Original Article

Downregulation of EGF Receptor Signaling in Pancreatic Islets Causes Diabetes Due to Impaired Postnatal β-Cell Growth

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Epidermal growth factor receptor (EGF-R) signaling is essential for proper fetal development and growth of pancreatic islets, and there is also evidence for its involvement in β-cell signal transduction in the adult. To study the functional roles of EGF-R in β-cell physiology in postnatal life, we have generated transgenic mice that carry a mutated EGF-R under the pancreatic duodenal homeobox-1 promoter (E1-DN mice). The transgene was expressed in islet β- and δ-cells but not in α-cells, as expected, and it resulted in an ~40% reduction in pancreatic EGF-R, extracellular signal–related kinase, and Akt phosphorylation. Homozygous E1-DN mice were overtly diabetic after the age of 2 weeks. The hyperglycemia was more pronounced in male than in female mice. The relative β-cell surface area of E1-DN mice was highly reduced at the age of 2 months, while α-cell surface area was not changed. This defect was essentially postnatal, since the differences in β-cell area of newborn mice were much smaller. An apparent explanation for this is impaired postnatal β-cell proliferation; the normal surge of β-cell proliferation during 2 weeks after birth was totally abolished in the transgenic mice. Heterozygous E1-DN mice were glucose intolerant in intraperitoneal glucose tests. This was associated with a reduced insulin response. However, downregulation of EGF-R signaling had no influence on the insulinotropic effect of glucagon-like peptide-1 analog exendin-4. In summary, our results show that even a modest attenuation of EGF-R signaling leads to a severe defect in postnatal growth of the β-cells, which leads to the development of diabetes. ***Diabetes*** **55:3299–3308, 2006**

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BTC, betacellulin; EGF-R, epidermal growth factor receptor; ERK, extracellular signal–related protein kinase; Glp, glucagon-like peptide; pdx-1, pancreatic duodenal homeobox-1.

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The pancreatic β-cell is the key player of the glucoregulatory machinery. It senses changes in blood glucose levels and immediately adjusts the release of insulin accordingly. Gene targeting experiments have revealed transcriptional networks required for β-cell differentiation (1,2). Achievement and maintenance of the correct β-cell mass is a crucial issue that is controlled at all stages of development. In the embryonic pancreas, this is controlled by an interplay between growth factors, such as fibroblast growth factor-10 (3), provided by the mesenchyme, and notch signaling between the epithelial cells to regulate the expression of the proendocrine transcription factor neurogenin-3 (4,5). Epidermal growth factor receptor (EGF-R) stimulation also promotes embryonic pancreatic epithelial proliferation and suppresses endocrine differentiation (6). The last days of gestation and the first postnatal weeks in the mouse are characterized by a dramatic growth and remodeling of the pancreatic islets. The β-cell mass increases by fourfold within 2 weeks, and this period is crucial for the determination of the functional β-cell mass for the remaining lifetime (7,8). The same is true in humans, where the major β-cell expansion occurs during the last third of gestation. The growth factors that are responsible in vivo for the control of this critical phase of β-cell expansion are poorly understood.

EGF-R is a tyrosine kinase receptor (9) belonging to the erbB gene family (EGF-R/erbB1, neu/erbB2, erbB3, and erbB4) (10,11). Binding of EGF family growth factors leads to EGF-R autophosphorylation and activation of downstream extracellular signal–related protein kinase (ERK) and phosphatidylinositol 3-kinase signaling pathways. EGF-R has been associated with cell proliferation and differentiation and is implicated in the development of organs undergoing branching morphogenesis. All erbBs are expressed in the developing pancreas in an overlapping manner (12,13). We previously have shown, using an EGF-R–deficient mouse model, that EGF-R signaling is essential for proper pancreatic development (14). In the absence of EGF-R, the nascent islet cells have a migration defect and the development of β-cells occurs at a later stage in EGF-R$^{-/-}$ mice than in wild-type littermates. Moreover, we showed that ligands of the EGF-R/erbB-1 and erbB-4 receptors regulate the lineage determination of islet cells during pancreatic development. Particularly, betacellulin (BTC), acting through EGF-R/erbB-1, is important for the differentiation of β-cells (12).

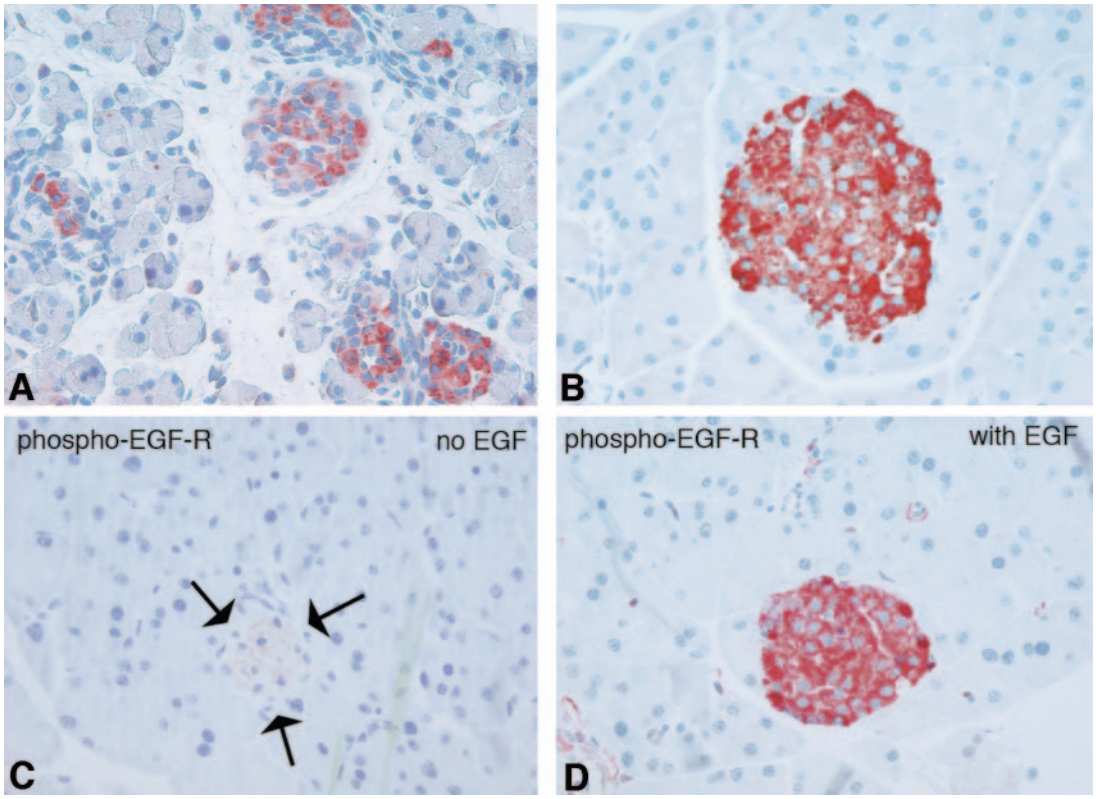

FIG. 1. Expression of EGF-R in mouse pancreas. Newborn (*A*) and 3-month-old (*B*) mouse pancreatic islets stain strongly for EGF-R immunoreactivity (red). To detect activated EGF-R, we used an antibody for phosphorylated EGF-R and analyzed mouse islets in animals injected with PBS or EGF (10 μg/g body wt). Without EGF stimulation, EGF-R is only weakly phophorylated in the islets (*C*; arrows). Ten minutes after EGF injection, strong immunoreactivity (red) is seen in islets and only faintly in vessel walls and ducts.

