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P53 Mediated Regulation of Metallothionein Transcription in Breast Cancer Cells

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Abstract Recent studies have shown that only breast cancer epithelial cells with intact p53 can induce metallothionein (MT) synthesis after exposure to metals. In this study, the potential role of p53 on regulation of MT was investigated. Results demonstrate that zinc and copper increased metal response elements (MREs) activity and MTF-1 expression in p53 positive MN1 and parental MCF7 cells. However, inactivation of p53 by treatment with pifithrin-α or the presence of inactive p53 inhibited MRE-dependent reporter gene expression in response to metals. MTF-1 levels remained unchanged after treatment with zinc in cells with nonfunctional p53. The introduction of wild-type p53 in MDD2 cells, containing nonfunctional p53, enhanced the ability of zinc to increase MRE-dependent reporter gene expression. The cellular level of $p21^{Cip1/WAF1}$ was increased in MDD2 cells after p53 transfection, confirming the presence of active p53. The treatment of MN1 and parental MCF7 with trichostatin A led to a sixfold increase in the MRE activity in response to zinc. On the contrary, MRE activity remained unaltered in MDD2 cells with inactive p53. The above results demonstrate that activation of p53 is an important factor in metal regulation of MT. J. Cell. Biochem. 102: 1571–1583, 2007.

Key words: metallothionein; p53; metal responsive elements; metal responsive transcription factor 1

The p53 tumor suppressor protein is an important regulator of cell cycle progression and apoptosis in response to stress. Phosphorylation and acetylation modify p53 which increase both stability and concentration of the protein in response to stress. The activated p53 promotes transcription of various target genes including $p21^{WAF1/Cip1}$, bax, mdm2, and Noxa [Miyashita and Reed, 1995; Oda et al., 2000; Rich et al., 2000; Stewart and Pietenpol, 2001; Yu et al., 2001] and interacts with several proteins involved in cell proliferation and apoptosis [Singh et al., 2002; Chipuk et al., 2003]. The metal-induced stress results in phosphorylation of p53 and up-regulation of $p21^{WAF1/Cip1}$, Bax, and PIG3 [Lag et al., 2002; Harris and Shi, 2003; Ostrakhovitch and Cherian, 2004, 2005]. Loss of p53 activity or mutation of tumor suppressor genes leads to disruption of normal cell homeostasis, chromosomal abnormalities, and malignant transformation [Levine, 1997; Harris and Levine, 2005]. It has been reported that mutations of p53 occur in more than half of all human tumors, and that this mutation is associated with unfavorable prognosis of survival for patients [Feki and Irminger-Finger, 2004; Olivier et al., 2004; Soussi and Lozano, 2005; Vousden and Prives, 2005].

Metallothioneins (MTs), 6–7 kDa proteins, have been proposed to be involved in the homeostasis of essential metals because of their metal binding properties. MT can modulate both cell proliferation and apoptosis [Abdel-Mageed and Agrawal, 1997; Jin et al., 2002], and can protect the cells from metal-induced stress and chemotherapeutic agents. Most mammalian tissues show low basal level of MT, whereas overexpression of MT has been reported in human tumors including breast cancer, ovarian cancer, renal cell carcinoma, and pancreatic carcinoma [Ohshio et al., 1996; Cherian et al., 2003; Jin et al., 2004; Gallicchio et al., 2005;

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Surowiak et al., 2005]. A strong association has been observed between MT expression and tumor progression and aggressiveness. The expression of MT genes is regulated by a number of regulatory elements, including cis-acting metal responsive element (MRE), which is present in multiple copies with a highly conserved sequence [Stuart et al., 1984]. In addition to the MREs, the MT gene promoters contain one or more copies of other elements that initiate transcription. Among them are sequences for AP-1, AP-2, and Sp-1 [Klaassen et al., 1999; Andrews, 2000]. The metal-responsive transcription factor 1 (MTF-1) regulates basal and metal-stimulated expression of MT via binding to MREs. However, it is also possible that there are other, as yet unknown transcriptional factors that may mediate the MT transcription.

The translocation of MT into the nucleus during G1-S phase of the cell cycle in tumor cells suggests that MT can facilitate cell proliferation by donating zinc ions to various transcription factors including the p53 tumor suppressor protein [Apostolova et al., 2000; Shimoda et al., 2003]. It has been suggested that p53 status can modulate MT expression [Jin et al., 2004], and that p53 mutated cell lines resistant to apoptosis are unable to induce MT after metal treatment [Fan and Cherian, 2002]. The stabilization and binding of p53 to specific DNA sequences entirely depend on zinc binding. It has been shown that refolding into the wild-type form of p53 (wt-p53) requires the presence of micromolar amount of zinc ions [Meplan et al., 2000a; Hainaut and Mann, 2001]. Incubation of p53 with recombinant MT in a ratio of 1:1 led to an activation of p53 suggesting that MT can act as a zinc donor and regulates functional activity of p53 [Meplan et al., 2000b].

This study was undertaken to investigate the potential role of p53 on regulation of MT expression. Our results indicate that in breast cancer epithelial cells, a functional p53 can modulate the activities of MREs and also factors such as MTF-1 which are required for MT transcription.

MATERIALS AND METHODS

Cell Culture

Human breast cancer epithelial cell line MCF7, estrogen receptor (ER)-positive (ER+) with wt-p53 (p53+) was obtained from ATCC. MCF7-E6 cells are derived from epithelial breast cancer MCF7 cells, constitutively expressing the E6 protein from human papilloma virus and have no p53 activity. MCF-E6 cells were obtained from Dr. Fornace (NIH, Bethesda, MD). The MN1 cell line, containing wt-p53 and the MDD2 cell line, a variant derived from MCF7 by transfection with an inactive p53 (pCMV-DD-p53 encodes the first 14 amino acids and residues 302–390) [Shaulian et al., 1992], were generously provided by C.M. Galmarini, (Laboratoire de Cytologie Analytique, Faculté de Médecine Rockefeller, France). MN1 cells were obtained by transfection of the MCF7 line with the empty plasmid. All cell lines were maintained in DMEM medium containing 10% fetal bovine serum (both from Gibco, Burlington, Canada), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin).

