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Knockdown of prolactin receptors in a pancreatic beta cell line: effects on DNA synthesis, apoptosis, and gene expression

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Abstract

Background—Prolactin (PRL) and placental lactogen stimulate beta cell replication and insulin production *in vitro* and *in vivo*. The molecular mechanisms by which lactogens promote beta cell expansion are unclear.

Methods—We treated rat insulinoma cells with a PRL receptor (PRLR) siRNA to determine if PRLR signaling is required for beta cell DNA synthesis and cell survival and to identify beta cell cycle genes whose expression depends upon lactogen action. Effects of PRLR knockdown were compared with those of PRL treatment.

Results—PRLR knockdown (−80%) reduced DNA synthesis, increased apoptosis, and inhibited expression of cyclins D2 and B2, IRS-2, Tph1, and the anti-apoptotic protein PTTG1; p21 and BCL6 mRNAs increased. Conversely, PRL treatment increased DNA synthesis, reduced apoptosis, and enhanced expression of A, B and D2 cyclins, CDK1, IRS-2, FoxM1, BCLxL, and PTTG1; BCL6 declined.

Conclusions—PRLR signaling is required for DNA synthesis and survival of rat insulinoma cells. The effects of lactogens are mediated by down-regulation of cell cycle inhibitors (BCL6, p21) and induction of A, B, and D2 cyclins, IRS-2, Tph1, FoxM1, and the anti- apoptotic proteins BCLxL and PTTG1.

Keywords

lactogen; cyclin; BCL6; PTTG1; IRS-2; Tph1; FoxM1; p21

INTRODUCTION

Beta cell mass and insulin production increase dramatically during two stages of the normal lifespan: the perinatal/early postnatal period and pregnancy [1-4]. A surge of beta cell replication and insulin secretion during the perinatal period is essential for neonatal glucose tolerance and establishment of life-long beta cell reserve: preterm and growth-retarded infants, who lack the beta cell surge, are at high risk for glucose intolerance in the newborn period and have double the risk of developing diabetes in adulthood [5] Likewise, beta cell

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expansion and increases in insulin secretion during mid and late pregnancy, when the mother develops severe insulin resistance, mitigate the risk of gestational diabetes mellitus (GDM) [6,7].

The factors controlling beta cell mass and function in the perinatal period and pregnancy have not been fully elucidated, but studies from this laboratory and others implicate a critical role for the lactogenic hormones of the pituitary gland (prolactin, PRL) and placenta (placental lactogen, PL). The lactogens circulate at high concentrations in the late-gestational fetus, newborn infant, and pregnant mother [6-9] and stimulate beta cell replication and glucose-stimulated insulin secretion (GSIS) and prolong cell survival in isolated human and rodent islets and rat and mouse insulinoma cells [10-17]. Over-expression of lactogenic hormones in isolated islets *in vitro* and in rats and mice *in vivo* induces beta cell replication and insulin production [18,19]. Conversely, global deletion (knockout/KO) of the PRLR, which mediates the actions of PL as well as PRL, reduces beta cell mass and GSIS in pregnant and non-pregnant mice [20,21].

The molecular mechanisms by which the lactogens promote beta cell expansion are poorly understood. Studies of the effects of lactogenic hormones on gene expression in isolated rodent islets have identified a variety of lactogen-responsive genes and potential lactogen signaling pathways [10,11,22-24]. However, the effects of lactogens in isolated islets may reflect changes in gene expression in non-beta as well as beta cells. Moreover, the effects of lactogen deficiency or lactogen resistance on beta cell gene expression have not been examined previously. To that end we treated rat insulinoma (beta) cells with an siRNA to the rat PRLR and examined the effects of the ensuing PRLR knockdown on beta cell DNA synthesis, survival, and gene expression. The effects of PRLR knockdown were compared with those of PRL treatment. Together with previous studies, our findings suggest diverse pathways by which the lactogens control beta cell expansion during the neonatal period and pregnancy.

METHODS

Adenoviral vectors

Small inhibitory RNAs (siRNAs) to the rat prolactin receptor (PRLR) were cloned into the adenoviral shuttle vector FF805 [25], using methods described previously [10]. Preliminary studies examined the effects of four different siRNAs on the expression of PRLRs in the rat beta cell line 832-13 (below). Three of the siRNAs reduced PRLR expression by at least 50%; the sequence of the most effective was 5′-GGA TGT GAC TTA CAT CGT T-3′); a scrambled siRNA (5′-GAG ACC CTA TCC GTG ATT A-3′) with no known homology to other protein sequences was used as a control.

Cell culture

Rat insulinoma cells (INS-1) with high glucose responsivity (832-13 cells, [26] were grown in RPMI 1640 (11.1 mM glucose) with 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, and 1% antibiotic/antimycotic solution (complete media).

