Glial cell line-derived neurotrophic factor enhances neurogenin3 gene expression and β-cell proliferation in the developing mouse pancreas

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Mwangi SM, Usta Y, Raja SM, Anitha M, Chandrasekharan B, Parsadanian A, Sitaraman SV, Srinivasan S. Glial cell line-derived neurotrophic factor enhances neurogenin3 gene expression and β-cell proliferation in the developing mouse pancreas. Am J Physiol Gastrointest Liver Physiol 299: G283–G292, 2010. First published May 6, 2010; doi:10.1152/ajpgi.00096.2010.—Glial cell line-derived neurotrophic factor (GDNF) is a factor produced by glial cells that is required for the development of the enteric nervous system. In transgenic mice that overexpress GDNF in the pancreas, GDNF has been shown to enhance β-cell mass and improve glucose control, but the transcriptional and cellular processes involved are not known. In this study we examined the influence of GDNF on the expression of neurogenin3 (Ngn3) and other transcription factors implicated in early β-cell development, as well as on β-cell proliferation during embryonic and early postnatal mouse pancreas development. Embryonic day 15.5 (E15.5) mouse pancreatic tissue when exposed to GDNF for 24 h showed higher Ngn3, pancreatic and duodenal homeobox gene 1 (Pdx1), neuroD1/β2, paired homeobox gene 4 (Pax4), and insulin mRNA expression than tissue exposed to vehicle only. Transgenic expression of GDNF in mouse pancreata was associated with increased numbers of Ngn3-expressing pancreatic cells and higher β-cell mass at embryonic day 18 (E18), as well as higher β-cell proliferation and Pdx1 expression in β-cells at E18 and postnatal day 1. In the HIT-T15 β-cell line, GDNF enhanced the expression of Pax6. This response was, however, blocked in the presence of Pdx1 small interfering RNA (siRNA). Chromatin immunoprecipitation studies using the HIT-T15 β-cell line demonstrated that GDNF can influence Pdx1 gene expression by enhancing the binding of Sox9 and neuroD1/β2 to the Pdx1 promoter. Our data provide evidence of a mechanism by which GDNF influences β-cell development. GDNF could be a potential therapeutic target for the treatment and prevention of diabetes.

Pancreatic and duodenal homeobox gene 1 (Pdx1) is a transcription factor expressed in pancreatic progenitor cells as well as mature β-cells and is essential for the development of all pancreatic cell types and maintenance of β-cell function (16, 37). These progenitors proliferate to enhance the progenitor pool and are maintained in an undifferentiated state through the expression of the transcription factor Sry/HMG box gene 9 (Sox9) and activation of the notch signaling (3, 22, 30). All endocrine cell types derive from Pdx1-positive progenitors that express the basic helix-loop-helix (bHLH) transcription factor neurogenin3 (Ngn3) (15, 19, 28). Genetic gain-and-loss-of-function experiments have shown that the transcription factors NeuroD1/β2, the NK-homeodomain genes NKx2.2 and NKx6.1, and paired homeobox genes 4 (Pax4) and 6 (Pax6) that are required for β-cell development are downstream of Ngn3 (19, 27, 28, 31).

Although the pancreas develops in close association with the enteric nervous system, the role that neurotrophic factors play in pancreas development has not been well characterized. Glial cell line-derived neurotrophic factor (GDNF), which belongs to the TGF-β family of growth factors, plays a critical role in the development of the enteric nervous system (2, 33). Our previous studies have shown evidence of expression of GDNF in and around the islets in mice, whereas GDNF transgenic mice that are engineered to overexpress GDNF in glia under the control of the glial fibrillary acidic protein promoter have higher β-cell mass and improved glucose control than their CF1 wild-type (WT) littermates (24). The role that GDNF plays in early β-cell development and the mechanisms involved is, however, not known.

In this study we examined the influence of GDNF on early β-cell development. Our studies demonstrate that GDNF enhances Pdx1, Ngn3, neuroD1/β2, and Pax4 gene expression in embryonic mouse pancreata and β-cell proliferation in embryonic and postnatal mouse pancreata. We also show that GDNF can enhance the binding of the transcription factors NeuroD1/β2 and Sox9 to the Pdx1 promoter to stimulate the gene. Our data provide evidence of a mechanism by which GDNF can influence β-cell development.

