Molecular and Integrative Toxicology

Anuradha Mudipalli
Judith T. Zelikoff *Editors*

Essential and Non-essential Metals

Carcinogenesis, Prevention and Cancer Therapeutics

Humana Press

Molecular and Integrative Toxicology

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Preface

Metals are constituents of vital proteins that drive numerous critical biochemical activities essential for life. In addition, metallic elements have become integral parts of human civilization by virtue of their diverse applications and utility throughout society.

For the purposes of human health, metals are classified as 'essential' (e.g. calcium, magnesium, manganese, selenium, zinc, iron, and copper) or non-essential. Non-essential metals include nickel, hexavalent chromium, cadmium, and arsenic (the latter, in the strict sense, a metalloid, but referred here as a metal for practical purposes). Both essential and non-essential metals can occur naturally in the environment or via diverse routes of exposure such as from personal products from agricultural, commercial, and industrial applications. Advances in metal research have also revealed that several essential metals can pose health threats at certain dietary concentrations. Recent research also shed light on the therapeutic role that metal-based drugs can play in a variety of disease conditions including cancer. Metals can also prove helpful for prevention of disease under different levels of dietary intake and/or through interactions with the internal environment of the host. Each chapter in this book describes underlying molecular mechanisms and gene pathways associated with one or more of these effects. In addition, consideration is given to the cross talk among these pathways as elucidating the multifarious biological role(s) of these metals is an active area of research.

Though there are a number of excellent books on metal biology and health, this book provides a multi-disciplinary and integrative look at the diverse molecular pathways of metals focusing on 'essential and non-essential metals, carcinogenesis, prevention and chemotherapy'. The nine chapters, contributed by renowned experts across the globe, detail the mechanistic pathways involved in metal carcinogenesis from an inter-disciplinary perspective including cancer prevention and applications in chemotherapy.

We believe this book caters to the intellectual appetite of metal biologists relentlessly pursuing the myriad of research and risk assessment questions which remain.

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Chapter 1
Calcium Channels and Pumps: Importance During Lactation as Potential Targets for Breast Cancer

Gregory R. Monteith and Teneale A. Stewart

Abstract The enrichment of milk with calcium is critical for the survival of mammals after birth. The process of transfer of calcium ions from the maternal blood supply into milk occurs through mammary alveolar epithelial cells. Recent research has provided deep mechanistic insight into these processes with candidates for the critical pathways involved in calcium transport identified. These proteins include the store-operated Ca^{2+} entry component Orai1 (basolateral Ca^{2+} influx), the secretory pathway Ca^{2+}-ATPase isoform 2 (SPCA2, secretion of Ca^{2+}), and the plasma membrane Ca^{2+}-ATPase isoform 2 (PMCA2, apical membrane Ca^{2+} efflux). Increased expression of Orai1, SPCA2, and PMCA2 has also been identified in breast cancer cells; however, the remodeling of these targets often demonstrates selectivity for specific clinical and/or molecular subtypes. Silencing of these targets has identified roles for these proteins in the proliferation and/or migration of some breast cancer cell lines.

Keywords ATPase • Calcium • Cancer • Channel • Epithelial • Lactation • Mammary • Milk • Pump

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CaSR	Calcium-sensing receptor
CYT	Cytosolic
HER2	Human epidermal growth factor receptor 2
IGF1R	Insulin-like growth factor 1 receptor
IP^3	Inositol 1,4,5-trisphosphate
MCU	Mitochondrial uniporter
mM	Millimolar
NCLX	Mitochondrial sodium calcium exchanger
NCX	Na^+/Ca^{2+} exchanger
nM	Nanomolar
PMCA	Plasma membrane Ca^{2+}-ATPase
SERCA	Sarco-/endoplasmic reticulum Ca^{2+}-ATPase
SPCA	Secretory pathway Ca^{2+}-ATPase
STIM1	Stromal interaction molecule 1
STIM2	Stromal interaction molecule 2
TRP	Transient receptor potential

1.1 Introduction

The Ca^{2+} ion is a key regulator of a variety of cellular processes. The events that the Ca^{2+} ion can regulate include muscle contraction, neurotransmitter release, and gene transcription. The ubiquity of the Ca^{2+} signal has required the evolution of pathways that can differentially respond to the diversity of the nature of increases in cytosolic-free Ca^{2+} ($[Ca^{2+}]_{CYT}$). This diversity in the nature of calcium signals is reflected in Fig. 1.1, where transient and sustained global increases in $[Ca^{2+}]_{CYT}$, $[Ca^{2+}]_{CYT}$ oscillations and highly localized increases in $[Ca^{2+}]_{CYT}$ are all illustrated. These differential changes in $[Ca^{2+}]_{CYT}$ can be decoded by the cell to achieve specific cellular events [1]. Examples include the ability of sustained elevations in $[Ca^{2+}]_{CYT}$ to induce cell death [2], the regulation of gene transcription by the frequency of $[Ca^{2+}]_{CYT}$ oscillations [3], and the relaxation of smooth muscle cells through highly localized Ca^{2+} changes just beneath the plasma membrane [4]. Arguably, calcium is perhaps best known for its importance in bone and teeth. The central role for calcium in the body means that there are complex mechanisms to transport Ca^{2+} ions across cell membranes. Indeed, epithelial cells of the gastrointestinal tract have pathways to ensure the absorption of calcium from the diet [5], and those in the mammary gland are responsible for the enrichment of milk with calcium, which is essential for the growing infant [6].

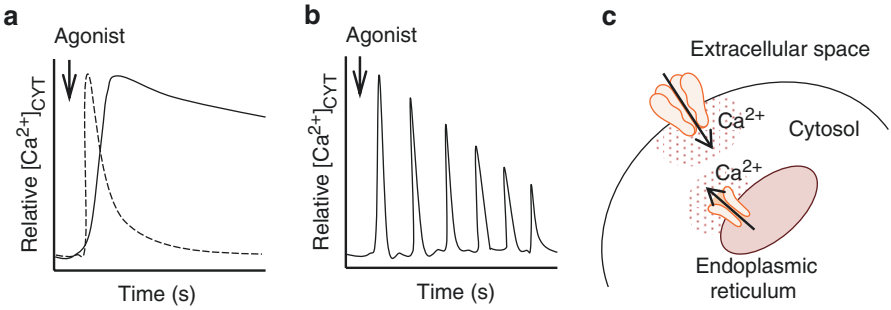

Fig. 1.1 Examples of the diverse nature of intracellular calcium signals in mammalian cells. Cytosolic free Ca^{2+} increases may be (**a**) transient (represented by the *dotted line*) or sustained (represented by the *solid line*), or (**b**) oscillatory in nature, (**c**) while others may be highly localized within the cytosol (represented by the *shaded area*). Adapted from [57–59]

In this chapter we will focus on the mechanisms by which milk is enriched with calcium. We will also discuss evidence that some of the proteins important in this biological process and/or their related isoforms, which have unique expression patterns and function in breast cancer tissue, have been proposed as novel targets for some breast cancer subtypes.

1.2 Ca^{2+} Transport Mechanisms in Epithelial Cells

Epithelial cells line the lumen of a variety of organs, including those of the intestine and mammary gland. Epithelial cells are often polarized with a basolateral and apical membrane. The transport of ions and molecules is a key capability of epithelial cells, and this transport is important in processes as diverse as the absorption of ions and nutrients from the diet, the reabsorption of ions in the kidney, to the transport of key components into milk. The calcium channels, pumps, and exchangers expressed by epithelial cells can vary significantly depending on the organ; Fig. 1.2 provides an overview of the general classes of proteins which are often present in epithelial cells and contribute to changes in Ca^{2+} across the membrane of organelles or the plasma membrane.

Like other mammalian cells, there is a maintained Ca^{2+} concentration gradient in epithelial cells (~1.8 mM extracellular free Ca^{2+} vs~100 nM intracellular free Ca^{2+}). An active Ca^{2+} transport protein, the plasma membrane Ca^{2+}-ATPase (PMCA), is critical in maintaining this Ca^{2+} gradient (Fig. 1.2). PMCAs extrude Ca^{2+} ions across the plasma membrane against a concentration gradient through energy derived from the cleavage of ATP [7]. PMCAs have been established as an important mechanism by which epithelial cells return to resting levels of $[Ca^{2+}]_{CYT}$. Such increases in $[Ca^{2+}]_{CYT}$ can occur following the release of Ca^{2+} from endoplasmic reticulum Ca^{2+} stores via inositol 1,4,5-trisphosphate (IP_3) sensitive ion channels after activation of

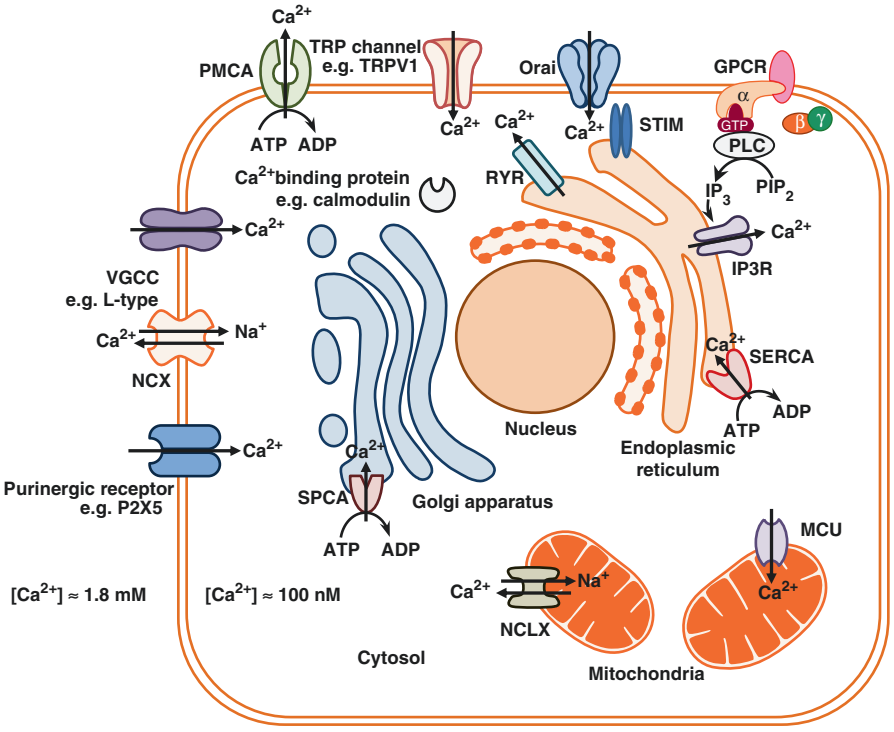

Fig. 1.2 Diagrammatic overview of the general classes of calcium signaling and transporting proteins, including channels, channel activators, pumps, and exchangers, often present in mammalian epithelial cells, which contribute to the regulation of cytosolic or organellar Ca^{2+}. Major classes of calcium influx channels include the transient receptor potential (TRP) family (e.g., TRPV1), and the Orai family of store-operated calcium influx channels, which are activated by STIM proteins in response to endoplasmic reticulum Ca^{2+} store emptying. Purinergic receptors (e.g., P2X5) and voltage-gated calcium channels (VGCCs) (e.g., L-type) may also regulate epithelial cell calcium influx. Activation of certain G-protein-coupled receptors (GPCRs) at the plasma membrane leads to the generation of the second messenger inositol 1,4,5-triphosphate (IP3) via protein lipase C (PLC), which subsequently activates endoplasmic reticulum localized IP_3 receptors (IP_3Rs) resulting in Ca^{2+} store emptying. Ryanodine receptor (RYR) activation also contributes to endoplasmic reticulum store emptying, while the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) facilitates store refilling. The plasma membrane Ca^{2+}-ATPase (PMCA) family and sodium calcium exchanger (NCX) represent major calcium efflux pathways. The secretory pathway Ca^{2+}-ATPase (SPCA) family and mitochondrial uniporter (MCU) and mitochondrial sodium calcium exchanger (NCLX) play key roles in Golgi and mitochondrial calcium regulation, respectively. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, *PIP2* phosphatidylinositol 4,5-bisphosphate. Adapted from [8, 9, 12, 59]

many G-protein-coupled receptors (Fig. 1.2) [8]. Exchangers, such as the Na^+/Ca^{2+} exchanger (NCX), can also expel Ca^{2+} from cells through a secondary active transport mechanism where the Na^+ gradient can drive Ca^{2+} efflux [8]; however, the contribution of NCX in epithelial cells seems to be less than in other cell types (e.g., cardiac cells and neurons).

Elevations in $[Ca^{2+}]_{CYT}$ can also occur through the opening of Ca^{2+} channels of the plasma membrane. Plasma membrane-localized channels can be activated by changes in membrane potential (voltage-gated) or via specific ligands such as the hot chili component capsaicin (transient receptor potential V1 ion channel) or ATP (P2X receptors), while other channels are activated by the depletion of endoplasmic reticulum Ca^{2+} stores (e.g., Orai1) [9] (Fig. 1.2). In addition to PMCAs and NCXs of the plasma membrane, normal resting $[Ca^{2+}]_{CYT}$ is returned in part through the sequestration of Ca^{2+} into the endoplasmic reticulum Ca^{2+} store via the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [10]. The secretory pathway (e.g., Golgi) also has active transport mechanisms for Ca^{2+} sequestration, namely, the secretory pathway Ca^{2+}-ATPase (SPCA), which also efficiently transports Mn^{2+} ions [10]. The mitochondria is also equipped with a complex system for regulating Ca^{2+} levels, and these recently defined processes have been reviewed elsewhere [11].

1.3 Lactation

The supply of nutrient- and energy-rich milk to growing infants is one of the defining features of mammalian development. The process of lactation requires significant developmental changes in the human breast. Milk is a rich source of lactose, lipids, vitamins, and calcium [6]. The components of milk can vary widely between mammalian species. The calcium content in milk is often high in animals with a fast-growing skeletal system, for example, the total calcium content of milk from whales exceeds 60 mM, while the calcium content of human milk is less than 10 mM [6].

The powerhouses of lactation in animals are the mammary gland epithelial cells. Milk components accumulate in the lumen of mammary alveolar epithelial cells, while contraction of the myoepithelial cells that form a mesh network around the alveoli leads to the movement of milk through the mammary duct system and eventual expulsion through the nipple [6, 12, 13]. The contraction of myoepithelial cells and the letdown process is triggered by oxytocin via suckling and even infant crying [14].

1.4 Ca^{2+} Transport During Lactation

Seminal early work on calcium transport during lactation provided compelling evidence that Ca^{2+} ions made their way into milk through direct transport across mammary gland epithelial cells [15]. Early studies provided evidence that a significant proportion of calcium is present in casein micelles, implicating an important role for the secretory pathway. However, the molecular components responsible for enabling the total calcium levels in milk (10–400 mM, depending on the species) [6] to exceed the total calcium level in the maternal blood supply are only now being fully identified and characterized.

The free Ca^{2+} concentration gradients between the maternal blood supply (~1.8 mM), the cytosol of the mammary alveolar epithelial cell (~100 nM), and milk (>3 mM [6]) provide insights into the types of proteins involved at each critical stage of the enrichment of milk with calcium. The higher concentration of free Ca^{2+} in the maternal blood supply, compared to that in the cytosol of alveolar epithelial cells, creates a Ca^{2+} gradient whereby the opening of a Ca^{2+} permeable ion channel on the basolateral membrane of alveolar epithelial cells represents the most efficient Ca^{2+} entry mechanism. The detection of Ca^{2+} in casein micelles of milk implicates involvement of secretory pathway Ca^{2+} pumps [6, 12]. The presence of free Ca^{2+} ions in milk at a level significantly higher than that of the cytosol of mammary alveolar epithelial cells is indicative of a plasma membrane localized active Ca^{2+} transport mechanism. Below, we outline and discuss the likely molecular candidates for the key processes required for the enrichment of milk with calcium, these are (1) Ca^{2+} influx across the basolateral membrane of mammary alveolar epithelial cells, (2) Ca^{2+} secretion into milk, and (3) the efflux of Ca^{2+} ions across the apical membrane of mammary alveolar epithelial cells into milk.

1.4.1 Basolateral Ca^{2+} Influx in Mammary Epithelial Cells During Lactation

Although it represents the first step in the transfer of calcium ions from the maternal blood supply to milk, the ion channel responsible for this step has been elusive [6]. Given its high Ca^{2+} selectivity and its essential role in the absorption of dietary Ca^{2+} [16], the transient receptor potential ion channel family member TRPV6 was proposed as a likely candidate. However, it now appears that the mammary gland engages a different mechanism. One pathway arguably uniquely suited to supplying Ca^{2+} influx during lactation is store-operated Ca^{2+} entry, a pathway activated upon depletion of Ca^{2+} stores, involving the Ca^{2+} channel component Orai1 and the endoplasmic reticulum Ca^{2+} sensor STIM1 [17] as depicted in Fig. 1.3. McAndrew et al. reported a significant increase in the mRNA levels of Orai1 in samples from the mouse mammary gland during lactation compared to samples isolated from nulliparous, pregnant, and weaning mice [18]. In contrast there was no significant increase in the related isoforms Orai2 and Orai3 [18]. In mouse HC11 mammary gland epithelial cells, differentiation to a lactation phenotype is associated with an increase in non-stimulated Ca^{2+} influx [19]. This augmented Ca^{2+} influx is eliminated by Orai1 silencing [19]. Similarly, non-stimulated Ca^{2+} influx is reduced by Orai1 silencing in mouse SCp2 mammary epithelial cells [20].

The most direct evidence for a role for Orai1 in basolateral Ca^{2+} influx during lactation are recent studies using Orai1 null mice [21]. As expected, the mammary luminal epithelial cells from Orai1 null mice have significantly reduced store-operated Ca^{2+} entry [21]. Moreover, milk produced by Orai1 null mice has less than 50% total calcium compared to wild-type mice. This effect is not due to general changes in Ca^{2+} homeostasis in the mother or milk quality, as maternal serum Ca^{2+}

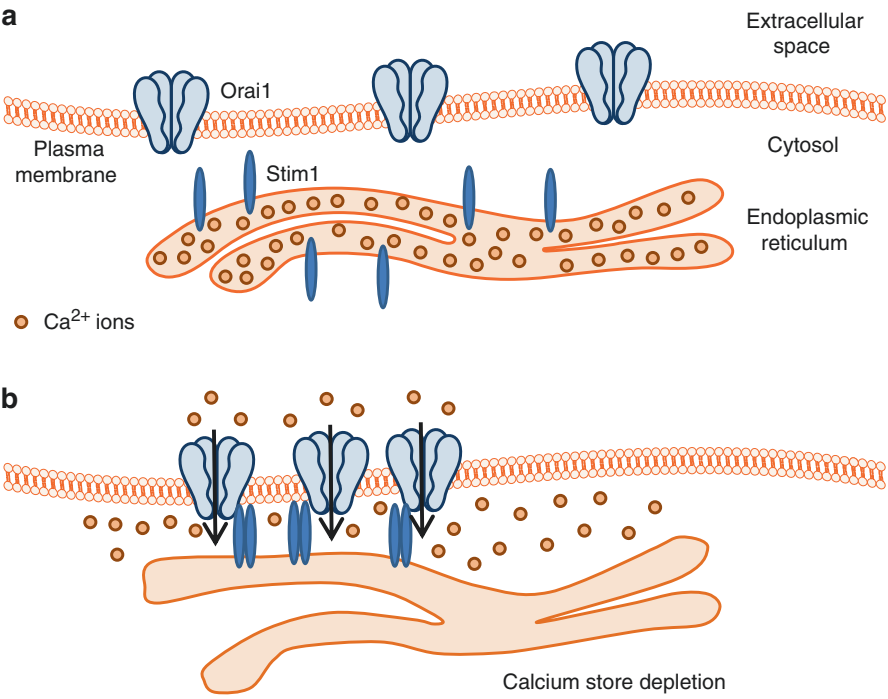

Fig. 1.3 Simplified overview of store-operated calcium entry. (**a**) At rest where calcium stores (i.e., endoplasmic reticulum) are replete, the store-operated calcium channel, Orai1, and calcium store sensor, STIM1, are found dispersed throughout the plasma and endoplasmic reticular membranes, respectively. (**b**) Following calcium store depletion (e.g., in response to receptor activation), Orai1 and STIM1 become juxtaposed, enabling Orai1 channel activation by STIM1, resulting in calcium influx and subsequent store refilling. Adapted from [60–63]

and milk protein levels were not significantly altered in Orai1 null mice compared with wild type [21]. This was further supported by the reduced calcium content in milk collected from mice where Orai1 knockdown was more targeted to the mammary gland using an MMTV-Cre system [21]. The studies of Davis et al. also identified an unexpected and vital role for Orai1 in lactation [21]. This work identified Orai1 as the mechanism for $[Ca^{2+}]_{CYT}$ oscillations and maintained contractions induced by oxytocin in mammary myoepithelial cells, the consequence of which was a reduced ability to expel milk in Orai1 null mice [21]. Collectively, the aforementioned studies identify Orai1-mediated Ca^{2+} influx as the mechanism for both the basolateral Ca^{2+} influx step for Ca^{2+} entry from the maternal blood supply and the contraction of myoepithelial cells required for milk expulsion (Fig. 1.4). Further studies are now required to explore any potential differences between mammals in this pathway and how such differences could be related to the variation in milk calcium levels seen between some species. It is also still unclear how Orai1 is activated during lactation, with a variety of candidates suggested based on expression, in vitro models or overexpression models.

