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Epidermal Growth Factor Receptor Signaling Promotes Pancreatic β-Cell Proliferation in Response to Nutrient Excess in Rats Through mTOR and FOXM1

The cellular and molecular mechanisms underpinning the compensatory increase in β-cell mass in response to insulin resistance are essentially unknown. We previously reported that a 72-h coinfusion of glucose and Intralipid (GLU+IL) induces insulin resistance and a marked increase in β-cell proliferation in 6-month-old, but not in 2-month-old, Wistar rats. The aim of the current study was to identify the mechanisms underlying nutrient-induced β-cell proliferation in this model. A transcriptomic analysis identified a central role for the forkhead transcription factor FOXM1 and its targets, and for heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), a ligand of the EGF receptor (EGFR), in nutrient-induced β-cell proliferation. Phosphorylation of ribosomal S6 kinase, a mammalian target of rapamycin (mTOR) target, was increased in islets from GLU+IL–infused 6-month-old rats. HB-EGF induced proliferation of insulin-secreting MIN6 cells and isolated rat islets, and this effect was blocked in MIN6 cells by the EGFR inhibitor AG1478 or the mTOR inhibitor rapamycin. Coinfusion of either AG1478 or rapamycin blocked the increase in FOXM1 signaling, β-cell proliferation, and β-cell mass and size in response to GLU+IL infusion in 6-month-old rats. We conclude that chronic nutrient excess promotes β-cell mass expansion via a pathway that involves EGFR signaling, mTOR activation, and FOXM1-mediated cell proliferation.

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Type 2 diabetes occurs when the pancreatic β-cell is unable to compensate for the increase in insulin demand due to insulin resistance in peripheral tissues. The compensatory response of the β-cell to insulin resistance occurs via the following two mechanisms: enhanced insulin secretion and increased β-cell mass. In obese humans, insulin secretion is considerably enhanced to maintain normoglycemia (1). In addition, investigations in human cadaveric pancreata have shown that β-cell mass is increased in obese nondiabetic individuals compared with lean individuals, but decreased in obese

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individuals with impaired fasting glucose and type 2 diabetic patients (2). These observations suggest that alterations of the anatomical compensation of the β-cell in obesity may contribute to the onset of type 2 diabetes.

In rodents, β-cell mass expansion is primarily driven by replication of existing cells (3) and is dynamically regulated in response to insulin resistance induced by obesity, high-fat feeding, or gestation (4). The molecular mechanisms underlying β-cell compensation to insulin resistance are essentially unknown; however, experimental evidence points to the importance of interorgan cross talk between insulin-resistant peripheral tissues and the β-cell. For instance, altered insulin signaling in the liver triggers a large increase in β-cell proliferation (5,6), and circulating factors likely play a role in β-cell adaptation to insulin resistance (7). Importantly, a recent study showed that human islets transplanted into mice subjected to an obesogenic diet undergo a compensatory increase in mass through enhanced proliferation (8).

We have established an in vivo model of chronic nutrient excess in rats, in which a 72-h coinfusion of glucose and Intralipid (GLU+IL) triggers a marked increase in β-cell mass and proliferation at 6 months of age, but not at 2 months of age, despite similar levels of hyperglycemia, hyperlipidemia, and hyperinsulinemia (9). We surmised that the β-cell proliferative response in this model is driven by insulin resistance in response to nutrient excess, which only occurs in 6-month-old rats. This model provides a unique opportunity to identify the molecular mechanisms underlying nutrient-induced β-cell proliferation. The aims of this study were therefore *1*) to characterize the changes in the islet transcriptome in response to the GLU+IL infusion in 6-month-old rats, and *2*) to identify the molecular mechanisms governing the adaptive increase in β-cell proliferation in response to nutrient excess.

RESEARCH DESIGN AND METHODS

Reagents and Solutions

RPMI-1640 and FBS were from Invitrogen (Burlington, ON, Canada). Dulbecco's modified Eagle's medium was from Wisent (Saint Bruno, QC, Canada). AG1478 and rapamycin were from LC Laboratories (Woburn, MA). Recombinant heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) was from R&D Systems (Minneapolis, MN). CRM197 was from Sigma-Aldrich (St. Louis, MO).

Animal Infusions and Drug Treatments

All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal. Infusions were performed as previously described (9). Male Wistar rats weighing 250–300 g (~2 months old) and 500–600 g (~6 months old) (Charles River, Saint-Constant, QC, Canada) were housed under controlled temperature on a 12-h light/dark cycle with unrestricted access to water and standard laboratory chow. The animals were randomized into two groups, receiving either 0.9% saline (SAL) (Baxter, Mississauga, ON, Canada) or 70% dextrose (McKesson Canada, Montréal, QC, Canada) plus 20% Intralipid (a soybean oil emulsion which generates a mixture of ~80% unsaturated/20% saturated fatty acids when coinfused with heparin; Fresenius Kabi, Bad Homburg, Germany) (10) with 20 units/mL heparin (Sandoz Canada, Boucherville, QC, Canada) (i.e., GLU+IL). Infusions were performed using Harvard Apparatus infusion pumps (Pump 33), independently operating two syringes simultaneously. During the infusions, all animals had unrestricted access to food and water. For drug administration, AG1478 or rapamycin were dissolved in N,N-dimethylacetamide (Sigma-Aldrich) to a concentration of 50 mg/mL and then diluted in propylene glycol to generate a 2 mg/mL stock solution. Daily, the stock solution was diluted to the working concentration in 1.2% Tween 80/27% polyethylene glycol 400 (Sigma-Aldrich) and injected intravenously at a dose of 0.5 mg/kg/day. The control group was injected with the same volume of vehicle.

Cell Culture

MIN6 cells (passages 25–30) were cultured as previously described (11). Cells were seeded in six-well plates at a density of 500,000 cells/well. The following day, the cells were cultured in complete medium with increasing concentrations of HB-EGF in the absence or presence of inhibitors for an additional 24 h. For cell counting, cells were fixed with 10% formaldehyde, nuclei were stained by Hoechst reagent (Sigma-Aldrich), and 10 images per well were taken randomly. Cell numbers were determined using ImageJ software (National Institutes of Health).

Rat Islet Proliferation

Isolated rat islets were dispersed and plated in 96-well plates coated with HTB-9 cell extracellular matrix as described previously (12). Dispersed rat islets were treated for 72 h in the absence or presence of 100 ng/mL HB-EGF in complete islet media. The media were changed every 24 h. At the end of treatment, cells were fixed and stained for the β-cell marker pancreatic duodenal homeobox-1 (PDX-1) and the proliferative marker Ki67, as described previously (13). Proliferation was calculated as the percentage of double-positive $Ki67^+$/$PDX\text{-}1^+$ cells over the total $PDX\text{-}1^+$ population.

Human Islets

The use of human islets was approved by the Institutional Ethics Committee of the Centre Hospitalier de l'Université de Montréal. Isolated islets from nondiabetic human cadaveric donors were provided by the Clinical Islet Laboratory at the University of Alberta and the Integrated Islet Distribution Program sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases and the Juvenile Diabetes Research Foundation International.

