High Glucose Is Necessary for Complete Maturation of Pdx1-VP16—Expressing Hepatic Cells into Functional Insulin-Producing Cells

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Pdx1 has been shown to convert hepatocytes into both exocrine and endocrine pancreatic cells in mice, but it fails to selectively convert hepatocytes into pure insulin-producing cells (IPCs). The molecular mechanisms underlying the transdifferentiation remain unclear. In this study, we generated a stably transfected rat hepatic cell line named WB-1 that expresses an active form of Pdx1 along with a reporter gene, RIP-eGFP. Our results demonstrate that Pdx1 induces the expression of multiple genes related to endocrine pancreas development and islet function in these liver cells. We do not however find any expression of the late-stage genes (Pax4, Pax6, Isl-1, and Mafa) related to β-cell development, and the cells do not secrete insulin upon the glucose challenge. Yet when WB-1 cells are transplanted into diabetic NOD-scid mice, these genes become activated and hyperglycemia is completely reversed. Detailed comparison of gene expression profiles between pre- and postransplanted WB-1 cells demonstrates that the WB-1 cells have similar properties as that seen in pancreatic β-cells. In addition, in vitro culture in high-glucose medium is sufficient to induce complete maturation of WB-1 cells into functional IPCs. In summary, we find that Pdx1-VP16 is able to selectively convert hepatic cells into pancreatic endocrine precursor cells. However, complete transdifferentiation into functional IPCs requires additional external factors, including high glucose or hyperglycemia. Thus, transdifferentiation of hepatocytes into functional IPCs may serve as a viable therapeutic option for patients with type 1 diabetes. Diabetes 53:3168–3178, 2004

The liver and pancreas have an intimate relationship during embryogenesis. Indeed, it has been proposed that these two organs are derived from a common progenitor cell (1), and transdifferentiation between the liver and pancreas has been demonstrated under certain conditions (2). We previously demonstrated that hepatic stem cells could be induced in vitro to transdifferentiate into insulin-producing pancreatic endocrine-like cells (3). Recent studies have shown that ectopic and transient expression of the transcription factor Pdx1 in the mouse liver induces transdifferentiation into pancreatic cells, including both exocrine and endocrine cells, and reduces hyperglycemia in chemically induced diabetic mice (4–7). However, the conversion from liver to endocrine pancreatic cells mediated by Pdx1 alone is incomplete and nonselective, resulting in severe hyperglycemia due to the production of by-products such as the exocrine enzymes amylase and trypsin (7).

