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RESEARCH ARTICLE

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Expression pattern and subcellular localization of p62/SQSTM 1 during mouse oocyte maturation

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Abstract

Objective: Autophagy is a survival mechanism, and it is initiated by several factors such as oxidative stress. Cells give autophagy response against oxidative stress through Nrf2-Keap1-p62 pathway. p62 or Sequestosome 1 (SQSTM 1) which takes place during autophagy, and it is showed that p62 plays critical role during primordial follicle formation. During oocyte maturation, Germinal Vesicle (GV) oocytes resume meiosis with the beginning of puberty and progress through Metaphase I and Metaphase II stages. Metaphase II (MII) oocytes are ready to be fertilized and until fertilization they are arrested at metaphase II stages. It is known that oxidative stress is increasing during oocyte maturation. However, it is not shown that spatial and temporal expression of p62 throughout oocyte maturation. Thus, the aim of the study was to reveal expression pattern and the subcellular localization of the p62 protein in mouse GV, MI and MII oocytes.

Methods: GV oocytes were received from female Balb/C mice at 4 weeks aged and GV oocytes were cultured for 8h to develop into MI oocytes and for 14h to mature into MII oocytes. Then, expression of p62 protein was observed in oocytes at GV, MI and MII maturation stages by using immunofluorescence method.

Results: Our results showed that there is a significant increasing p62 expression when GV oocytes are compared to MI and MII oocytes, but there is no difference between MI and MII oocytes. Moreover, we revealed that p62 is localized in cytoplasm of GV oocytes, while it is localized around chromosomes in MI and MII oocytes.

Conclusion: In conclusion, our results indicate that further study is mandatory to understand the participation of p62 during meiosis, and the impact of p62 during oocyte maturation.

Key words: Autophagy, oocyte maturation, p62, SQSTM 1

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INTRODUCTION

Autophagy is a survival mechanism of the cells as it is able to degrade and recycle the components of the cell in case of damaged organelles or unfunctional proteins, starving and oxidative stress (1). Autophagy helps cells to keep homeostasis in balance (2). In oocytes, autophagy is initiated with the increased ROS (Reactive oxygen species) level and disrupted antioxidant mechanisms of the cell (3). p62 or Sequestosome 1 (SQSTM 1) which has a critical role as an adaptor protein during autophagy mechanism (4). When there are ubiquitinated proteins or organelles, p62 is phosphorylated and p62 becomes more mobile and binds to ubiquitinated proteins (5). Also, mTORC 1 phosphorylates p62 and phosphorylated p62 shows more affinity against KEAP 1 protein (6). Phosphorylated p62 proteins which are bound to ubiquitinated protein aggregation interact with Keap1(7) , phosphorylated p62 and ubiquitinated proteins are recognized by LC3 on autophagosome, then all of them are degraded by autolysosome (8). p62 also takes place in oxidative stress response through Nrf2-Keap1 pathway (9). Unless there is an oxidative stress, p62 is self-oligomerized and found in the cytoplasm, and Keap1 which binds to Nrf2 prevents Nrf2 from entering the nucleus. When oxidative stress is present in the cell, p62 is phosphorylated and binds to Keap1, so it prevents Keap1 from binding to Nrf2 transcription factor and free Nrf2 can now move into the nucleus and activate transcription of genes which enable cells to deal with oxidative stress, surprisingly Nrf2 is also activate expression of p62, so a positive feedback is observed between Nrf2 and p62 (7, 10, 11).

Germ cells are differentiated from Primordial Germ Cells (PGCs). During embryonic development, PGCs migrate from the endoderm of yolk sac to the gonadal ridges, and they start differentiation in there and form oogonia (12). Oogonia cells enter meiosis, but they arrest at prophase I and they are called as Germinal Vesicle (GV) oocytes at this stage (13). With puberty, they resume their meiosis and proceed through Metaphase I and Metaphase II stages. Until fertilization, they are arrested at metaphase II stages (14).

During development of GV oocyte into MII oocyte, ROS level increases and it exceeds the level which could be compensated by natural cellular antioxidant, and homeostasis become imbalance and oxidative stress is formed (15). Therefore, since p62 is an adaptor protein which takes place in autophagy and the oxidative stress is one of the inducer for the autophagy (16), and according to our literature search there are no studies in the literature that show expression level of p62 during oocyte maturation, the study was designed to reveal the expression pattern and localization of the p62 protein in mouse GV, MI and MII oocytes.

