# The role of autophagy in follicular development and atresia in rat granulosa cells

*Jong Yeob Choi, Ph.D.,*[a] *Min Wha Jo, M.S.,*[b] *Eun Young Lee, M.S.,*[a] *Byung-Koo Yoon, M.D., Ph.D.,*[c] *and Doo Seok Choi, M.D., Ph.D.*[a]

[a] Infertility Clinic, Samsung Medical Center, School of Medicine, Sungkyunkwan University; [b] Center for Clinical Research, Samsung Biochemical Research Institute; and [c] Department of Obstetrics and Gynecology, Samsung Medical Center, School of Medicine, Sungkyunkwan University, Seoul, South Korea

**Objective:** To investigate the involvement of autophagy in folliculogenesis and its correlation with apoptosis.
**Design:** Animal model–based study.
**Setting:** University medical center.
**Animal(s):** Twenty-one day-old female Sprague-Dawley rats.
**Intervention(s):** Ovaries obtained from established immature rat models primed with pregnant mare serum gonadotropin (PMSG) were used for the induction of follicular development and atresia. Granulosa cells isolated from developing follicles were cultured in serum-free condition with or without follicle-stimulating hormone.
**Main Outcome Measure(s):** Microtubule-associated light-chain protein 3 (LC3) and autophagic vacuoles were used as autophagic markers, and cleaved caspase-3 was used as an apoptotic marker in ovaries and/or granulosa cells.
**Result(s):** The LC3 protein was expressed mainly in granulosa cells during all developmental stages. In granulosa cells isolated from PMSG-primed immature rat ovaries, LC3-II expression showed a similar expression pattern to cleaved caspase-3. In addition, granulosa cells of atretic follicles that showed intense cleaved caspase-3 staining also showed intense LC3 immunoreactivity. An in vitro culture experiment revealed that the levels of LC3-II and cleaved caspase-3 proteins were gonadotropin-dependent. The induction and the gonadotropin dependency of granulosa cell autophagy were confirmed by the observation of autophagic vacuoles under transmission electron microscopy.
**Conclusion(s):** These preliminary results suggest that autophagy is induced mainly in granulosa cells during folliculogenesis and shows good correlation with apoptosis. (Fertil Steril® 2010;93:2532–7. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Apoptosis, atresia, autophagy, follicular development, granulosa cell

In the mammalian ovary, only a small fraction of follicles mature fully and are involved in ovulation; most follicles become atretic and die. Although follicular atresia occurs repeatedly during the ovarian cycle, the precise mechanism underlying the massive cell death has not been fully elucidated. Recent studies have suggested that follicular atresia is associated with granulosa cell death by apoptosis, a form of programmed cell death (1–6). However, follicular atresia may not be under the exclusive control of apoptosis because nonapoptotic forms of programmed cell death such as autophagy and necrotic-like cell death have also been observed in the antral follicles of geese (7) and quails (8).

Autophagy is an intracellular bulk degradation system in which a portion of the cytoplasm is enveloped in double membrane-bound structures called autophagosomes, which undergo maturation and fusion with lysosomes for degradation (9, 10). It was originally thought to represent a survival response to nutrient deprivation and other forms of cellular stress (11); however, the results of many studies suggest that autophagy promotes cell death by excessive self-digestion and degradation of essential cellular constituents (12–17). Furthermore, recent studies have demonstrated that autophagy can be triggered by various stimuli that induce apoptosis (18–20). In human granulosa cells in particular, cell death by autophagy results from the exposure of cells to oxidized low-density lipoprotein that induces endothelial cell apoptosis (21). Therefore, autophagy may be involved in folliculogenesis, as granulosa cells are the primary site of apoptosis during follicle atresia (22). However, the involvement and induction of autophagy during follicular development and atresia have not been elucidated to date. We evaluated the involvement and induction of autophagy during follicular development and atresia, with a particular focus on granulosa cell apoptosis.

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Reprint requests: Doo Seok Choi, M.D., Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul, South Korea 135-710 (FAX: 82-2-3410-0044; E-mail: dooseok.choi@samsung.com).

## MATERIALS AND METHODS

### Animal Treatment

The animal experimentation protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Sungkyunkwan University School of Medicine. Immature female Sprague-Dawley rats aged 21 to 23 days were injected intraperitoneally with 15 IU of pregnant mare serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO) to induce ovarian follicular development and atresia (23, 24). Rats were killed by cervical dislocation 0, 1, 2, 3, 4, and 5 days after gonadotropin treatment, and the



