Chapter 10

Assessing Autophagy in Sertoli Cells

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Abstract

Autophagy is an important cellular homeostatic process, it degrades most long-lived proteins and some organelles by lysosome to provide raw materials for the survival of the cells during nutrient or energy deprivation condition. Autophagy is active in Sertoli cells and involved in many cellular processes. However, the precise role of autophagy in Sertoli cells is still largely unknown. Thus, the assessment of autophagy in Sertoli cells should be helpful for investigating the functional roles of autophagy in Sertoli cells. This chapter describes some methods for assessing autophagy in Sertoli cells, including detection of LC3 maturation/aggregation, transmission electron microscopy, half-life assessments of long-lived proteins, immunofluorescence microscopy, and co-localization of autophagy-targeted proteins with autophagy components or lysosomal proteins.

Key words Autophagy, Sertoli cell, Transmission electron microscopy, LC3, Immunoblotting, Immunofluorescence

1 Introduction

Autophagy is an evolutionarily conserved membrane trafficking process that delivers cytoplasmic material such as long-lived proteins and organelles to the lysosome for degradation [1, 2]. Autophagy has been implicated in various physiological processes including cell growth, adaptation to stress conditions, anti-aging mechanisms, intracellular quality control, renovation during development and differentiation, and the biogenesis of organelles such as cilia and acrosome [3–6]. At least three types of autophagy have been defined, including macroautophagy, microautophagy, and chaperone-mediated autophagy [2, 7, 8]. Microautophagy involves direct engulfment of cytoplasmic cargos through the invagination and vesicle scission into the lysosomal lumen, which is the nonselective lysosomal degradative process [7]. The direct translocation of cytoplasmic proteins across the lysosomal membrane, which requires protein unfolding by chaperone proteins but not vesicle, is known as chaperone-mediated autophagy [8]. The most extensively investigated is macroautophagy, which is mediated by a special

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double-membrane organelle termed the autophagosome. The canonical macroautophagy is initiated from an isolated membrane and followed by the formation of a double-membrane autophagosome to engulf cytoplasmic cargos that then fuses with lysosomes for degradation, and then, the amino acids and other small molecules generated by autophagic degradation are delivered back to the cytoplasm for recycling or energy production [1, 2, 9].

Macroautophagy will be the focus of this chapter and referred to as autophagy hereafter. The process of autophagy includes the initiation, nucleation, expansion, maturation, and degradation [2, 9]. More than 40 autophagy-related (ATG) proteins have been characterized during autophagy, which consist of several functional units: ULK1 kinase complex, the PI3K complex, ATG9-ATG2-WIPI1/ATG18 complex, and two ubiquitin-like conjugation systems [6, 10, 11]. Nutrient, carbon, or nucleic acid starvation and some physiological stress stimuli could trigger the induction of autophagy in many organisms through intricate regulatory mechanisms via numerous kinases and signaling pathways with the Ser/Thr kinase TOR (target of rapamycin) at its center [2, 9, 10]. TOR could modulate the ULK1 complex undergoing a series of phosphorylation/dephosphorylation events and lead to the activation of this complex and the initiation of autophagy [12, 13]. After the induction of autophagy, the phagophore, a cup-shaped double-membrane structure, is gradually formed [12–14]. In mammalian cells, the nucleation of the phagophore membrane takes place at the omegasome through the recruitment of the ULK1 and PIK3C3 complexes to the nucleation site containing the transmembrane protein VMP1 [12, 14, 15]. The phosphatidylinositol 3-phosphate (PtdIns3P) produced by membrane-bound PIK3C3 complex at the nucleation site further enhances membrane bending and reinforce the localization of the ULK1 complex and other ATG proteins at the growing membrane [12, 14, 16]. And then, an ubiquitin-activating E1-like enzyme, ATG7, activates ATG12 and then facilitates ATG12-ATG5-ATG16L1 complex formation, which works as an E3 ligase for the second ubiquitin-like conjugation system, to promote LC3 conjugating to the growing membrane. As a scaffold protein, the LC3-lipid-containing membrane drives membrane expansion and vesicle completion to form an autophagosome [2, 10, 12, 14]. Once fused with a lysosome, the inner proteins of the autophagosome are eventually degraded in autolysosomes [10]. To investigate the mechanism and functional roles of autophagy, many routine methods have been developed to detect different stages of the autophagy pathway, such as early autophagosome, autolysosome, and autophagic degradation products [2, 17]. And these methods could also be used coordinately with each other to determine whether an increase in intermediates in the pathway represents a true increase in autophagic degradation or a block in the completion of the autophagic pathway [2, 17].

