Fas-associated protein factor 1 is involved in meiotic resumption in mouse oocytes

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Abstract. Fas-associated protein factor 1 (FAF1) is a Fas-associated protein that functions in multiple cellular processes. Previous research showed that mutations in Faf1 led to the lethality of cleavage stage embryos in a mouse model. The aim of the present study was to analyze the expression pattern, localization, and function of FAF1 in meiotic resumption of mouse oocytes. FAF1 was exclusively expressed in oocytes at various follicular stages within the ovary and was predominantly localized in the cytoplasm of growing oocytes. Furthermore, Faf1 mRNA and protein were persistently present during oocyte maturation and Faf1 mRNA levels were similar in the germinal vesicle (GV), GV breakdown (GVBD), and metaphase II (MII) stages of oocytes. Moreover, knockdown of Faf1 in GV-stage oocytes led to a significantly decreased rate of GVBD. To our knowledge, these results provide the first evidence regarding a novel function of FAF1 in meiotic resumption in mouse oocytes.

Key words: FAF1 protein, Meiotic resumption, Mouse, Oocyte

Meiosis starts during the fetal period and is arrested at the diplotene stage in mammalian oocytes. After meiotic arrest, an extensive accumulation of mRNAs and proteins in oocytes occurs during the growth phase. These maternal factors play a role in meiotic arrest and resumption [1–3]. Fas-associated protein factor (FAF1) is expressed in growing oocytes [4], suggesting that FAF1 is a maternal factor that might play a role in oocyte meiosis.

Fas is a member of the tumor necrosis factor receptor family that induces apoptosis upon binding to Fas ligand [5]. FAF1 is a Fas-associated protein identified by using a yeast two-hybrid assay; FAF1 specifically interacts with the cytoplasmic domain of Fas [6]. Previous studies have demonstrated the functions of FAF1 in various processes such as apoptosis signaling [7, 8], ubiquitination [9], nuclear translocation [10, 11], and embryonic development [4, 12].

The multifunctional FAF1 consists of several protein interaction domains. A previous study reported that the region of FAF1 (amino acids 181-381) interacts with the death effector domains of caspase-8 and Fas-associated death domain protein, which are involved in apoptosis [13]. Additionally, this study showed that the overexpression of FAF1 in Jurkat cells led to a significant increase in apoptosis [13]. However, other studies have revealed that FAF1 plays a role in mammalian reproduction [4]. FAF1 is expressed in several tissues, especially in the testis and ovary, and the level of Faf1 mRNA gradually increases after post-natal development [4]. A mutation in Faf1 results in the death of Faf1GTGT embryos at the 2-cell stage, implying that FAF1 is required at early cleavage stages in the mouse embryos [4]. However, until date, the physiological function of FAF1, especially its role in oocyte meiosis, has not been reported.

In the present study, we used a mouse model to investigate the expression, localization, and function of FAF1 during folliculogenesis and oocyte meiosis I processes. This novel advancement in the characterization of the effect of FAF1 on GVBD indicates a potential mechanism of FAF1 action in regulating oocyte meiosis in mice.

Materials and Methods

Ethics statement for mouse model

All the experimental procedures were approved by the Animal Care Commission of the College of Animal Science, Fujian Agriculture and Forestry University. Adult male and female ICR mice were purchased from the Experimental Animal Center of Fujian Medical University (Fuzhou, P.R. China). Mice were provided with water and mouse chow ad libitum and maintained on a 14/10-h light/dark cycle in the Animal Facility of the College of Animal Science, Fujian Agriculture and Forestry University.

Chemicals

All the chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Collection of oocytes

To obtain GV-stage oocytes, female ICR mice were injected with 10 IU of pregnant serum gonadotropin (PMSG) at 48 h prior to ovary collection. The ovaries were isolated and placed in Hepes-buffered potassium simplex optimized medium (H-KSOM) containing 0.1 mM 3-isobutyl-methyl-xanthine (IBMX) to maintain the oocytes at the GV stage. Cumulus-oocyte complexes were recovered from
the oviducts of female mice that were administered with a second injection of 10 IU of human chorionic gonadotropin (hCG) at 48 h after PMSG injection. Metaphase II (MII) oocytes were collected at 16 h after the injection of hCG. Cumulus cell masses were treated with H-KSOM containing hyaluronidase (1 mg/ml) to release the oocytes.

