Oxygen Tension Regulates Pancreatic β-Cell Differentiation Through Hypoxia-Inducible Factor 1α

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OBJECTIVE—Recent evidence indicates that low oxygen tension (pO_2) or hypoxia controls the differentiation of several cell types during development. Variations of pO_2 are mediated through the hypoxia-inducible factor (HIF), a crucial mediator of the adaptative response of cells to hypoxia. The aim of this study was to investigate the role of pO_2 in β-cell differentiation.

RESEARCH DESIGN AND METHODS—We analyzed the capacity of β-cell differentiation in the rat embryonic pancreas using two in vitro assays. Pancreata were cultured either in collagen or on a filter at the air/liquid interface with various pO_2. An inhibitor of the prolyl hydroxylases, dimethyloxaloylglycine (DMOG), was used to stabilize HIF1α protein in normoxia.

RESULTS—When cultured in collagen, embryonic pancreatic cells were hypoxic and expressed HIF1α and rare β-cells differentiated. In pancreata cultured on filter (normoxia), HIF1α expression decreased and numerous β-cells developed. During pancreas development, HIF1α levels were elevated at early stages and decreased with time. To determine the effect of pO_2 on β-cell differentiation, pancreata were cultured in collagen at increasing concentrations of O_2. Such conditions repressed HIF1α expression, fostered development of *Ngn3*-positive endocrine progenitors, and induced β-cell differentiation by O_2 in a dose-dependent manner. By contrast, forced expression of HIF1α in normoxia using DMOG repressed *Ngn3* expression and blocked β-cell development. Finally, hypoxia requires hairy and enhancer of split (HES)1 expression to repress β-cell differentiation.

CONCLUSIONS—These data demonstrate that β-cell differentiation is controlled by pO_2 through HIF1α. Modifying pO_2 should now be tested in protocols aiming to differentiate β-cells from embryonic stem cells. ***Diabetes*** **59:662–669, 2010**

At low oxygen tension (pO_2), cells undergo adaptative changes, including increased angiogenesis and erythropoiesis, and a switch to glycolytic metabolism (1). The cellular response to hypoxia is tightly controlled by the hypoxia-inducible factor (HIF) complex, an (α/β) heterodimer (2). HIF is regulated in an O_2-dependent manner by hydroxylation of one of the three HIFα subunits (HIF1α, HIF2α, and HIF3α) (3,4). HIF1α was the original HIF isoform identified by affinity purification using oligonucleotides from the erythropoitin (EPO) locus, whereas HIF2α and HIF3α were identified by homology searches. These α-subunits are expressed differentely and independently in different time frames (5–7). In normoxia, hydroxylation of the proline HIF1α residues by prolyl hydroxylases leads to recognition and polyubiquitinylation by the Von Hippel-Lindau (VHL) E3 ligase complex, leading to proteasomal degradation of HIF1α (8,9). On the other hand, in the hypoxic state, prolyl hydroxylase activity is decreased and HIF1α is stabilized. During embryogenesis, cell oxygenation levels vary widely. In general, pO_2 is considered to occur at 2–9% O_2 decreasing to even lower levels in tissues such as kidney medulla, bone marrow, or thymus (10). Recent investigations of the role of hypoxia and HIF1α during development of several organs showed that hypoxia decreases the differentiation of neural precursor cells, myogenic cells, adipocytes, and endothelial cells in an HIF1α-dependent manner (11–13).

The pancreas originates from the dorsal and ventral regions of the foregut endoderm directly behind the stomach. Signals derived from adjacent mesodermal structures, notochord, dorsal aorta, and cardiac mesoderm induce initiation of pancreas development (14–17). Next, mesenchyme condenses around the underlying committed endoderm; mesenchyme is also involved in the control of pancreas development (18). Embryonic pancreatic epithelium contains progenitor cells that express the transcription factor pancreatic and duodenal homeobox factor one (PDX1), whose deletion leads to pancreatic agenesis (19). Other transcription factors are also important for pancreatic development (20). Interestingly, the transcription factor *Ngn3* is transitorily expressed in pancreatic endocrine progenitor cells during development, and its deletion leads to the absence of the four endocrine cell types (α, β, δ, and PP) that produce glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively (21).

Advances in the understanding of signals that control pancreatic development have been made using in vivo and in vitro reconstituted models (15,22–24). In the present study, we found that when rat embryonic pancreata were cultured in collagen gels, cells were hypoxic, HIF1α was stabilized, *Ngn3* expression was not induced, and consequently, β-cell differentiation was arrested. However, increasing pO_2 led to HIF1α degradation, upregulation of *Ngn3* expression, and β-cell differentiation. Similarly, when pancreata were cultured on filter at the air-medium interface, where tissues are not subject to hypoxia, HIF1α was not expressed, *Ngn3* was induced, and β-cells developed. Under such conditions, reduction of pO_2 stabilized HIF1α levels and decreased both *Ngn3* expression and β-cell development. Moreover, HIF1α stabilization using prolyl-hydroxylase inhibitors repressed β-cell develop-

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ment. We also found that expression of *Hes1*, a transcription factor involved in pancreas development (25), was induced both by hypoxia and by forced expression of HIF1α in normoxia. Moreover, inhibition of the *Hes1* expression by a γ-secretase inhibitor in hypoxic conditions restored normal development of *Ngn3*-expressing progenitor cells. Finally, we found that HIF1α is temporally regulated during pancreas development in vivo. Based on these findings, we hypothesized that differences in pO_2 were responsible for such changes in β-cell development. Thus, we propose that pO_2 is a regulator of pancreatic endocrine cell development, acting through HIF1α.

RESEARCH DESIGN AND METHODS

Pregnant Wistar rats were purchased from the Janvier breeding center (CERJ). The animals had free access to food pellets and water. The first day postcoitus was taken as embryonic day 0.5 (E0.5). At 13.5 days' gestation, pregnant female rats were killed by asphyxiation with carbon dioxide or by a lethal injection of ketamin/xylazin according to the guidelines of the French Animal Care Committee.

