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Julia Halperin, Sangeeta Y Devi, Shai Elizur, Carlos Stocco, Aurora Shehu, et al.. Prolactin signaling through the short form of its receptor represses forkhead transcription factor FOXO3 and its target gene galt causing a severe ovarian defect.. Molecular Endocrinology -Baltimore-, Endocrine Society, 2008, 22 (2), pp.513-22. <10.1210/me.2007-0399>. <hal-00475736>

HAL Id: hal-00475736
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Prolactin Signaling through the Short Form of Its Receptor Represses Forkhead Transcription Factor FOXO3 and Its Target Gene Galt Causing a Severe Ovarian Defect

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Prolactin (PRL) is a hormone with over 300 biological activities. Although the signaling pathway downstream of the long form of its receptor (RL) has been well characterized, little is known about PRL actions upon activation of the short form (RS). Here, we show that mice expressing only RS exhibit an ovarian phenotype of accelerated follicular recruitment followed by massive follicular death leading to premature ovarian failure. Consequently, RS-expressing ovaries of young adults are depleted of functional follicles and formed mostly by interstitium. We also show that activation of RS represses the expression of the transcription factor Forkhead box O3 (FOXO3) and that of the enzyme galactose-1-phosphate uridyltransferase (Galt), two proteins known to be essential for normal follicular development. Our finding that FOXO3 regulates the expression of Galt and enhances its transcriptional activity indicates that it is the repression of FOXO3 by PRL acting through RS that prevents Galt expression in the ovary and causes follicular death. Coexpression of RL with RS prevents PRL inhibition of Galt, and the ovarian defect is no longer seen in RS transgenic mice that coexpress RL, suggesting that RL prevents RS-induced ovarian impairment. In summary, we show that PRL signals through RS and causes, in the absence of RL, a severe ovarian pathology by repressing the expression of FOXO3 and that of its target gene Galt. We also provide evidence of a link between the premature ovarian failure seen in mice expressing RS and in mice with FOXO3 gene deletion as well as in human with Galt mutation. (Molecular Endocrinology 22: 513–522, 2008)
most established functions of PRL in reproduction is its key role in maintaining the ovarian corpus luteum (CL) and progesterone production (21, 22). Indeed, one of the defects seen in PRLR null females is an early involution of the CL and infertility due to insufficient levels of progesterone to support implantation and to maintain the uterus quiescent (23). Beside this defect in the CL of pregnancy, PRLR null ovaries are normal and do not present differences in either follicular development or ovulation rate when compared with wild types (24). Whereas PRL regulation of CL is thought, but yet never proven, to be through activation of RL (21, 22), the impact of RS activation on ovarian development is not at all known.

For the present study, we have generated PRLR<sup>-/-</sup> females overexpressing the RS as the only isoform of the receptor, which makes this animal an ideal model to examine the putative role of the RS in vivo. Our results show, for the first time, that in absence of RL, PRL signaling through RS causes a severe follicular impairment that leads to premature ovarian failure (POF). Furthermore, activation of RS induces down-regulation of Forkhead transcription factor (FOXO3) as well as galactose-1-phosphate uridyltransferase (Galt), two molecules known to be critical for normal ovarian development (25–27). Our results on FOXO3- or Galt mutation to the similar POF displayed by the FOXO3 null females get older, (Figs. 2C and 3C), the ovaries are almost completely depleted of functional follicles and disorganized and the oocytes devoid of cumulus. Those follicles that are in the process of degeneration are the result of follicular death (Figs. 2C and 3C). In females get older, (Figs. 2C and 3C), the ovaries are almost completely depleted of functional follicles and disorganized and the oocytes devoid of cumulus.

RESULTS

Effect of RS Expression on Follicular Development

For the present study, we used females bearing a null mutation in both alleles of the PRLR gene and over-expressing a transgenic construct containing the mouse PR-1 short isoform of the PRLR (PRLR<sup>-/-</sup>RS mice). Because expression of this construct is driven by the elongation factor 1 promoter, RS is ubiquitously expressed in all cell types (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). These mice were generated by crossing PRLR<sup>-/-</sup>RS females with fertile PRLR<sup>+/+</sup> males. C, Homozygous, heterozygous, or null PRLR mRNA expression was detected by using primers for exons 5 and for neo cassette on genomic DNA samples. Expression of transgenic RS was detected by using primers for the terminal block of human GH in the eF1α-PRLR-PR-1 transgenic construct.

