

Expression mechanism of tryptophan hydroxylase 1 in mouse islets during pregnancy

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Abstract

Serotonin signaling plays key roles in augmentation of pancreatic β -cell function during pregnancy. Increased expression of tryptophan hydroxylase 1 (Tph1), a rate-limiting enzyme for serotonin synthesis by lactogenic hormones, is involved in this phenomenon. To investigate its mechanisms, we here performed 5'-RACE and identified β -cell-specific transcription initiation sites for *Tph1*. Prolactin enhanced the expression of mRNA containing these exons; however, reporter gene plasmids containing the proximal 5'-flanking region of these exons did not show prolactin responsiveness in MIN6 cells. Prolactin-induced *Tph1* expression was inhibited by a Jak2 inhibitor and was partially inhibited by an MEK1/2 or PI3K inhibitor. Therefore, we analyzed interferon γ -activated sequences (GAS) and found GAS-A about 9-kbp upstream of the transcription start site. The reporter gene plasmid containing the GAS-A region linked to a heterologous promoter showed increased promoter activity by prolactin, which was inhibited by the forced expression of a dominant-negative mutant form of Stat5A and a Jak2 inhibitor. Chromatin immunoprecipitation analysis showed that prolactin treatment augmented Stat5 binding to the GAS-A region in MIN6 cells, as well as in isolated mouse islets, and that Stat5 recognized the GAS-A region in pregnant mouse islets. In addition, the transactivation activity of Stat5 was enhanced by prolactin through the Erk and PI3K pathways in MIN6 cells. Finally, serotonin expression was attenuated in islets of β -cell-specific *Stat5*-deficient mice compared with that of control littermates during pregnancy. Our findings suggest that prolactin-induced *Tph1* expression is mediated by the activation of Jak2/Stat5, Erk, and PI3K pathways in β cells.

Key Words

- ▶ β cell
- ▶ islet
- ▶ serotonin
- ▶ Tph1
- ▶ prolactin
- ▶ Stat
- ▶ Erk
- ▶ PI3-kinase
- ▶ pregnancy
- ▶ MIN6 cell

*Journal of Molecular
Endocrinology*
(2015) 55, 41–53

Introduction

Glucose homeostasis is maintained through the coordinate activities of the insulin-producing pancreas and glucose-storing tissues, including muscle, liver, and

adipose tissue. Pancreatic β cells respond to fluctuations in blood glucose to secrete appropriate amounts of insulin; therefore, disruption of insulin release causes glucose

intolerance. The capacity of insulin secretion from a pancreas is governed by its β -cell mass and the capacity of each β cell to release insulin. Type 1 diabetes is characterized as insulinitis triggered by an autoimmune disorder against β cells, leading to an absolute defect of insulin release. In type 2 diabetes, insufficient insulin secretion to compensate for insulin resistance causes hyperglycemia. Previous studies showed that a reduction in β -cell mass was observed in type 1 as well as type 2 diabetic patients, suggesting that the preservation of or increase in β -cell mass is a promising approach to maintain normoglycemia (Butler *et al.* 2003, Meier *et al.* 2005). Whereas islet mass increases during the prenatal and neonatal period, the appearance of proliferation markers in β cells decreases with ageing (Meier *et al.* 2005, 2008, Gregg *et al.* 2012, Saisho *et al.* 2013). Although pancreas or islet transplantation would be an effective approach to augment β -cell mass, there is limited availability of pancreata for transplantation.

Placental lactogens induce glucose intolerance during pregnancy, which leads to dramatic increases in insulin release to maintain blood glucose levels. Pathological analysis of pregnant rodent models demonstrated that β -cell mass increases daily (Buchanan & Xiang 2005, Rieck & Kaestner 2010). Numerous studies have shown that the prolactin receptor plays an important role in the augmentation of β -cell function in pregnant rodent models. Indeed, heterozygous prolactin receptor knockout mice exhibited mild glucose intolerance and failed increase in β -cell mass during pregnancy (Huang *et al.* 2009, Hughes & Huang 2011). In addition, treatment with prolactin or placental lactogens evoked β -cell proliferation in isolated islets. These data suggest that mechanisms of β -cell compensation during pregnancy are different from those during overnutrition (Chang-Chen *et al.* 2008). Recently, our group showed that serotonin (5-hydroxytryptamine (5-HT)) is highly expressed in mouse and human islets during pregnancy and contributes to increases of β -cell proliferation as well as insulin secretion (Kim *et al.* 2010). At the middle stage of pregnancy, expression of a rate-limiting enzyme for 5-HT synthesis, tryptophan hydroxylase 1 (*Tph1*), is upregulated \sim 500-fold. This dramatic change of gene expression results in the enhanced expression of 5-HT in islets during pregnancy. The treatment of isolated mouse islets with 5-HT increased the proliferation of β cells, at least in part through the $G\alpha(q)$ -linked 5-HT receptor called the 5-HT2b receptor (Kim *et al.* 2010). In addition, activation of the inotropic 5-HT receptor, 5-HT3a receptor, results

in enhanced glucose-induced Ca influx, which plays a central role in enhanced glucose-stimulating insulin secretion during pregnancy (Ohara-Imaizumi *et al.* 2013). Inhibition of 5-HT synthesis by dietary restriction of tryptophan or a *Tph1* inhibitor induced glucose intolerance in pregnant mice without affecting insulin sensitivity (Kim *et al.* 2010). The expression of *Tph1* was enhanced by prolactin; however, its precise mechanism has not been fully elucidated (Kim *et al.* 2010, Schraenen *et al.* 2010).

The mechanism involved in the prolactin-induced expression of *Tph1* in β cells is one of the key mechanisms that compensate for insulin resistance during pregnancy. In this study we investigated the mechanisms by which prolactin regulates *Tph1* mRNA expression in a β -cell line and in islets from pregnant mice.

Materials and methods

Cell culture and islet isolation

MIN6 mouse insulinoma cells were cultured in DMEM supplemented with 15% (v/v) FCS, 10 mM HEPES, and 1% (v/v) penicillin and streptomycin (Life Technologies) under 5% CO₂ at 37 °C. β TC3 mouse insulinoma cells were cultured in DMEM supplemented with 15% (v/v) horse serum, 2.5% (v/v) FCS, and 1% (v/v) penicillin and streptomycin. Isolation of mouse islets was performed as described previously (Iwashita *et al.* 2007). For chromatin immunoprecipitation (ChIP) analysis, islets were isolated from non-pregnant and pregnant mice at gestational age (G) 12.5 days. Mouse islets were cultured in RPMI1640 supplemented with 10% (v/v) FCS, and 1% (v/v) penicillin and streptomycin. After pretreatment with or without 100 μ M AG490 (a tyrosine kinase inhibitor of JAK2) (Sigma–Aldrich), 10 μ M U0126 (a selective inhibitor of MEK1/2) (WAKO, Tokyo, Japan), or 50 μ M LY-294002 (an inhibitor of PI3Ks) (Cell Signaling, Tokyo, Japan) for 30 min, MIN6 cells, β TC3 cells, or mouse islets were stimulated with or without 44.2 nM of prolactin (R&D Systems, Minneapolis, MN, USA) for the indicated times.

Quantitative RT-PCR

MIN6 cells (1×10^6), β TC3 cells (1×10^6), and \sim 50 isolated mouse islets were seeded in six-well plates and treated with or without 44.2 nM of prolactin for 24 h. Total RNA was extracted from the cells using RNeasy kit (Qiagen) according to the manufacturer's instructions.

Complementary DNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Reaction mixtures for PCR were prepared using FAST SYBR Green Master Mix (Life Technologies), and quantitative PCR was performed using the 7500 Real-Time PCR System (Life Technologies). Specific primers used for each gene are as follows: mouse *Tph1* (forward: 5'-TGGACATGCCAAAGTCAAGCCCTT-3', reverse: 5'-GTA CTTCAGTCCAAACGGGCGCTTC-3'), mouse prolactin receptor (gene symbol *Prlr*; forward: 5'-TCTCTGTGGAGGTGAGCATCGCA-3', reverse: 5'-TCAG-GCTGGCCCTTCTTCTTGC-3'), and mouse TATA-binding protein (gene symbol *Tbp*; forward: 5'-GCTGCAGTCAT-CATGAGAATAAGAG-3', reverse: 5'-CACCATGTTCTG-GATCTGAAGT-3'). The condition for PCR was 40 cycles of 95 °C for 3 s and 60 °C for 30 s.