Hypoxia in vivo. Pregant Wistar rats at 13.5 days postcoitus were subjected to hypoxia in the anaerobic workstation at 25°C and in a humidified atmosphere (95%) supplied with the gas mixture allowing it to adjust the hypoxic environment by gradually decreasing the O_2 level from 21 to 8 and 92% N_2. The rats were provided with food and water ad libitum. The animal study protocols were conducted according to institutional guidelines for animal use.

Dissection of pancreatic rudiments and organ culture. Embryos were harvested at E13.5. The dorsal pancreatic bud was dissected as described previously (23). For cultures at the air-medium interface, the dorsal pancreatic rudiments were laid on Millicell culture plate inserts (Millipore) (24). DMOG (Interchim) was used at concentrations of 1 and 2 mmol/l, and the γ-secretase inhibitor XXI (Calbiochem) was used at a concentration of 250 nmol/l. L-mimosine (Sigma) was used at a concentration of 400 μmol/l. For collagen cultures, pancreatic explants were embedded in 500-μl collagen gels as previously described (23). All cultures were maintained at 37°C in humidified 5% CO_2 in a hypoxia chamber at various levels of pO_2.

Detection of hypoxia. To detect hypoxic areas, the dorsal pancreatic rudiments were cultured for either 24 h or 7 days in collagen and at the air-medium interface. Pimonidazole (200 μmol/l; Hypoxyprobe, NPI) was added to culture medium during the last 2 h of the culture period. Pancreatic explants were analyzed with an anti-Mab1 fluorescent antibody (NPI) according to the manufacturer's instructions.

Immunohistochemistry. Tissues were fixed in 10% formalin and processed for immunohistochemistry as previously described (23,26). The following antibodies were used: mouse anti-insulin (1:2,000; Sigma-Aldrich), rabbit anti-PDX1 (1:1,000) (26), rabbit anti-carboxypeptidase A (1:600; Biogenesis), mouse anti–E-cadherin (1:100; BD Biosciences), rabbit anti-HES1 (1:1,000 [a gift from Dr. Tetsuo Sudo, Pharmaceutical Research Laboratories, Toray Industries, Tebiro, Kamakura, Japan]), mouse anti-BrdU (1:2; Amersham), rabbit anti-NGN3 (1:1,000) (27), and rabbit anti-HIF1α (9). The fluorescent secondary antibodies were fluorescein-isothiocyanate anti-rabbit and Texas Red anti-mouse antibodies (1:200; Jackson Immunoresearch) and Alexa-fluor anti-rabbit antibody (1:400; Biogenex). For NGN3, revelation was performed using the vectastain ABC kit (Vector). For HIF1α, revelation was performed with an anti-rabbit Dakocytomation Envision+ system HRP-DAB (Dako). Photographs were taken using a fluorescence microscope (Leica, Leitz DMRB) and digitized using a Hamamatsu C5810 cooled 3CCD camera. No signals were observed when the primary antibodies were omitted.

Transferase-mediated dUTP nick-end labeling experiments. The transferase-mediated dUTP nick-end labeling (TUNEL) procedure was performed using an in situ cell death detection kit (Roche) according to the manufacturer's instructions. Subsequently, E-cadherin (1:100; BD Biosciences) immunostaining was used to visualize the epithelium.

Quantification. To quantify the absolute numbers of insulin-expressing cells, all sections of each pancreatic rudiment were digitized. On every image, the surface area of insulin staining was quantified using IPLab Eval (version 3.2.4; Scanalytics), and the stained areas were tallied as previously described (24). Three to five rudiments were analyzed per condition.

To measure proliferation of early progenitors expressing PDX1, we counted the frequency of $BrdU^+$ nuclei among 1,000 $PDX1^+$ cells. The percentage of undifferentiated PDX1-expressing cells was then calculated. Three rudiments were analyzed per condition. Statistical significance was determined using Student's *t* test.

In situ hybridization. Tissues were fixed at 4°C in 4% paraformaldehyde, cryoprotected in 15% sucrose at 4°C overnight, embedded in 15% sucrose and 7.5% gelatin, and frozen in isopentane. Cryosections (14 μm thick) were prepared. An *Ngn3* probe (726 bp) was used and in situ hybridization performed as previously described (24). No signal was obtained using a sense riboprobe.

Real-time PCR. Total RNA was purified using the Rneasy microkit (Qiagen). The cDNA was generated using Superscript reagents (Invitrogen), and the real-time PCR was performed on a 7300 real-time PCR system (Applied Biosystem) with an SYBR Green PCR master mix. The oligonucleotide sequences for RT-PCR are available on request.

Western blotting. For Western blot analysis, cells were lysed in Laemmli buffer. Proteins (20 μg) were resolved by SDS/PAGE and electrophoretically transferred onto a polyvinylidine fluoride membrane (Biorad). After blocking with milk, membranes were probed with rabbit anti-HIF1α or anti-HIF2α antibodies (Novus) and mouse anti-actin (Sigma-Aldrich). Immunoreactive bands were visualized with the SuperSignal system (Pierce).

RESULTS

Pancreatic β-cell differentiation depends on cell oxygenation. We cultured E13.5 rat pancreata in collagen. While acinar cells expressing carboxypeptidase-A developed, NGN3 expression was poorly induced and β-cells did not develop. However, when we cultured them on a filter at the air-medium interface, NGN3 expression was induced and insulin-positive cells developed (Fig. 1*A*). When pimonidazole staining was applied, we found that collagen-cultured tissues were hypoxic from day 1 to day 7, which was not the case for pancreata cultured at the air-medium interface (Fig. 1*B*).

To investigate the role of O_2 in β-cell differentiation, we cultured pancreatic explants in collagen with increased pO_2. When pancreata were cultured at 21% O_2, very few β-cells were detected on day 7 (Fig. 2*A*). At 60 and 80% O_2, the surface of insulin staining increased 25.1- and 81.2-fold, respectively, compared with cultures at 21% O_2 (Fig. 2*A*). Next, we examined development of different types of endocrine cells (α, β, and δ cells) using quantitative real-time PCR (qPCR). When pancreatic buds were cultured at 80% O_2, glucagon-, insulin-, and somatostatin-mRNA steady-state levels were increased 8.3-, 175.1-, and 8.9-fold, respectively, compared with levels induced at 21% O_2 (supplementary Fig. S1, which can be found in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0891/DC1). Thus, pancreatic endocrine cell development is controlled by pO_2. Interestingly, oxygen tension had no effect on acinar cell development (Fig. 2*A*).