Determination of the Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Cells were treated with copper (100 μM) or zinc (100 μM) for 14 h, and the mitochondrial membrane potential was analyzed in the cells by staining them with 10 μM of JC-1 (Molecular Probes, Eugene, OR) for 10 min. The intensity of fluorescence was immediately measured at 527 and 590 nm emission wavelength (with excitation at 488 nm), corresponding to the fluorescence peak of the monomer and that of the aggregate, respectively. Proportion of cells with depolarized mitochondria was calculated as a ratio of the intensities at 590 nm (red) and 527 nm (green). Cells stained with JC-1 were visualized using a Zeiss Axiovert 100 TV fluorescence microscope (Carl Zeiss, Germany).

RNA Preparation and RT-PCR

Total RNA was extracted from MCF7, MN1, and MDD2 cells using RNeasy Mini kit (Qiagen). First strand cDNA was synthesized from 2 μg total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, Canada). Aliquots of cDNA were amplified with primers specific for MTF-1 (forward primer, 5′-CCACAACACAATGGATCAGAGGA-3′; reverse primer, 5′-GAGTTGGCACCCAGGGGCAG-3′) and β-actin (forward primer, 5′-CATCCTCACCCTGAAGTACC-3′; reverse primer, 5′-CACCATTCATGATGGACTTGA-3′). PCR was carried out as follows: one denaturation cycle at 94°C for 4 min, followed by

25 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C. Aliquots of the PCR mixtures were separated on 2% agarose gel, stained with ethidium bromide and photographed.

Plasmid Constructs

The p53-CMV-TnT expression vector was constructed using the human p53 gene product corresponding to the NM 000546 accession number. *EcoR1* was used to extract the insert of a p53-pBluescript KS(−), and the insert was ligated into a *EcoRI* pre-digested alkaline-phosphatase treated pCMV-TnT. Orientation of insert was determined by using *SmaI* and insert identity was confirmed by sequencing. The 6MRE containing promoter region (nucleotides −793 to +5) and the 4AP1 containing promoter region (nucleotides −867 to −785) and TATA box from the rainbow trout MTA gene were isolated using PCR with forward and reverse primers, containing a *KpnI* and *HindIII* site respectively, and inserted into the *KpnI-HindIII* site of the pGL3 basic vector (Promega, Madison, WI). The pRL-SV40 (Promega) containing the SV40 enhancer/promoter region, which provides strong, constitutive expression was used to normalize the expression level of 6MRE-pGL3 and 4AP1-pGL3. For visualization of MRE activation the 793 bp fragment was also inserted into the multiple cloning site of the pEGFP reporter vector (Stratagene).

Transfection Assay

Cells were transiently transfected with 0.8 μg of the 6MRE-Luc, the 6MRE-EGFP or the p53 expression vector and 1 ng of the control luciferase construct pRL-SMV (Promega) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were treated with Zn^{2+} or Cu^{2+} in complete DMEM medium for 14 h. MDD2 cells were also transfected with p53-pCMV-TnT 5 h before these cells were transfected with 6MRE-Luc. Twenty-four hours latter, medium was replaced and cells were exposed to Zn^{2+} for 14 h.

Luciferase Reporter Assay

Cells were transfected with the plasmids (0.8 μg 6MRE-Luc and 0.01 μg pRL-CMV) and were allowed to incubate in the presence of the transfection mixture in DMEM with 10% fetal bovine serum for 24 h followed by replacement with fresh medium. After 14 h exposure to either copper or zinc in complete DMEM medium, the cells were harvested in 1× lysis buffer (Promega) and luciferase activity was measured using the dual luciferase reporter system (Promega) in a luminometer (Microtiter plate Luminometer, MLX, Dynex Technologies, Chantilly). Results were expressed as the ratio of firefly luciferase activity to Renilla luciferase activity.

Western Blot Analysis

Western blotting was performed as described previously [Ostrakhovitch and Cherian, 2005]. The following antibodies were employed: anti-$p21^{Cip/WAF1}$ (Santa Cruz Biotechnology, Inc., CA), anti-MT (Dako Cytomation, Inc., Mississauga, Canada), and anti-MTF1 (a kind gift of G.K. Andrews, University of Kansas Medical Center, Kansas City, KS). Loading of equal amount of protein was confirmed by blotting with anti-GAPDH antibody (HyTest Ltd, Turku, Finland).

Immunofluorescence

Cells were fixed in ice-cold methanol for 10 min, washed twice and fixed with 4% paraformaldehyde for additional 20 min. These cells were blocked with 5% goat serum in PBS and incubated with anti-MT. Positive cells were visualized using goat anti-rabbit Alexa 488 conjugated secondary antibody (Molecular Probes) using a Zeiss Axiovert 100 TV fluorescence microscope (Carl Zeiss).

Zinc Measurement

The concentration of total zinc in MN1 and MDD2 cells was determined by using a Varian Spectra 800 atomic absorption spectrophotometer (AAS) (Varian, Australia) equipped with an air-acetylene flame. Briefly, cell lysates were digested in 100% nitric acid at room temperature overnight, and were then heated to 95°C for 1 h in order to facilitate digestion. Zn concentrations were recorded from a standard curve and normalized to protein concentration.

Data Analysis

Data are presented as means + SE of three to five independent experiments. Densitometric quantitation of Western immunoblots was performed using Scion Image software for Windows (Scion Corporation, Frederick, MD). Statistical analysis was performed using two-group

comparisons by means of Student's *t*-test; a *P*-value below 0.05 was considered statistically significant.

RESULTS

Effect of p53 Status on Sensitivity to Metal Ions

The sensitivity to metal-induced stress was compared in two cell lines derived from the human epithelial breast cancer MCF7 cells with different p53 status: the MN1 cell line containing wt-p53 and the MDD2 line, containing p53 miniprotein with the deletion of residues 15–301. In MDD2 cells, the existing short C-terminal stretch of p53 has been shown unable to exhibit any transcriptional activation potential [Shaulian et al., 1992]. To confirm that in MDD2 cells p53 miniprotein indeed is transcriptionally inactive the levels of $p21^{CIP1/Waf1}$ protein, which is transcriptionally regulated by p53, were analyzed (Fig. 1A). Exposure of MN1 cells to 100 μM of cadmium, zinc or copper led to an increase in p21 protein levels, whereas there was no increase in p21 after metal treatment in MDD2 cells, with inactivated p53 (Fig. 1A).

