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Involvement of a chromatin modifier in response to mono-(2-ethylhexyl) phthalate (MEHP)-induced Sertoli cell injury: Probably an indirect action via the regulation of NFκB/FasL circuitry

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ABSTRACT

The Fas/FasL signaling pathway, controlled by nuclear factor-κB (NFκB) at the transcriptional level, is critical for triggering germ cell apoptosis in response to mono-(2-ethylhexyl) phthalate (MEHP)-induced Sertoli cell (SC) injury, but the exact regulation mechanism remain unknown. Here, we discovered that expression level of Metastasis associated protein 1 (MTA1), a component of the Mi-2/nucleosome remodeling and deacetylase complex, was upregulated in SCs during the early recovery after MEHP exposure. This expression change was in line with the dynamic changes in germ cell apoptosis in response to MEHP treatment. Furthermore, a knockdown of MTA1 by RNAi in SCs was found to impair the MEHP-induced early activation of NFκB pathway and abolish the recruitment of NFκB onto *FasL* promoter, which consequently diminished the MEHP-triggered FasL induction. Considering that Fas/FasL is a well characterized apoptosis initiating signaling during SCs injury, our results point to a potential "switch on" effect of MTA1, which may govern the activation of NFκB/FasL cascade in MEHP-insulted SCs. Overall, the MTA1/NFκB/FasL circuit may serve as an important defensive/repairing mechanism to help to control the germ cell quality after SCs injury.

1. Introduction

Phthalic acid esters are widespread in the environment due to their use as plasticizers to produce plastic wraps, toys, and bags, and have been frequently shown to adversely affect spermatogenesis [1]. Among phthalic acid esters, di-(2-ethylhexyl) phthalate (DEHP) is widely dispersed throughout the environment due to its increased commercial use. Mono-(2-ethylhexyl) phthalate (MEHP), one of the active metabolites of DEHP, is a well known Sertoli cell (SC) toxicant [2]. The testicular toxicity is characterized with detachment and sloughing of spermatogenic cells due to increased germ cell apoptosis and atrophy in seminiferous tubules [3].

The FasL/Fas signaling pathway has been established to operate as an essential mechanism to regulate germ cell apoptosis in the testis as part of a physiologic pathway to match germ cell numbers to the SCs supportive capacity [4]. Recent studies indicate that the induction of FasL protein expression in germ cells by MEHP treatment is critical for initiating massive germ cell elimination by apoptosis after SCs injury [5]. MEHP-induced FasL expression requires the transcriptional regulation of transcription factors such as nuclear factor-κB (NFκB) as well as specificity protein-1 (Sp-1) [6]. However, the exact regulation of Sertoli cell NFκB/FasL expression in response to MEHP remains to be fully delineated.

Regulation of fundamental germ cell apoptosis demands dynamic coordinated participation of transcription factors and their coregulators at the target gene chromatin [7]. Metastasis associated protein 1 (MTA1), a component of the Mi-2/nucleosome remodeling and deacetylase complex, plays a central role in the regulation of divergent cellular pathways by associating and modifying the acetylation status of the target gene chromatin [8]. In testis, MTA1 is predominantly expressed in pachytene spermatocytes and weakly expressed in SCs [9–11]. Data from this lab have shown that MTA1 might operate as an indispensable modulator in the maintenance of the proper apoptotic balance of meiotic

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spermatocytes under certain pathologic conditions [12,13]. In addition, SC-expressing MTA1 is crucial to maintain the germ cell nursery function and normal anchoring junction formation in SCs [14]. Although MTA1 has been linked intimately with spermatogenesis and is widely regarded as a potential master coregulator, the role of MTA1 in SCs injury remains unrecognized and delineated here. For the first time, we provide evidence that NFκB controlled FasL expression after MEHP exposure is governed by MTA1 originated from SCs. Our results reiterate the importance of paracrine interactions between SCs and germ cells during SCs injury.

2. Materials and methods

2.1. Animal treatment

Adult male C57BL/6 mice, obtained from the Animal Research Center of our university, were given a single dose of MEHP (1 g/kg) (Sigma) by oral gavage. Control animals ($n = 5$) received a similar volume of vehicle (corn oil). Mice were then sacrificed at different time-points ($n = 5$ for each time-point) by CO_2 inhalation. All procedures involving animals were approved by the local ethical committee.

