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ORIGINAL ARTICLE

Study on pancreatic islet adaptation and gene expression during pregnancy in rats

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Abstract During pregnancy, the pancreatic islets undergo major structural and functional changes in response to increased peripheral resistance to insulin. In this study, we investigated the adaptive changes of the pancreatic islet beta-cell mass during pregnancy in rats, and explored profiles of islet gene expression at various stages of pregnancy. Some differentially expressed genes were verified by RT-PCR and Real-time PCR. Our results showed that compared with the non-pregnant control group, insulin synthesis, glucose-stimulated insulin secretion, islet beta-cell proliferation, and islet size were all increased in pregnant rats. The study also demonstrated that expression of several-hundred islet genes were changed during pregnancy, especially at day 14.5. The differentially expressed genes identified were distributed into eight main categories according to their biological functions: (1) genes involved in apoptosis or tumor; (2) genes related to binding; (3) genes involved in metabolism; (4) genes related to cell cycle; (5) genes for signal transducer activity; (6) genes related to structural molecule activity; (7) genes involved in transcription regulator activity; (8) genes for transporter activity. Among these genes, regenerating islet-derived 3 alpha (Reg3a) was remarkably increased during pregnancy. We hypothesize that differentially expressed genes may play an important role in adaptation of pancreatic islets during pregnancy in rats. In addition, the markedly increased expression of gene Reg3a is probably related to islet regeneration.

Keywords Pregnancy · Pancreatic islet · Islet adaptation · Reg3a · Islet regeneration

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Introduction

Changes in carbohydrate metabolism and insulin resistance occur during pregnancy to ensure a continuous supply of nutrients to the growing fetus [1]. Besides adaptation to increased body weight, the beta-cell mass can also compensate for the increased demand for insulin during pregnancy. In order to accommodate this increased requirement for insulin secretion, it is essential that the pancreatic islets undergo major structural and functional changes. Failure to compensate is thought to lead to the development of gestational diabetes.

At the end of pregnancy in rats, the beta-cell mass is 2.5-fold increased compared with non-pregnant females [2]. This results from both an increased beta-cell number and cellular hypertrophy. Islet enlargement and beta-cell hyperplasia have also been observed in autopsies from pregnant humans [3]. These studies demonstrate the capability of beta-cells to up-regulate their mass using the mechanisms of beta-cell replication and changes in beta-cell size, to achieve homeostasis in conditions of changing insulin demand. But the regulation of islet beta-cell mass is

still incompletely understood. We chose to study the endocrine pancreas of pregnant rats, in which a highly reproducible physiological proliferation of pancreatic islet beta-cells occurs [4].

In this study, we investigated adaptive changes of islets during pregnancy in rats, including changes of glucose tolerance, insulin release, glucose-stimulated insulin secretion, beta-cell proliferation, and beta-cell size. These adaptive changes in pregnant rats, as well as during growth, differentiation, and maturation of the fetal and neonatal endocrine pancreas, are thought to be mediated by a variety of hormonal, chemical, and neural signals to the islets [5–7]. Furthermore, there has been no comprehensive analysis of gene regulation in pancreatic islets during pregnancy in rats. Therefore, we used microarray to analyze pancreatic islet gene expression in pregnant rats. The identification of differentially expressed genes would help to elucidate the mechanisms underlying the structural and functional adaptations during pregnancy. Moreover, we identified several genes, in particular regenerating islet-derived 3 alpha (Reg3a), which was remarkably increased during pregnancy compared with non-pregnant rats.

Materials and methods

Animals

Healthy male Sprague–Dawley (SD) rats with a body mass of 300 ± 25 g and non-pregnant female SD rats with body mass of 280 ± 25 g were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Male and female rats were placed in the same cage overnight at a ratio of 1:1 for mating. Day 0.5 of pregnancy was defined as the day on which vaginal plugs were found. The gestational length of SD rats is 21–23 days. All pregnant rats were randomly divided into five groups that were pregnant day 10.5, 12.5, 14.5, 18.5, and 20.5. All animals which were raised at Animal Lab Center Building of Nanjing Medical University had free access to water and pelleted food throughout the study. Age-matched non-pregnant rats were used as controls. All experiments were approved by the local animal ethical committee and were in accordance with accepted standards of humane animal care.

Oral glucose tolerance test (OGTT) and insulin release test (IRT)

Non-pregnant female rats and pregnant rats at days 10.5, 12.5, 14.5, 18.5, and 20.5 (11 rats for each group) were investigated. All rats were fasted overnight for 12 h, then the following morning, blood samples were taken for determination of blood glucose and serum insulin. Rats received gastric perfusion of 50% glucose according to 2.0 g/kg body mass. Blood samples were collected before and 30, 60, and 120 min after glucose administration from the cut tip of the tail. Blood glucose was measured using a blood glucose monitor (Roche, Germany). Serum insulin concentrations were determined by ELISA technique (Rat Insulin ELISA kit; Linco, USA). Area under the glucose tolerance curve (GAUC) was calculated using the following equation: $GAUC = 0.5 \times 0$ min glucose + 30 min glucose + 1.5×60 min glucose + 120 min glucose. Area under the insulin release curve (IAUC) = 0.5×0 min insulin + 30 min insulin + 1.5×60 min insulin + 120 min insulin.

Islet isolation and culture

Rats were anesthetized with an intraperitoneal injection of 1% pentobarbitone (60 mg/kg). After laparotomy and occlusion of the pancreatic duct near the duodenum, blood was drawn. The pancreas was distended by intraductal injection of 10 ml cold collagenase V (568 U/mg, Sigma, USA) in D-Hanks medium. The distended pancreas was immediately excised and placed in a conical tube containing 5 ml of the cold collagenase solution. The cold ischemic time (4°C) was approximately 30 min. The resected pancreas was incubated in a 37–38°C water bath for 16–20 min. The pancreatic tissue was vortexed at full speed on a Vortex mixer 3 times, for 10 s each. After washing 3 times with Hanks solution supplemented with 10% newborn calf serum, the undigested fragments were carefully removed. The tissue suspension was then filtered through a 100 μm screen and washed three times with D-Hanks solution supplemented with 10% newborn calf serum. Islet purification was achieved by a 2-layer density gradient. The tissue pellet was resuspended in Histopaque-1077 (Pharmacia, USA) and placed at the bottom. D-Hanks solution was overlaid onto the bottom layer. After centrifugation at 2000 rpm for 20 min, islets were harvested from the interface between the Histopaque-1077 and D-Hanks solution layer. After washing two times, the islets were handpicked and cultured at 37°C (air/CO_2 95:5) in RPMI 1640 (GIBCO, USA) supplemented with L-glutamine (2 mmol/l), benzylpenicillin (100 U/ml), streptomycin (0.1 mg/ml), HEPES (12.5 mmol/l), and 10% fetal bovine serum, overnight to recover from the isolation, before starting the experimental procedures. Islet viability was estimated by using the trypan blue exclusion test.

Glucose-stimulated insulin secretion (GSIS) test

Islets were randomly hand-picked into Eppendorf tubes in triplicate (10 islets/tube), and preincubated in 0.5 ml basic

medium, RPMI 1640 without glucose (Sigma, USA) at 37°C (air/CO2 95:5) for 30 min. After centrifugation at 1000 rpm for 1 min, the supernatants were discarded. In the experiment, each tube was filled with 0.2 ml basic medium supplemented with 3.3 mmol/l glucose and incubated for 1 h. After centrifugation, the supernatants were harvested, and each tube was filled with 0.2 ml stimulation medium, RPMI 1640 without glucose supplemented with 16.7 mmol/l glucose. Afterward, the tubes were incubated for another hour at 37°C (air/CO_2 95:5). The supernatants were harvested after centrifugation at 1000 rpm for 1 min. The supernatant samples were stored at −80°C until insulin measurement using a Rat Insulin RIA kit (Linco, USA). Stimulation index was calculated as insulin concentration in the 16.7 mmol/l glucose stimulation samples divided by insulin concentration in the 3.3-mmol/l glucose stimulation samples.

