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Role of Metallothionein in Transcriptional Regulation by Metal-Responsive Element-Binding Transcription Factor 1

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Owing to the high metal binding affinity of metallothionein (MT), newly synthesized MT was speculated to attenuate the activity of metal-responsive element (MRE)-binding transcriptional factor 1 (MTF-1) by removing Zn from the activated MTF-1. To investigate the potential role of MT in the inactivation of MTF-1, we examined the transcriptional levels of reporter and endogenous MRE-dependent genes using mouse embryonic fibroblasts (MEFs) established from MT-knockout (KO) and wild-type (WT) mice. The activation of MTF-1 by the Cd exposure of MT-KO MEFs was sustained for 12 h, whereas that of MT-WT MEFs showed the rapid attenuation. Consequently, MT was found to negatively regulate MTF-1 activity, which can control the expression of MT itself.

Key words metallothionein, MTF-1, MRE, cadmium, zinc

INTRODUCTION

Metallothionein (MT) is a heavy metal-binding small protein with a molecular weight of *ca.* 6500 Da. About 30% of all amino acids in MT is cysteine.[1)] Because of these structural features, MT has high affinity and capacity to bind to heavy metals, such as zinc (Zn), copper (Cu), cadmium (Cd), and mercury (Hg). The metal binding affinity of MT contributes to both the minimization of the cytotoxicity of toxic heavy metals[2,3)] and the maintenance of the homeostasis of essential metals.[4)] In addition, MT has roles as an antioxidant and a radical scavenger.[5)]

The expression of classical MT proteins, namely, MT-1 and MT-2, is induced by metals,[6,7)] cytokines,[8,9)] and oxidative stress[10,11)] *via* transcriptional *cis* elements, such as glucocorticoid-responsive elements, antioxidant-responsive elements, and metal-responsive elements (MREs).[12)] The MRE sequence is 5'-TGCRCNC-3' (R, purine nucleotides; N, any nucleotides), which is well conserved among mammals and most frequently appears in MT promoter regions.[13,14)] Metal-responsive element-binding transcriptional factor 1 (MTF-1) is a transcriptional factor corresponding to MRE. Several studies suggest the activation of MTF-1 by processes such as phosphorylation[15)] and small ubiquitin-like modifier (SUMO) conjugation.[16)] However, the activation of MTF-1 is basically attributed to the binding of Zn to Zn finger motifs in MTF-1, which allows MTF-1 to bind to MRE.[17,18)] The lack of Zn finger motifs in MTF-1 affects not only the DNA binding but also the nuclear localization of MTF-1.[19)] In addition, the chelation of Zn with EDTA decreases MTF-1 activity,[20)] whereas treatment with Zn leads to a significant increase in MTF-1 activity (*i.e.*, the nuclear localization and DNA binding of MTF-1) with Zn dose.[14,17)] It is suggested that MTF-1 is an essential factor for the transcription of MT and other MRE-regulated genes.[21–25)] Therefore, since MTF-1 is required for the proper growth and development of mammals, MTF-1 null mutation is embryonically lethal owing to liver impairment, and MTF-1-knockout cells exhibit a higher susceptibility to Cd and H_2O_2 than control cells.[23)]

MT can also be induced by treatment with metal elements other than Zn, such as Cu, Cd, and Hg. Additionally, physiological conditions such as hypoxia[26)] or oxidative stress[11,27)] are inducers of MT. Regarding these inducers, MTF-1 is activated through the re-distribution of Zn in a cell, namely, the release of Zn from MT or other Zn-containing proteins, and the binding of released Zn with MTF-1.[28,29)]

After the induction of sufficient amounts of MT, the activation of MTF-1 should be switched-off. That is, the induced MT proteins can inactivate MTF-1 by removing Zn atoms from the activated MTF-1, followed by the downregulation of MT expression. However, little is known about the effect of MT on MTF-1 activity and the negative feedback regulation of MT itself. In this study, to elucidate the potential role of the MT protein in the regulation of MTF-1 activity, changes in the transcriptional levels of reporter and endogenous genes under MRE regulation were investigated using mouse embryonic fibroblasts (MEFs) established from MT-knockout (KO) and wild-type (WT) mice. Our results provide a new insight into the functions of MT on the activation/inactivation of MTF-1 and the subsequent regulation of MRE-dependent genes, including MT.

