

Chapter 2

Metallothioneins: Structure and Functions

2.1 The Structure of Metallothioneins

All metallothioneins (MTs) possess a highly conserved amino acid sequence and present only a few structural changes even when isolated from different animal species. In mammals, a single MT molecule is made up of 61–68 amino acids, depending on the isoform (the MT-1, MT-2, and MT-4 isoforms consist of 61–62 amino acids, whereas the MT-3 isoform comprises 68 amino acids), and the protein sequence is composed of up to 20 cysteine (Cys) residues (Vasak 2005; Vasak and Meloni 2011). Furthermore, in mammals, no aromatic amino acids are found in the MT molecules. Protein sequencing has revealed that the MT molecule is a single polypeptide chain, in which the Cys residues are organized in the sequences Cys-X-Cys, Cys-X-X-Cys, and Cys-Cys, where “X” denotes an amino acid other than Cys (Kojima et al. 1976; Huang and Yoshida 1977). The Cys residues are the metal-binding domains of the MT molecule, in which they are juxtaposed with lysine (Lys) and arginine (Arg) amino acid residues and arranged in two thiol-rich sites designated domains α and β (Fig. 2.1). The two metal-binding domains are separated by a non-cysteine-containing sequence often designated as the spacer or linker (Zangger et al. 2001; Babula et al. 2012). The α -domain consists of amino acids 31–68 and is located on the C-terminal edge, whereas the N-terminal β -domain contains amino acids 1–30 (Zangger et al. 2001; Dziegiel 2004). It has been demonstrated that the α -domain is capable of binding up to four, and the β -domain up to three, bivalent metal ions such as zinc, cadmium, mercury, or lead (Coyle et al. 2002b; Duncan et al. 2006). The part of the protein with no bound metal ions is termed apo-metallothionein (apo-MT) or thionein (Coyle et al. 2002b). Metallothioneins are also capable of reacting with up to 12 univalent metal ions (Palmiter 1998; Coyle et al. 2002b). Zinc ions, which naturally occur in the organism, are regarded as the main binding partner of apo-MT. However, other nonessential metal ions occurring pathologically in the organism—such as lead, copper, cadmium, mercury, platinum, chromate, bismuth, and silver—often possess

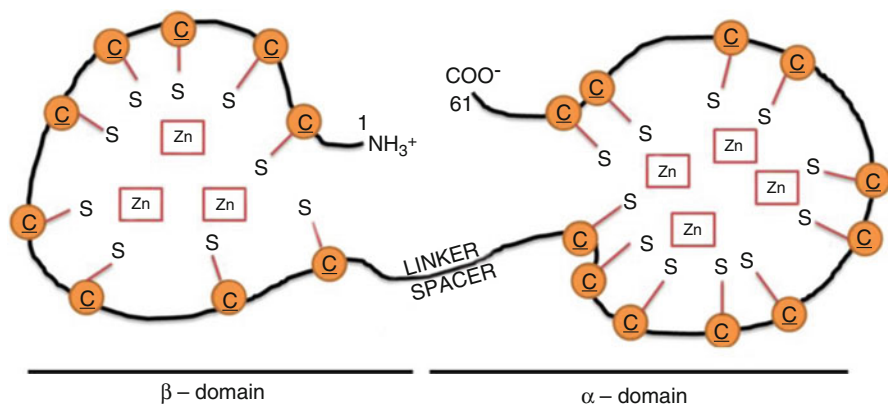


Fig. 2.1 Metal ion-binding sites of a metallothionein molecule. The α -domain is capable of binding of up to four, and the β -domain up to three, bivalent metal ions such as zinc, cadmium, mercury, or lead. Adopted and modified according to Nielsen et al. (2007)

higher affinity to the apo-MT-binding sites (Nordberg and Nordberg 2000; Ngu and Stillman 2009; Ngu et al. 2010b; Gumulec et al. 2011; Babula et al. 2012). So far, only iron ions (Fe^{2+}) have been identified to possess lower affinity to the metal-binding sites of the apo-MT domains (Foster and Robinson 2011). Interestingly, only a small proportion of MT molecules was found bound to zinc ions in various organisms. In rat tissues, apo-MT has been shown to constitute up to 54 % of the total amount of MT, whereas higher apo-MT levels were detected in rat cancer cells (Yang et al. 2001). Recent studies have also identified small amounts of sulfide ligands bound to recombinant MT-1 and MT-4 proteins overexpressed in *Escherichia coli* (Capdevila et al. 2005; Tio et al. 2006). Nevertheless, studies analyzing MT proteins in the cytoplasm of mammalian cells have failed to detect sulfide ligands bound to their molecules (Mounicou et al. 2010).

The structure of MT-3 shows a high degree of sequence similarity to other MT molecules (approximately 70 %). However, there are some differences in the sequence that might reflect its functional diversity in comparison to the MT-1 and MT-2 isoforms (Fig. 2.1). In MT-3, a glutamate-rich hexapeptide has been found near the C-terminus containing a Cys-Pro-Cys-Pro motif (amino acids 6–9), which is absent in other MT members (Uchida et al. 1991; Ding et al. 2010). The presence of this fragment, not apparent in other MT members, may result in the reported unique growth-inhibitory activity of the MT-3 molecule and may contribute to other functional differences between the MT-1 and MT-2 isoforms (Uchida and Tomonaga 1989; Uchida et al. 1991; Uchida 1994; Ding et al. 2010; Faller 2010). Taking into account the divergent nature of this isoform, the exact structure and biological functions of MT-3 will be reviewed in Chap. 3.

2.2 Metallothionein Gene Expression and the Regulation of Synthesis

Molecular studies of mouse and human MT genes have permitted the identification and recognition of their expression pattern in various tissues and have allowed the main regulatory mechanisms of its synthesis to be described. In mice, only four MT genes exist localized to chromosome 8 (MT-1, MT-2, MT-3, and MT-4). In humans, however, 17 MT genes have so far been identified in the q13 region of chromosome 16. Of these genes, 13 code for MT-1 and two for MT-2, while two single genes code for each of MT-3 and MT-4 isoforms (Palmiter et al. 1992; Quaife et al. 1994; Mididoddi et al. 1996). Abundant lines of evidence suggest that at least 10 genes encode functional MT proteins: MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3, and MT-4 (Mididoddi et al. 1996; Werynska et al. 2011) (Fig. 2.2).

Lately, a new functional MT-1M member was identified in the liver (Mao et al. 2012). *MT-1C*, *MT-1D*, *MT-1I*, *MT-1J*, *MT-1K*, *MT-1L*, and *MT-2B* are regarded as pseudogenes in humans, as no corresponding proteins have so far been identified. However, their functionality, in most cases, remains unknown (Stennard et al. 1994; Mididoddi et al. 1996). Additionally, a gene called MT-like 5 (MTL-5), which is closely related to the other MT isoforms, has been identified in the testes of mice in the q13 region of chromosome 11 (Olesen et al. 2004). The product of this gene, called tesmin, has been shown to differentially regulate meiosis in male and female cells (Olesen et al. 2004). In summary, the structure and possible MT gene regulatory expression mechanisms are presented in Fig. 2.3.

