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Cathepsin L is Required for Completion of Oocyte Meiotic Maturation in Mammals

Eman Awad[1*], Wael Bedir Eldomany[2], Abdelmonem Montaser[2], Samy Zaabel[2]

[1]Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt.
[2]Department of Theriogenology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt.

***Correspondence**
Eman Awad
Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt.
E-mail: emanawadsherif@gmail.com

Abstract

Infertility is a major concern affecting a huge population worldwide. Aneuploidy is one of the leading causes of the infertility, usually originated from errors that happens in meiosis I as chromosomal misalignment and abnormal chromosomal segregation. Recent research showed that cathepsins family play an important role in affecting the oocytes developmental competence in many species. Cathepsin B inhibition improved bovine developmental competence of the oocytes, especially in the low-quality oocytes. Here, we are the first to introduce the significance of another member of this family, cathepsin L (CTSL) in the process of oocyte maturation using mouse oocyte model. In this study we evaluated the expression of CTSL during oocyte meiotic maturation and the effect of CTSL inhibition using specific inhibitor "SCP110" on the subsequent maturation process. We found, CTSL is expressed in all stages of Meiosis I. In addition, inhibition of CTSL resulted in delaying the maturation time by elongation the time required for extrusion of the polar body (PBE). Moreover, CTSL inhibition reduced the maturation rate compared to the control group as we found a significant decrease in PBE percent. Trying to understand the reason of the lower maturation rates, we stained the chromosomes and spindles at metaphase I stage, we found a significant increase in chromosomal misalignment at metaphase plate. Finally, we evaluated matured oocytes quality after CTSL inhibition, importantly, aneuploidy incidence was increased significantly after CTSL inhibition. In summary, CTSL is required for normal meiosis completion in oocytes and production of healthy euploid egg.

KEYWORDS
CTSL, oocytes, meiosis, SCP110

INTRODUCTION

Cathepsin is originated from the word "Kathepsein" that describe the ability of enzyme to digest in acidic environment (Willstätter and Bamann, 1929). According to the catalytic mechanisms, cathepsins are divided into three different families: cysteine, aspartic and serine proteases. Cysteine cathepsins are a group of eleven lysosomal enzymes including cathepsin B, C and L. Aspartic proteases as D and E while the serine proteases include A and G (Burster *et al*., 2010; Turk *et al*., 2012). Cysteine cathepsins are mainly lysosomal proteases that require acidic environment to optimize their activity (Turk *et al*., 2000; Turk *et al*., 2001).

Cathepsin L (CTSL), one of the most powerful peptidase as it has the ability to degrade nearly all the proteins and polypeptides (Barrett and Kirschke, 1981). Active CTSL can localize inside the nucleus and regulate the cell cycle dynamics and progression (Duncan *et al*., 2008; Goulet *et al*., 2004; Maubach *et al*., 2008; Santos-Rosa *et al*., 2009). In addition to the role of CTSL in proteolysis, it also has a diverse of another roles such as tissue regeneration, metastasis, bone apoptosis, and many other critical activities (Chen *et al*., 2020; Dohchin *et al*., 2000; Furuyama and Fujisawa, 2000).

A significant research has been done to study cathepsins relation with oocytes and preimplantation developmental competence especially cathepsin B and its implication in apoptotic pathways (Aboelenain *et al*., 2017; Balboula *et al*., 2022; Balboula *et al*., 2010b). Cathepsin B activity is inversely related to the quality of the oocytes and preimplantation embryos (Balboula *et al*., 2010b). Also, other cathepsins are now more involved in bovine reproduction as cathepsin S, D, Z and K (Balboula *et al*., 2020; Li *et al*., 2020), CTSL roles and involvement in oocytes development and maturation has not been evaluated yet.

Recently CTSL has shown to have a role in regulating the ovary maturation in Macrobrachium nipponense (Jiang *et al*., 2022), However CTSL role in regulating oocytes maturation and developmental competence in mammals has never been studied before. This study showed for the first time to our knowledge, the important role of CTSL in completion of meiosis I and production of healthy and euploid eggs using the mouse oocyte in vitro maturation system as a model.

