Glucose Is Necessary for Embryonic Pancreatic Endocrine Cell Differentiation*

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Mature pancreatic cells develop during embryonic life from endodermal progenitors, and this developmental process depends on activation of a hierarchy of transcription factors. While information is available on mesodermal signals controlling pancreas development, little is known about environmental factors, such as the levels of nutrients including glucose, that may control this process. Here, we studied the effects of glucose on pancreatic cell development. We used an in vitro model where both endocrine and acinar cells develop from early pancreatic and duodenal homeobox-1 (PDX1)-positive embryonic pancreatic progenitors. We first showed that glucose does not have a major effect on global pancreatic cell proliferation, survival, and acinar cell development. On the other hand, glucose controlled both alpha and beta cell development. Specifically, the surface occupied by insulin-positive cells was 20-fold higher in pancreases cultured in presence than in absence of glucose, and this effect was dose-dependent over the range 0.5–10 mM. Glucose did not appear to control beta cell development by activating the proliferation of early progenitors or beta cells themselves but instead tightly regulated cell differentiation. Thus, glucose did not modify the pattern of expression of Neurogenin3, the earliest marker of endocrine progenitor cells, but was necessary for the expression of the transcription factor NeuroD, a direct target of Neurogenin3 known to be important for proper pancreatic endocrine cell development. We conclude that glucose interferes with the pancreatic endocrine cells development by regulating the transition between Ngn3 and upstream NeuroD.

The mature mammalian pancreas contains two types of tissues: endocrine islet cells that produce hormones such as insulin (beta cells) and glucagon (alpha cells) and exocrine tissue whose acinar cells produce enzymes (e.g. amylase) that are secreted via the pancreatic ducts into the intestine. The pancreas originates from the dorsal and ventral regions of the foregut endoderm directly posterior to the stomach (1). Research conducted in the last few years has shed light on the processes controlling pancreatic endocrine-cell development. Studies of genetically engineered mice have identified a hierarchy of transcription factors regulating pancreas organogenesis and islet-cell differentiation (2, 3). The endodermal region committed to a pancreatic fate expresses the transcription factor Pancreatic and duodenal homeobox-1 (Pdx-1) (4, 5). The basic helix-loop-helix factor Neurogenin3 (Ngn3) is then expressed in epithelial pancreatic progenitor cells prior to endocrine differentiation (6). Ngn3 is necessary for pancreatic endocrine cell development, and Ngn3-deficient mice lack pancreatic endocrine cells (7). Lineage tracing experiments have also provided direct evidence that NGN3-expressing cells are islet progenitors (8). Thus, Ngn3 is a valuable marker for monitoring pancreatic endocrine-cell differentiation. NGN3 controls the expression of another member of the basic helix-loop-helix transcription factor family, NeuroD (9). In mice lacking NeuroD, islet development is strongly perturbed, demonstrating the requirement of this transcription factor for a normal endocrine pancreas development (10).

The process of pancreatic development is tightly controlled. Permissive signals derived from adjacent mesodermal structures such as the notochord and dorsal aorta control the first step of this process (11, 12). Signals from the mesenchyme, which condenses around the underlying committed endoderm, control the subsequent steps (13, 14). While different molecules produced by mesodermal structures and acting on pancreas such as Sonic Hedgehog (Shh), activins, sphingosine 1-phosphate, fibroblast growth factors have now been characterized (15–17), less information is available concerning the role of environmental factors during early steps of pancreas development.

Several recent reports have shown that the nutritional environment during prenatal life is crucial for proper development of pancreatic beta cells. These results have been obtained from a large panel of intrauterine growth retardation animal models and showed that undernutrition during prenatal life gives rise to a decrease in the number of beta cells that will form (18–21). However, these studies were performed in vivo, thus restricting both the accessibility to the molecular process giving rise to this pancreatic phenotype and the ability to study the precise effect of specific nutrient on pancreatic development.

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Recently, we developed an in vitro model of embryonic pancreas development. We showed that in E13.5 rat embryonic pancreas cultured on a filter at the air-liquid interface, endocrine and acinar cells develop in a way that perfectly replicates pancreas development occurring in vivo (22). Specifically, under such conditions, early PDX1-positive progenitor cells first proliferate and next differentiate into acinar or endocrine cells. This model was used in the present study to analyze the effect of nutritional factors, specifically the effect of glucose, on pancreatic cell development. We focused on the effects of glucose, as glucose is the preferred carbon source of most eukaryotic cells, and regulates the expression of a large number of genes in many cell types, especially in liver and pancreatic beta cells (23–25). In adult pancreatic beta cells, glucose is the major insulin secretagogue and has been shown to play an important role in beta cell proliferation (26).

Here we demonstrate that glucose is crucial for both alpha and beta cell development by regulating the transition between Ngn3 and NeuroD.

**EXPERIMENTAL PROCEDURES**

**Animals**—Pregnant Wistar rats were purchased from the Janvier breeding center (Centre d’élevage René Janvier, Le Genest, France). The first day post-coitum was taken as embryonic day 0.5 (E0.5). The animals had free access to food pellets and water. Pregnant female rats at 13.5 days of gestation were killed by CO₂ asphyxiation, according to the guidelines of the French Animal Care Committee.