5'-RACE

Total RNA was extracted from MIN6 cells as described above, and the 5' ends of the mRNA were analyzed by the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. Briefly, total RNA was treated with calf intestinal alkaline phosphatase to remove 5' phosphates, followed by treatment with tobacco acid pyrophosphatase to remove the 5'-cap structure. Decapped mRNA was ligated to the GeneRacer RNA oligo by T4 DNA ligase. mRNA was transcribed into cDNA using SuperScript III Reverse Transcriptase, and then the cDNA was amplified by PCR using GeneRacer 5' primer and the *Tph1* gene specific reverse primer (5'-GAGAGGACGGTGGCGT-GCGA-3'). After purifying the PCR products, nested PCR was performed using GeneRacer 5' primer and the nested *Tph1* gene-specific reverse primer (5'-CTGATCGGGC-GAGTCCACCGA-3'). After purification, PCR products were subcloned into the pCR2.1-TOPO plasmid vector (Invitrogen) and sequenced. The newly determined *Tph1* exon 1 variant was deposited in the DNA Databank of Japan (accession number: LC008513).

Transcriptional variants of *Tph1* were analyzed by amplifying its cDNA from MIN6 cells and mouse islets using each specific forward primer for exon 1a (5'-GAAGTATGTCCACGGGCCTC-3'), exon 1b (5'-ATTG-GCCGTTCTGACGATGC-3'), exon 1c (5'-CAGCAAG-GACGGGATCAACT-3'), and the reverse primer designed in exon 3 (5'-TCCGGGACTCGATGTGTAAC-3'). β -actin (gene symbol; *Actb*) was amplified as an internal control (forward: 5'-CATCCGTAAGACCTCTATGCCAAC-3', reverse: 5'-ATGGAGCCACCGATCCACA-3'). Total RNA of mouse brain stem cells was purchased from Takara Bio, Inc. (Tokyo, Japan) as a positive control.

Plasmid constructs

Upstream sequences of various lengths of mouse *Tph1* exon 1 or exon 2 were amplified by PCR using each primer set as described in the [Supplementary Table](#), see section on [supplementary data](#) given at the end of this article, and subcloned into the reporter gene plasmid pFox Luc One (pFLO) (Smith *et al.* 1999) or pGL3 (Promega). Two putative Stat5-binding elements and a mutated element were constructed by annealing two oligonucleotides (GAS-A forward: 5'-GATCTGTCCCCAGCTCTTCCCAGAA-AGCCCTGAGGTG-3', reverse: 5'-GATCCACCTCAGGGC-TTCTGGGAAGAGCTGGGGACA-3', GAS-B forward: 5'-GATCTCTTCTTGGCTTACGGGAAGACGGTTTAG-3', reverse: 5'-GATCCTAAACCGTCTTCCCCTAAGCCAA-GAAGA-3', GAS-A Mutation forward: 5'-GATCTGTCCC-CAGCTCTGACCAATAAGCCCTGAGGTG-3', reverse: 5'-GATCCACCTCAGGGCTTATTGGTCAGAGCTGGGGA-CA-3'). 6×GAS-A, 6×GAS-B, or 6×GAS-A Mutation were subcloned into the reporter gene plasmid containing the prolactin minimal promoter, pFLO-Prl (Deering *et al.* 2009), and pFLO-*Tph1* ex1b-760bp via BglII and BamHI restriction sites as described in a previous report (Smith *et al.* 1999). The reporter gene plasmid containing upstream activating sequences for galactose and TATA box (UAS-TATA-Luciferase) was a generous gift from Dr Kajimura (University of San Francisco) (Seale *et al.* 2007). The *Stat5A* cDNA sequence was amplified using the primer set described in [Supplementary Table](#), and subcloned into the expression vector for GAL4-DBD (pM) (Takara Bio, Inc.) via EcoRI and MluI restriction sites. The expression vectors for Stat5A and a dominant-negative form of Stat5A were a generous gift from Dr Shannon L Kellenher (Pennsylvania State University, USA) (Qian *et al.* 2009). Cloned DNA fragments that were amplified by PCR were validated by direct sequencing.

Transient transfection and luciferase assay

MIN6 cells (1×10^6) were seeded in six-well plates, transfected with 1 μ g of reporter gene plasmid, and cotransfected with or without 0.2 μ g of expression vector encoding Stat5A or a dominant-negative form of Stat5A using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. At 24 h after transfection, the cells were treated with 44.2 nM of prolactin. The cells were then harvested and lysed in Reporter Lysis buffer (Promega). The luciferase assay was performed using Luciferase Assay Substrate (Promega). The relative firefly luciferase activity was normalized by the protein

concentration, which was measured using the Pierce 660 nm Protein Assay kit (Thermo, Yokohama, Japan). Each experiment was repeated at least four times.

Electrophoretic mobility shift assay

MIN6 cells were seeded in 10-cm dishes, and isolated nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) according to the procedure described previously (Sadowski *et al.* 1993). The GAS-A oligonucleotides (5'-GATCTGTCCCCAGCTCTTCCCAGAAAGCCCTGAGGTG-3') and GAS-A Mutation oligonucleotides (5'-GATCTGTCCCCAGCTCTGACCAATAAGCCCTGAGGTG-3') labeled by IRDye700 were purchased from Integrated DNA Technologies (Coralville, IA, USA). One microgram of nuclear protein extract was incubated in EMSA buffer (10 mM HEPES (pH 7.9), 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 3% (w/v) Ficoll, 0.05% (w/w) poly(dI-dC), 7.35 μ M BSA) for 10 min at room temperature with 5 μ l rabbit serum or 5 μ l of a STAT5 antibody (Santa Cruz). For competitive analysis, we used a 50-fold or 100-fold excess of non-labeled oligonucleotides (5'-CTGTCGTTTCTGGAATTGGCCATACCTGGAAGAGGCC-3'). Reaction mixtures were electrophoresed through a 5% non-denaturing polyacrylamide gel. After electrophoresis, the gels were dried and scanned by the Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA).

ChIP assay

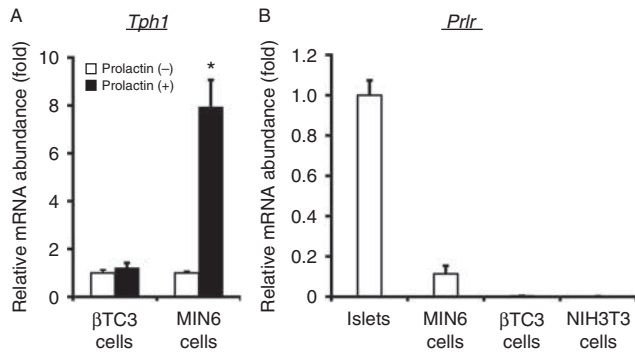
MIN6 cells (3×10^7) were treated with trypsin/EDTA, and ~150 mouse islets were suspended in Ca²⁺-free Hank's balanced salt solution for 30 min at 37 °C, followed by adjusting to 1 mM EGTA for dissociation, and resuspended in PBS. DNA-protein complexes were cross-linked by 1% (w/v) formaldehyde and incubated at room temperature for 10 min, followed by the addition of 0.2 M glycine to stop the cross-linking reaction. The cells were washed twice with ice-cold PBS and three times with 10 ml of MC lysis buffer (10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, and 0.5% (v/v) NP-40). The pellet was resuspended in 1 ml of MNase buffer (10 mM Tris-Cl (pH7.5), 10 mM NaCl, 3 mM MgCl₂, 1 mM CaCl₂, 4% (v/v) NP-40, and 1 mM phenylmethylsulphonyl fluoride (PMSF)), to a final volume of 1.5 ml. DNA was fragmented by 10–100 U of Micrococcal Nuclease for 10 min at 37 °C. To stop the enzymatic reaction, the sample was adjusted to 3 mM EGTA, 1 mM PMSF, 1 \times Halt protease and phosphatase inhibitor cocktail (Life Technologies), 1% (v/v) SDS and 200 mM NaCl. The nuclei were subsequently sheared on

ice with 15 pulses (MIN6 cells) or 40 pulses (mouse islets) of 5 s sonication with a cooling period 15 s, using Sonicator (Qsonica, Newtown, CT, USA). Supernatants were diluted 1:5 by adding FA lysis buffer (50 mM HEPES adjusted to pH 7.5 with KOH, 150 mM NaCl, 1 mM EDTA, 1% (v/v) TritonX-100, 0.1% (w/v) sodium deoxycholate, and 0.1% (v/v) SDS) containing 1 mM PMSF and 1 \times Halt protease and phosphatase inhibitor cocktail. Then 50–100 μ l of protein A/G PLUS-Agarose beads (Santa Cruz) were added and incubated at 4 °C for 4 h to preclean the chromatin. After this incubation, the samples were centrifuged at 300 g for 5 min at room temperature, and 10% of the supernatant was taken as the input. The samples were incubated with protein A/G PLUS-Agarose beads (50–100 μ l) conjugated to 1 μ g of a STAT5 antibody (sc-835x; Santa Cruz) overnight at 4 °C on an end-over-end rotator. The STAT5 antibody-conjugated beads were washed and the chromatin was eluted by 100 μ l of ChIP elution buffer. Two percent (w/w) of Pronase (Roche) was added and the eluted chromatin fragments were reverse cross-linked by incubation at 42 °C for 2 h, followed by incubation at 65 °C for 6 h. The DNA was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

The relative amount of Stat5 binding was determined by PCR using the primers specific for the mouse *Tph1* promoter (GAS-A forward: 5'-ACGTGTCCCCAGCTCTTCCCA-3', and reverse: 5'-TGCTCCAAGCCCTGTCCGAT-3'; GAS-B forward: 5'-TGCTGTGAGCGCATAACGAGAAA-3', and reverse: 5'-AAACCGTCTTCCCGTAAGCCAA-3'), the mouse *Alb* promoter (forward: 5'-TGGGAAAACCTGGAAAACCATC-3', and reverse: 5'-CACTCTCACACATACTCTCTGCTG-3').

