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Expression and function of leptin and its receptor in mouse mammary gland

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Leptin is an autocrine and paracrine factor which affects the development of duct, formation of gland alveolus, expression of milk protein gene and onset involution of mammary gland. In order to know the function and mechanism of leptin in mammary gland, the protein expression and localization of leptin and its long form receptor (OB-Rb) were detected by a confocal laser scanning microscope. To study the impacts of leptin on mammary gland and leptin signal transduction pathway in pregnancy-, lactation- and involution-stage mammary gland, explants were cultured and Western blotting was used. The results showed that in the whole development cycle of mammary gland, the expression of leptin and OB-Rb was in positive correlation. In virgin the leptin expression was the highest and then decreased in pregnancy. In lactation the expression of leptin was low and upgraded in involution, and recovered to the original level about virgin on involution 13 d. The localization of leptin and OB-Rb revealed that leptin induced the expression of OB-Rb specifically and controlled the development and physiological function of the mammary gland by binding to OB-Rb. In pregnancy stage, leptin stimulated proliferation and differentiation of ductal epithelial cells by JAK-MAPK signal pathway. In lactation, leptin induced gene expression of β-casein by JAK-STAT5 signal pathway, and in involution leptin induced mammary epithelial cell apoptosis and mammary gland restitution by JAK-STAT3 signal pathway.

mammary gland, leptin, OB-Rb, expression, signal transduction

Leptin is mainly, but not exclusively, an adipocyte-secreted protein[1] that is involved in the regulation of food intake and energy balance[2], as well as in normal sexual maturation and reproduction[3]. Recently, there is increasing evidence that it also acts as an autocrine and paracrine factor to influence the development and lactation of mammary gland. It promotes the development of duct[4], formation of gland alveolus, expression of milk protein genes and onset involution, which is necessary for the development and function of mammary gland. At present, the research about leptin is only limited to the expression of leptin on mRNA[5] and the impacts of leptin on proliferation and differentiation of mammary epithelial cells simply[6]. Only in ovine mammary gland, was leptin protein located by immunohistochemical analysis partly during pregnancy and lactation[7]. There is no research about leptin and its receptor's expression in protein on development, lactation, and involution mammary gland of mouse systematically.

In order to reveal the relationship between the expression and localization of leptin and its long form receptor (OB-Rb) in the whole development cycle of mammary gland, and make clear the impact and mechanism of leptin on mammary gland in pregnancy, lactation and involution, mouse was used to investigate the expression and localization of leptin and OB-Rb in protein in the whole development cycle by a confocal laser scanning microscope. By culturing mammary gland *in vitro*, the impacts of leptin on duct development in pregnancy, milk protein expression in lactation and

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mammary gland involution after withdrawing offspring were investigated. In the end, the specific signal transduction pathways of leptin via OB-Rb in pregnancy, lactation and involution mammary gland were elucidated by immunoblotting.

1 Materials and methods

1.1 Materials

Female mice were purchased from Harbin Medical University. Ob antibody (sc-9014) was purchased from Santa Cruz Biotechnology; Rabbit Anti-Leptin receptor (long form), FITC-conjugated goat anti-rabbit IgG and goat serum were from Boster; PhosphoPlus Stat3 (Tyr705) Antibody Kit, PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) Antibody Kit and PhosphoPlus Stat5 (Tyr694) Antibody Kit were from Cell Signaling Technology; Waymouth's MB 752/1 was purchased from Gibco; Collagenase III was from Worthington Biochemical Corporation; Recombinant murine letpin was purchased from Peprotech; β-casein (from bovine milk), insulin, prolactin, hydrocortisone and propidium iodide were purchased from Sigma.

The following instruments were used in our experiments: confocal laser scanning microscope (Leica TCS SP2), capillary electrophoresis (Beckman System5000), refrigerated centrifuge (Beckman), ultrapure water apparatus (Poll), CO_2 incubator (Sanyo), semi-dry transfer electrophoresis (Bio-Rad).

