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Epidermal Growth Factor Signaling Regulates the Expression of Metastasis Tumor Antigen 1 in Mouse Pachytene Spermatocyte

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Abstract

Selective regulation of gene transcription is clearly important for cells to orchestrate an adaptive response to heat stress. We previously showed that Metastasis Associated Protein 1 (MTA1), a component of the nucleosome remodeling and deacetylase (NuRD) complex, operates as a negative coregulator of p53 in the maintenance of the apoptotic balance in pachytene spermatocytes (PS) after heat stress, although specific mechanisms are not well defined. The purpose of the current study was to investigate potential upstream signaling events activating MTA1 pathway in murine PS. Using murine sialoadenectomy model, it was demonstrated that deprivation of circulated EGF significantly impaired the *in vivo* expression of MTA1 in the PS. The upstream regulation of MTA1 expression during meiosis by endogenous EGF was further confirmed *in vitro* using a selective Epidermal Growth Factor Receptor (EGFR) inhibitor, tryphostin AG1478. Moreover, inhibition of EGF signaling by AG1478 treatment significantly suppressed the heat stress-induced MTA1 in PS. The available data collectively suggest that EGF signaling regulates the expression of MTA1 in PS, and early activation of EGF/MTA1 cascade in PS in response to heat stress may serve as an intrinsic self-defensive mechanism maintaining apoptotic balance during meiotic heat stress.

Keywords: Metastasis associated protein 1 (MTA1); Pachytene spermatocytes; EGF; Meiosis

Introduction

Normal testicular function occurs in a low-temperature environment. However, the temperature of scrotum can be easily affected by numerous external factors such as posture, clothing, lifestyle, occupation and season [1]. During the complicated process of spermatogenesis, the DNA of the spermatocytes in meiosis is most vulnerable to the introduction of a range of errors upon hyperthermal stimulations. In this regard, multifactorial protective mechanisms are warranted in spermatocytes to ensure the maintenance of cellular integrity during stress condition [2].

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 modifying the acetylation status of crucial target genes [3]. In testis, MTA1 expression is gradually increased in tetraploid primary spermatocytes since 14 days postnatal. During this course, where continuous meiosis takes place, messenger RNA storage is a common phenomenon seen In Pachytene Spermatocytes (PS), where the messengers are saved for utilization during spermatogenesis. In this regard, the accumulation of MTA1 may imply its role in testicular development [4]. Our group has provided the evidence that overexpression of MTA1 *in vitro* could remarkably elevate the capability of spermatogenic derived cells against heat-induced apoptosis and endogenous MTA1 operates as a negative co-regulator of p53 in maintenance of apoptotic balance during early phase after hyperthermal stress [5,6]. Because MTA1 is localized in the nuclear site, however, the molecular events by which the rise in extracellular temperature is transduced into intracellular responses of MTA1 remain to be defined.

Epidermal Growth Factor (EGF), a polypeptide of 53 amino acids, was first isolated and purified from the submandibular glands of male mice. EGF activates epidermal growth factor receptor (EGFR or ErbB1) tyrosine kinase, which in turn activates intracellular signal transduction, enhances transcription of growth related genes, and usually promotes cell growth [7]. Considerable reports exist to suggest that EGF influences spermatogenesis directly. For example, ablation of the submandibular gland (sialoadenectomy) in adult male mice led to a marked decrease of epididymal spermatozoa; in turn, this effect was specifically reversed by daily administration of EGF [8]. Further, EGF administration to the cryptorchid testis significantly decreases the number of apoptotic germ cells. Supplement of exogenous EGF after testicular torsion improves bilateral testicular injury. Thus, EGF is an important anti-apoptotic/pro-survival factor during testicular stress [9].

The complexity of the cellular heat shock response is quite apparent, and involves multiple signal cascades, among which activation of EGF signaling has been identified as one of the earliest extracellular transduction events. To this end, we hypothesize that the EGF pathway may be potentially involved in the upstream regulation of MTA1 expression and function in pachytene spermatocytes. An experimental study was therefore designed to evaluate the effect of deprivation of EGF on testicular MTA1 expression.