Fig. 1. Mice Genotyping by RT-PCR

A, Females expressing only the short form of the prolactin receptor (PRLR<sup>-/-</sup>RS) have been generated by mating PRLR<sup>+/+</sup>RS females with fertile PRLR<sup>-/-</sup> males. Arrows indicate hybridization site for the primers used for genotyping. B, PRLR<sup>+/+</sup> females were obtained by mating PRLR<sup>-/-</sup> females with PRLR<sup>-/-</sup> males. C, Homozygous, heterozygous, or null PRLR mRNA expression was detected by using primers for exons 5 and for neo cassette on genomic DNA samples. Expression of transgenic RS was detected by using primers for the terminal block of human GH in the eF1α-PRLR-PR-1 transgenic construct.

null or the wild-type mice (Fig. 2A). At 2 months of age, the number of secondary, preantral, and antral follicles is markedly increased in PRLR<sup>-/-</sup>RS ovaries (Fig. 2B), indicating that premature follicular development occurs at an early age in these females. Interestingly, however, a severe follicular death begins from 4 wk of age, and by the time these PRLR<sup>-/-</sup>RS females are 4 months old, the ovaries appear severely pathological (Fig. 2C). At this age, these females still cycle and accept the male, yet they cannot be superovulated (Fig. 3). Histological examination shows ovaries with severe follicular impairment and numerous holes that are the result of follicular death (Figs. 2C and 3C). In those follicles that are in the process of degeneration (Fig. 3D, upper panel), the mural granulosa cells are disorganized and the oocytes devoid of cumulus. Without the surrounding granulosa, the oocytes degenerate and lose their content. Finally, the zona pellucida collapses and remains surrounded by theca/interstitial cells (Fig. 3D, upper panel). As PRLR<sup>-/-</sup>RS females get older, (Figs. 2C and 3C), the ovaries are almost completely depleted of functional follicles and are formed mostly by theca and interstitial cells surrounding numerous holes containing collapsed zona pellucida that are the remnant of dead oocytes (Fig.

null or the wild-type mice (Fig. 2A). At 2 months of age, the number of secondary, preantral, and antral follicles is markedly increased in PRLR<sup>-/-</sup>RS ovaries (Fig. 2B), indicating that premature follicular development occurs at an early age in these females. Interestingly, however, a severe follicular death begins from 4 wk of age, and by the time these PRLR<sup>-/-</sup>RS females are 4 months old, the ovaries appear severely pathological (Fig. 2C). At this age, these females still cycle and accept the male, yet they cannot be superovulated (Fig. 3). Histological examination shows ovaries with severe follicular impairment and numerous holes that are the result of follicular death (Figs. 2C and 3C). In those follicles that are in the process of degeneration (Fig. 3D, upper panel), the mural granulosa cells are disorganized and the oocytes devoid of cumulus. Without the surrounding granulosa, the oocytes degenerate and lose their content. Finally, the zona pellucida collapses and remains surrounded by theca/interstitial cells (Fig. 3D, upper panel). As PRLR<sup>-/-</sup>RS females get older, (Figs. 2C and 3C), the ovaries are almost completely depleted of functional follicles and are formed mostly by theca and interstitial cells surrounding numerous holes containing collapsed zona pellucida that are the remnant of dead oocytes (Fig.
Although morphologically the theca/interstitial cells do not seem affected, expression of cytochrome P450 17α-hydroxylase (P450c17), a key enzyme for androgen biosynthesis, is completely absent in PRLR−/− RS ovaries compared with PRLR+/−, indicating that activation of RS has also a negative impact on the steroidogenic capacity of the theca/interstitial cells (Fig. 4A, upper panel).