The EGF-R$^{-/-}$ mice do not survive beyond the perinatal period. To further clarify the role of EGF-R signaling in postnatal growth and function of the β-cells, we have generated transgenic mice that express kinase-negative EGF-R under the pancreatic duodenal homeobox-1 (pdx-1) promoter. Using this mouse model, we show that an intact EGF-R signaling pathway is required for the development of a sufficient and functional β-cell mass and that even a partial inactivation of EGF-R function in the β-cells leads to diabetes.

RESEARCH DESIGN AND METHODS

Generation of pdx-1–EGF-R dominant-negative mice (E1-DN). The E1-DN transgene (Fig. 2*A*) consists of the mouse pdx-1 promoter (a kind gift from Dr. Pedro Herrera, University of Geneva), followed by a rabbit β-globin second intron (15), the human kinase–deficient EGF-R cDNA (CD533; Schlessinger) with a myc-tag (GEQKLISEEDLN), and a growth hormone polyA-tail (16). The EGF-R deletion mutant previously has been shown to function effectively as a dominant-negative manner in mice (17). The transgene was injected into pronuclei, and eventually two transgenic mouse lines were obtained. The transgenic animals were genotyped by Southern blot or dot blot analysis (16). The study protocol was approved by the animal ethics committee of the University of Helsinki. Wild-type controls were derived from nonrelated litters to exclude the effect of intrauterine hyperglycemia.

Blood glucose measurements and in vivo testing of glucose tolerance, insulin secretion, and insulin sensitivity. Random blood glucose values were measured from wild-type (n = 4–8), E1-DN heterozygous (n = 6–30), and E1-DN homozygous (n = 15–60) mice at various ages from tail veins using a OneTouch Ultra glucometer (Lifescan). The intraperitoneal glucose tolerance test was done on 3- to 8-month-old mice (n = 12 for the transgenic and n = 12 for the control group) by intraperitoneally injecting glucose (1 mg/g body wt). Blood samples were collected at 0, 30, 60, and 120 min, and glucose (OneTouch) and insulin (Ultrasensitive Mouse Insulin ELISA; Mercodia, Uppsala, Sweden) concentrations were measured. Exendin-4 (100 ng/mouse; Bachem) was intraperitoneally injected immediately before glucose loading on the contralateral side. To exclude differences in insulin tolerance, 0.75 IU/g insulin was intraperitoneally injected to wild-type and E1-DN mice (twice for n = 3 for both groups) and blood glucose measured at 0, 15, 30, and 60 min. All in vivo experiments were done on homozygous or heterozygous male mice, as males had the most pronounced phenotype. The results are expressed as the mean of different experiments ± SE.

Histological and morphometrical analysis. Newborn and 2- to 8-month-old E1-DN transgenic and wild-type mice were killed by cervical dislocation, and the pancreata were dissected, weighed, and fixed overnight in 4% paraformaldehyde or Bouin's fixative. The tissues were processed into paraffin using routine procedures. For morphometrical analysis, the pancreata were serially sectioned through, and five 3 μm sections per every 100 μm were collected, deparaffinized, stained with insulin or glucagon antibodies, counterstained with hematoxylin, and morphometrically analyzed directly under light microscope using Image-Pro Plus 4.5 (version 0.19) software as previously described (14).

Total pancreatic insulin content. Pancreata were weighed and then homogenized in acid ethanol (75% ethanol, 23.5% distilled water, and 1.5% concentrated HCl) at 10 ml/g of tissue. After overnight incubation at 4°C, the suspensions were centrifuged at 2,000*g* for 10 min and the supernatants were collected and analyzed for insulin content using RIA (DPC, Los Angeles, CA).

Immunohistochemistry. Paraffin sections from wild-type and E1-DN transgenic mice were stained as described (12) with the following antibodies: amylin (mouse anti-human; Labvision, Fremont, CA), insulin (guinea pig anti-swine; DakoCytomation, Glostrup, Denmark), glucagon (rabbit anti-human; DakoCytomation), somatostatin (rabbit anti-human; DakoCytomation), PP (rabbit anti-human; DakoCytomation), EGF-R (E3138 [Sigma] and no. 100-401-149 [Rockland] for both mouse and human EGF-R and Ab-10 [Neomarkers, Fremont, CA] for human EGF-R), phospho-specific EGF-R (P845-EGF-R; Biosources), pdx-1 (kind gift from Dr. Christopher Wright, Vanderbuilt University, Nashville, TN), myc (9E10.3 mouse anti-human; Neomarkers/Labvision), and GLUT-2 (no. sc-7580, goat anti-human; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactions were visualized under either light or fluorescence microscope and digitally photographed.

Cell proliferation and apoptosis. Paraformaldehyde-fixed pancreatic sections from newborn, postnatal day 7 (D7), and day 14 (D14) wild-type and E1-DN pancreata were double stained with antibodies to insulin (DakoCytomation) and either Ki67 (Novocastra, Newcastle upon Tyne, U.K.) or cleaved caspase 3 (Cell Signaling). Briefly, paraffin sections were microwave treated in 10 mmol/l citrate or in 1 mmol/l EDTA for 15 min. Nonspecific binding was

