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ORIGINAL ARTICLE

Molecular mechanism of pancreatic β-cell adaptive proliferation: studies during pregnancy in rats and in vitro

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Abstract There is a widespread interest in defining factors and mechanisms that stimulate proliferation of pancreatic islet β-cells. Pregnancy is a special periods when the pancreatic islet display a highly reproducible physiological proliferation. However, the molecular mechanism of β-cell proliferation during pregnancy is unclear. Here, we used cDNA expression array to explore gene expression profiles of islet at various stages of pregnancy in rats. Differentially expressed genes related to islet proliferation were screened by bioinformatics methods, and further verified by real-time PCR, RT-PCR, and Western blotting. Compared with control group, expressions of hundreds of genes were changed during pregnancy. The differentially expressed genes related to islet proliferation were mainly distributed in three groups: genes involved in transcription regulator activity, genes involved in apoptosis or tumor, and genes for Wnt signaling pathway. Among these genes, expressions of Nupr1, Atf3, Btg2, β-catenin, and c-Myc mRNA were up-regulated during pregnancy. A prominent expression of Nupr1 and Atf3 protein was observed in islets on day 10.5 of pregnancy, i.e., with earlier time phases than proliferation peak. Moreover, we found that prolactin (PRL) can increase the proliferation of β-cell in vitro, which is accompanied by up-regulation of Atf3 and Nupr1, indicating that they may play a crucial role in PRL-induced pancreatic β-cell growth. In conclusion, our results suggest that the transcription factor Nupr1, Atf3, and Wnt pathway may play an important role in adaptive proliferation of pancreatic islets during pregnancy in rats.

Keywords Pregnancy · Pancreatic islet β-cell · Proliferation · Gene chips

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Introduction

Absolute or relative deficiency of pancreatic islet β-cells underlies the pathogenesis of both type 1 and type 2 diabetes [1, 2]. Thus, a primary goal for diabetes research is to develop ways to increase the number of functional β-cells [3].

In the past, most authors believed that the mature, differentiated β-cell was unable to replicate, or did so only very rarely. Since Swenne [4] reported that β-cell could replicate, a number of groups have begun to focus on this field. Evidence suggests that β-cell replication, rather than stem-cell differentiation, is the primary mechanism for maintaining postnatal β-cell mass [5, 6]. Although the rate of baseline replication is very slow, under certain conditions, it can be stimulated in vitro and in vivo. For example, glucose infusion, partial or subtotal pancreatectomy, obesity, gestation and the neonatal periods, are all associated with increased rate of β-cell proliferation. Until recently, the cellular and molecular mechanisms through which β-cell replication is controlled remain incompletely understood. It is the goal of this study to elucidate molecular mechanism of pancreatic islet β-cell proliferation during pregnancy in rats.

There is a physiological insulin resistance in pregnant maternal [7, 8]. Islets undergo major structural and functional changes such as enhancement of glucose-stimulated insulin secretion, increased β-cell proliferation and islet volume, to maintain normal glucose levels [9, 10]. We have determined that pancreatic islet proliferation is considerably increased in middle and late pregnancy, with a peak at day 14.5 of pregnancy in rats, by double-stained for 5-bromodeoxyuridine (BrdU) and insulin [11]. Therefore, pregnancy is a good physiological model to study the molecular mechanisms of islet β-cell proliferation. In this report, we used microarray to analyze gene expression profiles in pancreatic islet during pregnancy in rats. Data, especially genes related to islet proliferation, were analyzed by bioinformatics methods and then verified by real-time PCR, RT-PCR and Western blotting. Furthermore, we studied proliferation of INS-1 cells under the treatment of prolactin in vitro.

Results

Gene expression profiles in pancreatic islets of pregnant SD rats

As a first step to understand the molecular mechanism underlying islets adaptative proliferation during pregnancy, we compared the genomic profiles of islets of pregnant rats at days 10.5 and 14.5, at which time proliferation increased to a peak, and age-matched virgin rats as control. The cDNA arrays consist of 31,099 rat genes. RNA was prepared from the islets of pregnant rats and age-matched virgin rats. Only changes in gene expression higher than 2-fold were selected for further analysis.

We found 277 genes up-regulated and 278 genes down-regulated in P10.5 compared with control, and for P14.5 668 genes were up-regulated and 431 genes were down-regulated. There were 176 genes up-regulated and 60 genes down-regulated both in P10.5 and P14.5, compared with control.

Functional classification of microarray outliers

Clustering based on known or likely functions indicated that major categories of the significantly up- or down-expressed genes included: (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, and (8) genes for transporter activity.

Some interesting regulatory genes which might be related to islets proliferation were analyzed. These genes could be divided into several categories according to their biological functions: (1) genes involved in transcription regulator activity (Table 1), (2) genes involved in apoptosis or tumor (Table 2), and (3) genes for Wnt signaling pathway (Table 3). Among them, Nupr1, Atf3, Btg2, β-catenin, and c-Myc were chosen for further study by bioinformatics methods and database analysis.

RT-PCR and real-time PCR analyses

Semi-quantitative RT-PCR and quantitative real-time PCR analyses were done for five selected genes to confirm the microarray results. According to the gene chip, the signals of selected genes were all increased during pregnancy. Genes analyzed by RT-PCR confirmed the results obtained in the array, but there were still slight differences. Quantitative PCR was then performed afterward to validate the expression.

Both semi-quantitative and quantitative PCR suggested that the expression of Atf3, Nupr1, and Btg2 mRNA were up-regulated during pregnancy, with a peak at day 10.5 except for Atf3 which decreased to control level at day 14.5. The expression of β-catenin and c-Myc were also increased during pregnancy, with a peak at day 14.5 (Fig. 1; Tables 4, 5).

