Acta Histochemica 118 (2016) 632–639

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Expression and distribution of forkhead activin signal transducer 2 (FAST2) during follicle development in mouse ovaries and pre-implantation embryos

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ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form 6 July 2016
Accepted 7 July 2016

Keywords:
FAST2
Ovaries
Pre-implantation embryos
CLs

ABSTRACT

Xenopus forkhead activin signal transducer 1 (xFAST 1) was first characterized in *Xenopus* as the transcriptional partner for Smad proteins. FAST2, which is the xFAST 1 homologues in mouse, is expressed during early developmental stages of the organism. However, the function of FAST2 in mouse ovaries and pre-implantation embryos is unclear. Therefore, we investigated its expression during these processes. In postnatal mice, FAST2 was expressed in oocytes and thecal cells from postnatal day (PD) 1 to PD 21. In gonadotropin-induced immature mice, FAST2 was expressed in oocytes, thecal cells and newly formed corpora lutea (CLs), but was expressed at a lower level in degenerated CLs. Similar results were observed upon western blot analyses. In meloxicam-treated immature mice, ovulation was inhibited and FAST2 was expressed in thecal cells, luteinized granulosa cells and entrapped oocytes. Immunofluorescence results showed that FAST2 was expressed in the cytoplasm and nucleus but not the nucleolus from the zygote to 8-cell embryo stage, after which it was localized to the cytoplasm of the morulae and inner cell mass of the blastocysts. Taken together, these observations suggest that FAST2 is expressed in a cell-specific manner during ovarian follicle development, ovulation, luteinization and early embryonic development, and that FAST2 might play important roles in these physiological processes.

1. Introduction

A follicle is the basic functional unit of mammalian ovaries, consisting of an innermost oocyte, surrounding granulosa cells (GCs), and outer layers of thecal cells (TCs). Follicle development can be described in stages, beginning as primordial follicles each containing a small oocyte surrounded by a layer of fibroblast-like GCs, through primary, preantral, antral, to the preovulatory or Graafian stage containing a fully grown oocyte with multiple layers of GCs (Edson et al., 2009; McGee and Hsueh, 2000). The preovulatory LH surge causes a few of the antral follicles to ovulate and rapidly initiate a program of terminal differentiation. Within a few hours the remaining granulosa and thecal cells of ovulated follicles transform into corpora lutea (CLs) through a process known as luteinization, which sustains the pregnancy in a fertile cycle (Edson et al., 2009; Stocco et al., 2007). If the oocytes are fertilized, the mouse embryos develop from the zygote to the blastocyst stage within about three days (Bowman and McLaren, 1970). Embryos develop from zygotes to morulae in the oviducts, while the development of blastocysts occurs in the uterus. Prior to implantation in the uterus, the blastocysts undergo a series of development, consisting of cell division, apoptosis and differentiation (Hardy and Spanos, 2002).

Xenopus forkhead activin signal transducer 1 (xFAST 1) was first characterized in *Xenopus* as the transcriptional partner for Smad proteins by forming the activin response factor and binding to the *Mix.2* promoter (Chen et al., 1996). Human FAST1 and mouse FAST2 are the mammalian homologues of xFAST 1 (Labbe et al., 1998; Liu et al., 1999; Zhou et al., 1998). Mouse FAST2 has two functional domains, a conserved N-terminal forkhead domain and a distinct C-terminal domain that is essential for DNA binding, regulation of transcription, and interaction with various Smad proteins including Smad2, Smad3 and Smad4 (Chen et al., 1997; Nagarajan et al., 1999).

Previous studies have demonstrated that FAST, encoded by *foxh1* gene, is expressed primarily during early developmental stages in

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http://dx.doi.org/10.1016/j.acthis.2016.07.003
0065-1281/

Xenopus and mouse (Weisberg et al., 1998; Yeo et al., 1999). Overexpression of *xFAST* 1 induces the expression of a broad range of genes downstream of *activin* and leads to abnormal axis formation in *Xenopus* (Watanabe and Whitman, 1999). In zebrafish, FAST1 is involved in vascular formation by negatively modulating *flk1* gene expression and is required for the development of dorsal axial structures and gastrulation (Choi et al., 2007; Pei et al., 2007; Sirotkin et al., 2000). *FAST2* has been examined via in situ hybridization analysis in mouse embryos from pregastrulation stages [approximately 6.0 day post coitus (dpc)] to 9.5 dpc. The results indicated that *FAST2* is predominantly expressed at the epiblast stage before gastrulation and then declines as development progresses. It specializes the developmental process through the TGF-β superfamily signals (Weisberg et al., 1998). Yet another in situ hybridization study showed that *FAST2* is expressed in the inner cell mass (ICM) at embryonic day 3.5 (E 3.5) as well as in both the epiblast and primitive endoderm between E 4.25 and E 4.75 (Takaoka et al., 2006). Furthermore, deletion of *foxh1* in mice results in various abnormalities, including lack of anterior primitive streak and failure to form node (Hoodless et al., 2001; Yamamoto et al., 2001). Although the distribution of *FAST2* has been examined via in situ hybridization and northern analyses (Labbe et al., 1998; Takaoka et al., 2006; Weisberg et al., 1998), little is known about its effects on the development of mouse ovaries and pre-implantation embryos. The purpose of the present study was to examine the spatiotemporal expression patterns of FAST2 at these stages and to further elucidate its biological roles. Our results indicate that FAST2 is expressed in a stage-specific manner during follicle development, luteinization and early embryonic development, and therefore may be involved in these processes.