METHODS

Animals

The approval of experiment protocol was received from the Animal Care and Usage Committee of Ankara University (Protocol no: 2020-2-19). The female Balb/C mice at 4 weeks-aged (n=12) were purchased from the Research Animal Laboratory Unit of Ankara University. Hosting of the mice was under freely accessing to food and water conditions and hold in a 12 hr light-dark cycle.

Oocyte Collection and in vitro Culture

Cumulus-enclosed GV oocytes were obtained by using the ovaries of 4-week-old Balb/C female mice following superovulation with 5 IU pregnant mare's serum gonadotropin (PMSG, Intervet). Adipose tissue of the ovaries was removed, cumulus- oocytes complexes (COC) comprising GV oocytes were released through puncturing the ovaries by using the 23-gauge needle in morpholinepropanesulfonic acid (MOPS) buffered medium (G-MOPS^{TM}) (Vitrolife, Sweden). The cumulus cells were got rid of by pipetting. Following denuding, GV oocytes were placed into the culture medium (G-TL^{TM}; Vitrolife, Sweden) as 50 µL of culture drops inside 35 mm culture dishes (Corning, USA) that were filled with 3 mL Ovoil (10029, Vitrolife). GV oocytes (0 hr) were cultured up to Metaphase I (MI) oocytes (8 hr) and Metaphase II (MII) oocytes (14 hr) at 37 °C in 5% CO_2. Around 100 oocytes were considered for each group, and all experiments were carried out at least in three replicates.

Immunofluorescence (IF) Staining

Following fixation of GV, MI and MII oocytes in 4% paraformaldehyde (Sigma-Aldrich, USA), permeabilization was performed with 1% Triton X-100 (Thermo Fischer Scientific, USA) at room temperature (RT), afterwards blocking with 20% normal goat serum (Vector Laboratory, USA) blocking solution, IF was performed to determine the spatial temporal expression pattern of the p62 in the GV, MI and MII stage oocytes. In summary, oocytes were placed overnight at +4°C with rabbit monoclonal antibody for p62 (Cell Signaling, 23214) After a triple wash with 1x PBS consisting of 2% bovine serum albumin (BSA) for 10 min (PBS-BSA; Sigma-Aldrich, USA), incubation of oocytes was performed with anti-rabbit Alexa 488 secondary antibody (Invitrogen, USA) throughout 1 hr at RT, then washing of the oocytes was performed three times with 1x PBS-BSA solution for 10 min each. Control group was prepared by omission of primary antibody application. Mini well trays (Thermo Fisher Scientific, USA) were used for the staining part of the procedure in a humidified chamber. Then, oocytes were placed into a 4 µLdrop of PBS-based mounting medium consisting of 1 µg/mL Hoechst (Thermo Fisher Scientific, USA) indicating DNA on glass-bottomed 35-mm Petri dishes. The drops were overlaid with paraffin oil.

Oocytes were intact in terms of their 3D spherical shape and then micrographs were taken by using a Zeiss LSM-880 Airyscan® system (Zeiss, Germany). Staining of oocytes for all groups was performed at least five times and cumulated images were analyzed. Measurement of the signal intensity was performed with using Image J software (National Institutes of Health, Bethesda, Maryland, USA), and the signal intensities of the p62 has been quantified relatively.

Statistical Analysis

Analysis of the experimental results were performed by using one-way analysis of variance (one-way ANOVA) followed by Dunn's post hoc test. Statistical analyses were conducted with SigmaStat for Windows, version 3.5 (Jandel Scientific Corp). For all tests, $P < 0.05$ was evaluated as statistically significant.

