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Expression and localization of Luman/CREB3 in mouse embryos during the pre-implantation period

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ABSTRACT. Luman/CREB3 is a transcription factor that is a member of the cAMP-response-element-binding protein family of basic region-leucine zipper transcription factors. This protein interacts with host cell factor 1, which also associates with the herpes simplex virus protein VP16 to induce the transcription of herpes simplex virus. Currently, the physiological function of Luman/CREB3 in reproductive processes remains unclear. In this study, quantitative real-time PCR and immunofluorescence assays were used to investigate the expression and localization of Luman in mouse oocytes as well as in early embryonic development. Luman protein was detected in the germinal vesicle and metaphase II stage oocytes, and was distributed in the cytoplasm, nucleus, and polar body of the oocyte stage. However, Luman protein and mRNA expression levels were significantly ($P < 0.05$) increased before activation of the zygotic genome, and expression levels peaked in 4-cell embryos. Expression levels were significantly ($P < 0.05$) decreased following the 8-cell stage throughout the blastocyst stage.

The Luman protein was also distributed in the nucleus and cytoplasm in the early preimplantation embryo and showed enhanced nuclear staining starting from the 2-cell stage embryo up to the 8-cell stage embryo. The differences in the expression and localization of Luman in mouse oocytes and early embryo suggested that Luman plays an important role in oocyte maturation and early embryonic development processes.

Key words: Early embryo; Development; Luman; Mice; Oocyte

INTRODUCTION

The process of the preimplantation development embryos in mammals includes the growth of the oocyte, its fertilization, and implantation of the blastocyst into the receptive endometrium. Development of mammalian oocyte into an embryo involves of a period of transcriptional silence that mainly depends on maternal RNAs and proteins produced during the oocyte growth stage. It is well-known that the signaling pathways control the timing of transcription and translation of RNA as well as post-translational modification of proteins (Harwood et al., 2008). The normal development and survival of the mammalian preimplantation embryo requires the activation of related gene expression (Telford et al., 1990; Schultz, 1993; Latham and Schultz, 2001). To date, the specific transcription factors that are active within the oocytes and preimplantation embryo remain unknown.

Luman (also known as LZIP and CREB3) is a basic leucine zipper transcription factor of the CREB/ATF gene family (Freiman and Herr, 1997), which is also an endoplasmic reticulum (ER)-combined transmembrane glycoprotein that is proteolytically cleaved from the ER during the response of the unfolded protein to ER stress (Lu et al., 1997; Raggo et al., 2002; Liang et al., 2006). It has a potent N-terminal acidic activation domain and a basic-leucine zipper motif (Freiman and Herr, 1997; Lu et al., 1997; Lu et al., 1998; Lu and Misra, 2000). The Luman primary structure was found to be highly conserved, and its homologs have been detected in fruit flies (dCREB-A/BBF-2) (Abel et al., 1992; Smolik et al., 1992) and in mice (LZIP) (Burbelo, 1994). Luman was initially recognized while screening for cellular ligands of the host cell factor (also called C1 factor), a protein required by the herpes simplex virus (HSV) VP16 transactivator (Kristie and Sharp, 1993; Freiman and Herr, 1997; Lu et al., 1997). In addition to HSV latency and reactivation (Lu and Misra, 2000), Luman is also involved in the gene regulation of human immunodeficiency virus (Blot et al., 2006), migration and function of leukocytes (Jang et al., 2007; Sung et al., 2008), signaling of glucocorticoid receptor (Kang et al., 2009), maturation of dendritic cells (Eleveld-Trancikova et al., 2010), and suppression of tumors (Jin et al., 2000; Kim et al., 2010). All family members of CREB3 appear to play a role in the ER stress response or in the unfolded protein response (Schröder and Kaufman, 2005; Bernales et al., 2006). Although Luman has been reported to regulate cell growth (Jin et al., 2000), the mechanism involved in this process has not been clearly defined.

Luman mRNA has been reported in a large range of human adult and fetal tissues (Lu et al., 1997). However, it is not clear whether its encoding protein is ubiquitous. Luman was also detected in the mouse testis, ovaries, and uterus (Audas et al., 2009; Lan et al., 2013), but its functions during the early stages of mouse pregnancy remain unclear.

No previous studies have investigated the expression and localization of Luman in the mammalian oocytes and preimplantaion embryos. Therefore, we investigated the expression and localization of Luman/CREB3 in mouse oocytes and preimplantation embryos at different developmental stages.

