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Elicitation of metastasis associated protein 2 expression in the phagocytosis by murine testicular Sertoli cells

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ABSTRACT

Efficient phagocytic clearance of apoptotic spermatogenic cells and residual bodies (RBs) by Sertoli cells (SCs) is crucial for functional mature spermatogenesis. However, little is known about the molecular mechanisms underlying this SCs function. Herein, we reported for the first time that SCs-expressing metastasis associated protein 2 (Mta2), a chromatin modifier playing a critical role in modifying DNA accessibility for transcriptional regulation, was steadily up-regulated when SCs were co-cultured with RBs. The most efficient stimulatory substrates for the inducement of phagocytosis-elicited Mta2 expression were RBs and fragments from apoptotic spermatocytes. Furthermore, one major result of this response is the transcriptional repression of follicle-stimulating hormone receptor gene (*Fshr*) expression during phagocytosis, which should lead to a low level of circulated FSH because effects of FSH on spermatogenesis is fundamentally regulated by the down-regulation of *Fshr* after exposure to FSH. Given that high concentration of circulated FSH inhibits SCs phagocytic activity and impairment of MTA2 expression is associated with the abnormal high level of serum FSH, our present results suggest that the FSH/MTA2/*Fshr* cascade may serve as an indispensable negative feedback mechanism to help to maintain low level of circulated FSH, which is required for the normal occurrence of SCs phagocytosis.

1. Introduction

Mammalian spermatogenesis is a precisely controlled and cyclical timed process consisting of a series of events, including mitotic proliferation of spermatogonia, meiotic divisions of spermatocytes, and the maturation and differentiation of haploid spermatids [1]. During this complex process, more than 75% of developing germ cells (GCs) are estimated to die under physiological condition, probably due to apoptosis, before they mature into spermatozoa [2]. Moreover, the cytoplasmic compartments of elongated spermatids are shed and form residual bodies (RBs) before releasing of differentiated sperm into the lumen of the seminiferous tubules during the late stage of spermatogenesis [3]. Both apoptotic GCs and RBs are phagocytosed and degraded by Sertoli cells (SCs). This phagocytic action, comprising the recognition and engulfment of apoptotic cells, processing of engulfed apoptotic cells and the alteration of gene expression in engulfing SCs, has been shown to be necessary for healthy spermatogenic cells to proceed through spermatogenesis [4]. Although the investigation of these phenomena has recently become more intensive, most mechanisms still remain obscure.

Metastasis associated protein 2 (Mta2), an integral component of the nucleosome remodeling and histone deacetylation (NuRD) complex, functions in conjunction with distinct histone deacetylase (HDAC) to mediate transcriptional repression as it facilitates the association of repressor molecules with the chromatin [5]. In testis, Mta2 is exclusively expressed in SCs and its expression is regulated directly by testosterone or indirectly by follicle-stimulating hormone (FSH). We previously demonstrated that Mta2 associated with HDAC1 can bind to the *Fshr* gene and mediate its transcriptional repression in response to FSH treatment. Failure to do so will result in the inevitable reduction of competency of Fshr to bind FSH, followed by an unusual elevation of the serum FSH level. Thus, the FSH/Mta2/Fshr cascade may serve as an important mechanism to modulate the timing and magnitude of subsequent signal transduction of SCs in response to FSH [6]. Nevertheless, the role of this chromatin modifier in other SCs functions remains largely unknown.

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Accumulated evidence suggests that SCs phagocytosis is subjected to an elaborate regulation by multiple factors, such as epithelium growth factor and FSH signaling [7]. On the other hand, emerging data point to a potential involvement of deacetylases in the phagocytic activity [8–10]. Considering the close relationship between Mta2 function and recruitment of histone deacetylation complexes in response to FSH treatment [6], we hypothesized that Mta2 may be potentially involved in the SCs phagocytic function. An expression and mechanistic study comprising multiple analyses was therefore designed to elucidate the potential links between this chromatin modifier and the complicated nature of this unique activity. Our systematic analysis will pave the way for a better understanding of the role of Mta2 in SCs biology.