Mouse experiments

Floxed *Stat5* mice were a generous gift from Dr Lothar Hennighausen (National Institute of Health, USA) (Lee *et al.* 2007). Tamoxifen-inducible islet-specific Stat5 knockout mice were generated by crossing *Pdx1*-Cre-ER mice with floxed *Stat5* mice (Lee *et al.* 2007). Tamoxifen (75 mg/kg body weight) or corn oil was injected intraperitoneally into 6-week-old mice. These mice were mated at 12 weeks old, and sacrificed at G12.5. After perfusion with 4% (w/v) paraformaldehyde, pancreata were isolated from the mice and embedded in paraffin. Immunofluorescent staining was performed using an anti-5-HT antibody (1:1000, Immunostar, Hudson, WI, USA), an anti-Ki-67 antibody (1:200, BD Biosciences, Tokyo, Japan), and an anti-insulin antibody (1:1000 dilution, Dako, Tokyo,

**Figure 1**

Prolactin induces the expression of *Tph1* in MIN6 insulinoma cells. (A) β TC3 cells and MIN6 cells were incubated with 44.2 nM of prolactin for 24 h. *Tph1* expression was evaluated by qRT-PCR and normalized to *Tbp* expression ($n \geq 3$). * $P < 0.05$ compared with cells treated without prolactin. (B) Expression of *Prlr* in mouse islets, MIN6 cells, β TC3 cells, and NIH3T3 cells was evaluated by qRT-PCR and normalized to *Tbp* expression ($n \geq 3$). Results are shown as the means \pm s.e.m.

Japan) as described previously (Kim *et al.* 2010). Primary antibodies were detected with Alexa-fluor 488- and 555-conjugated secondary antibodies and visualized using TCS-SP5 (Leica, Tokyo, Japan) and the Leica application Suite Version 2.6.0 software. Animal experiments were performed with the approval of the Ethics Review Committee for Animal Experimentation of Juntendo

University and Korea Advanced Institute of Science and Technology.

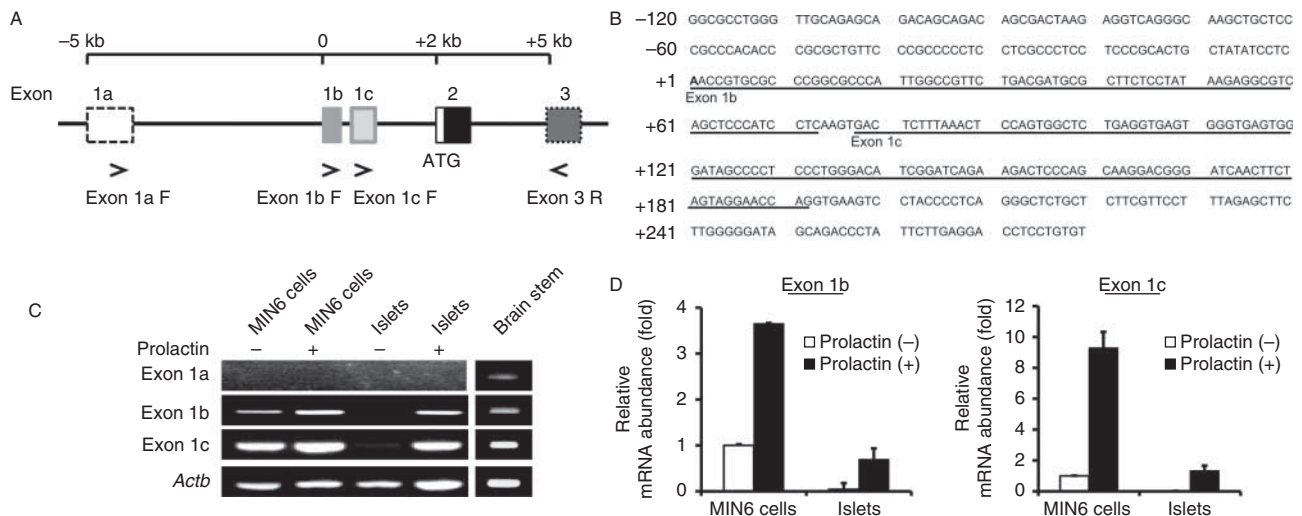
Statistical analysis

All data are presented as the means \pm s.e.m. The Student's *t*-test was used for comparisons involving two conditions. One-way ANOVA followed by the Tukey–Kramer test, or two-way ANOVA was used for comparisons among more than three groups. $P < 0.05$ was considered to indicate a statistically significant difference between two groups.

Results

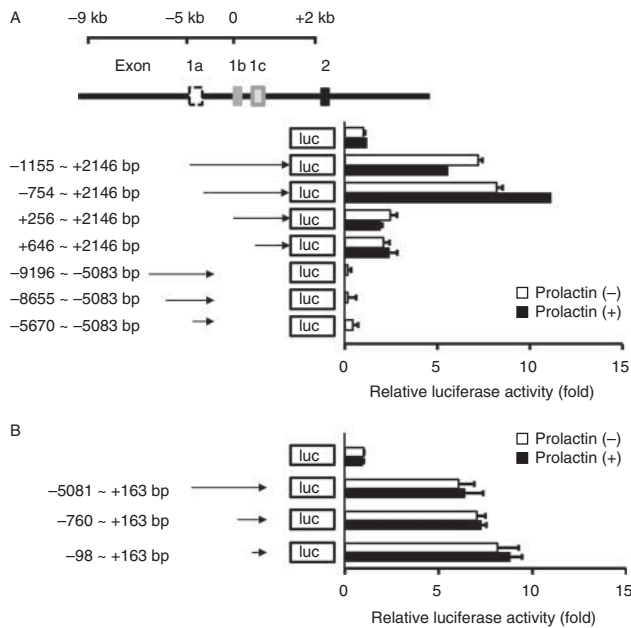
Prolactin-induced *Tph1* expression in the mouse insulinoma cell line MIN6

As the first step toward investigating the mechanisms of *Tph1* expression in β cells, we evaluated the prolactin-induced expression of *Tph1* in mouse insulinoma cell lines MIN6 and β TC3. In MIN6 cells, prolactin induced an eightfold increase in expression of *Tph1* mRNA (Fig. 1A). On the other hand, although β TC3 cells also expressed *Tph1* mRNA, prolactin did not alter its expression. Thus, we used MIN6 cells to investigate prolactin-responsive *Tph1* expression. It is of note that the expression level of the

**Figure 2**

Exon 1b and exon 1c exhibit β -cell-specific transcriptional activity of *Tph1*. 5'-RACE was performed using total RNA isolated from MIN6 cells to identify the β -cell-specific transcription start site for *Tph1*. (A) Diagram of the structure of the *Tph1* gene. Arrows indicate the location of the primers used to amplify each exon 1. F, forward primer; R, reverse primer. (B) Sequences of the β -cell-specific transcription start site, exon 1b and exon 1c. The transcription start site for exon 1b is set as +1. (C) Total RNA

was isolated from MIN6 cells and mouse islets treated with or without 44.2 nM of prolactin. Reverse transcribed cDNA was amplified using each exon 1 primer set as indicated in A. Mouse brain stem was used as positive control for each exon 1. (D) Amounts of cDNA transcribed from exon 1b and exon 1c were evaluated by qRT-PCR and normalized to *Tbp* expression ($n \geq 3$). Results are shown as the means \pm s.e.m.

**Figure 3**

Reporter gene plasmid containing the 5' flanking region of exon 1b and exon 1c demonstrates transcriptional activity, but not prolactin responsiveness in MIN6 cells. Reporter gene plasmids were constructed containing various-length fragments of the mouse *Tph1* gene, as indicated in A and B, and then transfected into MIN6 cells. At 24 h after transfection, MIN6 cells were treated with or without 44.2 nM of prolactin for 24 h and then harvested for the luciferase assay. Luciferase activity was standardized by protein concentration. Relative luciferase activities were calculated with the activity of cells transfected with the pGL3 plasmid alone treated without prolactin set as 1 in (A), and with the pFLO plasmid alone treated without prolactin set as 1 in (B). All experiments were repeated at least three times. All data are shown as the mean \pm s.e.m.