Early during development, PRLR−/− as well as PRLR−/− RS females ovulate and form CL of pregnancy; however, a regression of these CLs is seen within 2.5 d, and pregnancy cannot be sustained. Both genotypes show similar levels of apoptosis in the regressing CLs (Fig. 4A, lower panel). Subcutaneous implantation of progesterone pellets in PRLR−/− and PRLR−/− RS females allowed a partial rescue of embryos with no significant differences between the two genotypes (Fig. 4B). Because similar expression of both isoforms were found in CL (7, 9), a role for RS on CL was suggested (9). However, the inability of the RS to rescue the CL in pregnant PRLR−/− RS females clearly establishes a key role for RL in the PRL maintenance of a functionally progesterone-producing CL.

The absence of RL expression and the inability to maintain the CL and pregnancy are probably the only similarities between PRLR−/− and PRLR−/− RS ovaries. In contrast to PRLR−/− RS, histological analysis of PRLR−/− ovaries shows normal follicular development (Fig. 3B), suggesting that the follicular defect displayed by PRLR−/− RS ovaries is due entirely to activation and signaling through RS. Interestingly, RS transgenic females that are wild type or heterozygous for the PRLR gene (PRLR+/− RS or PRLR+/−) do not present any sort of ovarian impairment. These
Number of fully developed embryos counted in uterus of brown and hematoxylin-counterstained nuclei in high circulating PRL in PRLR are in agreement with data previously reported for the els as compared with wild-type females. These results males (Fig. 4D), both groups presented high PRL lev-

females are fertile and have normal litters. Although they highly express RS, they display a normal follicular development (Fig. 4C), suggesting that the RL reverses the detrimental effect of RS on follicular development.

Even though no difference in the serum PRL levels was found between PRLR−/− and PRLR−/+ RS females (Fig. 4D), both groups presented high PRL levels as compared with wild-type females. These results are in agreement with data previously reported for the high circulating PRL in PRLR−/− females (28) and support the finding that PRL through RL down-regulates its own synthesis and/or secretion at the hypothalamic and/or pituitary level (29).

Galt Expression Is Repressed by PRL Signaling through RS

Microarray analysis performed with ovarian tissue shows that PRL through RS significantly regulates the expression of more than 80 genes that participate in different biological processes such as immune response, protein metabolism, transport, signal transduction, and cell communication (the entire list of RS-regulated genes can be found in supplemental Table 1). This shows that PRL indeed signals through RS in the ovary and actively regulates the expression of several genes. Interestingly, Galt, whose mutation was shown to induce galactosemia and POF in women (30), is down-regulated in PRLR−/− RS ovaries.

To further analyze the down-regulation of Galt by RS, we examined Galt mRNA levels in ovaries of PRLR+/+, PRLR−/+ and PRLR−/− RS mice by semiquantitative RT-PCR. As shown in Fig. 5A, Galt expression is completely abolished in ovaries of PRLR−/− RS females in contrast to their PRLR null littermates (Fig. 5A). We also examined the ability of PRL to regulate Galt transcription in a subclone of UIII cells (31) that does not express any PRLR. Cells were transiently transfected with Galt promoter-reporter vector and RS expression vector. PRL treatment induced a marked decrease in Galt promoter activity, indicating clearly that PRL acting through RS represses the transcriptional activity of
this enzyme (Fig. 5B). Remarkably, when these cells were transfected with increasing doses of RL expression vector, a clear RL dose-dependent up-regulation of the Galt promoter was observed after PRL treatment (Fig. 5C). Moreover, RS-mediated repression of Galt transcription is reversed by expression of RL (Fig. 5D).

**FOXO3 Expression Is Repressed by PRL Signaling through RS**

The analysis of the full-length mouse Galt promoter sequence revealed 16 putative forkhead transcription factor sites, five of them being FOXO3 sites (Fig. 6A). This attracted our attention because deletion of FOXO3 gene causes an ovarian defect similar to that seen in PRLR−/−;RS mice (25, 26) as well as in women with Galt mutation (24). This finding together with the fact that FOXO3 regulates transcriptional activity of genes involved in glucose metabolism (32) led us to examine whether FOXO3 is repressed by PRL through RS, and whether FOXO3 regulates Galt transcription. As shown in Fig. 6B, FOXO3 is profoundly repressed at mRNA level in the ovaries of PRLR−/−;RS females as compared with their PRLR−/− littersmates.