Metallothionein genes consist of three exons encoding the α -domain (exon 1) and the β -domain (exons 2–3). The regulatory mechanisms of the MT-1 and MT-2 isoforms are the best recognized so far. It has been shown that these isoforms are inducible by several substances and agents, e.g., heavy metals, steroids, cytokines, growth factors, free oxygen, and nitric radicals (Ghoshal et al. 1998, 1999; Jacob et al. 1999; Ghoshal and Jacob 2001; Haq et al. 2003). Indeed, several metal response elements (MRE) (Koizumi et al. 1999; Langmade et al. 2000; Otsuka et al. 2000; Saydam et al. 2002), glucocorticoid-response elements (GRE)

Protein	Aa	1	10	20	30	40	50	60	68
MT-1A	61	MDPNCSCATG	GSCCTCTGSK	CKECKCTSK	KSCCSCCPMS	CAKCAQGCIC	KGASEKSCC	A	
MT-1B	61	MDPNCSCTTG	GSCACAGSCK	CKECKCTSK	KCCSCCPVG	CAKCAQGCVC	KGSSEKCRCC	A	
MT-1E	61	MDPNCSCATG	GSCTCAGSCK	CKECKCTSK	KSCCSCCPVG	CAKCAQGCVC	KGASEKSCC	A	
MT-1F	61	MDPNCSCAAG	VSCCTCAGSCK	CKECKCTSK	KSCCSCCPVG	CSKCAQGCVC	KGASEKSCC	D	
MT-1G	62	MDPNCSCAAA	GVSCTCASSC	KCKECKCTSC	KKSCCSCCPV	GCAKCAQGCIC	CKGASEKSCC	CA	
MT-1H	61	MDPNCSCAAG	GSCACAGSCK	CKCKCKCTSK	KSCCSCCPVG	CAKCAQGCIC	KGASEKSCC	A	
MT-1X	61	MDPNCSCSPV	GSCACAGSCK	CKECKCTSK	KSCCSCCPVG	CAKCAQGCIC	KGASEKSCC	A	
MT-2A	61	MDPNCSCAAG	DSCTCAGSCK	CKECKCTSK	KSCCSCCPVG	CAKCAQGCIC	KGASEKSCC	A	
MT-3	68	MDPETCPCPS	GGSCCTCADSC	KCEGCKCTSC	KKSCCSCCPA	ECEKCAQGCIC	CKGGEAAEAE	AEKSCCQ	
MT-4	62	MDPRECVCMS	GGICMCGDNC	KCTTCNCKTY	WKSCCPCPP	GCAKCAQGCIC	CKGSEKSCC	CP	

β -domain

α -domain

Fig. 2.2 Comprehensive presentation of amino acid sequences of all isoforms of metallothioneins with noticeable division on α - and β -domain. Aa amino acids. According to UniProt service website

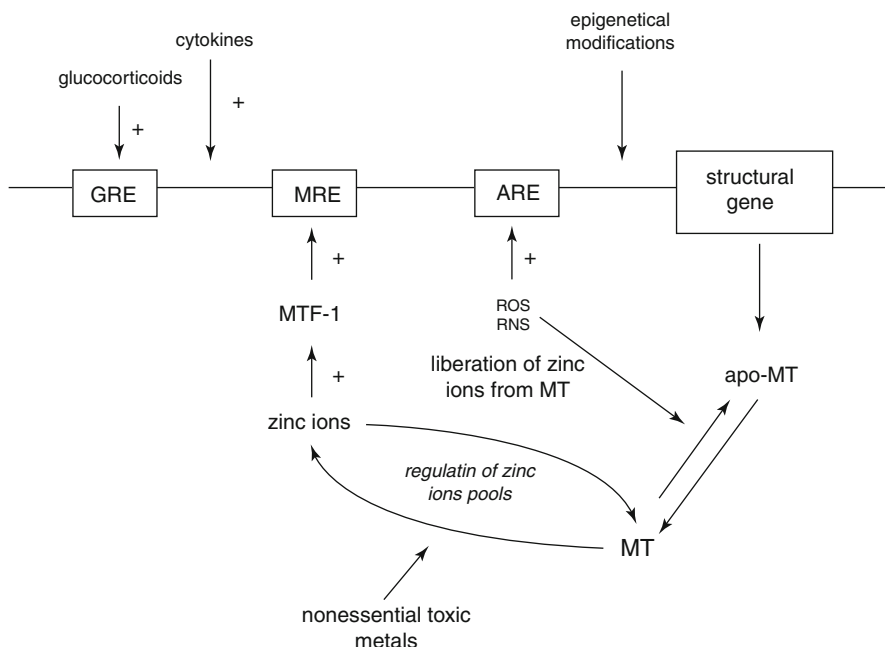


Fig. 2.3 Possible mechanisms of the regulation of metallothionein gene expression. *apo-MT* apo-metallothionein, *ARE* antioxidant response element, *GRE* glucocorticoid-responsive element, *MTF-1* metal-regulatory transcription factor-1, *MRE* metal response element, *ROS* reactive oxygen species, *RNS* reactive nitric species. Detailed description in the text. Adopted and modified according to Sato and Kondoh (2002)

(Hernandez et al. 2000), and antioxidant response elements (ARE) have been noted in the promoter region of the MT-1 and MT-2 genes (Campagne et al. 2000; Bi et al. 2004).

Metal ions, especially zinc ions that occur naturally in the organism, seem to be the most potent inducers of MT-1/2 expression. They have been shown to bind to metal-regulatory transcription factor-1 (MTF-1), which interacts with the DNA via its six zinc finger C2H2 domains to the MRE sequence in the promoter regions of MT-1/2 genes. This binding subsequently results in the initiation of gene transcription (Langmade et al. 2000; Otsuka et al. 2000; Saydam et al. 2002). MRE elements have also been identified in the promoter regions of the MT-3 gene, though contrary results exist concerning possible expression induction of this isoform by metal ions (Heuchel et al. 1994; Chapman et al. 1999; Garrett et al. 2002). MTF-1 is also responsible for the basal expression of MT genes and so far is the only identified mediator of their sensitivity to metal ions (Heuchel et al. 1994; Ghoshal et al. 1999; Ghoshal and Jacob 2001).

Toxicological studies have revealed that metal ions other than zinc may also induce MT-1/2 gene expression. Increased amounts of MT-1/2 were found in the liver, kidney, and intestines of experimental animals following parental or dietary

exposure to cadmium, mercury, or zinc (Vasak and Meloni 2011). Although metal ions other than zinc are capable of inducing MT-1/2 expression, this mechanism differs from the induction by zinc ions, discussed above, since they bind directly to MTF-1. The nonessential metal ions cannot activate the MTF-1, but due to their higher affinity to MT-1/2 proteins, they are capable of displacing zinc ions from MT-1/2 molecules and increasing free intracellular zinc levels (Koizumi et al. 1999; Murata et al. 1999). Subsequently, the free zinc ions bind to the MTF-1, leading to activation of MT-1/2 gene transcription (Koizumi et al. 1999; Murata et al. 1999; Lichtlen and Schaffner 2001).

