Regulation of pancreatic islet gene expression in mouse islets by pregnancy

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Abstract

Pancreatic β cells adapt to pregnancy-induced insulin resistance by unclear mechanisms. This study sought to identify genes involved in β cell adaptation during pregnancy. To examine changes in global RNA expression during pregnancy, murine islets were isolated at a time point of increased β cell proliferation (E13·5), and RNA levels were determined by two different assays (global gene expression array and G-protein-coupled receptor (GPCR) array). Follow-up studies confirmed the findings for select genes. Differential expression of 110 genes was identified and follow-up studies confirmed the changes in select genes at both the RNA and protein level. Surfactant protein D (SP-D) mRNA and protein levels exhibited large increases, which were confirmed in murine islets. Cytokine-induced expression of SP-D in islets was also demonstrated, suggesting a possible role as an anti-inflammatory molecule. Complementing these studies, an expression array was performed to define pregnancy-induced changes in expression of GPCRs that are known to impact islet cell function and proliferation. This assay, the results of which were confirmed using real-time reverse transcription-PCR assays, demonstrated that free fatty acid receptor 2 and cholecystokinin receptor A mRNA levels were increased at E13·5. This study has identified multiple novel targets that may be important for the adaptation of islets to pregnancy.

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Introduction

Pancreatic β cells within the islets of Langerhans play a critical role in the regulation of glucose homeostasis by sensing glucose levels and secreting insulin. Compensation in response to increased demands for insulin occurs through changes in both insulin synthesis and release, as well as changes in β cell mass. These changes observed in β cells play an important role in regulating glucose homeostasis during different physiological and pathological states such as the insulin resistant states of pregnancy and obesity (Butler *et al.* 2007). Studying states in which insulin needs change can provide insight into pathways mediating β cell adaptations.

In mice, β cell mass increases about twofold during pregnancy, with a maximum rate of β cell proliferation near day 14 post coitum. This response is dynamic, as β cell mass decreases in the *postpartum* period (Karnik *et al.* 2007). Although the activation of the prolactin receptor (PRLR) by prolactin and placental lactogens contributes to the regulation of β cell mass during pregnancy (Brelje *et al.* 1993), the mechanisms controlling the expansion of β cell mass during pregnancy remain incompletely defined, and it is likely that additional signaling pathways contribute to or are directly involved in the PRLR-mediated proliferative response (Karnik *et al.* 2007, Sorenson & Brelje 2009).

This study was designed to begin to elucidate the molecular mechanisms responsible for pregnancy-induced changes in β cell mass. This was accomplished by comparing the results of whole genome RNA expression analysis using islets from pregnant mice on day E13·5 and control mice. We identified 110 genes that are differentially expressed in pregnant islets, including the gene encoding surfactant protein D (SP-D). Furthermore, the impact of pregnancy on the expression of G-protein-coupled receptors (GPCRs), a family of proteins involved in the regulation of β cell function and mass and effectors for a number of therapeutic agents (Vasavada *et al.* 1996, Holz & Chepurny 2005, Xiao *et al.* 2008), was examined in detail using a separate but complementary microarray approach. Together, the results of our studies suggest multiple novel targets that will facilitate further studies seeking to define molecular changes characteristic of the pregnancy-induced increase in β cell mass.

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Materials and Methods

Islet isolation

C57BL/6 pregnant (day 13·5 of pregnancy) and control (age- and sex-matched) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Islets were isolated using standard protocols as described previously (Chen *et al.* 2006), and RNA was isolated using RNeasy (Qiagen). For RNA analyses, islets isolated from three mice were pooled for each sample to reduce interindividual variability in the RNA expression analyses. All procedures performed on animals were approved by the Northwestern University Animal Care and Use Committee.

Microarray analysis

RNA expression analysis was performed using the Illumina MouseWG-6 BeadChip, which provides coverage of over 45 200 genes and expressed sequence tags. Islet RNA from pregnant and control mice was labeled using a commercial kit (TargetAmp 1-Round Aminoallyl-aRNA Ki; Epicentre, Madison, WI, USA). To reduce changes related to interindividual variability, each sample of islet RNA was prepared using pooled islets from three pregnant or control mice. Labeled RNA was hybridized to microarrays. Raw signal intensities of each probe were obtained and processed as described previously (Du *et al.* 2008). Differentially expressed genes were identified using an ANOVA model with empirical Bayesian variance estimation. The problem of multiple comparisons was corrected using the false discovery rate (FDR). Genes were identified as being differentially expressed on the basis of a statistical significance (P value $<0{\cdot}01$), FDR $<5\%$, and 1·5-fold change in expression level in pregnant compared with control islets. Functional classification was performed using the ingenuity pathway analysis (IPA) software, based on statistical significance (IPA pathway test, $P<0{\cdot}01$) and biological importance.

GPCR RNA array

A commercially available GPCR-specific 384 well TaqMan real-time reverse transcription (RT)-PCR microarray (Applied Biosystems, Carlsbad, CA, USA), which allows for the detection of mRNAs encoding 343 GPCRs (not including the odorant, olfactory, gustatory, and pheromone receptors), was used with RNA extracted as described above. cDNA was prepared using the high-capacity reverse transcriptase kit (Applied Biosystems) from the RNA that had contaminating DNA removed by DNAase I (Ambion, Applied Biosystems, Austin, TX, USA), and each port was loaded with cDNA (from 1 μg RNA) and the TaqMan Gene Expression Master Mix (Applied Biosystems). The resulting plate was analyzed on the 7900HT ABI PRISM, and the data were analyzed using SDS 2·3 and RQ Manager 1.2 software provided by Applied Biosystems. The results for each individual GPCR were examined, and if the sample had a calculated threshold $<0{\cdot}1$, it was defined as undetectable (this generally indicated no discernible melt curve) and for data processing, the cycle time (C_t) was set at 40·0. A comparative C_t approach was utilized to quantify the relative levels of mRNA. C_ts were normalized to the geometric mean of three housekeeping (HK) genes. Each HK gene on the microarray was evaluated for stability by geNorm (Vandesompele *et al.* 2002), and the three most stable HK genes were used. This experiment was performed on two independent samples, where each sample consisted of islets pooled from three mice.

Real-time RT-PCR

RNA was isolated and cDNA was prepared as described above. cDNA samples were examined by real-time RT-PCR and were normalized to the geometric mean of the three most stable HK genes as determined by geNorm (Vandesompele *et al.* 2002). These were hypoxanthine–guanine phosphoribosyltransferase (*HPRT*), TATA-binding protein (*TBP*), and peptidylprolyl isomerase A (*PPIA*). Sequences of primers used in the different assays are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. A comparative C_t analysis was utilized to determine fold change in RNA expression based on the following approach: fold change $= 2^{(\Delta\Delta C_t)} = 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ control})}$ (Fleige *et al.* 2006). For each real-time RT-PCR experiment, three independent replicates were used.

Western blot

Islets from the control or pregnant mice (day 13·5 of pregnancy) were isolated as described above, and proteins were extracted in RIPA cell lysis buffer containing a cocktail of protease inhibitors (Calbiochem, LaJolla, CA, USA). Primary antibodies for free fatty acid receptor 2 (FFAR2) and PRLR were from Santa Cruz (Santa Cruz, CA, USA) and for tryptophan hydroxylase 1 (TPH1) from Thermo Scientific (Rockford, IL, USA). Mouse monoclonal antibodies to SP-D (hyb-246) were prepared as described earlier (Sorensen *et al.* 2009). The protein content of the lysate was determined using the Coomassie blue protein assay. Twenty micrograms of protein were separated by 10% SDS–PAGE and transferred to a polyvinylidene fluoride membrane in a semidry apparatus. Western blot analyses were performed as previously described (El Muayed *et al.* 2010). For SP-D samples, both reduced and nonreduced protein samples were examined. For reduced samples for SP-D, protein preparations were prepared by mixing the protein samples 1:2 with Laemmli sample buffer with 5% 2-mercaptoethanol and heating at 100 °C for 1 min (nonreduced preparations were heated in sample buffer with the addition of iodoacetamide, final concentration of 90 mM) as previously described (Madsen *et al.* 2000, Sørensen *et al.* 2009). Each assay was repeated with two or three independent samples.

