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Delineating Biological Pathways Unique to Embryonic Stem Cell-Derived Insulin-Producing Cell Lines from Their Noninsulin-Producing Progenitor Cell Lines

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To identify unique biochemical pathways in embryonic stem cell-derived insulin-producing cells as potential therapeutic targets to prevent or delay β-cell dysfunction or death in diabetic patients, comparative genome-wide gene expression studies of recently derived mouse insulin-producing cell lines and their progenitor cell lines were performed using microarray technology. Differentially expressed genes were functionally clustered to identify important biochemical pathways in these insulin-producing cell lines. Biochemical or cellular assays were then performed to assess the relevance of these pathways to the biology of these cells. A total of 185 genes were highly expressed in the insulin-producing cell lines, and computational analysis predicted the pentose phosphate pathway (PPP), clathrin-mediated endocytosis, and the peroxisome proliferator-activated receptor (PPAR) signaling pathway as important pathways in these cell lines. Insulin-producing ERoSHK cells were more resistant to hydrogen peroxide (H2O2)-induced oxidative stress. Inhibition of PPP by dehydroepiandrosterone and 6-aminonicotinamide abrogated this H2O2 resistance with a concomitant decrease in PPP activity as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Clathrin-mediated endocytosis, which is essential in maintaining membrane homeostasis in secreting cells, was up-regulated by glucose in ERoSHK but not in their progenitor ERoSH cells. Its inhibition by chlorpromazine at high glucose concentration was toxic to the cells. Troglitazone, a PPARG agonist, up-regulated expression of Ins1 and Ins2 but not Glut2. Gene expression analysis has identified the PPP, clathrin-mediated endocytosis, and the PPAR signaling pathway as the major delineating pathways in these insulin-producing cell lines, and their biological relevance was confirmed by biochemical and cellular assays. (Endocrinology 151: 3600–3610, 2010)

Diabetes is a severe metabolic syndrome of global epidemic proportions. Although diabetes is traditionally classified into two forms, type 1 and type 2 diabetes, there is sufficient commonality to envisage a common universal therapeutic strategy for both types of diabetes (1, 2). Both types involve a loss or eventual loss of insulin-producing β-cells. Fortunately, in both types of diabetes, early diagnosis provides a window of opportunity for therapeutic intervention to reverse or delay disease progression. To systematically search for potential therapeutic targets that are important in improving β-cell survival, function, and regeneration and that could also be used synergistically in a multitarget treatment regimen, we performed a comparative gene expression analysis using two groups of cell lines. The first group was insulin-producing cell lines consisting of mouse embryonic stem cell (mESC)-derived...
ERoSHK as well as mouse embryo-derived RoSH2.K and MEPI (3, 4). The second group encompassed progenitor cell lines, namely mESC-derived ERoSH cell lines and mouse embryo-derived RoSH2 cell lines (5, 6).

The group of insulin-producing cell lines was recently derived in a sequential manner from in vitro differentiation of three different but related cell sources. ERoSHK cell lines were derived from the mESC-derived E-RoSH2.1 cell line, RoSH2.K cell line was derived from embryo-derived RoSH2 cell lines, and MEPI cell lines were derived from a primary embryo culture (3, 4). Despite being derived from different cell sources, all the insulin-producing lines are highly similar in their morphology and other functional properties. These cell lines are amenable to subcloning from single cells, can be stably propagated and expanded, and can withstand repeated cycles of freeze-thaw without loss of cell morphology or insulin production. Gene expression in RoSH2.K, ERoSHK, and MEPI cells is highly similar. We have to date generated eight ERoSHK lines (named ERoSHK1-8), two RoSH2.K lines (of which one was lost), and 14 MEPI lines (named MEPI1-14). The ERoSHK lines have insulin content of 0.2–3.4 μg/10^6 cells, the RoSH2.K cell line has insulin content of 0.5 μg/10^6 cells, and the MEPI lines have insulin content of 0.3–5.8 μg/10^6 cells. Their growth rate and insulin content were stable for up to 9 months of cultures for those cell lines that were tested. They have typical insulin storage vesicles, and their mechanism of insulin release is similar to that of adult pancreatic β-cells. They secrete an equimolar ratio of insulin and C-peptide when stimulated by 15 mm glucose, 100 μmol/liter tolbutamide, or 100 nmol/liter glibenclamide in a stimulus-secretion coupling resembling that of primary pancreatic β-cells. We also demonstrated that these stimuli trigger the closure of ATP-sensitive K(+) channels leading to the depolarization of the cell membrane and opening of the voltage-gated Ca^{2+} channels in these cells. Most importantly, these insulin-producing cells can reverse hyperglycemia in streptozotocin-treated mice.

