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.

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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% NaN3 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 (Vector) re-tinylated. Lumi-Phos WB (Thermo) and Plus-ECL (Perkin Elmer) were used as the secondary antibody. Anti-rabbit IgG (NA934; GE Healthcare) as the secondary antibody. (A2066; Sigma) was used as the primary antibody with AP-labeled goat anti-rabbit antibody (sc-2066; Sigma) was used as the primary antibody with AP-conjugated goat IgG antibody (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 tube fragments, anti-β-actin antibody (A5441; Sigma), anti-WT1 antibody (sc-192; Santa Cruz), TRA98 antibody (73-003; Bio Academia), anti-cytokyme P450 side chain cleavage enzyme antibody (P450ccc) antibody (ab1244; Chemicon), and anti-Str8 antibody (ab49602; Abcam) were also used as the primary antibody (Supplementary Table S1).

2.3. Western blotting

Tests from 14-days post partum (dpp) and 60-dpp mouse were homogenized with RIPA buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA (pH8.0), 1 mM Na2VO4, 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 nitrocellulose membrane. The membranes were incubated with 7.5 μg/ml collagenase type I A (Sigma), 0.25 mg/ml enzyme solution (1 mg/ml collagenase type IA [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% CO2 with 10% fetal bovine serum (FBS; Hyclone), 5 mM (+)-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 × 105 cells/cm2. 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 PBS. 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% CO2 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 P450ccc (Leydig cell marker), indicated that each type of cell was harvested. The signal of TRA98 was also detected in the Ledyig 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 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 control by the addition of 20 ng/ml TNF-α (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 is involved in spermatogonial proliferation [20]. To determine the FSH-signaling effect without the cAMP signaling factor, 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 0.6-fold by the addition of 0.5 mM cAMP alone, increased 2.4-fold by the addition of 0.5 mM cAMP alone, increased 2.4-fold by cAMP and increased 2.3-fold by TNF-α (Fig. 3C). These results indicated that the addition of cAMP or TNF-α can control the expression of Jagged1 in Sertoli cells in reconstructed testes.

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 is involved in spermatogonial proliferation [20]. 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,
suggested 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. Stage VII–VIII is the last stage that spermatocytes contact Sertoli cells, and undifferentiated spermatogonia differentiate to differentiated spermatogonia [19]. RA signaling is activated at this stage, but Jagged1
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 NFκB [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 IkB-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 IkB-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 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.

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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.

References

[1] Russell LD, Ettlin RA, Hikim AP, Clegg ED. Histological and histopathological evaluation of the testis. Caché River Press: 1990.
[2] Oatley JM, Brinster RL. Regulation of spermatogenic stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 2008;24:263–86.
[3] Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, et al. In vitro production of functional sperm in cultured neonatal mouse testes. Nature 2011;471(7339):504–7.
[4] Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, et al. In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2011;2:472.
[5] Zhang J, Hatakeyama J, Eto K, Abe S. Reconstruction of a seminiferous tubule-like structure in a 3 dimensional culture system of re-aggregated mouse neonatal testicular cells within a collagen matrix. Gen Comp Endocrinol 2014;205:121–32.
[6] Yokonishi T, Sato T, Katagiri K, Komeya M, Kubota Y, Ogawa T. Vitro reconstruction of mouse seminiferous tubules supporting germ cell differentiation. Biol Reprod 2013;89(1):15:1–6.
[7] Legendre A, Froment P, Desmots S, Lecomte A, Habert R, Lemazurier E. An engineered 3D blood-testis barrier model for the assessment of reproductive toxicity potential. Biomaterials 2010;31(16):4492–505.
[8] Koch U, Lehal R, Radtke F. Stem cells living with a Notch. Development 2013;140(4):689–704.
[9] Kimble J, Crittenden SL. Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans. Annu Rev Cell Dev Biol 2007;23:405–33.
[10] Campese AF, Grazioli P, de Cesaris P, Riccioli A, Bellavia D, Pelullo M, et al. Mouse Sertoli cells sustain de novo generation of regulatory T cells by triggering the notch pathway through soluble Jagged1. Biol Reprod 2014;90(3):53.
[11] Dirami AF, Ravidranathan N, Achi MV, Dym M. Expression of Notch pathway components in spermatogonia and Sertoli cells of neonatal mice. J Androl 2001;22(6):944–52.
[12] Haegawa K, Okamura Y, Saga Y. Notch signaling in Sertoli cells regulates cyclical gene expression of Hey1 but is dispensable for mouse spermatogenesis. Mol Cell Biol 2012;32(1):206–15.
[13] Mori S, Kadokawa Y, Hoshinaga K, Marunouchi T. Sequential activation of Notch family receptors during mouse spermatogenesis. Dev Growth Differ 2003;45(1):7–13.
[14] Murta D, Batista M, Silva E, Trindade A, Henrique D, Duarte A, et al. Dynamics of Notch pathway expression during mouse testis post-natal development and along the spermatogenic cycle. PLoS One 2013;8(8):e72767.
[15] Garcia TX, Farinha JK, Kow S, Hofmann MC. RBP5 in mouse Sertoli cells is required for proper regulation of the testis stem cell niche. Development 2014;141(23):4668–78.
[16] Huang J, Rivas B, Agoulnik AI. NOTCH1 gain of function in germ cells causes failure of spermatogenesis in male mice. PLoS One 2013;8(7):e71213.
[17] Murta D, Batista M, Trindade A, Silva E, Henrique D, Duarte A, et al. In vivo notch signaling blockade induces abnormal spermatogenesis in the mouse. PLoS One 2014;9(11):e113305.
Kotaja N, Kimmins S, Brancorsini S, Hentsch D, Vonesch JL, Davidson I, et al. Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. Nat Methods 2004;1(3):249–54.