To assess the effects of PRLR knockdown, the cells were washed and incubated for 24-72 hr with the PRLR or scrambled siRNAs (10^{6} infectious particles/million cells) in complete medium containing 10% FBS. The inclusion of FBS, which contains bovine prolactin (~50 ng/ml) and bovine placental lactogen (~10 ng/ml) [27], allowed us to determine if the PRLR siRNA could modulate beta cell growth and survival in the presence of endogenous lactogens and other growth factors. The complete medium with 10% FBS contains ~5 ng/ml (~0.2 nM) PRL and ~1 ng/ml (~0.04 nM) placental lactogen.

To assess the effects of PRL treatment, cells were washed and incubated for 24hr with 20 nM rat PRL or diluent in serum-free 'basal medium' (RPMI with 11 mM glucose, 0.1% human serum albumin, 10 μg/ml human transferrin, 50 μM ethanolamine, 0.1 nM tri-iodothyronine, 50 μM phosphoethanolamine, and 1% antibiotic/antimycotic solution).

Quantification of mRNA levels in 832-13 cells

832-13 cell RNA was isolated and reverse transcribed as described previously [10]. Oligonucleotide primers for quantitative real-time PCR (Q-RTPCR) were designed using Primer Express (Applied Biosystems, Foster City, CA). Amplicon lengths averaged 60bp; all primer pairs spanned introns. 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 levels of actin and quantified using the comparative threshold cycle (CT) method. Table 1 shows the sequences of primers used for Q-RTPCR and mean baseline CT values in control cells incubated in FBS or serum-free medium.

Western blot analysis

The cells were washed in PBS and centrifuged at 5000 × *g; w*hole cell lysates were prepared as described previously [11]. The blots were incubated with primary antibodies (a rabbit polyclonal PRLR antibody (1:200) (Santa Cruz Biotechnology, Dallas, TX), a rabbit polyclonal IRS-2 antibody (1:1000) (Cell Signaling technology, Danvers, MA.), or a mouse monoclonal cyclin D2 antibody (1:1000) (ThermoFisher Scientific, Fremont, CA). The blots were exposed to chemiluminescent substrate (ECL Advance Western blotting detection kit; GE Health Care, Piscataway, NJ), and imaged using the VersaDoc 4000 imaging system (Bio-Rad, Los Angeles, CA). Mouse monoclonal anti-tubulin antibody was used to detect tubulin as an internal control. Proteins were quantified by densitometric analysis of the blots using Image lab software (Bio-Rad, Los Angeles, CA).

DNA synthesis

Cells were treated with the PRLR siRNA or the scrambled siRNA in complete medium (10% FBS). Methyl-[^{3}H] thymidine was added (0.25 μCi/ml) during the last hour of incubation. In parallel experiments the 832-13 cells were washed and incubated for 24h in serum-free basal medium (11 mM glucose) containing rat PRL (20nM) or diluent. Methyl-[^{3}H] thymidine was added (0.25 μCi/ml) during the last hour of culture. Cellular DNA was precipitated with cold 10% TCA, washed, and dissolved in 0.3N NaOH. Methyl-[^{3}H] thymidine incorporated into DNA was normalized to total cellular protein.

Apoptosis

To assess the effects of the PRLR siRNA on cell survival the 832-13 cells were grown to 50% confluence on glass cover slips in complete media. The cells were incubated with the PRLR siRNA or scrambled siRNA in complete medium (10% FBS) for 72 hr. In parallel experiments the 832-13 cells were incubated with rat PRL (20 nM) or diluent for 24 hr in serum-free basal medium (11 mM glucose). The cells were fixed in 4% paraformaldehyde and followed the In situ Cell Death kit protocol (Roche, Indianapolis, Indiana).

The cells were incubated for 5 min with DAPI for nuclear staining. Five photographs at 20X magnification were taken for each experimental condition; images were viewed in Adobe Photoshop. TUNEL positive, DAPI positive, and double positive cells were counted. A total of at least 10,000 cells were counted for each experimental condition; apoptosis was expressed as % of cells with TUNEL-positive nuclei.

Cell counts were measured after a 72 hr incubation with the PRLR siRNA or scrambled siRNA in complete medium. After washing extensively the cells were trypsinized to single cell populations and counted by hemocytometry. Non-viable cells were identified by uptake of trypan blue.

RESULTS

1. PRLR knockdown in 832-13 cells

As shown in Figures 1a, b and c, the PRLR siRNA reduced PRLR mRNA levels in 832-13 cells by 75-80%, with a corresponding reduction in PRLR protein as assessed by Western blot. Maximal effects of the PRLR siRNA were achieved during an incubation of 72 hr.

Beta cell PRLR expression is induced by treatment with PRL [10]. As shown in Figure 1d, pretreatment of the 832-13 cells with the PRLR siRNA prevented the up-regulation of PRLR expression by subsequent PRL treatment. This finding demonstrates that the siRNA specifically inhibits PRL action in the beta cell line.