This study essentially described a transcriptome comparison between homogeneous populations of nontumorigenic insulin-producing cells against homogenous populations of their noninsulin-producing progenitor cells. The insulin-producing cells were represented by three clonal insulin-producing cell lines, namely ERoSHK, RoSH2.K, and MEPI, that were independently derived from different sources of progenitor cell lines or embryo culture. The noninsulin-producing progenitor cells were represented by two clonal progenitor cell lines from which the insulin-producing cell lines were derived, namely E-RoSH2 (5) and RoSH2 (6). These progenitor cell lines were independently derived from mESC and mouse embryos, respectively. The availability of highly similar, scalable, and clonal insulin-producing lines from three different but related sources, of which two are also highly scalable clonal cell lines, overcome two current major limitations in performing gene expression analysis of insulin-producing β-cells. These limitations are the lack of large homogeneous preparations of insulin-producing cells and closely related but noninsulin-producing cells for comparison. Therefore, our insulin-producing cell lines and their noninsulin-producing progenitor cell lines confer an unprecedented robustness on transcriptome analysis and the identification of genes and biochemical or cellular pathways that determine and delineate the unique biology of the insulin-producing cell lines from that of their progenitor cell lines.

Materials and Methods

Cell culture

Insulin-producing RoSH2.K, MEPI-1, and E-RoSHK-2, -4, and -6 cells as well as the progenitor cells, RoSH2 and E-RoSH2, were cultured as described previously (3, 5).

Microarray analysis of gene expression profile

Total RNA (2 μg) from two biological replicates of RoSH2, RoSH2.K, MEPI-1, E-RoSH2, and E-RoSHK-2, -4, and -6 cells were converted to biotinylated cRNA, amplified, and purified using the Illumina TotalPrep RNA amplification kit (Ambion Inc., Austin, TX) according to the manufacturer’s directions. Hybridization to the Illumina Sentrix MouseRef-8 Expression BeadChip version 1.0 and Illumina Sentrix MouseRef-8 Expression BeadChip version 1.1 chips (Illumina Inc., San Diego, CA), washing, and scanning were performed according to the Illumina BeadStation 500x manual. The progenitor cell lines were analyzed using BeadChip version 1.0, whereas the insulin-producing cells were analyzed using both Beadchip versions 1.0 and 1.1. The data were extracted, normalized, and analyzed using Illumina BeadStudio provided by the manufacturer. Transcript signals that were below the limit of detection at 99% confidence were defined as genes not expressed. Microarray analysis was performed on a GeneSpring GX 10.0 platform.

Gene datasets specific for each group of cells were uploaded onto Ingenuity Pathway Analysis platform (Ingenuity Systems, Mountain View, CA) and PANTHER (protein analysis through evolutionary relationships) Classification System (7, 8) for analysis of biological processes, molecular functions, and pathways.

Cell treatment

For the study involving H_2O_2 and dehydroepiandrosterone (DHEA), 20,000 ERoSH2.1 or 60,000 ERoSHK-6 cells were plated in each well of a 96-well plate and cultured at 37 C for 24 h. Subsequently, the cells were exposed to 0, 200, 400, or 800 μmol/liter H_2O_2 in the presence and absence of 100 μmol/liter DHEA for 3 h. Cell viability was determined by trypan blue dye exclusion. ERoSH2.1 and ERoSHK-6 cells were then exposed to 100 μmol/liter DHEA or 5 μmol/liter 6-aminonicotinamide (6-AN) in the presence or absence of H_2O_2 for 3 h. Cell susceptibility to H_2O_2 in the presence and absence of DHEA or 6-AN was
verified using the CytoTox-Fluor cytotoxicity assay (Promega Corp., Madison, WI) according to the manufacturer’s protocol. Fluorescence intensity of the cleaved fluorogenic peptide substrate bis-AAF-rhodamine 110 indicating dead cell protease activity was measured using the Infinite M200 microplate reader (Tecan Group Ltd., Männedorf, Zürich). Changes in reduced nicotinamide adenine dinucleotide (NADH) and NADH phosphate (NADPH) levels were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. For the troglitazone study, 200,000 cells were seeded in each well of a six-well plate and incubated at 37 °C for 24 h. Troglitazone was added at final concentrations of 0, 2.5, 5, 10, or 20 μM/liter for a period of 3 d, after which RNA was extracted from the cells and the expression levels of Ins1, Ins2, and Glut2 were quantified by quantitative RT-PCR (qRT-PCR). For vehicle controls, chloroform and dimethylsulfoxide were added in place of DHEA and troglitazone, respectively.

