HB-EGF Signaling Is Required for Glucose-Induced Pancreatic β-Cell Proliferation in Rats

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The molecular mechanisms of β-cell compensation to metabolic stress are poorly understood. We previously observed that nutrient-induced β-cell proliferation in rats is dependent on epidermal growth factor receptor (EGFR) signaling. The aim of this study was to determine the role of the EGFR ligand heparin-binding EGF-like growth factor (HB-EGF) in the β-cell proliferative response to glucose, a β-cell mitogen and key regulator of β-cell mass in response to increased insulin demand. We show that exposure of isolated rat and human islets to HB-EGF stimulates β-cell proliferation. In rat islets, inhibition of EGFR or HB-EGF blocks the proliferative response not only to HB-EGF but also to glucose. Furthermore, knockdown of HB-EGF in rat islets blocks β-cell proliferation in response to glucose ex vivo and in vivo in transplanted glucose-infused rats. Mechanistically, we demonstrate that HB-EGF mRNA levels are increased in β-cells in response to glucose in a carbohydrate-response element–binding protein (ChREBP)–dependent manner. In addition, chromatin immunoprecipitation studies identified ChREBP binding sites in proximity to the HB-EGF gene. Finally, inhibition of Src family kinases, known to be involved in HB-EGF processing, abrogated glucose-induced β-cell proliferation. Our findings identify a novel glucose/HB-EGF/EGFR axis implicated in β-cell compensation to increased metabolic demand.

In obesity, the maintenance of glucose homeostasis is dependent on the capacity of the pancreatic β-cell to meet the increased insulin requirements that arise due to insulin resistance. Failure of this mechanism leads to type 2 diabetes (1). Hence, understanding how the β-cell compensates for insulin resistance is a critical prerequisite to defining the pathogenesis of type 2 diabetes.

β-Cell compensation involves both an increase in the capacity to secrete insulin and an increase in mass. In adult rodents, β-cell expansion arises primarily from replication of existing β-cells (2,3). Over the last decade, modeling metabolic stress in rodents has led to the identification of an array of factors, including the insulin receptor (4), neurotransmitters (5), epidermal growth factor receptors (EGFRs) (6), serpin B1 (7), and nutrients (8) that control β-cell proliferation. Prominent among these factors, glucose controls β-cell replication in rodent (9–12) and human (13) islets. Glucose-induced β-cell proliferation requires glucokinase, ATP-sensitive potassium channel closure, and membrane depolarization (10,11). While several studies implicated insulin receptor signaling in glucose-induced β-cell replication (14,15), this observation has been challenged by evidence supporting a role for insulin receptor substrate 2 (IRS2), mammalian target of rapamycin (mTOR) (16), and the carbohydrate-response element–binding protein (ChREBP) (17,18). ChREBP is a glucose-sensing transcription factor that binds DNA with its partner Mlx at carbohydrate-response elements to stimulate glucose-responsive genes (19). Thus, the precise mechanisms underlying glucose-induced β-cell proliferation remain debated.

We established an in vivo model of nutrient excess in rats, in which a 72-h coinfusion of glucose and a lipid

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emulsion triggers a marked increase in β-cell proliferation and mass (20). Subsequent studies identified a signaling cascade involving EGFR–mTOR–FoxM1 that underlies the β-cell response to nutrient infusion (21). In support of these findings, EGFR loss-of-function prevents compensatory β-cell mass expansion in adult rodents under conditions of physiological (pregnancy) and pathophysiological (high-fat feeding) insulin resistance (6) as well as following partial pancreatectomy (22). However, the identity of the EGFR ligand mediating this effect remains unknown. In previous studies, we discovered that expression of the heparin-binding EGF-like growth factor (HB-EGF) is upregulated in islets from nutrient-infused rats and that exogenous HB-EGF stimulates replication of MIN6 cells and primary rat β-cells (21). HB-EGF is synthesized as a membrane-anchored precursor (proHB-EGF) that is processed by the action of a disintegrin and metalloproteinase (ADAM) to release the soluble active form (23). HB-EGF induces phosphorylation of EGFR and subsequent activation of a downstream signaling cascade, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT.

The aims of this study were *1*) to determine the role of HB-EGF in the β-cell proliferative response to glucose in rat islets ex vivo and in vivo and *2*) to investigate the mechanisms linking glucose to an HB-EGF/EGFR signaling pathway promoting β-cell proliferation.

RESEARCH DESIGN AND METHODS

Reagents and Solutions

RPMI 1640 and qualified FBS were from Invitrogen (Carlsbad, CA). Recombinant HB-EGF and betacellulin (BTC) were from R&D Systems (Minneapolis, MN). The HB-EGF inhibitor CRM197 and the Src family kinase inhibitor PP1 were from Sigma-Aldrich (St. Louis, MO). The EGFR tyrosine kinase inhibitor AG1478 and the mTOR complex 1 inhibitor rapamycin were from LC Laboratories (Woburn, MA). Adenoviruses expressing shRNAs against HB-EGF (Adv-shHBEGF) and control scrambled shRNA (Adv-shCTL) were from Vector Biolabs (Malvern, PA). SmartPool siRNA duplexes against rat ChREBP and control siRNA were obtained from Dharmacon (Lafeyette, CO). Primary antibodies and dilutions are listed in Supplementary Table 1.

Rat Islet Isolation and Adenoviral Infection

All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). Islets were isolated from 2-month-old male Wistar or Lewis rats (Charles River Laboratories, Saint-Constant, Quebec, Canada) by collagenase digestion and dextran density-gradient centrifugation as described (24). For adenoviral infections, isolated islets were partially dissociated and then infected with 100 plaque-forming units of adenoviruses per cell overnight as described (25), after which the medium was replaced with complete medium and cultured for an additional 24 h prior to stimulation ex vivo or transplantation. To ensure that the HB-EGF knockdown was sustained for a period compatible with our ex vivo and in vivo experiments, we measured HB-EGF expression 5 days after infection. In Adv-shHBEGF–infected islets exposed to 16.7 mmol/L glucose, HB-EGF mRNA was reduced by 32 ± 8% ($P < 0.05$; $n = 5$) versus Adv-shCTL–infected islets.

Human Islets

Islets from human donors without diabetes were provided by the Alberta Diabetes Institute IsletCore and the Integrated Islet Distribution Program. The use of human islets was approved by the Institutional Ethics Committee of the CRCHUM (protocol number ND-05–035; Montreal, Quebec, Canada).

Islet Proliferation Ex Vivo

Rat islets were cultured in RPMI 1640 with 10% (v/v) qualified FBS (complete medium) for 72 h in the presence of glucose, 100 ng/mL HB-EGF, or 50 ng/mL BTC as indicated in the figure legends. 5-Ethynyl-2'-deoxyuridine (EdU) (10 μmol/L) was added as indicated. The media were changed every 24 h. At the end of treatment, islets were embedded in optimal cutting temperature (OCT) compound, frozen, sectioned at 8 μm, and mounted on Superfrost Plus slides (Life Technologies Inc., Burlington, Ontario, Canada). Sections were immunostained for insulin (Ins) or Nkx6.1 to mark β-cells and for the proliferative markers Ki67, phospho-histone H3 (pH3), or EdU (Click-iT EdU Imaging Kit; Life Technologies Inc.). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Images were acquired with a fluorescence microscope (Zeiss, Thornwood, NY). Proliferation was calculated as the percentage of double-positive $Ki67^+$ (or $pH3^+$ or EdU^+) and Ins^+ (or $Nkx6.1^+$) cells over the total Ins^+ (or $Nkx6.1^+$) population. At least 1,500 β-cells from 7–17 individual islets were manually counted per condition.