2. Materials and methods

2.1. Cell preparation and treatment

All procedures involving animals were approved by the local ethical committee. SCs and spermatogenic cells were individually isolated from the testes of C57BL/6 mice purchased from the Animal Research Center of our university, as described previously [11,12]. Briefly, testes were decapsulated and digested for 15 min in 0.25% (w/v) collagenase (type IX, Sigma) at room temperature with constant shaking, followed by digestion in minimum essential medium containing 1 mg/ml trypsin for 30 min at 30 °C. Digestion was stopped by adding 10% fetal calf serum and the released GCs were collected after sedimentation (10 min at room temperature) of tissue debris. GCs were then filtered through a 50-mm nylon mesh and resuspended in 20 ml of elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ ($7H_2O$), 1.3 mM $CaCl_2$, 11 mM glucose, 1× essential amino acid, penicillin, streptomycin, 0.5% bovine serum albumin). Different GCs were separated by velocity sedimentation at unit gravity on 2–4% BSA gradients [13]. For isolation of SCs, after being dispersed (but not fragmented) in 0.1% collagenase (type IV) and 0.04% DNase I in 1× Hanks fluid (pH 7.4) at 34 °C for 10–15 min, the seminiferous tubules were incubated in 1× Hanks fluid (pH 7.4) containing 0.04% DNase I, 0.05% hyaluronidase, and 0.5% trypsin for at least 10 min at 34 °C with agitation. The fragmented tubules were allowed to settle and cells were subsequently centrifuged at 200×*g* for 3 min. Cells in the supernatant were collected and cultured (in DMEM/F12 medium containing 5% FCS) overnight. SCs attached to the bottom and acquired an irregular shape, whereas GCs did not attach and could easily be removed by repeated washing. The purity of isolated testicular cells could reach 80% (spermatogonia, Spg), 85% (pachytene spermatocytes, Spc), 90% (round spermatids, Rsd) and 90% (SCs), respectively. Further confirmation of the cell types were carried out using quantitative RT-PCR according to our previous report [11,14]. Subsequently, Spg and Spc suspending in medium were collected and cultured alone for another 2 days to induce spontaneous apoptosis [15].

RBs were isolated from testes of 70-day-old mice and were then biotin labeled as described elsewhere [7]. Briefly, seminiferous tubules were incubated in 25 ml of 0.01 M PBS (pH 7.2) containing 0.1% glucose, 3 mM lactate, 0.25 mg/ml trypsin for 20 min at 33 °C, and then cells were dispersed by gentle pipetting. The suspension was filtered through sterile surgical gauze. Cells were pelleted by centrifugation at 800×*g* for 10 min and resuspended in 40 ml of 0.01 M PBS (pH 7.2) containing 0.1% glucose, 3 mM lactate, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. Supernatants were collected gently by pipetting and were pelleted twice at 200×*g* for 3 min. The resulting suspension containing RBs and GCs was allowed to sediment overnight at 4 °C, and then was centrifuged at 800×*g* for 10 min. The supernatant was centrifuged again at 800×*g* for 30 min. The quality of RBs preparations were monitored by microscopic observation. RBs were transferred into labeling buffer (10 mM Na borate [pH 8.8], 150 mM NaCl), followed by incubation in biotin labeling solution (50 μg/ml in DMSO) at 20 °C for 15 min. The labeling was ended by adding 10 mM NH_4Cl. To measure RB phagocytosis activity, SCs seeded in eight-well Labtek chambers were incubated with 1.2×10^7 biotinylated RBs for 2, 4, or 6 at 34 °C in a humidified atmosphere of 5% CO_2. Unbound RBs were washed away with DMEM, and cells were fixed for 5 min with 4% paraformaldehyde/PBS (pH 7.4). Subsequently, RBs were labeled with avidin-FITC for 1 h at 25 °C. The ingested RBs were observed using an inverted microscope (Axio Imager M1 microscope, Zeiss) and phagocytic index was calculated as the ratio of RB/nuclei number in each well.

