-free KRBB buffer containing 103.45 mM NaCl, 5.33 mM KCl, 5.63 mM Na2HPO4, 0.407 mM MgSO4, 1.28 mM CaCl2, 10 mM HEPES and 17.86 mM NaHCO3 and then incubated in KRBB buffer with 0.05% bovine serum albumin (BSA) and 2.5 mM glucose for 30 min at 37°C. Then, cells were washed once more with glucose-free KRBB buffer and treated with 500 - μL KRBB buffer containing 25 mM glucose and with or without the indicated concentrations of reagents in DMSO (final DMSO concentration of 0.1%). After 2 h, supernatant from each plate was collected and the insulin level determined using an Insulin (Rat) High-range ELISA (ALPCO, Windham, NH). The data were expressed as fold changes compared to the amount of insulin secreted (ng/ml insulin vs. mg/ml total protein).
Gene Expression Profiles in GPR40 Activation

Western blot analysis

Whole cells were lysed in Pro-prep protein extraction solution (Intron Biotechnology, Seoul, Korea), and the protein concentration in the lysates was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (10 μg) were run on a 10% SDS-PAGE gel and transferred onto a PVDF membrane by electroblotting. The membranes were then washed with TBST containing 5% BSA at room temperature and incubated for 2 h with the following antibodies: 1:1000 anti-ERK1/2 and anti-phospho-ERK1/2 (Cell Signaling, Beverly, MA, USA), 1:500 anti-GPR40 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 1:5000 anti-β-actin (AbFrontier, Seoul, Korea), which was used as an internal control. The membranes were washed in TBST and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin antibodies (1:1000) under the same conditions. After washing with TBST, signals were detected using an enhanced chemiluminescence detection reagent (AbFrontier, Seoul, Korea), visualized, and measured by densitometry using a ChemiDoc XRS digital imaging system and the Quantity One software (ver. 4.4.1; Bio-Rad Laboratories, Hercules, CA, USA).

Treatment of drug and RNA extraction for microarray

The RIN-5f and RIN-40 cells were seeded (10^6/well) in six-well plates for 48 h and were made quiescent in serum-free medium for 24 h, followed by treating with 30 μM linoleic acid to RIN-40 for an additional 2 h. Total RNA was isolated using a HiYield Total RNA Mini Kit according to the manufacturer’s protocol (RBCBioscience, New Taipei, Taiwan). The quality of total RNA was confirmed by running samples on an Agilent 2100 Bioanalyzer (Agilent Technology, CA, USA). RIN (RNA Integrity Number, cut-off threshold: 7.0) values were 9.6 for RIN-5f and RIN-40, and 9.7 for RIN-40 treated linoleic acid. Agilent’s Rat Oligo Microarray (44K) analysis was carried out at eBiogen Inc. (Seoul, Korea).

Microarray data analysis and bioinformatics analysis

The sample quality control was based on the Pearson correlation of a sample with other samples in the whole experiment. Hybridized images were scanned using an Agilent DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology, Palo Alto, CA, USA). Probe features were divided with ‘present,’ ‘marginal,’ and ‘absent’ flags, and the present and marginal flags were retained for further analyses. The genes flagged as ‘present’ and ‘marginal’ were selected as reliable for further analysis. All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, USA). To assess functional relationships between genes, DAVID (http://david.abcc.ncifcrf.gov/) as a web-based singular enrichment analysis (SEA), was used, providing mainly annotation and gene ontology (GO) term enrichment analysis to highlight the most relevant GO terms associated with a given gene list. The enrichment p value was calculated for each term from the list of genes of interest with a single-linkage method. Then, enriched terms were listed in a simple linear text format. In the DAVID annotation system, the EASE score and a modified Fisher’s exact test between ‘in pathway’ and ‘not in pathway’ were used to measure gene enrichment in terms of annotation. Due to the redundant nature of annotations, a Functional Annotation Chart was used to present similar/relevant annotations repeatedly. The grouping algorithm is based on the hypothesis that similar annotations should have similar gene members. Functional Annotation Clustering integrates the same techniques as kappa statistics to measure the degree of common genes between two annotations, and fuzzy heuristic clustering (as used in the Gene Functional Classification Tool) to classify the groups of similar annotations according to kappa values. In this sense, the greater the number of common genes shared by annotations, the greater the likelihood they will be grouped together. The Group Enrichment Score, the geometric mean of members’ p-values in a corresponding annotation cluster, is used to rank their biological significance. Thus, the members of the top-ranked annotation groups most likely have consistently lower p-values. To determine whether certain functional categories were over-represented in the gene lists determined in the microarray experiments, reliable genes were first filtered using the ‘present’ and ‘marginal’ flags and re-filtered according to being 1.5-, 2-, and 3-fold up- and downregulated. The filtered gene lists were applied to DAVID and annotated functional pathways in GO and KEGG, which can identify the locations of genes in related pathways. Annotated pathways were displayed with statistical significance values in terms of enrichment scores, nominal p value, and FDR (p<0.05). For discrete genes in certain pathway we identified biological functions in the KEGG pathways and verified their functions using GeneBank and literature searches using PubMed (http://www.ncbi.nlm.nih.gov/).