MTT assay

MTT (catalog item M5655; Sigma-Aldrich, St. Louis, MO) was dissolved in sterile PBS at a concentration of 10 mg/ml and sterile-filtered through a 0.22-μm filter. The MTT solution was stored at 4 °C, protected from light. MTT solution (10 μl) and culture medium (100 μl) were added to each well of cells in 96-well plates and incubated overnight. Solubilization buffer (100 μl) from the cell QuantiMT-assay kit (catalog item CQMT-500; BioAssay Systems, Hayward, CA) was added to each well, and the plates were incubated at 37 °C for 3 h to dissolve the purple formazan. Absorbance at 570 nm was measured using a Benchmark Plus microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA).

Transferrin uptake

Cells were first trypsinized and counted, and 1 × 10⁶ cells were washed with 1× PBS twice and serum starved by resuspending cells in 2 ml 2 g/liter glucose, serum-free INS medium for 1 h at 37 °C with rotation to remove free transferrin. The medium was replaced with either 2 g/liter or 6 g/liter glucose (high) serum-free INS medium and incubated for an additional 45 min. The medium was subsequently replaced with the addition of 5 g/liter glucose (NADPH) levels were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. For the troglitazone study, 200,000 cells were seeded in each well of a six-well plate and incubated at 37 °C for 24 h. Troglitazone was added at final concentrations of 0, 2.5, 5, 10, or 20 μM/liter for a period of 3 d, after which RNA was extracted from the cells and the expression levels of Ins1, Ins2, and Glut2 were quantified by quantitative RT-PCR (qRT-PCR). For vehicle controls, chloroform and dimethylsulfoxide were added in place of DHEA and troglitazone, respectively.

RT-PCR and qRT-PCR

Total RNA was extracted using the Nucleospin RNA/protein kit (Macherey-Nagel, Düren, Germany). Total RNA (1 μg) was converted to cDNA using random primers in a 20-μl reaction volume using a high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). For PCR amplification of Ppara, Ppard, and Pparg transcripts, PCR mixes were prepared using 1 μl cDNA (20 ng), 1 μl primer pair (20 μM/liter), and 18 μl Platinum PCR Super-mix (Invitrogen Corp., Carlsbad, CA). They were denatured at 92 °C for 2 min and subsequently underwent 40 cycles of 92 °C for 15 sec, 58 °C for 30 sec, and 72 °C for 45 sec. PCR were completed with a final extension at 72 °C for 5 min. The PCR products were then resolved by agarose gel electrophoresis. The primers used were 1) Ppard (5'-ACT TCA GGC GCC TCT TCC TCA-3' and 5'-TGT CCT GGA TGG CTT CTA CCT-3'), 2) Ppara (5'-CCA CGA AGC CTA CCT GAA GAA-3' and 5'-AAT CGG ACC TCT GCC TCT TTG-3'), 3) Pparg (5'-AAG TGc GAT CAA AGT AGA ACC-3' and 5'-AAA CcT GAT GcC TTT GTG AGA AGA-3'), and 4) Actb (5'-TAT CCT GAC CcCT GAA GTA CCC-3' and 5'-GGC CAT CTC TTG CTC CAA GTc-3').

For real-time PCR, a mixture of 1 μl cDNA (10 ng), 1 μl TaqMan primer, 8 μl TaqMan 2× PCR master mix, and 10 μl distilled water was subjected to amplification on an ABI StepOne Plus Real Time PCR system (Applied Biosystems). The cycle threshold values obtained were used to calculate the relative transcript levels. Each sample was performed in technical and biological triplicates. The TaqMan primer ID for the genes analyzed were Actb, Mm00607939_s1; Ins1, Mm01259683_g1; Ins2, Mm00731595_gh; Glut2, Mm00446224_m1; Rbks, Mm00462703_m1; Ncoa1, Mm00447958_m1; G6pdx, Mm00656735_g1; Pfkp, Mm00444792_m1; Pik3r3, Mm00725021_m1; Pdgfa, Mm01205760_m1; Sh3g12, Mm00490393_m1; Rab5b, Mm00834147_g1; and Sh3kbp1, Mm00451715_m1 (Applied Biosystems).

