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**REVIEW ARTICLE**

# Metallothioneins and zinc in cancer diagnosis and therapy

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**Abstract**

Metallothioneins (MTs) are involved in protection against oxidative stress (OS) and toxic metals and they participate in zinc metabolism and its homeostasis. Disturbing of zinc homeostasis can lead to formation of reactive oxygen species, which can result in OS causing alterations in immunity, aging, and civilization diseases, but also in cancer development. It is not surprising that altered zinc metabolism and expression of MTs are of great interest in the case of studying of oncogenesis and cancer prognosis. The role of MTs and zinc in cancer development is tightly connected, and the structure and function of MTs are strongly dependent on $Zn^{2+}$ redox state and its binding to proteins. Antiapoptic effects of MTs and their interactions with proteins nuclear factor kappa B, protein kinase C, esophageal cancer-related gene, and p53 as well as the role of MTs in their proliferation, immunomodulation, enzyme activation, and interaction with nitric oxide are reviewed. Utilization of MTs in cancer diagnosis and therapy is summarized and their importance for chemoresistance is also mentioned.

**Keywords:** Apoptosis, cancer, resistance, metallothioneins, zinc, NF-κB, protein p53, prognostic factor, tumor marker

## Zinc and its role in cancer and noncancer cells

Zn(II) ions contribute to a number of biological processes, including DNA synthesis, gene expression, enzymatic catalysis, neurotransmission, and apoptosis. It is not surprising that zinc, as a trace element, is the second-most abundant metal in humans. Approximately 90% of zinc(II) ions are tightly bound, mostly by cysteine, histidine, and asparagine residues of peptides and proteins, and the rest (10%) is bound with relatively low affinities, forming a reactive zinc(II) pool able to interact with other intracellular substances and compartments (Franklin and Costello, 2009). The last, very small fraction (approximately <0.01% of total cellular zinc(II), ranging from pM to single-digit nM) includes free zinc(II) ions (Colvin et al., 2010). Because of the abundance of zinc(II) ions, more than 10% of mammalian proteome consists of zinc-containing proteins involved in cell signaling, gene expression, membrane-structure stability and function, cell respiration, and modulation of the redox state. From those, zinc is required for the activity of more than 300 enzymes, interacting with zinc-binding domains, such as zinc fingers, RING fingers, and LIM domains (John et al., 2010a).

$Zn^{2+}$ dysregulation, deficiency, and oversupply are connected with various pathologies in the form of diseases of the immune, gastrointestinal (GI), endocrine, and nervous systems, heart failures, hematologic diseases, wound healing, ocular functions, and neoplasms (Gumulec et al., 2011; Babula et al., 2011; Cummings and Kovacic, 2009). Displacement of $Zn^{2+}$ from zinc-binding structures, as zinc fingers in DNA-repairing enzymes, may even be a major mechanism for the carcinogenicity of other metals, such as cadmium, cobalt, nickel, and

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arsenic (Beyersmann and Hartwig, 2008). The role of zinc in cancer has received increasing attention. A link between zinc deficiency and cancer has been shown in numerous clinical studies (Ames, 2001; Fang et al., 2002), and it was found that zinc status is compromised in cancer patients, compared to healthy people (Dhawan and Chadha, 2010). This is clearly indicated in the ecological study, which was conducted using state-averaged cancer-mortality rate data for white Americans for 1970–1994 with indices for alcohol consumption, smoking, Hispanic heritage, urban residence, and dietary factors for four large U.S. regions. The dietary zinc index was inversely correlated with 12 types of cancer (Hodgkin's lymphoma, bladder, breast, colon, esophageal, gastric, rectal, laryngeal, nasopharyngeal, oral, skin, and vulvar cancers) (Grant, 2008). These results were also confirmed by Zuo et al. and Unal et al., who found decreased zinc levels in serum of leukemic patients (Zuo et al., 2006) and in serum of Hodgkin's disease patients, respectively (Unal et al., 2001). Moreover, decreased blood levels of antioxidants (i.e., retinol, α-tocopherol, and β-carotene) and zinc were found in childhood malignancies (Malvy et al., 1993, 1997), which could be one of the negative effects of zinc-metabolism alterations. Zinc deficiency has also been associated with oxidative stress (OS) (Eide, 2011) and esophageal, head and neck cancer, and prostate and other cancer types, especially in their development and progression (Franklin and Costello, 2007; Hogstrand et al., 2009; Ostrakhovitch, 2011; Fukada et al., 2011; Pedersen et al., 2009). The cancer risk resulting from zinc deficiency and OS was also positivelly correlated with a lack in DNA repairing (Ho, 2004). One of the possible explanations is that the OS under the low zinc concentrations is caused by damaging of the mitochondrial functions, because zinc is necessary for mitochondrial functions (Eide, 2011). In addition, cancer patients with zinc deficiency exhibited increased paraneoplastic cachexia and treatment-associated morbidity (Cummings and Kovacic, 2009).

One may suggest that excessive zinc supplementation would be beneficial, but this feature depends on the type of tumor disease, because Plum et al. and Ko et al. showed that excessive zinc supplementation may be immunosuppressive and may therefore increase the risk of prostate cancer (Plum et al., 2010; Ko et al., 2010) because of unique prostate cancer zinc metabolism (Gumulec et al., 2011). In spite of the situation in prostate cancer, it could be concluded that some supplementation with zinc(II) ions could be beneficial (Prasad and Kucuk, 2002). However, this is limited by the absence of a marker of its deficiency, because decreased serum-zinc concentrations are a late sign of its deficiency. Depression of immune-stimulated tumor necrosis factor alpha (TNF-α) secretion by leukocytes seems to be such a marker, but there are some technical disadvantages of this method (Ryu et al., 2011).