MATERIALS AND METHODS

Experimental animals. Embryonic day 15.5 (E15.5) and 18 (E18) and postnatal day 1 and 7 mice were obtained from crossing CF1 WT mice with GDNF transgenic (GDNF-tg) mice that were on a CF1 background and were engineered to overexpress GDNF in glia under the control of the glial fibrillary acidic protein promoter (36). Previous studies in these mice showed increased GDNF expression in the brain and spinal cord and enhanced motor neuron survival during embryonic and postnatal development (36). Mid-day on which a vaginal plug was detected was considered as stage E0.5 for embryo collection.

THE VERTEBRATE PANCREAS ORIGINATES from the foregut as two protrusions of specialized endodermal epithelial cells and goes through three developmental transitions in which there are extensive cell proliferation and differentiation culminating in the formation of the exocrine pancreas (consisting of acinar tissue and ductal epithelium) and endocrine pancreas (consisting of glucagon-secreting α-cells, insulin-secreting β-cells, somatostatin-secreting δ-cells, and pancreatic polypeptide-secreting PP-cells) (20). These waves of cell proliferation and differentiation are driven by signals such as activin-β and fibroblast growth factors that derive from the mesoderm and notochord, and by the sequential expression of transcription factors (10, 15, 18, 27, 29).

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(26). The genotypes of the mice were determined by polymerase chain reaction (PCR) using DNA extracted from mouse tail by using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO) according to recommended procedure. Forward (5'-AGACGGCATCACCCTCGGCT-3') and reverse (5'-TGACGTCACTAAACTGCTTACGGG-3') primers designed to amplify the transgene sequence were used. All animal studies were approved by the Emory University Animal Care and Use Committee.

Cell and tissue culture and transfection. CF1 E15.5 embryos were obtained according to approved protocol. The embryos were removed and the entire gut including the stomach, pancreatic buds, and small and large bowel were isolated. The pancreatic buds containing β-cell clusters were identified after staining with the pancreatic cell DTZ detection assay kit (Chemicon International, Temecula, CA), teased out from the rest of the gut tissue, split into two groups of at least five pancreatic rudiments per group, and cultured for 24 h at 37°C in a CO2 incubator in Kight’s modification of Ham’s (F-12K) medium (ATCC, Manassas, VA) supplemented with or without 100 ng/ml recombinant rat GDNF obtained as previously described (9).

HIT-T15 hamster β-cells (ATCC) were cultured in F-12K medium supplemented with 10% horse serum (ATCC) and 2.5% fetal bovine serum (ATCC). For GDNF stimulation studies, the cells were serum deprived for 24 h followed by incubation with serum-free F-12K medium alone or serum-free F-12K medium supplemented with 100 ng/ml recombinant rat GDNF. HIT-T15 cells grown in complete medium were transfected with 30 nM Pdx1 small interfering RNA (siRNA) (CAG CUC CCU UUC CCG UGG AUG AAA U) or control siRNA by use of Lipofectamine RNAiMax (Invitrogen). The siRNA was designed based on published Mescocritus auratus PDX1 mRNA sequence (accession no. U73854.1). The cells were washed three times with F-12K medium without serum (incomplete F-12K medium) 24 h after transfection and cultured for an additional 48 h in incomplete F-12K medium supplemented with or without 100 ng/ml DNDF/ml.

PCR. GDNF gene expression in embryonic and postnatal mouse pancreas was confirmed by RT-PCR. Total RNA was isolated from frozen sections from mouse pancreata by use of the PicoPure RNA isolation kit (Arcturus, Mountainview, CA) and first-strand cDNA generated with the Sensiscript Reverse Transcription kit (Qiagen, Hilden, Germany). Analyses of transcription factor gene expression in cultured E15.5 mouse embryonic pancreatic tissue were performed by real-time PCR. Total RNA was isolated from cultured tissue by using the RNeasy Mini kit (Qiagen) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The integrity of isolated RNA was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) equipped with Agilent’s RNA 6000 Nano Chips. RNA integrity numbers (RINs) were generated by using the 2100 Bioanalyzer Expert software (Agilent Technologies), and samples with a RIN of 6.0 or higher were used for downstream assays whereas those with lower RINs were discarded. Real-time PCR reactions were set up by using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and thermal cycling was performed on an iCycler (Bio-Rad) equipped with an iCycler real-time PCR detection system. The sequences of the oligonucleotide primers used are presented in Table 1. All primers were designed such that at least one primer in each pair spanned an intron to prevent it from priming on genomic DNA. The inability of these primers to amplify genomic DNA was confirmed by PCR. GAPDH was used as an endogenous control for RT-PCR experiments to analyze the expression of GDNF in pancreatic tissue whereas β2-microglobulin was used as an endogenous control in real-time PCR analyses of gene expression in embryonic and postnatal mouse pancreas.