**Immunohistochemistry**

Ovaries were isolated from 4-week-old mice, fixed (overnight) in 4% paraformaldehyde at 4°C, and processed for paraffin wax embedding. Sections were cut at 6-µm thickness and processed for deparaffinizing and rehydrating. All the sections were heated in 10 mM sodium citrate buffer (pH 6.0) by using a microwave oven for antigen retrieval step. Immunohistochemistry was performed on the ovarian tissues using UltraSensitive™ SAP IHC kits (Maixin-Bio, Fuzhou, P. R. China) according to the manufacturer’s instructions. In brief, sections were incubated with 10% non-immune goat serum (Maixin-Bio) to block non-specific sites, followed by incubation (overnight) with primary antibody (1:50, anti-FAF1 antibody, Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4°C. Sections were rinsed in phosphate buffered saline (PBS) and incubated with a biotinylated goat anti-rabbit secondary antibody (Maixin-Bio). After performing several washes in PBS, sections were incubated with streptavidin-linked alkaline phosphatase (Maixin-Bio) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution (Maixin-Bio) for 10–30 min at room temperature. Negative controls were obtained by processing the sections according to the aforementioned method in the absence of primary antibody. After the final wash in PBS, sections were observed by using a Nikon Eclipse Ti-S microscope equipped with a 198 Nikon DS-R1i digital camera (Nikon, Tokyo, Japan).

**Reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was extracted from pooled GV-stage, GVBD, or MII oocytes (n = 100) using an RNasy Mini Kit (Qiagen, Valencia, CA, USA). The PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan) was used to synthesize cDNA. Oocytes (n = 10) at different stages were pooled, lysed, and qRT-PCR was performed using Power SYBR® Green Cells-to-Ct™ Kit (Thermo Fisher Scientific, Waltham, MA, USA). The primer pairs used for RT-PCR and qRT-PCR were (i) Faf1: 5’-AACCTGGGGCTTTGGATCGAC-3’ (forward) and 5’-GTCCAATAACGCTGCAAAGTG-3’ (reverse) and (ii) β-Actin: 5’-GAAGTGTGACGTTGACATCGAC-3’ (forward) and 5’-ACTTGCCGTACGATCGAGG-3’ (reverse). The annealing temperatures of both the primer sets were 60°C. Electrophoresis was performed to evaluate the specificity of primer sets and the additional size of RT-PCR products. Amplification was conducted in triplicate for each individual sample on an ABI PRISM 7700 Sequence Detection System for qRT-PCR analysis. Relative expression levels were normalized using the levels of β-Actin mRNA. Variations in Faf1 mRNA expression levels were calculated using the established $2^{-\Delta\Delta CT}$ formula [14].

**Western blotting**

Oocytes (n = 50) at different stages were pooled and lysed in RIPA lysis buffer (Beyotime, Jiangsu, P. R. China). Total protein lysates were resolved on 10% polyacrylamide gels containing 0.1% SDS and transferred onto hydrophobic PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk diluted in Tris-buffered saline (TBS; pH 7.4)/0.1% Tween 20 (TBST) for 4 h at room temperature and incubated overnight with a primary antibody against FAF1 (1:200, Santa Cruz Biotechnology) at 4°C. Membranes were then washed thrice in TBST and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000, Thermo Fisher Scientific). The enhanced chemiluminescence (ECL) Advanced Western Blotting Detection System (Thermo Fisher Scientific) was used to detect FAF1 bands on membranes. β-Actin was used as a loading control (1:1000, Santa Cruz Biotechnology).

**Immunofluorescence**

All the procedures were performed at room temperature unless otherwise stated. Oocytes were fixed in 4% paraformaldehyde in PBS for 1 h and permeabilized by treatment with 0.2% Triton X-100 in PBS for 20 min. Samples were blocked in PBS containing 3% BSA for 4 h and incubated (overnight) with a primary antibody against FAF1 (1:100, Santa Cruz Biotechnology) at 4°C. Oocytes were washed using PBS and incubated with a secondary antibody of Alexa Fluor 488-conjugated goat anti-rabbit (Beyotime) at 1:500 dilution for 2 h. Nuclei were stained with DAPI (Beyotime) for 5 min. Fluorescence was detected using a Zeiss LSM 510 confocal microscope equipped with differential interference contrast optics (Carl Zeiss Inc., Thornwood, NY, USA).