To determine whether the increased β-cell development was due to higher cell survival or increased differentiation, we compared the rates of cell apoptosis at day 1 in pancreata cultured at 21 and 80% O_2. Results of TUNEL staining detected very few apoptotic cells in the epithelia of pancreata treated in either culture (Fig. 2*B*). Some apoptotic cells were found in the mesenchyme; however, the numbers of apoptotic cells were similar for tissues in both cultures. Next, we used qPCR to compare *Ngn3* expression in pancreata cultured at 21 and 80% O_2 for 1, 3, and 5 days. At all time points, increased pO_2 induced *Ngn3* expression (Fig. 2*C*). Immunohistochemistry analysis confirmed these results (supplementary Fig. S2). Altogether, these data indicate that increased oxygen tension activates β-cell differentiation as opposed to enhancing cell survival.

HIF1α signaling pathway is induced by hypoxia in the pancreas. In various cell types, hypoxia stabilizes HIF1α and activates the HIF transcription complex. In our study, we used Western blot analysis to determine whether HIF1α was stabilized in pancreata cultured in collagen.

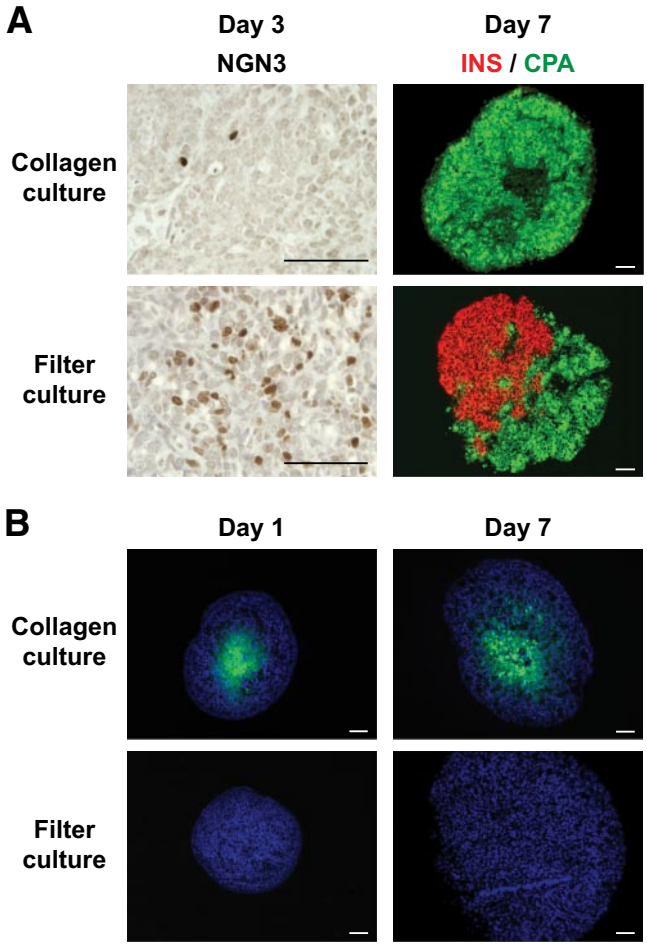

FIG. 1. Differential development of β-cells between pancreata cultured in collagen and at the air-medium interface. *A*: E13.5 pancreata were cultured in collagen or on a filter at the air-medium interface for 3 or 7 days. At day 3, NGN3 was detected by immunohistochemistry (in brown). At day 7, anti-insulin (INS) (in red) and anti-carboxypeptidase A (CPA) (in green) antibodies were used to detect β- and acinar cell development, respectively. *B*: Hypoxia was analyzed in pancreata cultured in collagen and at the air-medium interface. After 1 or 7 days, pimonidazole was added to the medium for the last 2 h of the culture period and hypoxia was analyzed using anti-Mab1 antibodies (in green). Bar: 50 μm. (A high-quality digital representation of this figure is available in the online issue.)