1.2 Expression and localization of leptin and OB-Rb in mammary gland

The fourth pairs of abdominal mouse mammary glands from virgin, pregnancy, lactation and involution mice were made into frozen sections of about 8 μm thick, and then fixed with cold acetone. Fixed sections were rinsed with PBS (pH 7.4) for three times, and then incubated with 10% goat serum for 2 h at 37℃. For labeling leptin and OB-Rb protein, tissue sections (n = 3) were incubated with rabbit anti-mouse leptin antibody and rabbit anti-leptin receptor (long form) antibody separately overnight at 4℃, and then rinsed with PBS (pH 7.4) for three times. After being incubated with goat anti-rabbit IgG for 30 min at 37℃, tissue sections were rinsed with PBS (pH 7.4), and then the nucleus was stained with pI. After rinsing with deionized water twice and mounting with DABCO, the sections were observed by a confocal laser scanning microscope.

1.3 Mammary gland organ culture

The fourth pairs of abdominal mouse mammary glands from pregnancy 12 d, lactation 12 d and involution 5 d mice were prepared under sterile condition. The left mammary gland was used as control and the right was treated with leptin. Each individual gland was placed in a 3.5-cm culture well and cut into 6 uniformly-sized pieces to facilitate growth factor access. Glands as control were cultured in medium consisting of Waymouth's MB 752/1, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL PRL and 5 μg/mL hydrocortisone. Glands as treatment were cultured in medium consisting of Waymouth's MB 752/1, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL PRL, 5 μg/mL hydrocortisone and 5 μg/mL leptin. The culture wells were maintained at 37℃ with dioxide of 95% oxygen and 5% carbon. The medium was changed on the 3rd day and the cultures were terminated on the 5th day.

After being cultured, the mammary glands of pregnancy 12 d and involution 5 d were made into paraffin sections and the impact of leptin on the structure was observed; for lactation mammary glands, the medium was used to detect the protein level of β-casein after being cultured for 24, 48, 72, 96 and 120 h.

For leptin signal transduction pathway research, mammary glands were cultured for 48 h in medium Waymouth's MB 752/1 with or without leptin (5 μ/mL), and then the total protein was extracted from the mammary epithelial cells.

1.4 Total protein extraction from mammary epithelial cells

Mammary tissues were removed from culture and rinsed in cold PBS. To enrich epithelial cells, tissues were minced and digested with 0.2% collagenase III for 3 h at 37℃. The digested tissues were then centrifuged at 6000×g for 5 min at 4℃, and the floating adipocytes and supernatant were discarded. The remaining epithelial-enriched cell pellet was washed thoroughly with PBS to remove collagenase and homogenized on ice in 100—200 μL of lysis buffer containing SDS. Tissue lysates were sonicated on ice. Then 15 μL of lysate was boiled for 5 min, and electrophoresed through SDS polyacrylamide gel.

1.5 Western blot analysis

The lysate was electrophoresed through an 8% SDS

polyacrylamide gel, and transferred onto nitrocellulose. Membranes with transferred protein were incubated for 1 h in blocking solution, and then treated according to the introduction of PhosphoPlus Stat3(Tyr705) Antibody Kit, PhosphoPlus p44/42 MAPKinase (Thr202/Tyr204) Antibody Kit and PhosphoPlus Stat5(Tyr694) Antibody Kit. Bands were visualized using enhanced chemiluminescence.

1.6 Statistical analysis

Expression of leptin and OB-Rb was analyzed by using Image Pro 5.0 Plus and the data were analyzed with SPSS. The data comparisons came from the same sample which was treated by different methods by *t*-test. Multitude group data comparisons were analyzed by analysis of variance.

2 Results

2.1 Expression and localization of leptin and OB-Rb

Expression of leptin and OB-Rb is shown in Figure 1.

In the whole development cycle, the tendency of leptin and OB-Rb expression was similar and the expression level of leptin was higher than that of OB-Rb.

In details, the expression of leptin and OB-Rb was the highest in virgin, and then decreased in pregnancy. On pregnancy 16 d, the end of branch ducts formed gland alveolus mostly, so the expression of leptin and OB-Rb upgraded slightly and then decreased. In the whole lactation, leptin and OB-Rb expression kept lowest. From lactation 16 d, the expression of leptin and OB-Rb upgraded significantly and recovered to the original level about virgin on involution 13 d. Statistical analysis showed that the protein expression of leptin and OB-Rb was in positive correlation and coefficient correlation was 0.941.

