Effects of luteinizing hormone on peroxisome proliferator-activated receptor γ in the rat ovary before and after the gonadotropin surge

Jayeeta Banerjee and Carolyn M Komar

Department of Animal Science, Iowa State University, 2356 Kildee Hall, Ames, Iowa 50011-3150, USA

Correspondence should be addressed to Carolyn M Komar; Email: ckomar@iastate.edu

Abstract

We have shown previously that mRNA for peroxisome proliferator-activated receptor γ (PPARγ) is expressed in granulosa cells and downregulated by the luteinizing hormone (LH) surge. The current studies were undertaken to test the hypothesis that LH stimulates a decrease in the expression of PPARγ, as well as its activity, in granulosa cells. Ovaries were collected from immature rats 0 and 48 h after they received pregnant mares’ serum gonadotropin (PMSG), and 4 and 24 h after administration of human chorionic gonadotropin (hCG), and used for protein isolation or processed for immunolocalization of PPARγ. The amount of phosphorylated PPARγ was measured by immunoblot analysis to determine how LH affects the phosphorylation status, and therefore the activity, of PPARγ. Granulosa cells were also collected from immature rats 48 h after PMSG. Cells were cultured with LH in the absence and presence of H89 and cycloheximide to investigate the role of PKA and protein synthesis in the LH-mediated decline in mRNA for PPARγ respectively. Protein corresponding to PPARγ was localized to nuclei of granulosa cells 0 and 48 h after PMSG. Expression was greatly reduced by 4 h after hCG, with expression in mural granulosa cells lost before that in cumulus cells. The amount of phosphorylated PPARγ did not change during the periovulatory period. Blocking PKA activity had no effect on levels of mRNA for PPARγ. However, levels of mRNA for PPARγ were significantly increased in cells treated with cycloheximide (P < 0.05, ANOVA followed by Tukey’s HSD). These data suggest that PPARγ is tightly regulated in the ovary and that its expression is the primary mechanism by which LH influences the activity of PPARγ. In addition, protein synthesis may be involved in modulating levels of PPARγ in granulosa cells.


Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) has been identified in ovarian tissue of man (Lambe & Tugwood 1996), cattle (Sundvold et al. 1997, Lührke et al. 1998), sheep (Froment et al. 2003), pigs (Schoppe et al. 2002), rats (Braissant et al. 1996, Komar et al. 2001) and mice (Cui et al. 2002). Mohan et al. (2002) reported that PPARγ is also expressed in bovine oocytes. Our laboratory (Komar et al. 2001) and others (Lührke et al. 1998, Mu et al. 2000, Schoppe et al. 2002, Veldhuis et al. 2002, Froment et al. 2003, Lovekamp-Swan et al. 2003) have shown that PPARγ can also influence ovarian steroid production. Agonists of PPARγ have been reported to modify steroid production by both follicular (Komar et al. 2001, Schoppe et al. 2002, Veldhuis et al. 2002, Froment et al. 2003, Lovekamp-Swan et al. 2003) and luteal cells (Lührke et al. 1998, Mu et al. 2000).

PPARγ is a member of the PPAR family of nuclear hormone receptors. This transcription factor is predominantly known for its role as an adipocyte differentiation factor (reviewed by Debril et al. 2001). PPARγ also plays a role in lipid and glucose homeostasis, inflammation, the cell cycle, and cancer (reviewed by Escher & Wahli 2000, Berger & Moller 2002). It plays a critical role in placental development and function, as illustrated by disrupted trophoblast differentiation and placental vascularization in mice lacking PPARγ (Barak et al. 1999).

Aside from the roles mentioned above, there are several other mechanisms by which PPARγ could affect ovarian function. PPARs can directly regulate the expression of the rate-limiting enzyme involved in prostaglandin production, cyclooxygenase-2 (COX-2) (Meade et al. 1999), and the proteolytic enzymes, MMP-3 (Yee et al. 1997) and MMP-9 (Marx et al. 1998, 1999, Shu et al. 2000). PPARγ can also regulate the expression of vascular endothelial...
growth factor (Yamakawa et al. 2000), and could thereby affect angiogenesis, which is critical for follicular and luteal development. Genes involved with cell-cycle regulation (reviewed by Theocharis et al. 2004) and cell survival (Butts et al. 2004) are also regulated by PPARγ.