Statistical analyses

Statistical significance between two samples was determined using unpaired Student’s t tests, with P < 0.05 as the level of significance.

FIG. 1. Gene expression. A, Hierarchical clustering of expressed genes in E14 mESC, E-RoSH and RoSH2 progenitor cells, and E-RoSH, RoSH2.K, and MEPI insulin-producing cells; B, distribution profile of expressed genes in insulin-producing cells and their progenitor cells as represented by Venn diagram. A total of 5541 genes were highly expressed (≥2.0-fold) in the progenitor cells, 2347 genes were expressed at similar levels in both insulin-producing cells and their progenitor cells, and 185 genes were highly expressed (≥2.0-fold) in the insulin-producing cells.
**Results**

**Gene expression analysis**

Gene expression profiles of the three groups of cells, namely insulin-producing cell lines (ERoSHK, RoSH2.K, and MEPI), the progenitor cell lines (mESC-derived ERoSH and mouse embryo-derived RoSH cell lines), and mESC were prepared by hybridization of labeled cRNA to Illumina BeadArray containing about 24,000 unique features as described in Materials and Methods.

Hierarchical clustering of expressed genes in the insulin-producing cell lines revealed that, as expected, the gene expression profile of the three insulin-producing cell lines clustered with each other, whereas that of the progenitor cell lines clustered with each other. Not unexpectedly, the gene expression profile of mESC was more closely related to that of the progenitor cells than to the insulin-producing cells (Fig. 1A). A total of 7888 genes were expressed by the progenitor cells, and 2532 genes were expressed by the insulin-producing cells. Of these, 2347 genes were commonly expressed at similar levels by both the progenitor and insulin-producing cells. The progenitor cells expressed 5541 genes at a more than 2-fold higher level, and the insulin-producing cells expressed 185 genes at a more than 2-fold higher level (Fig. 1B) (Supplemental Table 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). The 5541, 2347, and 185 genes were analyzed by PANTHER (protein analysis through evolutionary relationships) (7, 8) in which the observed frequency of genes for each biological process in each gene set was compared with the reference frequency, which in this case, is the frequency of genes for that biological process in the NCBI database. The analysis disclosed that three sets of genes were overrepresented in 64, 19, and 26 biological processes, respectively (Fig. 2). Comparison of these three sets of biological processes revealed that three biological processes, cell structure and

![PANTHER analysis of expressed genes](image-url)
motility, monosaccharide metabolism, and carbohydrate metabolism, were present in all three sets (Fig. 2A); another three processes were overrepresented in both sets of genes highly expressed by the insulin progenitor cells and the insulin-producing cells (Fig. 2B); and 16 processes were overrepresented in the two sets of genes (Fig. 2C) that were highly expressed by the progenitor cells and the set of commonly expressed genes. After removing these redundancies, the set of highly expressed genes in the progenitor cells was overrepresented in 42 unique processes, whereas that in the insulin-producing cells was overrepresented in 20 unique biological processes (Fig. 2, D and E). The 42 unique biological processes driven by genes that were highly expressed in the progenitor cells were largely involved in cell growth and proliferation such as DNA replication, cell cycle control, gene expression, and metabolism (Fig. 2D). On the other hand, the 20 unique biological processes driven by genes that were highly expressed by insulin-producing cells were involved in development and function of insulin-producing cells. Of these 20 unique biological processes, seven were underrepresented in the progenitor cell-specific gene set, and these seven processes were signal transduction, neurogenesis, cell communication, ectoderm development, neuronal activities, cell adhesion, and ion transport (Fig. 3). To identify those biochemical or molecular pathways that are responsible for the unique biology of pancreatic β-cells, the 185 genes that were highly expressed in the insulin-producing cells were analyzed by Ingenuity Pathway Analysis (Ingenuity Systems). Eight pathways with a P < 0.05 were identified. The three most significant pathways were the pentose phosphate pathway (PPP), clathrin-mediated endocytosis, and peroxisome proliferator-activated receptor (PPAR) signaling (Fig. 4A). The expression levels of 10 genes randomly selected from genes that were classified into these three pathways (Supplemental Table 2) were further evaluated by qRT-PCR. The 10 genes were Rbks, G6pd, and Pfkp in the PPP; Pik3r3, Sb3khp1, Pdgfa, Rab5b, Sb3g12, and Ins2 in clathrin-mediated endocytosis; and Pdgfa, Ins2, and Ncoa1 in PPAR signaling. Seven of the 10 genes were up-regulated by more than 2-fold in the insulin-producing ERoSHK cell line compared with its progenitor ERoSH cell line (Fig. 4B), in concordance with the microarray analysis.