MATERIALS AND METHODS

Animal handling, mouse oocyte collection and maturation

Animal handling, ethics, experiments, and the protocol of this

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study has been sent and approved from the Scientific Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University, Egypt. This study was conducted and guided with the supervision of Theriogenology department, Faculty of Veterinary medicine, Mansoura University. Mouse oocytes were collected from CF1 mice (around 8 weeks) after retrieval of the ovaries. In vitro maturation (IVM) was performed with a small modification from (Li *et al*., 2022), briefly, prophase-I arrested oocytes collected after smashing the ovaries in minimal essential medium (MEM) containing 2.5 µM milrinone (Sigma-Aldrich, M4659) to prevent spontaneous meiotic resumption. Mice, were primed by pregnant mare serum gonadotropin (PMSG, Lee Biosolutions, 493-10, 5 I.U.) two days before collection. Oocytes matured in Chatot, Ziomek and Bavister (CZB) medium for the desired time of the experiment (7.5 hours for Metaphase I and 16 hours for Metaphase II) in a condition of 5% CO_2 and 37°C.

Western blotting

Western blotting was performed as describe before (Gai *et al*., 2022), total protein from around 50 oocytes lysed using sodium dodecyl sulphate sample buffer (Laemmli Sample Buffer; Bio-Rad, 1610737) and denatured at 95°C for 5 minutes. Membranes were incubated with anti-cathepsin L antibody (Cathepsin L Antibody, cell signaling technology, #71298, 1:1000) for 1 h at room temperature. Alpha tubulin antibody was used as a loading control (α-Tubulin, DM1A, cell signaling technology, Mouse mAb #3873). Secondary antibodies horseradish peroxidase-conjugated (anti-Rabbit-HRP, R1006) were used for 1 h at room temperature. Imaging of the results and detecting of the protein bands was done using ECL Substrate Solution following the manufacturer's protocol.

Immunocytochemistry

For immunocytochemistry, as described before (Gai *et al*., 2022) oocytes from different meiotic stages were fixed in paraformaldehyde (2% PFA; Sigma-Aldrich, P6148) for 20 minutes at room temperature. After fixation, oocytes were washed in a blocking solution that contain bovine serum albumin (BSA) and tween 20 dissolved in PBS. Permeabilization was done by incubating the oocytes in the permeabilization solution containing BSA and triton X-100 (w/v) for at least 20 minutes, then followed by two consecutive washes through blocking buffer. Incubation with the primary antibodies; human anti-ACA (1:30; Antibodies Incorporated, 15-234). Secondary antibodies as goat anti-Mouse IgG (H+L) Cross-Adsorbed, Alexa Fluor™ 568 (1:200; Life Technologies, A11004), and goat anti-Human IgG (H+L) Cross-adsorbed, Alexa Fluor™ 633 (1:100; ST john's laboratory, STJS000510) were used. All secondary antibodies incubation was used for 1 hour at room temperature. Then oocytes were mounted on a slide using vectashield prior imaging.

Chromosome counting assay

To check the ploidy status of the matured oocytes, as described before (Shimoi *et al*., 2022), IVM oocytes "eggs" were treated with 100 µM Monastrol (Sigma-Aldrich, M8515), for at least two hours. Then were stained against the anti-centromeric antigen protein "ACA" and mounted with DAPI to label the chromosomes. After counting of the chromosomes, the normal number is 20 pair of the sister chromatids. Any deviation from this number will be considered as aneuploid eggs. Imaging was done using a high-quality confocal microscope confocal with 0.5 µm z-intervals.

Live imaging

To assess the timing of maturation, oocytes were matured in the imaging system using iBright imaging system (Thermofisher scientific) for at least 20 hours in 5% CO2 and 37°C to detect the time of extrusion of the polar bodies (PBE timing). Images were captured every 20 minutes.