MATERIALS AND METHODS

Reagents Milli-Q water with a specific resistance of 18.3 MΩ cm (Millipore, Tokyo, Japan) was used throughout the study. High-purity cadmium acetate dihydrate (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used to

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activate MTF-1 in cells. Phosphate buffer saline (PBS, pH 7.4) for cell washing was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture Fibroblasts originating from both WT and MT-KO mouse embryos (hereafter, MT-WT MEFs and MT-KO MEFs, respectively) were kindly provided by Professor Seiichiro Himeno and Dr. Hitomi Fujishiro (Tokushima Bunri University, Japan).[30] Both the MT-WT MEFs and MT-KO MEFs were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Biosera, Nuaille, France) and a mixture of 100 U/mL penicillin and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemical Corporation) at 37°C under 5% CO_2 atmospheric condition. The cells were passaged until they reached about 80% confluence using 0.025% trypsin/EDTA in PBS. In this study, the cells were cultured in metal-free DMEM for 24 h before Cd exposure to enhance the effect of Cd on the activation of MTF-1 and to minimize the differences in initial amounts of MT between MT-WT MEFs and MT-KO MEFs. Metal-free DMEM was prepared by removing divalent or trivalent metals from FBS through chelation with Chelex® 100 resin (Bio-Rad, Hercules, CA) before adding the FBS to DMEM. Serum-free DMEM was also prepared by mixing DMEM and antibiotics noted above and used in recovery culture after Cd exposure to evaluate the inactivation of MTF-1 without dilution of cellular Cd concentration through cell proliferation.

Cell Viability Assay of Cd-exposed MT-WT and MT-KO MEFs Cell viability was measured and compared between two cell lines, namely, MT-WT, and MT-KO MEFs. In this experiment, MT-WT and MT-KO MEFs were seeded in a 96-well plate at a density of 1.0×10^4 cells in each well, and cultured in metal-free DMEM. After incubation for 24 h, metal-free DMEM was removed, and the cells were washed with PBS. Both MT-WT and MT-KO MEFs were exposed to Cd at concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, and 50 μM in serum-free DMEM, and incubated for 3 h in serum-free DMEM. Then, the Cd-containing medium was removed, and the cells were cultured in serum-free DMEM for another 3 h. Cell viability was determined by MTS assay as follows. A 20 μL aliquot of MTS reagent (Celltiter 96® Aqueous One Solution Cell Proliferation Assay, Promega) was added to each of the wells, and incubated for another 2 h. Absorbance at 490 nm was measured using a microplate reader (EMax, Molecular Devices, San Jose, CA, USA).

Evaluation of MTF-1 Activity by Luciferase Reporter Assay Both MT-WT and MT-KO MEFs suspended in metal-free DMEM were seeded in a 96-well plate at a density of 1.0×10^4 cells in each well. A plasmid vector encoding luciferase (pGL4.22-FTpro) in the downstream of the human metallothionein 2A (hMT-2A) promoter was kindly provided by Professor Fuminori Otsuka and Dr. Tae Shimoyama (Teikyo University, Tokyo Japan). MT-KO MEFs were transfected with the plasmid by lipofection using FuGENE® HD (Promega, Fitchburg, WI, USA) for 24 h. The transfected MT-WT and MT-KO MEFs were cultured in serum-free medium containing Cd with 0, 0.5, 1.0, and 2.5 μM. After the Cd exposure for 6 h, the intensity of luminescence of luciferin was measured using a multimode microplate reader (FilterMax F5, Molecular Devices) with Bright-Glo luciferase assay reagent (Promega). In addition, the luminescence of luciferin was measured in the two cell lines exposed to Cd at 0.5 μM at different exposure times (*i.e.*, 0, 3, 6, 12, and 24 h).