It is clear that the role of zinc in tumor diseases is still not satisfactorily answered and needs to be further investigated. One piece of the puzzle could be the maintaining of zinc homeostasis, which is critical under healthy and disease conditions (Murakami and Hirano, 2008), because zinc must be available in proper concentrations in the right place at the right time. There are no specific zinc-storage systems, therefore its homeostasis is achieved by regulation of zinc uptake, distribution, and excretion (Maret and Krezel, 2007). Membrane transporters and intracellular regulators, from which (apo)metallothioneins (apo-MTs) seem to be the most important ones, are of great interest (Hao and Maret, 2005). Various types of tumor cell lines have been used to investigate the cellular effects of zinc(II) ions and its connection with metallothioneins (MTs) (Figure 1), because it still has not been clarified whether zinc may directly act on cancer cells and what the molecular mechanisms are that are involved in this effect. Apoptosis belongs to the mostly targeted issue relating to Zn(II). It was found that zinc concentrations within the range from 33.7 to 75 μM induced apoptosis in mouse TS/A mammary adenocarcinoma cells, and that the induced apoptosis was associated with the increased production of intracellular reactive oxygen species (ROS), as well as p53 and Fas/Fas ligand (FasL) messenger RNA (mRNA) and protein. $Zn^{2+}$ induced only a faint MT response in cancer cells, in comparison with mouse lymphocytes. The treatment of tumor cells with the antioxidant, *N*-acetylcysteine, was able to prevent zinc-induced apoptosis, as well as the increase in p53 and FasL proteins induced by zinc. This indicates that zinc exerts a direct action on mammary cancer cells inducing ROS-mediated apoptosis, and that the effect may be mediated by the ROS-dependent

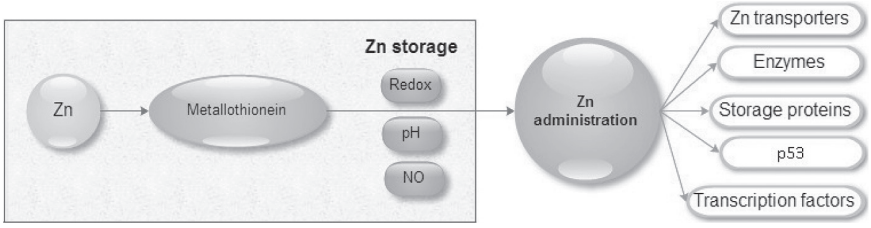


Figure 1. Interactions of MTs with zinc(II) ions are influenced by three main conditions: 1) redox state of MT; 2) pH of the environment; and 3) the presence of NO. If these conditions support the formation of the zinc/metallothionein complex, which is, under the healthy physiological state, a common phenomenon, MTs can serve as "administrators" of zinc(II) ions for zinc transporters, enzymes, storage proteins, and transcription factors. As one of the highlighted issues, interactions of Zn-MTs with protein p53 are mentioned.

induction of p53 and Fas/FasL (Provinciali et al., 2002). Variability of responses to increased external zinc concentration was shown in three colon cancer cell lines, with different sensitivities to zinc representing different stages of carcinogenesis. The most sensitive cell lines exhibited the increased levels of the intracellular free zinc and the inability to overexpress MTs. Mechanisms of zinc-induced cell injury and cell death revealed OS as the most important underlying mechanism activating stress kinase-dependent signaling, perturbation of mitochondria, and plasma membrane damage. In addition, observed cell death in individual cell populations was cell-line–dependent and variable, including cells displaying features of apoptosis, necrosis, autophagy, and other mixed types of cell death (John et al., 2010b). Based on the above-mentioned facts, there are some connections between MTs and zinc and/or OS, which are summarized below.

## MTs

MTs are ubiquitous metal-binding proteins that have been highly conserved throughout evolution. These proteins were discovered by Margoshes and Valee as cadmium-binding proteins isolated from horse kidney in 1957 (Margoshes and Vallee, 1957). Subsequently, their being involved in heavy metal homeostasis, OS coping, gene-expression and transcription regulation, enzyme activation, apoptosis, and cell proliferation have been found (Thirumoorthy et al., 2007; Theocharis et al., 2003). Four major isoforms (MT-1 through MT-4) have been identified in mammals (Miles et al., 2000; Simpkins, 2000). MT genes are tightly linked, and, at a minimum, they consist of 11 MT-1 genes (MT-1A, -B, -E, -F, -G, -H, -I, -J, -K, -L, and -X) encoding functional or nonfunctional RNAs, and one gene for each of the other MTs isoforms (the MT-2 A, MT-3, and MT-4 genes) (Ghoshal and Jacob, 2001). The nomenclature for MT isoforms has not been standardized until recently (Ghoshal and Jacob, 2001). A gene called MT-like 5 (MTL-5) that encodes a testis-specific MT-like protein called tesmin was described in the q13 region of chromosome 11 (Olesen et al., 2004). Tesmin plays a specific role in both male and female meiotic prophasis (Olesen et al., 2004). The specific functional roles of MT isoforms and their molecular interactions are still unclear (Kagi and Schaffer, 1988). MT-1 and MT-2 are the most widely distributed MT isoforms. They are expressed in many cell types in different tissues and organs. Contrarywise, MT-3 and MT-4 demonstrate a very limited cell-specific pattern of expression. MT-3 represents a unique metalloprotein called also neuronal-growth inhibitory factor, which inhibits the outgrowth of neuronal cells (Huang, 2010). In comparison with MT-1 and MT-2, MT-3 shows distinct chemical, structural, and biological properties (Faller, 2010; Ding et al., 2010; Brewer, 2009; Bofill et al., 2009; Ba et al., 2009). Moreover, the connection of MT-3 to neurodegenerative processes is discussed. In addition, MT-4 belongs to noninducible proteins, with its expression primarily confined to certain squamous epithelia (Vasak and Meloni, 2011).

These cysteine-rich proteins are localized in cytoplasm and some organelles, predominantly in mitochondria, where their presence is sensitively and strictly regulated by the oxidative state induced by mitochondrial respiration (Banerjee et al., 1982). Reciprocal regulation of mitochondrial ROS production is evident (Suzuki et al., 2005; Futakawa et al., 2006). In addition, MTs are involved in the regulation of the permeability of the inner mitochondrial membrane (Simpkins et al., 1998). Cell- and tissue-specific regulation of cellular respiratory and energy metabolism in liver mitochondria are still discussed (Ye et al., 2001; Lindeque et al., 2010; Chiaverini and De Ley, 2010). From mitochondria, whose outer membrane pores admit molecules up to 10 kDa, MTs can be transported to cytoplasm and other target organelles. Lysosomes represent other places of MTs localization. The presence of MT, namely MT-3, is related to lysosomal changes and cell death in neurons under OS (Lee et al., 2010). The relation between iron-catalyzed intralysosomal peroxidative reactions, MT-protective effect, and OS is suggested in a study by Baird et al. (2006). Depending on the cell state, but especially the presence of OS, MTs are rapidly translocated to the nucleus through nuclear pore complexes (Nzengue et al., 2009). MT localized in the nuclei is oxidized there and is transported to the cytosol; this system is balanced (Takahashi et al., 2005). The translocation of MTs to the nucleus is probably connected with the prevention of cells against DNA damage and apoptosis as well as gene transcription during different stages of the cell cycle (Gunes et al., 1998; Chen et al., 2004; Formigare et al., 2007a, 2007b; Chen et al., 2007; Cherian and Apostolova, 2000; Müller, 2010), but also with the high extracellular concentration of glucose in certain cell types, as was demonstrated using human umbilical vein endothelial cells (Apostolova et al., 2001; Chen and Song, 2009). In these cells, MT expression is regulated by endothelin ET-1, whereof elevated levels were evidenced in diabetes mellitus patients.