2. Effect of PRLR knockdown and PRL treatment on DNA synthesis and apoptosis in 832-13 cells

DNA synthesis and apoptosis were assessed in 832-13 cells following a 72 hr incubation with the PRLR or scrambled siRNAs in medium containing 10% FBS. In cells treated with the scrambled siRNA, the rate of DNA synthesis, asassessed by [^{3}H]-thymidine incorporation, was 116474±5405 cpm/mg protein (mean ± SEM), and the percent of TUNEL+ cells was 2.81 +/− 0.22, mean ± SEM). As shown in Figures 2a, b and c, the PRLR siRNA reduced thymidine incorporation by 16% ($p<0.01$) and increased by 2.2-fold the rate of apoptosis as assessed by TUNEL staining ($p<0.001$). Total cell counts were reduced by 30% ($p<0.001$).

Parallel experiments examined the effects of rat PRL (20 nM) or diluent for 24h in basal (serum-free) medium. [^{3}H]-thymidine incorporation in the diluent controls was 97775±2953 cpm/mg protein (mean ± SEM), and the percent of TUNEL+ cells was 1.61 +/− 0.11 (mean SEM). Treatment of 832-13 cells with rat PRL for 24 hr stimulated a 42.5% increase

($p<0.001$) in the incorporation of [^{3}H]-thymidine and reduced by 25% ($p<0.05$) the rate of apoptosis during the 24 hr incubation (Figures 2d and e).

3. Effects of PRL and PRLR knockdown on beta cell gene expression

To explore the molecular mechanisms by which lactogens control beta cell expansion we compared the effects of PRL treatment and PRLR knockdown on the expression of genes critical for beta cell replication and survival. As shown in Table 2, PRL decreased BCL6 mRNA (−67%) but increased mRNAs for cyclin A2 (+28%), cyclin B1 (+30 %), cyclin B2 (+18%), cyclin D2 (+42%), cyclin dependent kinase 1 (CDK1, +16%), insulin receptor substrate 2 (IRS−2, +46%), FoxM1 (+34%) BCLxL (+22%), and PTTG1 (securin, +11%). There was no significant effect on menin or Tph1 mRNAs. Conversely, PRLR knockdown reduced expression of cyclin D2 (−48%), IRS-2 (−37%) and, to a lesser extent, cyclin B2 (−15%), PTTG1 (−17%), and Tph1 (−25%). p21 (+43%) and cyclin D1 (+26%) mRNAs increased, while menin was slightly decreased. Similar or identical effects on gene expression were noted with a second PRLR siRNA (not shown).

Under the experimental conditions outlined in the Methods section, neither PRL treatment nor PRLR knockdown altered significantly the expression of other genes postulated to modulate beta cell replication or survival, including p18, p19, Cyclin D3, CDK2, CDK4, CDK6, IGF2, BAX, or Toll-like receptor 4.

4. Effects of PRLR knockdown on cyclin D2 and IRS-2 protein levels

To determine if changes in gene expression at the mRNA level are also present at the protein level we examined two critical proteins whose mRNAs are down-regulated by the PRLR siRNA and up-regulated by PRL treatment. As shown in Figure 3, the levels of IRS-2 and cyclin D2 proteins mirror the levels of IRS-2 and cyclin D2 after treatment with the PRLR siRNA or with PRL: PRLR knockdown reduced cellular levels of cyclin D2 (−35%, $p<0.05$) and IRS-2 (−40.8%, $p<0.01$, Figures 3a and 3b), while PRL treatment increased levels of cyclin D2 (+39.7%, $p<0.05$) and IRS-2 (+ 65.4%, $p<0.01$, Figures 3c and 3d)

DISCUSSION

In this study we treated rat insulinoma cells with a PRLR siRNA to determine if PRL signaling is required for beta cell DNA synthesis and cell survival and to identify beta cell cycle genes whose expression depends upon lactogen action. The effects of PRLR knockdown were compared with those of PRL treatment.

Studies of the PRLR siRNA were conducted in the presence of fetal bovine serum, which contains bovine prolactin and bovine placental lactogen. This allowed us to determine if the PRLR-siRNA could modulate beta cell growth and survival in the presence of endogenous lactogens and other growth factors. We showed that PRLR knockdown inhibits beta cell DNA synthesis and increases beta cell apoptosis, reducing total beta cell number; in contrast, PRL treatment increased beta cell DNA synthesis in serum-free medium and reduced beta cell apoptosis. These findings suggest that PRLR signaling is required for beta cell expansion and maintenance of beta cell reserve, a hypothesis supported by reductions in beta cell replication and beta cell mass in global PRLR knockout mice [20].