2.2. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. Routine DNase (Applied Biosystems/Ambion, Austin, TX, USA) treatment (1 U DNaseI per μg total RNA) was performed before reverse transcription. First-strand cDNA was synthesized using 1 μg RNA with Superscript III (Rnase H-Reverse Transcriptase; Invitrogen) and PCR was set up according to Promega's reverse transcription system protocol. Primer sequences used were reported previously [6]. PCR products were then quantified by SYBR Green intercalation using the MiniOpticon™ system (Bio-Rad). The relative abundance of each target transcript was quantified using the comparative $\Delta\Delta Ct$ method, with *Gapdh* as an internal control.

2.3. Western blotting

Protein samples were prepared according to our previous report [16]. Protein was separated on SDS–PAGE and transferred to nitrocellulose membrane (Millipore, Bedford, MA). Membranes were then incubated with primary antibodies, including anti-Mta2 (dilution 1:1000; Santa Cruz Biotechnology, Inc.) and anti-Tubulin (dilution 1:1000; Santa Cruz Biotechnology, Inc.) in blocking solution overnight at 4 °C. Positive signals were finally detected by using an ECL kit (Amersham Biosciences). Densitometric analyses of immunoblots were performed using Image J software.

2.4. Measurement of HDAC activity

Nuclear extracts were prepared as reported [17]. Subsequently, 50 mg of nuclear extract was applied to measurement of HDAC activity using a colorimetric assay kit (BioVision, Milpitas, California). Activity was analyzed as the relative optical density value per mg of protein sample and was recalculated using the deacetylated standard as △OD/μM.

2.5. Immunohistochemistry

A Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining according to the protocol recommended by the manufacturer. The primary anti-MTA2 antibody was used at a dilution of 1:150. Control slides were incubated with a preabsorbed serum instead of primary antibody.

2.6. Statistical analysis

Experiments were repeated at least three times, and one representative result from at least three similar results is presented. Quantitative data are presented as mean ± S.D. Results were

analyzed for statistically significant differences using Student's *t* test, with $P < 0.05$ being considered as statistically significant.

3. Results

3.1. Stage-specific expression of Mta2 in mouse testis

By immunohistochemical analysis, an exclusive nuclear staining for Mta2 was observed in SCs of mouse testis. In particular, SCs-expressing Mta2 first appeared at stages I–II and maintained the low intensity at stages III–VI, increasing to highest at stages VIII–XII (Fig. 1).

3.2. Inducement of Mta2 expression by RBs phagocytosis

To study the effects of phagocytosis on Mta2 expression, we firstly analyzed the time-dependent rate of RBs ingestion by SCs. The number of ingested RBs increased from 2 to 6 h of incubation, with the highest phagocytic index being observed 6 h after RBs addition (Fig. 2A and B). In line with this, the expression level of *Mta2* in primary cultured SCs began to increase from 4 h of RBs incubation onwards. Normalized expression level of *Mta2* using qRT-PCR revealed a 126.5% and a 187.3% increase in the SCs at 4 or 6 h of RBs incubation, respectively ($P < 0.05$, $n = 3$) (Fig. 2C). Western blotting also demonstrated a time-dependent stimulation of Mta2 protein by RBs treatment (Fig. 2D). Because Mta2 is an essential component of the NuRD complex, we next sought to determine the influence of phagocytosis on the status of HDAC activity using a colorimetric assay. The addition of RBs to SCs was accompanied by an expected increase in the HDAC activity at 4 h (2.761 ± 0.388 versus 1.374 ± 0.092, $P < 0.05$ when compared with 0 h group) and 6 h (3.105 ± 0.206 versus 1.374 ± 0.092, $P < 0.05$ when compared with 0 h group), respectively (Fig. 2E).

