Immunoreactivities of PPARγ2, leptin and leptin receptor in oviduct of Chinese brown frog during breeding period and pre-hibernation

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Abstract

The Chinese brown frog (*Rana dybowskii*) is a special amphibian with one unique physiological phenomenon, which is that its oviduct expands prior to hibernation, instead of during the breeding period. In this study, we investigate the localization and expression level of PPARγ2, leptin and leptin receptor proteins in oviduct of *Rana dybowskii* during breeding period and pre-hibernation. There were significant variations in oviductal weight and size, with values much lower in the breeding period than in pre-hibernation. PPARγ2 was observed in stromal and epithelial cells in both periods. Leptin was immunolocalized in epithelial cells in both periods, whereas leptin receptor was detected only in stromal cells. Consistently, the protein levels of PPARγ2, leptin and leptin receptor were higher in pre-hibernation as compared to the breeding period. These results suggested that oviduct was the target organ of leptin, which may play an important paracrine role in regulating the oviductal hypertrophy during pre-hibernation.

Introduction

Oviduct is a dynamic organ that goes through significant morphological, biochemical, and physiological modifications throughout the reproductive cycle. It is not only a passive channel for gamete and embryo transport, but also encompasses a highly active secretory organ involved in several critical reproductive events, such as estrous cycle and ovulation.[1] Oviduct is regulated by a wide variety of factors, including locally synthesized molecules working in an collaborative, synergistic, or antagonistic manner to regulate different oviductal functions, such as gene expression, protein synthesis, morphology with sexual maturation and reproductive activity.[2] Oviductal fluid is essential for the oviduct to perform its reproductive functions,[3] which offers an optimal microenvironment for biological functions that contribute to sperm capacitation, final oocyte maturation, fertilization and early embryo development.[4,5] Oviductal fluid is consisted of hundreds of macromolecules which can be secreted from oviduct epithelium or serum transudate.[6,7]

The Chinese brown frog (*Rana dybowskii*) is a unique amphibian species in northeastern China. It is a seasonal breeder with the habit of seasonal migration between mountain and wetland. *Rana dybowskii* is a famous economic species which has been used widely in the Traditional Chinese Medicine.[8] The hibernation for *Rana dybowskii* takes place from October to February, which is followed by the breeding period ranging from February to June depending on the latitude and altitude. Interestingly, one unique physiological phenomenon of *Rana dybowskii* is that its oviduct expands during pre-hibernation but not during the breeding period. Besides, dried oviduct of the female *Rana dybowskii*, Oviductus Ranae, is one of the best-known and highly valued oriental foods and Chinese crude drugs, which is recorded in the Pharmacopoeia of the People's Republic of China.[9] Traditional Chinese medicine holds that Oviductus Ranae can nourish the yin, moisten the lung and replenish the kidney essence.[10] Meanwhile, Oviductus Ranae is mainly composed of proteins and lipid, which are up to 50% or more.[11]

Vast published literature has been made in our understanding of adipocyte differentiation and adipocyte-specific gene expression. Peroxisome proliferator-activated receptor (PPAR)-γ is a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily. It has been proven to be involved in directing expression of fat-specific genes and in activating the program of adipocyte differentiation.[12] PPARγ can also induce trans-differentiation from fibroblasts and myoblasts to adipocytes.[13,14] Besides, PPARγ has two main isoforms: PPARγ1 and PPARγ2.[15] Adipose tissue is the most abundant site for both isoforms.[16] The difference is that PPARγ2 is restricted in fat,[17] whereas PPARγ1 is predominant in other tissues.[18] Adipose tissue contributes to the regulation of energy homeostasis, secreting a large number of active adipokines.[19] Leptin is one of the best-characterized adipokines, which affect carbohydrate and lipid metabolism and energy.[20] Leptin is a 16-kDa polypeptide hormone coded by the *obese (ob)* gene.[21] In addition to regulating energy homeostasis, leptin is also a vital hormone/cytokine for a number of diverse physiological processes such as reproduction, angiogenesis, inflammation, and immune function.[22] The leptin receptor (Ob-R, also known as LEP-R, LR) was first isolated from mouse choroid plexus by expression cloning and is a member of the interleukin-6 receptor family of class I cytokine receptors.[23] Ob-Rb is the long form of the leptin receptor and has a long cytoplasmic region containing several motifs required for signal transduction.[24] Leptin binding to this receptor induces conformational changes in the intracellular receptor domain.[25] Many studies have shown that dietary intake and fat stores regulate the production of leptin, which then enters the circulatory system and binds to leptin receptor in multiple tissues, modulating numerous other physiological processes, including body temperature, energy regulation, immune reaction, reproduction and development.[26,27] Leptin and its receptor have also been shown to express in ovary and testis,[28,29] as well as in several other tissues of the reproductive tract.[30]