Sertoli cell is an important somatic cell in the seminiferous epithelium and plays key roles in the control of spermatogenesis [18, 19]. Autophagy is also active in Sertoli cells [20–23], where it is involved in the clearance of SHBG/ABP (sex hormone-binding globulin) that is selectively regulated by testosterone and ectoplasmic specialization assembly [20, 24]. However, the role of autophagy in Sertoli cells is still largely unknown. The assessment of autophagy in Sertoli cells should be helpful for further investigating the functional roles of autophagy in Sertoli cells. For instance, the transmission electron microscopy (TEM) is the most reliable and conventional technique to visualize autophagic vacuolization; biochemical methods and techniques could be designed to measure the aggregation of autophagosome markers (such as LC3 and SQSTM1/p62) and allow for the monitoring of autophagy in Sertoli cells. Here, we will provide an overview of routine methods for assessing autophagy in Sertoli cells.

2 Materials

2.1 Immunofluorescence Analysis of Autophagy Components

1. PBS (1×): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 in deionized water (dH_2O), adjust pH to 7.4 with 2 N NaOH.
2. 4% PFA: 4% paraformaldehyde in PBS, adjust pH to 7.4 with 2 N NaOH.
3. Sodium citrate buffer: 1.8 mM citric acid, 8.2 mM sodium citrate, adjust pH to 6.0 with citric acid.
4. 5% BSA: To PBS add 5% (w/v) bovine serum albumen (BSA) and store at 4 °C.
5. Primary antibody solution: predicted primary antibody of autophagy components, 1.5% (w/v) BSA in PBS.
6. Secondary antibody solution: predicted secondary antibody in PBS.
7. DAPI: Stock solution, 5 mg/mL in distilled water. Store in 40 mL aliquots at −20 °C in Eppendorf tubes in the dark. Working stain, 0.5 mg/mL in PBS in the dark.
8. Mounting medium.
9. 3-Aminopropyl-triethoxysilane (APES).

2.2 Immunoblotting Analysis of LC3

1. F12-DMEM: Dulbecco's Modified Eagle's Medium-Ham's Nutrient Mixture F-12.
2. Collagenase IV from *Clostridium histolyticum*, type IV.
3. DNase I from bovine pancreas, lyophilized.
4. Hyaluronidase type III.

5. Trypsin from bovine pancreas.
6. Sertoli cells culture medium: F12-DMEM containing 15% fetal calf serum and penicillin-streptomycin.
7. RIPA-like buffer: 25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate.
8. Phenylmethylsulfonyl fluoride (PMSF): 1 M stock solution in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM.
9. Protease inhibitor cocktail.
10. 2× Loading buffer: 100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM dithiothreitol (DTT), and 0.2% bromophenol blue.
11. 15% Running gel: 2.3 mL of distilled water, 5.0 mL of 30% acrylamide solution, 2.5 mL of 1.5 M Tris–HCl, pH 8.8, 100 μL of 10% SDS, 100 μL of 10% APS, and 4 μL of TEMED.
12. Stacking gel: 2.7 mL of distilled water, 0.67 mL of 30% acrylamide solution, 0.5 mL of 1.0 M Tris–HCl, pH 6.8 [at 25 °C], 40 μL of 10% SDS, 40 μL of 10% APS, and 4 μL of TEMED.
13. Running buffer: 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS. Store at room temperature.
14. Transfer buffer: 48 mM Tris, 39 mM glycine, 20% methanol (analytical grade).
15. Blocking buffer: 5% (w/v) nonfat dry milk in PBS.
16. Immunoblotting primary antibody solution: 1:1000 anti-LC3 (Sigma, L7543), 1% (w/v) BSA, 20 mM Tris–HCl, pH 7.5 at 25 °C, 0.1% NaN3, and 150 mM NaCl.
17. Immunoblotting secondary antibody solution: predicted secondary antibody in blocking buffer.

2.3 Transmission Electron Microscopy

1. 0.1 M cacodylate buffer: 5.35 g Na-cacodylic acid in 220 mL distilled water, adjust pH to 7.3 with 1 N HCl, and complete to 250 mL with distilled water.
2. 2.5% glutaraldehyde: 25% glutaraldehyde. Dilute to a final concentration of 2.5%.
3. 1.5% paraformaldehyde: 10% paraformaldehyde in distilled water, adjust pH to 7.3 with 2 N NaOH. Dilute to a final concentration of 1.5%.
4. 1% osmium tetroxide: dissolve 1 g osmium tetroxide (Sigma, 419494) in 100 mL distilled water. Store at 4 °C.
5. 2% uranyl acetate: dissolve 1 mL uranyl acetate (Zhong Jing Ke Yi, GS02625) in 49 mL distilled water. Store powder at RT. Radioactive.