The cells undergoing apoptosis can be detected by an increase in the PARP fragmentation. The amount of cleaved PARP (89 kDa) was elevated in MN1 exposed to 100 μM copper and zinc (Fig. 1B). However, no cleavage of PARP was observed in copper and zinc treated MDD2 cells. An important characteristic of early apoptosis is changes in the mitochondrial membrane potential ($\Delta\Psi_m$) that can result in mitochondrial membrane permeabilization [Zamzami et al., 1996]. Therefore, we monitored the cells with a specific probe JC-1. Loss of mitochondrial membrane potential was observed only in MN1 cells after 14 h of exposure to copper and zinc at

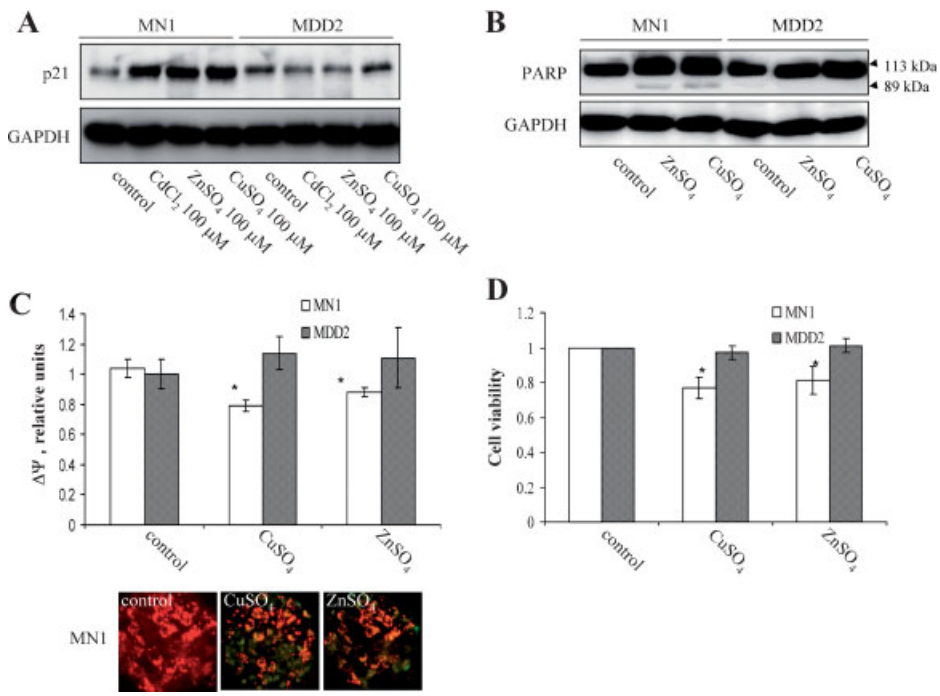

Fig. 1. Analysis of $p21^{Cip1/WAF1}$, mitochondrial membrane potential and PARP cleavage. **A**: Immunoblot analysis of $p21^{Cip1/WAF1}$ expression levels in MN1 and MDD2 cells exposed to cadmium, zinc, and copper. The cells were treated with indicated concentration of metal ions for 14 h. The results are representative of three different experiments. **B**: An increase in PARP fragmentation in MN1 cells. PARP cleavage following exposure to 100 μM copper or 100 μM zinc for 14 h in MN1 cells. Cell lysates were analyzed by Western blotting using anti-PARP antibody. **C**: Changes in mitochondrial membrane potential ($\Delta\Psi_m$) following exposure to 100 μM of copper or zinc for 14 h in MN1 and MDD2 cells. $\Delta\Psi_m$ was analyzed in the cells by staining them with 10 μM of JC-1. The asterisk sign (*) denotes values versus control ($P < 0.05$). Mitochondrial membrane potential in MN1 cells treated with copper and zinc stained with JC-1 and analyzed with fluorescent microscopy. The untreated control shows a strong red fluorescence with a high membrane potential (J-aggregate form of JC-1), whereas incubation with the metal ions results in a decrease in the membrane potential and cells showing green fluorescence due to the monomer form of JC-1. **D**: MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was used to test the cell viability. MN1 and MDD2 cells were treated with copper or zinc in complete DMEM medium for 14 h. Results are presented as means + SE of four independent experiments. The asterisk sign (*) denotes values versus control ($P < 0.05$).

100 μM (Fig. 1C). Taken together, these data indicate that both metals induce apoptosis in MN1 cells expressing functional p53, whereas MDD2 cells with nonfunctional p53 were resistant to metal-induced stress. The viability of MN1 cells was also decreased from 100% to 77% at 100 μM $CuSO_4$ exposure (Fig. 1D). About 20% decrease in cell viability was observed when MN1 cells were exposed to 100 μM $ZnSO_4$. The MDD2 cell line was resistant to both copper and zinc as compared to MN1 cell line and showed no change in viability after exposure to metals. In agreement with our previous data, these results suggest that cell sensitivity to metal ions depends on p53 status of cells [Ostrakhovitch and Cherian, 2005; Fan and Cherian, 2002].

Comparison of MT Expression in p53 Positive MN1 and MCF7 Cells, and in p53 Inactive MDD2 Cells

In MN1 cells and in their parental MCF7 cells, the basal level of MT protein was low, but an increase in MT protein was detected after metal treatment for 14 h. Zinc at 100 μM was the most potent inducer of MT protein (Fig. 2B). Unlike MCF7 and MN1 cells, the MDD2 cells, with inactive p53, had high basal levels of MT but the induction of MT synthesis was small after exposure to metals, and was apparently not comparable to high levels of MT induction in MN1 cells. This was confirmed by the basal level of MT-2A mRNA determined by semi-quantitative RT-PCR and was higher in MDD2 cells than in MN1 cells. The mRNA levels did not change to metal exposure (Fig. 2A). Zinc even at high concentrations (300, 500 μM) did not activate MT transcription in MDD2 cells. In MN1 cells the expression of MT mRNA was significantly enhanced in response to treatment with metals. Localization of MT in MN1 and MDD2 cells using immunocytochemistry was in agreement with results from Western blot analysis. Low MT immunostaining was detected in untreated MN1 cells, whereas intense MT staining was detected in untreated MDD2 cells (Fig. 2C). Notably, MT was mainly localized in the nucleus in MDD2 cells. To demonstrate that high basal level of MT in MDD2 cells was not due to alterations in concentration of intracellular zinc, we measured the level of zinc in