Our previous study has shown that *c-kit* and proliferating cell nuclear antigen (PCNA) had higher expressions in the pre-hibernation oviduct, which suggested that as the intrinsic regulator including the *c-kit* receptor might play a regulatory role in oviductal cell proliferation.[31] In order to elucidate the relationship between leptin and oviductal hypertrophy during pre-hibernation, this study investigated the expression of PPARγ2, leptin and leptin receptor proteins in the oviduct of *Rana dybowskii* during the breeding and pre-hibernation periods.

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Materials and Methods

Animals

Totally, 40 adult female Chinese brown frogs were obtained in April (n=20), and October (n=20), 2012 from Jilin Paektu Mountain Chinese Brown Frog Breeding Farm, Jilin Province (125°40 E-127°56 E, 42°31 N-44°40 N), China. All these animals were treated in accordance with the National Animal Welfare Legislation. All experimental procedures were carried out in accordance with the guidelines established by the Beijing Forestry University. The animals were euthanized by 4% isoflurane before tissue removal. The weight of each female frog was measured using scales. Each pair of oviducts was obtained from each body, and the weight of oviduct was measured after necropsy. One side of oviduct was immediately fixed for 24 h in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 M PBS, pH 7.4 for histological and immunohistochemical observations; the other side of oviduct was immediately frozen and stored at -20°C for Western blotting detection.

Histology

The oviduct samples were dehydrated in ethanol series and embedded in paraffin wax. Serial sections (4 μm) were mounted on slides coated with poly-L-lysine (Sigma). Sections were stained with hematoxylin-eosin (HE) for observations of general histology.

Immunohistochemistry

The serial sections of the oviduct tissues were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with primary polyclonal antibody (1:500) against leptin (Y-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), leptin receptor (H-300) (Santa Cruz Biotechnology) and PPARγ2 (bs-7114R) (Beijing Biosynthesis Biotechnology Co., Beijing, China) for 12 h at 4°C. The control sections were treated with normal rabbit serum (Sigma) instead of the primary antisera. The sections were then incubated with a second antibody for 0.5 h at room temperature, goat anti-rabbit lgG conjugated with biotin and peroxidase with avidin, The sections were visualized using a rabbit ExtrAvidin™ staining kit (Sigma) in 150 mL of 0.05 M Tris-HCl buffer, pH 7.6 containing 30 mg 3, 3-diaminobenzidine (Wako, Tokyo, Japan) plus 30 μL H_2O_2. Finally, the reacted sections for leptin and leptin receptor were counterstained with hematoxylin solution (Merck, Tokyo, Japan).

Western blot

The oviducts were weighed and diced into small pieces using a sterilized razor blade, respectively. The tissue was homogenized in a homogenizer containing 300 μL of 10 mg/mL PMSF stock and incubated on ice for 30 min while maintaining the temperature at 4°C throughout all the procedures. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C. Protein extracts (25 μg) were mixed with an equal volume of 2× Laemmli sample buffer. Equal amounts of each sample were loaded and run on an 8% and 13% SDS-PAGE gel at 18V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The concentration of SDS used for protein gel and protein sample buffer was 10%. The membranes were blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membranes was carried out using a 1:500 dilution of rabbit anti-rat leptin antibody, rabbit anti-rat leptin receptor and rabbit anti-rat PPARγ2 for 1 h. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 1 h. Finally, the membrane was colored with 10 mg 3,3-diaminobenzidine (Wako) solution in 50 mL phosphate buffer (0.03