There are various mechanisms regulating the activity of PPARγ. PPARγ can be ubiquinated (Hauser et al. 2000), sumoylated (Ohshima et al. 2004) and nitrated (Shibuya et al. 2002), reducing its ability to affect gene transcription. The activity of PPARγ can also be modified by phosphorylation. The A/B domain in the amino-terminus of this receptor contains mitogen-activated protein kinase sites at serines 84 (Adams et al. 1997) and 112 (Hu et al. 1996). Phosphorylation of PPARγ decreases its activity (Hu et al. 1996, Adams et al. 1997, Reginato et al. 1998, Camp et al. 1999, Hsi et al. 2001), and increases its degradation by facilitating the targeting of PPARγ to the cellular ubiquitin-proteosome system (Floyd & Stephens 2002).

Although a great deal has been learned about PPARγ since its discovery over a decade ago, not much is known about the role of PPARγ in the ovary. Previous work from our laboratory has shown that mRNA for PPARγ is primarily localized in granulosa cells, and is downregulated in response to the luteinizing hormone (LH) surge (Komar et al. 2001). Interestingly, cells that responded to the LH surge lost expression of mRNA for PPARγ, whereas those that did not respond to the surge maintained PPARγ expression (Komar & Curry 2003).

The LH surge plays a pivotal role in the ovarian cycle. Genes essential for ovulation and luteinization are turned on by the LH surge (i.e., progesterone receptor) (Lydon et al. 1995), whereas genes not required for these processes, or that may interfere with ovulation or luteinization, are downregulated (i.e., cyclin D2) (Robker & Richards 1998). One mechanism that may result in amplification of the effects of the LH surge on ovarian gene expression is regulation of PPARγ, and therefore PPARγ-regulated genes. Therefore, the following experiments were conducted to investigate the effects of the LH surge on the expression and activity of PPARγ in the ovary. We tested the hypothesis that the LH surge stimulates a decrease in the amount of PPARγ, as well as its activity, in periovulatory granulosa cells. Our findings show that the expression pattern of PPARγ closely parallels that of its mRNA, and that protein synthesis may be involved in regulating the expression of PPARγ.

Materials and Methods

Materials

Unless otherwise noted, all chemicals came from Sigma or Fisher Scientific (Pittsburgh, PA, USA). Antibodies against PPARγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antiphosphoserine antibody was purchased from Upstate (Lake Placid, NY, USA), and the antiaCTin antibody was obtained from Oncogene Research Products (San Diego, CA, USA). Pregnant mares’ serum gonadotropin (PMSG) and LH were purchased from the NIDDK’s National Hormone and Peptide Program (Torrance, CA, USA). H89 was obtained from Alexis Biochemicals (San Diego, CA, USA).

Animals

All animal procedures were approved by Iowa State University’s Animal Care and Use Committee. Animals were housed in a controlled environment with a 14:10 light:dark cycle, and had free access to food and water. Female, Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) were injected with 10 IU PMSG subcutaneously on day 23 of age. Forty-eight hours later, animals received 10 IU human chorionic gonadotropin (hCG) subcutaneously to stimulate ovulation and luteal development. Animals were killed and their ovaries collected 0 (no PMSG) and 48 h after receiving PMSG, and 4 and 24 h after hCG (n = 4–5 animals/time point). Ovaries were frozen and stored at −70°C until used for protein extraction, or fixed in 4% paraformaldehyde overnight at 4°C. Fixed tissues were embedded in paraffin for immunohistochemical analysis.

The role of protein kinase A (PKA) and protein synthesis in the LH-mediated decline in PPARγ was investigated with cultured granulosa cells collected from PMSG-primed animals. On day 23 of age, female rats received 10 IU PMSG subcutaneously. After 48 h, granulosa cells were collected as described previously (Mann et al. 1991).