The mechanisms by which the lactogens promote beta cell expansion are unclear. Previous studies from this and other labs have used a variety of experimental paradigms to identify lactogen-responsive beta cell genes [10-12,22-24]. Here we found that PRL induced expression of the A and B cyclins, cyclin D2, CDK1, IRS-2, FoxM1, BCLxL, and PTTG1 (securin) and reduced expression of the transcriptional inhibitor BCL6. Conversely, PRLR knockdown reduced expression of cyclin D2, IRS-2, and (to a lesser extent) cyclin B2, CDK1, Tph1, and PTTG1, and up-regulated expression of cyclin D1 and the cyclin-dependent kinase inhibitor p21. BCL6 mRNA levels increased slightly. It should be noted that a number of these and other beta cell genes are regulated at the post-transcriptional as well as the transcriptional levels; analysis of post-transcriptional effects of lactogens on beta cell gene expression will require additional investigation.

That cyclin D2 plays a central role in lactogen action in the rodent beta cell is suggested by three lines of evidence. First, the cyclin D2 promoter [28] contains a consensus binding sequence for STAT5, which mediates effects of lactogenic hormones in most tissues. Second, cyclin D2 mRNA and protein levels in islets and insulinoma cells are up-regulated by PRL and down-regulated by PRLR knockdown. And third, knockdown of cyclin D2 expression attenuates PRL induction of beta cell [^{3}H]-thymidine incorporation in isolated rat islets [10]. PRL induction of cyclin D2 is physiologically relevant, because a global knockout of cyclin D2 causes beta cell hypoplasia and glucose intolerance in postnatal mice [29,30]. Still, cyclin D2 knockdown did not abolish lactogen induction of islet DNA synthesis in rat islets [10], suggesting roles for other transcriptional mediators including the A and B cyclins, which are up-regulated in pregnancy [31], FoxM1, shown previously to be up-regulated by placental lactogen [32], and IRS-2, which is essential for the beta cell compensatory response to insulin resistance [33].

In contrast to Kim et al [23], we found no effect of PRL on Tph1 mRNA levels in 832-13 cells (or isolated islets [10]); however, PRLR knockdown reduced Tph1 mRNA, suggesting that PRL signaling is required for normal Tph1 expression. Tph1 is postulated to play a critical role in beta cell replication during pregnancy [23], though serotonin-deficient mice with global Tph1 KO have normal beta cell mass [24].

Previous studies showed that lactogens up-regulate the anti-apoptotic protein BCLxL [34,35]. The induction of the anti-apoptotic protein PTTG1 (securin) by PRL and its suppression by the PRLR siRNA identifies PTTG1 as a novel target of lactogen action. PTTG1 plays a critical role in beta cell preservation: PTTG1 knockout mice develop beta cell hypoplasia, insulinopenia, and glucose intolerance resulting from beta cell apoptosis and senescence, up-regulation of p21, and marked reductions in beta cell proliferation and neogenesis [36,37]. Future studies will determine if induction of PTTG1 by lactogenic hormones contributes to beta cell expansion during pregnancy or the perinatal period or if down-regulation of PTTG1 following delivery of the infant promotes maternal beta cell apoptosis.

In sum, our findings suggest that PRLR signaling is required for beta cell DNA synthesis and survival; these effects appear to be mediated by a number of cell cycle genes, transcriptional mediators, and anti-apoptotic proteins including the A, B and D2 cyclins,

IRS-2, FoxM1, Tph1, BCLxL, and PTTG1. The mechanisms by which the lactogens regulate the expression of the various genes are not entirely clear, though induction of STAT5 likely plays an important role. As noted previously, STAT5 mediates PRL induction of cyclin D2. In addition, PRL treatment or constitutive expression of STAT5a down-regulated BCL6 in human mammary tumor cell lines and B-lymphoma cells [38,39]. Conversely, STAT5 knockdown up-regulated BCL6 in human colorectal cancer cells [40]. STAT5 binds to a consensus sequence in the first untranslated exon of the human and rat BCL6 genes and inhibits BCL6 transcription in B-lymphoma cells and mammary cancer cells [38,39,41].

Our findings suggest a paradigm whereby PRL down-regulates expression of genes that inhibit beta cell replication (e.g. BCL6). Subsequent induction of cyclin D2, the A and B cyclins, CDK1, IRS-2, FoxM1, and Tph1 provides the stimulus for PRL-dependent beta cell expansion, while induction of BCLxL and PTTG1 maintain the increase in beta cell mass.

The ability of PRL to up-regulate beta cell glucose transport and utilization [12,16] adds complexity to this paradigm. Glucose promotes FoxO1 phosphorylation and cytoplasmic re-distribution [42], up-regulates cyclin D1, IRS-2, IGF-2, and Tph1 in isolated rat islets [10], and acts synergistically with PRL to increase islet DNA synthesis and gene expression [10]. Thus, certain effects of PRL on beta cell growth or function could be mediated indirectly through induction of glucose uptake and utilization and/or insulin production; this may be particularly relevant during the perinatal period and pregnancy, when circulating glucose concentrations rise and beta cell glucose availability increases.

