Regenerative Therapy 3 (2016) 75–81

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original article

The mechanism and control of Jagged1 expression in Sertoli cells

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
8 February 2016
Accepted 15 February 2016

Keywords:
Sertoli cell
Jagged1
Seminiferous epithelial cycle
TNF-α
cAMP

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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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

http://dx.doi.org/10.1016/j.reth.2016.02.005

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

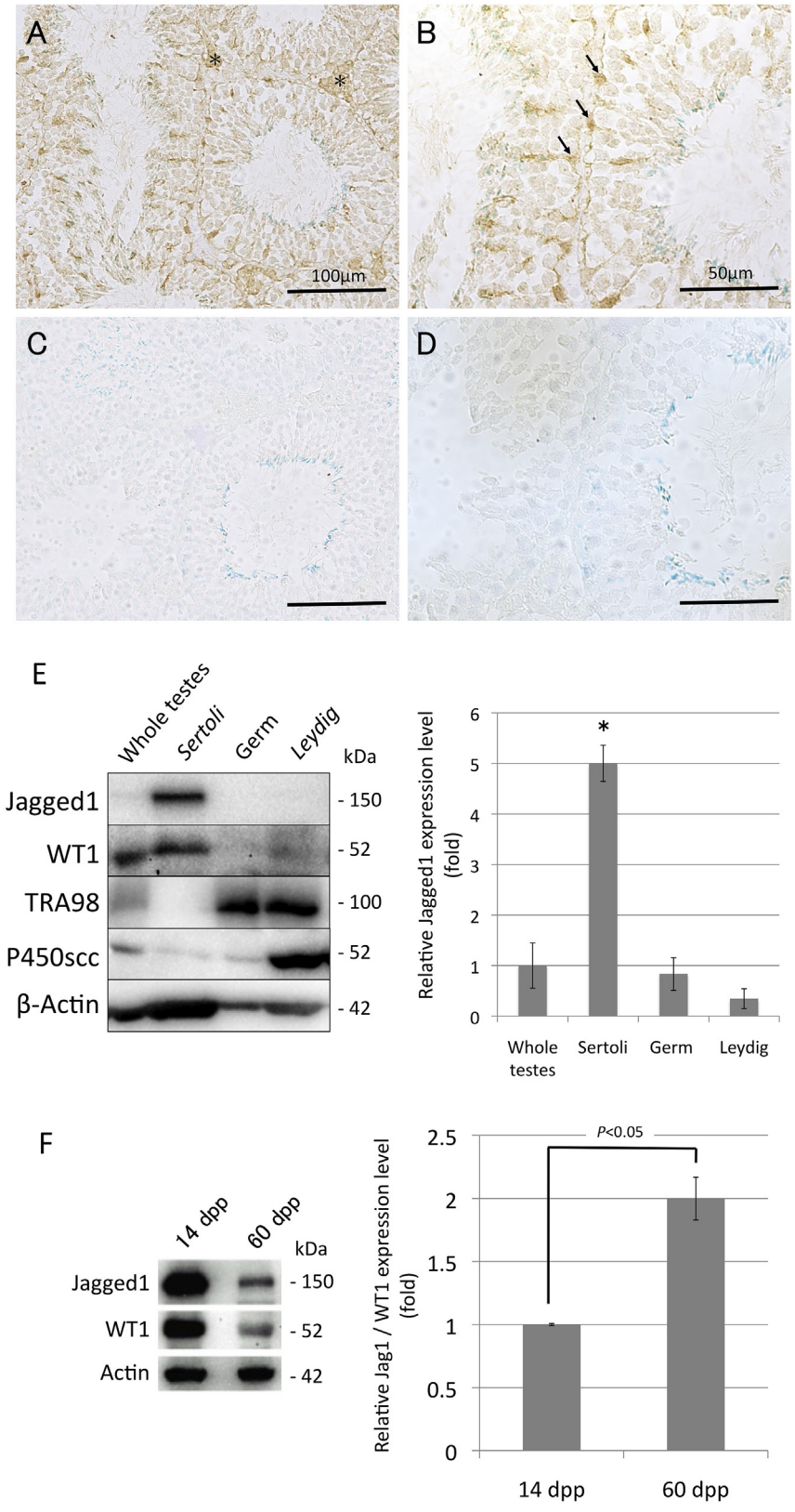

Fig. 1. Localization of the Jagged1 ligand in the mouse testis. A–D) Immunostaining with anti-Jagged1 antibody in a 60-dpp mouse testis, counterstained with methyl green (200× and 400× magnification). Interstitial cells and Sertoli cells were stained (A, B). Control IgG (C, D). Asterisks mark interstitial cells; arrows mark Jagged1-positive Sertoli cells. E) Western blotting of cell lysates from 60-dpp whole testes, primary Sertoli cells, primary germ cells, and Leydig cell-rich fraction ($n = 3$). Jagged1 expression was normalized to β-actin, and the fold increase is plotted (value of whole testes = 1). Full-length Jagged1 was mainly detected in primary Sertoli cells. WT1, Sertoli cell marker; TRA98, germ cell marker; P450scc, Leydig cell marker. Asterisk indicates a statistically significant difference ($P < 0.05$). F) Western blotting of cell lysates from 14-dpp and 60-dpp whole testes ($n = 3$). Jagged1 expression was normalized to actin and WT1, and fold increase is plotted. Jagged1 expression in Sertoli cells significantly increased during testicular development. Error bars represent the standard error of the mean.

analyzed the expression of Jagged1 in developing testes. Jagged1 expression was detected from 14-dpp to adult testis (Fig. 1F). Immunostaining of WT1 in developing testes revealed that WT1 expression was constant in Sertoli cells throughout aging, from pup to adult (Fig. S2). The expression level of Jagged1 per Sertoli cell, which had a value that was normalized by the expression level of WT1, was significantly higher in adult testes than 14-dpp testes.

3.2. The expression of Jagged1 is controlled by or related to seminiferous epithelial cyclic regulation

In the adult testis, spermatogenesis shows a wave-like manner in tubules, which is called the seminiferous epithelial cycle [1]. This cycle in mouse is divided into 12 stages relating to types of differentiation of germ cells in each section of the tubule. The seminiferous epithelial cycle supports stable and continuous spermatogenesis, suggesting the existence of precise cyclic programs of gene expression. Therefore, we analyzed the expression pattern of Jagged1 at each stage of the cycle using transillumination-assisted microdissection [18]. The protein level of Jagged1 at stage VII–VIII, which was Stra8 positive region, was 2.2-fold higher than that of stage IX–XI and XII–I (Fig. 2). These results show that the expression of Jagged1 is related to the stage of the cycle and the possibility that Jagged1 has a role in the cyclic regulation of spermatogenesis.