Statistical analysis

All the experiments repeated at least three times. The data are expressed as the means ± standard error of mean (SEM). The statistical significance was analyzed by Student's t-tests or one-way ANOVA using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). *P < 0.05 was considered statistically significant. Number of oocytes and statistical significance labelling is mentioned in the figure legends section for each figure.

RESULTS

Meiosis I starts with prophase I arrested oocytes and ended by a successful chromosome segregation and cytokinesis resulted in metaphase II oocytes "egg". To check the expression of CTSL during oocyte meiosis in mouse oocytes, we did a western blotting against CTSL in the membranes containing proteins extracted from oocytes at Prophase I, Pro-metaphase, Metaphase I and Metaphase II stages at "0h, 3h, 7.5h and 16h, respectively". We found an even expression of the CTSL protein in all these stages of the meiosis I (Fig. 1). This result suggests a requirement and importance of CTSL protein during meiosis I.

Then to evaluate the CTSL importance during meiosis I, we inhibited the activity of CTSL using, SCP110 (Sigma-Aldrich), specific CTSL inhibitor. SCP110 was supplemented during IVM throughout the full time of maturation then then, maturation rate was calculated based on the polar body extrusion (PBE) percent. We tried different SCP110 concentrations, (Dimethyl sulfoxide (DMSO) "Control", 1uM, 5uM and 10uM SCP110), a significant reduction in PBE% was noticed with 5 and 10 uM concentrations, suggesting that the activity of CTSL is required for a successful completion of meiosis I (Fig. 2 A and B). Then we select the dose of 5uM of SCP110 to be used for the rest of the evaluation experiments. In addition to lower maturation rate, the timing of the PBE is significantly delayed after CTSL inhibition in the population of the oocytes that could proceed meiosis I and extrude polar bodies (Fig. 2C and D).

Trying to understand how CTSL inhibition resulted in a reduction in maturation rates, we evaluated the spindle and chromosomes morphology at the metaphase I stage. For completion of meiosis, chromosomes must align properly at the metaphase plate allowing the spindle microtubule to attach to chromosomes kinetochores. Interestingly, CTSL inhibition resulted in a significant increase in the chromosomal misalignment incidence (Fig. 3A-C), suggesting that this alignment defect could be the reason for activating the spindle assembly checkpoint (SAC) and preventing cell from starting anaphase onset leading to reduction the maturation rate after CTSL inhibition.

Finally, we evaluated the quality of the matured oocytes "egg" after CTSL inhibition in the pool of the oocytes that could proceed meiosis I and expel PB. We measured the chromosomes number to evaluate the ploidy status. Importantly, CTSL inhibition resulted in a significant increase in the incidence of aneuploidy compared to the control group (Fig. 3D-E) suggesting that CTSL is required for meiosis completion and production of healthy and

euploid eggs.

DISCUSSION

Meiotic maturation of the oocytes is a critical process for generating healthy euploid egg able to be fertilized by healthy viable haploid sperm. This study emphasized a novel requirement of CTSL during meiotic maturation of mouse oocytes. Initially, we found an even CTSL expression in all the stages of meiosis as represented in (Fig. 1). This finding suggests the requirement of CTSL for completion of meiosis I. Many other cathepsin were known to be expressed in oocytes as CTSB (Balboula *et al.*, 2010b) and CTSK (Balboula *et al.*, 2020). CTSB, CTSD and CTSZ gene expression

