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Original article

The mechanism and control of Jagged1 expression in Sertoli cells

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ABSTRACT

The regulation of Sertoli cells by some hormones and signaling factors is important for normal spermatogenesis. Notch signaling is considered to be necessary for normal spermatogenesis in mouse. In this study, we revealed two new facts about Sertoli cells by western blotting experiments on different types of primary cells and microdissected tubules. The first is that Sertoli cells express the Jagged1 ligand in mice testes. The second is that the expression level of Jagged1 oscillates in the seminiferous epithelial cycle. Therefore, we inferred that Jagged1 in Sertoli cells contributes to the Notch signaling involved in spermatogenesis. Furthermore, we examined the regulation of Jagged1 expression and found that Jagged1 expression was suppressed by cAMP signaling and was promoted by TNF-α signaling in Sertoli cells. When cAMP and TNF-α were simultaneously added to Sertoli cells, Jagged1 expression was suppressed. Therefore, cAMP signaling dominates Jagged1 expression over TNF-α signaling. These results suggest that cAMP signaling may cause the periodicity of Jagged1 expression in the seminiferous epithelial cycle, and controlling Jagged1 expression by adding TNF-α or cAMP may contribute to normal spermatogenesis *in vitro*.

1. Introduction

Homeostatic and stable spermatogenesis is supported by the strict regulation of precise proliferation, differentiation, and meiosis of germ cells [1,2]. In 2011, Sato et al. found that *in vitro* organ culture with the gas–liquid interphase method can lead spermatogonia and germline stem cells to fertile sperm [3,4]. Furthermore, three-dimensional culture methods could reconstruct testicular cells to the structure of the seminiferous tubule, and collagen in matrigel promoted reconstruction of testes [5]. These *in vitro* culture methods might be a prospective application for regenerative therapy of infertility but could not lead germ cells to elongated spermatids in the reconstructed testis [5–7]. Therefore, an unknown factor is necessary for normal spermatogenesis in a reconstructed testis.

Notch signaling, which is highly conserved from insect to vertebrate, relates to fate determination, lateral inhibition, and differentiation [8]. In *Caenorhabditis elegans*, Notch signaling promotes proliferation and inhibits differentiation of germline stem cells [9]. In the mammalian testis, localization of Notch signaling components was reported in several studies [10–14]. Notch1 has been shown to be expressed in undifferentiated spermatogonia and Sertoli cells. Notch2 and Notch3 are ubiquitously expressed in germ cells, and Jagged1 and Delta-like4 are expressed in elongated spermatids in adult testes [14]. However, other studies have reported Notch1 is expressed only in Sertoli cells [12], and Jagged1 is expressed in Sertoli cells [10,11]. Thus, the expression profiles of Notch signaling components in testes are controversial. On the other hand, loss- and gain-of-function analyses of Notch signaling in mouse testes have been reported [12,15,16]. Notch signaling in Sertoli cells inactivated by deletion of the protein O-fucosyltransferase1 showed normal spermatogenesis [12], whereas RBPj knockout in Sertoli cells led to abnormal spermatogenesis and an atrophic tubule [15]. Blocking Notch signaling in all testicular cells by injection of γ-secretase inhibitor into the tubule induced the collapse of the spermatogenic cycle and abnormal spermatozoa [17]. These studies suggest that Notch signaling in mouse testes is necessary for normal spermatogenesis. Therefore, we inferred that elucidation of the role of Notch signaling for spermatogenesis would contribute to the reconstruction of testes and *in vitro* spermatogenesis.

Abbreviations: RA, retinoic acid; FSH, follicle-stimulating hormone; cAMP, cyclic adenosine monophosphate; TNF-α, tumor necrosis factor-alpha; WT1, Wilm's tumor 1; P450scc, cytochrome P450 side-chain cleavage enzyme; Stra8, stimulated by retinoic acid gene 8.

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In this study, we analyzed the Jagged1 ligand in mice testes to identify Notch signaling in spermatogenesis and tried to find the controlling factor of Jagged1 expression in testes.

2. Methods

2.1. Mice

Male Slc:ICR mice were purchased from Japan SLC, Inc. and maintained in our animal facility on a 12-h light–dark cycle and were given access to food (MF; Oriental Yeast Co., Ltd.) and water ad libitum. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Immunohistochemistry

Cryosections were fixed with 4% paraformaldehyde for 5 min and then blocked with 5% normal horse serum for 1 h at room temperature. Sections were incubated for 72 h at 4 °C with either 2 μg/ml anti-Jagged1 goat polyclonal antibody (sc-6011; Santa Cruz) or normal goat IgG diluted in blocking solution: 3% BSA, 0.1% NaN_3 in PBS. Subsequently, sections were washed with PBS and then incubated for 1 h at room temperature with 7.5 μg/ml biotinylated horse anti-goat IgG antibody (BA-9500; Vector) in blocking solution followed by Vectastain ABC kit (Vectastain) reaction and incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dojindo). Sections were counterstained slightly with 25% methyl green.