2.2. Cell preparation and treatment

Sertoli cells (SCs) were prepared as described elsewhere [14]. SCs Cultures were hypotonically treated with 20 mM Tris (pH 7.4) for 2.5 min to lyse residual germ cells. The purity was monitored by RT-PCR analyses using primer sets specific to marker genes. To evaluate the effect of MEHP-triggered SCs injury on MTA1 expression, cells were dosed with 200 μM MEHP diluted in Me_2SO for various time periods as indicated in Figure legends. In another experimental setting, SCs were incubated with 5 μg/ml actinomycin D (ActD, Sigma) and 200 μM MEHP together for 12 h before being subjected to the following biochemical analyses of MTA1 expression level.

To determine the effect of MTA1 knockdown on NFκB signaling in response to MEHP treatment, SCs receiving siRNA treatment were dosed with 200 μM MEHP diluted in Me_2SO for 12 h before being harvested for further analysis.

2.3. Detection of apoptosis by in situ end labeling of fragmented DNA (TUNEL)

TUNEL assay was done using In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) following instructions of the manufacturer. To assess apoptosis in testicular cells, 100 seminiferous tubules were observed in each section at a magnification of 400×. A histogram of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL-positive germ cells were analyzed.

2.4. RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from SCs using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA). For RT-PCR, first-strand cDNA was synthesized with Superscript III (Rnase H-Reverse Transcriptase; Invitrogen) and PCR was set up according to Promega's protocol. The primers used for detection *MTA1*, *Rhox5*, *Sycp3*, *Dbil5* and *Gapdh* were chosen according to the previous report [14]. Amplification of *Gapdh* was served as the internal control. PCR products were quantified by SYBR green intercalation using the MiniOpticon™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). *Gapdh* was used to obtain the ΔΔCt values for the calculation of fold increases.

2.5. Western blotting

Western blotting was carried out as described previously [11,13]. Briefly, protein samples were prepared in ice-cold RIPA buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 1% vol/vol, sodium deoxycholate 1% wt/vol, and SDS 0.1% wt/vol pH 7.5) supplemented with complete proteinase-inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany). 20 μg of protein sample were separated by SDS/PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes were then incubated with different primary antibodies including goat anti-MTA1, rabbit anti-FasL, rabbit anti-β-actin and rabbit anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology, Inc.); rabbit anti-pho-Iκκ α/β, rabbit anti-Iκκ α/β, rabbit anti-pho-p65 and rabbit anti-p65 (Cell Signaling Technology) and goat anti-rabbit IgG-HRP secondary antibody (Abcam). β-actin serves as an internal control. Immunostained bands were finally detected by using an ECL kit (Amersham Biosciences, Buckinghamshire, UK).

2.6. In vitro siRNA treatment

We designed and synthesized chemically two siRNA sequences targeting MTA1 (GenBank accession number: AF463504.1) (Ruibo Co., Shanghai, China). The oligo sequences used were as follows: si-1 (sense: 5′-AGUAGAAGAAGAAAUCCUCACdTdT-3′, antisense: 3′-dTdT GAGGAUUUCUUCUUCUACUCU-5′); si-2 (sense: 5′- AAUAU GUCCAACAAGAUGCGCdTdT-3′; antisense: 3′-dTdT GCAUCUUGU UGGACAUAUUGG-5′). MTA1-siRNAs or a non-silencing scrambled control siRNA (Altogen Biosystems, NY) were transfected into SCs using Lipofectamine 2000. 48 h after transfection, cells were collected and subjected to other experiments.

2.7. NFκB activity monitored by NFκB filter assay

48 h after siRNA treatment, SCs were dosed with 200 μM MEHP diluted in Me_2SO for 12 h. Subsequent nuclear extraction was carried out using a commercial kit from Signosis (Zhongzhi Biotech Development Co., Ltd. Wuhan, China). Signosis' NFκB filter assay was performed to evaluate the activity of NFκB pathway as instructed by the manufacturer. The bound NFkB probe was finally measured with luminescence using GloMax™ 20/20 Luminometer (Promega).