Human islets were handpicked, washed with PBS, and dispersed in accutase (Innovative Cell Technologies, Inc., San Diego, CA) for 10 min at 37°C. At the end of the digestion, cells were washed, resuspended, and plated in 96-well plates (PerkinElmer, Waltham, MA) treated with poly-D-lysine hydrobromide (Sigma-Aldrich). After 24 h, dispersed human islets were cultured in RPMI 1640 with 1% (v/v) human albumin serum (Celprogen, Torrance, CA) for 72 h in the presence of glucose and 100 ng/mL HB-EGF as indicated in the figure legends. The medium was changed every 24 h. At the end of treatment, cells were fixed and immunostained for Ins and EdU. Images were acquired with an Operetta high-content imaging system (PerkinElmer) at ×20 magnification. Approximately 1,500 cells were manually counted per condition.

Static Incubations

Triplicate batches of 10 islets each were sequentially incubated twice with Krebs-Ringer bicarbonate solution

containing 0.1% (w/v) BSA and 2.8 mmol/L glucose for 20 min at 37°C and then incubated for 1 h with 2.8 or 16.7 mmol/L glucose. Intracellular insulin content was measured following acid–alcohol extraction. Insulin was measured by radioimmunoassay using a rat insulin RIA kit (Millipore, Billerica, MA).

Islet Transplantation and Glucose Infusions in Rats

Male Lewis rats weighing 250–350 g (~2-month-old) (Charles River Laboratories) underwent catheterization of the jugular vein for infusion and the carotid artery for sampling as described (26). For islet transplantation, 500 islets isolated from 2-month-old male Lewis rats

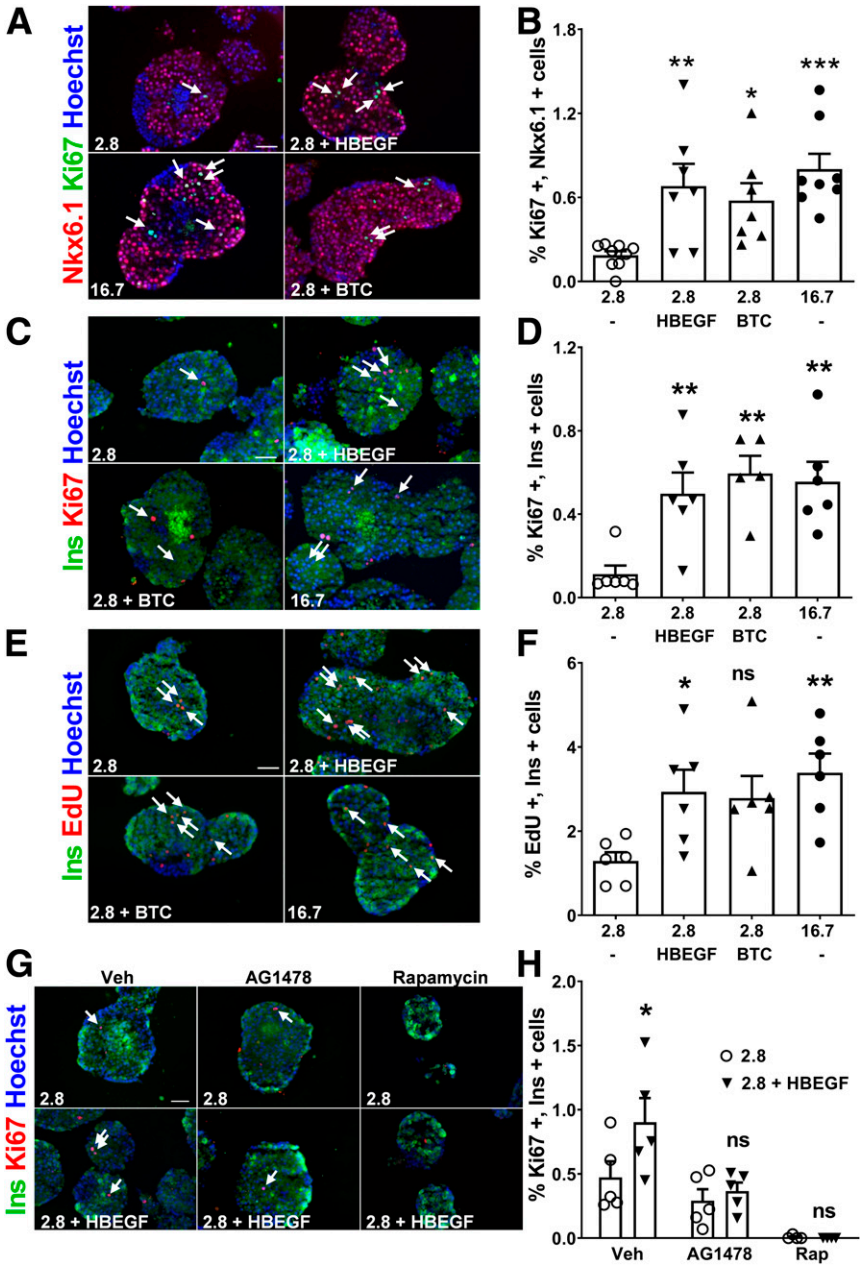

Figure 1—HB-EGF stimulates β-cell proliferation via the EGFR. *A–F*: Isolated rat islets were exposed to 2.8 mmol/L glucose, 16.7 mmol/L glucose, or HB-EGF (100 ng/mL) or BTC (50 ng/mL) in the presence of 2.8 mmol/L glucose for 72 h. *G* and *H*: Isolated rat islets were exposed to 2.8 mmol/L glucose and left untreated or treated with HB-EGF (100 ng/mL) with or without AG1478 (300 nmol/L) or rapamycin (Rap; 10 nmol/L) for 72 h. Proliferation was assessed by Ki67 (*A–D*, *G*, and *H*) or EdU (*E* and *F*) staining and Nkx6.1 (*A* and *B*) or Ins (*C–H*). Representative images of Nkx6.1 (red), Ki67 (green), and nuclei (blue) (*A*) or Ins (green), Ki67 or EdU (red), and nuclei (blue) (*C*, *E*, and *G*) staining. Arrows show nuclei positive for Ki67 and EdU. *B*, *D*, and *H*: The percentage of $Ki67^+Ins^+$ (or $Nkx6.1^+$) cells of total Ins^+ (or $Nkx6.1^+$) cells. *F*: Percentage of EdU^+Ins^+ cells of total Ins^+ cells. Data represent individual values and are expressed as means ± SEM (n = 4–6). Scale bars, 50 μm. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the 2.8 mmol/L glucose control condition. ns, not significant; Veh, vehicle.

