Transgenic expression of the human growth hormone minigene promotes pancreatic β-cell proliferation

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Baan M, Kibbe CR, Bushkofsky JR, Harris TW, Sherman DS, Davis DB. Transgenic expression of the human growth hormone minigene promotes pancreatic β-cell proliferation. Am J Physiol Regul Integr Comp Physiol 309: R788–R794, 2015. First published July 22, 2015; doi:10.1152/ajpregu.00244.2015.—Transgenic mouse models are designed to study the role of specific proteins. To increase transgene expression the human growth hormone (hGH) minigene, including introns, has been included in many transgenic constructs. Until recently, it was thought that the hGH gene was not spliced, transcribed, and translated to produce functional hGH protein. We generated a transgenic mouse with the transcription factor Forkhead box M1 (FoxM1) followed by the hGH minigene, under control of the mouse insulin promoter (MIP) to target expression specifically in the pancreatic β-cell. Expression of FoxM1 in isolated pancreatic islets in vitro stimulates β-cell proliferation. We aimed to investigate the effect of FoxM1 on β-cell mass in a mouse model for diabetes mellitus. However, we found inadvertent coexpression of hGH protein from a spliced, bicistronic mRNA. MIP-FoxM1-hGH mice had lower blood glucose and higher pancreatic insulin content, due to increased β-cell proliferation. hGH signals through the murine prolactin receptor, and expression of its downstream targets tryptophan hydroxylase-1 (Tph1), tryptophan hydroxylase-2 (Tph2), and cytokine-inducible SH2 containing protein (Cish) was increased. Conversely, transcriptional targets of FoxM1 were not upregulated. Our data suggest that the phenotype of MIP-FoxM1-hGH mice is due primarily to hGH activity and that the FoxM1 protein remains largely inactive. Over the past decades, multiple transgenic mouse strains were generated that make use of the hGH minigene to increase transgene expression. Our work suggests that each will need to be carefully screened for inadvertent hGH production and critically evaluated for the use of proper controls.

human growth hormone; transgene expression; FoxM1; β-cell; proliferation

TRANSGENIC MOUSE MODELS ARE DESIGNED to study the effects of enhanced expression of specific proteins in vivo. It was discovered that the inclusion of introns increased transgene expression (2, 21). These initial studies were done with the rat or human growth hormone (hGH) gene using various combinations of introns and exons (2, 20, 21). The hGH construct starting within exon 1 and containing all of the introns resulted in the highest expression. Subsequently, investigators found that the hGH minigene could also increase the expression of other intronless transgenes (21, 26). This hGH minigene was then included downstream of numerous promoter-transgene sequences to increase expression (23, 33, 36). The expression of hGH itself, as the second open reading frame, was often not examined. Among the constructs containing hGH, there are many in which transcription is driven by the mouse or rat insulin promoter (MIP, RIP) to target the pancreatic β-cell specifically (3, 8–10, 22, 23, 31, 39). These β-cell transgenics express various proteins implicated in β-cell growth and development, and they provide tools for development of reporter and knockout cell lines, e.g., for green fluorescent protein (GFP) and Cre-recombinase.

Recently, hGH protein production was described in three mouse lines with β-cell-specific promoters and an hGH minigene (Pdx1-Cre<sup>latc</sup>, RIP-Cre, and MIP-GFP mice) (3). The Pdx1-Cre<sup>latc</sup> mice have lower blood glucose, increased β-cell mass, and pancreatic insulin content, and are protected from streptozotocin-induced β-cell death. These phenotypes may be related to hGH signaling, through its action on the prolactin receptor (PRLR) to stimulate serotonin production in the β-cell.