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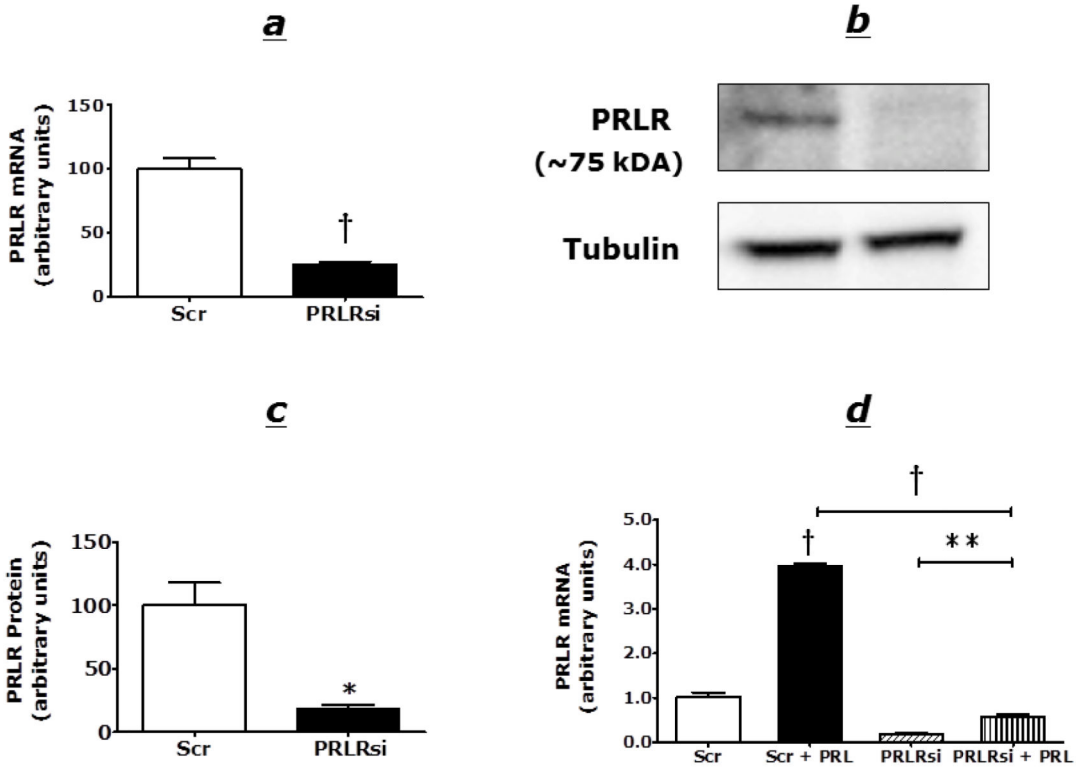

FIG 1. PRLR knockdown in 832-13 cells

a. 832-13 cells were treated with adenoviruses containing scrambled (Scr) or prolactin receptor (PRLR) siRNAs. PRLR mRNA was assessed after a 72 hr incubation in medium containing 10% FBS. ***b and c***. PRLR protein was detected in 832-13 whole cell lysates using a polyclonal antirabbit antibody to the PRLR. ***d***. 832-13 cells were treated with adenoviruses containing Scr or PRLR siRNAs and incubated for 52 hr in medium containing 10% FBS. The cells were then washed and incubated in serum-free medium with PRL (20nM) or diluent for an additional 24 hr. PRLR mRNA was measured at the end of the final incubation period. * $p < 0.05$, **$p<0.01$, † $p < 0.001$ vs scrambled controls. Similar findings were obtained in 3 or more experiments.