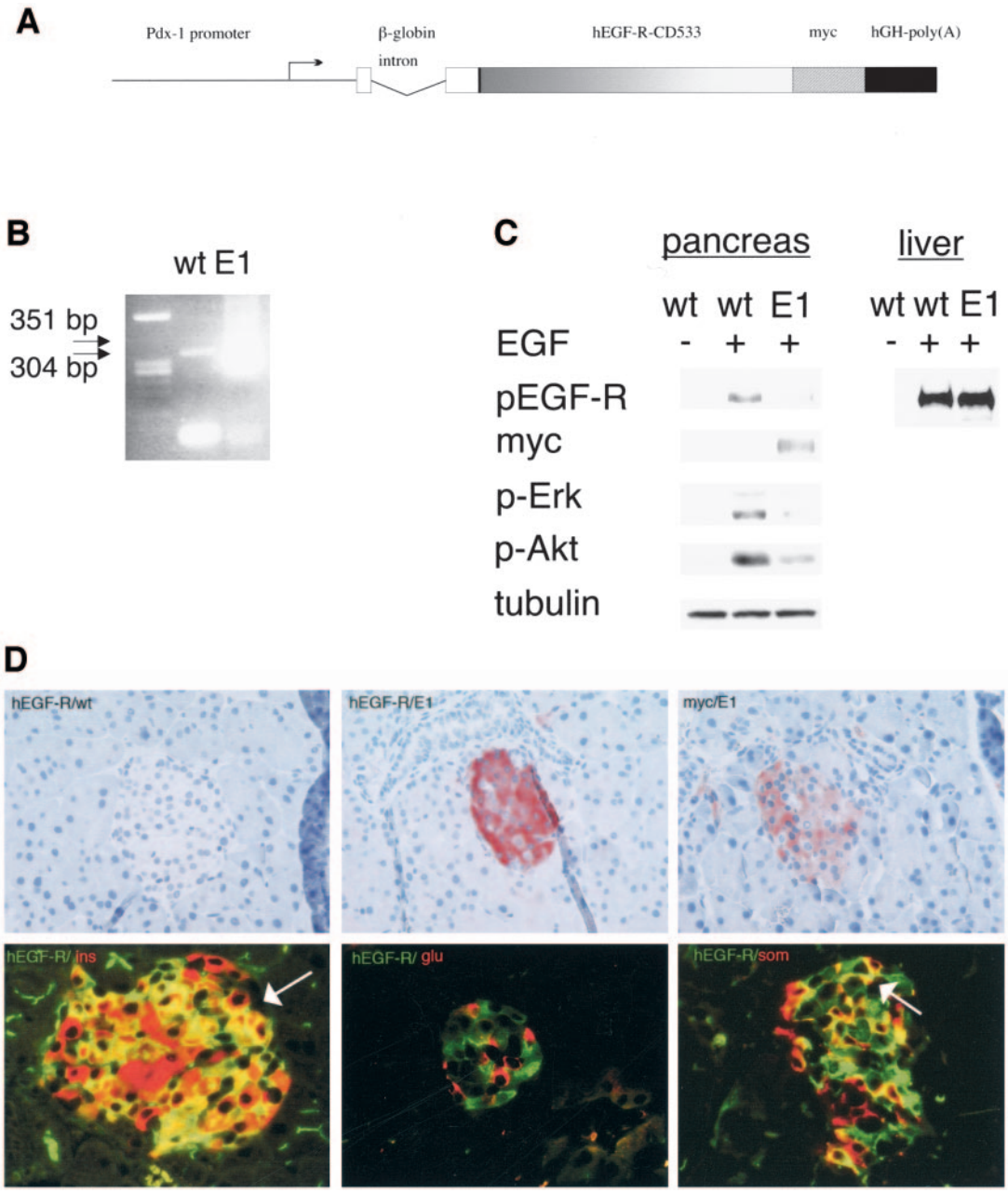

FIG. 2. Structure and expression of the pdx1–EGF-R dominant-negative (E1-DN) transgene. *A*: The E1-DN transgene consists of the mouse pdx-1 promoter driving the expression of a kinase-negative human EGF-R and a myc-tag. *B*: Expression of the E1-DN transgene was analyzed in wild-type (wt) and E1-DN islets by RT-PCR using primers for both the endogenous and mutated EGF-R. E1-DN islets abundantly express the truncated EGF-R (304 bp). *C*: EGF (10 μg/g body wt) was injected intraperitoneally into wild-type (wt) and E1-DN mice. Protein lysates from pancreas and liver were subjected to Western analysis. Upon EGF stimulation, EGF-R, mitogen-activated protein kinase (ERK), and Akt signaling pathways are phosphorylated and activated in the wild-type pancreas but only weakly in the E1-DN pancreas. Level of E1-DN protein expression is shown is by anti-myc Western analysis. The E1-DN transgene is present and active only in pancreas as shown by equally strong EGF-R phosphorylation between wild-type and E1-DN liver. Tubulin protein expression is shown as a loading control. *D*: E1-DN transgene is expressed abundantly in the islets as shown by transgene-specific immunostaining using anti-hEGFR or anti-myc (*upper row*). Anti–hEGF-R does not stain anything in the wild-type pancreas. E1-DN transgene (green) is located mainly in the cell membrane (arrow) with some cytoplasmic expression (*lower row*). It is expressed in the β-cells and in some δ-cells as shown by hEGF/insulin (red) and hEGF-R/somatostatin (red) double staining (yellow cells). No E1-DN transgene can be detected in α-cells by hEGF-R/glucagon double staining (only green or red cells). Magnification ×40.

blocked by preincubation in 4% normal donkey serum followed by incubation of primary antibodies overnight at 4°C. As secondary antibodies we used, TRITC (tetramethylrhodamine isothiocyanate) donkey anti–guinea pig and FITC (fluorescein isothiocyanate) donkey anti-rabbit (Jackson Immunoresearch).

Immunoprecipitation and Western analysis. Pancreata, livers, or isolated islets were homogenized in lysis buffer (25 mmol/l HEPES, pH 7.4, 1% Triton X-100, 10% glyserol, 0.5 mmol/l EGTA, 10 mg/ml approtinin, 10 mg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, and 2 mmol/l Na-orthovanadate) and protein concentration determined by the Bio-Rad DC Protein Assay (Bio-Rad). Lysates (50 μg protein) were run into 10% SDS-PAGE and analyzed by Western analysis with antibodies to EGF-R, phospho–EGF-R, myc, phosphor-ERK (Promega), phospho-Akt (no. 9271; Cell Signaling), and tubulin (no. T5168; Sigma) as described (12).

In vivo phosphorylation assay. Wild-type and E1-DN transgenic mice (2–4 months old) were intraperitoneally injected with recombinant EGF (1–10 μg/g body wt; R&D Systems) and killed after 10 min. The pancreata were dissected, split in half, and either snap frozen in liquid nitrogen for EGF-R phosphorylation analyses by Western analysis or fixed in 4% paraformaldehyde for

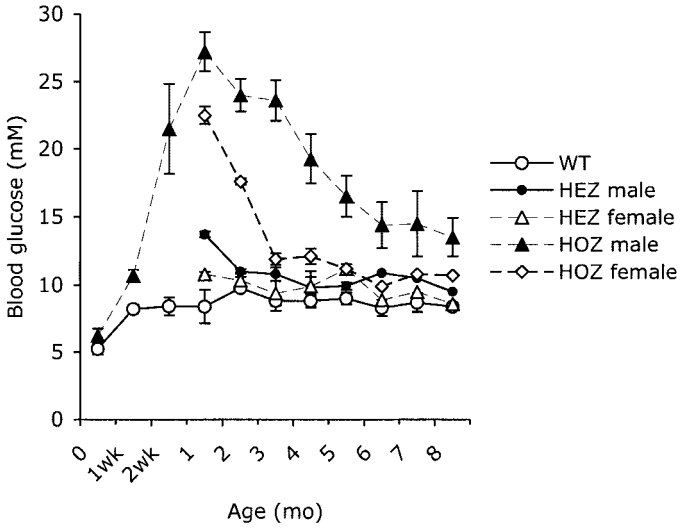

FIG. 3. E1-DN mice are diabetic. Random blood glucose values were measured from wild-type (WT; $n = 4–8$) and E1-DN mice ($n = 6–30$ for heterozygous [HEZ] and $n = 15–60$ for homozygous [HOZ]) at indicated ages. The E1-DN mice are clearly hyperglycemic from D7 onwards. Blood glucose of the homozygous E1-DN mice rises very steeply and remains high. Heterozygous E1-DN mice present with moderate hyperglycemia showing a clear gene-dose effect. Blood glucose of male mice is consistently higher than female mice.

immunohistochemistry. Liver was used a positive control tissue for EGF-induced phosphorylation (18).

Islet isolation and in vitro insulin release. Islets from 3- to 6-month-old wild-type and E1-DN transgenic mice were isolated by standard collagenase digestion (Collagenase P; Roche Diagnostics, Mannheim, Germany) and subsequently handpicked under a stereomicroscope. After an overnight culture in RPMI-1640 (Life Technologies) medium with 10% FCS, the islets were first preincubated for 60 min at 37°C in Krebs-Ringer bicarbonate buffer with 1.7 mmol/l glucose. Groups of 5–10 islets per well were then incubated in a 24-well plate in 300 μl of the low glucose (1.7 mmol/l) Krebs-Ringer bicarbonate buffer for the 1st h and in 11.2 mmol/l glucose with or without 10 nmol/l exendin-4 for the 2nd h. Insulin was measured from the supernatants by radioimmunoassay (DPC, Los Angeles, CA). Intracellular insulin content of the islets was measured after sonication in 300 μl distilled deionized water and overnight extraction in acid ethanol.