Evaluation of Expression Levels of Endogenous MRE-dependent Genes The expression of the genes in the downstream of functional MREs such as zinc transporter 1 (*ZnT1*), γ-glutamylcysteine synthetase (*γ-GCS*), and Zrt/Irt-like protein 10 (*Zip10*) was evaluated by the real-time RT-PCR method. Zn transporters such as ZnT7 and Zip1, which were independently regulated from MREs, were also evaluated. Both MT-WT and MT-KO MEFs suspended in metal-free DMEM were seeded in a 6-well plate at 1.0×10^5 cells in each well and cultured for 24 h. After washing with PBS, the cells were exposed to Cd at 1 μM for 3 h in serum-free DMEM. Then, the Cd-containing medium was removed from the wells and cells were cultured with a fresh serum-free DMEM (*i.e.*, recovery culture). Recovery culture was conducted for monitoring the change in gene expression levels which can be affected by the MT proteins newly synthesized after the Cd exposure. The cell samples were collected 0, 3, 6, 9, and 12 h after the start of recovery culture. After washing the cells with PBS, total RNA was isolated from the cells with ISOGEN II (NIPPON GENE, Tokyo) on the basis of the manufacturer's protocol. Using the extracted mRNAs, reverse transcription reaction was carried out at 37°C for 15 min and at 50°C for 5 min with ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka). Gene-specific primers for amplification of cDNAs are as follows: *ZnT1*-forward (F), 5′-TCCTGATCCCTGCAAATCGTC-3′; *ZnT1*-reverse (R), 5′-GTTGGGTCTAGGTAGAGCACC-3′; *γ-GCS*-F, 5′-GGACAAACCCCAACCATCC-3′; *γ-GCS*-R, 5′-GTTGAACTCAGACATCGTTCC-3′; *Zip10*-F, 5′-GCTGAACTGCCCATCAAAGC-3′; *Zip10*-R, 5′-TCACTGTGAGCAACGGAGTC-3′; *ZnT7*-F, 5′-TGTTCGGCAAGATCTCAGGC-3′; *ZnT7*-R, 5′-AGTTGCTCCAGATGCCGTAG-3′; *Zip1*-F, 5′-CCTGGCTGCCATAGATGAGG-3′; *Zip1*-R, 5′-CCAGGACCAGGAAGAAACCC-3′; *MT2*-F, 5′- GATCTCGAGCCACCATGGACCCCAACTGC-3′; *MT2*-R, 5′- CTAGTCGACTCAGGCACAGCAGC-3′ *β-actin*-F, 5′-CCTAAGGCCAACCGTGAAAA-3′; *β-actin*-R, 5′-AGGCATACAGGGACAGCACA-3′. PCR was repeated 40 times under the following conditions: 98°C for 5 s for denaturation, 60°C for 20 s for annealing and extension. The amount of cDNA was measured by detecting SYBR® Green fluorescence (Brilliant III Ultra-Fast SYBR Green QPCR Master Mix, Agilent Technologies, Tokyo, Japan) using a plate reader (Mx3000P, Agilent Technologies). The measurements were performed in triplicate for each sample, and the relative expression levels of the target genes against *β-actin* were normalized by the same ratio data for the MT-WT MEFs that were not exposed to Cd at 0 h after the start of recovery culture.

Statistical Analyses The uncertainties in luciferase activity, mRNA expression level, and cell viability were defined by the standard deviation (SD) calculated from the data of replicate analyses (n=3–5) for each sample. In this study, differences between groups were examined by one-way analysis of variance followed by Student's t-test. When the two-sided p-value was less than 0.05, the differences between the groups were considered statistically significant.

RESULTS AND DISCUSSION

Cell Viability of MT-WT and MT-KO Exposed to Cd The MT-KO was more susceptible to Cd (LC_{50} = 4.5 μM) than the MT-WT (LC_{50} = 13 μM) (Fig. 1), which was consist-

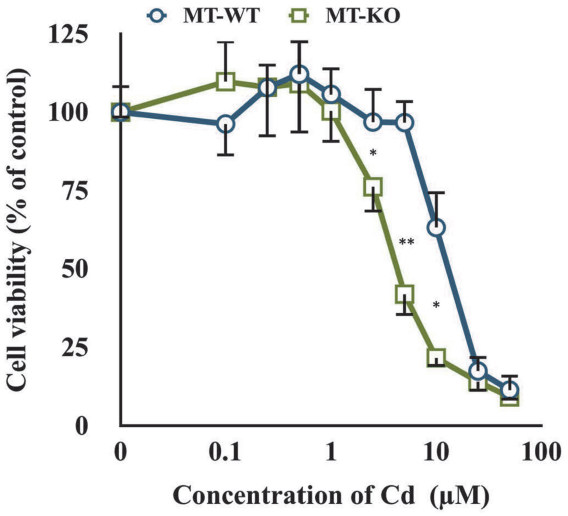

Fig. 1. Cell Viability in MT-WT and MT-KO

Cells were exposed to Cd for 3 h. Points and bars are means ± SD for 5 wells. Statistical analysis included the one-way analysis of variance followed by the Student's t-test. The level of significance was donated as *($p < 0.05$) and **($p < 0.01$) in comparison between MT-WT and MT-KO.