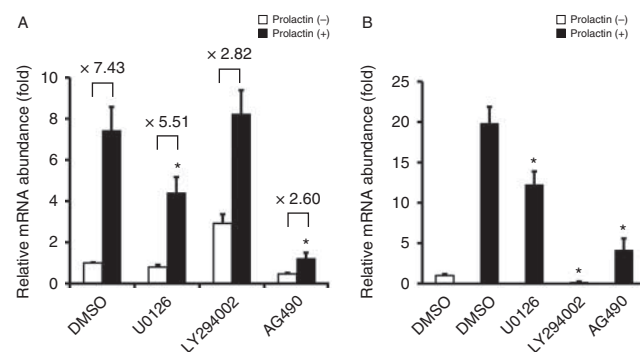
prolactin receptor was \sim 100 times lower in β TC3 cells than in MIN6 cells (Fig. 1B). According to the NCBI database (<http://www.ncbi.nlm.nih.gov/>), the mouse *Tph1* gene has at least two alternatively spliced exon 1s (shown as exon 1a and exon 1c in Fig. 2A). To identify the *Tph1* gene transcripts that are expressed in β cells, we performed 5'-RACE using RNA isolated from MIN6 cells, and found a transcript encoding another form of exon 1 located between 2 exons (shown as exon 1b in Fig. 2A and B). Using primers to identify the expression of transcripts encoding each exon 1 (1a, 1b, and 1c), we performed RT-PCR (Fig. 2A). In MIN6 cells and islets, expression of transcripts encoding exon 1b and 1c, but not that encoding exon 1a were observed and the expression of both of these transcripts was increased by prolactin (Fig. 2C and D).

To investigate whether the proximal 5'-flanking region of each exon contains promoter activity, we cloned various lengths of the 5'-flanking region of exon 1a, and exon 1b+1c, and inserted them upstream of the firefly

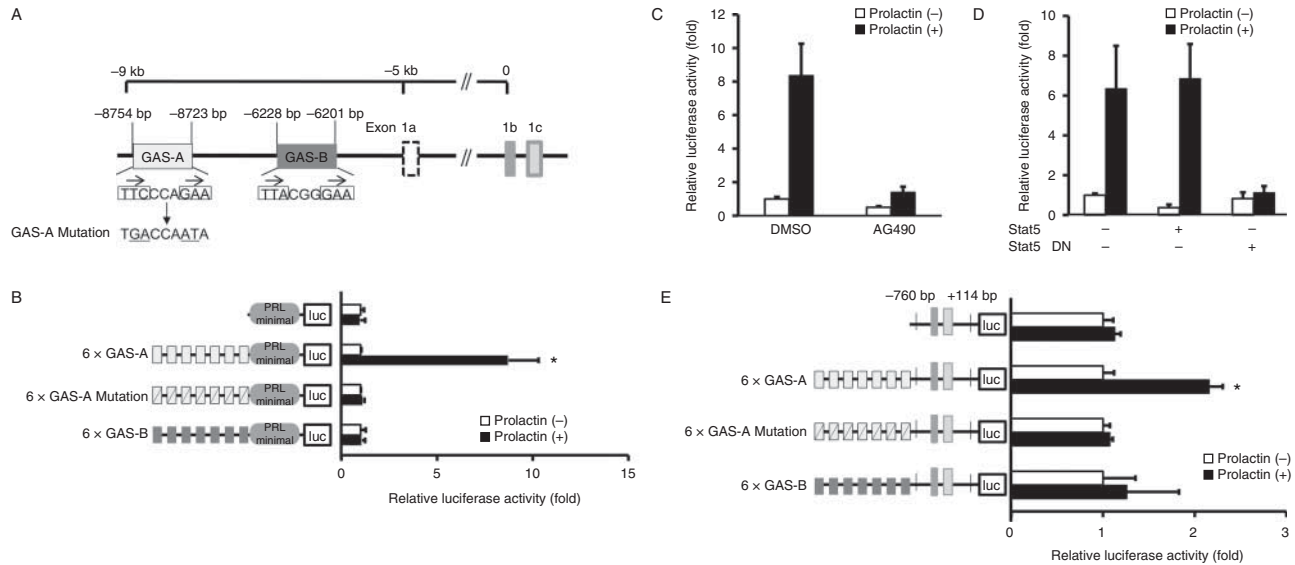
luciferase gene in a reporter gene construct (Fig. 3A and B). After transfection of a series of constructs in MIN6 cells, we evaluated their transcriptional activity by the luciferase assay and found that the plasmids containing the 5' flanking region of exon 1b+1c have promoter activity in MIN6 cells, but the plasmids containing the 5'-flanking region of exon 1a did not show any promoter activity (Fig. 3A). However, stimulation with prolactin did not increase the promoter activity of any of the constructs that we generated (Fig. 3A and B). These results suggest that prolactin responsive elements are not located in the proximal 5'-flanking region of exon 1b+1c.

Prolactin-induced expression of *Tph1* is disrupted by treatment with a JAK2 inhibitor

Reporter gene analyses have suggested that prolactin-responsive elements are located at a distal region from the transcription start site. Thus, we next analyzed the signaling pathway that is essential for prolactin-responsive *Tph1* expression. Prolactin binds the prolactin receptor and drives several downstream protein kinase cascades, such as Jak2, Erk, and PI3K (Buchanan & Xiang 2005, Bernichtein *et al.* 2010). After pretreatment with an inhibitor for each cascade, we treated MIN6 cells with or without prolactin and evaluated *Tph1* expression by quantitative RT-PCR (qRT-PCR). The prolactin-induced expression of *Tph1* was greatly reduced by treatment with the Jak2 inhibitor AG490 and partially reduced by the selective MEK1/2 inhibitor U0126 (Fig. 4A). On the other

**Figure 4**

Prolactin-induced expression of *Tph1* is inhibited by treatment with prolactin receptor signaling inhibitors. (A) MIN6 cells were treated with 44.2 nM of prolactin for 24 h in the presence or absence of 10 μ M U0126, 50 μ M LY294002, or 100 μ M AG490 ($n \geq 3$). (B) Mouse islets were treated with or without 44.2 nM of prolactin for 24 h in the presence of each of the inhibitors ($n = 3$). *Tph1* expression was evaluated by qRT-PCR and normalized to *Tbp* expression. * $P < 0.05$ compared with cells treated with prolactin in the absence of inhibitors. Results are shown as the means \pm s.e.m.

**Figure 5**

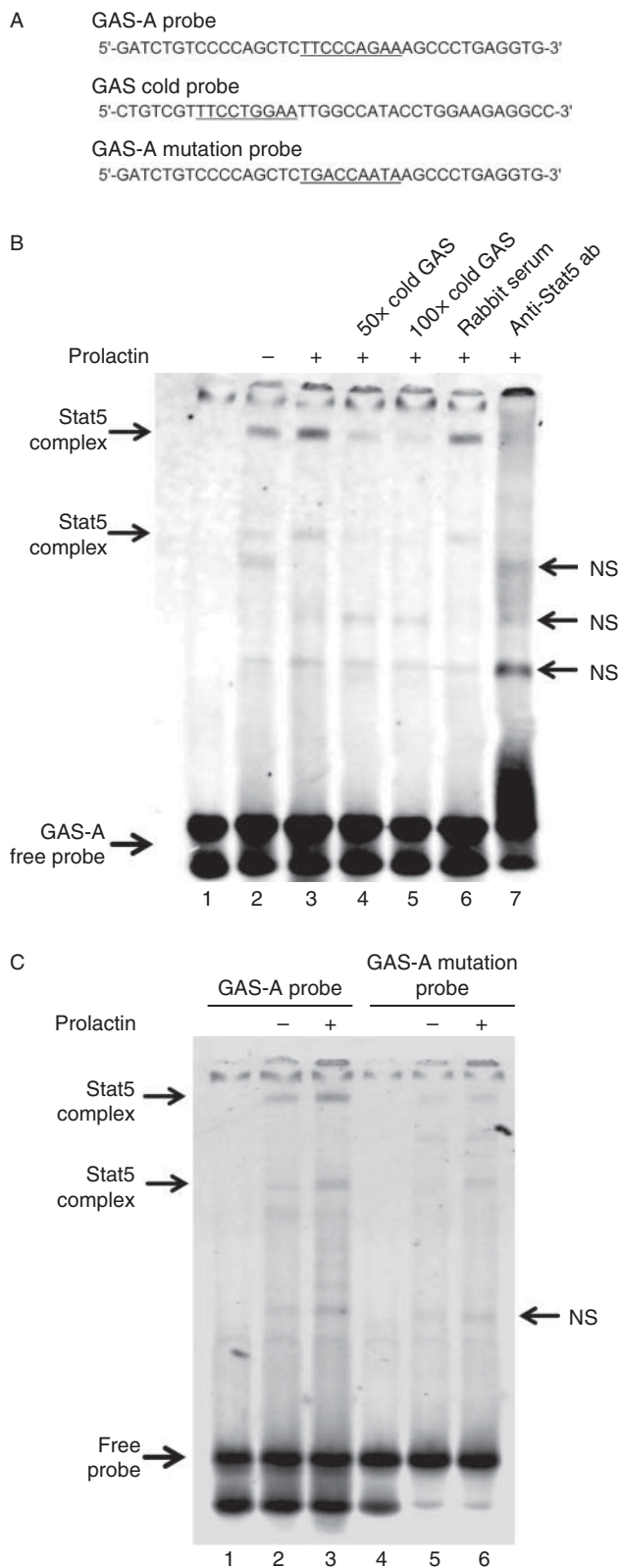
Putative interferon- γ -activated sequence shows prolactin responsiveness in MIN6 cells. (A) Diagram of the mouse *Tph1* gene. Potential binding sites for Stat5 are shown as GAS-A and GAS-B. Mutated GAS-A sequences are indicated by underlines. (B) Reporter gene plasmids with 6 \times GAS-A, 6 \times GAS-A Mutation, or the 6 \times GAS-B fragment inserted upstream of the heterologous prolactin minimal promoter were transfected into MIN6 cells. At 24 h after transfection, MIN6 cells were treated with or without 44.2 nM of prolactin for 24 h, and harvested for the luciferase assay. (C) The 6 \times GAS-A plasmid was transfected into MIN6 cells. At 24 h after transfection, MIN6 cells were treated with or without 44.2 nM of prolactin in the presence or absence of 100 μ M AG490 for 24 h. (D) Plasmids encoding