**PPP protected ERoSHK-6 but not ERoSH2.1 cells from H₂O₂-mediated insult**

PPP is a major metabolic pathway for the metabolism of pentose sugars and the generation of NADPH. NADPH acts mainly as a reducing agent to reduce the coenzyme glutathione, which in turn converts reactive H₂O₂ into H₂O and prevents conversion of H₂O₂ into cytotoxic hydroxyl free radicals. Because PPP was predicted as the most significant pathway based on the 185 genes that were highly expressed in insulin-producing cells, we hypothesized that PPP has an important role in their cellular response to H₂O₂-induced oxidative stress.

To elucidate this role, insulin-producing ERoSHK-6 cells and their progenitor cells, ERoSH2.1 cells, were treated with H₂O₂ in the absence or presence of DHEA, an inhibitor of glucose-6-phosphate dehydrogenase (G6PD) (9), the rate-limiting enzyme in the PPP. Exposure of both cell types to increasing concentrations of H₂O₂ to 400 μmol/liter caused a corresponding decrease in viability of both cell types (Fig. 5A). However, ERoSHK-6 cells were more resistant to H₂O₂ toxicity and had a significantly higher survival rate at 400 and 800 μmol/liter (P = 0.02 and 0.016, respectively). We postulated that PPP mediated this resistance, and consistent with this, DHEA, an inhibitor of PPP abolished this resistance such that low H₂O₂ concentration at 200 μmol/
liter was highly toxic to EROSHK-6 cells but not EROSH2.1 cells (Fig. 5B). Exposure to DHEA alone without \( \text{H}_2\text{O}_2 \) was not toxic to either cell type, as evidenced by the cell viability trypan blue dye exclusion assay (Fig. 5C), but it significantly reduced the level of MTT activity in EROSHK-6 cells (\( P < 0.05 \)). This discrepancy between the two cell viability assays is because MTT assay measures cell viability by assessing NADPH and NADH levels (10). The reduction in MTT activity but not trypan blue dye exclusion during DHEA treatment was therefore consistent with the inhibitory effect of DHEA on PPP, the major source of NADPH in a cell (11), and a subsequent reduction in NADPH level. Our observation that DHEA had little effect on MTT activity in EROSH2.1 cells also suggested that PPP-generated NADPH did not contribute significantly to the MTT activity in EROSH2.1 cells. To verify that DHEA enhanced the \( \text{H}_2\text{O}_2 \) cytotoxicity on insulin-producing EROSHK-6 cells through the inhibition of PPP, a second PPP inhibitor, 6-AN, was used. 6-AN is a nonmetabolizable analog of NADP and competitive inhibitor of G6PD in the PPP (12). Like exposure to 100 \( \mu \text{mol/liter} \) DHEA, 5 \( \mu \text{mol/liter} \) 6-AN reduced MTT activity in EROSHK-6 cells by 39% but not in EROSH2.1 cells (Fig. 5D). However, when the effect of 6-AN or DHEA exposure was assessed by a cytotoxic assay that measures enzyme released by dying cells, we observed that 6-AN or DHEA was slightly cytotoxic to EROSH2.1 cells but not to EROSHK-6 cells (Fig. 5E). These observations were therefore consistent with our above observations that PPP inhibitors reduced MTT activity in EROSHK-6 cells through the depletion of NADPH without a deleterious effect on cell viability. In addition, the cytotoxicity of 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) in EROSHK-6 cells was also enhanced 4.0- and 2.9-fold (\( P < 0.001 \)) by 6-AN or DHEA, respectively. No significant enhancement was observed for EROSH2.1 cells (Fig. 5E).

