Yang Jiao,[1] John Le Lay,[1] Ming Yu,[2] Ali Naji,[2] and Klaus H. Kaestner[1]

Elevated Mouse Hepatic Betatrophin Expression Does Not Increase Human β-Cell Replication in the Transplant Setting

The recent discovery of betatrophin, a protein secreted by the liver and white adipose tissue in conditions of insulin resistance and shown to dramatically stimulate replication of mouse insulin-producing β-cells, has raised high hopes for the rapid development of a novel therapeutic approach for the treatment of diabetes. At present, however, the effects of betatrophin on human β-cells are not known. Here we use administration of the insulin receptor antagonist S961, shown to increase betatrophin gene expression and stimulate β-cell replication in mice, to test its effect on human β-cells. Although mouse β-cells, in their normal location in the pancreas or when transplanted under the kidney capsule, respond with a dramatic increase in β-cell DNA replication, human β-cells are completely unresponsive. These results put into question whether betatrophin can be developed as a therapeutic approach for treating human diabetes.

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A common feature of type 1 and 2 diabetes is an insufficient number of insulin-producing β-cells in the endocrine pancreas. Although promising in principle, targeted expansion of human β-cells as a therapeutic strategy for diabetes has not been possible (1). After β-cells have been specified from panendocrine precursors in fetal life, postnatal β-cell mass expansion is the result of replication of preexisting β-cells and is not dependent on tissue-resident progenitor cells, at least in rodents (2). The basic proliferation rate of β-cells in adult mammals is very low under normal physiological conditions, typically less than 1% (3). However, when metabolically challenged, such as during pregnancy, diet-induced insulin resistance, and experimental β-cell ablation, β-cells have the capacity to expand by proliferation, at least in rodents (4). In particular, hepatic insulin resistance was shown decades ago to be a powerful promoter of β-cell replication in mice (5–7). Increased glycolytic flux in β-cells, as occurs when blood glucose levels are elevated, was recently proposed as one mediator of increased β-cell replication (8). Whether additional signals contribute to β-cell replication during the insulin-resistant state remains unknown.

Using a pharmacological inhibitor of insulin action, Yi et al. (9) screened for a potential secreted molecule that might represent an additional mitogenic signal to β-cells in conditions of insulin resistance. They identified betatrophin, also known as lipasin (10) or angiopoietin-like 8 (11), a secreted protein synthesized in the liver and to

[1]Department of Genetics and Institute for Diabetes, Obesity and Metabolism, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA
[2]Department of Surgery and Institute for Diabetes, Obesity and Metabolism, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Corresponding author: Klaus H. Kaestner, kaestner@mail.med.upenn.edu.

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a lesser extent in white adipose tissue, as a potential β-cell mitogen (9). Betatrophin mRNA expression was induced by four- or sixfold in response to treatment of mice with the insulin receptor antagonist S961 in white adipose tissue or the liver, respectively (12). Most strikingly, overexpression of a *myc*-tagged betatrophin protein in the liver via hydrodynamic injection resulted in a 17-fold increase in β-cell replication in mice, a stimulation of replication similar to that seen by inducing insulin resistance through treatment with S961 (9). Although the mechanism of action of betatrophin is currently unknown, its description has led to widespread hopes that this hormone might represent a new therapeutic avenue for diabetes. An important question remaining is whether betatrophin can also stimulate replication of human β-cells, which we addressed here through transplantation of human islets into immunodeficient mice with elevated betatrophin expression in the liver.

RESEARCH DESIGN AND METHODS

Mice and Glucose Measurements

Immunodeficient NOD-Scid (NOD.CB17-Prkdc^{scid}/J) female mice were purchased from The Jackson Laboratory (Cat No. 001303) and used for experiments at 8 weeks of age. Random blood glucose levels were measured from tail vein blood using the Breeze2 glucometer (Bayer AG, Leverkusen, Germany).

Islet Transplantation

Adult C57BL6/J mice were used for islet isolation as described (3), with three preparations obtained from 2-month-old mice and two preparations from 12-month-old mice. In each mouse, 500 human islets were transplanted under the left kidney capsule and 100 mouse islets were transplanted under the right kidney capsule.

S961 Treatment

S961 was received from Dr. Lauge Schäffer (Novo Nordisk) (12). Vehicle (H_2O) or 10-nmol S961 was loaded into Alzet 2001 osmotic pumps that were implanted subcutaneously on the back of mice 1 week after islet transplantation. Mice were killed for tissue harvesting 7 days after S961 or vehicle treatment.