MATERIAL AND METHODS

Mice

Mature female mice of the Kunming white strain aged 8-10 weeks old were purchased from the laboratory animal center of Xian Jiao Tong University (Shaanxi, China). All animal manipulation procedures were performed according to the ethical guidelines established by the committee for the animal care and experiments at the Northwest A&F University. The female mice were group-housed with the same sex and maintained in a temperature- and light-controlled room (12-h light/dark cycle).Food and water were provided *ad libitium*.

Oocytes collection

Female mice were subjected to hormonal treatments to induce superovulation. Mice were injected intraperitoneally with 10 IU pregnant mare serum gonadotropin (Ningbo Sansheng Pharmaceutical, Zhejiang, China) between 15:00 and 17:00 h, and 10 IU human chorionic gonadotropin (hCG; Ningbo Sansheng Pharmaceutical) 48 h later. Immature germinal vesicle (GV) stage oocytes were collected at 48 h after pregnant mare were injected with serum gonadotropin; metaphase II (MII) stage oocytes were collected at 16 h after hCG injection. After hormonal treatments, animals were sacrificed by cervical dislocation, ovaries were removed and placed in 0.01M phosphate-buffered saline (PBS), and then the follicles were punctured using a 1-mL syringe (27-gauge needle) under a microscope. GV and mature oocytes were picked using hand pipette suction; next, the cells were washed with PBS. Finally, the oocytes were subjected to immunofluorescence staining and were determined by quantitative real-time-polymerase chain reaction (qRT-PCR) to determine the presence and expression of the Luman gene. The samples were frozen at -80°C until further use.

Embryo collection

Preimplantation embryos were recovered from mice that had been subjected to superovulation treatment. Females were injected intraperitoneally with 10 IU pregnant mare serum gonadotropin followed by 10 IU hCG 48 h later. After hCG injection, females were immediately mated with fertile males of the same strain to induce pregnancy. The pregnancy on days 1 (day 1, presence of a vaginal plug) to 4 were confirmed by recovering the embryos from the reproductive tracts. Preimplantation embryos were collected at different periods as follows (presence of a vaginal plug was considered0h): zygote (1-cell) 9 h from the oviduct, 2-cell: 33 h from the oviduct, 4-cell: 41 h from the oviduct, 8-cell: 53 h from the oviduct, morula: 72 h from the oviduct and uterus, and blastocyst: 82 h from the uterus.

Luman mRNA expression and protein localization in preimplantation embryos were qRT-PCR and stained with immunofluorescence. Samples were stored at -80°C until further analysis.

RNA extraction and cDNA synthesis

Total RNA from mouse oocytes and embryos was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. Briefly, 250 µL Trizol was added to oocytes or embryos in 1.5-mL Eppendorf tubes, mixed, and incubated for 5 min. To this mixture,

approximately 300 µL chloroform was added, mixed, and incubated with Trizol reagent and purified for another 10 min. The tubes were centrifuged at 15294 *g* for 15 min at 4°C and the supernatant was collected. To the supernatant, an equal amount of isopropanol was added and incubated at room temperature for 10min. The samples were centrifuged again at 15294 *g* for 20 min and the pellet was collected. The pellet was washed twice with 75% ethanol by centrifugation at 15294 *g* for 2 min at 4°C. The tubes were dried for 5-10 min. The RNA pellet was dissolved in 20 µL RNase-free water.

The cDNA was reverse-transcribed using a Prime Script RT reagent kit (Takara, Shiga, Japan) according to the manufacturer protocols. The final reaction volume was 10 µL, including 400 ng total RNA. The reverse transcription product was stored at -80°C.

qRT-PCR

qRT-PCR was conducted by using a Light Cycler system (iQ5, Bio-Rad, Inc., Hercules, CA, USA). Detection of qRT-PCR products was conducted using a SYBR® Premix Ex Taq™ II Kit (Takara) following the manufacturer guidelines. Each PCR was performed in a 20.0 µL reaction mixture containing 10.0 µL SYBR Premix Ex Taq II (2*), 2.0 µL template cDNA (equivalent of 20 ng total RNA), 0.8 µL each primer, and 6.4 µL nuclease-free water. The specific primers sequences used are shown in Table 1. Cycling conditions were set as follows: denaturation for 30 s at 95°C, and then 40 cycles for 5 s at 95°C, followed by a final extension for 20 s at 60°C. Melting curve analysis was used to confirm the specificity of the qRT-PCR products. Experiments were performed in triplicate for each data point; the mean was used for final analysis. mRNA was quantified using the $2^{-\Delta\Delta Ct}$ method and the amount of transcripts in each sample was determined to confirm the expression levels by qRT-PCR, normalized to glyceraldehyde-3-phosphate dehydrogenase as an internal control gene.