**Microinjection of customized short interfering RNA (siRNA)**

GV-stage oocytes were microinjected with approximately 10 pL of Faf1 siRNA (2 µM, Santa Cruz Biotechnology) in M2 medium, as previously described in the corresponding citation [15]. Deionized water was used as a blank control (BC). Negative control siRNA (2 µM, Santa Cruz Biotechnology) was microinjected into GV-stage oocytes as a control (NC) for the siRNA experiments. After microinjection, GV-stage oocytes were washed thrice and cultured in KSOMaa-BSA medium containing 0.1 mM IBMX in a humidified atmosphere of 5% CO2/95% air at 37°C for 24 h. The microinjected GV-stage oocytes were washed and cultured in KSOMaa-BSA medium for 14 h. To assess the GVBD rate, microinjected oocytes were observed and counted at 2, 4, 6, and 12 h after removal of IBMX from the medium.

**Statistical analysis**

All the experiments were independently replicated at least thrice and the data are presented as mean ± standard error of the mean (SEM). The normal distribution of the data and the equality of variances were tested before analysis. Data concerning the Faf1 mRNA expression level and GVBD rate after Faf1 siRNA injection into GV-stage oocytes were normally distributed and were analyzed by one-way analysis of variance (ANOVA) and the least significant difference (LSD) test using SPSS version 13.0 software (IBM, Armonk, NY, USA). P values < 0.05 were considered statistically significant.
Results

Expression of FAF1 during folliculogenesis

To determine the expression level of FAF1 during folliculogenesis, we conducted immunohistochemistry analyses on ovary sections from 4-week-old mice. FAF1 immunoreactivity was exclusively found in oocytes at various follicular stages within the ovary (Fig. 1). Positive staining was not observed in the oocytes that were processed to obtain negative controls.

Expression of Faf1 mRNA and protein during oocyte maturation

RT-PCR and qRT-PCR analysis confirmed the presence of Faf1 mRNA; moreover, its level was similar in GV-stage, GVBD, and MII oocytes (Fig. 2A and 2B). Furthermore, western blot analysis demonstrated that FAF1 was expressed during oocyte maturation (Fig. 2C).

Localization of FAF1 in oocytes

To evaluate the localization of FAF1 in oocytes, immunofluorescence was performed and oocytes were examined by confocal microscopy. The results indicated that FAF1 is uniformly present in the GV and cytoplasm in fully grown immature oocytes (Fig. 3), while in growing oocytes it is localized in the cytoplasm (Fig. 1).

Effect of Faf1 knockdown on the GVBD rate in oocytes

To analyze the function of Faf1 during meiotic resumption in oocytes, GV-stage oocytes were microinjected with Faf1 siRNA. As shown in Fig. 4A, the microinjection of Faf1 siRNA led to a distinct reduction in Faf1 mRNA levels compared with that in oocytes injected with BC and NC siRNA. Immunofluorescence of FAF1 in oocytes with FAF1 knockdown was lower than that in BC and NC oocyte groups (Fig. 4B). Moreover, we confirmed that the introduction of Faf1 siRNA led to a significant decrease in FAF1 level (Fig. 4C). These data confirmed that Faf1 siRNA successfully downregulated Faf1 mRNA and protein levels in oocytes.

Further, we observed the microinjected oocytes at 2, 4, 6, and 12 h after removal of IBMX from the medium and found that the GVBD rate was significantly decreased in Faf1 siRNA-microinjected oocytes compared to that in BC and NC oocyte groups (Fig. 4D). On comparison, the microinjected oocytes derived from BC and NC groups showed a normal GVBD rate.

Discussion

Previous research showed that Faf1 mutation led to the lethality of early cleavage stage embryos in the mouse model [4]. However, the role of Faf1 in meiosis mostly remains unknown. The present study showed a spatio-temporal distribution of Faf1 during folliculogenesis as well as oocyte maturation and demonstrated that FAF1 is involved in meiotic resumption in mouse oocytes.

Immunohistochemical analysis showed that FAF1 presence was restricted to oocytes at various follicular stages, implying that FAF1 is a maternal factor that might play a key role in oogenesis and preimplantation embryo development. In the ovary, GV-stage oocytes reinitiate meiosis and undergo GVBD to become MII oocytes. Even during the maturation process, Faf1 mRNA and protein was persistently present in oocytes. Moreover, confocal microscopy experiments revealed that the distribution of FAF1 was uniform in full-grown oocytes and it was localized mainly in the cytoplasm of growing oocytes. The expression and localization of FAF1 during oocyte maturation suggests a possible role of FAF1 in this process. In addition, the expression pattern of Faf1 is consistent with that of other previously reported maternal factors [16–18].