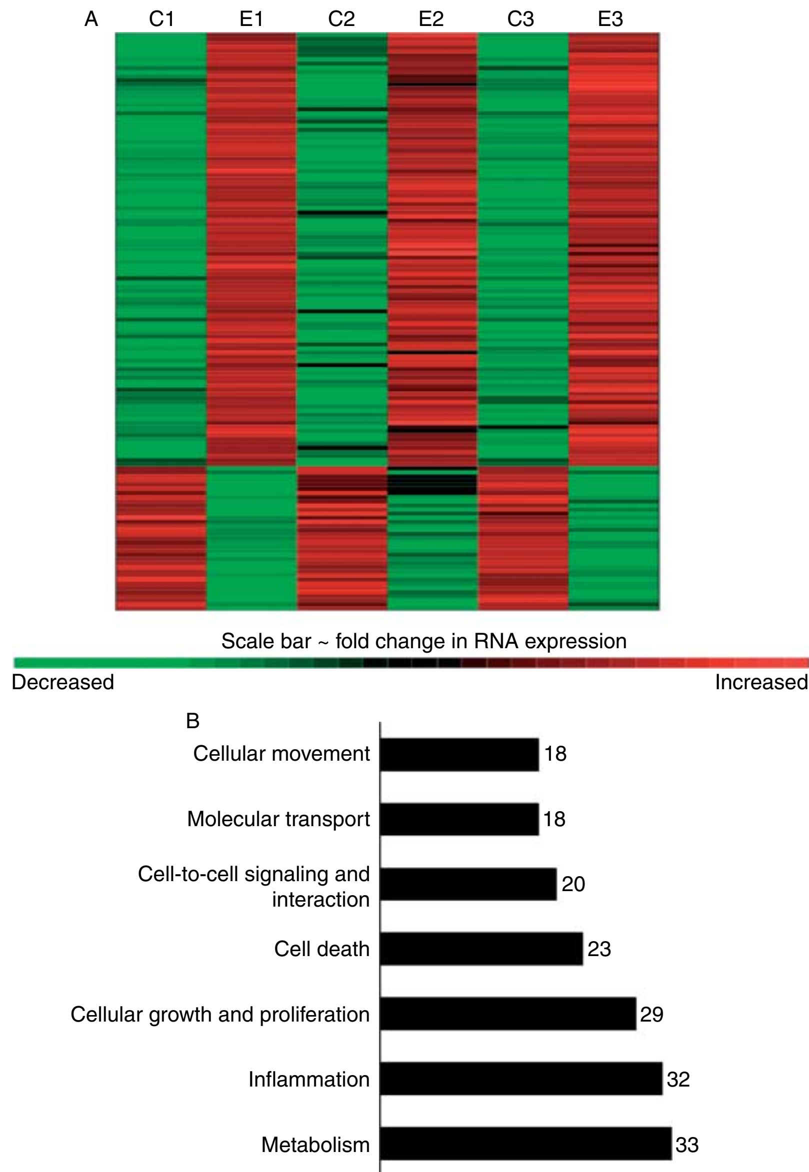

Figure 1 Global gene expression analysis of pancreatic islets at day 13·5 of pregnancy. (A) Heat map of differentially regulated pancreatic islet genes in pooled islets from either pregnant mice on day 13·5 (E1–E3) or control mice. E1, E2, C1, and C2 represent independent samples prepared from separate pools of pregnant (E1 and 2) and control (C1 and 2) islets. E3 and C3 are data obtained using the same RNA as E1/C1 respectively but prepared for microarray analysis independently. Scale bar: relative RNA expression (mean centered, standardized). (B) Graphical representation of the 110 differentially regulated genes from islets of pregnant mice compared with those of control mice using a functional classification scheme from ingenuity. Genes could be included in more than one category. The number of genes in each category is shown. *P* values for each category were determined by a category overrepresentation *P* test to examine the relative proportion of the genes in each category among the differentially regulated genes compared with the chip as a whole. The *P* values were as follows: cellular movement ($P=2{\cdot}9\times10^{-3}$), molecular transport ($P=4{\cdot}5\times10^{-4}$), cell-to-cell signaling and interaction ($P=4{\cdot}5\times10^{-4}$), cell death ($P=9{\cdot}3\times10^{-4}$), cellular growth and proliferation ($P=4{\cdot}5\times10^{-4}$), inflammation ($P=1{\cdot}5\times10^{-2}$), and metabolism ($P=3{\cdot}4\times10^{-3}$).

Table 1 Global expression analysis of RNA isolated from pregnant mice (E13·5) compared with that from control mice (age- and sex-matched)

Genes	Description	Fold	*P* value
Cell-to-cell signaling and interaction			
Sftpd	Surfactant-associated protein D	12·8	<0·0005
Tph1	Tryptophan hydroxylase 1	6·1	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	<0·0005
Vip	Vasoactive intestinal polypeptide	2·9	0·002
Tac1	Tachykinin 1	2·6	<0·0005
Prlr	Prolactin receptor	2·2	<0·0005
C3	Complement component 3	2·1	<0·0005
Cartpt	CART prepropeptide	2·1	<0·0005
Glycam1	Glycosylation-dependent cell adhesion molecule 1	2·1	0·002
Grp	Gastrin-releasing peptide	2·1	0·001
C1qa	Complement component 1, q subcomponent, alpha polypeptide	1·7	<0·0005
Ttyh1	Tweety homolog 1 (*Drosophila*)	1·7	<0·0005
Ffar2	Free fatty acid receptor 2	1·6	<0·0005
Ptpre	Protein tyrosine phosphatase, receptor type, E	1·6	0·003
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1·5	<0·0005
Lpl	Lipoprotein lipase	−1·5	<0·0005
Pcsk9	Proprotein convertase subtilisin/kexin type 9	−1·5	0·0001
Rap2a	RAS-related protein 2a	−1·6	0·003
Ibsp	Integrin-binding sialoprotein	−1·7	0·002
Spon2	Spondin 2, extracellular matrix protein	−1·7	<0·0005
Cellular growth and proliferation			
Sftpd	Surfactant-associated protein D	12·8	<0·0005
Cish	Cytokine-inducible SH2-containing protein	7·5	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	<0·0005
Vip	Vasoactive intestinal polypeptide	2·9	0·002
Nupr1	Nuclear protein 1	2·9	0·001
Ovol2	Ovo-like 2 (*Drosophila*)	2·7	<0·0005
Tac1	Tachykinin 1	2·6	<0·0005
Socs2	Suppressor of cytokine signaling 2	2·6	<0·0005
Igfbp-5	Insulin-like growth factor-binding protein-5	2·5	<0·0005
Hopx	HOP homeobox	2·3	<0·0005
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2·3	<0·0005
Prlr	Prolactin receptor	2·2	<0·0005
C3	Complement component 3	2·1	<0·0005
Grp	Gastrin-releasing peptide	2·1	0·001
Gadd45α	Growth arrest and DNA-damage-inducible 45 alpha	2·0	<0·0005
Apobec1	Apolipoprotein B editing complex 1	1·7	0·0003
Dnmt3b	DNA methyltransferase 3B	1·7	<0·0005
Mcfd2	Multiple coagulation factor deficiency 2	1·7	0·0001
Eif4ebp-1	Eukaryotic translation initiation factor 4E-binding protein-1	1·6	0·003
Cited2	Cbp/p300-interacting transactivator, w/Glu/Asp carb.-terminaldom.2	1·6	0·0008
Ptpre	Protein tyrosine phosphatase, receptor type, E	1·6	0·003
Fgl2	Fibrinogen-like protein 2	1·6	<0·0005
Fhit	Fragile histidine triad gene	1·6	<0·0005
Isg20	Interferon-stimulated protein	1·6	0·0002
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1·5	<0·0005
Defb1	Defensin beta 1	−1·5	0·0002
Tox	Thymocyte selection-associated high-mobility group box	−1·5	<0·0005
Fosb	FBJ osteosarcoma oncogene B	−1·5	<0·0005
Tm4sf4	Transmembrane 4 superfamily member 4	−1·7	<0·0005
Molecular transport			
Sftpd	Surfactant-associated protein D	12·8	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	<0·0005
Vip	Vasoactive intestinal polypeptide	2·9	0·002
Tac1	Tachykinin 1	2·6	<0·0005
Igfbp-5	Insulin-like growth factor-binding protein-5	2·5	<0·0005
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2·3	<0·0005
Prlr	Prolactin receptor	2·2	<0·0005
C3	Complement component 3	2·1	<0·0005
Grp	Gastrin-releasing peptide	2·1	0·001

(continued)