In the postgenomic era, it is becoming increasingly clear that MTs fulfil multiple functions, including the involvement in zinc and copper homeostasis, protection against heavy metal toxicity, and oxidative damage (Adam et al., 2010). The regulatory power of the redox environment lies in its capacity to control growth behavior, spread, and differentiation (McGee et al., 2010; Cai et al., 1999; Sato and Bremner, 1993). Neoplastic cells adapt to a wide variety of environmental conditions, including persistent OS and genomic instability, by shifting their redox environment to more-reductive conditions, which, in turn, triggers the upregulation of various redox-sensitive prosurvival pathways, including MT transcription and translation (Ostrakhovitch, 2011). Therefore, there must exist some mechanisms for how tumor cells use MTs for their pathological behavior, including using MTs as transporters, inhibitors of apoptosis through interactions with some antiapoptotic proteins, stimulators of

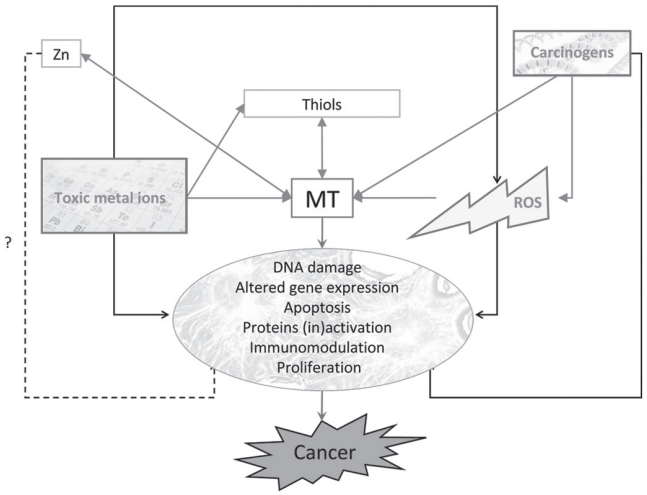


Figure 2. Scheme of connections of MTs with DNA damage, altered gene expression, apoptosis, protein (in)activation, immunomodulation, and proliferation leading to carcinogenesis. Indicated also are mutual associations with ROS, carcinogens, and low-molecular-mass thiols, including cysteine and reduced glutathione, toxic metal ions, and zinc.

proliferation, and as immunomodulator and enzyme activators (Figure 2).

## MTs as a binder of metal ions involved in carcenogenesis

The genes for MTs are clustered and are located on chromosome 16q12-22 in humans (Karin et al., 1984). Gene transcription is initiated when zinc(II) ions associate with metal-regulatory transcription factor-1 (MTF-1). MTF-1 is the only known mediator of the metal responsiveness of MTs (Ghoshal and Jacob, 2001; Gunes et al., 1998; Klassen et al., 2004). MTF-1 binds to metal-responsive elements (MREs) that regulate MT expression. MREs are located in the promoter regions of MT genes (Gunes et al., 1998) and are present in multiple copies in the promoter/enhancer regions of almost all metal-inducible MTs (Culotta and Hamer, 1989; Searle et al., 1985). However, there have been identified other metals, which are able to induce MT transcription and also pose a threat to cells because of their ability to promote carcinogenetic processes, such as $Cd^{2+}$ (Il'yasova and Schwartz, 2005; Waalkes and Rehm, 1994), $Cr^{6+}$, and $Ni^{2+}$ (Seo et al., 2005; Gumulec et al., 2011). One of their adverse actions is displacing zinc from zinc-saturated MTs or other zinc-binding proteins. Zinc binds to MTF-1 and promotes the formation of a complex containing MTF-1 and p300. The MTF-p300 complex then binds to DNA and triggers a transcription apparatus, leading to MT gene transcription (Kimura, 2010). Further, $Zn^{2+}$ released as a result of its replacement with carcinogenic metals then disrupts zinc-controlled processes (Waisberg et al., 2003; Liu et al., 2009). Proteins with $Zn^{2+}$ replaced by other metals are not able to perform their biological functions as zinc-fingers, in which zinc replacing by cadmium exhibits up to 10 times the decreased affinity to DNA that is caused by different conformation of key amino acids (Namdarghanbari et al., 2011). The generation of ROS and the altering of protein complex formation belong to other mechanisms of heavy metal carcinogenity. Cadmium can also serve as a transcription modulator, which influences the transcription of the genes involved in apoptosis, cell metabolism, cell signaling, and the expression of stress-response proteins, including MTs (Luparello et al., 2011; Koizumi and Yamada, 2003). $Cr^{6+}$, unlike other heavy metals, can inhibit MT transcription by preventing MTF-1/p300 complex formation, although increases in intracellular labile zinc concentration led to increased binding of MTF-1 to DNA. The decreased concentration of MTs then causes DNA lesions and decreases the direct reduction of $Cr^{6+}$ to $Cr^{3+}$ that is not carcinogenic (Kimura, 2010). Moreover, it was found that MTF-1 is elevated in human breast, lung, and cervical carcinoma-derived cell lines (Shi et al., 2010). This finding supports the importance of MTs in carcinogenesis.