Islet beta-cell proliferation

Rats were fasted overnight for 12 h, then the following morning were injected intraperitoneally with 5-bromodeoxyuridine (BrdU) (100 mg/kg body weight; Sigma, USA) and killed 6 h later. The pancreatic glands were carefully dissected free from surrounding tissues, removed, fixed in a 10% (vol/vol) formaldehyde solution, dehydrated, and embedded in paraffin. Sections (3 μm thick) randomly chosen from all parts of the pancreas, were prepared and mounted on poly-L-lysine glass slides. Sections were then incubated for 60 min with a monoclonal guinea pig anti-insulin antibody (DAKO, Denmark). Subsequently, they were incubated for 45 min with a peroxidase-conjugated rabbit anti-guinea pig antibody (EnVisionTM; DAKO, Denmark). Immunoreactive cells were stained by 3,30-diaminobenzidine (DAB). Afterward, sections were microwaved for 10 min at 95–98°C to repair antigens. Following cooling at room temperature, sections were stained for BrdU [5-Bromo-2′-deoxy-uridine Labeling and Detection Kit II [(Roche, Germany)] by incubating with a mouse monoclonal anti-BrdU antibody diluted in a nuclease solution (according to the kit protocol) for 45 min at 37°C and an anti-mouse Ig-alkaline phosphatase for 45 minutes at 37°C. Thereafter, sections were incubated with Buffer III solution for 5 min (0.1 M Tris–HCl solution 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). Then, they were stained with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP). On these sections, beta-cells could be identified with a brownish yellow cytosol, and BrdU positive cells appeared with purple black nuclei. Negative control sections were prepared using PBS solution instead of primary antibody. The proportion of BrdU + beta-cell nuclei to total beta-cell nuclei (proliferative index, PI) was calculated. The result represents the beta-cell replicative rate in a 6 h interval.

Measurement of islet area

Areas of insulin-positive cells were measured by Super-Image Analysis System on histological sections stained with DAB. A total of 10 different fields were viewed randomly in each section. The insulin-positive ratio (the islet beta-cell area as a fraction of the whole pancreas in a view) was also estimated.

RNA extraction and purification

Total RNA was extracted from islets isolated from eight rats, using Trizol Reagent (Roche, USA) following the manufacturers recommended procedures. The ratio of the optical densities measured at 260 and 280 nm was used to evaluate nucleic acid purity. Total RNA concentrations were determined by DNA/RNA Analyzer (GeneQuant, USA). The quality of total RNA was estimated based on the integrity of 28S and 18S rRNA. rRNA was separated using 1% agarose gel electrophoresis and good RNA quality was indicated by the 28 s rRNA banding being twice the intensity of the 18 s rRNA, without significant smearing of the rRNA bands. Samples of total RNA from approximately 2000 cultured islets were pooled for subsequent use in the GeneChip analysis, prior to which the pooled total RNA samples were purified using the manufacturers recommended procedures of the RNeasy Total RNA Mini Kit (Qiagen, Valencia, CA, USA).

Microarray analysis

Single- and double-stranded cDNA was synthesized from total RNA samples using a One-cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA). After double-stranded cDNA clean up and quality check, an in vitro transcription reaction was conducted with GeneChip IVT Labeling Kit (Affymetrix, Santa Clara, CA, USA) to produce biotin-labeled cRNA from the cDNA. The cRNA was then purified with a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and fragmented for hybridization analysis. 15 μg aliquots of the fragmented cRNA was hybridized with the Rat Genome 230 2.0 array (Affymetrix, Santa Clara, CA, USA) in a hybridization cocktail (0.05 μg/μl cRNA, 50 pM control oligonucleotide B2, 1.5 pM bioB, 5 pM bioC, 25 pM bioD, 100 pM cre, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100 mM MES, 1 M Na^+, 20 mM EDTA, 0.01% Tween 2). Hybridization was allowed to proceed overnight (16 h) at 45°C, followed by washing and staining with streptavidin–phycoerythrin (SAPE). Hybridization assay procedures, including preparation of solutions, were carried out as described in the Affymetrix GeneChip Expression Analysis Technical Manual. The distribution of fluorescent material on the

array was obtained using G2500A GeneArray Scanner (Affymetrix, Santa Clara, CA, USA). Microarray Suite (MAS) version 5.0 and GeneChip Operating Software (GCOS) supplied by Affymetrix were used to perform gene expression analysis. Only changes in gene expression that were reproducible and higher than the cut-off of twofold in three independent experiments were considered.

RT-PCR analysis

Semi-quantitative RT-PCR using specific primers was performed to confirm the differential expression of four mRNAs detected with the microarray analysis. Reverse transcription was done with 1 μg of total RNA using Rever Tra Ace and oligo(dt) 20, according to manufacturer's instructions (RT-PCR Kit; TOYOBO, Japan). RT-PCR assays were done using KOD Dash (RT-PCR Kit; TOYOBO, Japan) and 10 pmol of each primer in a master mix of 20 μl. The primer sets used in RT-PCR analysis are shown in Table 1. The number of cycles for each gene was defined after titration using 20 to 42 cycles and was within the logarithmic phase of amplification. PCR products were separated on 2% agarose gels and the band intensities were determined by digital scanning followed by quantification using KDS1D2.0 analysis software (Kodak, Japan). The results were expressed as a ratio of target to GAPDH signals. The RNAs used for RT-PCR analysis were obtained from three to four sets of experiments.

Real-time PCR analysis

Regenerating islet-derived 3 alpha (Reg3a) was amplified using the gene-specific primers: Reg3a pF: TTCATA TTTCAGGTACGAGGTGAAGA, Reg3a-rF: GCAGTAA GAACGATAAGCCTTGGA, Reg3a-Probe:CCAGAAGG CAGTGCCCTCTACACGAA. cDNA was used to perform fluorescent-based Real-time PCR quantification using the ABI Prism 7900HT LightCycler PCR apparatus (ABI, USA). The conditions for the PCR reactions were: denaturation at 95°C for 15 s, annealing and elongation at 60°C for 60 s. The data were normalized using the mRNA expression levels of the housekeeping gene beta-actin as an internal standard. The mRNA expression levels were expressed as number of copies/μg total RNA using a standard curve of Cp versus logarithm of the quantity. All samples were run in duplicates and quantification of each target gene expression was done three times. The cycle threshold (Ct) was used to calculate relative amounts of target RNA. The Cts of target gene were normalized to the levels of beta-actin as an endogenous control in each group. The average Ct for each gene is calculated by subtracting the Ct of the endogenous control RNA from that of the sample RNA for the same time measurement. This value is known as the ΔCt and reflects the relative expression of the sample compared with the control, and becomes the exponent in the calculation for amplification $2^{\Delta Ctcontrol-\Delta Ctsample}$, the equivalent to fold change in expression. ANOVA with LSD test was used for statistical analysis.

Statistical analysis

All values are given as means ± SEM. Multiple comparisons of parametric values with control values were performed by ANOVA with LSD test (SPSS10.0). For all comparisons, the difference was considered statistically significant if $P < 0.05$.

Results

Glucose homeostasis and insulin release

Compared with the non-pregnant control group, levels of fasting glucose and fasting insulin were significantly decreased, and glucose levels after glucose load were also

Table 1 Primers sequences and reaction conditions

Gene		Primers sequences	Amplicon (bp)	Annealing (°C)	Extending (s)	Cycle
GAPDH	Forward	5′-CACCCTGTTGCTGTAGCCATATTC-3'	196	59	15	26
	Reverse	5'-GACATCAAGAAGGTGGTGAAGCAG-3'				
Insulin 1	Forward	5′-CCGTCGTGAAGTGGAG-3'	156	62	20	28
	Reverse	5'-CAGTTGGTAGAGGGAGCAG-3'				
Insulin 2	Forward	5′-ATGGCCCTGTGGATCCGCTT-3'	333	58	22	21
	Reverse	5'-CTAGTTGCAGTAGTTCTCCA-3'				
PDX-1	Forward	5′-GGTGCCAGAGTTCAGTGCTAA-3'	249	59	25	28
	Reverse	5'-CCAGTCTCGGTTCCATTCG-3'				
Reg3a	Forward	5′-GCTGCTCTACTACCTGTTC-3'	253	56	30	35
	Reverse	5'-GTTGTTCACTCTGCCTGT-3'				

decreased during pregnancy in rats ($P < 0.05$). The areas under the curve for blood glucose were decreased remarkably ($P < 0.05$). Moreover, insulin secretion after glucose load and the areas under the insulin release curve were increased from day 14.5 to day 20.5 of pregnancy ($P < 0.05$) (Tables 2 and 3).