RT-PCR. RNA from wild-type and E1-DN islets was isolated using the NucleoSpinRNAII kit according to the manufacturer's instructions (Macherey-Nagel, Dureer, Germany). Reverse transcription and PCR were done as previously described (12). The upstream primer was the same for both mouse EGF-R and the transgene (5′-cca gtg tgc cca cta cat tg-3′), while the downstream primers were separate (for mouse EGF-R 5′-ctg ggt gtg aga ggt tcc ac-3′ and for the E1-DN transgene myc-specific 5′-cct cgg ata tca gct tct gc-3′). These primers created a 351-bp fragment for the mouse EGF-R and a 304-bp fragment for the E1-DN transgene.

Statistical analysis. All data are expressed as means ± SE, unless otherwise indicated. Significance of the differences between two groups was tested with Student's unpaired t test. Differences between more than two groups were tested using the one-way ANOVA and Fisher's least significant differences test. $P < 0.05$ was used as the limit for statistical significance.

RESULTS

Expression of EGF-R in mouse pancreas. We previously have shown that targeted inactivation of EGF-R leads to streak-like islets and a poorly branched pancreas. Normally, EGF-R is strongly expressed in mouse islets at all postnatal ages (Fig. 1*A* and *B* for newborn and 3-month-old islets). Without EGF stimulation, EGF-R is phosphorylated only at a low level as shown by immunostaining with anti–P845-EGF-R (Fig. 1*C*). However, after a subcutaneous EGF injection, phosphorylated EGF-R is abundantly expressed in the islets (Fig. 1*D*) and only weakly in exocrine pancreas.

Generation of pdx-1–EGF-R dominant-negative (E1-DN) mice. The pdx-1 promoter was used to drive the expression of a kinase-negative human EGF-R into developing β- and δ-cells (Fig. 2*A*). For transgene expression studies, a myc-tag was added to the cytoplasmic tail of the receptor. The E1-DN transgenic mice were generated through pronucleus injections and routine transgenic animal techniques. Two founder lines were bred and analyzed for transgene expression and phenotype. Heterozygotes and homozygotes were identified by Southern and dot blot analysis and by PCR. As shown, the E1-DN mRNA is readily detectable from transgenic islets (Fig. 2*B*). The effect of the transgene on the endogenous EGF-R signaling was studied by injecting wild-type and E1-DN mice with EGF (1–10 μg/g body wt i.p.). In the homozygous E1-DN mice, autophosphorylation of the pancreatic EGF-R was 37% of the wild-type level (Fig. 1*C*), and phosphorylation of the downstream signaling components ERK1/2 and Akt were 43% of the level seen in the wild-type pancreata (Fig. 2*C*). As expected, the hepatic EGF-R of the E1-DN animals was phosphorylated similarly to that in the wild-type mice.

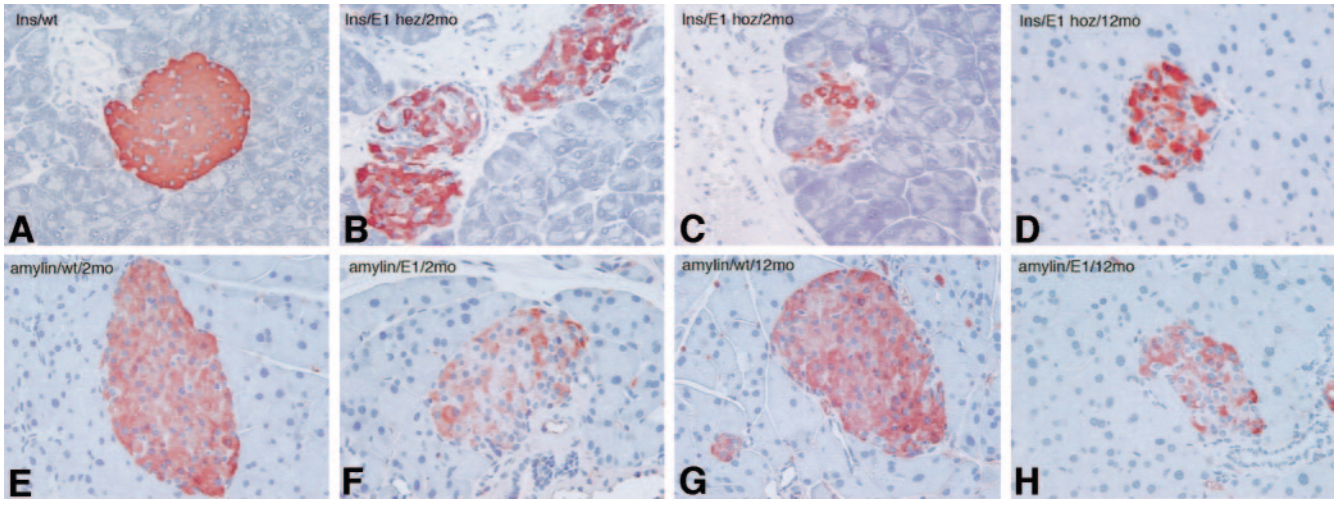

FIG. 4. Characterization of the E1-DN β-cells. *A–D*: Insulin immunohistochemistry (red) shows a patchy staining pattern in the E1-DN pancreata from 2- and 12-month-old heterozygous (hez) and homozygous (hoz) mice. β-Cells stain for insulin in a varying intensity, and there are many insulin-negative cells in the islet mantle. The staining pattern for amylin correlates to insulin-like immunoreactivity (*E* and *F*; red). Original magnification ×40.

