Autophagy is a major intracellular proteolytic process that contributes to the maintenance of protein homeostasis and the supply of amino acids under conditions of starvation (1). In the first step of autophagy, the formation and extension of the isolation membrane occurs in the vicinity of the endoplasmic reticulum, and then an autophagosome is formed. Part of the cytoplasm and organelle are sequestered in the autophagosome. In the final step of autophagy, the intracellular components sequestered by the autophagosomal membrane are degraded by lysosomal enzymes via fusion of the autophagosome and lysosome (1).

Ovariectomized (OVX) rats are widely used as model animals for menopausal disorders caused by deficiencies in ovarian hormones. Recent studies reported that autophagy in the uterus and proximal tibias were induced in OVX rodents (2–4). Tan et al. reported that ovariectomy (OVX) had no effect on autophagy in mouse liver (5). Conversely, Kim et al. reported a decrease in AMP-activated protein kinase (AMPK) phosphorylation in the liver of OVX mice (6). AMPK acts as an energy sensor in cells and is phosphorylated when there is an increase in the intracellular AMP/ATP ratio (7). The phosphorylation of AMPK promotes autophagy via mTOR complex 1 (mTORC1) inhibition (7). The observed decrease in AMPK phosphorylation in the liver of OVX mice indicates the possibility that autophagy in the liver is decreased by OVX. Therefore, it is necessary to verify the effect of OVX on hepatic autophagy.

In the uterus and proximal tibias of OVX rodents, autophagy was suppressed by the administration of β-estradiol and progesterone (2, 4). In cultured uterine endometrial cells, autophagy was promoted by the addition of an estrogen inhibitor and this promoting effect was blocked by β-estradiol (3). In contrast, ovarian hormones (β-estradiol and progesterone) promoted autophagy in bovine mammary epithelial cells (8). In addition, β-estradiol promoted the phosphorylation of AMPK in C2C12 myotube cells (9). Thus, the effect of ovarian hormones on autophagy might vary by organ type.

Recently, it was revealed that lipid droplets are an organelle selectively degraded by autophagy (10). In liver-specific Atg7-knockout mice, an increase in triglycerides in the liver was observed (11), suggesting autophagy is closely involved in hepatic lipid metabolism. The accumulation of triglycerides in the liver is a representative phenotype of OVX rats (12). Therefore, the reduction of hepatic autophagy by OVX may be responsible for the increase of triglycerides, through a decrease in lipid droplet degradation. The purpose of this study was to investigate the influence of OVX on autophagy in the rat liver as a first step to clarify the relationship between hepatic fat accumulation and the change in autophagy caused by OVX. We found that hepatic autophagy tended to be lower in OVX rats than in sham-operated rats. In addition, hepatic autophagy in OVX rats was induced by short-term exposure to ovarian hormones, especially progesterone. This study suggests that autophagy in the rat liver is suppressed by OVX and activated by progesterone. However, the autophagy-promoting effect of β-estradiol was not clarified.

**Materials and Methods**

Animals and experimental design. All animals were maintained in accordance with the guidelines of the Management of Laboratory Animals in Chiba Prefectural University of Health Sciences (Chiba, Japan). The
study was approved by the Institutional Animal Care and Use Committee (Approval number 2013-A004, 2015-A005, 2016-A004).

During the experimental period, rats were housed individually in wire-bottomed stainless-steel cages at 23 ± 1°C with a 12 h/12 h light/dark cycle (7:00–19:00, light), with free access to food and water. A 20% casein diet based on AIN93G was prepared and used in this study. Rats were euthanized under non-starvation conditions between 10:00 and 12:00.

In experiment 1, 6-wk-old female Sprague-Dawley (SD) rats (CLEA Japan, Inc., Tokyo, Japan) were randomly divided into a sham-operated rat group (Sham: n = 6) and an OVX rat group (OVX: n = 6). Bilateral ovariectomy and sham operations were performed under anesthesia with sodium pentobarbital (Somnopentyl, 40 mg/kg body weight, Kyoritsusuiyaku Corp., Tokyo, Japan). At 2 wk (23–25 d) after OVX, rats were anesthetized by intraperitoneal injection of sodium pentobarbital, and liver samples were collected.