2.8. Chromatin immunoprecipitation (ChIP)

48 h after siRNA treatment, SCs were dosed with 200 μM MEHP diluted in Me_2SO for 12 h. Subsequent ChIP assays were carried out as described elsewhere by using a kit from Upstate Biotechnologies (Lake Placid, NY) [15]. Briefly, SCs were cross-linked with 1% formaldehyde and sonicated at 30% output setting (Misonix Sonicator Q700, OpticsPlanet, Inc., IL) followed by centrifugation for 15 min at 16,000×*g* at 4 °C. Supernatant (50 μl) was taken as an input control and the rest of the sample was immunoprecipitated with antibodies against NFκB and normal rabbit-IgG (both from Santa Cruz Biotechnology Inc.). After overnight incubation at 4 °C, precipitates were eluted and incubated at 65 °C overnight to reverse formaldehyde cross-linking. DNA was purified by phenol/chloroform extraction and ethanol precipitation. The chromatin fragments were amplified by PCR using primers flanking the NFκB binding sites of murine *FasL* promoter as follows: (forward) ACAGGCCTCTCAGGACACAC and (reverse) TAAGGTTTCCGCAGTCAAGG [16].

2.9. Immunohistochemistry

Testicular tissues were fixed in Bouin's solution, embedded in paraffin and further processed into 5-μm-thick sections. The avidin–biotin–peroxidase (ABC) method was employed in the immunohistochemical assay as previously described [11,12].

2.10. Statistical analysis

All graphed data represent three independent experiments performed in triplicate. Data are presented as mean ± SEM with probability determined by Student's *t* test, with $P < 0.05$ being considered as statistically significant.

3. Results

3.1. MTA1 expression in mouse SCs is induced by MEHP in a time-dependent manner

Immunohistochemistry was firstly employed to evaluate the influence of MEHP on MTA1 expression level in adult mouse testis. Mice were given a single dose of MEHP by oral gavage and testicular tissues were then collected at different time-points after treatment. MTA1 staining in SCs was significantly increased from the postoperative 12 h onwards (*black arrows* in Fig. 1A). In contrast, positive signals in pachytene spermatocytes (pachy) remained relatively constant and began to decrease at postoperative 24 h