To establish an in vitro system to study the molecular mechanism of selective liver to endocrine pancreas transdifferentiation, we generated a stably transfected rat hepatic cell line (WB-1) that overexpresses an activated form of Pdx1 (Pdx1-VP16) along with a reporter gene, RIP-eGFP, to monitor insulin gene expression. Pdx1-VP16 is a fusion of mouse Pdx1 with the VP16 activation domain from the Herpes simplex virus that creates a super active version of Pdx1 (5). In this study, we investigated the profile of gene expression induced by Pdx1-VP16-mediated transdifferentiation of WB-1 cells in vitro and during in vivo cell transplantation. We find that expression of Pdx1-VP16 can transdifferentiate hepatic WB cells into glucose-insensitive endocrine precursor cells expressing multiple genes related to β-cell development and function but with no evidence of pancreatic exocrine gene expression. However, these precursor cells require either in vivo hyperglycemia or in vitro long-term high-glucose culture to become fully functional pancreatic endocrine cells. Thus, the conversion of hepatocytes into endocrine pancreatic cells may be a viable option for cell replacement therapy in patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Plasmids and plasmid construction. Pdx1-VP16 was constructed by fusing the activation domain of VP16 (80 amino acids) to the mouse COOH-terminus of Pdx1 as follows. Full-length Pdx1 was isolated from IPF1-pcDNA3 (a gift from H.-P. Huang) using the T7 primer and a 3′ primer that included a ClaI site, 5′-TCG CAG TGG ATC GAT GCT GGA G-3′. The product was cut with HindIII and ClaI and subcloned into VP16-N in pCS2+ (a gift from Dr. Kessler) (8). Pdx1-VP16 was then subcloned into the HindIII and XbaI sites of pcDNA3. A vector containing a chimeric gene of the zeocin resistance gene (neo) and a green fluorescence protein gene under the control of rat insulin-1 promoter–enhanced green fluorescence protein (RIP-eGFP) was constructed in our laboratory. To construct pRIP-eGFP, a 645-bp fragment of the 5′ untranslated (promoter) region of the rat insulin 1 gene was amplified from DNA of rat pancreas. The PCR product was first cloned into pGEM-T vector...
Hyperglycemic by intraperitoneal injections of streptozotocin (STZ) at 50 mg/kg body wt daily for 5 days, as previously described (14). When blood glucose levels reached levels in the nonfasting condition. Three of the six WB-1 cell–implanted tissues were fixed and embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). Sections were incubated with antibodies against insulin, glucagon, and albumin (Dako), and amylase (Santa Cruz Biotechnology) according to our previously published procedure (3) with minor modifications. Cytospin slides were made from cultured WB-1 and WB cells and fixed with methanol for 10 min. For Pdx1 staining, cytospin slides were incubated sequentially with rabbit anti-Pdx1 (1:5,000) antibody for 1 h and then biotinylated secondary anti-rabbit polyclonal antibody for 30 min. The antigen-antibody complex was visualized with DAB reagent (Vector Laboratories).

Electron microscopy with immunogold labeling. For immunogold localization of insulin, the cultured WB-1 cells and rat pancreas were embedded as previously described (14). For immunogold labeling, ultra-thin sections were blocked with 5% BSA and 5% normal goat serum in PBS and then incubated overnight at 4°C with rabbit anti-insulin antibody (Santa Cruz Biotechnology) diluted 1:50 in PBS containing 0.2% BSA and 10 mmol/l NaN3. After washing, the samples were incubated for 1.5 h at room temperature with the secondary goat anti-rabbit IgG antibody conjugated to 0.8 nm colloidal gold particles (Auron EM Grade Ultra Small, EM Sciences). The gold particles were silver-enhanced for 45 min at room temperature (Auron R-Gent SE EM, EM Sciences). The samples were counterstained and then viewed using a Zeiss EM-10A transmission electron microscope.

Statistical analysis. Statistical analyses were performed using an independent sample t test. A value of P < 0.05 was considered significant.

RESULTS
Generation and characterization of the WB-1 cell line. To establish an in vitro system to study the molecular mechanisms of the transdifferentiation of hepatic cells into insulin-producing cells (IPCs), rat WB cells were cotransfected with CMV-Pdx1-V16/neo and RIP-eGFP/neo plasmids. Five single cell–derived green fluorescence protein–positive clones (Fig. 1A, part a) were isolated and named WB-1 to -5. These clones were further expanded (Fig. 1A, b and c), and the cells were found to exhibit an intense cytoplasmic green fluorescence, indicating activation of the insulin promoter by Pdx1-V16 (Fig. 1A, d). To confirm the nuclear localization of the Pdx1 protein, WB-1 cells were subjected to immunocytochemistry with anti-Pdx1 antibodies. As expected, Pdx1 protein was mainly distributed in the nuclei, as shown by dark brown nuclear staining in WB-1 cells (Fig. 1B, left), and there was no staining in WB cells (Fig. 1B, right). Cells stained with control antibody showed no detectable staining (data not shown). Western blot analysis of whole-cell lysates from WB-1, and INS-1 cells demonstrated abundant expression of activated 46-kDa Pdx1 phosphorylated protein in WB-1 and INS-1 cells, but not in parental WB cells (Fig. 1C, WB-1). Similar expression of the Pdx1 protein was also observed in other WB-derived clones 2–5 (data not shown). Because all five Pdx1-V16–expressing WB clones expressed similar amounts of Pdx1-V16 protein and RIP-eGFP, the remaining studies were performed with the WB-1 clone.

