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MicroRNA let-7g regulates mouse granulosa cell autophagy by targeting insulin-like growth factor 1 receptor

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ABSTRACT

As an important type of somatic cell, granulosa cells play a major role in deciding the fate of follicles. Therefore, analyses of granulosa cell apoptosis and follicular atresia have become hotspots of animal research. Autophagy is a cellular catabolic mechanism that protects cells from stress conditions, including starvation, hypoxia, and accumulation of misfolded proteins. However, the relationship between autophagy and apoptosis in granulosa cells is not well known. Here, we demonstrate that let-7g regulates the mouse granulosa cell autophagy signaling pathway by inhibiting insulin-like growth factor 1 receptor expression and affecting the phosphorylation of protein kinase B/mammalian target of rapamycin. Small interference-mediated knockdown of insulin-like growth factor 1 receptor significantly promoted autophagy signaling of mouse granulosa cells. In contrast, overexpression of insulin-like growth factor 1 receptor in mouse granulosa cells attenuated autophagy activity in the presence of let-7g. In addition, overexpression of let-7g increased the apoptosis rate, as indicated by an increased number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-positive cells. Finally, 3-methyladenine as well as the lysosomal enzyme inhibitor chloroquine partially blocked apoptosis. In summary, this study demonstrates that let-7g regulates autophagy in mouse granulosa cells by targeting insulin-like growth factor 1 receptor and downregulating protein kinase B/mammalian target of rapamycin signaling, and that mouse granulosa cell autophagy induced by let-7g participates in apoptosis.

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1. Introduction

Autophagy is a highly conserved mechanism involved in degradation of long-lived proteins and damaged organelles (Mizushima, 2007). It promotes cell survival and can be rapidly activated upon exposure to conditions of stress, including starvation (Sato et al., 2007), hypoxia (Azad et al., 2008), and interruption of growth signaling (Lum et al., 2005). Moreover, it is associated with resistance to diverse ailments, including cancer, neurodegenerative diseases, and infections (Mizushima et al., 2008; Ravikumar et al., 2010). During autophagy, cytoplasmic components are sequestered into a double-membrane vesicle, namely autophagic vesicles or autophagosomes. Subsequently, the completed autophagosome acidifies and fuses with late endosomes or lysosomes to become autolysosomes, resulting in degradation of cytoplasmic cargo. Following cargo degradation, the products are recycled for further use (Gozuacik and Kimchi, 2004; Nakatogawa et al., 2009).

Insulin-like growth factor 1 (IGF-1) is a key regulator of cell growth and development (Bach et al., 1991). The binding of IGF-1 to its receptor (IGF1R) triggers the recruitment and activation of several intracellular kinases, including phosphatidylinositol-3-kinase (PI3K). PI3K phosphorylates and converts the membrane phospholipid phosphatidylinositol-4, 5-bisphosphate into phosphatidylinositol-3, 4, 5-trisphosphate, generating a lipid-binding site on the cell membrane for the serine/threonine kinase Akt (or PKB-protein kinase B). Activated Akt regulates mammalian target of rapamycin (mTOR) signaling through the tuberous scle-

Abbreviations: miRNA, microRNA; IGF1R, insulin-like growth factor 1 receptor; Akt, v-akt murine thymoma viral oncogene homolog 1; mTOR, mammalian target of rapamycin; MGC, mouse granulosa cell; LC3, microtubule-associated protein 1 light chain 3; p-Akt, phosphorylated-Akt; p-mTOR, phosphorylated-mTOR; siRNA, small interference RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling terminal; UTR, untranslated region; FACS, fluorescence-activated cell sorting; 3-MA, 3-methyladenine; CQ, chloroquine; IGF-1, insulin-like growth factor 1; PI3K, phosphatidylinositol-3-kinase; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; AMP, adenosine monophosphate; ATP, adenosine-triphosphate; ICR, Institute of Cancer Research; PMSG, pregnant mare serum gonadotropin; PBS, phosphate buffered saline; miR-scr, miRNA-scramble; MDC, Monodansylcadaverine; MRE, miRNA response element; WT, wide type; MT, mutant type; siRNA-CNT, control-siRNA; IL-33, interleukin-33; ox-LDL, oxidized low density lipoprotein; FoxO, Forkhead box O.