6. Resin: SPI-Pon 812 Epoxy Embedding Kit (Spi Supplies, 02635-AB). Mix 13 mL SPI-Pon 812, 7 mL NMA, 8 mL DDSA, and 10–12 drops DMP-30. Store at 4 °C and keep dry.
7. Uranyl acetate for staining: dissolve 1 mL uranyl acetate in 99 mL of distilled water. Store in 10 mL Norm-Ject syringes with a 0.22 μm filter at the tip of the syringe. Light sensitive, cover syringe with aluminum foil. Stable for several months at 4 °C.
8. Lead citrate for staining of thin sections: Solution A: lead nitrate 1 M, i.e., 3.3 g in 10 mL water. Solution B: trisodic sodium citrate 1 M, i.e., 3.57 g in 10 mL water. Solution C: NaOH 1 N, i.e., 1 g in 25 mL water. To 16 mL of boiled distilled water, add 3 mL of solution B and mix gently with hand. Add 2 mL of solution A and mix gently with hand. A milky precipitate will form. Add, dropwise, 4 mL of solution C while mixing gently with hand. The precipitate must disappear completely. If not, discard and start over. Dispatch in 2 or 5 mL syringes with a 0.22 μm filter at the tip of the syringe. Stable for several months at 4 °C.

2.4 GFP-LC3 Lysosomal Delivery and Proteolysis

1. Opti-MEM: Opti-MEM reduced serum medium.
2. Lipofectamine 2000.

2.5 Turnover of Autophagic Substrate Proteins

1. Cycloheximide.
2. TAP buffer: 10% glycerol, 50 mM HEPES–KOH, pH 7.5, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 10 mM NaF, 0.25 mM Na_3VO_4, 50 mM β-glycerolphosphate, 2 mM DTT.
3. IP buffer: 20 mM Tris–HCl, pH 7.4, 2 mM EGTA, 1% NP-40.
4. Protein A-Sepharose.

2.6 Antibody

1. LC3 for immunofluorescence.
2. LAMP2 for immunofluorescence.
3. LC3 for immunoblotting.
4. SQSTM1/p62 for immunoblotting.
5. LC3 for immunoprecipitation.
6. Secondary antibodies for immunofluorescence: goat anti-rabbit FITC-conjugated secondary antibody, goat anti-mouse FITC-conjugated secondary antibody, goat anti-mouse TRITC-conjugated secondary antibody, goat anti-rabbit TRITC-conjugated secondary antibody, goat anti-rat TRITC-conjugated secondary antibody.
7. Secondary antibodies for immunoblotting: Alexa Fluor 680-conjugated goat anti-mouse and Alexa Fluor 680-conjugated goat anti-rabbit.

3 Methods

3.1 Immunofluorescence Analysis of Autophagy Components

As for the autophagic pathway, many molecules are very good marker proteins for the detection of autophagic membranes, such as ATG12-ATG5 and ATG16L which are specific markers for the isolation membrane and microtubule-associated protein 1A/1B-light chain 3 (LC3) which is a general marker for autophagosome membranes [2, 17, 25]. Their localizations are easily examined by the immunofluorescence analysis.

1. Kill adult male mice by cervical dislocation immediately. Lay each animal on its back; sterilize the ventral surface with 70% ethanol. Remove testes from the abdomen with a pair of surgical scissors. Wash testes with PBS immediately, and repeat twice. Fix testes in 4% paraformaldehyde (PFA) in PBS pH 7.4 at room temperature (RT) for up to 24 h, stored in 70% ethanol.
2. Embed testes in paraffin.
3. Cut 5-μm-thick testis sections and mount on 3-aminopropyltriethoxysilane (APE) precoated glass slide.
4. Deparaffinize in xylene 3 × 10 min and hydrate in an ethanol series (3 × 5 min of 100%, 5 min each of 95%, 80%, 70%).
5. Rinse once in PBS and boil for 15 min in sodium citrate buffer for antigen retrieval. Chill the samples at RT.
6. Rinse 3 × 5 min in PBS at RT.
7. Incubate in 5% BSA in PBS (pH 7.4) at RT for 30 min.
8. Rinse 3 × 5 min in PBS at RT.
9. Incubate in primary antibody solution at 4 °C overnight (*see* **Note 1**).
10. Rinse 3 × 5 min in PBS at RT.
11. Incubate in secondary antibody solution at 37 °C for 60 min.
12. Rinse 3 × 5 min in PBS at RT.
13. Incubate in 1 × DAPI solution at RT for 5 min.
14. Rinse 2 × 5 min in PBS at RT.
15. Add one drop mounting medium on the samples. Put the slide facedown on the top of the cover slip, and then turn the slide over. Remove excess mounting medium and seal cover slip with nail polish to prevent drying. Store in dark at −20 °C or 4 °C.
16. Fluorescent images are obtained using a fluorescence microscope.