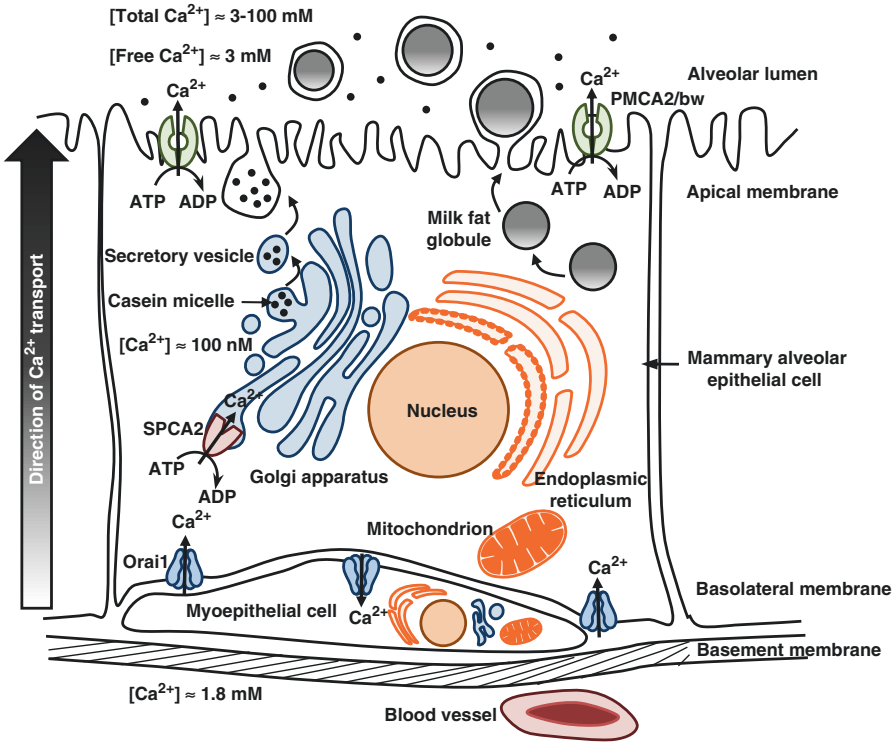

Fig. 1.4 Diagrammatic representation of a mammary alveolar epithelial cell and myoepithelial cell indicating localization of specific calcium channels and pumps known to play a role in lactation. The plasma membrane Ca^{2+}-ATPase 2 (PMCA2) and its splice variant PMCA2bw play a key role in regulating calcium flux across the apical membrane and the enrichment of milk with calcium during lactation. The calcium channel Orai1 plays a dual role in lactation by facilitating basolateral calcium entry into mammary alveolar epithelial cells and playing a critical role in myoepithelial cell contraction, a requirement for efficient milk ejection. Expression studies suggest that the secretory pathway Ca^{2+}-ATPase 2 (SPCA2) contributes to the secretion of Ca^{2+} into milk. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate. Adapted from [12, 21, 23, 26]

1.4.2 Ca^{2+} Secretion from Mammary Epithelial Cells During Lactation

The presence of calcium in casein micelles in milk is consistent with an important role for the secretory pathway [6, 12] and hence points to a pivotal role for SPCAs in lactation. Indeed, until the studies of PMCAs in lactation (discussed below), it was predicted that the vast majority of calcium that was present in milk was obtained through the secretory pathway. Early studies of lactation identified an upregulation of the calcium pump now known as SPCA1 [22]. SPCA1 has a wide tissue distribution and is present in most cells. However, the more recently identified and less widely expressed SPCA2 isoform has been found to undergo greater increases in

expression in the mammary glands of mice during lactation [23]. SPCA2 expression is highly localized to mammary epithelial cells, whereas SPCA1 expression is present in a variety of cell types in the mammary gland.

SPCA1 and SPCA2 levels undergo pronounced decreases in expression after cessation of milk production, and this downregulation has been proposed to contribute (in addition to the downregulation of PMCA2, discussed below) to a Ca^{2+}-dependent apoptosis cascade during mammary gland involution [24]. SPCA2 has also been linked to other roles in lactation beyond Ca^{2+} accumulation in the Golgi. The reported ability of fragments of SPCA2 to activate Orai1 calcium influx (the protein linked to store-operated Ca^{2+} influx) has been put forward as a potential novel mechanism to match the Ca^{2+} supply (Ca^{2+} influx) with demand (Ca^{2+} secretion) in mammary gland epithelial cells. In this context, SPCA2 has also been reported to regulate Orai1 trafficking [20]. However, further work is required given the inconsistent results in different in vitro lactation models. It should also be noted that in addition to SPCAs, the temporal upregulation of expression of the Ca^{2+}/H^{+} antiporter TMEM165 has been proposed as indicative of a role for this protein in calcium transport in the secretory pathway during lactation [25]. Further studies focused on TMEM165 are now warranted, as are studies of lactation in $SPCA2^{(-/-)}$ and $SPCA1^{(-/+)}$ mice and the role of SPCA2 in Orai1 trafficking and activity during lactation in vivo.

1.4.3 Ca^{2+} Efflux from Mammary Epithelial Cells During Lactation

The identification of pronounced upregulation of the PMCA isoform PMCA2 during lactation [22], and the reduced (~60%) Ca^{2+} content of milk from PMCA2 null mice [26], defined a role for direct Ca^{2+} efflux and specifically PMCA2 in lactation. PMCA2, up until this time, had been associated with very specific processes consistent with its relatively restricted tissue distribution [27]. For example, PMCA2 null mice were associated with hearing and balance defects but were otherwise relatively healthy [28]. Links between PMCA2 and hearing were further strengthened by the association between a PMCA2 mutation and a type of hereditary deafness in humans [29]. The use of PMCA2 in mammary alveolar epithelial cells during lactation is again indicative of the specific Ca^{2+} transport demands during lactation. Indeed the PMCA2 splice variant identified in the mammary gland during lactation (PMCA2bw) is a particularly specialized variant [26].

The association between PMCA2 and the transport of Ca^{2+} during lactation has been explored in mouse cell culture models [30] and a human sample undergoing lactational change [31]. There is also evidence that the calcium-sensing receptor (CaSR) is a regulator of PMCA2, suggesting a mechanism by which the maternal Ca^{2+} levels (supply) may be sensed by the mammary gland and Ca^{2+} transfer to milk adjusted accordingly [30]. The downregulation of PMCA2 expression during weaning is one of the key mechanisms by which mammary gland epithelial cells become

sensitive to apoptotic signals and undergo subsequent involution [32]. The recent identification of the sensitivity of PMCA2 expression during lactation to serotonin deficiency [33] provides compelling evidence that the role of PMCA2 in processes in the breast is dynamic and needs to be further investigated.

In summary, the last decade has seen a much clearer picture of the proteins responsible for calcium transfer from the maternal blood supply to milk for infant development (Fig. 1.4). The proteins used in this process are those which have previously been linked to the immune system (Orai1) and the nervous system (PMCA2) or had physiological roles which were poorly understood (SPCA2). The use of these proteins in lactation highlights the unique demands that lactation places on calcium transport.

1.5 Breast Cancer

The introduction of hormone-based therapy and the advent of agents targeting the human epidermal growth factor receptor 2 (HER2) oncogene have resulted in breast cancer now having one of the best survival rates of all cancers. However, triple-negative breast cancers, many of which overlap with the molecularly defined basal subtype, lack effective molecularly targeted therapies and are associated with treatments with severe side effects and which often have poor long-term effectiveness. The recent identification of a number of triple-negative breast cancer molecular subtypes [34] suggests that this type of breast cancer may require a variety of specific therapies, rather than a "one size fits all" approach, and may continue to represent treatment challenges. In the text below, we outline some of the recent work that has identified proteins involved in Ca^{2+} transport during lactation as potential therapeutic targets in breast cancer.

1.6 Ca^{2+} Signaling and Transport and Breast Cancer

The regulation of many of the defined hallmarks of cancer [35] by calcium signaling (e.g., proliferation, death, metastasis [36]), and the remodeling of calcium signaling and/or calcium channel or pump expression in specific cancers [37], has led to the proposal that specific Ca^{2+} channels and pumps may represent novel therapeutic targets [38, 39].

A variety of calcium channels and pumps have been reported to have alterations in breast cancer. Examples include TRPV6, where gene copy number is increased in estrogen receptor-negative breast cancers and whose silencing reduces the proliferation of a breast cancer cell line with endogenously high levels of TRPV6 [40]. However, in this chapter we will focus on studies of Ca^{2+} transport proteins with roles in calcium transport during lactation. Proteins involved in lactation may represent unique ways to target proliferative breast cancer cells in patients. Indeed,

such an approach has been highlighted in the context of iodide transporters in breast cancer [41]. As outlined below, the proteins involved in Ca^{2+} transport during lactation have been identified as characterizing features of some breast cancer subtypes.

1.6.1 ORAI1 and Breast Cancer

Silencing of Orai1 has been shown to reduce the proliferation and migration of human breast cancer cells in vitro [18, 42] and the growth and metastasis of human breast cancer in vivo [42, 43]. Pharmacological inhibition of store-operated Ca^{2+} entry using SKF96365 also inhibits the development of lung metastasis in a mouse breast cancer model where immune function is intact [42].

Some proteins involved in breast cancer metastasis may in part be due to effects on Orai1-mediated Ca^{2+} influx. Such an association is evidenced by studies of the ether-à-go-go (hEag1) K^+ channel in triple-negative basal-like MDA-MB-231 breast cancer cells [44]. The activation of receptors linked to migration (e.g., PAR-2) produces Ca^{2+} influx that is Orai1 sensitive in breast cancer cell lines [18]. Other examples of this link include the pro-migratory effects of metalloprotease-cleaved CD95L in basal-like MDA-MB-468 breast cancer cells, which involves an Orai1-mediated Ca^{2+} influx pathway [45]. Similarly the pro-migratory effect of cell surface-associated enolase-1 in MDA-MB-231 involves effects on Orai1-mediated Ca^{2+} influx [46].

Store-operated Ca^{2+} entry and specifically Orai1-mediated Ca^{2+} influx may be particularly important in basal breast cancers. This association goes beyond the aforementioned studies of Orai1 in triple-negative breast cancer cell lines such as MDA-MB-231. Orai1 mRNA levels are higher in basal vs non-basal breast cancers, and basal breast cancers are associated with a significant relationship between STIM1 and STIM2 (activators of Orai1) compared to other breast cancer molecular subtypes, with high levels of STIM1 relative to STIM2 [18]. Indeed, breast cancers with high levels of STIM1 relative to STIM2 levels are associated with a poorer prognosis [18]. The association between basal breast cancers and Orai1 was at first surprising given Orai1's proposed role in basolateral Ca^{2+} influx in alveolar mammary epithelial cells during lactation, which could suggest a more luminal breast cancer molecular subtype expression signature. However, the identification of Orai1 as a key regulator of the contraction of myoepithelial cells during lactation [21] and the association between basal breast cancers and some features of myoepithelial cells [47] may in part explain the association between the Orai1 Ca^{2+} influx pathway and basal breast cancers. The potential link between Orai1-mediated Ca^{2+} influx and triple-negative breast cancers is mirrored somewhat by the association between Orai3 and estrogen receptor-positive breast cancer cells. Orai3 has enhanced protein expression in estrogen receptor-positive breast cancer cell lines compared to estrogen receptor-negative breast cancer cell lines and is regulated by the estrogen receptor [48]. Moreover, store-operated Ca^{2+} entry in estrogen receptor-positive but not

estrogen receptor-negative breast cancer cell lines is sensitive to Orai3 silencing [49]. Collectively these results highlight the need for the full characterization of Orai isoforms (their expression, regulation, and role) in different breast cancer subtype models and in clinical samples.

1.6.2 SPCA2 and Breast Cancer

SPCA2 represents the most recently identified P-type Ca^{2+}-ATPase, and soon after the first studies of SPCA2 [50, 51] came the first report of its upregulation in the mammary gland during lactation in mice [23]. SPCA2 is overexpressed in some luminal-like breast cancer cell lines (such as MCF-7) and a subset of clinical breast cancer cells compared to matched normal surrounding tissue [43]. Elevated SPCA2 mRNA levels appear to be most associated with the ERBB2 molecular phenotype and are not associated with the basal breast cancer molecular subtype. Silencing of SPCA2 reduces the proliferation and growth in soft agar of MCF-7 breast cancer cells [43]. Consistent with a pro-proliferative role for SPCA2, overexpression in the nonmalignant MCF-10A breast cell line promotes proliferation and growth in soft agar [43]. Silencing of SPCA2 also reduces the in vivo growth of MCF-7 cells [43]. In addition to these effects being potentially mediated by effects on Ca^{2+} levels within the Golgi, the pro-proliferative effects of SPCA2 when overexpressed appear to occur in part through a novel mechanism involving activation of Orai1-mediated Ca^{2+} influx (also linked to breast cancer cell proliferation) [43]. SPCA2 activation of Orai1 occurs independently of the endoplasmic reticulum Ca^{2+} store sensors STIM1 and STIM2 and involves the direct interaction between the amino terminus of SPCA2 with Orai1 [43]. It is interesting to note that the related isoform SPCA1 has been linked to the basal breast cancer molecular phenotype [52]. In this case, the antiproliferative effects of SPCA1 silencing in MDA-MB-231 triple-negative breast cancer cells are likely due to effects on the activity of Ca^{2+}-dependent enzymes residing in the Golgi lumen. These enzymes include proprotein convertases, which regulate key proteins such as insulin-like growth factor 1 receptor (IGF1R) [52]. Collectively these studies suggest that some breast cancers are associated with a remodeling of SPCAs and that this may be an opportunity for future therapeutic intervention.

1.6.3 PMCA2 and Breast Cancer

The first Ca^{2+} transporter associated with lactation that was assessed in breast cancer was PMCA2. PMCA2 was found in a number of breast cancer cell lines [53], with elevated levels in breast cancer cell lines assessed compared to nonmalignant breast cell lines [54]. PMCA2 is expressed in a significant proportion of breast cancers, with reports of either a significant increase or trend toward greater PMCA2 protein levels in HER2 (ERBB2)-positive breast cancers [31, 32]. However, elevated mRNA levels

appear to be a feature of the basal molecular subtype [31]. Forced overexpression of PMCA2 in luminal T47D breast cancer cells increases their ability to recover from ionomycin-mediated increases in $[Ca^{2+}]_{CYT}$, reduces both ionomycin- and docetaxel-induced cell death [32], and increases T47D growth in vivo [55]. PMCA2 silencing also reduces the proliferation of MDA-MB-231 cells and augments the anti-proliferative effects of low concentrations of doxorubicin [31]. The identification that PMCA2 is a regulator of HER2 protein localization in breast cancer cells and the observation that MMTV-Neu mice (a model of Erbb2-induced mammary cancer) are less likely to develop tumors if they are null for PMCA2 [55] provide more compelling evidence that PMCA2 inhibitors could have therapeutic potential in some breast cancers.

Despite the promising signs for PMCA2 as a drug target or biomarker in breast cancer (which include the viability of PMCA2 knockout animals and the highly restricted tissue distribution of PMCA2 [28]), there are still some key issues that will need to be addressed over the next decade. Firstly, PMCA2 inhibitors could have effects on balance and hearing [28], which may require targeting of specific splice variants or the use of agents more directed toward breast cancer cells. The relationship between PMCA2 and prognosis appears complex and related to specific patient cohorts. This is exemplified by high levels of PMCA2 being associated with poor prognosis in women under 50 years of age [32], yet in women with the basal-like or claudin-low breast cancer subtype, high levels of PMCA2 are associated with better survival [31]. Another layer of complexity regarding the potential targeting of PMCA2 in breast cancer is found in the ability of overexpressed PMCA2 fragments to reduce the viability of MCF-7, MDA-MB-231, T47-D, and ZR-75-1 breast cancer cells [56]. Hence, more studies are required to provide insight into the role and mechanism of the contribution of PMCA2 in breast cancer progression and the most effective way to therapeutically target this protein.

1.7 Summary

Over recent years the components responsible for the accumulation of calcium into milk have slowly been identified. The use of Orai1 and Pmca2 null mice have helped identify a role for these proteins in basolateral Ca^{2+} influx and apical membrane Ca^{2+} efflux, respectively. Expression studies also suggest that SPCA2 is a key mechanism for Ca^{2+} secretion into milk. Orai1, PMCA2, and SPCA2 have also been linked to breast cancer, but their contributions appear to be very different, and studies so far suggest that their roles may be more evident in particular breast cancer subtypes.

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Conflict of Interest Prof Monteith is associated with QUE-Oncology Inc.®

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Chapter 2
Tumor Development Through the Mg^{2+}nifying Glass

Valentina Trapani and Federica I. Wolf

Abstract The last decades have witnessed a greater appreciation of the importance of magnesium for human health, but the relationship between magnesium and cancer development remains controversial. Here we review the current knowledge on the cellular and molecular mechanisms that underlie the manifold effects of magnesium during tumor progression. A complex picture emerges where the positive consequences of low magnesium availability on tumor growth seem to be counterbalanced by negative outcomes in the very early and late stages of tumorigenesis; a concurrent immune-inflammatory response appears to contribute throughout the natural history of a tumor. We also discuss the debated interaction between magnesium status and the response to therapy and the potential application of the TRPM7 magnesium channel as a prognostic marker and a therapeutic target. A deeper understanding of magnesium homeostasis through the interaction of fundamental and clinical researchers is key to develop new strategies of cancer prevention and treatment.

Keywords Carcinogenesis • Cancer therapy • Inflammation • Magnesium • Metastasis • TRPM7 • Tumor progression

2.1 Introduction

Magnesium (Mg) has been recognized as an essential element for all living beings for over a century. An enormous amount is known about Mg: its requirement as a nutrient in all species; its role in enzymatic processes; its unique function in all reactions that depend on ATP and nucleotides, including phosphorylation; its structural role in tissues, proteins, and membranes; and its role in modulating intracellular metabolism [1]. Our knowledge on the role of Mg in biology has advanced rapidly, particularly in the past two decades, and now encompasses fields of knowledge

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from nutrition to molecular biology. This has paralleled a deeper understanding of the importance of Mg for human health and disease.

Mg has an important physiological role in many tissues, and not surprisingly, disturbances of Mg homeostasis have been implicated in the pathophysiology of a variety of diseases [2]. The World Health Organization listed magnesium among the nutrients consumed in amounts low enough to be of concern [3]. In particular, this occurs in Western (or "Westernized") countries, where a modest to mild hypomagnesemia is thought to be widespread [4], due to soil impoverishment, but also to extensive food processing, and the preference for calorie-rich, micronutrient-poor foods.

Among the major public health concerns worldwide is the continuing increase in the global burden of cancer, which in the first place results from an aging population, but also from the adoption of cancer-causing lifestyles, including diet [5]. Several epidemiological studies have found an inverse correlation between magnesium intake and the incidence of some types of cancer (for a review, see [6]), and a recent meta-analysis confirmed that a high dietary magnesium intake could have protective effects against the overall risk of developing a cancer, in particular for colorectal cancer [7].