Table 1 *Continued*

Genes	Description	Fold	*P* value
Cartpt	CART prepropeptide	2·1	<0·0005
Slc6a8	Solute carrier family 6 (neurotransmitter transporter, creatine), member 8	1·8	<0·0005
Apobec1	Apolipoprotein B editing complex 1	1·7	0·0003
Hba-α1	Hemoglobin alpha, adult chain 1	1·6	0·0008
Hbb-β1	Hemoglobin, beta adult major chain	1·6	<0·0005
Lpl	Lipoprotein lipase	−1·5	<0·0005
Pcsk9	Proprotein convertase subtilisin/kexin type 9	−1·5	0·0001
Adh1	Alcohol dehydrogenase 1 (class I)	−1·7	0·0002
Ibsp	Integrin-binding sialoprotein	−1·7	0·002
Cell death			
Sftpd	Surfactant-associated protein D	12·8	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	<0·0005
Vip	Vasoactive intestinal polypeptide	2·9	0·002
Nupr1	Nuclear protein 1	2·9	0·001
Tac1	Tachykinin 1	2·6	<0·0005
Socs2	Suppressor of cytokine signaling 2	2·6	<0·0005
Igfbp-5	Insulin-like growth factor-binding protein-5	2·5	<0·0005
Prlr	Prolactin receptor	2·2	<0·0005
C3	Complement component 3	2·1	<0·0005
Gadd45α	Growth arrest and DNA-damage-inducible 45 alpha	2·0	<0·0005
Dnmt3b	DNA methyltransferase 3B	1·7	<0·0005
C1qa	Complement component 1, q subcomponent, alpha polypeptide	1·7	<0·0005
Fgl2	Fibrinogen-like protein 2	1·6	<0·0005
Fhit	Fragile histidine triad gene	1·6	<0·0005
Ptpre	Protein tyrosine phosphatase, receptor type, E	1·6	0·003
Ezr	Ezrin	1·6	<0·0005
Ern1	Endoplasmic reticulum (ER) to nucleus signaling 1	1·6	0·0003
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	1·6	0·003
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1·5	<0·0005
Pcsk9	Proprotein convertase subtilisin/kexin type 9	−1·5	0·0001
Defb1	Defensin beta 1	−1·5	0·0002
Anpep	Alanyl (membrane) aminopeptidase	−1·5	<0·0005
Fosb	FBJ osteosarcoma oncogene B	−1·5	<0·0005
Cellular movement			
Sftpd	Surfactant-associated protein D	12·8	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	<0·0005
Vip	Vasoactive intestinal polypeptide	2·9	0·002
Tac1	Tachykinin 1	2·6	<0·0005
Socs2	Suppressor of cytokine signaling 2	2·6	<0·0005
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2·3	<0·0005
Cartpt	CART prepropeptide	2·1	<0·0005
Grp	Gastrin-releasing peptide	2·1	0·001
C3	Complement component 3	2·1	<0·0005
Gadd45α	Growth arrest and DNA-damage-inducible 45 alpha	2·0	<0·0005
Dnmt3b	DNA methyltransferase 3B	1·7	<0·0005
Ezr	Ezrin	1·6	<0·0005
Fgl2	Fibrinogen-like protein 2	1·6	<0·0005
Fhit	Fragile histidine triad gene	1·6	<0·0005
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1·5	<0·0005
Defb1	Defensin beta 1	−1·5	0·0002
Anpep	Alanyl (membrane) aminopeptidase	−1·5	<0·0005
Rap2a	RAS-related protein 2a	−1·6	0·003
Metabolism			
Tph2	Tryptophan hydroxylase 2	3·9	<0·0005
Ivd	Isovaleryl coenzyme A dehydrogenase	3·7	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	<0·0005
Tac1	Tachykinin 1	2·6	<0·0005
Igfbp-5	Insulin-like growth factor-binding protein-5	2·5	<0·0005
Prlr	Prolactin receptor	2·2	<0·0005

(continued)

Table 1 *Continued*

Genes	Description	Fold	*P* value
Prlr	Prolactin receptor	2·2	<0·0005
Grp	Gastrin-releasing peptide	2·1	0·001
C3	Complement component 3	2·1	<0·0005
Cartpt	CART prepropeptide	2·1	<0·0005
Slc40a1	Solute carrier family 40, member 1	2·1	<0·0005
Grhl1	Grainyhead-like 1 (*Drosophila*)	2·0	<0·0005
Slc2a13	Solute carrier family 2 (facilitated glucose transporter), 13	2·0	<0·0005
Enox1	Ecto-NOX disulfide-thiol exchanger 1	1·8	<0·0005
Slc6a8	Solute carrier family 6 (neurotransmitter transporter, creatine), member 8	1·8	<0·0005
Cdh7	Cadherin 7, type 2	1·7	<0·0005
Matn2	Matrilin 2	1·7	<0·0005
Gcdh	Glutaryl-coenzyme A dehydrogenase	1·6	<0·0005
Fhit	Fragile histidine triad gene	1·6	<0·0005
Ptpre	Protein tyrosine phosphatase, receptor type, E	1·6	0·003
Slc39a11	Solute carrier family 39 (metal ion transporter), member 11	1·6	<0·0005
Uhrf2	Ubiquitin-like, containing PHD and RING finger, 2	1·6	<0·0005
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1·5	<0·0005
Atp8a1	ATPase, aminophospholipid transporter	1·5	<0·0005
Edem2	ER degradation enhancer, mannosidase alpha-like 2	1·5	0·0003
Tmem2	Transmembrane protein 2	1·5	<0·0005
Lpl	Lipoprotein lipase	−1·5	<0·0005
Pcsk9	Proprotein convertase subtilisin/kexin type 9	−1·5	0·0001
Neurog3	Neurogenin 3	−1·5	<0·0005
Tox	Thymocyte selection-associated high-mobility group box	−1·5	<0·0005
Ddc	DOPA decarboxylase	−1·6	<0·0005
Hmgn3	High-mobility group nucleosomal-binding domain 3	−1·6	<0·0005
Scg3	Secretogranin III	−1·6	<0·0005
Inflammation			
Sftpd	Surfactant-associated protein D	12·8	<0·0005
Tph2	Tryptophan hydroxylase 2	3·9	<0·0005
Tnfrsf11b	Osteoprotegerin	3·7	< 0·0005
Chgb	Chromogranin B	2·9	<0·0005
Tac1	Tachykinin 1	2·6	<0·0005
Hopx	HOP homeobox	2·3	<0·0005
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2·3	<0·0005
Prlr	Prolactin receptor	2·2	<0·0005
C3	Complement component 3	2·1	<0·0005
Grp	Gastrin-releasing peptide	2·1	0·001
Slc2a13	Solute carrier family 2 (facilitated glucose transporter), 13	2·0	<0·0005
Sgip1	SH3-domain GRB2-like (endophilin) interacting protein 1	2·0	<0·0005
Gadd45α	Growth arrest and DNA-damage-inducible 45 alpha	2·0	<0·0005
Enox1	Ecto-NOX disulfide-thiol exchanger 1	1·8	<0·0005
C1qa	Complement component 1, q subcomponent, alpha polypeptide	1·7	<0·0005
Matn2	Matrilin 2	1·7	<0·0005
Hba-α1	Hemoglobin alpha, adult chain 1	1·6	0·0008
Hbb-β1	Hemoglobin, beta adult major chain	1·6	<0·0005
Slc39a11	Solute carrier family 39 (metal ion transporter), member 11	1·6	<0·0005
Fhit	Fragile histidine triad gene	1·6	<0·0005
Ern1	Endoplasmic reticulum (ER) to nucleus signaling 1	1·6	0·0003
Atp8a1	ATPase, aminophospholipid transporter	1·5	<0·0005
Tspan14	Tetraspanin 14	1·5	<0·0005
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1·5	<0·0005
Edem2	ER degradation enhancer, mannosidase alpha-like 2	1·5	0·0003
Tox	Thymocyte selection-associated high-mobility group box	−1·5	<0·0005
Anpep	Alanyl (membrane) aminopeptidase	−1·5	<0·0005
Fosb	FBJ osteosarcoma oncogene B	−1·5	<0·0005
Acpl2	Acid phosphatase-like 2	−1·6	<0·0005
Sphkap	SPHK1 interactor, AKAP domain containing	−1·6	0·0005
Ddc	DOPA decarboxylase	−1·6	<0·0005
Aard	Alanine and arginine-rich domain-containing protein	−1·7	<0·0005

(continued)