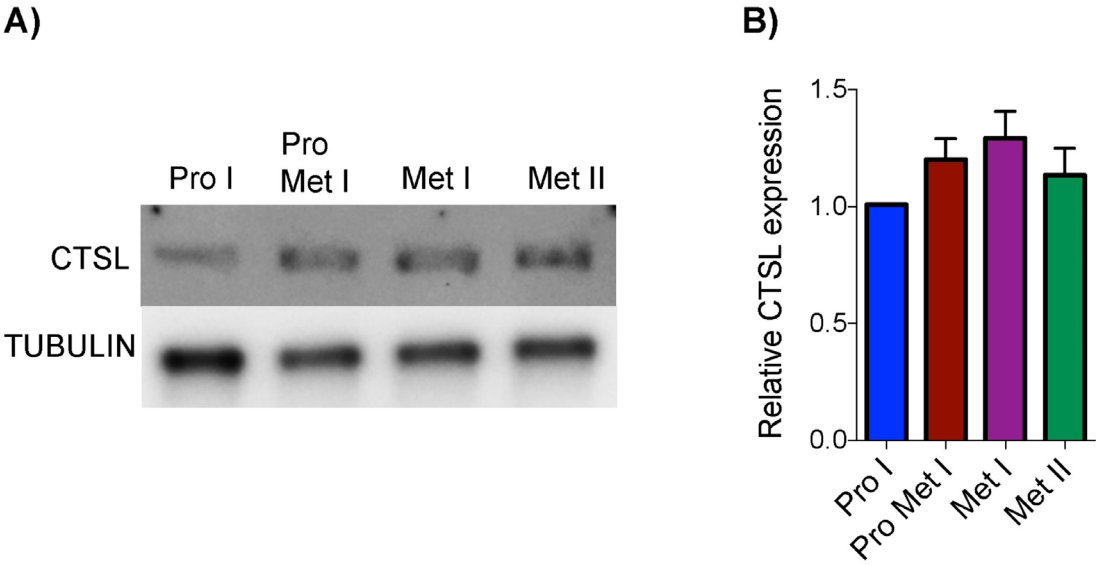

Fig. 1. Detection of CTSL expression during oocyte meiosis. A) Mouse oocytes were matured in CZB medium and lysed in different meiotic stages "prophase I, Pro-metaphase I, Metaphase I and Metaphase II" stages. Number of oocytes per lane is 50. SDS-PAGE were done and western blotting against CTSL. This experiment was replicated 3 times. B) quantification of the relative CTSL to Tubulin expression levels. Data was expressed as the means ± SEM. Number of replicates is 3. $P < 0.05$ was considered statistically significant (no labels means non-significant).