In the face of a consistent and growing body of epidemiological evidence, the role of magnesium in cancer biology and therapy is nonetheless highly debated. In this chapter, we will focus on the cellular and molecular mechanisms that underlie the manifold effects of magnesium on tumor development. We will explore in detail each stage of this multistep process, namely, initiation, growth at the primary site, and formation of distant metastases. This will serve a thorough discussion on the still controversial function of magnesium in the response to therapy and the potential clinical implications. Finally, we will review the newest findings on the involvement of the TRPM7 cation channel, which seems to offer the best therapeutic and diagnostic potential at the moment.

2.2 Magnesium and Carcinogenesis

The protective effect of Mg in the early stages of carcinogenesis can be carried out via two main mechanisms: (1) by affecting oxidative stress and consequent oxidative DNA modifications that might lead to mutagenesis and (2) by affecting DNA repair mechanisms that maintain genomic stability [8].

The first lines of evidence that link low magnesium availability to increased oxidative stress date back to the early 1990s. Since then only sparse in vitro investigations have directly shown that depletion of extracellular Mg affected the levels of intracellular reactive oxygen species [9]. We found indirect evidence by cDNA array analysis of genes modulated by changes in extracellular magnesium availability [10]. Among these genes, some were involved in antioxidant defense. Of particular interest was an inverse relation between magnesium availability and expression/activity of radical scavenger enzymes such as glutathione S-transferase (GST),

which is consistent with the notion that low magnesium availability induces a pro-oxidant condition. Overall, however, in vitro studies provided conflicting results, suggesting that the cellular response to Mg deficiency could be highly dependent on cell type or on other ancillary determinants ([10] and references therein). On the other hand, numerous in vivo investigations have consistently reported indexes of oxidative stress in Mg-deficient animals: enhanced tissue, erythrocyte and lipoprotein peroxidation, oxidative modifications of proteins, reduced antioxidant status, and increased plasma nitric oxide ([11] and references therein). The current view is that the major origin of the oxidative stress in vivo is the inflammatory response triggered by Mg deficiency. After a few days on an Mg-deprived diet, rodents develop an intense inflammatory syndrome characterized by leukocyte and macrophage activation, release of pro-inflammatory cytokines and acute-phase proteins, and excessive production of free radicals [11]. The molecular bases for this phenomenon are not completely understood, but several mechanisms have been proposed. A possible explanation might lie in a reduced extracellular magnesium/calcium antagonism as a result of decreased plasma magnesium concentration. NMDA receptors have a threshold of activation that is lowered when extracellular Mg levels decrease. In addition, magnesium binds to the regulatory gates of calcium channels and limits calcium influx into the cell. Therefore, conditions of low Mg availability will lead to an increase in calcium influx through NMDA receptors and calcium channels; in turn, this will result in increased production and/or release of inflammatory mediators and neurotransmitters such as substance P, which will amplify the response [12]. Another major event involved in the initiation of the inflammatory response due to Mg deficiency could be the activation of NFκB. The NFκB family consists of a group of inducible transcription factors implicated in the regulation of crucial steps of immune and inflammatory responses through regulation of the gene expression of a large number of cytokines and other immune response genes [13]. Indeed, activation of NFκB in conditions of low Mg availability has been shown in endothelial [14] and vascular smooth muscle cells [15]. Most importantly, a low Mg status has been clearly associated with increased inflammatory stress in humans [16], and the inflammation-cancer connection is a well-established paradigm [13]. Inflammation is involved both in the early and late stages of tumor development. We will discuss in the following sections the contribution of the low Mg-induced inflammation to the creation of a favorable milieu for tumor progression. Here, in the context of carcinogenesis, we underline that inflammatory mediators, some of which are direct mutagens, also directly or indirectly downregulate DNA repair pathways and cell cycle checkpoints, thus destabilizing cell genome and contributing to the accumulation of random genetic alterations [17].

In addition to the indirect inflammation-mediated effects on genome stability, Mg could also have a direct role in maintaining genome fidelity. Magnesium has dual effects on nucleic acids: it stabilizes their structure [18] and serves an essential cofactor in almost all enzymatic systems involved in DNA processing, as well as in nucleotide excision repair, base excision repair, and mismatch repair [19]. In practice, however, very few investigations have been designed to explore the effect(s) of Mg deficiency on DNA repair and tumorigenesis in cellular or in vivo systems.

Among these, a case-control study found that low Mg dietary intake was associated with poorer DNA repair capacity in lymphocytes and increased risk of lung cancer [20]. Nonetheless, most of the current knowledge is limited to structure-activity determinants of the interaction of Mg with defined repair enzymes (for recent examples, see [21, 22]).

In conclusion, low Mg availability might contribute to carcinogenesis by different mechanisms, all of which converge onto genome instability. In vivo and clinical studies suggest that this could be ascribed to the generalized inflammatory condition driven by Mg deficiency. However, it must be noted that even though low magnesium determines inflammation and increases the levels of free radicals, which both generate genetic instability, it is more likely that a low Mg status only contributes to tumorigenesis by synergizing with other factors [23].

2.3 Magnesium and Primary Tumor Growth

The relationship between magnesium and cell proliferation has been well established since the 1970s, thanks to the seminal work by Harry Rubin, who postulated the theory of "the coordinated control of cell proliferation" and proposed Mg as the key factor that regulated the different steps of this complex process [24]. The molecular determinants were not identified until the late 1990s and later. It was shown that low magnesium availability inhibits cell cycle progression leading to a G_0/G_1 arrest through the upregulation of p27 [25, 26], p21 [27, 28], and p16 [28]. Magnesium-dependent growth arrest is reversible: upon reintroducing magnesium, the percentage of cells in S-phase increases, and the levels of cell cycle inhibitory proteins decrease, which leads to an increase in the proliferation rate [10]. Proliferating cells contain more magnesium than resting ones, and the required amount can be retrieved irrespectively of extracellular availability over a wide range of concentrations [29]. In other words, no proliferation can occur without an adequate magnesium supply.

Extracellular Mg availability is likely to affect progression through the cell cycle by determining sizeable modifications of intracellular Mg pools that might alter Mg-dependent enzymatic activities. Rubin proposed that mTOR (mammalian target of rapamycin), a PI3K (phosphoinositide 3-kinase)-related kinase which initiates protein synthesis, might be the key activity modulating G_1-phase protein synthesis, based on biochemical characteristics [30]. Since $Mg\text{-}ATP^{2-}$ is the only active form of ATP, the limiting step of mTOR-specific kinase reaction would be the full availability of Mg to form $Mg\text{-}ATP^{2-}$ at a concentration close to its K_m for ATP, i.e., 1 mmol/L [30]. Amazingly, more recent research findings support the view that extracellular magnesium availability is sensed and transduced into cell proliferative behavior by transporter molecules and that mTOR could be the magnesium-sensitive key regulator of protein synthesis associated with proliferative signals. In particular, the transient receptor potential melastatin type 7 (TRPM7) channel has been identified as the gatekeeper of cellular Mg homeostasis and has been shown to be essential for cell viability and growth [31]. Most relevantly to the present discussion,

TRPM7 is important for cell cycle progression during G_1 phase [32], and TRPM7-mediated Mg^{2+} influx seems to be associated to receptor-mediated mitogenic signaling along the PI3K/Akt/mTOR protein translation cascade [33, 34].

The absolute requirement of Mg for cell proliferation seems to imply that highly proliferating tumor cells should be extremely avid for their Mg supply. Indeed, tumor cells have a higher intracellular magnesium content; however, they are more refractory to the growth inhibition induced by low Mg availability in comparison with normal cells [8, 23]. This apparent contradiction can be reconciled by increasing experimental evidence showing that overexpression of TRPM7 is a common feature shared by several types of cancer and has an involvement in tumor development and progression, as we will discuss in the dedicated section.

The role of magnesium in tumor growth is probably not confined just to the regulation of cell proliferation, but might also be linked to cancer metabolic reprogramming. The importance of metabolic alterations in proliferating tumor cells was first described by Otto Warburg in the 1920s [35] and has been lately reappraised [36]. Tumor cells display a high glucose consumption through the glycolytic pathway, even in the presence of oxygen (aerobic glycolysis), which results in increased lactate production leading to acidification. Indeed, all dividing cells consume far more glucose than resting ones, as glycolysis provides extremely rapid ATP synthesis compared with oxidative phosphorylation, in spite of lower efficiency. Notably, most of the enzymes involved in glycolysis, the Krebs cycle, and the respiratory chain depend on magnesium as either an allosteric modulator or a cofactor [9]. An association between glucose transport and Mg fluxes across the cell membrane was suggested in early reports [1], and the underlying molecular mechanisms have been recently reviewed [37]. Alterations in tumor metabolism, referred to as "metabolic reprogramming," address not only the need for rapid energy generation but also two other equally important needs: (1) increased biosynthesis of macromolecules and (2) tightened maintenance of appropriate cellular redox status [36]. Interestingly, aerobic glycolysis is associated with massive de novo expansion of the adenine nucleotide pool, resulting in generation of new molecules of ADP, each of which requires a new Mg^{2+} ion to be taken up from the extracellular milieu. Indeed, Mg uptake has been linked to metabolic transitions occurring in lymphocytes switching from a quiescent state to activation via PI3K/Akt/mTOR-dependent growth signaling [33, 34]. It is intriguing to speculate that the same metabolic transitions associated with rapid proliferation might occur during malignant transformation. Furthermore, as we discussed in the previous section, magnesium might contribute to the maintenance of a proper redox balance.

A comprehensive overview of the role of Mg in tumor biology must nonetheless consider that a growing tumor is not simply a mass of proliferating tumor cells. Over the past decade, tumors have increasingly been recognized as organs whose complexity approaches and may even exceed that of normal healthy tissues. They are composed of multiple distinct cell types that participate in heterotypic interactions with one another. Therefore, the biology of a tumor can only be understood by studying the individual specialized cell types within it, as well as the tumor microenvironment that they construct during the course of multistep tumorigenesis [38].

It is therefore worth noting that magnesium availability can modulate the functions of a variety of normal cells present in the tumor microenvironment. In particular, endothelial cells cultured in low magnesium release higher amounts of matrix metalloproteases (MMPs) and growth factors [14]. Similar results were obtained in cultured human fibroblasts [39]. In addition, low magnesium promotes endothelial and fibroblast senescence [39], and senescent cells can modify the tissue environment in a way that synergizes with oncogenic mutations to promote the progression of cancers [40]. On the other hand, however, one of the first needs that an enlarging tumor must address is the provision of nutrients and oxygen, as well as an ability to evacuate metabolic wastes and carbon dioxide, via induction of neo-angiogenesis, which is mainly carried out by microvascular endothelial cells. Low extracellular magnesium impairs acquisition of the angiogenic phenotype by microvascular endothelial cells, as it retards their proliferation, migration, and differentiation, without affecting MMP production and 3D organization [41]. Moreover, it has been shown that low Mg suppresses cellular response to hypoxia [42], which is a recognized hallmark of cancer modulating angiogenesis as well as metabolic reprogramming [43].

Keeping in mind these premises, it could be envisaged that overall magnesium availability should correlate with the rate of tumor growth also in vivo. Indeed, Mg-deficient mice exhibited a striking 60% reduction in the growth of primary tumors in comparison with Mg-sufficient controls, which was reversed by reintroduction of Mg in the diet [44]. These experiments provided persuasive evidence that low magnesium availability does inhibit tumor growth in vivo; further investigations indicated that inhibition of tumor growth was accompanied by a decreased number of tumor vessels and an increased oxidative damage to DNA [45]. Such findings fit well with the role of Mg as a key regulator of cell proliferation, redox balance, and angiogenic switch, as emerged from in vitro studies. However, as discussed in the previous section, hypomagnesemia in rodents is always accompanied by leukocytosis and other markers of systemic inflammation [11], and it cannot be ruled out that such inflammatory condition could contribute to the overall inhibition of tumor growth.

2.4 Magnesium and Tumor Metastasis

Both in vitro and in vivo data concur to delineate a sound picture in which magnesium promotes tumor growth, in line with its well-established role in modulating cell proliferation. Unexpectedly, the same animal studies drew attention to an alarming twist in the story: in spite of the smaller size of primary tumors and the low degree of neovascularization therein, mice on a low magnesium diet developed far more lung metastases than controls [44]. The multistep process of invasion and metastasis envisions a succession of events, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape

of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed "colonization" [38]. Of note, magnesium deficiency triggers expression of several proteases both in the tumor [45] and the vasculature [46]. For example, low Mg conditions induce a marked increase in the total amounts and activity of MMP-2 and MMP-9 in endothelial cells [46]. These enzymes are able to degrade the matrix network and create tiny holes in the basal membrane and interstitial stroma that allow the cells to migrate. Thus, low magnesium availability lays the requisites for an improved migration of tumor cells in the surrounding environment, by promoting local invasion and intravasation. Once in the bloodstream, tumor cells must interact with the endothelium of target organs before they extravasate and grow. In the previous sections, we have already recalled the occurrence of an intense inflammatory response in Mg-deficient rodents [11] and the contribution of such condition to the initiation and growth of the primary tumor. Inflammation could also foster further cancer progression, due to the presence of inflammatory cells and mediators, forging the tumor microenvironment [13]. Tumor necrosis factor (TNF) α, interleukins (IL) 1 and 6, all induced under magnesium deprivation [11], augment the capacity of cancer cells to metastasize [13]. Lungs from mice on a magnesium-deficient diet exhibited upregulation of several genes of the inflammatory response [47]. With particular regard to extravasation, both micro- and macrovascular endothelial cells cultured in low magnesium were found to upregulate vascular cell adhesion molecules (VCAMs) [27], which can facilitate the tethering of metastatic cells to the vessel wall, and their subsequent transmigration to and colonization of the adjacent tissues.

On a biochemical note, it should be kept in mind that magnesium ion is essential for the activity of countless enzymes and proteins. A possible link between low magnesium and metastasization is suggested by the absolute requirement of Mg for the function of the metastasis suppressor gene product NM23-H1 [48]. Hypomagnesemia might therefore mimic what happens in NM23-H1 knockout mice, which show accelerated and massive lung metastasis [49].

An intriguing association has been recently found between phosphatase of regenerating liver protein tyrosine phosphatase 2 (PRL-2) and the magnesium transporter CNNM3; a functional heterodimer of the two proteins seems to be involved in the regulation of intracellular magnesium homeostasis and the promotion of oncogenesis [50]. PRL tyrosine phosphatases are known to promote invasion and motility via modulation of rho family GTPases [51]. In particular, expression levels of PRL-2 correlate with tumor progression, being markedly elevated in metastatic lymph nodes compared with primary tumors [52]. Since PRL-2 expression is inversely regulated by dietary Mg levels [53], it can be hypothesized that low Mg conditions may boost metastatic potential by upregulating PRL-2.

In conclusion, while the near future could provide further molecular details linking magnesium to tumor progression, the current state of the art delineates a complex picture where the positive consequences of a low Mg availability (i.e., inhibition of primary tumor growth and neo-angiogenesis) seem to be counterbalanced by

negative outcomes in the very early and late stages of tumorigenesis (i.e., tumor initiation and stimulation of invasion and metastasis formation). The immune-inflammatory response that complicates Mg deficiency appears as a recurrent theme playing throughout the natural history of a tumor, as demonstrated by animal studies and confirmed by clinical findings.

2.5 Magnesium and Cancer Therapy

In the face of extensive in vitro and in vivo investigations on the role of Mg in tumor development, there is still very little information on this relationship in clinical settings. In particular, because of the avidity of rapidly proliferating cells for Mg, it is often assumed that tumor growth can alter systemic Mg availability in a patient. Indeed, some reports showed that serum Mg concentrations are decreased in patients with solid tumors and that such decrease correlates to the stage of malignancy [54, 55]. More relevantly, compelling evidence is accumulating that diverse therapeutic agents can affect serum Mg levels, but it is not clear whether and how treatment-associated changes of magnesemia may influence tumor growth and dissemination. This is not a trivial point, as it raises a clinical dilemma circa the opportunity of correcting or not hypomagnesemia in cancer patients [56].

Body Mg balance can be disturbed by a broad variety of drugs, which can potentially lead to symptomatic hypomagnesemia with tetany, seizures, and cardiac arrhythmias [57]. Among anticancer drugs, the widely used cisplatin has long been known to cause severe hypomagnesemia, due to its nephrotoxic effect, which results in renal magnesium wasting [58]. More recently, hypomagnesemia has also been found to occur in patients treated with the anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab, with a severity proportional to the dose and the duration of treatment [58]. In contrast to cisplatin, cetuximab impairs renal Mg reabsorption with no evident tubular damage. Cetuximab-induced Mg wasting is instead due to a specific molecular cross talk between EGF and the transient receptor potential melastatin TRPM 6 channel that plays a key role in Mg reabsorption in the distal convoluted tubule [59]. TRPM6 activity is increased upon binding of EGF to EGFR; thus, inhibition of EGFR activation by cetuximab results in decreased Mg uptake [60]. Panitumumab, which also targets the extracellular ligand binding domain of the EGFR, induces hypomagnesemia as well [61]. To date there are no reports of hypomagnesemia in patients treated with inhibitors of the intracellular tyrosine kinase domain of the EGFR (such as erlotinib and gefitinib). However, a couple of animal studies have indicated that erlotinib may have a small effect on serum Mg levels [62, 63].

The effects of cisplatin or EGFR inhibitors on systemic Mg availability upturned the existing view, showing that treatment-related hypomagnesemia is caused by pharmacologic disturbances of Mg reabsorption rather than by magnesium sequestration and metabolic utilization by the tumor. Since tumor growth and Mg availability seem to be so strictly interdependent, we are confronted with an unavoidable

catch-22: should hypomagnesemia be corrected by appropriate supplementation to alleviate severe symptoms, or should it be considered an important factor contributing to the anticancer action of chemotherapeutics and as such tolerated? A possible association between hypomagnesemia and cetuximab efficacy was indeed proposed in advanced colorectal cancer patients. It was found that patients showing an early ≥20% decrease in serum Mg levels during treatment with cetuximab plus irinotecan displayed longer median time to progression and overall survival compared to patients with a lower reduction in serum Mg [64]. These findings were confirmed in a cohort of patients selected for wild-type KRAS status, but stratified for a ≥50% decrease in serum Mg levels [65], and collectively led to the proposal that hypomagnesemia could be employed as an easy and inexpensive biomarker of efficacy and outcome for cetuximab therapy. However, this assumption has been challenged by following reports, in which higher grade of hypomagnesemia during cetuximab monotherapy seemed to predict worse survival in a much larger group of patients [66]. Regardless of the potential application of hypomagnesemia as a prognostic marker, clinical studies do not address the fundamental question as to whether reduced serum Mg levels have a causal implication in the progression of the disease. For example, the effects of Mg supplementation have not been investigated. Ad hoc experimental studies are needed to fully explore the contribution of Mg to the response to treatment, keeping in mind that the outcome might differ substantially depending on the mode of action of a given drug and/or the pathophysiology of a given tumor. Two recent publications have cast some light on how altered Mg levels can affect efficacy and/or side effects of cisplatin [67, 68]. As already mentioned, the main dose-limiting toxicity of cisplatin is kidney injury that is responsible for the insurgence of hypomagnesemia. The first paper by Dr. Metz and collaborators demonstrated that magnesium deficiency synergistically contributes to cisplatin nephrotoxicity, as cisplatin accumulation in kidney cells is amplified in Mg-deficient mice and is inhibited or reversed following Mg supplementation, via modulating expression of the cisplatin efflux transporter *Abcc6* in renal cells [67]. Intriguingly, Mg status was found to differentially affect tumor vs. kidney cells in an ovarian tumor xenograft model: in addition to confirming that Mg supplementation improved renal function following cisplatin treatment, it was also shown that Mg status did not interfere with the chemotherapeutic efficacy of cisplatin [68]. These findings support the notion that Mg deficiency might affect the host rather than the neoplasia and that it might be recommended to supplement hypomagnesemic cancer patients to prevent or minimize serious complications. Nonetheless, the pleiotropic effects of Mg and the diversity among drug classes should call for an extreme caution in generalizing the conclusions drawn from a single study. In contrast to the data on cisplatin, another recent report highlighted that magnesium supplementation can reduce intracellular doxorubicin accumulation in breast cancer cells and thus contributes to diminish cell sensitivity to the drug [69]. The discrepancy might be explained by bearing in mind that although in both cases Mg affects intracellular drug accumulation, the underlying mechanisms are different and strictly dependent on the chemical characteristics of the drug molecule.