Insulin secretion after glucose stimulation

The results of glucose-stimulated insulin secretion tests in islets are shown in Fig. 1. A significant insulin release in response to stimulation at 16.7 mmol/l glucose was seen in islets isolated from pregnant rats, particularly at day 18.5, with a glucose stimulation index of 4.89 ± 1.36.

Islet beta-cell proliferation

Islet beta-cell proliferation index (Figs. 2 and 3) increased significantly at day 12.5 of pregnancy, rose continuously to a peak at day 14.5, and then gradually returned to the control level. In addition, areas of insulin-positive cells were much greater during pregnancy at day 14.5 to day 20.5, than those in non-pregnant rats (Table 4).

Micrroarray analysis

There were 31099 probes included in the Affymetrix Rat Genome 230 2.0 Array. Scanning profiles of samples were obtained from three groups including non-pregnant group (N), pregnant group at day 10.5 (P10.5) and day 14.5 (P14.5) after being hybridized with microarray. The detection rates in each group were 45.3, 38.2, and 37.2%, respectively. Comparison analyses of the expression profiles were performed between control rats and pregnant rats using GeneChip data.

The scatter plots are shown in Fig. 4. The vertical position of each gene represents its expression level in the experimental group, and the horizontal position represents its control condition. Expressions of over 500 genes were significantly altered by at least twofold at P10.5 compared with non-pregnant group, and at P14.5 expressions of over 1000 genes were altered (Table 5). The use of twofold cut-

Table 2 Changes of glucose tolerance during pregnancy in rats ($\bar{X} \pm s$)

Group	Glucose (mmol/l)				GAUC
	0 min	30 min	60 min	120 min	
Non-pregnant control	4.27 ± 0.87	7.42 ± 0.98	6.30 ± 1.08	5.00 ± 0.76	24.01 ± 3.15
Day 10.5	3.83 ± 0.42	7.12 ± 1.84	5.60 ± 0.70	4.51 ± 0.45	21.95 ± 2.44
Day 12.5	3.11 ± 0.52**	6.17 ± 0.84*	5.61 ± 0.77	3.76 ± 0.74**	19.89 ± 1.30**
Day 14.5	3.09 ± 0.36**	6.21 ± 0.83*	5.33 ± 0.69*	3.69 ± 0.87**	19.44 ± 2.09**
Day 18.5	2.57 ± 0.50**	4.99 ± 1.17**	4.43 ± 0.75**	3.43 ± 0.68**	16.35 ± 2.61**
Day 20.5	2.59 ± 0.38**	6.10 ± 0.92*	5.13 ± 0.61**	3.34 ± 0.55**	18.42 ± 1.99**

GAUC Glucose area under the curve

All values are mean ± SEM ($n = 11$ per group)

* $P < 0.05$, ** $P < 0.01$ vs. control

Table 3 Changes of insulin release during gestation in rats ($\bar{X} \pm s$)

Group	Insulin (ng/ml)				IAUC
	0 min	30 min	60 min	120 min	
Non-pregnant control	0.363 ± 0.114	0.480 ± 0.161	0.399 ± 0.081	0.381 ± 0.094	1.565 ± 0.200
Day 10.5	0.269 ± 0.117	0.496 ± 0.254	0.405 ± 0.137	0.298 ± 0.083	1.584 ± 0.600
Day 12.5	0.181 ± 0.081*	0.682 ± 0.146	0.464 ± 0.178	0.403 ± 0.096	1.877 ± 0.425
Day 14.5	0.178 ± 0.095*	0.798 ± 0.185*	0.682 ± 0.175**	0.572 ± 0.159*	2.507 ± 0.727*
Day 18.5	0.196 ± 0.113*	0.993 ± 0.113**	0.693 ± 0.217*	0.568 ± 0.175*	2.731 ± 0.451**
Day 20.5	0.296 ± 0.112	0.825 ± 0.209*	0.729 ± 0.182**	0.570 ± 0.141*	2.657 ± 0.555**

IAUC Insulin area under the curve

All values are mean ± SEM ($n = 11$ per group)

* $P < 0.05$, ** $P < 0.01$ vs. control

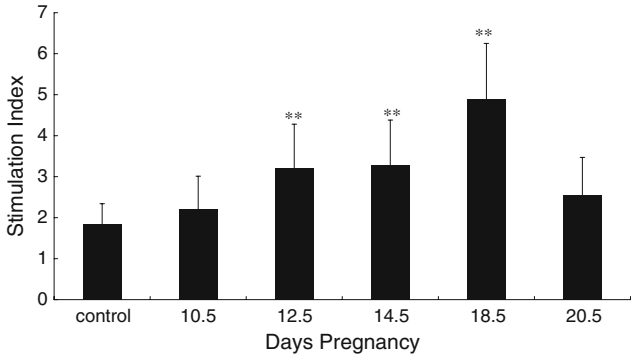

Fig. 1 Insulin secretion of islets during pregnancy in response to glucose stimulation. Stimulation index(SI) was calculated as the insulin concentration in 16.7 mmol/l glucose stimulation medium divided by the insulin concentration in 3.3 mmol/l glucose stimulation medium. Each bar represents SI expressed as mean ± SEM ($n = 8$ per group). ** $P < 0.01$ vs control

off for significance has been conventionally employed in other similar studies.

Based on known or likely functions, differentially expressed genes could be divided into eight categories: (1) genes involved in apoptosis or tumor; (2) genes related to binding; (3) genes involved in metabolism; (4) genes related to cell cycle; (5) genes for signal transducer activity; (6) genes related to structural molecule activity; (7) genes involved in transcription regulator activity; (8) genes for transporter activity. The major changes in gene expression during pregnancy are listed in Tables 6 and 7 (Table 6 only shows the top 20 of up-regulated genes. The full table is placed in an online repository that is referenced in the manuscript). Genes for which the annotation was unclear, such as expressed sequence tags (ESTs), were excluded from the table.