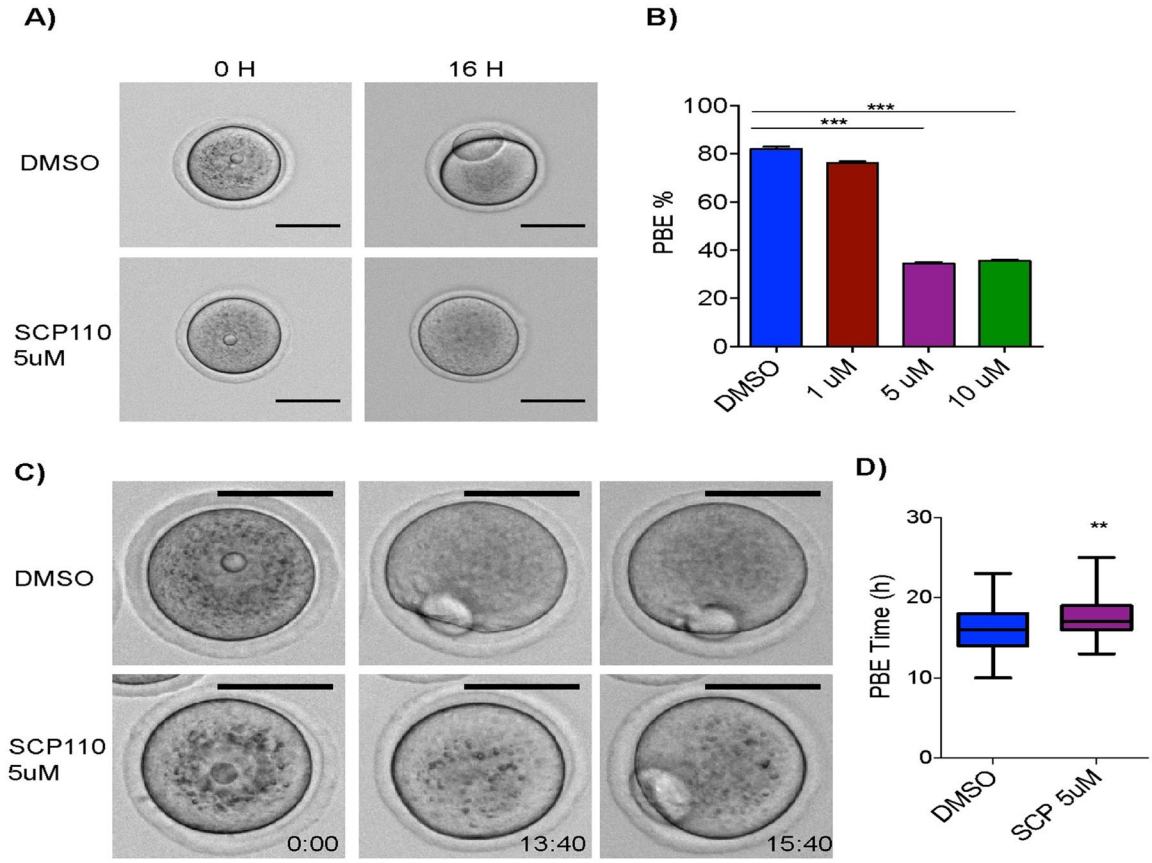

Fig. 2. CTSL inhibition decreases and delays the maturation of mouse oocytes. A) Mouse oocytes were matured in presence of CTSL inhibitor, SCP110 in different doses. Maturation rate was calculated in all groups. Representative oocytes after finishing the 16 hours of IVM from control "DMSO" and SCP110 5uM. B) Maturation rates in all groups. Total number of analyzed oocytes was 315. C) The proportion of oocytes showed polar bodies after CTSL inhibition showed a prolonged time to expel PB. D) PBE time quantification. Scale bar is 10um. Data was expressed as the means ± SEM. Number of replicates is 3. $P < 0.05$ was considered statistically significant (**$P < 0.01$ and ***$P<0.001$).