## Inhibition of apoptosis

The ability of MTs to bind toxic heavy metals and detoxify free radicals can be both beneficial and deleterious. According to the titles of two popular reviews, MT is a multipurpose protein with two faces (Coyle et al., 2002; McGee et al., 2010). In healthy cells, MTs protect cells against heavy metals and ROS action (Klaassen et al., 2009), but in cancer cells, increased expression of MTs during chemotherapy or irradiation allows cells to survive and develop a resistance to chemo- and radiotherapy (Knipp, 2009; Boulikas and Vougiouka, 2003). MTs can also help cancer cells to survive by inhibition of apoptosis (McGee et al., 2010; Dutsch-Wicherek et al., 2008). The two main roles of MTs are regulation of intracellular zinc concentration and interaction of MTs with some proteins involved in apoptosis. Zinc is an intracellular mediator of apoptosis, which can interfere with the action of $Ca^{2+}$. Zinc addition prevents DNA fragmentation and inhibits many proteins connected to apoptosis, such as caspases and calcium-magnesium–dependent proteases (Dhawan and Chadha, 2010). Moreover zinc induces a transcription of the p53 gene, with increased expression of p53 mRNA and protein (Fan and Cherian, 2002). Some interactions between zinc, MTs, and some genes and proteins involved in apoptosis must be considered, because MTs interact with the p50 subunits of a nuclear factor kappa B (NF-κB), with a kinase domain of PKCl, with GTPase Rab3A and protein p53. Those interactions are important for tumor growth, because activation and/or inactivation of these proteins may mediate the antiapoptotic effect of MTs.

*NF-κB* MT-1 and MT-2 regulate the level, activity, and cellular location of the transcription factor, NF-κB (Butcher et al., 2004; Kim et al., 2003; Abdel-Mageed and Agrawal, 1998; Wang et al., 1999). NF-κB is necessary to ensure cell protection from the apoptotic cascade induced by TNF and other stimuli through activation of antiapoptotic genes and proto-oncogenes, such as

breast cancer lymphoma 2, c-myc, and TNF receptor-asscoiated factor 1. Overexpression of MT-2 sensitized rodent cells to apoptosis induced by a DNA cross-linking agent through inhibition of NF-κB activation (Papouli et al., 2002). Zinc has been suggested to be an important regulator of NF-κB. In HeLa cells, pyrrolidinedithiocarbamate (PDTC), a zinc ionophore, and zinc itself inhibited NF-κB activity. When cells were pretreated with MT inducers, PDTC did not inhibit NF-κB activity. HeLa cells overexpressing MT-2A did not exhibit an inhibition of NF-κB activity by PDTC. These results implicate MTs in the zinc regulation of NF-κB and identify MTs as one of the potential intracellular modulators of NF-κB activation (Kim et al., 2003).

*Zinc fingers* MTs can transfer zinc to zinc fingers of transcription factors. Therefore, MTs influence the binding of transcription factors to DNA and thus regulate transcription (Vallee et al., 1991). Zinc transfer from transcription factor IIIA fingers to thionein clusters was observed (Zeng et al., 1991; Huang et al., 2004), as well as at transcription factor IIA (Zeng et al., 1991), zinc-finger transcription factor Zn(3)-SP1 (Kothinti et al., 2010), and estrogen receptor zinc finger (Canogauci and Sarkar, 1996). Zinc fingers, in which zinc is replaced with cadmium, exhibit lower affinity to DNA as a result of a different conformation of DNA-binding domains (Malgieri et al., 2011). It was observed that binding of zinc-finger peptides to DNA can be modulated by the MT/thionein conjugate pair (Roesijadi et al., 1998).

*Protein kinase C (PKC)* PKCs contain zinc-finger–like domains (Kuroda et al., 1996). Interaction of MTs with zinc fingers is known through zinc administration. It was found that MT-2A interacts with the kinase domain of PKC in prostate cancer (Rao et al., 2003). It is believed that this interaction contributes to the induction of chemoresistance and/or androgen independence of prostate cancer cells. Moreover, zinc enhances the activity of PKC that leads to the induction of MT mRNA (Ebadi et al., 1993).

*Esophageal cancer-related gene (ECRG)* ECRG2, a novel candidate of a tumor-suppressor gene in esophageal carcinoma, interacts directly with MT-2A and links to apoptosis (Cui et al., 2003). The interaction of ECRG2 and MT-2A was confirmed by glutathione *S*-transferase pull-down assays *in vitro* and coimmunoprecipitation experiments *in vivo*. ECRG2 colocalized with MT-2A mostly to nuclei and slightly to cytoplasm, as shown by confocal microscopy. Transfection of the ECRG2 gene inhibited cell proliferation and induced apoptosis in esophageal cancer cells (Cui et al., 2003).

*p53* The p53 tumor-suppressor protein has achieved stardom in molecular oncology because of its frequent inactivation in a large range of cancers. This apoptosis-inducing guardian of genome integrity binds to DNA through a sequence-specific, DNA-binding domain and is stabilized by the coordination of an atom of zinc within a $Cys_3His_1$ cluster (Meplan and Hainaut, 1999). This binding to DNA is necessary for its function. The structure and function of p53 is controlled by zinc-binding and redox conditions (Hainaut and Mann, 2001). Properties of p53 can be regulated through two integrated biochemical systems: the redox-sensing capacity of the p53 protein (as a result of its structural features and its regulation by redox factors, such as thioredoxin, MTs, or the redox-repair enzyme, APE1/ref-1) and the expression of p53 as multiple isoforms with antagonist effects (Hafsi and Hainaut, 2011). Surface plasmon resonance was used to study the specific interaction of p53 with apo-MT (the metal-free form of MT). Interaction was originated from the high binding affinity of free sulfhydryl groups of apo-MT with $Zn^{2+}$ of p53, causing p53 to adopt a "mutant like" form with loss of sequence-specific, DNA-binding activity (Xia et al., 2009). In other *in vitro* studies, apo-MT-1, but not MT-1 (MT-1 with metal ion), forms a complex with p53 (Abdel-Mageed and Agrawal; 1998, Knipp et al., 2005; Ostrakhovitch et al., 2006). Further, recombinant MT, a metal-chelator protein, was found to modulate p53 conformation *in vitro* (Meplan et al., 2000). One may conclude that in cultured cells, transfection with the MT gene could modulate p53 transcriptional activity. Therefore, some data to support this speculation are needed. Thus, the potential role of p53 in the regulation of MTs in p53-positive MN1 and parental MCF7 cells was investigated. Zinc and copper increased the activity of MREs and MTF-1 expression, and the inactivation of p53 or the presence of inactive p53 inhibited MRE-dependent reporter gene expression in response to metals (Figure 1). This indicates that activation of p53 is an important factor in the metal regulation of MTs (Ostrakhovitch et al., 2007). In addition, the removal of $Zn^{2+}$ from protein p53 by MTs leads to its inactivation and therefore to inhibition of p53-mediated apoptosis (Fan and Cherian, 2002) by inhibition of its binding to DNA (Palecek et al., 1999) and proteins (Wang and Yang, 2010). Persistent apo-MT overexpression in cells may therefore promote their accelerated growth through the induction of a p53-null state (Kondo et al., 1997).