Transcriptomic Profiling and Quantitative RT-PCR Validation

Rat islets were isolated by collagenase digestion and dextran density gradient centrifugation as described previously (14). Total RNA was extracted from 150 islets with TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy MinElute cleanup kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Microarray analysis was performed on total RNA using the GeneChip Rat Gene 1.0 ST microarrays (five arrays per group; Affymetrix, Santa Clara, CA). One hundred nanograms of total RNA was processed using the Ambion WT Expression Kit (Invitrogen). The resulting fragmented and labeled single-stranded cDNA was processed according to the Affymetrix protocol. The Partek (St. Louis, MO) Genomics Suite was used for data analysis. The data were normalized by Robust Multichip Average algorithm, which uses background adjustment, quantile normalization, and summarization. After correction of statistical significance for multiple comparisons using false discovery rate ($P < 0.01$), the transcripts significantly and differentially expressed by >20% between the GLU+IL and control group were processed for gene set enrichment analyses using Ingenuity Pathways Analysis (application build 192063, content build 14400082; Ingenuity Systems, Redwood City, CA). Selected genes were validated by quantitative RT-PCR (qRT-PCR) as previously described (9). All qRT-PCR results were normalized to cyclophilin A mRNA levels. Primer sequences are described in Supplementary Table 1.

Analytical Measurements

Plasma glucose and free fatty acid (FFA) levels were measured using kits from Wako Chemical (Osaka, Japan). Insulin and glucagon were measured by ELISA (Alpco, Windham, NH).

Immunostaining of Pancreatic Sections

Pancreata were trimmed of fat, weighed, fixed for 3–4 h in 4% paraformaldehyde, and cryoprotected overnight in 30% sucrose. Pancreata were then embedded in OCT (TissueTek), and 8-μm sections were obtained by cryosection (Leica). Antigen retrieval was performed using sodium citrate buffer. Primary antibodies and dilutions are listed in Supplementary Table 2. Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Images were taken with a fluorescence microscope (Zeiss, Thornwood, NY). β-Cell mass and size were determined as previously described (9,15). To assess β-cell proliferation, insulin$^+$ and double-positive insulin$^+$/Ki67$^+$ cells were counted manually for at least 2,000 β-cells per animal.

Immunoblotting

Twenty micrograms of proteins from cells or islets were extracted and resolved by SDS-PAGE as described previously (11). Primary antibodies are listed in Supplementary Table 2. Signals were detected using a horseradish peroxidase–labeled IgG (Bio-Rad, Richmond, CA) and enhanced chemiluminescence (PerkinElmer Canada, Woodbridge, ON, Canada) on Kodak BioMax XAR films (Kodak, Rochester, NY). The bands were quantified by densitometry and ImageJ software (National Institutes of Health).

Expression of Data and Statistics

Data are expressed as the mean ± SEM. Statistical analyses were performed using the Student *t* test or ANOVA followed by two-by-two comparisons using Bonferroni post hoc adjustments, as appropriate using Instat software (GraphPad Software). A P value <0.05 was considered to be significant.

RESULTS

Infusion of GLU+IL Induces Insulin Resistance in 6-Month-Old Wistar Rats

In response to the 72-h GLU+IL infusion, circulating blood glucose and FFA levels were raised to similar levels in 2- and 6-month-old animals (n = 4; difference not significant; Fig. 1*A* and *B*). Insulin levels also increased in response to the GLU+IL infusion but were not different between the two age-groups (n = 4; difference not significant; Fig. 1*C*). Consistent with our previous observation in this model (9), significantly less glucose was required to maintain the same level of glycemia in 6- vs. 2-month-old rats, suggestive of insulin resistance ($P < 0.001$; n = 11–18; Fig. 1*D*). Circulating glucagon levels decreased to the same extent in response to the GLU+IL infusion in both age-groups (Fig. 1*E*). Expression of Ki67, a marker of cell proliferation, was strongly induced in response to the GLU+IL infusion in 6-month-old rats only (Fig. 1*F*). This is also in accordance with the previously observed increase in β-cell mass and number of Ki67-positive β-cells in this model (9).

Transcriptional Profiling of GLU+IL–Infused 6-Month-Old Rat Islets Identifies a Transcriptional Network Involved in Cell Cycle Progression

To gain insight into the molecular mechanisms involved in the regulation of pancreatic β-cell proliferation in response to nutrient-induced insulin resistance, we performed a microarray-based transcriptomic analysis of the islets of 6-month-old rats. Six-month-old Wistar rats were infused with either SAL or GLU+IL for 72 h, and islets were isolated from five animals in each group matched for body weight at the end of the infusion. As shown in Fig. 2*A*, with a false discovery rate <0.01, ~3,000 genes were significantly modulated by the GLU+IL infusion. Overall, half of the genes were upregulated, and half were downregulated. When the cutoff was set to a twofold difference between the SAL group and the GLU+IL group, 300 genes were significantly upregulated, whereas only 30 genes were downregulated by GLU+IL infusion. Within the 300 upregulated genes, a group of genes expressed at low levels in the SAL group were

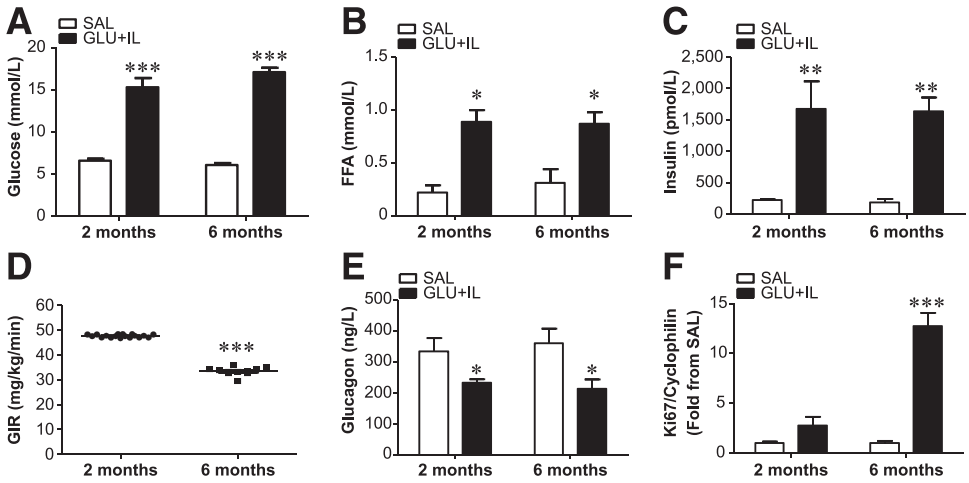

Figure 1—Metabolic parameters in GLU+IL–infused 6-month-old rats. The 2- and 6-month-old Wistar rats were infused with SAL or GLU+IL for 72 h. *A–C*: Plasma glucose, FFA, and insulin levels at the end of the infusion ($n = 4$). *D*: Average glucose infusion rate (GIR) during the infusion ($n = 11–18$). *E*: Plasma glucagon ($n = 4$). *F*: Relative Ki67 mRNA expression in islets isolated at the end of the infusion (normalized to cyclophilin A mRNA and relative to the levels in SAL-infused animals for each age-group; $n = 4$). Data are expressed as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