Oxidative stress induced by various factors and conditions has been shown to elevate MT expression, independently of the mechanism involving the MRE (Vasak and Meloni 2011; Babula et al. 2012). The generation of free radicals such as hydrogen peroxide (H_2O_2) by various factors results in the oxidation of the MT molecule and the subsequent release of its bound zinc ions, which ultimately lead to MTF-1 activation (Andrews 2000; Nguyen et al. 2003). It has been shown that catecholamines (Gauthier et al. 2008; Eibl et al. 2010), tissue hypoxia (Murphy et al. 2008; Kojima et al. 2009), physical exercise (Podhorska-Okolow et al. 2006), or hypothermia (Park et al. 2013) may induce MT-1 and MT-2 gene expression. MT-1/2 transcription may be also regulated by glucocorticoids via their direct binding to the GRE in the promoter region of MT genes (Davis and Cousins 2000; Hernandez et al. 2000). High glucose levels have recently been found to induce MT-1 and MT-2 expression in human umbilical vein endothelial cells (HUVECs) on stimulation of endothelin receptor-1 (ET1) (Apostolova et al. 2001). Furthermore, epigenetic regulation of MT-1, MT-2, and MT-3 gene expression via DNA methylation or histone modifications has been observed in the cancer cells of neoplastic diseases such as esophageal, gastric, and prostate cancers (Deng et al. 2003a, b; Smith et al. 2005; Han et al. 2013).

2.3 Localization and Main Functions of MT Proteins

Metallothioneins have been detected in various normal and pathological cells (Dziegiel 2004; Pula et al. 2012; Werynska et al. 2013a), as well as in blood serum (Nordberg et al. 1982; Ghoshal et al. 1998; Adam et al. 2010; Kruseova et al. 2013), where they are capable of regulating and mediating several important cellular processes (Fig. 2.4). Functional MT-1/2 isoforms have mainly been found in cellular cytoplasm and in some organelles, with their expression predominantly noted in mitochondria (Banerjee et al. 1982). Their concentration is strongly dependent on the oxidative status of these organelles. Due to the small molecular size of MTs, they can be transported through the outer membrane of mitochondria and regulate the permeability of their inner membrane (Simpkins et al. 1998). The presence of MT isoforms has also been observed in lysosomes: MT-1 and MT-2 have been shown to protect this organelle from oxidative stress by reducing the iron-catalyzed intralysosomal peroxidative reactions (Baird et al. 2006). Moreover,

MT-3 has been shown to mediate proper lysosome function in astrocytes and neurons by affecting lysosomal function and cell viability (Lee and Koh 2010; Lee et al. 2010). The presence of MT-1/2, as well as of MT-3, has been demonstrated in the nuclei of many cells, e.g., hepatocytes (Fig. 2.5) (Cherian and Apostolova 2000; Werynska et al. 2013a). It has been shown that, under conditions of oxidative stress, the MT-1/2 isoforms are rapidly translocated to the cells' nucleus through nuclear pore complexes. It is further known on the basis of many studies that, once localized in the nucleus, MT-1/2 molecules play an important role

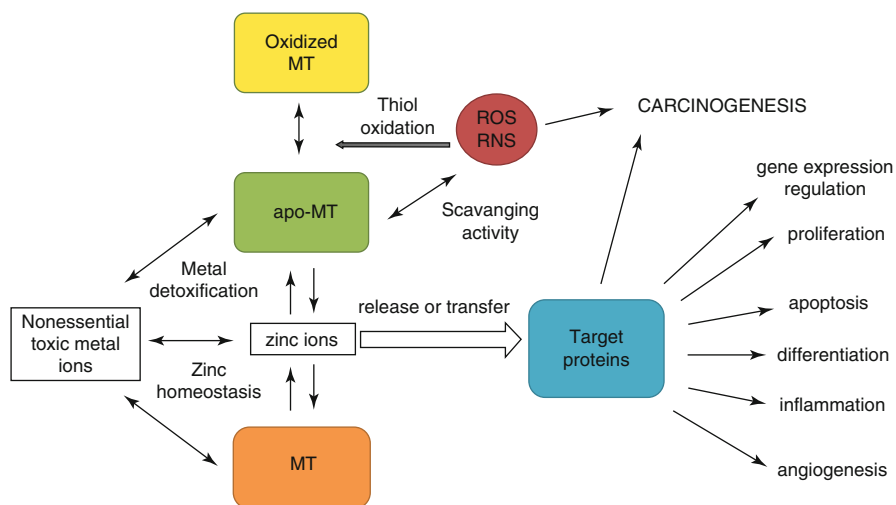


Fig. 2.4 Cellular processes regulated or mediated by metallothioneins. apo-MT apo-metallothionein, ROS reactive oxygen species, RNS reactive nitrogen species

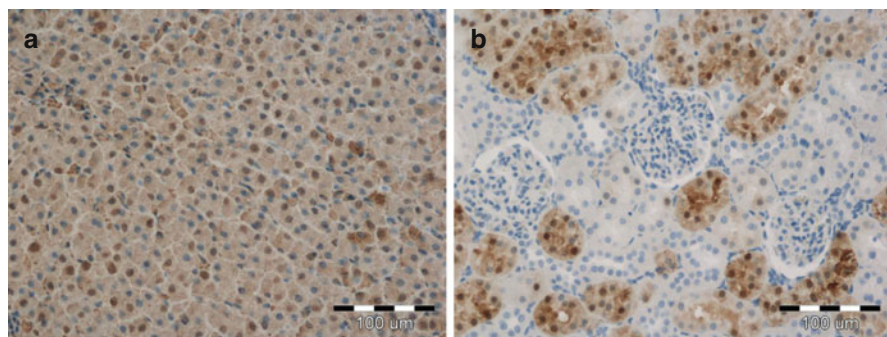


Fig. 2.5 Immunohistochemical demonstration of metallothionein 1/2 in cytoplasm and nuclei of normal human hepatocytes (a) and proximal tubule cells in human kidney (b). Staining was performed according to Cherian et al. (2003). Archival sections from the Department of Histology and Embryology, Wrocław Medical University, Wrocław, Poland

in cell proliferation and differentiation (Apostolova et al. 2000; Cherian and Apostolova 2000; Chen et al. 2004; Nzungue et al. 2009), as well as in genotoxicity and cell apoptosis (Gunes et al. 1998; Apostolova et al. 1999; Santon et al. 2006). Moreover, nuclear MT-1/2 expression has been noted in hepatocytes (Tsujikawa et al. 1991), myoblasts (Apostolova et al. 2000), and tumor cells of various malignancies (Surowiak et al. 2007; Szelachowska et al. 2008). Similarly, the observed different localizations of MT-3 in the cellular cytoplasm and the nucleus seem to play a significant role in protection against DNA damage and in regulation of transcription (Chen et al. 2002; Werynska et al. 2013a).

Aside from the expression of MTs in various cells, the presence of these proteins has also been detected in blood, with concentrations varying from 0.01–1.0 ng/l in serum (Nordberg et al. 1982) to 0.51–1.86 ng/ml in plasma (Milnerowicz et al. 2009). The assessment of MT levels in patients' blood may become a promising marker for diagnostic and prognostic estimation of therapy efficacy for childhood tumors—in the case of which serum MT levels have been observed to be elevated approximately five times over those of healthy children controls (Krizkova et al. 2010). Sabolic et al. suggest that blood MTs may originate from damaged cells via protein leakage through cell membranes or in the complete demise of cells (Sabolic et al. 2010). However, the functional significance of blood MTs still needs to be determined.