Table 1 *Continued*

Genes	Description	Fold	*P* value
Miscellaneous			
Fmo1	Flavin-containing monooxygenase 1	10·7	<0·0005
B3galnt1	UDP-beta 1, 3-galactosaminyltransferase, polypep-1	2·9	0·001
EG244911	Predicted gene, EG244911	2·8	<0·0005
H21Rik	RIKEN cDNA 64 305 50H21 gene	2·8	<0·0005
Hsbp-1	Heat shock factor-binding protein-1	2·7	0·0007
Wipi1	WD repeat domain, phosphoinositide interacting 1	2·6	<0·0005
Znrf2	Zinc and ring finger 2	2·4	0·0009
Car15	Carbonic anhydrase 15	2·4	0·003
Chac1	ChaC, cation transport regulator-like 1 (*Escherichia coli*)	2·2	0·002
Aqp4	Aquaporin 4	2·2	0·0009
Txnrd2	Thioredoxin reductase 2	2·0	<0·0005
Fkbp-11	FK506-binding protein-11	2·0	0·001
Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl CoA dehydrogenase	1·9	<0·0005
Igfals	Insulin-like growth factor-binding protein, acid labile subunit	1·9	0·0005
Rag1ap1	Recombination activating gene 1 activating protein 1	1·9	0·02
Aldh1l2	Aldehyde dehydrogenase 1 family, member L2	1·9	0·002
Pycr1	Pyrroline-5-carboxylate reductase 1	1·8	0·001
BC006662	cDNA sequence BC006662	1·7	0·0005
C ldn8	Claudin 8	1·7	0·0005
Gnβ1l	G-protein, beta polypeptide 1-like	1·7	0·0009
Rnf182	Ring finger protein 182	1·7	0·001
Hapln4	Hyaluronan and proteoglycan link protein 4	1·7	0·006
Tmem28	Transmembrane protein 28	1·7	0·006
Lsm6	LSM6 homolog, U6 small nuclear RNA associated	1·6	<0·0005
Ugt1a7c	UDP glucuronosyltransferase 1 family, polypeptide A7C	1·6	0·006
Ugt1a6b	UDP glucuronosyltransferase 1 family, polypeptide A6B	1·5	0·001
Edem1	ER degradation enhancer, mannosidase alpha-like 1	1·5	0·002
Uck2	Uridine-cytidine kinase 2	1·5	0·01
D01Rik	RIKEN cDNA 22 104 12D01 gene	−1·5	0·002
H3f3b	H3 histone, family 3B	−1·5	0·04
Mlxipl	MLX interacting protein-like	−1·5	0·001
Slc30a8	Solute carrier family 30 (zinc transporter), member 8	−1·5	0·0006
Mdm1	Transformed mouse 3T3 cell double minute 1	−1·5	<0·0005
Slc29a4	Solute carrier family 29 (nucleoside transporters), member 4	−1·6	0·001
Nt5dc2	5′-nucleotidase domain-containing 2	−1·7	0·02
Sult1d1	Sulfotransferase family 1D, member 1	−1·7	0·01
Msx3	Homeobox, msh-like 3	−1·8	0·04
Cib-3	Calcium and integrin-binding family member-3	−1·8	0·01
CryβA2	Crystallin, beta A2	−1·8	0·0009

[a]Shown are the 110 unique total transcripts that were identified, where each of these genes demonstrated at least 1·5-fold change, being statistically significant (P value <0·01) with false discovery rate (P<0·05). These 110 genes were categorized based on a classification scheme from ingenuity, and could be classified into more than one category. The miscellaneous category corresponds to genes that did not appear in any category shown here.

Cytokine treatment

For studies with murine islets, purified islets were incubated for 24 h in RPMI 1640 (ATCC, Manassas, VA, USA) supplemented with 1% nonheat inactivated FBS. The medium was supplemented with or without 5 ng/ml interleukin 1β (IL1β) or 10 ng/ml tumor necrosis factor-α (TNF-α, R&D Systems, Minneapolis, MN, USA), and RNA was isolated as described above. Three independent experiments were performed.

Statistical analysis

P values were calculated by Student's *t*-test (one tailed) with a significance level at P<0·05 using Excel software. To determine the statistical significance for a change in the real-time RT-PCR studies, the fold change was used relative to the control condition, which was set at 1·0. For TPH1, statistical analysis was performed using logarithmic transformed values.

Results

Global RNA expression analysis of mouse islets during pregnancy

Pregnancy-induced changes in islet gene expression were examined by RNA expression analysis using RNA prepared from islets isolated from pregnant (day 13·5) and control

(age- and sex-matched) mice. In all, 110 differentially expressed transcripts were identified (Fig. 1A), as defined by a statistically significant ($P<0·01$, FDR $<5\%$) $>1·5$-fold change in expression. Table 1 lists the 110 differentially regulated transcripts using a classification scheme from ingenuity (see Materials and Methods). In Fig. 1B, these 110 differentially regulated genes are categorized into seven groups. An overrepresentation *P* test, which is a hypergeometric test and provides a representation of the enrichment of the genes in each category compared with the chip as a whole, demonstrated that the genes in each of the categories were overrepresented relative to the genes on the chip as a whole.

To confirm the results of the RNA expression analysis, real-time RT-PCR was performed on a select group of the differentially regulated genes (rationale for their selection is provided below). The gene encoding SP-D was examined as it demonstrated the greatest pregnancy-induced change in expression in the global expression array (12·8-fold increase in SP-D mRNA levels). This finding was verified by real-time RT-PCR (Fig. 2A) where the level of SP-D mRNA increased 22·4±1·7-fold (mean±s.e.m., $n=3$, $P<0·05$) in pregnant compared with that in control islets. Real-time RT-PCR studies were performed on additional genes that demonstrated significant pregnancy-induced increases in mRNA levels and have been directly or indirectly implicated in islet function: osteoprotegrin (Schrader *et al.* 2007), TPH1 (Paulmann *et al.* 2009), TPH2 (Paulmann *et al.* 2009), cytokine-inducible SH2-containing protein (Dif *et al.* 2001), and isovaleryl-CoA dehydrogenase (MacDonald 2007). The pregnancy-induced changes in the expression were confirmed for each of these genes (Fig. 2A). In addition, the expression of two ligands for GPCRs, gastrin-releasing peptide (GRP), and vasoactive intestinal polypeptide (VIP) was increased; however, in follow-up real-time RT-PCR analyses, the changes in GRP and VIP mRNA levels, while trending in the same direction, did not reach statistical significance (Fig. 2A). In addition to the above mRNAs, the levels of mRNA encoding the PRLR, which has a well described role in β cells during pregnancy, as well as suppressor of cytokine signaling 2, which is known to attenuate PRLR signaling (Sutherland *et al.* 2007), were also increased by pregnancy in the global expression analysis and confirmed in real-time RT-PCR studies.

Among the mRNAs with a decreased level of expression in pregnant compared with that in control islets were mRNAs encoding zinc transporter-8 (ZnT8), neurogenin 3 (NGN3), and MLX interacting protein-like 1 (Mlxipl). ZnT8 and NGN3 are important for islet cell function and/or development (Gu *et al.* 2002, Lemaire *et al.* 2009), while the role of Mlxip1 in islets is unclear, although an association with NGN3 has been reported (Soyer *et al.* 2010). The pregnancy-induced changes in the expression for each of these genes were confirmed by real-time RT-PCR (Fig. 2B).

Figure 2C shows the results of western blot analyses that examined changes in the protein level of select genes in islets from pregnant mice compared with those in control mice.

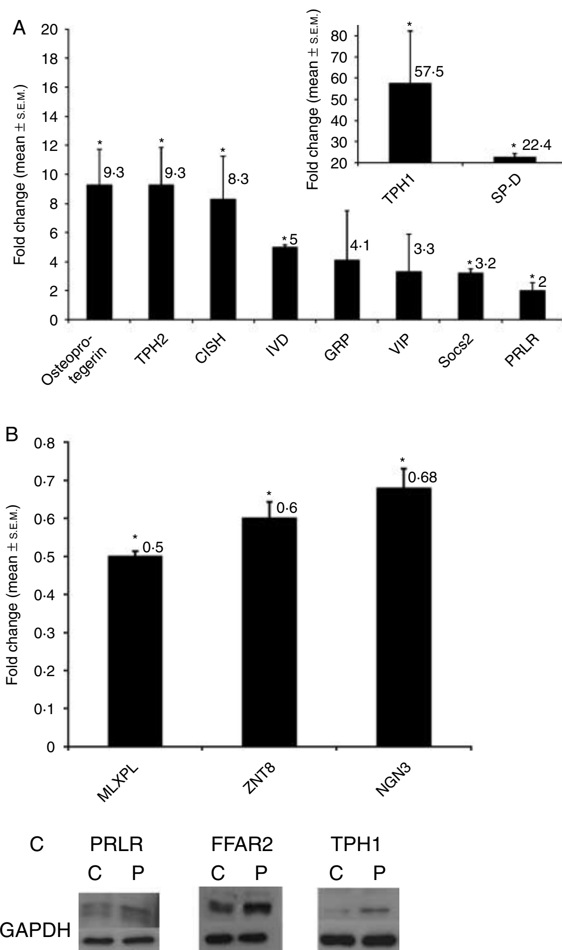

Figure 2 Pregnancy induced changes in expression of select genes by real-time RT-PCR and western blot analysis. (A and B) Fold change in the level of select mRNAs in islets isolated from pregnant mice at day E13·5 compared with that in age- and sex-matched control mice. (A) Shows the results for genes with increased mRNA levels and (B) shows the results for genes with deceased mRNA levels in pregnant compared with the control islets. The change in the level of each of the transcripts in both (A) and (B) was statistically significant at $P<0·05$ (indicated by *), except for GRP ($P=0·13$) and VIP ($P=0·13$). Values are the mean fold change ±s.e.m. from three independently prepared samples (each individual sample was a pooled sample from three mice). Fold change was calculated by a comparative cycle time (C_t) approach (see Materials and Methods) where the housekeeping genes (*HPRT, TBP,* and *PPIA*) were determined by geNORM (Vandesompele *et al.* 2002) using an approach defined in section 'Materials and Methods'. The housekeeping C_ts were used to derive the geometric means for the comparative C_t analyses. (C) Changes in the level of proteins encoded by three genes that exhibited pregnancy-induced changes in expression. Proteins were extracted from isolated islets from pregnant mice at day E13·5 and age- and sex-matched control mice. Representative immunoblots for prolactin receptor (PRLR), FFAR2, and TPH1 are shown. Shown are the results of a single experiment. Three independent experiments were performed. Each experiment was performed using islets prepared from a different control and pregnant mouse. After hybridization, the blots were stripped and re-probed with antibody against GAPDH.