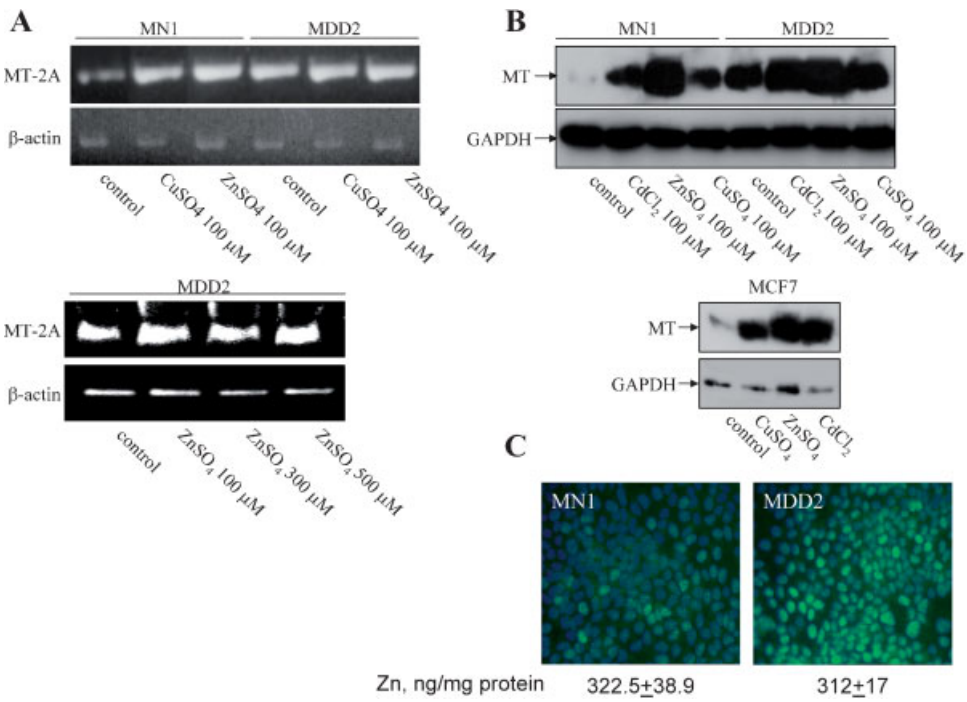

Fig. 2. Metallothionein expression following inactivation of p53. Metal-induced expression of metallothionein in MN1, MCF7, and MDD2 cells. The cells were exposed to 100 μM $CdCl_2$, 100 μM $ZnSO_4$, or 100 μM $CuSO_4$ for 14 h. **A**: RT-PCR analysis of MT-2A mRNA levels. Total RNA was subjected to RT-PCR analysis with MT2A and β-actin-specific primers. **B**: Metallothionein protein levels following different treatments. The results are representative of three individual experiments. **C**: Subcellular localization of metallothionein and total level of zinc in MN1 and MDD2 cells. The cells were fixed and stained with anti-MT antibody. The cells were counterstained with Hoechst 33342 and analyzed by fluorescent microscopy. Total zinc level in cells was measured by atomic absorption spectrometry (AAS).

both cell lines. The total level of zinc did not differ between MDD2 and MN1 cells (Fig. 2C). These results suggest that regulation of MT by metal ions requires the presence of an active p53 and that high basal level of MT in p53 deficient MDD2 cells is not due to modulation in intracellular concentration of zinc.

Expression of MTF-1 in p53 Positive and p53 Inactive Cells

Previous reports have shown that MTF-1 transcription factor activity is exclusively regulated by zinc, whereas other metals like cadmium and copper indirectly activate it by mobilization of intracellular zinc [Koizumi et al., 1999; Smirnova et al., 2000; LaRochelle et al., 2001]. The MN1 and parental MCF7 cells had low basal level of MTF-1 mRNA (Fig. 3A,C) but treatment with 100 μM $ZnSO_4$ for 14 h strongly induced MTF-1 (Fig. 3A). While a significant basal level of MTF-1 mRNA was expressed in p53 inactive MDD2 cells, the MTF-1 transcriptional level remained unchanged following zinc treatment. To further explore the role of p53 in metal-induced MT expression, we examined the ability of pifithrin-α, an inhibitor of p53, to reduce MTF-1 expression. Treatment of parental MCF7 cells with pifithrin-α resulted in a slight increase in basal level of MTF-1 expression and an inhibition of MTF-1 mRNA expression induced by zinc (Fig. 3B). The basal MT protein level was slightly increased in pifithrin-α treated MCF7 cells. However, pretreatment with pifithrin-α suppressed the MT expression in response to 100 μM $ZnSO_4$. To gain more insight into p53 mediated MT expression, we determined MTF-1 protein and observed that in wt-p53 MCF7 and MN1 cells treatment with zinc led to increase in MTF-1 protein levels (Fig. 3C). MCF7-E6 and MDD2 cells, with disrupted p53 activity, were not responsive to zinc treatment as compared to wt-p53 parental cells.

Comparison of MRE Activation in p53 Positive Cells and in Cells With Inactive p53

In p53 positive MN1 cells, there was a significant dose-dependent increase in 6MRE-Luc mediated luciferase activity in response to zinc and copper (Fig. 4A). In contrast, in p53 inactive MDD2 cells, only weak 6MRE-Luc reporter activity was observed in response to zinc and copper. The basal level of MRE luciferase activity in MDD2 cells was slightly less as compared with basal level in MN1 cells ($1.4 + 0.17$ in MN1 cells vs. $0.92 + 0.09$, $P < 0.005$). These data were verified by monitoring MRE activation using a 6MRE-GFP expression vector (Fig. 4B). In parental p53 positive MCF7 cells, the maximal activation of MRE activity was obtained with $ZnSO_4$ (Fig. 4C). Treatment of MCF7 cells with an inhibitor of p53, pifithrin-α, at 30 μM concentration blocked MRE activity in response to metals. The basal level of MRE luciferase activity was decreased when MCF7 cells were treated with pifithrin. Thus, these results suggest that transactivation of MREs by metals, in particular by zinc and copper, requires the presence of an active p53.