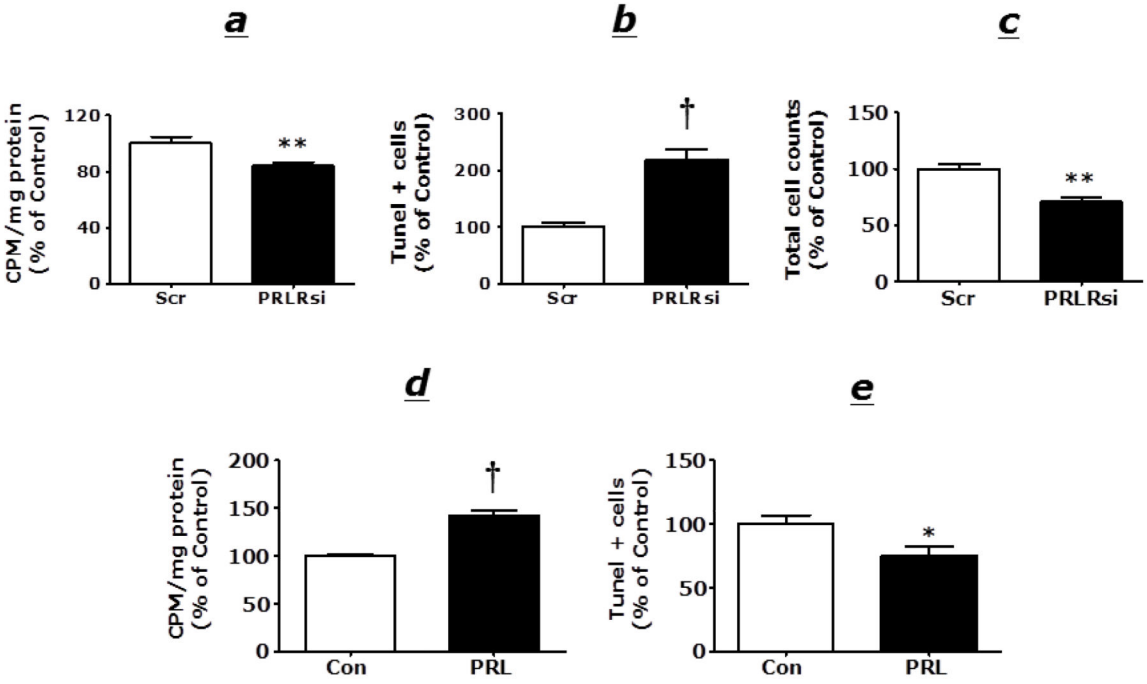

FIG 2. Effects of PRLR knockdown and PRL treatment on DNA synthesis and apoptosis

DNA synthesis (a).832-13 cells were incubated for 72 hr with adenoviruses containing scrambled (Scr) or PRLR siRNAs in medium containing 10% FBS. ***(d)***. Parallel experiments examined the effects of PRL or diluent in serum-free basal medium during a 24 hr incubation. In both cases, [^{3}H]-thymidine was added for the final hour of incubation. [^{3}H]-thymidine in control cells incubated with the scrambled siRNA (Scr) and in control cells incubated with diluent (Con) was 116474±5405 cpm/mg protein and 97775±2953 cpm/mg protein, respectively. The results represent the data from 3 independent experiments (n=12 in each group). **p<0.01, † p < 0.001 vs controls

Apoptosis (b, e). Apoptosis was estimated by TUNEL staining, fluorophore tagged TUNEL positive cells were counted. The results represent mean ± SEM of 12 samples in each group. Ten thousand cells were counted in each group. The percent of TUNEL+ cells in the scrambled siRNA (Scr) and diluent (Con) groups was 2.81 and 1.61, respectively. † p < 0.001 and * p < 0.05 vs. controls

Cell counts (c). Total cell counts were obtained following 72 hr incubation with the PRLR siRNAs or scrambled siRNAs. Values represent the mean ± SEM of 12 samples in each group. **p<0.01 vs controls