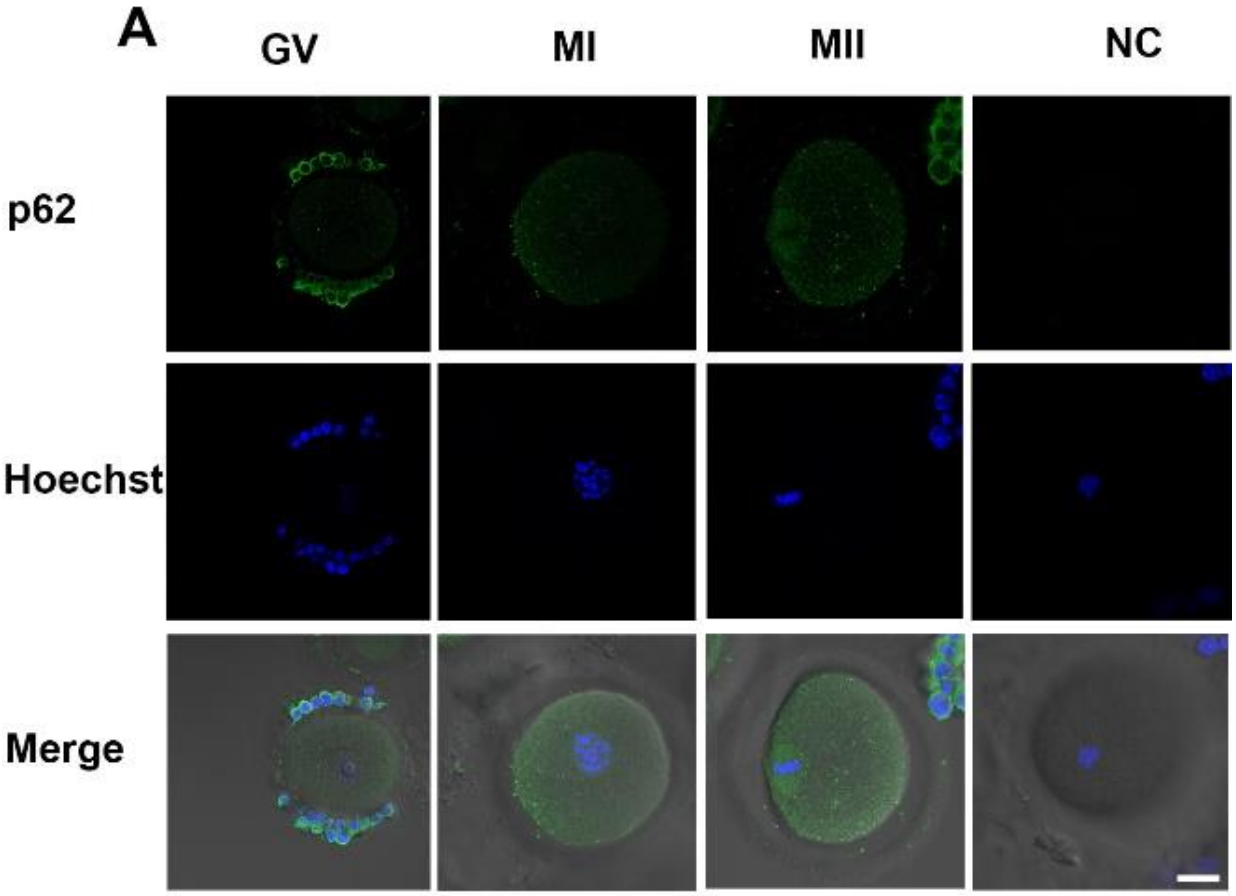
RESULTS

In the study, we aimed to show p62 expression pattern during the oocyte maturation. When the p62 expression level is considered temporally, signal intensity of the MI and MII oocytes is significantly increased compared with the signal intensity of GV oocytes. However, there is not statistically meaningful difference in p62 expression level between MI and MII oocytes (Fig. 1B). In addition to that, we aimed to reveal p62 expression spatially. In GV oocytes, p62 signal was observed in cytoplasm and particularly in granulosa cells, while in MI and MII oocytes it was observed around the chromosomes (Fig. 1A and Fig. 2). Moreover, in polar body of MII oocytes, p62 signal was observed around the chromosomes (Fig. 2).

DISCUSSION

In this study, this is the first-time; we have shown that expression pattern of the p62 protein during mouse oocyte maturation. We found that expression level of p62 was significantly increased when GV oocytes were compared with both MI and MII oocytes, but there was no difference between MI and MII oocytes. In literature, it is showed that mitophagy, selective autophagy, inhibition results in decreased quality and viability of vitrified porcine oocytes and unsuccessful embryonic development (17). Moreover, autophagy is responsible for the primordial follicle formation and oocyte reserve (8). Also, it is showed that p62 expression level is changed during the primordial follicle formation and activated autophagy is related with more oocyte reserve as there is a crosstalk between apoptosis and autophagy (18). Furthermore, in post maturation oocyte aging (PMOA), autophagy is activated during in vitro mouse oocyte maturation until 12h incubation, but its activity turns to basal level between 12h and 18h. p62 level is decreased during first 12h but increased between 12h and 18h. It is suggested that autophagy prevents apoptosis during early PMOA, but in late PMOA, it cannot prevent

oocytes from the entering apoptosis since higher activity of the caspases inhibit autophagy (19). However, during oocyte maturation, expression level of p62, such an important protein for the autophagy, is not investigated.