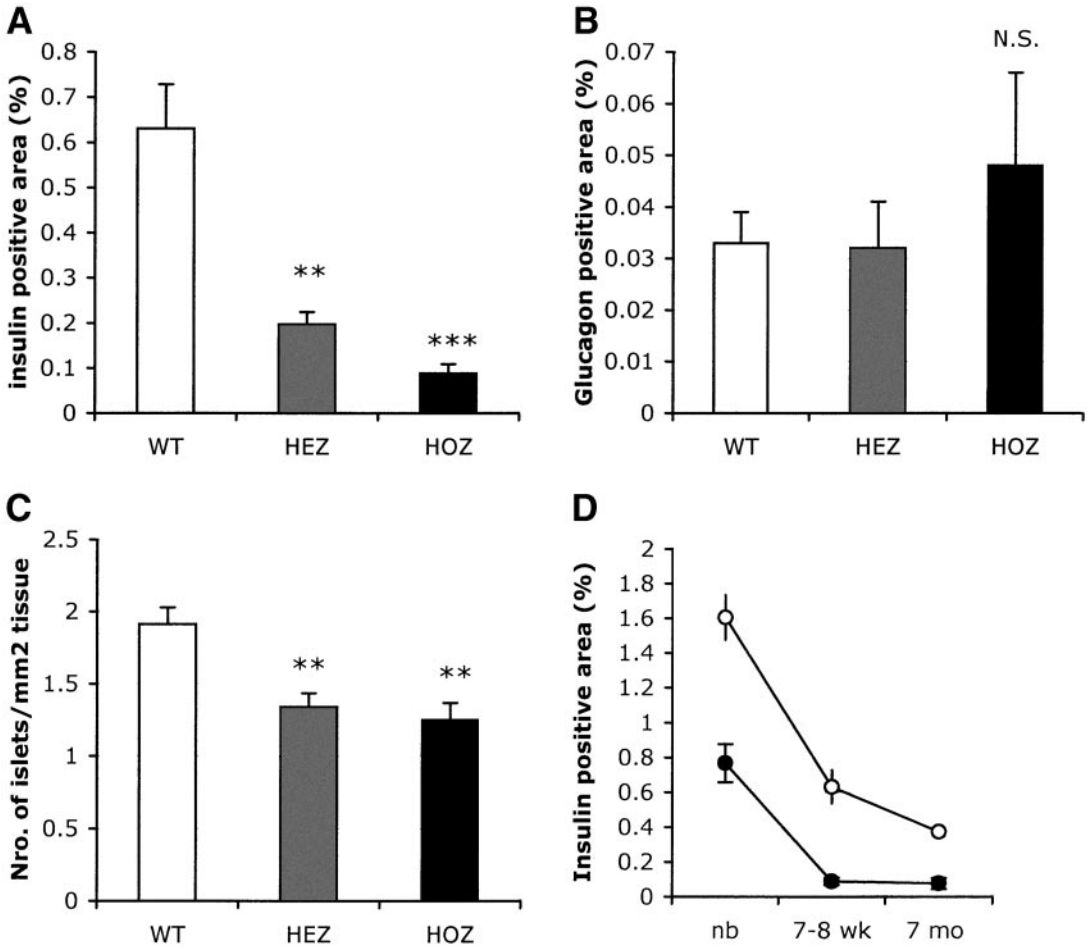

FIG. 5. Reduced β-cell mass in E1-DN mice. *A*: Morphometric quantitation of insulin-positive area relative to the whole pancreatic area ($n = 4$ mice for each genotype) shows that the heterozygous (HEZ) (▩) and homozygous (HOZ) (■) E1-DN mice have 70–85% less β-cells when compared with the wild-type (WT) (□) mouse at the age of 2 months. Results are expressed as means ± SE. *B*: There is no difference in the expression of glucagon between wild-type and E1-DN mice at this age. *C*: The number of islets per pancreatic area is reduced by 30% in the E1-DN mice. *D*: The relative β-cell surface area from birth until the age of 7 months. *$P < 0.05$; **$P < 0.01$. ○, wild type; ●, homozygous.

Cellular location of the E1-DN protein was studied by immunohistochemistry using transgene-specific antibodies (anti-myc and anti-human EGF-R, which does not cross-react with mouse EGF-R). As shown (Fig. 2*D*), the E1-DN β- and also some δ-cells displayed a strong membranous and also a cytoplasmic staining pattern for the transgene, whereas α-cells did not express the transgene. There was some variability in the intensity of the transgene-specific immunoractivity between individual β-cells. The expression pattern remained similar at all ages studied (newborn and 2- and 8-month-old mice; data not shown). To conclude, the kinase-negative EGF-R is expressed in the β- and δ-cells and inhibits endogenous EGF-R function.

E1-DN mice are hyperglycemic. The transgenic E1-DN mice were fertile, and their weights did not differ from the wild-type animals at any time point (data not shown). However, although normoglycemic at birth, they were increasingly hyperglycemic since the age of 7 days (Fig. 3). Homozygous E1-DN mice were overtly diabetic from the age of 2 weeks. The mean fed blood glucose of male mice peaked at 27 mmol/l at the age of 1 month. After the peak, it later stabilized at ~15 mmol/l but remained significantly elevated compared with wild-type mice (at 8 months: 13.5 vs. 8.4 mmol/l, $P < 0.01$). Female homozygous mice consistently were less hyperglycemic than the homozygous males, but they also remained clearly diabetic. Also, heterozygous male mice were constantly slightly hyperglycemic (mean random blood glucose 11.1 vs. 8.8 mmol/l in male 1- to 6-month-old heterozygous versus wild-type mice; $P < 0.05$). These results suggest a clear gene-dose effect and sexual dimorphism on the glucoregulatory role for EGF-R signaling in the β-cell.

The age-related gradual decrease in blood glucose levels seen in all E1-DN mice was not due to a loss of transgene expression, because the islets of the 7- to 8-month-old transgenic animals were still strongly transgene positive when stained for hEGF-R or c-Myc immunoreactivity (data not shown). Nor did it appear to be due to increased insulin sensitivity, since there were no differences in the hypoglycemic responses of wild-type and E1-DN animals in an insulin tolerance test (data not shown).

Islet structure, β-cell proliferation, and apoptosis. Reduced β-cell mass would be a logical explanation for the development of hyperglycemia in the E1-DN mice. To study this, newborn and 2-month-old pancreata were dissected, weighed, and fixed in paraformaldehyde and processed through routine histology. The weight of the pancreas per body weight did not differ between the wild-type and E1-DN mice (1.33 ± 0.069% vs. 1.37 ± 0.060%, respectively), allowing the use of relative insulin positive area as a reliable reflection of total pancreatic

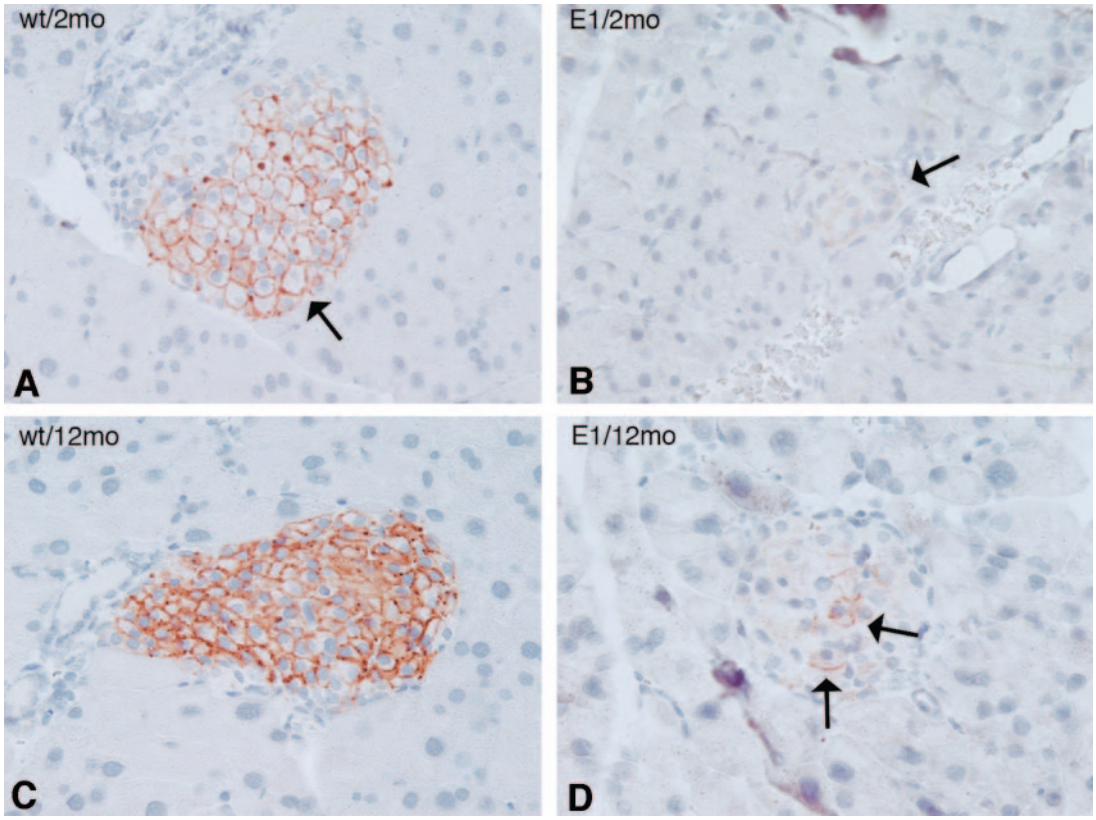

FIG. 6. Reduced GLUT-2 expression in E1-DN pancreata. GLUT-2 immunoreactivity (brown; arrow) is strong at cell membranes in the 2- and 12-month-old wild-type (wt) islets (*A* and *C*), while in the E1-DN islets (*B* and *D*) only weak cytoplasmic staining is seen (arrow) with occational membranous reactivity in the older islets. Magnification ×40.