To confirm that the WB-1 cells indeed expressed the Pdx1-V16 fusion protein, we performed a Western blot using anti-V16 antibody. A single band at ~58 kDa of the Pdx1-V16 fusion protein was detected in WB-1 cells, but not in parental WB cells (Fig. 1D, left). When a Pdx1 antibody was used to examine the Pdx1 expression, we were able to not only detect the Pdx1-V16 fusion protein, but also a phosphorylated/active (46-kDa) and an unphosphorylated/inactive (31-kDa) endogenous Pdx1 protein.
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that did not normally express in WB cells (Fig. 1D, right). These results indicate that Pdx1-VP16 activates the expression of the endogenous Pdx1 during the transdifferentiation to pancreatic endocrine cells. In conclusion, we have successfully established an in vitro system consisting of a single hepatic cell–derived clone expressing Pdx1-VP16 with a built-in reporter RIP-eGFP gene to reflect the activity of the insulin promoter.

To characterize the gene expression profile of the newly generated WB-1 cells, we examined the expression of various genes related to pancreatic development and β-cell function by RT-PCR and compared the results with parental WB cells, rat insulinoma cells (INS-1), and normal pancreas (Fig. 2). Expression of Pdx1-VP16 in WB cells resulted in the transcription of multiple genes related to endocrine pancreas development and β-cell function. These include HNF1, endogenous Pdx1, NeuroD/Beta2, Ngn3, NKx2.2, NKx6.1, Glut-2, GK, insulin I and II, and glucagon. This gene expression profile is similar to that seen in the INS-1 cells and rat pancreas. However, there was no detectable expression of the genes Pax4, Pax6, Isl-1, or MafA, which are involved in the late stages of differentiation of pancreatic endocrine cells (15). To determine whether the newly generated WB-1 cells are capable of glucose-responsive insulin release, they were challenged with 20 mmol/l glucose for 2 h, and insulin secretion was determined by ELISA. We found that although the WB-1 cells express multiple pancreatic endocrine genes, including insulin, they do not respond to glucose stimulation by releasing insulin (data not shown). Moreover, there was no detectable mature insulin by Western blot with an anti-insulin antibody (data not shown). Taken together, these results indicate that Pdx1-VP16–expressing WB-1 cells are precursors of pancreatic endocrine cells that do not exhibit mature β-cell function in the absence of further differentiation.

Reversal of diabetes in WB-1 cell–transplanted diabetic mice. To determine whether the WB-1 cells possess the ability to further differentiate into mature functional pancreatic endocrine β-like cells, they were transplanted into the left renal subcapsular space of STZ-induced diabetic NOD-scid mice. As demonstrated in Fig. 3, WB-1 cells are capable of reducing blood glucose levels from ~400 to ~200 mg/dl in the diabetic mice within 2–3 weeks after cell transplantation, and by day 60, blood glucose levels were normalized (70–100 mg/dl). In contrast, mice...
implanted with WB cells did not show any reduction in blood glucose levels and remained hyperglycemic during the entire observation period. As expected, mice receiving INS-1 cell transplantation showed a sharp reduction in blood glucose levels near 150–200 mg/dl for a long time, but eventually became hypoglycemic (~30–40 mg/dl). Furthermore, removal of implanted WB-1 cells by left nephrectomy induced a rebound persistent hyperglycemia, confirming that the implanted WB-1 cells are indeed responsible for the reduction of blood glucose levels. To evaluate the long-term effects of the implanted WB-1 and INS-1 cells, the remaining mice from the two groups were continuously observed for 4 months. WB-1–transplanted mice displayed a consistent euglycemia (70–100 mg/dl), whereas INS-1–transplanted mice had persistent hypoglycemia (30–40 mg/dl). All mice maintained normal body weight comparable to the aged-matched normal mice without diabetes (data not shown). These results demonstrate that although WB-1 cells do not appear to be mature endocrine cells in vitro, they are able to further differentiate and mature in vivo, as well as function like β-cells and rescue diabetic mice.