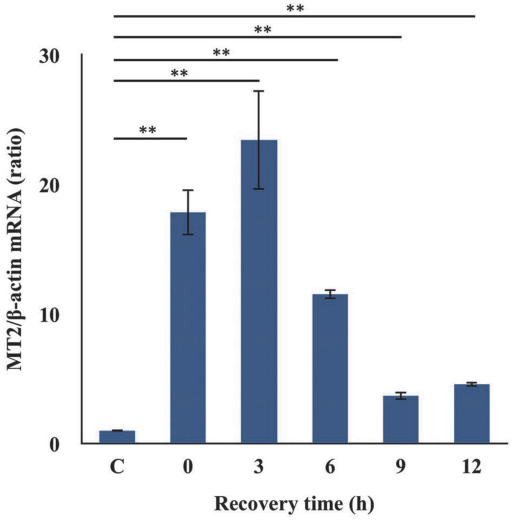

Fig. 2. mRNA Expression Levels of MT2 for MT-WT MEFs

The level of significance was set at **($p < 0.01$) in the comparison between control (without Cd, 0 h recovery culture) and Cd exposed cells collected at 0, 3, 6, 9, and 12 h after the start of recovery culture.

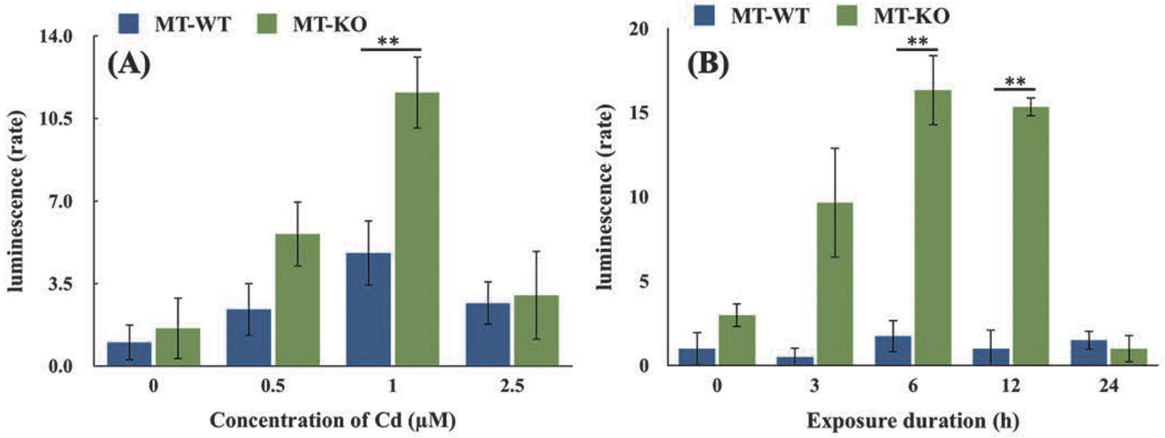

Fig. 3. MRE-Driven Reporter Assay of MT-WT and MT-KO MEFs

A) Cd-concentration-dependent luminescence intensity in MT-WT and MT-KO MEFs exposed to Cd for 6 h. B) Cd-exposure-duration-dependent luminescence intensity in MT-WT and MT-KO MEFs exposed to 0.5 μM Cd. Values and bars are means ± SD for 5 wells. Statistical analyses included one-way analysis of variance followed by Student's t-test. The level of significance was set at **($p < 0.01$) in the comparison between MT-WT and MT-KO MEFs.

ent with the previously reported data by Kondo *et al*.[30] These results indicated that MT plays a key role on detoxification against Cd.[31,32] Cd exposure induces MT at a transcriptional level through activation of MTF-1.[29] Indeed,, the expression levels of *MT-2* for MT-WT MEFs were increased by Cd exposure and remained high level through the recovery culture for 6 h (Fig. 2). Therefore, both endogenous and induced MT contribute to increase in the cell viability of MT-WT. Based on these results, MT-WT and MT-KO MEFs are useful cell lines to evaluate effects of MT on mechanisms for the activation/inactivation of MTF-1.