Stat5A or a dominant-negative form of Stat5A were co-transfected with the 6 \times GAS-A plasmid into MIN6 cells. At 24 h after transfection, MIN6 cells were treated with or without 44.2 nM of prolactin for 24 h. (E) The reporter gene plasmids containing a fragment from -760 bp to +114 bp of the *Tph1* gene linked to the 6 \times GAS-A, 6 \times GAS-A Mutation, or the 6 \times GAS-B fragment were transfected into MIN6 cells. At 24 h after transfection, the cells were incubated with or without 44.2 nM of prolactin for 24 h and harvested. Luciferase activity was standardized by the protein concentration. Luciferase activity in cells without prolactin treatment was set as 1. * $P < 0.05$ compared with cells treated without prolactin. All experiments were repeated at least three times. All data are shown as means \pm s.e.m.

hand, addition of the PI3K inhibitor LY294002 increased the basal expression of *Tph1*; however, its expression was not altered upon treatment with prolactin, and hence resulted in decreased prolactin responsiveness. We also evaluated the effect of these inhibitors on isolated mouse islets treated with prolactin and found that *Tph1* expression was attenuated by treatment with the MEK1/2 inhibitor, the PI3K inhibitor, or the JAK2 inhibitor (Fig. 4B). These data suggest that each pathway contributes to prolactin-induced *Tph1* expression in mouse islets. As activation of Jak2 plays a central role in *Tph1* expression in MIN6 cells as well as in mouse islets, we further investigated the Jak2/Stat5 pathway. The prolactin receptor activates Jak2, which induces the phosphorylation and dimerization of Stat5 (Sorenson & Brelje 2009, Binart *et al.* 2010). Dimerized Stat5 translocates from the cytosol into the nucleus and recognizes interferon γ -activated sequence (GAS) (Berg 2008, Muller *et al.* 2008). Therefore, we surveyed the GAS consensus sequence in the 5'-flanking region of the *Tph1* gene and found two sequences that perfectly match the GAS consensus sequence (GAS-A and GAS-B shown in

Fig. 5A). These sequences are located at about 9 and 6 kbp upstream of the transcription start site, respectively.

To investigate whether these potential Stat5 binding regions are sufficient to exhibit prolactin responsiveness, we analyzed reporter gene constructs containing each potential Stat5 binding region upstream of a heterologous promoter, the prolactin minimal promoter. Transcriptional activity of the GAS-A plasmid, but not the GAS-B plasmid, was enhanced by treatment with prolactin in MIN6 cells (Fig. 5B). Insertion of mutations into the GAS-A sequences disrupted prolactin-induced transactivation of the GAS-A plasmid (Fig. 5B). Prolactin-induced transcriptional activity was disrupted by the forced expression of a dominant-negative form of Stat5A as well as by treatment with a Jak2 inhibitor (Fig. 5C and D). Furthermore, a reporter gene construct containing the 760 bp upstream of the 5'-flanking region of exon 1b showed prolactin responsiveness upon insertion of the GAS-A sequence, but not the GAS-B nor the mutated GAS-A sequence (Fig. 5E). These data support the idea that GAS-A is a functional prolactin-responsive element that is activated by Jak2 and Stat5.



Stat5 binds a potential Stat5 binding region in the *Tph1* gene

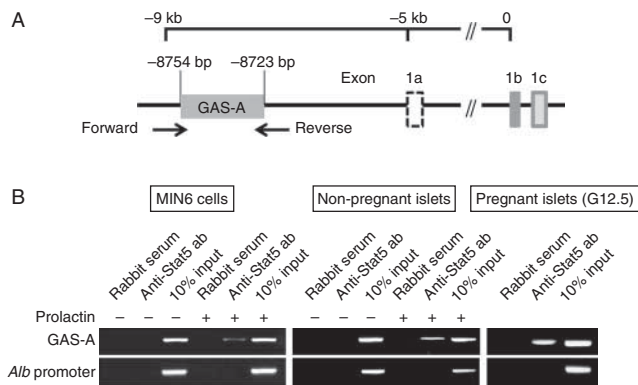
To investigate the prolactin-responsive binding of Stat5 to GAS-A, we isolated nuclear proteins from MIN6 cells and performed the EMSA using sequences of the GAS-A region as a probe. The GAS-A probe identified several nuclear proteins, and the amount of these nuclear proteins bound to the GAS-A probe further increased by treatment with prolactin (Fig. 6A and B). The cold probe of the consensus sequence for GAS (Soldaini *et al.* 2000) disrupted the association between the GAS-A probe and the nuclear proteins that were recognized by the anti-serum for Stat5. On the other hand, the mutated GAS-A probe did not efficiently recognize Stat5 (Fig. 6C). Next, we designed primers recognizing the GAS-A region and performed the ChIP assay using anti-serum for Stat5 (Fig. 7A). Prolactin treatment enhanced the enrichment of Stat5 in the GAS-A region of MIN6 cells as well as of isolated mouse islets (Fig. 7B). In addition, we performed ChIP analysis in islets isolated from pregnant mice and found that Stat5 recognized the GAS-A region in mouse islets during pregnancy (Fig. 7B). As there are no GAS consensus sequences except the GAS-A sequence around this region, our results suggest that Stat5 binds to the GAS-A site in islets during pregnancy.

Prolactin increases the transcriptional activity of Stat5 through Erk and PI3K

Recently, prolactin was reported to enhance the transactivation activity of Stat5 after inducing the binding of dimerized Stat5 to GAS (Moriggl *et al.* 1996). Therefore, we generated a plasmid encoding Stat5A conjugated with the DNA-binding domain (DBD) of GAL4, and cotransfected this with a reporter gene construct containing GAL4

Figure 6

Putative interferon- γ -activated sequence identifies Stat5. (A) Sequences of the IR Dye-labeled probes and non-labeled cold probe. (B) Electrophoretic mobility shift assays (EMSAs) were performed using nuclear extracts from MIN6 cells treated with or without 44.2 nM of prolactin for 1 h. Nuclear protein-DNA complexes were resolved in 5% non-denaturing polyacrylamide gels (lanes 2–3). Competition assays were performed in the presence of a 50-fold or 100-fold molar excess of the GAS consensus sequences (lanes 4–5). The supershift assay was performed in the presence of rabbit serum (lane 6) and STAT5 antibody (lane 7). (C) Binding activity of the GAS-A probe (lanes 1–3) and the GAS-A Mutation probe (lanes 4–6) were evaluated using nuclear extracts from MIN6 cells. All experiments were repeated at least three times. 'Stat5 complex' indicates the protein and probe complex, including Stat5. 'NS' indicates a non-specific band.

