Involvement of Metastasis Tumor Antigen 1 in Hepatic Regeneration and Proliferation

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Key Words

Metastasis tumor antigen 1 (MTA1) • Nucleosome remodeling and histone deacetylation (NuRD) complexes • Deacetylation • Partial hepatectomy (PHx) • Liver regeneration

Abstract

Background/Aims: Metastasis tumor antigen 1 (MTA1), an integral part of nucleosome remodeling and histone deacetylation (NuRD) complexes, is well correlated with the potential of metastasis, with the ability to regulate divergent cellular pathways by modifying the acetylation status of crucial target genes. However, additional biological functions of this molecule remain largely unexplored. This study was undertaken to explore the potential role of this molecule in mouse liver. **Methods:** MTA1 expression was firstly explored in mouse partial hepatectomy model (PHx). The effect of overexpression of MTA1 on hepatic proliferation and differentiation was then examined *in vivo* by hydrodynamic-based gene transfer method and *in vitro* using transformed cell line AML12 overexpressing MTA1, respectively. **Results:** Consistent with the hepatic regeneration, MTA1 expression was significantly increased 24h

post-PHx, with a maximum level at 48h after PHx. MTA1 immunoreactivity was generally elevated right after PHx and the staining appeared to experience a cytoplasm-to-nuclear transition. Overexpression of exogenous MTA1 could notably stimulate hepatic proliferation *in vivo* and could also accelerate hepatocyte differentiation *in vitro*. **Conclusion:** These data underscore a hepatocellular facet of this recently defined molecule, which may represent as a novel regulator and a new therapeutic target for the treatment of impaired liver.

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Introduction

The liver has the unique property of changing its volume depending on pathophysiologic status [1]. Fragmentary information has previously suggested several mechanisms responsible for hepatic regeneration, but the resultant mechanism has not been elucidated [2]. Therefore, the studies on specific gene expression patterns of liver regeneration are of great significance for understanding this process and for the treatment of liver injury.

MTA1, the founding member of the MTA family of genes, was originally identified through differential

screening of a cDNA library from rat metastatic breast tumors [3]. Subsequent studies indicated that the 80 kD-peptide, as a part of the NuRD complex (nuclear remodeling and deacetylation complex), could enhance the aggressive course of several carcinomas by recruiting histone deacetylase and changing the status of chromatin remodeling [4]. Besides a strong correlation between MTA1 upregulation and cancer, growing evidences strongly suggest that MTA1 could regulate divergent cellular pathways by modifying the acetylation status of crucial target genes under both pathological and physiological status [5]. For example, inactivation of the MTA1 homologues *egl-27* and *egr-1* (also known as *lin-40*) in *C. elegans* leads to abnormal patterning of cells in the embryo and forced overexpression of MTA1 in the mouse mammary gland epithelium was accompanied by extensive ductal branching and proliferation in virgin glands, suggesting a role for MTA1 in both embryonic development and mammary gland development [6, 7]. However, the physiological characteristics of MTA1 are still poorly addressed.

Recently, the functional consequence of MTA1 overexpression on p53-induced apoptosis of cancer cells has been identified. MTA1 had deacetylation activity on p53 in human hepatoma cells SK-Hep1, and MTA1 could inhibit p53-induced apoptosis by deacetylating p53 [8]. Furthermore, activation of the heregulin/HER2 (one of epidermal growth factor (EGF) receptors) pathway can remarkably stimulate the expression of MTA1 and lead to suppression of histone acetylation and enhancement of deacetylase activity [9]. Since p53 and growth factors are both important modulators during hepatic regeneration [2], we speculate that MTA1 have certain distinct regulatory role in liver. Thus, the present study was initiated to investigate the potential involvement of MTA1 in hepatic function. MTA1 expression level was firstly explored in mouse partial hepatectomy model (PHx). Moreover, the possible participation of MTA1 in hepatic proliferation was examined *in vivo* on the basis of hydrodynamic-based gene transfer method and *in vitro* using transformed cell line AML12 overexpressing MTA1. Our combined analysis would help to understand the biological relevance of this newly characterized molecule.