**Dissection of Pancreatic Rudiment**—The embryos were harvested on E13.5. The dorsal pancreatic bud was dissected as described previously (27). Briefly, the stomach, the pancreas, and a small portion of the intestine were dissected together; then, the pancreatic primordium was dissected in Hank’s balanced salt solution (Invitrogen, Cergy-Pontoise, France).

**Organ Culture**—Dorsal pancreatic rudiments were laid on Millicell® culture plate inserts (Millipore, St-Quentin-en-Yvelines, France) at the air-liquid interface in sterile Petri dish of 35 mm diameter, containing 2 ml of RPMI 1640 without glucose (Cambrex, Emerainville, France) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), Hepes (10 mmol/liter), non-essential amino acids (1X, Invitrogen), 10% heat-inactivated calf serum (HyClone, Logan, UT). D-Glucose, L-glucose, and xylose (Sigma-Aldrich, Lyon, France) were used at the indicated concentrations. Cultures were maintained at 37 °C in humidified 95% air, 5% CO₂. Medium was changed every other day. At the end of the culture period, the pancreatic rudiments were photographed and fixed as described below or harvested for RNA extraction.

**Immunohistochemistry and Quantification**—Tissues were fixed in 10% formalin, pre-embedded in agarose gel (4% of type VII low gelling temperature agarose Sigma-Aldrich) and embedded in paraffin. Sections (4 µm thick) were collected and processed for immunohistochemistry, as described previously (27, 28). Antibodies were used at the following dilutions: mouse anti-human insulin (Sigma-Aldrich, 1/2000), rabbit anti-amyglase (Sigma-Aldrich, 1/300), guinea pig anti-insulin (Dako, Trappes, France, 1/500), mouse anti-BrdUrd⁵ (Amersham Biosciences, Buckinghamshire, UK; 1/2), rabbit anti-PDX1 (Ref. 28; 1/1000). The fluorescent secondary antibodies were: fluorescein isothiocyanate anti-rabbit antibody (Jackson Immunoresearch, Baltimore, MD; 1/200), Texas Red anti-mouse antibody (Jackson ImmunoResearch; 1/200) and Alexa Fluor anti-rabbit antibody (Biogenex, San Ramon, CA; 1/400). Nuclei were stained in blue using Hoechst 33342 (0.3 µg/ml, Invitrogen). Photographs were taken using a fluorescence microscope (Leica, Leitz DMRB, Reuil-Malmaison, France) and digitized using a Hamamatsu (Middlesex, NJ) C5810 cooled 3CCD camera.

For NGN3 detection, tissue sections were heat-treated, permeabilized in Tris-buffered saline-1% Triton for 10 min at room temperature and incubated with anti-NGN3 antibody (1/1000) overnight at 4 °C. The rabbit anti-NGN3 antibody was raised against the synthetic peptide YCLLPTGTLVFSDFL conjugated to keyhole limpet hemocyanin. Revelation was performed using secondary biotinylated antibody (diluted 1/200 in BioGenex buffer (Biogenex)) and the Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s recommendations. The NGN3 antibody was produced in collaboration with Dr. Eric Quemeneur, CEA, Bagnoles sur Céze, France and validated in our laboratory (data not shown).

**TUNEL Experiments**—TUNEL experiment was performed using in situ cell death detection kit (Roche, Neuilly-sur-Seine, France) following manufacturer’s instructions. This experiment was followed by insulin immunostaining. To determine the percentage of apoptotic beta cells, we counted the frequency of TUNEL-positive cells among 500 insulin-positive cells.

**In Situ Hybridization**—Tissues were fixed at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS), cryoprotected in 15% sucrose, PBS at 4 °C overnight, embedded in 15% sucrose-7.5% gelatin in PBS, and frozen at −50 °C in isopentane. Cryosections 14 mm in thickness were prepared. The Ngn3 probe (726 bp) was prepared as previously described (29). Plasmids were linearized and used as templates for synthesizing sense or antisense riboprobe using T7 or SP6 RNA polymerase (Roche Diagnostics, Meylan, France), in the presence of digoxigenin-UTP (Roche Diagnostics). In situ hybridization was done as described previously (30), and colorimetric revelation was performed with 5-bromo-4-chloro-3-indolyl phosphate (Promega, Charbonnière, France) and nitro blue tetrazolium (Roche Diagnostics) to obtain a blue precipitate. Photographs were digitized using a Hamamatsu (Middlesex, NJ) C5810 cooled 3CCD camera. No signal was obtained when a sense riboprobe was used.

**Quantification**—To quantify the surface area of insulin-, glucagon-, amylase-expressing cells, all sections of each pancreatic rudiment were digitized. One out of two consecutive sections was analyzed by immunohistochemistry for a given antigen to avoid counting of the same cell twice. The surfaces of insulin, amylase, glucagon, and Hoechst stainings were quantified using

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⁵ The abbreviations used are: BrdUrd, bromodeoxyuridine; TUNEL, dUTP nick end-labeling assay; PBS, phosphate-buffered saline.
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ImageJ 1.34s, and the stained areas were summed and presented in μm².