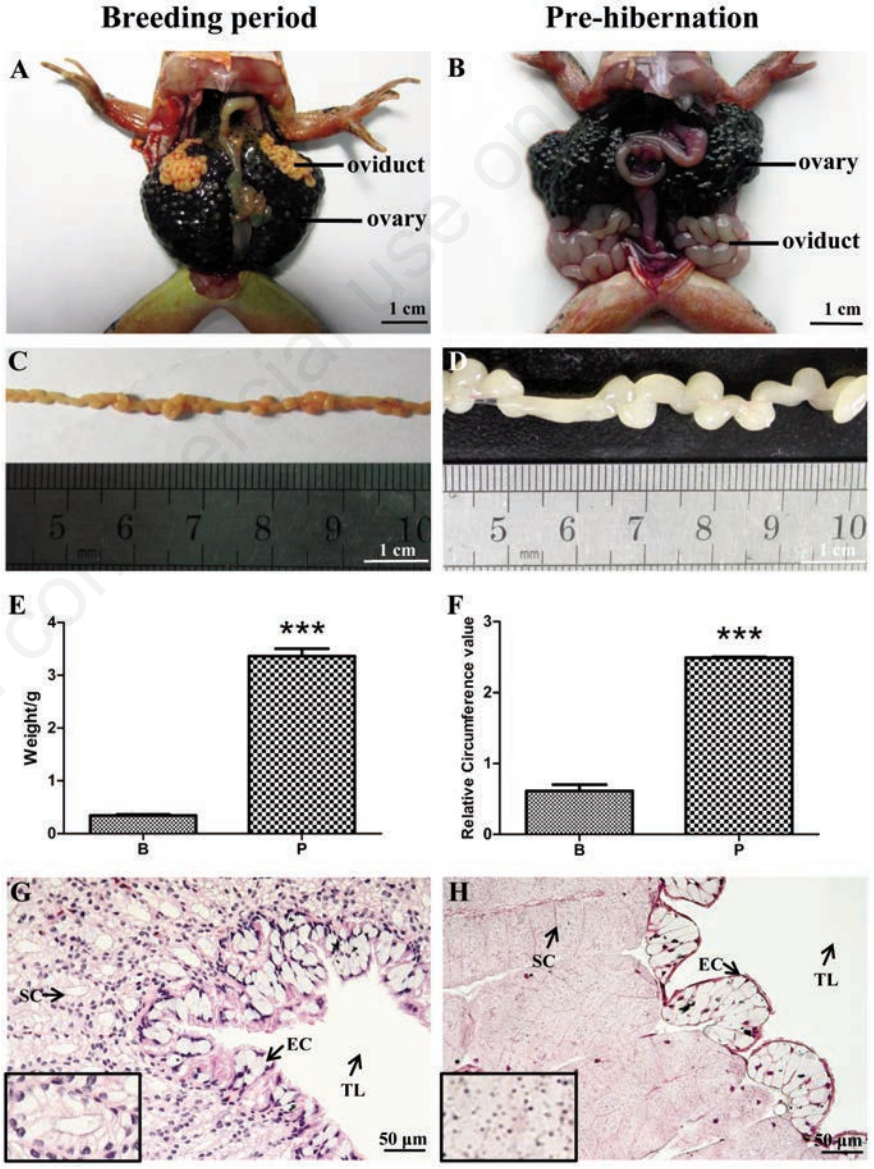

Figure 1. Seasonal changes in oviductal morphology of *Rana dybowskii*. Topography and morphology of the oviduct during the breeding period (A,C) and pre-hibernation (B,D). Changes in weight (E) and relative circumference (F) of oviduct between the breeding period and pre-hibernation. Histological structure of the oviducts in the breeding period (G) and pre-hibernation (H). EC, epithelial cell; SC, stromal cell; TL, tubule lumen.