In virgin and early pregnancy, leptin was found in adipocytes, ductal epithelial cells and basal lamina around the ducts, OB-Rb was expressed in adipocytes and ductal epithelial cells; in late pregnancy and lactation, leptin was detected in alveolus epithelial cells near the basal lamina and extracellular matrix, OB-Rb was detected on the membrane of alveolus epithelial cells near the basal lamina; in involution, leptin and OB-Rb were localized in adipocytes and ductal epithelial cells again (Figure 2).

2.2 Impact of leptin on mammary gland

In pregnancy, leptin stimulated duct branches increased (Figure 3).

In lactation, the expression of β-casein increased by treatment with leptin (Figure 4). Culturing lactation mammary gland *in vitro*, the content of β-casein in medium increased from 24-h culture to 96-h culture ($P < 0.05$), and then decreased at 120 h culture ($P<0.05$). There was no significant change in control medium ($P > 0.05$).

In involution, mammary duct lumina disappeared and gland epithelial cells cords formed in treatment with leptin. But in the control group, the branch ducts remained (Figure 5).

2.3 Leptin signal transduction pathway in mammary gland

The results of Western blot showed that in pregnancy, leptin induced the phosphorylation of p44/p42MAPK specifically but not STAT3 and STAT5 (Figure 6).

In lactation, leptin induced the phosphorylation of

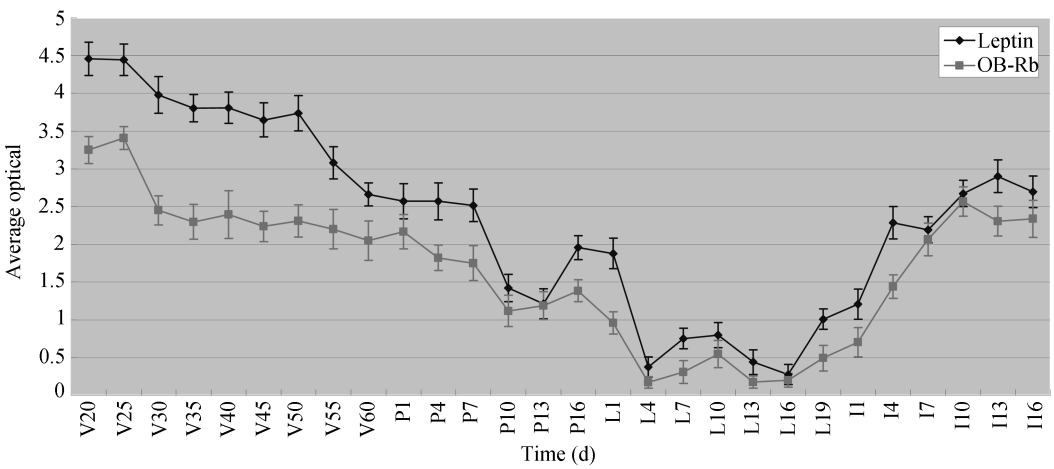

Figure 1 Expression of leptin and OB-Rb in mammary gland of mouse. V, P, L and I stand for virgin, pregnancy, lactation, and involution, respectively.