To measure proliferation of either beta cell or early progenitors expressing PDX1, we counted the frequency of BrdUrd-positive nuclei among 1000 insulin-positive cells or 1000 early progenitors expressing PDX1 per rudiment. To quantify the absolute number of NGN3-expressing cells, pancreatic rudiments were sectioned and all sections were stained with an anti-NGN3 antibody. Positive cells were counted on all sections of each pancreatic rudiment. A minimum of three rudiments was analyzed per condition.

Real-time PCR—To determine the transcript level of insulin2, insulin1, Ngn3, and NeuroD in pancreatic rudiments before (E13.5) and after 1, 3, 5, and 7 days of culture, in absence or in presence of glucose 10 mM, real-time PCR was performed with the 7300 Fast real-time PCR system (Applied Biosystem, Paris, France). Total RNA was isolated using the Qiagen RNeasy Microkit (Qiagen, Courtaboeuf, France) and treated with DNase I to eliminate genomic contamination. Two-hundred and fifty nanograms of each RNA sample were reverse transcribed as described above. The synthesized cDNA was diluted to 1/20 and 5 μl were used per PCR reaction. Each reaction consisted of a mix of Taqman® universal PCR master mix (Applied Biosystem) with primers and Taqman-labeled probe specific for each gene (Applied Biosystem) and was run following universal thermal cycling protocol (95 °C, 10 min followed by 40 cycles of 95 °C, 15 s and 65 °C, 1 min). Peptidylprolyl isomerase A was used as endogenous control. E16.5 pancreatic cDNA was used as calibrator sample. The data were analyzed by comparative cycle threshold method and presented in a Dose-dependent Manner.

Determination of ATP Levels—Tissue was extracted in ice 200 μl of ice-cold perchloric acid (20% v/v), rapidly frozen and stored at −80 °C. Determination of ATP was based on procedures published in (32, 33). Briefly samples were thawed and adjusted to pH 7.4 with a known volume of neutralization mixture (0.5 M triethanolamine, 2 M KOH, 100 mM EDTA). Precipitated potassium perchlorate was removed by centrifugation (10,000 × g, 2 min) and the supernatant stored on ice prior to assay. Neutralized sample (10 μl) was added to 1 ml of assay buffer (130 mM NaHAsO₄, 17 mM MgSO₄, 4 mM NaHPO₄, pH 7.4), and the reaction was initiated with 10 μg of firefly luciferase (10 mg/ml stock, Sigma). Light emission was recorded for 30 s using a photon counting luminometer. [ATP] was obtained by comparison to standards prepared in parallel with the tissue extracts.

Statistics—Results are expressed as means ± S.E. Statistical significance was determined using Student’s t test when only two sets of data were compared. For larger analysis, an one-way analysis of variance test followed by a Tukey’s test was used.

RESULTS
Pancreatic Endocrine Cell Development Is Glucose-dependent while Acinar Cell Development Is Glucose-independent—To study the role of glucose in pancreatic development, pancreases at E13.5 were dissected. At this stage, the pancreas is composed of an epithelium surrounded by mesenchymal tissue. The pancreatic epithelium mainly contains early PDX1-positive progenitor cells. We cultured pancreatic rudiments at the air/liquid interface on filters floating on culture medium. Under such conditions, endocrine and acinar cells develop in a way that replicates pancreas development occurring in vivo (22, 34). To test the effects of glucose on pancreas development, pancreases were first cultured with or without 10 mM of glucose in the presence of 10% fetal calf serum, plus a mixture of essential and non-essential amino acids added to maintain basal intracellular metabolism and ATP content (see below and Ref. 35). Under both conditions, the epithelium grew rapidly, spread into the mesenchyme, and developed lobules, and after 7 days of culture, pancreatic growth was similar in the presence or absence of glucose (Fig. 1, A, upper panel, and B, top left panel for quantification). Immunohistochemistry was next performed in order to analyze endocrine (insulin- and glucagon-expressing cells) and acinar (amylase-positive cells) differentiation. Nuclei, stained using Hoechst dye (Fig. 1A), showed no difference in apoptosis in pancreases cultured in the presence or absence of glucose. Moreover, we specifically analyzed apoptosis of insulin positive cells using TUNEL method. The percentage of insulin-positive cells that stained positive for TUNEL was not statistically different in both conditions (1.76% ± 0.22 in absence of glucose versus 1.62% ± 0.19 in presence of glucose). Quantification of the surface occupied by amylase-positive cells indicated that acinar cell development was identical in pancreases cultured with or without glucose (Fig. 1, A and B, top right panel for quantification). On the other hand, the surface area occupied by insulin-expressing cells that developed was glucose-dependent. Specifically, the area occupied by the insulin staining was 20-fold higher in pancreases cultured in the presence of 10 mM of glucose, when compared with pancreases cultured without glucose (Fig. 1, A and B, bottom left panel, for quantification). A similar positive effect of glucose was observed on surface area of glucagon-positive cells (Fig. 1B, bottom right panel).