Materials and Methods

Animals and 70% PHx Surgery

The Ethics Committee for Animal Experiments of the Fourth Military Medical University approved all animal work

and the experimental protocols complied with the institutional guidelines and the criteria outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 86-123). Male C57BL/6 mice, purchased from Animal Research Centre of Fourth Military Medical University (Xi'an, China), were housed under standard conditions in conventional cages with free access to water and food.

Adult mice were subjected to PHx operations to induce liver regeneration. The sham operated mice were served as the control group. Five mice at each time point were killed using cervical dislocation at the following intervals after PHx: 12, 24, 36, 48, and 72 h. The regenerating livers were harvested and divided into three portions (*i*) to isolate total RNA, (*ii*) to isolate total protein extract, and (*iii*) for paraffin embedding. The liver regeneration rate was calculated measuring histological proliferating cell nuclear antigen (PCNA) expression [10]. Briefly, the sections of liver specimens were dewaxed and incubated with polyclonal antibody against mouse PCNA (Santa Cruz Biotechnology) in a 1:80 dilution for 1h. After washing with TBST (50 mM Tris-HCl, pH 7.6, 0.15M NaCl + 0.05% Tween), the sections were incubated with the secondary antibody and immunostained by the EnVision (Dako, Denmark) method according to the manufacturer's instructions. By using three regenerating livers per time point, we counted the number of PCNA-positive cells per 1,000 hepatocytes to calculate the PCNA labeling index and the results were expressed as percentage ratios. Aspartate transaminase (AST), alanine transaminase (ALT) and bilirubin levels were determined in serum samples with a standard clinical automatic analyzer (type 726, Hitachi, Tokyo, Japan).

Cytoplasmic and nucleonic fractionation analysis

Cells were isolated from mice of different groups using the two-step collagenase perfusion method [11]. After being perfusion with Hepatocyte Liver Perfusion Medium (1x) (Gibco), the livers were excised and pelleted into small pieces in Hepatocyte Liver Digest Medium (1x) (Gibco) and then incubated at 37°C for 30 minutes. Cell lysis was prepared by adding Nonidet P-40 to a final concentration of 0.3% and homogenization using a Dounce homogenizer with a tight pestle until over 90% cells were broken and nuclei released. Dounced nuclei were centrifuged at 1200×g for 5 min at 4°C. The nuclear pellet was resuspended in 10 volumes of 0.25 M sucrose with 10 mM MgCl₂. The supernatant fraction was harvested as the cytoplasm and stored for further analysis. Cytoplasm and nuclei were all lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.02% sodium azide, 100 µg/ml PMSF, 1 µg/ml Aprotinin) for 30 min on ice. 50 µg total proteins were loaded on each lane and separated on a 10-15% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Millipore, Bedford, MA, USA) for the further western blot analysis as described below. To control for the fidelity of the fractionation, blots were probed with antibodies to proteins of preestablished subcellular localization: cytoplasmic marker GAPDH (1: 500, Sigma) and nuclear marker histone H1 (1:450, Santa Cruz).

RT-PCR, western blot and immunohistochemical analyses

Total RNA was prepared from mouse liver at indicated hours after PHx and RT-PCR was conducted according to our previous report [12]. For western blot analysis, 50 µg of total liver protein was separated on SDS/PAGE and transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). The signals from primary MTA1 (1:500, Santa Cruz, CA) antibody was amplified by anti-goat IgG: Alexa Fluor 680 rabbit anti-goat IgG (Molecular Probes, Invitrogen, #A21088) and detected with Odyssey™ Infrared Imaging System. Immunohistochemical detection of paraffin-embedded 4-µm sections of regenerating liver was performed as described [13].