Real-Time PCR Analysis and Immunohistochemistry

Total RNA isolation and real-time PCR were performed as described (3). Liver and gonadal white adipose tissues were dissected and snap-frozen for subsequent RNA isolation. Mice were administered 1 g/L BrdU (Sigma-Aldrich Co., LLC, St. Louis, MO) in the drinking water 72 h before they were killed. Kidneys were dissected and fixed with 10% formalin (Protocol 10% formaldehyde; Fisher Scientific, Pittsburgh, PA). The pancreas was dissected, flattened by forceps, fixed in 4% paraformaldehyde, and embedded in paraffin. Tissues were sectioned to 5-μm thickness. Deparaffinized and rehydrated slides were subjected to antigen retrieval by pressure cooker in 10 mmol/L citric acid buffer (pH 6.0).

Simultaneous immunofluorescent staining was performed for BrdU and insulin. The primary antibodies used were guinea pig anti-insulin (1:1,000 dilution; Dako North America, Inc., Carpinteria, CA) and rat anti-BrdU (1:500 dilution; AbD Serotec, Raleigh, NC). Secondary antibodies were Cy2–anti-guinea pig (1:200) and Cy3–anti-rat (1:200). The β-cell proliferation rate was quantified as the number of BrdU$^+$/insulin$^+$ double-positive cells divided by the number of insulin$^+$ cells.

Similar double-immunofluorescent staining was performed for the proliferation marker Ki67 and insulin. The primary antibodies used were mouse anti-Ki67 (1:500; BD Biosciences, San Jose, CA) and guinea pig anti-insulin. Secondary antibodies were Cy2–anti-guinea pig (1:200) and Cy3–anti-mouse (1:200). The β-cell proliferation rate was quantified as the number of Ki67$^+$/insulin$^+$ double-positive cells divided by the number of insulin$^+$ cells. At least 1,000 β-cells were counted, respectively, for human islets under the kidney capsule, mouse islets under the kidney capsule, and endogenous mouse islets in the pancreas.

TUNEL Assessment

Apoptosis was assessed by TUNEL staining (Roche Applied Science, Minneapolis, MN) according to the manufacturer's instruction. More than 1,000 cells each were counted for endogenous islets, transplanted mouse islets, and transplanted human islets.

RESULTS

We asked whether the proliferation rate of human islets transplanted under the kidney capsule of immunodeficient mice would increase in response to increased betatrophin levels. First, we established that immunodeficient NOD-Scid (NOD.CB17-Prkdc^{scid}/J) mice respond to the insulin receptor inhibitor S961 in a fashion similar to that seen by Yi et al. (9) in C57BL/6J mice. As shown in Fig. 1*A*, delivery of S961 using osmotic minipumps indeed caused hyperglycemic in NOD-Scid mice within 24 h, consistent with prior findings (9), and this effect persisted for the entire treatment period. Next, we evaluated whether expression of betatrophin is induced in the liver of NOD-Scid mice. Messenger RNA levels of betatrophin were induced about fivefold as determined by quantitative RT-PCR (Fig. 1*B*), similar to what was published previously for C57BL/6 mice (9). However, betatrophin mRNA levels were not increased in gonadal white adipose tissue after S961 treatment as had been reported by Yi et al. (9), possibly due to the different mouse strains used or fat depots analyzed (Fig. 1*C*). It is worth noting that the increase in adipose tissue betatrophin mRNA reported previously was not statistically significant (9).

Next, we assayed replication of β-cells in the endocrine pancreas of S961-treated NOD-Scid mice by determination of incorporation of the thymidine analog