The metal-binding abilities of MTs and their differentiated and vast cellular localizations (due to their small molecular size) give rise to their multifunctionality in various cellular processes (Sabolic et al. 2010; Vasak and Meloni 2011; Babula et al. 2012). Apart from their regulatory and protective roles in numerous normal cells, in some circumstances—such as during carcinogenesis—they may contribute to tumor progression (Dziegiel 2004). MTs are thus often referred to as multipurpose proteins with two faces (Coyle et al. 2002b; McGee et al. 2010). Abundant lines of evidence indicate that these small proteins are involved in several key processes, such as detoxification of heavy metal ions, scavenging of reactive oxygen species (ROS) and of reactive nitric species (RNS), differentiation, proliferation, regulation of cell death, migration, and invasiveness of cancer cells, as well as angiogenesis (Lee and Koh 2010; McGee et al. 2010; Sabolic et al. 2010; Zbinden et al. 2010; Vasak and Meloni 2011). Interestingly, although MTs exert numerous functions, mice lacking MT-1 and MT-2 proteins (MT-null mice), as well as transgenic mice overexpressing these isoforms under normal conditions, show no gross phenotypic or reproductive abnormalities (Sabolic et al. 2010). The significance of MT-1 and MT-2 expression in the abovementioned processes seems to become first apparent under conditions of pathological stress (Takano et al. 2004).

2.4 Detoxification of Metal Ions

MT-1 and MT-2 have been shown to bind various metal ions, univalent as well as bivalent. An ability to detoxify such compounds has thus been ascribed to these proteins (Sabolic 2006; Sabolic et al. 2010). It has been noted that apo-MT molecules may connect with 7–9 bivalent ions (e.g., zinc or cadmium). However, they may also bind up to 12 copper or even 18 mercury ions in experimental conditions (Nielson et al. 1985; Palumaa et al. 2002, 2003, 2005; Meloni et al. 2006). MT molecules may bind different metal ions at the same time (Palumaa et al. 2002; Romero-Isart and Vasak 2002; Palacios et al. 2011). Spontaneous oligomerization via the disulfide bonds of MT molecules' α -domains has been also reported (Zangger et al. 2001). Such aggregates, isolated from horse and rabbit kidney, have been found to be capable of binding more metal ions than could be predicted from their single polypeptide chain structure (Zangger et al. 2001; Wilhelmsen et al. 2002). The exact affinity of particular metal ions has been determined in various studies. However, most toxic ions (such as cadmium, lead, and mercury) have been shown to possess higher affinity toward MT molecules in the majority of studies (Waalkes et al. 1984; Nielson et al. 1985; Sabolic et al. 2010). These metal ions may displace zinc or other lower affinity metal ions bound to MTs, which may lead to alterations in crucial cellular process such as transcription or translation (McGee et al. 2010). The protective effect of MT-1/2 in the majority of circumstances was mainly mediated by the release of zinc ions from MT-1/2 molecules and by the subsequent activation of MTF-1, leading to an increase in the synthesis of new MT proteins. Furthermore, the released zinc ions antagonize the pro-oxidative effect of other toxic metals, such as cadmium, by shifting the redox state toward the reducing/antioxidative effect of the MT-1/2 molecules (Sabolic et al. 2010).

As mentioned above, metal ions are capable of inducing MT expression in many mammalian tissues, including the liver, kidney, testes, and intestine (Sabolic et al. 2010; Babula et al. 2012). Increased expression of MTs has been noted in other organisms. It has been shown that the assessment of MT levels in the gills of various animal species may be a useful and effective biomarker for monitoring environmental contamination with toxic metals (Hamza-Chaffai et al. 1999; Hayes et al. 2004; Smaoui-Damak et al. 2009). Moreover, the protective role of MTs against heavy metal intoxication is additionally supported by their ROS scavenging ability (Chiaverini and De Ley 2010). Abundant experimental data underlie the importance of MT-1 and MT-2 as protective agents against intoxication with nonessential heavy metal ions.

2.4.1 Role of Metallothioneins in Preventing Cell and Organ Toxicity Induced by Cadmium Ions

Hitherto, the relationship between MTs and cadmium, a widely occurring environmental pollutant, has been best studied in relation to cytotoxic effects. Cadmium ions are taken up by animals and humans in contaminated food or inhaled in pollen to the lungs. Upon ingestion, cadmium cations are redistributed from the gastrointestinal tract and transferred to the liver, kidney, and testes. In intoxicated organs, cadmium ions exert their cytotoxic effects by activating ROS generation, subsequently leading to lipid peroxidation, DNA damage, and protein denaturation (Rani et al. 2014). The acute effects of cadmium intoxication, e.g., pulmonary edema, hemorrhage, fulminate hepatitis, and testicular lesions, as well as its chronic effects (nephrotoxicity, osteotoxicity, and immunotoxicity) have been described (Rani et al. 2014).

Experimental data show that pretreatment of animals with a small amount of cadmium increases their resistance to very high doses of cadmium ions, which in normal conditions could induce cell death (Goering and Klaassen 1983). Isolation of hepatic subcellular fractions in these animals 2 h after injection of the lethal cadmium dose revealed a diminished level of cadmium ions in nuclei, mitochondria, and endoplasmic reticulum and increased levels of cadmium ions in cytosol. Cadmium ions in the cytoplasm were bound to MTs, which had been markedly induced by cadmium pretreatment of the animals (Goering and Klaassen 1983). Further studies confirmed that high liver MT levels are capable of diminishing the hepatotoxic effect of cadmium ion administration to both newborn and adult rats (Goering and Klaassen 1984; Mukhopadhyay et al. 2009). Genetically modified MT-1/2 null animals (Liu et al. 1996; Zheng et al. 1996a, b; Habeebu et al. 2000a) and experimental models based on MT overexpression (Liu et al. 1995) confirmed the critical role of MT-1/2 in protecting against the acute and chronic effects of cadmium ions on liver toxicity.

Moreover, increases in MT-1 and MT-2 levels have also been reported in the lung following cadmium treatment via inhalation or intratracheal instillations (Hart et al. 1989, 1995, 2001; Kenaga et al. 1996; Potts et al. 2001). As was shown in the liver, pre-exposure to low doses of cadmium induces MT levels in the lungs of male Lewis rats, resulting in an increased tolerance of these animals to higher toxic doses of this metal ion (Hart et al. 1989). Potts et al. suggested that the most prominent change in cadmium-treated epithelial lung cells is the upregulation of MT expression, which may sequester cadmium ions and diminish the subsequent cytotoxic effects of generated ROS (Hart et al. 2001; Potts et al. 2001). Although the cells adapted to cadmium challenge by increasing MT-1/2 expression, they were also characterized by reduced ability of DNA repair and became more resistant to apoptotic stimuli (Hart et al. 2001; Potts et al. 2001). Human chronic cadmium-treated lung cells (CCT-LC) were shown to acquire a phenotype similar to that of cancer cells and this phenomenon was accompanied by an increase in MT-1/2 expression. This transformation occurs despite the cells' ability to adapt to chronic

cadmium exposure (Person et al. 2013). Indeed, the increase in MT-1/2 expression in lung alveolar cells was accompanied by decreased apoptosis of these cells and may therefore promote tumor development (Hart et al. 2001). Similarly, a decrease in E-cadherin expression in human lung cells was reported by Pearson et al. (2003) and was regarded as a significant risk factor for lung cancer development. Chronic cadmium intoxication and induction of MT-1/2 expression could thus contribute to the carcinogenesis process, quite apart from the protective role of these proteins in normal cells (Hart et al. 2001).