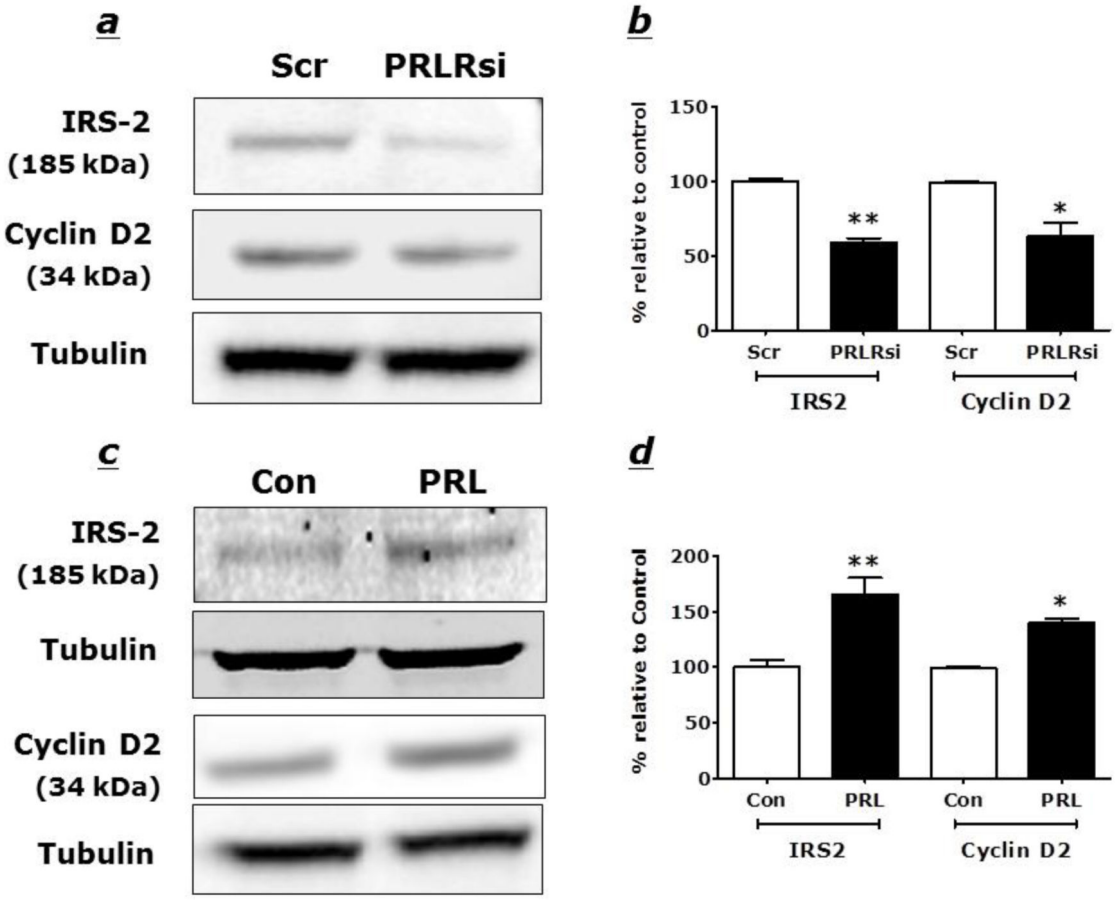

FIG 3. Effects of PRLR knockdown and PRL treatment on I R S-2 and cyclin D2 protein levels in 832-13 cells

(a and b) 832-13 cells were treated with adenoviruses containing PRLR or scrambled siRNAs and incubated for 72 hr in medium containing 10% FBS. Additional cells **(c and d)** were incubated for 24 hr in serum-free medium in the presence of PRL (20 nM) or diluent. Cyclin D2 and IRS-2 levels were measured in whole cell lysates. Cellular proteins were analyzed by densitometric analysis of Western blots and normalized to tubulin levels. Control values were assigned a mean value of 100%; experimental values were expressed relative to control values. Molecular weights of cyclin D2, IRS-2, and tubulin were 34, 185, and 40 kDa, respectively. Similar results were obtained in three experiments. * $p<0.05$, **$p<0.01$ vs controls

Table 1
Analysis of gene expression in 832-13 cells by quantitative real time PCR

The table shows the oligonucleotide primer pairs of rat genes used for Q-PCR; mean baseline CT values were obtained in 832-13 cells incubated with a scrambled siRNA (Scr) for 72 hr in RPMI 1640 (11 mM glucose) supplemented with 10% FCS ("serum"); and (b) diluent-treated cells incubated for 24 hr in serum-free RPMI (11mM glucose, "serum-free").

Gene	Accession #	Primer sequence (top forward, bottom reverse)	Mean CT (serum)	Mean CT (serum-free)
PRLR-L	NM_001034111	5′ CCT GCA TCT TTC CAC CAG TTC 3′ 5′ GCA CTC AGC AGT TCT TCA GAC TTG 3′	21	22
p18 Ink4c	NM_131902	5′ ACT CTG AAA TTC TGC CTC AAG TCA 3′ 5′ CCA AGG CTC GGC CAT TCT 3′	21	22
p21 CIP1	NM_080782	5′ GAC ATC TCA GGG CCG AAA AC 3′ 5′ AAT CGG CGC TTG GAG TGA TA 3′	21	24
Menin	NM_019208	5′ TTC AGC TTC ATC ACA GGC ACT AA 3′ 5′ TGG CAG GCC CCT ACC A 3′	22	21
Cyclin A2	NM_053702	5′ CCG CAG CAG AGG CTC AAG 3′ 5′ CAT CGT TTA TAG GAA GGT CCT TAA GAG 3′	21	23
Cyclin B1	NM_171991	5′ TGC TGC AGG AGA CCA TGT ACA 3′ 5′ GCA CAC AAC TGT CCT GCA TGA 3′	20	22
Cyclin B2	NM_001009470	5′ CAG GCA GCT TGA GGC TTT G 3′ 5′ CGT CCG TTT ATG TCT CTT CCA TCT 3′	21	23
Cyclin D1	NM_171992	5′ CGC AAA CAT GCA CAG ACC TT 3′ 5′ GAG GGT GGG TTG GAA ATG AA 3′	22	23
Cyclin D2	NM_022267	5′ GCT ACC TGG ATG CTA GAG GTC TGT 3′ 5′ GGT AAT TCA TGG CCA GAG GAA A 3′	20	20
Cyclin D3	NM_012766	5′ GCC CTC TGT GCC ACA GAT TAC 3′ 5′ GCC CGT GGC GAT CAT G 3′	23	22
CDK1	NM_019296	5′ CGC GTC CCA CGT CAA GA 3′ 5′ AGA CCA GCA TTT TCG AGA GCA A 3′	19	21
CDK2	NM_199501	5′ AAG ACG GAC GGA GCT TGT TAT C 3′ 5′ TTG GCT GAA ATC CGC TTG TT 3′	21	24
CDK4	NM_053593	5′ GTT GGC TGT ATC TTC GCA GAG A 3′ 5′ CAG CCT CAG AGT TCC CAC AGA 3′	19	20
IRS2	NM_001168633	5′ CGC AAG CAT CGA CTT CTT GTC 3′ 5′ GCC CGC AGC ACT TTA CTC TT 3′	20	23
IGF2	NM_001190163	5′ TGT CTA CCT CTC AGG CCG TAC TT 3′ 5′ CCA GGT GTC GAA TTT GAA GAA CTT G 3′	19	22
PDX1	NM_022852	5′ GCC AGT GGG CAG GAG GTG C 3′ 5′ CCT TCT CCA GCT CCA GCA GCT 3′	19	20
FoxMl	NM_031633	5′ TGG CAC CAG GCC TAC CA 3′ 5′ TGG AGA GAA AGG TTG TGA CGA A 3′	22	23
PTTG1	NM_022391	5′ TGC CTA CCC AGA AAT AGA AAA GTT C 3′ 5′ CAG GCA GGT CAA AAC TCT CAA A 3′	20	21
BCL6	NM_001107084	5′ CAA GGC ATT GGG CAA ACA C 3′ 5′ GGC GGG TGA ACT GGA TAC AG 3′	24	25
BCL-XL	X_82537	5′ AAT GAC CAC CTA GAG CCT TGG A 3′ 5′ GCT GCA TTG TTC CCG TAG AGA 3′	21	22
BAX	NM_047558	5′ CCG CCC CAG GAT GCA 3′ 5′ TCC AAT TCG CCG GAG ACA 3′	19	22