The hypothesis of interactions between MTs and protein p53 has also been verified using animal experiments. In p53-deficient mice with zinc deficiency, which were exposed to carcinogen 4-nitroquinoline 1-oxide, a significantly greater development of tumors was observed. In those tumors and in preneoplastic lingual and esophageal lesions, overexpression of cytokeratin 14, cyclooxygenase-2, and MT, which correlates with increased cellular proliferation, was detected (Fong et al., 2006). MTs and p53 were colocalized in the nuclei of canine mammary tumors, subcellular accumulation of p53 protein and MTs was associated with tumor malignancy, and, in some benign tumors, low to moderate intensity of MTs and p53 protein was detected. The investigators found that these results speculated that this expression in benign tumors would evolve into malignant ones (Vural et al., 2009).

Analysis of human cancer patients also showed interesting correlations between p53 and MT gene

expressions. In pancreatic serous cystadenomas, the increased expressions of MTs and p53 were observed in the less-differentiated tumors. Thus, the mRNA expression of MTs may be a potential prognostic marker for these tumors (Sliwinska-Mosson et al., 2009). In oral squamous cell carcinoma, frequent localization of MTs in nuclei was associated with the increased expression of the TP53 gene. MT and p53 coexpression can therefore be considered a sign of the shorter survival of patients with advanced disease (Cardoso et al., 2009).

### Proliferation

Proliferation is one of the key processes for a cell. In the multicolor phenomenon, numerous tightly connected cascades and pathways are involved. Based on the published results, it can be concluded that elevated levels of MTs found in rapidly growing tissues, such as the neonatal liver, have suggested a role of MTs in cell proliferation, even under physiological conditions (Cherian and Apostolova, 2000; Ogra and Suzuki, 2000). This is clearly supported by a study where the investigators located MTs in the proliferating cells of hair follicles of healthy skin (Karasawa et al., 1991). There was also found some connections between MTs and zinc in the proliferating epidermis during wound healing in mouse skin (Iwata et al., 1999). The induction of MTs was also associated with erythropoietin (EPO)-induced cellular proliferation and inhibition of cell differentiation in the erythroid progenitor K562 cell line. EPO induced a 3-fold increase in MT transcripts in K562 cells. Further, the MT-induced inhibition of differentiation was associated with the downregulation of EPO receptor transcripts in K562 cells (Abdel-Mageed et al., 2003). The kinetics of the induction of MTs is in good correlation with the transition of the cells from $G_0$ to the proliferative fraction. Cells entering the cell cycle require higher amounts of Zn, whereas most transcription factors required for the transcription of enzymes necessary to initiate DNA synthesis need zinc to form their zinc fingers (Kuppens and De Ley, 2006). Modulation of MT isoforms was also associated with collagen deposition in proliferating keloid fibroblasts *in vitro* (Toh et al., 2010). It can be concluded that a role of MTs is connected with redox processes (Wlostowski, 1993) and with protection of DNA from oxidative damage (Chubatsu and Meneghini, 1993) in cell proliferation.

Considering the fact that MTs are somewhat associated with cell proliferation, the involvment of MTs in proliferation has been studied intensively in carcinogenesis. MT overexpression in basal cell carcinomas was correlated with an infiltrative growth pattern (Rossen et al., 1997). Based on these results, it was found that expression of MTs in breast cancer cells was related to cell-proliferative activity, and dedifferentiation of carcinoma cells may play a role in the induction of MT expression (Oyama et al., 1996). The results found from breast cancer cells are supported by experiments with cell-cycle regulation of MTs in human colonic cancer cells (HT-29). The investigators observed that the oscillation of cytoplasmic MTs reached a maximum in the late $G_1$ phases and at the $G_1$/S transition, which shows in the involvment of MTs in the most important cell-cycle stages (Nagel and Vallee, 1995). Moreover, correlation with a proliferative potential, tissue-zinc levels, and MT expression were found in nasopharyngeal carcinoma (Jayasurya et al., 2000). From the point of view of MT isoforms and their specific role in the cell cycle, it was found that MT-2A participates in cellular-differentiation processes in megakaryoblastic leukemia DAMI cells. Cells exhibited differentiation and increased MT-2A expression after incubation with PMA (phorbol 12-myristate 13-acetate), which serves as an inducer of differentiation and polyploidization in this cell line. Cells exhibited an increase in size, intracellular granulation, and megakaryocytic-specific antigen expression, such as CD41 and CD42, and arresting cell proliferation, which have validated the role of MTs in differentiation in this cell line (Bagheri et al., 2011). Moreover, MT-2A expression was also associated with cell proliferation in invasive ductal breast cancer (Jin et al., 2002). This suggests the great importance of the study of MT-2A in the case of cell proliferation. In addition, one may suggest that some stimulation of cancer development may influence zinc and MT levels. This was confirmed by a study in which treatment of cells with a carcinogen (diethylnitrosamine) led to an alteration of $Zn^{2+}$ and $Cu^{2+}$ levels, resulting in early DNA damage, along with an increase in the expression of MTs. Those changes may ultimately lead to hepatic cell proliferation (Chakraborty et al., 2007).