Involvement of p53 in Zinc-Induced Response of MRE and MTF-1 Expression

To further ascertain the relationship between p53 and accumulation of MTF-1 protein in response to zinc treatment, MDD2 cells were transiently transfected with the wt-p53 expression vector containing the complete coding sequence of wt-p53. The transfection of MDD2 cells with wt-p53-pCMV-TnT plasmid enhanced the ability of Zn^{2+} to increase MRE activity (Fig. 5A). In order to confirm the activity of p53 in MDD2 cells after transfection with p53 expression vector, the cellular levels of $p21^{Cip1/WAF1}$ were examined and it was observed that p53 expression partially restored the ability of Zn^{2+} to enhance the $p21^{Cip1/WAF1}$ expression (Fig. 5B). The p53 dependence on zinc-induced increase of MRE activity was also confirmed by 6MRE-GFP co-transfected with wt-p53 expression vector in MDD2 cells. In contrast to nontransfected MDD2 cells, a strong increase in MTF-1 protein level was detected after zinc treatment of wt-p53 transfected MMD2 cells (Fig. 5C). The introduction of wild-type p53 into MDD2 cells resulted in reduction of MT-2A mRNA basal level, and the expression of MT mRNA was enhanced in response to zinc treatment (Fig. 5D). Taken together, these results demonstrate that inactivation of p53 can diminish accumulation of MTF-1 protein after treatment with zinc ions.

Inhibitor of Histone Deacetylase, Trichostatin A (TSA), Activate MRE in Response to Copper and Zinc in p53 Positive Cells But Fail to Induce MRE in p53 Negative Cells

Treatment of MN1 and parental MCF7 containing wt-p53 with 300 nM TSA significantly

induced MRE expression in the presence of Zn^{2+} (Fig. 6). There was about sixfold increase in the MRE activity in response to zinc in the presence of TSA. In contrary, MRE activity remained unaltered in MDD2 cells with inactive p53, after treatment with zinc in the presence of TSA (Fig. 6A). Pretreatment of MCF7 cells with 30 μM of pifithrin-α suppressed MRE-dependent