To further examine the PRL-mediated inhibition of FOXO3, PRLR−/−;RS females were sc injected with 100 μl CB-154 (1 μg/μl 70% ethanol) to block the endogenously produced PRL. Six hours later, they were injected ip with 100 μl PRL (60 μg/100 μl saline), and ovaries were isolated at different times thereafter. Results shown in Fig. 6C (first lane) indicate that FOXO3 is highly expressed in the PRLR−/−;RS ovaries 6 h after CB-154 treatment and before PRL administration. Injection of PRL induced a drop in FOXO3 protein, and within 2 h of PRL treatment, FOXO3 expression was almost completely inhibited.

**Fig. 6.** Activation of RS Represses FOXO3 Activity

A, Schematic diagram shows putative FOXO3 response elements found in the −2879/+391-bp Galt promoter sequence (black arrows). Other forkhead response elements are indicated with gray arrows. The position of each forkhead response element as well as their core similarity is indicated in the table. B, FOXO3a mRNA levels were measured by RT-PCR in PRLR−/− and PRLR−/−;RS ovaries; L19 was used for loading control. Densitometric analysis (right) shows a significant decrease in FOXO3 mRNA levels in PRLR−/−;RS vs. PRLR−/− ovaries: *, *P < 0.001, t test. C, FOXO3 protein levels were analyzed by Western blot in ovaries of PRLR−/−;RS and PRL−/− females injected with PRL for 0, 15, 30, and 120 min. β-Actin was used as an internal loading control. Densitometric analysis shows that whereas PRL causes in vivo a significant decrease in FOXO3 expression in PRLR−/−;RS ovaries (top right; *, P < 0.001 by Dunnett’s multiple comparison post test), it has no inhibitory effect on FOXO3 protein levels in PRLR−/− ovaries (bottom right, P > 0.5).
We examined whether PRL can repress FOXO3 in ovaries expressing both RS and RL. For this experiment, PRL null mice were used because they express both types of receptors but do not produce PRL. As shown in Fig. 6C, PRL has no detectable effect on FOXO3 protein levels in the ovaries of these mice. This suggests that expression of RL may prevent the down-regulation of FOXO3 induced by PRL signaling through RS.

**FOXO3 Enhances Galt Transcription**

To evaluate the role of FOXO3 as a regulator of Galt transcription, the full-length mouse Galt promoter was transfected into HepG2 cells in the presence or absence of either wild-type or constitutively active FOXO3, also known as triple-mutant nonphosphorylatable FOXO3 (33). As shown in Fig. 7A, both FOXO3 expression vectors up-regulate Galt promoter activity. A serial 5′-deletion of Galt promoter revealed that the essential site for FOXO3 stimulation is located between −613 and +21 bp, a region that contains a putative FOXO3 site at −584 bp (Fig. 7B). Surprisingly, mutation of −584-bp FOXO3 response element did not prevent FOXO3-induced stimulation of the promoter. To examine whether this putative FOXO3 site binds to its cognate transcription factor, EMSAs were performed using oligonucleotides containing the −584-bp FOXO3 response element either intact or mutated. The sequence of the FOXO3 consensus binding site-containing oligonucleotide from the IGF-binding protein IGFBP-1 gene [termed insulin-responsive sequence (IRS)] was used as a positive control (33). Incubation of the oligonucleotides with nuclear extract from HepG2 cells revealed the formation of two complexes (Fig. 7C). Competition with excess unlabeled wild-type oligonucleotides and supershift with anti-FOXO3 antibody indicate the specificity of FOXO3 binding to Galt promoter. Interestingly, mutation of the −584-bp FOXO3 response element did not prevent the binding of this transcription factor to this piece of DNA, which suggests that FOXO3 could be stimulating Galt promoter either by binding to a novel response element not yet reported or by associating with a cofactor that binds to Galt promoter, stimulating its activity. In either case, it is clear from these results that FOXO3 enhances Galt transcriptional activity.

**DISCUSSION**

In the present study, we report that mice expressing only RS, specifically the PR-1 isoform of the receptor, have a severe ovarian impairment. From the three short isoforms reported in mouse (8), PR-1 has the highest homology with the rat and, more importantly, is the one that shows specific binding of PRL and mitogenic responsiveness (20). Whether the other two short isoforms have the same deleterious effect on the ovary remains to be investigated.