3.3. Differential effects of different GCs fragments on phagocytosis-induced Mta2 expression

Because SCs show strong phagocytic activities toward different substrates: apoptotic GCs and RBs [7], we next tried to determine whether the elevated Mta2 expression in SCs was a RBs-specific induction by incubating primary cultured SCs with different cellular compartments. Interestingly, the 6 h incubation of apoptotic Spc and RBs both substantially increased the phagocytic activities of SCs, with the highest phagocytic index being detected in the latter. In contrast, the addition of apoptotic Spg only exerted a slightly stimulation on SCs phagocytosis (Fig. 3A). Consistently, incubation of SCs with apoptotic Spc and RBs both significantly increased Mta2 expression, and this stimulatory effect was barely observed in the SCs incubated with apoptotic Spg (Fig. 3B and C).

3.4. Repression of follicle-stimulating hormone receptor (Fshr) expression by phagocytosis-induced Mta2

FSH regulates mammalian spermatogenesis at a fundamental level by controlling the number of Fshr present at the surface of SCs. One underlying mechanism is the down-regulation of the expression levels of the *Fshr* gene via deacetylation modification [6]. Previously we have demonstrated that Mta2 represses FSH-mediated *Fshr* transcription by recruiting HDAC1, so we were then curious whether the up-regulated Mta2 expression could exert any effects on *Fshr* expression during phagocytosis. In contrast to the up-regulation of Mta2 expression, the relative expression level of *Fshr* mRNA in SCs was gradually down-regulated along the phagocytosis, with the lowest value being detectable at 6 h following RBs incubation (Fig. 4A).

4. Discussion

Previous histological studies revealed a stage-dependent formation of SCs phagocytosis. The highest phagocytic activity has been reported at the stages VIII and IX within the seminiferous

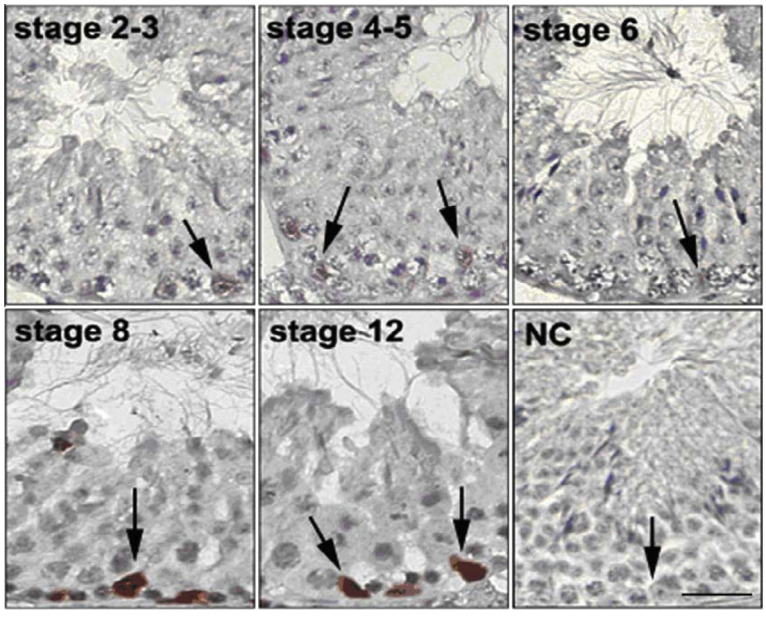

Fig. 1. Sections of testicular seminiferous tubules from adult mouse testis, at different stages of the epithelial cycle, were immunostained with a goat polyclonal antibody against Mta2. Positive signals in Sertoli cells (SCs) were denoted using black arrows. Replacement of primary antibody with preabsorbed IgG abolished immunostaining in tissues, confirming specificity of assay (negative control, NC). Bar = 25 μm.