Plasmid constructions and intravenous injection of plasmid DNA

Expression of MTA1 full-length sequences was achieved using pcDNA3 (Invitrogen). The following primers, forward 5'-ATG GCC GCC AAC ATG TAC AGG-3' and reverse 5'-CTA GTC CTC GAT GAC GAT GG-3', were used to amplify, from human breast cancer cDNA (Clontech), a 2.2 kb fragment encoding for human MTA1 [14]. The PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min. MTA1 cDNA containing the entire reading frame was subcloned into pcDNA3 vector using restriction sites BamHI and XhoI. The resultant recombinant MTA1 expression plasmid, designated pCMV-MTA1, was characterized by detailed restriction digestion and by sequencing (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. China).

Plasmid DNA was administrated into mice by a hydrodynamic-based gene transfer technique as previously described [15]. Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg body weight), and then 50 µg DNA/mouse of plasmid DNA (pCMV-MTA1 or control pCMV) in lactated Ringer's solution (0.1 ml/g body weight), was injected into the tail vein using a syringe with a 27-gauge needle. DNA injection was completed within 5-10 s. To achieve a long-term expression of exogenous MTA1 gene *in vivo*, we performed repeated weekly injections of plasmid DNA into mice for 2 weeks. On day 16 after the first injection, the mice were subjected to PHx operations to induce liver regeneration. Western blot and immunohistochemical analyses using rabbit anti-hMTA1 antibody (M7693, Sigma) were employed to assess the human MTA1 protein expression *in vivo*.

Bromodeoxyuridine labeling and immunofluorescence detection

For *in vivo* measurement of hepatocyte DNA synthesis, an intraperitoneal (IP) injection of a PBS solution containing 10 mg/ml of 5-bromo-2'-deoxyuridine (BrdU, Sigma; 50mg/g body weight) was administered two hours prior to harvesting the remnant regenerating liver. Animals were then sacrificed and liver specimens were obtained. Liver tissues were fixed in 4% paraformaldehyde for 24 hours, processed for histological analysis, and stained using BrdU Immunofluorescence Assay Kit (Roche Applied Science, US). Three animals were

used per treatment group per timepoint, and five separate low-power fields were assessed per animal group per timepoint.

Construction of cell lines stably expressing MTA1 and cell culture

AML12 cells overexpressing His-tagged MTA1 were constructed according to our previous work [16]. The expression of exogenous MTA1 was verified by immunofluorescence and immunoblotting assays using anti-His monoclonal antibody (Abcam, Cambridge, UK). Upon immunofluorescence staining, the transfected cells fixed on coverslips were incubated with the primary antibody (anti-His monoclonal antibody, Abcam, Cambridge, UK; 1:400 diluted) in PBST supplemented with 3% BSA at 4°C overnight. Final confocal analysis was carried out using a Zeiss laser-scanning confocal microscope. Nuclei were stained with a nuclear marker (DAPI).

Reconstructed cells (viability > 80%) were cultured for 48 h as a monolayer in a medium and at a density either stimulating proliferation or favoring differentiation. The former, referred to as "proliferation stimulating medium (PSM)", was consisted of MEM/M199 (3:1, v/v), supplemented with 1 mg/ml BSA, 5 µg/ml bovine insulin, 2 mM L-glutamine, antibiotics (7.3 IU/ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 50 µg/ml kanamycin monosulphate and 10 µg/ml sodium ampicillin) and 10% (v/v) FBS at a density of 0.5×10^5 cells/cm² [17]. For measuring the metabolic functionality, hepatocytes were maintained in differentiation stimulating medium (DSM) composed of WEM, supplemented with 2 mM L-glutamine, 7 ng/ml glucagon, antibiotics and 10% (v/v) FBS at a density of 0.6×10^5 cells/cm² [18].

In vitro BrdU labeling and examination of proliferative hepatocytes

For identification of cells in S-phase *in vitro* by BrdU labeling, AML12 cells overexpressing MTA1 were pulsed with BrdU at final dilution 1:50 for the last 4 h of the final 48 h of incubation. Subsequent immunocytochemical staining was performed exactly according to the manufacturers' instructions. For *in situ* quantification, 50 unit areas in five visual fields in three independent groups were measured using a $\times 40$ objective. The number of cells staining positively for BrdU were counted and expressed as mean $\pm$ SEM for each group.

