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

Running Head: Inadvertent hGH expression in MIP-FoxM1 transgenics

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Abstract

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 co-expression of hGH protein from a spliced, bicistronic mRNA.

MIP-FoxM1-hGH mice had lower blood glucose and higher pancreatic insulin content, due to increased beta-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 generally 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.
Introduction

Transgenic mouse models are designed to study the effects of enhanced expression of specific proteins \textit{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 (GH) 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 this hGH minigene could also increase the expression of another 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 are many where transcription is driven by the mouse or rat insulin promoter (MIP, RIP) to target the pancreatic $\beta$-cell specifically (3, 8-10, 22, 23, 31, 39). These $\beta$-cell transgenics express various proteins implicated in $\beta$-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 $\beta$-cell specific promoters and an hGH minigene (Pdx1-Cre\textsuperscript{late}, RIP-Cre, and MIP-GFP mice)(3). The Pdx1-Cre\textsuperscript{late} mice have lower blood glucose, increased $\beta$-cell mass and pancreatic insulin content, and are protected from streptozotocin-induced $\beta$-cell death. These phenotypes may be related to hGH signaling, through its action on the prolactin receptor (PRLR) to stimulate serotonin production in the $\beta$-cell.

Identifying factors that regulate pancreatic $\beta$-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 if FoxM1 could increase β-cell proliferation \textit{in vivo}.

As our construct contained the hGH minigene, we investigated our mouse model for the production of hGH. We found that islets of MIP-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.

\textbf{Research Design and Methods}

\textit{Mice}

Mouse protocols were approved by the University of Wisconsin Animal Care and Use Committee. The MIP-FoxM1-myc-hGH (MIP-FoxM1) mice were derived from the original MIP-GFP-hGH construct (9), which was obtained in a pGEM11z backbone plasmid vector (Promega Corporation, Madison, WI). From this vector, GFP was
removed with XhoI cleavage, and a 47 bp sequence (MCS) 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 (2281 bp, derived from NM_008021) with a Kozak sequence inserted before the ATG transcriptional start site, and a 29 bp myc tag followed by two stop codons at the 3’ end. The Kozak-FoxM1-myc-stop-stop construct (total of 2321 bp) was cloned into the MIP-hGH construct using XhoI and ClaI restriction sites in the newly created 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 1A,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 beta cell proliferation response in the face of obesity-induced insulin resistance (5,11). Only one founder line was obtained. Non-transgenic littermates served as controls. PCR was used for FoxM1-hGH genotyping (Fig. 1A, primers 2B: F-cttctagacatcagtttccctggc, R-tggccaaatactgggcttac).

Mice were housed in aseptic disposable ventilated cages (Innovive, San Diego, CA), on a 12h light/12h dark cycle, at 22±2°C ambient temperature, and 30-70% humidity, with 1/8 corncob bedding, and enviro-dry and an igloo for cage enrichment. Mice were fed 2920x Irradiated Harlan Teklad Global Soy Protein-Free Extruded Rodent Diet.

**Phenotyping**
Glucose homeostasis was evaluated by retro-orbital eye bleeds in conscious male mice performed after a 4-h fast. Intra-peritoneal glucose tolerance testing (IP-GTT) was performed using 2 mg of dextrose/gram of body weight injected intraperitoneally, after a 4-h fast, with sampling at 0, 15, 60 and 120 minutes. Glucose was measured with a colorimetric assay according to manufacturer’s instructions (Infinity Glucose Oxidase Method, Cat#TR15221, ThermoScientific, West-Sussex, UK). Insulin was measured with a sandwich ELISA as described (14), with the following modifications: the coating antibody was a monoclonal mouse antibody to insulin and proinsulin (Cat# 10R-I136a, Fitzgerald Industries International, Acton, MA, USA), the secondary antibody was a biotin-conjugated monoclonal mouse antibody to insulin and proinsulin (Cat# 61R-I136bBT, Fitzgerald).

**mRNA quantification**

Islets were isolated as described previously (19). Islet RNA was extracted with the RNeasy kit (Qiagen). cDNA was synthesized with a Reverse Transcription Kit (Applied Biosystems). Real-time PCR was done with Power SYBR Green (Applied Biosystems). All values were normalized to $\beta$-actin (F-acctctcataatgctgcg, R-agcctggatgctacgtaca). FoxM1: primers 1A (Fig. 1A), F-cacaatgatgcttcctca, R-cagatcctctcttctgaga. Tph1: F-ttccaggagaatgatcgcgtg, R-cataacgtcttctcttctgagt. Tph2: F-ccecggaaccagatcagtc, R-atctgatgtgccagagcc. hGH (primers 3C, Fig. 1A), Cish, and Glut2 primers were as in (3).

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

**hGH measurement**

hGH was measured with an ELISA (Roche Life Science) in media from 200 islets cultured in 2 mL of media (RPMI, 10% HI-FBS, 1% penicillin/streptomycin) for 24h at 8 mmol/L glucose or for 1h in 20 mmol/L glucose, and in islet lysate.