Quantitative RT-PCR

RIN-5f and RIN-40 cells were seeded (10^6/well) in six-well plates for 48 h, and exposed to various reagents. Total RNA was isolated using a HiYield Total RNA Mini Kit according to the manufacturer’s protocol (RBCBioscience, New Taipei, Taiwan). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a 20-μL reaction volume using the AccuPower CycleScript RT PreMix, as recommended by the manufacturer (Bioneer, Daejeon, Korea). The cDNA synthesis thermal cycling program included the following three steps: 37°C for 1 min, 47°C for 3 min, 55°C for 1 min, then 95°C for 5 min. Using the PCR mixture, initial DNA polymerase activation was carried out at 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 min. A final extension was carried out at 72°C for 5 min. The PCR products were visualized by ethidium bromide staining after separation by electrophoresis in a 2% agarose gel in Tris borate ethylenediamine tetra-acetic acid (TBE) buffer (pH 8.3). Images were captured using a Fluor-S Max MultImaging system and band densities were assessed with the Quantity One 1-D software (ver. 4.6.0). The reaction mixture for real-time quantitative reverse transcription PCR (qRT-PCR) contained cDNA, 1X SYBR green Taq polymerase mixture (Toyobo, Osaka, Japan), and primers, and was performed using an Exicycler 96 instrument (Bioneer, Daejeon, Korea). Relative gene expression levels were compared using an invariant endogenous control (β-actin). The ΔΔCT method was used for relative quantification according to the manufacturer’s guidelines. The sequences of the primers

Fisher's exact test between 'in pathway' and 'not in pathway' were used to measure gene enrichment in terms of annotation. Due to the redundant nature of annotations, a Functional Annotation Chart was used to present similar/relevant annotations repeatedly. The grouping algorithm is based on the hypothesis that similar annotations should have similar gene members. Functional Annotation Clustering integrates the same techniques as kappa statistics to measure the degree of common genes between two annotations, and fuzzy heuristic clustering (as used in the Gene Functional Classification Tool) to classify the groups of similar annotations according to kappa values. In this sense, the greater the number of common genes shared by annotations, the greater the likelihood they will be grouped together. The Group Enrichment Score, the geometric mean of members' p-values in a corresponding annotation cluster, is used to rank their biological significance. Thus, the members of the top-ranked annotation groups most likely have consistently lower p-values. To determine whether certain functional categories were over-represented in the gene lists determined in the microarray experiments, reliable genes were first filtered using the 'present' and 'marginal' flags and re-filtered according to being 1.5-, 2-, and 3-fold up- and downregulated. The filtered gene lists were applied to DAVID and annotated functional pathways in GO and KEGG, which can identify the locations of genes in related pathways. Annotated pathways were displayed with statistical significance values in terms of enrichment scores, nominal p value, and FDR (p<0.05). For discrete genes in certain pathway we identified biological functions in the KEGG pathways and verified their functions using GeneBank and literature searches using PubMed (http://www.ncbi.nlm.nih.gov/).
used for PCR amplification are shown in Table 1.