**Figure 7**

Stat5 binds to the GAS-A region in MIN6 cells and isolated mouse islets. Chromatin immunoprecipitation analysis was performed to demonstrate binding of Stat5 on the *Tph1* promoter in MIN6 cells and mouse islets. (A) Diagram of the region in the *Tph1* locus amplified by PCR. (B) MIN6 cells and isolated mouse islets were treated with or without 44.2 nM of prolactin for 1 h. Pregnant mouse islets were isolated at G12.5. Cross-linked protein-DNA complexes were immunoprecipitated with the anti-Stat5 antibody or normal rabbit IgG used as a non-specific control. Input DNA was determined using 10% of the total sample as the template. The proximal promoter region of the *Alb* gene was used as a negative control. All experiments were repeated at least three times.

binding sequences (upstream activating sequences of galactose (UASG)) into MIN6 cells. Forced expression of GAL4 DBD-Stat5A increased luciferase activity to a higher level than forced expression of the GAL4 DBD, suggesting the existence of the transactivation activity of Stat5A (Fig. 8A). Treatment with prolactin further enhanced the transactivation activity of Stat5A in MIN6 cells, and its activity was partially attenuated by pretreatment with an MEK1/2 inhibitor or a PI3K inhibitor, but not a Jak2 inhibitor (Fig. 8B). These results suggest that the Erk and PI3K pathways partially regulate prolactin-induced Stat5 transcriptional activity.

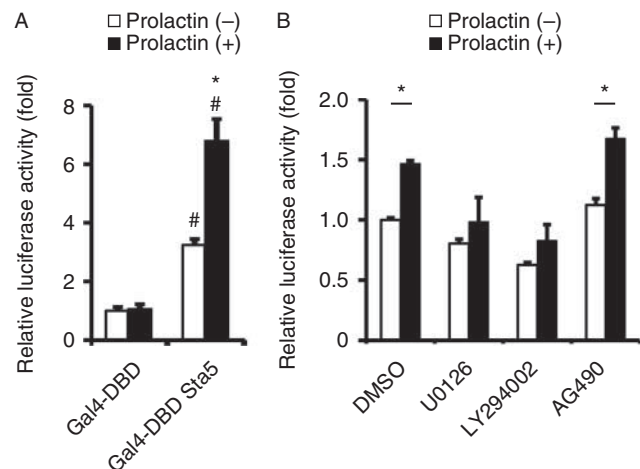
Serotonin expression is decreased in islets of β -cell specific *Stat5*-deficient mice

To confirm the contribution of Stat5 on *Tph1* expression in islets, we generated tamoxifen-inducible β -cell specific *Stat5*-deficient mice (β -*Stat5* K/O) by crossing mice expressing tamoxifen-inducible Cre driven by the mouse *Pdx1* promoter and mice with floxed *Stat5*. After the injection of tamoxifen or corn oil, β -*Stat5* K/O mice were mated, and the pancreata of the resulting pregnant mice were isolated at G12.5. Immunohistochemical analysis was then performed to evaluate the expression of 5-HT in the isolated islets. Stat5 inactivation led to a decrease in the expression of 5-HT in islets, which was accompanied

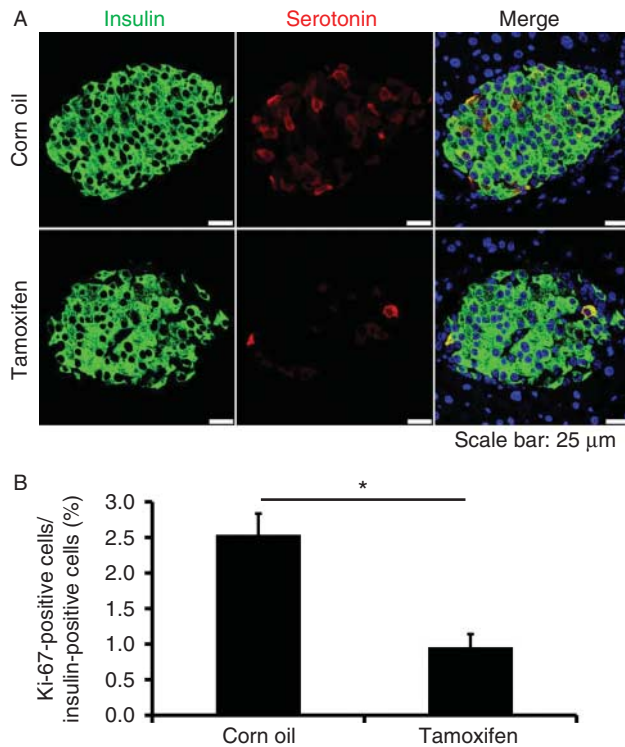
with decreases in the percentage of Ki-67-positive cells among insulin-positive cells (Fig. 9A and B).

Discussion

Pregnancy greatly alters hormonal release patterns, causing physiological changes including insulin resistance. The insufficient compensation of insulin secretion contributes to the development of gestational diabetes. Meanwhile, numerous studies performed in pregnant mice have shown that proliferation of β cells is augmented in response to pregnancy (Zhang *et al.* 2010, Demirci *et al.* 2012, Jacovetti *et al.* 2012). The prolactin receptor is the target of prolactin and placental lactogen, and is expressed in pancreatic β cells to contribute for augmentation of insulin release during pregnancy (Buchanan & Xiang 2005). Although overnutrition also causes insulin resistance and an increase in islet mass, the mechanism of the β -cell proliferation is thought to be different from that which occurs during pregnancy. Pregnancy augments the

**Figure 8**

Prolactin enhances the transactivation of Stat5A in MIN6 cells. (A) One microgram of UAS-TATA-Luciferase was cotransfected with 0.2 μ g of the expression plasmid encoding GAL4-DBD or the GAL4 DBD-Stat5A fusion protein into MIN6 cells. At 24 h after transfection, the cells were incubated with or without 44.2 nM of prolactin. At 48 h after transfection, cells were harvested and the luciferase assay was performed. Relative luciferase activities were calculated with the activity of the cells transfected with pGAL4-DBD without prolactin treatment set as 1. (B) At 30 min after preincubation with 10 μ M U0126, 50 μ M LY294002, or 100 μ M AG490, MIN6 cells were treated with or without 44.2 nM of prolactin. The luciferase activity was standardized by the protein concentration. Relative luciferase activities were calculated with the activity of the cells transfected with pGAL4 DBD-Stat5A without prolactin treatment set as 1. # P <0.05 compared with cells transfected with pGAL4-DBD in the absence of prolactin; * P <0.05 compared with cells transfected with pGAL4 DBD-Stat5A in the absence of prolactin. Experiments were repeated at least four times. All data are shown as means \pm s.e.m.

**Figure 9**

5-HT expression is reduced in islets of β -cell specific *Stat5* knockout mice. Tamoxifen-inducible β -cell specific *Stat5* knockout mice (*Pdx1-Cre-ER* mice \times floxed *Stat5* mice) were treated with tamoxifen or corn oil at 6 weeks of age and were mated at 12 weeks of age. Their pancreata were isolated at G12.5. (A) Immunofluorescent staining was performed in pancreatic sections (green: insulin; red: 5-HT; blue: DAPI). (B) The percentage of Ki-67-positive cells per insulin-positive cells was calculated. Approximately $1\text{--}2 \times 10^3$ insulin-positive cells were counted in 1 or 2 pancreatic sections of both the tamoxifen-injected group ($n=8$) and the corn-oil-injected group ($n=5$). * $P < 0.05$ compared with the corn-oil-injected group. All data are shown as means \pm s.e.m.

secretion of various hormones that may regulate β -cell function or survival, such as estrogen, placental lactogen, and prolactin. Previously, our group showed that prolactin increases the expression of the rate-limiting enzyme for 5-HT synthase, *Tph1*, and that 5-HT contributes to the increase in β -cell mass through upregulation of *Ccna1*, *Ccnb1*, and *Ccnb2* (Kim *et al.* 2010).

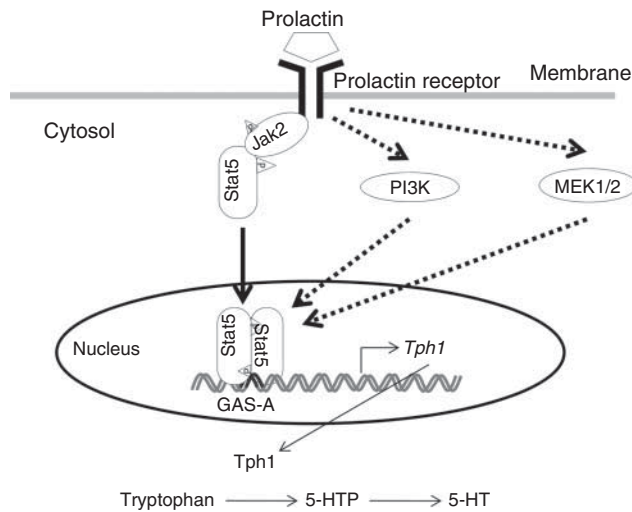
In this study, we identified the β -cell-specific transcriptional start site of *Tph1* as exon 1b and exon 1c, and evaluated the precise mechanisms of prolactin-responsive *Tph1* expression by reporter gene analysis. Whereas proximal 5'-flanking regions of exon 1b and exon 1c exhibited promoter activity in β -cells, they did not show prolactin responsiveness. Because prolactin-induced transcription of *Tph1* was greatly disrupted by treatment with a Jak2 inhibitor in MIN6 cells as well as mouse islets, we

analyzed potential GAS regions in the 5'-flanking region of exon 1s and identified the GAS-A region located 9 kb upstream of exon 1b. The GAS-A region exhibited prolactin responsiveness and was recognized by Stat5 in MIN6 cells. We did not detect the expression of mRNA containing exon 1a in MIN6 cells or in mouse islets. Consistent with the results of RT-PCR analysis, the luciferase activity of reporter gene plasmids containing the 5'-flanking region of exon 1a was repressed compared to that of the control reporter vector, suggesting that repressors might block the transcriptional activity of exon 1a in β cells. Although the reporter gene constructs containing the 5'-flanking region of exon 1a have the GAS-A region, they failed to respond to prolactin (Fig. 3A). We speculated that this was due to the binding of repressor proteins to these constructs.