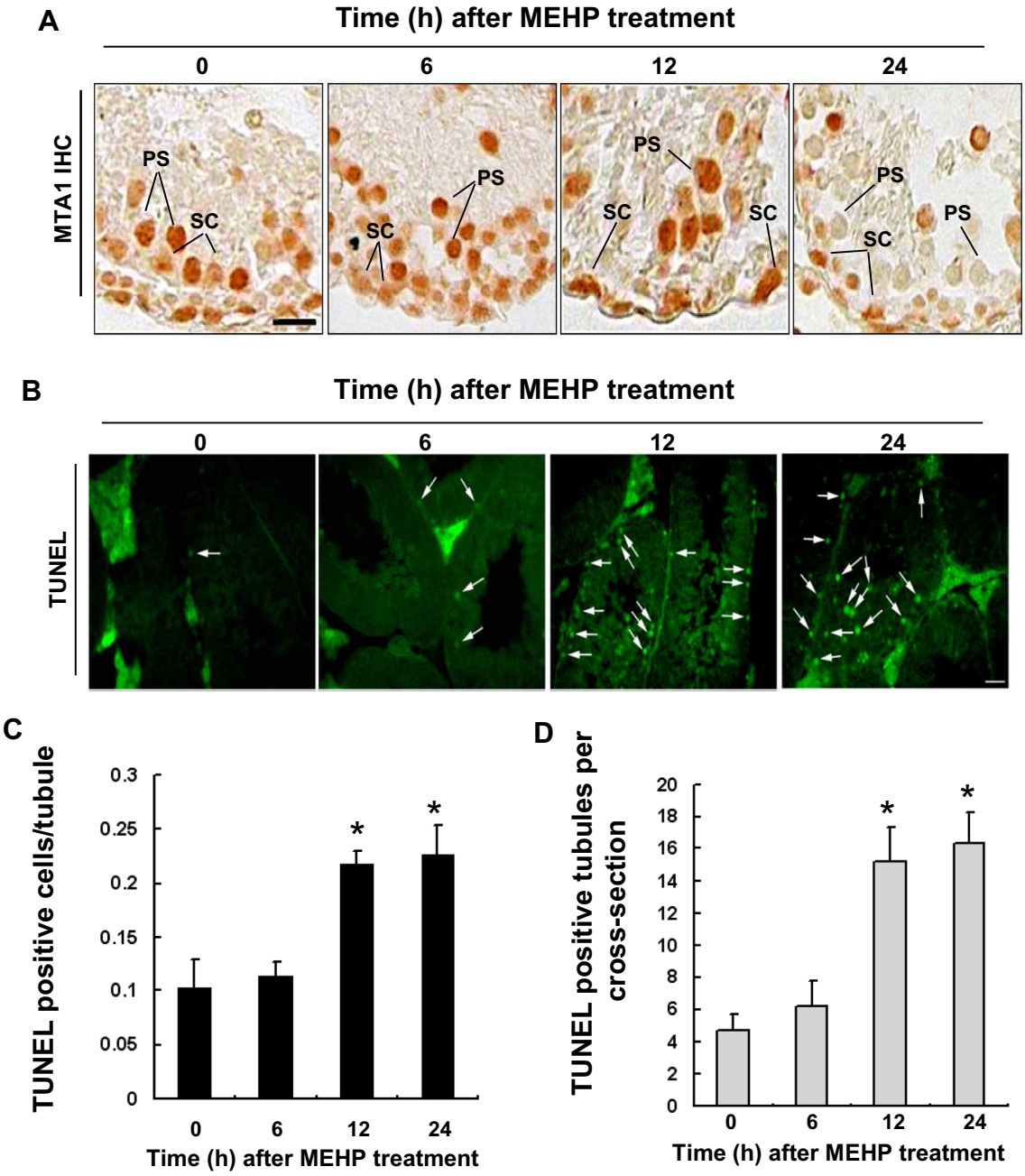

Fig. 1. *In vivo* effect of MEHP treatment on MTA1 expression in Sertoli cells (SCs) and testicular apoptosis. (A) Mice were given a single dose of MEHP treatment as described in Section 2 and testicular tissues were collected at different postoperative time-points as indicated. Expression level of MTA1 was then evaluated using immunohistochemistry. PS, pachytene spermatocytes; SC, Sertoli cell. Bar = 10 μm. (B) Germ cell apoptosis at different postoperative time-points was identified as spermatogonia/pachytene spermatocytes (arrows) in TUNEL assay of testicular sections. Bar = 10 μm. (C) The number of TUNEL positive germ cells per seminiferous tubule. (D) The percentages of seminiferous tubule with TUNEL positive germ cells. Data were presented as mean ± SEM (*$P < 0.05$, Student's *t*-test).

(Fig. 1A). The upregulation of MTA1 expression in SCs was well consistent with the dynamic changes in germ cell apoptosis in response to MEHP treatment (Fig. 1B–D).

3.2. In vitro evidence of MTA1 participation in response to MEHP-induced SCs injury

To further confirm the expression change of MTA1 in SCs in response to MEHP treatment, we examined the MTA1 expression level in primary cultured SCs in the presence of MEHP. To determine the purity of the cell populations, we conducted RT-PCR analyses using primer sets specific to pachytene spermatocyte (pachy: *Sycp3*), round/elongating spermatid (RS/ES: *Dbil5*), and SCs (*Rhox5*). Measurement of the relative expression of each gene after normalizing against the internal control further confirmed the enrichment of SCs. Cells were then exposed to 200 μM MEHP for the indicated time points (Fig. 2B). *MTA1* mRNA expression level was significantly increased (~4-fold) after incubation for 12 h as revealed by *qRT-PCR* analyses (Fig. 2B). Similar changes in protein levels (~3-fold at 12 h) were observed via western blotting analyses using whole cell lysates from MEHP-treated SCs (Fig. 2C). ActD, a transcriptional inhibitor, effectively blocked both MEHP-inducible expression of MTA1 mRNA and protein at the end of the 12-h treatment (Fig. 2D and E). These findings suggested that MTA1 may be a target of MEHP-induced SCs injury.