Gene	Accession #	Primer sequence (top forward, bottom reverse)	Mean CT (serum)	Mean CT (serum-free)
TLR4	NM_019178	5′ TGA GAC CAG GAA GCT TGA ATC C 3′ 5′ GAG ATT CTG ATC CAT GCA TTG G 3′	28	29
Tph1	NM_001100634	5′ TCA GAA ACT GGC AAC GTG CTA 3′ 5′ CTG CCC ATC TTG CTT GCA 3′	28	29
Actin	NM_031144	5′ CGT GAA AAG ATG ACC CAG ATC A 3′ 5′ GCC TGG ATG GCT ACG TAC ATG 3′	16	16

Table 2

Effects of PRLR knockdown and PRL treatment on gene expression in 832-13 cells

The table shows the effects of PRLR knockdown and PRL treatment on the mRNA levels of various genes demonstrated previously [10,11,22-24] to play important roles in beta cell replication and survival. 832-13 cells were treated with adenoviruses containing scrambled or PRLR siRNAs and incubated in medium containing 10% FBS. mRNA levels were measured after a 72 hr incubation. The effects of PRLR knockdown are compared with the effects of PRL treatment (24 hr in serum-free basal medium) relative to diluent controls. Scrambled and diluent control values were normalized to a mean of 1.0; values in other groups were calculated as a function of control values, as previously described [10,11]. The data are expressed as mean ± SEM of all values obtained in 3-5 independent experiments (n = 17-23). *p< 0.05, ** p<0.01, † p < 0 . 0 0 1 vs controls. Neither PRLR knockdown nor PRL treatment altered significantly the expression of other genes postulated to modulate beta cell replication or survival, including p18, p19, Cyclin D3, CDK2, CDK4, CDK6, IGF2, BAX, or Toll-like receptor 4.

Genes	Scrambled siRNA	PRLR siRNA	Control	PRL
PRLR	1.00±0.02	0.22±0.01 †	1.00±0.04	3.93±0.06 †
p21	1.00±0.02	1.43±0.06 **	1.00±0.07	1.17±0.08
Menin	1.00±0.02	0.84±0.04	1.00±0.02	1.00±0.02
BCL6	1.02±0.06	1.17±0.08 *	1.00+0.04	0.33±0.02 †
BCLXL	1.00±0.04	1.04±0.02	1.00±0.03	1.22±0.02 **
PTTG1	1.00±0.02	0.83±0.03 *	1.00±0.02	1.11±0.02 *
Cyclin A2	1.00±0.01	0.97±0.02	1.00±0.04	1.28±0.04 **
Cyclin B1	1.00±0.01	0.87±0.02 *	1.01±0.06	1.30±0.04 **
Cyclin B2	1.00±0.01	0.85±0.01 *	1.00±0.02	1.18±0.03 *
Cyclin D1	1.00±0.03	1.26±0.03 **	1.00±0.03	0.71±0.03 **
Cyclin D2	1.00±0.01	0.52±0.02 †	1.00±0.01	1.42±0.02 †
Cyclin D3	1.01±0.04	1.00±0.02	1.00±0.02	0.97±0.08
Cyclin E1	1.00±0.01	0.88±0.02	1.00±0.02	1.14±0.09
CDK1	1.00±0.02	0.84±0.02 *	1.00±0.01	1.16±0.03 *
CDK4	1.00±0.01	0.99±0.02	1.00±0.01	1.05±0.02
CDK6	1.00±0.02	0.84±0.02	1.00±0.02	0.97±0.03
IRS-2	1.00±0.01	0.67±0.03 **	1.02±0.09	1.46±0.05 †
Tph1	1.01±0.04	0.75±0.02 **	1.00±0.02	0.97±0.04
FoxM1	1.00±0.02	0.90±0.03	1.00±0.03	1.34±0.05 **