M) plus 3 μL H_2O_2. β-actin was used for the endogenous control. Preabsorptions of the antibodies were performed with an excess of relative antigens (Sigma) for the negative control.

Statistical analysis

Statistical comparisons were made with the Students *t*-test. A value of P<0.01 was considered indication of statistical significance.

Results

Seasonal changes in oviductal morphology and histology between breeding period and pre-hibernation

Morphological differences were observed in regard to the oviduct of *Rana dybowskii* during breeding period and pre-hibernation (Figure 1 A-D). The weight of the oviduct in pre-hibernation (3.37±0.14) was significantly larger than that of the breeding period (0.34±0.02) (Figure 1E). Similar differences between pre-hibernation (2.49±0.01) and breeding period (0.61±0.09) were also seen for relative circumference values (Figure 1F). The HE staining also revealed a significant histological variance. As shown in Figure 1 G,H, the oviduct of *Rana dybowskii* mainly comprises epithelial cells and lobules that consisted of stromal cells.

Immunohistochemical localization of PPARγ2, leptin and leptin receptor in *Rana dybowskii* oviduct during breeding period and pre-hibernation

PPARγ2, leptin and leptin receptor were detected in oviduct of *Rana dybowskii* during breeding period and pre-hibernation (Figure 2). Positive signal of PPARγ2 was localized in the nucleoli in both epithelial and stromal cells of breeding period and pre-hibernation (Figure 2 A,B). Immunohistochemical reaction for leptin was observed in the cytoplasm of epithelial cells of breeding period and pre-hibernation (Figure 2 C,D), whereas leptin receptor was immunostained only in stromal cells (Figure 2 E,F). The intensities of the immunohistochemical signals for PPARγ2, leptin and leptin receptor were all higher in the pre-hibernation than those in the breeding period (Table 1).

Expression level of PPARγ2, leptin and leptin receptor proteins in *Rana dybowskii* oviduct of the breeding period and pre-hibernation

The results of Western blot analysis for PPARγ2, leptin and leptin receptor in *Rana dybowskii* oviduct of the breeding period and pre-hibernation are shown in Figure 3. Bands

Table 1. Relative abundance of PPARγ2, leptin and leptin receptor in *Rana dybowskii* oviduct during breeding period and pre-hibernation.

	PPARγ2		Leptin		Leptin receptor	
	B	P	B	P	B	P
Epithelial cell	+	+	++	++/+	-	-
Stromal cell	++	++/+	-	-	++	+++

B, Breeding period; P, pre-hibernation. The immunohistochemical staining was determined as positive (+), strong positive (++), very strong positive (+++), and negative (-). Staining that was weak but higher than control was set as positive (+); the highest intensity staining was set as very strong positive (+++); staining intensity between + and +++ was set as strong positive (++).