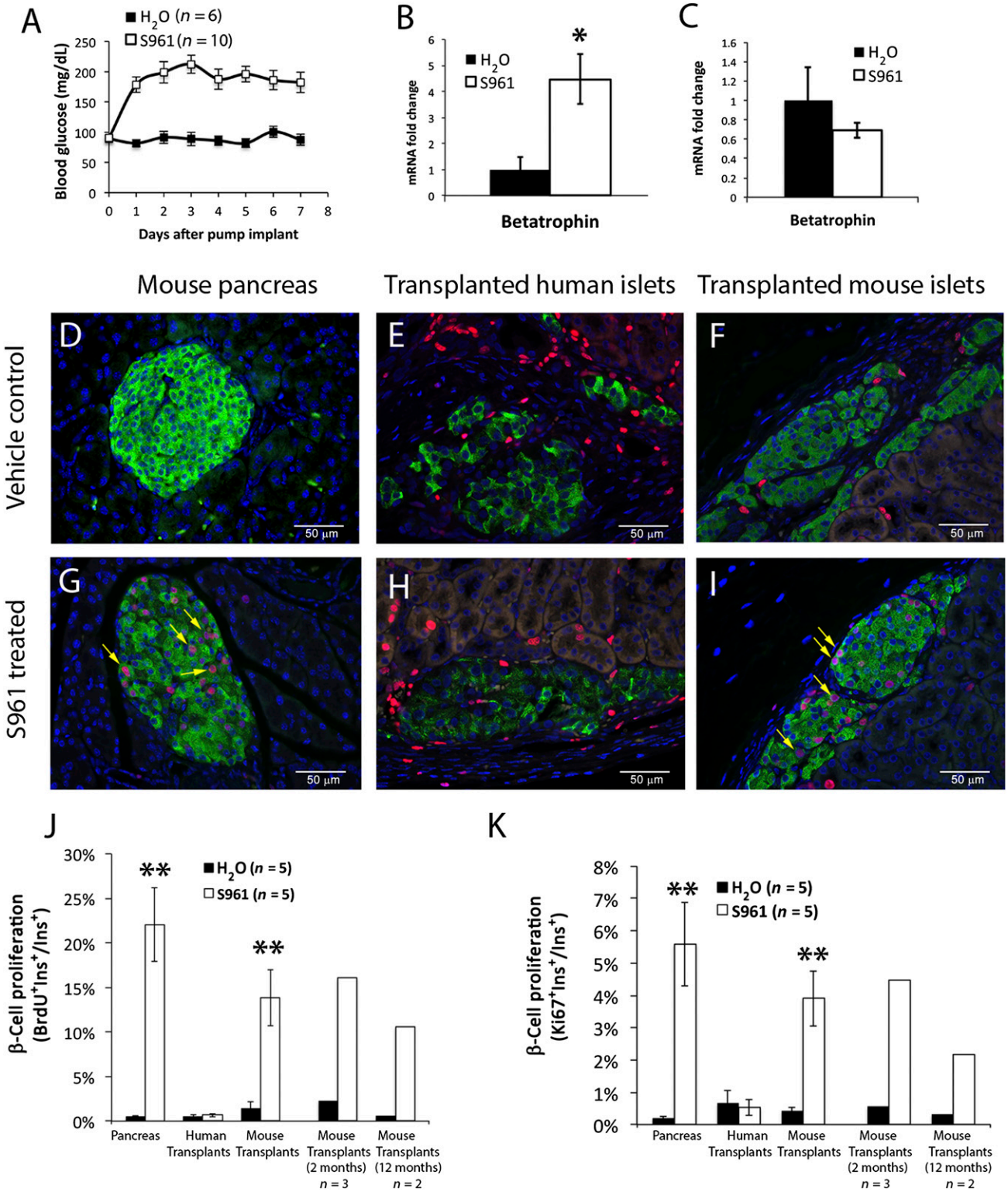

Figure 1—Elevated betatrophin expression does not stimulate replication of human β-cells. *A*: Random blood glucose levels of NOD-Scid mice treated with vehicle (n = 6) or the insulin receptor antagonist S961 (n = 10). Glucose levels in S961-treated mice were significantly elevated from day 1 to 7 ($P < 0.001$). *B*: Hepatic betatrophin mRNA levels in mice treated with vehicle (n = 3) or S961 (n = 4) as determined by quantitative RT-PCR. Levels of mRNA were normalized to those of TATA-box binding protein as the internal control and are expressed as fold over vehicle. *$P < 0.05$. *C*: Gonadal white adipose tissue betatrophin mRNA levels in mice treated with vehicle (n = 8) or S961 (n = 6) as determined by quantitative RT-PCR. β-Cell replication in NOD-Scid mice treated with vehicle or S961 was determined by BrdU incorporation (red signal). β-Cells were identified by insulin immunofluorescence staining (green). *D*: β-Cells in the endocrine pancreas of vehicle-treated mice. *E*: Transplanted human islets recovered from the kidney capsule of vehicle-treated mice. *F*: Transplanted mouse islets recovered from the kidney capsule of vehicle-treated mice. *G*: β-Cells in the endocrine pancreas of S961-treated mice. Note the frequent $BrdU^+$ β-cells (yellow arrows). *H*: Transplanted human islets recovered from the kidney capsule of S961-treated mice.