strongly upregulated in the GLU+IL group (Fig. 2*A*, lower left corner of the graph). Gene set enrichment analysis performed using Ingenuity Pathway Analysis software revealed that these genes were, for the most part, related to cell cycle progression and mitosis (Fig. 2*B*). A more detailed analysis revealed that within this group, most of the direct downstream targets of the forkhead transcription factor FOXM1, a key regulator of the G2/M cell cycle transition, were upregulated by the GLU+IL infusion (Fig. 2*C*). Interestingly, expression of the forkhead transcription factor FOXO3a, a repressor of FOXM1 (16,17), was decreased upon GLU+IL infusion (Fig. 2*C*). In addition, expression of the gene encoding HB-EGF, a potential autocrine/paracrine growth factor inducing β-cell proliferation (18) (Fig. 2*C*), was also upregulated in the GLU+IL group. These changes in expression were validated by qRT-PCR analysis. First, we verified that expression of the proliferation marker Ki67 was enhanced in the GLU+IL group to the same extent as shown in Fig. 1 (data not shown). Second, we confirmed that expression of FOXO3a was repressed by the GLU+IL infusion (Fig. 3*A*), while that of HB-EGF, FOXM1, and the direct targets of FOXM1 Aurora kinase B (AURKB) and Polo-like kinase 1 (PLK1) was markedly induced (Fig. 3*B–E*). FOXM1 protein levels were also significantly increased in the GLU+IL group (Fig. 3*F*). In accordance with the role of HB-EGF as a trophic factor, its expression in individual samples was positively correlated with that of Ki67, FOXM1, AURKB, and PLK1; and negatively correlated with FOXO3a (data not shown). The expression of all these genes remained unchanged in the islet of 2-month-old rats upon GLU+IL infusion (Fig. 3*A–E*). To confirm that this transcriptional network is activated in a more chronic model of nutrient excess, we measured the expression of these genes in islets from C57BL/6 mice fed a high-fat diet for 8 weeks and stratified into the following two groups according to body weight gain: low-diet responders and high-diet responders (19). The expression of FOXM1, AURKB, and PLK1 was significantly increased in islets from high-diet responder mice, albeit to a lesser extent than in infused rats (Supplementary Fig. 1).

HB-EGF Promotes β-Cell Proliferation Through EGF Receptor and Mammalian Target of Rapamycin

To determine the mitogenic potential of HB-EGF in β-cells, we evaluated its ability to stimulate the proliferation of MIN6 cells and dispersed rat islets. Exogenous HB-EGF dose-dependently increased MIN6 cell proliferation after a 24-h treatment (Fig. 4*A*), and this effect was also observed in dispersed rat islets (Fig. 4*B*). This was associated with a time-dependent phosphorylation of S6 ribosomal kinase (S6R), a downstream target of mammalian target of rapamycin (mTOR), in both MIN6 cells (Fig. 4*C*) and human islets (Fig. 4*D*). The phosphorylation of S6R by HB-EGF in human islets was abolished in the presence of the phosphatidylinositol 3 (PI3) kinase inhibitor LY294002 (Fig. 4*E* and *F*). The ability of HB-EGF to enhance the proliferation of MIN6 cells was similar to that of betacellulin, an EGF receptor (EGFR) ligand that has been reported to promote β-cell replication both in vitro and in vivo (20,21) (Fig. 4*G*). Induction of MIN6 cell proliferation in response to HB-EGF was completely blocked in the presence of the EGFR inhibitor AG1478 or the mTOR inhibitor rapamycin (Fig. 4*G* and Supplementary Fig. 2), as well as in the presence of CRM197, a diphtheria toxin mutant that binds to and neutralizes HB-EGF (Fig. 4*H*).

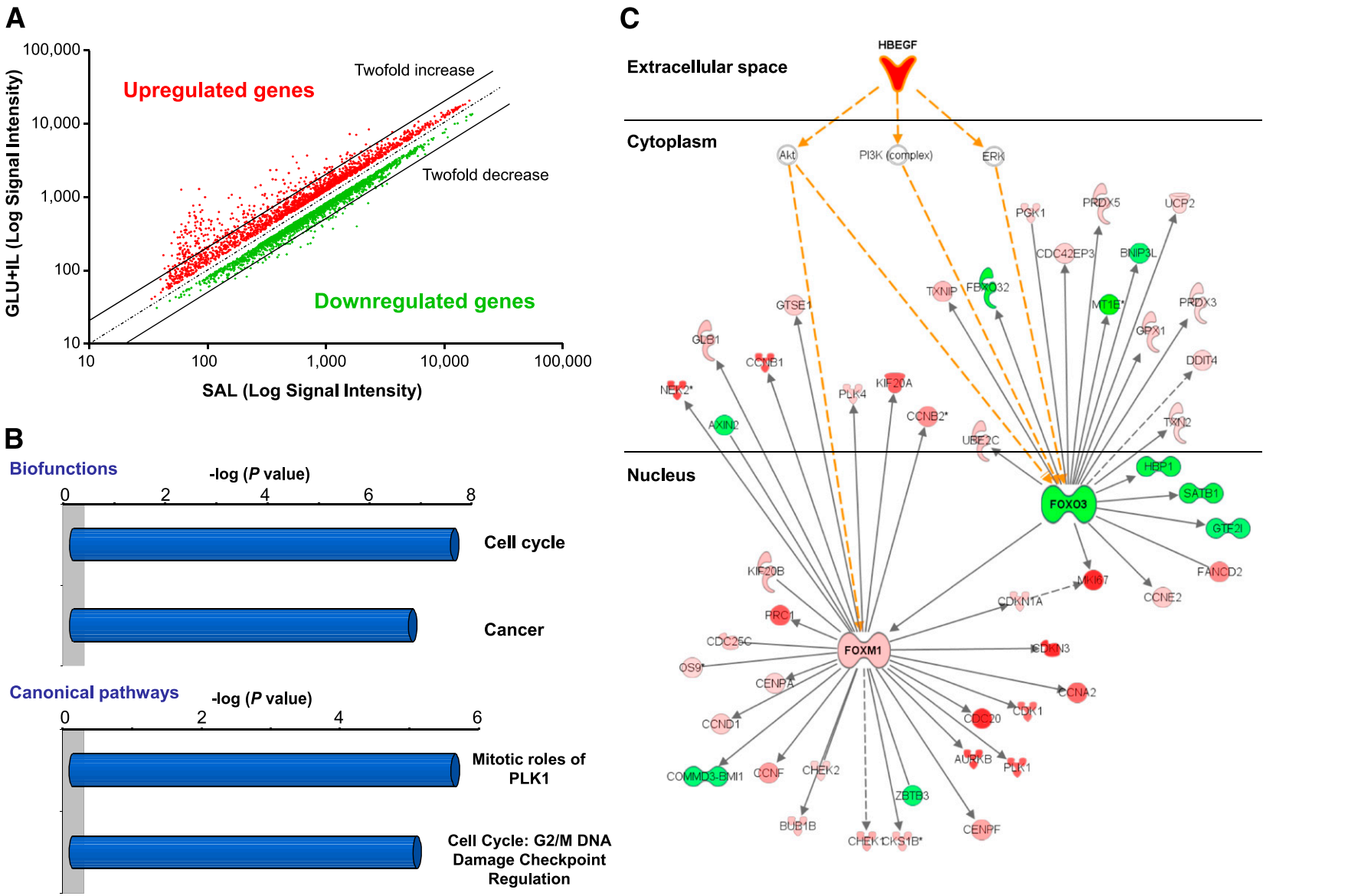

Figure 2—Transcriptomic analysis of isolated islets from GLU+IL–infused 6-month-old rats. *A*: Differentially expressed genes represented in scatter plot graph. *B*: Top-ranked biofunctions and canonical pathways annotated by the Ingenuity Pathway Analysis software (Fisher exact test *P* values are shown). *C*: Representative network of genes involved in cell cycle progression, showing a central role for HB-EGF, FOXM1, and FOXO3a. Upregulated genes are shown in red, and downregulated genes are shown in green. Data were obtained from five animals in each group.