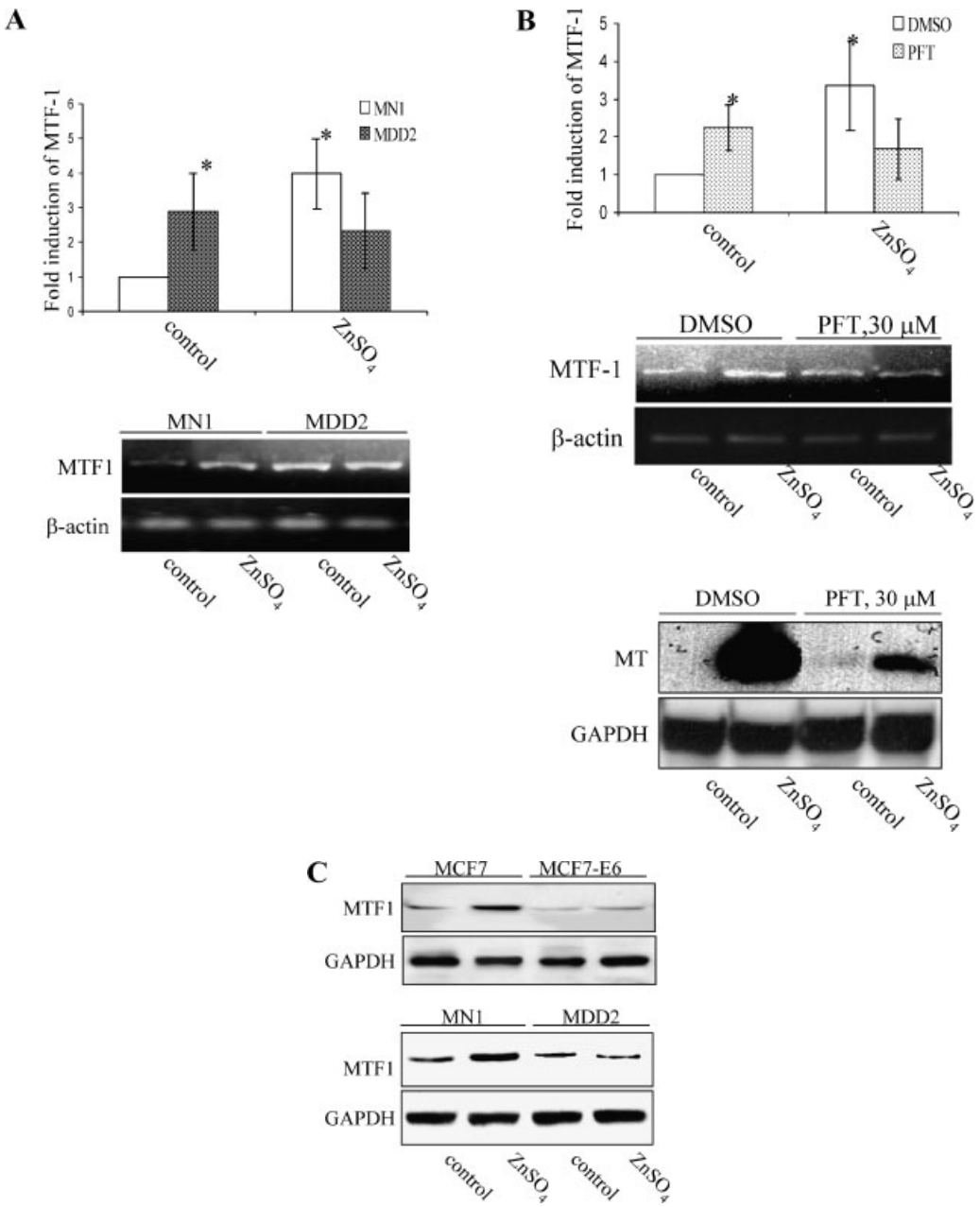

Fig. 3. Differential expression of MTF-1 in cells with wt-p53 and cells with inactive p53. RT-PCR analysis of MTF-1 mRNA expression in MN1 and MDD2 cells (**A**), and MCF7 cells (**B**). Either inactivation of p53 activity in MDD2 cells or inhibition by pifithrin-α in MCF7 cells results in increase in the accumulation of MTF-1 mRNA. A: Comparison of control MN1 and MDD2 cells and cells treated with 100 μM $ZnSO_4$ for 14 h. B: MCF7 cells were pretreated with DMSO or 15 μM of pifithrin-α for 2 h, followed by 14 h exposure to 100 μM $ZnSO_4$. Total RNA was subjected to RT-PCR analysis with MTF-1 and β-actin-specific primers. The densitometric analysis of MTF-1 mRNA levels is presented as means ± SE of three individual experiments. The asterisk sign (*) denotes values versus control ($P < 0.01$). Metallothionein protein levels following treatments with pifithrin-α and metal ions were analyzed by Western blot. **C**: Comparison of expression of MTF-1 in MN1 and MCF7 cells with wild-type p53 and MDD2 and MCF7-E6 with inactive p53. Cells were treated with 100 μM $ZnSO_4$ for 14 h. The results are representative of three independent experiments. Equal protein loading was confirmed by analysis of GAPDH protein level.

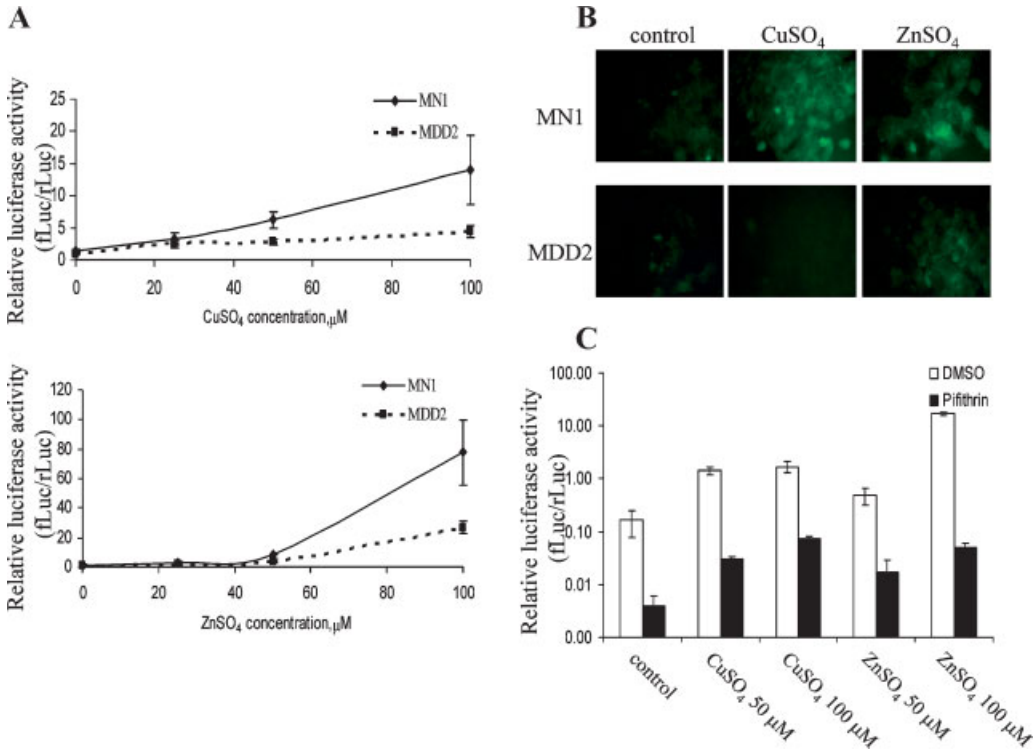

Fig. 4. Comparison of MRE activity in p53 positive and p53 negative cells. Cells were transfected with either the 6MRE-Luc (**A**) or 6MRE-EGFP (**B**) construct. Cells were exposed to $ZnSO_4$ and $CuSO_4$ for 14 h. A: Time course of induction of 6MRE-Luc in response to metal treatment of MN1 and MDD2 cells. Renila luciferase was included as an internal control. Results are expressed as the ratio of firefly luciferase activity to Renilla luciferase activity. The average values with SE from four to five independent experiments are shown. B: Visualization of MRE activity using MN1 and MDD2 cells transfected with 6MRE-EGFP construct. MRE was examined with Carl Zeiss Axiovert 100 fluorescence microscope. **C**: Suppression of p53 activity by pifithrin-α in MCF7 cells. MCF7 cells were transfected with luciferase reporter plasmid 6MRE-Luc. These cells were pre-treated with or without 15 μM of pifithrin-α for 2 h followed by exposure to $ZnSO_4$ and $CuSO_4$ for 14 h. Results are expressed as the ratio of firefly luciferase activity to Renilla luciferase activity. The average values with SE from three to five independent experiments are shown.

luciferase expression after treatment with zinc. Furthermore, TSA was unable to increase the MRE activity in these cells (Fig. 6B).

Comparison of AP-1 Activation in p53 Positive Cells and in Cells With Inactive p53

Activator protein-1 (AP-1) induced by metals can regulate the MT gene expression through AP-1 binding sites in MT promoter region [Lee et al., 1987]. To study the involvement of AP-1 in p53-mediated regulation of MT, MN1, parental MCF7, and MDD2 cells were transfected with the AP1-Luc reporter construct. In p53 positive MN1 and MCF7 cells, there was a significant increase in luciferase activity in response to added zinc (Fig. 7). The basal level of AP1 activity in MDD2 cells with inactive p53 was similar to that in MN1 cells. However, in the MDD2 cells, transfection with the AP1-Luc reporter resulted in much smaller degree of response to zinc exposure. These data suggest that increase in the basal level of MT expression in cells with inactive p53 is not due to the action of AP1.