Table 1. Sequences of specific primers.

Gene	Sequence No.	Primer sequences
Luman	NM_006368	F: 5'-CTTCTCCGACTCCAACCTTC-3' R: 5'-CCACATCCTCACACCTAACC-3'
GAPDH	NM_008084	F: 5'-TCACTGCCACCCAGAAGA-3' R: 5'-GACGGACACATTGGGGGTAG-3'

Immunofluorescence assay

A confocal laser-scanning microscope was used to detect the localization of Luman protein. Briefly, the oocytes and preimplantation embryos were washed 3 times in PBS and immediately fixed in 4% paraformaldehyde for 30 min. After fixation, the cells were washed with PBS 3 times for 5 min each; next, they were transferred into 0.5% Triton in PBS solution, pH 7.4pH, containing 0.5% Triton X-100 for 30 min. After washing 3 times for 5 min each, the cells were placed into a plate and coated with PBS containing 1% bovine serum albumin and incubated for 60 min at 37°C. The cells were then incubated with 1: 150 anti-Luman monoclonal antibody overnight at 4°C and then washed 3 times for 5 min each with PBS, followed by incubation for 60 min with 1: 100 anti-rabbit secondary antibody diluted with PBS solution containing. The samples were washed 3 times for 5 min each. The nuclei were stained with 4,6'-diamidino-2-phenylindole dihydrochloride for 15 min and washed 3 times for 5 min each time. Then the cells were then transferred into slides, observed using a confocal laser-scanning microscope, and photos were acquired.

Statistical analysis

Data were statistically analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data were analyzed by analysis of variance and, if significant differences were found ($P < 0.05$), Fisher's least significant differences test was applied.

RESULTS

Expression of Luman mRNA in mouse oocytes and preimplantation embryo

The expression of Luman mRNA in mouse oocytes and pre-implantation embryos was examined using qRT-PCR. The results showed that Luman mRNA was expressed in mouse oocytes and preimplantation embryos collected from the reproductive tract at all developmental stages, with the highest level observed at the 4-cell stage of the embryo. Luman mRNA expression was significantly ($P < 0.05$) decreased following the 8-cell stage up to the blastocyst stage, but the mRNA level in the blastocyst stage was higher compared with that in the zygote stage (Figure 1). There were no significantly differences in the Luman mRNA levels between the MII and zygote stage embryos, or between the morula and blastocyst stage embryos.

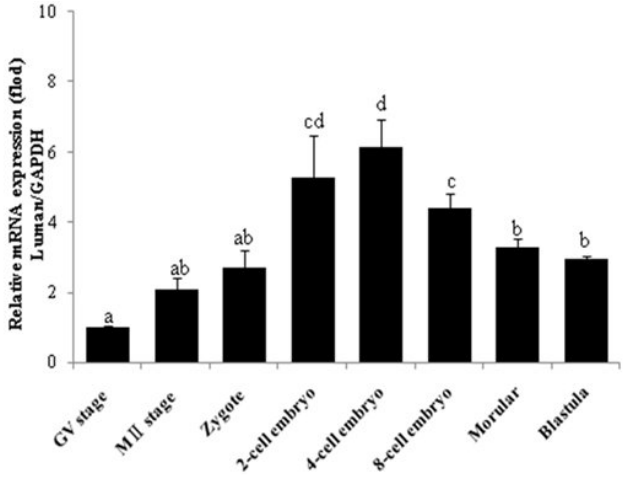

Figure 1. Relative expression of Luman mRNA in mouse oocytes and preimplantaion embryos. The values represent the mean ± SEM from 3 duplicates, and the bar bearing different superscript indicates a significant difference between the mean values.