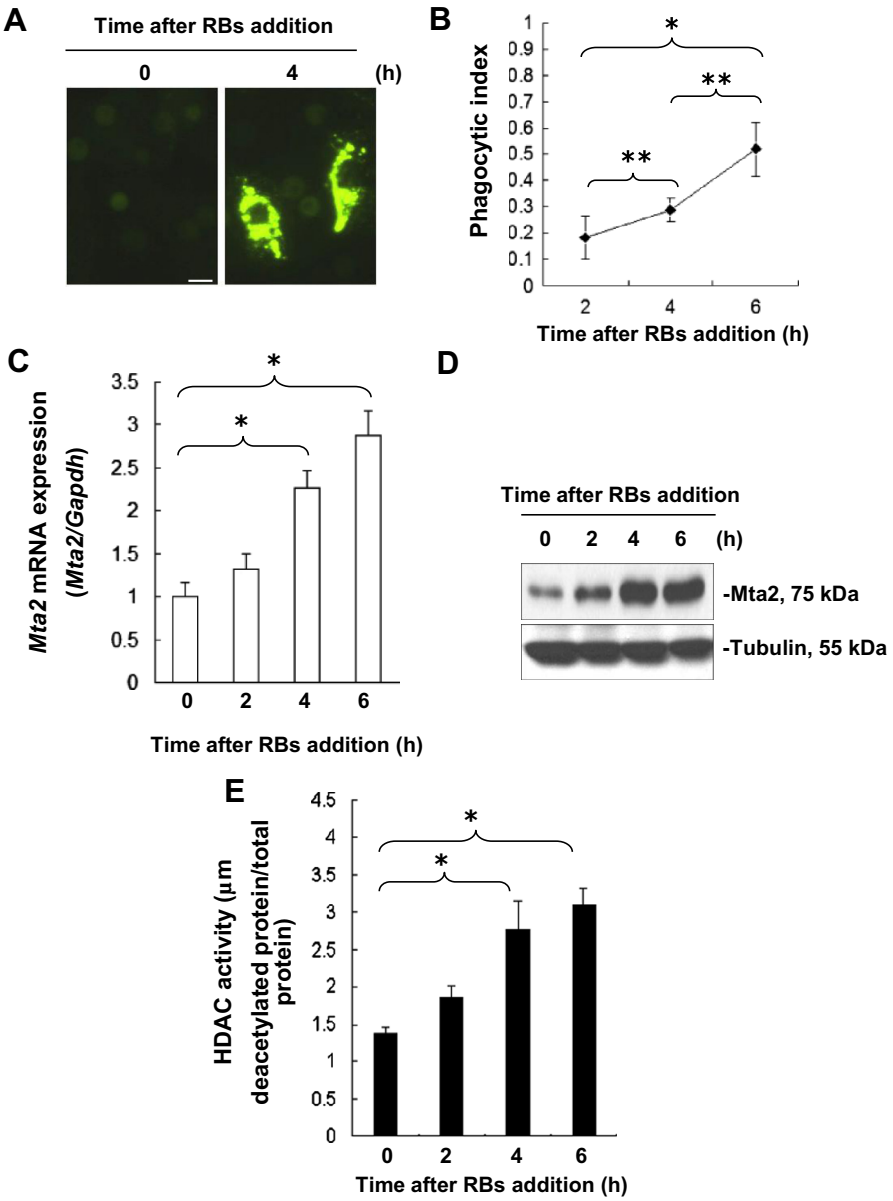

Fig. 2. Inducement of Mta2 expression by SCs phagocytosis. (A) Representative pictures of residual bodies (RBs) phagocytosis by SCs 4 h after RBs addition. Bar = 10 μm. (B) Kinetics of RBs phagocytosis by SCs (phagocytosis index) were calculated as the ratio of RBs/nuclei number in each well. Values represent the mean ± S.D. of three independent experiments. (C) The expression levels of *Mta2* mRNA in SCs at different time-points after RBs incubation were monitored using qRT-PCR (*$P < 0.05$; $n = 3$). (D) Western blotting analysis of Mta2 protein in SCs at different time-points after RBs incubation. Tubulin was used as a loading control. (E) Dynamic change in histone deacetylase (HDAC) activity at different time-points after RBs incubation was measured colorimetrically as described in Section 2 (*$P < 0.05$; $n = 3$).