Materials and Methods

Animal model

A total of 148 male mice (C57BL/6), obtained from the Animal

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Research Center of the university, were housed, maintained, and handled in compliance with the National Institutes of Health guidelines and the rules for the care and handling of laboratory animals (NIH publication No. 85-23, revised 1996). Animals were divided initially into three groups: sham operated controls, sialoadenectomy, and sialoadenectomy+EGF treated (Sigma Aldrich, St Louis, 30 or 100 μg/kg/day subcutaneously for 7 days). Sialoadenectomy was carried out in mice under sodium pentobarbital anesthesia [0.04-0.05 mg/g of body weight, i.p. (intraperitoneally)] as described previously [10]. Briefly, a small incision was made to expose the submandibular salivary glands and the tissues were then ligated and excised. The sham-operated mice received the same operation except that the tissues were not ligated and excised. Six males from each group were sacrificed by CO_2 asphyxiation at different time-points after surgery as indicated. Blood of some sialoadenectomized mice was collected from the inferior vena cava. Blood collection was completed within 28 days. Plasma and intratesticular EGF were determined as described elsewhere [10]. For histological studies, testes were fixed in Bouin's solution for about 24 hours. All animal work was approved by the Animal Care and Use Committee of our university.

Cell culture and treatment

Primary PS were isolated according to our previous work and cells were cultured in minimum essential medium (Gibco) and supplemented with 0.5% BSA (Sigma-Aldrich), 1 mM sodium pyruvate, and 2 mM lactate at 32°C in a humidified atmosphere containing 95% air and 5% CO_2 [5]. Cells were stimulated for 10 min with EGF (50 or 200 ng/ml) in the presence or absence of AG1478 (10^{-7}). For heat stressed conditions, cells were cultured at 43°C for 1 h followed by incubation at 32°C.

Flow cytometry

Germ cells were released from seminiferous tubules in PBS as described [11]. After germ cells were stained with 25 mg/l ethidium bromide (Sangon Biotech, Shanghai, China), the samples ($\times 10^6$) were analyzed by a high speed cell sorter flow cytometer (BD Biosciences, San Jose, CA, USA) with an excitation wavelength of 488 nm.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as described previously [5]. Primer sequences used were *MTA1*, 5'-CAGTGTCGCCTCTGCGCATC-3' and 5'-TCCACTGCTCCGAGCTGGAA-3'; *GAPDH*, 5'-GGGTGAGGCCGGTGCTGAGT-3' and 5'-TGACCCGTTTGGCTCCACCCT-3'.

Amplification of *GAPDH* mRNA was served as an internal control. PCR products were then quantified by SYBR green intercalation using the MiniOpticon™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative abundance of *MTA1* transcript was quantified using the comparative ΔΔCt method.

Western blotting

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). Membranes were then incubated with different primary antibodies in blocking solution overnight at 4°C (Table 1). Final signals were detected using an ECL kit (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry

The Avidin-Biotin-Peroxidase (ABC) method was employed in the immunohistochemical assay on serial 5-μm sections as previously described [5]. Briefly, after endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min, slides were incubated with the anti-MTA1 goat antibody, diluted in PBS at 4°C overnight in a moist box. Biotinylated rabbit anti-goat IgG (1:500 dilution) was incubated for 1 h at RT and detected with streptavidin–peroxidase complex. Peroxidases were detected with 0.7 mg/mL 3-3'-diaminobenzidine tetrahydrochloride (Sigma) in 1.6 mg/mL urea hydrogen peroxide (60 mmol/L Tris buffer, pH 7.6). Negative control slides were incubated with pre-absorbing serum.

Quantification of the apoptotic cells

An apoptosis ELISA kit (Roche Diagnostics, Mannheim, Germany) was used to quantitatively measure cytoplasmic histone-associated DNA fragments as previously reported [5].

Statistical analysis

Experiments were repeated at least three times, and one representative from at least three similar results was presented. Quantitative data are presented as mean ± SEM. Results were analyzed for statistically significant differences using ANOVA, followed by Tukey's test. $P<0.05$ was considered statistically significant. Statistical analyses were performed by using SPSS 15.0 software.