Our data show that PRLR−/− RS ovaries have a premature follicular development followed by massive follicular cell death. Although the signaling pathway downstream of RS still needs to be determined, we clearly show that PRL signaling through RS represses FOXO3 and Galt, which are important for normal follicular development. Normally, Galt is highly expressed in ovaries and liver, which are the major sites of expression for this enzyme (34). Galt participates in the metabolism of galactose to glucose (35). Deficiency in Galt activity leads to accumulation of galactose metabolites, which causes ovarian toxicity (27, 34). This toxicity is explained by the synergism of two metabolites, galactose-1-phosphate and galactitol. Accumulation of galactose-1-phosphate is thought to inhibit enzymes involved in glucose metabolism, leading to deficient glycosylation reactions and decrease in energy production in the ovarian cells. As to galactitol, it is a galactose metabolite that cannot pass through the cellular membrane, and its accumulation into the cells causes an osmotic disequilibrium that
leads to water influx and ultimately to cell death. In women, either mutations in Galt gene or a deficiency in enzyme activity causes a disease known as galactosemia associated with POF (27, 30, 34). Young women with this disease are fertile early in life but due, in some patients, to a failure of the RL is intriguing and deserves further investigation. We have generated a mouse model for this type of POF and provide an explanation as to how PRL acting through its short cognate receptor can lead to POF.

Our findings that activation of RS by PRL represses FOXO3 and Galt and that FOXO3 stimulates Galt transcriptional activity provide an interesting and novel link between the POF seen in mice expressing RS and mice with FOXO3 gene deletion and in women with Galt mutation.

MATERIALS AND METHODS

Animal Model

RS transgenic females were originally generated by microinjecting the eF1α-PRLR-PR-1 transgenic construct encoding the mouse cDNA for RS into fertilized PRLR+/− oocytes derived from 129 Sv pure background mice (20). This construct is driven by the elongation factor 1 promoter, which makes it ubiquitously expressed in the tissues along all stages of development. These PRLR+/− RS females are fertile, and by overexpressing the RS, they can rescue the mammary development defect displayed by PRLR+/− females (20).

For the present study, we have generated females expressing only the short form of the prolactin receptor (PRLR−/− RS) by mating PRLR+/− RS females with fertile PRLR−/− males. The PRLR+/− females were obtained by mating PRLR+/− females with PRLR+/− males.

Animals were identified by RT-PCR on genomic DNA purified from tail using direct PCR lysis reagent (Viagen Biotech, Inc., Los Angeles, CA) (Fig. 1). For PRLR gene expression, the forward primers were 5′-GAG GAG CAA GAT CTC AAG AAC CC A-3′ for the wild type and 5′-CCA GTC CCT TCC CGC TTC AGT-3′ for the mutated (Neo) strand, and the reverse primer was 5′-TGG ACC CAA GAT TTC AGT-3′. For RS transgenic expression, forward 5′-GAG TGG ACC CAA GTC AAC-3′ and reverse 5′-ACT GAG TGG ACC CAA CGC AT-3′ primers for the human GH terminator present in the eF1α-PRLR-PR-1 transgenic construct were used (Fig. 1). The cycling parameters for the PRLR consisted of one cycle of 94°C for 5 min and then 35 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 45 sec followed by a single cycle of 5 min at 72°C for extension. The molecular size for the wild-type product is 350 and 580 bp for the mutant. For the RS transgenic construct, the cycling parameters were 94°C for 5 min and then 30 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min followed by a single cycle of 5 min at 72°C for extension, and the molecular size is 750 bp. RT-PCR products were electrophoresed on a 1% agarose gel using 100-bp PCR markers (Invitrogen, Carlsbad, CA) as standards to determine the molecular size.

Animals were kept under conditions of controlled light (0700–1900 h) and temperature (22–24°C) with free access to standard rodent chow and water.

Experimental Animals

All experimental procedures were performed in accordance with the Guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.
Tissue Preparation and Histology

For histological analysis, cycling females at different ages were killed at estrus. The ovaries were dissected and either frozen in liquid nitrogen for RNA and protein extraction or fixed either in Bouin or 10% formalin for histological examination. Tissues were serially sectioned (5 μm) and stained with hematoxylin-eosin. Follicular counting was performed in all sections of each ovary. Follicles that contained oocytes with clearly visible nuclei were scored, and the total number of follicles at any particular developmental stage was calculated as the sum of follicles from all sections of an ovary.