To rule out the possibility that the lack of endocrine differentiation in absence of glucose was due to an osmotic change, we compared the effect of D-glucose, L-glucose, a non-transported sugar and xylose, a sugar transported by GLUT3, a glucose transporter not expressed in the embryonic pancreas (36). After 7 days of culture in the presence of such sugars, insulin expression was analyzed by real-time PCR and compared with pancreases developed in the absence of glucose. As shown in Fig. 2A, insulin gene expression was strongly activated only in pancreases cultured in the presence of D-glucose. Such an effect of glucose on beta cell differentiation could be mimicked neither by L-glucose nor by xylose. We next asked whether the lack of endocrine cell differentiation in the absence of glucose could be explained by a decrease in ATP content. As shown in Fig. 2B, glucose did not significantly modify ATP contents in pancreatic explants.

The above first set of results thus indicated that glucose selectively controls pancreatic endocrine cell development.

Glucose Specifically Activates Pancreatic Endocrine Development in a Dose-dependent Manner—Embryonic pancreases were cultured for 7 days across a range of glucose concentrations (0–10 mM) and insulin expression was analyzed by real-
time PCR. At the beginning of the culture (E13.5), Insulin gene expression was below the limit of detection in agreement with previous data (37). Insulin gene expression was weakly turned on with 0.75 mM glucose. With higher glucose concentrations, the levels of Insulin mRNA were significantly increased in a dose-dependent manner. A near-maximum Insulin expression level was reached at 5 mM glucose, with no further increase when embryonic pancreases were cultured with 10 mM glucose (Fig. 3). The levels of Glucagon mRNA also increased in a dose-dependent manner in response to glucose (data not shown). At the same time, there was no effect of glucose on acinar cell development (Fig. 1 and data not shown), further indicating that glucose had a selective effect on pancreatic endocrine cell development.

**Glucose Does Not Control Pancreatic Progenitor Cell Proliferation**—Lineage tracing studies have demonstrated that pancreatic endocrine and exocrine cells derive from a pool of early PDX1-positive progenitor cells present in the pancreatic epithelium (8, 38). Such progenitor cells have a high proliferative potential before they differentiate into mature cells and
perturbation of their proliferation has a strong impact on the final number of beta cells that develops (17). Here, we first asked whether glucose controls beta cell development by acting on pancreatic progenitor cell proliferation. Embryonic pancreases were cultured for 1 day in the presence or absence of glucose and pulsed with BrdUrd during the last hour of culture. The percentage of early PDX-1-positive progenitor cells incorporating BrdUrd was quantified by immunohistochemistry. In pancreases cultured in the absence of glucose, the proliferative rate of PDX-1+ cells was high, as about 25% of the cells incorporated BrdUrd, further demonstrating the high proliferative potential of such cells. Such proliferation was not modified in pancreases cultured in the absence of glucose (Fig. 4). This set of data indicates that the effect of glucose on beta cell development cannot be explained by an action on precursor cells proliferation.

Glucose Controls Specific Steps of Pancreatic Progenitor Cell Differentiation—Our data described above indicate that beta cell development was strongly dependent on the presence of glucose, a process that could not be explained by an effect on progenitor cell proliferation. We therefore tested whether glucose controls beta cell differentiation. We first analyzed the effect of glucose on the expression of Neurogenin3 (Ngn3), the first transcription factor involved in the engagement in the pancreatic endocrine fate (7). Real-time PCR was performed on pancreases cultured for 0, 1, 3, 5, or 7 days to quantify Ngn3 expression in the presence or absence of 10 mM glucose. Ngn3 mRNA level was low at E13.5. After 1 day of culture in the presence or absence of glucose, Ngn3 expression increased dramatically, reaching a peak at day 3, and decreased thereafter (Fig. 5A). Thus glucose does not control Ngn3 expression.

We next analyzed the expression of NeuroD, a direct target of Ngn3 known to be important for beta cell differentiation (9, 10). In the absence of glucose, while Ngn3 mRNA expression was strongly induced, NeuroD expression was poorly activated in correlation with a poor induction of Insulin mRNA (Fig. 5, B and C). On the other hand, in the presence of glucose, both Ngn3, NeuroD, and Insulin mRNA were induced (Fig. 5).

As NeuroD is a direct target of Ngn3, a simple explanation could be that Ngn3 mRNA is not translated or rapidly degraded in the absence of glucose. To test this hypothesis, Ngn3 expression was analyzed by immunohistochemistry, and the number of Ngn3-expressing cells was compared in pancreases grown for 5 days in the presence or absence of glucose. As shown in Fig. 6, the number of Ngn3-positive cells that developed was identical in pancreases grown in the presence or absence of glucose, indicating that the lack of NeuroD expression in the absence of glucose is not the consequence of a weak expression of Ngn3. Taken together, such results indicate that glucose controls beta cell differentiation by regulating the expression of NeuroD.