3.3. Coactivator activity of MTA1 on NFκB signaling is required for MEHP-induced FasL expression in SCs

Because NFκB has been shown to play an important role in response to MEHP stimulation [6] and MTA1 acts as an essential component of the NFκB circuitry [17], we next investigated the effect of ablation of endogenous MTA1 on MEHP-induced NFκB response. To select the effective MTA1-siRNA, two different siRNAs against MTA1 were tested, with a non-silencing scrambled siRNA as a negative control. Immunoblotting analysis demonstrated that si-2 was more effective in suppressing the expression of MTA1 (Fig. 3A), and it was therefore selected for further studies. Interestingly, selective knockdown of MTA1 in SCs abolished MEHP-induced early activation of pho-Ικκα/β, and also delayed p65 phosphorylation as compared with those observed in cells transfected with the control siRNA at the end of 12-h MEHP treatment (Fig. 3B). Consistent with these observations, there was a reduced NFκB activity in MEHP-stimulated MTA1 knockdown SCs as compared with cells treated with control siRNA (Fig. 3C). These findings suggested that MTA1 status is required for NFκB activation in MEHP-stimulated SCs. Because the Fas/FasL signaling pathway has previously been demonstrated to be critical for triggering germ cell apoptosis in response to MEHP-induced SCs injury and FasL expression is tightly regulated by NFκB activation [6], we were then eager to know whether MTA1 plays a role in driving the MEHP-inducible expression of FasL in SCs. MTA1 knockdown in