Western blotting analysis

Although PCR was done, we validated the expression of two transcripts (Atf3 and Nupr1) by Western blotting for the corresponding proteins. In control group, Atf3 was expressed at a stable low level, while Nupr1 almost showed no expression. In pregnant groups, the results were confirmed with mRNA verified by PCR, with an expression peak at day 10.5 (Fig. 2).

Effect of PRL on INS-1 cell proliferation

We further examined the effect of lactogens (PRL) on β-cell proliferation in vitro. INS-1 cells were treated with different concentrations of PRL for 24 h. As measured by the MTT assay, PRL did not significantly alter cell proliferation at low level (10 ng/ml). With a range of 50–1000 ng/ml PRL culture, there was a significant dose-dependent increase in INS-1 cell proliferation compared with controls, with a peak at 500 ng/ml (Fig. 3). Thus, these results indicate that lactogens such as PRL can indeed promote β-cells proliferation in vitro, and that INS-1 cells may serve as a useful model system for studying intracellular signaling pathways and molecular mechanisms mediating the pro-replication effect of lactogens in β-cells.

Table 1 Expressions of genes involved in transcription regulator activity

Genebank ID	Gene title	Detection (N/P10.5/P14.5)	Change
113900	Nuclear protein 1 (Nupr1)	PPP	P10.5 vs. N ↑2.46
			P14.5 vs. N ↑4.00
25389	Activating transcription factor 3 (Atf3)	APP	P14.5 vs. N ↑5.66
114490	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (Cited2)	PPP	P10.5 vs. N ↑2.30
			P14.5 vs. N ↑4.00
81673	General transcription factor IIB (Gtf2b)	PPP	P14.5 vs. N ↑2.00
171361	Eukaryotic translation elongation factor 1 alpha 1 (Eef1a1)	PPP	P14.5 vs. N ↑2.14
25231	One cut domain, family member 1 (Onecut1, Hnf6)	PPP	P14.5 vs. N ↑2.64
29535	Pancreatic and duodenal homeobox gene 1 (Pdx1)	PPP	–
65193	NK6 transcription factor related, locus 1 (Drosophila) (Nkx6.1)	PPP	P10.5 vs. N ↓0.81
			P14.5 vs. N ↓0.44
366214	NK2 transcription factor related, locus 2 (Drosophila) (predicted) (Nkx2.2)	PPP	P10.5 vs. N ↓0.71
			P14.5 vs. N ↓0.47
25509	Paired box gene 6 (Pax6)	PPP	–
83630	Paired box gene 4 (Pax4)	AAA	–
29458	Neurogenic differentiation 1 (Neurod1)	PPP	–
24817	Transcription factor 1 (Tcf1/Hnf1a)	AAA	–
25735	Hepatocyte nuclear factor 4, alpha (Hnf4a)	AAA	–
25099	Forkhead box A1 (Foxa2)	PPP	–

N normal, *P10.5* pregnant day 10.5, *P14.5* pregnant day 14.5, *P* present, *A* absent, ↑ up-regulated, ↓ down-regulated

Table 2 Expressions of genes involved in apoptosis or tumor

Genebank ID	Gene title	Detection (N/P10.5/P14.5)	Change
24887	Bcl2-associated X protein (Bax)	PAM	–
64639	Bcl-associated death promoter (Bad)	AAA	–
116502	Bcl-antagonist/killer 1 (Bak1)	AAA	–
64625	BH3 interacting domain death agonist (Bid)	PAA	–
78963	Apoptotic peptidase activating factor 1 (Apaf1)	PPP	–
266610	Fas (TNFRSF6)-associated via death domain (Fadd)	AAA	–
246756	TNFRSF1A-associated via death domain (Tradd)	AAA	–
25402	Caspase3	AAA	–
64026	Caspase7	PPA	–
58918	Caspase9	PPP	–
24224	B-cell leukemia/lymphoma 2 (Bcl2)	AAA	–
29619	B-cell translocation gene 2, anti-proliferative (Btg2)	PPP	P10.5 vs. N ↑2.14
			P14.5 vs. N ↑2.46

N normal, *P10.5* pregnant day 10.5, *P14.5* pregnant day 14.5, *P* present, *A* absent, *M* marginal, ↑ up-regulated, ↓ down-regulated

Effect of PRL on Atf3 and Nupr1 production in INS-1 cell

INS-1 cells were then cultured with 200 ng/ml PRL to detect Atf3 and Nupr1 transcripts. After cultured with PRL, the proliferation of INS-1 cell enhanced with the time prolonged (data not show), which was paralleled by an increase in Atf3 and Nupr1 protein expression. Expression of Atf3 protein started to increase at 6 h, with a significant increase at 12 h, which continued to 24 h, while Nupr1 protein expression had a continuous rise up to 12.6-fold after 24 h (Fig. 4).