Immunohistochemistry

PPARγ was immunolocalized in fixed ovarian tissues collected from rats 0 and 48 h after PMSG, and 4 and 24 h after hCG. Tissues were sectioned at 4–5 μm, dewaxed, and rehydrated. After quenching endogenous peroxidase activity in 1.5% hydrogen peroxide in methanol, tissue sections were blocked with 10% normal goat serum. The PPARγ antibody (E-8; 1:100 dilution) was applied overnight at 4°C. After rinsing, sections were incubated with a biotinylated secondary antibody (Amersham) and subsequently treated with a peroxidase/streptavidin conjugate (Vector Laboratories, Burlingame, CA, USA). The immunocomplex was detected by incubating the tissue sections with a dianinobenzidine solution (Zymed Laboratories, San Francisco, CA, USA). Normal goat serum was used in place of the anti-PPARγ antibody as a control. At least nine tissue sections from each of 3–4 animals/time point were analyzed.

Immunoprecipitation and immunoblot analysis of PPARγ

Protein corresponding to PPARγ was immunoprecipitated from frozen rat ovarian tissue (n = 3–4/time point). Ovaries were homogenized in RIPA buffer (1× phosphate buffered saline (PBS), 1% NP-40, 0.5% sodium deoxycho
late and 0.1% sodium dodecyl sulfate (SDS) containing phenylmethylsulfonyl fluoride (100 μM) and sodium orthovanadate (1 mM). The homogenate was centrifuged, and 50 μl/ml protein A agarose (Repligen, Waltham, MA, USA) were added to the supernatant. After incubation and subsequent centrifugation, the supernatant was collected and 2 μg/ml anti-PPARγ antibody (H-100) was added and incubated. A volume of 50 μl protein A was added to the solution, and, after incubation, the solution was centrifuged. The supernatant was discarded and the pellet washed with RIPA buffer. The resultant pellet was resuspended in 40 μl SDS buffer (0.019 M Tris–HCl, 0.186 M glycine and 0.05% SDS).

To determine the phosphorylation status of PPARγ, equal amounts of immunoprecipitate from ovarian tissue samples collected 0 and 48 h after PMSG, and 4 and 24 h after hCG were loaded onto a 10% polyacrylamide gel, separated by electrophoresis and transferred to a nitrocellulose membrane (Amersham). Membranes were blocked with 5% milk and incubated with an antiphosphoserine antibody (MPM-2; 1:500 dilution). After washing, membranes were treated with a horseradish peroxidase-labeled secondary antibody (1:1000 dilution), and the immunocomplex was visualized with an enhanced chemiluminescence (ECL) kit (Amersham). Membranes were stripped and subsequently processed for immunodetection of PPARγ (E-B) to verify immunoprecipitation. Membranes were exposed to Kodak X-OMAT autoradiography film from 15 s to 5 min to ensure linearity of the signal. Films were scanned, and densitometry of the bands was conducted with AlphaEase 4.0 (Alpha Innotech, San Leandro, CA, USA).

**Cell culture**

Granulosa cells were cultured as previously described (Komar et al. 2001) with the following modifications. To investigate the role of PKA in the LH-mediated decline in mRNA for PPARγ, granulosa cells were cultured with LH (100 ng/ml) in the presence and absence of H89 (10 or 30 μM), an inhibitor of PKA. LH and H89 were added to the cells at the time of plating. Cells were collected after 24 h culture and lysed with lysis buffer (Ambion, Austin, TX, USA). Cellular lysate was stored at −70°C until analyzed by a direct lysate RNA protection assay. Progesterone was measured in conditioned media by RIA (Diagnostic Products, Los Angeles, CA, USA) (Komar et al. 2001). Treatment with H89 resulted in approximately 24% and 72% decreases in basal and LH-stimulated progesterone production, respectively.

Cells were also treated with cycloheximide to determine the role of protein synthesis in the LH-stimulated decrease in mRNA for PPARγ. Fetal calf serum (5%) was used in the culture media in place of BSA. Cycloheximide (10 μg/ml) was added to cells at the time of plating, and LH (100 ng/ml) was administered to the cells 30 min later. After 5-h culture, cells were collected and processed as described above.

**Quantification of mRNA for PPARγ**

Plasmids containing cDNAs for PPARγ (generously provided by Dr Walter Wahli, Université de Lausanne, Lausanne, Switzerland) and the ribosomal protein L32 (a gift from Dr O-K Park-Sarge, University of Kentucky, Lexington, KY, USA) were linearized with EcoRI. Antisense riboprobes were transcribed with Ambion’s Maxiscript kit and α-32P-UTP. Lysate ribonuclease protection assays (RPA) were carried out as described previously (Jo et al. 2002). Briefly, granulosa cell lysates were hybridized overnight with excess radiolabeled antisense riboprobes for PPARγ and L32. Protected fragments were analyzed by PAGE. Relative levels of mRNA for PPARγ and L32 were quantified with a phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA). The band intensity of mRNA for PPARγ was normalized to the corresponding band for L32 per sample.