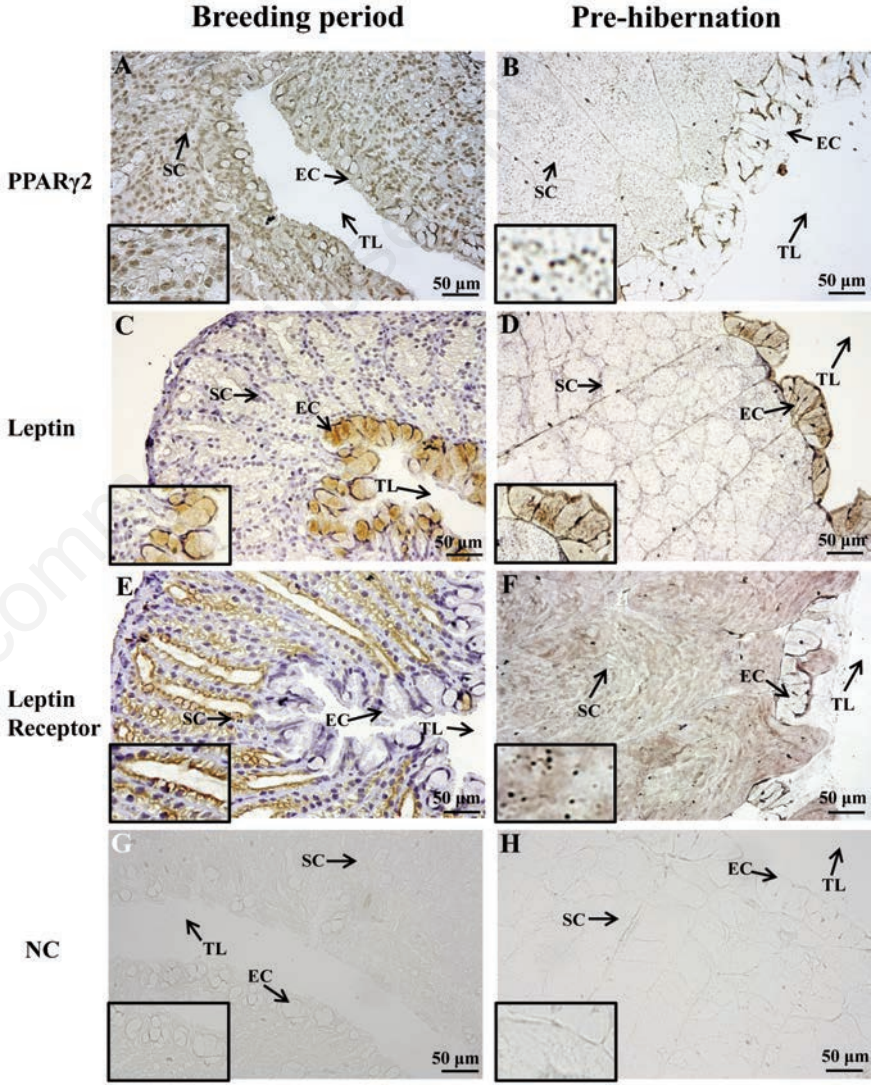

Figure 2. Immunohistochemical localization of PPARγ2, leptin and leptin receptor in *Rana dybowskii* oviduct during breeding period and pre-hibernation. Positive signal of PPARγ2 was localized in the nucleoli of both epithelial and stromal cells in breeding period and pre-hibernation (A,B), and stronger positive-staining was shown in pre-hibernation (B). Immunohistochemical reaction for leptin was observed in the cytoplasm of epithelial cells of breeding period and pre-hibernation (C,D), whereas leptin receptor was immunostained only in stromal cells (E,F). G,H) Negative controls. EC, epithelial cell; SC, stromal cell; TL, tubule lumen; NC, negative control.

of approximately 52kDa for PPARγ2 (Figure 3A), 16kDa for leptin (Figure 3B) and 132kDa for leptin receptor (Figure 3C) were identified in lysates, in consistent with previously reported in rat.[12,32] The results were normalized to the expression level of β-actin. The expression of PPARγ2, leptin and leptin receptor was significantly high in pre-hibernation as compared to the breeding period. Protein extracted from the fat tissue of rat was used for the positive control (Figure 3, lane PC). The primary antibodies preabsorbed with an excess amount of the antigens were used for the negative control (Figure 3, lane NC).

Discussion

The present study indicated for the first time that PPARγ2, leptin and its receptor proteins were expressed in oviduct of *Rana dybowskii* and revealed different expression patterns of these proteins during breeding period and pre-hibernation. Moreover, leptin was immunolocalized in epithelial cells of oviduct in both periods, whereas leptin receptor was immunostained only in stromal cells. These findings suggested that leptin was produced in oviduct of *Rana dybowskii* and may play an important paracrine regulatory role in oviductal hypertrophy during pre-hibernation. The present results provided a basis for future investigation of other regulatory factors in oviductal hypertrophy of *Rana dybowskii*.