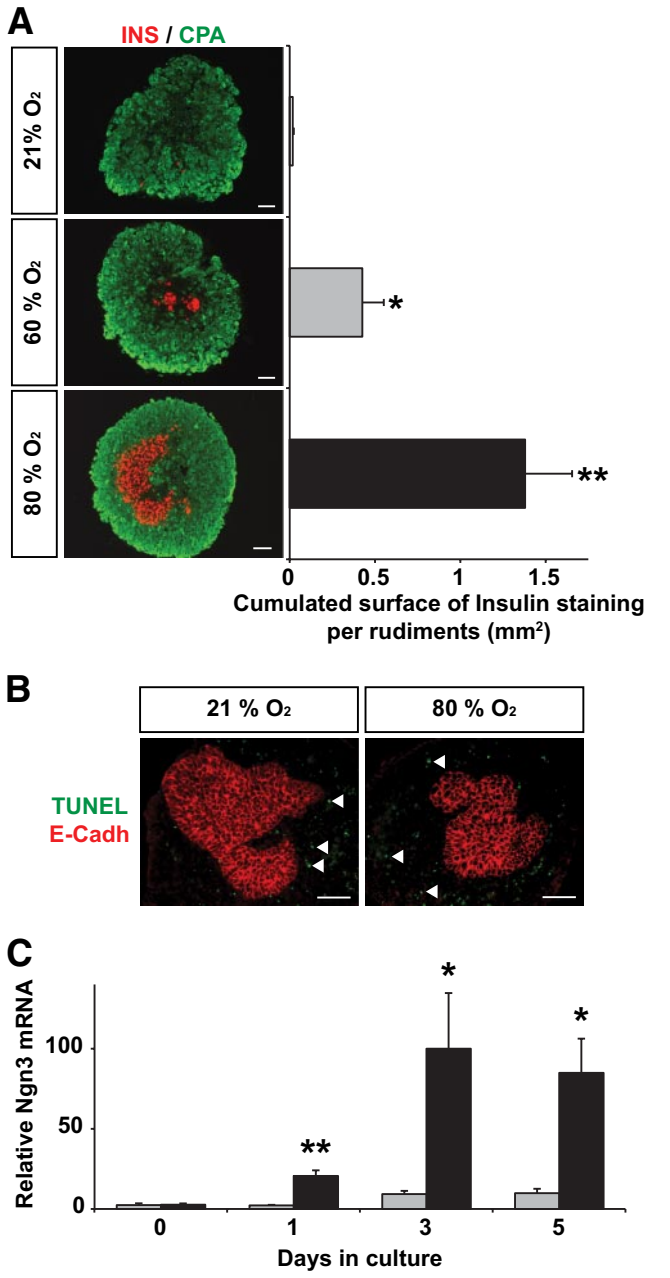

FIG. 2. Induction of β-cell differentiation by increasing pO_2 in collagen. *A*: Pancreata were cultured for 7 days at 21, 60, and 80% O_2. β- and acinar cells were detected by immunohistochemistry with antibodies directed against insulin (INS) (in red) and carboxypeptidase A (CPA) (in green). Next, the absolute surface area occupied by insulin-positive cells was quantified. *B*: Apoptotic cells (in green) were detected using a TUNEL reaction assay in pancreata cultured for 1 day at 21 or 80% O_2. The epithelial cells were detected using an anti–E-cadherin antibody (in red). Note the absence of apoptotic cells within the epithelium. *C*: Real-time PCR quantification of *Ngn3* mRNA in pancreata cultured at 21% (□) or 80% (■) O_2 at different time points. Gene expression is presented as a percentage of the highest sample. Each point represents the mean ± SEM of three individual data pools. *P < 0.05; **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)

After one day in collagen culture, HIF1α levels at 21% O_2 increased dramatically compared with those cultured at 80% O_2 (Fig. 3*A*). To confirm these results, we used immunohistochemistry. Both HIF1α and pimonidazole were detected at low but not high oxygen tension (Fig. 3*B*). We also analyzed the expression of HIF1α targets. Using qPCR, we found that expression of the glycolytic enzymes *Pgk1* and *LdhA*, as well as expression of *Glut1*, *Vegf*, and *Redd1*, increased 9.1-, 8.4-, 11.3-, 4.1-, 4.7-fold, respectively, when cultured at 21% O_2 compared with 80% O_2 (Fig. 3*C*). Taken together, these results demonstrate that the HIF1α signaling pathway is active in collagen culture at 21% O_2, but not at 80% O_2.

Stabilization of HIF1α at normoxia mimicks the effects of hypoxia on β-cell differentiation. To determine whether HIF1α was responsible for the arrest of β-cell differentiation under hypoxic conditions, we developed an approach to stabilize HIF1α under normoxic conditions. To this end, we first showed that when pancreata were cultured on a filter at the air-medium interface, low pO_2 induced HIF1α but not HIF2α accumulation (Fig. 4*A* and supplementary Fig. S3) and increased transcription of the HIF1α target genes *Pgk1*, *LdhA*, *Glut1*, *Vegf*, and *Redd1* (supplementary Fig. S4). HIF1α was expressed in the pimonidazole-positive areas (supplementary Fig. S5*A*), as was the case when pancreata were cultured in collagen (Fig. 3*B*). Moreover, under hypoxic conditions, *Ngn3* expression decreased both in mRNA

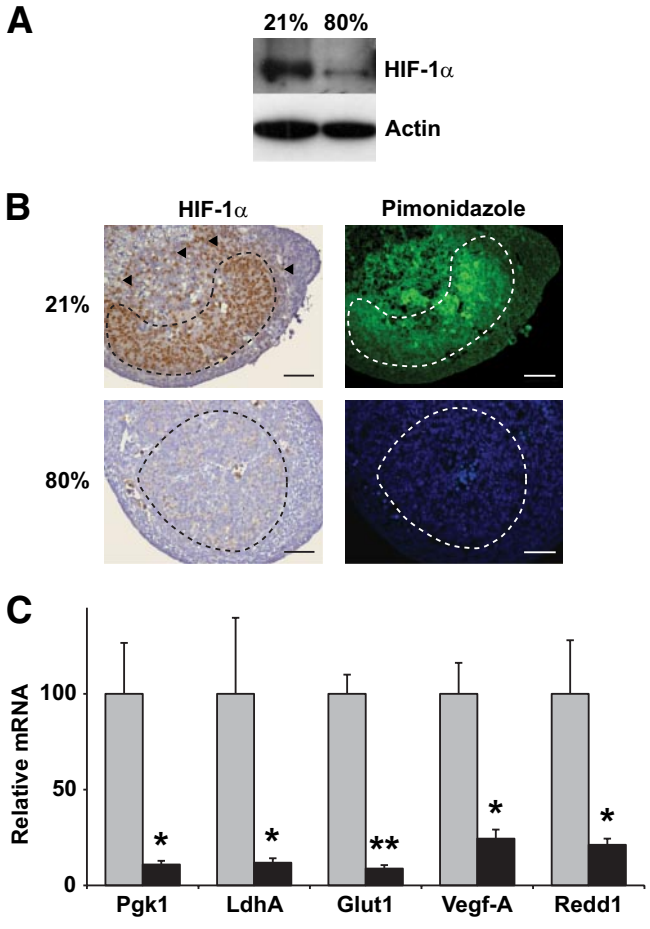

FIG. 3. The HIF1α signaling pathway is induced by hypoxia in the pancreas. *A*: Protein extracts from pancreata cultured in collagen for 1 day at 21 or 80% O_2 were analyzed by Western blot to quantify HIF1α expression and β-actin (loading control). *B*: Pancreata were cultured for 1 day at 21 or 80% O_2 and pimonidazole was added in the culture medium during the last 2 h of the culture. HIF1α-positive cells were detected by immunohistochemistry (in brown). On serial sections, hypoxia was analyzed using anti-Mab1 antibodies (in green). Note the topographical relationship between HIF activation and pimonidazole adduct formation. Bar: 50 μm. *C*: Expression of HIF1α target genes *Pgk1*, *LdhA*, *Glut1*, *Vegf*, *and Redd1* was analyzed by qPCR in pancreata cultured in collagen for 1 day at 21% (□) or 80% (■) O_2. Gene expression is presented as a percentage of the highest sample. Each point represents the mean ± SEM of three individual pools. **P* < 0.05; ***P* < 0.01. (A high-quality digital representation of this figure is available in the online issue.)