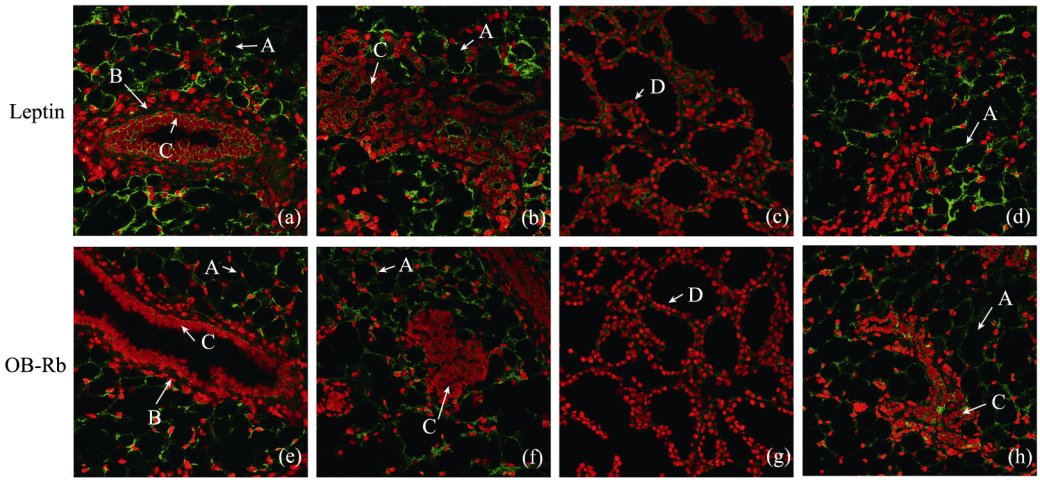

Figure 2 Localization of leptin and OB-Rb in mammary gland of mouse(40×). Leptin expression: (a) Virgin; (b) pregnancy; (c) lactation; (d) involution. OB-Rb expression: (e) Virgin; (f) pregnancy; (g) lactation; (h) involution. A, Adipoctye; B, basal lamina; C, ductal epithelial cell; D, alveolus epithelial cell.

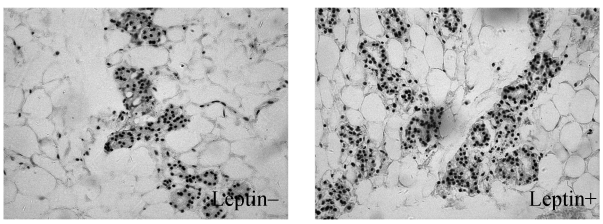

Figure 3 Paraffin sections of pregnancy mouse mammary gland cultured for 5 d (40×).

STAT3 and STAT5 (Figure 7).

In involution, leptin induced the phosphorylation of STAT3 specifically (Figure 8).

3 Discussion

Leptin was mainly synthesized and secreted by adipocytes. So the amount of leptin in mammary gland depended on the protein secreted by fat tissues. In virgin, there were few parenchymas and most areas in mouse mammary gland were occupied by fat pad. In this period, the expression of leptin was the highest. In pregnancy, by the action of estrogen and progestin, mammary ducts began branching. With mammary fat pad regressed, the expression of leptin decreased. This coincided with the fact that in mice with the decreasing in mammary leptin mRNA level around the first third of pregnancy, mammary adipocyte numbers strongly decreased[8]. On pregnancy 16 d, the end of ducts became functional gland alveolus. In our study, expression and localization of leptin showed that mammary epithelial cells produced and secreted leptin in late pregnancy and lactation. So on pregnancy 16 d, the expression of leptin increased slightly. On the contrary of the impact of prolactin on leptin expression in lactation cow mammary gland[9], prolactin inhibited the expression of leptin in mouse mammary gland[5]. In lactation, prolactin was high, so the expression of leptin kept low. After pup withdrawal, involution was onset. With the gland alveolus collapsing, mammary fat pad generated again. The expression of leptin upgraded and recovered to the original level of virgin mammary gland on involution 13 d. The tendency of leptin expression in protein was in agreement with the expression of leptin on mRNA in mammary gland[5]. The expression of OB-Rb was similar to that of leptin in the whole development cycle. The expression of leptin and OB-Rb showing positive correlation and the localization of leptin and OB-Rb in the development cycle revealed that leptin induced the expression of OB-Rb specifically and controlled the development and physiological function by binding to OB-Rb.

OB-Rb was a member of interleukin (IL)-6 receptor families which belonged to class I cytokine receptors[10,11]. This long form isoform was commonly believed to be functional because it was the only isoform that contained intracellular tyrosine residues[12], while the shorter cytoplasmic domain isoform mainly expressed in peripheral tissues[13] was generally considered ineffective in exerting a functional activity, in particular in activating STATs pathway[14]. In mammary gland, leptin binding to OB-Rb resulted in the activation of JAK2 by transphosphorylation and subsequent tyrosine phos-

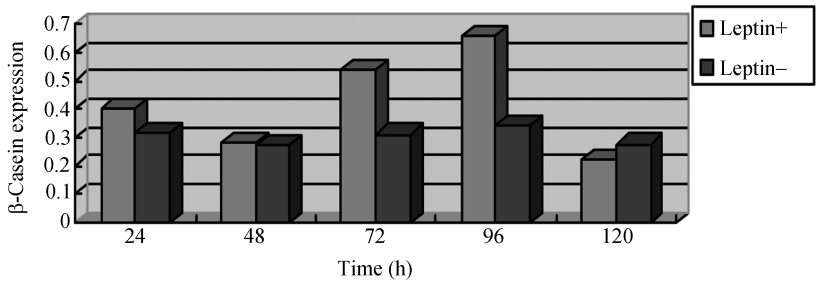

Figure 4 β-casein in medium of lactation mammary gland. The level of β-casein in control and treatment was different at 72 h culture (P<0.05) and 96 h culture (P<0.01).