Preparation of cell lysates were conducted as previously reported [19]. Immunoblot analysis was performed as described above using anti-CDK1 (Santa Cruz Biotechnology, CA) and anti-cyclin D1 (Neomarkers, CA, USA) antibodies [20, 21].

For measuring albumin secretion, medium samples (PSM) were collected and analyzed for its albumin content by an ELISA technique [22]. For the determination of urea secretion, the hepatocyte monolayers maintained in DSM were incubated with 10 mM NH₄Cl, 6 h prior to medium collection and cell harvesting. Urea concentration in the medium was analyzed spectrophotometrically using a Quantichrom Urea Assay kit (BioAssay Systems, CA, USA). Total cellular protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Germany), using BSA as a standard.

Fig. 1. Construction of mouse PHx model was monitored by assessing Aspartate transaminase (AST) (A), alanine transaminase (ALT) (B) and bilirubin levels (C) in serum samples at the following intervals after PHx: 12, 24, 36, 48, and 72 h. The liver regeneration rate was also calculated measuring histological proliferating cell nuclear antigen (PCNA) expression (D). Subsequent PCNA labeling index was evaluated counting the number of PCNA-positive cells per 1,000 hepatocytes using three regenerating livers per time point and the results were expressed as percentage ratios (E). Statistically significant differences: * $p < 0.05$, ** $p < 0.01$.

Statistical analysis

Quantitative data are presented as mean $\pm$ s.e.m. Results were analyzed for statistically significant differences using ANOVA, followed by Tukey's test. $P < 0.05$ was considered significant.

Results

Characterization of the expression pattern of MTA1 in mouse PHx model

To investigate the potential relationship between the expression of MTA1 and the hepatic regeneration, we

firstly employed the mouse PHx model. In this model, the hepatic regeneration is activated within 2 days after operation, thus providing an optimal experimental background in which to test hepatic cell-specific expression of liver-derived factors [2]. As demonstrated in Fig 1, serum AST and ALT levels increased at 24h after PH (40.12 and 18.69 U/L, compared to 21.23 and 17.48 U/L of the control group, respectively, $p < 0.05$) and comparably peaked at 48h (91.08 U/L and 43.89 U/L, $p < 0.01$) after PH. Serum total bilirubin level notably increased at 12h (1.12 mg/dl, $p < 0.05$ when compared to 0.93 mg/dl of the control group) and peaked at 48h after

Fig. 2. Analysis of MTA1 expression level in hepatic specimens obtained at 12, 24, 36, 48, and 72 h after PHx, was conducted using semi-quantitative RT-PCR (A) and Western blot (B). Parallel amplification of GAPDH mRNA and loading of β -actin were served as internal control. The representative expression profile of MTA1 after PHx was summarized in panel C. The values of both analyses are the mean $\pm$ s.e.m. of at least four independent determinations. Asterisk denote groups that are statistically different (* p <0.05, ** p <0.01; ANOVA followed by Tukey's test).

PH (1.78mg/dl, p < 0.01). The serum levels of representative liver enzymes, particularly ALT, reflected the liver tissue damage (loss) following PHx [23]. Number of PCNA-positive cells, averaging at cells per 1,000 hepatocytes, was also significantly increased in regenerating livers versus quiescent liver tissue since 24h after PHx (Proliferation Index =24.7%, p < 0.01). These data confirmed the correctness of our PHx model.

Semi-quantitative RT-PCR and western blot assays of MTA1 were then conducted in hepatic samples at different time-points after PHx. In this setting (Fig 2), persistent high expression of MTA1 (both mRNA and protein levels) was demonstrated throughout a 72-h period after PHx, with a significant increased levels observed at 24h and a maximum level at 48h after PHx, which was followed by a succedent decreasing trend. The pattern of MTA1 immunoreactivity was evaluated in hepatic tissue sections as mentioned above. Particularly, the intense staining was confined to the cytoplasm of hepatocytes closed to the central vein (Fig 3A). MTA1 expression was generally elevated right after PHx and the staining appeared to experience a cytoplasm-to-nuclear transition after PHx as shown in Fig 3B. To further confirm the subcellular localization of target protein in different experimental settings, the extracts from isolated

hepatocytes were also examined by western blot with cytoplasmic marker GAPDH and nuclear marker histone H1. As shown in Fig 3C, the marker proteins were found in the expected fractions and the anti-MTA1 antibody also detected a band with a molecular mass of about 80 kDa on different lines, further confirming the subcellular localization demonstrated by immunohistochemical means.