Ovarian estrogens control development of sex accessory structures such as the hypertrophy of oviduct prior to sexual maturation and during each season prior to ovulation.[33] The oviducts regress when estrogen synthesis declines after breeding.[33] In *Rana dybowskii*, the oviductal hypertrophy took place during pre-hibernation, instead of the breeding period. Histological observation showed that epithelial cells and a large number of stromal cells were observed in the expanded oviductal tissues of pre-hibernation. Moreover, significantly higher values of oviductal weight and size (relative circumference value) were also found in pre-hibernation when compared to those of the breeding period. These findings implied that the hypertrophy of oviduct was independent of ovarian steroid hormones, which might be speculated that locally produced intrinsic regulators might play a regulatory role in the oviductal hypertrophy of *Rana dybowskii.* PPARγ is a ligand-dependent transcription factor that is predominantly expressed in adipose tissue, both white and brown.[34] PPARγ plays an important promoting role in the differentiation of adipocytes.[35] Recent reports have showed that PPARγ was also involved in processes that were critical to normal female reproductive function, for example, PPARγ was expressed strongly in the granulosa cells of rat, mouse, and sheep, as well as in oocytes from cattle, zebrafish, Xenopus, and human,[36] indicating that PPARγ may be an important player regulating ovarian gene expression. Since PPARγ1 distributed widely in many tissues, and only PPARγ2 can express in fat,[17,18] we focus on PPARγ2 to investigate oviductal hypertrophy. In the present study, PPARγ2 was immunolocalized in stromal and epithelial cells of oviduct during the breeding period and pre-hibernation. The expression level of PPARγ2 protein was significantly higher in pre-hibernation as compared to the breeding period. In addition, our previous studies have shown that higher contents of *c-kit* receptor and PCNA proteins were present in pre-hibernation oviductal tissues.[31] These results implied that as an intrinsic regulator, PPARγ2 might play a regulative role in oviductal hypertrophy of *Rana dybowskii* during pre-hibernation.

Previous studies showed that changes in the level of PPARγ expression could cause or contribute to the regulation of leptin expression, or that both genes are responding to a common pathway or transacting factor(s).[12] More recently, leptin has been shown to play a role in other target reproductive organs, such as the endometrium, placenta, and mammary gland, with corresponding influences on important physiologic processes such as menstruation, pregnancy, and lactation.[37] In this study, leptin was immunolocalized in epithelial cells of oviducts during the breeding period and pre-hibernation, whereas leptin receptor was observed only in stromal cells. The presence of leptin and leptin receptor proteins were further confirmed by Western blot, suggesting *in situ* synthesis and secretion of leptin and leptin receptor in this organ, as well as the possibility of a paracrine regulatory role of leptin in the *Rana dybowskii* oviducts. These findings are not limited to this amphibian though, as similar observations have also been reported in other species. By using an *in vitro* model, leptin was produced in the porcine oviduct, making it spatially available to interact with its receptor during pre-implantation development.[38] In mice, leptin is expressed in both the oviduct and uterus during early pregnancy, and