Although MTs are primarily cytoplasmic proteins, they are also located in the nuclei under several pathological and physiological conditions. Its nuclear location was found in malignant tumor cells, fetal liver, cells in a high-glucose medium, and proliferating and differentiating cells (Nartey et al., 1987; Tsujikawa et al., 1991; Apostolova and Cherian, 2000; Apostolova et al., 2001) and is also cell-cycle or proliferation-stimuli dependent (Nagano et al., 2000). This suggests an existence of the specific nuclear MT-transporting mechanism. It was suggested that this mechanism could be based on the redox interaction of MTs and cytosolic proteins (Takahashi et al., 2005). In addition, the presence of MTs outside of cells as a result of the influence of stressors suggests that this protein may make important contributions as a "danger signal" that influences the management of responses to cellular damage. Extracellular MTs may operate as a chemotactic factor that governs the trafficking of inflammatory cells that move to resolve damaged tissues, as a counter to extracellular oxidant-mediated damage, and as a signal that influences the functional behavior of wounded cells. A thorough understanding of the mechanisms of MT release from cells, the conditions under which MTs are released to the extracellular environment, and the ways in which MTs interact with sensitive cells may illuminate our understanding of mechanisms that control stress. Such knowledge should indicate new opportunities for therapeutic management

by the manipulation of this pool of extracellular MTs (Lynes et al., 2006).

### Immunomodulation

It was shown that MTs can interact with the cellular membrane of lymphocytes and modulate their functions (Dutsch-Wicherek et al., 2008). The augmented humoral immune function in MT-null mice (Crowthers et al., 2000), MT-mediated leukocyte chemotaxis (Yin et al., 2005), and B-cell-stimulating activity of MT *in vitro* (Sugiura and Yamashita, 2000) were observed. MTs have an effect on the production of antibodies (Lynes et al., 1993) and functions of macrophages (Youn et al., 1995). MTs can be also induced by some of the acute phase cytokines [interleukin (IL)-1, IL-6, TNF-α, and interferon-gamma]. It is possible to consider MTs as regulators of the immune response suppressing the autoimmune attack on self-tissues (Krizkova et al., 2009). The effects of MTs on specific lymphocyte subpopulations characterize the mechanism of MT-mediated alterations of immune activity. MTs bind to the membrane of both T and B lymphocytes and induce the lymphoproliferation of B cells; for the proliferation of T lymphocytes, costimulation is necessary. Moreover, MTs enhance the capacity of naïve B lymphocytes to differentiate into plasma cells (Borghesi et al., 1996).

Effects of extracellular MTs on T-cell function may contribute to the immunosuppression of cell-mediated immunity. MTs decreased antigen-specific humoral responses *in vivo* and inhibited the ability of T cells to proliferate in response to antigen presented *in vitro* (Borghesi and Lynes, 1996). MTs caused dramatic decreases in murine cytotoxic T lymphocyte (CTL) activity against allogeneic target cells and reduced the proliferative response of a murine cytotoxic T-cell-derived line CTLL-2 to cytokines. They also decreased the level of the major histocompatibility complex class I and CD8 molecules detectable on the surface of lymphocytes, whereas they had no significant effect on the level of CD4. These findings suggested that the immunosuppressive effects of MTs may, at least in part, interfere with cell-cell interactions that are important for cell-mediated immunity. Despite this suppressive effect on CTL functioning, MTs were found to augment mixed lymphocyte reactions (MLRs) in concert with increased IL-2 receptor (IL-2R) expression. This MT-augmented proliferation was observed in both allogeneic and syngeneic MLRs. Taken together, these results indicate that MTs may increase the number of immature T cells, but decrease their differentiation to the effector CTL stage (Youn and Lynes, 1999).

The antioxidant, zinc-transport, and regulatory functions of MT are also involved in immunity and aging. MTs protect from oxidative damage during transient stress conditions at the young-adult age. This protection no longer exists in the elderly and in age-related diseases (e.g., cancer and infections), because the stress condition is constant, as suggested by a free radical theory of aging. As such, MTs may constantly deplete zinc from plasma and tissues. This phenomenon causes increased MT levels on the one hand, but, on the other hand, induces low zinc-ion bioavailability for healthy immune responses. This may be particularly relevant for thymic functions and natural-killer activity. Therefore, MTs, which are protective in young adults, may become dangerous. Physiological supplementation of zinc in aging may correct central and peripheral immune defects, resulting in prolonged survival and decreased mortality (50%) from infections and tumors, especially during middle age (Mocchegiani et al., 2000).

### Interactions of MTs with enzymes, zinc-containing proteins, and nitric oxide (NO)

MTs may interact either with low-molecular compounds (e.g., heavy metals, free radicals, NO, or proteins) either directly through –SH groups or indirectly through $Zn^{2+}$. A scheme of MT interaction is shown in Figure 3.

*Enzymes* Thionein/MT controls $Zn^{2+}$ availability and the activity of enzymes (Krezel and Maret, 2008). Possible mechanisms are by zinc administration, interaction with proteins by thiol groups, or reduction/oxidation (Maret et al., 1999). It is not surprising that aconitase (Feng et al., 2005), carboxypeptidase (Maret et al., 2001), glycerol phosphate dehydrogenase (Maret et al., 2001), carbonic anhydrase (Krezel and Maret, 2008; Shi et al., 2002; Ejnik et al., 1999), and superoxide dismutase (Krezel and Maret, 2008) belong to enzymes, where their activation by MTs has been studied and found. D-penicillamine catalyzes $Zn^{2+}$ transfer from carboxypeptidase A to chelators, such as thionein and ethylenediaminetetraacetic acid, at a rate constant up to 400-fold faster than the uncatalyzed release (Chong and Auld, 2007). There were also observed differences between apo-MTs and MTs containing metal

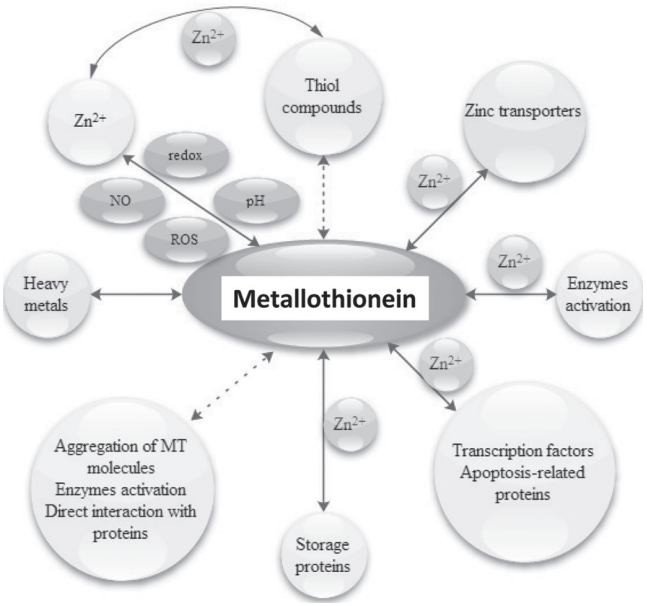


Figure 3. Schematic representation of the relation between MT and thiols, zinc transporters, enzymes, transcription factors, other proteins, and heavy metals. Many of these interactions are driven by zinc(II) ions.