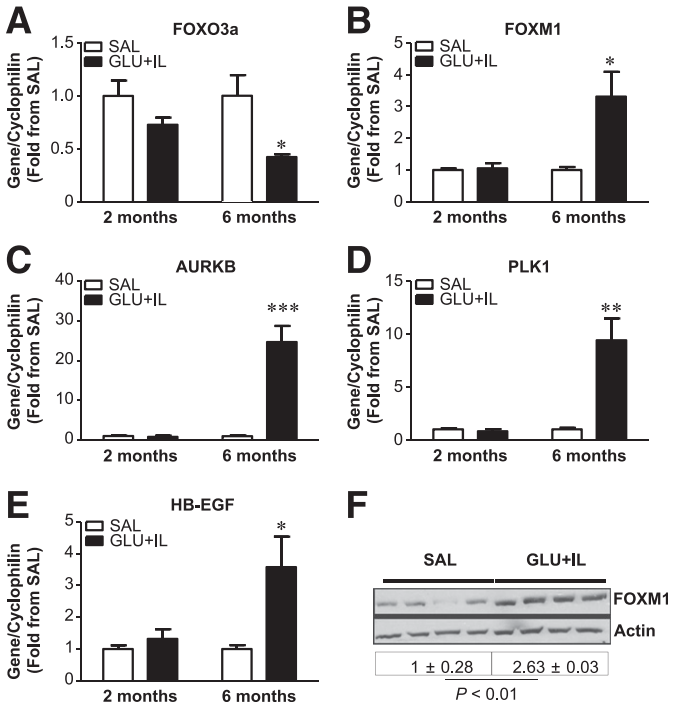

Figure 3—Expression of FOXM1 and its targets is induced in islets from GLU+IL–infused 6-month-old rats. *A–E*: qRT-PCR measurements of mRNA expression normalized to cyclophilin A and relative to SAL-infused animals in each age-group. Data are expressed as the mean ± SEM of 5–11 animals in each group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *F*: Representative Western blot for FOXM1 in four individual 6-month-old rats in each infusion group.

Inhibition of EGFR Signaling and mTOR Inhibits β-Cell Mass Expansion, β-Cell Proliferation, and Prevents Upregulation of FOXM1 and Its Targets in Islets From GLU+IL–Infused 6-Month-Old Rats

To ascertain the implication of the mTOR pathway in GLU+IL–induced β-cell proliferation, we examined S6R phosphorylation in pancreatic sections from 6-month-old GLU+IL–infused rats. As shown in Fig. 5, phosphorylation of S6R was strongly increased in islets from GLU+IL–infused rats (Fig. 5*B*), concomitant with a marked increase in the number of cells staining positive for Ki67 (Fig. 5*A*).

To test the importance of the EGFR and mTOR in β-cell proliferation in response to GLU+IL, we treated another group of 6-month-old rats with AG1478 or rapamycin during the SAL or GLU+IL infusion. In GLU+IL–infused animals, blood glucose and FFA levels were maintained at a similar level, irrespective of the drug treatment (Table 1). The glucose infusion rate was not affected by AG1478 but was significantly reduced in the animals treated with rapamycin (Table 1), which is consistent with the known effect of this drug on insulin resistance (22,23). In accordance with our previous report in this model (9), β-cell mass was markedly increased in GLU+IL–infused 6-month-old rats (Fig. 6*A*). This was also associated with a significant increase in β-cell size (Fig. 6*B*). Remarkably, treatment of the rats with either AG1478 or rapamycin completely prevented both β-cell hyperplasia (Fig. 6*A*) and hypertrophy (Fig. 6*B*).

Consistent with the data shown in Fig. 5, the number of KI67$^+$ β-cells was markedly increased in islets from GLU+IL–infused animals (Fig. 7*A* and *B*). This was associated with increased nuclear staining of FOXM1 (Fig. 7*C*) and AURKB (Fig. 7*D*). Importantly, the increase in Ki67, FOXM1, and AURKB was totally abolished upon treatment of GLU+IL–infused rats with AG1478 or rapamycin (Fig. 7*A–D*). Finally, both AG1478 and rapamycin prevented the decrease in FOXO3a, and the increase in FOXM1, AURKB, and Ki67 mRNA expression in islets from GLU+IL–infused rats (Fig. 7*E*).

DISCUSSION

This study was aimed to characterize the changes in the islet transcriptome in response to nutrient excess and to identify the molecular mechanisms governing the adaptive increase in β-cell proliferation in response to nutrients. Our results show that a 72-h combined infusion of GLU+IL in 6-month-old rats activates a signaling cascade that leads to the induction of FOXM1 and its downstream targets, and is dependent upon EGFR signaling and mTOR activation (Fig. 7*F*). A potential role of HB-EGF as a mitogenic signal acting through the EGFR and mTOR is supported by both its increased expression in islets from GLU+IL–infused 6-month-old rats, and its ability to activate mTOR and enhance proliferation in MIN6 cells and dispersed rat islets. Importantly, HB-EGF also activates the mTOR pathway in human islets in a PI3 kinase–dependent manner.