Table 1—Summary information for the deceased organ donors used for the human islet transplantation studies

Donor	ID	Age (years)	Sex	BMI (kg/m^2)	Race	Cause of death
1	AAER055	53	M	29	Hispanic	Head trauma
2	AAFC089	46	M	28.8	White	NA
3	ICRH-51	4	F	16.1	Hispanic	Head trauma
4	AAGW388	43	M	30.6	White	Head trauma
5	ICRH-52	18	M	23.5	White	Head trauma

F, female; M, male; NA, not available.

BrdU. As shown in Fig. 1*G*, the number of replicating β-cells was dramatically increased in S961-treated mice compared with vehicle-treated controls (Fig. 1*D*). Quantification of the data showed a mitogenic effect exceeding 20-fold, in line with what had been observed in C57BL/6J mice (Fig. 1*I*) (9). Coimmunostaining for insulin and Ki67, another proliferation marker, also showed a 20-fold increase in replication rate after S961 treatment (Fig. 1*K*), similar to what had been observed in C57BL/6J mice (9). The difference in the labeling index between the two methods is explained by the fact that BrdU captures all cells proliferating during the 72-h period of BrdU administration, whereas Ki67 staining only detects those cells that are replicating at the time of tissue harvest.

Having validated the experimental model, we were now in the position to test the effects of S961 treatment and elevated betatrophin levels on human β-cell replication. To this end, we transplanted islets obtained from five deceased, nondiabetic organ donors aged 4 to 53 years (see Table 1 for donor information) under the kidney capsule of S961-treated NOD-Scid mice. In contrast to the dramatic increase in proliferation seen in the mouse endocrine pancreas documented above, human β-cells did not respond with increased BrdU incorporation to S961 treatment and elevated betatrophin expression (Fig. 1*H* vs. Fig. 1*E*). Quantification of BrdU$^+$/insulin$^+$ double-positive cells confirmed that the percentage of replicating human β-cells was less than 0.5% in vehicle and S961-treated mice (Fig. 1*J*). Coimmunostaining for insulin and Ki67 also confirmed that the engrafted human β-cells did not respond to insulin resistance in the same manner as the β-cells of the mouse pancreas (Fig. 1*K*).

An important caveat of this study is that we could not analyze human β-cells in their normal location, but only after transplantation under the kidney capsule. One could envision, for instance, that the betatrophin produced in the liver does not reach the islets in the kidney capsule in the same concentration as is seen by β-cells in the endocrine pancreas. In addition, it is possible that betatrophin exerts its effects not directly by binding to a receptor on β-cells, but indirectly, for instance, by altering β-cell innervation or by activating another cell or signal not present under the kidney capsule. To address this issue, we simultaneously transplanted mouse islets under the right kidney capsule and human islets under the left kidney capsule of the same NOD-Scid mouse. Strikingly, mouse islets were responsive to S961 treatment regardless of location and showed a robust increase in β-cell replication even when placed under the kidney capsule (Fig. 1*F*, *I–K*). In fact, the S961-stimulated replication rate of transplanted mouse β-cells was similar to that seen in β-cells in the endocrine pancreas of the same mice. Coimmunostaining for Ki67 and insulin confirmed that mouse islets transplanted under the kidney capsule respond to S961 treatment with a significantly increased replication rate. Because human β-cells might be more susceptible to hyperglycemia, we performed TUNEL staining to test if these cells had become apoptotic, but found no evidence of increased β-cell death (Fig. 2).

DISCUSSION

In summary, we have confirmed that the insulin-resistant state produced by treating mice with the insulin receptor antagonist S961 causes a dramatic increase in murine β-cell replication and that this effect occurs regardless of whether β-cells are in their native environment in the endocrine pancreas or in the ectopic location under the kidney capsule. This dramatic increase in replication rate is accompanied by the previously documented increase in betatrophin expression in the liver

Insulin/BrdU double-positive cells are very rare. *I*: Transplanted mouse islets recovered from the kidney capsule of S961-treated mice. Note the frequent BrdU$^+$ β-cells (yellow arrows). *J*: β-Cell replication was quantified as the percentage of BrdU$^+$/insulin (Ins)$^+$ double-positive cells of the total number of insulin$^+$ cells from the six conditions shown in *D–I*. $n = 5$ for vehicle and for S961. $^{**}P < 0.01$. β-Cell replication in transplanted mouse islets from 2-month-old or 12-month-old donor mice was also quantified separately. *K*: β-Cell replication was determined as the percentage of Ki67$^+$/insulin$^+$ double-positive cells of the total number of insulin$^+$ cells in the six conditions shown in *D–I*. $n = 5$ for vehicle and for S961. $^{**}P < 0.01$.