ions from the point of view of their interactions with enzymes. Relative abilities of cathepsins B, C, and D to degrade $Zn_7$-MT, $Cd_7$-MT, and apo-MT *in vitro* were studied. Although apo-MT was rapidly degraded by all three cathepsins, cathepsin B degraded apo-MT approximately 36-fold more rapidly than cathepsin C and 45-fold more rapidly than cathepsin D. Therefore, under the *in vitro* conditions used in this study, the relative potency of the cathepsins tested was cathepsin B⋙cathepsin C>cathepsin D. In comparison, metal-saturated MT was more than 1,000-fold more resistant to degradation by the cathepsins tested (McKim et al., 1992; Klaassen et al., 1994).

*Zinc-containing proteins* Zinc-binding proteins function as storage proteins, transcription factors, and replication proteins besides their enzymatic role (Coleman, 1992; Frederickson et al., 2005; Jamieson et al., 2003; Porteus and Carroll, 2005). Their structure and function is dependent on zinc binding in their structure (Maret, 2005; Vallee et al., 1991). MTs can transfer zinc to these zinc fingers of transcription factors. Moreover, MTs would act as a zinc reservoir for proteases, such as matrix metalloproteinases (MMPs) (Ribeiro et al., 2011). MMPs are important for tumor biology, particularly for angiogenesis and metastasis. Activation of MMPs by MTs is also discussed (Haga et al., 1996; Zitka et al., 2010), but the mechanism of this activation is unknown. MTs may either interact with pro-MMPs through its thiol groups, leading to uncovering its active site, or directly administer zinc to MMP molecules (Haga et al., 1996, 1997). MT-2A overexpression increases the expression of MMT-9 and thus invasivity of breast cancer cells (Kim et al., 2011). One may suggest applying this mechanism even for other cancer types. Moreover, zinc and MMPs are also involved in inflammation and wound healing. It was found that exogenous MT-2A promotes accelerated healing after a burn wound in mice (Morellini et al., 2008).

*Interaction of MTs with NO* NO is an important low-molecular cellular signaling molecule involved in many physiological and pathological processes, such as vasodilatation and neurotransmission, and is also produced by phagocytes as a part of immune response. There has been described several mechanisms by which NO affects the biology of living cells. These include oxidation of iron-containing proteins, such as ribonucleotide reductase and aconitase, activation of the soluble guanylate cyclase, adenosine diphosphate ribosylation of proteins, protein sulfhydryl group nitrosylation, and iron-regulatory factor activation (Shami et al., 1995). NO has been demonstrated to activate NF-κB in peripheral blood mononuclear cells (Kaibori et al., 1999).

It was also found that NO releases intracellular zinc from prokaryotic MT in *Escherichia coli* (Binet et al., 2002), from eukaryotic MTs (Kroncke et al., 1994), and that it mediates intracytoplasmic and nuclear zinc release (Berendji et al., 1997). St Croix et al. proved that NO-induced changes in intracellular zinc homeostasis are mediated by MT/thionein (St Croix et al., 2002). Zinc-finger proteins may serve as molecular targets for NO-mediated gene regulation (Kroncke, 2001).

## MTs in cancer diagnosis

Current knowledge of MTs is juxtaposed with our understanding of the pathogenesis of disease (Capdevila et al., 2012). MT is known to modulate three fundamental processes: 1) the release of gaseous mediators, such as hydroxyl radicals or NO; 2) apoptosis; and 3) the binding and exchange of metals, such as zinc, cadmium, or copper. Associations of MTs with several diseases, including cancer, circulatory and septic shock, coronary artery disease, and Alzheimer's disease, have been found. Further, strong evidence exists that MTs modulate the immune system, as stated above (Simpkins, 2000).

A tumor stimulates the remodeling of its microenvironment for its own survival. To protect its own growth and induce angiogenesis, the tumor changes the structure of extracellular matrix and the function of existing cells; it thus chemoattracts immune-system cells, altering their function. MT, because of its antiapoptotic, pro-proliferative, and immunomodulating functions, is discussed as a potential marker of tumor microenvironment remodeling. Most likely, the expression of this protein by the fibroblasts of the tumor microenvironment is related to the remodeled phenotype of these cells because of the tumor influence on cancer-associated fibroblasts (Dutsch-Wicherek, 2010).

Great attention is paid to the study of MTs in tumors (Jasani and Schmid, 1997; Theocharis et al., 2004; Cherian et al., 2003; Eckschlager et al., 2009). Most work is focused on the immunohistochemical determination of MTs in tumors (Adam et al., 2008; Babula et al., 2011; Eckschlager et al., 2009; Gumulec et al., 2011; Krejcova et al., 2012; Krizkova et al., 2008, 2009, 2010, 2012; Sochor et al., 2012), but also on its determination in serum by various methods, such as Brdicka's reaction or enzyme-linked immonosorbent assay (Eckschlager et al., 2009; Krizkova et al., 2010). MT can serve as a prognostic marker in central nervous system tumors of childhood and adolescence (Rickert and Paulus, 2005; Rickert, 2004), osteosarcoma (Trieb and Kotz, 2001), breast cancer (Woolston et al., 2010; Jin et al., 2004; Gomulkiewicz et al., 2010), pancreatic islet cell tumors (Tomita, 2002), and tongue squamous-cell carcinoma (Theocharis et al., 2011). MT can also serve as a serum tumor marker in prostate cancer (Krizkova et al., 2011; Masarik et al., 2011), head and neck tumors (Krejcova et al., 2012), childhood solid tumors (Krizkova et al., 2010), and melanoma (Krizkova et al., 2008). Expression of MT may help to distinguish between benign and malignant tumor, as shown in thyroid tumors (Krolicka et al., 2010), prostatic lesions (El Sharkawy et al., 2006), GI stromal tumors, and gastric carcinomas (Soo et al., 2011). At isoform levels, MT expression in breast and prostate cancers, renal tumors, and papillary thyroid cancer was reviewed by Thirumoorthy et al., who found that expression of MT

isoforms was down- and upregulated differentially in dependence on a cancer type (Thirumoorthy et al., 2011; Ghoshal et al., 2002). Arriaga et al. have described that expression of different MT isoforms in colorectal cancer is relevant for tumor progression and patient survival (Arriaga et al., 2012).