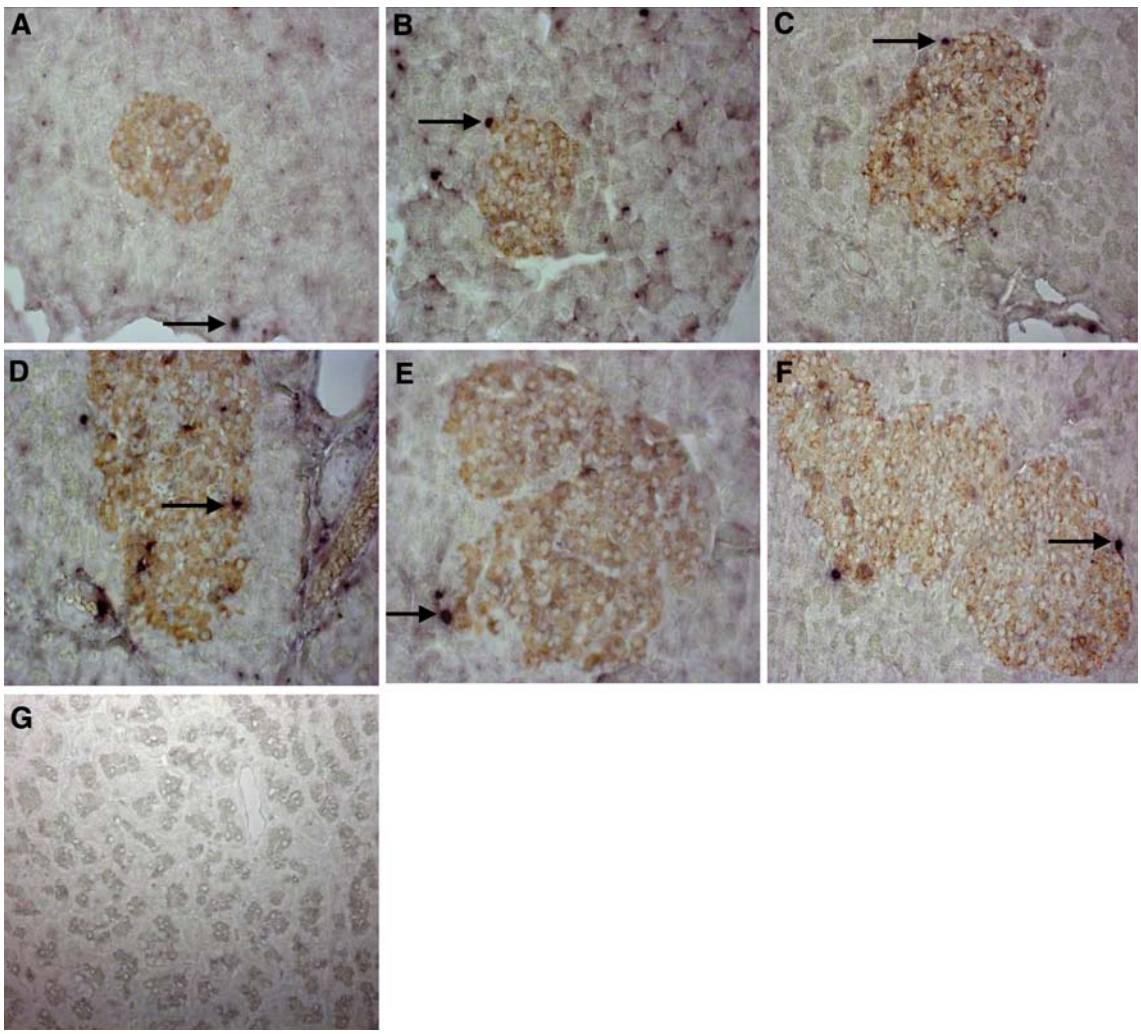

Fig. 2 Changes of pancreatic islet morphology and proliferation during pregnancy in rats. Double-staining for 5-bromodeoxyuridine(BrdU) and insulin by immunohistochemistry technique. Beta-cells showed *brownish yellow* cytosol and BrdU positive cells appeared with *purple black* nuclei (*arrowheads*). **a** Non-pregnant control; **b** Pregnant day 10.5; **c** Pregnant day 12.5; **d** Pregnant day 14.5; **e** Pregnant day 18.5; **f** Pregnant day 20.5; **g** Negative control (×400 magnification)

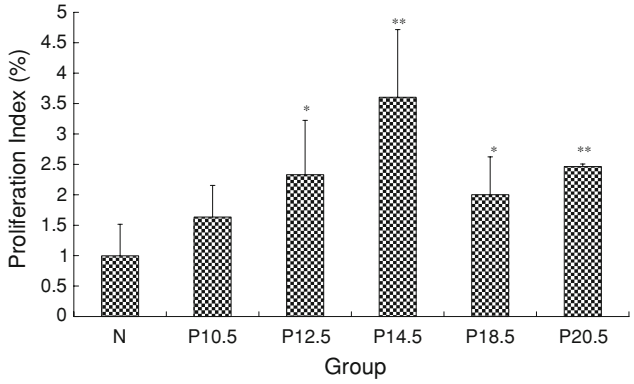

Fig. 3 Changes of pancreatic islet beta-cell mass proliferation index during pregnancy in rats. Proliferative index (PI) was calculated as the proportion of $BrdU^{+}$ beta-cell nuclei to total beta-cell nuclei. The result represents the percentage of beta-cell replicative rate in a 6 h interval. Values represent mean ± SEM of PI for samples from 6 to 8 rats per group. * $P < 0.05$, ** $P < 0.01$ vs. control

Some regulatory genes in pancreatic islets were analyzed which could be divided into several categories according to their biological functions: pancreatic-hormone-related genes (Table 8), insulin-release-related genes (Table 9), and islet proliferation related genes. Among these genes, expression of insulin 1 and insulin 2 genes was up-regulated during pregnancy, especially at day 14.5. Gene expressions of potassium inwardly rectifying channel (Kir6.2) and Rab3a were significantly decreased at day 14.5 of pregnancy compared with non-pregnant group, while T-voltage-dependent calcium channel (Cacnalh) was up-regulated during pregnancy. In addition, Reg3a was also remarkably increased.

RT-PCR and real-time PCR analysis

Semi-quantitative RT-PCR analysis was performed for four selected genes to confirm the microarray results. The selected genes and their sequences of primers are listed in Table 1. Genes analyzed by RT-PCR approximately confirmed the results obtained from the array (Table 10, Fig. 5), but there were still slight differences, particularly for Reg3a. Quantitative PCR was then performed afterward to validate its expression (Fig. 6).

Discussion

In this study, we investigated structural and functional changes of islets during pregnancy in rats. We found that levels of serum insulin were significantly increased during pregnancy compared with the non-pregnant group, which may be associated with enhanced glucose-stimulated insulin secretion, increased beta-cell proliferation, and enlarged beta-cell size. All of these adaptations are mechanisms for accommodating the increased demand for insulin.

During early pregnancy, glucose tolerance is normal or slightly improved and peripheral (muscle) sensitivity to insulin remains normal [8]. Insulin responses to oral glucose are also greater in the first trimester than before pregnancy. However, glucose tolerance and insulin release are different in middle and later pregnancy. Our study indicated that compared with non-pregnant rats, fasting glucose level was significantly decreased, whereas glucose tolerance was not impaired in pregnant rats. Pregnancy is associated with lower glucose levels because of decreased glucose uptake as a result of fasting for 12 h before OGTT and gradually enhanced glucose utilization due to diversion of energy to the fetus. Besides, urinary glucose excretion is increased because of the decreased renal glucose threshold. Insulin secretion after glucose load was also found to be increased from gestational days 14.5–20.5, which may be associated with reduced peripheral insulin sensitivity. The hyperinsulinemic–euglycemic clamp technique and computer-assisted intravenous-glucose-tolerance test indicate that insulin action in late normal pregnancy is 50–70% lower than that of non-pregnant women [9]. Our study confirmed a progressive increase in postprandial insulin concentrations with advancing pregnancy, whereas the level of basal insulin was found decreased compared with the pregravid value. Although the precise mechanism is uncertain, lower fasting glucose during pregnancy is

Table 4 Changes of islet volume and insulin content during pregnancy in rats ($\bar{X} \pm s$)

Group	Insulin-stained positive ratio (%)	Insulin positive cell area (μm^2)
Non-pregnant control	14.39 ± 11.43	11658.77 ± 10696.88
Day 10.5	22.65 ± 8.69	18973.22 ± 7146.82
Day 12.5	23.28 ± 8.93*	16002.60 ± 7215.09
Day 14.5	26.75 ± 12.74**	21267.06 ± 9831.24*
Day 18.5	27.52 ± 11.30**	20713.29 ± 11201.54*
Day 20.5	30.96 ± 15.09**	23816.55 ± 12871.75**

