Expression mechanism of tryptophan hydroxylase 1 in mouse islets during pregnancy

Hitoshi Iida1, Takeshi Ogihara1, Mun-kyeong Min5, Akemi Hara1, Yeong Gi Kim5, Kyoko Fujimaki1, Motoyuki Tamaki6, Yoshio Fujitani1,4, Hail Kim5 and Hirotaka Watada1,2,3

1Department of Metabolism and Endocrinology, 2Center for Molecular Diabetology, 3Center for Therapeutic Innovations in Diabetes, and 4Japan Science and Technology Agency-Core Research for Evolutionary Science and Technology Program, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
5Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea
6Diabetes Therapeutics and Research Center, Tokushima University, Tokushima 770-8503, Japan

Correspondence should be addressed to T Ogihara
Email take@juntendo.ac.jp

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. 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

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 insulitis 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 in 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 ~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 Gα(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, and 1% (v/v) penicillin and streptomycin (Life Technologies) under 5% CO2 at 37 °C. βTC3 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 streptomyacin. 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 streptomyacin. After pretreatment with or without 100 μM AG490 (a tyrosine kinase inhibitor of JAK2) (Sigma–Aldrich), 10 μM U0126 (a selective inhibitor of MEK1/2) (WAKO, Tokyo, Japan), or 50 μM LY-294002 (an inhibitor of PI3Ks) (Cell Signaling, Tokyo, Japan) for 30 min, MIN6 cells, βTC3 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 × 10⁶), βTC3 cells (1 × 10⁶), and ~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'-TGGAGCATGCAGATGACCATGCA-3', reverse: 5'-TGACCAATAAGCCCTGAGGTG-3'), mouse prolactin receptor (gene symbol Ptrl; forward: 5'-TCTCTGTGGAGGTGAGCATCGCA-3', reverse: 5'-TCAGGCTGGCCCTTCTTGC-3'), mouse TATA-binding protein (gene symbol Tbp; forward: 5'-GCTGGCCCTTCTTCTTGC-3', reverse: 5'-GATCTTGAAGT-3'), mouse brain stem cells was purchased from Takara Bio, Inc.

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'-GATCTGTCCACCAGCTCTCAGGACGTGGAGGTG-3', reverse: 5'-GATCCACCCAGCCGTTTCTGGGAAGAGCTGGGGACA-3', GAS-B forward: 5'-GATCTCTTCTGACAGGTAGGTG-3', reverse: 5'-GATCCACCTGAGCAGGTCAGAAGACGGTTTAG-3'), and mouse TATA-binding protein (gene symbol Tbp; forward: 5'-GCTGAGATCACATGATGCA-3', reverse: 5'-CACCATGTTCGATCATTTGAAGT-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'-GAGAGGGCTGGGCCTGCGGA-3'). After purifying the PCR products, nested PCR was performed using GeneRacer 5' primer and the nested Tph1 gene specific reverse primer (5'-GAGAGGGCTGGGCCTGCGGA-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'-ATTGCGGTCTCAGGATGC-3'), exon 1c (5'-CAGCAAGGAGGATGCTGACCA-3'), and the reverse primer designed in exon 3 (5'-TCCGGGACTCGATGTAAC-3'). β-actin (gene symbol; Actb) was amplified as an internal control (forward: 5'-CACATGTTCTGACGATGC-3', reverse: 5'-ATGGAGGCCACGGATCCACA-3'). Total RNA of mouse brain stem cells was purchased from Takara Bio, Inc. (Tokyo, Japan) as a positive control.
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′-GATCTGTCCTCCCCAGCTCTTCCCCAGAAAGCCCTGAGGTG-3′) and GAS-A Mutation oligonucleotides (5′-GATCTGTCCTCCCCAGCTCTTCCCCAAATAGCCTTGAGGTG-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) poly(dI-dC), 7.35 μM BSA) for 10 min at room temperature with 5 μl rabbit serum or 5 μl of a STAT5 antibody (Santa Cruz). For competitive analysis, we used a 50-fold or 100-fold excess of non-labeled oligonucleotides (5′-CTGTGGCTTTTCTGGAAATTGGCCATACCTGGAAGAGGCCC-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 × 10⁷) 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 1 ml of MC lysis buffer (10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 2 mM MgCl2, and 0.5% (v/v) NP-40). The pellet was resuspended in 1 ml of MNase buffer (10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, 4% (v/v) NP-40, and 1 mM phenylmethylsulfonyl 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×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×Halt protease and phosphatase inhibitor cocktail. Then 50–100 μl of protein A/G PLUS-Agarose beads (Santa Cruz) were added and incubated at 4 °C for 4 h to preclene 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 μl) conjugated to 1 μg of a STAT5 antibody (sc-835x; Santa Cruz) overnight at 4°C. The GAS-A oligonucleotides (5′-CTGCTGTGAGCGCATACGAGAAA-3′) and GAS-A Mutation oligonucleotides (5′-CTGCTGTGAGCGCATACGAGAAA-3′) were added and incubated at 4°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′-ACGTGTGCCCCAGCTCTTCCCCCA-3′, and reverse: 5′-TGCTCCAAGCCTGTCCGAT-3′; GAS-B forward: 5′-TGCTGTGAGCCATACCGAGAAA-3′, and reverse: 5′-AAACCGTCTTCCCCAGCCAA-3′), the mouse Alb promoter (forward: 5′-TGGGAAAAGCTGGGAAAACCATC-3′, and reverse: 5′-CCTGCTACACATACATCCTGCGT-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-SH2 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,
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 ± 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 \( \beta \) cells, we evaluated the prolactin-induced expression of \( Tph1 \) in mouse insulinoma cell lines MIN6 and \( \beta TC3 \). In MIN6 cells, prolactin induced an eightfold increase in expression of \( Tph1 \) mRNA (Fig. 1A). On the other hand, although \( \beta TC3 \) 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 1](http://jme.endocrinology-journals.org/C209/)

Figure 1

Prolactin induces the expression of \( Tph1 \) in MIN6 insulinoma cells. (A) \( \beta TC3 \) 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, \( \beta TC3 \) cells, and NIH3T3 cells was evaluated by qRT-PCR and normalized to \( Tbp \) expression (\( n \geq 3 \)). Results are shown as the means ± S.E.M.
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 ± S.E.M.

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
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 (Sorensen & Brejle 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. 4B).

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× GAS-A, 6× GAS-A Mutation, or the 6× 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× GAS-A Mutation 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 μM AG490 for 24 h. (D) Plasmids encoding Stat5A or a dominant-negative form of Stat5A were co-transfected with the 6× 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× GAS-A, 6× GAS-A Mutation, or the 6× 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±S.E.M.
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 (EMSA) 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.
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
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 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
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 GAs are located around the Igf1 gene and regulate the expression of Igf1 in response to GH, suggesting that multiple GAs 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.

**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.

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**

**Author contribution statement**
T O, H W, and H K designed 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


Sadowski HB, Shuai K, Darnell JE Jr & Gilman MZ 1993 A common nuclear signal transducer and activator of transcription 5a (STAT5a) and STAT5b: impact on STAT5 transcriptional activity. *Molecular Endocrinology* 15 2157–2171. (doi:10.1210/med.15.12.0744)


Received in final form 29 June 2015
Accepted 1 July 2015
Accepted Preprint published online 1 July 2015