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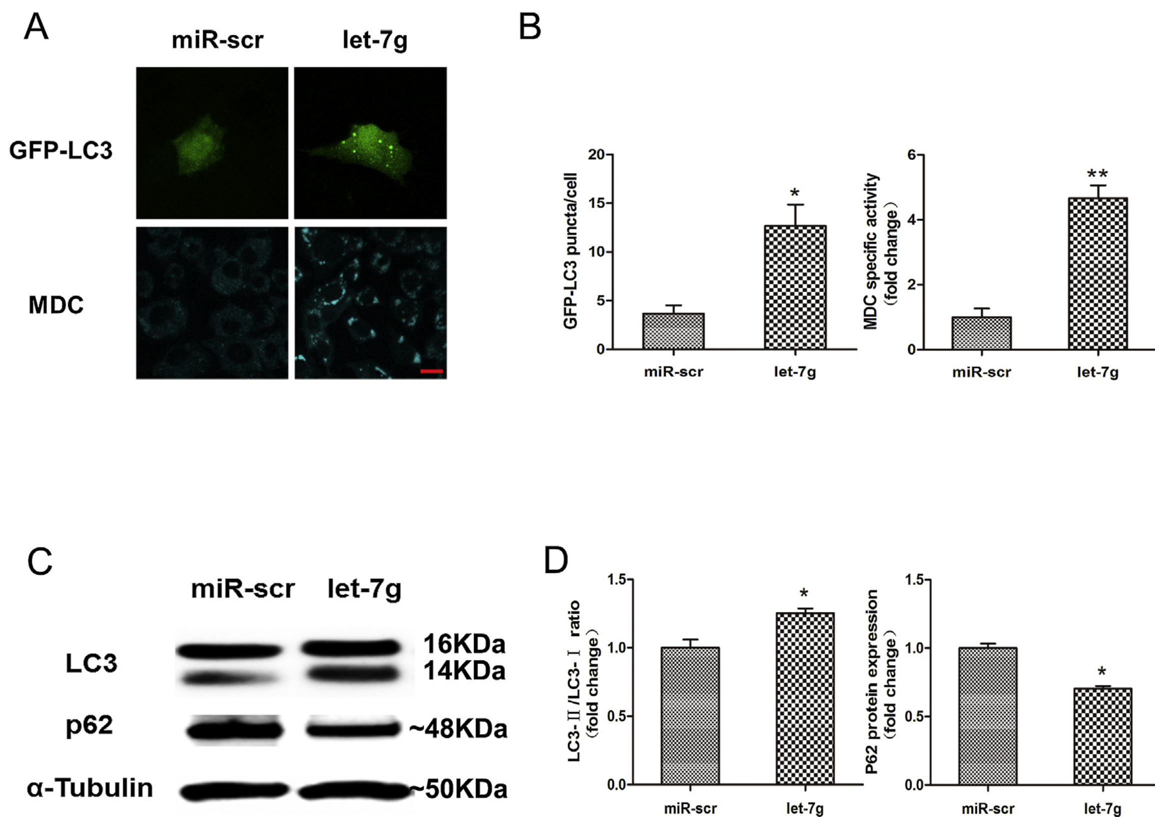

Fig. 1. Let-7g induces MGC autophagy.
(A) *let-7g* increased GFP-LC3 puncta formation and MDC activity in MGCs. Cells were cotransfected with *let-7g* or scramble miRNA (*miR-scr*) together with the GFP-LC3 plasmid, and autophagy was assessed after 48 h. Bar = 10 μm. (B) Quantitative analysis of the data in (A). Overexpression of *let-7g*, but not the control (*miR-scr*), increased autophagy activity in MGCs. (C) *let-7g* increased the conversion of LC3-I into LC3-II and decreased the P62 protein level in MGCs. Western blot results of extracts from cells transfected with *let-7g* or control (*miR-scr*) (n = 3). α-*Tubulin* was used as a loading control. (D) Quantitative analysis of the data in (C) (mean ± SD of independent experiments, n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$).

rosis proteins TSC1 (hamartin) and TSC2 (tuberin) (Edinger and Thompson, 2002). Recent work has suggested that Akt can also signal to mTOR by decreasing the AMP/ATP ratio and thus preventing AMPK from inhibiting TSC1/2 (Hahn-Windgassen et al., 2005). mTOR acts as a central sensor of growth factors, nutritional condition, and energy status, and is a master regulator of autophagy (Codogno and Meijer, 2005). The mTOR inhibitor rapamycin upregulates autophagy even under normal conditions (Blommaart et al., 1995; Noda and Ohsumi, 1998).

MicroRNAs (miRNAs) are small (20–22 nucleotides long), noncoding RNAs that are expressed endogenously in various organisms from plants to mammals (Ambros, 2004). They control various fundamental biological processes including cell development, proliferation, differentiation, migration, and apoptosis, and an important and central role in autophagy regulation (Fu et al., 2012; Xu et al., 2012). Let-7 miRNA, one of the first miRNAs discovered, was initially shown to have a relationship with autophagy (Dubinsky et al., 2014). let-7g is a member of the let-7 family with an important role in animal reproduction (Shinoda et al., 2013; West et al., 2009). We have found that let-7g is the only miRNA significantly upregulated during follicular atresia and that it promotes apoptosis in granulosa cells (GCs) (Lin et al., 2012; Zhou et al., 2015). Autophagy is induced mainly in GCs and regulates apoptosis (Choi et al., 2010, 2011). However, the relationship between let-7g and autophagy is unclear. Here, we demonstrated the function and epigenetic mechanism of let-7g in MGCs autophagy, and confirmed its role in the regulation of autophagy in GCs.

2. Materials and methods

2.1. Cell culture

Female ICR mice (Nanjing Qinglongshan Experemental Animal Center) were injected intraperitoneally with 10 units of PMSG (Nicolson et al., 1975) and sacrificed 44 h later. Superovulated mouse ovaries were obtained and transferred to Petri dishes (35 × 15 mm) filled with PBS and then punctured with a syringe to release MGCs from DFs (>200 μm in diameter) under a surgical dissecting microscope. MGCs (1×10^6) were seeded into T25 flasks in 4 mL Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (1:1; Life Technologies, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Life Technologies) and 1% antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin; Life Technologies). Before treatment, cells were incubated at 37 °C and 5% CO_2 for 3 days. Human embryonic kidney (HEK) 293 cells (Institute of Biochemistry and Cell Biology, CAS, Shanghai, China) were cultured in DMEM high-glucose medium (Invitrogen) with 10% FBS (Gibco) following previously reported protocols.

2.2. Plasmid construction

The 3′UTR of IGF1R containing the predicted let-7g binding site was synthesized by Shanghai Generay (Appendix: Supplementary table S1). A double-stranded linker DNA with *Sac*I and *Xho*I sites was cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The plasmid encoding GFP-LC3 was kindly provided by Prof. Jiyong Zhou of Zhejiang University, Zhejiang, China.

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