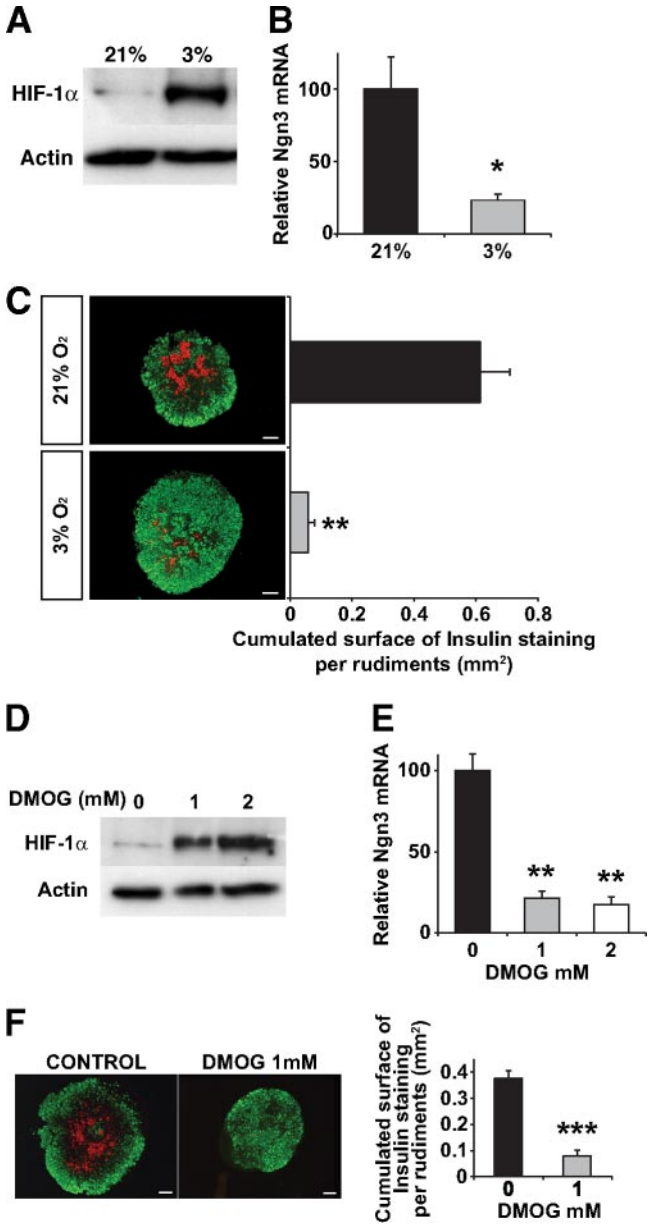

FIG. 4. DMOG, an inhibitor of prolyl hydroxylases, stabilizes HIF1α and represses β-cell differentiation. *A*: Pancreata at E13.5 were cultured at the air-medium interface for 1 day at 21 or 3% O_2, and HIF1α was analyzed by Western blot. *B*: Real-time PCR quantification of *Ngn3* mRNA in pancreata cultured for 1 day at 21% (■) or 3% (□) O_2. Gene expression is presented as a percentage of the highest sample. Each point represents the mean ± SEM of three individual pools. **P* < 0.05. *C*: Pancreata were cultured for 7 days at the air-medium interface at 21 or 3% O_2. Immunohistochemistry was used to detect β- and acinar cells using antibodies directed against insulin (in red) and carboxypeptidase A (in green). Next, the absolute surface occupied by insulin-positive cells was quantified. Black and gray bars represent the surface of insulin-positive cells in pancreata cultured at 21 and 3% O_2, respectively. ***P* < 0.01. *D*: Pancreata at E13.5 were cultured at the air-medium interface for 1 day in the presence or absence of 1 or 2 mmol/l DMOG, and HIF1α was analyzed by Western blot. *E*: Real-time PCR quantification of *Ngn3* mRNA in pancreata cultured for 1 day with 0 (■), 1 (□), or 2 (□) mmol/l DMOG at 21% O_2. ***P* < 0.01. *F*: Pancreata cultured in the presence or absence of 1 mmol/l DMOG for 7 days were analyzed by immunohistochemistry. Acinar cells were detected using anti–carboxypeptidase A antibody (in green) and β-cells using anti-insulin antibody (in red). Next, the absolute surface occupied by insulin-positive cells was quantified. Gray and black bars represent the surface of insulin-positive cells in pancreata cultured at 21% O_2 with or without DMOG, respectively. ****P* < 0.001. (A high-quality digital representation of this figure is available in the online issue.)

(Fig. 4*B*) and protein (supplementary Fig. S5*B*), and β-cell mass dramatically decreased (Fig. 4*C*). To evaluate the impact of oxygen tension on the proliferation of the precursor cells, we measured BrdU incorporation in pancreata cultured with 21 or 3% O_2 for 1 day (supplementary Fig. S6). There was a twofold decrease of BrdU incorporation in Pdx1-positive progenitors in the explants in hypoxia compared with normoxia. Thus, oxygen tension controls the proliferation of the progenitor cells. This effect should participate in the control of the number of NGN3-positive cells and the final β-cell mass. Next, we stabilized HIF1α at normoxia with dimethyloxaloylglycine (DMOG), a competitive inhibitor of prolyl hydroxylases (28). As shown in Fig. 4*D*, DMOG treatment caused HIF1α accumulation and induction of the HIF1α target genes in a dose-dependent manner (supplementary Fig. S7). On the contrary, DMOG did not stabilize HIF2α (supplementary Fig. S3). Interestingly, under normoxic conditions, DMOG