Effect of the overexpression of MTA1 in vivo on hepatic regenerative status after PHx

To efficiently deliver exogenous MTA1 gene, we have adapted an *in vivo* gene transfection procedure by rapid injection of a large volume of plasmid DNA solution via the tail vein. This hydrodynamic-based approach has been identified as a far more efficient gene transfer approach than others such as intramuscular injection and the liver is the organ with the highest level of exogenous gene expression [24]. As shown in western blot analysis (Fig 4A), systemic administration of the pCMV-MTA1 plasmid resulted in a marked expression of local human MTA1 *in vivo* as expected. Like the endogenous MTA1, the exogenous MTA1 was mainly expressed in the cytoplasm of hepatocytes closed to the central vein (Fig 4B). To be noted, because the M7963 antibody only detects MTA1 protein specific for human and does not

Fig. 3. Subcellular localization of MTA1 protein in adult mouse liver. (A) A representative reaction after immunolabeling with the specific anti-MTA1 primary antibody was conducted in sham operated mouse liver. Specific MTA1 immunoreactivity was clearly evident in the cytoplasm of hepatocytes closed to the central vein (arrows). (B) Sections from PHx group displayed a diffuse nuclear staining regardless of the cell distribution (arrows heads). A negative control using preabsorption of the primary antibody was presented in the inserted window (C). Distribution of MTA1 among the subcellular fractions was also analyzed in the isolated hepatocytes of different groups as described in Materials and Methods (D). Hepatocytes were fractionated into cytoplasmic and nuclear fractions. A 50- μ g sample of each fraction was separated by electrophoresis on 10-15% SDS polyacrylamide gradient gel and then analyzed by Western blotting. Blots, prepared in parallel, were probed with the antibodies indicated to the right. To control for the fidelity of the fractionation, blots were probed with antibodies to proteins of preestablished subcellular localization: cytoplasmic marker GAPDH and nuclear marker histone H1. Western blotting demonstrates an anti-MTA1 immunoreactive band of about 80 kDa in the different fractions. Bar = 25 μ m.

cross-react with the endogenous mouse MTA1, the level of tissue MTA1 protein detected by the immunoblotting assay represents only the exogenous human MTA1 derived from transgene expression. As expected, no human MTA1 protein was detected in the liver samples from the mice injected with pCMV control plasmid (Fig 4A, B).

We next investigated the effect of exogenous MTA1 expression on the hepatic regeneration after hepatectomy. Immunofluorescence studies were performed using a commercial kit directed against BrdU staining. As shown in Fig 4C, a few cells were stained positive in mice injected with pCMV-MTA1 24h after hepatectomy (BrdU positive cells = 24/low-power field; $p < 0.01$ when

compared to 7/low-power field of the control group). DNA synthesis reaches its maximum 48h after hepatectomy ($p < 0.01$ when compared to the control group). A persistent high positive staining was demonstrated during a 72-h labeling period. In contrast, a significant increase of positive staining was detected in the control group injected with pCMV plasmid 48h after hepatectomy (BrdU positive cells = 16/low-power field). Changes in DNA synthesis found in the control group were not intense as the mice injected with pCMV-MTA1 at 24, 48h after hepatectomy (Fig 4D). These results revealed that *in vivo* overexpression of MTA1 could promote cell cycle progression after PHx through the G1/Sphase checkpoint.