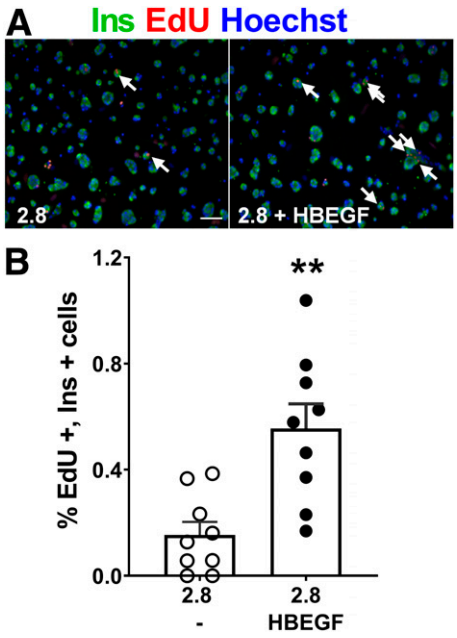

Figure 2—HB-EGF stimulates β-cell proliferation in human islets. Human islets were dispersed and then subjected to 2.8 or 16.7 mmol/L glucose or HB-EGF (100 ng/mL) in the presence of 2.8 mmol/L glucose for 72 h. Proliferation was assessed by EdU and Ins to mark β-cells. *A*: Representative images of Ins (green), EdU (red), and nuclei (blue) staining. Arrows show nuclei positive for EdU. *B*: The percentage of EdU^+Ins^+ cells of total Ins^+ cells. Data represent individual values and are expressed as means ± SEM ($n = 9$). Scale bar, 50 μm. $^{**}P < 0.01$ as compared with the 2.8 mmol/L glucose control condition.

were infected with Adv-shHBEGF or Adv-shCTL, as described above, and injected via a cannula under the left kidney capsule during the catheterization surgery. Animals were allowed to recover for 72 h followed by intravenous infusions of either saline (0.9% w/v NaCl) (Baxter, Mississauga, Ontario, Canada) or 70% (w/v) glucose (McKesson, Montreal, Quebec, Canada) for an additional 72 h. The glucose infusion rate was adjusted to maintain plasma glucose at 13.9–19.4 mmol/L throughout the 72-h infusion.

Immunostaining of Tissue Sections

Transplanted kidneys and pancreata were fixed for 4 h in 4% paraformaldehyde and cryoprotected overnight in 30% sucrose. Tissues were then embedded in OCT, frozen, sectioned at 8 μm, and mounted on Superfrost Plus slides (Life Technologies Inc.). Antigen retrieval was performed using sodium citrate buffer, and β-cell proliferation was assessed as described above.

Flow Cytometry of β-Cells

Islets were isolated from male RIP7-RLuc-YFP transgenic rats (27), washed in PBS, and dispersed in accutase for 10 min at 37°C. At the end of the digestion, cells were washed, resuspended in PBS, and passed through a 40-μm filter prior to sorting. Flow cytometric sorting of yellow fluorescent protein (YFP)–positive and –negative cells was carried out using an FACSAria II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA). YFP-expressing cells were detected using the 488-nm laser and 530/30-nm bandpass filter.

Quantitative RT-PCR

Total RNA was extracted from 150 to 200 whole islets or 100,000 sorted islet cells using the RNeasy Micro kit (Qiagen, Valencia, CA). RNA was quantified by spectrophotometry using a NanoDrop 2000 (Life Technologies Inc.), and 1 μg of RNA was reverse transcribed. Real-time PCR was performed by using the QuantiTect SYBR Green PCR kit (Qiagen). Results were normalized to cyclophilin A RNA levels.

Chromatin Immunoprecipitation and Chromatin Confirmation Capture

INS-1 832/13 cell culture, siRNA treatment, RNA isolation, and RT-PCR were performed as described (17). The pool of siRNA duplexes directed against ChREBP was previously shown to significantly decrease ChREBP mRNA (65%) and protein (70%) levels (17). Chromatin immunoprecipitation (ChIP) was performed as previously described (18). Briefly, INS-1 cells were cultured for 16 h in 2 mmol/L glucose followed by 6 h at 2 or 20 mmol/L glucose. An anti-ChREBP or normal rabbit IgG was used for immunoprecipitation, and a genomic region 30 kb downstream from the transcription start site of the HB-EGF gene known to bind ChREBP (28) was amplified by RT-PCR. Chromatin confirmation capture (3C) was performed essentially as described in Hagège et al. (29). INS-1 cells were treated as for ChIP. The sequences of primers used for RT-PCR, ChIP, and 3C are shown in Supplementary Table 2.

Immunoblotting and ELISA

For immunoblotting, proteins were extracted from rat islets and subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies against phospho-EGFR, phospho–S6 ribosomal protein (S6RP), and α-tubulin in 5% (w/v) milk. Signals were revealed using horseradish peroxidase–conjugated anti-rabbit IgG secondary antibodies (Bio-Rad, Hercules, CA) in 5% (w/v) milk and visualized using Western Lighting Plus-ECL (PerkinElmer). Band intensity was quantified using ImageJ software (National Institutes of Health).

HB-EGF was measured by ELISA (MyBioSource, San Diego, CA) in protein extracts from 200 to 300 rat islets treated with glucose for 1 h.

Statistical Analyses

Data are expressed as means ± SEM. Significance was tested using one-way ANOVA with Tukey or Dunnett post hoc test or two-way ANOVA with post hoc adjustment for multiple comparisons, as appropriate, using GraphPad InStat (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