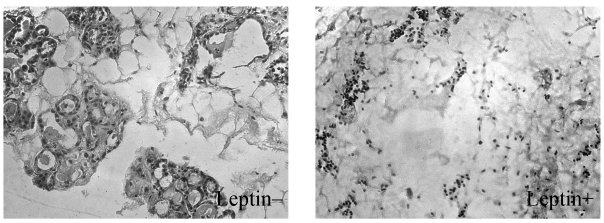

Figure 5 Paraffin sections of involution mouse mammary gland cultured for 5 d (40×).

phorylation of tyrosine residues on the intracellular OB-Rb[15]. Murine OB-Rb contained three tyrosine residues (Tyr^{985}, Tyr^{1077} and Tyr^{1138}). Of these, only Tyr^{985} and Tyr^{1138} residing in hydrophilic motifs were likely to be accessible to the JAK2 tyrosine kinase and activate intracellular signal molecules.

Tyr^{985} recruited tyrosine phosphatase SHP-2[16] and made it associate with GRB-2, then ERK serine/threonine kinase was activated[17]. ERK was extracellular signal-regulated kinase and participated in cell proliferation and differentiation[18]. Leptin about physiological concentration could activate ERK/AP-1 signal pathway[4]. ERK signal pathway was the main signal pathway which was activated by leptin to induce cell proliferation and differentiation. The result of our study showed that in pregnant mouse mammary gland, leptin induced the phosphorylation of p44MAPK/p42MAPK specifically and activated JAK-MAPK signal transduction pathway. Mammary gland cultured *in vitro* also demonstrated that in pregnancy mammary gland, ducts increased by being treated with leptin. Therefore, it was clear that leptin induced proliferation and differentiation of mammary duct epithelial cells by activating JAK-MAPK signal pathway.

Tyr^{1138} recruited signal transducer and transcription activators STAT3[19] and STAT5 and induced the phosphorylation of their tyrosine residues. Then phosphorylated STATs dimerized and translocated to the nucleus and functioned as transcription factors[20]. STAT5 was reported to be critical in the differentiation of mammary alveolar epithelium[21]. In our experiment, we found that leptin induced the phosphorylation of STAT5 in lactation mouse mammary gland and then activated JAK-STAT5 signal transduction pathway. This was adapted to the function of STAT5 in lactation because milk protein genes were the target genes of STAT5 and at least one STAT5-binding site was found in the promoters of β-casein genes[22,23]. In mouse mammary epithelial cell line HC11, leptin at low concentration was shown to influence β-casein expression positively both in the presence and in the absence of prolactin[6], and ob/ob female mice were effective in feeding newborns as a result of the increased milk production induced by continuous leptin treatment[24]. Mammary gland cultured *in vitro* also showed that in lactation, leptin-induced β-casein synthesis and secretion increased. This revealed that maybe leptin exerted its activity acting on the same intracellular pathway of prolactin to induce the expression of milk protein genes.

However, in lactation, leptin also induced the phosphorylation of STAT3 and activated JAK-STAT3 signal pathway. JAK-STAT3 signal pathway inhibited the development of mammary gland. On one hand, the phosphorylated STAT3 induced the transcription of cytokine suppressor SOCS3. SOCS3 contained SH-2 domain and could bind to OB-Rb which was phosphorylated on Tyr^{985}. This could inhibit JAK-MAPK signal transduction pathway[25]. But overexpression of SHP-2 prevented the inhibition of SOCS3 to leptin signal. On the other hand, STAT3 induced the apoptosis of mammary epithelial cells. In mouse mammary gland, the expression of leptin kept lowest in lactation. So leptin did not obviously induce the apoptosis of mammary epithelial cells by JAK-STAT3. Besides that, in lactation, there were a lot of hormones and cytokines to promote mam-