epithelium [7]. Interestingly, we also observed a stage-specific pattern of expression of Mta2 peptide throughout the seminiferous epithelial cycle, with maximum values at stages VIII–XII and decreased expression thereafter. The physiological meaning of such a staged pattern of expression awaits further investigation, but the variations in Mta2 expression that occur during the highest phagocytic course of the spermatogenic cycle do suggest the physiological relevance of a potential involvement for Mta2 in SCs phagocytosis. This contention is further supported by our present data using *in vitro* systems, in which Mta2 expression was found to be substantially up-regulated when SCs were co-cultured with RBs. Of note, Mta2 up-regulation was simultaneously accompanied by an elevation of HDAC activity (Fig. 2E). Considering that Mta2 signaling represents a novel master coregulator among a variety of important cellular events including hormonal action, cell differentiation and cell fate programs, our current data collectively substantiate a putative role of Mta2 in the control of SCs phagocytic function.

Emerging evidence points to a frequent participation of deacetylation in phagocytic activity. For example, a transcriptional coregulator belonging to the family of type III histone deacetylases, has been shown to play a key role in calorie restriction-induced macrophage function by inhibiting suppresses activator protein-1 transcriptional activity and cyclooxygenase-2 expression [10]. Similarly, deacetylation of Foxo by Sirt1 plays an essential role in mediating starvation-induced autophagy in cardiac myocytes [9]. More recently, Contreras et al. reported that in interferon-γ (IFN-γ)-exposed immortalized human bronchial epithelial cells, deacetylation of p53 suppresses the expression of the BH3-only domain protein (Bmf) and thus facilitates autophagy [18]. Although the mechanisms whereby Mta2 expression is up-regulated in phagocytic SCs remain to be defined, several regulators