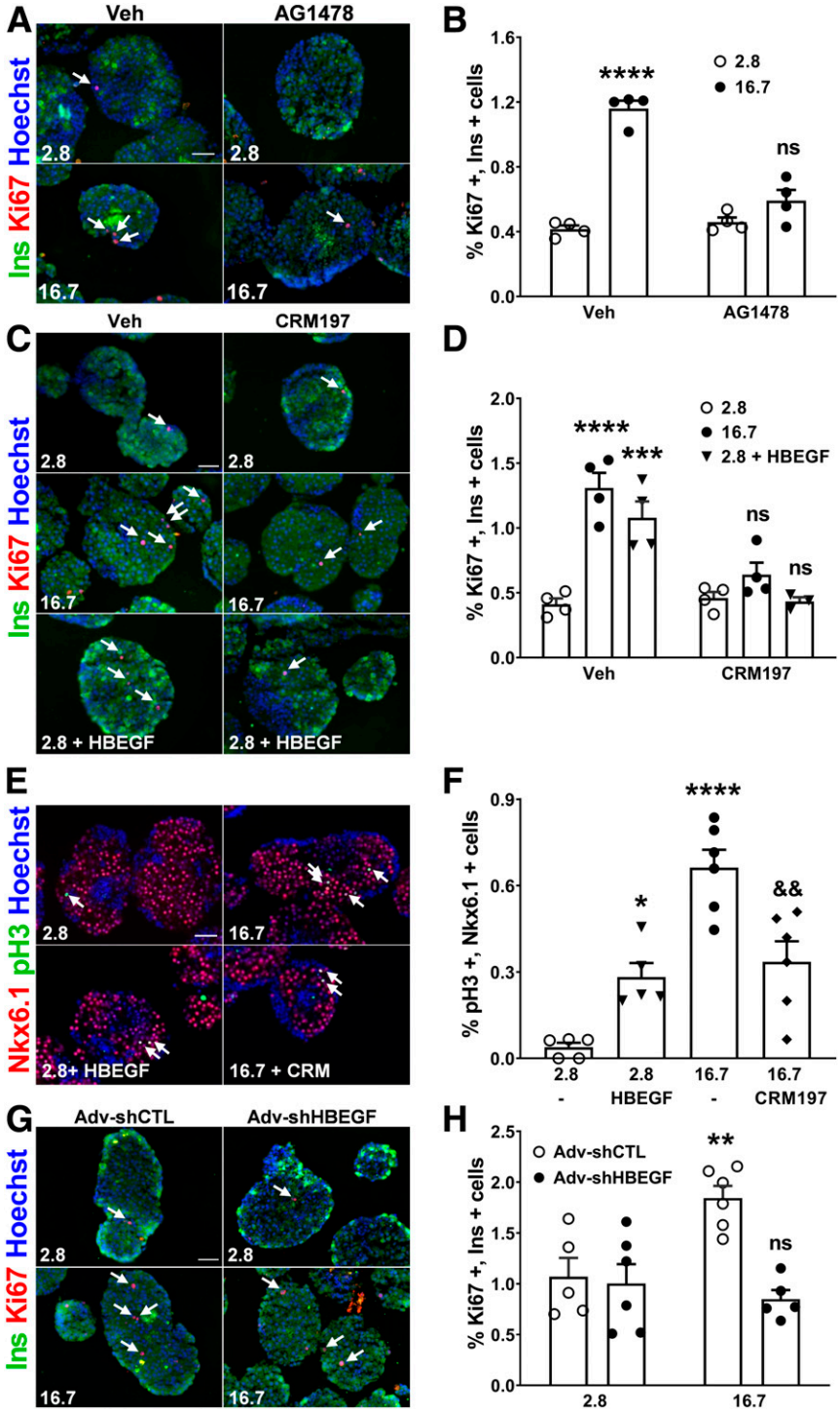

Figure 3— Glucose stimulates β-cell proliferation via HB-EGF/EGFR signaling. *A* and *B*: Isolated rat islets were exposed to 2.8 or 16.7 mmol/L glucose with or without AG1478 (300 nmol/L) for 72 h. *C* and *D*: Isolated rat islets were exposed to 2.8 mmol/L glucose, 16.7 mmol/L glucose, or 2.8 mmol/L glucose plus 100 ng/mL HB-EGF in the absence or presence of 10 μg/mL CRM197 for 72 h. *E* and *F*: Isolated rat islets were exposed to 2.8 mmol/L glucose with or without 100 ng/mL HB-EGF or 16.7 mmol/L glucose with or without 10 μg/mL CRM197 for 72 h. *G* and *H*: Isolated rat islets were infected with Adv-shHBEGF or Adv-shCTL and exposed to 2.8 or 16.7 mmol/L glucose for 72 h. Proliferation was assessed by Ki67 (*A–D*, *G*, and *H*) or pH3 (*E* and *F*) staining and Ins (*A–D*, *G*, *H*) or Nkx6.1 (*E* and *F*) staining to mark β-cells. *A*, *C*, *E*, and *G*: Representative images of Ins (green) or Nkx6.1 (red), Ki67 (red) or pH3 (green), and nuclei (blue) staining. Arrows show nuclei positive for Ki67 or pH3. *B*, *D*, and *H*: Percentage of $Ki67^{+}Ins^{+}$ cells of total Ins^{+} cells. *F*: Percentage of $pH3^{+}Nkx6.1^{+}$ cells of total $Nkx6.1^{+}$ cells. Data represent individual values and means ± SEM ($n = 4–6$). Scale bars, 50 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as compared with the 2.8 mmol/L glucose condition; $^{\&\&}P < 0.01$ as compared with 16.7 mmol/L glucose. ns, not significant; Veh, vehicle.