**Gene expression profiles of pre- and posttransplanted WB-1 cells.** To explore the molecular mechanism responsible for the functional shift of WB-1 cells from being glucose insensitive in vitro to being functional in vivo, we compared the gene expression profiles of posttransplanted WB-1 cells at 40 days and 4 months to that of the functional rat insulinoma INS-1 cells and to pretransplanted WB-1 cells as well as to their parental WB cells. We observed several noticeable changes in the expression of some genes related to β-cell development and function (Fig. 4A). First, after 40 days in vivo, the WB-1 cells now express the genes Pax4, Pax6, and Isl-1, which were not expressed before transplantation. Second, Ngn3, a key transcription factor that is transiently expressed in the pancreatic endocrine precursors but not in mature pancreatic endocrine cells (15–17), was now undetectable. Third, we found increased expression of NKx2.2, GK, and insulin 2 genes in the day 40 posttransplanted WB-1 cells. Fourth, MafA and PP genes became activated in WB-1 cells 4 months after transplantation. Last, we confirmed that the exogenous Pdx1-VP16 fusion gene was persistently expressed in the explanted WB-1 cells throughout the entire observation period. (see supplemental figure [available at http://diabetes.diabetesjournals.org]). The profile of gene expression at 4 months posttransplantation is similar to that of INS-1 cells. Taken together, the changes in gene expression profiles of WB-1 cells suggest a correlation of the sequence of gene activation, β-cell-like differentiation and maturation, and the ability of glucose-regulating function in the WB-1 cells.

To characterize the molecular components of the glucose sensing, insulin receptor–coupling machinery, and β-cell function, we investigated the expression profiles of genes known to be involved in β-cell function: SURI, Kir6.2, Snare 25, PC1/3, PC2, IAPP, and chromogranin A (Chrom A). SURI and Kir6.2 are ATP-sensitive K⁺ channel proteins, Snare 25 is involved in coupling and fusing vesicles to the cell membrane, IAPP is colocalized with insulin in secretory granules, and Chrom A, an abundant protein, is present in all islet cells. We also examined the gene expression of hexokinase (HK) to compare the levels to glucokinase (GK) during various stages of WB-1 cell maturation. Several interesting findings are demonstrated in Fig. 4B. 1) The gene expression of GLP-1R and PC2 is weak in WB-1 cells (pretransplantation) but becomes strong in mature (posttransplantation) WB-1 cells at 40 days and 40 months posttransplantation. 2) Several genes including SURI, Kir6.2, Snare 25, and IAPP are not expressed in immature WB-1 cells but become highly expressed in mature WB-1 cells, indicating that these proteins are related to mature β-cell functions. 3) HK gene expression appears to gradually decrease as the cells become mature. In contrast, GK gene expression increases as the WB-1 cells become mature during their in vivo differentiation. These results suggest that the estimated
GK/HK ratio increases as the WB-1 cells undergo maturation. Chrom A, a widely distributed protein in all islet cells, is expressed at all stages of WB-1 cell maturation, but is not detected in the parental liver epithelial WB cells. As expected, the rat insulinoma cell line INS-1 (823/13) expresses all genes with the exception of HK, whereas the parental WB cells express GK, HK, PC1/3, and PC2. These results demonstrate that upon maturation, the WB-1 cells indeed express many of the molecular components involved in regulated insulin secretion in mature β-cells.

