Biochemical and Biophysical Research Communications 474 (2016) 667–672

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Nek11 regulates asymmetric cell division during mouse oocyte meiotic maturation

Lei Guo, Zhen-Bo Wang, Hong-Hui Wang, Teng Zhang, Shu-Tao Qi, Ying-Chun Ouyang, Yi Hou, Qing-Yuan Sun*

State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China

ARTICLE INFO

Article history:
Received 15 April 2016
Accepted 1 May 2016
Available online 2 May 2016

Keywords:
Nek11
Symmetric division
Meiosis
Oocyte maturation

ABSTRACT

Nek11, a member of the never in mitosis gene A (NIMA) family, is activated in somatic cells associated with G1/S or G2/M arrest. However, its function in meiosis is unknown. In this research, the expression, localization and functions of NEK11 in the mouse oocyte meiotic maturation were examined. Western blotting indicated that NEK11S was the major NEK11 protein in mouse oocyte. MYC-tagged *Nek11* mRNA microinjection and immunofluorescent staining showed that NEK11 was localized to the meiotic spindles at MI and MII stage. Knockdown of *Nek11* by microinjection of siRNA did not affect germinal vesicle breakdown (GVBD) and the first polar body extrusion, but caused formation of 2-cell-like eggs. These results demonstrate that *Nek11* regulates asymmetric cell division during oocyte meiotic maturation.

1. Introduction

Meiosis is a distinctive process to produce haploid gametes. During meiosis, mammalian oocyte undergoes twice successive cell divisions with once replication of maternal DNA. Germinal vesicle breakdown (GVBD) is the hallmark of the resumption of meiotic maturation. After GVBD, microtubules assemble around chromosomes, and then the bipolar spindle forms and chromosomes migrate to the central plate of the spindle. Subsequently, the spindle migrates to the cortex and the first polar body is emitted. After that, the metaphase II (MII) spindle assembles, and the matured oocytes are arrested at MII stage until fertilization.

Cell cycle progression is regulated by protein phosphorylation/dephosphrtylation which is catalyzed by protein kinases and phosphatases. The functions of never in mitosis-gene A (NIMA)-related kinases (*Neks*) in the regulation of the cell cycle are not well known [1]. The first discovered member of this family of kinases is the *Aspergillus nidulans* NIMA, which is functionally involved in the initiation of mitosis and promotes the chromosome condensation by phosphorylation of histone H3 [2]. A total of 11 members of *Neks* have been found in human [3]. These kinases share approximately 40–45% identity with NIMA in their N-terminal catalytic kinase domains, while the C-terminal non-catalytic regions are highly divergent suggesting that each kinase might have different functions [4]. NEK11 in human has two isoforms: the longer isoform (NEK11L) is composed of 645 residues and the shorter one (NEK11S) contains only 470 residues [5]. *Nek11* could participate G1/S-arreste in U2OS cells [6]. In the presence of genotoxic agents, *Nek11* showed both increased expression and activity at the G2/M transition in HelaS3 cells [5]. NEK11 could phosphorylate CDC25A, leading to proteasomal degradation and subsequent inhibition of cyclins followed by a cell cycle arrest at the G2/M transition in U2OS cells treated with ionizing radiation [7]. NEK11was co-stained with β-TUBULIN in metaphase cells [5], which suggests that NEK11 may have other functions in cell cycle.

Although numerous studies have shown the key role of NEK11 in mitosis, the expression and roles of NEK11 in meiosis remain unknown. In this study, we examined the roles of *Nek11* during oocyte meiotic maturation using RNAi approach. The results showed that, unlike in mitotic cells, *Nek11* knockdown did not cause G2/M transition failure, but resulted in defects in asymmetric cell division in meiotic mouse oocyte.

2. Materials and methods

All chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned.

* Corresponding author. State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, #1 Beichen West Road, Chaoyang District, Beijing 100101, PR China.
E-mail address: sunqy@ioz.ac.cn (Q.-Y. Sun).

http://dx.doi.org/10.1016/j.bbrc.2016.05.002
0006-291X/

2.1. Construction of plasmids for MYC-tagged Nek11 and in vitro transcription

The full length *Nek11* coding sequence (NCBI Reference Sequence: NM_172461.3) was amplified by Nested PCR with the following primers: F1: 5′-ATGCCCAACTGTTGTTTCTGTG-3′; R1: 5′-GAATCAAGGCTTTAATGCTCCA-3′; F2: 5′-GTTGAATTCATGCTGAAATTCCAAGAGACTG-3′; R2: 5′-GTTGGCGCGCCTCATGGATTGTCACAGAAATGA-3′, and then subcloned to pCS2 plus vector which contains MYC-tag coding sequence. The *Nek11*-pCS2 plus vector was then used for *in vitro* transcription of polyadenylated mRNA from SP6 promoter. *In vitro* synthesis of capped mRNA was performed using linearized plasmids with the mMessage mMachine SP6 kit (Ambion). The mRNAs were purified with RNeasy micro kit (QIAGEN) and eluted in H_2O.

2.2. Oocyte collection and culture

Mice care and handling were conducted in accordance with the Animal Research Committee guidelines of the Institute of Zoology, Chinese Academy of Sciences. Oocytes at GV stage were collected from the ovaries of 6- to 8-week-old CD-1 mice in M2 medium containing 2.5 μm milrinone. For maturation, oocytes were washed and cultured in milrinone-free M16 medium under mineral oil at 37 °C, in an atmosphere of 5% CO2 in air.