To examine whether the interstitial/thecal tissue left in the ovaries of mice expressing only RS express P450c17, ovarian sections were incubated overnight at 4°C with a primary polyclonal antibody to P450c17 and then incubated with a secondary biotinylated goat antirabbit IgG according to the manufacturer’s instructions (Vectorstain ABC kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was developed with Nova Red solution (Vector) in a humidified atmosphere of 5% CO2 at 37°C.

Superoovulation Protocol and Progesterone Pellet Implantation

To induce superovulation, PRLR+/+, PRLR−/−, and PRLR−/RS female mice were injected ip with 5 IU pregnant mare serum [equine chorionic gonadotropin (eCG)] (Sigma Chemical Co., St. Louis, MO) followed by 5 IU hCG (Sigma) 48 h later. Ovaries were then isolated for histological examination. The difference in the number of released oocytes was determined by one-way ANOVA followed by Dunnett’s multiple comparisons post test that allows comparisons against a control (wild type).

To determine whether PRLR+/+ and PRLR−/−RS females can maintain pregnancy, they were mated with fertile PRLR+/+ males, and the day that a vaginal plug was found, a progesterone pellet (25 mg; Innovative Research of America, Sarasota, FL) was sc implanted. These females were maintained until the time of normal parturition. The difference in the number of fully developed embryos for each genotype was determined by one-way ANOVA followed by Dunnett’s multiple comparisons post test.

PRL Hormone Assay

After anesthesia, retroorbital blood samples were taken from cycling PRLR−/−, PRLR+/−, RS, and PRLR−/− females, and PRL levels were measured by RIA, at the National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA. The statistical differences were determined by one-way ANOVA followed by Dunnett multiple comparisons post test.

RNA Extraction and RT-PCR

RNA was extracted using TRIzol reagent (Life Technologies, Rockville, MD) following the manufacturer’s protocol. Ovarian RNA from 2-month-old females was transcribed into cDNA by superscript polymerase II. Custom oligonucleotide primers were obtained from Life Technologies and used to amplify the appropriate cDNA templates by PCR. Mouse Galt, FOXO3, and L19 mRNA expression was detected using 5'-CAG TAC CCG GTG TCG CAT AGT-3' (forward), 5'-GGG CTG CTG ACA GTC TCT GC-3' (reverse); and 5'-AGC GCC TTC AGG CCA AGG-3' (forward), 5'-CCA GGC CGC TAT GTA CAG ACA CGA-3' (reverse) primers, respectively. PCR product size for Galt, FOXO3, and L19 were 217, 400, and 100 bp, respectively.

Conditions for each template were optimized so that signals were in the linear range of detection. The PCR products with DNA loading buffer were then separated by gel electrophoresis on a 0.7% agarose gel. L19 concentrations were used as internal control for comparison.

Western Blot Analysis

Total ovary lysates were prepared by homogenizing the tissues in RIPA buffer (1× PBS, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 μM sodium orthovanadate, 10 μg/ml Bovine serum, and 30 μl/mi aprotinin. Proteins were resolved on 8.5% denaturing polyacrylamide. After gel electrophoresis, proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The blots were incubated 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (pH 8.0) containing 0.1% Tween 20. Blots were washed and incubated overnight at 4°C with FOXO3 monoclonal antibody (1:10,000 dilution; Upstate Biotechnology, Lake Placid, NY) and then incubated with a secondary antibody linked to horseradish peroxidase for 1 h at room temperature. β-Actin (Abcam Inc., Cambridge, MA) was used as internal loading control. Complexes were visualized using the West Pico chemiluminescence detection kit (Pierce Biotechnology, Inc., Rockford, IL).