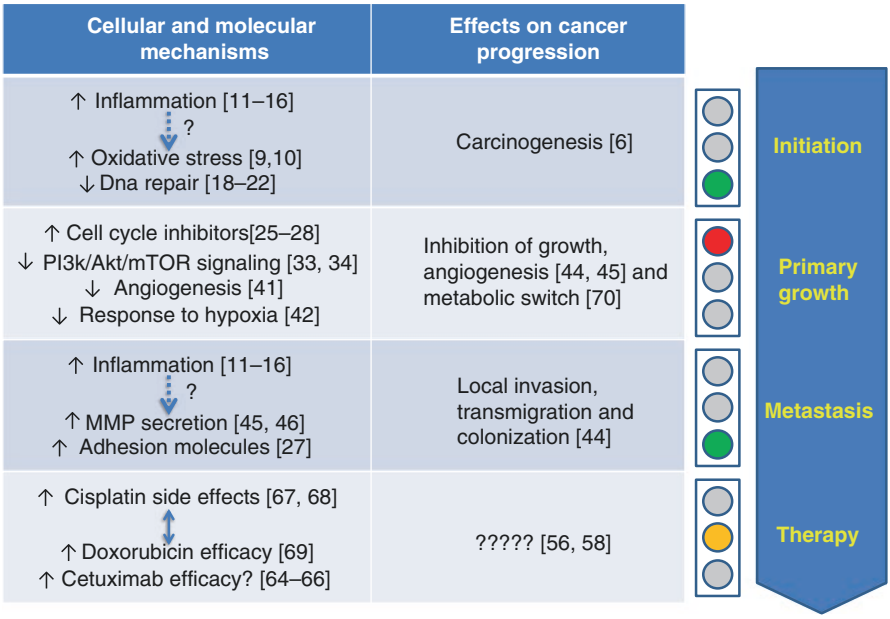

Fig. 2.1 Low magnesium availability can have both pro- and anticancer effects, depending on tumor stage. Magnesium deficiency is associated to increased cancer risk, due to direct or inflammation-mediated oxidative damage and impaired DNA repair capacity. Low magnesium conditions hinder primary tumor growth mainly by inhibiting cell proliferation and angiogenesis, but can result in increased formation of metastases, likely via induction of inflammatory cytokines. The contribution of magnesium to therapy outcome is still debated

In conclusion, little doubt remains that Mg acts as a chemopreventive agent, and optimizing Mg intake or normalizing Mg homeostasis when deranged could be an effective and low-cost measure to reduce cancer risk. However, when a tumor does develop, the picture becomes much more complex: Mg seems to exert both pro- and anticancer effects, and tipping of the balance could possibly depend on tumor stage, among other factors. The latest findings add still another layer of complexity, i.e., a potential interaction between Mg status and the response to therapy, which is likely modulated according to the specific characteristics of the drug (e.g., chemical structure, cellular access route, intracellular mode of action) and/or the tumor (e.g., tissue origin, proliferative index, invasiveness, etc.). Figure 2.1 summarizes our current knowledge on how magnesium availability impacts on cancer progression. Clearly, we need more preclinical models of Mg deficiency and tumor progression, and more clinical investigations specifically designed to address these delicate issues, in order to translate our knowledge on the cancer-Mg connection into successful clinical practice. In particular, the points that should be clarified include the following:

1. Does inflammation play the same dual role in Mg-deficient human cancer patients as seen in rodent models [23]? Inflammation does occur in hypomagnesemic subjects, but we do not know whether hypomagnesemia and inflammation

correlate in cancer patients, nor do we know whether and how this impacts on tumor progression.
2. Would normalization of magnesemia in cancer patients lead to a burst of tumor growth similar to what happens in mice upon reintroducing magnesium in the diet [44]? At present, all clinical data derive from trials that were designed before knowledge of treatment-induced hypomagnesemia, and Mg supplementation was not mandated in the protocol. For the same reason, we do not know whether serum Mg levels really affect response to cetuximab, as it has been proposed [64, 65].
3. Is Mg status a determinant for tumor response in patients treated with cisplatin and doxorubicin, as suggested by experimental models [68, 69]? What about chemotherapeutic drugs belonging to different classes?

2.6 Involvement of the TRPM7 Cation Channel

As reviewed so far, the involvement of magnesium in the modulation of tumor development has long been postulated, and in the last decades, more and more epidemiological, experimental, and clinical data have accumulated and contributed to better define its pleiotropic effect. However, in many cases underlying molecular mechanisms have remained elusive. Extracellular magnesium availability is translated into intracellular Mg content (and eventual signaling) by specific molecules that regulate ion transport through the plasma membrane. Therefore, the absolute requirement of magnesium for cell growth implies that in tumor cells the regulation of magnesium transport must be more efficient to guarantee sufficient magnesium availability and to sustain cell proliferation. Recently this concept has been corroborated and expanded by an ever-increasing number of studies showing that the TRPM7 ion channel is involved in the regulation of numerous key features of cancer cells, including proliferation, adhesion, migration, and invasion, and suggesting that altered expression and/or activity of this channel could be a common signature of human tumors [70].

TRPM7 is permeable to Mg^{2+} as well as Ca^{2+} and is most unusual in having a carboxy-terminal atypical alpha-kinase domain coupled to the transmembrane channel pore; functional channels are most likely organized as either homo- or heterotetramers with its close homologue TRPM6, which have distinct electrophysical properties and functions [31]. These unique features caught the attention of researchers worldwide, as they offer fascinating avenues to explore that could combine protein expression, ion entry, and signal transduction events. A plethora of functions have been ascribed to TRPM7 in normal cell physiology, but discussion of this issue is beyond the scope of the present chapter; for recent reviews, see, for example, [31, 71]. More relevant to our context, a role for TRPM7 has been invoked in each phase of multistep tumor development.

As to carcinogenesis, a single nucleotide polymorphism that substitutes TRPM7 threonine 1482 (T1482) to isoleucine (T1482I) has been linked to the development

of adenomatous and hyperplastic polyps, which might eventually progress to carcinoma [72]. The same SNP was found to be associated with breast cancer risk in a Chinese population [73]. TRPM7 T1482 is a potential site of autophosphorylation or phosphorylation by TRPM6. In vitro studies found that heterologously expressed T1482I leads to an elevated sensitivity to inhibition by intracellular Mg^{2+} [74], which suggests that (re)absorption of magnesium is more subject to inhibition among subjects bearing this substitution. A genomic analysis of 210 diverse human cancers found somatic mutations of TRPM7 in breast, gastric, and ovarian carcinoma; out of the 518 protein kinase genes that were screened, TRPM7 figured among the approximately 130 genes showing evidence for bearing "driver" mutations contributing to the development of the cancers studied [75]. Unfortunately, the functional consequences of the newly identified mutations have not been investigated yet.

The role of TRPM7 in cancer development has been further supported by comparative transcriptomic analyses of TRPM7 expression in healthy vs. cancerous human tissues: altered expression of TRPM7 was detected in several carcinomas, being up- or downregulated depending on the tissue [76]. Overexpression of TRPM7 in human tumors has been validated by other approaches (e.g., Western blot or immunohistochemistry) in prostatic [77, 78], nasopharyngeal [79, 80], pancreatic [81, 82], breast [83, 84], and ovarian [85] cancers as well as in glioblastoma [86]. Moreover, in most of these studies, TRPM7 expression levels were correlated to clinical parameters such as Ki67 staining, tumor size, grade, or stage, and, most importantly, patient survival. In view of such findings, TRPM7 expression was actually proposed as a potential prognostic factor [87]. Not surprisingly, TRPM7 expression was also found to be in correlation with metastatic potential in nasopharyngeal [79], pancreatic [81], and breast [88, 89] carcinomas.

The dual nature of the TRPM7 molecule opens up intriguing scenarios with regard to the mechanisms that underlie its involvement in cancer growth and progression. It is still unclear whether the manifold roles of TRPM7 are to be attributed to channel activity or to kinase function, or rather to a combined action of cation transconductance and substrate phosphorylation. TRPM7 was shown to be essential for the proliferation of different cancer cells, including retinoblastoma, glioblastoma, leukemia, head and neck, lung, pancreas, stomach, and breast cancer cells, and TRPM7-like currents were convincingly associated to proliferation (for reviews, see [70, 87]). However, the transported cation species was not always identified. In many cases, Ca^{2+} fluxes received most of the scrutiny, as Ca^{2+} signaling is central in normal as well as cancer cells [90]. Nonetheless, in some studies, Mg supplementation rescued the growth arrest induced by TRPM7 disruption, which strongly argues for an involvement of an Mg^{2+} influx [70]. It should be noted that recent research developments suggest that the extracellular Ca^{2+}/Mg^{2+} ratio could be more important than Ca^{2+} and Mg^{2+} concentrations on their own [91]; intriguingly the T1482I SNP is associated to greater risk of adenomas and hyperplastic polyps especially in subjects consuming a diet with high Ca^{2+}/Mg^{2+} intake [72], and an increase in the extracellular Ca^{2+}/Mg^{2+} ratio activates TRPM7 channel in prostate cancer cells [77]. Inhibition of channel expression and/or

activity by RNA interference and/or channel blockers disrupts cell cycle and proliferative signals through various signaling cascades, including PI3K/Akt, MEK/MAPK, JAK2/STAT3, and/or Notch pathways, depending on the cell type [87]. Of note, TRPM7-mediated Mg^{2+} influx is required for sustained PI3K/Akt/mTOR-dependent growth signaling, leading to rapid quiescent/proliferative metabolic transitions [33, 34].

TRPM7-mediated fluxes were also found to modulate cell migration, in particular a Ca^{2+} influx in prostate [78] and nasopharyngeal [79] cancer cells, and an Mg^{2+} influx in pancreatic adenocarcinoma [82], but the latest findings indicate that modulation of cell plasticity/motility by TRPM7 might be more dependent on its α-kinase activity. The relationship between the kinase activity and the channel function is still a matter of debate. The consensus in the field is that the kinase activity is not essential for opening of TRPM7 channels, but opening of TRPM7 channels could affect kinase function by causing a local increase in Ca^{2+} and/or Mg^{2+} concentration, which could possibly regulate kinase activity and/or the recruitment/targeting of TRPM7 kinase substrates [31]. Interestingly, TRPM7 kinase substrates include the three mammalian myosin II heavy chain isoforms, MHC-A, B, and C [92]. Consequently, TRPM7 kinase activity can affect actomyosin contractility that plays a key role in cell migration and invasion. Indeed, in a mouse xenograft model of human breast cancer, TRPM7 knockdown interfered with the metastatic potential of triple negative cells; mechanistic investigation revealed that TRPM7 regulated myosin II-based cellular tension, thereby modifying the number of focal adhesions, cell-cell adhesion, and polarized cell movement [88]. These results were confirmed by an independent study, which provided further evidence for the involvement of TRPM7 kinase domain and MHC-A phosphorylation [93]. In addition, in breast cancer cells, TRPM7 seems to play a role in the epithelial-mesenchymal transition (EMT), which represents a crucial switch toward an invasive phenotype [94]. TRPM7 also contributes to the invasive properties of neuroblastoma cells by affecting invadosome formation [95]. Intriguingly, in the last two cited papers, although the role of TRPM7 kinase domain and/or activity was not directly investigated, the authors ruled out an involvement of Ca^{2+} fluxes. Thus, we are presented with two possibilities: (1) cation influx is dissociated from phosphotransferase activity, and the two different domains of the TRPM7 molecule simply coexist for an accidental evolutional step, but they in fact regulate different functions independently; or, more excitingly, (2) the fusion of a channel pore with a kinase domain represents an optimized and integrated unit, being able to couple extracellular sensing to intracellular signaling. In this regard, it is worth recalling that Mg^{2+} is essential for transphosphorylation reactions, which are an integral part of signal transduction. One last remark concerns a possible involvement of the TRPM7 channel and/or kinase also in the response to doxorubicin. Both protein expression and Mg^{2+} fluxes were correlated to cell sensitivity to doxorubicin in two different cellular models [69, 96]. At present, the underlying molecular mechanisms are unknown, though two hypotheses have been put forward: TRPM7 kinase could affect intracellular drug trafficking [69], or Mg availability modulated by TRPM7 could influence activity of drug efflux pumps [96].

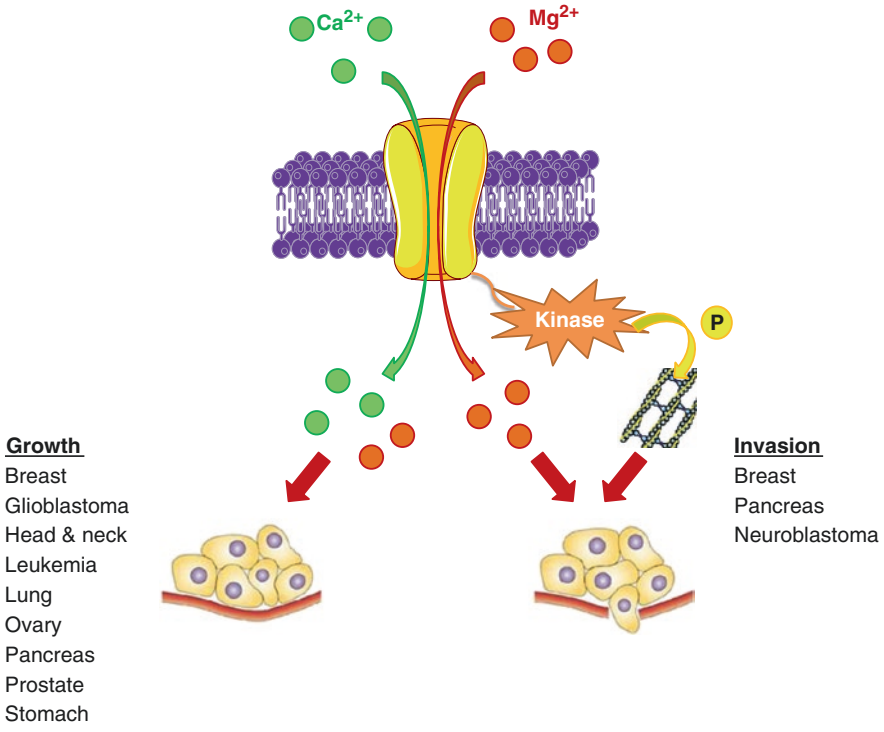

Fig. 2.2 The bifunctional TRPM7 chanzyme has been involved in the regulation of cell proliferation mainly through cation (Ca^{2+} and/or Mg^{2+}) homeostasis control in several types of cancer cell lines and human tumors. It has also been shown to play a role in cell migration and invasion through its kinase activity and interaction with cytoskeletal proteins; kinase activity could depend on local Mg^{2+} concentrations

In conclusion, TRPM7 involvement seems to change during cancer progression, as shown in Fig. 2.2: in early-stage tumors, TRPM7 is involved in the regulation of cell proliferation mainly through cation homeostasis control, while cell migration and invasion in advanced-stage and aggressive tumors require TRPM7 kinase activity and interaction with cytoskeletal proteins, which could nonetheless depend on local ion concentrations [87]. Although more research efforts are certainly needed to fully elucidate the underlying molecular mechanisms, not only does TRPM7 appear as a promising prognostic marker but also as a potential therapeutic target to hamper cancer progression.

2.7 Conclusion

The importance of magnesium homeostasis in tumor development has been disregarded for decades, often overwhelmed by an encumbering interest in calcium. The recently revived interest in the relationship between magnesium and tumors, both in

experimental and clinical oncology, has expanded our knowledge, but, at the same time, it has raised new urgent issues:

1. As all available data concur to indicate Mg as a cancer-preventing agent, more public awareness on individual Mg status should be promoted. Mg levels should be determined routinely in daily clinical practice, possibly by identifying novel parameters for the determination of status and dietary requirements for Mg. In addition, appropriate supplementation protocols should be defined.
2. The sparse and controversial findings about the influence of Mg status on disease progression and therapy outcome call for more studies specifically designed to address this issue.
3. The promising potential of TRPM7 as a therapeutic target needs to be explored by developing specific and efficient pharmacological tools to inhibit channel and/or kinase function to be tested in vitro and in vivo.

In tackling these points, interaction of fundamental and clinical researchers can be an extremely powerful engine to push forward our knowledge of Mg homeostasis and to exploit the possibility that optimizing Mg homeostasis might prevent cancer or help in its treatment.

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Chapter 3
Selenium: Roles in Cancer Prevention and Therapies

Xiangrong Geng, Liu Liu, Kan-Jen Tsai, and Zijuan Liu

Abstract Selenium is an essential mineral for all animals including humans. It is found in amino acids selenocysteine and selenomethionine, which usually forms active sites in selenoproteins. In humans, 25 selenoproteins have been identified, and most of them function as important antioxidant enzymes and play important roles in the detoxification of reactive oxygen species (ROS). Selenoproteins are critical for normal physiology. Dysregulation and malfunction of selenoproteins are associated with numerous human diseases, mostly cancers. The selenium levels are found to be associated with frequency of multiple cancer types, by clinical studies and experimental data. In addition, multiple forms of small selenium compounds, such as inorganic selenite and selenate and organic selenomethionine, Se-methyl-selenocysteine, and methyl-selenic acid, have been applied clinically for cancer prevention and treatment. These small selenium molecules share distinct function and mechanisms in the cellular signaling and effects. In this chapter, mechanisms involved in small selenium molecule cellular metabolism, regulation of cancer cell signaling, and their application in the cancer prevention and therapies are discussed.

Keywords Selenium • Glutathione • Chemotherapeutics • Selenoproteins • Antioxidant • Cancer • Prostate cancer • Inflammation

Abbreviations

GSH	Glutathionine
MSA	Methyl-selenic acid

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MSC Se-methyl-selenocysteine
Se Selenium
SeCys Selenocysteine
SeMet Selenomethionine

3.1 Selenium Is an Essential Element for Animals, and Selenium Deficiency Is Associated with Multiple Diseases

Selenium, as a nonmetal mineral, is an essential element for most eukaryotes and mammals. Selenium homeostasis affects immune system response, thyroid hormone synthesis, and antioxidant effects in humans [1]. Selenium level in serum varies by areas and corresponds to intake, at least 8 μg/dL or higher in healthy people. Daily uptake of selenium is recommended to be 60 μg/day for men and 53 μg/day for women [1]. Both excess and deficient uptakes of selenium cause diseases in animals and humans. Although selenium toxicity is scarce and only happened accidentally, chronic selenium overdose has been observed in animals consuming selenium accumulator plants, with a symptom of alkali and blind stagger disease [2]. Selenium toxicity in humans, with symptoms of brittle hair, nail discoloration, and gastrointestinal problems, often occurs when selenium is not appropriately added as nutrient supplement and in area with high selenium soil [3].

Selenium deficiency happens in human when diet lacks sufficient selenium, usually compromised with other factors. For example, an epidemiological disease reported in Northeast China, named Keshan disease, was identified to be associated with selenium-deficient local diet and the presence of coxsackievirus B infection. The patients show different degrees of heart function insufficiency such as cardiogenic shock, severe arrhythmia, or more severe heart enlargement. Large scale of Se supplementation dramatically reduced disease incidence [4]. Moreover, selenium deficiency has been associated with disorders of the immune system (e.g., chronic inflammatory disorders), thyroid hormone metabolism (e.g., hypothyroidism), reproduction (e.g., preeclampsia), and

Table 3.1 Diseases associated with insufficient selenium intake in human and animals

Victims	Disease	Symptom	Treatment	Ref.
Human	Keshan disease	Multiple focal myocardial necrosis, various degrees of fibrosis, and pulmonary edema	Sodium selenite	[4]
Human	Kashin-Beck disease	Stunting feet and hands or dwarfism	Sodium selenite Vitamins A, C, and E or iodine	[6]
Human	Hypothyroidism	Thyroiditis	Selenium with or without iodide	[7]
Livestock	White muscle disease	Muscular weakness and muscular dystrophy	Sodium selenite and vitamin E	[8]
Sheep/cattle/lambs	Weaner ill thrift	Poor growth rates, profuse diarrhea, unable to use feed	Sodium selenite or sodium selenate	[9]

neurologic system (e.g., intractable epileptic seizures and Alzheimer's) [5]. In general, selenium deficiency is epidemic and limited to regions with insufficient level of selenium intake. In most selenium deficiency symptom, parental nutritional supplementation including selenium and antioxidants was used to prevent or mitigate the symptom [5]. We summarize the common diseases associated with selenium deficiency along with the treatment that can effectively prevent or reverse the symptom in Table 3.1.