The expression of each of the proteins was increased in pregnant compared with that in control islets, consistent with the pregnancy-induced change in the level of their mRNA.

A potential role of SP-D in pancreatic β cells

The transcript encoding SP-D exhibited the largest pregnancy-induced increase in expression in the global expression analysis, which was confirmed by the real-time RT-PCR studies. As the expression of SP-D in adult islets has not been observed before, we examined SP-D expression in islets during pregnancy. Using mouse monoclonal antibodies, SP-D was detected in the mouse lung tissue (Fig. 3A, lane 2, at ∼40–45 kDa) but not in the brain (lane 1), consistent with the known pattern of SP-D expression (Motwani *et al.* 1995). The immunoreactive SP-D detected in mouse lung was consistent with monomeric SP-D, which is ∼45 kDa (Sørensen *et al.* 2009). However, SP-D is commonly present in large complex structures (Sørensen *et al.* 2009). In unreduced preparations, the antibody typically recognizes proteins with a molecular weight of ∼120 kDa and above, which likely represent trimers and other multimers. In our studies, using unreduced protein preparations, the anti-SP-D antibodies recognized proteins of ∼120 and 180 kDa (see Fig. 3A, lane 3) consistent with previous reports (Sørensen *et al.* 2009). Interestingly, in murine islets, a single band of ∼100 kDa was detected and in the nonreduced state, a band of ∼180 kDa was observed (Fig. 3A, lanes 4 and 5). It has been previously described that differences in covalent binding patterns between SP-D monomers exist and can result in the formation of nonreducible dimers (Crouch *et al.* 1993, Guo *et al.* 2008, Matalon *et al.* 2009, Sørensen *et al.* 2009) that are detected by western blots. Therefore, this band at 100 kDa likely represents a dimer. To further explore this, we examined SP-D expression in the lung, pancreas, spleen, and islets (Fig. 3A, lanes 6–9). All tissues exhibited immunoreactive bands at both 45 and 100 kDa, although the relative intensities of the bands varied widely between tissues. These data are consistent with differences in SP-D complexes between the tissues. Finally, we compared the level of SP-D in islets from control and pregnant mice, and found increased expression in pregnant islets, consistent with the pregnancy-induced increase in the level of SP-D mRNA (Fig. 3B).

In addition to its role in surfactant biology, SP-D may have an anti-inflammatory role in the lung tissue (Sørensen *et al.* 2007). As pregnancy is characterized by increased levels of pro-inflammatory peptides (Richardson & Carpenter 2007), we hypothesized that SP-D mRNA levels may be regulated by pro-inflammatory molecules in islets. To address this possibility, the impact of treating isolated native murine islets for 48 h with IL1β or TNF-α on SP-D expression was examined. As can be seen (Fig. 3C), IL1β and TNF-α each increased SP-D mRNA levels in isolated murine islets.

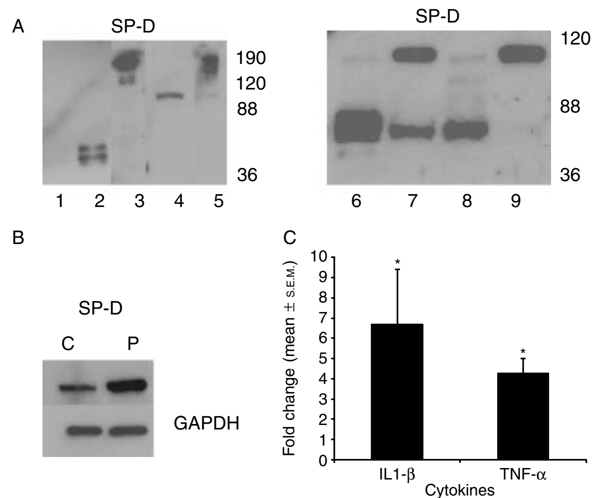

Figure 3 Surfactant protein D (SP-D) expression in mouse islets. (A) Expression of SP-D in the lungs and islets. Proteins were extracted from mouse lung and islets. Samples from the brain (lane 1), lung (lane 2), mouse lung – not reduced (lane 3), islets (lane 4), islets – not reduced (lane 5), lung (lane 6), pancreas (lane 7), spleen (lane 8), and islets (lane 9) were either reduced or not reduced prior to separation by PAGE. The results are representative of the results of two to three independent experiments. (B) SP-D expression in pregnant islets. Protein extracts were prepared from islets isolated from pregnant mice at day E13·5 and age- and sex-matched control mice. Western blot analysis was performed as described in section 'Materials and Methods.' C, control; P, pregnant. After hybridization, the blots were stripped and re-probed with antibody against GAPDH. The findings are representative of the results of three independent experiments. (C) Effect of cytokines on SP-D mRNA levels in murine islets. RNA was prepared from murine islets treated for 48 h with 5 ng/ml IL1β or 10 ng/ml TNF-α. The fold change in SP-D mRNA was determined by real-time RT-PCR, using the comparative method and the housekeeping gene, *18S*. Values represent the mean ± S.E.M. (three independent samples, $n=3$) and are the relative level of SP-D mRNA compared with the level in control islets not treated with cytokines. *$P<0·05$ compared with control mice.

Analysis of GPCR-specific expression in mouse islets during pregnancy

In the global RNA expression analysis, one GPCR, FFAR2, was differentially regulated by pregnancy (>1·5-fold change in expression, $P<0·01$). As the genes for GPCRs tend to be underrepresented in the global RNA expression analyses, potentially leading to missed targets (Fredriksson & Schioth 2005), further studies were performed to determine whether pregnancy induced changes in the expression of genes encoding members of this receptor family. To do this, a secondary approach, a real-time RT-PCR array, was used to examine the expression of transcripts encoding 343 different GPCRs. The expression of GPCRs in the mouse islets during pregnancy at E13·5 was compared with the expression in islets isolated from nonpregnant female mice. Of the 343 GPCRs probed in the real-time array, the mRNAs encoding 216 of these GPCRs were detectable in the islets, as defined by the

detection of a discernible melt curve in the control islets. Of the 343 GPCR transcripts that were examined in the real-time RT-PCR array, 92 were detected in the global RNA expression array. For this study, we focused on the 100 most highly expressed GPCR mRNAs in control islets (Table 2). Three of these GPCRs (FFAR2; GAL3R, galanin receptor 3; and CCKAR, cholecystokinin receptor A) exhibited a >2·5-fold change in the expression in pregnant compared with those in control islets. In the global RNA expression analysis, the mRNA encoding CCKAR was not detected. In contrast, FFAR2 mRNA exhibited a significant 1·5-fold increase in expression in the global RNA expression analysis and GAL3R mRNA was increased 1·3-fold ($P=0·19$). Follow-up real-time RT-PCR studies of these three GPCRs were performed (Fig. 4), and a significant pregnancy-induced increase in mRNA levels was observed for only FFAR2 and CCKAR ($P<0·05$).

Discussion

The global RNA expression analysis and the confirmatory real-time RT-PCR assays demonstrated a marked increase in the level of the mRNA encoding SP-D, a protein best known for its role in lung function (Botas *et al*. 1998). A clear role for SP-D in lung and surfactant homeostasis has been described (Sørensen *et al*. 2007), but expression of this gene in the adult islets has not been previously reported. However, a recent study identified SP-D as a marker of new β cell formation during the perinatal period (Aye *et al*. 2010). A global SP-D knockout has been created (Botas *et al*. 1998), although alterations in glucose metabolism and/or islet biology in these mice have not been reported. A possible metabolic role of SP-D is suggested by the observation that SP-D impacts systemic lipid levels and atherosclerosis (Sørensen *et al*. 2006*b*). Consistent with that is the observation that circulating SP-D levels are inversely associated with obesity in humans (Zhao *et al*. 2007). Furthermore, mice with a null mutation of the *SP-D* gene maintained on a normal diet exhibited more weight gain and increased adiposity compared with control mice (Sørensen *et al*. 2006*a*).

The novel observation that SP-D mRNA is expressed in β cells was confirmed by western blot and immunohistochemical analyses (see Fig. 3A and B). Through a combination of studies, it is apparent that SP-D in islets exists in a form that leads to nonreducible dimers. This is not surprising, considering the complex nature of this protein. First, SP-D ranges in size from 37 to 50 kDa in the monomeric form because of different glycosylation patterns (Leth-Larsen *et al*. 1999, Sørensen *et al*. 2009). Furthermore, SP-D trimers contain multiple disulfide bonds between cysteine residues as well as nondisulfide covalent bonds, and these nondisulfide covalent bonds result in dimers resistant to reduction (Crouch *et al*. 1993, Guo *et al*. 2008, Matalon *et al*. 2009, Sørensen *et al*. 2009). As the function of SP-D is dependent on these modifications (Leth-Larsen *et al*. 1999, Sørensen *et al*. 2009), the modifications of SP-D in islets may have important implications for its function.