**Statistical analysis**

Differences in levels of mRNA and protein corresponding to PPARγ, and the amount of phosphorylated PPARγ in ovarian tissues were analyzed by ANOVA. Post-hoc comparisons were made with Tukey’s HSD test. P < 0.05 denoted significant differences.

**Results**

Previously, we reported that mRNA for PPARγ was localized primarily to granulosa cells of developing follicles, and that administration of hCG to PMSG-primed rats resulted in a significant decrease in levels of mRNA for PPARγ within 4 h (Komar et al. 2001). In the current study, protein corresponding to PPARγ was analyzed in ovarian tissue collected at defined times before and after the LH surge to determine whether the gonadotropin surge modified its expression and/or functional status.

PPARγ was immunolocalized in ovarian tissue collected from immature rats 0 and 48 h after PMSG, and 4 and 24 h after hCG. PPARγ was expressed almost exclusively in granulosa cells of developing follicles, and was immunolocalized to the nucleus (Fig. 1). Expression of PPARγ was relatively high in granulosa cells during follicular development (0 and 48 h after PMSG) (Fig. 1A and B). However, by 4 h after hCG, granulosa cells in some follicles lost expression of PPARγ, while PPARγ expression remained high in other follicles (Fig. 1C). Interestingly, after the gonadotropin surge, the expression of PPARγ within the follicle appeared to decline in a progressive manner. PPARγ was highly expressed in some granulosa cells, but absent from others within the same follicle (indicated by arrows in Fig. 1C). The cumulus granulosa cells appeared to retain expression of PPARγ longer than mural.
granulosa cells (Fig. 1C). By 24 h after hCG, expression of PPARγ in luteal tissue was very low (Figs 1D and 2A). Cells within the newly forming luteal tissue did express PPARγ, albeit at a much lower level than what was observed in follicular cells (Fig. 2A).

Interestingly, in tissue collected from one animal 48 h after PMSG, the theca externa of a few follicles expressed PPARγ at a high level in a relatively uniform pattern (data not shown). Aside from certain follicles in this one animal, the expression of PPARγ in the theca externa of other follicles in this animal, and in the other animals studied, was inconsistent and nonuniform (Fig. 1).

As mentioned above, the phosphorylation status of PPARγ can influence its activity. To determine whether the LH surge influences the activity of PPARγ by modifying the phosphorylation status of this transcription factor, PPARγ was immunoprecipitated from ovarian tissue collected during the periovulatory period. The phosphorylation status of PPARγ was determined both before and after the gonadotropin surge, and was expressed as a ratio of the amount of total PPARγ in the sample. Levels of phosphorylated PPARγ tended to decrease 0–48 h after PMSG (reduced by 25%; $P > 0.05$), and then remained steady throughout the remainder of the periovulatory period (data not shown).

Figure 1 Immunolocalization of PPARγ in ovarian tissue sections collected from rats 0 (A, E) and 48 (B) h after PMSG, and 4 (C) and 24 (D) h after hCG ($n = 3–4$ animals/time point). Tissue sections (4–5 μm) were processed as described in Materials and Methods. Protein corresponding to PPARγ was identified by the brown reaction product. The tissue section pictured in (E) was treated with normal goat serum in place of the anti-PPARγ antibody. Arrows indicate granulosa cells expressing high levels of PPARγ (black) versus those that no longer express PPARγ (gray) within the same follicle. CL: corpus luteum. × 100.
The LH surge sets in motion a cascade of events that culminates in ovulation and luteinization. To begin investigating the second messenger systems involved in the LH-mediated decline in the expression of PPARγ in follicular cells, granulosa cells were treated with LH in conjunction with H89, an inhibitor of PKA. As seen in Fig. 3, H89 had no effect on the basal expression of mRNA for PPARγ. However, administration of LH to the cultured cells reduced levels of mRNA for PPARγ by 54% compared with controls ($P < 0.05$) (Fig. 3). This reduction in mRNA for PPARγ in response to LH was not affected by cotreating the cells with H89 (Fig. 3B).