3.2 Immunoblotting Analysis of LC3

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is an important autophagic marker protein. During autophagy, a cytosolic form of LC3 (LC3-I, approximately 18 kDa) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine

conjugate (LC3-II, approximately 16 kDa), which is recruited to autophagosomal membranes (Fig. 1). Thus, the detection of LC3 by immunoblotting has become a reliable method for monitoring autophagy and autophagy-related processes [2, 17, 25, 26].

3.2.1 Primary Sertoli Cell Isolation

1. Testes are dissected from mice at 18 to 22 days of age immediately after euthanasia. Wash testes with PBS immediately. Repeat twice.
2. Decapsulate the testes under the dissection microscope. Wash the seminiferous tubules with PBS three times.
3. Incubate with 2 mg/mL collagenase IV and 0.5 mg/mL DNase I in F12/DMEM medium for 30 min at 37 °C on a shaker at 85 rpm.
4. Wash twice with F12-DMEM medium.
5. Incubate with 2 mg/mL collagenase IV, 0.5 mg/mL DNase I, and 1 mg/mL hyaluronidase type III for 10 min at 37 °C on a shaker at 85 rpm.

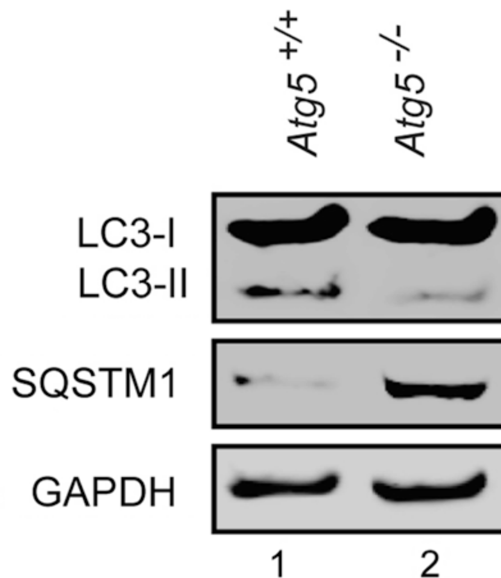

Fig. 1 Immunoblotting analysis of SQSTM1 and LC3 to monitor autophagic flux in Sertoli cells. LC3 is an important autophagic marker protein, and level of LC3-phosphatidylethanolamine conjugate (LC3-II) could indicate the activity of autophagy. In *Atg5*$^{+/+}$ Sertoli cells, LC3 II could be detected by immunoblotting (lane 1), suggesting autophagy is active in Sertoli cells. ATG5 is required for the initiation of autophagy. In *Atg5*$^{-/-}$ Sertoli cells, LC3B-II is reduced (lane 2), indicating the deficiency of autophagy initiation. The autophagic substrate SQSTM1/p62 is accumulated in *Atg5*-deficient Sertoli cells (lane 2), suggesting the autophagic flux is disrupted in *Atg5* knockout Sertoli cells. Thus, the immunoblotting analysis of SQSTM1 and LC3 could monitor autophagic flux in Sertoli cells

6. Centrifuge 500 × *g* for 1 min at RT and wash with F12-DMEM medium. Repeat twice.
7. Incubate with 2 mg/mL collagenase I, 0.5 mg/mL DNase I, 2 mg/mL hyaluronidase, and 1 mg/mL trypsin for 30 min at 37 °C on a shaker at 85 rpm (*see* **Note 2**).
8. Add equal volume of Sertoli cell culture medium. Using a pipette, gently pipette samples up and down to single cell suspension (*see* **Note 3**).
9. Filter the cell suspension through a 100 μm filter to remove any cell aggregates.
10. Centrifuge 1000 × *g* for 3 min at RT and wash with F12-DMEM medium. Repeat twice.
11. Place the dispersed cells into culture dishes, and incubate with Sertoli cell culture medium at 34 °C and 5% CO_2. Spermatogonia are unable to attach to the dish and removed during the medium change after 48 h.