Identifying factors that regulate pancreatic β-cell proliferation is important in the development of new treatments for Type 1 and Type 2 diabetes mellitus. Both diseases result from inadequate functional β-cell mass. β-cells have low rates of basal proliferation, but increased proliferation and expansion of β-cell mass can occur in response to demand from insulin resistance. We have previously shown that expression of the transcription factor Forkhead box M1 (FoxM1) in isolated mouse and human islets stimulates β-cell proliferation (6). We generated MIP-FoxM1-hGH transgenic (FoxM1-hGH) mice to study the effects of FoxM1 in pancreatic β-cells and to determine whether FoxM1 could increase β-cell proliferation in vivo.

As our construct contained the hGH minigene, we investigated our mouse model for the production of hGH. We found that islets of FoxM1-hGH mice express hGH protein and also show evidence of enhanced PRLR signaling. MIP-FoxM1-hGH mice have lower glucose concentrations, higher pancreatic insulin content, and increased β-cell proliferation. Several other transgenic models have used a similar construct to study β-cell mass and growth (7, 31, 32), and our work suggests that each will need to be carefully screened for hGH production or critically evaluated for the use of appropriate controls.

METHODS

Mice. Mouse protocols were approved by the University of Wisconsin Animal Care and Use Committee. The MIP-FoxM1-myc-hGH (FoxM1-hGH) mice were derived from the original MIP-GFP-hGH construct (9), which was obtained in a pGEM11z backbone plasmid vector (Promega, Madison, WI). From this vector, GFP was removed with Xhol cleavage, and a 47-bp sequence containing multiple additional restriction enzyme sites was inserted between the MIP and the hGH for ease of future cloning. The transgene construct was assembled by amplifying the mouse FoxM1 coding sequence (2,281 bp, derived from NM_008021) with a Kozak sequence inserted before the

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ATG transcriptional start site, and a 29 bp myc tag following by two stop codons at the 3’ end. The Kozak-FoxM1-myc-stop-stop construct (total of 2,321 bp) was cloned into the MIP-hGH construct using XhoI and ClaI restriction sites in the newly created multiple cloning site (MCS). This left 15 bp of DNA sequence after the stop codons, which was not present in the original MIP-GFP construct, upstream of the hGH minigene (Fig. 1, A and B). The transgene construct was fully sequenced from mFoxM1 through the hGH gene. Linearized transgene DNA was microinjected into pronuclei of BTBR embryos. We chose the BTBR mouse specifically, because they fail to upregulate FoxM1 expression and lack a compensatory β-cell proliferation response in the face of obesity-induced insulin resistance (5, 11). Only one founder line was obtained. Nontransgenic littermates served as controls. PCR was used for FoxM1-hGH genotyping (Fig. 1A, primers 2B: forward (F): ttctagacatcagtttccctggc and reverse (R): tggc-gaagatgatagcctca and R, cagatcctcttctgagatga; primers 3C, Fig. 1A). Primers were as in Ref. 3.

Mice were housed in aseptic disposable ventilated cages (Innovive, San Diego, CA), on a 12:12-h light-dark cycle, at 22 ± 2°C ambient temperature, and 30–70% humidity, with 1/8 corn cob bedding, envirodri, and an igloo for cage enrichment. Mice were fed Harlan Teklad Global Soy Protein-Free Extruded Rodent Diet. Dry, and an igloo for cage enrichment. Mice were fed Irradiated dry, and an igloo for cage enrichment. Mice were fed 2920x Irradiated controls. PCR was used for quantification. For each islet, the percentage of BrdU-positive cells was calculated (19–36 islets per mouse).

Real-time PCR was done with Power SYBR Green (Applied Biosystems). All values were normalized to actin

mRNA quantification. Islets were isolated, as described previously (19). Islet RNA was extracted with the RNeasy kit (Qiagen). cDNA was synthesized with a reverse transcriptase kit (Applied Biosystems). Real-time PCR was done with Power SYBR Green (Applied Biosystems). All values were normalized to actin

Insulin secretion and content. Insulin secretion was measured in individual islets (11–20 per condition per mouse) from 10-wk-old mice cultured at 1.7 and 17 mM glucose (n = 2) (18). The pancreas was collected at 10 wk, and insulin content was measured as previously reported (5).