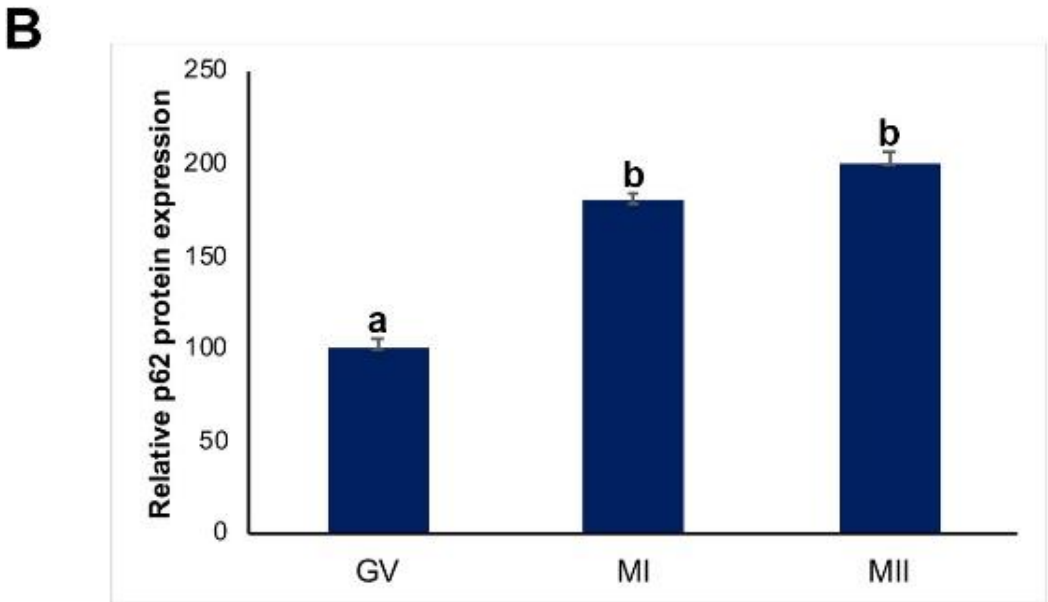


Figure 1. Immunofluorescent (IF) staining. **A.** p62 expression (green) and DNA (blue) in Germinal Vesicle (GV), Metaphase I (MI) and Metaphase II (MII) oocytes (n=100). **B.** Relative p62 protein expression from IF during oocyte maturation. Different letters are indicator for $p<0.05$. NC, negative control. Scale bar: 20 µm.