Results

Characterization of murine sialoadenectomy model

To explore the relationship between EGF signaling and testicular MTA1 expression at the *in vivo* level, we established the murine sialoadenectomy model. Despite the fact that the majority of circulated EGF originate from submandibular, many other tissues are also in the list of synthesis [12]. To this end, we firstly studied the evolution of the plasma and intratesticular EGF levels after sialoadenectomy. The plasma EGF concentration was fairly constant in the sham-operated mice throughout the study period. In contrast, the plasma EGF concentration began to decline 1 d after sialoadenectomy and remained relatively low along the experimental period, with the lowest level detected (about 26.3% of the control level) at the 7th d after surgery (Figure 1A). Similarly, the testicular EGF concentration began to decrease at the 3rd d and started to restore 14th d after sialoadenectomy, with the minimal level observed at the 7th d after surgery (Figure 1B). We were then curious about the deleterious effect of sialoadenectomy on germ cell

Antibody	Host species	Vendor	Catalog number	Application(s)/ dilution(s)
MTA1	Goat	Santa Cruz Biotechnology	sc-9446	IB (1:250) IHC (1:100)
Actin	Goat	Santa Cruz Biotechnology	sc-1616	IB (1:1000)
EGF	Goat	Santa Cruz Biotechnology	sc-1342	IB (1:500)
pEGFR	Rabbit	Cell Signaling Technology, Inc. (Beverly, MA)	# 2231	IB (1:1000)

IB: Immunoblotting/western blotting; IHC: Immunohistochemistry.
It is noted that all the antibodies listed herein immunologically reacted with the corresponding proteins in mouse.

Table 1: Antibodies used for various experiments in this report.