Fig. 4. Effect of *in vivo* overexpression of exogenous MTA1 on the BrdU incorporation. (A) Western blot analysis using rabbit anti-hMTA1 antibody (M7693, Sigma) was employed to assess the exogenous MTA1 protein levels in different experimental groups after hydrodynamic-based gene transfer. (B) Localization of the exogenous MTA1 protein was demonstrated by immunohistochemical staining using rabbit anti-hMTA1 antibody (M7693, Sigma). The exogenous MTA1 was mainly expressed in the cytoplasm of hepatocytes closed to the central vein as expected (arrow). Bar = 25 μ m (C) An intraperitoneal (IP) injection of a PBS solution containing 10 mg/ml of 5-bromo-2'-deoxyuridine (BrdU, Sigma; 50mg/g body weight) was administered two hours prior to harvesting the remnant liver. Histological analysis was performed using commercial BrdU Immunofluorescence Assay Kit. Bar = 50 μ m (D) Relative BrdU-positive signals were quantitated by counting positive cell number per low-power field. Three animals were used per treatment group per timepoint, and five separate low-power fields were assessed per animal group per timepoint. Statistically significant differences: * p <0.05, ** p <0.01.

Effect of the overexpression of MTA1 in vitro on hepatic proliferative status

To further confirm the biological influence of the overexpression of exogenous MTA1 on hepatocellular proliferation, we established AML12 cells stably expressing His tagged MTA1. AML12 are nontransformed hepatocytes which have been immortalized without viral oncogenes, and have retained its differentiated feature [25]. The construction was confirmed by both immunofluorescence and immunoblotting assays as demonstrated in Fig 5A, B.

The reconstructed AML12 cells were maintained in

PSM to stimulate proliferation [17]. The expression of cell cycle-specific markers was investigated by immunoblot assay to confirm the progression of the hepatocytes through the different subsequent cell cycle phases in PSM. As demonstrated in Fig 6B, in reconstructed AML cells overexpressing His-tagged MTA1, both the G1 and S phase cell cycle markers cyclin D1 and cyclin-dependent kinase 1 (CDK1), respectively, were notably expressed. In contrast, a weak expression of both cyclin D1 and CDK1 protein was detected in the AML cells transfected with empty vector and in wild cells. To further assess cell proliferation and to identify the

Fig. 5. Construction of AML12 cells overexpressing MTA1. (A) Cells transfected with pcDNA3.1-His-MTA1 vector, pcDNA3.1-His vector and untransfected cells (wild) were all subjected to western blotting analysis using specific anti-His antibody, respectively. (B) Immunofluorescent staining of AML12 overexpressing pcDNA3.1-His-MTA1 by anti-His monoclonal antibody. Nuclei were stained with DAPI (blue). Localization of the target protein is indicated by the presence of viridescence color as a result of overlapping green and blue pixels (white arrows). Instead in the negative control cells (transfected with pcDNA3.1-His-vector), the immunofluorescence was only merged with DAPI staining (blue). Bar = 5 μ m.

Fig. 6. (A) Identification of cells in S-phase *in vitro* by BrdU labeling. AML12 cells overexpressing MTA1 were pulsed with BrdU at final dilution 1:50 for the last 4 h of the final 48 h of incubation. For in situ quantification, 50 unit areas in five visual fields in three independent groups were measured using a $\times 40$ objective. The number of cells staining positively for BrdU were counted and expressed as mean $\pm$ SEM for each group. Statistically significant differences: * $p < 0.05$. (B) Immunoblot analysis of the G1 and S phase cell cycle proteins cyclin D1 and CDK1 in the different hepatocytes cultured under conditions that stimulate proliferation (PSM), respectively. Results of three independent experiments are presented.

proliferating cells, labeling with BrdU *in vitro* and immunocytochemical staining were used. After 48 h of culture, the reconstructed AML cells showed a nearly twofold increase of BrdU labeled cells compared with the control (Fig 6A). We next investigated whether the progression through the cell cycle in the hepatocytes overexpressing MTA1 affected the expression of hepatic functionality by measuring the secretion rate of albumin and urea into the medium. Hepatocytes cultured for 48 h

in modified PSM (to examine the level of albumin) or DSM (to examine the level of urea) exhibited significantly higher levels of either albumin or urea secretion as shown in Fig 7, but the stimulating effect on albumin and urea secretion was much more pronounced in the AML cells overexpressing His-tagged MTA1 (0.68 and 17.41 μ g/ml/h per mg protein for albumin and urea secretion, respectively; $p < 0.05$ when compared to 0.37 and 11.84 μ g/ml/h per mg protein for albumin and urea secretion of