Animal models and in vitro studies allow the possible actions of MT-1/2 in protecting the kidneys against cadmium intoxication to be recognized. Cadmium ions may enter the tubular cells of kidneys via the basal and the luminal cell membranes (Zalups and Ahmad 2003). Acute nephrotoxicity has been observed upon *i.v.* injection of the cadmium-MT (Cd-MT) complex in mice (Nordberg et al. 1975). However, in this experimental model, the acute effects of cadmium intoxication were caused by the damaging effect exerted by the cadmium-MT complex upon its uptake from primary urine into proximal tubule cells (Nordberg et al. 1975). Nevertheless, under normal conditions, this mechanism is not observed, as free cadmium ions that are not bound to a complex with MTs are mostly absorbed from the interstitial fluid. Interestingly, it has been shown that MT-null mice, although presenting much lower cadmium accumulation in the kidney, manifest much more rapid and severe impairment in kidney function, as compared to wild-type mice, indicating a protective function of endogenously expressed MTs (Liu et al. 1998). Moreover, it seems that most protective effects can be seen in renal tubular proximal cells, which express higher levels of MT-1/2 as compared to tubular distal cells (Fig. 2.4). In addition, an in vitro study showed that the bound Cd-MT complex was less toxic than CdCl₂ to the cultured rat proximal tubule cells, as well as to pig renal proximal tubular cells (LLC-PK1 cell line), contradicting the observations of Nordberg et al. (Liu et al. 1994). The role of cadmium and Cd-MT complex in chronic nephrotoxicity was investigated by administering equal amounts of cadmium ions in a form of CdCl₂ or Cd-MT complex for 10 months to male Wistar rats (Groten et al. 1994). In this experimental setting, animals treated with CdCl₂ were characterized by a much higher nephrotoxicity, whereas the Cd-MT group only showed a slight increase in urinary gamma-glutamyl transpeptidase activity (a sensitive indicator of ischemic experimental injury) at the end of the experiment (Groten et al. 1994). It therefore seems that it is not the Cd-MT molecules, but the free intracellular cadmium ions, that are responsible for the observed nephrotoxic effects. The increased MT-1/2 expression in rat proximal tubular cells could also protect the cells from exercise-induced apoptosis by scavenging ROS (Podhorska-Okolow et al. 2006).

MTs have also been shown to protect bone tissue from cadmium-induced toxic effects. As in the case of hepato- and nephrotoxicity, MT knock-out mice were characterized by hypersensitivity to cadmium-induced bone injury, as compared to wild-type controls (Habeebu et al. 2000b; Regunathan et al. 2003). Osteocytes were identified to be the cells most affected by cadmium exposure, as increasing levels of

MT-1/2 in response to this toxic metal have been noted in this cell type (Oda et al. 2001).

2.4.2 Role of Metallothioneins in Preventing Cell and Organ Toxicity Induced by Other Metal Ions

Apart from cadmium ions, MT molecules have been shown to mediate resistance toward several other toxic metal ions, such as arsenic, lead, mercury, copper, and chromate. However, in comparison to cadmium ions, the protection provided by MTs against these toxic ions has been far less closely studied. Lines of evidence suggest that MTs are involved in protection against arsenic, which is responsible for numerous toxic and carcinogenic effects (Schuhmacher-Wolz et al. 2009). Inorganic arsenic can be transformed into more toxic methylated arsenicals, which are potent carcinogens causing tumors of the skin, lungs, and urinary bladder (Schuhmacher-Wolz et al. 2009). Arsenic compounds are capable of binding to MT's thiol groups at experimental low pH values of 3.5, as well as at physiological pH 7.0. This indicates that MT-1/2 may detoxify arsenic metal ions (Ngu and Stillman 2006; Ngu et al. 2010a). Moreover, it has been shown that arsenate induces MT-1/2 synthesis (Kreppel et al. 1993). MT-1/2 molecules have been shown to protect kidneys (Liu et al. 2000b), liver, and lungs (Jia et al. 2004a) against arsenic compounds in experimental conditions. In both studies, the severity of the lesions in these organs was significantly higher in the MT knock-out mice compared to the control animals (Liu et al. 2000b; Jia et al. 2004a).

Increases in MT-1 expression have also been noted in response to lead challenge, although a dual effect on expression of this isoform in mice was noted (Yu et al. 2009). An enhancement of MT-1 gene transcription was observed in the liver and kidney; however, MT synthesis was suppressed in the kidneys of the experimental animals (Yu et al. 2009). MT knock-out mice were also more susceptible to lead-induced toxicity, as exposure to this metal significantly impaired renal function in comparison to wild-type mice (Qu et al. 2002). Moreover, MT knock-out mice accumulated less lead in kidneys than did wild-type mice and did not form lead inclusion bodies (Qu et al. 2002). Consistent with the protective role of MTs in lead toxicity, Tokar et al. showed that MT knock-out mice are more sensitive to early-life lead exposure with regard to the frequent generation of testes tumors and of renal and urinary bladder preneoplastic lesions (Tokar et al. 2010).

MT-1 and MT-2 have also been shown to act as protective agents against mercury, a potent neurotoxin (Monnet-Tschudi et al. 2006). Mercury ions possess a high affinity to the thiol groups of various proteins, leading to their inactivation. Thus, the observed induction of MT-1/2 synthesis following mercury intoxication seems to counteract its toxic effects (Chan et al. 1992). So far, MT-1 and MT-2 have been shown to reduce the toxic effects of copper intoxication in the liver, central

nervous system, kidneys, and skin (Liu et al. 2000a; Aschner et al. 2006; Brandao et al. 2006; Peixoto et al. 2007; Hwang et al. 2013).

MT-1 and MT-2 have also been shown to sequester copper ions in two hereditary diseases affecting its metabolism, namely, Wilson's disease and Menke's disease (Nartey et al. 1987b; Suzuki-Kurasaki et al. 1997; Klein et al. 1998). Wilson's disease is a rare inherited syndrome presenting with altered copper metabolism characterized by copper deposition in the liver, brain, and cornea. Analysis of the liver of a patient affected by Wilson's disease revealed that copper ions were bound to MT-1/2 molecules (Nartey et al. 1987b). Experimental data from Long–Evans Cinnamon (LEC) rats, which bear a mutation similar to that noted in Wilson's disease, confirmed that MT-1 and MT-2 bind the majority of free copper ions in the lysosomes, where they are transformed over time, leading to subsequent hepatocyte necrosis via oxidative damage (Klein et al. 1998). This knowledge allowed a preventive therapy to be designed, based on chronic administration of zinc, which acts indirectly as a competitive inhibitor of copper ions' entry into the bloodstream. In this therapy setting, zinc ions increase the enterocyte pool of MT-1/2 molecules, which intercept the excess of absorbed copper ions. This prevents their entry into the bloodstream and the subsequent damage of liver and central nervous system. Chronic zinc therapy has replaced penicillamine (a potent metal-ion chelator) as the first-line therapy option in Wilson's disease (Hoogenraad 2006).