In experiment 2, 8-wk-old OVX SD rats, 2 wk after surgery, were purchased from Japan SLC, Inc. (Hamasatsu, Japan) and housed for 10 d. At 3 wk (24 d) after OVX, the rats were divided into three groups either injected subcutaneously with β-estradiol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, 5 μg/kg body weight; OVX-E2: n = 4) or progesterone (Nacalai Tesque, Inc., Kyoto, Japan, 10 mg/kg body weight; OVX-P4: n = 4) suspended in propylene glycol, or a control group of OVX rats receiving propylene glycol (OVX: n = 3). A liver sample was taken from OVX rats at 2 h after hormone injection.

In experiment 3, 8-wk-old female SD rats (CLEA Japan, Inc.) were randomly divided into two groups (Control: n = 4, Fulvestrant: n = 3) and housed for 10 d. Fulvestrant (LKT Labs, Inc., Canada), an estrogen receptor antagonist, was suspended in propylene glycol and subcutaneously administered (≤8 mg/kg body weight), and liver samples were taken from the rats at 2 h after injection.

Measurement of autophagy. To detect LC3 and p62, which are indicators of autophagy, liver samples were homogenized in lysis buffer (0.25 M sucrose, 5 mM Tris-HCl [pH 7.5], 2% Triton X-100) supplemented with a protease inhibitor mixture (Complete, Roche Diagnostics, Tokyo, Japan) and centrifuged at 700 × g for 10 min. The supernatants were subjected to SDS-PAGE and then immunoblotting with LC3B antibody (#2775, Cell Signaling Technology Japan K.K., Tokyo, Japan) and p62 antibody (anti-p62 (SQSTM1) pAb, PM045, MBL, Nagoya, Japan). α-Tubulin and β-actin were measured by immunoblotting with α-tubulin antibody (anti-α-tubulin pAb, PM054, MBL) and β-actin antibody (anti-β-actin pAb, PM053, MBL) as markers of housekeeping proteins. The immunoreactive signal was detected using X-ray film or a chemiluminescence detection system (LumiCube, Liponics, Inc., Tokyo, Japan) and then quantified by densitometric analysis software (ImageJ, NIH, USA).

Statistical analysis. All data are presented as the mean ± SE. Statistical analysis was performed by Student’s t-test and Dunnett’s test (p < 0.05).

Results and Discussion

Effect of OVX on autophagy in the rat liver (experiment 1)

In experiment 1, the effect of OVX on autophagy in the female rat liver was investigated. LC3 is a specific marker of autophagy and exists as two different forms (LC3-I and LC3-II). LC3-I is a cytosolic form of LC3 that is converted to LC3-II, which is the autophagosomal membrane-bound form, by covalently binding with phosphatidylethanolamine (13). Because the amount of LC3-II correlates with the number of autophagosomes, LC3 conversion (LC3-II/LC3-I ratio) and the amount of LC3-II are widely used as indicators of autophagy (14, 15). Figure 1A shows the changes in the amounts of LC3-I, LC3-II, and total LC3 in the rat livers after OVX. The amounts of LC3-I and total LC3 were significantly decreased in OVX rats compared with Sham rats, but there was no difference in the amount of LC3-II, an indi-
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In experiment 1, although the number of autophagic cells of mice fed a high-fat diet, an accumulation of LC3-II was reported by a suppression of late stage autophagy was reported in OVX rats, an accumulation of LC3-II was lower than that in Sham rats. In the liver, accumulation of p62 indicates that hepatic autophagy was greater in OVX rats than in Sham rats (Fig. 1B). The amount of p62 was significantly degraded (16). The result for LC3-II/LC3-I ratio indicated the induction of autophagy by OVX. In experiment 1, it was difficult to evaluate an increase or decrease in autophagy based only on the LC3-II/LC3-I ratio, as the LC3-II/LC3-I ratio was significantly increased in OVX rats compared with Sham rats (Fig. 1A). The increase of the LC3-II/LC3-I ratio was significantly higher than that in the control OVX rats (Fig. 2A). Accordingly, the LC3-II/LC3-I ratio in progesterone-treated OVX rats was significantly higher than that in the control OVX rats (Fig. 2A). The administration of β-estradiol did not significantly increase the LC3 level, but significantly increased the LC3-II/LC3-I ratio (Fig. 2A). The amount of p62 was significantly lowered in progesterone-treated OVX rats than in control OVX rats (Fig. 2B). However, the administration of β-estradiol did not affect the amount of p62 in the OVX rat liver (Fig. 2B).