β-cell mass in morphometric analysis (see below). However, the pattern of insulin immunoreactivity was dramatically abnormal in the E1-DN mice. In homozygous mice, the islets were clearly smaller and this was due to a marked loss of insulin-positive cells (Fig. 4). In the heterozygous mice, the islets were somewhat larger, but instead of the homogenous insulin staining seen in wild-type islets, there was a large variation in the staining intensity, and many of the centrally located cells lacked insulin immunoreactivity (Fig. 4*B–D*). Another β-cell–specific protein, amylin, showed a similar expression pattern at all ages studied (Fig. 4*E–H*). The relative β-cell surface area (Fig. 5*A*) of the E1-DN mice was highly reduced at the age of 2 months (wild type 0.63%, heterozygous 0.20%, homozygous 0.09% of the whole pancreatic area; $P < 0.001$). The E1-DN pancreata also contained less insulin (E1-DN heterozygous 196 ± 20 μIU/mg tissue vs. wild type 408 ± 20 μIU/mg tissue), corresponding to the results of the morphometric analysis. There was no change in the relative surface area of glucagon-positive α-cells correlating with the specificity of the pdx-1 promoter (Fig. 5*B*). The reduced β-cell mass was mainly due to a reduction of this cell type in each islet because there was only a marginal decrease in the number of islets per pancreatic area (Fig. 5*C*). The β-cell mass defect clearly developed primarily during the early postnatal expansion phase (Fig. 5*D*). In the newborn mice, the relative β-cell surface area was reduced by 52% but at 3 months by 86% compared with wild-type littermates. Thereafter, the difference remained fairly constant. Expression of the β-cell glucose transporter GLUT-2 was studied immunohistochemically at 3- and 12-month-old wild-type and E1-DN mice. In the wild-type islets, GLUT-2 immunoreactivity was specifically located at the islet cell membrane. In sharp contrast, only faint and diffuse cytoplasmic immunoreactivity was seen in the transgenic islets at both time points, although some membrane-bound GLUT-2 staining was occasionally visible in the older animals (Fig. 6).

Proliferation of β-cells was studied by insulin/Ki67 double immunohistochemistry during the first 2 postnatal weeks (Fig. 7*A*). The labeling index of E1-DN β-cells was already reduced in the newborn mice by ~50% ($P < 0.05$). At postnatal day 7 (D7), the proliferation rate had increased in the wild-type mice but decreased in the E1-DN mice (wild-type 7.44%, homozygous 2.41%; $P < 0.001$). At postnatal day 14, the proliferation of wild-type β-cells had decreased to 4.6%, while the proliferation of E1-DN β-cells remained at a low level (2.6%; $P < 0.05$). Apoptosis was studied by immunostaining newborn and 1- and 2-week-old pancreata for active (i.e., cleaved) caspase-3 and double staining for insulin. Interestingly, the number of apoptotic cells was lower in the postnatal E1-DN homozygous pancreata compared with wild-type pancreata when studied at the whole-pancreas level (Fig. 7*B*; $P < 0.05$). However, no difference could be detected in the rate of β-cell–specific apoptosis (0.50% in the wild-type vs. 0.47% in the E1-DN pancreata).

β-Cell function. An intraperitoneal glucose tolerance test was used to study insulin secretion in vivo. Before the test, the mice fasted for 16 h. As can be seen from Fig. 8*A*, the E1-DN animals were hyperglycemic throughout the test, while the wild-type mice returned to normoglycemia by 2 h. The increase of circulating insulin in response to the glucose challenge was blunted and delayed in the E1-DN

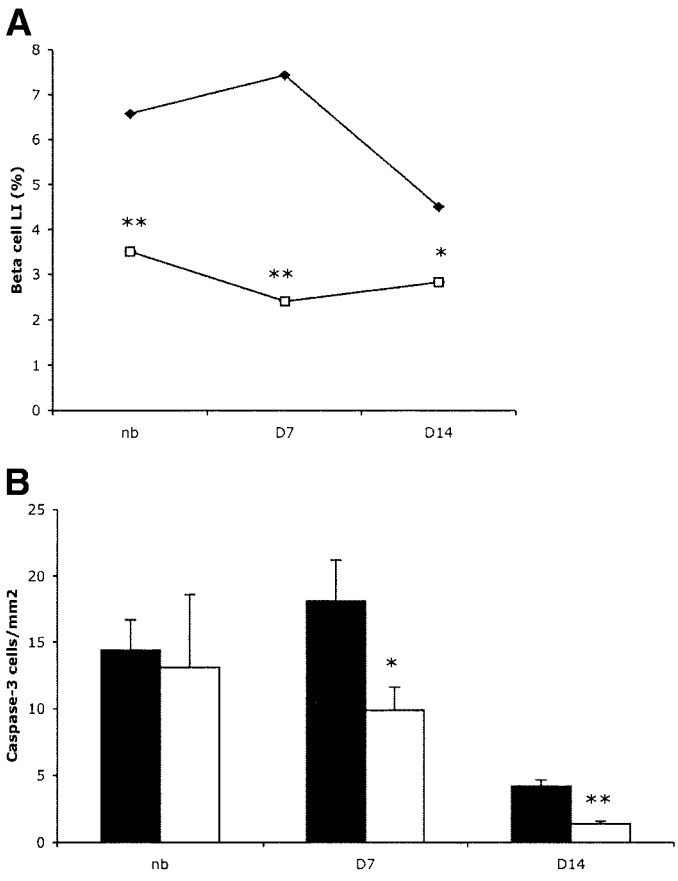

FIG. 7. β-Cell proliferation and pancreatic cell apoptosis. *A*: Cell proliferation was quantitated by insulin and Ki67 immunohistochemistry from newborn and postnatal day 7 (D7) and 14 (D14) pancreata. Double-positive cells are expressed relative to insulin positive cells. There are ~40% less proliferating β-cells in the E1-DN pancreas already at newborn stage, and no enhanced cell proliferation can be seen in the wild-type β-cells cells. ♦, wild-type [WT]; □, homozygous [HOZ]. *B*: Apoptotic cells were visualized by caspase 3 immunohistochemistry and expressed relative to the whole pancreatic area. No difference can be seen in apoptotic rate at the newborn stage. However, at D7 the E1-DN pancreas contains 50% less apoptotic cells and at D14 67% less apoptotic cells. No difference could be seen in β-cell apoptosis (data not shown). *$P < 0.05$; **$P < 0.01$. ■, wild-type; □, homozygous.

mice (Fig. 8*B*). Interestingly, the fasting insulin level of the E1-DN heterozygous mice was within the range of wild-type mice and significantly higher than in the E1-DN homozygous animals. However, the immediate insulin response was absent also in the E1-DN heterozygous mice, and only a delayed, low-magnitude response between 60 and 120 min was detected.