The role of protein synthesis in the LH-stimulated reduction in PPARγ was also investigated by treating granulosa cells in vitro with cycloheximide. As seen in the previous experiment, treating the cells with LH caused a significant decrease in levels of mRNA for PPARγ ($P < 0.05$) (Fig. 4). When the cells were treated with both LH and cycloheximide, the levels of mRNA for PPARγ were not different from those in control cells. Interestingly, granulosa cells treated with cycloheximide alone had 30% more mRNA for PPARγ than did controls ($P < 0.05$) (Fig. 4B).

Figure 3 Levels of mRNA for PPARγ in granulosa cells collected 48 h after PMSG and cultured with vehicle (control), LH (100 ng/ml) and/or H89 (10 or 30 μM; $n = 4$ independent experiments). (A) Representative autoradiograph of direct lysate RNase protection assay demonstrating the protected fragments of mRNA corresponding to PPARγ and the ribosomal protein L32. (B) Relative levels of mRNA for PPARγ after correcting for the amount of L32 in each sample. Data are expressed as means ± S.E.M. Bars with no common superscripts are significantly different ($P < 0.05$). C: control.
Discussion

Data from various studies suggest that PPARγ plays a critical role in normal ovarian function. Using cre/loxP technology, Cui et al. (2002) disrupted the expression of PPARγ in granulosa cells, as well as B and T cells, and the mammary gland. One-third of these transgenic females were infertile, and the remaining two-thirds suffered from subfertility (Cui et al. 2002). The authors hypothesized that the fertility problems may have been caused by disruption of PPARγ in the ovary, resulting in insufficient ovarian function to support implantation. In addition, studies by Froment et al. (2003) and Swan and Chaffin (2004) have shown that endogenous PPARγ is functional in granulosa cells. In these studies, granulosa cells were transiently transfected with reporter constructs whose expression was driven by peroxisome proliferator-activated receptor response elements. Both in the absence and presence of agonists for PPARγ, there was an increase in reporter activity (Froment et al. 2003, Swan & Chaffin 2004), indicating that PPARγ is functional in granulosa cells and that endogenous ligand is also present within these cells (Swan & Chaffin 2004).

Although PPARγ has been identified in ovarian cells from various species, there is a paucity of information regarding how this transcription factor is regulated in the ovary, and what genes it targets in ovarian cells. We reported previously that LH regulates the expression of mRNA for PPARγ in granulosa cells of developing follicles (Komar et al. 2001). In the current study, we show that the expression of protein corresponding to PPARγ parallels that of its mRNA. Taken together, these findings indicate that levels of both mRNA and protein corresponding to this transcription factor are tightly regulated in the ovary by LH.

Further evidence supporting tight regulation of PPARγ in the ovary comes from the observed increase in basal levels of mRNA for PPARγ in granulosa cells treated with cycloheximide. Blocking protein synthesis led to significant accumulation of mRNA for PPARγ. This finding shows that there is rapid turnover of mRNA for PPARγ in granulosa cells.

The high level of PPARγ in developing follicles, both mRNA (Komar et al. 2001) and protein (current study), implies that this transcription factor is important in granulosa cell development and function. Since these processes depend on sequential and temporal patterns of gene expression, regulation of transcription factors would be
one way to ensure that gene expression is tightly controlled. The current study demonstrates that LH decreases the expression of PPARγ, but does not affect its phosphorylation status. Therefore, the activity of PPARγ in ovarian cells may be regulated moreso by controlling its expression, or by modifications other than phosphorylation.

LH can stimulate various second-messenger systems, including PKA and phospholipase C (Davis et al. 1986). The current study shows that the decline in PPARγ is not mediated by the activity of PKA. High doses of LH, such as those attained during the LH surge, are associated with the activation of phospholipase C as well as PKA (Morris & Richards 1993, Flores et al. 1998, and references therein). Therefore, the effect of LH on the expression of PPARγ may be mediated by activation of phospholipase C. Tyrosine kinases are also activated by LH (Davis 1994) and may be involved in regulating expression of PPARγ. Studies are currently underway in our laboratory to determine the intracellular signaling mechanisms behind the LH-induced decline in expression of PPARγ.