If SP-D indeed has a role in islet function, it could be hypothesized that, similar to endothelial cells where SP-D has an anti-inflammatory role (Snyder *et al*. 2008), it may function as an anti-inflammatory molecule in islets. As pregnancy is characterized by increased levels of circulating pro-inflammatory molecules (Richardson & Carpenter 2007), we examined the impact of the pro-inflammatory cytokines, IL1β and TNF-α, on SP-D expression in islets. We demonstrated that not only SP-D mRNA is expressed in islets but also its expression is regulated by cytokines. This is consistent with the possibility that SP-D serves as an anti-inflammatory factor in islets during pregnancy, although future studies will be needed to more fully examine this possibility. As with another anti-inflammatory molecule, cytokine IL1 receptor antagonist, which protects mice fed with high-fat diets from hyperglycemia (Sauter *et al*. 2008), this type of molecule could have unique therapeutic potential.

Recent genome-wide association studies have reported association of a missense mutation in *SLC30A8*, the gene that encodes the ZnT8, with type 2 diabetes (Chimienti *et al*. 2006, Sladek *et al*. 2007). ZnT8 may also be an autoantigen in type 1 diabetes (Wenzlau *et al*. 2007). Expression of *SLC30A8* is largely localized to islets and, more specifically, β cells (Chimienti *et al*. 2006). ZnT8 is thought to play an important role in zinc transport into the insulin secretory granules (Nicolson *et al*. 2009). Thus, the decrease in ZnT8 mRNA levels in islets from pregnant compared with those in control mice was unexpected given the increased insulin secretory demands in pregnancy. However, pregnancy is a pro-inflammatory state, and recent observations (Egefjord *et al*. 2009, El Muayed *et al*. 2010) have shown that cytokines decrease the level of ZnT8 mRNA in islet cells. This may explain, in part, the pregnancy-induced decrease in ZnT8 mRNA levels.

In addition to the global RNA expression analysis, we examined genes encoding the GPCRs in greater detail. Multiple GPCRs are known to be expressed in islets (Winzell & Ahren 2007), but the impact of pregnancy on GPCR expression has not been previously examined. These receptors are well-known targets for multiple medicinal therapies. Moreover, given the important role of some GPCRs (e.g. the glucagon-like peptide 1 and parathyroid hormone-1 receptors) in the regulation of islet function and mass (De Leon *et al*. 2003), we explored the impact of pregnancy on the expression of mRNAs encoding GPCRs in islets using a real-time RT-PCR array. This array examined the level of mRNAs encoding 343 GPCRs in pregnant compared with that in control islets and was complementary to the global RNA expression analyses. Low levels of GPCR mRNA (Fredriksson & Schioth 2005) and the stringent requirements for statistical significance can lead to missed targets with the global RNA expression analysis. A recent study reported a role for serotonin in the regulation of β cell mass in pregnancy (Kim *et al*. 2010). The expression of mRNA encoding the serotonin

Table 2 Comparison of the 100 most highly expressed G-protein-coupled receptors (GPCRs) in the GPCR-specific real-time reverse transcripton-PCR array compared with the global expression array for RNA from islets isolated from pregnant mice (E13·5) compared with control mice (age- and sex-matched)

	GPCR array		Global array	
	Relative expression[a]	Fold change[a]	Fold change[a]	*P*<value
GPCRs				
Glp1r	32 988	1·7	1·2	0·01
Sstr3	11 663	1·1	−1·1	0·03
Adra2a	9473	1·1	−1·0	0·59
Gabbr2	5441	2·1	ND	–
Gpr56	5441	1·4	−1·0	0·89
Gipr	5077	1·3	ND	–
Gpr116	5077	0·6	ND	–
Lphn1	5077	0·9	ND	–
Casr	4737	0·8	−1·0	0·29
Ffar1	4419	1·1	−1·1	0·14
Gpr108	4419	1·4	1·2	0·01
Gpr119	4123	2·0	ND	–
Vipr1	4123	0·7	1·0	0·52
Adora1	2720	0·7	−1·1	0·10
Gpr137b	2683	0·9	ND	–
Chrm3	2538	1·3	−1·0	0·40
Galr1	2368	1·0	−1·1	0·04
Gpr137	2210	1·2	ND	–
P2ry6	2062	1·6	1·0	0·56
Crcp	1924	0·8	−1·1	0·34
F2rl1	1795	0·7	−1·3	$7{\cdot}04\times10^{-5}$
P2ry5	1795	1·1	1·2	0·004
Ptger3	1360	0·9	ND	–
Bai3	1269	0·5	−1·0	0·31
Tm7sf3	1269	1·3	ND	–
Eltd1	1184	0·9	ND	–
Gabbr1	1184	0·9	ND	–
Gcgr	1184	1·2	−1·2	0·01
Gprc5c	1184	1·6	ND	–
Lgr4	1184	1·2	ND	–
Adcyap1r1	1105	0·7	ND	–
Fzd4	1105	0·9	1·0	0·58
Fzd3	1031	1·4	ND	–
Fzd6	1031	1·2	−1·1	0·01
Gpr125	1031	1·1	−1·0	0·12
Fzd7	962	1·0	−1·1	0·07
Taar1	962	1·0	−1·1	0·03
F2r	897	0·9	−1·1	0·07
Edg2	837	0·6	−1·1	0·06
Ednra	837	0·6	ND	–
Lphn3	837	0·8	1·0	0·54
Ccrl2	729	1·2	−1·0	0·40
Calcrl	680	1·1	ND	–
Cd97	680	1·2	ND	–
Gpr146	680	1·2	−1·0	0·10
Chrm4	635	0·6	ND	–
Fzd5	635	1·0	ND	–
Gpr120	635	1·1	−1·3	$2{\cdot}67\times10^{-5}$
Oxtr	635	1·6	ND	–
Celsr3	592	1·3	−1·2	0·04
Ednrb	592	1·4	1·0	0·53
Gprc5b	592	0·8	−1·1	0·01
Lphn2	592	1·1	ND	–
Fzd8	552	1·3	ND	–
Gpr85	552	0·8	−1·2	0·03
Cnr1	515	0·7	−1·0	0·58
Galr3	**515**	**6·5**	**1·1**	**0·01**
Gpr39	515	0·9	ND	–

(continued)

Table 2 *Continued*

	GPCR array		Global array	
	Relative expression[a]	Fold change[a]	Fold change[a]	*P*<value
P2ry1	515	0·8	ND	–
Gpr161	481	1·1	ND	–
Gpr75	449	0·7	ND	–
Gpr81	449	0·7	ND	–
Ffar2	**419**	**2·8**	**1·6**	**$3{\cdot}96\times10^{-5}$**
Mrgpre	364	1·8	ND	–
Ghsr	317	0·7	−1·1	0·06
Agtrl1	296	2·2	−1·0	0·66
Gpr41	296	1·5	ND	–
Npy1r	296	0·6	−1·0	0·93
Adora2b	258	0·7	−1·1	0·26
Gpr124	258	0·9	−1·0	0·20
Gpr63	258	0·8	ND	–
Cckar	**240**	**11·1**	**ND**	–
Adora2a	224	0·7	ND	–
Gpr19	224	1·1	1·1	0·03
Admr	209	1·2	ND	–
Avpr1b	209	0·9	ND	–
Cx3cr1	209	1·1	−1·0	0·51
Edg1	209	1·7	ND	–
Gprc5a	209	0·6	−1·0	0·82
Ebi2	195	0·8	ND	–
Gpr4	195	1·7	ND	–
Sstr1	195	1·0	ND	–
Celsr1	182	2·2	1·1	0·03
Cxcr3	182	1·3	ND	–
Gpr153	182	1·2	ND	–
Avpr1a	170	0·3	−1·0	0·61
Chrm1	170	0·5	ND	–
Crhr1	170	0·7	−1·0	0·13
Gprc6a	170	0·6	1·0	0·50
P2ry2	159	0·4	ND	–
Ccr2	148	1·1	ND	–
Gpr135	148	1·1	1·0	0·46
Tbxa2r	148	1·4	−1·1	0·01
Chrm2	138	0·6	ND	–
Emr1	138	1·6	−1·0	0·24
Fzd1	129	1·4	ND	–
Gpr109a	129	1·4	1·0	0·30
Gpr65	129	0·8	−1·0	0·28
Galr2	129	1·3	ND	–
Celsr2	129	0·6	ND	–

Indicated in bold are the G-protein-coupled receptors that demonstrated a fold change of >2·5.
[a]Shown are the 100 GPCRs with the greatest expression and their corresponding fold change in pregnant islets compared with those in control. Relative expression was calculated by $2^{C_t(\text{GPCR})-C_t(\text{housekeeping gene})}\times10^5$. Fold change for the GPCR array and global array were calculated as outlined in section 'Materials and Methods'.

receptor, Htr2b, was increased at mid-gestation while that encoding Htr1d was increased at the end of gestation. In our studies, Htr2b was only weakly expressed in islets from both pregnant and control mice and a clear pregnancy-induced change in expression was not observed; Htr1d mRNA was detectable on day E13·5 but a pregnancy-induced change in the expression was not evident at that time point during pregnancy.