### Statistical analysis

The DAVID web-based tool was used to extract the major biological features in the large gene lists. Statistical significance was expressed as nominal p values and the corrected p value though FDR. For the insulin secretion test, statistical analyses were conducted using a Kruskal-Wallis test, followed by Dunn’s post hoc test or Mann-Whitney U-test using the Prism software (GraphPad Software, San Diego, CA, USA). p values<0.05 were considered to indicate statistical significance.

### RESULTS

#### Identification of a GPR40-overexpressing stable cell line

To evaluate the effects of overexpressing GPR40, we established RIN-40 from the original rat pancreatic β-cell line RIN-5f. Microscopic observation of RIN-40 cells revealed that they were morphologically similar in overall appearance to RIN-5f (data not shown). By RT-PCR, the RIN-40 cell line showed abundant expression of GPR40 mRNA (human GPR40), but little endogenous Gpr40 mRNA (human GPR40; Fig. 1A). Neither GPR40 nor Gpr40 mRNA was detected in the RIN-5f cell line. As expected, the GPR40 protein level in RIN-40 was considerably greater than that in RIN-5f (Fig. 1B). To determine whether insulin secretion was mediated by RIN-40 activation, the dose-response relationship between insulin secretion and cell differentiation represented >30% of the total. Upregulated and downregulated genes showed similar distributions in each biological pathway. Genes associated with signal transduction pathways whose expression changed by at least threefold were presented in Table 2. To identify the signaling pathways in which genes were involved, the genes applied on DAVID with at least a 1.5fold change were matched to specific KEGG pathways and classified by cellular function with statistical values, based on frequencies of genes in each pathway (Table 3).

In the various biological pathways affected by overexpression of GPR40, many genes were related to chemokines (pcorrected=0.028), mitogen-activated protein (MAP) kinase (pcorrected=0.030), maturity onset diabetes of the young (pcorrected=0.031), mTOR (pcorrected=0.033), and the dilated cardiomyopathy signaling pathway (pcorrected=0.049).
Table 2. Frequencies of differentially expressed genes in RIN-5f vs. RIN-40 and RIN-40 vs. RIN-40 treated with linoleic acid

| Biological pathway                  | RIN-5f vs. RIN-40 | RIN-40 vs. RIN-40 LA* |
|-------------------------------------|-------------------|-----------------------|
|                                     | Up% (genes)       | Down% (genes)         | Up% (genes)       | Down% (genes)         |
| Aging                               | 1.5 (7)           | 3.4 (18)              | 0 (0)             | 0 (0)                 |
| Angiogenesis                        | 1.7 (6)           | 0.4 (2)               | 6.1 (4)           | 3.7 (1)               |
| Apoptosis                           | 5.9 (29)          | 10.4 (55)             | 9.1 (6)           | 7.4 (2)               |
| Cell cycle                          | 6.5 (31)          | 2.3 (12)              | 3 (2)             | 14.8 (4)              |
| Cell differentiation                | 15.8 (75)         | 14.7 (78)             | 12.1 (8)          | 11.1 (3)              |
| Cell proliferation                  | 8.6 (41)          | 6.6 (35)              | 12.1 (8)          | 11.1 (3)              |
| DNA repair                          | 1.1 (5)           | 0 (0)                 | 3 (2)             | 0 (0)                 |
| Extracellular matrix                | 2.7 (13)          | 3.4 (18)              | 1.5 (1)           | 3.7 (1)               |
| Immune response                     | 2.9 (14)          | 7 (37)                | 3 (2)             | 3.7 (1)               |
| Inflammatory response               | 1.5 (7)           | 5.9 (31)              | 3 (2)             | 3.7 (1)               |
| Neurogenesis                        | 10.1 (48)         | 5.7 (30)              | 4.5 (3)           | 3.7 (1)               |
| Response to oxidative stress        | 4 (19)            | 6.2 (33)              | 3 (2)             | 0 (0)                 |
| Regulation of cellular protein      | 6.9 (35)          | 5.7 (30)              | 4.5 (3)           | 7.4 (2)               |
| RNA splicing                        | 0.8 (4)           | 0.4 (2)               | 0 (0)             | 0 (0)                 |
| Signal transduction                 | 18.3 (87)         | 19.1 (101)            | 22.7 (15)         | 25.9 (7)              |
| Transcription                       | 11.6 (55)         | 8.9 (47)              | 12.1 (8)          | 3.7 (1)               |
| Total                               | 100 (475)         | 100 (529)             | 100 (66)          | 100 (27)              |