To evaluate the GAS-A region in islets, we performed ChIP analysis using isolated mouse islets, and demonstrated that prolactin induced Stat5 binding on GAS-A region and that Stat5 recognized the GAS-A region in islets isolated from pregnant mice. Furthermore, we analyzed the pancreata of β -cell-specific *Stat5* knockout mice that were shown to exhibit glucose intolerance (Lee *et al.* 2007). During pregnancy, β -cell-specific *Stat5* knockout mice demonstrated decreases of 5-HT expression and decreases in the number of Ki-67-positive cells in islets. Our results suggest that a reduction of 5-HT expression may contribute to the glucose intolerance in β -cell-specific *Stat5* knockout mice.

Prolactin induces the phosphorylation and dimerization of STAT5, which increases the binding activity of STAT5 on GAS (Karnik *et al.* 2007, Qian *et al.* 2009). Although the C-terminal domain of Stat5 is not sufficient for the DNA binding of dimerized Stat5, it still contributes to the enhancement of transactivation in response to prolactin or growth hormone (GH) (Moriggl *et al.* 1996). To assess the transactivation activity of Stat5, we generated the fusion protein GAL4 DBD-Stat5A and evaluated its transcriptional activity using a reporter gene plasmid containing UASG. GAL4 DBD-Stat5A bound UASG, and treatment with prolactin further enhanced the luciferase activity in MIN6 cells. Our results indicate that prolactin augments the transcriptional activity of Stat5 through increases of both DNA binding and transactivation activity in β cells.

Previous studies showed several mechanisms of transactivation in the C-terminal domain of Stat5. Disruption of serine phosphorylation in the C-terminal domain of Stat5 impairs prolactin and GH responsiveness (Yamashita *et al.* 1998, Park *et al.* 2001). Nonacidic residues of the C-terminal domain contribute to prolactin responsiveness in NIH3T3

**Figure 10**

Proposed model of 5-HT synthesis in response to prolactin in β cells during pregnancy. Prolactin binds to its receptor on β cells, which induces the phosphorylation of Jak2 and Stat5. The dimerized Stat5 is translocated into the nucleus and binds to the GAS-A region in the *Tph1* promoter to induce *Tph1* expression. Stat5-induced *Tph1* expression is partially mediated by Erk and PI3K in response to prolactin. Tph1 is the rate-limiting enzyme for the synthesis of 5-HT that contributes to β -cell proliferation during pregnancy. 5-HTP, 5-hydroxytryptophan.

cells (Callus & Mathey-Prevot 2000). Litterst *et al.* (2003) demonstrated that an interaction between the C-terminal domain and a cofactor is important for prolactin-induced β -casein expression in HeLa cells. However, the upstream cascade of the C-terminal domain of Stat5 is not clear. Our qRT-PCR analysis showed that pretreatment with an MEK1/2 inhibitor or PI3K inhibitor partially impairs prolactin responsiveness in *Tph1* expression. Reporter gene analysis using GAL4 DBD-Stat5 demonstrated that prolactin-induced enhancement of the transactivation of Stat5 was disrupted by treatment with an MEK1/2 inhibitor or PI3K inhibitor, but not with a Jak2 inhibitor. These results suggest that the Erk and PI3K cascades enhance the transactivation of Stat5 in β cells (Fig. 10). Schraenen *et al.* (2010) showed that treatment with an MEK1/2 inhibitor or PI3K inhibitor did not impair prolactin-induced *Tph1* expression in MIN6 cells. MIN6 cells are heterogeneous and can be subcloned into several populations with slightly different characteristics (Lilla *et al.* 2003, Yamato *et al.* 2013). The discrepancy between our results and those of Schraenen *et al.* might hence be a result of using different subclones of MIN6 cells, in which the expression profiles of cofactors may differ. Therefore, the signaling cascade that utilizes Erk or PI3K appears to contribute to the prolactin-induced enhancement of Stat5 transactivation in the

MIN6 cell subclone that was used in our present study, and importantly, in mouse islets. Further experiments should be performed to address the relationship between the Stat5, Erk, and PI3K pathways for *Tph1* expression.

Satyanaryana *et al.* showed that several GASs are located around the *Igf1* gene and regulate the expression of *Igf1* in response to GH, suggesting that multiple GASs may contribute to *Tph1* expression in islets (Eleswarapu *et al.* 2008). The recently developed tool for ChIP analysis linked to next-generation DNA sequencing should allow us to identify numerous *cis*-elements in the whole genome. This technique should provide more comprehensive data on the role of Stat5 in *Tph1* expression, as well as comprehensive information on the mechanism by which prolactin regulates the compensation of β cells during pregnancy. Further studies are required to fully clarify the role of 5-HT in β -cell proliferation, which should contribute to developing new strategies to increase β -cell mass for the treatment of type 1 as well as type 2 diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0299>.

Declaration of interest

H W has received lecture fees from Astellas Pharma, AstraZeneca, Boehringer Ingelheim, Daiichi Sankyo, Inc., Eli Lilly and Company, Kissei Pharmaceutical Co., Kowa Pharmaceutical Co., Kyowa Hakko Kirin Co., MSD (Merck Sharp and Dohme), Novartis Pharmaceuticals, Novo Nordisk Pharma, Ono Pharmaceutical Co., Mitsubishi Tanabe Pharma, Sanofi-Aventis, Sanwakagaku Kenkyusho, and Takeda Pharmaceutical Co., and research funds from Astellas Pharma, AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Daiichi Sankyo, Inc., Dainippon Sumitomo Pharma, Eli Lilly, Johnson and Johnson, Kissei Pharmaceutical Co., Kowa Pharmaceutical Co., Kyowa Hakko Kirin Co., MSD, Mitsubishi Tanabe Pharma, Mochida Pharmaceutical Co., Novartis Pharmaceuticals, Novo Nordisk Pharma, Pfizer, Sanwakagaku Kenkyusho, Sanofi-Aventis, and Takeda Pharmaceutical Co. Y F has received a lecture fee from Novartis and Eli Lilly, research funding from Novartis, MSD, and Takeda Pharmaceutical Company.

Author contribution statement

T O, H W, and H K designed research; H I, M M, A H, K F, Y G K, and M T conducted research; T O, H W, and Y F analyzed the data; T O, H I, and H W wrote the paper; H W had primary responsibility for final content. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr Lothar Hennighausen (National Institute of Health, USA) for providing the floxed *Stat5* mouse. We thank Dr Shannon L Kelleher (Pennsylvania State University, USA) for providing plasmids encoding

Stat5A and Stat5A DN. We gratefully acknowledge Ms Naoko Daimaru and Ms Hiroko Hibino for assistance with the experiments.