**Histology and pancreatic hormone production in the explanted tissues.** To further characterize the identity of the transplanted WB-1 cells, we examined their histological appearance and insulin protein expression after in vivo differentiation at 40 and 120 days posttransplantation. The explanted WB-1 cells formed glandular or islet-like clusters with a rich network of microvasculature (Fig. 5A and B, H&E). The cytology of the posttransplanted WB-1 cells at 40 days revealed large nuclei and relatively scant cytoplasm, which is morphologically consistent with less mature cells (Fig. 5A). In contrast, at 120 days, the cells showed a decreased nucleus/cytoplasm ratio with “salt and pepper” chromatin and abundant cytoplasm, which is characteristic of mature pancreatic endocrine cells (Fig. 5B, H&E). Immunostaining for insulin protein showed that >95% of the implanted cells expressed insulin (Fig. 5A, e and 5B, d) with no detectable glucagon-positive cells present (data not shown). Neither amylase nor albumin was detectable in WB-1 and INS-1 cells in comparison to positive controls (Fig. 5C). These results demonstrate that in vivo, Pdx1-VP16–expressing WB-1 cells selectively differentiate into mature functional IPCs with no detectable pancreatic exocrine or liver proteins.

**Effects of high-glucose culture on WB-1 cell maturation.** Based on the results of cell transplantation, as well as our previous studies on hepatic oval cells (3) and bone marrow–derived stem cells (14), we hypothesize that high-glucose condition is a critical factor to promote further differentiation of precursor WB-1 cells. To determine whether WB-1 cells could be induced to mature in vitro into functional IPCs, we cultured them in high-glucose media for 4 weeks. Table 2 summarizes the insulin content and release in WB, WB-1, and INS-1 cells upon stimulation with 20 mmol/l glucose. We found a 1.8-fold increase in insulin release in WB-1 cells in response to glucose stimulation when compared with unstimulated WB-1 cells. A similar ratio is seen with INS-1 cells under our experimental conditions, demonstrating that high glucose can indeed promote the maturation of nonfunctional WB-1 cells into functional IPCs. These results support our hypothesis that Pdx1-VP16–expressing hepatic cells are endocrine precursor cells that selectively differentiate into mature functional IPCs only when placed in the proper microenvironment, such as in high-glucose culture or in a hyperglycemic diabetic mouse.

To examine whether the functional WB-1 cells can maintain their differentiated state when glucose levels are switched back to basic levels, we continuously followed the WB-1 cells in the basic medium (11.1 mmol/l glucose) and measured static insulin levels in the culture medium. We observed that the static insulin secretion in the medium was maintained at a high level (>1.7 ng/ml culture medium) for five passages, decreased to 0.8 ng/ml for the next two passages, and then remained at 0.3 ng/ml for six additional passages before it became undetectable. This phenomenon of changing sensitivity to glucose is commonly observed and has been well documented, even in true β-cell lines or genetically engineered β-cell lines (18,19) when they were cultured in vitro for a long time. These results indicate that functional WB-1 cells can indeed maintain their differentiated state during in vitro culture conditions.

**Selective transdifferentiation of WB-1 into IPCs without evidence of exocrine differentiation.** Previ-
ously, we showed that amylase protein is not detected in the explanted WB-1 cells. To confirm that transdifferentiation is selective toward an endocrine cell fate, we examined the expression of both early (p48) and late exocrine (amylase, elastase, and carboxypeptidase) genes in various stages of WB-1 cells by RT-PCR. In agreement with our hypothesis that WB-1 cells are pancreatic endocrine precursors, we found no detectable expression of exocrine markers (Fig. 6A). In contrast, we do find expression of hepatic mRNA, though we do not detect protein expression (Fig. 6B). These results indicate that expression of Pdx1-VP16 selectively transdifferentiates hepatic cells into pancreatic endocrine IPCs cells and the gene expression profile that we examined in WB-1 cells become identical to
that of mature β-cell line INS-1 cells after transplantation into diabetic mice.