Table 3 Expressions of genes related to Wnt signaling pathway

Genebank ID	Gene title	Detection (N/P10.5/P14.5)	Change
24881	Wingless-type MMTV integration site family, member 1 (Wnt1)	PAP	P14.5 vs. N ↑2.83
303181	Wingless-related MMTV integration site A (predicted) (Wnt3a)	AAA	–
84353	Catenin (cadherin associated protein), beta 1, 88 kDa (β-catenin)	AAA	–
94201	Cyclin-dependent kinase 4 (Cdk4)	PPP	P10.5 vs. N ↓0.76
79257	Axin 1 (Axin1)	PPP	–
84027	Glycogen synthase kinase 3 beta (GSK-3β)	PAA	P14.5 vs. N ↓0.43
24577	Myelocytomatosis viral oncogene homolog (avian) (c-Myc)	APP	P10.5 vs. N ↑4.29 P14.5 vs. N ↑2.64

N normal, *P10.5* pregnant day 10.5, *P14.5* pregnant day 14.5, *P* present, *A* absent, ↑ up-regulated, ↓ down-regulated

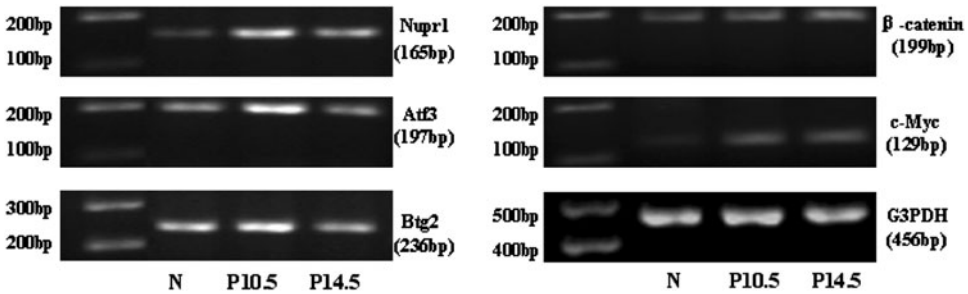

Fig. 1 Gene expressions of Nupr1, Atf3, Btg2, β-catenin, and c-Myc in pancreatic islets during pregnancy in rats. G3PDH served as control for equal total RNA input into RT-PCR reactions. Shown are representative semi-quantitative RT-PCR assays ($n = 5$)

Table 4 Gene expressions of Atf3, Nupr1, Btg2, β-catenin, and c-Myc during pregnancy in rats (real-time PCR, $2^{-\Delta\Delta Ct}$)

	Atf3	Nupr1	Btg2	β-catenin	c-Myc
N	1.00 ± 0.39	1.00 ± 0.72	1.00 ± 0.09	1.00 ± 0.34	1.00 ± 0.11
P10.5	5.36 ± 0.86*	27.06 ± 1.40**	9.74 ± 0.71**	3.38 ± 0.46*	1.64 ± 0.13
P14.5	1.19 ± 0.27$^{\#}$	10.53 ± 1.54*$^{\#\#}$	5.05 ± 0.52*$^{\#\#}$	4.76 ± 0.44**	3.47 ± 0.31*

vs. *N*, * $P < 0.05$, ** $P < 0.01$; vs. *P10.5*, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$

Table 5 Mean Ct of Atf3, Nupr1, Btg2, β-catenin, and c-Myc during pregnancy in rats (real-time PCR)

Ct value	G3PDH	Atf3	Nupr1	Btg2	β-catenin	c-Myc
N	20.21	21.38	20.02	19.33	27.26	26.12
P10.5	20.70	18.87	16.84	18.15	25.24	25.48
P14.5	20.84	20.75	17.90	18.79	23.89	23.58

Discussion

There is widespread interest in defining factors and mechanisms that regulate regeneration (neonatal or proliferation) of pancreatic islet β-cells, in order to find potential targets for therapy in diabetes [3, 12, 13]. Our previous study suggested that both maternal pancreatic islet function and proliferation are considerably increased in middle and late pregnancy to match the increased insulin demand and ensure maternal and fetal glucose homeostasis. To assess maternal islet cell proliferation, we performed double-labeling studies with 5-bromodeoxyuridine (BrdU) and insulin. Insulin-secreting β-cells proliferation increased in pregnant rat at day 12.5, rose continuously to day 20.5, with a peak at day 14.5 [11].

Although descriptive studies support the hypothesis that proliferation of islet β-cells is the principal mechanism of β-cell expansion in pregnancy [9], the molecular basis and genetic pathways of β-cell proliferation in this setting is poorly understood. To investigate the mechanisms controlling maternal islet β-cell proliferation, we have used a transcriptional profiling approach to characterize the expression of differential genes in isolated pancreatic islets from normal and pregnant rats. Our results reveal that transcription factors may play an important role in islet β-cell proliferation during pregnancy.

Transcription factors related to islet development and differentiation, as well as to β-cell function were well studied in the past years [14, 15], while transcriptional