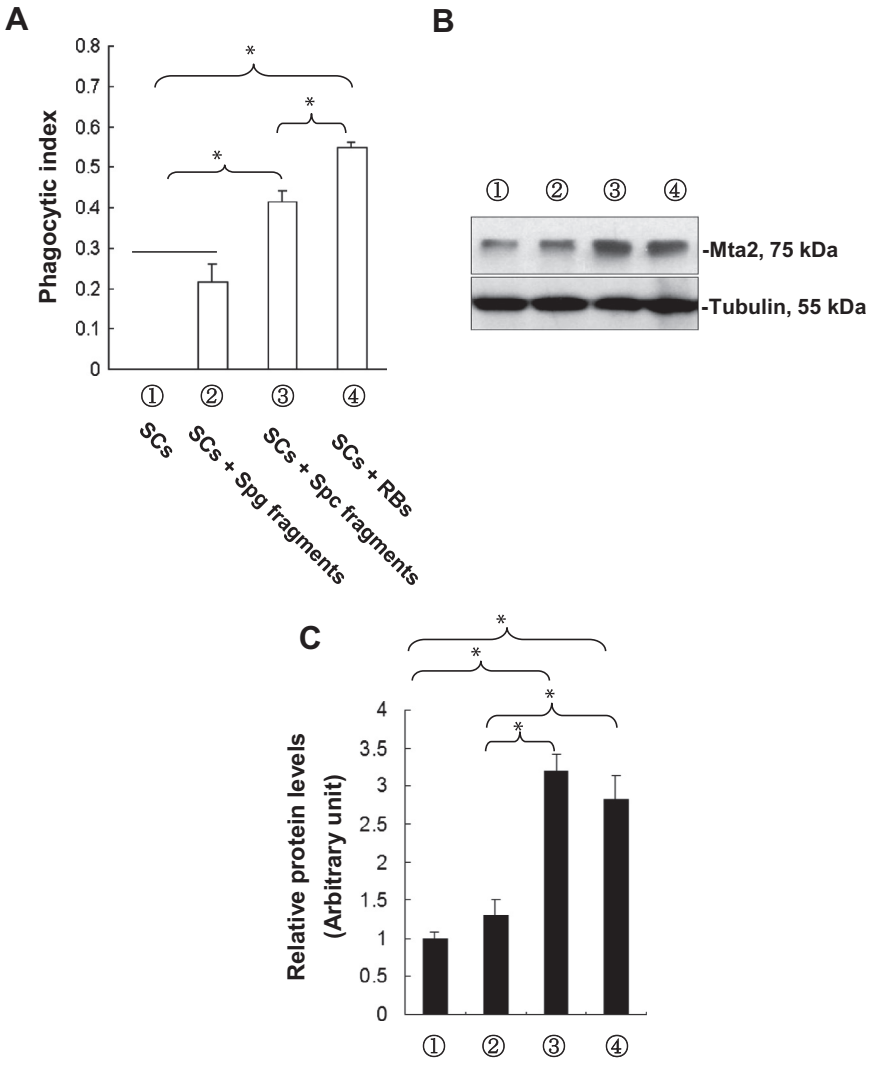

Fig. 3. Differential effects of different GCs fragments on phagocytosis-elicited Mta2 expression. (A) Differential phagocytosis indexes were determined 6 h following the incubation with different phagocytic substrates including RBs, fragments from apoptotic spermatogonia (Spg) and spermatocytes (Spc). Values represent the mean ± S.D. of three independent experiments. (B) Western blotting analysis of Mta2 protein in SCs at 6 h after with different phagocytic substrates as indicated. Tubulin was used as a loading control. (C) Densitometric scanning of immunoblots was performed in which the level of a target protein was normalized against the protein level in SCs cultured alone, which was arbitrarily set at 1. Each bar represents the mean ± S.D. of results from three experiments using different batches of cells. Each experiment had replicate cultures (*$P < 0.05$).

have been so far reported to contribute to the level of Mta2 expression. For example, the up-regulation of estrogen receptor α (ERα), but not ERβ, has been associated with functional signals compatible with autophagic cytoprotection triggering and leading to cell survival when mouse neuronal cells are exposed to chronic minimal peroxide treatment [19]. MTA2 is a repressor of ERα, and overexpression of the former leads to estrogen-independent growth of human breast cancer cells [20]. In addition, MTA2 specifically interacts with p53 both *in vitro* and *in vivo*, and its expression reduces significantly the steady-state levels of acetylated p53. MTA2 expression strongly represses p53-dependent transcriptional activation, and, notably, it modulates p53-mediated cell growth arrest and apoptosis [5]. Thus, deregulation of ERα or p53 signaling may at least in part explain the elevated expression of Mta2 during the SCs phagocytosis.

Germ cells exist in an environment created by SCs; therefore, paracrine signaling between these intimately associated cells must regulate the process of germ cell death. In turn, SCs functions are also critically influenced by the status of adjacent germ cells [11]. In our study, the addition of apoptotic Spc and RBs both substantially increased the phagocytic activities of SCs, with the highest phagocytic index being detected in the latter. Consistently, the most significant stimulatory effects of phagocytosis on Mta2 expression levels were observed in the SCs co-cultured with RBs and apoptotic Spc. These results were in line with the previous report that SCs functions are crucially regulated by the postmeiotic spermatids and meiotic spermatocytes [21]. Moreover, our data also suggest that a novel paracrine feed-back loop between meiotic spermatocytes and SCs in phagocytosis likely operates via Mta2/HDAC cascade.