DISCUSSION

A potential interaction between MT and p53 has been demonstrated in a recent study [Ostrakhovitch et al., 2006]. It has been reported that MT can modulate functional activity of p53 by zinc transfer and facilitate DNA-binding conformation of p53 [Meplan et al., 2000a]. On the other hand, p53 may be involved in MT induction by metals in epithelial breast cancer cells [Fan et al., 2002; Cherian et al., 2003]. However, there is limited evidence on a direct role of p53 on regulation of MT synthesis. We have analyzed the activation of MRE after exposure to MT inducing metals such as zinc and copper in cells with different p53 status, and show that the presence of a

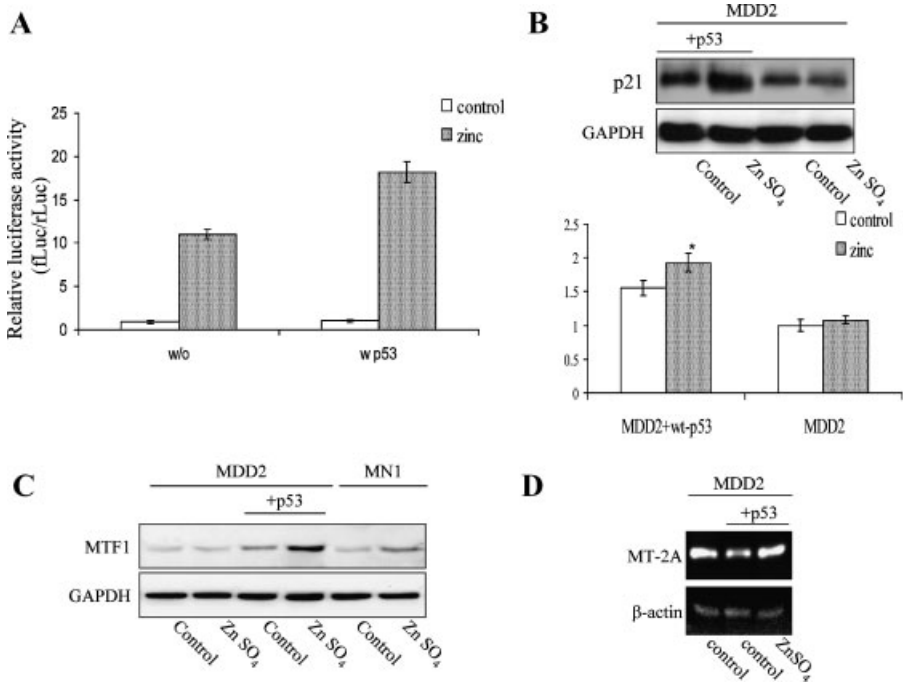

Fig. 5. Overexpression of p53 restores zinc-induced MRE activation. Cells were treated with 100 μM $ZnSO_4$ for 14 h. **A**: MDD2 cells were co-transfected with 6MRE-Luc plasmid and p53-pCMV-TnT plasmid containing the complete coding sequence of wt-p53. Results are expressed as the ratio of firefly luciferase activity to Renilla luciferase activity. The average values with SE from three independent experiments are shown. **B**: The expression of $p21^{Cip1/WAF1}$ was analyzed in MN1 and MDD2 cells, nontransfected or transfected with wt-p53 expression vector. The asterisk sign (*) denotes values versus control ($P < 0.05$). **C**: Expression of MTF-1 in MN1 with wt-p53 was compared to nontransfected and p53-pCMV-TnT transfected MDD2 cells. **D**: RT-PCR analysis of MTF-1 mRNA levels in MDD2 cells, nontransfected or transfected with wt-p53 expression vector.

functional p53 is required in metal-induced expression of MT in breast cancer cells. Furthermore, we observed that inactivation/mutation of p53 results in elevated nonmetal-regulated levels of MT whereas the restitution of wt-p53 function led to the decrease in basal level of MT mRNA and increase in induction of MT by metals. It has been reported that increased MT expression and translocation to the nucleus increase the rate of cell proliferation [Abdel-Mageed and Agrawal, 1997; Apostolova et al., 2000; Shimoda et al., 2003] and the elevation of MT levels following p53 inactivation may contribute to the unfavorable prognosis of these tumors. These results suggest a new biological function for p53 in MRE-mediated control of MT expression. The MRE is a binding site for the transcription factor MTF-1, which is essential for basal and metal-induced MT expression [Heuchel et al., 1994]. To further characterize the role of p53 in expression of MT, we examined the induction of MTF-1 in cell lines containing active or nonfunctional p53. During metal induced MT synthesis, MTF-1 is activated and translocated to nucleus. Moreover, phosphorylation of MTF-1 is essential for it transactivation function [LaRochelle et al., 2001]. However, we also observed changes in MTF-1 mRNA along with metal-induced MT protein expression and MRE activation. MN1 and parental MCF7 cells with functional p53 had low basal levels of MTF-1 which were increased with zinc treatment. In agreement with our results, Hasumi et al. [2003] demonstrated the metal inducibility of MTF-1 mRNA in prostate cancer cells. In comparison to cells with functional p53, a significant basal level of MTF-1 mRNA was observed in p53 inactive MDD2 cells and that level remained unchanged with exposure to zinc. It is known that MTF-1 requires an elevated concentration of zinc for strong binding to DNA and facilitation of MT expression [Bittel et al., 1998; Chen et al., 1999]. However, the high basal levels of MT and MTF-1 were not due to increase in zinc concentration in MDD2 cells since the total zinc levels have remained relatively the same as in MN1 cells. Moreover,

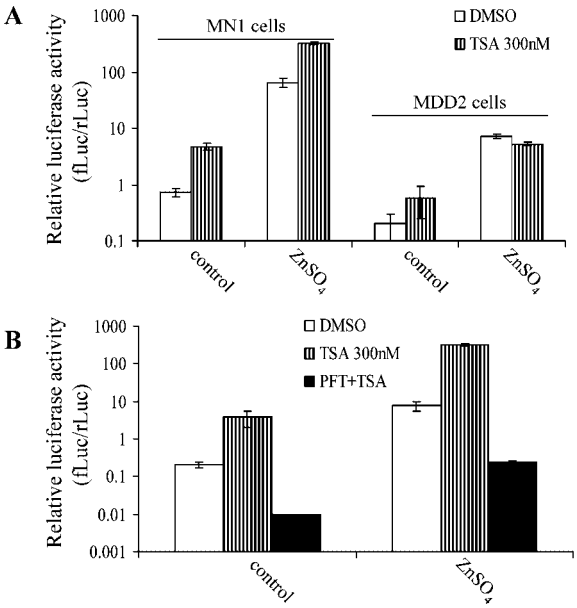

Fig. 6. MRE activity following TSA exposure. MN1 and MDD2 (**A**), and MCF7 (**B**) cells were transfected with luciferase reporter plasmid 6MRE-Luc. Cells were exposed to 100 μM $ZnSO_4$ or $CuSO_4$ for 14 h after pre-treatment with 300 nM TSA for 2 h. MCF7 cells were also pretreated with 15 μM of pifithrin-α combined with 300 nM TSA. Results are expressed as the ratio of luciferase activity to Renilla luciferase activity. The average values with SE from three to five independent experiments are shown.