Our studies did exhibit a reproducible pregnancy-induced change in two GPCRs, FFAR2 and CCKAR. Of the two, the role of CCKAR in islets has been more fully examined, with initial studies suggesting that CCKAR plays a role in mediating insulin secretion through CCK binding (Winzell & Ahren 2007). FFAR2, which exhibited a pregnancy-induced increase in mRNA and protein levels in islets, is a member of a recently identified family of GPCRs that are activated by free fatty acids. This family can be divided by the chain length (long versus short) of the free fatty acids that bind to the receptors. GPCRs that are specific for long-chain free fatty acids include GPR40 (FFAR1) and

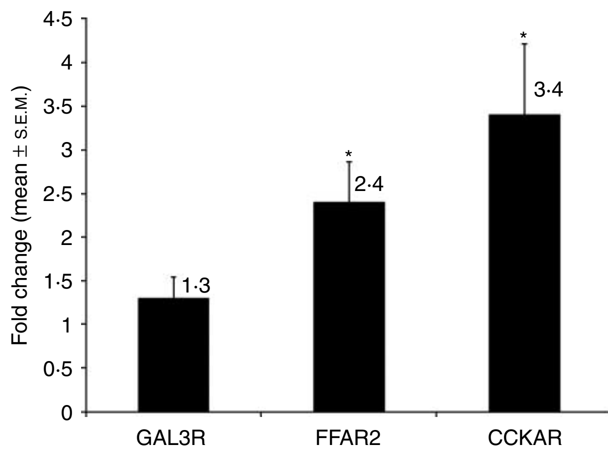

Figure 4 Real-time RT-PCR analysis of GPCR mRNAs regulated by pregnancy in mouse islets at day 13·5 of pregnancy. The fold change in the level of mRNA encoding the following GPCRs, CCKAR, FFAR2, and GAL3R, in islets isolated from pregnant mice (day E13·5) compared with that in age- and sex-matched control mice was determined by real-time RT-PCR. Values are the mean ± S.E.M. from three independent experiments. The change in the level of each transcript was statistically significant at $P<0·05$ (indicated by *), except for GAL3R ($P=0·19$).

GPR120. In contrast, FFAR2 binds specifically to short-chain fatty acids (Kebede *et al.* 2009). Pregnancy-induced changes in the expression of the mRNAs encoding GPR40 and GPR120 were not observed. The role of FFAR2 in islets is unclear, although a previous study has demonstrated the expression of FFAR2 in islets (Regard *et al.* 2007). Moreover, evidence exists that FFAR2 is expressed in β cells, at least in pancreatic β cell lines (Kebede *et al.* 2009). Taken together, these data suggest that FFAR2 may be involved in the regulation of islet cell function or mass in pregnancy, although future studies will be required to define its role in islet cell biology.

During the preparation of this manuscript, a study exploring the global RNA expression analysis in islets from pregnant mice was reported (Rieck *et al.* 2009). These investigators identified 1907 genes that exhibited differential regulation compared with 110 in our study. This difference relates to the use of different analytic approaches. Using an approach similar to that of Rieck *et al.*, we also detected ∼1500 differentially regulated genes. Of the 110 differentially regulated genes identified in our study, 37 were not shown to be differentially regulated (Rieck *et al.* 2009). A few of the notable exceptions were suppressor of cytokine signaling 2, *Ngn3* and *ZnT8*, genes in which a pregnancy-induced change in expression was verified in our study. These differences may be explained, in part, by differences in the gene expression platforms used and the day of gestation studied, E13·5 vs E14·5.

To date, there are three genes that have been specifically identified as playing a role in gestational diabetes using mouse models. The *PRLR*, as discussed below, has a clearly established role. The other two genes suggested to be involved in gestational diabetes are *FoxM1* and *menin*. The level of FoxM1 mRNA was shown by Zhang *et al.* (2010) to be increased on E14·5. Our data show that FoxM1 mRNA levels were increased on E13·5; however, this increase was not statistically significant. Zhang *et al.* (2010) also developed mice with pancreatic specific null mutations for *FoxM1* and found that these mice developed gestational diabetes. The other gene identified as having an important role in gestational diabetes was *menin* (Karnik *et al.* 2007). Karnik *et al.* demonstrated decreased menin mRNA levels in islets during pregnancy, while overexpression of menin led to hyperglycemia during pregnancy. Finally, expression of menin was shown to be regulated by prolactin (Karnik *et al.* 2007). In our study, a pregnancy-induced change in menin mRNA levels was not demonstrated, although only a single time point in pregnancy was examined.

Increased PRLR expression in islets during pregnancy was demonstrated over 10 years ago (Sorenson & Brelje 2009). In this report, we have confirmed those findings using the global RNA expression analysis and follow-up confirmation by real-time RT-PCR. Furthermore, a transgenic mouse model has confirmed the role of the PRLR in glucose homeostasis during pregnancy (Huang *et al.* 2009). Although prolactin signaling undoubtedly contributes to pregnancy-induced changes in β cell mass (Sorenson & Brelje 2009), other signaling pathways and downstream molecules are likely important in this process. Together, with the recently published study by Rieck *et al.* (2009), our study demonstrates multiple novel proteins that warrant further investigation for their role in pregnancy-induced changes in β cells. Many of these genes were not previously known to be regulated by pregnancy, and, in some cases, expression of the gene in islets had not been previously demonstrated. These data should lay the foundation upon which select genes can be more fully examined for their role in pregnancy-induced changes in pancreatic β cell function.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/JOE-10-0298.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Aye T, Toschi E, Sharma A, Sgroi D & Bonner-Weir S 2010 Identification of markers for newly formed β-cells in the perinatal period: a time of recognized β-cell immaturity. *Journal of Histochemistry and Cytochemistry* **58** 369–376. (doi:10.1369/jhc.2009.954909)

Botas C, Poulain F, Akiyama J, Brown C, Allen L, Goerke J, Clements J, Carlson E, Gillespie AM, Epstein C *et al*. 1998 Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. *PNAS* **95** 11869–11874. (doi:10.1073/pnas.95.20.11869)

Brelje TC, Scharp DW, Lacy PE, Ogren L, Talamantes F, Robertson M, Friesen HG & Sorenson RL 1993 Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology* **132** 879–887. (doi:10.1210/en.132.2.879)

Butler PC, Meier JJ, Butler AE & Bhushan A 2007 The replication of β cells in normal physiology, in disease and for therapy. *Nature Clinical Practice. Endocrinology and Metabolism* **3** 758–768. (doi:10.1038/ncpendmet0647)

Chen X, Zhang X, Larson CS, Baker MS & Kaufman DB 2006 *In vivo* bioluminescence imaging of transplanted islets and early detection of graft rejection. *Transplantation* **81** 1421–1427. (doi:10.1097/01.tp.0000206109.71181.bf)

Chimienti F, Devergnas S, Pattou F, Schuit F, Garcia-Cuenca R, Vandewalle B, Kerr-Conte J, Van Lommel L, Grunwald D, Favier A *et al*. 2006 *In vivo* expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. *Journal of Cell Science* **119** 4199–4206. (doi:10.1242/jcs.03164)

Crouch E, Persson A & Chang D 1993 Accumulation of surfactant protein D in human pulmonary alveolar proteinosis. *American Journal of Pathology* **142** 241–248.

De Leon DD, Deng S, Madani R, Ahima RS, Drucker DJ & Stoffers DA 2003 Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. *Diabetes* **52** 365–371. (doi:10.2337/diabetes.52.2.365)

Dif F, Saunier E, Demeneix B, Kelly PA & Edery M 2001 Cytokine-inducible SH2-containing protein suppresses PRL signaling by binding the PRL receptor. *Endocrinology* **142** 5286–5293. (doi:10.1210/en.142.12.5286)

Du P, Kibbe WA & Lin SM 2008 Lumi: a pipeline for processing Illumina microarray. *Bioinformatics* **24** 1547–1548. (doi:10.1093/bioinformatics/btn224)

Egefjord L, Jensen JL, Bang-Berthelsen CH, Petersen AB, Smidt K, Schmitz O, Karlsen AE, Pociot F, Chimienti F, Rungby J *et al*. 2009 Zinc transporter gene expression is regulated by pro-inflammatory cytokines: a potential role for zinc transporters in β-cell apoptosis? *BMC Endocrine Disorders* **9** 7. (doi:10.1186/1472-6823-9-7)

El Muayed M, Billings LK, Raja MR, Zhang X, Park PJ, Newman MV, Kaufman DB, O'Halloran TV & Lowe WL Jr 2010 Acute cytokine-mediated downregulation of the zinc transporter ZnT8 alters pancreatic β-cell function. *Journal of Endocrinology* **206** 159–169. (doi:10.1677/JOE-09-0420)

Fleige S, Walf V, Huch S, Prgomet C, Sehm J & Pfaffl MW 2006 Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnology Letters* **28** 1601–1613. (doi:10.1007/s10529-006-9127-2)

Fredriksson R & Schioth HB 2005 The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Molecular Pharmacology* **67** 1414–1425. (doi:10.1124/mol.104.009001)

Gu G, Dubauskaite J & Melton DA 2002 Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129** 2447–2457.