3.2 Naturally Occurring Selenium Compounds in Diet and Selenium in Supplements

Selenium (Se) is a rare element naturally found in soil and water system. It exists in many different forms with four oxidation states: selenate [Se(VI)], selenite [Se(IV)], elemental selenium [Se(0)], and selenide [Se(II)]. The most common natural organic selenium forms include selenomethionine (SeMet), selenocysteine (SeCys), γ-glutamyl-selenium-methyl-selenocysteine (GGMSC), and selenium-methyl-selenocysteine (Se-MSC) [10].

Both inorganic and organic forms of Se can be utilized as mineral nutrition and some are the important components in Se supplement agents (reviewed in [11]). Naturally, the content and amount of selenium in the dietary supplements depend on foods consumed and the soil selenium content [12]. Animals and plants are able to nonspecifically incorporate SeMet into protein by replacement of methionine. SeCys is integrated into selenoproteins via the protein translation process, which is genetically encoded by a specific UGA codon [13]. In addition to dietary plants and

Table 3.2 Selenium species in dietary plant, animals, fish, and selenoyeast

	Plant sources	Fish	Animals	Selenoyeast
Selenium species	Selenomethionine (SeMet)	SeCys	SeMet- and SeCys-bounded proteins	Selenomethionine
	Selenocysteine (SeCys)	SeMet		Selenocysteine
	Se-containing proteins	Selenite		GGMSC
	Se-methyl-selenocysteine (Se-MSC)	Selenate		Se-MSC
	γ-Glutamyl-Se-methyl-selenocysteine (GGMSC)	Selenobetaine		Selenite
	Selenate			Selenate
	Selenite			
	Selenoxide			
	γ-Glutamyl-Se-methionine			

animals which contain a variety of different inorganic and organic selenium compounds, selenium fortified yeast, named selenoyeast, is frequently used commercial product as an organic food-form selenium supplement for human and farming animals [11]. Table 3.2 lists the major selenium species being discovered from diet and applied as nutritional supplement.

3.3 Cellular Metabolism of Selenium: Transport, Incorporation, Methylation, and Glutathiolation

Inorganic selenite and selenate, as small selenium molecules, are quickly transformed and metabolized in tissues. Most of the inorganic and organic selenium molecules can be metabolized by reduction and methylation and eventually incorporated into selenoproteins [14]. However, there is limited knowledge about the biochemical conversion of the different selenium species in mammals, and selenium metabolic pathway in tissue specificity remains uncertain. The major metabolic pathway of Se has been assumed to be analogous to the metabolic pathway of sulfur based on the similarity of the two elements thought without being experimentally verified. In the following, we summarize the major steps involved in selenium metabolism.

3.3.1 Selenium Uptake

Uptake of selenium compounds is the first and the rate-limiting step for selenium function in cells. Organic selenium amino acids, SeMet and SeCys, are transported by the corresponding amino acid analog transporters. SeMet, the methionine analog, is found to be taken up by the b^{0+}rBAT system in intestinal cells and B^0 in renal cells [15]. Selenolate, the physiological form of SeCys, competes with cysteine for excitatory amino acid transporters [16].

For inorganic selenium species selenite, a monocarboxylate transporter, Jen1, has been identified to control cellular accumulation and toxicity of selenite in yeast, serving as a direct selenite transporter [17]. In mammal cells, extracellular redox state could facilitate the selenite uptake based on observations that high-affinity uptake of selenite is achieved through the addition of extracellular glutathione (GSH) [18]. It is predicted that selenite is reduced to selenide HSe^- by extracellular thiols and then transported by X_c^- cysteine/glutamate antiporter [19]. Selenite can also be symported with zinc and bicarbonate into mammal cells via a zinc transporter ZIP8 [20]. Modification of ZIP8 activity or expression determined selenite toxicity and tolerance in mammal cells and mouse models, indicating ZIP8 is a major selenite transporter for mammals.

Inorganic selenate is predicted to be transported by sulfate transporters because selenate shares similar molecular structure with sulfate in microbes and plants [21]. Selenate uptake is via the sulfate ABC transporter complex in *E. coli* [22], and two

sulfate transporters *Sul1p* and *Sul2p* are good candidates for the selenate uptake in yeast based on the observation of the selenate resistance in sulfate transport mutants [23]. In mammal cells, selenate might be uptaken by the *SLC26* multifunctional anion exchanger families in intestinal cells since the *SLC26* inhibitor could inhibit selenate transport [24].

3.3.2 Incorporation to Selenoproteins

Incorporation of selenium into protein produces physiological essential proteins: selenoproteins and selenoenzymes. The details involved in selenium incorporation into selenoproteins have been thoroughly studied and discussed in details [25, 26]). To date, 25 human selenoproteins have been identified [27]. Instead of serving as a cofactor, selenium is cotranslationally incorporated into the polypeptide chain as part of the amino acid selenocysteine (SeCys). SeCys shares the common UGA codon, which, in most circumstances, signals translational termination. Though as a cysteine analog, the pKa of SeCys (pKa = 5.2) is different from that of cysteine (pKa = 8.3) under physiological condition [28]. Thus, SeCys, forming a negative-charged selenolate, is more reactive than cysteine with a protonated thiol group and a good redox candidate, making most selenoproteins functioning in the antioxidant system.

3.3.3 Reduction and Conjugation of Selenium with GSH

Glutathione protects cells from oxidative damage via reduction and conjugation reactions with its function group sulfhydryl group (−SH) [29]. Selenide, as the most reduced form of selenium, is the converge point for protein assimilation of selenite and selenate. In mammals, inorganic selenite or selenate is reduced by GSH to selenodiglutathione $Se(GS)_2$, which is then converted to hydrogen selenide in reactions catalyzed by glutathione reductase or by thioredoxin reductase [30, 31], and selenite can also be directly reduced to selenide by the Trx system [14]. However, GSH can enhance the toxicity of seleno-compounds based on the observation that selenite-mediated inhibition of protein and nucleic acid synthesis is potentiated by the addition of GSH [32].

3.3.4 Methylation of Selenium

Selenium reduction by GSH generates reactive intermediates that are substrates for subsequent methylation. The methylation process is reviewed in [33]. In vivo, reductive metabolism and methylation of selenium are linked. The

reduced selenium intermediates may become precursors for methylation. Both selenite and selenate can be reduced by glutathione to yield selenodiglutathione $Se(GS)_2$ and then to hydrogen selenide. Hydrogen selenide is thought to be an intermediary metabolite that serves as a precursor for the synthesis of selenocysteine and/or is methylated to methylselenol, CH_3SeH; dimethylselenide, $(CH_3)_2Se$; and trimethylselenonium cation, $(CH_3)_3Se^+$ [33]. CH_3SeH is the first intermediate leading to other methylated metabolites. $(CH_3)_2Se$ is a volatile metabolite that is expired from the lungs when the capacity for synthesis of urinary metabolite $(CH_3)_3Se^+$ is exceeded [34, 35].

3.4 Selenoproteins and Their Expression and Function in Cancers

3.4.1 Selenoprotein Cellular Function

Many selenoproteins are oxidoreductases with unique synthesis mechanisms [36]. Selenocysteine is specifically incorporated into the active site of essential selenoproteins. Selenoproteins are of critical importance for normal cell function. Some well-studied selenoproteins include glutathione peroxidases (GPx), iodothyronine deiodinases (DIOs), and thioredoxin reductases (TrxR). Some selenoproteins have yet to have a characterized function. Here, we briefly summarize the major selenoproteins and their functions identified in cells and model animals.

Glutathione peroxidase (GPX): Glutathione peroxidase plays an essential role in removing intracellular hydrogen peroxide, thus decreasing levels of reactive oxygen species (ROS). GPX1 is the major antioxidant protein in mammals. It is involved in reduction of inflammation and infection-induced oxidant stress as well as regulation of glucose homeostasis and maintenance of intracellular oxidation states [37–39]. GXP1 knockout mice were more susceptible to the diquat-induced oxidative stress, suggesting role of GPX1 under oxidative stress [40]. GPX2 is highly expressed in gastrointestinal epithelium and cancers of epithelial origin [41–43]. GPX2 plays a physiological role in the self-renewal of the intestinal epithelium [44]. In GPX2 knockout mice, an increase of apoptotic cells at colonic crypt bases was observed and accompanied by an increase in GPX1 [44]. The plasma GPX3 is the antioxidant and a tumor suppressor gene in prostate cancer [45]. GPX4 is a membrane-associated protein and has the ability to reduce hydroperoxides [46].

Thioredoxin reductases (TrxR or TRNRD): Thioredoxin reductases (TrxR) catalyze reaction of reducing thioredoxin (TR). Three TrxRs have been identified in mammals and each contains a selenocysteine in the C-terminal. TrxR1 is a cytoplasmic and nuclear protein. It has multiple physiological roles including regulation activity of transcription factors, cell proliferation, apoptosis, and tumor cell growth [47].

Iodothyronine deiodinases (DIOs): There are three types of Se-dependent iodothyronine deiodinases in mammals, which are involved in the maturation of thyroid hormones. Thyroid hormones are produced mainly in the inactive form, thyroxine (T4). Enzymes including DIO1 and DIO2 catalyze the formation of active hormone triiodothyronine (T3) [48]. On the contrary, DIO3 inactivates T3 and T4, producing inactive T2 and reverse T3 (rT3), respectively. DIO3 demonstrates opposite effects on thyroid hormone function. Overexpression of DIO3 in basal cell carcinomas (BCCs) promoted cell proliferation due to inactivation of T3 and in vivo DIO3 knockdown in basal cell carcinoma cells decreased tumor formation in nude mice [49].

Selenoprotein P (Sel P): Selenoprotein P is a plasma selenoprotein, containing ten selenocysteine residues, and responsible for whole-body selenium metabolism [50]. "Sel P" bound to endothelial cells protected cells against *tert*-butylhydroperoxide (*t*-BHP)-induced oxidative stress via upregulating glutathione peroxidase and thioredoxin reductase activity [51]. SelP knockout mice showed decreased selenium levels in the testis, brain, and kidney with no change in the liver, indicating SelP involvement in transport hepatic selenium to other tissues [52]. The low-level concentration of SelP in plasma was related to the cancer morbidity [53].

Other selenoproteins: 15-kDa selenoprotein (Sep15) locates in the endoplasmic reticulum (ER) and affects glycoprotein folding by interacting with UDP-glucose/glycoprotein glucosyltransferase (UGT) [54]. Mice deficient of Sep15 developed cataracts caused by the improper lens glycoprotein folding [55]. Selenoprotein S (SelS) has anti-inflammatory role and links to the metabolic disease [56, 57]. It is involved in the retro-translocation process, removing the misfolded proteins from ER [58]. Selenoprotein N (SelN) is an ER glycoprotein. It is imperative for the muscle development through modulating ryanodine receptor calcium release channel [59]. Mutations in SelN cause multiminicore disease [60]. Selenoprotein M (SelM) is a low molecular weight Se-containing protein, located in ER, and reduces methionine-R-sulfoxide [61]. In primary neuronal cells, SelM can decrease the hydrogen peroxide-induced reactive oxygen species and apoptotic cell death as well as calcium influx [62]. SelM knockout mice exhibited increased weight gain and adiposity, suggesting the role of SelM in regulation of body weight and energy metabolism [63]. Selenoprotein R (SelR) is the methionine sulfoxide reductase, reducing methionine-R-sulfoxides [64]. In vitro overexpression of SelR and clusterin (Clu) in Alzheimer's disease model cell lines discovered the synergic interaction between these two proteins and significantly reduced the level of intracellular ROS [65]. Selenophosphate synthetase 2 (SPS2) serves as a selenium donor. It is an indispensable protein for the selenophosphate formation in mammals [66]. Selenoprotein W (SelW), a cytoplasmic protein, can decrease intracellular ROS using glutathione as an electron donor [67]. In developing myoblasts, SelW was engaged in muscle growth and differentiation, brain development, and embryogenesis serving as an antioxidant [68, 69].

3.4.2 Roles of Selenoproteins in Cancer

Numerous studies and reviews have reported the expression, function, and pathological relation of selenoproteins [70, 71]. Selenoproteins collectively play an essential role in human physiology and function as important scavengers for reactive oxygen species (ROS). They also play a role in the prevention of cancer through their function as antioxidants. Lower level of selenium and abnormal selenoprotein expression are often observed to have association with multiple cancer incidence due to lack of protection of cells from excessive oxidation. Such protective roles of selenoproteins have been investigated in cells and animal models. For example, mice with knockout of glutathione peroxidases (GPX) were more susceptible to colon cancer induced by oxidative stress. GPX2 knockout mice were more susceptible to develop UV-induced squamous cell carcinoma [72]. However, GPX2 was found to be upregulated in colon adenocarcinoma, Barrett's esophagus, and squamous cell carcinoma [73–75], which implies its dual roles in cancer development. Mice with double knockout of GPX1 and GPX2 developed leo-colitis and intestinal cancer [76]. In prostate cancer, GPX3 was downregulated. Overexpression of GXP3 in prostate cancer cells decreased cell growth and invasiveness, which implies GPX3 might serve as a tumor suppressor [45].

Recent studies have shown that some selenoproteins may promote cancer growth. For example, selenoproteins serving as antioxidants could promote cancer cell proliferation and protect them from chemotherapy. There are reports that some selenoproteins are upregulated in cancer cells, which makes them as drug target(s) in the treatment. For example, selenium-binding protein 1 seems to be a tumor suppressor, and it can serve as a prognostic indicator of clinical outcome [77]. Two selenoproteins, thioredoxin reductase 1 (TrxR1) and selenoprotein 15 (Sep15), were proposed to have dual roles in preventing and promoting cancer development. Similar to GPX2, TrxR1 expression was also increased in cancer cell lines and cancer tissues [78, 79]. Knockdown of TrxR1 expression in mouse Lewis lung carcinoma (LLC1) cells decreased tumor growth and metastasis in vivo [80]. Sep15 is involved in progressing of colorectal cancer. Inhibition of Sep15 in murine colon cancer cells reduced tumor formation in vivo [81]. Moreover, when these Sep15-downregulated cells are injected into BALB/c mice, lung metastatic lesions were significantly reduced. These examples implied that selenoproteins have different functions in different stages of cancer development.

Cell culture and knockout mice have been created and used to investigate roles of these proteins through gain and loss of function. Salient among these researches are summarized in Table 3.3. For more information on the function of all selenoproteins, refer to other review articles [36].

Table 3.3 Function of selenoproteins, tissue expression, function, and phenotypes in knockout mouse model

Gene/ protein	Tissue expression	Function	Phenotype in knockout animals	References
GPX1	Prostate, liver, kidney, heart, blood	Reduction of peroxides	Lesions associated with prostate cancer progression	[82, 83]
GPX2	Colon, intestine, liver	Reduction of intracellular peroxides	UV-induced squamous cell carcinoma	[72]
GPX3	Plasma, kidney	Reduction of peroxides	Increased histological injury in *Gpx3*$^{-/-}$ colons	[84]
GPX4	Heart, liver, kidney, brain, testes	Reduction of phospholipid hydroperoxides	Sensitive to gamma irradiation	[85]
GPX6	Embryos and olfactory epithelium	Reduction of hydroperoxides	N/A	[86]
TrxR1	Liver, embryos	Cytoplasmic thioredoxin reductase	Increased liver/body ratio and hepatic lipidosis, defective morphology in Tr*xR1*$^{-/-}$ embryos	[87]
TrxR2	Mitochondria	Mitochondrial thioredoxin reductase	Absence of a discernible phenotype in heterozygous mice, homozygous mutant embryos were lethal	[88]
TrxR3	Testis	Thioredoxin/glutathione reductase	Unavailable	[89]
Dio1	Liver, kidney, thyroid, and pituitary	Production of iodothyronamine compounds, regulation serum T4 level	Increased body weight	[90]
Dio2	Brown adipose tissue	Production of T3	Diet-induced obesity, liver steatosis, and glucose intolerance	[91, 92]
Dio3	Cerebrum, cerebellum, skin, liver, kidney, placenta, and intestine	Production of T3	Unavailable	[93]
Sel P	Brain, liver, kidney, testis, plasma, heart	Maintaining selenium content	Neurological defects	[94]
SPS2	Embryos	Selenophosphate synthetase	N/A	[95]

(continued)

Table 3.3 (continued)

Gene/ protein	Tissue expression	Function	Phenotype in knockout animals	References
Sep15	Colon, prostate, liver, kidney, testis, and brain	Glycoprotein folding	Less aberrant crypt formation upon carcinogen treatment	[96, 97]
Sel K	Intestine and spleen	Promoting Ca^{2+} flux	Specific immune cell defects (T cells, neutrophils, and macrophages)	[98]
Sel N	Skeletal muscle, heart, lung, brain, and kidney	Calcium and redox homeostasis, muscle development	Limited motility and body rigidity	[77]
Sel T	Brain	Brain ontogenesis, development	Neurodevelopmental abnormalities and hyperactive behavior	[99]
Sel V	Testes	Glutathione peroxidase and thioredoxin reductase	Unavailable	[100]
Sel W	Developing nervous system, muscles, heart, spleen, kidney, lung, liver, intestine, testis	Antioxidant actions and involved in muscle development	Unavailable	[69]
Sel H	Neuronal cells	Mitochondrial biogenesis and reducing superoxide formation	Unavailable	[101]
Sel I	Neuronal cells	Reduction of the levels of reactive oxygen species and apoptotic cell death, regulation of cytosolic calcium level	Unavailable	[62]
Sel M	Neuronal cells	Protection of the brain against oxidative damage	Unavailable	[62]
Sel O	Mitochondria	Kinase function	Unavailable	[102]
Sel R	Liver, heart, lung, kidney, and testis	Redox control	Increased levels of malondialdehyde, protein carbonyls, protein methionine sulfoxide, and oxidized glutathione; reduced levels of free and protein thiols in the liver and kidney	[103]
Sel S	Adipose tissue, muscle, and liver	Associated with metabolic disease, elimination of misfolded proteins from ER	Unavailable	[56, 104]

3.5 Association of Selenium Level/Selenoprotein SNPs with Cancer Pathologies

Epidemiological studies have showed a reverse correlation between selenium level and cancer risk. A large range of data from epidemiological studies, animal studies, and in vitro studies support the protective role of Se against cancer development. Those results form the basis of parental selenium administration in cancer prevention.

Prostate cancer: Several studies showed that selenium reduces the risk of prostate cancer [105]. In health professional follow-up study, high selenium, as indicated by toenail selenium levels, reduced the risk of prostate cancer [106]. In another prospective cohort study, high toenail selenium level was associated with decreased risk of prostate cancer [107]. But in a case-control study in British men, there was no association between selenium concentrations in toenail and prostate cancer risk, although protective association was found in advanced prostate cancer patients with high selenium quintile compared with that in the lowest quintile (OR = 0.78, 95% CI, 0.27–2.25) [108]. The protective effect of serum selenium on risk of prostate cancer was found at concentration >135 μg/L in US men and especially in men with low serum α-tocopherol concentrations [109]. Physicians' Health Study discovered the pre-diagnostic selenium levels could significantly reduce the risk of prostate cancer when prostate-specific antigen (PSA) level was >4 ng/mL, implying the effect of selenium on prostate cancer progression [110]. However, in Carotene and Retinol Efficacy Trial (CARET), there was no association between serum selenium and prostate cancer incidence [111]. Nevertheless, most studies support the inverse relationship between low plasma selenium level and prostate cancer risk [112, 113]. Smoking is a factor that affects the protective role of Se. In ex-smokers with low toenail selenium level, Se has its strongest protective role [107]. And the inverse association between serum selenium and prostate cancer risk was pronounced in current and ex-smokers [112, 114].