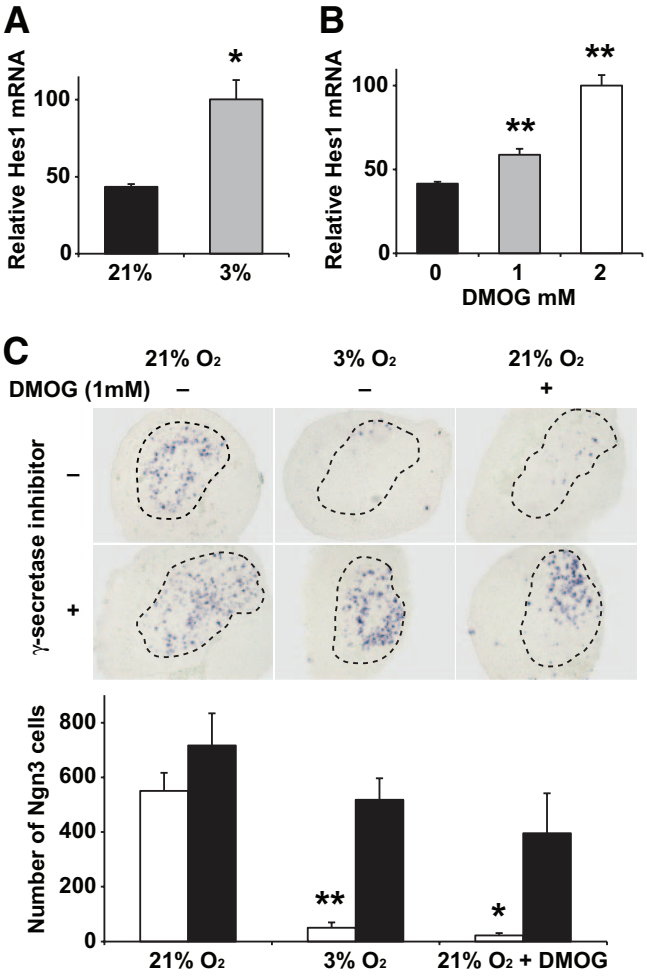

FIG. 5. Regulation of *Hes1* expression by hypoxia. *A*: Real-time PCR quantification of *Hes1* mRNA in pancreata cultured at the air-medium interface with 21% (■) or 3% (▒) O_2 for 1 day. *$P < 0.05$. *B*: Real-time PCR quantification of *Hes1* mRNA in pancreata cultured at the air-medium interface with 0 (■), 1 (▒), or 2 (□) mmol/l DMOG for 1 day. **$P < 0.01$. *C*: Detection of *Ngn3* transcripts by in situ hybridization in pancreata cultured for 1 day at 21% O_2, 21%O_2 with DMOG, or 3% O_2 with or without γ-secretase inhibitor treatment. Epithelium is circled in black in each panel. The absolute number of *Ngn3*-positive cells was quantified in each condition in the presence (■) or absence (□) of γ-secretase inhibitor treatment. *$P < 0.05$; **$P < 0.01$. (A high-quality digital representation of this figure is available in the online issue.)

treatment decreased *Ngn3* expression both in mRNA (Fig. 4*E*) and protein (supplementary Fig. S8). We further validated such results using L-mimosine, another inhibitor of prolyl hydroxylases (29–31). L-mimosine treatment resulted in HIF1α stabilization and repression of *Ngn3* expression (supplementary Fig. S9). In addition, quantification of insulin staining indicated a sixfold decrease in β-cell development after 7 days of culture with DMOG compared with a culture without DMOG (Fig. 4*F*). Taken together, these results indicate that HIF1α represses *Ngn3* expression and, consequently, negatively regulates β-cell differentiation.

Hypoxia regulates *Hes1* expression via HIF1α. Based on our present discovery that collagen-cultured tissues are hypoxic, we hypothesized that hypoxia could control the expression of *Hes1*, *a* transcription factor involved in pancreas development (25). To prove this, we cultured E13.5 rat pancreata on filter with 3 and 21% O_2 and quantified expression of *Hes1*. We found that *Hes1* expression is activated by hypoxia (Fig. 5*A*). Results were similar when DMOG was added to pancreata cultured at normoxia (Fig. 5*B*). To investigate whether hypoxia inhibits β-cell differentiation by regulating *Hes1* expression, we cultured pancreata under hypoxic conditions in the presence and absence of a γ-secretase inhibitor that inhibits the cleavage of Notch. In several cell types, *Hes1* has been described as a target of the Notch-activated receptor (11,32), but such a finding remains to be demonstrated in the pancreas (33,34). We thus first tested the effect of γ-secretase inhibitor XXI (Calbiochem) on HES1. Cultures of pancreatic explants were performed in the presence or absence of γ-secretase inhibitor, and HES1 protein expression was visualized using immunohistochemistry. γ-Secretase inhibitor abolished HES1 protein expression (supplementary Fig. S10). Interestingly, whereas in the absence of the γ-secretase inhibitor hypoxia decreased *Ngn3* expression, the presence of the γ-secretase inhibitor restored *Ngn3* expression (Fig. 5*C*). To further compare the effects of hypoxia and DMOG treatment, we cultured pancreata with DMOG in the presence or absence of γ-secretase inhibitor. As shown in Fig. 5*C*, γ-secretase inhibitor also abolishes the repression of *Ngn3* in DMOG-treated explants. Thus, these results demonstrate that both hypoxia and DMOG require expression of *Hes1* to repress *Ngn3*.

HIF1α protein expression decreases during pancreatic development. Immunohistochemistry results showed that at E11.5 and E12.5, HIF1α is present in both pancreatic epithelium and mesenchyme (Fig. 6*A*). Interestingly, HIF1α levels are reduced at E16.5. By E20.5, HIF1α expression decreased sharply and was rarely detected (Fig. 6*A*) and pancreatic islet cells stained negative for HIF1α (Fig. 6A). Finally, HIF1α is absent in the adult pancreas (Fig. 6*A*). Such a specific temporal pattern of expression was confirmed using Western blot analysis (supplementary Fig. S11).