3.2.2 Preparation of Sertoli Cell Lysates

1. Collect primary cultured Sertoli cells by trypsinization and wash once in cold (4 °C) PBS. Centrifuge at 4 °C, 500 × *g* for 5 min (*see* **Note 4**).
2. Discard supernatant, and resuspend the pellets by using 30–50 μL of RIPA-like buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protein inhibitor cocktail. Incubate on ice for 30 min.
3. Centrifuge the homogenates at 4 °C, 13,523 × *g* for 15 min. Remove the supernatant to a new tube.
4. Determine the protein concentration of lysates by using the Bio-Rad DC Protein Assay (*see* **Note 5**).
5. Add equal volume of 2× loading buffer to proteins from each lysate, and then incubate for 15 min at 65 °C (*see* **Note 6**).
6. Chill the samples on ice and centrifuge at 4 °C, 13,523 × *g* for 10 min. Store at −20 °C or −80 °C.

3.2.3 SDS-PAGE

1. Prepare 15% running gel and pour into the space between the glass plates with1.0 mm thickness (*see* **Note 7**).
2. Gently overlay the solution with water to ensure a flat surface and exclude air (*see* **Note 8**).
3. After the polymerization (*see* **Note 9**), pour off water and gently wash the top of the gel with water.
4. Prepare the stacking gel and pour on top of the polymerized running gel. Insert the comb for the creation of wells.
5. After the polymerization, mount the gel into the SDS-PAGE electrophoresis chamber, and add running buffer.

6. Gently remove the comb and wash the wells with running buffer.
7. Load sample into the well. Run the gel by connecting the gel unit to power supply (*see* **Note 10**).

3.2.4 Immunoblotting

1. Incubate two sponges, four sheets of Whatman paper, and one nitrocellulose membrane of approximately the same size as the running gel in transfer buffer (1×) for 1–2 min at RT.
2. Disassemble the gel unit and discard the stacking gel.
3. Prepare the transfer "sandwich" by overlaying the following components: one sponge, two sheets of Whatman paper, the nitrocellulose membrane, the running gel, two sheets of Whatman paper, and one sponge.
4. Place the transfer "sandwich" in a transfer cassette and insert into the transfer apparatus. The transfer is accomplished at the constant voltage of 90 V for 2 h (*see* **Note 11**).
5. Once the transfer is complete, disassemble the transfer cassette. Incubate the nitrocellulose membrane in 10 mL of blocking buffer (45–40 min, RT) on a rocking platform.
6. Rinse the membrane with PBS twice, and incubate with the immunoblotting primary antibody solution at 4 °C overnight on the rocking platform.
7. Recover the immunoblotting primary antibody solution, and rinse the membrane 3 × 5 min in PBST on the rocking platform.
8. Incubate with the immunoblotting secondary antibody solution at RT for 1 h on the rocking platform.
9. Remove the secondary antibody solution and rinse the membrane 3 × 5 min in PBS on the rocking platform.
10. The membrane is scanned using an Odyssey infrared imager (9120, LI-COR Biosciences, Lincoln, NE).

3.3 Transmission Electron Microscopy

The transmission electron microscopy (TEM) is a reliable method to monitor autophagy [2, 17, 25, 27]. The fine structure of autophagic vacuoles could be identified by morphology such as the following: autophagosome is a double-membrane structure containing undigested cytoplasmic material including organelles, while the autolysosome is a single-membrane structure containing cytoplasmic components at various stages of degradation (Fig. 2).

3.3.1 Resin Flat Embedding of Aldehyde-Fixed Mouse Testes

1. Kill adult male mice by cervical dislocation immediately. Lay each animal on its back; sterilize the ventral surface with 70% ethanol. Remove testes from the abdomen with a pair of surgical scissors. Take the testis as soon as possible. Wash testes with PBS immediately. Repeat twice.