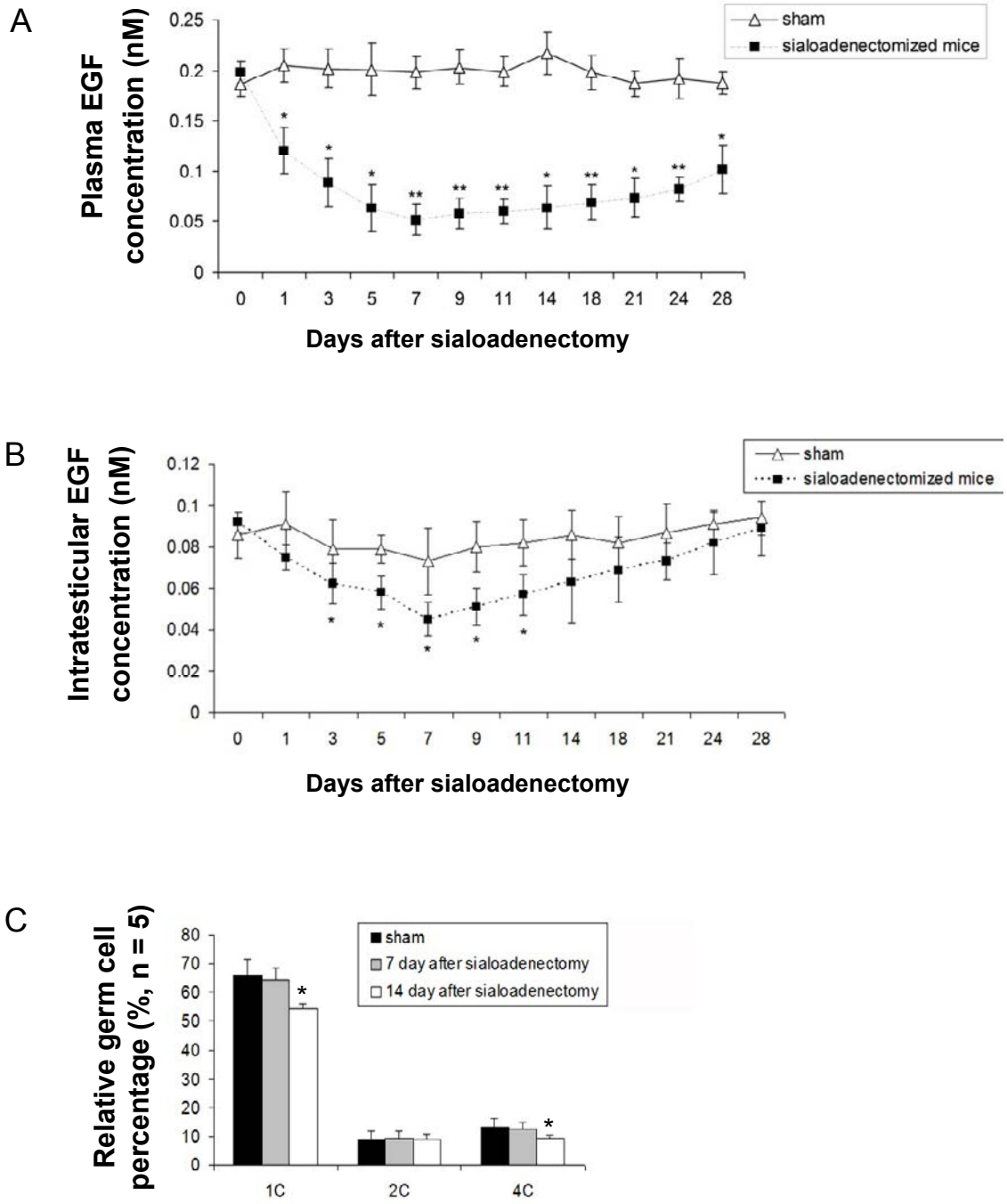


Figure 1: Characterization of murine sialoadenectomy model. Effect of sialoadenectomy on plasma (A) and testicular (B) EGF concentration was determined during a 28-day experimental period, respectively. Results are the mean ± SEM of 6 animals per group. $^{*}P<0.05$ or $^{**}P<0.01$ between sialoadenectomized and paired-time control value. (C) Alteration in the relative germ cell percentages by sialoadenectomy was evaluated by flow cytometry at the end of 7 or 14 days after surgery. $^{*}P<0.05$ between sialoadenectomized and paired-time control value.

composition. The flow cytometric DNA content distribution of various germ cells in the control rats is well characterized by the presence of three main distinct peaks, representing spermatid (1C, including elongated spermatid and round spermatid), diploid spermatogonia (2C), and tetraploid primary spermatocyte (4C) populations [11]. As shown in Figure 1C, the percentages of 1C and the percentages of 4C populations were both significantly decreased at the 14th d after surgery. This is understandable since spermatid differentiation in mice last for at least 8.6 days. All together, the available data prompted us to choose Postoperative 7 days as the experimental duration for the subsequent study, because (i) both the circulated and local EGF concentrations reached the minimum at the 7th d and (ii) the quantity of germ cells was

relatively stable within this time-window. In this way, we may exclude the influence of the diminishment of PS induced by sialoadenectomy on the expression level of MTA1.

Regulation of MTA1 expression in PS by EGF signaling: the *in vivo* evidence

In the first attempt to understand the relationship between MTA1 expression and EGF signaling, we studied the *MTA1* mRNA expression after depletion of circulated EGF using qRT-PCR. Sialoadenectomy began to attenuate the *MTA1* mRNA expression at the 5th day after surgery, with a lowest level of *MTA1* observed at postoperative 7th d. This decrease could be prevented by replacement with exogenous EGF and this prevention was also dose-dependent (Figure 2A). Such an expression profile was confirmed by western blotting analysis of representative testicular samples at the translational level (Figure 2B). In keeping with these data, our immunohistochemical assays demonstrated clear-cut suppression of MTA1 immunoreactivity in PS from sialoadenectomized mice that was partially restored by EGF replacement (Figure 3).

EGF regulates MTA1 expression in the isolated PS

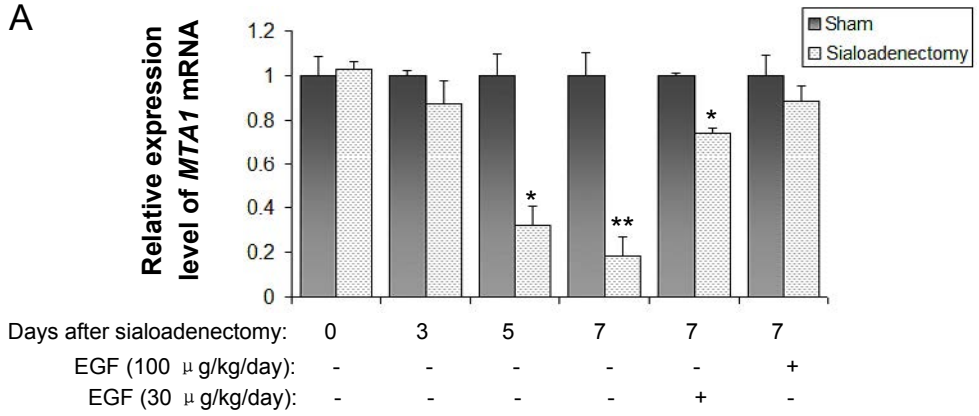
To further confirm the above-mentioned observation, we treated primary cultured PS with different dose of exogenous EGF. As expected, higher concentration of EGF evoked more dramatic increase in MTA1 protein level, which was completely compromised by application of selective EGFR inhibitor, AG1478 (Figure 4).