The Default of Endocrine Differentiation Observed in the Absence of Glucose Is Reversible—PDX1 is expressed both in pancreatic progenitor cells and in mature beta cells where it activates Insulin gene transcription (5, 39). After 7 days of culture with glucose, a large number of beta cells developed and

**FIGURE 3. Glucose activates beta cell development in a dose-dependent manner.** Quantification by real-time PCR of insulin2 mRNA expression in E13.5 pancreases before (Day 0) and after 7 days of culture with increasing concentrations of glucose (0, 0.75, 1.25, 2.5, 5, or 10 mM). Values are means ± S.E. of three independent experiments. Identical results were obtained when insulin1 mRNA were quantified (data not shown).

**FIGURE 4. Glucose does not control pancreatic progenitor cell proliferation.** A, pancreatic rudiments were grown for 24 h with (panels a and a’) or without (panels b and b’) glucose and pulsed with BrdUrd during the last hour of culture to ensure labeling of cells in S phase. The tissues were analyzed by immunohistochemistry using anti-PDX1 (in green) and anti-BrdUrd (in red) antibodies. Proliferative PDX1 cell are seen in yellow. On a and b, the epithelium and the entire pancreas are both circled. Panels a’ and b’ are a larger view of panels a and b. Scale bar: 100 μm for panels a and b and 25 μm for a’ and b’. B, quantification of the number of PDX1-positive cells in S phase after 24 h of culture in presence (gray bar) or in absence (white bar) of glucose, indicating that glucose did not activate the proliferation of early progenitors. Values are means ± S.E. of three independent experiments. NS, no significant difference.
nearly all PDX1-positive cells stained positive for insulin (Fig. 7A, middle panel). On the other hand, in cultures performed without glucose, while rare insulin-positive cells developed, numerous cells stained positive for PDX1 after 7 days of culture and stained negative either for insulin and glucagon, suggesting that pancreatic progenitors remained present in cultures performed in the absence of glucose (Fig. 7A, left panel). We thus asked whether after a 7-day culture period in the absence of glucose beta cell differentiation could occur upon addition of glucose. When pancreases were cultured 7 days in absence and then 7 days in presence of glucose, the surface area occupied by insulin-expressing cells was equivalent to what was obtained in pancreases cultured 7 days with glucose (Fig. 7B for quantification). Such an increase in beta cell development was not observed when pancreases were cultured 14 days in the absence of glucose (Fig. 7B). These results strongly suggest that undifferentiated cells are still present after culture without glucose and can differentiate into beta cells upon glucose addition.

Addition of Glucose Does Not Re-induce Ngn3 Expression but Stimulates NeuroD Expression—The next step was to define how re-addition of glucose induces beta cell development. A first hypothesis was that newly formed insulin cells were derived from the proliferation of the rare insulin-positive cells present after 7 days of culture without glucose. To test this first hypothesis, beta cell proliferation was compared in pancreases cultured either 14 days in the presence of glucose or 7 days in absence of glucose followed by 7 days in presence of glucose. At days 8, 10, 12, and 14, pancreases were pulsed with BrdUrd for 1 h, and beta cell proliferation was analyzed at each time point. As shown in Fig. 8A, addition of glucose did not modify the number of proliferative beta cells at any time point, suggesting that the newly formed beta cells were not derived from the proliferation of pre-existing beta cells. We thus asked whether Ngn3 expression was induced upon the addition of glucose. To answer this question, in situ hybridization was performed at different time points of culture performed with or without glucose. While Ngn3 expression could be detected in pancreases cultured for 3 days in the presence or absence of glucose (Fig. 8B), Ngn3 expression was not reinduced upon the addition of glucose (Fig. 8B). Such results were further confirmed by real-
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FIGURE 7. The default of endocrine differentiation can be reversed by the addition of glucose. A, E13.5 rat pancreases were cultured 7 days in absence or in presence of glucose or 7 days without glucose followed by 7 days with glucose. PDX1 was revealed in green and insulin in red. Scale bar: 100 μm. B, quantification of the absolute surface areas occupied by insulin-positive cells in pancreases cultured 7 or 14 days in absence of glucose (white bar), 7 days in presence of glucose 10 mM (gray bar), or 7 days in absence followed by 7 days in presence of glucose (black bar). Values are means ± S.E. of three independent experiments. **, p < 0.001.

time PCR, performed 1, 3, 5, 7 days after the addition of glucose (data not shown).

The expression of NeuroD was next analyzed by real time PCR at days 0, 7, 10, and 14 of culture (Fig. 8C). In the absence of glucose, NeuroD expression was never induced. Interestingly, when pancreases were cultured 7 days in absence and then 3 or 7 days in presence of glucose, the expression of NeuroD was re-induced, to the same extent to what was obtained when pancreases were cultured in the presence of glucose. This result strongly suggests that addition of glucose stimulates beta cell development by re-inducing the process of development downstream of Ngn3 and upstream of NeuroD.