Changes in Gene Expression Induced by Cd Exposure

The luminescence intensity of luciferin increased with Cd concentration (Fig. 3A) and exposure duration (Fig. 3B) in both Cd-exposed MT-WT and MT-KO MEFs. When Cd was taken up by cells, free Cd ions are bound to basal MT and other proteins to minimize cellular damage.[31,32] In addition, oxidative stress is enhanced by Cd exposure through the consumption of antioxidants such as glutathione and the inhibition of the mitochondrial respiratory chain,[33,34] resulting in the oxidation of thiols (-SH). These toxicological responses lead to the release of Zn from basal MT and other Zn-containing proteins, followed by the activation of MTF-1 by the released Zn.[29] Indeed, the increased luminescence intensity in MT-WT and MT-KO MEFs exposed to Cd was attributable to MTF-1 activated through these processes. The luminescence intensity in 1 μM-Cd-exposed MT-KO MEFs was significantly higher than that in 1 μM-Cd-exposed MT-WT MEFs, indicating that MTF-1 activity became higher in MT-KO MEFs than in MT-WT MEFs following the Cd exposure (Fig. 3A). Since MT-KO MEFs have no basal MT, greater oxidative stress could be induced, contributing to the MTF-1 activation. In fact, MT-KO MEFs generated more reactive oxygen species than MT-WT MEFs under physiological condition, even without Cd exposure.[35]

In addition to the oxidative stress-mediated MTF-1 activa-

tion, direct interaction between MT and MTF-1 can be considered. One possible explanation is that MT proteins inactivate MTF-1 by the removal of Zn atoms from the activated MTF-1. The time-course experiments showed that the MTF-1 activity in MT-KO MEFs became significantly high 6 and 12 h after the Cd exposure at 0.5 μM, whereas the MTF-1 activity in MT-WT MEFs was not significantly increased at this Cd concentration (Fig. 3B). The elevated MTF-1 activity in MT-KO MEFs 6 and 12 h after the Cd exposure indicates that Zn is not removed from MTF-1 due to the lack of MT proteins. It should be noted that transcriptional levels of MTF-1 is not affected by Cd exposure.[36] Thus, the presence/absence of MT proteins indeed affects the difference in luminescence intensity, namely difference in MTF-1 activity between MT-WT and MT-KO MEFs. The luciferase activity in MT-KO MEFs was reduced to the same level as that in MT-WT MEFs 24 h after the exposure. During this exposure period, Cd toxicity appeared in MT-KO MEFs but not in MT-WT MEFs.

The luciferase activity was not significantly increased for MT-WT MEFs under Cd exposure at 0.5 μM. This result implies that the MTF-1 activity in MT-WT MEFs is reduced faster than that in MT-KO MEFs. Then, we examined the reduction rate of MTF-1 activity based on the changes in transcriptional levels of endogenous MRE-dependent genes through the recovery culture.

