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

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ABSTRACT

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 co-infusion of glucose and Intralipid (GLU+IL) induces insulin resistance and a marked increase in β-cell proliferation in 6-mo-old but not in 2-mo-old Wistar rats. The aim of the present 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 EGF-like growth factor (HB-EGF), a ligand of the EGF receptor (EGFR), in nutrient-induced β-cell proliferation. Phosphorylation of ribosomal S6 kinase, an mTOR target, was increased in islets from GLU+IL-infused 6-mo-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. Co-infusion 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-mo-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.
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 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 non-diabetic compared to lean individuals, but decreased in obese individuals with impaired fasting glucose and type 2 diabetic patients (2). These observations suggest that alterations of the anatomical compensation of the β-cell to 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 inter-organ crosstalk 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 co-infusion of glucose and Intralipid (GLU+IL) triggers a marked increase in β-cell mass
and proliferation at 6 mo. but not at 2 mo. 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-mo-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-mo-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). Dulbecco's Modified Eagle's Medium (DMEM) was from Wisent (Saint Bruno, QC). AG1478 and Rapamycin were from LC Laboratories (Woburn, MA, USA). Recombinant Heparin-Binding EGF-like Growth Factor (HB-EGF) was from R&D systems (Minneapolis, MN, USA). CRM197 was from Sigma Aldrich (Saint Louis, MO, USA).

**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-mo-old) and 500-600 g (~ 6-mo-old) (Charles River, St.-Constant, QC) 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 (Baxter, Mississauga, ON) (SAL) or 70% dextrose (McKesson Canada Corp, Montreal, QC) plus 20% Intralipid (Fresenius Kabi, Sweden; a soybean oil emulsion which generates a mixture of approximately 80% unsaturated / 20% saturated fatty acids when co-infused with heparin (10)) with 20 U/ml heparin (Sandoz Canada, Boucherville, QC) (GLU+IL). Infusions were performed using Harvard infusion pumps (Pump 33; Harvard Apparatus) 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, Saint Louis, MO, USA) 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, Saint Louis, MO, USA) and injected intravenously at a dose of 0.5 mg/kg/d. The control group was injected with the same volume of vehicle.

Cell culture

MIN6 cells (passage 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,
Saint Louis, MO, USA), and ten images per well were taken randomly. Cell numbers were determined using ImageJ software (NIH).

**Rat islet proliferation**

Isolated rat islets were dispersed and plated in 96-well plates coated with HTB-9 cell extracellular matrix as described (12). Dispersed rat islets were treated for 72h in absence or presence of 100 ng/mL HBEGF in complete islet media. The media was changed every 24 hours. At the end of treatment, cells were fixed and stained for the β-cell marker pancreas-duodenum homeobox-1 (PDX-1) and the proliferative marker Ki67 as described (13). Proliferation was calculated as percentage of double-positive Ki67⁺/PDX-1⁺ cells over total PDX-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 non-diabetic human cadaveric donors were provided by the Clinical Islet Laboratory at the University of Alberta and the Integrated Islet Distribution Program (IIDP) sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the Juvenile Diabetes Research Foundation International (JDRFI).

**Transcriptomic profiling and qRT-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 (5 arrays per group, Affymetrix, Santa Clara, CA). One hundred ng of total RNA was processed using the Ambion® WT Expression Kit (Invitrogen). The resulting fragmented and labeled single-stranded cDNA was processed according to Affymetrix protocol. Partek Genomics Suite (Partek, St. Louis, Missouri) was used for data analysis. The data were normalized by Robust Multichip Average (RMA) algorithm, which uses background adjustment, quantile normalization and summarization. After correction of statistical significance for multiple comparisons using false discovery rate (FDR, p < 0.01), the transcripts significantly and differentially expressed by more than 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, Inc., Redwood City, CA). Selected genes were validated by qRT-PCR as previously described (9). All qRT-PCR results were normalized to cyclophilin A mRNA levels. Primer sequences are described in Suppl. Table 1.

Analytical measurements

Plasma glucose and free fatty-acid (FFA) levels were measured using Wako kits (Wako Chemical, Osaka, Japan). Insulin and glucagon were measured by ELISA (Alpc0, Windham, NH, USA).
**Immunostaining of pancreatic sections**

Pancreata were trimmed of fat, weighed, fixed for 3-4 hours 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 Suppl. Table 2. Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Images were taken with a fluorescence microscope (Zeiss, Thornwood, NY). Beta-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 µg of proteins from cells or islets were extracted and resolved by SDS-PAGE as described (11). Primary antibodies are listed in Suppl. Table 2. Signals were detected using a horseradish peroxidase–labeled IgG (BioRad, Richmond, CA) and enhanced chemiluminescence (ECL; PerkinElmer Las Canada, Woodbridge, ON) on Kodak BioMax XAR films (Kodak, Rochester, NY). The bands were quantified by densitometry and Image J software (NIH).