References

- Berg T 2008 Signal transducers and activators of transcription as targets for small organic molecules. *Chembiochem* **9** 2039–2044. (doi:10.1002/cbic.200800274)
- Bernichtein S, Touraine P & Goffin V 2010 New concepts in prolactin biology. *Journal of Endocrinology* **206** 1–11. (doi:10.1677/JOE-10-0069)
- Binart N, Bachelot A & Bouilly J 2010 Impact of prolactin receptor isoforms on reproduction. *Trends in Endocrinology and Metabolism* **21** 362–368. (doi:10.1016/j.tem.2010.01.008)
- Buchanan TA & Xiang AH 2005 Gestational diabetes mellitus. *Journal of Clinical Investigation* **115** 485–491. (doi:10.1172/JCI200524531)
- Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA & Butler PC 2003 β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* **52** 102–110. (doi:10.2337/diabetes.52.1.102)
- Callus BA & Mathey-Prevot B 2000 Hydrophobic residues Phe751 and Leu753 are essential for STAT5 transcriptional activity. *Journal of Biological Chemistry* **275** 16954–16962. (doi:10.1074/jbc.M909976199)
- Chang-Chen KJ, Mullur R & Bernal-Mizrachi E 2008 β -cell failure as a complication of diabetes. *Reviews in Endocrine & Metabolic Disorders* **9** 329–343. (doi:10.1007/s11514-008-9101-5)
- Deering TG, Ogihara T, Trace AP, Maier B & Mirmira RG 2009 Methyltransferase Set7/9 maintains transcription and euchromatin structure at islet-enriched genes. *Diabetes* **58** 185–193. (doi:10.2337/db08-1150)
- Demirci C, Ernst S, Alvarez-Perez JC, Rosa T, Valle S, Shridhar V, Casinelli GP, Alonso LC, Vasavada RC & Garcia-Ocana A 2012 Loss of HGF/c-Met signaling in pancreatic β -cells leads to incomplete maternal β -cell adaptation and gestational diabetes mellitus. *Diabetes* **61** 1143–1152. (doi:10.2337/db11-1154)
- Eleswarapu S, Gu Z & Jiang H 2008 Growth hormone regulation of insulin-like growth factor-I gene expression may be mediated by multiple distal signal transducer and activator of transcription 5 binding sites. *Endocrinology* **149** 2230–2240. (doi:10.1210/en.2007-1344)
- Gregg BE, Moore PC, Demozay D, Hall BA, Li M, Husain A, Wright AJ, Atkinson MA & Rhodes CJ 2012 Formation of a human β -cell population within pancreatic islets is set early in life. *Journal of Clinical Endocrinology and Metabolism* **97** 3197–3206. (doi:10.1210/jc.2012-1206)
- Huang C, Snider F & Cross JC 2009 Prolactin receptor is required for normal glucose homeostasis and modulation of β -cell mass during pregnancy. *Endocrinology* **150** 1618–1626. (doi:10.1210/en.2008-1003)
- Hughes E & Huang C 2011 Participation of Akt, menin, and p21 in pregnancy-induced β -cell proliferation. *Endocrinology* **152** 847–855. (doi:10.1210/en.2010-1250)
- Iwashita N, Uchida T, Choi JB, Azuma K, Ogihara T, Ferrara N, Gerber H, Kawamori R, Inoue M & Wataha H 2007 Impaired insulin secretion *in vivo* but enhanced insulin secretion from isolated islets in pancreatic β cell-specific vascular endothelial growth factor-A knock-out mice. *Diabetologia* **50** 380–389. (doi:10.1007/s00125-006-0512-0)
- Jacovetti C, Abderrahmani A, Parnaud G, Jonas JC, Peyot ML, Cornu M, Laybutt R, Meugnier E, Rome S, Thorens B *et al.* 2012 MicroRNAs contribute to compensatory β cell expansion during pregnancy and obesity. *Journal of Clinical Investigation* **122** 3541–3551. (doi:10.1172/JCI64151)
- Karnik SK, Chen H, McLean GW, Heit JJ, Gu X, Zhang AY, Fontaine M, Yen MH & Kim SK 2007 Menin controls growth of pancreatic β -cells in pregnant mice and promotes gestational diabetes mellitus. *Science* **318** 806–809. (doi:10.1126/science.1146812)
- Kim H, Toyofuku Y, Lynn FC, Chak E, Uchida T, Mizukami H, Fujitani Y, Kawamori R, Miyatsuka T, Kosaka Y *et al.* 2010 Serotonin regulates pancreatic β cell mass during pregnancy. *Nature Medicine* **16** 804–808. (doi:10.1038/nm.2173)
- Lee JY, Gavrilova O, Davani B, Na R, Robinson GW & Hennighausen L 2007 The transcription factors Stat5a/b are not required for islet development but modulate pancreatic β -cell physiology upon aging. *Biochimica et Biophysica Acta* **1773** 1455–1461. (doi:10.1016/j.bbamcr.2007.05.010)
- Lilla V, Webb G, Rickenbach K, Maturana A, Steiner DF, Halban PA & Irminger JC 2003 Differential gene expression in well-regulated and dysregulated pancreatic β -cell (MIN6) sublines. *Endocrinology* **144** 1368–1379. (doi:10.1210/en.2002-220916)
- Litterst CM, Kliem S, Marilley D & Pfitzner E 2003 NCoA-1/SRC-1 is an essential coactivator of STAT5 that binds to the FDL motif in the α -helical region of the STAT5 transactivation domain. *Journal of Biological Chemistry* **278** 45340–45351. (doi:10.1074/jbc.M303644200)
- Meier JJ, Bhushan A, Butler AE, Rizza RA & Butler PC 2005 Sustained β cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? *Diabetologia* **48** 2221–2228. (doi:10.1007/s00125-005-1949-2)
- Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA & Butler PC 2008 β -cell replication is the primary mechanism subserving the postnatal expansion of β -cell mass in humans. *Diabetes* **57** 1584–1594. (doi:10.2337/db07-1369)
- Moriggl R, Gouilleux-Gruart V, Jahne R, Berchtold S, Gartmann C, Liu X, Hennighausen L, Sotiropoulos A, Groner B & Gouilleux F 1996 Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. *Molecular and Cellular Biology* **16** 5691–5700.
- Muller J, Sperl B, Reindl W, Kiessling A & Berg T 2008 Discovery of chromone-based inhibitors of the transcription factor STAT5. *Chembiochem* **9** 723–727. (doi:10.1002/cbic.200700701)
- Ohara-Imaizumi M, Kim H, Yoshida M, Fujiwara T, Aoyagi K, Toyofuku Y, Nakamichi Y, Nishiwaki C, Okamura T, Uchida T *et al.* 2013 Serotonin regulates glucose-stimulated insulin secretion from pancreatic β cells during pregnancy. *PNAS* **110** 19420–19425. (doi:10.1073/pnas.1310953110)
- Park SH, Yamashita H, Rui H & Waxman DJ 2001 Serine phosphorylation of GH-activated signal transducer and activator of transcription 5a (STAT5a) and STAT5b: impact on STAT5 transcriptional activity. *Molecular Endocrinology* **15** 2157–2171. (doi:10.1210/mend.15.12.0746)
- Qian L, Lopez V, Seo YA & Kelleher SL 2009 Prolactin regulates ZNT2 expression through the JAK2/STAT5 signaling pathway in mammary cells. *American Journal of Physiology. Cell Physiology* **297** C369–C377. (doi:10.1152/ajpcell.00589.2008)
- Rieck S & Kaestner KH 2010 Expansion of β -cell mass in response to pregnancy. *Trends in Endocrinology and Metabolism* **21** 151–158. (doi:10.1016/j.tem.2009.11.001)
- Sadowski HB, Shuai K, Darnell JE Jr & Gilman MZ 1993 A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* **261** 1739–1744. (doi:10.1126/science.8397445)
- Saisho Y, Butler AE, Manesso E, Elashoff D, Rizza RA & Butler PC 2013 β -cell mass and turnover in humans: effects of obesity and aging. *Diabetes Care* **36** 111–117. (doi:10.2337/dc12-0421)
- Schraenen A, Lemaire K, de Fauteur G, Hendrickx N, Granvik M, Van Lommel L, Mallet J, Vodjdani G, Gilon P, Binart N *et al.* 2010 Placental lactogens induce serotonin biosynthesis in a subset of mouse β cells during pregnancy. *Diabetologia* **53** 2589–2599. (doi:10.1007/s00125-010-1913-7)
- Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, Tavernier G, Langin D & Spiegelman BM 2007 Transcriptional control of brown fat determination by PRDM16. *Cell Metabolism* **6** 38–54. (doi:10.1016/j.cmet.2007.06.001)
- Smith SB, Ee HC, Connors JR & German MS 1999 Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. *Molecular and Cellular Biology* **19** 8272–8280.

- Soldaini E, John S, Moro S, Bollenbacher J, Schindler U & Leonard WJ 2000 DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. *Molecular and Cellular Biology* **20** 389–401. (doi:10.1128/MCB.20.1.389-401.2000)
- Sorenson RL & Brelje TC 2009 Prolactin receptors are critical to the adaptation of islets to pregnancy. *Endocrinology* **150** 1566–1569. (doi:10.1210/en.2008-1710)
- Yamashita H, Xu J, Erwin RA, Farrar WL, Kirken RA & Rui H 1998 Differential control of the phosphorylation state of proline-juxtaposed serine residues Ser725 of Stat5a and Ser730 of Stat5b in prolactin-sensitive cells. *Journal of Biological Chemistry* **273** 30218–30224. (doi:10.1074/jbc.273.46.30218)
- Yamato E, Tashiro F & Miyazaki J 2013 Microarray analysis of novel candidate genes responsible for glucose-stimulated insulin secretion in mouse pancreatic β cell line MIN6. *PLoS ONE* **8** e61211. (doi:10.1371/journal.pone.0061211)
- Zhang H, Zhang J, Pope CF, Crawford LA, Vasavada RC, Jagasia SM & Gannon M 2010 Gestational diabetes mellitus resulting from impaired β -cell compensation in the absence of FoxM1, a novel downstream effector of placental lactogen. *Diabetes* **59** 143–152. (doi:10.2337/db09-0050)

Received in final form 29 June 2015

Accepted 1 July 2015

Accepted Preprint published online 1 July 2015