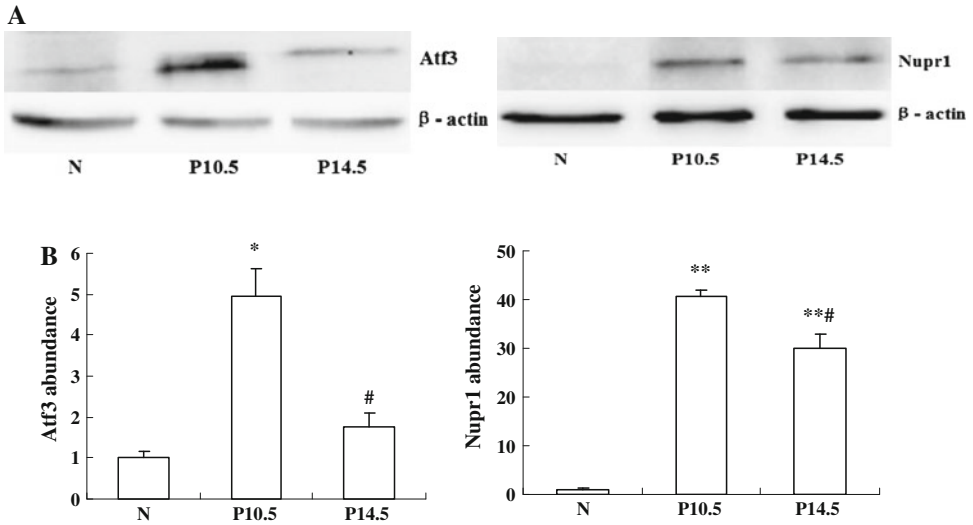

Fig. 2 Expressions of Atf3 and Nupr1 protein in pancreatic islets during pregnancy in rats. The total protein of each group (normal, pregnant days 10.5 and 14.5) was subjected to Western blotting. Level of Atf3 and Nupr1 protein expression was determined by optical density measurements and expressed as a fold of the control group after normalization to β-actin (42 kD). **a** Both Atf3 (21 kD) and Nupr1 (9 kD) proteins were increased during pregnant day 10.5. **b** Densitometric analysis showed accurately changes of Atf3 and Nupr1 protein during early pregnant. Values are results from three separate experiments. vs. N, * $P < 0.05$, ** $P < 0.01$; vs. P10.5, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$

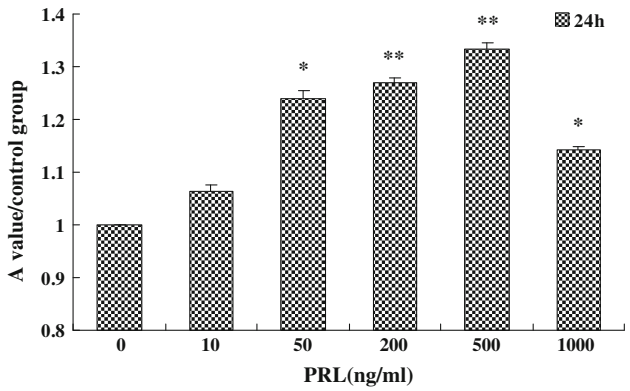

Fig. 3 Prolactin-induced INS-1 cells proliferation in a dose-dependent manner. Increase of the oPRL concentration from 0 to 500 ng/ml enhanced proliferation of INS-1 cells cultured for 24 h. vs. 0 ng/ml oPRL, * $P < 0.05$, ** $P < 0.01$

regulators related to islet proliferation were poorly understood. We first focused on the transcriptional regulators with differential expression during pregnancy, and found that Nupr1, Atf3, Btg2, and some eukaryotic general transcription factors were significantly up-regulated during pregnancy. Wnt signaling is an important regulator of organ growth and cell fates, and genes encoding Wnt-signaling factors are expressed in the pancreas. In our experiment, gene expression for Wnt signaling pathway was also changed specially, suggested that Wnt signaling may participant in adaptive structure change of pregnant islet β-cell. There was no significant change in expression of transcriptional regulator related to islet development and differentiation (Foxa2, Pdx1, Nkx6.1, Nkx2.2, Pax6, Pax4, and Neurod1) (Table 1). Thus, we verified the hypothesis that pancreatic β-cell expansion during pregnancy is formed by self-duplication rather than stem-cell or progenitor-cell differentiation.

Activating transcription factor 3 (Atf3) encodes a member of the ATF/CREB family of transcription factors [16]. Atf3 gene is induced in many tissues by a variety of stress signals, including in the pancreatic β-cells by pro-inflammatory cytokines, reactive oxygen species, and high concentrations of glucose or fatty acids [17–19]. Despite overwhelming evidence indicating that Atf3 is a stress-inducible gene, the physiological consequence of expressing Atf3 is not clear. To date, both protective and detrimental effects of Atf3 expression have been reported. In this study, we tried to elucidate the physiological significance of Atf3 in proliferation-related signaling pathways. Both Atf3 gene and protein expressed at a low stable level in islet of control rats, while up-regulated in pregnant rats at day 10.5, and then decreased to normal level at day 14.5. It suggested that Atf3 has an early effect in regulating the proliferation of islet during pregnancy, and may play a role in upstream of Nupr1. Tamura et al. [20] reported that ectopic expression of Atf3 promotes proliferation of c-Myc-deficient cells, whereas Atf3 knockdown significantly suppresses proliferation of wild-type cells. They demonstrate that Atf3 is downstream of the c-Myc signaling pathway and plays a role in mediating the cell proliferation function of c-Myc.

c-Myc is the downstream target gene of Wnt signaling pathway. Results from gene chip and verification by