MT-1 and MT-2 have also been shown to bind chromate ions. Unlike other nonessential metals, the apo-MT molecule possesses a much higher binding affinity to this potent carcinogen and forms a stable complex once bound (Krepkiy et al. 2003). The mode of action of chromate ions in cells may involve generation of ROS and disruption of protein–DNA interactions (Borthiry et al. 2007, 2008). Chromium (Cr^{6+}) may thus also inhibit MT synthesis by interfering with the formation of the complex of MTF-1 and histone acetyltransferase p300/CBP, which is crucial for MT-1/2 transcription initiation. The decreased cellular levels of MT-1/2 may result in potentiation of the carcinogenic effects of Cr^{6+} due to the decreased antioxidant potential of the cell (Krizkova et al. 2012).

The high affinity of MT-1/2 to metal ions may also be the reason for the inactivation of alkylating drugs whose cytotoxic effect depends on the presence of heavy metal compounds (e.g., cisplatin, carboplatin) (Andrews et al. 1987; Shimoda et al. 2003; Choi et al. 2004). This effect may contribute to chemotherapy failure in some cancer types (Surowiak et al. 2003, 2007).

2.5 The Role of Metallothioneins Under the Conditions of Oxidative Stress

Abundant lines of evidence point to the role of MT-1/2 in diminishing the effects of oxidative stress, due to the action of free radicals such as ROS or RNS (Valko et al. 2006). These shortly lived molecules are characterized by the presence of at

least one unpaired electron and may be generated upon action of different physical factors (UV, gamma or X-ray radiation, chemical reactions catalyzed by metals) or during various biological processes (inflammatory reactions, mitochondrial respiration). Free radicals may be beneficial in inflammatory and immune reactions, but, produced in excess, they may lead to damage of different cellular structures, ultimately leading to cell death or neoplastic transformation (Valko et al. 2006). It was demonstrated that their damaging effects may be counterbalanced by antioxidant molecules such as MT-1 and MT-2 isoforms (Krizkova et al. 2009a, 2012; Chiaverini and De Ley 2010).

The thiolate cluster of MT-1/2 proteins is responsible for these molecules' redox potential, and is dependent on the stability of the zinc/thiolate binding, which in turn modulates the mobility of zinc ions and their transfer to other zinc-dependent proteins (Maret and Vallee 1998). The observed induction of MT-1/2 expression by free radicals has led to the suggestion that these proteins may protect cells from oxidative stress (Andrews 2000; Nguyen et al. 2003). The antioxidant properties of MT-1/2 have been confirmed in numerous *in vitro* and *in vivo* studies. Thornalley and Vasak observed for the first time that, in rabbit liver, MT-1 scavenged free hydroxyl and superoxide radicals much more effectively than bovine serum albumin, which was used as a control in this cell-free experimental setting (Thornalley and Vasak 1985). The antioxidative properties enable MT-1/2 to decrease the DNA damage caused by hydroxyl radicals (Abel and de Ruiter 1989). Furthermore, induction of MT-1/2 expression by ZnCl_2 pretreatment of HL-60 human promyelocytic leukemia cells and V79 Chinese hamster cells confirmed the ROS-scavenging ability of MT-1/2 (Chubatsu et al. 1992; Quesada et al. 1996). In both cell types, elevated MT-1/2 levels abrogated oxidative stress and reduced the DNA damage in comparison to the control cells (Chubatsu et al. 1992; Quesada et al. 1996). MT-1/2 also protected mouse embryonic fibroblasts (NIH 3T3) from tert-butyl hydroperoxide toxicity—a potent inducer of free radicals (Schwarz et al. 1995). Moreover, MT-2A, overexpressed in lymphocytes, has been shown to protect them from UV radiation-induced damage (Yang et al. 2007).

The ability of MT molecules to transfer from cytoplasm to nucleus has been found to be strongly dependent on the redox state of the nucleus (Apostolova et al. 2000; Ogra and Suzuki 2000). Since the cell cycle may be affected by redox status, it has been suggested that MT-1/2 molecules may protect the cell's nucleus from excess free radicals and thus regulate and warrant the progress of the cell cycle (Takahashi et al. 2005).

Interestingly, although *in vitro* studies point to the antioxidant role of MT-1/2, *in vivo* experiments have not confirmed the results of the *in vitro* experiments. The use of MT knock-out mice permitted an analysis of the role of MT isoforms under *in vivo* conditions. The levels of antioxidant proteins and molecules (superoxide dismutase, catalase, or glutathione peroxidase and glutathione) did not differ in MT-null mice exposed to oxidative stress induced by gamma-irradiation or 2-nitropropane, compared to control wild-type mice (Conrad et al. 2000). The extent of oxidative damage to DNA, lipids, and proteins in the liver of both mouse types was comparable, although the levels of MT-1/2 in the liver of the

wild-type mice increased significantly. Furthermore, when mice were exposed to whole-body irradiation, no differences were observed in their survival time, even when the animals were subjected to zinc pretreatment in order to increase MT-1/2 expression levels (Conrad et al. 2000). Also the study of Davis et al., who used MT knock-out and MT-1/2 overexpressing mice also did not produce convincing results regarding the antioxidative role of MT-1/2 in vivo (Davis et al. 2001). Hepatotoxicity, as measured by serum alanine aminotransferase activity, histological analyses, and hepatic thiol levels, was greater in the knock-out mice than in the controls 12 h after carbon tetrachloride treatment. However, no differences were observed at later time points up to 48 h. Hepatotoxicity was also similar between MT-overexpressing and control mice, and the dietary zinc treatment provided no further protection (Davis et al. 2001).

Although the abovementioned studies do not confirm the antioxidant role of MT-1/2, several other in vivo experiments have noted significant differences, suggestive of the protective role of MT expression in some diseases. In a model of acetaminophen-induced liver injury, MT-1/2 prevented the generation of ROS, rather than functioning as a ROS scavenger (Saito et al. 2010). The reactive acetaminophen metabolite, *N*-acetyl-*p*-benzoquinone imine, was effectively trapped by MT-1/2 molecules by covalent bindings. This prevented the binding of *N*-acetyl-*p*-benzoquinone imine to cellular proteins and thus blocked the further actions that would lead to mitochondrial dysfunction and nuclear DNA damage (Saito et al. 2010). In addition, MT-1 and MT-2 have been shown to protect the cell against DNA damage in liver and bone marrow in mice with high fat-diets (Higashimoto et al. 2009). MT-1 and MT-2 also have protective effects on gastric mucosa during inflammation caused by infection with *Helicobacter pylori* (Mita et al. 2008). MT-1/2 inhibited the activation of cyclooxygenase-2 (COX-2) in an experimental model of collagen-induced arthritis (Youn et al. 2002), and inducible nitric oxide synthase (iNOS) in a model of cryogenic brain cortex injury (Penkowa et al. 2006), which supports the antioxidant role of these MT isoforms. Mice overexpressing the MT-1/2 isoforms were relatively resistant to oxidative stress. However, MT knock-out mice have been shown to be sensitive to oxidative stress-induced carcinogenesis of the skin and liver cells in vivo (Suzuki et al. 2003; Waalkes et al. 2006). Due to the scavenging ability of MT-1/2, these molecules have been shown to mediate cancer cells' resistance to certain chemotherapeutics, such as irinotecan (Chun et al. 2004), adriamycin (Hatcher et al. 1997), and doxorubicin (Yap et al. 2009), as well as radiotherapy (Cai et al. 1999; Smith et al. 2006).

