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Microarray Analysis of Novel Candidate Genes Responsible for Glucose-Stimulated Insulin Secretion in Mouse Pancreatic β Cell Line MIN6

Eiji Yamato*, Fumi Tashiro, Jun-ichi Miyazaki*

Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

Abstract

Elucidating the regulation of glucose-stimulated insulin secretion (GSIS) in pancreatic islet β cells is important for understanding and treating diabetes. MIN6 cells, a transformed β-cell line derived from a mouse insulinoma, retain GSIS and are a popular *in vitro* model for insulin secretion. However, in long-term culture, MIN6 cells' GSIS capacity is lost. We previously isolated a subclone, MIN6 clone 4, from the parental MIN6 cells, that shows well-regulated insulin secretion in response to glucose, glybenclamide, and KCl, even after prolonged culture. To investigate the molecular mechanisms responsible for preserving GSIS in this subclone, we compared four groups of MIN6 cells: Pr-LP (parental MIN6, low passage number), Pr-HP (parental MIN6, high passage number), C4-LP (MIN6 clone 4, low passage number), and C4-HP (MIN6 clone 4, high passage number). Based on their capacity for GSIS, we designated the Pr-LP, C4-LP, and C4-HP cells as "responder cells." In a DNA microarray analysis, we identified a group of genes with high expression in responder cells ("responder genes"), but extremely low expression in the Pr-HP cells. Another group of genes ("non-responder genes") was expressed at high levels in the Pr-HP cells, but at extremely low levels in the responder cells. Some of the responder genes were involved in secretory machinery or glucose metabolism, including *Chrebp*, *Scgn*, and *Syt7*. Among the non-responder genes were *Car2*, *Maf*, and *Gcg*, which are not normally expressed in islet β cells. Interestingly, we found a disproportionate number of known imprinted genes among the responder genes. Our findings suggest that the global expression profiling of GSIS-competent and GSIS-incompetent MIN6 cells will help delineate the gene regulatory networks for insulin secretion.

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* E-mail: yamato@nutri.med.osaka-u.ac.jp (EY); jimiyaza@nutri.med.osaka-u.ac.jp (JM)

Introduction

MIN6 cells, which were isolated from an insulinoma of a transgenic mouse expressing the SV40 T antigen in pancreatic islet β cells, retain some features of differentiated pancreatic β cells [1]. However, long-term culture of MIN6 cells results in the loss of their insulin secretory capacity in response to glucose [2–4]. Several reports have described the isolation of glucose-responsive and -unresponsive MIN6 cell subclones, and these subclones have been used to identify genes associated with glucose-stimulated insulin secretion (GSIS) [5,6]. However, these reports did not rule out the possibility that this differential gene expression was attributable simply to clonal variation. In addition, further analyses of the phenotypic stability of the glucose-responsive MIN6 subclones during long-term culture have not been performed.

Gene expression profile analysis has been performed to compare MIN6 cells after short-term and long-term culture [4]. In addition, comparative proteomic analyses of the cell lysates [7] and the conditioned media [8] were used to investigate the relative changes in the proteome that accompany the loss of GSIS over time in culture. Recently, impairment of glucose and lipid oxidation was suggested to be involved in the loss of GSIS in high passage MIN6 cells [9]. However, these studies did not verify that the differentially expressed genes and proteins were directly involved in the mechanisms for GSIS.

In the present study, we used a MIN6 subclone, designated MIN6 clone 4, which retains GSIS even after long-term culture. To identify the genes involved in the maintenance of GSIS capacity, we compared the gene expression profiles among parental MIN6 cells after short-term culture (Pr-LP) and long-term culture (Pr-HP) and of MIN6 clone 4 cells after short-term culture (C4-LP) and long-term culture (C4-HP). The results revealed one group of genes whose expression was high in well-regulated (i.e., low passage number) parental MIN6 cells and MIN6 clone 4 cells, but extremely low in the dysregulated (high passage number) parental MIN6 cells. Another group of genes was expressed at extremely low levels in the well-regulated MIN6 cells, but at high levels in the dysregulated ones. We discuss the roles of these differentially expressed genes in insulin secretion.

Materials and Methods

Culture of MIN6 parental cells and subclone

MIN6 cells, which we have previously established [1], 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% CO_2 at 37°C [1]. MIN6 clone 4 cells are a subclone isolated from low-passage-number parental MIN6 cells by the limiting dilution method. This subclone was maintained in the same culture conditions as the parental cells, and retained good GSIS even after 6 months of continuous culture. For the low-passage-number parental MIN6 cells (Pr-LP), we used cells passaged 17–20 times; for the high-passage-number MIN6 cells (Pr-HP), we used cells passaged 35–40 times. Seventeen to 20 passages were also used for the low-passage-number MIN6 clone 4 cells (C4-LP), and the high-passage ones (C4-HP) were used after 40 to 50 passages. For all of the following methods sections, the parental MIN6 cells and their subclone were treated identically.

Measurement of insulin secretion and insulin content

MIN6 cells were cultured in 24-well plates for 4 days. Prior to the insulin secretion assay, the cells were starved in Krebs Ringer solution containing 0.1% bovine serum albumin (BSA) with 3 mM glucose for 30 min, and the wells were washed twice with the same buffer. The cells were then incubated in Krebs Ringer Solution with 3, 8, 15, or 25 mM glucose, 100 μM glybenclamide+3 mM glucose, or 30 mM KCl+3 mM glucose for 1 hr.

Insulin secreted into the medium and contained in the MIN6 cells was measured. For the secreted insulin, medium was collected and the insulin measured using an ELISA kit (Cat.#10-1250-01; Mercodia, Uppsala, Sweden). To normalize the amount of secreted insulin to the protein content of each well, the cells in each well were lysed with RIPA buffer, and the protein concentration of the cell lysates was measured by the Bradford method (Cat.#500-0006; Bio-Rad, Hercules, CA). To measure the insulin content of MIN6 cells, we lysed cells in a different set of wells with acid-ethanol, and centrifuged the cell extracts. The amount of insulin in the supernatants was assayed using the ELISA kit. Statistical analysis was performed by Student's *t*-test.

DNA microarray analyses

Total RNA was extracted from MIN6 cells using Trizol reagent (Invitrogen, Carlsbad, CA) and subjected to double-strand cDNA synthesis using the Superscript Choice system (Cat.#18090-019; Invitrogen) and the T7-$(dT)_{24}$ reverse transcription primer (Cat.#72-1591-01; Amersham Biosciences, Piscataway, NJ). Synthesis of biotin-labeled cRNA was carried out by *in-vitro* transcription using the Bio Array RNA Transcript Labeling Kit (Cat.#900182; Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. The biotin-labeled cRNA was purified using RNeasy spin columns (Cat.#74106; Qiagen GmbH, Hilden, Germany) and fragmented in a reaction mixture. The biotin-labeled and fragmented cRNA was hybridized to the murine genome U74 version 2 GeneChip array (Affymetrix), incubated, and washed according to the manufacturer's instructions. The GeneChip arrays were then scanned with a Gene Array Scanner (Hewlett-Packard, Santa Clara, CA) and analyzed by GeneChip 5.1 software (Affymetrix). The microarray dataset has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE43774.