Clathrin-mediated endocytosis was glucose sensitive and critical for the survival of insulin-producing EROSH cells

Secretory cells such as insulin-secreting \( \beta \)-cells compensate for excess surface membrane that occurs after a stimulated burst of exocytotic activity by retrieving membrane by endocytosis (13). This coupled exocytosis-endocytosis activity is a well-documented phenomenon in many secretory cell types (14–16). In insulin-producing cells, \( \text{Ca}^{2+} \)-dependent clathrin-mediated endocytosis has been identified as one of the two types of membrane retrieval processes (17). Therefore, we hypothesized that the computationally predicted importance of clathrin-mediated endocytosis in insulin-producing cells was coupled to their main biological function of insulin secretion during glucose stimulation. To assess this importance, the rate of transferrin uptake, a classical marker of clathrin-mediated
endocytic pathway was measured in both ERoSHK-6 cells and their progenitor cells, E-RoSH2.1 cells, in the presence and absence of glucose stimulation. We have previously demonstrated that ERoSHK-6 cells increased insulin secretion upon glucose stimulation (3). When the glucose concentration was increased from 2 to 6 g/liter, ERoSHK-6 cells exhibited an 89% increase in transferrin internalization \((P < 0.001)\) (Fig. 6A). In contrast, transferrin uptake in E-RoSH2.1 cells exhibited a much smaller increase of 12% \((P < 0.005)\). These observations suggested that there was coupling of glucose-stimulated exocytosis of insulin with clathrin-mediated endocytosis. Because this coupling is important in maintaining membrane homeostasis and therefore cell viability, we next postulated that decoupling of clathrin-mediated endocytosis with glucose-stimulated insulin exocytosis will lead to expansion of cell membrane with deleterious consequences. To test this, ERoSHK-6 cells were treated with chlorpromazine, a drug that inhibits clathrin-mediated endocytosis by preventing the formation of coated pits on the cell surface (18). At 7.5 and 15 \(\mu\)mol/liter chlorpromazine, ERoSHK-6 cells in 6 g/liter glucose had a significantly reduced cell viability relative to cells in 2 g/liter glucose \((P < 0.001)\) (Fig. 6B).

**PPARG activation increased transcription of \(\beta\)-cell-specific genes**

PPAR signaling pathway was predicted as the third important pathway in determining the unique biological properties of the insulin-producing cells. PPARs are members of a nuclear receptor superfamily that include PPARA, PPARD, and PPARG. Here, the PPAR signaling pathway could therefore include signaling through any of these receptors. Although transcripts for each of the three receptors were not detected in our microarray analysis, they were readily detected in both insulin-producing cells and their progenitors by RT-PCR (Fig. 7A). Because PPARG was reported to up-regulate transcription of Pdx-1, insulin, Nkx6.1, Glut2, and glucokinase in pancreatic \(\beta\)-cells (19, 20), we postulated that the importance of PPAR signaling in the insulin-producing cells derived partly from transcriptional regulation by PPARG. To test this hypothesis, ERoSHK-6 and
ERoSH2.1 cells were treated with troglitazone, a PPARγ agonist, and the transcript levels of Ins1, Ins2, and Glut2 in the cells before and after treatment were determined by real-time RT-PCR (Fig. 7B). Ins1 transcript level was significantly increased at 5 μmol/liter troglitazone, whereas Ins2 transcript level was significantly increased at 10 μmol/liter troglitazone. However, troglitazone did not have an effect on Glut2 transcript level. Troglitazone had no significant effect on the transcript level of all three genes in the progenitor E-RoSH2.1 cells (Fig. 7B).

Discussion

Our starting premise in this study was that by comparing the gene expression profiles of insulin-producing cell lines and their progenitor cells, we would identify genes that were highly expressed in the insulin-producing cells and that these genes would inform us of the unique biological processes that delineate insulin-producing cells from their noninsulin-producing progenitor cells. These unique biological processes may provide opportunities for exploitation as therapeutic targets to specifically manipulate the survival and function of β-cells.