**Expression of data and statistics**

Data are expressed as mean ± SEM. Statistical analyses were performed using Student’s t-test or ANOVA followed by two-by-two comparisons using Bonferroni post-hoc
adjustments, as appropriate using GraphPad Instat (GraphPad Software). $P < 0.05$ was considered significant.

RESULTS

**Infusion of glucose + Intralipid induces insulin resistance in 6-mo-old Wistar rats**

In response to the 72-h GLU+IL infusion, circulating blood glucose and FFA raised to similar levels in 2-mo-old and 6-mo-old animals ($n=4$; NS; Fig 1A&B). Insulin levels also increased in response to the GLU+IL infusion but were not different between the two age groups ($n=4$, NS; Fig 1C). Consistent with our previous observation in this model (9), significantly less glucose was required to maintain the same level of glycemia in 6-mo-old vs. 2-mo-old rats, suggestive of insulin resistance ($p<0.001$; $n=11-18$; Fig 1D). Circulating glucagon levels decreased to the same extent in response to the GLU+IL infusion in both age groups (Fig 1E). Expression of Ki67, a marker of cell proliferation, was strongly induced in response to the GLU+IL infusion in 6-mo-old rats only (Fig 1F). 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-mo-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-mo-old rats. Six-mo-old Wistar rats were infused with either SAL or GLU+IL for 72 h and islets were isolated.
from 5 animals in each group matched for body weight at the end of the infusion. As shown in Fig. 2A, with a false discovery rate < 0.01, approximately 3,000 genes were significantly modulated by the GLU+IL infusion. Overall, half of the genes was up-regulated and half was down-regulated. When the cut-off was set to a 2-fold difference between the SAL group and the GLU+IL group, 300 genes were significantly up-regulated, whereas only 30 genes were down-regulated by GLU+IL infusion. Within the 300 up-regulated genes, a group of genes expressed at low levels in the SAL group were strongly up-regulated in the GLU+IL group (lower left corner of the graph in Fig. 2A). 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. 2B). 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 up-regulated by the GLU+IL infusion (Fig. 2C). Interestingly, expression of the forkhead transcription factor FOXO3a, a repressor of FOXM1 (16; 17), was decreased upon GLU+IL infusion (Fig. 2C). In addition, expression of the gene encoding HB-EGF, a potential autocrine/paracrine growth factor inducing β-cell proliferation ((18) and Fig. 2C), was also up-regulated 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 extend as shown in Fig. 1 (data not shown). Second, we confirmed that expression of FOXO3a was repressed by the GLU+IL infusion (Fig. 3A), 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. 3B-E). FOXM1 protein levels
were also significantly increased in the GLU+IL group (Fig. 3F). 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). Expression of all these genes remained unchanged in the islet of 2-mo-old rats upon GLU+IL infusion (Fig. 3A-E). To confirm that this transcriptional network is activated in a more chronic model of nutrient excess, we measured expression of these genes in islets from C57Bl/6 mice fed a high-fat diet for 8 weeks and stratified in two groups according to body weight gain: Low-Diet Responders (LDR) and High-Diet Responders (HDR) (19). Expression of FOXM1, AURKB, and PLK1 was significantly increased in islets from HDR mice, albeit to a lesser extent than in infused rats (Suppl. Fig. 1).

**HB-EGF promotes β-cell proliferation through EGFR and mTOR**

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

**Inhibition of EGFR signaling and mTOR inhibits β-cell mass expansion, β-cell proliferation, and prevents up-regulation of FOXM1 and its targets in islets from GLU+IL-infused 6-mo-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-mo-old GLU+IL-infused rats. As shown in Fig. 5, phosphorylation of S6R was strongly increased in islets from GLU+IL-infused rats (Fig. 5B), concomitant with a marked increase in the number of cells staining positive for Ki67 (Fig. 5A).

To test the importance of the EGFR and mTOR in β-cell proliferation in response to GLU+IL, we treated another group of 6-mo-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), 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-mo-old rats (Fig. 6A). This was also associated with a significant increase in β-cell size (Fig. 6B). Remarkably, treatment
of the rats with either AG1478 or rapamycin completely prevented both β-cell hyperplasia (Fig. 6A) and hypertrophy (Fig. 6B).

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. 7A-B). This was associated with increased nuclear staining of FOXM1 (Fig. 7C) and AURKB (Fig. 7D). Importantly, the increase in Ki67, FOXM1 and AURKB was totally abolished upon treatment of GLU+IL-infused rats with AG1478 or rapamycin (Fig. 7A-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. 7E).