Fig. 7. Effects of overexpression of MTA1 on albumin secretion and urea production in reconstructed hepatocytes cultured for 48 h under PSM or DSM conditions. Results are expressed as $\mu\text{g/ml/h}$ per mg total cellular protein. Values represent mean $\pm$ SD of three independent experiments. Statistically significant differences: * $p < 0.05$.

the control group). Collectively, these results indicated that the overexpression of exogenous MTA1 has a specific role in the regulation of mitotic activity in hepatocytes.

Discussion

Previous studies have demonstrated that stimulation of MTA1 exhibits the significant functions in epithelial proliferation and differentiation [26]. In this study, we have extended our investigations to the functions of MTA1 in regulating hepatic regeneration and proliferation under both pathological and physiological status. Initially, we examined the expression level of MTA1 in mouse PHx model and found that MTA1 expression was significantly upregulated 24h post-PHx and peaked at 48h after PHx. This observation was consistent with the time point at which hepatic regeneration occurs, suggesting that MTA1 may play some roles in the acute hepatic injury [27]. Of note, MTA1 immunoreactivity was selectively detected

at defined area under different pathophysiological status. In normal adult mouse, MTA1 expression was predominantly located in the cytoplasm of hepatocytes around central vein while after PHx, the positive signals were diffusely relocated in the nucleoli of hepatocytes regardless of their distributions. Three main points should be emphasized on the basis of this observation. (i) The elucidation of our results should exclude the promiscuous staining of a naturally occurring MTA1 variant, namely MTA1s. The latter is generated by alternative splicing at a cryptic splice site located in (or near) exon 14, resulting in the deletion of 47 nucleotides and the entire C-terminal portion of MTA1. MTA1s localizes in the cytoplasm, sequesters ER in the cytoplasm using an NR box motif (LRILL), and thus could enhance non-genomic responses of ER [28]. Our primary antibody employed in this study is an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of MTA1, so theoretically it could not recognize MTA1s (Actually, it has been confirmed in our lab that this antibody could not recognize *in vitro* translated or *in vivo* extracted MTA1s. Data not shown.) (ii) It has been demonstrated that hepatocellular carcinoma (HCC) specimens showed both cytoplasmic and nuclear immunoreactivity for MTA1, which raises the possibility of a NuRD complex-independent function of MTA1 [29]. Our results were consistent with this observation since the promotions of regeneration after PHx might be considered as adverse effects like the progression of tumorigenesis. MTA1 is an integral subunit of the Mi-2/NuRD complex. Because the latter contains both histone deacetylase and chromatin-remodeling ATPase activities, it is thought that such complexes are involved in the hypoacetylation of core histones and that HDAC-containing complexes participate in transcriptional repression. To date, most direct targets of MTA1 identified are nuclear proteins such as ER- α , HDAC1, MTA1-interacting coactivator (MICOA) and nuclear receptor-interacting factor 3 (NRIF3) [4]. In this context, it is tempting to presume that MTA1 should have to translocate from cytoplasm to nucleus to fulfill its transcriptional regulatory role upon proliferative stimulation such as hepatic regeneration induced by PHx. (iii) Mature hepatocytes are fully differentiated and remain quiescent (G0). Upon isolation, however, they enter the cell cycle and progress to mid-late G1 phase, where they are arrested at the mitogen-dependent restriction point. Upon stimulation by mitogens, such as growth factors in culture medium, hepatocytes pass beyond this restriction point and DNA synthesis is reactivated [30]. In our study, immunofluorescence assay detected a nuclear localization