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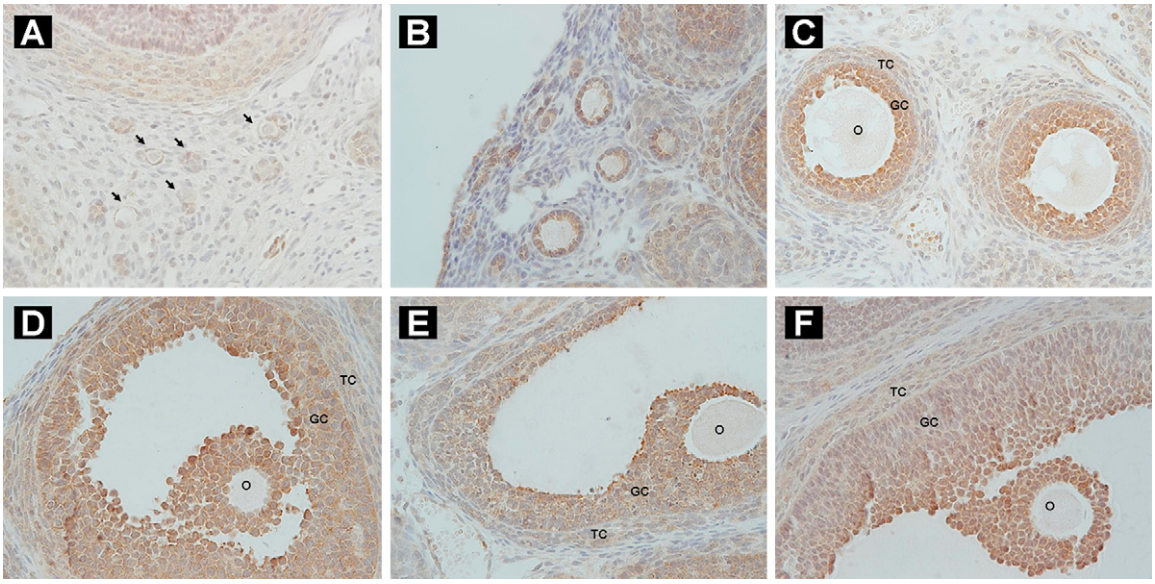
## FIGURE 1

Immunolocalization of LC3 protein in rat ovarian follicles at different stages of development: (**A**) primordial, (**B**) primary, (**C**) preantral, (**D**) early antral, (**E**) medium antral, and (**F**) large antral follicles. GC, granulosa cells; TC, thecal cells; O, oocytes. Magnification ×400.

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ovaries were excised. The ovaries were either immediately fixed in 10% neutral buffered formalin for histologic processing or used for granulosa cell collection.

### Immunohistochemistry

Paraffin-embedded whole ovarian sections were deparaffinized, rehydrated, and placed in a steamer for 30 minutes in 10 mM citric buffer for antigen retrieval. Endogenous peroxide was reduced by incubation of the sections with 3% $H_2O_2$ for 30 minutes. Nonspecific binding was blocked with 5% bovine serum albumin (BSA; Sigma Chemical) in phosphate-buffered saline (PBS) for 30 minutes. After washing, sections were incubated overnight at 4°C with anti-LC3 (light-chain protein 3) rabbit polyclonal antibody (diluted 1:300; Novus Biologicals, Littleton, CO) or cleaved caspase-3 rabbit polyclonal antibody (diluted 1:200; Cell Signaling Technology, Boston, MA), followed by incubation with a biotinylated secondary antibody (DAKO, Carpinteria, CA) for 1 hour at a dilution of 1:2000. After incubation with a streptavidin-peroxidase conjugate, antibody complexes were visualized with diaminobenzidine tetrahydrochloride chromogen. The sections were counterstained with hematoxylin, then dehydrated and mounted.

### Granulosa Cell Collection and Culture

Ovaries were excised 0, 1, 2, 3, 4, and 5 days after PMSG injection and placed in Dulbecco's modified Eagle's/F12 medium (DMEM/F12; GIBCO-BRL, Grand Island, NY) that was supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL), 10 mg/mL of streptomycin sulfate (Sigma Chemical), and 75 mg/mL of penicillin G (Sigma Chemical). Granulosa cells were harvested by follicle puncture using a 25-gauge needle (25). After follicle puncture, granulosa cells were suspended in the appropriate solution for immunoblotting.

For in vitro culture of granulosa cell under serum-free conditions, ovaries were collected 24 hours after PMSG injection (15 IU, intraperitoneally), and granulosa cells were collected by follicle puncture as described previously. The cells were seeded in 10-cm culture dishes or six-well plates and were allowed to attach overnight. The next morning, the medium and unattached cells were removed and replaced with serum-free media supplemented with 100 ng/mL of follicle-stimulating hormone (FSH; Sigma Chemical) or without FSH. Twenty-four hours later, the granulosa cells were scraped to extract the proteins or fixed for immunofluorescence and electron microscopy analysis.

### Western Blot Analysis

The freshly isolated and cultured granulosa cells were lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer that was supplemented with a protease inhibitor cocktail (Sigma Chemical). To facilitate the complete solubilization of the cellular proteins, the cell lysates were incubated on ice for 30 minutes then centrifuged (13,000 × *g*, 4°C, 30 minutes). The whole-cell lysates (20 $\mu$g/lane) were separated on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a polyvinylidene diflouride (PVFD) membrane (Bio-Rad Laboratories, Richmond, CA). After the nonspecific binding sites were blocked with 5% skim milk, the membrane was treated with anti-LC3 rabbit polyclonal antibody (diluted 1:5000; Novus Biologicals) or caspase-3 rabbit polyclonal antibody (diluted 1:1000; Cell Signaling Technology) overnight at 4°C. The immunoreactive bands were demonstrated by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1.5 hours. Peroxidase activity was visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). Integrated optical intensities of the immunoreactive protein bands were quantified by imaging (Gel Doc 2000; Bio-Rad Laboratories) and the analysis software Quantity One version 4.0.3 (Bio-Rad Laboratories), and they were normalized to $\beta$-actin values.

### Immunofluorescence Staining

Granulosa cells were cultured on sterilized glass coverslips, fixed with 4% paraformaldehyde, and blocked with 0.1% BSA in PBS. Cells were incubated with 1:500 diluted primary antibody (MAPLC3; Novus Biologicals) in PBS and reacted with 1:5000 diluted Alexa 488-conjugated secondary