2.6 Metallothioneins and Apoptosis

Apoptosis plays an important role in numerous physiological as well as pathological processes. Under normal conditions, unnecessary cells are eliminated from tissues, allowing normal functioning. Abnormalities and inhibition of this process

may lead to the development of many diseases—especially autoimmune or neoplastic disorders (Khan et al. 2014). Apoptosis has been shown to be modulated by the actions of ROS and zinc ions (Formigari et al. 2007, 2013). This essential metal ion has been shown in eukaryotic cells to regulate the activity of various enzymes and transcription factors, thus influencing several key processes, such as differentiation, cell growth, and apoptosis (MacDonald 2000). Zinc ions also function as protective agents for enzymes by inhibiting the oxidation of their sulfhydryl groups (Powell 2000). Due to the free radical scavenging and the metal-binding ability of MT-1/2, these proteins have been shown to strongly influence apoptosis (Coyle et al. 2002b; McGee et al. 2010). Depending on the cell type, MT-1/2 may protect DNA from UV-induced damage as a health-promoting effect (McGee et al. 2010). However, from another point of view, the elevated expression of MT-1/2 in cancer cells has been shown to protect them from chemotherapy and radiotherapy (Andrews et al. 1987; Hishikawa et al. 1997; Cai et al. 1999; Bedrnicek et al. 2005).

One of the major regulators of apoptosis is the p53 protein. In many studies, its expression has been shown to strongly depend on zinc ion concentration and ROS levels (Meplan et al. 2000; Fan and Cherian 2002; Ostrakhovitch and Cherian 2004). Furthermore, zinc is required for the proper functioning of the p53 protein, as it stabilizes the protein structure via its binding to the Cys₃His₁ cluster of the DNA-binding domain of the molecule (Meplan et al. 1999). Additionally, zinc ions have been shown to be essential for p53-mediated transcription regulation (Meplan et al. 2000). Taking into account the fact that MT-1/2 may regulate cell zinc and ROS content, they are also capable of regulating the activity of the p53 protein in several ways.

Direct interaction of apo-MT with the p53 protein has also been documented (Ostrakhovitch et al. 2006; Xia et al. 2009). However, MT (apo-MT bound with zinc ions) did not behave in this way and did not affect the transcriptional activity of p53 (Ostrakhovitch et al. 2006). Further studies identified that the interaction is mediated by the free sulfhydryl groups of apo-MT, which interacted with the zinc ion of the p53 protein. Such binding results in the acquisition of a mutant-like phenotype by p53, resulting in the loss of its DNA-binding abilities and in subsequent apoptosis inhibition (Xia et al. 2009). Moreover, recombinant MT molecules have been found to modulate the structure of the p53 protein in experimental *in vitro* conditions (Meplan et al. 2000). Furthermore, high levels of MT-1/2 could hypothetically intercept zinc ions from the p53 molecule, inhibiting its DNA-binding capabilities and suppressing its pro-apoptotic function (Palecek et al. 1999). On the other hand, cells lacking MT-1/2 were characterized by high levels of the p53 protein and demonstrated a high sensitivity toward apoptosis-inducing factors (Kondo et al. 1997). Interestingly, Ostrakhovitch et al. demonstrated that only cells with intact function of the p53 protein are capable of inducing MTs' expression upon treatment with metal ions (Ostrakhovitch and Cherian 2004; Ostrakhovitch et al. 2007). In an earlier study, the authors demonstrated that copper-induced apoptosis in MCF-7 cells was strongly dependent on the activity of the p53 protein, whereas the triple negative MDA-MB-231 cells, lacking functional p53, did not respond to such treatment (Ostrakhovitch and Cherian

2004). These results have been confirmed in an experiment performed on the p53 positive MN1 and parental MCF-7 breast cancer cells, which responded to zinc and copper treatment by increasing MRE activity and MTF-1 expression (Ostrakhovitch et al. 2006). Inactivation of p53 in these cells rendered them unresponsive to copper and zinc treatment, and no increase in MTF-1 expression could be noted. Furthermore, the introduction of functional wild-type p53 into the MDD2 breast cancer cell line with a dominant-negative p53 enhanced the ability of zinc ions to increase MTF-1 gene expression (Ostrakhovitch et al. 2007). These results suggest that, in addition to the presented interactions of MT and p53, an additional regulatory loop exists between these proteins, but further research is necessary to corroborate these findings.

Abundant lines of evidence point to the role of MT-1/2 in mediating the activity of *NF-κB*-related pathways. *NF-κB* is a DNA-binding protein complex found in almost all animal cells and involved in the regulation of numerous processes. Its activation has been observed in stress conditions, where it protects cells by activating antiapoptotic genes and proto-oncogenes, allowing cell survival (Gilmore 2006; Perkins 2007). It has been shown that MT-1/2 interacts with the p50 subunit of *NF-κB* and positively regulates its activity in murine fibroblasts (Butcher et al. 2004), cervical cancer HeLa cells (Kim et al. 2003), and MCF-7 breast cancer cells (Abdel-Mageed and Agrawal 1998). On the other hand, it was found that overexpression of MT-2 impaired *NF-κB* activation and sensitized the V-H4 hamster mutant cell line to mitomycin-C (Papouli et al. 2002). The inhibition of MT-2 in these cells, utilizing antisense nucleotides, restored the chemoresistance of the cell line to this therapeutic agent (Papouli et al. 2002). Similarly, Sakurai et al. demonstrated MT-1/2 to be a negative regulator of *NF-κB* activity in mouse embryonic cells (Sakurai et al. 1999). Taking into account the results of all studies performed so far, it seems that the modulation of *NF-κB* activity by MTs is strongly dependent on the cell type, which points to the involvement of yet undiscovered mechanisms. Nevertheless, the increased activity of *NF-κB* in cancer cells on induction of MT-1/2 expression may contribute to carcinogenesis and disease progression (Abdel-Mageed and Agrawal 1998; Kim et al. 2003).