Hypoxia decreases β-cell development in vivo. To investigate whether pO_2 regulates endocrine differentiation in vivo, we exposed pregnant rats at 13.5 days postcoitus to hypoxia at 8% O_2 or normoxia at 21% O_2 during 24 h as previously described (9). We next dissected the embryonic pancreases at E14.5. Expression of NGN3 was examined using immunohistochemistry, and expression of the HIF1α target genes were quantified using qPCR. We found that NGN3 expression was dramatically reduced in hypoxia compared with that in normoxia while the HIF1α target genes were induced (Fig. 6*B*). We thus conclude that experimental hypoxia downregulates endocrine differentiation in vivo as well as in vitro.

DISCUSSION

Previously, we showed that in vitro, mature β-cells develop efficiently from rat embryonic pancreas grown at the air-medium interface, whereas β-cell development is poor when the same tissue is grown in collagen (23,24). Our working hypothesis was that hypoxia occurred in collagen gel, activating the Notch pathway and inhibiting endocrine differentiation (23,24,35). In our present study, we used pimonidazole staining to demonstrate that pancreata cultured in collagen were hypoxic compared with cultures grown at the air-medium interface. We also found that in vitro, the level of HIF1α—a factor involved in regulation of many hypoxic responses (36)—increased in the pancreas as O_2 tension decreased and that it repressed pancreatic β-cell differentiation. At the difference to HIF1α, the factor

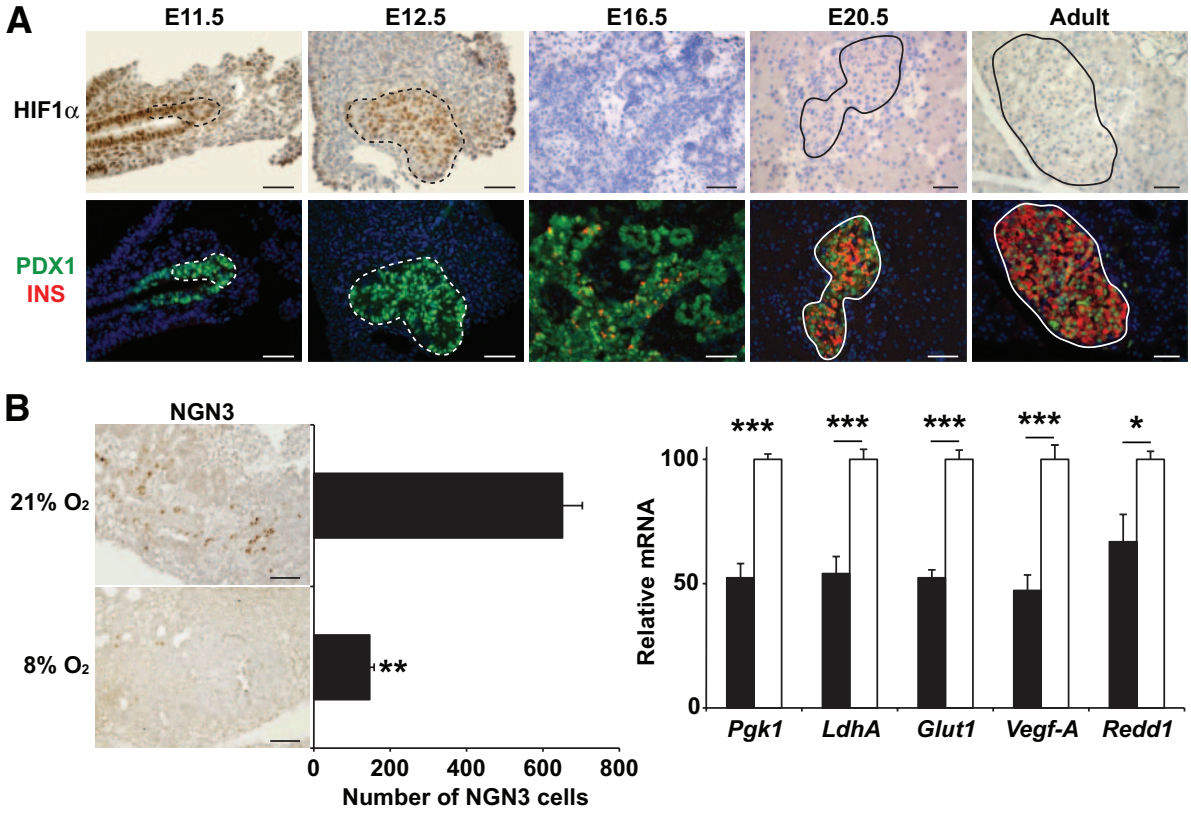

FIG. 6. HIF1α protein expression during pancreatic development in vivo. *A*: Immunohistochemical analysis of HIF1α protein expression (in brown) in pancreata at E11.5, E12.5, E16.5, E20.5, and adult phases. In the *left panel*, epithelium is circled in black. In the *right panel*, a pancreatic islet is circled in black. Antibodies against PDX1 (in green) and insulin (INS) (in red) were used on serial sections to visualize progenitors and β-cells, respectively. *B*: NGN3 expression is repressed in the pancreas of embryos exposed to hypoxia. Pregnant rats were maintained with 21 or 8% O_2 for 24 h. Animal weight did not differ between the hypoxic and normoxic groups. The pancreata were harvested at E14.5 and analyzed by immunohistochemistry and qPCR. Detection of NGN3 (brown; $^{**}P < 0.01$). Expression of HIF1α target genes, *Pgk1*, *LdhA*, *Glut-1*, *Vegf-A*, and *Redd1* in pancreata in hypoxia (□) or normoxia (■). $^{*}P < 0.05$; $^{***}P < 0.001$. (A high-quality digital representation of this figure is available in the online issue.)

HIF2α was not detected in the pancreas even in stimulated culture conditions (supplementary Fig. 3) or in VHL mutant mice (37). Data from literature indicate that HIF2α is expressed during embryonic development mainly in endothelial cells (7,38,39). It is thus possible that variations of HIF2α in the pancreatic endothelial cells may have influenced the development of endocrine cells in our experiments (17). However, our results indicating that hypoxia and DMOG do not stabilize HIF2α do not favor this hypothesis.