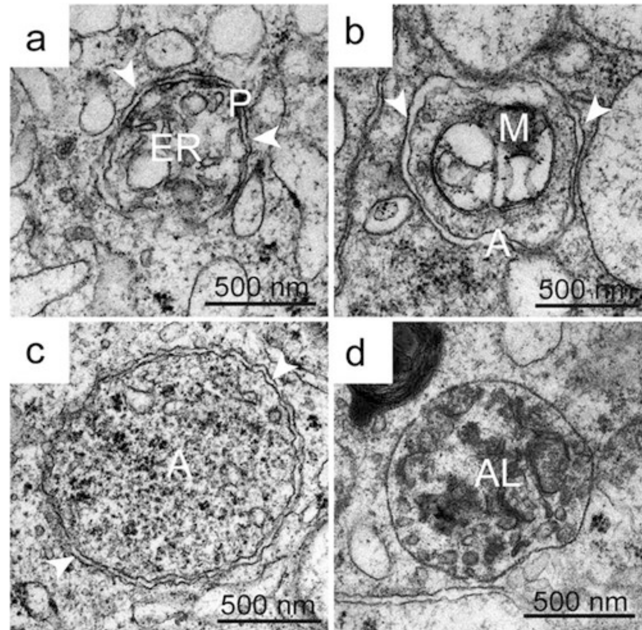

Fig. 2 Fine structure of phagophore, autophagosome, and autolysosome in Sertoli cells. (**a**) One putative phagophore seems to be in the process of wrapping around the endoplasmic reticulum (ER) with double membrane (arrow heads). P indicates phagophore. (**b**) The autophagosome contains a damaged mitochondrion. The limiting membrane (large arrowheads) is visible partially as a double membrane (arrow heads). M indicates mitochondrion, and A indicates autophagosome. (**c**) The autophagosome contains ER and ribosomes. The limiting membrane (large arrowheads) is visible as a double membrane (arrow heads). Ribosomes become more electron dense as the consequence of degradation. A indicates autophagosome. (**d**) One putative autolysosome seems to contain cytoplasmic components at various stages of degradation with a single-membrane structure. AL indicates autolysosome

2. Gently puncture testes in three to four areas with a 25G syringe needle and immersion fix in 2.5% (vol/vol) glutaraldehyde and 1.5% (vol/vol) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2 h at room temperature (RT) (*see* **Note 12**).
3. Cut testes into small pieces (1–2 mm cubes or pellets), and then fix the samples overnight at 4 °C.
4. Wash 5 × 15 min in 0.1 M cacodylate buffer (pH 7.3) at RT. It could be stored at 4 °C for up to some weeks.
5. Postfix in 1% osmium tetroxide in water for 1 h at 4 °C. As osmium is very toxic and volatile, use a good fume cupboard and gloves.
6. Wash 5 × 15 min in 0.1 M cacodylate buffer at RT.
7. Stain the cells in 2% uranyl acetate in water at RT, in the dark, for 1 h (*see* **Note 13**)

8. Wash 3 × 15 min in water at RT.
9. Dehydrate in a graded acetone series at RT (5 min each of 50%, 60%, 70%, 80%, 90%, 95% acetone in water). Incubate with 100% acetone 3 × 5 min in a new tube.
10. Incubate for 1.5 h in 3:1100% acetone and resin, followed by 1.5 h in 1:1100% acetone and resin at RT. And then incubate for 6 h in 1:3100% acetone and resin at RT.
11. Incubate for 3 × 12 h in pure resin at RT.
12. Embed the tissue in freshly made resin in a silicone mold (*see* **Note 14**). Polymerize the resin at 35 °C for 24 h, 45 °C for 24 h, and then at 65 °C for 24 h.

3.3.2 Sectioning and Collection on Grids

1. Cut 60 nm sections on an ultramicrotome and float on the water surface behind the diamond knife.
2. Collect sections on 100 mesh copper grids and stain the sections with uranyl acetate and lead citrate.
3. Image can be observed using a JEM-1400 transmission electron microscope (*see* **Note 15**).

3.4 GFP-LC3 Lysosomal Delivery and Proteolysis

GFP-LC3 is very useful to follow autophagy flux, as the LC3 part of the chimera is sensitive to degradation, whereas the GFP protein is relatively resistant to hydrolysis. The appearance of free GFP on western blots can be used to monitor lysis of the inner autophagosome membrane and breakdown of the cargo [2, 17].

1. Dilute 4 μg of GFP-LC3 plasmid in 100 μL Opti-MEM and 10 μL of lipofectamine 2000 in 100 μL Opti-MEM. After a first incubation of 5 min, gently mix the diluted plasmid solution and diluted lipofectamine 2000 solution, and incubate for another 20 min (*see* **Note 16**).
2. Add 200 μL of solution containing the lipofectamine 2000 and GFP-LC3 plasmid to 10^5 primary cultured Sertoli cells in 30 mm cell culture plates. Incubated at 34 °C, 5% CO_2 for 24 h.
3. Remove the medium and wash Sertoli cells with PBS.
4. Add fresh Sertoli cell culture medium and culture cell at 34 °C, 5% CO_2 for 24 h.
5. Collect primary cultured Sertoli cells by trypsinization, and perform the immunoblotting analysis of GFP as described in Subheading 3.2 (*see* **Note 17**).