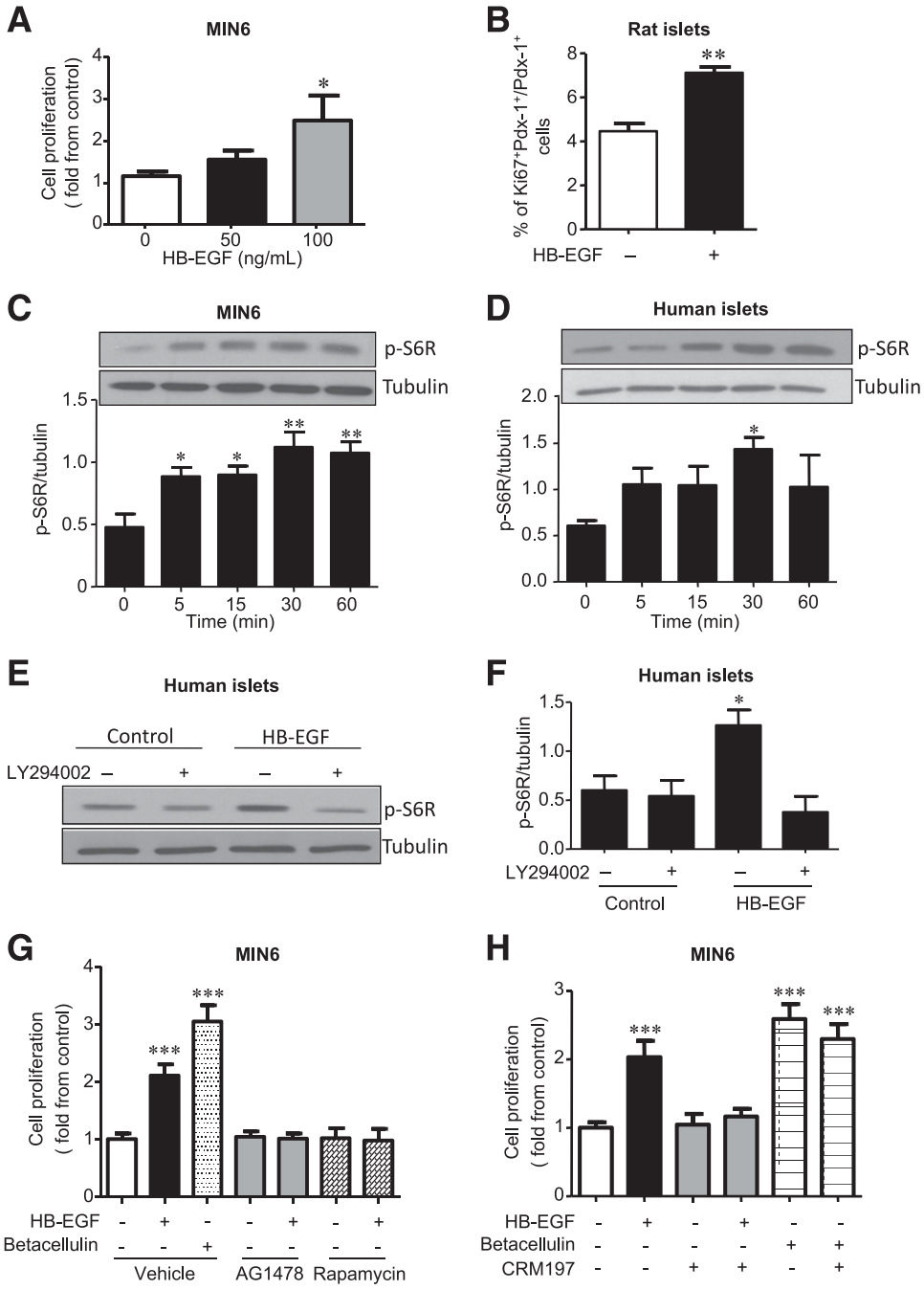

Figure 4—HB-EGF promotes β-cell proliferation in an EGFR/mTOR–dependent manner. *A*, *G*, and *H*: MIN6 cell proliferation was measured by counting nuclei after 24-h treatment with HB-EGF with or without AG1478 (300 nmol/L) or rapamycin (10 nmol/L) and the HB-EGF antagonist CRM197 (10 μg/mL). Data are expressed as the mean ± SEM of three to six experiments. $^{*}P < 0.05$, $^{***}P < 0.001$. *B*: Dispersed rat islets were treated for 72 h in the absence or presence of HB-EGF (100 ng/mL), and cells were fixed and stained for Ki67 and PDX-1. β-Cell proliferation was calculated as the percentage of double-positive $Ki67^{+}/PDX\text{-}1^{+}$ cells over the total $PDX\text{-}1^{+}$ population. Data are expressed as the mean ± SEM of four replicate experiments. $^{**}P < 0.01$. Representative Western blot and densitometric quantification of phospho-S6R (p-S6R) upon HB-EGF treatment in MIN6 cells (*C*) and human islets (*D*). Data are expressed as the mean ± SEM of three replicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. *E* and *F*: Representative Western blot and densitometric quantification of p-S6R upon HB-EGF treatment in the absence (−) or presence (+) of the PI3 kinase inhibitor LY294002 (10 μmol/L). Data are expressed as the mean ± SEM of three replicate experiments. $^{*}P < 0.05$.

In addition to a large increase in insulin secretion, the pancreatic β-cell response to physiological (e.g., pregnancy) or pathological (e.g., obesity) conditions of insulin resistance involves an expansion of β-cell mass, which results, at least in part, from replication of existing β-cells (3). This compensatory response is crucial to maintain normoglycemia in the face of insulin resistance and thereby prevents the development of type 2 diabetes

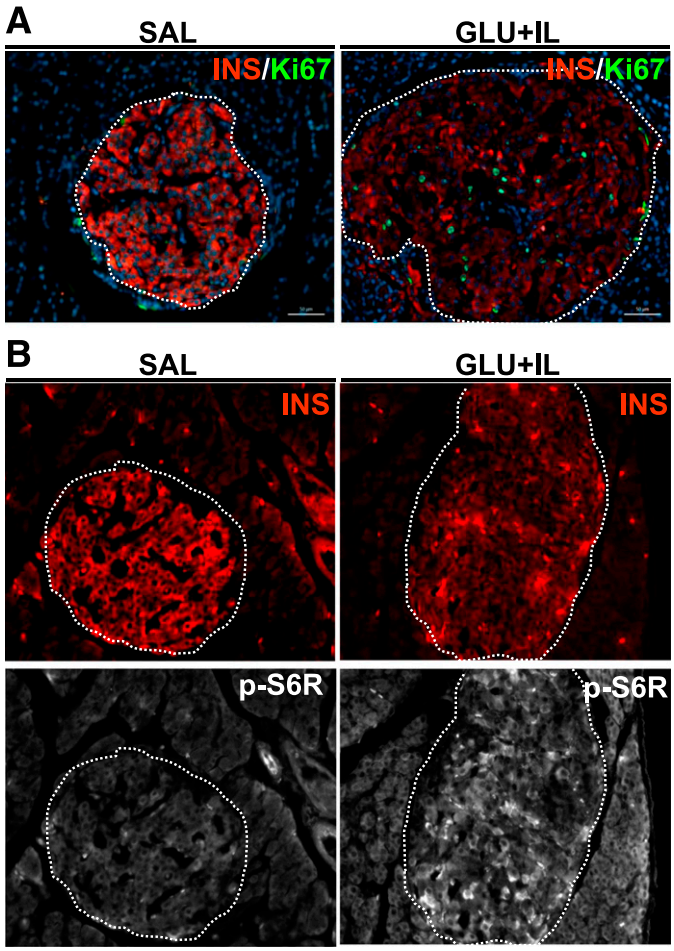

Figure 5—β-Cell proliferation induced by GLU+IL infusions in 6-month-old rats is associated with mTOR activation. *A*: Pancreatic sections from 6-month-old infused rats were stained for insulin (INS; red), and proliferation was assessed by Ki67 staining (green). *B*: Staining for phospho-S6R (p-S6R; white) was measured as an index of mTOR activation. Images are representative of three animals in each group.