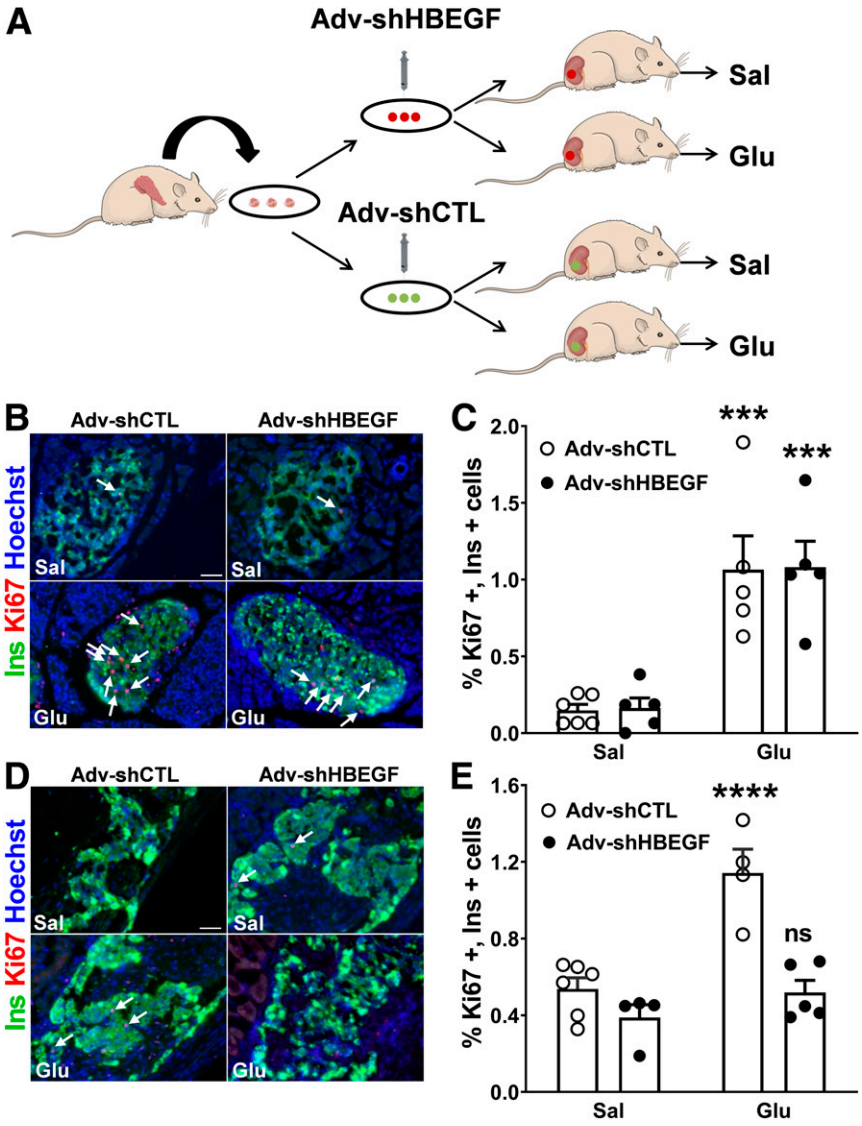

Figure 4—HB-EGF is required for glucose (Glu)–induced β-cell proliferation in vivo. *A*: Isolated rat islets were infected with Adv-shHBEGF or Adv-shCTL and transplanted under the kidney capsule of 2-month-old Lewis rats infused with saline (Sal) or Glu for 72 h. *B–E*: Proliferation was assessed by Ki67 and Ins staining. *B* and *D*: Representative images of Ins (green), Ki67 (red), and nuclei (blue) staining in the pancreas (*B*) or transplanted islets (*D*). Arrows show nuclei positive for Ki67. *C* and *E*: The percentage of $Ki67^{+}Ins^{+}$ cells of total Ins^{+} cells in the pancreas (*C*) and transplanted islets (*E*). Data represent individual values and means ± SEM (n = 4–6). Scale bars, 50 μm. ***$P < 0.001$, ****$P < 0.0001$ as compared with the Sal condition. ns, not significant.

Data and Resource Availability

All data generated or analyzed during this study are included in the published article (and the Supplementary Data). No applicable resources were generated or analyzed during the current study.

RESULTS

HB-EGF Induces β-Cell Proliferation via EGFR–mTOR Signaling

We previously showed that HB-EGF stimulates β-cell proliferation in dispersed rat islets (21). To confirm and extend these findings, we assessed the β-cell proliferative response to HB-EGF in intact rat islets after a 72-h exposure using either Ki67 or EdU labeling to mark proliferating cells and Ins or Nkx6.1 to mark β-cells (Fig. 1). In the presence of 2.8 mmol/L glucose, 100 ng/mL HB-EGF or 50 ng/mL BTC increased the percentage of Ki67-positive β-cells to levels comparable to those detected in response to 16.7 mmol/L glucose (Fig. 1*A–D*). Similar results were obtained when using EdU as a proliferative marker (Fig. 1*E* and *F*). Exposing islets to the EGFR tyrosine kinase inhibitor AG1478 (300 nmol/L) or the mTOR complex 1 inhibitor rapamycin (10 nmol/L) abrogated HB-EGF–induced β-cell proliferation (Fig. 1*G* and *H*). In isolated dispersed human islets, exposure to HB-EGF for 72 h also induced β-cell proliferation (Fig. 2).

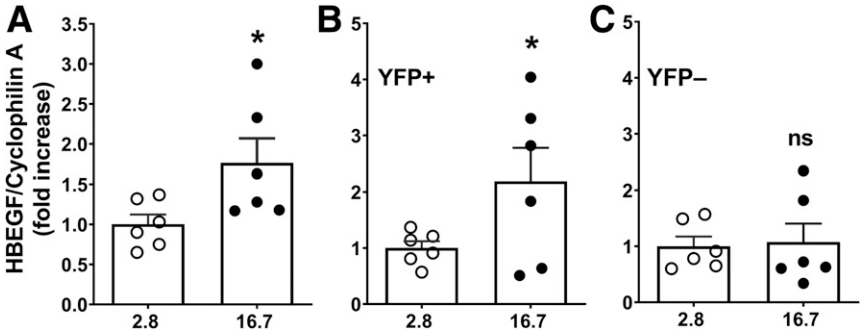

Figure 5— Glucose increases HB-EGF gene expression in the β-cell. HB-EGF mRNA was measured in isolated intact rat islets (*A*) or in FACS-sorted YFP-positive (*B*) and YFP-negative (*C*) cells from isolated RIP7-RLuc-YFP islets following exposure to 2.8 or 16.7 mmol/L glucose for 24 h. mRNA was determined by quantitative RT-PCR and normalized to cyclophilin A. Data are presented as the fold increase over the 2.8 mmol/L glucose condition and represent individual values and means ± SEM ($n = 5$ to 6). *$P < 0.05$ as compared with the 2.8 mmol/L glucose condition. ns, not significant.

We then asked whether HB-EGF affects insulin secretion in rat islets. Isolated islets were exposed to HB-EGF either simultaneously with glucose during a 1-h static incubation to measure insulin secretion or during the 24-h period preceding the static incubation. Neither acute nor prolonged exposure to HB-EGF significantly affected insulin secretion or insulin content (Supplementary Fig. 1). These results indicate that exogenous HB-EGF promotes rat β-cell proliferation via EGFR and mTOR without significantly affecting insulin secretion.

Glucose-Induced β-Cell Proliferation in Isolated Rat Islets Requires HB-EGF/EGFR Signaling

Given that glucose is a known β-cell mitogen (9–12), we next examined the contribution of HB-EGF/EGFR signaling to glucose-induced β-cell proliferation. Treatment of rat islets for 72 h with 16.7 mmol/L glucose led to an approximately threefold increase in Ki67 staining compared with 2.8 mmol/L glucose (Fig. 3*A* and *B*). Addition of AG1478 completely prevented the glucose-induced increase in β-cell proliferation (Fig. 3*A* and *B*). Likewise, the HB-EGF inhibitor CRM197 (10 μg/mL) blocked the stimulatory effect of glucose on β-cell proliferation (Fig. 3*C* and *D*). Similar findings were obtained by labeling rat islets with Nkx6.1 and the M-phase marker pH3 after exposure to HB-EGF, 16.7 mmol/L glucose, or 16.7 mmol/L glucose^{+} CRM197 (Fig. 3*E* and *F*). To further substantiate the implication of HB-EGF in glucose-induced β-cell proliferation, we infected isolated rat islets with Adv-shHBEGF or Adv-shCTL (Fig. 3*G* and *H*). Following a 72-h exposure to 16.7 mmol/L glucose, Adv-shHBEGF–infected islets did not display any increase in β-cell proliferation (Fig. 3*G* and *H*). Collectively, these results demonstrate that HB-EGF/EGFR signaling is required for glucose-induced β-cell proliferation in isolated rat islets.

Glucose-Induced β-Cell Proliferation in Transplanted Rat Islets Requires HB-EGF

To test whether islet-derived HB-EGF is necessary for glucose-induced β-cell proliferation in vivo, islets infected with either Adv-shHBEGF or Adv-shCTL were transplanted under the kidney capsule of Lewis rats. The rats were then infused with saline or glucose for 72 h (Fig. 4*A*). Average blood glucose levels and glucose infusion rates were not different between both groups (Supplementary Fig. 2). As expected, the glucose infusion increased the percentage of Ki67-positive β-cells in the endogenous pancreas to the same extent in Adv-shCTL and Adv-shHBEGF transplant recipients (Fig. 4*B* and *C*). Adv-shCTL–infected islet grafts also showed increased β-cell proliferation in response to glucose infusion (Fig. 4*D* and *E*). In contrast, Adv-shHBEGF–infected islets were unresponsive to glucose (Fig. 4*D* and *E*). These data demonstrate that, as observed in isolated islets (Fig. 3), HB-EGF/EGFR signaling is required for glucose-induced β-cell proliferation in vivo.