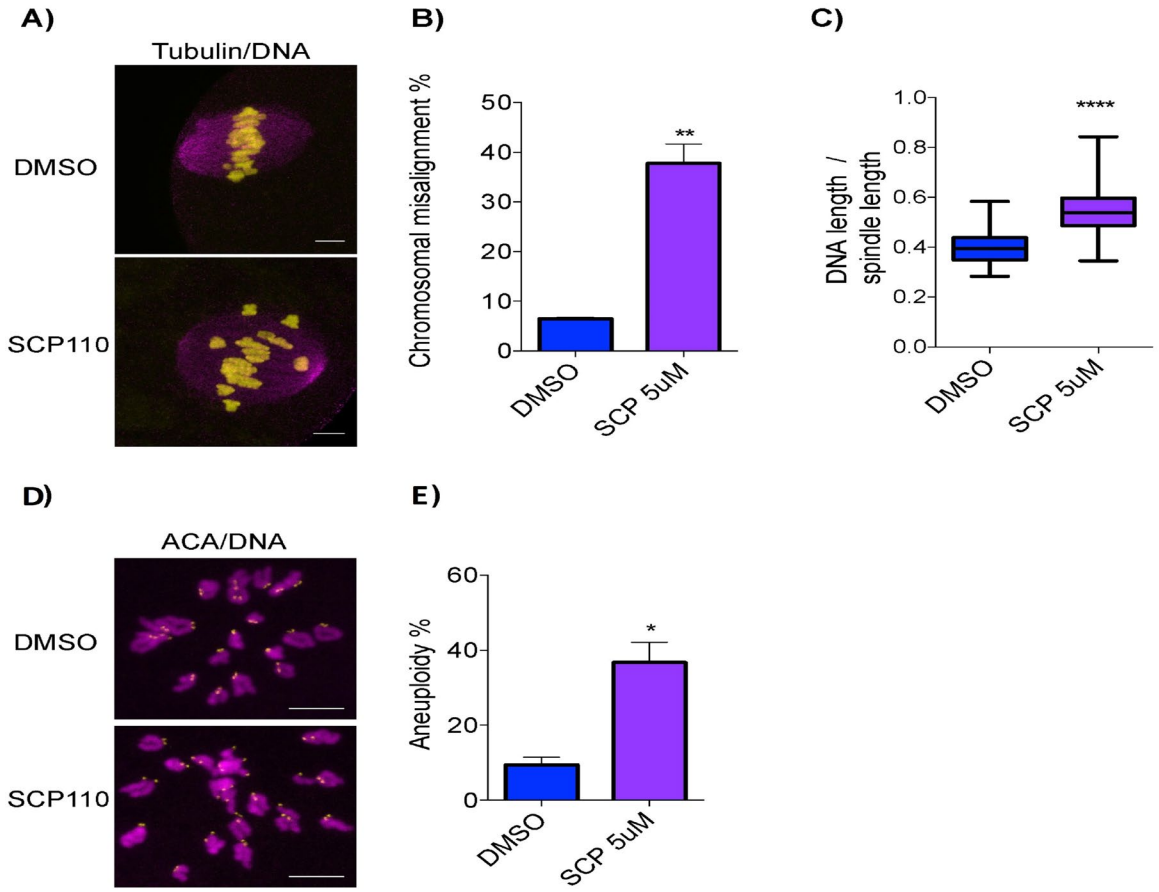

Fig. 3. CTSL inhibition disturbs meiosis by inducing chromosomal alignment and produces more aneuploid eggs. A) Mouse oocytes were matured in presence or absence of CTSL inhibitor SCP110, 5uM. Fixed oocytes at Metaphase I stages were stained to detect the spindles and chromosomes morphology. Chromosomes are perfectly aligned in the control group while they are misaligned after CTSL inhibition. B) Percentage of the oocytes with misaligned chromosomes. C) Severity of the misalignment was measured by calculating metaphase plate length to spindle length ratio. Number of replicates is 3. Total number of examined oocytes was 92. Scale bar is 3um. D) Prophase I oocytes were matured in in presence or absence of CTSL inhibitor SCP110, 5uM. Fixed oocytes at Metaphase II stage after doing the monastrol treatment were done and stained against ACA. Representative for chromosome spread in both groups. E) Aneuploidy incidence quantification (E). Data was expressed as the means ± SEM. Number of replicates is 3. Scale bar is 5um. Total number of examined eggs was 84. $P < 0.05$ was considered statistically significant (****P<0.0001).

levels were highest in matured oocytes followed by a significant decrease from the 8-cell embryo stage (Li *et al*., 2020). However, the CTSL expression in mouse oocytes has not been evaluated before, so to our knowledge, we are the first study to highlight this expression in oocytes.

Cathepsin family, especially CTSB is usually associated with apoptotic pathways and cell death of lower competent cells. Cathepsin B activity is a well-established marker for low quality oocytes and embryos, in addition, CTSB activity is involved and closely related in heat shock-induced dysfunction in bovine cumulus oocytes complexes (COCs) embryo development (Balboula *et al*., 2010a, 2010b; Balboula *et al*., 2013; Yamanaka *et al*., 2018). Inhibition of CTSB activity improves the developmental competence of oocytes and increases the yield of high-quality embryos in several species as bovine, ovine and porcine (Aboelenain *et al*., 2017; Balboula *et al*., 2022; Balboula *et al*., 2010a, 2010b; Liang *et al*., 2018; Pezhman *et al*., 2017). Unlike other cathepsins that has a bad effect on the oocytes and embryos quality and development, our result showed the opposite, the beneficial effect of CTSL during oocyte meiosis, we found that CTSL inhibition using SCP110 reduced the maturation rate by significant reduction of the PBE percent (Fig. 2) suggesting that CTSL doing a necessary role required for meiosis opposite to CTSB or CTSK as mentioned in the previous studies. In addition, CTSL inhibition prolonged the time required for PBE (Fig. 2), suggesting that CTSL activity is associated with high competent oocytes that can mature in the correct time unlike CTSB which is more active in the lower quality oocytes.