Anti-apoptotic effect of EGF signaling upon heat stress in PS may be executed partially via MTA1 functionality

In a next step, we analyzed the ability of EGF to regulate the MTA1 function in PS. In favor of the latter, it has been reported that MTA1 operates as a negative co-regulator of apoptotic balance in PS in response to heat stress [5]. To examine whether suppression of EGF activation could influence MTA1 expression in response to heat insult, we treated primary cultured PS with AG1478. One hour after heat stimulation, we observed that blockage of EGF pathway activation effectively abolished the MTA1 expression in PS upon heat insult (Figure 5A). In addition, apoptotic ELISA analysis revealed a simultaneous increase of heat-induced apoptosis in the cells treated with AG1478 (Figure 5B). These results suggested that activation of EGF pathway upon heat stress is anti-apoptotic in PS and this protective effect is exerted at least partially through its downstream effector, MTA1.

Discussion

Mature EGF has been immunolocalized in Sertoli cells, PS and round spermatids [13]. Low-level EGFR expression was also detected in these cell types [14]. These pioneering studies had left open the possibility that EGF could exert functions on testis physiology by acting at different levels. Accumulated evidences have demonstrated a participation of EGF in the direct control of Sertoli cell activity [15]. In



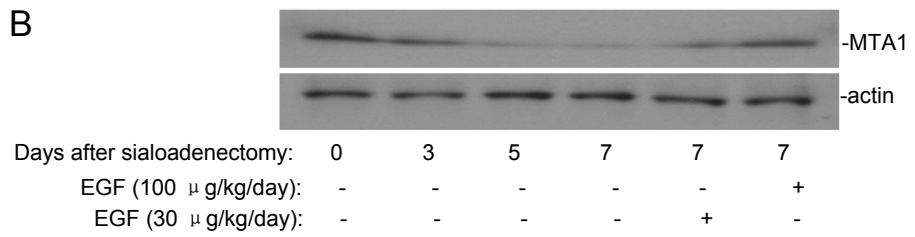


Figure 2: Ablation of endogenous EGF by sialoadenectomy reduced the MTA1 expression in the pachytene spermatocytes. Testicular expression of MTA1 in sham operation, sialoadenectomy and sialoadenectomy + EGF supplement groups were evaluated using qRT-PCR (A) and western blotting analyses (B) along a 7-day period. Values are the mean ± SEM of at least three independent determinations. $^{*}P<0.05$ or $^{**}P<0.01$ between sialoadenectomized and paired-time control value.

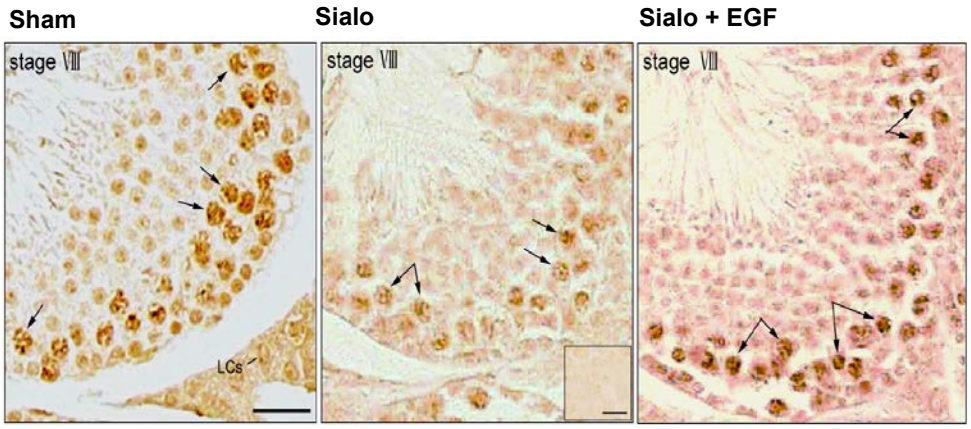


Figure 3: An impaired expression pattern of MTA1 in pachytene spermatocytes (arrows) was also confirmed by immunohistochemical staining. Replacement of the primary antibody with preabsorbed IgG abolished the immunostaining in the tissues, confirming the specificity of the assay (inserted window). LCs: Leydig cells. Bar=25 μm.