Colorectal, esophageal cancer: In colorectal cancer patients, low serum selenium level was related to a lower mean survival time and a lower cumulative cancer-related survival rate compared with high selenium level [115]. A pooled analysis of three studies (Wheat Bran Fiber Trial, Polyp Prevention Trial, and Polyp Prevention Study) proved high plasma selenium level could significantly reduce the recurrences of colorectal adenoma, supporting the protective and beneficial effects of high serum selenium level on the colorectal cancer risk [116].

Esophageal squamous cell carcinoma (ESCC) and gastric cardia cancer (GCC): One study conducted in Linxian, China, in which area populations had a high mortality rate of ESCC and GCC and low selenium concentration, found a significant inverse association between low baseline serum selenium concentrations (mean = 73 μg/L) and high mortality for ESCC and GCC [117].

Support of studies on selenoprotein gene variants associated with cancer growth and progression has emerged. Single nucleotide polymorphisms (SNPs), which affect expression and activity of selenoproteins, are associated with changes in selenium metabolism and found to be associated with cancer risks [118]. For example, a case-control study including 832 patients with colorectal cancer (CRC) and 705 controls in Czech populations discovered three SNPs (rs7579 (SEPP1), rs713041 (GPX4), and rs34713741 (SELS)) had an association with CRC risk [119]. In addition, two-loci interactions between rs4880 (SOD2), rs713041 (GPX4), and rs960531 (TXNRD2) and between SEPP1 and either SEP15 or GPX4 were statistically significant between patients and healthy controls, indicating interactions of selenoproteins involved in disease risk [119]. In a study conducted in Korean population, one SNP in promoter region of SELS (rs34713741) in men and two SNPs in 3'UTR of SEP15 (rs5845 and rs5859) in women might have influenced CRC risk [120]. Also in Prostate, Lung, Colorectal, and Ovarian Trial, four SNPs in SEPP1 and one SNP in TrxR1 were significantly associated with the advanced colorectal adenoma [121]. In addition, the association of colorectal cancer risk and SNPs in GPX1–4 and SEPP1 were assayed in a case-control study nested within the Women's Health Initiative Study [122]. Results identified one SNP in GPX4 gene (rs8178974) and it was statistically significantly associated with colorectal cancer risk. However, no association was observed between the overall variation in GPX4 gene and the risk of colorectal cancer. Individuals carrying T allele in GPX4 (rs34713741) had a greater risk of gastric cancer in Chinese and Japanese population [123, 124]. Using a tagged SNP method, Wang et al. found G allele of rs3805435, T allele of rs3828599, and A allele of rs2070593 in GPX3 could lower the risk for gastric cancer [125]. Interestingly, rs3828599, rs736775, and rs8177447 in GPX3 and rs4902347 in GPX2 could significantly decrease the risk of rectal cancer, but not colon cancer, suggesting different mechanisms of the function of selenoproteins in rectal and colon cancer [126]. rs1050450 (leucine to proline polymorphism at codon 198) in GPX1 is linked to the risk of lung, breast, and bladder cancer [127–129]. This variant could modulate the relationship between prostate cancer risk and serum selenium level due to altered GPX1 activity [130]. Moreover, the risk of prostate cancer was increased by the low serum selenium level together with the interaction between rs4880 in SOD2 and rs3877899 in SEPP1. Data from EPIC-Heidelberg study including 248 prostate cancer patients and 492 controls reported that the risk of prostate cancer was modified by a combination of low selenium level and SNPs in SELK (rs9880056), TXNRD2 (rs9605030 and rs9605031), and TXNRD1 (rs7310505) [131]. rs230812 and rs6865453 in SEPP1 were associated with the risk of breast cancer in women with a greater Native American ancestry [132].

These GWAS studies discover that SNPs in multiple selenoproteins are related to cancer risks. With the development of next-generation sequencing (NGS) technique, it is expected more association studies will be performed and will form bases for future personalized selenium application.

3.6 Clinical Application of Small Selenium Compounds in Cancer Prevention and Treatment

3.6.1 Selenium Compounds Applied in Cancer Prevention and Treatment as Antioxidant

There is increasing evidence suggesting the vulnerability of cancer cells to oxidative stress; therefore, targeting the antioxidant capacity of tumor cells becomes promising therapeutic strategy [133]. Among cancer cell redox modulators, selenium compounds gained substantial attention. Several human clinical trials testing the ability of Se supplementation to prevent cancer had the mixed results. Selenium molecules have shown promises in the prevention and treatment of prostate, colon, liver, and lung cancer. A small-scale trial using pure selenite as the Se form found supplementation decreased hepatocellular carcinoma incidence in patients with hepatitis [134]. A landmark trial in selenium cancer prevention was the Nutritional Prevention of Cancer (NPC) trial, published in 1996, in 1312 individuals, a 63% reduction in prostate cancer incidence, a 58% reduction in colon cancer, and a 46% reduction in lung cancer, as well as a 41% reduction in overall cancer-associated mortality [135]. The NPC trial was a double-blind placebo-controlled study that used selenized yeast, administered orally, and followed patients over 13 years at 200ug/day dosage. In a follow-up clinical trial, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) tested selenomethionine and vitamin E, another nutrient believed to reduce prostate cancer incidence, together or alone. SELECT showed no difference in prostate cancer incidence between selenomethionine supplementation groups with the placebo group. It is believed that usage of selenomethionine as the Se form in SELECT demonstrated that the choice of chemical form of selenium is a crucial factor in cancer prevention [136]. These mixed results suggest that more study into the basic biology of Se is needed before the clinical benefits of Se can be understood and realized. In addition to these larger scales of clinical trials, some smaller size of trials were initiated in different countries in cancer prevention and treatment. For example, in 2013, a clinical trial, SECAR, using selenite to treat existing cancer was initiated in Sweden [137].

Selenoprotein synthesis becomes saturated at nutritional levels below those required for the effects of clinical applications of selenium to occur; thus, low molecular weight selenium compounds are believed to play a role in selenium-based therapy. As discussed above, multiple selenium compounds have been applied in cancer prevention or treatment, adjuvant treatment at elevated concentrations to exert the chemopreventive benefit independent of selenoprotein function. Each of their application with antiproliferative properties, their tumor selectivity, and mechanism of action through regulation of cell signaling are distinct. Clinical trials performed or ongoing along with their treatment effects are summarized in Table 3.4.

Table 3.4 Clinical trials involve selenium compounds. Selenoyeast, selenomethione, and selenite have been applied in multiple clinical trials through dietary administration

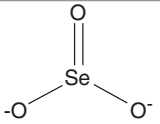
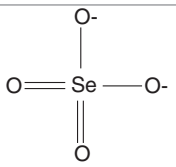
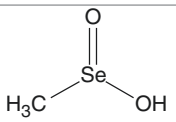
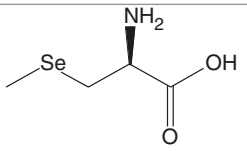
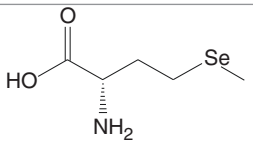
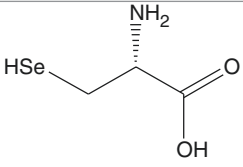
	Diseases	Selenium species	Results	Dosage and adjuvant	Starting year
NPC	Prostate, colorectal, lung cancer	Selenoyeast	Reduce cancer mortality and incidence in male participants	200 μg selenium/day	1983 [138]
Cancer prevention in skin carcinoma	Lung, prostate cancer	Selenoyeast	Reduce the secondary end point incidence of cancer	200ug selenium/day	1983 [135]
Nutrition intervention trials in Linxian, China	Stomach, esophagus, esophageal/cardia cancer	Selenoyeast	Reduce total cancer mortality, especially stomach cancer	50ug selenium, 15 mg β-carotene and α-tocopherol/day	1985 [139]
Se prevention trial in Qidong County, China	Liver cancer	Selenized yeast	Se had the protective role against hepatitis B virus (HBV) infection and primary liver cancer (PLC)	200 μg selenized yeast tablets/day	1993 [134]
SU.VI.MAX trial	Prostate cancer	N/A	Reduce the rate of prostate cancer	120 mg vitamin C, 30 mgα- tocopherol, 6 mg 15 mg β-carotene, 100 μg selenium, and 20 mg zinc/day	1994 [140]
SELECT	Prostate cancer	SeMet	Fail to reduce the risk of prostate cancer (medium serum selenium 136 μg/L)	200 μg selenium/day	2001 [141]
SECAR	Carcinoma	Selenite	No symptoms of toxicity at dose <3 mg/m^2, side effects at dose >4.5 mg/m^2	N/A	2013 [137]

3.6.2 Small-Molecule Selenium Compounds Used in Cancer Prevention and Therapies

Except from serving as a micronutrient supplement, small-molecule selenium compounds, including inorganic selenite, selenate, organic SeMet, SeCys, Se-MSC, MSA, and selenoyeast, can exert potential anticancer properties when used in high concentration. The concentration of toxicity on cancer cells and the mechanisms of anticancer activity are dependent on the selenium species [142].

Selenite, as one of the most applied anticancer selenium compounds, has shown antitumor and anti-inflammation effects on numerous cancer cells and mouse models and has been applied in clinic. In vitro assays have confirmed its chemotherapeutic potential against on a wide range of malignant cells, such as prostate cancer cells, lung cancer cells, colon cancer cells, ovarian cancer cells, leukemia cells, and cervical cancer cells (Table 3.5). Selenite can attack proteins with reduced thiol groups expressed on surface of tumor cells, inducing ROS-dependent cell apoptosis [173]. Inorganic selenate, a less reported and used anticancer form, has general lower cell toxicity than selenite and is quickly reduced to selenite to exert cellular function in cells with reducing environment.

Table 3.5 Selenium compounds used in cancer research and mouse models

Selenium species	Used in studies	Structures	References
Selenite	Lung cancer cells, prostate cancer cells, breast cancer cells, colorectal cancer cells, myeloid leukemia cells, malignant glioma cells, mesothelioma cells, colorectal carcinoma xenograft		[143–148]
Selenate	Oral squamous cancer cells, prostate cancer, colon cancer cells		[149–151]
Methylseleninic acid (MSA)	Prostate cancer cells, lung cancer cells, mouse mammary epithelial tumor cells, pancreatic cancer cells, triple-negative breast cancer, and colon cancer xenograft		[143, 152–156]
Se-methyl-selenocysteine (Se-MSC)	Ovarian cancer cells, prostate cancer cells, oral squamous cells, colon cancer cells, breast cancer cells, mouse mammary epithelial tumor cells, head and neck squamous cell xenograft		[157–161]
Selenomethionine	Prostate cancer cells, colon cancer cells, lung cancer cells, breast cancer cells, melanoma cells, colorectal carcinoma xenograft, squamous cell xenograft, and colon cancer xenograft		[148, 162–166]
Selenocysteine	Melanoma cells and lung tumors in mouse		[167, 168]
Selenoyeast	Mammary cancer, primary liver cancer, esophageal cancer, oral cancer, skin cancer		[135, 169–172]

Compared to inorganic selenium compounds, organic selenium compounds, including SeMet, SeCys, Se-MSC, and MSA, usually exhibit antitumor activity at much higher concentrations. Though SeMet is not an oxidizing agent, it can be metabolized to methylselenol, thus inducing cell cycle arrest, programmed cell death, and immune response [162, 174]. Methylselenol, as the common intermediate SeMet, Se-MSC, and MSA metabolism, can induce mitochondria dependent via producing oxidative stress. SeCys is a much safer reagent than SeMet in cancer research because SeMet can nonspecifically incorporate into proteins to replace methionine [175]. However, SeCys is chemically active, and thus, few chemotherapeutic and chemopreventive researches focus on SeCys but on SeCys derivatives [176]. Se-MSC, a highly bioavailable selenium form, is effective in inhibiting cell proliferation in both solid and metastatic tumors via its anti-angiogenic effects [177]. In addition, Se-MSC is an excellent therapeutic adjuvant to enhance the efficacy of anticancer drug since its anti-angiogenic effect is specific for tumors but not for normal organs [178]. MSA, an intermediate of SeMet and SeCys metabolism, can also be processed to methylselenol readily via cellular redox system, exerting significant chemopreventive effects and modulating tumor metastatic processes at low dosage [179]. Based on its ability to antitumor at a low dose, selenite could also serve as a therapeutic synergy compound to enhance the efficiency of other anticancer drugs, such as paclitaxel effects on breast cancers [152].

Selenoyeast, cultured in selenium-enriched medium, has achieved success in anticancer clinical trials. It contains a mixture of different selenium compounds including organic selenomethionine and a moiety of inorganic selenite along with uncharacterized selenium species [11]. However, the mechanism of selenoyeasts' effects on anticancer activity is limited by the poor control of product speciation and purity [180]. The following table summarizes the selenium compounds and their usages in cancer cells and mouse models.

3.7 Regulation of Oncology Signals by Selenium Compounds

Small selenium molecules show different effects in their clinical functions due to their distinct regulatory mechanisms in tumor and normal cells. Thus, the choice of selenium species is an important factor in the outcome of clinical trials. Meanwhile, dosage is another important clinical factor for treatment outcome. In this part, we summarize the mechanisms of major selenium molecules that have been clinically applied on cancers.

3.7.1 Inorganic Selenite Signaling

The inorganic selenite exerts different effects on cells in a dose-dependent manner. In the high nanomolar to low micromolar range, selenite supports proliferation in normal conditions and cell survival in stress conditions, such as ischemia [181]. In high

micromolar, selenite inhibits many transcription factors involved in oncogenic pathways, such as vascular endothelial growth factor (VEGF)-mediated angiogenesis.

Selenite can oxidize both redox-active and redox-inert thiols in a wide range of cysteine containing proteins and produce ROS, leading to activation or inactivation of various pathways. Selenite can bind to redox-inert thiols in zinc finger proteins, which contain zinc-thiolate cysteines and are protected from oxidation by the intracellular GSH or antioxidant protein thioredoxin [173]. For example, selenite can replace the zinc-bound thiols in SP1 and transcription factor IIIA, resulting in inhibition of cell growth [182]. Its interaction with reactive thiol clusters has been identified in diverse signal proteins, such as inactivation of caspase-3 to protect normal cells from stress [183] and alteration of redox-sensitive factors activator protein 1 (AP-1), NF-κB, and p53 to inhibit cell growth and induce caspase-mediated apoptosis in cancer cells [184, 185].

Selenite can also affect other signaling pathways through unknown and possibly different mechanisms. Selenite affects the phosphoinositide 3-kinase (PI3k)-serine-threonine kinase Akt pathway and three major mammalian mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase(JNK), and p38 in a dose-dependent manner [179, 186]. Though the basis for the interaction between selenite and these pathways is not well understood, it is known that MAPKs and PI3k can be activated by (1) ROS generation, which is mediated by superoxide production after oxidation of GSH; (2) the oxidation thioredoxin, which negatively regulates p38 and other factors; or (3) possibly the inhibition of certain phosphatases [187]. As for the dosage effects, selenite activates ERK1/2-mediated MAPK pathway and protects cell survival at dosage of 2–8 μM [72] while selenite induces the p38/JNK pathway and initiates cell apoptosis at higher doses of 5–10 μM [186]. These data show that selenite can produce incompatible, sometimes directly opposite effects, depending on its concentration. However, the reasons for dose-dependent activation of certain signal pathways are complex, and there is no evidence that selenite preferentially reacts with certain pathways at lower concentration levels. It is possible that there are factors that affect the access of selenite to thiol proteins, such as their conformational state. And there must exist other unknown mechanisms of the concentration-dependent manner of selenite, given the multiple targets of selenite.

3.7.2 Selenomethione (SeMet) Action in Cancer Cells

SeMet, the major component in selenoyeast, is non-redox active and non-DNA damaging compared to inorganic selenium, thus inducing antitumor activity at much higher concentration than that of selenite [176]. SeMet can achieve antitumor activity via inducing different pathways from that of selenite because of distinct metabolic pathways between selenite and organic selenium compounds in vivo. The major intermediate for SeMet in the liver is the redox-active methylselenol, which is toxic to cells by producing reactive oxygen species and inducing ROS-dependent

apoptosis [188]. The anticancer activity of SeMet is dependent on its ability to induce cell cycle arrest and apoptosis via p53-dependent pathway and associated with persistent activation of MAPK cascade and PARP cleavage in a dose-dependent manner [176]. In colon cancer cells, SeMet can induce S-G2/M cycle arrest at low concentration (22 μM) via ERK pathway-dependent histone H3 phosphorylation and induction of chromatin remodeling [189], while SeMet inhibit cell growth and induce apoptosis at higher doses (50–100 μM) by increasing p53 expression [164]. In addition, methioninase (METase) can enhance the anticancer effect of SeMet in prostate cancers by producing redox-active metabolites including methylselenol, thus increasing ROS and p53-dependent apoptosis [166]. Moreover, methylselenol generated from SeMet by METase in melanoma cells can induce cell detachment by decreasing integrin expression via activation of p38, protein kinase C (PKC)-δ, and NF-κB, leading to caspase-mediated apoptosis [162]. Except from studies in vitro, SeMet has been shown to be a low-toxic antitumor agent and a selective toxicity modulator of other anticancer drugs in human tumor xenografts [148, 163]. On colorectal carcinoma xenograft mice, SeMet treatment induces cell apoptosis in tumor tissue not in normal tissue by suppressing anti-apoptotic protein B-cell lymphoma-extra large (Bcl-xL) and activating caspase-9 cascade [148]. In xenografts bearing human squamous cell carcinoma of the head and neck and colon carcinoma, SeMet, at nontoxic dose, protects normal tissues against toxicity induced by chemotherapeutic agents without hampering their antitumor activities, such as irinotecan-induced programmed cell death [163].

3.7.3 Se-Methyl-selenocysteine (Se-MSC) Action in Cancer Cells

The methylated selenium form MSC has been shown to be a potential chemopreventive and chemotherapy agent in a wide variety of cancer cells and has attracted great attention as one of the most effective antitumor forms of selenium because of its good bioavailability, low toxicity, and readily metabolizing to redox-active methylselenol. The anticarcinogenic effect of MSC has been shown to block cell growth in S phase in mouse mammary epithelial tumor cells via deactivating PI3K-Akt and RAF kinase-mediated ERK signaling pathways [160, 190]. Cell apoptosis induced by death receptor (extrinsic) and mitochondrial (intrinsic) apoptotic pathways also contributes to the antitumor effect of MSC in dose- and time-dependent manners. At medium to high micromolar concentrations (100–400 μM), MSC has been reported to induce cytochrome c-independent apoptosis in ovarian cancer cells [161], while MSC induces ROS production and initiates cytochrome c-mediated caspase activation at 50 μM in promyelocytic leukemia cells [158]. Moreover, MSC, at 200 μM, induces apoptosis in colorectal adenocarcinoma cells by simultaneously increasing death receptor FasL-mediated, mitochondrial-dependent, and ER-stress-induced caspase activation [191]. In addition to stimulation of cell cycle arrest and apoptotic pathways, MSC exerts anti-angiogenic effects on cancer models both *in vitro* and

in vivo. In the colorectal cancer xenografts, non-toxic dose of MSC has been shown to inhibit tumor growth, reduce vessel formation, and increase chemotherapeutic efficacy of doxorubicin and irinotecan [177]. The anti-angiogenic effect of MSC is specific for tumor, and MSC can protect normal organs from toxicity of chemotherapeutic active agents, indicating MSC as a potent chemotherapeutic synergy for drug delivery to the tumor with no side effects [178]. When applied with irinotecan in human squamous carcinoma models, MSC enhances the cytotoxic activity of irinotecan in vitro and in vivo by inhibition of cyclooxygenase COX-2, iNOS, and HIF-1α-mediated neoangiogenesis [157, 192].