Using insulin-producing ERoSHK-2, -4, and -6 cell lines, the RoSH2.K cell line and MEPI-1 cell lines that we have previously derived from the mESC-derived E-RoSH2.1 cell line, embryo-derived RoSH2 cell lines, and a primary embryo culture, respectively (3, 4), we performed a comparative gene expression study using microarray technology. Based on genes that were highly expressed by the progenitors, we identified 42 unique biological processes that were largely involved in cell growth and proliferation such as DNA replication, cell cycle control, gene expression, and metabolism. This is highly consistent with our previously reported observation that the progenitor cells have a much faster population doubling time of 12–15 h than that of 3–4 d in the insulin-producing cells (3–6). Notably, the 20 unique biological processes based on genes that were highly expressed by insulin-producing cells indicated a strong neuronal component. Some of these processes include neurogenesis, synaptic transmission, neurotransmitter release, and neuronal activities. Such strong similarities between the insulin-secreting β-cells of the pancreas and neurons had long been observed and were responsible for earlier erroneous postulations that insulin-secreting β-cells have a neuroectodermal origin (reviewed in Ref. 21). Notably, the most prominent similarity lies in the most important β-cell function, glucose-sensitive insulin secretion. This process is essentially triggered by glucose-sensitive membrane depolarization leading to a Ca2+ -dependent exocytosis of secretory granules involving soluble N-ethylmaleimide-sensitive fusion-attachment protein receptor proteins. This process resembles many aspects of neurotransmitter release where the plasma membrane is also depolarized leading to an increased influx of Ca2+ ion through L-type Ca2+ channels and subsequent exocytosis of secretory granules involving soluble N-ethylmaleimide-sensitive fusion-attachment protein receptor proteins (22). β-Cells also express many neurotransmitter biosynthetic enzymes such as tyrosine hydroxylase, dopa decarboxylase, and glutamate decarboxylase as well as neurofilament protein and the neural cell adhesion molecule N-CAM (reviewed in Ref. 21). Aside from the neuronal characteristic, the other biological processes are also consistent with the unique biology of pancreatic β-cells. For example, cell communication, calcium homeostasis, and...
exocytosis are integral to glucose-sensitive insulin secretion. It is notable that the seven biological processes that were overrepresented in the insulin-producing cell-specific gene set but underrepresented in the progenitor cell-specific gene set were signal transduction, neurogenesis, cell communication, ectoderm development, neuronal activities, cell adhesion, and ion transport. As discussed, these processes are highly relevant to the unique biology of pancreatic β-cells. Together, this exercise demonstrated the robustness of the 185 insulin-producing cell-specific genes in delineating the unique biology of these cells from their noninsulin-producing progenitor cells.

Therefore, these 185 genes were then further analyzed by Ingenuity to identify biological pathways. Unlike the biological pathways predicted using PANTHER, the biological pathways predicted by Ingenuity were more amenable to the generation of a testable hypothesis about the biology of insulin-producing cells. Three pathways, PPP, clathrin-mediated endocytosis and PPAR signaling, were predicted to distinguish the insulin-producing cells from their noninsulin-producing progenitor cells.

The PPP functions primarily to produce reductive equivalents and pentoses for anabolic activities and is not known to contribute significantly to glucose metabolism in pancreatic islet cells (23). The PPP in most cell types is the major source of NADPH, and this in turn is responsible for reduction of glutathione disulfide to glutathione. One function of glutathione is to neutralize toxic oxidants such as lipid hydroperoxides and free hydrogen peroxide to their corresponding alcohols or water, respectively. Abnormal glutathione status has been associated with β-cell dysfunction and the pathogenesis of long-term complications of diabetes (24), whereas exposure to glutathione significantly improved β-cell function and reduced oxidative stress (25). Inhibition of G6PD, a rate-limiting enzyme in the PPP, has also been shown to cause β-cell dysfunction and death (26). Based on these reports, we postulated that the prominence of the PPP in the insulin-producing cells relative to their non-insulin-producing progenitor cells reflected a central role in protecting insulin-producing cells against oxidative stress. Consistent with our postulation, inhibition of the PPP by nontoxic concentration of G6PD inhibitors such as DHEA or 6-AN during H2O2 treatment was highly toxic to the insulin-producing cells but not their progenitor cells. We demonstrated here that nontoxic concentrations of PPP inhibitors significantly reduced MTT activity of the insulin-producing cell line ERoSHK-6 but not in its progenitor ERoSH2 cell line. Because the nontoxicity of the two inhibitors was verified by two different cytotoxic assays, the reduction in MTT activity was thus attributed to a reduction in NADPH level as a result of G6PD inhibition and not cell viability. Together, these experimental observations suggested that, like erythrocytes, insulin-producing cells rely predominantly on the PPP to neutralize oxidative stress. Indeed, a recent study revealed that neonatal rat islets are resistant to H2O2 because of a greater use of the PPP to produce NAD(P)H from glucose (27). Interestingly, epidemiological studies of several populations have reported a significant direct correlation between G6PD deficiency and risk of diabetes (28–31).