**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-mo-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. 7F). 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-mo-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.
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 (24), but its underlying mechanisms are poorly understood. We previously reported that a 72-h infusion of GLU+IL in 6-mo-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-mo-old animals. Since circulating levels of glucose, FFA, and insulin were similar in 2- and 6-mo-old animals during the infusion, we reasoned that the increase in β-cell mass likely occurs in response to the insulin resistance that only develops in 6-mo-old animals in response to the GLU+IL infusion. In the present study, we confirmed that GLU+IL infusions in 6-mo-old rats led to a marked increase in the expression of the proliferation marker Ki67 (Figs. 1 and 7E) as well as Ki67+ β-cells (Figs. 5A and 7A-B), resulting in β-cell hyperplasia and hypertrophy (Fig. 6). Our results may appear in contradiction with a recent study in which glucose-induced β-cell proliferation in mice was inhibited by co-infusion 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 much lower 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-mo-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) and 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. 4G) and in response to the GLU+IL infusion in 6-mo-old rats (Fig. 7). Consistent with our results, a cell surface proteomic analysis revealed that EGFR is amongst 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 to 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, over-expression 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&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-mo-old infused rats revealed a central role of the forkhead transcription factor FOXM1 in mediating the mitotic progression of the β-cell in response to GLU+IL infusion (Figs. 2&3). Importantly, a similar pattern was observed in high-fat fed mice, a more chronic and milder model of nutrient-induced β-cell proliferation (Suppl. 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 (Fig. 2 & 3). In rat cardiomyocytes, expression of FOXM1 is negatively correlated with that of FOXO3a (16), and induction of FOXO3a inhibits proliferation through repression of FOXM1 (17).

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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### TABLE 1. Metabolic parameters following drug treatment in vivo.

<table>
<thead>
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<th>Vehicle</th>
<th>AG1478</th>
<th>Rapamycin</th>
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<tr>
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<td>SAL</td>
<td>GLU+IL</td>
<td>SAL</td>
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<tr>
<td>Glucose (mmol/l)</td>
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<td>14.1 ± 1.1***</td>
<td>5.6 ± 0.1</td>
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<tr>
<td>FFA (mmol/l)</td>
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<td>0.9 ± 0.1*</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>Insulin (pmol/l)</td>
<td>256 ± 108</td>
<td>2709 ± 686***</td>
<td>240 ± 126</td>
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<tr>
<td>Glucagon (ng/l)</td>
<td>504 ± 33</td>
<td>214 ± 7***</td>
<td>344 ± 39</td>
</tr>
<tr>
<td>GIR (mg/Kg/min)</td>
<td>-</td>
<td>32.6 ± 0.4</td>
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During the course of the SAL or GLU+IL infusion, 6-mo-old rats were injected daily with either AG1478 or rapamycin intravenously at a dose of 0.5 mg/kg/d. Plasma glucose levels were monitored and the glucose infusion rate (GIR) adjusted to maintain glycemia at a similar levels in all GLU+IL groups. Plasma FFA, insulin and glucagon were measured at the end of the infusion. Results are mean ± SEM of 4-12 animals per group. *P < 0.05; **P < 0.01; ***P < 0.001.
FIGURE LEGENDS

Figure 1: Metabolic parameters in glucose + Intralipid (GLU+IL)-infused 6-mo-old rats. 2- and 6-mo-old Wistar rats were infused with SAL or GLU+IL for 72h. A-C: Plasma glucose, free fatty acids (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 mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2: Transcriptomic analysis of isolated islets from GLU + IL-infused 6-mo-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's 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. Up-regulated genes are colored in red and down-regulated genes are colored in green. Data were obtained from 5 animals in each group.

Figure 3: Expression of FOXM1 and its targets is induced in islets from GLU+IL-infused 6-mo-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 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 4 individual 6-mo-old rats in each infusion group.
**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 24h-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 mean ± SEM of 3-6 experiments. *P < 0.05; ***P < 0.001. B: Dispersed rat islets were treated for 72 hours in absence or presence of HB-EGF (100 ng/mL) and cells were fixed and stained for Ki67 and PDX-1. B-cell proliferation was calculated as the percentage of double-positive Ki67⁺ / PDX-1⁺ cells over the total PDX-1⁺ population. Data are expressed as mean ± SEM of 4 replicate experiments. **P < 0.01. C,D: Representative Western blot and densitometric quantification of phospho-S6 ribosomal kinase (p-S6R) upon HB-EGF treatment in MIN6 cells (C) and human islets (D). Data are expressed as mean ± SEM of 3 replicate experiments. *P < 0.05; **P < 0.01. E-F: Representative Western blot and densitometric quantification of phospho-S6 ribosomal kinase (p-S6R) upon HB-EGF treatment in the absence (-) or presence (+) of the PI3 kinase inhibitor, LY294002 (10 µM). Data are expressed as mean ± SEM of 3 replicate experiments. *P < 0.05.