HB-EGF Gene Expression Is Upregulated in β-Cells in Response to Glucose

As we previously showed that infusion of glucose and lipids in rats increases HB-EGF mRNA levels in islets (21), we asked whether glucose alone was sufficient to stimulate HB-EGF expression in isolated islets. Indeed, isolated rat islets exposed to 16.7 mmol/L glucose for 24 h displayed a 1.5-fold increase of HB-EGF mRNA compared with 2.8 mmol/L glucose (Fig. 5*A*). To determine whether the increase in islet HB-EGF gene expression was primarily in β-cells, we used a transgenic rat expressing YFP under the control of the Ins2 promoter (RIP7-RLuc-YFP) (27) to enrich for β-cells by flow cytometry after glucose treatment. Glucose augmented HB-EGF mRNA levels in the YFP-positive (β-cell enriched) (Fig. 5*B*) cells, but not the YFP-negative (Fig. 5*C*) fraction, suggesting that glucose stimulates HB-EGF gene expression in rat β-cells.

Glucose Stimulates HB-EGF Gene Expression via ChREBP

ChREBP is a key mediator of glucose-induced transcriptional changes (28). Therefore, we asked whether HB-EGF is a direct target of ChREBP. Consistent with the results shown in Fig. 5, glucose increased HB-EGF expression in

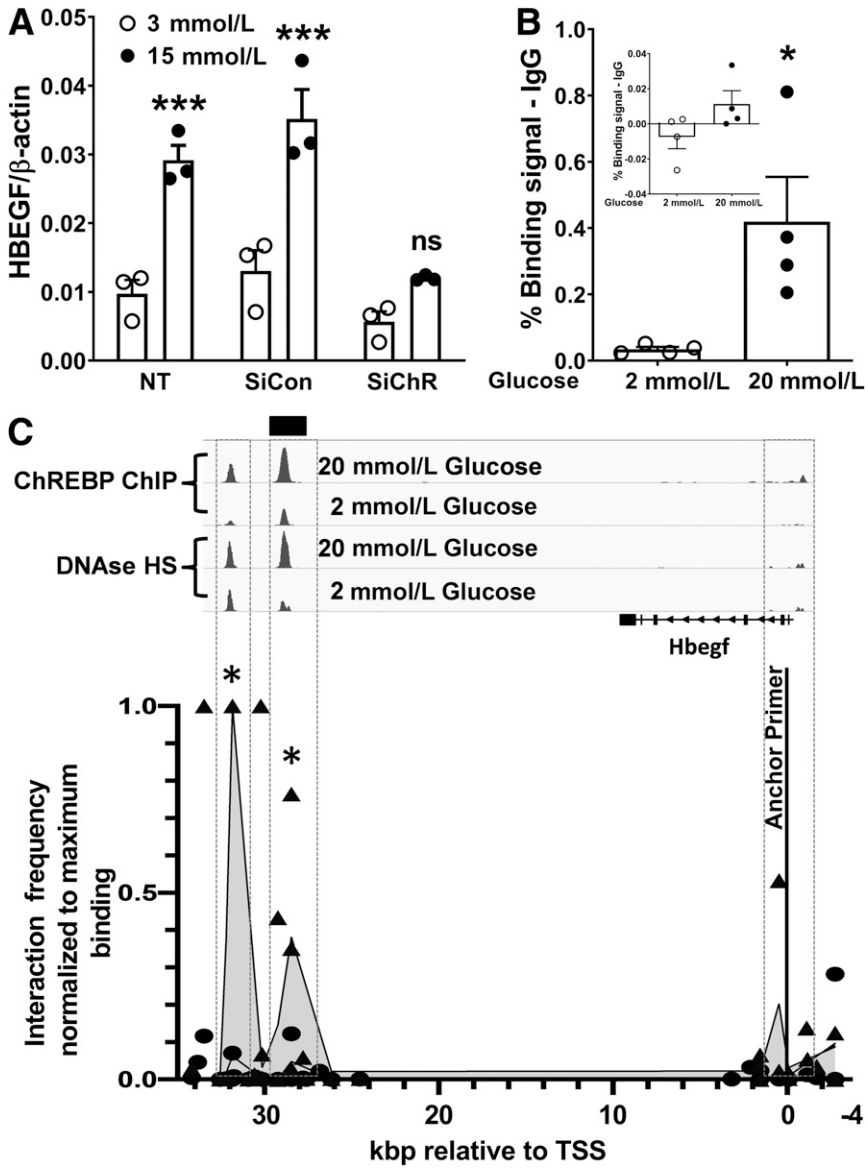

Figure 6—ChREBP mediates glucose-induced HB-EGF gene expression in INS-1 cells. *A*: HB-EGF RNA was measured in INS-1 cells exposed to 3 or 15 mmol/L glucose for 18 h in the presence of a control siRNA (SiCon) or an siRNA directed against ChREBP (SiChR) ($n = 3$). mRNA was determined by quantitative RT-PCR and normalized to β-actin. *B*: ChREBP binding to a genomic region 30 kb downstream from the transcription start site of the HB-EGF gene known to bind ChREBP (black bar in *C*, top panel) was assessed in INS-1 cells exposed to 2 or 20 mmol/L glucose for 6 h followed by ChIP using an antibody against ChREBP or control IgG ($n = 3$). Data indicate the percent binding after subtraction of the IgG control. *B* (inset): Pklr coding region serves as a negative control. *C* (top): Genome browser view of 38,000 bp of the genomic locus spanning the transcription start site (TSS) of the HB-EGF gene showing the ChREBP binding (ChREBP ChIP) and DNAse hypersensitivity sites (DNAse HS) downstream of the gene (28). Black bar is the region amplified in *B*. *C* (bottom): 3C data from INS-1 cells treated as in *B*, aligned to the genome browser and expressed as interaction frequency normalized to maximum interaction ($n = 3$). Black line, anchor primer. Shaded gray added for clarity represents interaction frequency after 20 mmol/L glucose treatment. Data are expressed as means ± SEM. $^*P < 0.05$, $^{***}P < 0.001$ as compared with the control condition. kbp, kilobase pair; ns, not significant; NT, nontransfected.

untransfected INS-1 cells and in cells transfected with a control siRNA (Fig. 6*A*). In contrast, siRNA-mediated knockdown of ChREBP abolished the glucose response (Fig. 6*A*). ChREBP ChIP-sequencing and DNase-sequencing analyses of INS-1 cells exposed to glucose identified putative enhancer elements containing canonical ChREBP binding sites located ~30 kb downstream of the HB-EGF transcription start site (28). ChIP analysis for one of these elements showed that a 6-h exposure to 20 mmol/L glucose significantly increased ChREBP binding, whereas binding to a control region was unchanged (Fig. 6*B*). Furthermore, 3C analysis revealed increased interactions between these enhancers and the HB-EGF promoter in the presence of 20 mmol/L glucose (Fig. 6*C*). These results show that glucose-induced HB-EGF gene expression is mediated by direct binding of ChREBP to enhancers located 3′ to the HB-EGF gene.