In experiment 2, the effect of ovarian hormones (β-estradiol and progesterone) on autophagy in the rat liver was examined using OVX rats. At 2 h after progesterone injection, a decrease in the LC3-I level and an increase in the LC3-II level were observed in progesterone-treated OVX rats (Fig. 2A). Accordingly, the LC3-II/LC3-I ratio in progesterone-treated OVX rats was significantly higher than that in the control OVX rats (Fig. 2A). The administration of β-estradiol did not significantly increase the LC3 level, but significantly increased the LC3-II/LC3-I ratio (Fig. 2A). The amount of p62 was significantly lowered in progesterone-treated OVX rats than in control OVX rats (Fig. 2B). However, the administration of β-estradiol did not affect the amount of p62 in the OVX rat liver (Fig. 2B).

In experiment 3, to confirm the effect of β-estradiol on autophagy, the influence of an estrogen antagonist on hepatic autophagy was examined using normal female rats. Fulvestrant is an estrogen antagonist that inhibits the actions of estrogen. The amounts of LC3-I and LC3-II were significantly decreased in fulvestrant-treated rats compared with control rats (Fig. 3A). Although no significant difference in the LC3-II/LC3-I ratio was observed (Fig. 3A), the decrease in LC3-II can be interpreted as indicating autophagy suppression. This suggests that β-estradiol treatment might accelerate hepatic autophagy. However, there was no significant difference in the p62 level in fulvestrant-treated rats (Fig. 3B). Several studies using cultured cell lines reported that the effect of ovarian hormones on autophagy varied by organ type (2, 3, 8). The result of experiment 2 indicated that progesterone activated autophagy in the rat liver. However, because the effects of β-estradiol and fulvestrant on LC3 and p62 were not consistent, the autophagy-promoting effect of β-estradiol was not clarified.

In experiment 1, the amount of total LC3 but not the LC3-II/LC3-I ratio between OVX rats and Sham rats was changed. In contrast, a change in the LC3-II/LC3-I ratio between control OVX rats and hormone-treatment OVX rats was observed in experiment 2. To
explain this discrepancy, the following hypothesis can be considered. Under conditions of long-term hormonal decline because of OVX, regulation of the total amount of LC3 via mRNA expression may occur. In contrast, after short-term hormone supplementation by a single administration, regulation of the conversion of LC3-I to LC3-II may occur. In the present study, the results of experiment 2 clearly revealed that short-term exposure to progesterone induced autophagy in the rat liver. However, the results of this study did not reveal the effect of progesterone on hepatic autophagy by OVX leads to fat accumulation in the liver needs to be examined.

In conclusion, the present study suggests that hepatic autophagy in female rats is reduced by OVX. Furthermore, hepatic autophagy in OVX rats was promoted by short-term exposure to progesterone. This suggests that progesterone promotes hepatic autophagy. However, the autophagy-promoting effect of β-estradiol was not clarified.

Disclosure of State of COI
No conflicts of interest to be declared.

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Fig. 3. Effect of fulvestrant administration on autophagy in female rat liver (experiment 3). (A) Fulvestrant and propylene glycol were subcutaneously administered to normal female SD rats at 2 h before sampling liver tissues. Liver tissues were homogenized and centrifuged at 700 × g at 4 °C for 10 min. LC3-I and LC3-II in the livers were analyzed by immunoblot analysis. Image data are representative of each experimental group. The graph represents densitometric data for LC3-I, LC3-II and total LC3, calculated as LC3-I+LC3-II. The LC3-II/LC3-I ratio was calculated from the densitometric data for LC3-I and LC3-II. (B) p62 in the livers was analyzed by immunoblot analysis. Image data are representative of each experimental group. The graph represents densitometric data for p62. Each value is the mean ± SE (Control: n = 4, Fulvestrant: n = 3). The statistical significance of differences between groups was analyzed by Student’s t-test (* p<0.05, ** p<0.01 vs Control).
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