All values are mean ± SEM for samples from 6 to 8 rats per group

* $P < 0.05$, ** $P < 0.01$ vs. control

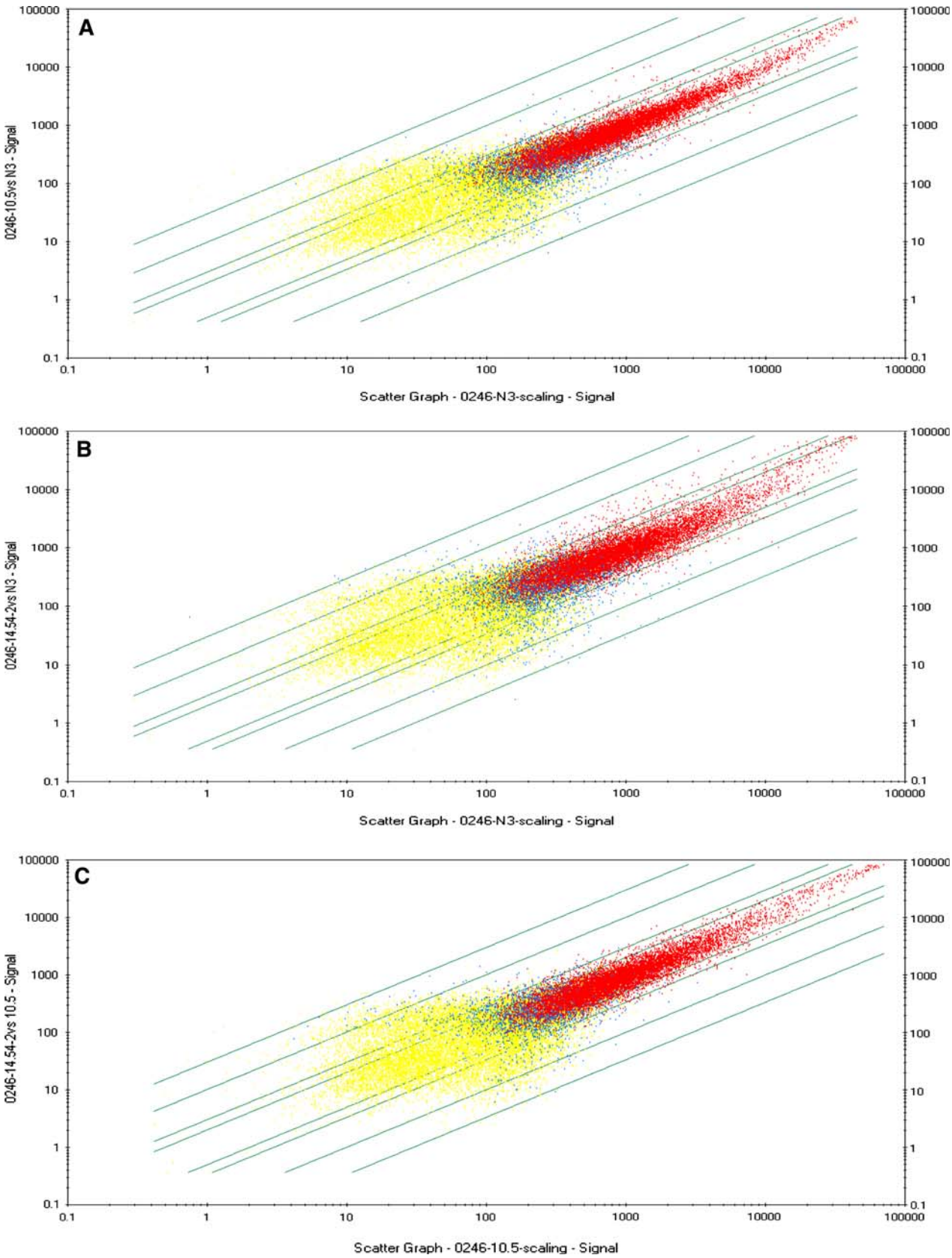

Fig. 4 Scatter plot of gene expression comparisons in three groups. **a** Pregnant day 10.5 compared with non-pregnant control; **b** pregnant day 14.5 compared with non-pregnant control; **c** pregnant day 14.5 compared with day 10.5

Table 5 Differentially expressed gene number in N,P10.5,P14.5

Group	Up-regulated	Down-regulated	Total
P10.5 vs. N	277	278	555
P14.5 vs. N	668	431	1099
P14.5 vs. P10.5	356	118	474
P14.5 and P10.5 vs. N	176	60	236

N Non-pregnant, *P10.5* Pregnant day 10.5, *P14.5* Pregnant day 14.5

probably responsible for the reduced basal insulin secretion. Only by reduced fasting insulin concentration can pregnant rats keep enough supply of glucose for both mother and fetus.

Consistent with insulin secretion in vivo, in vitro islet function stimulated by glucose was also enhanced in middle and later pregnancy, which was probably the adaptive change in order to meet maternal demand. Changes in beta-cell function occur in parallel with growth of the fetoplacental unit and its elaboration of hormones, which is believed to be the result of increased blood levels of several gestational hormones such as human chorionic somatomammotropin (HCS), progesterone, estrogens, cortisol, and prolactin (PRL) [10, 11].

It appears that in the absence of major external stimuli, the beta-cell population has only a very limited potential for regeneration. It can, however, increase in response to an augmented demand, resulting from obesity, pregnancy, or insulin resistance [12]. Islet mass was found to be increased in our study, mainly as a consequence of beta-cell hypertrophy and hyperplasia. Proliferation index (PI) of pancreatic islets was significantly increased at day 14.5 of pregnancy. In addition, areas of insulin-positive cells were much greater during pregnancy at days 14.5–20.5, than in non-pregnant rats, which has also been reported in previous studies [4, 13]. Changes of beta-cell mass may also be helpful for accommodating the increased demand for insulin. Several important genes are hypothesized to be involved in the adaptations of pancreatic islets.

To be able to increase the number of functional beta-cells would, however, be advantageous in several clinical settings such as clinical islet transplantation and new treatments for type 2 diabetes. Until now, regulatory genes which may be related to islet regeneration were still incompletely understood. Therefore, we chose to study the endocrine pancreas of pregnant rats, in which adaptive changes, especially a highly reproducible physiological proliferation of pancreatic islet beta-cells, occur. Genechips from Affymetrix were applied to explore gene expression profiles of islets from pregnant day 10.5, when changes took place (but with no significance), and day 14.5, when adaptation, in particular PI, rose to a peak. Compared with the non-pregnant group, expression of several hundred islet genes was changed during pregnancy, particularly at day 14.5. The most significantly induced genes were those involved in binding and metabolism. Other notably induced genes were involved in apoptosis or tumor, cell cycle and structural molecule activity, transcription regulator activity, and transporter activity. The most significantly suppressed genes were those related to binding, apoptosis, and signal transducer activity. These differentially expressed genes may play an important role in adaptation of pancreatic islets during pregnancy in rats.

Our results suggest that the expression of insulin 1 and insulin 2 genes was up-regulated during pregnancy at day 14.5, which may contribute to enhanced insulin secretion. We also focused on the insulin-release-related genes that may exert a predominant effect on serum insulin concentration. Among these genes, Kir6.2 and Rab3a were significantly decreased at day 14.5 of pregnancy compared with non-pregnant group, while Cacnalh was increased during pregnancy. The beta-cell is equipped with at least six voltage-gated Ca^{2+} (CaV) channel alpha1-subunits designated CaV1.2, CaV1.3, CaV2.1, CaV2.2, CaV2.3, and CaV3.1. These principal subunits, together with certain auxiliary subunits, assemble into different types of voltage-dependent calcium channels (VDCCs) conducting L-, P/Q-, N-, R-, and T-type Ca^{2+} currents, respectively [14, 15]. Decreased gene expression of Kir6.2 and increased expression of Cacnalh at day 14.5 of pregnancy may be conducive to inhibition of the ATP-sensitive K^+ channels and activation of VDCCs in beta-cells. This process results in Ca^{2+} influx. The rise in intracellular Ca^{2+} concentrations triggers the exocytosis of insulin secretory granules and the release of insulin into the extracellular space and into the circulation. However, L-voltage-dependent calcium channel (Cacnald) which was considered to have a key effect on insulin secretion was unchanged during gestation. Therefore, we hypothesize that Cacnalh may play a critical role in the enhanced insulin secretion seen in pregnant rats. Previous studies have indicated that the GTP-binding protein, Rab3a, plays a mechanistic role in insulin secretion. Rab3a, which was localized on the plasma membrane of beta-cells was found to reverse the enhancing effect of Rim (Rab3a-interacting molecule) on Ca^{2+}-stimulated insulin exocytosis [16]. Therefore, the decreased expression of Rab3a at pregnant day 14.5 found in our study could indicate an enhanced insulin secretion in pregnancy.