**Figure 5:** Beta-cell proliferation induced by GLU+IL infusions in 6-mo-old rats is associated with mTOR activation. Pancreatic sections from 6-mo-old infused rats were stained for insulin (red) and proliferation was assessed by Ki67 staining (green) (A). Staining for p-S6R (white) was measured as an index of mTOR activation (B). Images are representative of 3 animals in each group.
Figure 6: Inhibition of EGFR and mTOR prevents β-cell hyperplasia and hypertrophy in response to GLU+IL infusions in 6-mo-old rats. A: Morphometric quantification of β-cell mass in 6-mo-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+ cells contained in this area. Data are mean ± SEM of 4-5 animals in each group. *P < 0.05; ***P < 0.001.

Figure 7: Inhibition of EGFR or mTOR blocks β-cell proliferation and FOXM1 signaling in response to GLU+IL infusions in 6-mo-old rats. Six-mo-old rats were infused with GLU+IL and treated with vehicle, AG1478, or rapamycin (0.5 mg/kg/d). A-B: Pancreatic sections were stained for insulin (red) and proliferation was assessed by Ki67 staining (green) by manually counting at least 2,000 insulin positive (Ins+) cells per animal (A). Proliferation was determined as the percentage of double-positive Ki67+/Ins+ cells over total Ins+ cells (B). Data are mean ± SEM of 5 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 5 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 mean ± SEM of 4-5 animals in each group. **P < 0.01; ***P < 0.001. F: Proposed model for nutrient-induced β-cell proliferation. GLU+IL infusions in 6-mo-old rats.
induces 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.
Fig. 1

A. Glucose (mmol/l)

B. FFA (mmol/l)

C. Insulin (pmol/l)

D. GIR (mg/Kg/min)

E. Glucagon (ng/l)

F. Ki67/Cyclophilin (Fold from SAL)
Fig. 2

A

Up-regulated genes

Down-regulated genes

SAL (Log Signal Intensity)

GLU+IL (Log Signal Intensity)

2 Fold increase

2 Fold decrease

B

Biofunctions

-Log (p-value)

Cell cycle

Cancer

Canonical pathways

-Log (p-value)

Mitotic roles of polo-like kinase (PLK1)

Cell Cycle: G2/M DNA Damage Checkpoint Regulation

C

Extracellular space

Cytoplasm

Nucleus

HBEGF

FOXO3

FOXO1

Mitotic roles of polo-like kinase (PLK1)

Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Fig. 3

A) FOXO3a

B) FOXM1

C) AURKB

D) PLK1

E) HB-EGF

F) Western blot for FOXM1

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<td>GLU+IL</td>
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1 ±0.28
2.63 ±0.03
p < 0.01

SAL
GLU+IL

FOXM1 Actin

1 ± 0.28
2.63 ± 0.03
p < 0.01
**Fig. 4**

**A** MIN6

Cell proliferation (fold from control)

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**B** Rat islets

% of Ki67/Pdx-1-1 cells

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**C** MIN6

p-S6R/tubulin

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**D** Human islets

p-S6R/tubulin

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**E** Human islets

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**G** MIN6

Cell proliferation (fold from control)

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**H** MIN6

Cell proliferation (fold from control)

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* * *
Fig. 6

A

ß-cell mass (mg)

SAL  Vehicle  AG1478  Rapamycin

GLU+IL

B

ß-cell size (µm²)

SAL  Vehicle  AG1478  Rapamycin

GLU+IL
Supplementary Figures and tables:

Supplementary Figure 1: FOXM1 signaling is induced in mouse islets after 8 w of high fat feeding. Mice were separated in two groups according to body weight gain induced by the high-fat diet: Low- and High-Diet Responders (LDR and HDR (1)) and compared to normal-diet (ND) fed mice. At the end of the 8 weeks of diet, islets were isolated and gene expression was assessed by qRT-PCR. Expression levels were normalized by cyclophilin A. Data are mean ± SEM of 8 animals in each group. ***P <0.001.

Supplementary Figure 2: AG1478 dose dependently inhibits HB-EGF-induced β-cell proliferation in MIN6 cells. MIN6 cell proliferation was measured by counting nuclei after 24h-treatment with HB-EGF with or without AG1478 (3-300 nmol/l). Data are expressed as mean ± SEM of 4 replicate experiments. *P < 0.05; ***P < 0.001.

References:

Supplementary Table 1: Primers sequences for qRT-PCR.

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**Supplementary Table 2:** List and references of primary antibodies.

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Supplementary Fig. 1

A) Ki67

Gene/Cyclophilin (Fold change from ND)

B) FOXM1

Gene/Cyclophilin (Fold change from ND)

C) AURKB

Gene/Cyclophilin (Fold change from ND)

D) PLK1

Gene/Cyclophilin (Fold change from ND)