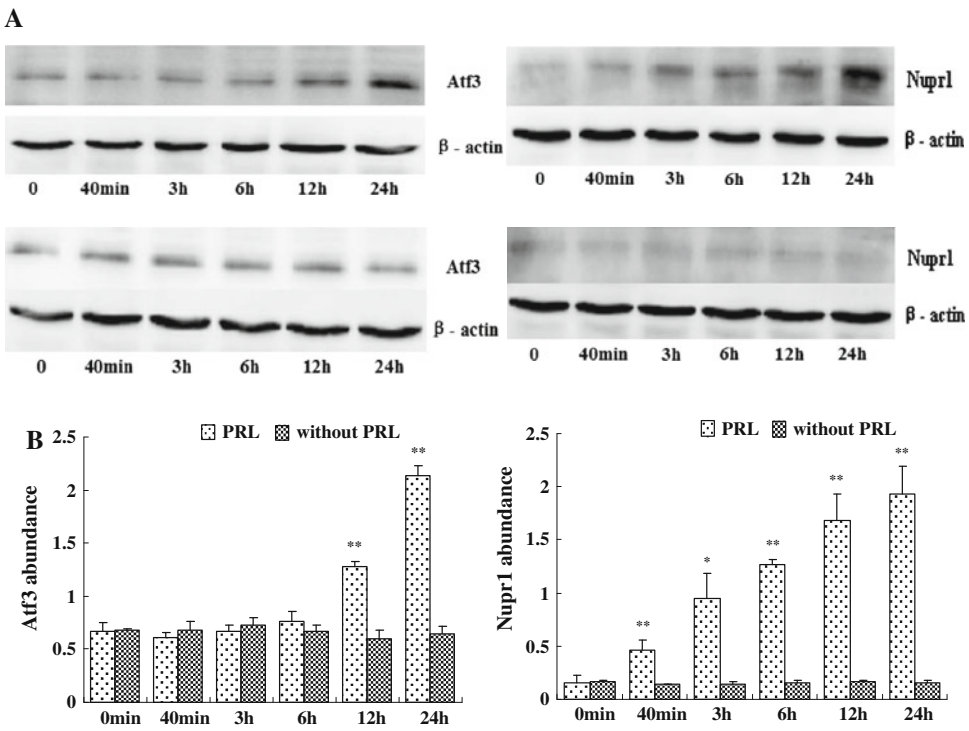

Fig. 4 Expressions of Atf3 and Nupr1 protein in INS-1 cells incubation with 200 ng/ml oPRL for various periods. **a** Prolactin enhanced both Atf3 and Nupr1 expressions of INS-1 cells. *Up* PRL group, *down* without PRL. **b** Densitometric analysis showed accurate changes of Atf3 and Nupr1 protein. vs. 0 min, * $P < 0.05$, ** $P < 0.01$. Three independent experiments were performed

RT-PCR and real-time PCR demonstrated that the expression of Wnt signaling pathway-related genes such as Wnt2, β-catenin,and c-Myc increased significantly during gestation, which indicated they may also play an important role in pancreatic islet adaption during gestation. Our results provide a novel insight into the functional link of the stress response gene ATF3 and the proto-oncogene c-Myc. We surmise, during pregnancy, Wnt signaling pathway is activated first, then stimulate downstream gene c-Myc and Atf3, and further promote islet β-cell proliferation. Wnt signaling is an important regulator of organ growth and cell fate determination. Prior studies provided evidence that pancreatic growth and differentiation are regulated by Wnt signaling [21–23]. Rulifson et al. [24] provided evidence that Wnt signaling lead to β-cell expansion, increased insulin production and serum levels, and enhanced glucose handling. Our results show its pro-proliferation effect in a physiological situation, and provide previously unrecognized evidence of a mechanism governing endocrine pancreas proliferation during pregnancy.

Nupr1 was reported to be one of the downstream genes of Atf3, and whether this Atf3/Nupr1 signal pathway makes sense in proliferation of pancreatic islet is not clear. Here, we report on β-cell-expanding properties of the protein Nupr1, which is considered to be a member of the HLH transcription factor family. In rats, high fetal pancreatic Nupr1 mRNA levels progressively decline during the postnatal period to reach low levels in the adult, indicating a role for Nupr1 during development and organogenesis [25]. Within the pancreas, Nupr1 expression had been reported only in exocrine cells [25, 26] until Path et al. [27] demonstrated that Nupr1 is also expressed in primary human islets and several pancreatic cell lines including ductal cells and differentiated β-cells (such as PANC-1, AR42 J, INS-1, β-TC6 cells). We further found that Nupr1 is expressed in rat islets as well, with a very low level in control rats and increased significantly during pregnancy, especially at pregnant day 10.5. It is suggested that Nupr1 is activated in maternal islet due to variation of hormone level, and participant in islet β-cells expansion during pregnancy. Expression of Nupr1 at pregnant day 14.5 was still above control group, while Atf3 was down to control group, which indicated that Nupr1 is located in downstream of Atf3 and play a more direct role in moderate islet β-cells proliferation by earlier time phase. Our results, together with existing studies demonstrating augmented growth of several Nupr1-overexpressing cell lines, indicate that Nupr1 protein is a mediator of β-cell expansion on its own.

Expression of pro-apoptosis gene (Bax, Bak, and Bid), death receptor-related gene (TRADD, FADD), and caspase were not changed overt, partial of them had a down trade during pregnant, without statistically significance. Expression of anti-apoptosis gene such as Bcl-2 was also not changed during pregnancy. However, Btg2 had an

obviously increased expression during pregnant, especially at day 10.5, which similar with Atf3 and Nupr1. Btg2 is important in regulating cell growth, difference and apoptosis, its over-expression in PC12 cell lines could inhibiter cell apoptosis [28]. We consider that up-regulated Btg2 may play an anti-apoptosis effect on islet β-cell during pregnancy and thereby lead to β-cell proliferation indirectly.

During pregnancy, pancreatic β-cells undergo changes that are probably due to an increase in the lactogenic hormones prolactin and placental lactogen. Several experiments performed both in vitro and in vivo have shown that lactogenic hormone can induces the same changes in β-cells as those observed during pregnancy, and PRL may plays a key role in adaptation of islets during pregnancy. The results of our study suggest that PRL can stimulate INS-1 β-cells proliferation in vitro. Since the transcription factors Atf3 and Nupr1 were involved in the regulation of the β-cell proliferation during pregnancy, we tested the possibility that whether the effect of PRL on INS-1 β-cells was mediated by Atf3 and Nupr1. We found exposure of β-cells to PRL in vitro resulted in increased levels of Atf3 and Nupr1 proteins, with earlier starting than proliferation effect. These observations confirm an association between PRL-stimulated transcriptional factor expression and expansion of rat INS-1 β-cells, and support the hypothesis that transcriptional factor Atf3 and Nupr1 may be involved in pancreatic β-cell mass expansion in response to PRL. Previous literature reported that the effect of PRL on INS-1 β-cells proliferation and function may be mediated by activation of the JAK–STAT pathway [29, 30]. Here, we indicate that the mechanisms by which PRL exerts its effects in INS-1 β-cells may be in part by activation of Atf3/Nupr1 pathway.