It was also shown that a member of the regenerating gene (Reg) family, Reg3a was significantly up-regulated in islets during pregnancy, especially when islet cells were undergoing regeneration. This finding was subsequently confirmed by RT-PCR and Real-time PCR, in which Reg3a mRNA levels were increased more than 2-fold, which

Table 6 List of up-regulated genes in pancreatic islets during pregnancy

Category or functions	Genbank ID	Gene descriptions	Gene symbol	Signal log ratio (P10.5 vs. N)	Fold change (P10.5 vs. N)	Signal log ratio (P14.5 vs. N)	Fold change (P14.5 vs. N)
Apoptosis or tumor	AF411318	Metallothionein	Mt1a	3.6	12.126	3.4	10.556
	NM_012674	Serine protease inhibitor, Kazal type 1	Spink1	1.7	3.249	3	8
Binding	X15679	Preprotrypsinogen IV	RGD:70 8585	2.7	6.498	3.2	9.19
	AA893484	Fibronectin 1	Fn1	1.5	2.828	2.6	6.063
	L10229	Regenerating islet-derived 3 alpha	Reg3a	1.4	2.639	0.7	1.625
Metabolism	NM_017074	CTL target antigen	Cth	1.7	3.249	2.8	6.964
	NM_053840	Gamma-glutamyltransferase 1	Ggt1	1.8	3.482	2.6	6.063
Cell cycle	BI288619	v-jun sarcoma virus 17 oncogene homolog (avian)	Jun	1	2	2.7	6.498
	NM_012842	Epidermal growth factor	Egf	1	2	1.7	3.249
Enzyme regulator activity	NM_023103	Alpha-1-inhibitor III///	LOC297				
		Murinoglobulin 1 homolog (mouse)	568///	3.1	8.574	4.4	21.112
			Mug1				
	NM_133612	Serine (or cysteine) proteinase inhibitor, clade I, member 2	Serpini2	1.1	2.144	1.8	3.482
Signal transducer activity	BF417079	Prostaglandin E receptor 3 (subtype EP3)	Ptger3	1.8	3.482	2.7	6.498
	NM_031523	Nerve growth factor, gamma	Ngfg	1.1	2.144	1.8	3.482
	U76206	G protein-coupled receptor 105	Gpr105	1.1	2.144	1.6	3.031
Structural molecule activity	NM_053844	Trefoil factor 2 (spasmolytic protein 1)	Tff2	1.3	2.462	2	4
	BI275716	Collagen, type III, alpha 1	Col3a1	2	4	1.1	2.144
Transcription regulator activity	NM_053964	Pancreas specific transcription factor, 1a	Ptf1a	2	4	2.4	5.278
	NM_053611	Nuclear protein 1	Nupr1	1.3	2.462	2	4
Transporter activity	AF231010	Purinergic receptor P2X, ligand-gated ion channel, 1	P2rx1	1.1	2.144	2.4	5.278
	AI179953	Gap junction membrane channel protein beta 2	Gjb2	1.2	2.297	2.1	4.287

N Non-pregnant, *P10.5* pregnant day 10.5, *P14.5* pregnant day 14.5, fold change=$2^{\text{Signal Log Ratio}}$

Table 7 List of down-regulated genes in pancreatic islets during pregnancy

Category or functions	Genbank ID	Gene descriptions	Gene symbol	Signal log ratio (P10.5 vs. N)	Fold change (P10.5 vs. N)	Signal log ratio (P14.5 vs. N)	Fold change (P14.5 vs. N)
Apoptosis or tumor	NM_013069	CD74 antigen (invariant polypeptide of major histocompatibility class II antigen-associated)	Cd74	−1	0.5	−1.6	0.33
Binding	BG371810	Chloride intracellular channel 1	Clic1	−4.3	0.051	−1.2	0.435
	NM_019161	Cadherin 22	Cdh22	−1.1	0.467	−1.4	0.379
	NM_130411	Coronin, actin binding protein 1A	Coro1a	−1.3	0.406	−2.2	0.218
	NM_133533	CD79B antigen	Cd79b	−2.6	0.165	−4.1	0.058
Metabolism	NM_017073	Glutamine synthetase 1	Glul	−1.1	0.467	−1.1	0.467
	NM_057188	Guanosine monophosphate reductase	Gmpr	−1.2	0.435	−1.6	0.33
	NM_080767	Proteosome (prosome, macropain) subunit, beta type 8	Psmb8	−1	0.5	−2.2	0.218
Cell cycle	NM_133589	Protein phosphatase V	Ppp6c	−1.3	0.406	−3	0.125
Enzyme regulator activity	AI535169	Rap2 interacting protein	Rap2ip	−1.2	0.435	−1.3	0.406
Signal transducer activity	AF155236	Mitogen activated protein kinase 3	Mapk3	−1.6	0.33	−1	0.5
Transcription regulator activity	NM_017339	ISL1 transcription factor, LIM/ homeodomain 1	Isl1	−1.1	0.467	−1.8	0.287
Transporter activity	AF021923	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	Slc24a2	−1.3	0.406	−1.1	0.467
	AA858962	Retinol binding protein 4, plasma	Rbp4	−1.3	0.406	−1.9	0.268

N Non-pregnant, *P10.5* pregnant day 10.5, *P14.5* pregnant day 14.5, fold change=$2^{\text{Signal Log Ratio}}$

Table 8 Expressions of pancreatic hormone related genes

Probe set ID	Gene title	Detection (N/P10.5/P14.5)	Fold change
1387815_at (NM_019129)	Insulin 1///insulin 2 (Ins1///Ins2)	PPP	**10.5 vs. N 1.625**
			14.5 vs. N 2
1370077_at (NM_019130)	Insulin 2 (Ins2)	PPP	**10.5 vs. N 1.625**
			14.5 vs. N 2
1387660_at (M25390)	Islet amyloid polypeptide (Iapp)	PPP	–
1369888_at (NM_012707)	Glucagon (Gcg)	PPP	–
1367762_at (NM_012659)	Somatostatin (Sst)	PPP	–
1369196_at (NM_012626)	Pancreatic polypeptide (Ppy)	PPP	–

suggests a potential role for Reg3a in beta-cell proliferation in pregnant rats. Reg, first isolated from a regenerating islet-derived cDNA library in the rat, encodes a secretory protein with a growth stimulating effect on pancreatic beta-cells. Reg and Reg-related genes have been revealed to constitute a multigene family, the Reg family, which consists of four subtypes (types 1, 2, 3, and 4) based on the primary structures of the encoded proteins of the genes [17–21]. While several Reg proteins (Reg1 and islet neogenesis-associated protein, i.e., INGAP) have been linked temporally and spatially with the induction of islet neogenesis in vitro and in animal models of diabetes and partial pancreatectomy [22–28], the roles of other Reg members have been shown to be different. Among them, Reg2 was recently proposed to serve as an autoantigen on beta-cells that elicits T-cell attack in type 1 diabetes

Table 9 Expressions of insulin release related genes

Probe set ID	Gene title	Detection (N/P10.5/P14.5)	Fold change
1387698_at (AB043638)	Potassium inwardly rectifying channel,	PPP	**14.5 vs. N 0.354**
1391007_s_at (BE113377)	subfamily J, member 11 (KIR6.2)		**14.5 vs. 10.5 0.5**
1371005_at (AI059506)	ATP-binding cassette, subfamily C (CFTR/MRP), member 1(SUR)	PPP	–
1383458_at (BF403759)	Calcium channel, voltage dependent, L type, alpha 1D subunit (Cacna1d)	PPP	**–**
1379197_at (BF414167)	Calcium channel, voltage dependent, T type, alpha 1H subunit (Cacna1h)	AAP	**14.5 vs N 2.297** **14.5 vs 10.5 2.297**
1388556_at (BG381589)	Syntaxin 6 (Stx6)	PPP	–
1369974_at (NM_012663)	Vesicle-associated membrane protein 2(Vamp2)	PPP	–
1387073_at (NM_030991)	Synaptosomal-associated protein 25 (Snap25)	PPP	–
1369414_at (NM_053637)	Syntaxin binding protein 3 (Munc18-c)	PPP	–
1369330_at (NM_022861)	Unc-13 homolog A (Munc13-1)	AAA	–
1369816_at (NM_013018)	RAB3A, member RAS oncogene family (Rab3a)	PPP	**14.5 vs N 0.435**
1391434_at (BF389910)			
1369332_a_at (NM_052829)	Regulating synaptic membrane exocytosis 1(RIMS1)	AAA	-
1369754_a_at (NM_053295)			
1373466_at (AA946474)	Calpastatin (Cast)	PPP	-
1370686_at (U32575)			
1374656_at (AA851443)	SEC6-like 1 (Sec6l1)	PPP	-
1375877_at (AI454612)	Synaptotagmin 4 (Syt4)	PPP	-
1375182_at (BE109671)	Similar to RIKEN Cdna D030028O16 (Nbat)	PPP	-
1369690_at (AI547471)	N-ethylmaleimide sensitive fusion protein (Nsf)	PPP	-
1369813_at (U39320)			
1371666_at (BM385852)	Cysteine string protein (Dnajc5)	PPP	-
1370257_at (AI234860)	Phospholipase A2, group IB(Pla2g1b)	PPP	-
1387228_at (NM_012879)	Solute carrier family 2 (facilitated glucose transporter), member 2 (GLUT2)	PPP	-