Microarray Analysis

Total RNA was extracted from 2-month-old PRLR null (control) and PRLR+/− RS ovaries using Atlas Glass Total RNA Isolation Kit and reverse-transcribed with PowerScript reverse transcriptase (BD Biosciences, San Diego, CA). cDNA was purified with the Atlas NucleoSpin, and hybridized overnight at 60°C with Atlas plastic mouse 5K oligo microarrays membranes (BD Biosciences) carrying cDNA probes for approximately 5000 known mouse genes according to the manufacturer’s instructions. Membranes were washed and exposed to a phosphorimaging screen overnight. The intensity of spots was assessed using Atlas Image 2.0 software. The intensity of each gene was averaged from two individual spots. A cDNA synthesis control was used as a positive control and for grid template alignment. The values were normalized using six housekeeping genes (ubiquitin, tyrosine 3-monoxygenase, ornithine decarboxylase, gyceraldehyde-3-phosphate dehydrogenase, cytoplasmic β-actin, and 40S ribosomal protein S29). Genes were excluded if they were detected in only one spot or at levels near or below background. Differences over 2-fold in the intensity of the spots were considered significant.

Cell Lines and Culture

Human hepatic carcinoma cells (HepG2) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Eagle’s MEM (with Eagle’s balanced salt solution and L-glutamine) supplemented with 10% fetal bovine serum, 1000 U/ml penicillin G, 2.5 μg/ml amphotericin B, 1000 μg/ml streptomycin, 1 mM sodium pyruvate, and 1× nonessential amino acids. Rat uterine stromal cells (UllI) were cultured in M199 medium (with phenol red and L-glutamine) supplemented with 10% fetal bovine serum, 1000 U/ml penicillin G, 1000 μg/ml streptomycin, 1 mM sodium pyruvate, and 1× nonessential amino acids. Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C.
Galt Promoter Reporter Constructs

The −21/−342-bp Galt promoter-reporter truncation was cloned by PCR from genomic DNA using primers generated with the Primer3 software (supplemental Table 2). Restriction sites for Smal and HindIII were added to the primers. PCR was performed using 1 μg mouse genomic DNA as a template. PCR product was cloned into pGEMTeasy (Promega, Madison WI) according to the manufacturer’s instructions. After sequencing, it was digested with Smal and HindIII (dual digestion in OnePhor All Buffer; Amersham), purified using the GeneClean II kit (Qbiogene Inc., Irvine, CA) according to the manufacturer’s instructions, and subcloned in pGL3 basic luciferase reporter vector (Promega).

To generate the −613/−379-bp Galt promoter-reporter construct, the full-length promoter-constructor was digested with VspI, Klenow blunt-ended (Invtrogen), purified by phenol extraction, digested with Ncol, and run in a 1.5% agarose gel. The construct was purified using the GeneClean II kit (Qiogene Inc.) and ligated in pGL3 basic luciferase reporter vector (Promega). Mutation of the putative FOXO3 response element located at −584 bp of this promoter-reporter truncation was created using the Stratagen (La Jolla, CA) QuikChange II kit according to the manufacturer’s instructions and confirmed by sequence analysis after cloning into pGEMTeasy and subcloning into pGL3 basic. Four mutations (underlined letters) were introduced into the primer with three of them in the core sequence of the FOXO3 response element (5′-GGT GTG CAC CAC CAC TGG CCG TT 3′), respectively, with the underlined letters showing the mutation positions) were end-labeled with [γ-32P]ATP (Amersham). Nuclear extracts (1 μg) were incubated for 30 min in binding buffer together with 1 × 106 cpm labeled oligonucleotides. Six micrograms FOXO3 antibody (Upstate) were added to binding buffer for the supershift. Samples were electrophoresed for 2.5 h on 4% nondenaturing polyacrylamide gels. Gels were dried and exposed to x-ray film for 18–48 h.

Acknowledgments

We thank P. A. Kelly for helpful comments, N. D. Horsemann for the PRL null mice, D. Linzer for the FOXO3 construct; B. Hales for the P450c17 polyclonal antibody, and K. Heretis for excellent technical help.

Received August 20, 2007. Accepted October 25, 2007.

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This work has been supported by National Institutes of Health HD11119, U54 HD 40093, and HD 12356 (G.G.), Institut National de la Santé et de la Recherche Médicale (N.B.), and an American Physiological Society Postdoctoral Fellowship in Physiological Genomics (J.H.).

Disclosure Summary: The authors have nothing to disclose.

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