Quantitative RT-PCR

Total RNA was extracted from MIN6 cells by the acid guanidinium-phenol-chloroform (AGPC) method and subjected to cDNA synthesis using ReverTra Ace α (Cat.#FSK-101; Toyobo, Tokyo, Japan). Quantitative RT-PCR analysis was carried out using SYBR Premix Ex Taq (Cat.#RR041A; Takara, Otsu, Japan). The reaction was performed with 1 μl cDNA per 25 μl reaction in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following thermal cycling conditions: 95°C for 10 sec followed by 40 cycles at 95°C for 5 sec and 60°C for 31 sec. The relative expression levels of the target genes were normalized to that of *Rpl32*. Statistical analysis was performed by Student's *t*-test. Primer sequences are listed in **Table S1**.

Immunocytochemistry and FACS (fluorescence-activated cell sorting) analysis

For immunocytochemistry, the MIN6 cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min, rinsed with PBS, incubated for 5 min in PBS with 1% Triton X-100, washed again with PBS, and incubated in blocking reagent (Blocking One, Cat.#03953-95; Nacalai Tesque, Kyoto, Japan). The samples were incubated with rat anti-CD24 antibody (Cat.#14-0241-81; eBioscience, San Diego, CA) for 1 hr at room temperature, washed with PBS, and then incubated with Alexa Fluor 488-conjugated anti-rat IgG (Cat.#A-11006; Molecular Probes, Eugene, OR) for 1 hr at room temperature. For FACS analysis, the cells were washed twice with PBS and then suspended in SM buffer (HEPES-buffered saline with 0.1% sodium azide and 1% BSA). CD24 staining was performed at 4°C for 30 min with rat anti-CD24 antibody, followed by incubation with Alexa Fluor 488-conjugated anti-rat IgG. FACS analyses were performed with a FACScan (Becton Dickinson, Franklin Lakes, NJ) using the CellQuest (Becton Dickinson) evaluation program.

In situ hybridization

Part of the mouse *Hepacam2* cDNA (NM_178899; sequence position 58–699) was subcloned into the pGEMT-Easy vector (Cat.#A1360; Promega, Madison, WI) and used to generate sense and antisense RNA probes. Digoxigenin (DIG)-labeled RNA probes were prepared with the DIG RNA Labeling Mix (Cat.#1277073; Roche, Mannheim, Germany) according to the manufacturer's instructions. *In situ* hybridization was performed according to the protocol of Genostaff (Tokyo, Japan). In brief, a pancreas of a C57BL/6J mouse was dissected out after perfusion and fixation with Tissue Fixative (Genostaff, Tokyo, Japan), embedded in paraffin, and sectioned at 6 μm. The tissue sections were de-waxed and fixed with 4% paraformaldehyde in PBS. The sections were then hybridized with sense and antisense *Hepacam2* probes. After treatment with blocking reagent in TTBS (10 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) for 30 min, the sections were incubated with anti-DIG alkaline phosphatase (AP) conjugate (Cat.#1093274; Roche) diluted 1:1000 with TTBS for 2 hr at room temperature, washed twice with TTBS, and then incubated in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Tween 20 for 30 min. Coloring reactions were performed with NBT/BCIP solution (Cat.#B6404; Sigma, St. Louis, MO) overnight, and the sections were then washed with PBS and counterstained with Kernechtrot stain solution (Cat.#40872; Mutoh Chemical Co., Tokyo, Japan).

Bisulfite sequencing

Bisulfite treatment of the genomic DNA isolated from Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells was performed using the EpiTect Bisulfite Kit (Cat.#59104; Qiagen) according to the manufacturer's instructions. The CpG islands in the first intron of the *Plagl1* gene (33 CpGs) and those in the DMR region of the *Dlk1* gene (24 CpGs) were chosen for analysis. The primers for the *Plagl1* gene were: forward, 5′-GGGTAGGTAAGTAGTGATAA-3′; reverse, 5′-CCTAAAACACCAAAATAACA-3′ [10]. The primers for the *Dlk1* gene were: forward, 5′-GATTAGTGATT-

TATAATTTGTGTTTTGGTT-3′; reverse, 5′-AAACTCACC-TAAATATACTAAAAACAAATA-3′ [11]. The PCR products were cloned into pBluescript and sequenced.

Statistical analysis

Results are presented as the mean ± SD. Statistical analyses were carried out by Student's *t*-test except for that of the distribution of the percentages of methylated CpGs in the *Plagl1* locus, which was carried out by the F-test. A value of $P<0.05$ was considered statistically significant.

Results

Insulin secretion and insulin content

Insulin secretory capacity was compared by static incubation among parental MIN6 cells at 17–20 passages (Pr-LP) and 35–40 passages (Pr-HP) and MIN6 clone 4 cells at 17–20 passages (C4-LP) and 40–50 passages (C4-HP) (**Figure 1 and 2**). Pr-HP cells showed a higher basal insulin secretion at 3 mM glucose than did the other groups, but the insulin secretion did not increase with higher glucose concentrations or the addition of glybenclamide (SU) or KCl, in contrast with the other three groups (**Figure 1, 2B, and 2C**). In addition, the insulin content of the Pr-HP cells was much lower than that of the Pr-LP, C4-LP, or C4-HP cells (**Figure 2A**). Therefore, we designated the C4-LP, C4-HP, and Pr-LP cells as "responder" cells and the Pr-HP cells as "non-responder" cells.

Furthermore, both C4-LP and C4-HP cells showed a better insulin secretory response to glucose and glybenclamide than did the Pr-LP cells (**Figure 1**), suggesting that the MIN6 clone 4 cells maintained stable regulatory mechanisms for insulin secretion. Interestingly, the insulin secretion induced with KCl was even higher in the C4-HP cells than in the C4-LP cells (**Figure 1D and 2C**).

DNA Microarray analysis

To identify genes involved in the regulation of the insulin secretory pathway, a comparative DNA microarray analysis was performed, using the Pr-LP, Pr-HP, C4-LP, and C4-HP cells. The results revealed one group of genes, which we call "responder genes," that were highly expressed in the responder cells (Pr-LP, C4-LP, and C4-HP), but only weakly in the non-responder cells (Pr-HP). A different group of genes ("non-responder genes") was highly expressed in the Pr-HP cells, but only weakly in the Pr-LP, C4-LP, and C4-HP cells. To compare the responder and non-responder results, we compared the mean value of the gene expression levels in the Pr-LP, C4-LP, and C4-HP cells with the expression level in the Pr-HP cells. Genes that showed a more than 5-fold difference in expression level are listed in **Table 1** (40 responder genes) and **Table 2** (41 non-responder genes). An extended list, including all the genes that showed a more than 3-fold difference, is provided in **Table S2** (60 responder and 62 non-responder genes). Genes that were differentially expressed