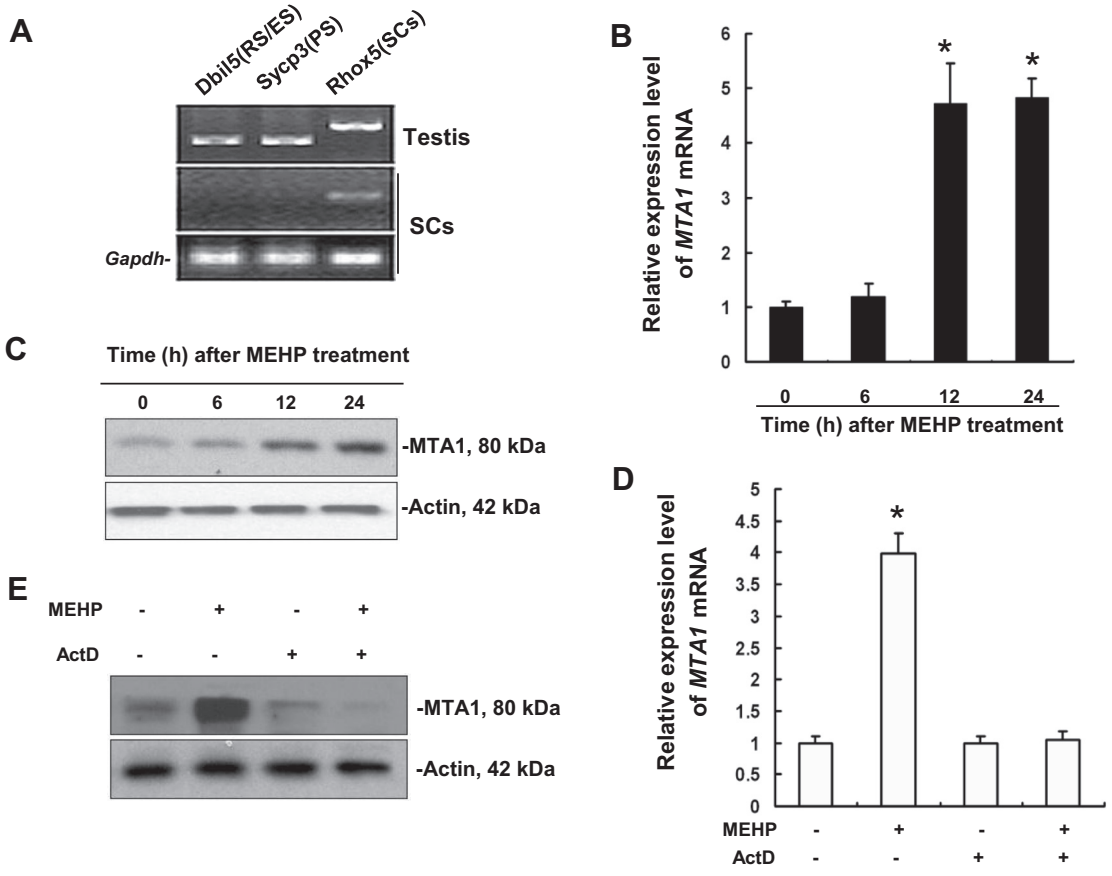

Fig. 2. *In vitro* effect of MEHP exposure on MTA1 expression in SCs. (A) Purity of isolated spermatogenic cell populations assessed by RT-PCR analyses using primer sets specific to PS, SC, and round/elongated spermatid (RS/ES) marker genes. Results presented as mean ± SEM of 3 independent experiments. Parallel amplification of *Gapdh* mRNA served as internal control. (B) The expression levels of *MTA1* mRNA in SCs at different time-points after MEHP treatment were monitored using qRT-PCR (*$P < 0.05$; $n = 3$). (C) Western blotting analysis of MTA1 protein in SCs at different time-points after MEHP treatment. Actin was used as a loading control. (D) Upregulation of *MTA1* mRNA by MEHP treatment in SCs was inhibited by blockage in transcription (*$P < 0.05$; $n = 3$). ActD, actinomycin D. (E) Expression level of MTA1 protein in SCs treated with MEHP and/or ActD was determined using western blotting analysis. Actin was used as a loading control.