2.3. Western blotting

Testes from 14-days post partum (dpp) and 60-dpp mouse were homogenized with RIPA buffer: 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1 mM EDTA (pH8.0), 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin. Following 10% SDS-PAGE, proteins were electro-transferred to a PVDF membrane (Immobilon-P; Millipore). Anti-Jagged1 goat antibody (sc-6011; Santa Cruz) or anti-actin rabbit antibody (A2066; Sigma) was used as the primary antibody with AP-conjugated anti-goat IgG (AP-5000; Vector) or HRP-conjugated anti-rabbit IgG (NA934; GE Healthcare) as the secondary antibody. Lumi-Phos WB (Thermo) and Plus-ECL (Perkin Elmer) were used to detect target proteins.

For analysis of primary cells and tubule fragments, anti-β-actin antibody (A5441; Sigma), anti-WT1 antibody (sc-192; Santa Cruz), TRA98 antibody (73-003; Bio Academia), anti-cytochrome P450 side chain cleavage enzyme (P450scc) antibody (ab1244; Chemicon), and anti-Stra8 antibody (ab49602; Abcam) were also used as the primary antibody (Supplementary Table S1).

2.4. Transillumination-assisted microdissection

Three-month testes without tunica albuginea were loosened in cold PBS. Seminiferous tubules were categorized by light transmittance to four compartments of pale zone (IX–XI), weak spot (XII–I), strong spot (II–VI), and dark zone (VII–VIII) [18]. The 30 pieces of 2-mm tubule for each compartment were harvested and homogenized with RIPA buffer.

2.5. Isolation and culture of primary Sertoli cells

Two-week testes without tunica albuginea were treated with enzyme solution (1 mg/ml collagenase type I A [Sigma], 0.25 mg/ml DNase I [DN25; Sigma], and 1 mg/ml hyaluronidase [Sigma] in Dulbecco modified Eagle medium [DMEM]), for 10 min at 37 °C to remove interstitial cells. Then tubules were washed with DMEM followed by re-incubation with the enzyme solution for 10 min at 37 °C. The tubules were cleaved into small pieces by gently pipetting with a blue cut tip (diameter 2–3 mm). The tubule pieces were cultured on a 0.1% gelatin-coated dish at 37 °C, 5% CO_2 with 10% fetal bovine serum (FBS; Hyclone), 5 mM L(+)-glutamine (Wako), 1 mM sodium-pyruvate (Wako), 0.1% sodium DL-lactate (Nacalai), and penicillin and streptomycin in DMEM. Two days after culture, the cells were subjected to hypoosmotic shock with 10 mM Tris–HCl (pH 7.4) for 10 min at room temperature to remove residual germ cells. At day 4, the cells were treated with 0.125% trypsin (Gibco) in PBS and replated as 2.5×10^5 cells/cm^2. At day 6, the medium was changed and the following were added: *all-trans*-retinoic acid (RA; Wako), dibutyryl cAMP (cAMP) (D0627; Sigma), forskolin (Sigma), or mouse TNF-α (Roche). Total RNA and cell lysates were harvested after 24 h.

2.6. Isolation of the Leydig cell-rich fraction and primary germ cells

Two-month testes without tunica albuginea were treated with the enzyme solution to dissociate stromal cells from the tubule. The supernatant was washed with PBS and harvested as a Leydig cell-rich fraction. The tubules were re-incubated with the enzyme solution and then digested by 0.25% trypsin for 10 min at 37 °C. The trypsin reaction was stopped by adding a 10–20% volume of FBS. The dissociated cells were filtered through a 40-μm cell strainer (352340; Falcon) and cultured on a 0.1% gelatin-coated dish at 37 °C, 5% CO_2 overnight. The next day, the supernatant including primary germ cells was harvested and dissolved with RIPA buffer.

2.7. Quantitative RT-PCR

Total RNA was isolated from the primary Sertoli cells by using RNeasy (Qiagen). One microgram total RNA was reverse transcribed using AMV Reverse Transcriptase XL (Takara) with Oligo dT primer (Invitrogen). Quantitative RT-PCR analysis (qRT-PCR) was performed in duplicate using gene-specific primers (Supplementary Table S2) with Power SYBR Green PCR Master Mix (Thermo) by the StepOnePlus realtime PCR system (Thermo).

3. Results

3.1. Sertoli cells in mouse testes express Jagged1

To define the contribution of Notch signaling to spermatogenesis in mouse, we investigated the localization of Notch signaling factors in testes. Immunohistochemistry experiments revealed that Jagged1, one of the Notch ligands, was expressed in Sertoli cells and was also detected in Leydig cells (Fig. 1A, B). Hasegawa et al. reported that elongated spermatid expressed Jagged1 mRNA by *in situ* hybridization [12]; we therefore tried to analyze cell-type specificity of Jagged1 expression by using several primary culture cells, such as the Sertoli cell, germ cell, and Leydig cell (Fig. 1E). The signals of the marker protein, such as WT1 (Sertoli cell marker), TRA98 (germ cell marker) and P450scc (Leydig cell marker), indicated that each type of cell was harvested. The signal of TRA98 was also detected in the Leydig cell-rich fraction and indicated that the fraction included some germ cell contaminants. Full-length Jagged1 protein (150 kDa) was detected in primary Sertoli cells but not in primary germ cells or Leydig cells. Some extra bands (<75 kDa) of Jagged1 were also detected in all samples (Fig. S1), but we considered that they were non-specific signals or might be degradation products. These results suggest that Jagged1 ligand in the mouse testis is expressed only in Sertoli cells. Subsequently, we