Localization of Luman protein in mouse oocytes and preimplantation embryo

A confocal fluorescence microscope was used to detect the localization of Luman in the GV-oocyte, mature oocyte, and all preimplantation stage embryos collected from the reproductive tract (Figure 2). Negative Luman staining was observed in nonimmune control samples (8-cell shown as example). There was uniform cytoplasmic, nuclear, and polar body Luman staining in oocytes and zygotes. Two-cell, 4-cell, and 8-cellstage embryos displayed cytoplasmic staining with enhanced staining in the nuclei. In the 8-cell stage embryo, the overall level of staining was

less than that in the 2-cell and 4-cell stage embryos. Cytoplasmic staining was lower than that in the nucleus. In the blastocyst, Luman was localized in the inner cell mass and trophectoderm. Less staining was observed in the nucleus than in the cytoplasm. Negative Luman staining was observed in nonimmune control samples (8-cell shown as example).

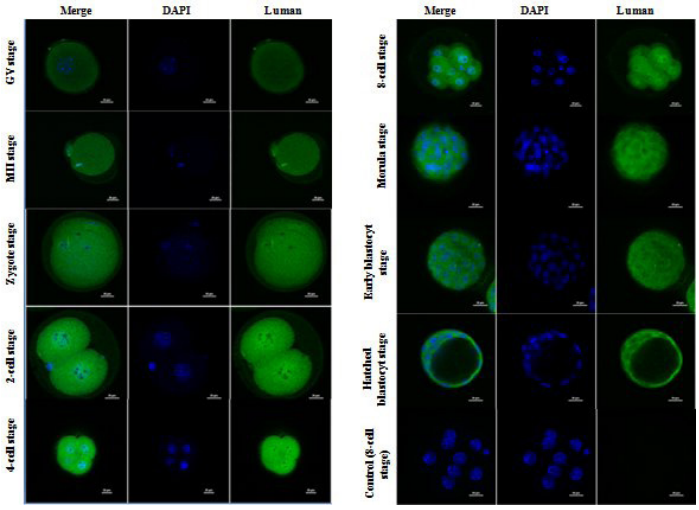

Figure 2. Immunofluorescence confocal results of Luman protein localization in oocytes and preimplantation embryos. The green color represents Luman staining, and the blue color indicates nuclear staining. Bar = 20 µm.

DISCUSSION

In this study, we investigated the Luman/CREB3 expression and localization in the mouse oocytes (GV and MII) and pre-implantation embryos. The results indicated that the expressed Luman protein was localized in the oocytes throughout the preimplantation phase of development. In the GV and MII stage oocytes, Luman/CREB3 protein expression was positive, with a marked increase in protein expression after the MII stage, and distributed throughout the cytoplasm, pronucleus, nucleus, and the polar body; Luman mRNA expression levels in the MII stage were significantly higher than that in the GV stage oocyte. The oocyte maturation and increasing expression of Luman indicated that Luman may play a substantial role in regulating oocyte maturation.No previous studies have been reported regarding the role of the Luman/CREB3 system in oocyte maturation. Luman/CREB3 is a transcription factor (Lu et al., 1998) that must be localized within the nucleus to be functional. Nuclear localization of Luman/CREB3 was observed to widen for the first time during the 2-cell stage. CREB family members are transcription factors known for their roles in cell proliferation, differentiation (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001; Sakamoto and Frank, 2009), cell survival, and normal preimplantation development (Bleckmann et al., 2002). The widening of the nuclear localization of Luman/CREB3 during this stage of development is thought to be initiated during early embryonic development (Latham and Schultz, 2001; Ma et al., 2001; Evsikov et al., 2004), which indicates that a transcription factor is involved in this first activation of transcription from the embryonic genome. However, the nuclear localization of Luman/CREB3 does not itself demonstrate that the transcription factor is active, and additional studies are needed to clarify this point. The expression of Luman/CREB3 was significantly decreased in the

8-cell stage embryo up to the blastocyst stage embryo when the embryo underwent compaction, indicating that Luman/CREB3 has an important role in early embryo cleavage. Further, Luman/CREB3 was not only found to be localized in the inner cell mass of the blastocyst stage embryo, but was also localized in trophectoderm cells, ensuring attachment to the endometrial luminal epithelium. Therefore, Luman/CREB3 appears to have an important role in implantation.

This is the first report to demonstrate the expression and localization of Luman/CREB3 in mammalian oocytes and preimplantation embryos. In conclusion, Luman/CREB3 may play an important role in oocyte maturation, early embryonic cleavage, and embryo implantation in mice.

Conflicts of interest

The authors declare no conflict of interest.

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