hGH measurement. hGH was measured with an ELISA (Roche Life Science, Madison, WI) in media from 200 islets cultured in 2 ml of media (RPMI, 10% HI-FBS, 1% penicillin-streptomycin) for 24 h at 8 mM/glucose or for 1 h in 20 mM/glucose, and in islet lysate. Brdu labeling. Bromodeoxyuridine (BrdU) (0.8 mg/ml) was added to the drinking water every 3 days for 2 wk prior to death. Pancreata were paraffin-embedded, and cryoembedded (5). Sections were stained with anti-insulin (1:100, cat. A0564; Dako, Glostrup, Denmark) and anti-BrdU (1:50, Cat#NA61; Calbiochem, Billerica, MA) antibodies, and appropriate secondary antibodies, and mounted in medium containing DAPI. Image-based Tool for Counting Nuclei (ImageJ plugin, National Institutes of Health, Bethesda, MD) was used for quantification. For each islet, the percentage of BrdU-positive β-cells was calculated (19–36 islets per mouse).

Analysis. Area under the curve was calculated with the trapezoidal method, starting from the time zero value as the baseline. Data were analyzed using GraphPad Prism. Comparisons were made with unpaired Student’s t-tests.

RESULTS

FoxM1-hGH mice were generated in the BTBR mouse strain, which has increased diabetes susceptibility (4, 14, 28). The transgene construct contains the MIP, the murine FoxM1 coding sequence, a myc tag, and the entire hGH gene (Fig. 1A). The FoxM1-myc sequence is followed by a double-stop codon (Fig. 1B). hGH was thought not to be transcribed, spliced, and translated. The original intent of this transgenic mouse was to overexpress FoxM1 in the β-cell to study its role in β-cell proliferation in vivo.

FoxM1-hGH islets express a FoxM1-myc fusion mRNA and protein (primers 1A, Fig. 1, A, C, D), as intended. Unexpectedly, they also express hGH mRNA, which is appropriately spliced, with the loss of the second intron (primers 3C, Fig. 1, A and C). The production of bicistronic mRNA with hGH intron splicing in FoxM1-hGH islets was confirmed using primers that span the FoxM1-myc-hGH junction (primers 2C, Fig. 1A, data not shown).

Furthermore, hGH protein was detected in FoxM1-hGH islet lysate (67.5 ± 7.4 pg/ml), whereas it was undetectable in controls (Fig. 1E). hGH was also present in the media of FoxM1-hGH islets cultured at 8 mM glucose for 24 h (7.0 ± 2.7 pg/ml) or at 20 mM glucose for 1 h (1.5 ± 0.23 pg/ml, Fig. 1E), suggesting that hGH protein is secreted. However, hGH was undetectable in the plasma of both control and MIP-FoxM1-hGH mice, indicating that β-cell production of hGH likely resulted in only local paracrine effects within the islet (data not shown).

hGH signals through the murine PRLR pathway and induces expression of downstream targets Tph1, Tph2, and Cish (3). These targets were upregulated in FoxM1-hGH islets (61-, 7.3-, and 7.6-fold, respectively; P < 0.05, Fig. 1F), indicating hGH-activated PRLR signaling in the islet. However, known transcriptional targets of FoxM1, including cyclin A2, B2, D1-3, and cyclin E2 (6, 35) were not upregulated (data not shown). Therefore, we did not see the anticipated downstream effects of increased FoxM1 transcriptional activity.

As expected, FoxM1-hGH males had normal body weight (Fig. 2A), further indicating that β-cell hGH and FoxM1 expression did not have systemic effects. Lean FoxM1-hGH males had lower 4-h fasting plasma glucose at all ages (Fig. 2B). Glucose (Fig. 2C) and insulin levels (data not shown) after IP-GTT were not different from littermate controls. Fasting insulin, as the baseline for the GTT, was not significantly different (data not shown). There was no evidence of a difference in overall insulin sensitivity, as suggested by the glucose-to-insulin ratios during GTT (Fig. 2D). Both FoxM1-hGH and controls exhibit hyperglycemia, consistent with the known diabetes-prone phenotype of BTBR mice.