The changes in the transcriptional levels of endogenous MRE-dependent genes were also investigated in both MT-WT and MT-KO MEFs exposed to 1 μM Cd (Figs. 4A–4C). The expression levels of the *ZnT1* and *γ-GCS* immediately increased following the Cd exposure because these two genes are positively regulated by the MTF-1 activation in both MT-WT and MT-KO MEFs (Figs. 4A and 4B). After the recovery culture to remove Cd, the induction of these genes rapidly attenuated in MT-WT MEFs. In contrast to MT-WT MEFs, the induction of the genes in MT-KO MEFs remained up to 12 h after the beginning of recovery culture. The expression level of Zip10 was reduced following the Cd exposure in both MT-WT and MT-KO MEFs because this gene was negatively regulated by the MTF-1 activation (Fig. 4C). The expression level of the *Zip10* in MT-WT MEFs rapidly increased to the same level as that in the control at the beginning of the recovery culture, whereas that in MT-KO MEFs remained low until 12 h after the beginning of the recovery culture. Since other transcriptional factors than MTF-1 can present for *ZnT1*, *γ-GCS*, and *Zip10*, the responses of transcriptional level through the recovery culture were different among the genes. Despite this, our data clearly indicated that MTF-1 activity was sustained in MT-KO MEFs, resulting in the prolonged regulations of not only the reporter gene but also the endogenous genes, whereas MTF-1 activity immediately recovered to the basal level in MT-WT MEFs. Although Cd is preferentially bound to MT proteins, due to the recovery culture with Cd-free medium, Cd was immediately removed from the cell and the effects of newly synthesized MT on MTF-1 activity were clearly observed in MT-WT MEFs. Our data show that MT proteins directly contributed to the regulation of the MTF-1 activity in the cells. The mechanism is speculated to be as follows. Newly synthesized MT can inactivate MTF-1 by removing the Zn atom from the Zn fingers of MTF-1. MTF-1 is a nuclear protein and is unable to freely localize between the nucleus and the cytoplasm owing to its molecular size (>80 kDa). In contrast to MTF-1, MT is a small protein whose size allows it to freely move between the nucleus and the cytoplasm. Since MTF-1 is a nuclear protein, its immediate inactivation outside of the nucleus seems to be difficult immediately after the induction or suppression of MRE-dependent genes. Hence, newly synthesized MT can inactivate MTF-1 by removing Zn from its critical Zn finger(s) owing to its free access to the nucleus.

ZnT7 and Zip1 also act as Zn transporters but are MRE-independently regulated, unlike MRE-dependent genes such as ZnT1 and Zip10; however, no apparent differences in the expression levels of these MRE-independent genes were found following the Cd exposure between MT-WT and MT-KO MEFs (Figs. 4D and 4E). These findings indicate that MT directly participates in the transcriptional regulation of MRE-dependent Zn transporters, and also suggest the possible effect of MT on the regulation of MT itself.

Potential Role of MT onto Transcriptional Regulation of MRE-dependent Genes In this study, we intend to propose a potential role of MT as a negative regulatory factor for MTF-1 activity functioning within the nucleus. It has been demonstrated that MT is present in not only the cytosol but also the nucleus.[37] The translocation of MT into the nucleus occurs during the early stage of cell differentiation, possibly owing to higher Zn requirements for the metabolism in the nucleus related to the differentiation.[38] MT is also concentrated in the nucleus in the S phase of the cell cycle.[39] Moreover, we previously reported that MT translocation into the nucleus reflects the oxidation status of cytoplasm suggesting that MT plays an important role in protection of the nucleus from oxidative stress.[40] In addition, a potential role of MT as a metal chelator to a nucleus has also been examined for various transcription factors with the Zn-binding motif, such as Sp1,[41] TFIIIA,[42] and the estrogen receptor.[43] These studies revealed that apo-MT removed Zn from the transcription factors and suppressed their Zn-dependent DNA binding. For MTF-1, Kimura *et al*. reported that transient reduction in the DNA binding activity of MTF-1 in WT mice was observed 8 h after the administration of lipopolysaccharide (LPS), while the reduction was not observed in MT-KO mice.[44] These findings support our scenario that MT newly synthesized enters to a nucleus via nuclear pores and inactivates MTF-1 as a Zn chelator.

CONCLUSION

We examined the effects of MT on the transcriptional regulation by MTF-1. MT can reduce the activity of MTF-1 increased by metals. As the mechanism of the role of MT in the inactivation of MTF-1, we propose that newly synthesized MT removes Zn from the Zn finger motifs of MTF-1. This interaction seems to facilitate the rapid inactivation of MTF-1. Namely, MT, which can freely move between the nucleus and the cytoplasm removes Zn from MTF-1 specifically localized in the nucleus. The precise mechanisms of the activation of MTF-1 is still unclear. Since MTF-1 in MT-KO MEFs is more continuously activated than MT-WT MEFs, MT-KO MEFs are expected to be a useful cell line for evaluating the mechanism of MTF-1 activation induced by various factors, such as heavy metals, oxidative stress, and glucocorticoids.