3.3. cAMP and TNF-α signaling control the expression of Jagged1 in Sertoli cells

The regulation of gene expression in Sertoli cells is controlled by the seminiferous epithelial cycle. It was previously reported that some signaling factors are secreted in a wave-like manner in the cycle [19]. For example, a concentration of RA in the tubule increases at stage VII–VIII according to the activated synthesis of RA. Meiosis of spermatocyte and the process of undifferentiated spermatogonia to differentiated spermatogonia are triggered by RA signaling at these RA-rich stages [19]. Accordingly, we analyzed what signaling factor regulates the expression of Jagged1 in Sertoli cells by the addition of some humoral factors to the primary Sertoli cells. Contrary to our expectation, Jagged1 mRNA expression was not significantly changed by RA signaling (Fig. 3A). Follicle-stimulating hormone (FSH) secreted from the pituitary stimulates Sertoli cells to produce the glial cell line-derived neurotrophic factor (GDNF) [20]. To determine the FSH-signaling effect without experimental dispersion of the expression level of the FSH receptor in the primary culture of Sertoli cells, we used cAMP, the second messenger of FSH signaling [21], as a powerful effector. cAMP decreased Jagged1 expression by less than 0.4-fold (Fig. 3B), which was significant, and the time course of inhibition revealed that Jagged1 expression was suppressed by cAMP for 6 h in the primary culture condition (Fig. 3C). Furthermore, the addition of forskolin, an activator of adenylate cyclase, showed the inhibition of Jagged1 expression, indicating that endogenous cAMP also could decrease Jagged1 expression in Sertoli cells (Fig. 3D). TNF-α, which is secreted from round spermatids in the mouse testis, is one of the major factors regulating Sertoli cells. For example, TNF-α signaling in Sertoli cells promotes secretion of transferrin and participates in BTB formation [22,23]. Furthermore, TNF-α signaling promoted Jagged1 expression in vascular endothelial cells [24]. As expected, Jagged1 expression in Sertoli cells was stimulated to twice that of the control by the addition of 20 ng/ml TNF-α (Fig. 3E). This TNF-α signaling increased the mean value of Jagged1 expression approximately fourfold up to 6 h, and thus the peak of induction of Jagged1 by TNF-α might be less than 24 h (Fig. 3F). mRNA analysis showed the expression level of Jagged1 protein decreased 0.6-fold by cAMP and increased 2.3-fold by TNF-α (Fig. 3G). These results indicated that the addition of cAMP or TNF-α can control the expression of Jagged1 in Sertoli cells in reconstructed testes.