*RIN-40 LA, RIN-40 cells treated with 30 μM Linoleic acid.

proliferation, signal transduction, and transcription were upregulated, ~63% of those related to the cell cycle, cell differentiation, cell proliferation, and signal transduction were downregulated by linoleic acid treatment. Table 4 shows representative genes related to signal transduction whose expression changed by at least twofold. In an analy-
sis of involvement by pathway, the gene sets annotated included olfactory transduction \((p=8.9\times10^{-23})\), neuroactive ligand-receptor interaction \((p=0.002)\), MAP kinase signaling pathway \((p=0.014)\), cytokine-cytokine receptor interactions \((p=0.024)\), and regulation of the actin cytoskeleton \((p=0.036)\). To confirm the microarray data, three upregulated genes [arrestin domain containing 3 (Arrdc3), early growth response 1 (Egr1), and serum/glucocorticoid-regulated kinase 1 (Sgk1)], and two downregulated genes [cAMP-specific phosphodiesterase 4B (Pde4b) and thrombospondin 1 (Tbpl1)] were selected and qRT-PCR was performed using RNA obtained independently of that used in the microarray. The mRNA levels of all selected genes in the qRT-PCR experiment showed patterns similar to those obtained by microarray analysis (Table 5).

### Table 3. Pathway and genes represented in GPR40 overexpressing RIN-40 cell compared with RIN-5f cell

| KEGG ID  | Pathway                                           | p-value | p corrected |
|----------|---------------------------------------------------|---------|-------------|
| rno04062 | Chemokine signaling pathway                       | 0.002   | 0.028       |
| rno04010 | MAPK signaling pathway                            | 0.001   | 0.030       |
| rno04950 | Maturity onset diabetes of the young               | 0.001   | 0.031       |
| rno04150 | mTOR signaling pathway                            | 0.002   | 0.033       |
| rno05414 | Dilated cardiomyopathy                            | 0.001   | 0.049       |
| rno04510 | Focal adhesion                                     | 0.013   | 0.133       |
| rno05200 | Pathways in cancer                                | 0.012   | 0.145       |
| rno04350 | TGF-beta signaling pathway                        | 0.012   | 0.165       |
| rno04810 | Regulation of actin cytoskeleton                  | 0.019   | 0.170       |
| rno04512 | ECM-receptor interaction                          | 0.041   | 0.310       |
| rno05410 | Hypertrophic cardiomyopathy (HCM)                 | 0.046   | 0.315       |
| rno04914 | Progesterone-mediated oocyte maturation           | 0.051   | 0.321       |
| rno05215 | Prostate cancer                                   | 0.056   | 0.328       |
| rno04620 | Toll-like receptor signaling pathway              | 0.056   | 0.328       |
| rno04200 | Calcium signaling pathway                         | 0.072   | 0.379       |
| rno05212 | Pancreatic cancer                                 | 0.099   | 0.462       |

p-value, nominal p value; p corrected value, corrected by Benjamini-Hochberg.

### Table 4. Pathway and genes represented in RIN-40 cell treated with linoleic acid

| KEGG ID  | Pathway                                           | p-value | p corrected |
|----------|---------------------------------------------------|---------|-------------|
| rno04740 | Olfactory transduction                            | <0.001  | <0.001      |
| rno04090 | Neuroactive ligand-receptor interaction            | 0.002   | 0.103       |
| rno04010 | MAPK signaling pathway                            | 0.014   | 0.340       |
| rno04060 | Cytokine-cytokine receptor interaction            | 0.024   | 0.415       |
| rno04810 | Regulation of actin cytoskeleton                  | 0.036   | 0.479       |
| rno05218 | Melanoma                                          | 0.036   | 0.420       |
| rno04662 | B cell receptor signaling pathway                 | 0.051   | 0.485       |
| rno05200 | Pathways in cancer                                | 0.060   | 0.499       |
| rno04200 | Calcium signaling pathway                         | 0.061   | 0.462       |