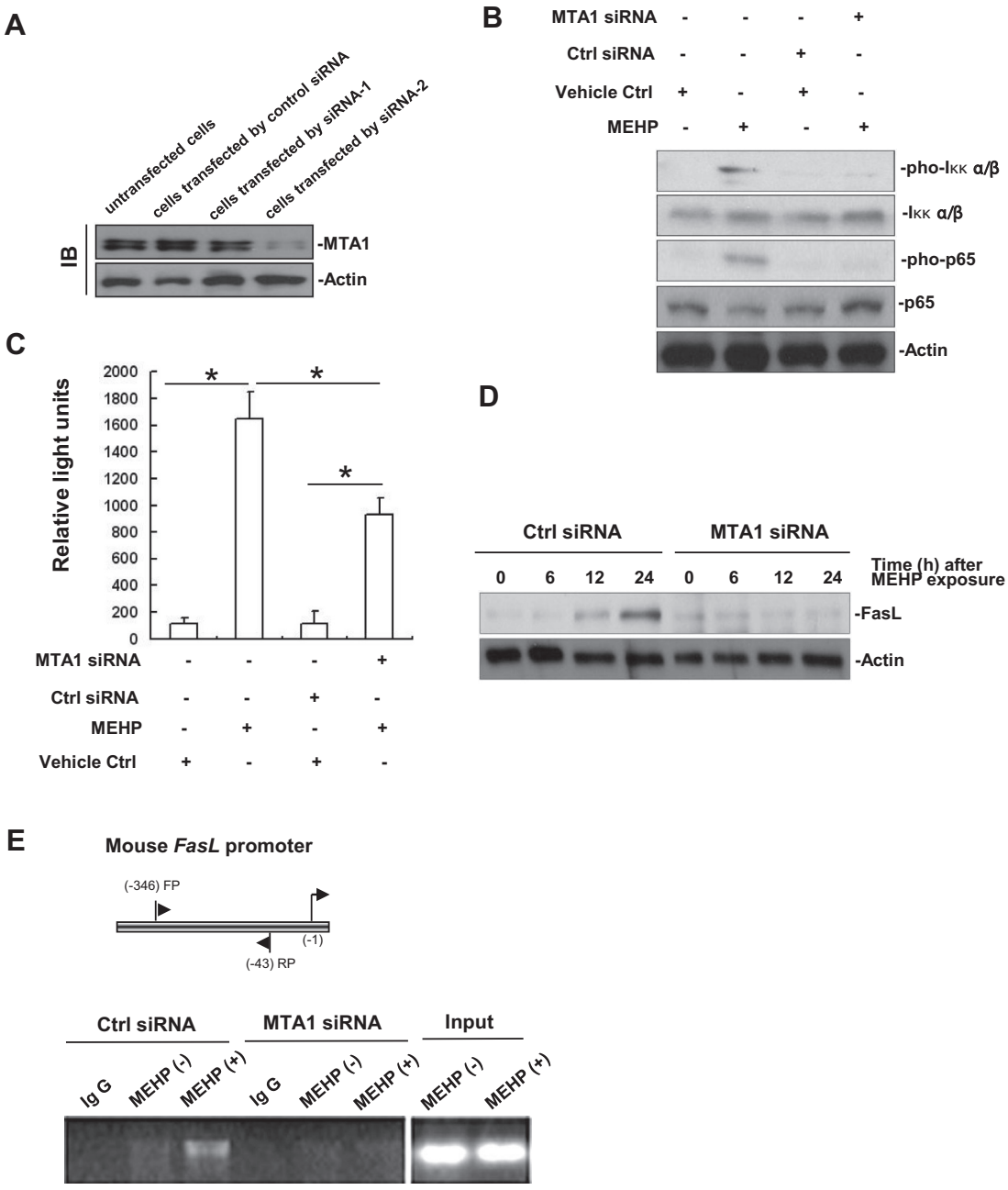

Fig. 3. Endogenous MTA1 is required for MEHP-induced FasL expression by transcriptional regulation of NFκB activation in SCs. (A) Verification of MTA1 knock down effect by siRNA treatment was performed using western blotting analyses. Actin was used as an internal control. IB, immunoblotting. (B) Effect of MTA1 knock down on MEHP-induced NFκB activation was evaluated by western blotting analysis. (C) MEHP-induced NFκB activity after MTA1 knock down was monitored by NFκB filter assay. Results presented as mean ± SEM of 3 independent experiments ($^*P < 0.05$; $n = 3$). (D) Effect of MTA1 knock down on MEHP-induced FasL expression was evaluated by western blotting analysis. (E) ChIP analysis revealed that recruitment of NFκB onto the mouse *FasL* promoter in MEHP-treated SCs was efficiently compromised after MTA1 knock down.

SCs impaired the effect of MEHP on FasL expression as compared with what was observed in control siRNA-transfected cells (Fig. 3D). To further examine the potential comodifying role of MTA1 in NFκB-mediated stimulation of FasL expression in response to MEHP exposure, we examined whether ablation of endogenous MTA1 could affect the recruitment of NFκB transcriptional regulator onto *FasL* promoter. By using ChIP assays, we observed that in SCs, MEHP stimulation promoted recruitment of the NFκB complex to the *FasL* promoter and this recruitment was effectively inhibited after MTA1 knockdown (Fig. 3E). Taken together, the available data indicate that MTA1 may act as an indispensible modulator of the NFκB-regulated FasL expression in MEHP-treated SCs.

4. Discussion

Accumulated evidence establishes MTA1 to be a valid DNA-damage responsive protein with a master co-regulatory role in maintaining the optimum DNA-repair activity in mammalian cells [18]. Genotoxic stimulations such as hyperthermia [12], ionizing radiation [19], chemotherapy and oxidative stress [20] could

significantly deregulate the expression level of MTA1. It is therefore a logical observation that this chromatin modifier was upregulated in response to MEHP treatment. Considering that MTA1 is predominantly expressed in pachytene spermatocytes and is only weakly expressed in SCs during normal spermatogenesis [11], however, it is of great interest to note that MEHP treatment failed to trigger MTA1 expression in meiotic spermatocytes. Instead, positive signals in meiosis remained relatively stable and were significantly downregulated from postoperative 24 h (Fig. 1A). These results suggest that MTA1 may serve distinct functions in different cell types, probably participating in a direct response to MEHP insult in SCs. Although the mechanisms whereby MTA1 expression is up-regulated in MEHP-treated SCs remain to be defined, one factor has been so far reported to determine the level of MTA1 expression. MEHP treatment triggers oxidative stress pathways during the pathogenesis [21], whereas oxidative stress may operates as an intrinsic inducer of MTA1 expression [20]. On the other hand, MTA1 belongs to the DNA repair genes family and genotoxic stress can trigger the biochemical and biological changes of MTA1 during the very early phase after insult [12]. MTA1 acts as a component of the NuRD complex and plays an essential role in chromatin remodeling and transcriptional regulation of genes [22]. Collectively, upregulation of this master transcriptional coregulator in the presence of MEHP treatment may endow SCs with the ability to promptly initiate a defensive or reparative response.

The observation that upregulation of MTA1 expression in SCs was positively coincident with the dynamic changes in germ cell apoptosis points to an pro-apoptotic effect of MTA1 in response to MEHP insult. Germ cell apoptosis in testis has been shown to be essential for functional spermatogenesis [23]. After MEHP exposure, germ cell numbers are actively reduced in the testis, which may compensate for the compromised supportive capacity of the MEHP-injured SCs to achieve populations of germ cells that can be supported by this somatic cell [6]. In this context, the pro-apoptotic effect of MTA1 upon genotoxic stress, if possible, may help to maintain a critical cell number ratio between germinal cell stages and SCs, whose normal functions and differentiation involve an elaborate network of communication.