In conclusion, transcription factors Atf3, Nupr1, and Wnt pathway-related genes were increased during pregnancy in pancreatic islets, with earlier time phases than proliferation peak occurs, suggesting that they may play an important role in adaptive proliferation of pancreatic islets during pregnancy in rats. The increased expression of critical Atf3 and Nupr1 proteins appears to be a predominant effect of PRL in islet β-cells. Our study is the first to show that Atf3/Nupr1 expression is modulated by PRL in both in vitro and in vivo model of increased β-cell proliferation. We also first describe the change of Wnt pathway and its significance in pancreatic islet β-cells during pregnancy. These results expand our understanding of mechanisms underlying β-cell proliferation and pathogenesis of diabetes, reveal potential targets for therapy in diabetes. However, it is not known whether these expression profiles are present in some other conditions and their physiological and pathological significance in diabetes mellitus, which needs further analysis.

Materials and methods

Animals

Healthy male Sprague–Dawley (SD) rats with 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. All pregnant rats were randomly divided into two groups that were pregnant at days 10.5 and 14.5, age-matched virgin rats were used as controls (P10.5, P14.5, N). 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. All experiments were approved by the local animal ethical committee and were in accordance with accepted standards of humane animal care.

Islet isolation and culture

For each set of experiments, islets from four adult female SD rats, obtained by collagenase V (568 U/mg, Sigma, St. Louis, MO, USA) digestion of pancreata and isolated by centrifugation in a two-layer density gradient (Histopaque-1077 at the bottom and D-Hanks solution overlaid onto the bottom), were maintained in cultured at 37°C in a 5% CO_2/air atmosphere for 2 h to recover from the isolation before starting the experimental procedures. The culture medium consisted of RPMI 1640 (GIBCO, Carlsbad, CA, USA) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), sodium pyruvate (1 mM), HEPES (12.5 mmol/l), and 10% fetal bovine serum.

RNA extraction, purification, and reverse transcription

For each group, total RNA was extracted from approximately 4,000 islets which were isolated from 16 rats using Trizol Reagent (Roche, San Francisco, CA, USA). The ratio of the optical densities at 260 and 280 nm from RNA samples were between 1.8 and 2.1 measured by Ultrospec 3100 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). Furthermore, the quality and purity of the RNA were checked by electrophoresis in a denaturing gel. Some RNA was used in the gene chip analysis, and the other used in semi-quantitative PCR and real-time PCR. 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, Osaka, Japan).

Microarray analysis

The gene expression profile was analyzed by Rat Genome 230 2.0 array (Affymetrix, Santa Clara, CA, USA) representing 31,099 genes. The whole procedure was complicated at Affymetrix "gene chip check station" which has been described previously [11]. 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 higher than the cut-off of 2-fold in the experiments were considered.

Semi-quantitative PCR

Semi-quantitative RT-PCR using specific primers was done to confirm the differential expression of five mRNAs detected with the microarray analysis. PCR assays were done using KOD Dash (RT-PCR Kit; TOYOBO, Osaka, Japan) and 10 pmol of each primer in a master mix of 20 µl. The number of cycles for each gene was defined after titration using 20 and 42 cycles and was within the logarithmic phase of amplification. PCR products were separated on 1.7% agarose gels and the band intensities were determined by digital scanning followed by quantification using Quantity One 1-D analysis software (Bio Rad, Hercules, CA, USA). The results were expressed as a ratio of target to G3PDH signals. The RNAs used for RT-PCR analysis were obtained from three to four sets of experiments.

Real-time PCR

Quantitative real-time PCR was performed with SYBR® Green Realtime PCR Master Mix-Plus (QPK-212) (TOYOBO, Osaka, Japan) using a Rotor-Gene 6.0.1 Real-time PCR System. PCR reactions were done in 20 µl volumes contained 2 µl cDNA. Thermal cycling conditions were 60 s at 95°C followed by 49 cycles for 15 s at 95°C, 15 s at 53–65°C (primer specific, Table 6) and 45 s at 72°C; SYBR green incorporation into a single peak was monitored using a dissociation curve. Negative controls were processed without reverse transcriptase. All samples from a single experiment were run using a single PCR mixture. Expression levels were normalized against the levels of housekeeping gene G3PDH. The primers used are shown in Table 6.

The levels of mRNA were quantified using the comparative threshold cycle (C_T) method. C_T was determined from a log-linear plot of the PCR signal versus cycle number. The amount of mRNA was normalized to that of the housekeeping gene (G3PDH) and expressed relative to controls by the formula $2^{-\Delta\Delta Ct}$, where $\Delta C_T = C_T$ (target)$-C_T$ (G3PDH) and $\Delta\Delta C_T = \Delta C_T$ (treated sample)$-\Delta C_T$ (untreated sample).