3.7.4 Methylseleninic Acid (MSA) Action in Cancer Cells

MSA, a synthetic simplified version of MSC, can bypass the beta-lyase action and be reduced to methylselenol by cellular-reducing system [193]. MSA can induce cell cycle arrest and cell apoptosis in the low micromolecular range. It has been shown that MSA induces apoptosis and G1 cell cycle arrest in lung cancer cells by perturbing PI3K through Akt kinase and forkhead box O proteins (FOXO) dephosphorylation [154]. In addition, MSA, at low micromolecular concentration, has been reported to change cell cycle-regulated genes on transcriptional level, inducing G0/G1 accumulation, and induce apoptosis by caspase-mediated cleavage of PAPR along with G1 cell cycle arrest in prostate cancer cells [179]. Its ability to induce G1 cell cycle arrest and cell apoptosis at low dosage has also been found in macrovascular and microvascular endothelial cells via modulating cell cycle kinases and PI3K and ERK1/2 kinases, thus inhibiting tumor angiogenesis [194, 195]. MSA can also inhibit tumor angiogenesis by downregulation of cellular HIF-1α and VEGF via inhibiting histone deacetylases (HDAC) activity in diffuse large B-cell lymphoma cell lines [196] and esophageal squamous cell carcinoma [197]. Moreover, MSA has been shown to hamper hormone androgen receptor signaling with redox-independent mechanism, making it a potential anticancer drug specific for prostate cancer cells with hormone refractory [143]. Compared to selenite and selenomethionine effects in vivo, MSA exerts superior inhibition of tumor proliferation and angiogenesis in lung carcinoma in mice and xenografts with human prostate cancer cells [198, 199].

3.8 Perspectives: Future of Selenium Application in Cancer

Selenium is an essential micronutrient, while multiple small-molecule selenium compounds exhibit promising potentials in the prevention and treatment of cancers. Although the largest clinical trial SELECT failed to demonstrate preventative effects for prostate cancer while using selenomethionine, success of prior trials hinted that this may relate to the choice of selenium chemical forms.

Because selenium treatment displays large interindividual variation in pharmacokinetics as well as treatment efficacy, more clinical trials with variation in choice of selenium compounds, dosages, etc. are required for future selenium application. The leading medical opinion among clinicians is an optimal selenite dosage and administration must be developed.

Therefore, more understanding of fundamental selenium mechanism is needed for selenium therapies. For example, what is the cascade of selenium binding and affinity to proteins? How labile selenium and bounded selenium converted? What are the detailed mechanisms of small selenium function? What is the function of all selenoproteins? How to control pharmaceutical selenium dosage for disease treatment? With the availability of personal genomic data from next-generation sequencing and improvement of proteomic identification of Se-binding proteins, along with better understanding in selenium biochemistry such as selenium uptake and metabolism, the factors affecting selenium pharmacokinetics can be explored and eventually integrated into selenium application. Overall, more studies are required in the understanding of selenium homeostasis and to maximize the application of selenium as essential element, treatment reagent, or therapeutic adjuvant.

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Chapter 4
Zinc and Zinc-Dependent Proteins in Cancer and Chemotherapeutics

Jenna Guynn and Elizabeth A.W. Chan

Abstract Zinc (Zn) is an essential element critical for numerous protein structures and catalytic functions. This chapter focuses on the importance of homeostatic concentrations and appropriate subcellular distributions of Zn within cells, as well as the structural and catalytic roles Zn plays for many important enzymes. The mechanisms and factors by which homeostatic levels of intracellular Zn are maintained are discussed, as well as means by which Zn is distributed within the cell. In addition, several important proteins that require Zn for catalytic activity, such as matrix metalloproteinases and lysine deacetylases, and structural functions, such as the transcription factor p53, are reviewed. Associations between the dysregulation of Zn-dependent proteins or intracellular Zn homeostasis and the development and progression of several cancers are detailed, with emphasis placed on mechanistic links related to Zn. Finally, the various chemotherapeutic strategies that have been developed to either modify intracellular Zn levels or directly target cancer-associated proteins that involve Zn and potential future chemotherapeutic targets are evaluated.

Keywords Zinc • Carcinogenesis • Zinc finger • Matrix metalloproteinase • Lysine deacetylase

Abbreviations

ECM	Extracellular matrix
ER	Estrogen receptor
FGF-2	Fibroblast growth factor 2

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GLI	Glioma-associated oncogene
HAT	Histone acetyltransferase
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
Hh	Hedgehog
IL-2	Interleukin-2
KDAC	Lysine deacetylase
MMP	Matrix metalloproteinase
MT	Metallothionein
mtp53	Mutant p53
PML	Promyelocytic leukemia gene
RAR-α	Retinoic acid receptor α
ROS	Reactive oxygen species
RRE	Ras-responsive element
RREB1	Ras-responsive element-binding protein 1
Snai1	Snail homolog 1
Sp1	Specificity protein 1
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
ZIP	Zrt-, Irt-related proteins
Zn	Zinc
ZnT	Zinc transporters

4.1 Introduction

Zinc (Zn) is a naturally occurring and nutritionally essential element [1]. Trace amounts are required for the basic cellular function of all living things for various structural, regulatory, and catalytic biological processes. Zn-binding motifs, common structural protein domains found in ~10% of all proteins [2, 3], play critical roles in numerous cellular processes, including protein folding, enzymatic activity, immune function, macromolecule syntheses, DNA repair, and oxidative stress responses [4–7]. Zn is also involved in multiple essential regulatory functions through the modulation of gene expression, protein binding, apoptosis, DNA and RNA metabolism, synaptic plasticity, redox status, and intracellular signaling [8, 9]. In addition, over 300 enzymes utilize Zn as a catalytic cofactor to stabilize negative charges for substrate activation [10, 11]. For additional details regarding Zn physiology, the reader is referred to other works on these matters [12–14].

Zn is not endogenously produced and must be obtained through diet. Humans consume Zn in a variety of foods, such as meats, seafood, dairy products, beans, and whole grains [15], or by dietary supplementation [16]. Within the average human, there is between 2 and 4 g of Zn, located mostly in the brain, eye, prostate, muscle, bones, kidney, and liver [3]. Intracellular influx of Zn is facilitated by Zrt-, Irt-related proteins (ZIP), while organelle distribution and efflux to the extracellular matrix (ECM) are mediated by Zn transporters (ZnT). In mammalian cells,

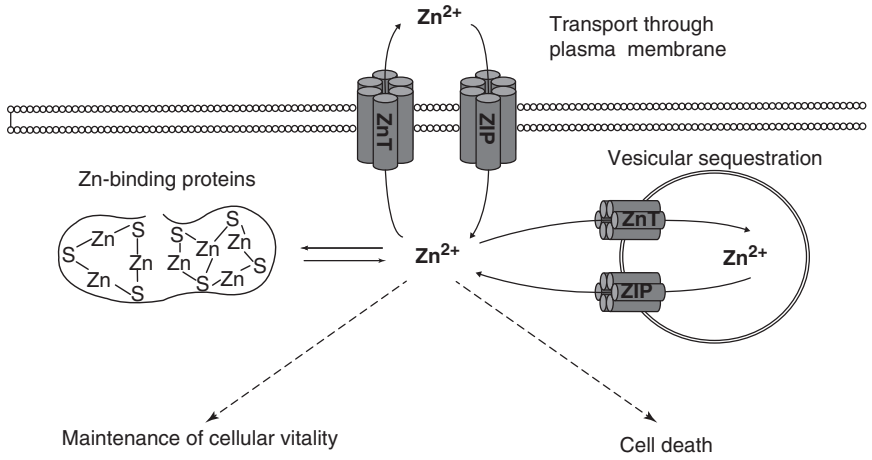

Fig. 4.1 Zn transport through the plasma membrane of cells and the maintenance of intracellular Zn homeostasis. Zn transport through the plasma membrane and distribution into organelles are facilitated by ZIP importers and ZnT exporters. Homeostasis is regulated by vesicle sequestration and Zn-binding proteins, such as MT (Reproduced with copyright permission. Plum et al. Int J Environ Res Public Health. 2010;7(4):1342–65)

approximately 30–45% of Zn is located in the nucleus, ~50% in the cytoplasm and other organelles, and the remaining ~5–15% is located in the cell membrane [17]. Total cellular Zn concentrations range from 100 to 500 μM, with most Zn tightly bound and unavailable for use in cellular reactions. In fact, only femto- or nanomolar quantities of Zn are in the form of free cytosolic Zn^{2+} [18]. Homeostasis of intracellular Zn is maintained through a buffering system of protein binding and vesicle sequestration. The metallothionein (MT) family proteins, which are cysteine rich and inducible, can bind Zn and other heavy metals in order to transport and buffer Zn levels, regulating various functions [19, 20]. Figure 4.1 summarizes Zn mobility inside and outside of cells, as well as certain Zn-related regulatory mechanisms.

The recommended daily allowance of Zn for adults in the United States is 11 and 8 mg for males and females, respectively, and the tolerable upper intake level for adults is 40 mg [21]. In general, there is approximately 2–4 g of Zn in each human, although this amount varies by age [3]. Zn intake below the recommended daily allowance may be insufficient to meet altered physiological demands, whereas excess Zn intake, either due to accidental ingestion in occupational settings or altered homeostasis, can have deleterious effects on human health [21–23]. Inadequate Zn intake can lead to reproductive, neurological, and skin abnormalities, whereas excess intake can lead to effects such as copper deficiency, bone growth retardation, and metal fume fever [1, 21, 24–26]. Either insufficient or excess intake of Zn can lead to immune system dysfunction [27]. The major routes of entry for Zn into the human body are inhalation, ingestion, and absorption [1]. In addition to diet, Zn exposure can occur through occupational settings, environmental contamination, and the use of personal care products [28]. In addition, Zn exposure as a component of air pollution has been associated with adverse respiratory and cardiovascular effects [29–31].

Abundant evidence implicates Zn dysregulation in the development and progression of cancer [32]. Compared to normal tissues, both increased and decreased Zn concentrations have been observed in malignant tissues. Moreover, the dysregulation of numerous proteins containing Zn^{2+} for catalytic and structural function is associated with the carcinogenic process in various cancers [33]. Taken together, these data provide a strong rationale for the development of chemotherapeutic strategies aimed at modulating intracellular Zn concentration and/or availability. Specific mechanisms and roles that Zn and Zn-related proteins play in carcinogenesis, as well as potential roles for chemotherapeutic strategies involving Zn, are discussed in the following sections.

4.2 Zn Homeostasis and Dysregulation

Homeostatic Zn levels, which differ by cell type, are required for appropriate cellular function, such as preventing apoptosis in healthy cells and promoting apoptosis when advantageous to the organism [34]. Aberrant proliferation, a hallmark of carcinogenesis, requires the evasion of protective cell death mechanisms such as apoptosis [35]. Excess intracellular Zn can mediate pro-oxidant and pro-apoptotic effects [36] and has resulted in activation of apoptosis in vitro [37]. In living cells under surplus Zn conditions, Zn^{2+} translocates into the mitochondria and induces ROS production, via inhibition of cytochrome C oxidase [38, 39]. Evidence also implicates the dysregulation of intracellular Zn^{2+} homeostasis early in the carcinogenic process, as a mechanism to facilitate proliferation through avoiding normal programmed cell death [40]. The following section addresses Zn dysregulation mediated by changes in Zn transport into and within cells.

4.2.1 Zn Transporters

Transport of Zn across the cellular membrane and between the cytosol and organelles is critical for cell function and survival. The two families of Zn transporters, ZIP and ZnT, belong to the solute carrier family [41, 42]. ZIP transporters mediate the movement of Zn both from the extracellular space and from organelles to the cytosol. Some of the 14 known ATP-dependent ZIP transporters (ZIP1–14) can also transport cadmium, iron, and manganese, in addition to Zn [43, 44]. Conversely, the movement of Zn from the cytosol to organelles or the extracellular space is mediated by ten known ZnT (ZnT-1–10). ZnT are not ATP dependent but instead function by Zn^{2+}/H^{+} exchange [45]. ZnT-1, the only known ZnT located primarily at the plasma membrane [46], is upregulated under high Zn conditions, presumably to facilitate Zn export [47, 48]. Interestingly, altered Zn levels in carcinogenic tumors as compared with healthy surrounding tissue have been reported, which suggested that Zn transporter expression may be involved in carcinogenesis [49, 50].

Table 4.1 Zn transporters deregulated in cancer

Transporter	Cancer type	Regulation	Change in Zn level	Reference
ZIP1	Prostate	Down	Reduced	[51–54]
ZIP2	Prostate	Down	Reduced	[55]
ZIP3	Prostate, pancreatic	Down	Reduced	[55–58]
ZIP14	Hepatocellular	Down	Reduced	[59]
ZIP6	Breast	Up	Increased	[60]
ZIP10	Breast	Up	Increased	[61]
ZnT-2	Breast	Up	Increased	[62, 63]

Additionally, several members of these transporter families are associated with specific cancers (Table 4.1).

During malignancy, Zn levels in prostate tissue, which are normally ~10× higher (~1 mg of Zn per gram of dry tissue) than levels in other soft tissues [64], can be markedly reduced [65–67]. This is unsurprising, as reduced Zn levels are commonly found in neoplastic tissue [65–67]. Abnormally low Zn concentrations have also been observed in prostate intraepithelial neoplasia, an established premalignant state [68, 69]. Interestingly, expression levels of human zinc transporter 1 (hZIP1) in prostate glands correlated with Zn levels, suggesting that hZIP1 may regulate Zn accumulation in malignant prostate glands [69].

Investigation into the molecular mechanism responsible for reduced Zn concentrations in malignant prostatic tumors revealed the downregulation of ZIP1–3 transporters [52, 55]. This downregulation was attributed to transcriptional inhibition of hZIP1 gene expression [53]. Interestingly, hZIP1 transcription is mediated by the Ras/Raf/MEK/ERK signaling pathway [52, 54], which is often upregulated in many types of cancer, including prostate [70]. However, in malignant prostatic tumor cells, reduced transcription is mediated by a Ras-responsive element (RRE) in the ZIP1 gene promoter region being bound by the Ras-responsive element-binding protein 1 (RREB1) [52, 54]. In turn, RREB1 itself is a Zn finger transcription factor, and can further reduced Zn levels in cells in which hZIP1 transcription. Furthermore, RREB1 can both activate and inhibit transcription of various target genes, depending on cell type, promoter, and co-binding proteins [71, 72].

Similar decreases in Zn levels have been found in pancreatic adenocarcinoma, an untreatable cancer with a low survival rate [56]. Zn deficiency is evident early in malignancy, for example, when pancreatic intraepithelial lesions first become present [57]. Decreased Zn levels in pancreatic adenocarcinoma tissue have been associated with reduced expression of the Zn importer ZIP3, which is present at the basilar membrane of normal ductal and acinar epithelium [56]. In contrast to prostate cancer, decreased RREB1 expression is associated with the downregulation of ZIP3 [57]. Zn supplementation inhibits malignant pancreatic cell proliferation, supporting the theory that reduced Zn levels protect cancerous cells from Zn-mediated cytotoxicity and apoptosis [73, 74]. With regard to the functional effects of RREB1, it had previously been demonstrated capable of both transcriptional activation and inhibition of various target genes, depending on cell type, promoter, and co-binding proteins [71, 72].

Altered Zn transporter levels have also been widely reported in hepatocellular carcinoma (HCC) tissue, as compared with normal liver samples [75]. Franklin et al. found reduced gene transcription and no expression of the Zn uptake transporter protein ZIP14 in a HCC cell line [59]. The same group also assessed a second HCC cell line that did express ZIP14 and found that treatment with physiological levels of Zn reduced cellular proliferation [59]. Zn supplementation also reduced mitochondrial function in the ZIP14-expressing HCC cell line, via aconitase inhibition [76]. Therefore, studies of Zn levels in cancer cell lines suggest that reduced Zn levels can prevent cytotoxic effects. Of note, in situ tumors may exhibit distinct ZIP gene deletions/mutations from the cell lines used for in vitro studies [40, 56, 59, 77].

Conversely, it has been well established that cancerous breast tissues typically have higher Zn levels compared to healthy surrounding tissue [78–82]. In addition, estrogen receptor-positive (ER+) breast tumor samples exhibited significantly increased Zn compared with ER− tumor samples [83]. Increased expression of certain Zn transporters mediating increased Zn levels and rapid cellular proliferation has arisen as an explanation behind breast carcinogenesis [61, 84, 85]. In support of this, higher expression of ZIP10 has been observed in invasive and metastatic breast cancer cell lines, as compared with those that are less metastatic [61]. In addition, ZIP6 was originally identified as an estrogen-inducible gene when elevated levels were associated with breast carcinogenesis [86, 87]. Increased Zn levels can inhibit glycogen synthase kinase 3β, which downregulates expression of the adherence gene E-cadherin via nuclear translocation of the unphosphorylated Zn finger protein, Snai1 [88]. This downregulation may promote invasion and metastasis of tumor cells. However, ZIP6 has also been associated with better survival outcomes [84]. Additionally, to combat potential cytotoxicity by increased Zn intracellular free Zn^{2+}, increased ZnT-2 may facilitate vesicle sequestration of Zn [62, 89].

4.2.2 Metallothionein

MTs are cysteine-rich, low-molecular weight, metal-binding proteins that support various cellular functions related to the redox cycle and heavy metal homeostasis. The four major MT isoforms, 1–4, and seven MT-1 subtypes are present in the cytoplasm and in certain organelles, including mitochondria and lysosomes [90]. While MTs were first discovered as cadmium-binding proteins [91], they can also bind other metals, including Zn, copper, selenium, arsenic, mercury, and silver, making their role in cellular pathophysiology very complex [92]. Regarding Zn, cysteine thiols in MTs allow for binding of up to seven Zn^{2+} atoms per MT and facilitate cellular distribution, as well as mitigate oxidative stress [92, 93]. Because MTs chelate Zn^{2+}, aberrant expression can lead to the loss of Zn^{2+} from critical tumor suppressor proteins, including p53 [94]. The role of Zn^{2+} in the function of p53 will be discussed in the section on proteins that utilize Zn for structural purposes.

Various lines of research have supported the idea that cancer cells exploit MT ability to prevent metal- or reactive oxygen species (ROS)-mediated toxicity, allowing

for increased cell survival by inhibiting apoptosis and promoting proliferation, especially when exposed to chemotherapeutic agents [33]. Some of the first evidence implicating expression of MT in the carcinogenic process came from studies that found increased apoptosis and sensitivity to chemotherapeutics in MT-null cancer cells [95, 96]. In addition, MTs are frequently overexpressed in human breast, colon, lung, ovary, salivary, and bladder cancers [19, 97]. In nasopharyngeal tumor samples, MT expression was also correlated with increased proliferation, as well as a lower ratio of both cell deaths and apoptotic events [98, 99].

MT overexpression may also confer resistance to antineoplastic drugs and radiotherapies [100–103]. For example, cultured cells with high MT levels were more resistant to treatment with a platinum-containing antitumor drug [100–103]. The mechanism behind this resistance may involve direct interaction with metal-containing therapies or scavenging for free radicals. Chemotherapeutic strategies to silence MT genes have been investigated, although no human treatments are currently in use [104, 105].

4.2.3 Role of Zn Homeostasis in Chemotherapeutics

Evidence has shown that Zn insufficiency is important for inhibiting apoptosis during the carcinogenic process, suggesting that increasing the intracellular concentration of Zn could treat cancer [65–67]. Zn can also inhibit thioredoxin reductase, a key oxidative stress mediator often overexpressed in human cancers [106]. To increase intracellular Zn levels in many cancers that exhibit downregulation of Zn importers, both supplementation with or without Zn-specific ionophores and compounds disrupting Zn homeostasis are used [40]. Zn supplementation alone in vivo increased tumor Zn levels and suppressed growth in a prostate cancer xenograft model [107]. While individual cases of Zn supplementation alone or in combination with other cancer therapeutics have been reported [108], no well-established treatments currently exist.

The Zn-specific ionophore clioquinol reversibly binds Zn^{2+} ions, shielding the charge, which allows for transport across cellular membranes leading to an increase in the intracellular concentration of Zn [109]. Clioquinol had been shown to inhibit cancer cell growth in vitro and in vivo, likely via the increase in intracellular Zn [110]. In vitro, combination of clioquinol and Zn supplementation increased intracellular Zn to cytotoxic levels and demonstrated that cytotoxicity was likely due to posttranscriptional effects of Zn, such as the downregulation of specific genes that regulate microRNA stability (e.g., Dicer and Ago2) and the global downregulated microRNA expression [111]. While the use of Zn and Zn-specific ionophores in the treatment of human cancers is currently not common, emerging evidence related to the safety of clioquinol and potential uses for Zn related to other carcinogenic mechanisms provides a promising avenue for treatment [112]. Many chemotherapeutics used presently are accompanied by debilitating side effects; however, clioquinol has little or no cytotoxic effects at the therapeutic dose (0.3–2 g/day) [113].