DISCUSSION

Here we demonstrate that glucose plays a major and specific role in pancreatic endocrine cell development. Using an in vitro model where both acinar and endocrine cells develop in culture from rat embryonic pancreas, we showed that glucose does not have major effect on pancreatic acinar cell development but is crucial for both alpha and beta cells development. Moreover, we showed that glucose controls endocrine cells development not by controlling cell proliferation but by regulating a specific step of pancreatic endocrine cell differentiation.

Our results indicate that glucose selectively controls pancreatic endocrine cell development. For example, the surface area of insulin-positive cells that developed was 20-fold higher in the presence than in the absence of glucose. While glucose is the preferred carbon source in mammals, an unlikely explanation of the poor endocrine differentiation observed in the absence of glucose for at least 4 reasons: first, the culture medium was supplemented with 10% fetal calf serum and a mix of amino acids, several of which are metabolized to generate ATP via the Krebs cycle (35); second, no difference in apoptotic nuclei could be observed in pancreases cultured in absence versus in presence of glucose; third, acinar cells developed properly whatever the extracellular glucose concentration was and, fourth, glucose did not modify the extractable ATP content of the pancreatic explants. On the other hand, we do not exclude the possibility that subtle or compartmentalized changes in intracellular free ATP concentration (40, 41), or in the levels of ADP or AMP may occur in response to glucose, and might regulate the activity of downstream targets including ATP-sensitive K⁺ channels (42) or AMP-activated protein kinase (43–45). These changes may in turn regulate cellular excitability and might affect gene expression through alterations in free Ca²⁺ concentrations (46) or via the release of insulin and an action via beta cell insulin receptors (47–49). On the other hand, a signaling role in the control of transcription for metabolites of glucose such as glucose 6-phosphate or xylulose 5-phosphate (50) may equally be involved (51), and future studies will be necessary to address this question. Interestingly, the dose dependence of the effects of glucose on insulin gene expression (Fig. 3) indicated that these were unlikely to be mediated largely or purely by “high affinity” glucose sensors, including hexokinases I-III, but are more compatible with the involvement of the “low affinity” glucose transporter GLUT2 (52) or the glucose phosphorylating activity, glucokinase (53). Importantly, the present results are consistent with the apparent impact in vivo of fetal glucokinase mutations on beta cell development and/or insulin secretion as indicated by the decreased birth weight of affected individuals (54).

The role of glucose on mature beta cell proliferation has been demonstrated both using pancreatic beta cell lines and primary islets (55, 56), and it was shown that glucose metabolism and activation of the phosphatidylinositol 3-kinase pathway were necessary for glucose stimulated beta cell proliferation (57). The importance of glucose in promoting pancreatic beta cell survival has also been dissected in detail and it has been shown that glucose activates a PI 3-kinase survival-signaling pathway in pancreatic beta cells (58, 59). Less information is available concerning the importance of glucose in beta cell differentiation. It has, however, been proposed that beta cell neogenesis plays an important role in beta cell regeneration that occurs in glucose infused rats (60, 61). However, how glucose controls beta cells neogenesis under such conditions remained unsolved. Here, we used an in vitro model of culture of rat embryonic pancreas to dissect the role of glucose on beta cell development by analyzing the pattern of changes in the expression of transcription factors, which are known to be pivotal to this process.

It is well established that during embryonic life, the final number of beta cells that will develop depends on the proliferation of early PDX1-positive pancreatic progenitor cells and their potential to differentiate into beta cells. For example, fibroblast growth factor 10 is necessary for the proliferation of...
Addition of glucose neither activates beta cells proliferation, nor re-induces Ngn3 expression, but stimulates NeuroD expression.

A, effect of glucose on beta cell proliferation. Gray bars, pancreatic rudiments were grown for different periods in presence of glucose 10 mM. Black bars, the rudiments were grown for 7 days without glucose followed by a culture with glucose. At days 8, 10, 12, and 14, pancreases were pulsed with BrdUrd during the last hour of culture. The tissues were analyzed by immunohistochemistry using anti-insulin and anti-BrdUrd antibodies. The percentage of insulin-positive cells in S phase was quantified. Values are means ± S.E. of three independent experiments.

B, effect of an addition of glucose on Ngn3 expression. Ngn3 expression was analyzed by in situ hybridization in E13.5 rat pancreases cultured in absence (G0) or in presence (G10) of glucose for 3 days (left panels) and 7 days (middle panels). Ngn3 expression was also analyzed in pancreases cultured 10 days in the presence of glucose or 7 days without followed by 3 days in presence of glucose (right panels). Note that Ngn3 is only expressed at day 3. Scale bar: 100 μm.

C, effect of an addition of glucose on NeuroD expression. Quantification by real-time PCR of NeuroD mRNA expression in E13.5 pancreases before (Day 0), after 7, 10, and 14 days of culture in absence of glucose (white bars), after 7 days in presence of glucose 10 mM (gray bar) or 7 days without followed by 3 or 7 days with glucose (black bars). Values are means ± S.E. of three independent experiments. ***, p < 0.001; NS: No significant difference.
early PDX1-positive pancreatic progenitor cells (17, 62) and mice deficient for this growth factor develop a highly reduced beta cell mass (17). We thus tested whether the poor endocrine development observed in the absence of glucose could be due to a lack of proliferation of early PDX1-positive pancreatic progenitor cells under such conditions. The fact that the proliferation of early PDX1-positive pancreatic progenitor cells could be glucose-dependent would not have been unexpected, due to the fact that such progenitor cells express during embryonic life the facilitative glucose transporter, GLUT2 (63). However, this was not the case, and glucose did not modify the rate of proliferation of early progenitors that remain high even in the absence of glucose.