Glucagon-like peptide-1 (Glp-1) has been shown to exert some of its biological functions through transactivation of EGF-R (19,20). To test whether this holds true for the insulinotropic effect of Glp-1, an intraperitoneal glucose tolerance test was also performed with the Glp-1 analog exendin-4 in the wild-type and E1-DN heterozygous mice. As seen in Fig. 8*C*, exendin-4 effectively improved the glucose tolerance in both the wild-type and E1-DN mice. The E1-DN heterozygous mice were able to respond to exendin-4 by increasing the insulin secretion (Fig. 8*D*) and obtained normoglycemia at the end of the glucose tolerance test. Finally, we studied the insulin release in islets isolated from wild-type and E1-DN heterozygous mice. Islets of homozygous mice could not be studied because they could not be isolated in sufficient numbers. As shown in Fig. 9, the insulin content and the absolute amounts of insulin released were significantly lower in the transgenic than wild-type islets. However, insulin release in response to stimulation by either glucose or glucose plus exendin-4 was well preserved in the transgenic islets. When related to the cellular insulin content, the E1-DN islets even released more insulin than control islets (Fig. 9*B*). Transgene expression was verified also in the isolated islets by immunostaining of human EGF-R. A uniform expression pattern was seen, thus excluding the possibility that only nontransgenic islet cells would have been selected in the isolation process. Also, GLUT-2 expression was similarly decreased in the isolated E1-DN islets as in the intact tissue (data not shown).

DISCUSSION

The present study shows that intact EGF receptor signaling in the pancreatic islet cells is essential for the achievement and maintenance of a sufficient β-cell mass and that defects in this pathway lead to diabetes. Based on our results, this appears to be mainly due to decreased β-cell proliferation and, to a lesser extent, depends on an early defect in β-cell neogenesis. We have previously shown that in the EGF-R–deficient mouse (EGF-R$^{-/-}$), fetal differentiation of β-cells is delayed (14). In the E1-DN mice, there is a partial tissue-specific defect in EGF-R signaling that does not lead to the severe neonatal islet phenotype seen in EGF-R$^{-/-}$ mice but leads to a 50% defect in the number of β-cells at the time of birth. However, the major effect is postnatal because at the age of 2 months the defect is >85%. This can clearly be explained by the lack of a postnatal surge in β-cell proliferation. Hyperglycemia develops within the first 2 weeks, consistent with the failure in β-cell mass expansion.

It is notable that the expression of GLUT-2 was clearly impaired in the transgenic islets. A similar finding was reported in transgenic mice with β-cell–targeted dominant negative fibroblast growth factor-R1 expression (21). However, this may not be of major functional importance, since there is no inherent defect in the capacity of E1-DN islets to release insulin in response to glucose in vitro. It thus seems likely that the hyperglycemia and low insulin secretion observed in vivo are principally caused by the reduced β-cell mass, resulting in a maximally stressed situation for the insufficient number of β-cells.

Intriguingly, there was a consistent gradual improvement of hyperglycemia in all animals after the age of 1 month. Yet, transgene expression remained stable, and there was no obvious recovery of the β-cell mass. What could be the explanation for this? It is known that the number of β-cells per body weight decreases with age in rats (22). Thus, it is conceivable that young rodents require more insulin for the rapid postnatal growth and metabolic changes associated with sexual maturation than later in life. This increased insulin requirement is normally met by the rapid expansion of β-cell mass. Since this does not occur in the E1-DN mice, diabetes develops. In later life, the discrepancy between insulin need and production gradually becomes less obvious and the animals present with a milder hyperglycemia. The insulin deficiency remained relative, since there was no difference in the body weight and no obvious effect on the lifespan of the transgenic diabetic animals. The E1-DN mice are overtly hyperglycemic but still viable and can thus be used as an excellent animal model to study long-term consequences

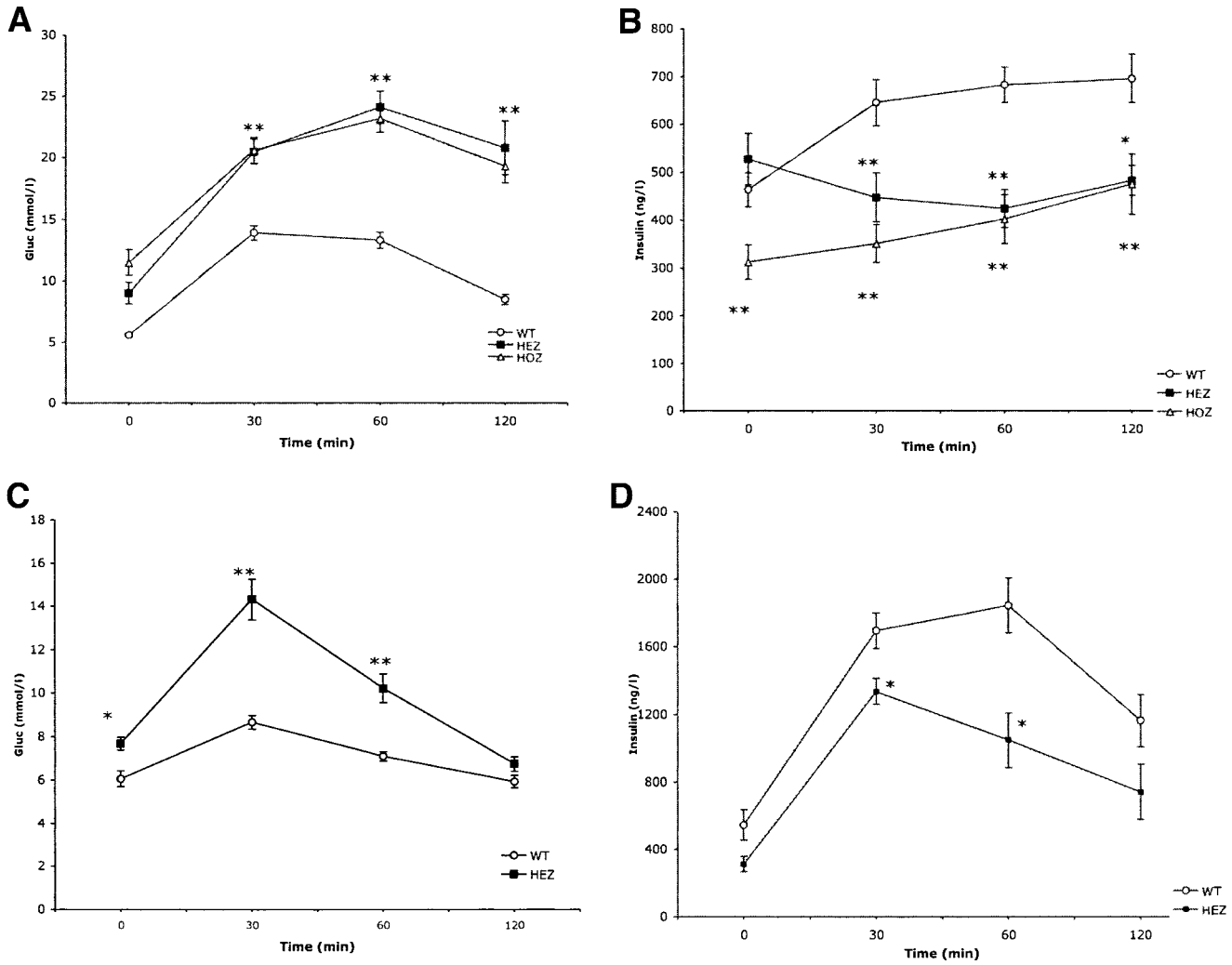

FIG. 8. Intraperitoneal glucose tolerance test. Four-month-old male mice (n = 12 for both wild-type [WT] and E1-DN mice) were injected intraperitoneally with glucose (1 mg/g body wt), and blood glucose (A) and serum insulin (B) values were measured at indicated time intervals. Alternatively, the mice (n = 6 for both wild-type and E1-DN) were injected with exendin-4 (100 ng) intraperitoneally followed by a glucose challenge (1 mg/g body wt), and blood glucose (C) and insulin (D) levels were measured at indicated time intervals. Results are shown as means ± SE. *P < 0.05; **P < 0.01. ○, wild-type; ■, heterozygous [HEZ]; △, homozygous [HOZ].

of hyperglycemia, such as diabetic nephropathy and retinopathy.