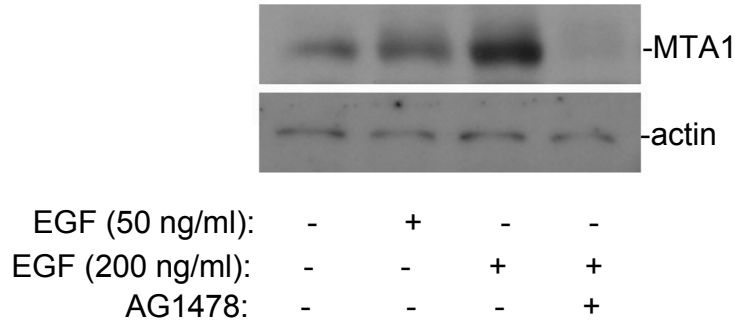


Figure 4: The stimulatory effect of EGF on MTA1 expression was confirmed in primary cultured pachytene spermatocytes using western blotting. Actin was served as internal controls.

this context, our study provides novel evidence that EGF may also serve as a protective mechanism against acute hyperthermal stimulation in PS, possibly through the modulation of MTA1 function. In favor of the latter, fragmentary evidence has pointed to a possible involvement of EGF signaling in the direct regulation of MTA1 expression. For example, activation of the Heregulin/Human Epidermal Growth-Factor Receptor 2 (HER2) signaling pathway in estrogen-receptor positive breast cancer cells stimulated MTA1 expression [16]. MTA1 immunostaining was widely and noticeably observed in the organs containing EGF-producing ability, including submandibular gland, kidney and testis [17]. In an EGF deficient background (i.e. the sialoadenectomy model) of the present study, a significant decrease in MTA1 expression levels was noted, whereas treatment with exogenous EGF was able to restore MTA1 expression. To note, however, in our immunohistochemical assay, we could still detect a relatively weak expression of MTA1 in PS of testis despite the withdrawal of circulated EGF. One tempting possibility is that the EGF synthesized by other tissues might provide compensatory effect to certain extent. To this end, the ability of EGF to stimulate MTA1 expression was further confirmed using a culture system of isolated primary PS. This approach demonstrated a clear-cut abolishment of MTA1 expression when EGF signaling was inhibited (Figure 4). Overall, the available data strongly suggest that expression of MTA1 in tetraploid population is subjected to precise regulation, involving stimulatory effects of EGF pathway.

EGF has been identified as a general pro-survival factor preventing the onset of apoptosis in response to heat shock. For example, heat induced damage in the intestinal epithelium is associated with the down-regulation of EGF signaling [18]. Production of EGF or EGF-like peptides during periods of cellular stress operates as an important survival mechanism in mammary epithelial cells [19]. EGF stimulates autophosphorylation of its receptor in both A-431 cells and human fibroblasts after heat shock [20]. Similarly in the male reproductive system, EGF administration in combination with orchiopexy is more effective for the restoration of spermatogenesis than orchiopexy alone in cryptorchid testis [9]. In good agreement with these reports, our study suggests that EGF may also serve as a local modulator of heat stress response in PS. This protective effect appeared very likely to be achieved through MTA1 function, because inhibition of EGF signaling significantly impaired the heat stress-induced MTA1 activation (Figure 5A).

Worthy to note, upregulation of MTA1 expression was transient and occurred only during the very early recovery after heat stress, whereas activation of EGF signaling was more persistent, indicating that EGF may act on the top of a pro-survival circuit in response to heat stress, which may harbor many other down-stream effectors. In favor of the latter, it has been shown that heat shock leads to the mitogenic activation of Mitogen-Activated Protein Kinase (MAPK) in A431 cells, which can be completely abrogated by treatment with EGFR inhibitor.