N Non-pregnant, *P* present, *A* absent

Table 10 Gene expressions of Insulin 1, Insulin 2, PDX-1, and Reg3a during pregnancy in rats (signals of genechips)

Gene title	Probe set ID	N	P10.5	P14.5
Insulin 1	1387815_at	P(31512.7)	P(52634.9)	P(66468.3)
Insulin 2	1370077_at	P(24289)	P(41681.4)	P(48483.7)
PDX-1	1369516_at	P(1300.30)	P(1880)	P(973.5)
Reg3a	1387930_at	P(11127)	P(28325.3)	P(16675)

N Non-pregnant, *P10.5* pregnant day 10.5, *P14.5* pregnant day 14.5, *P* present

mellitus [29]. Reg3a, which was not yet directly known to be implicated in the islet changes of pregnant rat, was significantly increased under conditions of islet overgrowth and regeneration during pregnancy. These findings may indicate a possible correlation between Reg3a and islet cell regeneration. Furthermore, the Reg family expression pattern is distinct. Endogenous Reg3δ predominates in the exocrine pancreas and is not expressed in normal islets [30].Recently, Gurr et al. confirmed the absence of Reg3δ in normal islets and identified the expression of Reg2 and Reg3a in the mature islet. However, Reg3a was found present in the non-beta-cell portion of mouse islets, while Reg2 was predominantly expressed in beta-cells [29]. For the differences of gene expressions between rat and mouse, the detection of individual Reg members in pregnant rat islets still need to be done. Elucidation of their actions may lead to the development of novel strategies aimed at promoting the survival and function of the pancreatic islets, therefore, may play a significant therapeutic role in diabetes.

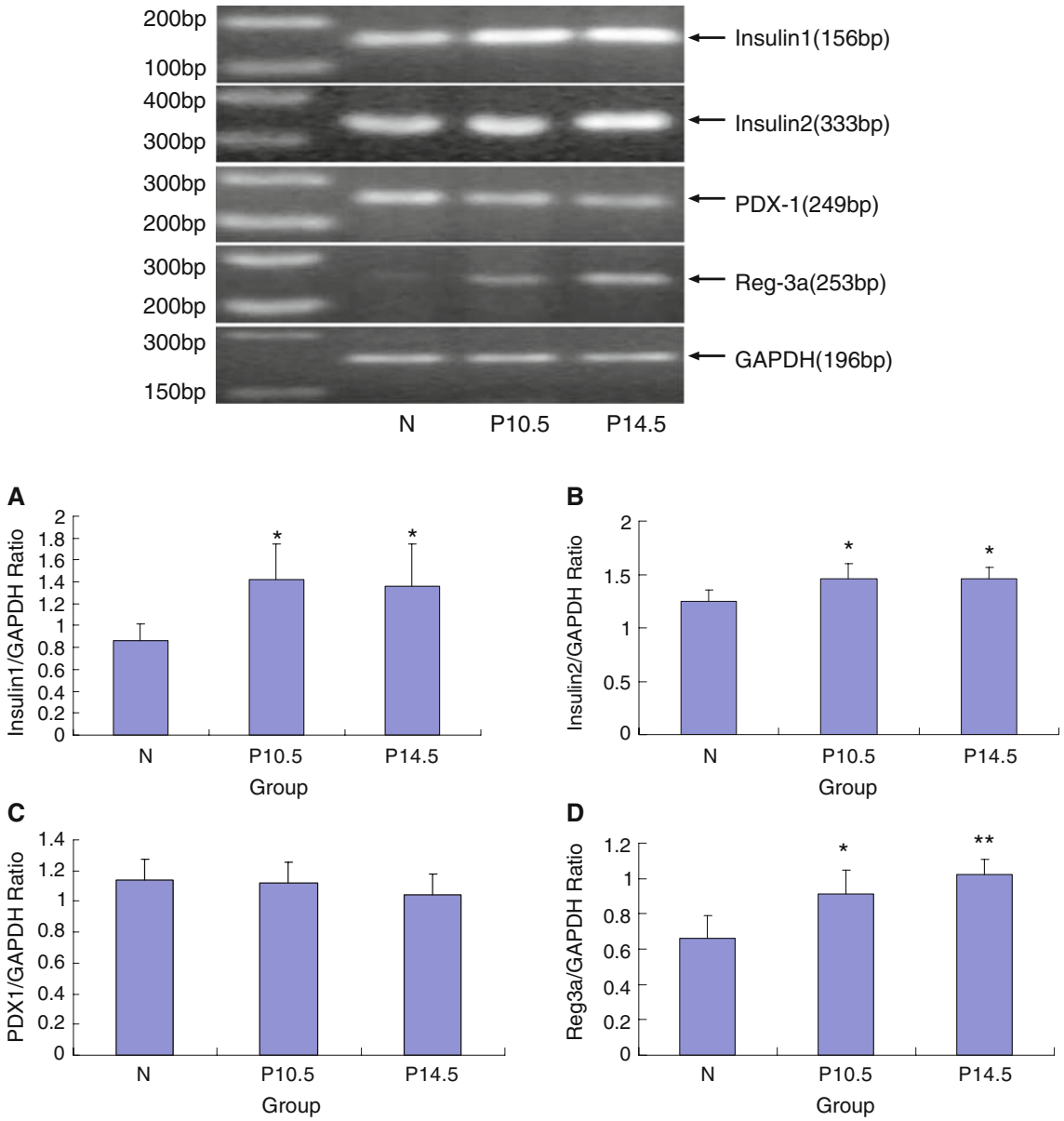

Fig. 5 Relative quantitation of GAPDH, Insulin 1, Insulin 2, PDX-1, and Reg3a mRNA from non-pregnant, pregnant day 10.5, pregnant day 14.5 rat islets (Semi-quantitative RT-PCR analysis). Multiple comparisons of parametric values with control values were performed by ANOVA with LSD test comparing day 10.5 to non-pregnant control and day 14.5 to non-pregnant. Samples were from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control. (A) Gene expression of insulin 1 was up-regulated during pregnancy at days 10.5 and 14.5. (B) Insulin 2 expression was increased during pregnancy. (C) PDX-1 level was unchanged at pregnant days 10.5 and 14.5. (D)Gene expression of Reg3a was significantly up-regulated during pregnancy, especially at day 14.5

In summary, we found that during pregnancy, pancreatic islets underwent several structural and functional changes in response to increased peripheral resistance to insulin, including : (1) augmented insulin synthesis; (2) enhanced glucose-stimulated insulin secretion; (3) increased beta-cell proliferation and enlarged islet size. Meanwhile, expressions of several hundred islet genes were changed during pregnancy, particularly at day 14.5. These differentially expressed genes may play an important role in the adaptations of pancreatic islets. For example, increased expression of genes involved in insulin release may be responsible for enhanced insulin secretion during pregnancy. In addition, we identified that gene expression of Reg3a was markedly increased during pregnancy at day 14.5, which may be related to islet regeneration. However, it is not known whether Reg3a is a potential candidate for islet regeneration therapy for diabetes. Further research on Reg3a protein still needs to be done.