The proportions of apoptotic islet cells were similar in young transgenic and wild-type mice. It can be speculated that we failed to observe an increase in β-cell apoptosis in the transgenic mice because of incorrect timing and the short duration of apoptosis in vivo. It is, however, intriguing that there were significantly fewer apoptotic cells in the exocrine compartment of the transgenic mice than in controls. Whether this could be linked with a compensatory mechanism aiming to restore the decreased β-cell mass remains speculative and requires further studies. Nevertheless, several lines of evidence suggest that pancreatic acinar cells may transdifferentiate into β-cells (23,24) and that this process requires EGF-R signaling (25,26).

Recent data have emphasized the role of β-cell proliferation, rather than neogenesis from precursors, as the major mechanism responsible for the control of postnatal β-cell mass (27,28). During late gestation and early postnatal period many organs, including pancreas, undergo massive cell proliferation. In mouse, this results in a fourfold increase in β-cell mass before weaning (28). Experiments with mice deficient in cyclin D2 have clearly demonstrated that this increase is mainly depending on proliferation. Mice deficient in cyclin D2 are born with a normal β-cell mass but are not able to expand their β-cell mass during second postnatal week and develop glucose intolerance (28). The postnatal pancreatic β-cell appears to be a unique cell type in its dependence on cyclin D2. The phenotype of cyclin $D2^{-/-}$ mice closely resembles that of the E1-DN mice. It is likely that the proliferation defect of our mice is directly linked with cell cycle regulation. EGF-R signaling has been shown to lead to activation of cyclin D/cdk4, subsequent Rb phosphorylation, and G1/S transition (29). Unpublished data from our laboratory suggest that an inhibitor of cdk4, p18 (INK4c), is upregulated and cyclin D2 downregulated in the E1-DN islets. Further studies are needed to verify a possible link between EGF-R and cyclin D2 via the p18 pathway.

Targeted inactivation of EGF superfamily members (i.e., EGF, heparin-binding EGF, transforming growth factor-α, amphiregulin, and BTC) in mouse models has shown that they have specific roles in cell proliferation and pattern formation of all germ layers (30–33). Yet, the resulting phenotypes are mild when compared with EGF-$R^{-/-}$ mice,

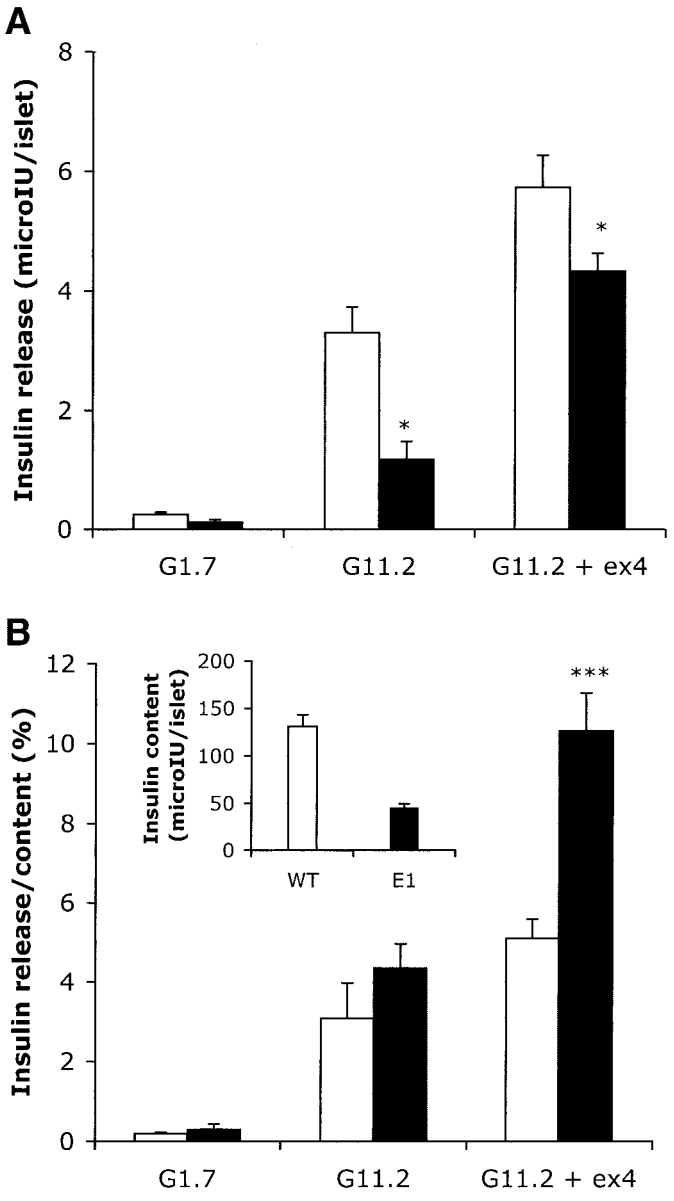

FIG. 9. Insulin release in vitro. Islets were isolated from wild-type (□) and E1-DN heterozygous (■) mice and their insulin release in response to low glucose (1.7 mmol/l; G1.7), high glucose (11.2 mmol/l; G11.2), and high glucose with exendin-4 (G11.2 + ex4) was measured. Intracellular insulin content (μU/islet) is shown in the insert. Data are presented as the means ± SE for four to six individual experiments performed in triplicate. *$P < 0.05$; ***$P < 0.001$.

suggesting excessive redundancy to ensure sufficient EGF-R activation. Accordingly, no single EGF-like growth factor seems to be vital for normal development. Even though BTC is strongly expressed in the pancreas and known to stimulate β-cell proliferation and differentiation (12,34), $BTC^{-/-}$ mice appear to have normal pancreatic differentiation and blood glucose levels (33). Recently, hormones and peptides other than those that are members of the EGF superfamily have been shown to be able to active EGF-R through metalloprotease-mediated ligand shedding (35). Glp-1 is one of these, and it is of particular interest in β-cell biology. It acts through its G-protein coupled receptor and stimulates insulin gene expression, secretion, and β-cell proliferation and inhibits β-cell apoptosis (36). The proliferative effect of Glp-1 has been shown to involve transactivation of EGF-R and phosphatidylinositol 3-kinase signaling (19,20). Our results show that expression of a kinase-negative EGF-R in the islets impairs the activation of the downstream mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways, as evidenced by reduced phosphorylation of ERK and Akt. It is thus possible that endogenous Glp-1 cannot efficiently stimulate β-cell proliferation, and this could partly explain the observed loss of postnatal β-cell expansion. Nevertheless, this was not tested in the current study. However, the potentiating effect of the Glp-1 analog exendin-4 on glucose-stimulated insulin secretion was intact in the E1-DN mice both in vivo and in vitro. This suggests that EGF-R signaling is not involved in the direct insulinotropic effects of Glp-1.

To conclude, our studies show that intact EGF-R signaling is crucial for the achievement of an adequate β-cell mass. It is likely that attenuation of EGF-R in the islets perturbs the actions of a number of growth factors required for both β-cell proliferation and neogenesis.

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