HIT-T15 cells grown to 80–90% confluency in incomplete F-12K medium in six-well plates were washed three times with serum-free F-12K medium and cultured for 24 h in serum-free F-12K medium. The medium was replaced with fresh serum-free F-12K medium or serum-free F-12K medium supplemented with 100 ng GDNF/ml, and the cells were grown for a further 24 h. The cells were fixed for 10 min with 1% paraformaldehyde, and chromatin immunoprecipitation (ChIP) assays were performed according to

**Table 1. Oligonucleotide primer sequences**

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<th>Oligo Name</th>
<th>Oligo Sequence</th>
<th>Temperature</th>
<th>Start Position</th>
<th>Location</th>
<th>Product Size, bp</th>
<th>Genomic Size, bp</th>
<th>Gene Accession No.</th>
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<td>220</td>
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<td>NM_011038.1</td>
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<tr>
<td>Insulin_FW</td>
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Fig. 1. Glial cell-derived neurotrophic factor (GDNF) enhances endocrine progenitor cell marker gene expression in mouse embryo pancreata. Real-time PCR analysis of neurogenin3 (Ngn3), NeuroD1, pancreatic and duodenal homeobox gene 1 (Pdx1), paired homeobox gene 4 (Pax4), and insulin gene expression in embryonic day 15.5 (E15.5) mouse embryo pancreata cultured for 24 h in vehicle or GDNF. Plotted are means ± SE. ***p < 0.001, N = 5 embryos per group.
recommended procedure with the EZ-ChIP Chromatin Immunoprecipitation kit (Millipore, Temecula, CA). Sonicated chromatin were incubated for 24 h at 4°C with 1:100 dilutions of rabbit antibodies to Pdx1 (Millipore), NeuroD1 (Abcam, Cambridge, MA), and Sox9 (Santa Cruz Biotechnology, Santa Cruz, CA). A rabbit antibody to Myc-Tag (Cell Signaling) was used as a negative control IgG. PCR to amplify immunoprecipitated DNA was performed using primer pairs 5′-TGGATTCAAGCGGAAATGCGT and 5′-CCCAGGTGTCTGTTAGCATTCT, and 5′-TCGCTCAACGTTGTCAACAA and 5′-TGCCAGTGACCCGTGTTTCT. The primers amplify portions of the mouse Pdx1 promoter that extent, respectively, from nt −2613 to −2084 and nt −1976 to −1279 upstream of the mouse Pdx1 gene transcription start site (accession no. NT_039324.7). These regions contain, respectively, potential neuroD1 and Sox9, and Pdx1 transcription factor binding sites as determined by use of the Transcription Element Search System software (TESS; Computational Biology and Informatics Laboratory, University of Pennsylvania); they lie within an area of the mouse Pdx1 promoter that has been shown to be required for β-cell-specific transcription of the Pdx1 gene (14).