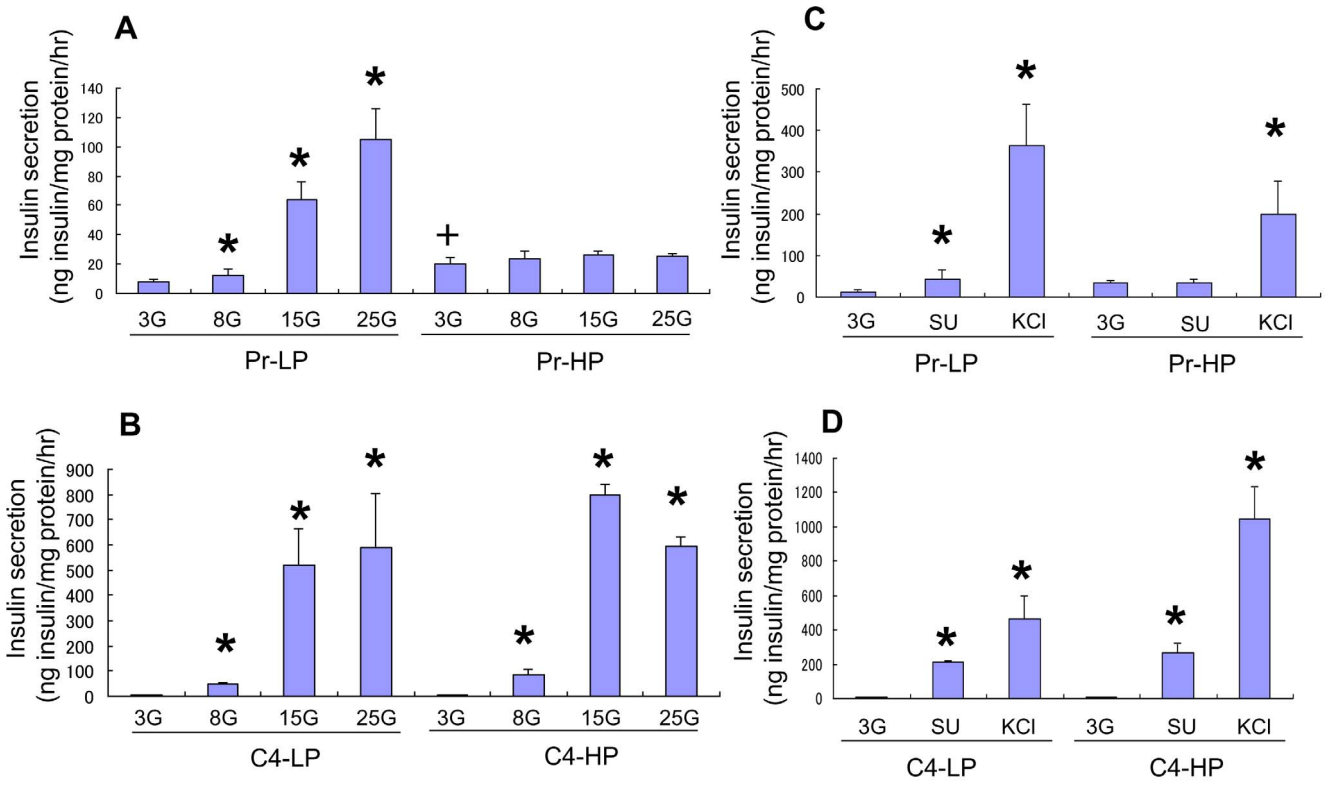

Figure 1. Insulin secretion from MIN6 cells. Insulin secretion from Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells stimulated with 3 mM (3G), 8 mM (8G), 15 mM (15G), or 25 mM (25G) glucose (A, B), 100 nM glybenclamide (SU), or 30 mM KCl (C, D). Pr-HP cells showed higher basal insulin secretion at 3 mM glucose compared with Pr-LP cells, but their insulin secretion did not increase further at higher glucose concentrations or with the addition of glybenclamide or KCl, whereas both C4-LP and C4-HP MIN6 cells showed a better insulin secretory response to glucose and glybenclamide than Pr-LP cells. Values are means ± SD and n = 5–6. *$P<0.05$ v.s. insulin secretion at 3 mM glucose.+$P<0.05$ v.s. insulin secretion of Pr-LP, C4-LP, and C4-HP cells at 3 mM glucose by Student's *t*-test.
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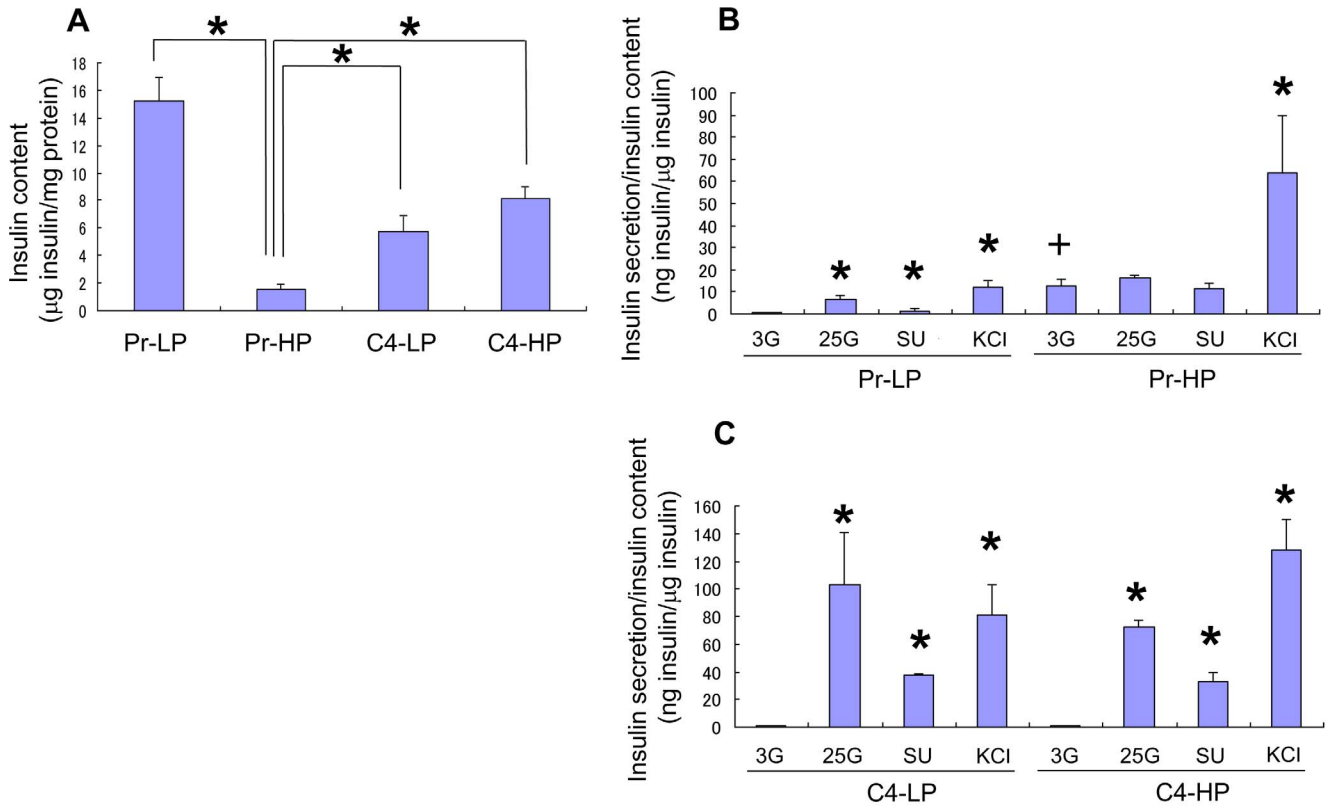

Figure 2. Insulin content and secretion of MIN6 cells. Insulin content of Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells (A). The insulin content of Pr-HP cells was lower than that of Pr-LP, C4-LP, or C4-HP cells. Values are means ± SD and n = 5–6. *$P<0.05$. Insulin secretion/insulin content from Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells stimulated with 3 mM (3G), 25 mM (25G) glucose, 100 nM glybenclamide (SU), or 30 mM KCl (B, C). Values are means ± SD and n = 5–6. *$P<0.05$ v.s. insulin secretion at 3 mM glucose.+$P<0.05$ v.s. insulin secretion of Pr-LP, C4-LP, and C4-HP cells at 3 mM glucose by Student's *t*-test.
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among Pr-LP, C4-LP, and C4-HP cells are also listed in **Table S3, S4, S5**.