**BrdU labeling**

Bromodeoxyuridine (BrdU) (0.8 mg/mL) was added to the drinking water every three days for 2 weeks prior to sacrifice. Pancreata were paraformaldehyde-fixed and cryo-embedded (5). Sections were stained with anti-insulin (1:400, Cat#A0564, Dako) and anti-BrdU (1:50, CA#NA61, Calbiochem) antibodies, and appropriate secondary antibodies, and mounted in medium containing DAPI. Image-based Tool for Counting Nuclei (ImageJ plugin, National Institutes of Health) 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 Graph Pad 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 \textit{in vivo}.

FoxM1-hGH islets express a FoxM1-myc fusion mRNA and protein (primers 1A, Fig. 1A,C,D), as intended. Unexpectedly, they also express \textit{hGH} mRNA, which is appropriately spliced, with the loss of the second intron (primers 3C, Fig. 1A,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 24h (7.0±2.7 pg/mL) or at 20 mM glucose for 1h (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 \textit{Tph1}, \textit{Tph2}, and \textit{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 4h-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 non-transgenic 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 Pdx1<sup>late</sup>-Cre-hGH mouse, has a reduction in glucose tolerance and GSIS, associated with a decreased expression of Glut2 (3). The Glut2 gene encodes for the glucokinase-coupled glucose transporter on the beta cell, which serves as a glucose sensor that triggers the release of insulin with increasing blood glucose concentrations. However, expression of <i>Glut2</i> 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 two weeks of supplementation in the drinking water (17±0.7 vs 13±0.7% BrdU-positive β-cells, Fig. 3B-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 (IRES) in the short sequence between the stop codons of the first cistron and the start codon of the second cistron (Fig. 1A,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 an 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 Pdx<sup>late</sup>-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 if the phenotype in FoxM1-hGH mice is solely dependent on hGH signaling, or if 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 where 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 where 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.

While 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 where 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 co-express 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 crossbreeding 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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Author contributions: MB and DBD conceived and designed the study, analyzed the data and wrote the manuscript. MB, CRK, JRB, TWH, and DSS were instrumental in data collection. CRK, JRB, TWH, and DSS edited the manuscript.

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Disclosures

The authors declare no conflicts of interests.

Figure Legends

Figure 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 coding 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 (mean±SEM, n=3, p=0.014 and 0.0054, respectively). (D) FoxM1 protein expression in islets from lean male FoxM1-hGH mice and wildtype non-transgenic controls. The FoxM1 transgene construct is expressed as a fusion protein with a Myc-tag (pooled islets from 3 mice). (E) FoxM1-hGH mice express high levels of hGH protein (black bars), whereas hGH is not detected in wildtype controls (white bars). hGH was detected in media from FoxM1-hGH islets (black bars) after 24h at 8mM glucose, and 1h at 20mM glucose (n=2). (F) FoxM1-hGH islets show upregulation of downstream targets of prolactin receptor signaling, Tph1, Tph2, and Cish (n=3).

Figure 2: Normal weight gain, improved glycemic phenotype, and normal GSIS in FoxM1-hGH mice.
(A) Body weight of FoxM1-hGH males does not differ from that of non-transgenic littermate controls (n=7-14, mean±SEM). (B) FoxM1-hGH males have lower 4h-fasted plasma glucose at 4, 6, and 8 weeks of age, with a trend toward lower levels at 10 weeks (p=0.069). (C) Plasma glucose concentrations (n=10-14, mean±SEM) and area under the curve after IP-GTT at 8 weeks were not different. (D) Glucose:insulin ratio during IP-GTT did not differ between groups (n=7-12, mean±SEM). (E) Glucose-stimulated insulin secretion (p=0.42) was not different in FoxM1-hGH mice compared with non-transgenic controls, which was consistent with unchanged Glut2 mRNA expression (F).

Figure 3: Pancreatic insulin content and β-cell proliferation in MIP-FoxM1-hGH mice. (A) FoxM1-hGH mice have higher pancreatic insulin content compared with non-transgenic controls. (B) Their islets show increased β-cell proliferation as measured by increased BrdU incorporation after supplementation for two weeks in the drinking water. (C, D) Representative images of BrdU staining. Blue: nuclei, Red: insulin, Green: BrdU.

References


Figure A: Schematic representation of the MIP-Mm FoxM1 construct.

Figure B: Nucleotide sequences of the FoxM1 and Myc promoters.

Figure C: mRNA expression levels of FoxM1-hGH normalized to β-actin in WT and FoxM1-hGH cells.

Figure D: Western blots showing protein expression levels of FoxM1, b-Tubulin, Myc Tag, and b-Actin in WT and FoxM1-hGH cells.

Figure E: hGH levels in different conditions: Islets, media 24h 8 mM Glu, and media 1h 20 mM Glu.

Figure F: Fold upregulation of Tph1, Tph2, and Cish in different conditions.