Immunofluorescence microscopy. Pancreata isolated from mouse embryos and postnatal day 1 and 7 mice were either fixed in 10% formalin solution and embedded in paraffin by standard techniques or frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA). Paraffin sections (5 μm thick) were dewaxed according to suggested protocols (Cell Signaling Technologies, Danvers, MA) and antigen retrieval achieved by boiling for 10 min at 125°C in 10 mM sodium citrate (pH 6.0) in a decloaking chamber (Biocare Medical, Concord, CA). Frozen sections (7 μm thick) and HIT-T15 cells were fixed for 15 min in 4% paraformaldehyde (Electron Microscopy Sciences, Fig. 2. GDNF transgenic (GDNF-tg) mouse embryos have more Ngn3-expressing pancreatic cells than wild-type (WT) littermates. A: analysis of GDNF and GAPDH (loading control) gene expression in pancreata from embryonic day 15.5 (E15.5) and 18 (E18) and postnatal day 1 (P1) and 7 (P7) GDNF-tg mice and WT littermates. MW, 100-bp molecular weight marker. B: pancreas sections from E18 GDNF-tg mice and WT littermates immunofluorescently stained for Ngn3 (red) with insulin (green) and 4′,6-diamidino-2-phenylindole (DAPI; blue) nuclear staining. Arrows show representative Ngn3+ cells. Scale 20 μm. Histogram shows a plot of Ngn3+ cell frequency expressed as a percentage of all the cells/field. Plotted are means ± SE (**P < 0.01), N = 3 mice.
hatfield, PA) and permeabilized for 5 min with 1% Igepal CA-630 (Sigma) in phosphate-buffered saline (pH 7.5). Blocking was performed for 1 h at room temperature in 3% bovine serum albumin in phosphate-buffered saline containing 0.02% Triton X-100 (Sigma). Immunostaining was performed as previously described (24) with use of the following antibodies: mouse anti-Ngn3 (Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa department of Biology, Iowa City, IA), guinea pig anti-insulin (Zymed Laboratories, San Francisco, CA), mouse anti-Ki67 (Novoceastra, Newcastle, UK), and rabbit anti-hairy enhancer-of-split (Hes)-1 (Millipore) at 1:100 dilution, rabbit anti-Pdx1 (Upstate Cell Signaling Solutions, Lake Placid, NY) at 1:200 dilution, and Alexa Fluor 488 and 594 donkey anti-mouse, anti-guinea pig and anti-rabbit IgG (Molecular Probes, Eugene, OR) secondary antibodies at 1:500 dilution. Primary and secondary antibodies for single labeling and antibody cocktails for double-labeling were prepared at their appropriate dilution in blocking buffer. Incubations with primary antibodies were performed overnight at 4°C in a humidified chamber, whereas incubations with secondary antibodies were for 1 h at room temperature. Nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI) was achieved by adding 300 nM DAPI (Invitrogen) to the second phosphate-buffered saline wash buffer after secondary antibody incubation.

Scoring procedure. Frozen sections from pancreatic tissues from E18 GDNF-tg mouse embryos and WT littermates were double labeled with antibodies to Ngn3 and insulin and nuclei counterstained with DAPI. Images were taken from at least three randomly selected fields from each section with use of a x40 objective and the number of Ngn3-positive cells as well as the total number of cells in each field determined. The proportion of Ngn3-positive cells was then expressed as a percentage of the total number of cells in each field.

Ki67 and Pdx1 scores were obtained from pancreas sections double labeled with antibodies to Ki67 and insulin and insulin, respectively, and nuclei were counterstained with DAPI. Images of all the islets in four sections (separated by 200 μm) from each mouse were taken and the number of Ki67 and Pdx1-positive β-cells as well as the total numbers of β-cells in each islet were determined. The numbers of Ki67- and Pdx1-positive β-cells were then expressed as a percentage of all β-cells in an islet.

Relative β-cell area. Four 5-μm pancreas sections (separated by 200 μm) per mouse were used to assess β-cell mass as previously described (4). Briefly, after staining of β-cells for insulin, each section was analyzed on a Zeiss Axioskop 2 plus fluorescent microscope (Carl Zeiss Werk, Gottingen, Germany) mounted with an AxioCam MRc 5 camera and images of the entire section were taken with the aid of the Axiosvision (Rel 4.5) software (Carl Zeiss Imaging System). The total area covered by β-cells and the total area of pancreatic tissue in each

![Fig. 3. GDNF enhances β-cell proliferation in E18 and postnatal day 1 mouse pancreata. Representative images of pancreas sections from E18 (A) and postnatal day 1 (B) GDNF-tg mice and WT littermates stained for Ki67 (red) and insulin (green) with DAPI nuclear staining. Scale bar: 20 μm. C: scatter chart showing percentages of Ki67 + β-cells in E18 WT and GDNF-tg mouse pancreata. Each point represents an islet. Horizontal lines show group means. ***P < 0.01, N = 3 WT and 6 GDNF-tg embryos. D: scatter chart showing percentages of Ki67 + β-cells in postnatal day 1 WT and GDNF-tg mouse pancreata. Each point represents an islet. Horizontal lines show group means. ***P < 0.001, N = 3 WT and 5 GDNF-tg mice.]
GDNF influences early β-cell development