The expression pattern of some responder and non-responder genes was examined by quantitative RT-PCR (**Figure 3; Figure S1**). The four responder genes chosen, *Chrebp*, *Syt7*, *Cplx2*, and *Scgn* (**Table 1**), were highly expressed in the responder cells, but only weakly in Pr-HP cells (**Figure S1A-D**). These genes are known to be expressed in islet β cells and probably have some roles in the regulation of GSIS in β cells (see Discussion). The three non-responder genes chosen, *Car2*, *Maf*, and *Gcg* (**Table 2**), were highly expressed in Pr-HP cells, but only weakly in the responder cells (**Figure 3A-C**). These genes are known to be expressed in the pancreas, but not in islet β cells. Thus, the Pr-HP cells can express genes that are not normally expressed in islet β cells.

Genes encoding β-cell-related transcription factors are listed in **Table 3**. *Pdx1*, *Nkx2-2*, *Nkx6-2*, *Foxa2*, *Pax4*, and *Pax6* were not differentially expressed among the four groups of MIN6 cells, but *Neurod1* showed a 1.5-fold higher expression in the responder cells than in Pr-HP cells. Because the *Mafa* gene was not included in the U74 version 2 GeneChip array used in our analysis, we performed a quantitative RT-PCR analysis, which showed that *Mafa* gene expression did not differ significantly among the four MIN6 cell groups (**Figure 3D**).

There was no significant difference in the expression of the insulin genes (*Ins1* and *Ins2*) as assessed by the DNA microarray analysis. However, because the expression levels of the *Ins1* and *Ins2* genes were dramatically higher than those of the other genes, their signal intensities might have exceeded the linear range of detection. Therefore, we analyzed the expression of the *Ins1* and *Ins2* genes by quantitative RT-PCR. We found that the level of *Ins2* gene expression was not significantly different among the four groups, but that of *Ins1* was much lower in the Pr-HP cells than in the responder cells (**Figure 3E and 3F**). Thus, the *Ins1* gene can be considered a responder gene.

Expression of a cell-surface protein, CD24

The DNA microarray data showed that *Cd24a* was expressed in the Pr-LP, C4-LP, and C4-HP cells, but not in the Pr-HP cells (**Figure 3G**). In addition, immunocytochemical analysis detected CD24 on the surface of Pr-LP, C4-LP, and C4-HP cells, but not on Pr-HP cells (**Figure 4A**). Flow-cytometric analysis also showed that CD24 was expressed similarly in Pr-LP, C4-LP, and C4-HP cells, but its expression was very low or lost in Pr-HP cells (**Figure 4B**). Interestingly, the *Cd24a* gene is expressed in the embryonic endocrine pancreas (E14.5), as shown by *in situ* hybridization (see http://www.genepaint.org/Frameset.html).

Analysis of the *Hepacam2* gene

Hepacam2 was highly expressed in the responder MIN6 cells, but not in the non-responder ones (**Figure 3H**). It encodes a putative membrane-anchored protein. *In situ* hybridization analysis showed that *Hepacam2* expression was restricted to the islets in the pancreas (**Figure 4C**). *Hepacam2* is also expressed in the embryonic pancreas (E14.5) [12].

Table 1. Genes preferentially expressed in responder MIN6 cells.

Gene name	Mean value for Pr-LP, C4-LP, and C4-HP*	Pr-HP*	Fold change
Tmem59l	3306.8	3.8	876.5
Mlxipl	750.0	<1.0	>750.0
Scgn	2686.6	10.3	261.6
Tmed6	107.7	<1.0	>107.7
Plagl1	4616.1	114.0	40.5
Hepacam2	2767.3	73.5	37.6
Dlk1	11245.0	323.7	34.7
Rps6kb1	119.0	5.4	22.2
Syt7	1327.9	65.9	20.1
Slc29a4	1531.1	78.9	19.4
Cd24a	4806.0	282.5	17.0
Rpgr	119.3	7.3	16.4
Meg3	2463.4	154.3	16.0
Glul	798.1	53.2	15.0
1700019D03Rik	210.7	15.2	13.9
Gm2115	607.8	44.8	13.6
Nnat	7113.3	637.7	11.2
Ndn	966.5	94.6	10.2
Ly6e	2512.2	250.9	10.0
Gucy2c	2118.9	217.6	9.7
3830403N18Rik	345.9	38.0	9.1
Kctd12	665.0	73.4	9.1
Cd200	508.3	61.7	8.2
Th	3705.1	477.8	7.8
Amdhd2	252.8	34.9	7.2
Blnk	2957.6	414.8	7.1
Celsr2	221.2	31.6	7.0
Cplx2	852.7	122.2	7.0
Tmod2	530.2	77.1	6.9
Cdhr1	1980.3	288.5	6.9
Ppp1r3d	495.8	73.8	6.7
Pparg	140.7	21.4	6.6
Alcam	278.1	42.5	6.6
Unc80	579.0	93.3	6.2
Epb4.1l4b	1667.5	270.6	6.2
Akr1c14	559.7	91.6	6.1
A830039N20Rik	285.8	48.3	5.9
Mirg	240.4	41.6	5.8
Fgf12	414.6	73.3	5.7
Slc44a1	218.2	39.1	5.6

*Raw values of expression intensities measured by Affymetrix arrays.
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Methylated genes

Interestingly, we found several imprinted genes among the differentially expressed genes. The responder genes included *Plagl1* (**Figure 5A**), *Dlk1* (**Figure 5B**), *Meg3*, *Nnat*, *Ndn*, *Mirg*, *Peg3*, and *Th*. *Cdkn1c* was a non-responder gene. Because only about 150 verified imprinted genes are known (http://igc.otago.ac.nz/1101Summary-table.pdf), the percentage of imprinted genes among the differentially expressed genes in our analysis seemed very high, especially for the responder genes. Therefore, we further investigated the expression of imprinted genes. *Dlk1*, *Meg3*, and *Mirg* are located in the well-known *Dlk1-Gtl1* imprinted gene cluster [13,14]. An analysis of the expression patterns of other imprinted genes in the *Dlk1-Gtl1* locus indicated that *Rian* (**Figure 5C**) and *Rtl1* (data not shown) are also responder genes.

Table 2. Genes preferentially expressed in non-responder MIN6 cells.

Gene name	Pr-HP*	Mean value for Pr-LP, C4-LP, and C4-HP*	Fold change
Cd68	2507.1	79.8	31.4
Car2	2841.2	93.0	30.6
Stmn2	1994.6	65.5	30.5
Maf	536.5	22.0	24.4
Il13ra1	237.9	11.1	21.4
Perp	998.0	52.7	19.0
Sh3bgrl2	512.9	30.6	16.8
Fgfr2	1035.3	66.6	15.6
Pqlc3	2170.1	171.1	12.7
Prr5l	969.2	81.1	12.0
Il4	217.4	19.9	10.9
Ptgs2	195.8	18.2	10.7
Scel	316.3	30.5	10.4
Lpl	1474.6	160.6	9.2
Rbfox1	491.0	54.5	9.0
Lcp2	710.5	86.2	8.2
Tbcel	397.9	50.7	7.9
Glyat	204.5	26.9	7.6
Tmcc3	879.6	117.5	7.5
Basp1	153.9	21.5	7.2
Pcdh7	357.1	52.0	6.9
Ppap2a	1835.0	271.5	6.8
Edil3	1365.9	207.3	6.6
S100a10	4357.1	661.4	6.6
Sox11	1180.7	188.5	6.3
Rpp25	346.5	55.8	6.2
Enpep	757.0	123.7	6.1
Bach2	216.5	35.9	6.0
1810011O10Rik	721.4	120.3	6.0
Gcg	8993.3	1553.4	5.8
Eif2ak4	634.5	110.2	5.8
Sash1	566.6	99.7	5.7
Hspbp1	603.6	106.8	5.7
Oat	1753.3	327.6	5.4
Ezr	1299.8	243.9	5.3
Gnai1	1080.2	202.5	5.3
Gnptab	1234.2	231.4	5.3
Fam132a	984.7	185.9	5.3
Zfp185	669.7	126.7	5.3
Ctsb	199.4	39.5	5.1
Hlf	379.4	75.8	5.0

*Raw values of expression intensities measured by Affymetrix arrays.