Meiotic maturation of the oocytes is the process of generation of haploid gamete with the correct number of the chromosomes to be ready for fertilization from a viable spermatozoon. Meiotic division is unique cell division and differ from mitotic cell division as it is consisting of two rounds of cell division without an intermediate step of DNA replication. In meiosis I many cytoplasmic and nuclear changes making it very unique and different from the robust mitotic division as spindle migration, homologues chromosomes segregation with mono-orientation of the sister chromatids. Homologs chromosomes should align together at the equatorial or metaphase plate to separate without separation of the sister chromatids. Half of the chromosomes and small part of cytoplasm will be extruded in the polar body at the cytokinesis part to complete the meiosis I. This unique separation makes meiosis I is highly error prone unlike mitosis which only sisters are separated with the opposite forces (Gruhn *et al*., 2019; Nagaoka *et al*., 2011). Chromosomal alignment should be tightly regulated and controlled in a spatiotemporal pattern for ensuring proper chromosome segregation. Kinetochores-microtubule (K-MT) attachment must be oriented and completed in a correct way to allow the normal chromosomal alignment in order to completion of meiosis I without chromosomal segregation errors (Homer *et al*., 2005; McGuinness *et al*., 2009; Musacchio, 2015; Wassmann *et al*., 2003). Chromosomal misalignment disturbs the microtubule-kinetochores attachment and activate the SAC and prevent the cell to go to anaphase stage to finish meiosis I (Musacchio and Salmon, 2007; Nagaoka *et al*., 2011). In this study, we found for the first time, that CTSL inhibition is associated with a significant increase in the chromosomal misalignment in mouse oocytes meiosis as shown in (Fig. 3A-B), suggesting a requirement of CTSL activity in regulation of the chromosomal alignment at the metaphase plate, or required for SAC silencing mechanism or cytokinesis regulation to go through anaphase stage. This finding opens a lot of interesting points for further

research to uncover the hidden mechanism for the CTSL significance in SAC and chromosomal alignment regulation.

Chromosomal misalignment or presence of unattached kinetochore leads to recruitment of the SAC mediators to kinetochore to form mitotic checkpoint complex (MCC) and prevents anaphase onset. MCC is composed of Bub3, BubR1, Cdc20 and Mad2. Formation of MCC inhibits anaphase promoting complex/cyclosome activation (APC/C) followed by inhibition of securin and cyclin B degradation and ended by blocking of anaphase (London and Biggins, 2014; Musacchio, 2015). However, if the cells went through the anaphase with weak SAC or misaligned chromosomes, the matured eggs will show a high incidence of aneuploidy, which is the leading cause of miscarriges and infertility in mammals (Gui and Homer, 2012; Jones and Lane, 2013; Kuhn and Dumont, 2019; Nagaoka *et al*., 2011; Sebestova *et al*., 2012). This is consistent with our study as we found as a consequence for the chromosomal misalignment, a higher incidence of aneuploid eggs after CTSL inhibition, confirming that CTSL activity is required for regulating chromosome segregation in a normal way to avoid generating of aneuploid eggs which are source of embryonic anomalies, miscarriges and infertility. This study is the first study to highlight the importance of CTSL in oocyte meiosis in mouse oocytes as a model for human infertility.

CONCLUSION

In conclusion, CTSL protein is expressed in all stages of meiosis I in mouse oocytes. CTSL inhibition reduced the maturation rates significantly. In addition, CTSL inhibition is associated with chromosomal misalignment that might attribute to the lower maturation rates. Furthermore, CTSL inhibition generates more aneuploid eggs. All of these conclude the importance of CTSL for meiosis I completion in mammals' oocytes. Further research is still required for more investigating the mechanisms behind these associated phenotypes.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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