RESULTS

GDNF enhances expression of genes influencing β-cell development in mouse embryo pancreata. The developmental period from about E13.5 to E16.5 in the mouse is characterized by intense pancreatic endocrine cell differentiation (20) and major changes in the expression pattern of several endocrine progenitor cell markers (20, 32). To investigate whether GDNF could exert its influence during this growth phase, the expression of mRNA for markers of pancreatic endocrine progenitors could be investigated (20, 32). To investigate whether GDNF might influence the number of β-cell precursors in vivo, sections from pancreatic tissues from E18 GDNF-tg mouse embryos and WT littermates were labeled with antibodies to Ngn3 and insulin and nuclei counterstained with DAPI (Fig. 2B). The percentage of Ngn3-positive cells in each section was then determined. The number of Ngn3-positive (Ngn3+) cells in pancreata from GDNF-tg mice (9.3 ± 0.3, N = 4) was significantly (P < 0.01) higher than that in pancreata from WT littermates (4.4 ± 1.1, N = 3) (Fig. 2B).

GDNF enhances β-cell proliferation in E18 and postnatal day 1 mouse pancreata. To further investigate the mechanisms by which GDNF could influence β-cell development, pancreata from E18 and postnatal day 1 GDNF-tg mice and WT littermates were analyzed for β-cell proliferation by staining for the Ki67 cell proliferation marker and insulin with DAPI nuclear counterstain-
ing (Fig. 3, A and B). Higher percentages of β-cells in each islet that were positive for Ki67 were observed in pancreata from GDNF-tg mice than from WT littermates at both E18 (44.7 ± 2.4 vs. 34.3 ± 2.8%) and postnatal day 1 (17.8 ± 1.3 vs. 9.3 ± 0.1%) (Fig. 3D).

GDNF-tg mouse embryos have higher relative β-cell area than WT littermates. To assess the impact of the higher β-cell proliferation rates observed in GDNF-tg mice, pancreata isolated from E18 GDNF-tg mice and WT littermates were stained for insulin (Fig. 4A) and β-cell areas expressed as a percentage of total pancreatic tissue area determined. As seen in Fig. 4B, GDNF-tg mouse embryos had a significantly higher β-cell area (17.09 ± 1.76%) than WT littermates (11.91 ± 1.69%) (P < 0.05).

GDNF-tg mouse embryos have more Pdx1-positive β-cells. Until about E15.5 in mice, Pdx1 expression occurs throughout the developing pancreas but later shifts to β-cells, where it plays a key role in insulin synthesis. To understand how GDNF influences Pdx1 expression, pancreata from E18 and postnatal day 1 GDNF-tg mice and WT littermates were immunofluorescently labeled with antibodies to Pdx1 and insulin followed by DAPI nuclear staining to identify β-cells (Fig. 5, A and B, respectively). The percentage of Pdx1-positive β-cells within each islet was then determined on the basis of intensity of

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![Fig. 5](image-url)

**Fig. 5.** Pancreata from E18 and postnatal day 1 GDNF-tg mice have more Pdx1+ β-cells. A: pancreata from E18 GDNF-tg and WT mouse embryos immunofluorescently stained for Pdx1 (red) and insulin (green) with DAPI nuclear staining (blue). B: pancreata from postnatal day 1 GDNF-tg and WT littermates immunofluorescently stained for Pdx1 (red) and insulin (green) with DAPI nuclear staining (blue). Scale bar: 20 μm. C: score of Pdx1+ β-cells in islets from E18 GDNF-tg mouse embryos and WT littermates. Each point represents an islet. Horizontal lines show group means. ***P < 0.001, N = 3 WT and 6 GDNF-tg embryos. D: score of Pdx1+ β-cells in islets from postnatal day 1 GDNF-tg mice and WT littermates. Each point represents an islet. Horizontal lines show group means. ***P < 0.001, N = 3 WT and 5 GDNF-tg mice. E: pancreata from E18 GDNF-tg and WT mouse embryos immunofluorescently stained for Hes-1 (red) and insulin (green) with DAPI nuclear staining (blue). Scale bar: 20 μm.
nuclear staining. Islets from E18 and postnatal day 1 GDNF-tg mice had 12.8 ± 3.6 and 9.95 ± 2.4%, respectively, more Pdx1-positive β-cells than islets from WT littermates (Fig. 5, C and D; P < 0.001).