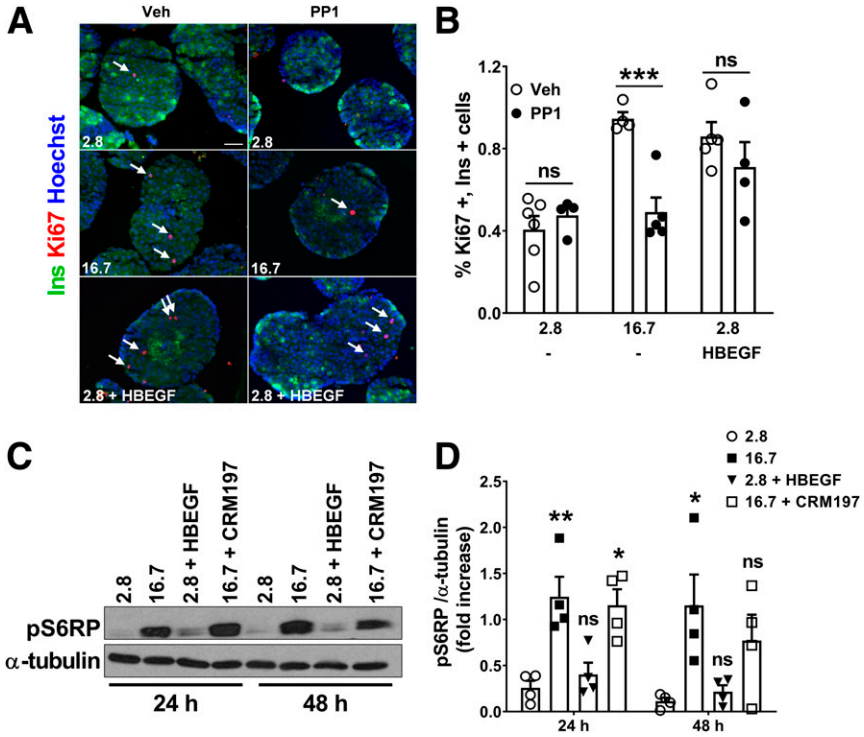

Figure 7— Src is required for glucose- but not HB-EGF–induced β-cell proliferation, and glucose-induced mTOR activation does not require HB-EGF. *A* and *B*: Isolated rat islets were exposed to 2.8 or 16.7 mmol/L glucose or HB-EGF (100 ng/mL) in the presence of 2.8 mmol/L glucose for 72 h with or without the Src inhibitor PP1 (1 μmol/L). Proliferation was assessed by Ki67 staining and Ins. *A*: Representative images of Ins (green), Ki67 (red), and nuclei (blue). Arrows show nuclei positive for Ki67. *B*: The percentage of $Ki67^{+}Ins^{+}$ cells of total Ins^{+} cells. Scale bar, 50 μm. *C* and *D*: Isolated rat islets were exposed to 2.8 or 16.7 mmol/L glucose or HB-EGF (100 ng/mL) in the presence of 2.8 mmol/L glucose with or without CRM197 (10 μg/mL) for 24 and 48 h. Representative Western blot (*C*) of phospho-S6RP (pS6RP) and α-tubulin and densitometric quantification (*D*) of pS6RP normalized to α-tubulin. Data represent individual values and means ± SEM (n = 4–6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the 2.8 mmol/L glucose condition or as indicated in the graph (*B*). ns, not significant; Veh, vehicle.

Glucose-Induced β-Cell Proliferation Is Dependent on Src Upstream of EGFR Activation, but Glucose-Induced mTOR Activation Does Not Require HB-EGF

Processing of proHB-EGF by ADAM proteins releases the active form that binds and activates EGFR (23). Previous studies in mesangial cells suggest that glucose-induced proteolytic processing of HB-EGF requires Src activation (30). Therefore, we investigated the role of Src family kinases in glucose-induced β-cell proliferation. Addition of the Src inhibitor PP1 abrogated the β-cell proliferative response to 16.7 mmol/L glucose but not to HB-EGF (Fig. 7*A* and *B*), consistent with the possibility that glucose promotes proHB-EGF cleavage via Src followed by HB-EGF activation of EGFR. To assess glucose-stimulated HB-EGF shedding, we attempted to measure HB-EGF levels in islet-conditioned media following a 1-h exposure to 16.7 mmol/L glucose. Unfortunately, HB-EGF levels in the samples were below the detection limit of the assay. However, we observed a trend toward an increase in total HB-EGF levels in islet extracts (Supplementary Fig. 3), which, although not statistically significant, is consistent with the glucose-induced HB-EGF expression shown in Figs. 5*A* and 6*A*.

Glucose-induced β-cell proliferation is dependent on mTOR activation (16). As the mitogenic effect of HB-EGF was also dependent on mTOR in rat islets (Fig. 1*G* and *H*), we asked whether mTOR activation by glucose is dependent on HB-EGF. Exposing islets for 24 and 48 h to 16.7 mmol/L glucose led to a significant increase in phosphorylation of the mTOR substrate S6RP (Fig. 7*C* and *D*). However, HB-EGF did not increase S6RP phosphorylation, and blocking HB-EGF with CRM197 did not affect glucose-induced mTOR activation (Fig. 7*C* and *D*). Hence, glucose activation of mTOR is independent of HB-EGF.

DISCUSSION

The results of this study demonstrate a critical role for HB-EGF in glucose-induced β-cell proliferation in rat β-cells. Exposing isolated islets to exogenous HB-EGF induced β-cell proliferation, whereas blocking HB-EGF signaling by inhibiting either EGFR or HB-EGF completely prevented the proliferative response. In vivo, silencing HB-EGF prevented the increase in β-cell proliferation in islets transplanted under the kidney capsule of glucose-infused rats. Taken together, our data identify

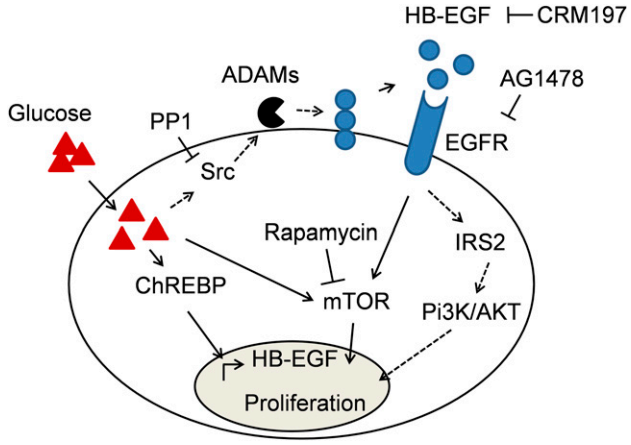

Figure 8—Proposed mechanism of glucose/HB-EGF/EGFR axis controlling β-cell proliferation. An increase in the soluble, active form of HB-EGF is mediated by glucose-induced ChREBP, which increases HB-EGF gene expression, and by glucose-induced Src, which is coupled to metalloprotease (ADAM)–dependent proHB-EGF processing. Subsequent binding of HB-EGF to the β-cell EGFR activates signaling pathways including mTOR but also possibly MAPK, PI3K/AKT, and IRS2 that together promote β-cell proliferation. Inhibitors used in this study to block glucose- and HB-EGF–induced β-cell proliferation are indicated.

a glucose/HB-EGF/EGFR axis that controls β-cell proliferation. Mechanistically, we showed that HB-EGF gene expression is induced by glucose in the β-cell through the action of ChREBP. In addition, we found that glucose-induced, but not HB-EGF–induced, β-cell proliferation is blocked by Src inhibition. As Src family kinases are involved in EGFR transactivation via ADAM metalloproteases, we propose a mechanism by which glucose activates ChREBP and Src to promote HB-EGF gene expression and HB-EGF membrane shedding, respectively, and subsequently EGFR downstream signaling and cell cycle activation (Fig. 8).