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Recent reports have shown that variants in *KCNQ1* are associated with susceptibility to human type 2 diabetes mellitus [15,16]. Interestingly, *Kcnq1* was reported to be an imprinted gene [13,14]. Our DNA microarray analysis showed that the *Kcnq1* expression in Pr-HP cells was 2.2-fold higher than in responder cells. Therefore, we analyzed the expression of *Kcnq1* by quantitative RT-PCR. As shown in **Figure 5D**, *Kcnq1* was a typical non-responder gene.

Genomic imprinting is an epigenetic form of gene regulation that involves differential DNA methylation of the paternal and maternal alleles of a gene. Such methylation is inherited in a parent-of-origin-specific manner. We investigated the methylation

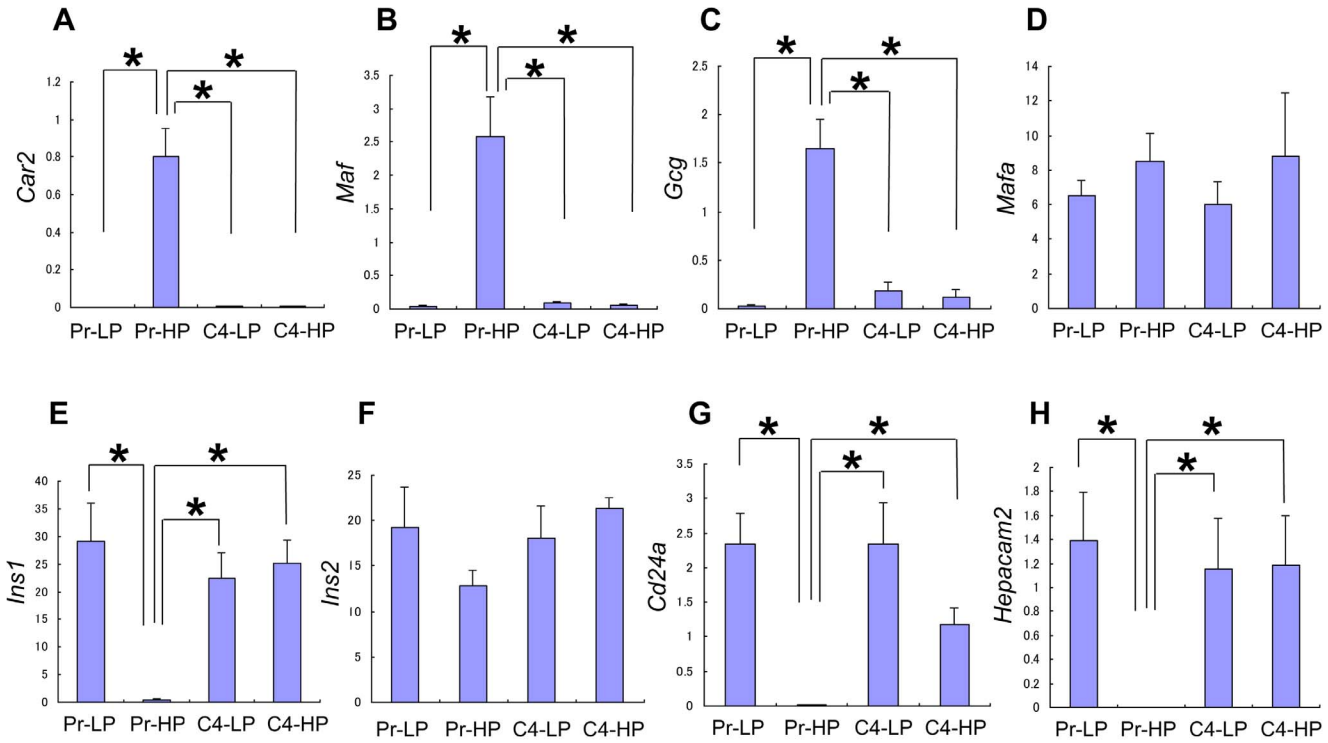

Figure 3. Quantitative RT-PCR analysis. Expression of the *Car2* (A), *Maf* (B), *Gcg* (C), *Mafa* (D), *Ins1* (E), *Ins2* (F), *Cd24a* (G), and *Hepacam2* (H) genes in Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells was examined by quantitative RT-PCR. *Car2*, *Maf*, and *Gcg* showed a non-responder gene expression pattern that was consistent with the DNA microarray analysis. *Ins2* and *Mafa* did not show significant differences in expression among the four groups of MIN6 cells, whereas *Ins1* was revealed to be a responder gene. *Cd24a* and *Hepacam2* showed a responder gene expression pattern that was consistent with the DNA microarray analysis. Values are means ± SD and n = 4–5. *$P<0.05$.
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status of the known regulatory regions of the *Plagl1* and *Dlk1* genes, which were highly expressed in the responder MIN6 cells, but hardly at all in the non-responder cells (**Figure 5B and 5C**). Bisulfite methylation analysis revealed that in the *Plagl1* gene locus, the pattern of methylation of CpG islands was quantitatively different between the responder MIN6 cells (Pr-LP, C4-LP, and C4-HP) and the non-responder cells (Pr-HP) (**Figure 6A**). In the responder cells, one allele was almost completely unmethylated and the other almost completely methylated, whereas in the Pr-HP cells, both alleles appeared to be methylated at random. In contrast, the *Dlk1* locus was significantly less methylated in Pr-HP cells than in the responder cells (**Figure 6B**). Thus, the methylation levels of the CpG islands of imprinted genes differed between the responder and non-responder MIN6 cells, but the pattern of methylation differed according to the gene locus.

Discussion

Although MIN6 cells are widely used as a model of pancreatic β cells, the GSIS is gradually lost with long-term culture. We previously isolated a subclone, MIN6 clone 4, from the parental MIN6 cells. This subclone showed stable GSIS even after long-term culture. To look for candidate genes for the maintenance of GSIS, we decided to analyze the gene expression patterns of Pr-LP, Pr-HP, C4-LP, and C4-HP cells. Our DNA microarray analysis extracted 60 differentially expressed responder genes (with consistently higher expression in Pr-LP, C4-LP, and C4-HP cells) and 62 non-responder genes (higher expression in the Pr-HP cells) (**Table S2**). Genes differentially expressed among Pr-LP, C4-LP, and C4-HP cells are also listed (Table S3, S4, S5). Among these genes, *Tmem59l* is also one of the responder genes (Table 1). As shown in Figure S1E, *Tmem59l* was highly expressed in the responder cells, but not in the non-responder cells. Its expression was higher in C4-LP and C4-HP cells than in Pr-LP cells. Further functional analysis is needed to know the roles of these differentially expressed genes in β cells.