The observed progression in the decline of PPARγ in periovulatory granulosa cells, mural cells losing expression before the cumulus cells, is most likely the result of a direct effect of LH. Because cumulus cells lack LH receptors (Peng et al. 1991), they do not respond directly to changes initiated by the LH surge, but rather indirectly via factors produced by neighboring mural granulosa cells.

Some effects of the LH surge are mediated by the progesterone receptor (Lydon et al. 1995, Robker et al. 2000, Shao et al. 2003). However, it is unlikely that the progesterone receptor plays a role in the effect of LH on the expression of PPARγ. Blocking activation of the progesterone receptor in cultured rat granulosa cells by treating them with ZK98299 did not affect the LH-mediated decline in PPARγ (unpublished data, M Jo and C Komar). In addition, there is an inverse relationship between the LH-mediated decrease in PPARγ and increase in the progesterone receptor; PPARγ declines by 4 h after hCG (Komar et al. 2001, current study), whereas the progesterone receptor increases 3–6 h after hCG (Park & Mayo 1991, Shao et al. 2003).

The LH-mediated reduction in PPARγ may be one mechanism by which the gonadotropin surge affects changes in gene transcription allowing for ovulation, and luteinization of follicular cells. The rapid decline in PPARγ in response to the LH surge, and low level of expression in developing luteal cells, indicates that genes transcriptionally regulated by PPARγ are not important agents in, and in fact may interfere with, ovulation and luteinization. For example, the downregulation of aromatase and cyclin D2, and upregulation of COX-2 in response to the LH surge, is important for the follicular/luteal phase shift and ovulation (reviewed by Richards 1994, Richards et al. 1998). Interestingly, these genes have been shown to be regulated by PPARγ in nonovarian tissues and cells (Meade et al. 1999, Laurora et al. 2003, Fan et al. 2005).

In cultured granulosa cells from PMSG-primed immature rats (Lovekamp-Swan et al. 2003) and human granulosa-lutein cells (Mu et al. 2000), activation of PPARγ reduced the expression of aromatase. The inhibitory effect of PPARγ on aromatase expression is through disrupting the interaction of NF-κB with the aromatase promoter II (Fan et al. 2005). Within 6 h of the gonadotropin surge, there is a significant decline in the expression of aromatase in granulosa cells (Hickey et al. 1988). We reported previously that there was no correlation between the expression of mRNAs for PPARγ and aromatase in granulosa cells during folliculogenesis or the periovulatory period (Komar & Curry 2003). These observations, taken together with the fact that PPARγ is expressed in granulosa cells coincidently with estradiol production, suggest that this transcription factor may not inhibit aromatase in the ovary under normal, physiologic conditions.

Cyclin D2 has a similar profile of expression to PPARγ. Like PPARγ, cyclin D2 is expressed in granulosa cells of developing follicles and downregulated within 4 h of the LH surge, but only in follicles that responded to the gonadotropin surge (Robker & Richards 1998). There are conflicting reports of how activation of PPARγ affects cyclin D2. In human leukemic cells, activation of PPARγ resulted in a decline in mRNA and protein for cyclin D2 (Laurora et al. 2003). However, administration of troglitazone, a PPARγ agonist, to cultured rat granulosa cells had no effect on cyclin D2 (Lovekamp-Swan & Chaffin 2005). Froment et al. (2003) reported that treating granulosa cells from sheep with a PPARγ agonist decreased granulosa cell proliferation. More work investigating the role of PPARγ in granulosa cell-cycle progression is needed to address the apparent paradox of PPARγ inhibiting cell proliferation yet being expressed at a high level in developing granulosa cells.

Because the function of PPARγ varies depending on cell type and experimental parameters, it is difficult to define a role for PPARγ in the ovary by extrapolating from studies in other systems. The current study shows that the expression of PPARγ is tightly regulated in the ovary, and that its expression appears to be the primary way this transcription factor is influenced by LH. The pattern of expression of PPARγ and its ability to affect a number of cellular functions suggest that its role in the ovary may be dictated by its relationship to other factors during the ovarian cycle. Because of its ability to influence many cellular functions, more work is needed to define the role of PPARγ in the ovary in order to clarify how it is regulated and what genes it regulates during the ovarian cycle.

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