Our previous (21) and current results are in agreement with studies showing that overexpression of HB-EGF by retrograde injection of adenoviruses into the pancreatic duct leads to proliferation of pre-existing β-cells in adult mice (31). In contrast, no increase in β-cell proliferation was observed following HB-EGF expression in developing mouse β-cells (32). However, the presence of pancreatic fibrosis, stromal expansion, and islet dysfunction in this model may have precluded such an effect. Interestingly, overexpression of HB-EGF (31) or BTC (33) in pancreatic ducts promotes β-cell neogenesis, and EGF gain-of-function studies in human duct cells (34,35) support a similar conclusion. Hence, we propose that the major effect of HB-EGF is to promote proliferation of existing β-cells, but that β-cell neogenesis could also contribute to its overall beneficial effects on β-cell mass. In contrast to its effects on β-cell proliferation, acute and extended (24-h) exposure to HB-EGF did not alter insulin secretion or insulin content in rat islets ex vivo. However, positive, antidiabetic effects of HB-EGF on the β-cell were demonstrated in multiple low-dose streptozotocin diabetic mice by which combined treatment of gastrin and HB-EGF led to improved islet function due in part to a reduction in insulitis (36). Further studies will be required to fully elucidate the pleotropic effects of HB-EGF on pancreatic islets.

We found that AG1478, a specific inhibitor of EGFR with minimal activity toward other ErbB isoforms, completely abrogates HB-EGF–induced β-cell proliferation. As HB-EGF signals via EGFR (ErbB1) and ErbB4 but not ErbB2 or ErbB3 (37), and EGFR is expressed in β-cells, whereas ErbB4 is only weakly expressed in rodent islets (38), we propose that HB-EGF acts predominantly via EGFR to promote β-cell proliferation.

EGFR inhibition, loss-of-function, and dominant-negative studies in adult rodents in the context of pathophysiological and physiological metabolic stress (6,21,39) and partial pancreatectomy (22) suggest that β-cell EGFR underlies the maintenance of glucose homeostasis by transducing signals that increase β-cell proliferation and mass. Notwithstanding a role for BTC downstream of glucagon-like peptide 1 (40), however, attempts to investigate the role of EGFR ligands in the regulation of β-cell mass and function have been limited to gain-of-function approaches (31,33,41,42), whereas the identification of endogenous ligands contributing to β-cell compensation is unknown. We found that glucose, a key effector of regulation of β-cell mass in the face of increased insulin demand (11), requires HB-EGF signaling. When rat islets were exposed to glucose ex vivo or in vivo, the β-cell mitogenic response was dependent on both EGFR and HB-EGF. Although HB-EGF was essential for the glucose response, whether HB-EGF is the sole endogenous EGFR ligand acting during β-cell compensation to metabolic stress remains an open question. BTC (41), epiregulin (43), transforming growth factor-α, and EGF (41,44) exert mitogenic effects on the β-cell and are expressed in developing (45) and adult (38) rodent islets and during β-cell neogenesis (46). Hence, different EGFR ligands likely contribute to β-cell compensation in a context-dependent manner.

In previous studies, we showed that HB-EGF gene expression is upregulated in islets following nutrient infusion in rats (21), and a similar trend was found in obese, diabetes-resistant (B6) mice (47). Our present results suggest that the increase in HB-EGF gene expression is due, at least in part, to the direct action of glucose. They are consistent with the time- and dose-dependent increase in HB-EGF gene expression observed in response to glucose in INS-1 cells (28) and rat islets (48). In addition, we found that ChREBP is necessary for HB-EGF gene expression and that ChREBP binds a 3′ HB-EGF gene enhancer element. Primary targets of ChREBP in the β-cell include RORγ and Myc (28), whereas the cell cycle regulatory cyclins and cyclin-dependent kinases, which lack ChREBP binding sites, respond to glucose in a delayed manner due to their dependency on first-phase factors (17,28). Hence, downstream of ChREBP, HB-EGF/EGFR signaling could play a role alongside first-phase transcription factors to drive

cell cycle regulators and initiate β-cell cycle progression in response to glucose (Fig. 8).

Although membrane-anchored proHB-EGF may be involved in juxtacrine signaling (49), the major effects of HB-EGF in the β-cell are likely mediated by the soluble form generated by proteolytic processing of proHB-EGF. In mesangial cells, glucose promotes HB-EGF shedding and EGFR transactivation through Src-dependent activation of metalloproteases (30). Our results showing that Src inhibition blocked glucose- but not HB-EGF–induced β-cell proliferation suggest that this phenomenon is also operative in β-cells. Consistent with this possibility, short-term exposure of MIN6 and human islets to glucose leads to phosphorylation of the Src family kinase YES (50). Overall, our data are consistent with the model proposed in Fig. 8, in which glucose promotes Src-dependent proHB-EGF processing, leading to HB-EGF shedding and stimulation of β-cell proliferation via paracrine and/or autocrine signaling through the EGFR.

mTOR is an essential mediator of mitogen-induced β-cell proliferation (51). Blocking mTOR activity prevents the mitogenic effects of glucose (16) and, as we showed in the current study, also mitigates HB-EGF–induced proliferation. Surprisingly, however, blocking HB-EGF had no effect on the increase in mTOR activity in response to glucose, yet HB-EGF inhibition completely prevented glucose-induced β-cell proliferation. Hence, we postulate the existence of a parallel signal emanating from EGFR acting alongside the mTOR pathway that is necessary for β-cell cycle engagement. A number of signaling effectors are known to act downstream of EGFR, including MAPK, PI3K/AKT (21), and IRS2 (52), that could contribute to the mitogenic response to HB-EGF (Fig. 8). Full characterization of the signaling pathway linking EGFR to the β-cell mitogenic response will require additional studies examining the potential implication of these kinases.

In conclusion, this study reveals a critical role of HB-EGF/EGFR signaling in glucose-induced β-cell proliferation in rat islets. Future studies will focus on further elucidating the underlying mechanism and assessing the importance of this pathway in human islet pathophysiology.

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Author Contributions. H.M. and M.R.M. designed the experiments and acquired the data. H.M., M.R.M., D.K.S., J.G., and V.P. researched data, analyzed the results, and wrote the manuscript. All authors revised the manuscript and approved the final version. V.P. is the guarantor of this work and, as such, takes full responsibility for the work.

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