Sugimoto R, Nabeshima Y, Yoshida S. Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. Mech Dev 2012;128(11–12):610–24.

Tadokoro Y, Yomogida K, Ohba H, Togha A, Nishimune Y. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech Dev 2002;113(1):29–39.

Walker WH, Cheng J. FSH and testosterone signaling in Sertoli cells. Reproduction 2005;130(1):15–28.

Sigillo F, Guillou F, Fontaine I, Benahmed M, Le Magueresse-Battistoni B. In vitro regulation of rat Sertoli cell transferrin expression by tumor necrosis factor alpha and retinoic acid. Mol Cell Endocrinol 1999;148(1–2):163–70.

Xia W, Wong EW, Mruk DD, Cheng CY. TGF-beta1 and TNFalpha perturb blood-testis barrier (BTB) dynamics by accelerating the clathrin-mediated endocytosis of integral membrane proteins: a new concept of BTB regulation during spermatogenesis. Dev Biol 2009;327(1):48–61.

Johnston DA, Dong B, Hughes CC. TNF induction of jagged-1 in endothelial cells is NFkappaB-dependent. Gene 2009;435(1–2):36–44.

Hasegawa K, Saga Y. Retinoic acid signaling in Sertoli cells regulates organization of the blood-testis barrier through cyclical changes in gene expression. Development 2012;139(23):4347–55.

van Pelt AM, de Rooij DG. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. Biol Reprod 1990;43(3):363–7.

De SK, Chen HL, Pace JL, Hunt JS, Terranova PF, Enders GC. Expression of tumor necrosis factor-alpha in mouse spermatogenic cells. Endocrinology 1993;133(1):389–96.

Gnassi L, Fabbri A, Spera G. Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. Endocr Rev 1997;18(4):541–600.

Kangasniemi M, Kaipia A, Mali P, Toppari J, Huhtaniemi I, Parvinen M. Modulation of basal and FSH-dependent cyclic AMP production in rat seminiferous tubules staged by an improved transillumination technique. Anat Rec 1990;227(1):62–76.

Johnston DS, Olivas E, DiCandelo P, Wright WW. Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. Biol Reprod 2011;85(4):763–9.

Zhang Z, Wu AZ, Feng ZM, Mruk D, Cheng CY, Chen CL. Gonadotropins, via cAMP, negatively regulate GATA-1 gene expression in testicular cells. Endocrinology 2002;143(3):829–36.

Scobey MJ, Fix CA, Walker WH. The Id2 transcriptional repressor is induced by follicle-stimulating hormone and cAMP. J Biol Chem 2004;279(16):16064–70.

Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. Circulation 2000;102(11):1296–301.

Kawada J, Kimura H, Akutsu H, Sakai Y, Fuji T. Spatiotemporally controlled delivery of soluble factors for stem cell differentiation. Lab Chip 2012;12:4508–15.