Some of the responder genes have been implicated in the function or development of pancreatic β cells. One responder gene, *Chrebp* (official name: *Mlxipl*), encodes a transcription factor called carbohydrate-responsive element binding protein (ChREBP), and its expression increases in response to extracellular glucose in β cells [17]. It is also involved in the differentiation [18] and proliferation of β cells [19]. *Chrebp* was also reported to be expressed in the embryonic pancreas and to be a target of FOXA1 and FOXA2, which are crucial for the mature β-cell phenotype [20]. Our data suggested that *Chrebp* is also important for the physiological function of β cells.

Genes known to be involved in exocytosis were also found among the responder genes. *Syt7* is a member of the synaptotagmin family, which is crucial for the exocytosis of vesicles [21]. Its protein product, Synaptotagmin VII, participates in the calcium-dependent insulin release from pancreatic β cells [22,23], and the ablation of *Syt7* impairs GSIS [24,25]. Complexin II, encoded by *Cplx2*, is a cytoplasmic pre-synaptic protein believed to regulate neurotransmitter release from pre-synaptic terminals [26]. In our results, the Complexin II level appeared to be high in pancreatic β cells. Because *Cplx2* is a homologue of *Cplx1*, which is implicated in insulin secretion [27,28], *Cplx2* may also play a role in insulin secretion. *Scgn*, which encodes a calcium-

Table 3. Expression levels of pancreas-related genes.

Gene Name	Pr-LP*	Pr-HP*	C4-LP*	C4-HP*	Mean**	Fold change***
Pdx1	701.4	519.5	591.7	570.0	621.0	0.84
Nkx2-2	2408.6	1395.5	1612.7	1501.1	1840.8	0.76
Nkx6-2	252.6	179.5	141.7	395.3	263.2	0.68
Pax4	606.7	507.0	487.6	581.9	558.8	0.91
Pax6	942.0	599.2	621.2	518.4	693.8	0.86
Isl1	936.3	516.3	850.3	715.7	834.1	0.62
Foxa2	1896.9	1349.5	1304.2	1318.7	1506.6	0.90
Insm1	1358.5	488.6	986.8	1078.4	1141.3	0.43
Neurod1	603.8	210.9	263.8	288.1	385.2	0.55
Neurog3	176.9	192.3	192.0	203.6	190.8	1.01
Pcsk2	4580.8	5429.3	5624.6	3924.2	4709.9	1.15
Slc2a2	478.5	499.2	463.8	1754.5	898.9	0.56
Gck	803.2	550.6	788.4	716.6	769.4	0.72
Abcc8	154.7	160.3	168.0	174.6	165.8	0.97
Kcnj11	815.8	644.0	1171.2	933.1	973.4	0.66
Ins1	28131.6	16852.5	27736.0	27214.7	27694.1	0.61
Ins1	19939.3	8937.0	18275.3	20041.0	19418.5	0.46
Ins2	14771.3	15525.9	15461.2	14919. 7	15050.7	1.03
Sst	206.1	206.4	211.3	191.4	203.0	1.02
Ppy	810.5	559.8	524.1	548.7	627.8	0.89
Iapp	8291.0	7443.6	9641.2	8110.5	8680.9	0.86

*Raw values of expression intensities measured by Affymetrix arrays. **Mean value of C4-LP, C4-HP, and Pr-LP. ***Ratio of Pr-HP to mean value.
doi:10.1371/journal.pone.0061211.t003

binding protein, is highly expressed in the pancreatic islets [29] and is also involved in the regulation of insulin secretion [30].

The Pr-HP MIN6 cells expressed some pancreas-related genes that are not normally expressed in mature β cells. For example, *Car2*, which encodes carbonic anhydrase 2, is expressed in duct cells and pancreatic α cells [31]. *Gcg*, encoding preproglucagon, is expressed in pancreatic α cells. *cMaf* (official name: *Maf*) encodes a transcription factor that belongs to the large Maf family, and the *cMaf* mRNA is detected in the embryonic pancreas [32], but it is not expressed in adult pancreatic β cells. *cMaf* was also reported to be expressed in pancreatic α cells and to have a role in their differentiation and maintenance [33–35].

The *Ins2* gene was expressed at high levels in all four MIN6 cell groups, whereas the *Ins1* gene expression was high in responder cells, but extremely low in Pr-HP cells (**Figure 3E**). *Ins1* and *Ins2* are regulated differently [36–39] and are under the control of different enhancer-promoter regions. The human insulin gene (*INS*) and the mouse *Ins2* gene have two introns, but the mouse *Ins1* gene lacks the second intron. Thus, the mouse *Ins2* gene appears more like the human insulin gene. Compensatory responses were reported in mice carrying a null mutation for *Ins1* or *Ins2* [40]. Therefore, the downregulation of *Ins1* might not account for the dysregulation of GSIS in Pr-HP cells. In pancreatic β-cell-specific *Neurod1* knockout mice, the *Ins1* gene expression is almost absent, whereas the *Ins2* gene expression is not much affected [41]. *Neurod1* expression in Pr-HP cells was approximately half that in the responder cells (**Table 3**), which may partly explain the low level of *Ins1* expression in the Pr-HP cells.

Our DNA microarray analysis identified *Cd24a* as a responder gene (**Figure 3G**). Immunohistochemical analysis confirmed that CD24 was expressed on the surface of Pr-LP cells and MIN6 clone 4 cells, but was low or absent in Pr-HP cells. Similarly, Cram *et al.* showed that the *Cd24* gene is expressed at higher levels in RIN-A12 insulinoma cells, which produce a larger amount of insulin, than in RIN-5AH insulinoma cells, which are low insulin producers [42]. Our present results may suggest that CD24 is a useful marker for β-cell lines that retain their differentiated phenotypes.

The *Hepacam2* gene was identified as a responder gene (**Figure 3H**). *Hepacacm2* encodes a putative membrane-anchored protein and was reported to be expressed in pancreatic endocrine cells, including β cells [43]. Our *in situ* hybridization analysis confirmed this (**Figure 4C**). It should be interesting to examine the possible roles of *Hepacam2* in the regulation of GSIS in β cells.

The present study also found a set of imprinted genes among the responder genes. The majority of known imprinted genes in mammals have roles in the control of embryonic growth and development, including development of the placenta [44]. Others are involved in post-natal development, with roles in suckling behavior and metabolism [44]. The imprinted responder genes *Plagl1*, *Dlk1*, and *Nnat* have been implicated in pancreatic β-cell function [45–51]. *Plagl1*, also called *Zac1*, was first isolated as a candidate gene for transient neonatal diabetes (TNDM) [45], which is a rare inherited diabetic syndrome apparent in the first weeks of human life and again during early adulthood. *Plagl1* encodes a proapoptotic zinc finger protein. To investigate its pancreatic function, Ma *et al.* developed a high-copy transgenic mouse line, *TNDM29*, carrying the human *TNDM* locus, including the *Plagl1* gene [46]. *TNDM29* neonates display hyperglycemia, and older adults have impaired glucose tolerance. *Plagl1* overexpression in β-cell lines impairs insulin secretion [47]. Combined