Glucose-stimulated insulin secretion from both FoxM1-hGH islets and BTBR nontransgenic control islets was low, but not significantly different between groups (P = 0.42, Fig. 2E), indicating that the insulin secretion response to glucose was not affected by hGH expression. Another transgenic model that produces hGH in β-cells, the Pdx1lataCre-hGH mouse, has a reduction in glucose tolerance and glucose stimulated insulin secretion (GSIS), associated with a decreased expression of Glut2 (3). The Glut2 gene encodes for the glucokinase-coupled

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Fig. 1. MIP-FoxM1-myc-hGH transgene construct and transgenic protein expression. (A) Organization of the MIP-FoxM1-myc-hGH transgene construct (not to scale). The hGH minigene contains introns and exons. Primer pairs indicated were used for genotyping and to evaluate transgene mRNA expression and processing. Kz, Kozak; FoxM1, Forkhead box M1 coding sequence; Myc, c-Myc tag; MCS, multiple cloning site; hGH, human growth hormone; TSS, transcription start site; ATG, translation start site. B: sequence of the FoxM1-Myc to hGH transition. C: FoxM1 and hGH mRNA expression (means ± SE; n = 3, P = 0.014 and 0.0054, respectively). D: FoxM1 protein expression in islets from lean male FoxM1-hGH mice and wild-type nontransgenic controls. The FoxM1 transgene construct is expressed as a fusion protein with a Myc-tag (pooled islets from three mice). E: FoxM1-hGH mice express high levels of hGH protein (black bars), whereas hGH is not detected in wild-type controls (white bars). hGH was detected in media from FoxM1-hGH islets (black bars) after 24 h at 8 mM glucose, and 1 h at 20 mM glucose (n = 2). F: FoxM1-hGH islets show upregulation of downstream targets of prolactin receptor signaling, Tph1, Tph2, and Cish (n = 3).
glucose transporter on the β-cell, which serves as a glucose sensor that triggers the release of insulin with increasing blood glucose concentrations. However, expression of Glut2 was unchanged in FoxM1-hGH islets, which suggests glucose sensing is not affected by hGH expression (Fig. 2F).

FoxM1-hGH mice had higher pancreatic insulin content than controls (649 ± 49 vs. 343 ± 75 μg insulin/g pancreas, P = 0.01, Fig. 3A). The endocrine pancreas makes up about 2% of total pancreatic mass, and pancreatic insulin content is considered a crude measure of β-cell mass (11, 29). Consistent with this, FoxM1-hGH islets had increased β-cell proliferation, measured by BrdU-incorporation in β-cells after 2 wk of supplementation in the drinking water (17 ± 0.7 vs. 13 ± 0.7% BrdU-positive β-cells, Fig. 3, B–D).

Therefore, FoxM1-hGH mice have higher pancreatic insulin content, as a result of increased β-cell proliferation. However, this is likely due to the role of inadvertent hGH signaling in the β-cell rather than to FoxM1 overexpression.

**DISCUSSION**

MIP-FoxM1-hGH mice are derived from the MIP-GFP-hGH construct (9), in which the hGH minigene was included to increase expression of GFP. In the original publication, there
was no phenotype in MIP-GFP-hGH mice, and hGH was not detected by immunohistochemistry (9). However, a more recent report found that MIP-GFP-hGH islets contained both hGH mRNA and protein, as shown in a Western blot (3). This resulted in activation of PRLR signaling and increased serotonin production. (3). Similar to our phenotype, the MIP-GFP-hGH mice also had reduced fasting glucose, no difference in glucose tolerance or insulin sensitivity, and, in fact, had diminished glucose-stimulated insulin secretion (3).