3.4. cAMP signaling is dominant in Jagged1 expression

To determine the dominant signaling factor in Jagged1 expression, we added both cAMP and TNF-α to Sertoli cells (Fig. 4). We used 20 ng/ml TNF-α, which was the concentration most effective at 24 h (Fig. 3E). The Jagged1 mRNA level in Sertoli cells decreased 0.5-fold by the addition of 0.5 mM cAMP alone, increased 2.4-fold by the addition of 20 ng/ml TNF-α alone, and decreased 0.6-fold by the addition of both. The expression level of Jagged1 with both cAMP and TNF-α was approximately equal to that with only cAMP,

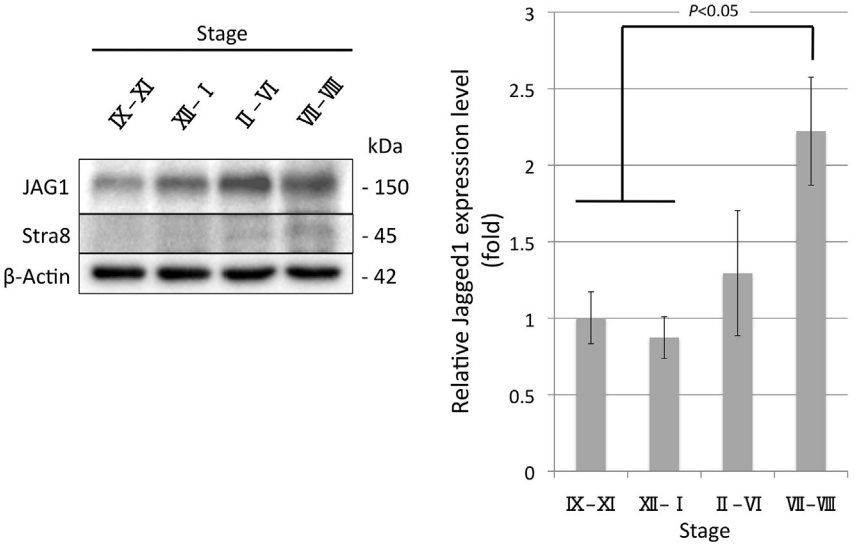

Fig. 2. Expression of Jagged1 during the seminiferous epithelial cycle. Western blotting of cell lysates from tubule fragments obtained by transillumination-assisted microdissection ($n = 3$). Jagged1 expression was normalized to β-actin and fold change is plotted (value of IX–XI = 1). Stra8, marker of stage VII–VIII. Error bars represent the standard error of the mean.