2.7 Metallothioneins and Cell Proliferation

High levels of MT-1/2 have been found in active and proliferating tissues, whether normal or affected by a specific disease process (Cherian and Apostolova 2000; Cherian et al. 2003; Dziegiel 2004; Krizkova et al. 2009b, 2012). In normal tissues, high MT-1/2 levels have been demonstrated by immunohistochemistry (IHC) in proliferating hair follicles of healthy skin (Karasawa et al. 1991) and in the basal cell layer of the epidermis (Zamirska et al. 2012). MT-1/2 has also been shown to mediate proper wound healing of the skin, which was accompanied by changes of zinc ion levels in proliferating cells as well as in serum (Iwata et al. 1999). Zinc ions have been shown to modulate several processes, as they function as cofactors for

many proteins, e.g., matrix metalloproteinases and transcription factors, assuring in this way their correct functioning (Chasapis et al. 2012; Krizkova et al. 2012). The metal-binding properties of MT-1/2 allow them to act as possible zinc ion donors for zinc-dependent enzymes and transcription factors functioning via their zinc finger domains. Proteins of the MT-1/2 family play a crucial role in processes such as replication, transcription, and translation (Ostrakhovitch et al. 2007; Pedersen et al. 2009). It has been shown that MT-1 and MT-2 act as metal chaperones, regulating the availability of zinc ions to the zinc finger domains of transcription factors. In many studies, it has been demonstrated that MT-1/2 regulates in this matter the activity of transcription factor IIIA (Zeng et al. 1991b; Petering et al. 2000; Huang et al. 2004), Gal4 (Maret et al. 1997), transcription factor Sp1 (Zeng et al. 1991a; Rana et al. 2008), tramtrack (Roesijadi et al. 1998), and the estrogen receptor (Cano-Gauci and Sarkar 1996).

The abovementioned observations correspond to MT-1/2 localization changes regarding cell cycle stages. In resting cells (G0 phase), MTs can be detected in the cytoplasm, whereas in cells undergoing division, these proteins have been observed to shift to the nucleus. Such a mechanism has been observed, e.g., in case of regenerating hepatocytes (Cherian and Apostolova 2000; Cherian and Kang 2006) and differentiating myoblasts (Apostolova et al. 2000). Furthermore, the high cytoplasmic expression of MTs has been noted in the late G1 phase and at the G1/S threshold, while the highest MT-1/2 concentration in the cell nucleus has been noted in the S and G2 phases (Cherian and Apostolova 2000; Levadoux-Martin et al. 2001). The data derived from normal cells have been confirmed by in vitro experiments on papillary thyroid carcinoma (KAT5) and anaplastic thyroid carcinoma (ARO) cell lines. Cadmium-induced MT-1/2 expression has been found to be related to alterations in the cell cycle, leading to increases in the proportion of cells in the S and G2-M phase and decreases in the number of cells in the G0/G1 phase (Liu et al. 2007, 2009). In human colonic HT-29 cancer cells, the highest MT-1/2 level has been noted in the G1 phase, confirming their proproliferative role (Nagel and Vallee 1995). Furthermore, the recent study of Lim et al. has revealed that downregulation of the MT-2A gene in MCF-7 cells by the use of siRNA particles inhibits cell growth by inducing cell cycle arrest in the G1-phase (G1-arrest), with a marginal increase in cells in sub-G1-phase (Lim et al. 2009). In addition, the MT-2A-silenced cells were characterized by a higher expression of the ataxia telangiectasia mutated (*ATM*) gene, concomitant with a lower expression of the *cdc25A* gene—suggesting that this MT isoform may mediate the progression from the G1 to the S phase of the cell cycle by regulating the activity of the ATM/Chk2/*cdc25A* pathway (Lim et al. 2009). The results obtained in in vitro experiments have been confirmed by studies on tumor sections. A positive correlation of MT-1/2 expression with that of Ki-67 antigen or PCNA (proliferating cell nuclear antigen) has been noted in formalin-fixed paraffin-embedded tumor sections of breast cancer (Jin et al. 2002; Gomulkiewicz et al. 2010; Wojnar et al. 2011), non-small cell lung cancers (Werynska et al. 2011), squamous cell cancers of the skin (Zamirska et al. 2012), and soft tissue sarcomas (Dziegiel et al. 2005), supporting their role in the regulation of proliferation.

2.8 The Structure and Role of the MT-4 Isoform

The MT-4 isoform is so far the least studied member of the MT family. The MT-4 gene is localized approximately 20 kb upstream from the 5' end of the MT-3 gene in the human and mouse genomes (Quaife et al. 1994). MT-4 is a 62 amino acid long, single-chain protein, similar in its structure to the members of the MT-1/2 isoforms. However, a glutamate insert is present at position 5, unlike in the MT-1 and MT-2 molecules (Quaife et al. 1994). Similarly to the MT-1 and MT-2 proteins, MT-4 is capable of binding up to seven bivalent metal ions. However, its metal-binding domains seem to be characterized by a higher demetallization resistance by EDTA, in comparison to that of the MT-1 molecule (Cai et al. 2005; Meloni et al. 2006). Interestingly, research conducted on *Escherichia coli* has shown that MT-4 has a higher affinity to monovalent copper ions than to bivalent zinc ions, which may significantly affect its function in cells. In turn, the MT-1 protein preferentially binds zinc ions (Tio et al. 2004). Moreover, MT-4 also possesses lower binding affinity to cadmium ions than MT-1 (Tio et al. 2004). Therefore, the MT-4 molecule has also been suggested to be a “copper thionein” in comparison to other family members, which are regarded as “zinc thioneins” (Tio et al. 2004; Vasak and Meloni 2011). The molecular studies underlie the significance of MT-4 expression in the skin, as copper and zinc ions are important in modulating the development of normal skin, wound healing, and the progression of skin diseases (Kumar et al. 2012; Ala et al. 2013).

It has already been shown that MT-4 is involved in the regulation of the development of the stratified squamous epithelium of the skin and upper respiratory tract, which might support this thesis (Quaife et al. 1994). In situ hybridization has shown that the most intense *MT-4* mRNA signals are present in the differentiating spinous layer of cornified epithelia. In turn, the MT-1 isoform expression has been noted predominantly in the proliferative basal layer of epidermis (Liang et al. 1996). Downregulation of *MT-4* gene expression in nude mice characterized by the lack of Whn transcription factor activity has also been noted (Schlake and Boehm 2001). These observations point to a different role of MT-4 than of other members of the MT family and indicate its important role in the differentiation process of the skin (Quaife et al. 1994; Schlake and Boehm 2001). In recent studies the presence of MT-4 was not found in squamous cell cancers of the lung. Thus, it seems that the MT-4 role in differentiation may be limited only to the skin (Werynska et al. 2011).

In addition, MT-4 has also been identified in mouse maternal decidua (Liang et al. 1996). *MT-1* and *MT-2* mRNA expression have been found in the visceral yolk sac, placenta, and fetal liver; however, no *MT-3* or *MT-4* mRNA expression has been noted in these tissues. In turn, *MT-3* and *MT-4* mRNAs are both abundant in the maternal decidua and in experimentally induced deciduoma at 7 and 8 days *post coitum*, in which high levels of *MT-1* and *MT-2* mRNA have also been observed (Liang et al. 1996).

Metallothioneins in Normal and Cancer Cells

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2016, XII, 117 p. 13 illus., 11 illus. in color., Softcover

ISBN: 978-3-319-27471-3