(24), but its underlying mechanisms are poorly understood. We previously reported that a 72-h infusion of GLU+IL in 6-month-old Wistar rats induces insulin resistance, β-cell dysfunction, and a marked increase in β-cell proliferation and mass (9). None of these abnormalities occurred in 2-month-old animals. Since circulating levels of glucose, FFA, and insulin were similar in 2- and 6-month-old animals during the infusion, we reasoned that the increase in β-cell mass likely occurs in response to the insulin resistance that develops only in

Table 1—Metabolic parameters following drug treatment in vivo

	Vehicle		AG1478		Rapamycin	
Parameters	SAL	GLU+IL	SAL	GLU+IL	SAL	GLU+IL
Glucose (mmol/L)	5.5 ± 0.3	14.1 ± 1.1*	5.6 ± 0.1	14.7 ± 1.0*	5.6 ± 0.1	17.5 ± 0.6*
FFA (mmol/L)	0.4 ± 0.1	0.9 ± 0.1**	0.3 ± 0.1	0.8 ± 0.1***	0.3 ± 0.1	1.2 ± 0.1*
Insulin (pmol/L)	256 ± 108	2,709 ± 686*	240 ± 126	2,590 ± 671*	404 ± 162	2,720 ± 437*
Glucagon (ng/L)	504 ± 33	214 ± 7*	344 ± 39	238 ± 25**	337 ± 18	229 ± 34**
GIR (mg/kg/min)	—	32.6 ± 0.4	—	30.6 ± 0.7	—	14.6 ± 0.5*

During the course of the SAL or GLU+IL infusion, 6-month-old rats were injected daily with either AG1478 or rapamycin intravenously at a dose of 0.5 mg/kg/day. Plasma glucose levels were monitored, and the glucose infusion rate (GIR) was adjusted to maintain glycemia at similar levels in all GLU+IL groups. Plasma FFA, insulin, and glucagon were measured at the end of the infusion. Results are the mean ± SEM of 4–12 animals per group. *$P < 0.001$. **$P < 0.05$. ***$P < 0.01$.

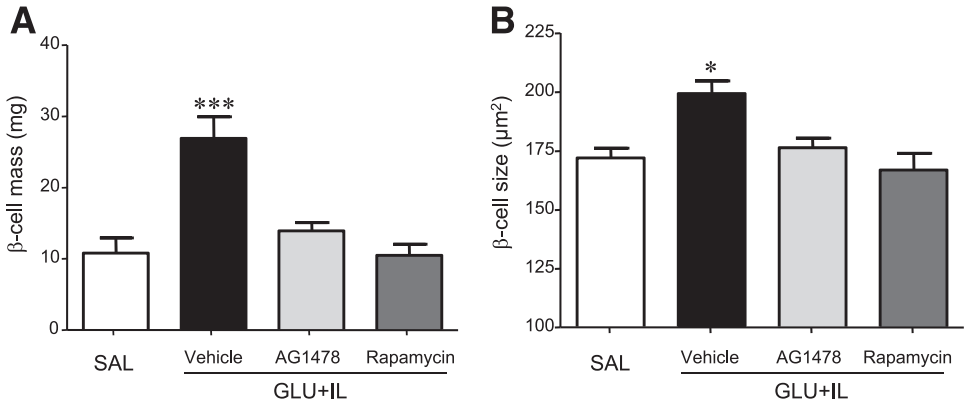

Figure 6—Inhibition of EGFR and mTOR prevents β-cell hyperplasia and hypertrophy in response to GLU+IL infusions in 6-month-old rats. *A*: Morphometric quantification of β-cell mass in 6-month-old rats infused with GLU+IL and treated with vehicle, AG1478, or rapamycin (0.5 mg/kg/d). *B*: β-Cell size was determined by dividing the surface of insulin-positive area by the number of insulin-positive cells contained in this area. Data are the mean ± SEM of four to five animals in each group. $^*P < 0.05$, $^{***}P < 0.001$.

6-month-old animals in response to the GLU+IL infusion. In the current study, we confirmed that GLU+IL infusions in 6-month-old rats led to a marked increase in the expression of the proliferation marker Ki67 (Figs. 1 and 7*E*) as well as Ki67$^+$ β-cells (Figs. 5*A* and 7*A* and *B*), resulting in β-cell hyperplasia and hypertrophy (Fig. 6). Our results may appear to be in contradiction with those of a recent study in which glucose-induced β-cell proliferation in mice was inhibited by coinfusion with lipids (25). However, besides possible species-related differences, the age of the animals and the lipid emulsion (Lyposin vs. Intralipid in our study) were different, and the levels of circulating glucose and insulin were much higher than those in the study of Pascoe et al. (25).

The question arises as to which signals are driving β-cell proliferation in response to the GLU+IL infusion in 6-month-old rats. In this regard, a recent study clearly demonstrated that circulating factors produced by the liver in insulin-resistant states can induce β-cell proliferation of not only rodent but also human islets (26). Our results are consistent with this possibility, although we have not measured liver insulin sensitivity in our model. The implication of neural signals has also been reported (27). Glucose itself is a potent inducer of β-cell proliferation in rodents (25,28–30), but marked β-cell proliferation can occur under normoglycemia, for instance, in liver-specific insulin receptor knock-out mice (6). Since a common feature of all these models is high circulating levels of insulin, it is conceivable that insulin is the β-cell mitogen. However, hyperinsulinemia is likely a permissive factor for β-cell proliferation (6) but is insufficient by itself (25) (Fig. 1). Here we have identified a key role for EGFR signaling and a potential involvement of its autocrine/paracrine ligand HB-EGF. Indeed, blocking EGFR signaling prevented the increase in β-cell proliferation both in response to HB-EGF in MIN6 cells (Fig. 4*G*) and in response to the GLU+IL infusion in 6-month-old rats (Fig. 7). Consistent with our results, a cell surface proteomic analysis revealed that EGFR is among the most highly expressed cell surface kinase receptors in the β-cell (31). EGFR is important for pancreatic β-cell development and postnatal growth (32), and is required for β-cell compensation for high-fat diet and pregnancy in mice (33). Betacellulin, an autocrine/paracrine ligand of the EGFR, is a potent inducer of β-cell proliferation both in vivo and in vitro (20,21,34,35). In addition, overexpression of HB-EGF in adult mice pancreas promotes β-cell proliferation and conversion of ductal cells to insulin-producing cells (18). Downstream of the EGFR, we showed that β-cell proliferation is associated with and dependent upon activation of the mTOR pathway both in vitro (Fig. 4) and in vivo (Figs. 5 and 7). Our findings are in accordance with the known role of the mTOR pathway in β-cell proliferation in rodent models (36–39) and establish, to our knowledge for the first time in β-cells, a link between the EGFR and mTOR.

Transcription profiling of islets of 6-month-old infused rats revealed a central role of FOXM1 in mediating the mitotic progression of the β-cell in response to GLU+IL infusion (Figs. 2 and 3). Importantly, a similar pattern was observed in high-fat–fed mice, a more chronic and milder model of nutrient-induced β-cell proliferation (Supplementary Fig. 1). This is in accordance with the reported role of FOXM1 in adult β-cell replication in response to pancreatectomy (40), pregnancy (41), and obesity (42). The intracellular mechanisms leading to FOXM1 activation remained unknown. In this regard, we have identified a possible reciprocal relationship between FOXM1 and the forkhead transcription factor FOXO3a, where FOXO3a might be acting as an upstream repressor of FOXM1 (Figs. 2 and 3). In rat cardiomyocytes, the expression of FOXM1 is negatively correlated with that of FOXO3a (16), and induction of FOXO3a inhibits proliferation through the repression of FOXM1 (17).