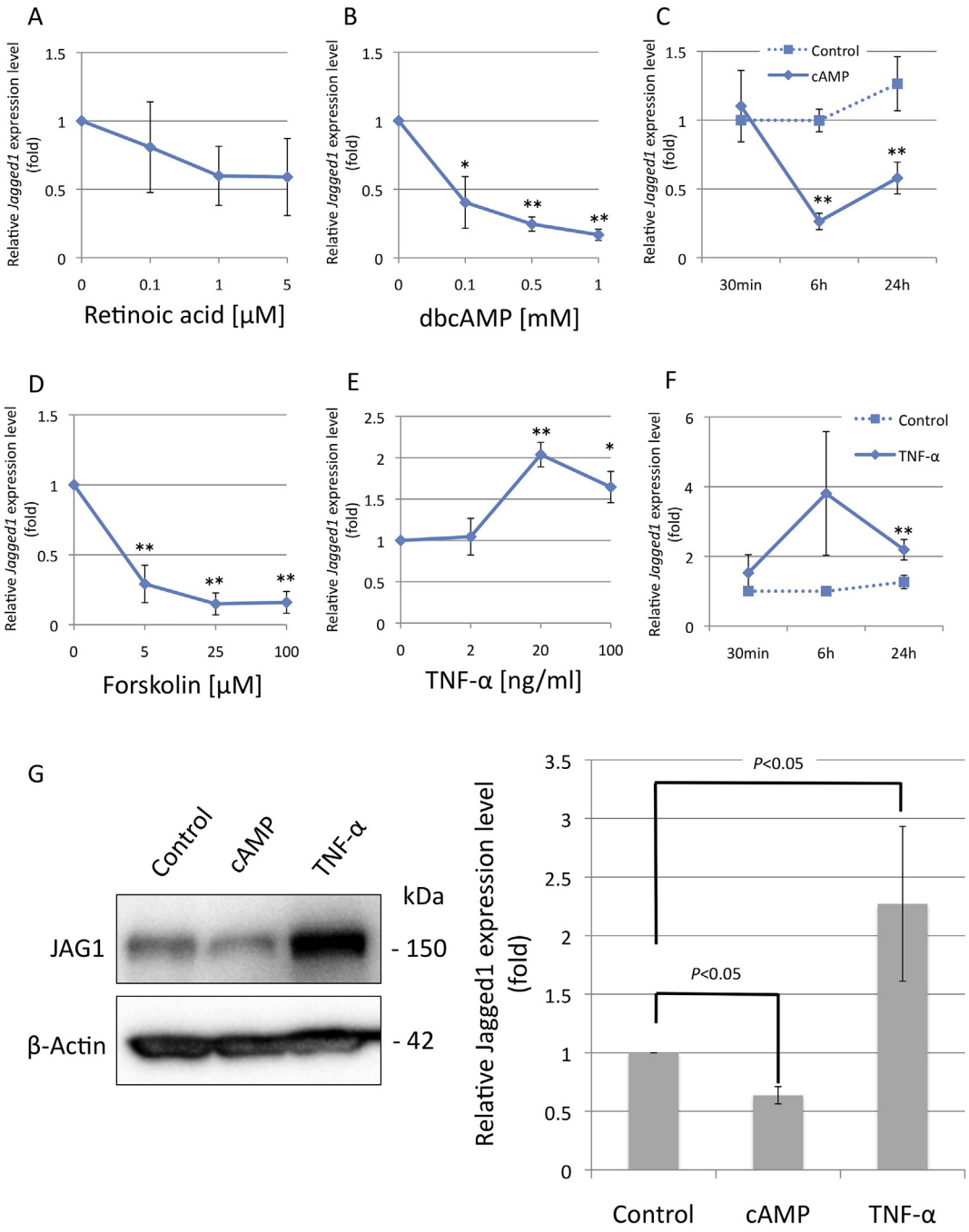

Fig. 3. Expression of Jagged1 in primary Sertoli cells treated with humoral factors A, B, D, E) Primary Sertoli cells were treated with each humoral factor for 24 h, and total RNA was harvested for qRT-PCR analysis ($n = 3$). C, F) Primary Sertoli cells were treated with either cAMP (0.5 mM) or TNF-α (20 ng/ml) for 30 min, 6 h and 24 h, and total RNA was harvested for qRT-PCR analysis ($n = 3$). Jagged1 expression was normalized to GAPDH and fold change is plotted. Error bars represent the standard error of the mean (SEM). $^{*}P < 0.05$, $^{**}P < 0.01$ by *t*-test, compared to control. G) Primary Sertoli cells were treated with either cAMP (0.5 mM) or TNF-α (20 ng/ml) for 24 h, and cell lysates were harvested for western blotting analysis ($n = 3$). Jagged1 expression was normalized to β-actin, and fold change is plotted. Error bars represent the SEM.

suggesting that signaling via cAMP dominated Jagged1 expression in Sertoli cells over TNF-α signaling.

4. Discussion

In the mammalian testis, various signaling factors are secreted with periodicity, and control of the periodicity supports normal spermatogenesis [25]. For example, spermatogenesis in the vitamin A-deficient mouse collapses by ablation of the seminiferous epithelial cycle [26]. Thus, we speculate that the reproduction of the seminiferous epithelial cycle is important for *in vitro* spermatogenesis to be successful.