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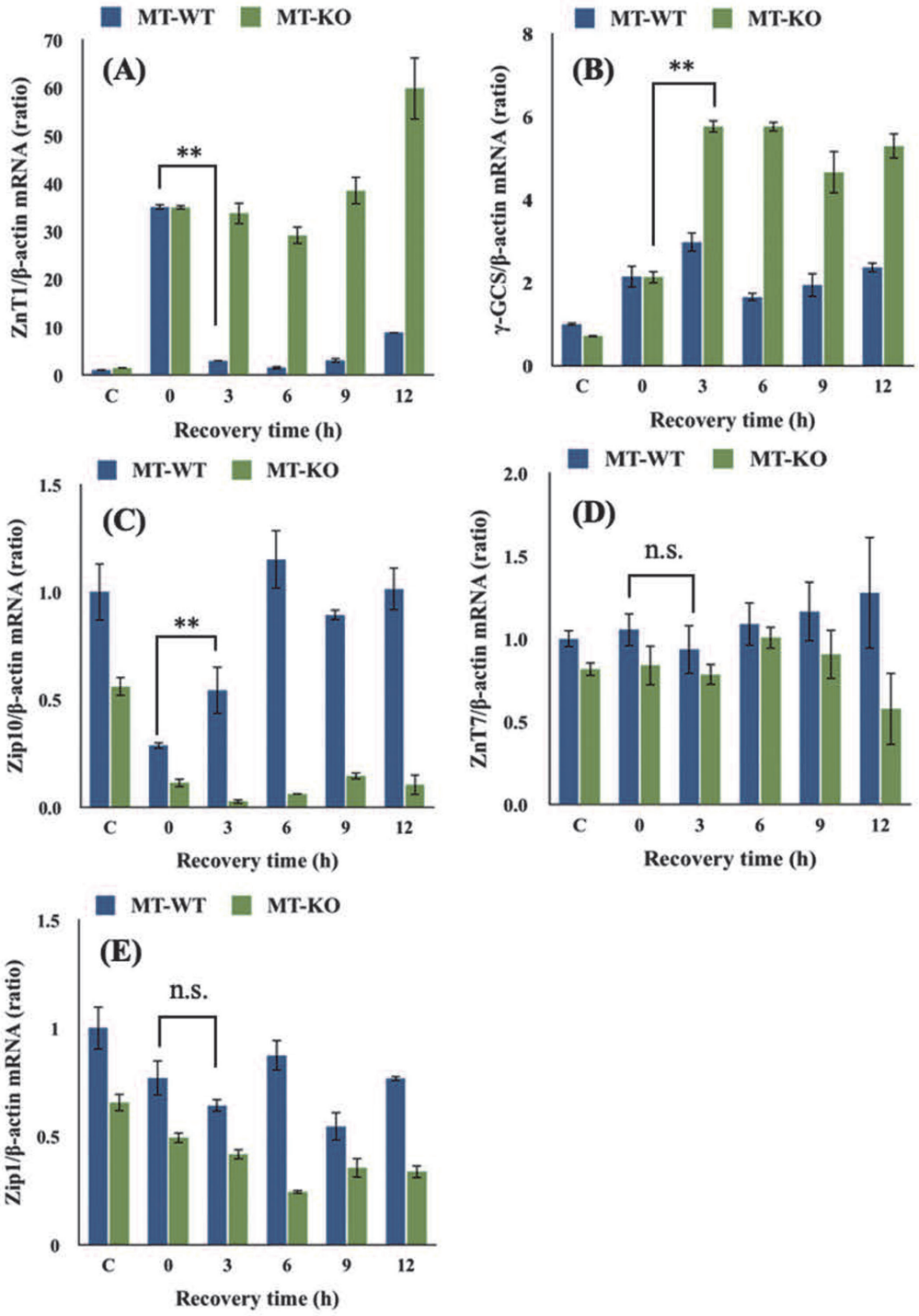

Fig. 4. mRNA Expression Levels of MRE-Dependent and MRE-Independent Genes

Cells were exposed to 1 μM Cd for 3 h before the start of recovery culture. C: No cadmium exposure. Values and bars are means ± SD for 3 wells. Statistical analysis included one-way analysis of variance followed by Student's *t*-test. The level of significance was set at ** ($p < 0.01$).

providing MT-WT and MT-KO MEFs. We also thank Professor Fuminori Otsuka and Dr. Tae Shimoyama for kindly providing the reporter plasmid.

Conflict of interest The authors declare no conflict of interest.

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