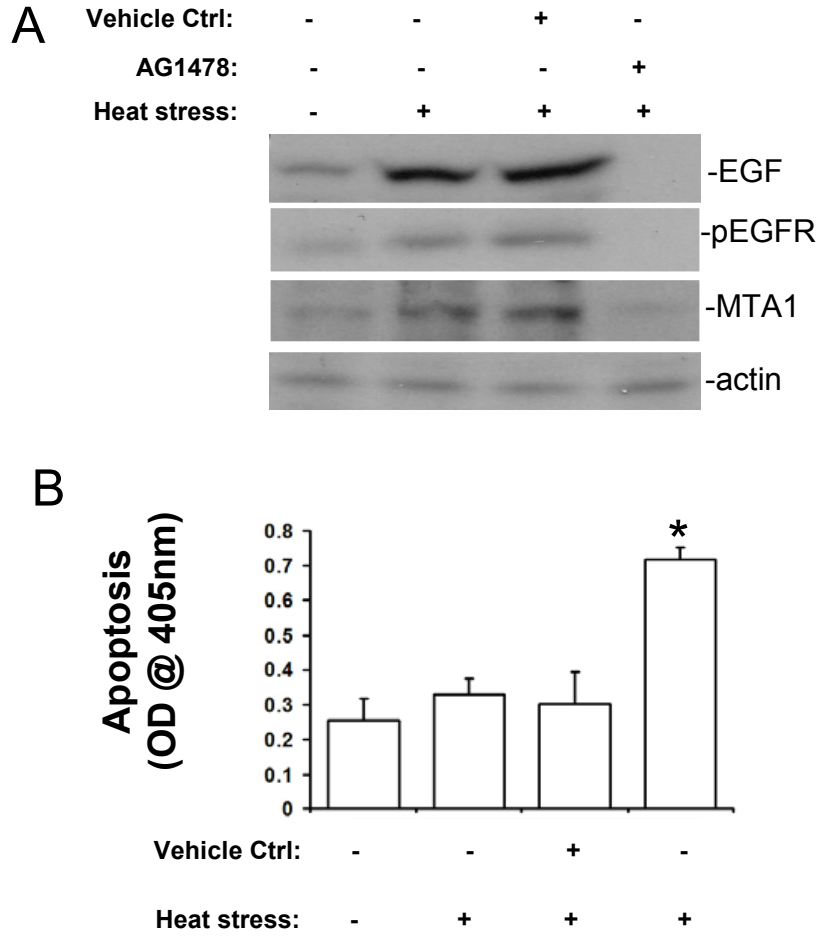


Figure 5: Regulation of MTA1 expression and function by EGF signaling: the *in vitro* evidence. (A) Effect of inhibition of EGF signaling by AG1478 treatment on MTA1 expression was determined by western blotting. Actin was served as internal controls. (B) Effect of inhibition of EGF signaling on the apoptosis in primary pachytene spermatocytes upon heat stress was assessed using ELISA method. $^{*}P<0.05$ vs. other groups.

In addition, MAPK, in conjunction with glycogen synthase kinase 3, phosphorylates heat shock factor-1 and represses transcriptional activation of the heat shock protein 70 promoter. Thus, MAPK may serve as a negative regulator of heat shock protein production and deactivate the heat shock response during recovery from stress under a fine tune of EGF pathway [20]. Nevertheless, the early down-stream signaling events in response to EGF stimulation upon testicular heat stress merit further investigation.

In summary, we propose a potential involvement of EGF/MTA1 cascade in the intrinsic process of meiotic recovery after heat stress. Heat stress induces a massive germ cell-specific apoptosis, which occurs mainly in meiotic spermatocytes and may help to remove the defected germ cells during the early recovery after insult. However, excessive apoptosis would lead to the disturbance of spermatogenic differentiation and thereafter impair the male fertility. To prevent this detrimental effect, activation of EGFR by local autocrine signal upregulates MTA1, which helps to maintain the proper apoptosis balance during early phase after heat shock in tetraploid spermatocytes. Overall, activation of EGF/MTA1 cascade appears to be a naturally occurring, indispensable defensive mechanism in response to meiotic heat stress.

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