**Analysis of insulin protein and insulin secretory granules.** To determine if the in vitro–differentiated WB-1 cells process proinsulin to insulin and if any insulin secretory granules present in these cells, we performed Western blotting to detect mature insulin and electron microscopy studies combined with immunogold labeling with anti-insulin antibody to detect insulin secretory granules. WB-1 cells were continuously cultured in high-glucose medium for further differentiation and maturation. The presence of mature insulin in high-glucose–cultured WB-1 cells was evaluated after the cells became glucose responsive to release insulin, as detected by ELISA. As indicated in Fig. 7A, WB-1 cells in high glucose culture (lane 2) produce mature insulin (arrow), compared with the positive control INS-1 (823/13) cells (lane 4). No insulin was detected in immature WB-1 (lane 3) or WB (lane 1) cells. At the ultrastructural level, these cultured mature WB-1 cells show scattered cytoplasmic globular structures containing insulin molecules, which were confirmed by immunogold-labeled anti-insulin antibody. As shown in Fig. 7B, insulin-containing electron dense granules were present in mature WB-1 cells (left) and were similar to that seen in β-cells (right). These results indicate that cultured mature WB-1 cells can indeed process insulin and form insulin secretory granules.

**DISCUSSION**

We have successfully generated stably transfected WB rat liver cell lines expressing an activated form of Pdx1 and containing the reporter construct RIP-eGFP. The parental WB cells are derived from a normal rat adult liver and are believed to represent rat liver epithelial stem-like cells, having the capacity to differentiate into both hepatocytes and bile ductal cells (10,11). The WB-1 cell line has a gene expression profile resembling endocrine pancreatic precursor cells and expresses several pancreatic transcription factors related to pancreatic endocrine development (HNF1, endogenous Pdx1, Ngn3, NeuroD/Beta2, NKx2.2, and NKx6.1) as well as numerous genes related to pancreatic endocrine function (insulin I and II, glucagon, Glut-2, GK, GLP-R, PC1/3, PC2, HK, and Chrom A. However, Pdx1-VP16 is unable to activate the late-stage pancreatic transcription factors Pax-4, Pax-6, Isl-1, and Mafa as well as β-cell function–related genes SUR1, Kir6.2, SNAP25, and IAPP. Moreover, although the WB-1 cells express insulin mRNA, they do not process and secrete insulin upon stimulation with glucose. It is possible that the lack of expression of Pax-4, Pax-6, Isl-1, SUR1, Kir6.2, SNAP25, and IAPP in early WB-1 cells may explain the results of the glucose insensitivity, since these genes are essential for late stages of β-cell differentiation and maintenance of mature β-cell function. In searching for external factors that can promote further differentiation of the WB-1 cells, we found that high-glucose environments (both in vitro and in vivo) are sufficient to induce the WB-1 cells to become fully functional IPCs.

**Cell transplantation into diabetic NOD-scid mice.** Although the WB-1 cells do not behave like mature β-cells, we found that they were able to differentiate further when transplanted into diabetic NOD-scid mice. Interestingly, the WB-1 cells were indeed capable of normalizing blood glucose levels and restoring weight loss in these mice, indicating that, in vivo, the implanted cells are fully functional. This pattern of reduction of blood glucose levels with WB-1 cells is similar to that seen with INS-1 cells, except that the INS-1 cells show a more rapid effect.

### Table 2

**Insulin content and insulin release after 2 h of glucose stimulation**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Insulin content (ng/ml)</th>
<th>20 mmol/l glucose (control)</th>
<th>20 mmol/l glucose (stimulated)</th>
<th>Fold of insulin release (stimulated/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
<td>—</td>
</tr>
<tr>
<td>WB</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
<td>—</td>
</tr>
<tr>
<td>WB-1</td>
<td>15.22 ± 1.549</td>
<td>10.38 ± 1.893</td>
<td>18.74 ± 0.51*</td>
<td>1.851 ± 0.373</td>
</tr>
<tr>
<td>INS-1</td>
<td>1225 ± 344.1</td>
<td>91.80 ± 6.184</td>
<td>165.7 ± 10.19*</td>
<td>1.666 ± 0.348</td>
</tr>
</tbody>
</table>

*P < 0.001. Note that 0.025 ng/ml is the lowest detectable level.