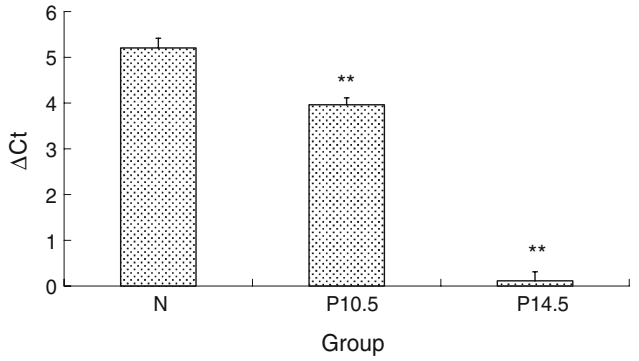

Fig. 6 Relative quantitation of Reg3a mRNA from non-pregnant, pregnant day 10.5, pregnant day 14.5 rat islets (Real-time PCR). Data are shown as the mean (±SEM) ΔCt. It is calculated by subtracting the Ct of the endogenous control RNA from that of the sample RNA for the same time measurement. ANOVA tests were run comparing day 10.5 to non-pregnant group and day 14.5 to non-pregnant. Three independent experiments were analyzed. Gene expression of Reg3a was significantly up-regulated during pregnancy, especially at day 14.5. ** $P < 0.01$ vs. control

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References

1. F.B. Nancy, Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. Am. J. Clin. Nutr. **71**(5 Suppl), 1256–1261 (2000)
2. B. Blondeau, A. Garofano, P. Czernichow, B. Breant, Age dependent inability of the endocrine pancreas to adapt to pregnancy: a long-term consequence of perinatal malnutrition in the rat. Endocrinology **140**, 4208–4213 (1999)
3. F.A. Van Assche, L. Aerts, F. De Prins, A morphological study of the endocrine pancreas in human pregnancy. Br. J. Obstet. Gynaecol. **85**, 818–820 (1978)
4. R.L. Sorenson, T.C. Brelje, Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm. Metab. Res. **29**, 301–307 (1997)
5. C. Bole-Feysot, V. Goffin, M. Edery, N. Binart, P.A. Kelly, Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr. Rev. **19**, 225–268 (1998)
6. S.P. Cousin, S.R. Hügl, M.G. Myers, M.F. White, A. Reifel-Miller, C.J. Rhodes, Stimulation of pancreatic beta-cell proliferation by growth hormone is glucose-dependent: signal transduction via janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) with no crosstalk to insulin receptor substrate-mediated mitogenic signalling. Biochem. J. **344**(3), 649–658 (1999)
7. M.E. Freeman, B. Kanyicska, A. Lerant, G. Nagy, Prolactin : structure, function, and regulation of secretion. Physiol. Rev. **80**, 1523–1631 (2000)
8. P.M. Catalano, E.D. Tyzbir, R.R. Wolfe, J. Calles, N.M. Roman, S.B. Amini, E.A. Sims, Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes. Am. J. Physiol. **264**, E60–E67 (1993)
9. P.M. Catalano, E.D. Tyzbir, N.M. Roman, Longitudinal changes in insulin release and insulin resistance in non-obese pregnant women. Am. J. Obstet. Gynecol. **165**, 1667–1672 (1991)
10. E. Sivan, X. Chen, C.J. Homko, E.A. Reece, G. Boden, Longitudinal study of carbohydrate metabolism in healthy obese pregnant women. Diabetes Care **20**, 1470–1475 (1997)
11. J.A. Parsons, T.C. Brelje, R.L. Sorenson, Adaptation of islets of langerhans to pregnancy: increased islets cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology **130**, 1459–1466 (1992)
12. S. Bonner-Weir, Perspective: postnatal pancreatic beta cell growth. Endocrinology **141**, 1926–1929 (2000)
13. M. Johansson, G. Mattsson, A. Andersson, L. Jansson, P.O. Carlsson, Islet endothelial cells and pancreatic beta-cell proliferation: studies in vitro and during pregnancy in adult rats. Endocrinology **147**(5), 2315–2324 (2006)
14. S.N. Yang, P.O. Berggren, The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology. Endocr. Rev. **27**(6), 621–676 (2006)
15. S.N. Yang, P.O. Berggren, Beta-cell CaV channel regulation in physiology and pathophysiology. Am. J. Physiol. Endocrinol. Metab. **288**(1), E16–E28 (2005)
16. M. Iezzi, R. Regazzi, C.B. Wollheim, The Rab3-interacting molecule RIM is expressed in pancreatic beta-cells and is implicated in insulin exocytosis. FEBS Lett. **474**(1), 66–70 (2000)
17. K. Terazono, H. Yamamoto, S. Takasawa, K. Shiga, Y. Yonemura, Y. Tochino, H. Okamoto, A novel gene activated in regenerating islets. J. Biol. Chem. **263**, 2111–2114 (1988)
18. T. Watanabe, H. Yonekura, K. Terazono, H. Yamamoto, H. Okamoto, Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues. The reg protein, pancreatic stone protein, and pancreatic thread protein are one and the same product of the gene. J. Biol. Chem. **265**, 7432–7439 (1990)
19. M. Unno, H. Yonekura, K. Nakagawara, T. Watanabe, H. Miyashita, S. Moriizumi, H. Okamoto, T. Itoh, H. Teraoka, Structure, chromosomal localization, and expression of mouse reg genes, reg I and reg II. A novel type of reg gene, reg II, exists in the mouse genome. J. Biol. Chem. **268**(21), 15974–15982 (1993)
20. H. Okamoto, S. Takasawa, Recent advances in the Okamoto Model: The CD38-Cyclic ADP-Ribose signal system and the regenerating gene protein (Reg)-Reg receptor system in ß-cells. Diabetes **51**(90003), S462–S473 (2002)
21. K. Nata, Y. Liu, L. Xu, T. Ikeda, T. Akiyama, N. Noguchi, S. Kawaguchi, A. Yamauchi, I. Takahashi, N.J. Shervani, T. Onogawa, S. Takasawa, H. Okamoto, Molecular cloning, expression and chromosomal localization of a novel human REG family gene, REG III. Gene **340**(1), 161–170 (2004)
22. M.E. Zenilman, T.H. Magnuson, K. Swinson, J. Egan, R. Perfetti, A.R. Shuldiner, Pancreatic thread protein is mitogenic to pancreatic-derived cells in culture. Gastroenterology **110**, 1208–1214 (1996)
23. H. Okamoto, The Reg gene family and Reg proteins: with special attention to the regeneration of pancreatic beta-cells. J. Hepatobiliary Pancreat. Surg. **6**(3), 254–262 (1999)
24. T. Yamaoka, K. Yoshino, T. Yamada, C. Idehara, M.O. Hoque, M. Moritani, K. Yoshimoto, J. Hata, M. Itakura, Diabetes and tumor formation in transgenic mice expressing Reg I. Biochem. Biophys. Res. Commun. **278**, 368–376 (2000)
25. L. Rosenberg, M. Lipsett, J.W. Yoon, M. Prentki, R. Wang, H.S. Jun, G.L. Pittenger, D. Taylor-Fishwick, A.I. Vinik, A pentadecapeptide fragment of islet neogenesis-associated protein increases beta-cell mass and reverses diabetes in C57BL/6 J mice. Ann. Surg. **240**(5), 875–884 (2004)
26. M. Lipsett, S. Hanley, M. Castellarin, E. Austin, W.L. Suarez-Pinzon, A. Rabinovitch, L. Rosenberg, The role of islet

neogenesis-associated protein (INGAP) in islet neogenesis. Cell Biochem. Biophys. **48**(2–3), 127–137 (2007)
27. S. Hanley, L. Rosenberg, Islet-derived progenitors as a source of in vitro islet regeneration. Methods Mol. Biol. **482**, 371–385 (2009)
28. L. Song, D.A. Taylor-Fishwick, I. Park, Chronological expression of INGAP (islet neogenesis associated protein) and clusterin during pancreas regeneration. Diabetes **54**, A650 (2005)
29. W. Gurr, M. Shaw, Y. Li, R. Sherwin, RegII is a beta-cell protein and autoantigen in diabetes of NOD mice. Diabetes **56**(1), 34–40 (2007)
30. K. Terazono, Y. Uchiyama, M. Ide, T. Watanabe, H. Yonekura, H. Yamamoto, H. Okamoto, Expression of reg protein in rat regenerating islets and its colocalization with insulin in the beta cell secretory granules. Diabetologia **33**, 250–252 (1990)