3.5 Immunoblotting Analysis of SQSTM1/p62

The SQSTM1/p62 protein serves as a link between LC3 and ubiquitinated substrates, and it is incorporated into the completed autophagosome and degraded in autolysosomes. The inhibition of autophagy correlates with increased levels of SQSTM1 (Fig. 1),

suggesting that steady-state levels of this protein reflect the autophagic status [2, 17].

1. Isolate the primary Sertoli cell from mouse testes as described in Subheading 3.2.1.
2. Prepare Sertoli cell lysates as described in Subheading 3.2.2.
3. Perform the immunoblotting analysis of SQSTM1 as described in Subheadings 3.2.3 and 3.2.4.

3.6 Turnover of Autophagic Substrate Proteins

3.6.1 Immunoblotting Analysis of Autophagic Substrate Proteins

1. Isolate the primary Sertoli cell from mouse testes as described in Subheading 3.2.1.
2. Prepare Sertoli cell lysates as described in Subheading 3.2.2.
3. Perform the immunoblotting analysis of autophagic substrate proteins as described in Subheadings 3.2.3 and 3.2.4.

3.6.2 Immunofluorescence Analysis of Autophagic Substrate Proteins in Primary Sertoli Cells

1. Seed primary Sertoli cells in 6-well plates in which sterile cover slips have been previously deposited.
2. Culture Sertoli cells for 24 h. Remove growth medium and wash cells twice with PBS.
3. Fix Sertoli cells in 4% PFA for 5 min at RT (*see* **Note 18**).
4. Remove fixative solution and rinse Sertoli cells 3 × 5 min in PBS at RT.
5. Perform the immunofluorescence experiment as described in Subheading 3.1, **steps 7–14**.
6. Mount the cover slips with Sertoli cells onto slides using mounting medium, and seal cover slip with nail polish to prevent drying.
7. Fluorescent images are obtained using a fluorescence microscope.

3.6.3 Immunofluorescence Analysis of Autophagic Substrate Proteins in Apical Ectoplasmic Specialization Region of Sertoli Cells

1. Testes are dissected from adult mice after euthanasia. Wash testes with PBS immediately. Repeat twice.
2. Fix testis in 4% PFA at room temperature for 2 h.
3. Wash the testis with PBS twice and decapsulate it in PBS.
4. Mince the seminiferous tubules into small pieces using scalpels, and then gently aspirate the small pieces, first through an 18-gauge needle and then a 21-gauge needle to fragment the seminiferous epithelium.
5. Sediment for 5 min (*see* **Note 19**). Remove the supernatant fraction to a new tube and concentrate at 800 × *g* for 5 min.
6. Remove the supernatant fraction and resuspend the pellet in a small amount of PBS.
7. Place drops of the material on APE precoated slides for 10 min.

8. Remove excess fluid, and immediately submerge the samples in cold acetone (−20 °C) for 5 min and then air dry.
9. Perform the immunofluorescence experiment as described in Subheading 3.1, **steps 7–15**.

3.6.4 Cycloheximide Chase Assay for Autophagic Protein

1. Isolate the primary Sertoli cell from mouse testes as described in Subheading 3.2.1. Plate Sertoli cells 1 day before the experiment (*see* **Note 20**).
2. Add the cycloheximide to the culture at 100 μg/mL to block new protein synthesis.
3. Collect primary cultured Sertoli cells by trypsinization at different time points after the cycloheximide treatment.
4. Prepare Sertoli cell lysates as described in Subheading 3.2.2.
5. Perform the immunoblotting analysis of the targeted protein as described in Subheadings 3.2.3 and 3.2.4.