In this study, western blotting analysis of the different cell types revealed that Sertoli cells expressed Jagged1 in mouse testes. Additionally, the expression level of Jagged1 oscillated in the seminiferous epithelial cycle. Therefore, we considered that this oscillation might have a role in spermatogenesis. Stage VII–VIII is the last stage that spermatozoa contact Sertoli cells, and undifferentiated spermatogonia differentiate to differentiated spermatogonia [19]. RA signaling is activated at this stage, but Jagged1

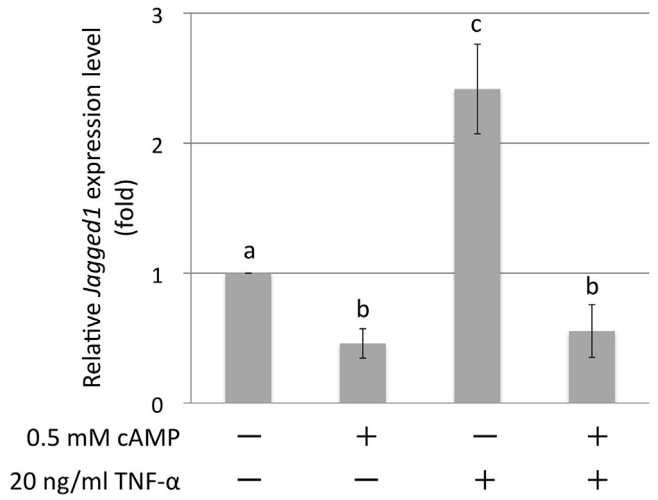

Fig. 4. Expression of Jagged1 in primary Sertoli cells with cAMP and TNF-α. Primary Sertoli cells were treated with cAMP (0.5 mM) and/or TNF-α (20 ng/ml) for 24 h, and total RNA was harvested for qRT-PCR analysis ($n = 3$). Jagged1 expression was normalized to GAPDH, and fold change is plotted. Error bars represent the SEM. Different letters indicate a significant difference ($P < 0.05$) by *t*-test.

expression in Sertoli cells was not stimulated by the addition of RA, suggesting that an unknown factor may induce Jagged1 expression at stage VII–VIII. In this study, we found an activator and inhibitor of Jagged1 expression in Sertoli cells. It is known that Jagged1 expression in vascular endothelial cells is increased by TNF-α signaling via NFκ-B [24], and TNF-α is secreted by round spermatids in the mouse testis [27,28]. Addition of TNF-α promoted Jagged1 expression in Sertoli cells, but the expression of TNF-α is highest at stage IX–XII [27]. Therefore, it remains unclear if TNF-α can stimulate Jagged1 expression in Sertoli cells *in vivo*. In rat testes, the concentration of cAMP in testicular cells is higher at stage I–VI and lower at stage VII–VIII compared to other stages, and this is the reason that the responsiveness in FSH signaling changes in a stage-dependent manner [29,30]. Our experiments revealed that signaling via cAMP downregulated Jagged1 expression in Sertoli cells. These results suggest that Jagged1 expression increased at stage VII–VIII with a low level of cAMP compared to the other stages. Transcription factor GATA-1 is downregulated by FSH signaling and cAMP in Sertoli cells [31]. The predicted promoter region at 3000 bp upstream of Jagged1 has several GATA-1 binding sites, and there is a possibility that GATA-1 promotes Jagged1 expression in these cells. Id2 transcriptional repressor is induced by FSH and cAMP signaling in Sertoli cells [32], and the Jagged1 promoter region has several Id2 binding sites as E-box. Inhibition of Jagged1 expression by cAMP may therefore occur by increasing the Id2 expression. By adding both cAMP and TNF-α to Sertoli cells, we showed that cAMP signaling is dominant in the downregulation of Jagged1 expression. Ouchi et al. reported that cAMP signaling inhibited TNF-α–induced IκB-α phosphorylation in human aortic endothelial cells [33]. Thus, in future studies we need to examine the Jagged1 gene promoter and the phosphorylation level of IκB-α in Sertoli cells.

In this study, we detected the Jagged1 protein in primary Sertoli cells (Fig. 3G), and in a previous study, Jagged1 expressed in Sertoli cells activated Notch signaling in T cells and promoted the expression of the target gene Hey1 [10]. We therefore considered that Jagged1 expressed in Sertoli cells has ligand activity.

Jagged1 periodicity in Sertoli cells may be important for stable and continuous spermatogenesis. Recently micro-fabrication technology has constructed a method to add humoral factors at specific times and in appropriate quantities by microfluidic devices. Using such a device, the differentiation of mouse-induced pluripotent stem cells was controlled by the spatiotemporally controlled delivery of RA and leukemia inhibitory factor [34]. It is expected that these devices and effective humoral factors will contribute to mimicking the cyclic-regulated testis in the reconstructed testis and promote spermatogenesis.

Conflict of Interest

All authors declare no conflict of interest associated with this manuscript.

Acknowledgments

This work was partly supported by Grant-in-Aid for Scientific Research on Innovative Areas “Bio Assembler” Grant Number 26106722 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2016.02.005.

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