## MTs in cancer therapy

MTs, because of their roles in tumors, can be targeted for cancer therapy (Lai et al., 2011). Silencing of MT by short interfering RNA (siRNA) was published by Tarapore et al., who used phage Phi29 motor pRNA as a vehicle to carry siRNA specifically targeted to MT-2A mRNA in ovarian cancers (Tarapore et al., 2011), and by Lai et al., who reported that silencing of the MT-2A gene by siRNA induces entosis (a process involving the invasion of one cell into another, and internalized cells are either degraded by lysosomal enzymes or released) in adherent MCF-7 breast cancer cells (Lai et al., 2010). Targeting unique mRNA molecules using antisense approaches, based on sequence specificity of double-stranded nucleic acid interactions, should, in theory, allow for the design of drugs with high specificity for intended targets. Antisense-induced degradation or inhibition of translation of a target mRNA is potentially capable of inhibiting the expression of any target proteins (Jason et al., 2004). Downregulation of MTs by antisense RNA is known to inhibit growth of the tumor cell. This strategy for downregulation of the MT gene in tumors is possible to inhibit their growth and metastasizing in breast cancer cells (Abdelmageed and Agrawal, 1997), leukemia P388 cells, Ehrlich's carcinoma, sarcoma 180 cells (Takeda et al., 1997), and nasopharyngeal cancer (Tan et al., 2005). Antisense MT mRNA may induce sensitivity of cancer cells to a heavy-metal–based cytostatic (Kennette et al., 2005). Some strategies are shown in Figure 4.

Aberrant DNA methylation in histologically healthy mucosae has attracted attention as an indicator of past exposure to carcinogens and as a marker for future risk prediction. Methylation and epigenetics of MT isoforms are also studied with their potential use in cancer therapy (Lee et al., 2011). MT-1M was methylated in squamous-cell carcinoma (Lee et al., 2011), and MT-1E was very commonly methylated in melanoma tissues. Increase in MT gene methylation was observed to correlate with melanoma development (i.e., only rarely is methylated in benign naevi), more frequently in primary tumors, and the most frequently in metastases. The resulting silencing might also play a role in the resistance of melanoma to cisplatin, as shown by *in vitro* experiments (Perez, 1998). This finding is at odds with most other studies, which, in turn, is increasing at MTH cells resistant to cisplatin (Faller et al., 2010).

Other roles of MT in cancer therapy is its protective action during chemotherapy (Volm, 1998). Cells with developed resistance to heavy-metal–based cytostatics have increased expression of MTs (Naito et al., 1999; Bredel, 2001; Perez, 1998; Chao, 1996; Scanlon et al., 1991). Targeting of MTs with antisense RNA for reversal of multidrug resistance was proposed (Gosland et al., 1996).

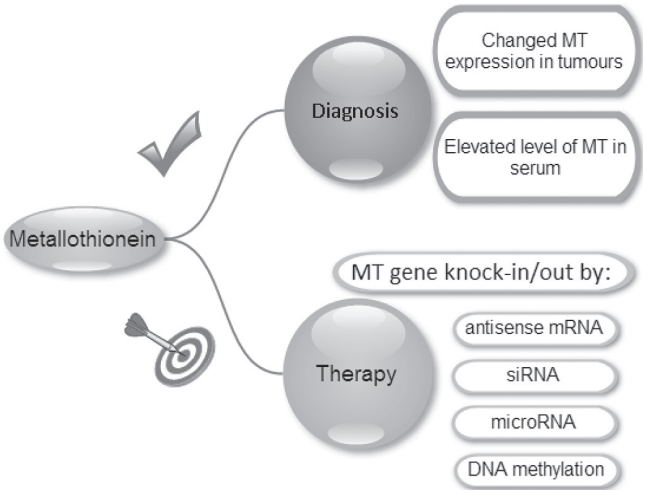


Figure 4. MT can be used in both the diagnosis and therapy of malignancies. In the case of therapy, targeted gene therapy, including using antisense mRNA, siRNA, microRNA, and DNA methylation, can be considered. Further, levels of MTs can be used for diagnostic purposes.

## Conclusions and future directions

Zinc(II) ions contribute to a number of biological processes, such as DNA synthesis, gene expression, enzymatic catalysis, neurotransmission, and apoptosis. Both zinc deficiency and zinc overload elicit OS, which can lead to cell death. These pro-oxidant functions contrast with pro-antioxidant functions in a range of physiological zinc concentrations. Oxidative or nitrosative stress can cause a release of zinc from proteins containing zinc fingers and cluster motifs and its redistribution, thereby altering the functions of those proteins, from which it is released and/or to which it binds. The transduction of redox signals into zinc signals, and vice versa, affects mitochondrial functions and signaling pathways, including alterations in NF-κB, p53, and AP-1 activities and levels. Zinc sensors for cellular and organ physiology, improved analytical tools to approach zinc proteome, biomarkers of zinc deficiencies, methods for zinc imaging, obtaining more complex information on polymorphisms in zinc transporters, importers, and binding proteins, and on methods of targeting specific subcellular pools of zinc will be needed to be developed for achieving this goal (John et al., 2010a; Krizkova et al., 2012).

Based on the above-mentioned facts, MTs belong to the important maintainers of the zinc pool and therefore could be considered as one of the targets for future diagnostic strategies. MT overexpression could be used as a predictive marker of worse prognosis and as a sign of higher grade in selected tumors. Changes of different MT isoforms seem to be important for carcinogenesis, and their detection may be useful in clinical diagnostics (Arriaga et al., 2012). The role of MTs in the development

of chemoresistance is not yet fully understood. Platinum drug resistance is caused by a combination of different mechanisms, and MT overexpression seems to be one of them. Prediction of chemoresistance to platinum drugs, based on MT expression, needs to be confirmed by experiments and by larger clinical studies. Examination of MT levels detected by the electroanalytical method in serum, which may be used as a tumor marker (Figure 4), seems to be promising for clinical practice. One may speculate that the pharmacological inhibition of MTs may reverse chemoresistance to platinum drugs and may induce cell death in some cancer cells.

## Declaration of interest

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