2.3. Microinjection of siRNAs or MYC-tagged Nek11 mRNA

Microinjection of siRNAs or mRNA was performed with Narishige micromanipulators (Narishige Inc., Sea Cliff, NY) under a Nikon inverted microscope TE 200 (Nikon UK Ltd., Kingston upon Thames, Surrey, UK) and finished in 30 min. The sequences of *Nek11* siRNAs were: siRNA-1: 5′- GGCUGCUCAUACGAUUAAUTT -3′; siRNA-2: 5′- CAGCUCCUGAUGUGUAAAUTT-3′. The concentration of each siRNA was 20 μm. The same amount of scrambled siRNAs was injected as a control. For RNAi and mRNA injection, oocytes were arrested at GV stage in M2 medium containing 2.5 μm milrinone for 24 h and 4 h, respectively. And then the oocytes were transferred to M16 medium and cultured for 14 h.

2.4. Real-time quantitative PCR analysis

Each sample contained 60 oocytes, and total RNA was extracted with RNeasy micro kit (Qiagen). PrimeScriptTM 1st strand cDNA synthesis kit (Takara) was used to generate cDNA. The primers used for the amplification of *Nek11* fragment are listed as follows: F: 5′-AGAAGACCCACTTGTTGCTGA-3′; R: 5′- CCTCTTTCGTGTCTCCTCCTG-3′. To measure mRNA levels, real-time PCR analyses were performed using the Roche LightCycler 480 real-time PCR system (Roche). Ultra-SYBR Mixture (CoWin Biotech) was used for monitoring amplification. The program for real-time quantitative PCR was 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression levels of different transcripts were normalized to the housekeeping gene *β-actin* within the log-linear phase of the amplification curve using the △△Ct method.

2.5. Immunofluorescence and confocal microscopy

Oocytes were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.5% Triton X-100 for 20 min at room temperature. After 2 h blocking in 1% BSA at room temperature, the oocytes were incubated with anti-MYC-FITC antibody (1:200, Invitrogen), Phalloidin-FITC (10 mg/ml), mouse monoclonal FITC-conjugated anti-α-TUBULIN antibody (1:200) or Alexa Fluor 647 conjugated rabbit monoclonal anti-α-TUBULIN antibody (1:100, Cell Signaling Technology) overnight at 4 °C, followed by washing three times with PBS supplemented with 0.1% Tween 20 and 0.01% Triton X-100. DNA was stained with Hoechst 33342 and the oocytes were mounted on glass slides for microscopy. The slides were examined under a confocal microscope (Carl Zeiss LSM780, Germany).

2.6. Western blotting

Samples (each contains 150 oocytes) were collected in SDS loading buffer and boiled for 5 min. Proteins were separated on 10% SDS-PAGE, then electrically transferred to polyvinylidene difluoride (PVDF) membranes. Then TBST containing 5% skimmed milk was used to block the membranes for 2 h. The membranes were incubated with rabbit polyclonal anti-NEK11 antibody (1:1000, Abcom) or mouse anti-β-ACTIN (1:1000, Zhongshanjinqiao) overnight at 4 °C. After three times of washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000, Zhongshanjinqiao) or HRP-conjugated anti-mouse IgG (1:1000, Zhongshanjinqiao) at 37 °C for 1 h. After that, the membranes were washed three times with TBST again and then examined with the enhanced chemiluminescence (ECL) detection system (Thermo Scientific).

2.7. Statistical analysis

At least 3 replications were performed for all experiments. Statistical evaluation was performed by using a chi square test or t -test, with SPSS software (SPSS, Chicago, IL, USA). Differences with $P < 0.05$ were considered as significant.

3. Results

3.1. Expression and localization of NEK11 during mouse oocyte maturation

Western blotting showed that NEK11 protein expressed during oocyte meiotic maturation, and the bands were completely consistent with the molecular weight of NEK11S (molecular weight 54 KDa), while NEK11L protein (molecular weight 74 KDa) was rarely detected (Fig. 1A). The result indicated that NEK11S is the major NEK11 protein in mouse oocyte. Due to the lack of working antibody for NEK11 immunostaining, we injected MYC-tagged *Nek11* mRNA to show the subcellular localization of NEK11. At GV stage, the NEK11 protein was in nucleus and dispersed in the cytoplasm at GVBD stage, then localized to the spindle at MI and MII stages (Fig. 1B) and colocalized with α-TUBULIN (Fig. 1C).

3.2. Knockdown of NEK11 increases symmetric cell division in mouse oocyte

RNAi was employed to detect the function of *Nek11* during mouse oocyte meiotic maturation. *Nek11* specific siRNAs (RNAi group) or negative control siRNA was microinjected into mouse oocytes at GV stage, and these oocytes were arrested at GV stage by culturing in M2 medium containing 2.5 μm milrinone. After 24 h culture, oocytes were collected for real-time PCR analysis or western blot analysis to examine the expression of *Nek11* at mRNA level and protein level, respectively. The expression level of *Nek11* mRNA in mouse oocytes after *Nek11* siRNAs injection was significantly decreased compared with that in the control group (scrambled siRNAs injected group, 30.1% *vs.* 100%, $P < 0.05$) (Fig. 2A). NEK11 protein level was also significantly declined in the *Nek11* RNAi group (0.46 *vs.* 1.00, $P < 0.05$) (Fig. 2B). Next, we want to figure out whether knockdown of NEK11 can affect the meiotic