Clathrin-mediated endocytosis was the second most significant pathway predicted to distinguish the insulin-producing cells from their progenitors. For cells such as the insulin-producing cells that are actively secreting through fusion of secretory granules with the plasma membrane, membrane retrieval is critical in maintaining membrane homeostasis. Although pancreatic β-cells have been shown to secrete insulin through kiss-and-run exocytosis where a fraction of vesicle membrane transiently fused with the plasma membrane to release the insulin contents of the vesicles, this form of exocytosis is estimated to constitute at most 20% of the exocytotic events (32). Therefore, most of the exocytosis in β-cells occurs through the classical membrane fusion where subsequent membrane retrieval is critical in removing excess membrane after exocytosis and in maintaining membrane homeostasis (33).

The pancreatic β-cell is thought to retrieve membrane by two mechanistically different endocytic processes: a fast clathrin-independent endocytosis and a slow clathrin-dependent endocytosis (17). Although the relative contribution of these two endocytic processes to membrane retrieval has not been determined, it was observed that activation of the clathrin-independent endocytosis requires a high concentration of cytoplasmic calcium (17).
Because a high concentration of cytoplasmic calcium can be maintained only transiently in cells, it is likely that slow clathrin-dependent endocytosis is the more dominant mechanism in membrane retrieval. Therefore, the prediction that clathrin-mediated endocytosis delineates the insulin-producing cells from their progenitor cells underlined the importance of coupling insulin secretion via exocytosis to clathrin-mediated endocytosis for membrane retrieval and maintenance of membrane homeostasis. Because exposure of EROSHK cells to a high glucose concentration of 6 g/liter stimulates insulin secretion (3), our observation that high glucose increased transferrin uptake in EROSHK cells much more than that in their progenitor EROSH cells was consistent with a coupling of insulin exocytosis to clathrin-mediated endocytosis. Decoupling of these two processes by inhibiting clathrin-mediated endocytosis with chlorpromazine (18) was more toxic to the insulin-producing EROSHK-6 cells at high glucose concentration when the cells are actively secreting insulin. Together, these observations indicated that clathrin-mediated endocytosis is vital to the viability of insulin-producing cells when they are performing their most critical function, secreting insulin in response to high glucose. Notably, chlorpromazine belongs to a class of antipsychotic drugs that has long been associated with type 2 diabetes and impaired glucose tolerance (34). In animal studies, chlorpromazine has been shown to induce significant hyperglycemia in mice (35) and attenuate pancreatic β-cell function and mass (36). This association between the use of chlorpromazine and incidence of diabetes, and also its effect on glucose regulation, and β-cell function and survival further implicate the importance of clathrin-mediated endocytosis in the biology of insulin-producing cells.

PPAR signaling was also predicted to be very important in insulin-producing cells. Because PPAR signaling could include signaling through any members of the PPAR nuclear receptor superfamily, and PPARA, PPARD, and PPARG were all expressed by both the insulin-producing cells and their progenitor cells, we validated this prediction by demonstrating that at least one of the receptors was important in the transcription of highly pancreatic β-cell-specific genes Ins1 and Ins2. Exposure to troglitazone significantly increased Ins1 and Ins2 transcript levels in ERSKH-6 cells but not their progenitor cells. This observation is consistent with the findings that PPAR agonists increased insulin content in islets and improved glucose-stimulated insulin secretion (37, 38).

In conclusion, this study demonstrated that mESC-derived insulin-producing cell lines and their nonsulin-producing progenitor cell lines provided a unique and robust system to delineate biological processes and pathways that are specifically relevant to insulin-producing cells. Because these cell lines are highly scalable, they are therefore amenable to the more efficient high-throughput analytical tools such as comparative genome- or proteome-wide analysis. This will facilitate identification of more candidate biological processes or pathways that define insulin-producing cells and potential therapeutic targets to improve survival and function of these cells in diabetic patients.