2. Materials and methods

2.1. Mice

Sexually mature (8 weeks old) female and male Kunming white strain mice were obtained from the Experimental Animal Center of Shandong University (Ji'nan, Shandong, China). All experiments were carried out with strict adherence to the Guidelines of Shandong Normal University for the Care and Use of Laboratory Animals. The mice were housed at a constant photoperiod (12 h light and 12 h dark cycle) and under controlled temperatures (23 ± 2 °C), with food and water provided ad libitum (Tang and Zhang, 2011).

2.2. Ovulatory response to gonadotropins and meloxicam (MEL)

To synchronize the stage of ovarian follicles, 5 IU pregnant mare serum gonadotropin (PMSG; Ningbo Sansheng Pharmaceutical, Zhejiang, China) was administered subcutaneously to the immature mice at 3 weeks of age (n = 27). At 48 h post PMSG administration, 5 IU human chorionic gonadotropin (hCG; Ningbo Sansheng Pharmaceutical) was injected. At 24 h and 48 h post PMSG administration, and also at 4 h, 10 h, 11 h, 12 h, 24 h, 48 h and 72 h post hCG treatment, the ovaries were dissected free from surrounding tissues, and were either immediately snap frozen in liquid nitrogen and stored at −80 °C for protein extraction or were embedded in OCT compound (Sakura Finetek Inc., Torrance, CA, USA) for immunohistochemistry or hematoxylin and eosin staining.

For meloxicam (MEL) treatment, immature female mice (n = 36) were treated with PMSG and hCG as described earlier. But at 6 h prior to hCG injection, the animals received 80 mg/kg MEL (prostaglandin synthase 2 (PTGS2) activity inhibitor; Baiweiling) to block ovulation (Jimenez et al., 2010; Salhab et al., 2003). Ovaries were collected at 18 h, 24 h and 36 h post hCG injection.

2.3. Immunohistochemical analysis of FAST2 in ovaries

The first day following parturition was designated as postnatal day (PD) 1. Ovaries were collected from untreated mice (n = 15) at PD 1, PD 4, PD 8, PD 13, PD 21. Ovaries were also collected from gonadotropin stimulated mice (n = 15) at 24 h and 48 h post PMSG injections, and at 24 h, 48 h and 72 h post hCG injections. 10 μm cryosections were fixed in acetone for 10 min at −20 °C and later washed thrice for 5 min each with 1 × PBS. The sections were then incubated with 0.3% Triton X-100 in PBS (pH 7.2) for 20 min. After the PBS washes, endogenous peroxidases were quenched by incubating in 0.3% H_2O_2 for 20 min. Non-specific binding was blocked by incubating the sections for 30 min at room temperature in IgG derived from goat, the same animal species as that of the primary antibody. The sections were then incubated overnight at 4 °C with goat polyclonal anti-FAST2 primary antibody (SC-12437, Santa Cruz, 1:50 dilution). Subsequently, the sections were incubated with peroxidase conjugated rabbit anti-goat IgG secondary antibody (SC-2774, Santa Cruz; 1:100 dilution) for 2 h at room temperature. Positive signals were developed using the DAB kit (ZLI-9033, ZSGB-BIO, Beijing, China). Finally the sections were counterstained with hematoxylin. The sections were then dehydrated, mounted, examined and photographed using an Olympus ML2000 microscope (Olympus, Tokyo, Japan). Negative controls were incubated with non-immune serum instead of the primary antibody.

2.4. Immunofluorescence analysis of FAST2 in oocytes and embryos

Female mice (6–8 weeks old, n = 36) were injected with 10 IU PMSG followed by 10 IU hCG at 48 h. The mice were then mated with proven fertile male mice. Germinal vesicle (GV) stage oocytes were obtained from ovaries at 46 h post PMSG injections. Zygotes, two cell, three-four cell, six-eight cell embryos and morulae were recovered at 19–21 h, 42 h, 55 h, 66 h and 72–88 h post hCG from oviducts; blastocysts were collected from uteri at 90–96 h post hCG as described earlier (Guo et al., 2014).

The eggs and embryos were fixed in 4% (w/v) paraformaldehyde for 30 min and permeabilized in incubation buffer (0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, and 0.02% NaN_3) for 30 min. The blastocysts were also fixed and permeabilized similarly but for 40 min each. Non-specific binding of antibodies was blocked by incubating the samples with 1% BSA in cleaning fluid (10 × PBS 1 ml, Tween-20 1 μl, 10% Triton X-100 1 μl, 9 ml distilled water) for 30 min. The embryos were then treated with the primary antibody mentioned above, followed by incubation with fluorescein-isothiocyanate-conjugated rabbit anti-goat IgG secondary antibody (SC-2777, Santa Cruz, CA, USA; 1:100 dilution) for 1 h at room temperature. The embryos were finally incubated with 10 μg/ml 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) for 10 min, mounted with 1, 4-diazabicyclo (2.2.2) octane hydrochloride (Sigma-Aldrich) medium and examined using a laser scanning confocal microscope (Leica TCS SPE, Germany) as described previously (Guo et al., 2014).

2.5. Western blot analysis of FAST2

Whole mouse ovaries were lysed using protein extraction reagent RIPA buffer (50 mM TRIS pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors (Sigma-Aldrich). 20 μg protein was separated by electrophoresis and blotted onto a polyvinylidene fluoride membrane (Hybond, Amersham Pharmacia Biotech, Piscataway, N.J., USA). The membranes were incubated with 5% nonfat dry milk powder at room temperature for 90 min and then with anti-FAST2 (1:500 dilution)