**FIG. 6.** Gene expression of pancreatic exocrine (A) and hepatic markers (B) by RT-PCR. Gene expression studies were performed on WB cells, WB-1 (newly generated), high-glucose–cultured WB-1 cells (WB-1 + HG), and 40-day explanted WB-1 cells (WB-1 Ex), as well as rat liver and pancreas as positive controls. Pancreatic exocrine genes include p48, amylase, carboxypeptidase, and elastase. The liver genes include three transcription factors (TGF-α, GATA-4, and HNF-4) and two genes of liver functional proteins (albumin [Alb] and transthyretin [TTR]).
on glucose reduction. Because the INS-1 cells are fully mature β-cells, the difference between the effects of WB-1 and INS-1 cells is most likely due to the time needed for the WB-1 cells to differentiate and mature. Furthermore, upon removal of the implanted WB-1 cells, a persistent hyperglycemia returned, confirming that the implanted WB-1 cells were indeed responsible for the reduction of blood glucose levels. In contrast to the results seen with WB-1 and INS-1 cells, WB cells had no effect on blood glucose levels. In contrast to the results seen with WB-1 cells, the difference between the effects of WB-1 and INS-1 cells after transplantation into diabetic mice. Other genes such as Nkx2.2, insulin II, GLP-1R, GK, and PC2 also showed increased expression in WB-1 cells after transplantation. In contrast, Ngn3 became undetectable after transplantation, consistent with the notion that Ngn3 is transiently expressed in early pancreatic precursor cells. Another interesting observation is GK and HK gene expression during the WB-1 cell in vivo maturation. In pancreatic β-cells, glucose is phosphorylated to glucose-6-phosphate by GK, whereas in most other cells (e.g., liver cells), the process is mediated by HK (19,20). The comparison of gene expression between GK and HK in various stages of the WB-1 cells suggests that as WB-1 cells become more differentiated, GK expression increases. Conversely, HK gene expression appears to decrease as the WB-1 cells differentiate and mature. The ratio of GK/HK increases at the transcriptional level, as the WB-1 cells become mature. These results indicate that the in vivo diabetic environment plays a key role in the differentiation and maturation of WB-1 cells into fully functional pancreatic endocrine cells.

**Selective pancreatic endocrine differentiation.** Several groups have overexpressed Pdx1 protein in the liver of STZ-induced diabetic mice but have not succeeded in producing selective pancreatic endocrine differentiation (4,6,7,21). Instead, severe hepatitis, presumably caused by the production of exocrine enzymes such as amylase, elastase, and chymotrypsin (7), and dysmorphogenesis, such as abnormal lobe structures and multiple cystic lesions (21), were recently reported. The conclusion drawn from these in vivo animal studies is that ectopic expression of Pdx1 alone in the liver initiates both endo-

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**FIG. 7.** Detection of insulin and insulin secretory granules. A: Mature insulin was detected in the differentiated WB-1 cells (lane 2) by Western blot with anti-insulin antibody (1:500; Santa Cruz). All lanes were loaded with 50 μg protein except for INS-1 (loading only one-tenth protein [5 μg] to the well). B: INS-1 secretory granules were detected by electron microscopy combined with immunogold labeling using anti-insulin antibody in an in vitro-differentiated WB-1 cell (B, left). Rat islet β-cells serve as the positive control (right). N represents the nucleus, and arrows indicate immunogold-labeled insulin particles.