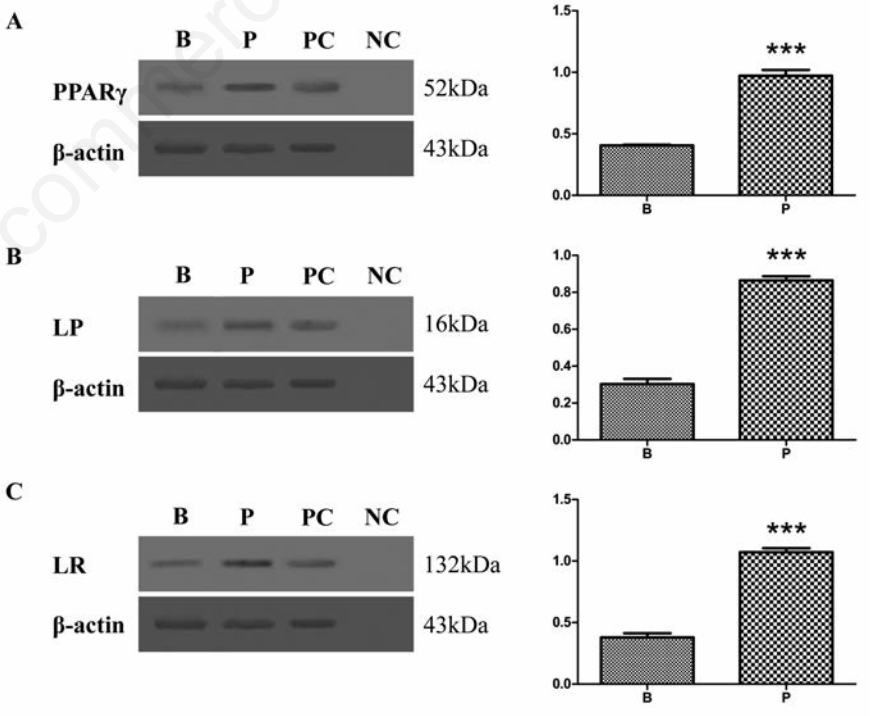

Figure 3. Western blot of of PPARγ2, leptin and leptin receptor in the oviduct tissues of *Rana dybowskii* during breeding period (B) and pre-hibernation (P). Positive bands of PPARγ2, leptin and leptin receptor were observed in the position of about 52kDa (A), 16kDa (B) and 132kDa (C) respectively. β-actin was used as controls to correct for loading in each lane. PC, positive control; NC, negative control. The expression levels were determined by densitometric analysis. Bars represent means ±SD for three independent experiments; asterisks indicate significant difference (P<0.01).

is thus temporally and spatially available for early embryo development.[39,40] Moreover, in the human oviduct, expression of leptin and its receptor has been detected by *in situ* hybridization and immunohistochemistry, suggesting that leptin could act in a paracrine manner to regulate biological functions of the oviduct.[41] Studies in domestic hen also suggested that oviduct may be a target tissue for leptin, where it may participate in egg formation and/or its transport through the oviduct.[42] Taken together, the present results were in accordance with the views that leptin may be produced by maternal organs such as oviduct and/or these embryos themselves, and act in a paracrine manner to regulate biological functions.[39]

Leptin can not only act directly on adipose tissue to increase preadipocyte proliferation,[43,44] but also induce an increase in body weight and the relative weights of the liver, spleen, pancreas, kidneys, and small intestine without any changes in triglycerides, glucose and cholesterol levels.[45,46] Previous studies have shown that oviduct and endometrial epithelium may produce and secrete leptin to the reproductive tract, and leptin can promote the development of mouse oviduct and preimplantation embryos through its receptor.[39,40] This study revealed that the mass change of oviducts and oviductal histological appearance were parallel to those in the expression patterns of oviductal leptin and its receptor proteins during the breeding period and pre-hibernation, implied that leptin might play a regulatory role in oviductal hypertrophy during pre-hibernation. Similar results were obtained in pigs and mice, the up-regulation of leptin and its receptor expression in the oviduct during early pregnancy compared to non-pregnant pigs and mice suggests that leptin expression was regulated in stage specific manner.[38,39] In humans, Leptin signaling appeared to be essential for endometrial cell adhesion, proliferation, survival and migration,[47,48] enhanced the proliferative activity of both the normal myometrium and myoma cells in primary culture.[49] In addition, the *in vitro* effects of leptin on the rabbit oviduct suggested that the oviduct could be a potential target for endocrine regulation by leptin, and leptin was involved in a large array of regulatory actions required for normal reproductive functions.[50] Thus, these observations suggested that given the wide distribution of leptin and its receptor in different sites of the reproductive organs, leptin may act as the critical link between adipose tissue and the reproductive system, indicating whether adequate energy reserves are present for normal reproductive function.[37] The present findings provided further evidence in support of these views.

This study demonstrated, for the first time, the seasonal expression of PPARγ2, leptin and leptin receptor proteins in oviductal tissues of *Rana dybowskii* during the breeding period and pre-hibernation. The dynamic regulation of PPARγ2 suggested that active cell differentiation might take place in the pre-hibernation oviduct. Furthermore, the distinct localization and immunoreactivity of leptin and leptin receptor indicate that oviduct might be the target organ of leptin, which possibly play an important paracrine role in regulating the oviductal hypertrophy of *Rana dybowskii* during pre-hibernation.

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