### Table 5. Confirmation of microarray by qRT-PCR representative genes related to signal transduction

| Name of genes                  | Entrez gene ID | Microarray | qRT-PCR |
|--------------------------------|----------------|------------|---------|
| Arrestin domain containing 3   | NM_001007797   | 3.12       | 3.59    |
| Early growth response 1        | NM_012551      | 2.64       | 4.36    |
| Serum/glucocorticoid regulated kinase 1 | NM_019252 | 2.12       | 2.69    |
| Phosphodiesterase 4B, cAMP specific | NM_017031 | 0.38       | 0.83    |
| Thrombospondin 1               | NM_001013062   | 0.24       | 0.6     |

**Involvement of ERK activation in linoleic-acid-induced insulin secretion**

To assess the involvement of ERK signaling in linoleic-acid-induced insulin secretion, the phosphorylation of ERK according to duration of incubation in the presence of linoleic acid and insulin secretion under GPR40 activation induced by linoleic acid treatment were measured. The data in Fig. 2A show that ERK phosphorylation was increased rapidly by treatment with 30 \(\mu\) M linoleic acid for 3 min, followed by a decrease, in RIN-40 cells. ERK activation coincided with the increase in insulin secretion in
**DISCUSSION**

This study has two novel findings: (1) GPR40-overexpressing pancreatic β-cells displayed altered expression of genes related to chemokines, MAP kinase, maturity onset diabetes of the young, mTOR, and dilated cardiomyopathy signaling pathways; and (2) GPR40-overexpressing pancreatic β-cells treated with linoleic acid showed significantly altered expression of genes associated with olfactory transduction, neuroactive ligand-receptor interaction, MAP kinase signaling pathway, cytokine-cytokine receptor interaction, and regulation of the actin cytoskeleton. The MAP kinase signaling pathway was altered in both GPR40-overexpressing pancreatic β-cells and GPR40-activated cells following treatment with linoleic acid. This study confirmed that ERK phosphorylation was significantly increased in GPR40-overexpressing pancreatic β-cells treated with linoleic acid; moreover, this coincided with an increase in insulin secretion. Although further studies are needed, this is the first report demonstrating an alteration in gene expression profiles mediated by GPR40 activation.

We identified differentially expressed transcripts involved in the MAP kinase pathway in both a GPR40-overexpressing cell line and GPR40-activated cells treated with linoleic acid. It has been suggested that glucose stimulation of insulin secretion is connected mainly to the activation of the MAP kinase pathway, which is mediated by L-type Ca\(^{2+}\) channels in pancreatic β-cells [6,22]. In our study, the differentially downregulated transcripts included those encoding RAS guanyl releasing protein 2 (Rasgrp2), neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2), dual specificity phosphatase 6 (Dusp6), and Myc under GPR40-overexpressing conditions. These results suggest that these downregulated genes may be involved in cell proliferation, cell survival, and apoptosis due to excessive signaling via GPR40 [23]. This study confirmed the relationship between linoleic acid treatment and activation of the ERK-MAP kinase pathway (Fig. 2), consistent with a previous report [21]. The microarray data showed that treatment with linoleic acid resulted in upregulation of transforming growth factor, beta receptor II (Tgfbr2), calcium channel, voltage-dependent, gamma subunit 5 (Cacng5), calcium channel, voltage-dependent, beta 4 subunit (Cacnb4), and protein kinase C, beta (PrkcB), and downregulation of ribosomal protein s6 kinase, 90 kDa, polypeptide 6 (Rps6ka6) and interleukin 1, alpha (Il1α). These data suggest that the reaction of linoleic acid via GPR40 resulted in changes in the expression of genes in the MAP kinase pathway, which are linked, directly or indirectly to Ca\(^{2+}\) channels and the signaling pathways that they mediate. Importantly, the alteration of gene expression by linoleic acid increased transcription of early response transcription factor (Egr-1), which stimulated a mitogenic signaling cascade (ERK) and enhanced the intracellular Ca\(^{2+}\) concentration by activating Gαq/11-coupled GPR40 and/or voltage-gated L-type Ca\(^{2+}\) channels. Moreover, the data suggest that Egr-1 can increase transcription of fibroblast growth factor (FGF), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) by the ERK-Elk1 pathway, and also expression of Fos by EKR-CREB pathways [24].
The activation of $\alpha_\omega$-coupled GPR40 can induce $\text{Ca}^{2+}$ signaling (Fig. 1D) via the activation of PLC and/or L-type $\text{Ca}^{2+}$ channels, subsequently leading to insulin release [11,18,25-27]. This is the mechanism of insulin exocytosis from pancreatic $\beta$-cells and glucose-stimulated insulinotropic action via GPR40. Interestingly, no significant change in the expression of genes related to $\text{Ca}^{2+}$ signaling pathways was evident under either GPR40-expressing or GPR40-activating conditions. This may be due to the complimentary regulation of $\text{Ca}^{2+}$ signaling pathways induced in the presence of GPR40 overexpression and excessive GPR40 activation. One possible explanation is that the changes in the expression levels of adenyl cyclase isoforms (Adcy) and protein kinase C (PKC) seen in the microarray data may be linked indirectly and cause unexpected functional outcomes.