Accumulated data from different model systems evidence that the rate of phagocytosis by SCs can be modulated *in vivo* and *in vitro* by numerous factors such as insulin, epithelium growth factor, FSH, hydrocortisone and β-endorphin [7]. Using *in vitro* settings, Charles et al. reported that high concentration of FSH accelerates binding of RBs but markedly reduces the number of RBs phagocytosed. Thus, FSH signaling may serve as a negative regulator of SCs phagocytosis [22]. Interestingly, MTA2 is an efficient corepressor of *FSHR* transcription, and it can recruit HDAC 1 onto the *FSHR* promoter and participates in the down-regulation of *FSHR*

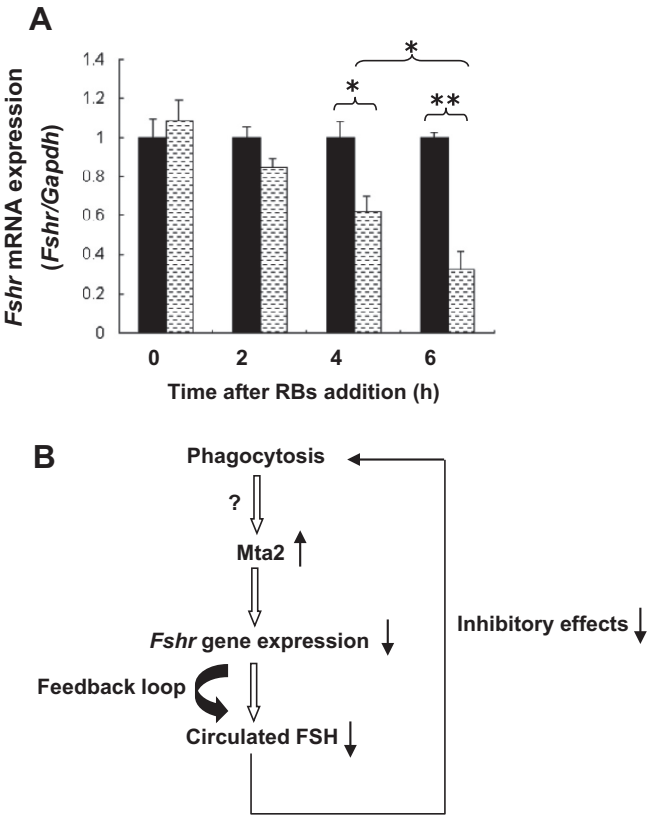

Fig. 4. Phagocytosis-induced Mta2 expression exerts inhibitory effects on follicle-stimulating hormone receptor gene (*Fshr*) expression. (A) Expression levels of *Fshr* mRNA in SCs incubated either with PBS (control) or RBs were assessed at 6 h of incubation using qRT-PCR (*$P < 0.05$; **$P < 0.01$; $n = 3$). (B) Summary diagram of the possible mechanisms related to up-regulation of Mta2 expression contributing to maintenance of the low level of circulated FSH during SCs phagocytosis.

expression upon FSH treatment. From a clinical standpoint, attenuated expression of MTA2 in SCs of human pathological testes negatively correlates to the abnormally high level of serum FSH [6]. We have showed that the expression level of *Fshr* mRNA was gradually down-regulated along the phagocytosis when SCs were incubated with RBs. It is therefore a logical hypothesis that the up-regulation of Mta2 expression (through unknown mechanism) may operate as a negative modulator to help to maintain the low level of circulated FSH, thus allowing the occurrence of functional engulfment (Fig. 4B). To this end, the available data broaden our understanding of the role of FSH signaling in SCs phagocytosis by providing a mechanistic explanation for the repression of *Fshr* expression by Mta2 corepressor associated with the HDAC complexes. The relevance of the potential crosstalk between Mta2 up-regulation and FSH signaling in the regulation of SCs phagocytic function is yet to be further defined from *in vivo* level.

In summary, our present data document for the first time that Mta2 expression is significantly elevated during SCs phagocytosis, and that the most efficient substrates for the induction of Mta2 up-regulation are RBs and cellular fragments from apoptotic spermatocytes. A major result of this response is the direct repression of *Fshr* transcription, which in turn may ensure a low level of circulated FSH that is required for the normal occurrence of functional phagocytosis.

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