NFκB activation in response to MEHP exposure in SCs has been previously established [6]. MEHP induces the nuclear translocation of NFκB and thereafter promotes the binding of NFκB and Sp-1 onto *FasL* promoter, which enhances MEHP-induced FasL expression. Nevertheless, the transcriptional or posttranscriptional mechanisms responsible for controlling NFκB activity in response to DNA damage are poorly understood. Our findings extend these understanding by identifying MTA1 as a potent coactivator of NFκB activation (Fig. 3B and C). Indeed, it has been shown that MTA1 depletion in LPS-stimulated macrophages impairs NFκB signaling and MTA1 knockdown in macrophages was accompanied by a distinct inhibition of NFκB-regulated genes [24,25]. Our results are important because it reflects that the reported homeostatic role of MTA1 as a component of the NFκB circuitry may exist on a broader relevance.

FasL, a type II transmembrane protein, is a well characterized apoptosis initiating protein [26]. Previous reports have demonstrated that the Fas/FasL paracrine signaling mechanism between SCs and germ cells plays a key role in mediating germ cell apoptosis in the MEHP-treated testis. SC-expressed FasL acts on Fas-expressing germ cells to initiate their elimination by apoptosis [5]. In our study, ablation of endogenous MTA1 by siRNA treatment significantly attenuated the MEHP-triggered FasL induction (Fig. 3D), indicative of a potential regulatory role for MTA1 as an upstream modulator of FasL expression. However, MTA1 should exert the influence on FasL expression via an indirect way because we did not detect a recruitment of MTA1 onto *FasL* promoter in MEHP-treated SCs (our unpublished data). Instead, we found that MEHP-induced recruitment of the NFκB complex to the *FasL* promoter was substantially compromised after MTA1 knockdown (Fig. 3E). These results collectively suggested that MTA1 may regulate FasL expression in a NFκB-dependent manner, providing an explanation for the coactivation of *FasL* transcription by MTA1-NFκB circuit at the molecular level. Furthermore, the available data support the idea that the *FasL* gene could indirectly utilize a mode of regulation that links the control of gene expression by signal transduction and chromatin structure.

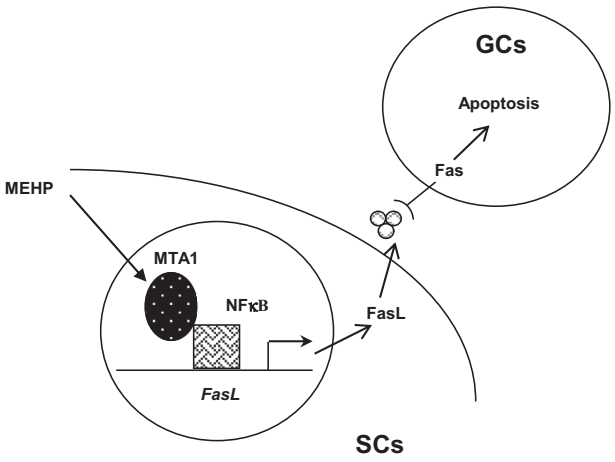

Fig. 4. Working model. SC-expressing MTA1 is upregulated upon MEHP treatment, and it in turn serves as a coactivator and help to maintain NFκB activation. Activated NFκB complex binds to *FasL* promoter and causes robust increases in FasL levels in SCs. SC-expressed FasL acts on Fas-expressing germ cells to initiate their elimination by apoptosis.

In summary, we propose a novel role of SCs-expressing MTA1. MEHP treatment induces the recruitment of NFκB onto *FasL* promoter in SCs. This interaction requires MTA1 activation, which may function as a coactivator of NFκB and helps to maintain the homeostasis of NFκB signaling network. Activation of MTA1-NFκB cascade consequently causes a robust induction of FasL expression in SCs. Subsequent germ cell apoptosis mediated by Fas/FasL system will help to remove the defective germ cells (Fig. 4). Overall, the MTA1/NFκB/FasL circuit may serve as an important defensive/repairing mechanism to help to control the germ cell quality after SCs injury.

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