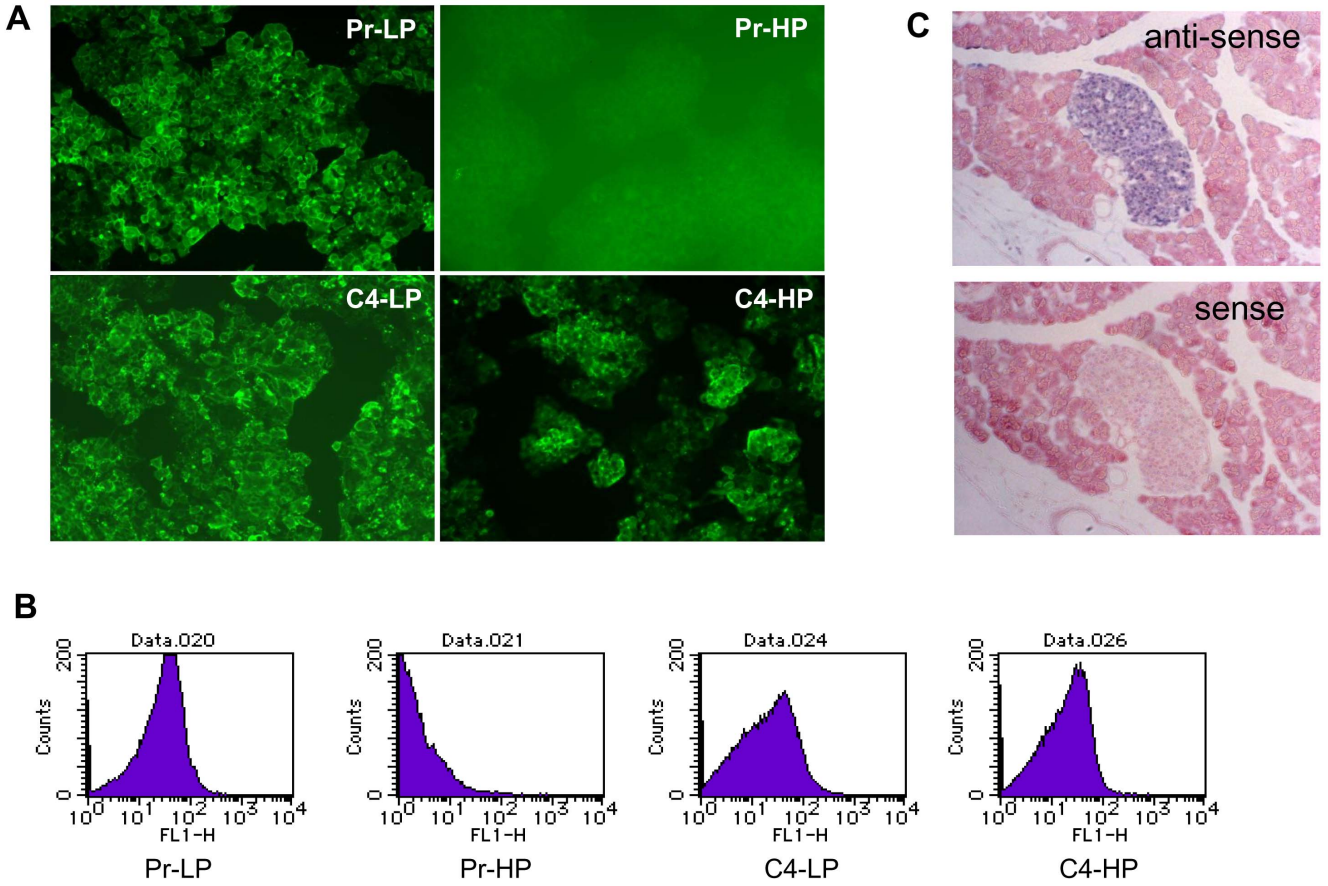

Figure 4. CD24 expression in MIN6 cells and analysis of *Hepacam2* expression by *in situ* hybridization. Immunocytochemical analysis (A) and flow cytometric analysis (B) of Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells with an anti-CD24 antibody. CD24 was not detected on the surface of Pr-HP cells. *In situ* hybridization analysis of *Hepacam2* expression in pancreatic islets (C). Pancreatic sections were hybridized with DIG-labeled anti-sense and sense RNA probes for *Hepacam2* transcripts (see **Materials and Methods**).
doi:10.1371/journal.pone.0061211.g004

with our data, these findings suggest that β-cell function requires the careful regulation of *Plagl1* expression levels.

Dlk1 is expressed in the embryonic pancreas and is gradually restricted to β cells during development [48]. Its protein, Dlk1, is secreted from islets and a β-cell line, and *Dlk1* overexpression in RINm5F cells attenuates their proliferation [49].

Nnat expression was first discovered in the brain and is regulated by *Neurod1*; its suppression by siRNA represses the GSIS by β cells [50]. A recent report also revealed that *Nnat* is involved in the insulin secretory mechanism and in apoptosis [51]. Thus, the low expression of *Nnat* may partly account for the impairment of insulin secretion in Pr-HP cells.

Two other imprinted genes, *Meg3* (*Gtl2*) and *Mirg*, were identified as responder genes. These genes are located in the *Dlk1-Gtl2* gene cluster. Upon examination, we found that other genes in this locus, *Rtl1* and *Rian*, were also responder genes. The human *DLK1-MEG3* (*GTL2*) gene region was recently reported to affect susceptibility for type 1 diabetes [52]. Thus, this locus may contribute to the regulation of β-cell function.

In contrast to the above imprinted genes, *Kcnq1* was expressed at very low levels in the responder cells, but at high levels in the non-responder, Pr-HP cells. Recently *Kcnq1* was reported to be a candidate gene for type 2 diabetes susceptibility [13,14]. *Kcnq1*-overexpressing MIN6 cells show a defect in insulin secretion in response to various secretagogues [53]. Thus, it is possible that high levels of *Kcnq1* expression lead to poor β-cell function.

DNA methylation is necessary for the proper expression of imprinted genes [13,14] and is believed to represent the primary imprinting mark. Imprinted genes can be divided into two classes based on whether they are activated or repressed by the loss of DNA methylation. To clarify the mechanism for the regulation of imprinted genes in MIN6 cells, we analyzed the methylation status of the known regulatory loci of the *Plagl1* and *Dlk1* genes. One allele of the *Plagl1* gene locus was unmethylated and the other was highly methylated in responder cells, whereas both alleles were randomly methylated at various levels in Pr-HP cells (**Figure 6A**). *Plagl1* expression is reported to be downregulated by methylation [54]. Therefore, the random methylation of the *Plagl1* locus may account for its reduced expression in Pr-HP cells. On the other hand, both alleles of the *Dlk1* locus were highly methylated in responder cells, but were less methylated in non-responder cells (**Figure 6B**). An analysis of $Dnmt^{-/-}$ embryos indicated that *Dlk1* is positively regulated by DNA methylation [55]. Therefore, the reduced methylation of the *Dlk1* locus may account for the lower *Dlk1* gene expression in the Pr-HP cells.