Hes-1 expression is similar in pancreata from E18 GDNF-tg mice and WT mice. The notch signaling pathway plays an important role in maintaining the pancreatic progenitor pool, and repression of notch signaling is required for the differentiation of pancreatic endocrine cells from their common Ngn3-expressing progenitor. We assessed the influence of GDNF on notch signaling activation in the pancreas by staining pancreata from E18 GDNF-tg mouse embryos and WT littermates for Hes-1, a downstream effector of notch signaling. Nearly all the cells in the islets, acinar tissue, and ductal tissue in GDNF-tg and WT embryo pancreata showed intense Hes-1 nuclear staining, and there was no difference in the staining pattern between the two groups of mice (Fig. 5E).

GDNF enhances the binding of Sox9 and neuroD1/β2 to the Pdx1 promoter. We next examined the effect of GDNF on the regulation of the Pdx1 gene. HIT-T15 hamster β-cells were cultured for 24 h in serum-free medium followed by a further 24 h in serum-free medium supplemented with or without 100 ng/ml GDNF. The binding of Sox9, neuroD1/β2, and Pdx1 to the Pdx1 promoter was then analyzed by the ChIP assay using antibodies to Sox9, neuroD1/β2, and Pdx1 and oligonucleotide primer pairs designed to amplify regions of the mouse Pdx1 gene promoter extending from nt −1886 to −1187 and nt −2521 to −1992 upstream of the published S1 transcription start site (14). These regions had been determined by using the Transcription Element Search System online software (TESS; Computational Biology and Informatics Laboratory, University of Pennsylvania) to contain potential Sox9 (nt −1722 to −1708), neuroD1/β2 (nt −1635 to −1627), and Pdx1 (nt −2046 to −1980) binding sites. PCR analyses of products amplified with the primer pair amplifying region −1886 to −1187 of the mouse Pdx1 promoter revealed 1.73 ± 0.01-fold (P < 0.001, N = 4) and 2.1 ± 0.02-fold (P < 0.001, N = 4) higher promoter DNA pull-down from lysates from cells exposed to GDNF by antibodies to neuroD1/β2 (Fig. 6Aa) and Sox9 (Fig. 6Bb), respectively, than from cells exposed to vehicle, whereas no DNA pull-down was observed with control IgG. In addition, DNA pull-down was detected using the primer pair amplifying region −2046 to −1980 of the Pdx1 promoter from samples precipitated from lysates from cells exposed to GDNF with anti-Pdx1 antibody whereas none was detected in samples from lysates from cells exposed to vehicle (Fig. 6C). These results suggest the ability of GDNF to promote the binding of transcription factors SOX9 and NeuroD1 to the Pdx1 promoter and result in enhanced Pdx1 transcription.

GDNF-stimulated Pax6 expression in β-cells is blocked by knockdown of Pdx1 gene expression. Pdx1 not only is essential for β-cell development but also plays a critical role in maintenance of differentiated β-cells by influencing insulin gene transcription. Pax6 is a key regulator in the terminal differentiation of the endocrine pancreas and β-cell function. To assess the role of Pdx1 in GDNF signaling in β-cells, Pdx1 gene expression in HIT-T15 hamster β-cells was knocked down by using Pdx1-specific siRNA and the cells were cultured for 48 h in medium supplemented with or without GDNF. The successful knockdown of Pdx1 was confirmed by Western blotting (Fig. 7A) and the expression of Pax6 was analyzed by immunofluorescence staining (Fig. 7B). GDNF increased the levels of PAX6 in the presence of control siRNA as well as in nontransfected cells (P < 0.001) but had no effects on cells transfected with Pdx1 siRNA (50.83 ± 3.35% Pax6-positive, nontransfected, GDNF supplemented vs. 3.0 ± 1.9% Pax6-positive, Pdx1 siRNA transfected, GDNF supplemented) (Fig. 7B).