Oxygen is known to control cell differentiation in different tissues. For example, hypoxia blocks myogenic and neuronal differentiation (11), and it arrests preadipocytes in an undifferentiated state (12,40). Thus, hypoxia seems to play a major role in maintaining a pool of undifferentiated stem/progenitor cells—a process important for their proper amplification. The fact that oxygen tension is low during early human and rodent development, when progenitor cell expansion is taking place (41,42), further supports this hypothesis. Recently, Fraker et al. (43) proposed that pO_2 plays a role in β-cell differentiation based on results with embryonic pancreata cultured on a silicone-perfluorocarbone membrane at different O_2 concentrations. However, the mechanism by which O_2 mediated its effects was not elucidated.

Recent data indicate that hypoxia, via HIF1α, regulates limb bud differentiation. In this tissue, Provot et al. (44) proposed that condensation of the mesenchyme leads to local hypoxia that stabilizes HIF1α levels. Pancreatic anatomy during development suggests that pancreatic endocrine cell development could be controlled in the same way. Here we show that in vivo, HIF1α levels are high at E11.5–E12.5 in the undifferentiated rat epithelium and in the mesenchyme, indicating that the pancreas is hypoxic at this early stage. This finding suggests that hypoxia could maintain the pool of progenitor cells in vivo. Moreover, when pregnant rats at 13.5 days postcoitus were exposed to hypoxia for 24 h, the developing embryonic pancreata failed to express NGN3 (Fig. 6*B*). This experimental finding supports the hypothesis that oxygen is a limiting parameter for β-cell differentiation in vivo. Moreover, cultures of pancreata with different physiological oxygen tensions showed that O_2 is required for β-cell development in vitro (Fig. 4 and data not shown). In fact, pO_2 influences both the proliferation of the progenitor cells (supplementary Fig. S6) and development of β-cells (Figs. 1 and 4). This finding should be considered when in vitro models are used to characterize the molecular mechanisms that control the development of the pancreas. Finally, we also found that increasing pO_2 to nonphysiological levels can enhance the development of β-cells in vitro (data not shown). This finding suggests that oxygen could be used as a new tool to generate β-cells in vitro. Recent studies have described the pancreatic phenotype of mice with different deletions of VHL or HIF1α (37,45–47). Interestingly, Cantley et al. (37) used a pPDX1-cre

recombinase (CRE) to delete VHL during embryonic life. They found that 12 weeks after birth, HIF1α was increased but β-cell mass was not modified. The difference between these results and ours could be due to the fact that β-cell mass was analyzed 12 weeks after birth, while VHL deletion had not occurred in all targeted cells (37). It is thus possible that compensation occurred during this developmental period. Moreover, Cantley et al. (37) used Pdx1-CRE recombinase to delete VHL in pancreatic progenitors. However, we found that at E11.5 and E12.5, HIF1α was expressed in PDX1-positive pancreatic progenitor cells but also in surrounding mesenchymal cells. This observation was found both in vivo and in vitro (Fig. 3*B*; supplementary Figs. 5*A* and 6). We thus cannot exclude the possibility that HIF1α, in addition to its role in progenitor cells, modifies epithelio-mesenchymal interactions that are known to be of major importance for pancreas development (24,48,49). One possibility could be that hypoxia modifies the signals from the mesenchyme, e.g., the ones mediated by FGF10 or Delta-like 1. Neither FGF10 nor delta-like 1 mRNA steady-state levels significantly differ in the explants cultured in hypoxia compared with those in normoxia (data not shown). Further genetic studies using conditional knockout of HIF1α in mice or siRNA in cultured pancreatic explants will be necessary to analyze whether the effects of hypoxia on the epithelium are direct or involve the pancreatic mesenchyme.

In the present study, we demonstrate that the level of expression of *Ngn3*, which is a basic helix-loop-helix transcription factor expressed in islet cell progenitors and which is necessary to initiate the endocrine differentiation program (21,50), was inversely related to HIF1α levels. Interestingly, *Ngn3* expression and pancreatic endocrine cell development are tightly regulated by *Hes1*. For example, deletion of *Hes1*, which is thought to be a target of the activated cleaved Notch, led to precocious endocrine cell differentiation, depletion of the progenitor cell pool, and a decrease in pancreatic cell mass (25). In the present study, we used a γ-secretase inhibitor to block the Notch pathway. While γ-secretase inhibitors have been used with success for this purpose (11,51,52), other substrates of the γ-secretase inhibitor have been listed, such as ErbB1 (epidermal growth factor receptor [EGFR]) and ErbB4, that are also involved in pancreas development (53–55) and we thus cannot exclude that γ-secretase inhibitor also acts on other pathways. Recent data indicate that γ-secretase inhibitor is able to repress HES1 expression within pancreatic explants (56). Our data confirmed this effect (supplementary Fig. S10). Importantly, in the presence of γ-secretase inhibitor, the effects of hypoxia and DMOG on *Ngn3* expression were alleviated. We thus concluded that HES1 was necessary for these effects, but the exact role of the Notch receptor should be clarified in further studies. Moreover, our data indicate that hypoxia activates *Hes1* through HIF1α. Recently, a direct link was found between hypoxia and Notch signaling, and it is now proposed that these two pathways interact to inhibit differentiation of myogenic and neuronal progenitors during early stages of embryogenesis (11,57). Gustafsson et al. (11) reported that pharmacological inhibition of Notch signaling by a γ-secretase inhibitor alleviates inhibition of differentiation under hypoxic conditions. In addition, they reported a direct physical interaction between HIF1α and the intracellular domain of the activated Notch receptor; HIF1α was recruited to Notch-responsive promoters upon Notch activation under hypoxic conditions (11). These data are consistent with our findings and suggest that the mechanism by which HIF1α activates the expression of *Hes1* under hypoxic conditions also applies to pancreatic endocrine cell development. The HIF1α-Hes1 pathway is thus a good candidate for an explanation of the effects of hypoxia.

Based on our observations, we speculate that hypoxia induces HIF1α in early pancreatic progenitor cells, delaying their differentiation into endocrine cells and thus preserving a sufficient pool of precursors necessary for attaining an adequate final β-cell mass. These findings will help shed light on the molecular mechanisms responsible for β-cell differentiation. In addition, they should help define new protocols for deriving insulin-producing cells, thereby improving options for cell replacement therapy in type 1 diabetes.

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