Previous studies suggested that FAF1 is required for the early cleavage stage of mouse embryos [4]. To determine the function of Faf1 during oocyte meiotic resumption, we used an RNAi approach, which is an efficient approach to knockdown the maternally expressed genes in oocytes [19, 20]. We found that the GVBD rate in Faf1-deficient oocytes was notably lower than that in control groups, suggesting that FAF1 maintains meiotic resumption in oocytes. Interestingly, the function of FAF1 is contrary to that of WEE1 homolog 2 (WEE2), which is an oocyte-specific meiosis inhibitor in mice and non-human primates [21, 22].

FAF1 contains a ubiquitin-associated domain (UBA, residues 1–57) and a ubiquitin-related domain (UB1, residues 99–180) [23]. FAF1 interacts with ubiquitin as well as ubiquitin-like proteins and is involved in ubiquitin-mediated protein degradation [24, 25]. Therefore, it is logical to hypothesize that knockdown of FAF1 might lead to an aberration in the ubiquitin proteasome pathway that reduces the efficiency to degrade maternal or other vital proteins and in turn disrupts the normal regulation of important developmental events. However, the precise mechanism of FAF1 participation in meiosis resumption requires further elucidation.

In conclusion, the present study demonstrates the expression of Faf1 during folliculogenesis and oocyte maturation, the localization of FAF1 in the growing oocyte cytoplasm, and the function of FAF1 in meiotic resumption of mouse oocytes.

Conflict of interest: The authors declare no conflict of interest that could influence the outcome of the study.

Acknowledgments

The authors thank Professor Wenchang Zhang for helpful discussion and Professor Xu Wu for generous technical assistance.

This work was supported by grants from the National Natural Science Foundation of P. R. China (grant numbers: 31402079 and 31672415) and FAFU Program for Distinguished Young Scholars (grant number: XJQ201509).
Fig. 1. Expression of FAF1 in the ovary. Immunohistochemical analysis of FAF1 in ovary sections obtained from 4-week-old mice. The representative oocytes at various follicular stages are indicated by red arrows (Original magnification × 100).

Fig. 3. Localization of FAF1 in oocytes. GV-stage, GVBD, and MII oocytes were fixed, permeabilized, and stained with specific antibodies against FAF1 (green). Each sample was counterstained with DAPI to visualize DNA (blue) (Original magnification × 200).

Fig. 4. Effect of Faf1 knockdown on the GVBD rate in GV-stage oocytes. (A) Relative abundance of Faf1 mRNA after microinjection of GV-stage oocytes with BC, NC or Faf1 siRNA and incubation for 38 h in the culture medium. Data were normalized to Faf1 mRNA levels in BC oocytes and expressed as mean ± SEM. Statistical comparisons were analyzed using ANOVA and LSD tests (* P < 0.05). (B) Immunofluorescence analysis of FAF1 after microinjection of GV-stage oocytes with BC, NC or Faf1 siRNA and incubation for 38 h in the culture medium. (C) Western blot analysis of FAF1 expression level after microinjection of GV-stage oocytes with BC, NC or Faf1 siRNA and incubation for 24 h in the culture medium. β-Actin was used as a loading control. (D) Determination of the GVBD rate after microinjection of GV-stage oocytes with BC, NC or Faf1 siRNA. Microinjected oocytes were observed and counted at 2, 4, 6, and 12 h after removal of IBMX. Statistical comparisons were analyzed using ANOVA and LSD tests (* P < 0.05).
**Fig. 2.** Expression of Faf1 mRNA and protein during oocyte maturation. (A) RT-PCR was performed to estimate Faf1 mRNA level by using RNA extracted from GV-stage, GVBD, and MII oocytes. β-actin was used as an internal control. (B) Faf1 mRNA expression level in GV-stage, GVBD, and MII oocytes was determined by qRT-PCR. Data were normalized to Faf1 mRNA levels in MII oocytes and expressed as mean ± SEM. (C) Western blot analysis of Faf1 expression level in GV-stage, GVBD, and MII oocytes. β-Actin was used as a loading control.

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