DISCUSSION

Decreased β-cell mass underlies the development and progression of both Type 1 and Type 2 diabetes mellitus in humans. Therefore, understanding how β-cell mass is regulated is imperative for both prevention and treatment (6). In our previous studies we showed that GDNF transgenic mice that are engineered to overexpress GDNF in glia under the control of the glial fibrillary acidic protein promoter have higher GDNF expression in the pancreas and increased β-cell mass and better glucose control than WT littermates (24). In our present study we demonstrate a role for GDNF in early β-cell development. Our data show that GDNF stimulates increased expression of transcription factors including Ngn3, Pdx1, neuroD1/β2, and Pax4 that are involved in early β-cell development, and enhances the number of Ngn3-expressing β-cell precursors during the embryonic stages of pancreas development. Our data also show higher proliferative activity and increased transcription of the insulin gene in islets during embryonic development in mice overexpressing GDNF.

Differentiation of endocrine progenitors into β-cells involves expression of a coordinated set of genes. One of these genes is the bHLH protein Ngn3, which plays an important role in specification of endocrine lineage and is sufficient to trigger endocrine differentiation in pancreatic progenitors and ductal cells (13). Recently, the expression of Ngn3 in the pancreas...
during embryonic development was shown to exhibit a biphasic pattern with the second wave preceding the second developmental transition (32). Data presented in this study show an ability of GDNF to significantly enhance Ngn3 expression in embryonic mouse pancreata.

In addition to Ngn3, the transcription factor Pdx1 plays an important role in β-cell differentiation. Targeted disruption of Pdx1 has been shown to lead to the development of overt diabetes (1, 5), whereas Pdx1 overexpression is associated with increased β-cell proliferation (12). Similarly, the development of Type 2 diabetes was recently shown to be associated with epigenetic silencing of Pdx1 (17, 25). Data from our study show that GDNF enhances Pdx1 expression during early development, whereas knockdown of Pdx1 expression prevents

Fig. 7. GDNF-stimulated Pax6 expression in β-cells is reduced by knockdown of Pdx1 gene expression. A: Western blot analysis of Pdx1 knockdown with Pdx1 small interfering RNA (siRNA). B: immunofluorescence staining for Pax6 (red) with DAPI nuclear staining (blue) in HIT-T15 cells transfected with Pdx1 or control siRNA and cultured for 48 h in the presence or absence of GDNF. Scale bar: 20 μm. Plotted are means ± SE. ***P < 0.001, **P < 0.01, *P < 0.05. N = 4.
β-cell differentiation induced by GDNF. Furthermore, GDNF enhances the binding to the Pdx1 promoter of transcription factors involved in β-cell differentiation. Transcriptional regulation of Pdx1 is critical to β-cell development. Thus data from our studies showing enhanced binding of regulators of the Pdx1 promoter in the presence of GDNF suggest a mechanism by which GDNF influences β-cell development.

Several growth factors including insulin-like growth factors, glucagon-like peptide 1 (GLP-1) and, recently, keratinocyte growth factor have been shown to improve β-cell mass (11, 34). GLP-1, for example, stimulates embryonic stem cells to differentiate into insulin-producing cells, and this stimulation is associated with increased expression of Pdx1 and Ngn3 (35). Moreover, embryonic rudiments from spontaneously diabetic GK rats when treated with IGF-II show improvement in β-cell mass (7). In our studies, addition of GDNF to embryonic pancreatic rudiments accelerated β-cell differentiation in vitro, whereas overexpression of GDNF in embryonic mouse pancreata was associated with increased cell proliferation within the islets, a higher number of Pdx1-positive β-cells per islet, as well as higher β-cell mass. Identification of new factors that can promote the development of insulin-producing cells is critical for the prevention and treatment of diabetes. Studies are ongoing for new sources of diabetes cell therapy (17). Islet cell transplant has been limited owing to shortage of donors. Thus development of methods to enhance the production of β-cells is important in islet transplantation. A recent review highlighted the role of Pdx1 and Ngn3 as important transcriptional therapeutic targets for diabetes (21). In our studies GDNF could enhance the expression of these transcriptional targets. Small molecules that differentiate embryonic stem cells into functional insulin secreting β-cells such as indolactam V have been identified (8). Future studies will focus on the development of an agonist similar to GDNF that can be administered to improve β-cell mass. Understanding how growth factors regulate normal β-cell development will also provide us with new insight on the development of β-cells from embryonic stem cells.

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DISCLOSURES

The authors have declared that no conflicts of interest exist.

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