3.6.5 Co-immuno-precipitation of LC3 and Autophagic Substrate Proteins

1. Collect primary cultured Sertoli cells by trypsinization and wash once in cold (4 °C) PBS. Centrifuge at 4 °C, 500 × *g* for 5 min.
2. Discard supernatant, and resuspend the pellets by using 150–200 μL of TAP buffer supplemented with 1 mM PMSF and a protein inhibitor cocktail. Incubate on ice for 30 min.
3. Centrifuge the homogenates at 4 °C, 13,523 × *g* for 15 min. Remove the supernatant to a new tube.
4. Incubate cell lysates with primary antibody at 4 °C overnight.
5. Incubate samples with protein A-Sepharose at 4 °C for 2 h.
6. Centrifuge at 4 °C, 1500 × *g* for 2 min. Remove the supernatant.
7. Wash the precipitants twice with IP buffer.
8. Add 30 μL of 2× loading buffer to proteins from each lysate, and then incubate for 15 min at 65 °C.
9. Chill the samples on ice and centrifuge at 4 °C, 13,523 × *g* for 10 min.
10. Perform the immunoblotting analysis of the targeted protein as described in Subheadings 3.2.3 and 3.2.4.

4 Notes

1. As many autophagy marker proteins localize on the membranes, such as LC3-II which is recruited to autophagosomal membranes, it is important to consider the hydrophobicity of autophagy marker proteins and avoid adding Triton or some other detergent in the primary antibody solution.

2. In **step 3**, the testes should be sufficiently digested to the seminiferous tubules which showed filamentous dispersion. In **step 5**, the seminiferous tubules should be sufficiently digested to short fragments. In **step 7**, the seminiferous tubules should be sufficiently digested to small dots.
3. This final digestion step resulted in a cell suspension containing primarily Sertoli cells and type A spermatogonia.
4. To minimize proteolytic degradation, samples should be kept on ice until adding loading buffer and boiling or storage.
5. As protein quantification is performed by interpolating a calibration curve that is built from a serial dilution of BSA and BSA standards are rarely included in the kits for quantification, thus BSA standards should be prepared shortly before use from the BSA stock solution.
6. As LC3-II is recruited to autophagosomal membranes, it is important to consider the hydrophobicity of LC3-II.
7. The percentage of acrylamide directly influence its separation range and resolution determines. As a guideline, proteins with smaller proteins can be separated on gels containing 10–15% acrylamide.
8. Pouring should be performed quickly, while avoiding the formation of bubbles.
9. Polymerization time depends on acrylamide concentration, but usually 30 min is sufficient.
10. Before entry in the running gel, voltage should be set at 50–70 mV. Thereafter, voltage can be augmented to 140 mV. It should be kept in mind that higher voltages result in reduced migration time but also in lower resolution.
11. To control temperature, an ice block is placed next to the transfer cassette.
12. After being fixed for 30 min to 1 h, if the testis appears firm, it can be sliced in half and then fixed for the additional hour. Avoid undue pressure on the organ.
13. This step gives contrast to autophagosome membranes.
14. Wash the silicone mold clearly as soon as possible.
15. The defined autophagosomes are double-membrane vacuoles contained undegraded cytoplasmic cargos. In some case, the two membranes are often too close to see them as one lay membrane. However, sometimes the membrane may appear to contain multiple layers. Occasionally, it is possible to observe putative phagophores, a cup-shaped double-membrane structure, which seem to be engulfing portions of the cytoplasm. The cytoplasmic contents of autophagosomes include organelles, such as ER membranes, mitochondria, and ribosomes.

The diameter of autophagosome profiles varies between 300 and 400 nm and several micrometers. Autophagosomes are frequently observed in fusion profiles with endosomal or lysosomal vesicles. In the fusion event, the outer limiting membrane fuses with the endo/lysosome limiting membrane. The contents, still surrounded by the inner limiting membrane, are delivered to the endo/lysosome lumen. It should be noted that special attention must be paid when identifying autophagic vacuoles in transmission electron microscopy. There are several examples in the current literature of different organelles, including multilamellar or multivesicular endosomes, even mitochondria, being claimed as autophagic vacuoles. Only vacuoles containing cytoplasmic material, in most cases ribosomes, can be claimed as autophagic.

16. As the transfection rate of Sertoli cells is lower, the concentration of plasmid and lipofectamine 2000 should be excessive.
17. As slight non-autophagic degradation of GFP-LC3 may seriously influence this experiment, operations should be quick as soon as possible, and samples should be always kept on ice until adding loading buffer.
18. As Sertoli cells are much thinner than the testis, the fixed time should not exceed 5 min.
19. During this time, larger tubule fragments settled to the bottom of the tube, and smaller epithelial fragments containing mature spermatids with associated Sertoli cell apical processes remained suspended in solution.
20. To avoid the influence caused by the operations collecting samples in different time point, 30 mm cell culture plates, but not 6-well cell culture plates, are encouraged to be used in this experiment.

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