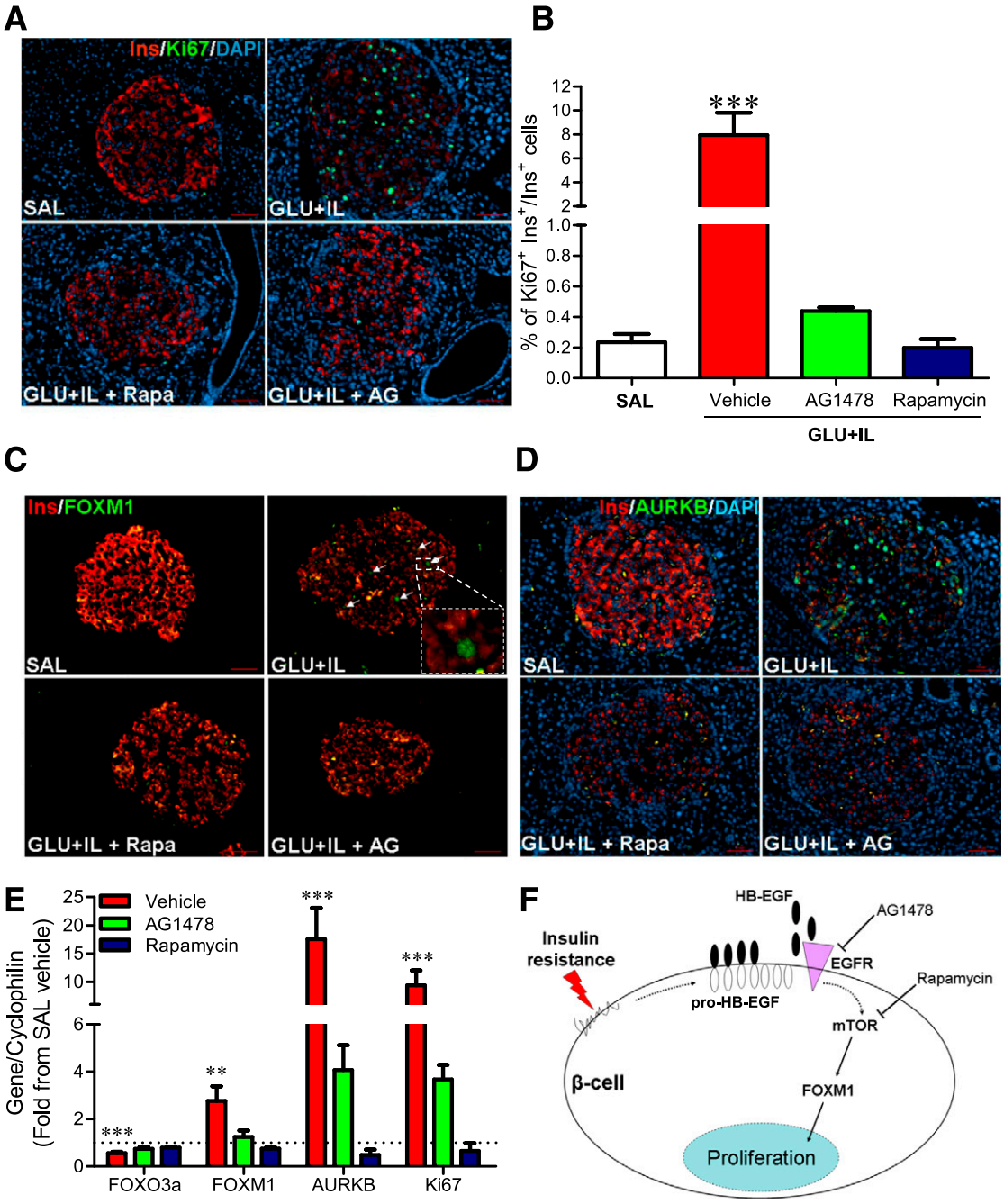

Figure 7—Inhibition of EGFR or mTOR blocks β-cell proliferation and FOXM1 signaling in response to GLU+IL infusions in 6-month-old rats. Six-month-old rats were infused with GLU+IL and treated with vehicle, AG1478 (AG), or rapamycin (0.5 mg/kg/day). *A*: Pancreatic sections were stained for insulin (Ins; red), and proliferation was assessed by Ki67 staining (green) by manually counting at least 2,000 insulin-positive (Ins^+) cells per animal. *B*: Proliferation was determined as the percentage of double-positive $Ki67^+/Ins^+$ cells over total Ins^+ cells. Data are the mean ± SEM of five animals in each group. ***$P < 0.001$. *C*: Pancreatic sections were stained for insulin (red) and FOXM1 (green). White arrows and the inset indicate expression and nuclear localization of FOXM1. *D*: Pancreatic sections were stained for insulin (red) and AURKB (green). Images in *A*, *C*, and *D* are representative of five animals in each group. *E*: qRT-PCR measurements of mRNA levels of FOXO3a, FOXM1, and AURKB. mRNA levels were normalized to cyclophilin A and are expressed relative to the vehicle-treated, SAL-infused group. Data are the mean ± SEM of four to five animals in each group. **$P < 0.01$, ***$P < 0.001$. *F*: Proposed model for nutrient-induced β-cell proliferation. GLU+IL infusions in 6-month-old rats induce insulin resistance, which leads to increased expression of pro-HB-EGF in islets. After cleavage by extracellular matrix proteases, HB-EGF activates the EGFR. Downstream of EGFR, activation of mTOR leads to FOXM1-induced β-cell proliferation. Rapa, rapamycin.

We acknowledge that there are limitations to the model used in this study. First, the relatively short duration of nutrient excess imposed here is clearly different from the natural history of nutrient-induced insulin resistance in human obesity, which occurs over several decades. Second, important differences exist between rodents and human islets regarding the control and mechanisms of β-cell proliferation (43). These limitations notwithstanding, our findings identify a signaling cascade leading to the activation of the FOXM1 transcription factor through activation of the EGFR/mTOR pathway, and a possible role of the autocrine/paracrine growth factor HB-EGF. These findings might provide new therapeutic approaches to enhance β-cell mass in insulin-resistant states and thereby prevent or delay the occurrence of type 2 diabetes. Further investigations are underway to identify the circulating factors activating the EGFR/mTOR/FOXM1 pathway in this model.

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Author Contributions. B.Z. performed the study, researched data, analyzed the results, and wrote the manuscript. I.B., G.F., M.-L.P., and O.S. performed the study, researched data, analyzed the results, and reviewed the manuscript. M.P. reviewed the manuscript. V.P. conceived the study, analyzed the results, and wrote the manuscript. V.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References