Protein analysis by Western blotting

Cultured islets were lysed in 100 µl lysis buffer (25 mM Hepes, pH 7.5, 5 mM EDTA, 0.5% Triton X-100, 1.5 mM sodium fluoride, 1 mM sodium vanadate, 10 µl/ml protease inhibitor mixture with aprotinin, leupeptin, and pepstatin) for 30 s using a Ultrasonic Instrument on ice, and centrifuged at 10,000×*g* at 4°C for 20 min. The protein concentration of the supernatants was assayed by the BCA method using the BCA assay (Pierce, Rockford, IL, USA). The proteins were treated with 6× sample buffer containing dithiothreitol and boiled for 5 min. An equal amount of protein (approximately 50 µg) from each sample was applied to a 15% polyacrylamide gel and separated by SDS-PAGE in a Bio-Rad miniature slab gel apparatus. The electrotransfer of proteins from the gel to polyvinylidene

Table 6 RT-PCR primer sets and reaction conditions

Gene	Primer sequences	Annealing temp. (°C)
G3PDH	F 5′-CACCCTGTTGCTGTAGCCATATTC-3′	59
	R 5′-GACATCAAGAAGGTGGTGAAGCAG-3′	
Atf3	F 5′-TGCTAACCTGACACCTTTTG-3′	63
	R 5′-ATTTTGTTTCTTTCCCGCC-3′	
Nupr1	F 5′-AGACATTTGGAAAACAGGC-3′	59
	R 5′-ACCGACGACATAAGATTGG- 3′	
Btg2	F 5′-GACCGATCATTACAAACACC-3′	59
	R 5′-CCTCATACAGCACGCAGA-3′	
β-catenin	F 5′-CAGTTGCTTTATTCTCCCATT-3′	53
	R 5′-GCTTGTCCTCAGACATTCG-3′	
C-Myc	F 5′-AACCCGACAGTCACGACG-3′	65
	R 5′-CTGCT-GTTGCTGGTGATAGA-3′	

F forward primer, *R* reverse primer

difluoride (PVDF) membranes was done at 300 mA for 1 h in a Bio-Rad miniature transfer apparatus. Before incubation with the primary antibody, the PVDF membranes were treated with a blocking buffer (3% BSA/TBST) for 2–4 h at room temperature. The membranes were incubated overnight at 4°C with antibodies against Atf3 (1:1000, 21kD) (Santa Cruz, CA, USA), Nupr1 (1:100, 9 kD) (Santa Cruz, CA, USA), or β-actin (1:1000, 42 kD) (Sigma, St. Louis, MO, USA) diluted in blocking buffer, and then washed for 30 min in TBST buffer. The blots were subsequently incubated with peroxidase-conjugated secondary antibody (1:1000 for Atf3 and Nupr1, 1:2000 for β-actin) for 1 h. Visualization of specific protein bands was done using commercial-enhanced chemiluminescence reagents with exposure by ChemiDocXRS system (Bio Rad, Hercules, CA, USA). The band intensities were quantified by Quantity One 1-D analysis software.

Rat insulinoma (INS-1) cell culture and related experiments

Rat insulinoma cells (INS-1) were grown in RPMI 1640 medium containing 11-mM D-glucose supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), sodium pyruvate (1 mM), β-mercaptoethanol (50 μM), HEPES (12.5 mmol/l), and 10% fetal bovine serum (INS-1 medium). Prior to any experiments, the cells were precultured in serum-free medium (SFM) for 4–6 h. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay in INS-1 cells (1×10^4) split in 96-well plates. After preculture, cells were cultured in medium with addition of ovine PRL (Sigma, St. Louis, MO, USA) made in PBS with 0.1% BSA (10, 50, 200, 500, 1000 ng/ml PRL), or PBS with 0.1% BSA alone as control for 24 h, and subsequently analyzed. MTT assay was performed by incubating the cells with 5 mg/ml MTT for 4 h at 37°C in 5% CO_2. After that, discarded supernatant, added each hole with 150 μl DMSO, oscillated 10 min, and read absorbance at 492 nm.

Furthermore, after preculture, cells were cultured in medium with or without addition of 200 ng/ml PRL for different periods (0 min, 40 min, 3 h, 6 h, 12 h and 24 h, respectively). Then, protein of cells for each group was extracted as mentioned above for Western blotting.

Statistical analysis

The data are expressed as the means ± SEM. Multiple comparisons of parametric values with control values were performed by ANOVA with LSD test (SPSS 10.0). For all comparisons, the difference was considered statistically significant if $P < 0.05$.