Because recent studies demonstrated that beta cell replication represents a major mechanism for maintaining beta cell mass (64, 65), and because, in different models, glucose has been shown to activate beta cell proliferation (26, 55, 56), we asked whether the observed effect of glucose on beta cell development could be explained by a direct effect of glucose on beta cell proliferation. Again, under our conditions, we could not detect major effects of glucose on the proliferation of beta cells themselves. Taken together, such results indicate that during the window of development we analyzed, glucose is not necessary for pancreatic cell proliferation and suggests that it could be specifically involved in the control on endocrine cell differentiation.

Interestingly, we found that glucose was not necessary for the first step of pancreatic endocrine cell development, visualized here by the induction of expression of Ngn3, the earliest known marker of pancreatic cells that enter the endocrine pathway (7, 8). However, while, in the presence of glucose, such Ngn3-positive cells developed into beta cells, this was not the case in the absence of glucose. Such results indicate that beta cell generation from Ngn3-expressing cells is a tightly controlled event, and glucose is important for this process. Such a point fits well with recent data where it was shown that knock-down of VEGF signaling in the embryonic pancreas does not seem to perturb the development of Ngn3-positive endocrine progenitors but completely represses their differentiation into beta cells.6 It is also supported by recent data indicating that while the expression of Ngn3 can be turned on in the embryonic pancreas using γ-secretase inhibitors, insulin-positive cells did not develop under such conditions (30). Thus, Ngn3 induction is necessary but not sufficient for beta cells differentiation and the cellular context and additional signals seem necessary to allow Ngn3-positive cells to develop into beta cells.

Here, we found that while Ngn3 expression was activated in the absence of glucose, the expression of NeuroD was not induced. Moreover, the phenotype of pancreases cultured in absence of glucose was similar to the one found in mice deficient for NeuroD (normal acinar development, altered endocrine development) (10). NeuroD is a basic helix-loop-helix transcription factor that has been proposed as a direct target of Ngn3 (9). NeuroD is important for pancreatic endocrine cell development and mice deficient for this transcription factor presented at birth a decrease number of alpha and beta cells (10). Until now, it has been generally accepted that expression of NGN3 in pancreatic precursor cells was sufficient for NeuroD induction and endocrine cell differentiation. Our data do not support this linear idea and indicate that endocrine cell development is more complex than expected. Additional factors are needed for pancreatic development, like glucose, shown in the present study to control NeuroD induction.

Recently, it was shown that first, FoxO1, a forkhead transcription factor of the FoxO family was a strong activator of NeuroD expression in pancreatic beta cells (67), second, that glucose regulates FoxO1 subcellular distribution in pancreatic beta cells (68) and third, that FoxO1 can bind to the preproglucagon gene in alpha cells and act then on their function (69). Thus, a future working hypothesis would be that glucose regulates pancreatic endocrine cell differentiation by modulating the expression of FoxO1.

Taken together, our data indicate that glucose controls in a dose-dependent manner pancreatic endocrine cell development, without a major effect on acinar cell development. This effect of glucose on endocrine cell development is not due to activation of cell proliferation but to a specific requirement of glucose to activate NeuroD, a direct target of NGN3. Such a specific role of glucose may therefore be important to increase beta cell development from embryonic stem cells.

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REFERENCES

1. Collombat, P., Hecksher-Sorensen, J., Serup, P., and Mansouri, A. (2006) Mech. Dev. 123, 501–512
2. Edlund, H. (1998) Diabetes 47, 1817–1823
3. Wilson, M. E., Scheel, D., and German, M. S. (2003) Mech. Dev. 120, 65–80
4. Ahlgren, U., Jonsson, J., and Edlund, H. (1996) Development (Camb.) 122, 1409–1416
5. Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L., and Wright, C. V. (1996) Development (Camb.) 122, 983–995
6. Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U., and Edlund, H. (1999) Nature 400, 877–881
7. Gadwohl, G., Dierich, A., LeMeur, M., and Guillemot, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1607–1611
8. Gu, G., Dubauskaite, J., and Melton, D. A. (2002) Development (Camb.) 129, 2447–2457
9. Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M., and Tsai, M. J. (2000) Mol. Cell. Biol. 20, 3292–3307
10. Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B., and Tsai, M. J. (1997) Genes Dev. 11, 2323–2334
11. Kim, S. K., Hrebok, M., and Melton, D. A. (1997) Development (Camb.) 124, 4243–4252
12. Lammert, E., Cleaver, O., and Melton, D. (2001) Science 294, 564–567
13. Pictet, R. L., Clark, W. R., Williams, R. H., and Rutter, W. J. (1972) Dev. Biol. 29, 436–467
14. Scharfmann, R. (2000) Diabetologia 43, 1083–1092
15. Hrebok, M. (2003) Mech. Dev. 120, 45–57
16. Edsbagge, J., Johansson, J. K., Esni, F., Luo, Y., Radice, G. L., and Semb, H. (2005) Development (Camb.) 132, 1085–1092
17. Bhushan, A., Itoh, N., Kato, S., Thiery, J. P., Czernichow, P., Belluscio, S.,