of exogenous MTA1 protein in reconstructed AML12 cells, also supporting the above presumption. Moreover, similar effects are also present in another transcription factor, namely “nuclear factor for the kappa chain of B cells” (NFκB). In liver cells, NFκB is a heterodimer composed of two protein subunits, referred to as p65 (or relA) and p50 [31]. The two subunits are assembled in the cytoplasm, but the heterodimer is inactive because an inhibitor, IK-B, binds to the p65 subunit. Free from the IK-B inhibitor, the NFκB heterodimer (p65/p50) migrates to the cell nucleus where it activates genes involved in inflammation, cell adhesion, stress responses, cell replication and apoptosis. After partial hepatectomy, NFκB is activated very rapidly, within 30 min, if not earlier, after the operation [32]. Taken together, our data suggest that the cytoplasmic-nuclear transition of the endogenous MTA1 may be physiologically important in the process to recover from acute hepatic injury.

Next, we were intrigued with the therapeutic effect of exogenous MTA1 on hepatic regeneration and tried to test whether the overexpression of MTA1 could exert positive effect on the mechanic injury of liver. We used a pharmacological strategy consisting in an *in vivo* transfection of human MTA1 directly to the mouse liver by the hydrodynamics-plasmid delivery procedure. MTA1 transfected mice exhibited increased hepatocyte proliferating levels compared to the empty vector transfected control group as determined by BrdU immunostaining of regenerating liver sections. This analysis further confirmed the hepato-stimulating effects of MTA1 after liver injury.

It is worthwhile to point out that MTA1 does not function directly as an accelerator of hepatic regeneration and hepatocyte proliferation until other factor(s) is preliminarily activated in response to stimuli for regeneration and proliferation. One possible explanation for this is that hepatocyte replication is a multistep process comprising distinct tightly regulated phases, namely the initiation, proliferation and termination phase. A large number of genes are either newly expressed or regularly increase their expression after PHx. Worthy to note that normal hepatocytes do not respond effectively to growth factors unless they are primed by activating the expression of immediate early genes. In response to physical damage, hepatocytes are primed by so called immediate early genes (such as c-fos, c-jun and c-myc), through the release of inflammatory cytokines, to increase the activation of transcriptional factors.

Subsequent gene expression is thought to occur only after activation by a number of identifiable hepatotrophic factors. Prominent among these are TNF-α, HGF, epidermal growth factor (EGF), transforming growth factor-α (TGF)-α, and IL-6 [33-35]. Interestingly, the upregulation of growth factor pathway could stimulate the expression of MTA1 *in vitro* [9]; MTA1 has also been identified as an essential downstream effector of c-myc signal pathway [36]. Thus, the transcriptional regulatory role of MTA1 during hepatic regeneration might be initiated only through the stimuli of the c-myc-growth factors pathway under physiological status although further studies should be carried out to substantiate this proposal.

Tissue loss upon cellular stress is always accompanied with an increased metabolic work of hepatocytes to maintain homeostasis. Namely, hepatocyte proliferation is certain to lead to the restoration of functional capacity [37]. In this regard, to further examine whether the hepato-stimulating property was intrinsic in hepatic function of MTA1, in the third experimental setting, we also demonstrated that AML12 cells stably expressing His-tagged MTA1, triggered to progress through the cell cycle in culture, displayed higher levels of albumin secretion, urea production and cell cycle-specific markers-indicated proliferative capacity compared with the control group. AML12 was a hepatocyte line derived from livers of transgenic mice overexpressing transforming growth factor α, a potent hepatocyte mitogen, which overcome hepatic proliferation limitations as mentioned above. Like normal hepatocytes, AML12 cells could express high levels of mRNA for serum (albumin, al-antitrypsin, and transferrin) and gap junction (connexins 26 and 32) proteins, secrete albumin during long-term culture, thus providing an ideal *in vitro* model to study liver-associated genes [38].

Our observations strongly suggested that MTA1 functions not only in hepatocyte growth but also in hepatic differentiation.

Overall, the data presented in this study indicate that MTA1 is positively involved in the hepatic regeneration process after PHx and overexpression of this histone modifier contributes to both the hepatocyte proliferation and the restoration of hepatocellular functional capacity. Therefore, the exact underlying mechanism of the accelerating role of MTA1 in liver regeneration is an area of great interest for the therapeutic treatment of impaired liver.

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