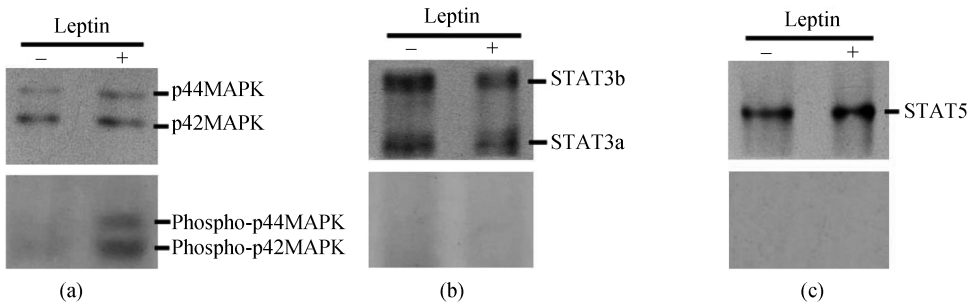

Figure 6 Tyrosine phosphorylation of MAPK (a), STAT3 (b) and STAT5 (c) by leptin in pregnancy mammary gland of mouse.

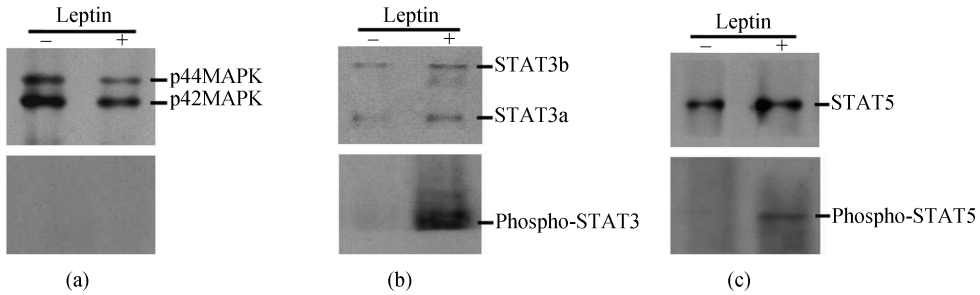

Figure 7 Tyrosine phosphorylation of MAPK (a), STAT3 (b) and STAT5 (c) by leptin in lactation mammary gland of mouse.

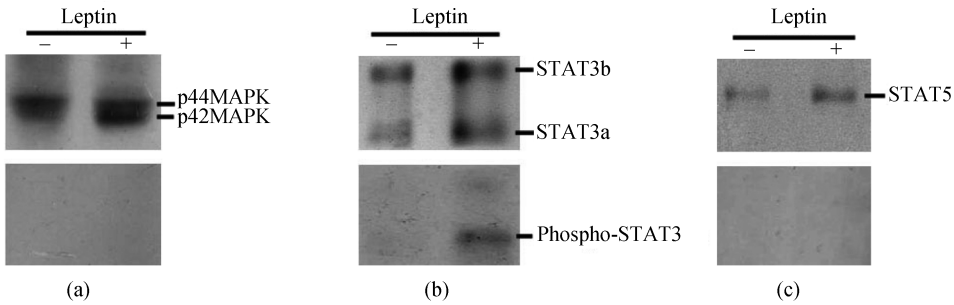

Figure 8 Tyrosine phosphorylation of MAPK (a), STAT3 (b) and STAT5 (c) by leptin in involution mammary gland of mouse.

mary gland cells proliferation and differentiation. They induced the high expression of SHP-2 and prevented the inhibition of SOCS3 to mammary epithelial cell proliferation competitively.

After pup withdrawal, the expression of leptin and OB-Rb recovered to the original level of virgin. At this time, leptin activated STAT3 specifically. STAT3 bound to C/EBPδ promoter and onset the apoptosis of mammary epithelial cells[26]. Now lots of SOCS3 were expressed and JAK-MAPK signal transduction pathway was inhibited. The experiment *in vitro* also showed that in involution, leptin induced the apoptosis of mammary epithelial cells by activating JAK-STAT3, and this was required for mammary gland reconstitution[27].

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