**TRANSDIFFERENTIATION OF LIVER CELLS INTO IPCs**

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**Novel points from gene expression studies.** Little is known about the molecular mechanism of Pdx1-induced transdifferentiation from liver to pancreatic endocrine cells. By examining the expression of numerous pancreatic transcription factors, we found that there are some important differences in the sequence of gene activation between normal pancreas development and Pdx1-induced transdifferentiation of liver cells. For example, a recent review by Wilson et al. (15) describes that Nkx6.1 is positioned as an immediate downstream target gene of Pax4 during embryogenesis. We find, however, that Nkx6.1 can be activated in the absence of Pax4, suggesting that Nkx6.1 may act upstream of Pax4. Interestingly, Pax4, along with the other transcription factors Pax6, Isl-1, and MafA and β-cell function–related genes SUR1, Kir6.2, SNAP25, and IAPP, did become activated in WB-1 cells after transplantation into diabetic mice. Other genes such as Nkx2.2, insulin II, GLP-1R, GK, and PC2 also showed increased expression in WB-1 cells after transplantation. In contrast, Ngn3 became undetectable after transplantation, consistent with the notion that Ngn3 is transiently expressed in early pancreatic precursor cells. Another interesting observation is GK and HK gene expression during the WB-1 cell in vivo maturation. In pancreatic β-cells, glucose is phosphorylated to glucose-6-phosphate by GK, whereas in most other cells (e.g., liver cells), the process is mediated by HK (19,20). The comparison of gene expression between GK and HK in various stages of the WB-1 cells suggests that as WB-1 cells become more differentiated, GK expression increases. Conversely, HK gene expression appears to decrease as the WB-1 cells differentiate and mature. The ratio of GK/HK increases at the transcriptional level, as the WB-1 cells become mature. These results indicate that the in vivo diabetic environment plays a key role in the differentiation and maturation of WB-1 cells into fully functional pancreatic endocrine cells.

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crine and exocrine pancreas differentiation and is insufficient to induce selective endocrine pancreas differentiation. Recent work by Zalzman et al. (22) has shown that introduction of Pdx1 into human fetal liver progenitor cells by lentivirus resulted in the expression of Ngn3, induced exocrine pancreas differentiation in WB-1 cells, as with Pdx1 alone, we found that Pdx1-VP16 does not give a daughter cell, allowing complete differentiation to take place. Therefore, the liver gene expression we detected by RT-PCR in the explanted WB-1 cells is probably due to residual precursor WB-1 cells that still maintain low levels of liver cell activity.

Effects of high glucose or hyperglycemia on cell differentiation. We have previously demonstrated that culture of hepatic stem cells and bone marrow–derived stem cells in high-glucose medium is critical to their transdifferentiation into insulin-producing cells (3,14). Our current study with WB-1 cells showed no detectable insulin protein or glucose-responsive insulin release even though the insulin genes were activated. Our results demonstrated that in vivo hyperglycemia or in vitro high-glucose culture is critical for promoting WB-1 cells to undergo further differentiation into functional β-like IPCs. Although the molecular mechanism of high-glucose–induced differentiation of stem/precursor cells into insulin-producing cells is unclear, it is well known that glucose is a growth factor for β-cells (26). It promotes β-cell replication in vitro and in vivo at the 20- to 30-mmol/l concentration (27). Those observations have been supported by two recent studies. Zalzman et al. (22) demonstrated that high-glucose (25 mmol/l) culture of immortalized Pdx1-expressing human fetal hepatocytes promoted the production, storage, and release of insulin in a regulated manner. Recently, Kojima et al. (28) showed that hyperglycemia produced by intraperitoneal injection of glucose into nondiabetic mice led to the appearance of proinsulin and somatostatin co-positive cells in the liver within 3 days, and mature insulin-positive cells were observed by day 15.

The results of our study demonstrate that hyperglycemic conditions in vivo turn on Pax4, Pax6, Isl-1, and MafA (late-stage genes in the developing endocrine pancreas) as well as β-cell function–related genes SUR1, Kir6.2, SNAP25, and IAPP, shut off Ngn3 expression, and upregulate multiple genes (Nkx2.2, GK, insulin II, PC2, and GLP-1R) that are related to glucose regulation in WB-1 pancreatic precursor cells. Based on these observations, we conclude that high glucose is a key factor in promoting the differentiation of WB-1 cells into functional IPCs. A detailed study of the molecular mechanism of the effect of a high-glucose condition on cell differentiation requires further investigation.

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