Here, we confirm hGH production in FoxM1-hGH mice and provide some insight into how the hGH transgene is expressed. We find that a single, bicistronic mRNA message is made, and the hGH mRNA is spliced to create an open reading frame for hGH protein. Exactly how this second hGH cistron is translated remains unclear. It seems unlikely that this bicistronic mRNA contains an internal ribosome reentry site in the short sequence between the stop codons of the first cistron and the start codon of the second cistron (Fig. 1, A and B). More likely, there is incomplete ribosomal detachment after translational termination of the first cistron.

Notably, our group has evaluated hGH expression in another transgenic mouse using a nearly identical construct (except with a different transgene). Despite upregulation of spliced, bicistronic hGH mRNA expression, this other transgenic mouse does not express hGH protein, and PRLR signaling is not upregulated (unpublished results). These data emphasize that hGH protein expression varies depending on unknown factors that affect either splicing efficiency or translational activity. Therefore, hGH protein expression needs to be separately evaluated in each transgenic model containing an hGH minigene.

While the phenotypes of hGH-expressing transgenics have been reported previously, our work adds to this literature and identifies some differences. First, we show direct evidence of increased β-cell proliferation in FoxM1-hGH mice. Secondly, we did not find diminished glucose tolerance nor GSIS, as described in the Pdx1floxed.Cre-hGH mice (3). Consistent with this, Glut2 expression was unaltered. Therefore, glucose intolerance may be unrelated to hGH expression and may be an effect of the expression of Cre protein itself, as has been previously reported (16).

It is unclear whether the phenotype in FoxM1-hGH mice is solely dependent on hGH signaling or whether transgenic expression of FoxM1 plays a role as well. Adenovirus-induced overexpression of human FoxM1b increases β-cell proliferation and transcription of multiple cell cycle genes in mouse and human islets ex vivo (6). Although FoxM1 transgenic protein was present in FoxM1-hGH mouse islets, key downstream transcriptional targets, such as cyclin A2, were not upregulated, suggesting that FoxM1 is not transcriptionally active. This is in concordance with prior studies in which overexpression of FoxM1 in vivo is not sufficient to drive proliferation (12, 13, 34, 38). Rather, only after a “second hit,” such as the infliction of tissue injury or treatment with mitogenic factors, does overexpressed FoxM1 become transcriptionally active and enhance cell proliferation (13, 38). Constitutive activity of inhibitors of FoxM1 may prevail under conditions in which there is no need for compensatory expansion of β-cell mass. In fact, we did not predict that transgenic FoxM1 would be active and lead to a phenotype in the absence of a proliferative stimulus such as obesity or β-cell destruction. Although we cannot fully rule out a partial role for FoxM1, we suspect that the phenotype in lean mice is due primarily to hGH activity.

As our transgenic mice are in the diabetes-prone BTBR strain, there are no appropriate control animals expressing hGH alone to allow us to tease apart the contribution of hGH separately from FoxM1. Other transgenic models exist in the diabetes literature in which transgene-hGH constructs similar to ours have been used to describe enhanced β-cell proliferation (7, 31, 32, 37). If these transgenics are found to coexpress hGH, appropriately controlled experiments will also be difficult to perform to isolate the direct in vivo effect of the transgene in β-cell proliferation.

The use of the human growth hormone minigene is common within the transgene literature. It has been used in transgenics designed to explore the function of proteins by overexpression in multiple tissues and in the development of multiple Cre-recombinase lines for generating knockouts (1, 15, 23, 30, 33, 36, 37). For example, Alb-Cre-hGH mice, which express the enzyme Cre-recombinase specifically in the liver under control of the albumin promoter, also produce high levels of hGH mRNA (23). Through extensive cross-breeding of these mice with various different floxed strains, the hGH gene is present in many mouse models (17, 24, 25, 27). Our findings raise important concerns about the potential impact of hGH expression on the phenotype of many other transgenic models, where appropriate control groups may not have been included.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