Recently, an epigenetic mechanism was reported to be involved in the dysfunction of pancreatic β cells *in vivo*, resulting in the development of diabetes mellitus [56–60]. The imprinted genes

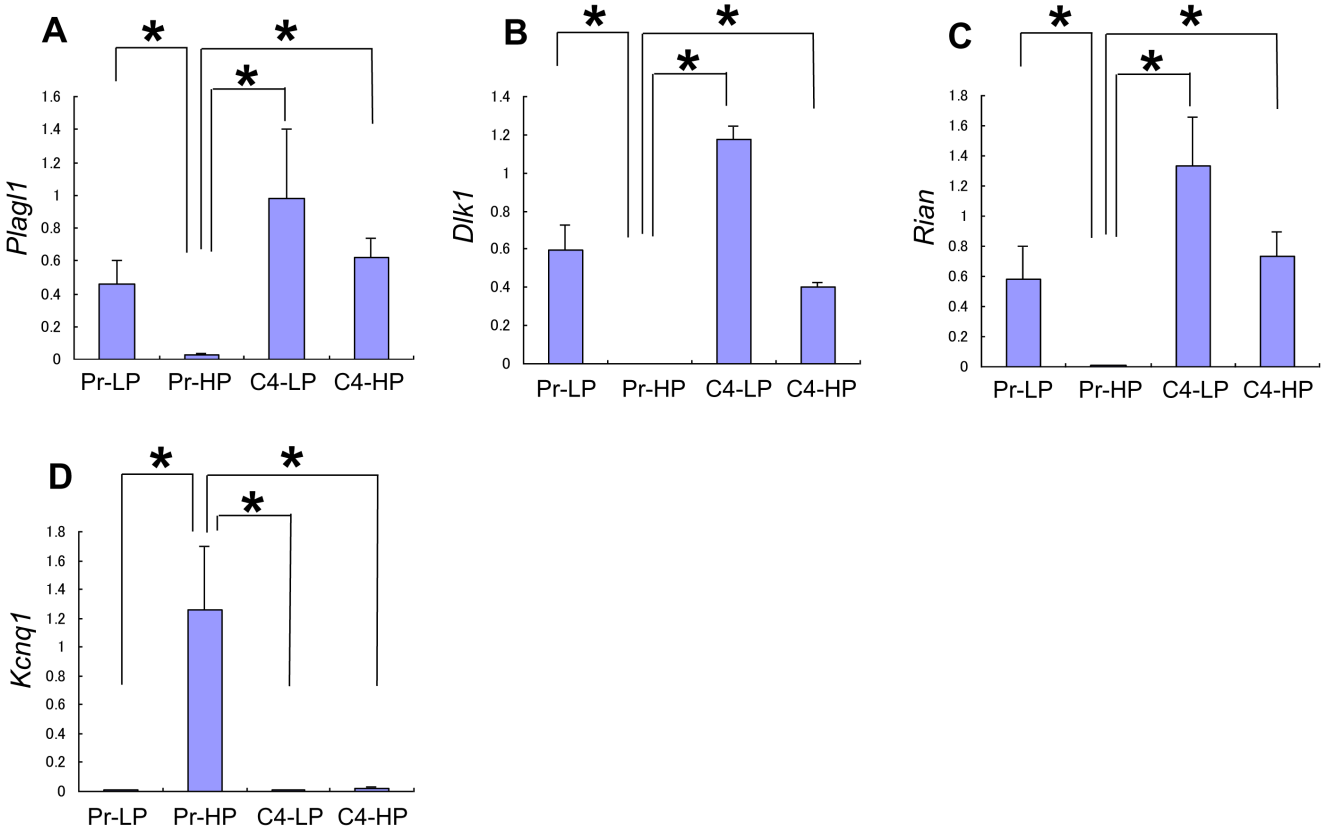

Figure 5. Quantitative RT-PCR analysis of imprinted genes. Expression of imprinted genes, *Rian* (A), *Plagl1* (B), *Dlk1* (C), and *Kcnq1* (D) in Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells. *Plagl1*, *Dlk1*, and *Rian* were confirmed to be responder genes, whereas *Kcnq1* was a non-responder gene. Values are means ± SD and n = 4–5. *$P<0.05$.
doi:10.1371/journal.pone.0061211.g005

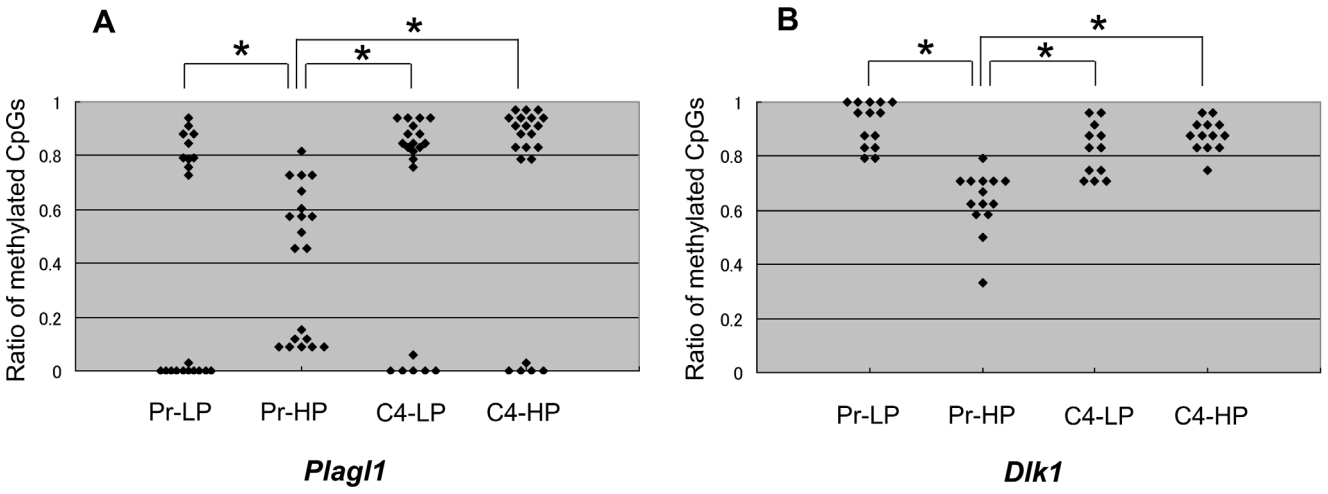

Figure 6. Analysis of CpG methylation of *Plagl1* and *Dlk1*. The ratio of methylated CpGs of each allele from Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells, in the *Plagl1* gene region (A) and the *Dlk1* gene region (B), by bisulfite sequencing. In the *Plagl1* gene locus, the pattern of methylation of CpG islands was quantitatively different between the responder MIN6 cells (Pr-LP, C4-LP, and C4-HP) and the non-responder cells (Pr-HP). In responder cells, one allele was either almost completely unmethylated and the other was almost completely methylated, whereas in the Pr-HP cells, both alleles were randomly methylated. In contrast, the *Dlk1* locus was significantly less methylated in Pr-HP cells than in the responder MIN6 cells. Values are means ± SD and n = 8-10. *$P<0$.
doi:10.1371/journal.pone.0061211.g006

found in the responder and non-responder genes may be important for the maintenance of β-cell functions. Further analysis of the genes identified by the global expression profiling of well-regulated and dysregulated MIN6 cells will help us to clarify the regulatory networks that control insulin secretion.

Supporting Information

Figure S1 Quantitative RT-PCR analysis. Expression of the genes of interest, *Chrebp* (A), *Syt7* (B), *Cplx2* (C), *Scgn* (D), and *Tmem59l* (E) in Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells was examined by quantitative RT-PCR. These genes were confirmed to be responder genes. n = 4–5. Values are means ± SD. *$P<0.05$.
(TIF)

Table S1 PCR primers used in the present study.
(PDF)

Table S2 Genes differentially expressed between responder and non-responder MIN6 cells.
(PDF)

Table S3 Genes differentially expressed between Pr-LP and clone 4 MIN6 cells.
(PDF)

Table S4 Genes differentially expressed between Pr-LP and C4-LP MIN6 cells.
(PDF)

Table S5 Genes differentially expressed between C4-LP and C4-HP MIN6 cells.
(PDF)

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Author Contributions

Conceived and designed the experiments: EY JM. Performed the experiments: EY FT JM. Analyzed the data: EY. Contributed reagents/materials/analysis tools: EY FT JM. Wrote the paper: EY JM.

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