1. Reaven GM, Chen YD, Hollenbeck CB, Sheu WH, Ostrega D, Polonsky KS. Plasma insulin, C-peptide, and proinsulin concentrations in obese and nonobese individuals with varying degrees of glucose tolerance. J Clin Endocrinol Metab 1993;76:44–48
2. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 2003;52:102–110
3. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41–46
4. Sachdeva MM, Stoffers DA. Minireview: meeting the demand for insulin: molecular mechanisms of adaptive postnatal beta-cell mass expansion. Mol Endocrinol 2009;23:747–758
5. Escribano O, Guillén C, Nevado C, Gómez-Hernández A, Kahn CR, Benito M. Beta-Cell hyperplasia induced by hepatic insulin resistance: role of a liver-pancreas endocrine axis through insulin receptor A isoform. Diabetes 2009;58:820–828
6. Okada T, Liew CW, Hu J, et al. Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance. Proc Natl Acad Sci USA 2007;104:8977–8982
7. Flier SN, Kulkarni RN, Kahn CR. Evidence for a circulating islet cell growth factor in insulin-resistant states. Proc Natl Acad Sci USA 2001;98:7475–7480
8. Gargani S, Thévenet J, Yuan JE, et al. Adaptive changes of human islets to an obesogenic environment in the mouse. Diabetologia 2013;56:350–358
9. Fontés G, Zarrouki B, Hagman DK, et al. Glucolipotoxicity age-dependently impairs beta cell function in rats despite a marked increase in beta cell mass. Diabetologia 2010;53:2369–2379
10. Stein DT, Esser V, Stevenson BE, et al. Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. J Clin Invest 1996;97:2728–2735
11. Fontés G, Semache M, Hagman DK, et al. Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. Diabetes 2009;58:2048–2058
12. Walpita D, Hasaka T, Spoonamore J, et al. A human islet cell culture system for high-throughput screening. J Biomol Screen 2012;17:509–518
13. Annes JP, Ryu JH, Lam K, et al. Adenosine kinase inhibition selectively promotes rodent and porcine islet β-cell replication. Proc Natl Acad Sci USA 2012;109:3915–3920
14. Kelpe CL, Johnson LM, Poitout V. Increasing triglyceride synthesis inhibits glucose-induced insulin secretion in isolated rat islets of langerhans: a study using adenoviral expression of diacylglycerol acyltransferase. Endocrinology 2002;143:3326–3332
15. Jetton TL, Lausier J, LaRock K, et al. Mechanisms of compensatory beta-cell growth in insulin-resistant rats: roles of Akt kinase. Diabetes 2005;54:2294–2304
16. Sengupta A, Kalinichenko VV, Yutzey KE. FoxO1 and FoxM1 transcription factors have antagonistic functions in neonatal cardiomyocyte cell-cycle withdrawal and IGF1 gene regulation. Circ Res 2013;112:267–277
17. Delpuech O, Griffiths B, East P, et al. Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol Cell Biol 2007;27:4917–4930
18. Kozawa J, Tokui Y, Moriwaki M, et al. Regenerative and therapeutic effects of heparin-binding epidermal growth factor-like growth factor on diabetes by gene transduction through retrograde pancreatic duct injection of adenovirus vector. Pancreas 2005;31:32–42
19. Peyot ML, Pepin E, Lamontagne J, et al. Beta-cell failure in diet-induced obese mice stratified according to body weight gain: secretory dysfunction and altered islet lipid metabolism without steatosis or reduced beta-cell mass. Diabetes 2010;59:2178–2187
20. Li L, Seno M, Yamada H, Kojima I. Promotion of beta-cell regeneration by betacellulin in ninety percent-pancreatectomized rats. Endocrinology 2001;142:5379–5385
21. Oh YS, Shin S, Lee YJ, Kim EH, Jun HS. Betacellulin-induced beta cell proliferation and regeneration is mediated by activation of ErbB-1 and ErbB-2 receptors. PLoS One 2011;6:e23894

22. Blättler SM, Cunningham JT, Verdeguer F, et al. Yin Yang 1 deficiency in skeletal muscle protects against rapamycin-induced diabetic-like symptoms through activation of insulin/IGF signaling. Cell Metab 2012;15:505–517
23. Lamming DW, Ye L, Katajisto P, et al. Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science 2012;335:1638–1643
24. Prentki M, Nolan CJ. Islet beta cell failure in type 2 diabetes. J Clin Invest 2006;116:1802–1812
25. Pascoe J, Hollern D, Stamateris R, et al. Free fatty acids block glucose-induced β-cell proliferation in mice by inducing cell cycle inhibitors p16 and p18. Diabetes 2012;61:632–641
26. El Ouaamari A, Kawamori D, Dirice E, et al. Liver-derived systemic factors drive β cell hyperplasia in insulin-resistant states. Cell Rep 2013;2:401–410
27. Imai J, Katagiri H, Yamada T, et al. Regulation of pancreatic beta cell mass by neuronal signals from the liver. Science 2008;322:1250–1254
28. Bonner-Weir S, Deery D, Leahy JL, Weir GC. Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. Diabetes 1989;38:49–53
29. Levitt HE, Cyphert TJ, Pascoe JL, et al. Glucose stimulates human beta cell replication in vivo in islets transplanted into NOD-severe combined immunodeficiency (SCID) mice. Diabetologia 2011;54:572–582
30. Alonso LC, Yokoe T, Zhang P, et al. Glucose infusion in mice: a new model to induce beta-cell replication. Diabetes 2007;56:1792–1801
31. Stützer I, Esterházy D, Stoffel M. The pancreatic beta cell surface proteome. Diabetologia 2012;55:1877–1889
32. Miettinen P, Ormio P, Hakonen E, Banerjee M, Otonkoski T. EGF receptor in pancreatic beta-cell mass regulation. Biochem Soc Trans 2008;36:280–285
33. Hakonen E, Ustinov J, Mathijs I, et al. Epidermal growth factor (EGF)-receptor signalling is needed for murine beta cell mass expansion in response to high-fat diet and pregnancy but not after pancreatic duct ligation. Diabetologia 2011;54:1735–1743
34. Kodera T, Yamada S, Yamamoto Y, et al. Administration of conophylline and betacellulin-delta4 increases the beta-cell mass in neonatal streptozotocin-treated rats. Endocr J 2009;56:799–806
35. Cho YM, Lim JM, Yoo DH, et al. Betacellulin and nicotinamide sustain PDX1 expression and induce pancreatic beta-cell differentiation in human embryonic stem cells. Biochem Biophys Res Commun 2008;366:129–134
36. Blandino-Rosano M, Chen AY, Scheys JO, et al. mTORC1 signaling and regulation of pancreatic β-cell mass. Cell Cycle 2012;11:1892–1902
37. Fraenkel M, Ketzinel-Gilad M, Ariav Y, et al. mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. Diabetes 2008;57:945–957
38. Balcazar N, Sathyamurthy A, Elghazi L, et al. mTORC1 activation regulates beta-cell mass and proliferation by modulation of cyclin D2 synthesis and stability. J Biol Chem 2009;284:7832–7842
39. Velazquez-Garcia S, Valle S, Rosa TC, et al. Activation of protein kinase C-ζ in pancreatic β-cells in vivo improves glucose tolerance and induces β-cell expansion via mTOR activation. Diabetes 2011;60:2546–2559
40. Ackermann Misfeldt A, Costa RH, Gannon M. Beta-cell proliferation, but not neogenesis, following 60% partial pancreatectomy is impaired in the absence of FoxM1. Diabetes 2008;57:3069–3077
41. Zhang H, Zhang J, Pope CF, et al. Gestational diabetes mellitus resulting from impaired beta-cell compensation in the absence of FoxM1, a novel downstream effector of placental lactogen. Diabetes 2010;59:143–152
42. Davis DB, Lavine JA, Suhonen JI, et al. FoxM1 is up-regulated by obesity and stimulates beta-cell proliferation. Mol Endocrinol 2010;24:1822–1834
43. Kulkarni RN, Mizrachi EB, Ocana AG, Stewart AF. Human β-cell proliferation and intracellular signaling: driving in the dark without a road map. Diabetes 2012;61:2205–2213