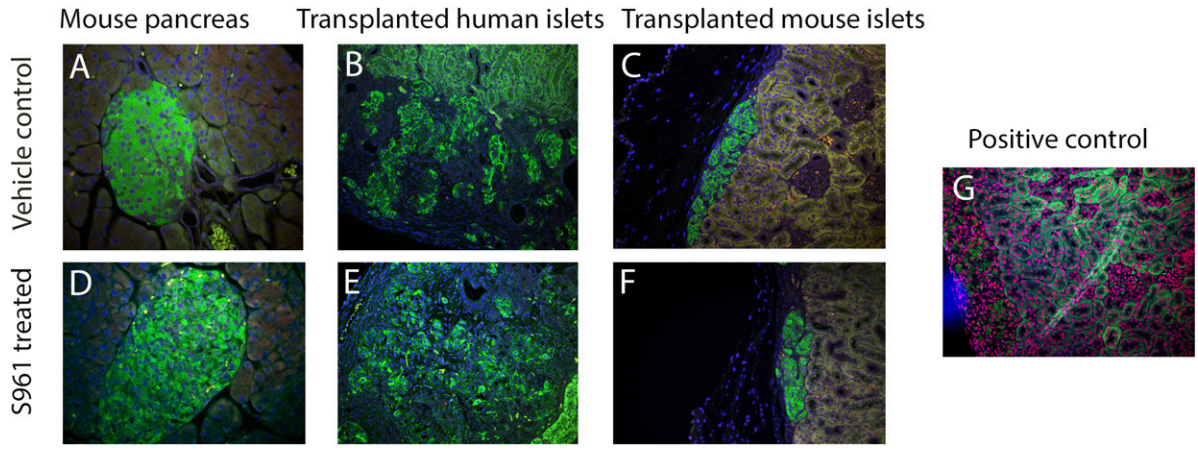

Figure 2—No evidence for increased β-cell apoptosis in S961-treated mice. Apoptosis in NOD-Scid mice treated with vehicle or S961 was determined by TUNEL staining (red signal). β-Cells were identified by insulin immunofluorescence staining (green). *A*: β-Cells in the endocrine pancreas of vehicle-treated mice. *B*: Transplanted human islets recovered from the kidney capsule of vehicle-treated mice. *C*: Transplanted mouse islets recovered from the kidney capsule of vehicle-treated mice. *D*: β-Cells in the endocrine pancreas of S961-treated mice. *E*: Transplanted human islets recovered from the kidney capsule of S961-treated mice. *F*: Transplanted mouse islets recovered from the kidney capsule of S961-treated mice. *G*: Positive control for the TUNEL staining assay using DNAse-treated tissue.

(9), although we did not determine the increase in plasma betatrophin levels. Human islets transplanted under the kidney capsule were not responsive and maintained negligible levels of β-cell replication even when betatrophin production was elevated. One caveat of the presented data is whether mouse betatrophin can act on its human receptor. Because this receptor is presently unknown, this issue cannot be addressed directly.

A second important issue is the age of the β-cell responding to a mitogenic signal, because there is a well-documented decline in β-cell replication rate with age in both mouse and human (13). Although the age of the recipient mice was 2 months, we used mouse islets from donor mice aged 2 months (young adult, postpuberty) or 12 months (aged mice, postfertile), which both responded to the S961 treatment with massively increased proliferation. The basal proliferation rate was fourfold lower in the 12-month-old mouse islets compared with the 2-month-old islets, but the fold induction by S961 treatment was the same (Fig. 1*J* and *K*). The human organ donors ranged in age from 4 to 53 years, thus including a donor of an even younger relative age than the mice used (Table 1), suggesting that even young human β-cells are not responsive to betatrophin. In conclusion, these data put into question whether betatrophin is a mitogen for human β-cells and whether betatrophin will be a useful therapeutic approach for human diabetes.

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Author Contributions. Y.J., J.L.L., and M.Y. researched data. A.N. contributed to discussion and reviewed and edited the manuscript. K.H.K. wrote the manuscript. K.H.K. 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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