6 J. Magenheim, E. Keshet, and Y. Dor, poster presented at the Programming Pancreatic Beta Cells Workshop, October 2006, El Perello, Spain.
and Scharffmann, R. (2001) Development (Camb.) 128, 5109–5117
18. Garofano, A., Czernichow, P., and Brent, B. (1998) Diabetologia 41, 1114–1120
19. Garofano, A., Czernichow, P., and Brent, B. (1997) Diabetologia 40, 1231–1234
20. Limesand, S. W., Jensen, J., Hutton, J. C., and Hay, W. W., Jr. (2005) Am. J. Physiol. 288, R1297–R1305
21. Petrlik, J., Reusens, B., Arany, E., Remacle, C., Coelho, C., Hoet, J. J., and Hill, D. I. (1999) Endocrinology 140, 4861–4873
22. Attali, M., Stetsyuk, V., Basmaciogullari, A., Aiello, V., Zanta-Boussif, A. M., Duvillie, B., and Scharffmann, R. (2007) Diabetes, in press
23. Girard, J., Ferre, P., and Foufelle, F. (1997) Annu. Rev. Nutr. 17, 325–352
24. Towle, H. C., Kaytor, E. N., and Shih, H. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8602–8605
25. Vaulont, S., Vasseur-Cognet, M., and Kahn, A. (2000) J. Biol. Chem. 275, 31555–31558
26. Bonner-Weir, S., Deery, D., Leahy, J. L., and Weir, G. C. (1989) Diabetes 38, 49–53
27. Miraftab, F., Czernichow, P., and Scharffmann, R. (1998) Development (Camb.) 125, 1017–1024
28. Duvillie, B., Attali, M., Aiello, V., Quemeneur, E., and Scharffmann, R. (2003) Diabetes 52, 2035–2042
29. Ravassard, P., Vallin, J., Mallet, J., and Icard-Liepkalns, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8602–8605
30. Duvillie, B., Attali, M., Bouuacer, A., Ravassard, P., Basmaciogullari, A., and Scharffmann, R. (2006) Diabetes 55, 582–589
31. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
32. Owen, M. R., and Halestrap, A. P. (1993) Biochim. Biophys. Acta 1142, 11–22
33. Stanley, P. E., and Williams, S. G. (1969) Anal. Biochem. 28, 381–392
34. van Eyll, J. M., Pierreux, C. E., Lemaigre, F. P., and Rousseau, G. G. (2004) J. Cell Sci. 117, 2077–2086
35. Leclerc, I., and Rutter, G. A. (2004) Diabetes 53, Suppl. 3, S67–S74
36. Gould, G. W., Thomas, H. M., Jess, T. J., and Bell, G. I. (1991) J. Biol. Chem. 266, 10213–10216
37. Jensen, J., Heller, R. S., Funder-Nielsen, T., Pedersen, E. E., Lindsell, C., and Rutter, G. A. (2000) Diabetes 49, 13281–13291
38. Herrera, P. L. (2000) J. Clin. Invest. 105, 17771–17779
39. Hoorens, A., Van de Casteele, M., Kloppel, G., and Pipeleers, D. (1996) J. Clin. Invest. 98, 1568–1574
40. Srinivasan, S., Bernal-Mizrachi, E., Obsugi, M., and Permutt, M. A. (2002) Am. J. Physiol. 283, E784–E793
41. Bernard, C., Thibault, C., Berthault, M. F., Magnan, C., Saulnier, C., Portha, B., Pralong, W. F., Penicaud, L., and Ktorza, A. (1998) Diabetes 47, 1058–1065
42. Bernhard, C., Berthault, M. F., Saulnier, C., and Ktorza, A. (1999) FASEB J. 13, 1195–1205
43. Hart, A., Papadopoulou, S., and Edlund, H. (2003) Dev. Dyn. 228, 185–193
44. Pank, K., Mukonoweshuro, C., and Wong, G. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9559–9563
45. Dor, Y., Brown, J., Martinez, O. I., and Melton, D. A. (2004) Nature 429, 41–46
46. Georgia, S., and Bhushan, A. (2004) J. Clin. Invest. 114, 963–968
47. Leclerc, I., and Rutter, G. A. (2000) Cell Metab. 2, 153–163
48. Martinez, S. C., Cras-Meneur, C., Bernal-Mizrachi, E., and Permutt, M. A. (2006) Diabetes 55, 1581–1591
49. McKinnon, C. M., Ravier, M. A., and Rutter, G. A. (2006) J. Biol. Chem. 281, 39358–39369