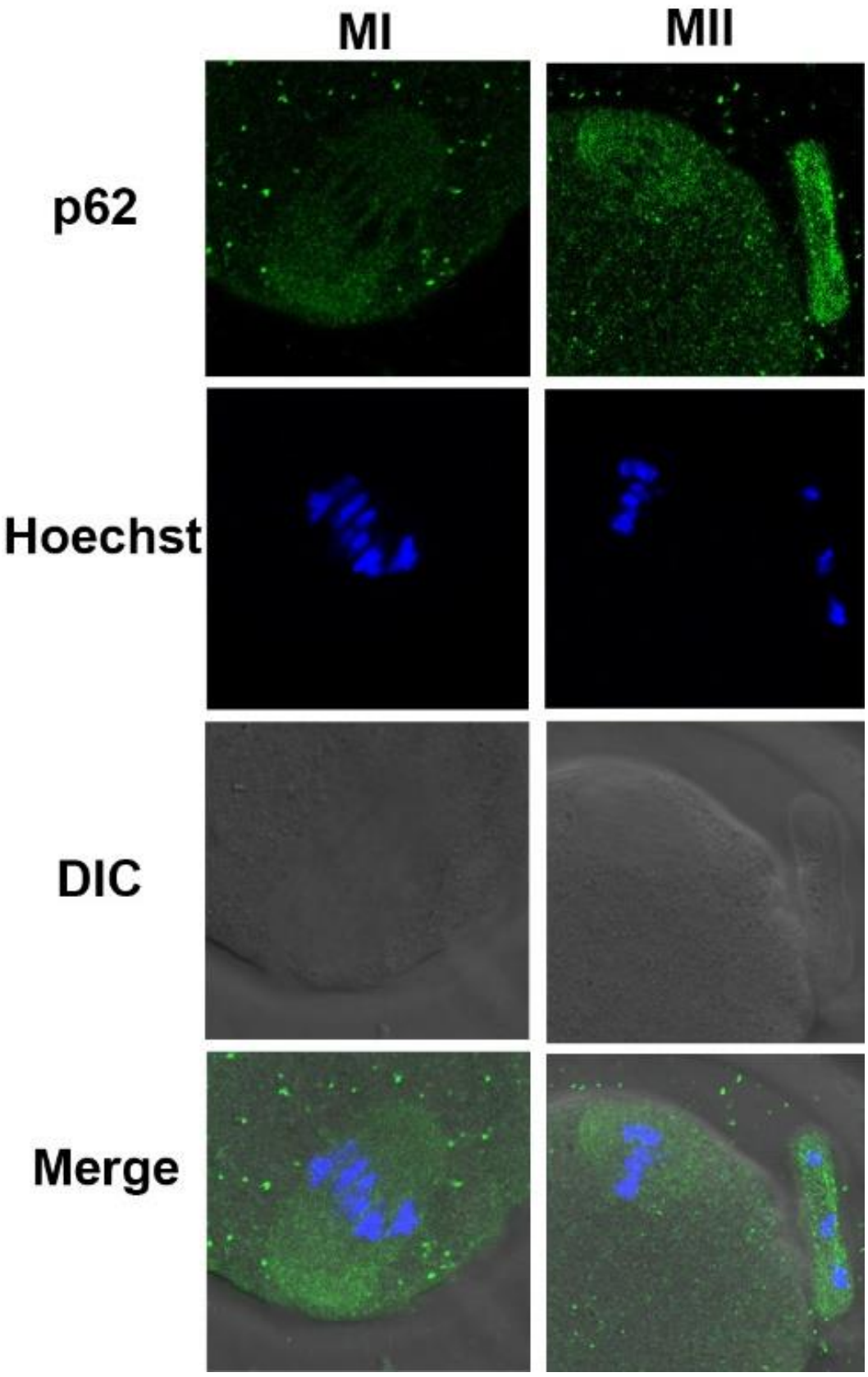


Figure 2. Immunofluorescent (IF) staining. p62 expression (green) is shown around chromosomes and meiotic spindles in Metaphase I (MI) and Metaphase II (MII) oocytes. Hoechst staining indicates chromosomes. DIC, differential interference contrast image

On the other hand, we showed cellular localization of the p62 during oocyte maturation. In GV oocytes, p62 was localized in cytoplasm and in surrounding granulosa cells (GCs). GCs support the maturation of oocytes (20). While p62 is accumulated and become unfunctional in cytoplasm, autophagy is decreasing in granulosa cells with aging, so promotion of oocyte maturation may be disrupted (21). We also observed that, p62 is located around the chromosomes and spindles in MI and MII oocytes and this result may bring the question about the role of p62 during meiosis. Previously, in sea urchin oocytes, it is demonstrated that p62 is bound to the chromatin at interphase stage while in clam germinal vesicle oocytes, p62 binding to chromatin is observed at prophase I (22).

Autophagy is crucial for homeostasis to maintain quality and function of cells and p62 takes place in autophagy as an adaptor protein (23). Moreover, it is known autophagy is activated in the case of oxidative stress and during oocyte maturation oxidative stress is increasing (24). Thus, investigating the expression level and subcellular localization of p62 protein may be important for understanding the compensation mechanisms of the oocytes against increased ROS level during oocyte maturation.

CONCLUSİON

In conclusion, the current work demonstrated the spatio-temporal expression of p62 protein in GV, MI and MII mouse oocytes. Our results give point of view about the relationship between autophagy, oocyte maturation and the potential role of p62 during meiosis. Additional studies, such as effect of silencing p62 during oocyte maturation, may reveal the function of p62 and its importance during oocyte maturation and meiosis.

Ethics Committee Approval: Approval of the experimental protocol was obtained from the Animal Care and Usage Committee of Ankara University (Protocol no: 2020-2-19).

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