Guo CJ, Atochina-Vasserman EN, Abramova E, Foley JP, Zaman A, Crouch E, Beers MF, Savani RC & Gow AJ 2008 *S*-nitrosylation of surfactant protein-D controls inflammatory function. *PLoS Biology* **6** e266. (doi:10.1371/journal.pbio.0060266)

Holz GG & Chepurny OG 2005 Diabetes outfoxed by GLP-1? *Science's STKE* **2005** pe2. (doi:10.1126/stke.2682005pe2)

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)

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)

Kebede MA, Alquier T, Latour MG & Poitout V 2009 Lipid receptors and islet function: therapeutic implications? *Diabetes, Obesity and Metabolism* **11** (Supplement 4) 10–20. (doi:10.1111/j.1463-1326.2009.01114.x)

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)

Lemaire K, Ravier MA, Schraenen A, Creemers JW, Van de Plas R, Granvik M, Van Lommel L, Waelkens E, Chimienti F, Rutter GA *et al*. 2009 Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. *PNAS* **106** 14872–14877. (doi:10.1073/pnas.0906587106)

Leth-Larsen R, Holmskov U & Hojrup P 1999 Structural characterization of human and bovine lung surfactant protein D. *Biochemical Journal* **343** 645–652. (doi:10.1042/0264-6021:3430645)

MacDonald MJ 2007 Synergistic potent insulin release by combinations of weak secretagogues in pancreatic islets and INS-1 cells. *Journal of Biological Chemistry* **282** 6043–6052. (doi:10.1074/jbc.M606652200)

Madsen J, Kliem A, Tornoe I, Skjodt K, Koch C & Holmskov U 2000 Localization of lung surfactant protein D on mucosal surfaces in human tissues. *Journal of Immunology* **164** 5866–5870.

Matalon S, Shrestha K, Kirk M, Waldheuser S, McDonald B, Smith K, Gao Z, Belaaouaj A & Crouch EC 2009 Modification of surfactant protein D by reactive oxygen–nitrogen intermediates is accompanied by loss of aggregating activity *in vitro* and *in vivo*. *FASEB Journal* **23** 1415–1430. (doi:10.1096/fj.08-120568)

Motwani M, White RA, Guo N, Dowler LL, Tauber AI & Sastry KN 1995 Mouse surfactant protein-D. cDNA cloning, characterization, and gene localization to chromosome 14. *Journal of Immunology* **155** 5671–5677.

Nicolson TJ, Bellomo EA, Wijesekara N, Loder MK, Baldwin JM, Gyulkhandanyan AV, Koshkin V, Tarasov AI, Carzaniga R, Kronenberger K *et al*. 2009 Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. *Diabetes* **58** 2070–2083. (doi:10.2337/db09-0551)

Paulmann N, Grohmann M, Voigt JP, Bert B, Vowinckel J, Bader M, Skelin M, Jevsek M, Fink H, Rupnik M *et al*. 2009 Intracellular serotonin modulates insulin secretion from pancreatic β-cells by protein serotonylation. *PLoS Biology* **7** e1000229. (doi:10.1371/journal.pbio.1000229)

Regard JB, Kataoka H, Cano DA, Camerer E, Yin L, Zheng YW, Scanlan TS, Hebrok M & Coughlin SR 2007 Probing cell type-specific functions of G(i) *in vivo* identifies GPCR regulators of insulin secretion. *Journal of Clinical Investigation* **117** 4034–4043. (doi:10.1172/JCI32994)

Richardson AC & Carpenter MW 2007 Inflammatory mediators in gestational diabetes mellitus. *Obstetrics and Gynecology Clinics of North America* **34** 213–224. (doi:10.1016/j.ogc.2007.04.001)

Rieck S, White P, Schug J, Fox AJ, Smirnova O, Gao N, Gupta RK, Wang ZV, Scherer PE, Keller MP *et al*. 2009 The transcriptional response of the islet to pregnancy in mice. *Molecular Endocrinology* **23** 1702–1710. (doi:10.1210/me.2009-0144)

Sauter NS, Schulthess FT, Galasso R, Castellani LW & Maedler K 2008 The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia. *Endocrinology* **149** 2208–2218. (doi:10.1210/en.2007-1059)

Schrader J, Rennekamp W, Niebergall U, Schoppet M, Jahr H, Brendel MD, Horsch D & Hofbauer LC 2007 Cytokine-induced osteoprotegerin expression protects pancreatic β cells through p38 mitogen-activated protein kinase signalling against cell death. *Diabetologia* **50** 1243–1247. (doi:10.1007/s00125-007-0672-6)

Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S *et al*. 2007 A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* **445** 881–885. (doi:10.1038/nature05616)

Snyder GD, Oberley-Deegan RE, Goss KL, Romig-Martin SA, Stoll LL, Snyder JM & Weintraub NL 2008 Surfactant protein D is expressed and modulates inflammatory responses in human coronary artery smooth muscle cells. *American Journal of Physiology. Heart and Circulatory Physiology* **294** H2053–H2059. (doi:10.1152/ajpheart.91529.2007)

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)

Sørensen GL, Hjelmborg JV, Leth-Larsen R, Schmidt V, Fenger M, Poulain F, Hawgood S, Sorensen TI, Kyvik KO & Holmskov U 2006*a* Surfactant protein D of the innate immune defence is inversely associated with human obesity and SP-D deficiency infers increased body weight in mice. *Scandinavian Journal of Immunology* **64** 633–638. (doi:10.1111/j.1365-3083.2006.01853.x)

Sørensen GL, Madsen J, Kejling K, Tornoe I, Nielsen O, Townsend P, Poulain F, Nielsen CH, Reid KB, Hawgood S *et al*. 2006*b* Surfactant protein D is proatherogenic in mice. *American Journal of Physiology. Heart and Circulatory Physiology* **290** H2286–H2294. (doi:10.1152/ajpheart.01105.2005)

Sørensen GL, Husby S & Holmskov U 2007 Surfactant protein A and surfactant protein D variation in pulmonary disease. *Immunobiology* **212** 381–416. (doi:10.1016/j.imbio.2007.01.003)

Sørensen GL, Hoegh SV, Leth-Larsen R, Thomsen TH, Floridon C, Smith K, Kejling K, Tornoe I, Crouch EC & Holmskov U 2009 Multimeric and trimeric subunit SP-D are interconvertible structures with distinct ligand interaction. *Molecular Immunology* **46** 3060–3069. (doi:10.1016/j.molimm.2009.06.005)

Soyer J, Flasse L, Raffelsberger W, Beucher A, Orvain C, Peers B, Ravassard P, Vermot J, Voz ML, Mellitzer G *et al*. 2010 Rfx6 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet cell development. *Development* **137** 203–212. (doi:10.1242/dev.041673)

Sutherland KD, Lindeman GJ & Visvader JE 2007 Knocking off SOCS genes in the mammary gland. *Cell Cycle* **6** 799–803.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (doi:10.1186/gb-2002-3-7-research0034)

Vasavada RC, Cavaliere C, D'Ercole AJ, Dann P, Burtis WJ, Madlener AL, Zawalich K, Zawalich W, Philbrick W & Stewart AF 1996 Overexpression of parathyroid hormone-related protein in the pancreatic islets of transgenic mice causes islet hyperplasia, hyperinsulinemia, and hypoglycemia. *Journal of Biological Chemistry* **271** 1200–1208. (doi:10.1074/jbc.271.40.24371)

Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW *et al*. 2007 The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *PNAS* **104** 17040–17045. (doi:10.1073/pnas.0705894104)

Winzell MS & Ahren B 2007 G-protein-coupled receptors and islet function – implications for treatment of type 2 diabetes. *Pharmacology and Therapeutics* **116** 437–448. (doi:10.1016/j.pharmthera.2007.08.002)

Xiao SH, Reagan JD, Lee PH, Fu A, Schwandner R, Zhao X, Knop J, Beckmann H & Young SW 2008 High throughput screening for orphan and liganded GPCRs. *Combinatorial Chemistry & High Throughput Screening* **11** 195–215. (doi:10.2174/138620708783877762)

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)

Zhao XM, Wu YP, Wei R, Cai HX, Tornoe I, Han JJ, Wang Y, de Groot PG, Holmskov U, Xia ZL *et al*. 2007 Plasma surfactant protein D levels and the relation to body mass index in a chinese population. *Scandinavian Journal of Immunology* **66** 71–76. (doi:10.1111/j.1365-3083.2007.01943.x)

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