In particular, differential transcript expression in GPR40-overexpressing cells showed a strong association with maturity onset diabetes of the young (MODY). Although the genes defined in our analysis are not the causal genes of MODY, including hepatocyte nuclear factor 1 homeobox A/B (HF1A/B), glucokinase, insulin promoter factor 1 (IPF1), and neurogenic differentiation 1 (NEUROD1) [28], excessive expression of GPR40 may affect insulin exocytosis in pancreatic $\beta$-cells by inducing changes in homotypically expressed homeobox (Hhex) [26,29], leading to granule docking, insulin 2 (Ins2) [30], and $\gamma$-maf musculoskeleto-neurotic fibrosarcoma oncogene homolog A (Mafa) [31].

Linoleic acid treatment of GPR40-overexpressing $\beta$-cells resulted in the differential expression of over 70 olfactory receptor (OLR) genes, related to ‘olfactory transduction’. Although highly statistically significant, the potential importance is unclear in pancreatic cells. Moreover, a large number of odor receptors, as many as 1,000, are present in the mammalian genome, representing ~5% of the total number of genes [32]. Because of the relatively large number of genes, OLR was annotated to olfactory transduction and achieved a high rank in the pathway enrichment test. However, it is possible that linoleic acid, as a free fatty acid, directly affects OLR and a GPCR receptor in $\beta$-cells, in addition to its systemic functions [33]. Linoleic acid, through GPR40, caused alterations in the expression of genes related to ‘neuroactive ligand-receptor interaction’ in our data. Islets are abundantly innervated by parasympathetic and sympathetic nerves, which produce neurotransmitters and neuropeptides. In pancreatic $\beta$-cells, the $\alpha_2$ adrenoreceptor influences insulin secretion, through inhibition of cAMP production induced by catecholamines [29]. The activation of muscarinic M3 receptors caused increased insulin secretion and glucose tolerance, and increased expression of CHRM3 (cholinergic receptor, muscarinic 3) genes led to a reduction in insulin secretion [34]. This seeming physiological antagonism between the sympathetic and parasympathetic nervous systems with regard to insulin secretion can be interpreted as an autoregulatory mechanism to maintain homeostasis against excessive activation of GPR40.

Our findings suggest several areas for future study. Cross-talk effects may be induced by altered cellular gene expression under GPR40-overexpressing and GPR40-activating conditions, and it is difficult to exclude the possibility of involvement of free fatty acid-mediated actions through GPCRs because of the complexity of the systems [35]. Moreover, genes whose expression is altered by treatment with FFAs, such as linoleic acid, in GPR40-overexpressing cells may be responsible for the development of FFA-induced lipotoxicity [36]. Thus, it is important to compare our current data with the gene expression patterns induced by treatment with non-fatty-acid agonists for GPR40, and systemic analyses of functional and phenotypic outcomes should be performed as part of well-designed animal studies.

In conclusion, this report provides the first demonstration that GPR40 in pancreatic $\beta$-cells influences differential gene expression under conditions in which it is overexpressed and in the presence of linoleic acid stimulation. Based on signaling pathway analysis, we provide evidence that will facilitate prediction of the inter- and intra-connectivity between gene sets. These data can be used to make predictions regarding the regulation of numerous pathways linked to GPR40, and further, possibly be used to alleviate the symptoms of T2DM via GPR40.

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