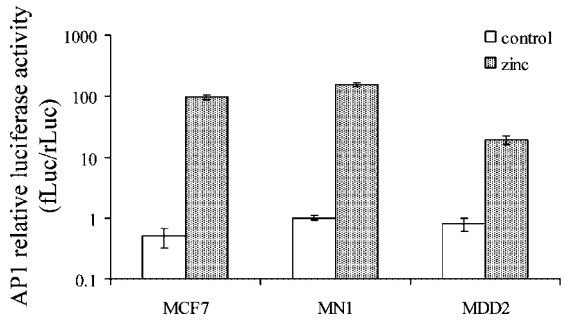

Fig. 7. AP1 activity in p53 positive and p53 negative cells. The AP1-Luc construct was introduced into MCF7, MN1, and MDD2 cells. Cells were exposed to 100 μM $ZnSO_4$ or $CuSO_4$ for 14 h. Results are expressed as the ratio of firefly luciferase activity to Renilla luciferase activity. The average values with SE from four-five independent experiments are shown.

zinc even at high concentrations (up to 500 μM) was unable to increase MT mRNA level in MMD2 cells. Consistent with our observations, Otsuka et al. [2000] also reported a significant level of expression of mRNA MTF-1 in HeLa cells; the treatment with either zinc or cadmium could not alter the level of mRNA MTF-1 expression. It should be pointed out that HeLa cells, which are HPV-18-positive human cervical carcinoma cells, contain very little of functional p53 protein, since generated p53 protein undergoes fast degradation promoted by E6 oncoprotein encoded by human papillomavirus 16 or 18 [Matlashewski et al., 1986; May et al., 1991]. Notably, although the basal level of MTF-1 mRNA was increased, the MTF-1 protein synthesis and 6MRE-Luc reporter activity were low in MDD2 cells. These results imply that up-regulation of MT in the absence of functional p53 is unlikely to be dependent on MTF-1. The rescue of p53 activity in MDD2 cells enhanced their ability to increase MRE activity and MTF-1 protein expression with zinc exposure. These results suggest that a functional p53 is required for metal-induced MRE-mediated activation of MT transcription. However, inactivation of p53 results in increased cellular accumulation of MT protein which is independent of metal accumulation, and MTF-1 and MRE activation.

In order to further investigate the mechanisms by which p53 can modulate metal-induced MT expression, we studied whether p53-dependent gene expression can be altered with changes in histone acetylation status. Since it has been reported that inhibition of histone deacetylases (HDAC) affects acetylation of promoters and increases both the expression on the MTF-1 gene and its DNA binding activity [Ghoshal et al., 2002], we investigated the effect of TSA on MRE binding activity in cells with different p53 status. In control cells with wt-p53, the histones associated with the MT-1 promoter are hypoacetylated and silenced the promoter [Ghoshal et al., 2002]. Treatment with TSA increased MRE activity in both control and metal-treated cells due to dissociation of HDAC associated with MT promoter, and hyperacetylation of the promoter. The inhibition of HDAC in cells with functional p53 also leads to stabilization of p53 [Roy et al., 2005] whereas stabilized p53 can displace HDAC1 from the promoter region of the p53 target gene, p21, resulting in up-regulation of p21 [Lagger et al., 2003]. In a similar mechanism, p53 may displace HDAC1 from the MT promoter and thereby facilitate MT expression in response to metals. Taken together, our results suggest that p53 together with HDAC1 is an important component of the machinery controlling MT expression. Furthermore, it has been recently shown that TFIID complex required for MT activation is recruited to the MT promoter and

remains in inactive state until the cofactor MED is recruited [Marr et al., 2006]. However, although binding to TATA box region is necessary, it is not sufficient for transactivation. On the other one hand, p53 has also been shown not only to stimulate TFIID-promoter complex assembly but activate the transcription by inducing conformational change in TFIID-promoter complex [Xing et al., 2001]. However, on the other hand, a recent study has shown that TFII undergoes degradation upon DNA damage in p53-dependent fashion [Desgranges et al., 2005]. It was also noted that TFII levels were higher in p53 null cells and cells expressing mutated p53. These data suggest that elevated basal MT expression in epithelial breast cancer cells expressing nonfunctional p53 might be due to an increase in TFII transcriptional activity. Therefore, these observations support our hypothesis of p53-mediated regulation of metal-induced MT expression.

Here, we demonstrated that the basal levels of MT expression in cells with nonfunctional p53 are independent of the metal ions. Moreover, the up-regulation of MT in cells with nonfunctional p53 was not regulated by AP1, the activity of which is increased under oxidative stress. Other possible triggers for metal-independent MT expression might be PI3 kinase-Akt signaling pathway because it has been shown that the presence of MT in the nucleus depends on the PI3 kinase-Akt signaling pathway [Apostolova et al., 2000]. In agreement with this hypothesis, recent report demonstrates that suppression of p27 up-regulates the MT expression implicating p53 and Akt on regulation of MT [Galizia et al., 2006]. The absence of active wild-type p53 can lead to up-regulation of Akt activity, thereby resulting in retaining of p27 in cytoplasm and disruption of cell cycle regulation that in its turn can affect expression of MT [Singh et al., 2002; Motti et al., 2004; Shin et al., 2005]. However, more research is needed to identify the mechanisms underlying the elevated basal levels of MT in cells with nonfunctional or mutated p53.

In summary, we provide evidence that p53 plays a crucial role in the transcriptional regulation of MT, which indicates a novel regulatory role for p53. Our results clearly demonstrate that active p53 is required for metal induced MRE and MTF-1-mediated activation of MT transcription whereas the high basal level of MT in the absence of active p53 is MRE, AP1, and MTF1 independent.

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