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References

1. A.E. Butler, J. Janson, S. Bonner-Weir, R. Ritzel, R.A. Rizza, P.C. Butler, β-Cell deficit, increased β-cell apoptosis in humans with type 2 diabetes. Diabetes **52**, 102–110 (2003)
2. E.A. Ryan, B.W. Paty, P.A. Senior, D. Bigam, E. Alfadhli, N.M. Kneteman, Five-year follow-up after clinical islet transplantation. Diabetes **54**, 2060–2069 (2005)
3. S. Bonner-Weir, G.C. Weir, New sources of pancreatic beta-cells. Nat. Biotechnol. **23**, 857–861 (2005)
4. I. Swenne, Glucose-stimulated DNA replication in the pancreatic islets during the development of the rat fetus. Diabetes **34**, 803–807 (1985)
5. S. Georgia, A. Bhushan, Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J. Clin. Invest. **114**, 963–968 (2004)
6. Y. Dor, J. Brown, O.I. Martinez, D.A. Melton, Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature **429**, 41–46 (2004)
7. E. Akbay, M.B. Tiras, I. Yetkin, F. Toruner, R. Ersoy, S. Uysal, Insulin secretion and insulin sensitivity in normal pregnancy and gestational diabetes mellitus. Gynecol. Endocrinol. **17**(2), 137–142 (2003)
8. E.A. Ryan, Hormones and insulin resistance during pregnancy. Lancet **362**(9398), 1777–1778 (2003)
9. A.M. Svensson, B. Bodin, A. Andersson, L. Jansson, Pancreatic islet blood flow during pregnancy in the rat: an increased islet mass is associated with decreased islet blood flow. J. Endocrinol. **180**(3), 409–415 (2004)
10. M. Johansson, G. Mattsson, A. Andersson, L. Jansson, P.O. Carlsson, Islet endothelial cells, pancreatic beta-cell proliferation: studies in vitro, during pregnancy in adult rats. Endocrinology **147**(5), 2315–2324 (2006)
11. Y. Xue, C.P. Liu, Y. Xu, Q.X. Yuan, X.D. Mao, G.F. Chen, C. Liu, Study on pancreatic islet adaptation, gene expression during pregnancy in rats. Endocrine **37**, 83–97 (2010)
12. L.C. Alonso, T. Yokoe, P. Zhang, D.K. Scott, S.K. Kim, C.P. O'Donnell, Glucose infusion in mice a new model to induce β-cell replication. Diabetes **56**, 1792–1801 (2007)
13. S. Fatrai, L. Elghazi, N. Balcazar, C. Cras-Meneur, I. Krits, H. Kiyokawa, Akt induces β-cell proliferation by regulating cyclin D1, cyclin D2, p21 levels, cyclin-dependent kinase-4 activity. Diabetes **55**, 318–325 (2006)
14. S.K. Chakrabarti, R.G. Mirmira, Transcription factors direct the development and function of pancreatic β cells. TEM **14**, 782–841 (2003)
15. E. Edlund, Factors controlling pancreatic cell differentiation and function. Diabetologia **44**, 1071–1079 (2001)
16. T. Hai, M.G. Hartman, The molecular biology and nomenclature of the ATF/CREB family of transcription factors: ATF proteins and homeostasis. Gene **273**, 1–11 (2001)
17. A.E. Allen-Jennings, M.G. Hartman, G.J. Kociba, T. Hai, The roles of ATF3 in glucose homeostasis: a transgenic mouse model with liver dysfunction and defects in endocrine pancreas. J. Biol. Chem **276**, 29507–29514 (2001)

18. M.G. Hartman, D. Lu, M.L. Kim, G.J. Kociba, T. Shukri, J. Buteau, Role for activating transcription factor 3 in stress-induced beta-cell apoptosis. Mol. Cell. Biol. **24**, 5721–5732 (2004)
19. A.K. Busch, D. Cordery, G.S. Denyer, T.J. Biden, Expression profiling of palmitate- and oleate-regulated genes provides novel insights into the effects of chronic lipid exposure on pancreatic beta-cell function. Diabetes **51**, 977–987 (2002)
20. K. Tamura, B. Hua, S. Adachi, I. Guney, J. Kawauchi, M. Morioka, Stress response gene ATF3 is a target of c-myc in serum-induced cell proliferation. EMBO **24**, 2590–2601 (2005)
21. L.C. Murtaugh, A.C. Law, Y. Dor, D.A. Melton, β-Catenin is essential for pancreatic acinar but not islet development. Development **132**, 4663–4674 (2005)
22. S. Papadopoulou, H. Edlund, Attenuated Wnt signaling perturbs pancreatic growth but not pancreatic function. Diabetes **54**, 2844–2851 (2005)
23. P.W. Heiser, J. Lau, M.M. Taketo, P.L. Herrera, M. Hebrok, Stabilization of β-catenin impacts pancreas growth. Development **133**, 2023–2032 (2006)
24. I.C. Rulifson, S.K. Karnik, P.W. Heiser, D. Berge, H. Chen, X. Gu, Wnt signaling regulates pancreatic β cell proliferation. PNAS **104**(15), 6247–6252 (2007)
25. G.V. Mallo, F. Fiedler, E.L. Calvo, E.M. Ortiz, S. Vasseur, V. Keim, Cloning and expression of the rat p8 cDNA, a new gene activated in pancreas during the acute phase of pancreatitis, pancreatic development, and regeneration, and which promotes cellular growth. J. Biol. Chem **272**, 32360–32369 (1997)
26. Y. Motoo, J.L. Iovanna, G.V. Mallo, S.B. Su, M.J. Xie, N. Sawabu, P8 expression is induced in acinar cells during chronic pancreatitis. Dig. Dis. Sci. **46**, 1640–1646 (2001)
27. G. Path, A. Opel, M. Gehlen, V. Rothhammer, M.D. Brendel, R.G. Bretzel, Glucose-dependent expansion of pancreatic β-cells by the protein p8 in vitro and in vivo. Am. J. Physiol. Endocrinol. Metab **291**, 1168–1176 (2006)
28. S. Wang, A.J. Dibenedetto, R.N. Pittman, Genes induced in programmed cell death of neuronal PC12 cells and developing sympathetic neurons in vivo. Dev. Biol. **188**, 322–336 (1997)
29. L.E. Stout, N.V. Bhagroo, R.L. Sorenson, Distinctive roles for prolactin and growth hormone in the activation of signal transducer and activator of transcription 5 in pancreatic islets of langerhans. Endocrinology **145**(9), 4162–4175 (2004)
30. Y. Fujinaka, K. Takane, H. Yamashita, R.C. Vasavada, Lactogens promote beta cell survival through JAK2/STAT5 activation and Bcl-XL upregulation. J. Biol. Chem **282**(42), 30707–30717 (2007)