4.3 Proteins Containing Catalytic Zn

4.3.1 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a class of >20 structurally similar endopeptidases that require Zn^{2+} for enzymatic activity [114]. MMPs are involved in a myriad of important biological processes, including the degradation of ECM proteins (proteins secreted outside of the cell to structurally and biochemically support adjacent cells), cleavage of cell surface receptors, and inactivation of chemokines. Beginning with the N-terminus, MMPs generally consist of a ~80 amino acid propeptide domain, a ~170 amino acid MP catalytic domain region dependent upon both Ca^{2+} and Zn^{2+}, a 15–65 amino acid linker/hinge region, and a ~200 amino acid-long hemopexin domain [115]. The hemopexin domain is involved in binding to collagen and to other MMPs [116]. Within the catalytic domain, MMPs utilize three histidine residues to hold the catalytic Zn^{2+} ion in a specific configuration, which allows for the Zn^{2+}-mediated catalytic cleavage of nonterminal amino acid peptide bonds [117]. There is also a second Zn^{2+} ion present in the catalytic domain, which stabilizes the overall structure [118]. Of the 24 human MMPs, 18 are secreted and six are membrane bound. Both secreted and membrane-bound forms exhibit overlapping substrate specificity for degrading chemicals, including cytokines, collagens, and other ECM proteins such as fibronectin, plasminogen, and E-cadherin [119].

MMP catalytic activity is tightly regulated. For example, all known MMPs are initially expressed as latent and inactive proenzymes [120], due to the presence of a "cysteine switch," composed of a cysteine-connected pro-domain sheath, which prevents substrate interaction with the catalytic Zn^{2+}. To become active, the switch domain must be cleaved by other MMPs or proteases to release the pro-domain. The switch domain also prevents Zn^{2+} interaction with a water molecule, a required step for Zn^{2+}-mediated catalysis. The water molecule, attracted to the Zn^{2+} cation, is efficiently deprotonated by the Zn^{2+} ion and produces a nucleophilic hydroxide ion. The nucleophilic hydroxyl ion then attacks and cleavages the peptide bond [118].

Even after switch cleavage, the Zn^{2+}-mediated catalytic activity of MMPs can be regulated. Tissue inhibitors of metalloproteinases (TIMPs) are constitutively produced, endogenous protein inhibitors that bind to MMPs and prevent enzymatic function [121]. There are only four TIMPs (1–4), which regulate all MMPs [118, 121]. Using X-ray crystallography techniques, TIMPs were demonstrated to be shaped like a wedge, which slide into the MMP active site and physically block Zn^{2+}-mediated catalytic activity [122, 123].

The regulation of MMP Zn^{2+} catalytic activity is ultimately important for growth, tissue remodeling, angiogenesis, and immune system modulation. With such varied and important regulatory functions, it is unsurprising that dysregulation of MMPs has been linked to several types of cancer. In fact, abnormal MMP activity has been associated with lung, prostate, breast, ovarian, hematological, and colorectal cancer progression and metastasis [124, 125]. One mechanism by which MMPs support

cancer progression and metastasis is through overactive MMPs degrading sizeable amounts of the ECM, thereby providing a clear and unobstructed path for cancer cells to metastasize to other areas [126, 127]. It has also been proposed that dysregulated MMPs can support cancer progression in other capacities, such as tumor growth and tumor angiogenesis [126].

The abundance of evidence implicating MMP activity in carcinogenesis and malignancy also suggests that MMP inhibitors could be used as a successful strategy to treat cancers and prevent metastasis. While MMP inhibition, via blocking catalytic activity through Zn^{2+} binding or altering protein conformation, has been investigated as a potential therapeutic strategy, little success has been achieved due to lack of MMP specificity and severe side effects [114, 128]. For example, several phase III clinical trials targeting pancreatic and non-small cell lung cancer with broad or limited specificity inhibitors showed no evidence of survival benefit or were halted due to reduced survival [129]. The lack of success has been attributed to the multifunctionality of MMPs [130], meaning that although the pathological activity of a subset of MMPs has been reduced or eliminated, there is also a loss of protective MMP functionality resulting from overall MMP inhibition.

4.3.2 Lysine Deacetylases

Homeostatic control of histone acetylation is regulated by the activity of lysine deacetylases (KDACs, also known as histone deacetylases [HDACs]) and histone acetyltransferases (HATs). Appropriate histone acetylation is critical for chromatin structure, transcription factor accessibility, and gene expression [131, 132]. KDACs catalyze the removal of acetyl groups from lysine residues on both histone and non-histone proteins [133, 134], whereas the acetylation of lysine residues can be performed by HATs. Nearly all KDACs and some HATs require Zn to function. In chromatin, DNA is organized around lysine-containing histone proteins (Fig. 4.2a). In addition to supporting chromatin structure by providing a scaffold for DNA organization and packaging, histones can also regulate gene expression [135]. KDAC and HAT activity on histones can effectively turn expression off or on as depicted in Fig. 4.2b. Structurally, class I, II, and IV KDACs have a narrow hydrophobic tunnel leading to an active site containing Zn^{2+} coordinated to a histidine and two aspartic acid residues [136]. Class III KDACs (sirtuins) require NAD^+ for catalytic activity and are implicated in cancer progression, as well as metabolism and aging [137, 138]; however, sirtuins do not require Zn for catalytic function.

The acetylation of lysine residues on histones reduces the positive charge, decreases interaction with the negatively charged DNA phosphate backbone, and creates a more transcriptionally "open" histone conformation, known as euchromatin [139]. Conversely, deacetylation by KDACs facilitates a tightly packed, "closed" heterochromatin structure, which can be unfavorable for gene transcription. Additionally, HATs and KDACs control the posttranslational modification of several proteins, including transcription factors and DNA repair enzymes [140].

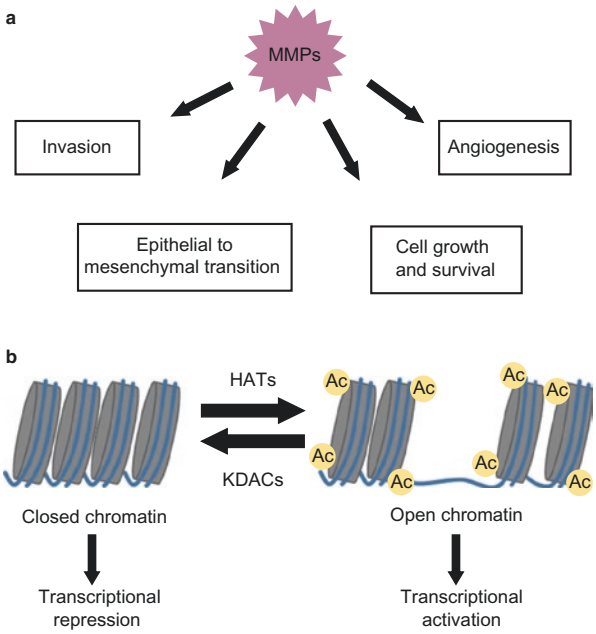

Fig. 4.2 The role of matrix metalloproteinases (MMPs) and histone acetylation in carcinogenesis. (**a**) Aberant MMP activity contributes to the activation of numerous pathways critical for tumor development and metastasis. (**b**) Decreased histone acetylation by KDACs results in transcriptional repression of tumor suppressor genes, which can contribute to the carcinogenic process

Most tumors and hematopoietic malignancies attributed to KDAC activity arise from deregulation of KDAC expression or recruitment to target genes [141]. Excessive KDAC expression can tip the balance of acetylation homeostasis, altering tumor suppressor gene expression and signaling [142, 143]. Genes associated with aberrant KDAC activity include those regulating cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis [144]. Several studies have reported increased expression of KDACs in tumors compared with normal tissue, which are often correlated with a worse prognosis [141]. For example, increased KDAC expression has been observed in gastric [145–148], colon [149–151], and breast [152–154] carcinomas.

Fusion proteins present in leukemia can promote gene silencing, through a mechanism involving KDAC recruitment to target gene promoters. In acute promyelocytic leukemia, a chromosomal translocation results in expression of a retinoic acid receptor α (RAR-α) and promyelocytic leukemia (PML) gene fusion. The PML-RARα fusion protein blocks transcription of genes critical for differentiation through enhanced recruiting of KDACs and nuclear corepressors [155]. Additionally, KDACs can contribute to carcinogenesis through interaction with nonhistone proteins. Acetylation of the tumor suppression protein p53 is involved in both protein activation and the prevention of subsequent ubiquitin-mediated degradation [156]. For example, HDAC1 deacetylation of p53 in vivo and in vitro has been demonstrated to alter p53 stability, leading to changes in downstream tumor suppressor functions [157, 158].

The role of KDACs in carcinogenicity has led to the development of KDAC inhibitors as an anticancer chemotherapeutic strategy [159, 160]. To date, four non-specific KDAC inhibitors have been approved for the treatment of hematopoietic malignancies, including multiple myeloma, peripheral T-cell lymphoma, and cutaneous T-cell lymphoma [161]. Of note, the hydroxamic acid vorinostat can inhibit enzymatic activity by binding to the active site of KDACs and acting as a Zn^{2+} chelator [136, 162]. However, lack of KDAC-specific selectivity and a narrow therapeutic window has presented challenges to the development and approval process of several other KDAC inhibitors [163, 164].

4.4 Proteins Containing Structural Zn

4.4.1 Zn Finger Proteins

Zn finger proteins require Zn^{2+} coordinated to Cys/His residue pairs to maintain proper structural conformation. The tertiary protein structure formed by the most common Cys_2His_2 tetrahedral Zn^{2+} binding facilitates hydrogen bonding of the α-helical side chain residues to the base pairs in the major groove of DNA [165]. Over 500 proteins contain the classical Zn finger structure. Most of these proteins are able to bind DNA and function as transcription factors or DNA repair proteins [166]. In general, transcription factors enhance transcription by binding to site-specific regions within gene promoters, where they interact with the RNA Pol II complex. Figure 4.3 depicts the Zn finger motif and Zn fingers binding DNA.

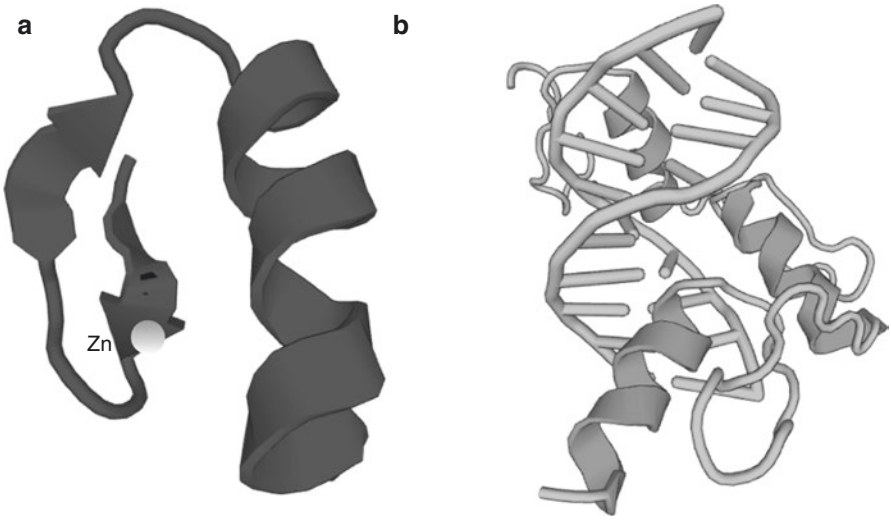

Fig. 4.3 Structure of Zn finger motif (PDB ID: 1ZNF) (**a**) and molecular model of the interaction between a domain composed of three Zn fingers and DNA (PDB ID: 1A1J) (**b**) generated using PV - JavaScript Protein Viewer (http://biasmv.github.io/pv/, Reproduced with copyright permission of Pleiades Publishing, Ltd. and Springer. Razin et al. Biochemistry-Moscow. 2012;77(3):217–26)

Numerous targeted therapies have been developed to counteract the aberrant gene activation resulting from the overexpression of Zn finger transcription factors [167]. For example, small-molecule therapeutics that interfere with RNA and DNA synthesis, primarily through inhibition of topoisomerase II, have been developed [168, 169]. Specific Zn finger proteins implicated in carcinogenesis are described below, as well as available targeted chemotherapeutic strategies.

Specificity Protein 1

Specificity protein 1 (Sp1) is a transcription factor that can activate or repress the expression of numerous genes related to cell growth, differentiation, angiogenesis, apoptosis, and tumorigenesis [170–172]. Sp1 proteins contain three Zn finger motifs at the carboxy-terminal end, which mediate DNA binding to GC-rich DNA elements often present in gene promoters [173]. Sp1 protein overexpression has been observed in several cancers and has been correlated with higher tumor stage and lower survival [174–177]. Sp1 binding activity was also increased in malignant breast tumor samples compared with benign lesions [178].

Reducing Sp1-associated gene expression via the inhibition of Sp1-DNA binding has been proposed as a potential chemotherapeutic strategy. Mithramycin A and newer analogs bind to the putative Sp1 binding site within gene promoter regions in DNA, blocking Sp1 binding [179, 180]. This strategy has also been used in a nonspecific fashion, using chemotherapeutics such as anthracyclines that bind to CG-rich sites and impede Sp1-mediated DNA binding [181, 182].

Snail Homolog 1

Snail homolog 1 (Snai1) is a Zn finger protein in the Snail family of transcriptional repressors [183]. While critical for normal epithelial to mesenchymal transition during development, Snai1 and other Snail family members have also been associated with carcinogenesis, tumor invasion, and metastasis. The role of Snai1 in the carcinogenic process likely primarily results from transcriptional repression of the E-cadherin gene, *CDH1*, a tumor suppressor glycoprotein involved in cell-cell adhesion [184, 185]. The expression of Snai1 has been observed in numerous carcinomas, including breast, ovarian, colon, squamous cell, and hepatocellular [186]. In breast carcinomas, Snai1 expression was correlated with reduced E-cadherin levels, metastasis, and reduced survival. Additionally, increased Snai1 expression and *CDH1* downregulation can be detected quite early in the carcinogenic process [187]. In addition to reduced E-cadherin, Snai1 expression has been correlated with reduced vitamin D receptor in colorectal cancer [188, 189]. Reestablishment of vitamin D receptor levels and supplementation with vitamin D have been explored as strategies for cancer prevention and treatment [190]. Snai1 expression can also initiate a positive feedback loop, as it can bind to its own gene promoter region and further activate transcription [191].

Snai1 interacts with other Zn-related proteins associated with carcinogenesis. For example, Snai1 has been correlated with increased MMP expression in squamous cell and HCC [192, 193]. In addition, Snail binding to target gene promoters has been shown to recruit HDACs and decrease gene transcription [186]. Despite the clear relationship between Snai1 and the carcinogenic process, no targeted chemotherapeutic strategies are under widespread investigation.

Glioma-Associated Oncogene

Aberrant activation of the hedgehog (Hh) signaling pathway, which is involved in normal development and tissue maintenance, has been associated with many cancers [194]. Glioma-associated oncogene (GLI) transcription factors are the final effectors of the Hh pathway. The three human GLI proteins, GLI1, GLI2, and GL3, each contain five conserved tandem Zn fingers. GLI activity is associated with expression of genes involved in epithelial to mesenchymal transition, survival, angiogenesis, and tumor cell invasion [168]. While GLI1 acts primarily as a transcriptional activator, GLI2 and GLI3 serve as both transcriptional activators and repressors [195]. GLI1 was first identified as overexpressed in a glioma tumor sample [196]. Since then, increased expression of GLI1 and other GLI transcription factors has been observed in brain, muscle, breast, lung, liver, stomach, prostate, pancreas, and colorectal tumors [197].

To counteract aberrant activation of GLI proteins, Hh antagonists, including those targeting signaling pathway intermediaries, have been developed [168]. For example, a smoothened (SMO) protein inhibitor, vismodegib, recently received approval for treatment of basal cell carcinoma in the United States [198]. Direct GLI antagonists have been investigated in vitro and in vivo with promising results in pancreatic, lung, and colon cancer models [199–201]. The small-molecule GANT-61 binds between the second and third Zn finger motifs of GLI1 and inhibits GLI1-mediated transcription [202]. Overall, direct GLI inhibitors are most promising because of resistance to side effects with SMO antagonists [197].

4.4.2 p53

p53, a tumor suppressor protein, is critical in protecting the genome from mutations resulting from normal cellular stress. Structurally, p53 is composed of four identical subunits, each of which requires one Zn^{2+} atom [203]. Zn^{2+} is required for the proper protein folding of p53, as well as for DNA binding and transcriptional activation by p53 [204]. The Zn^{2+} in p53 coordinates to Cys/His and Cys/Cys pairs, which are separated by a large section containing the minor-groove DNA-binding domain [205]. Relatively low p53 levels under normal conditions are mediated by MDM2, an E3 ubiquitin ligase that facilitates proteasomal degradation [206]. Under cellular stress conditions, p53 is activated though posttranslational modifications. Activated

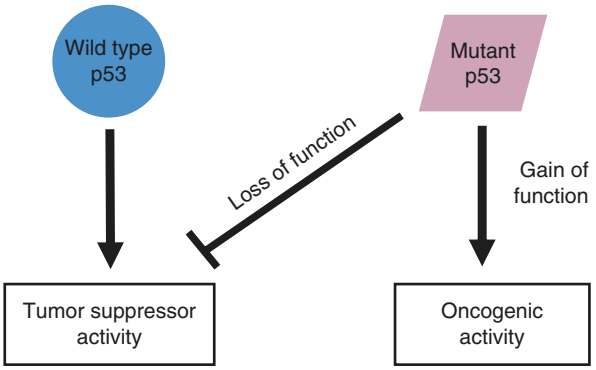

Fig. 4.4 The relationship between mutant p53 and development of cancer. Mutations in p53 protein often result in either the loss of normal tumor suppressor activity or the gain of new oncogenic functions, both of which can contribute to genomic instability and the carcinogenic process

p53 can then promote protection from deleterious mutations via either adaptation and recovery processes or cell death [207]. More than 50% of tumors contain mutant p53, suggesting that mutations of the *TP53* gene support carcinogenic transformation [208, 209]. Figure 4.4 summarizes the molecular mechanisms of p53 alterations related to carcinogenesis. Mutations or dysregulation of *TP53* resulting in loss- or gain of function in human cancers has also been associated with increased resistance to chemo- and radiotherapeutic strategies [210]. Notably, at least 90% of *TP53* mutations in tumor samples affect the DNA-binding domain [211].

Mutations resulting in the loss of p53 function inhibit tumor suppressor activities necessary for protection of the genome under cellular stress conditions, including the induction of genes responsible for cell cycle arrest and initiation of apoptosis. For example, many *TP53* mutations can reduce or eliminate Zn^{2+} binding. The absence of Zn^{2+} can alter p53 function by reducing DNA-binding affinity [212] leading to protein aggregation or misfolding, both of which can prevent normal function [213, 214]. The effects of *TP53* mutations altering Zn^{2+} binding residues are similar to the effects of removing Zn^{2+} through chelation, further highlighting the importance of Zn^{2+} for p53 function [215]. Notably, mutations in the Zn^{2+} binding loop of p53 are associated with poor prognosis in breast, prostate, non-small cell lung, and esophageal cancers [211].

Interestingly, gain-of-function *TP53* mutations also contribute to the carcinogenic process [216]. However, the mechanisms by which gain-of-function mutations promote tumor formation and invasion differ from those of loss-of-function mutations. For example, pro-oncogenic mutations can lead to p53-p73 interactions, which prevent p73-mediated apoptosis [217, 218]. Mutant p53 (mtp53) can also induce expression of the tumor-promoting chemokine CXCL1, as well as other pro-proliferative mediators such as c-MYC, MDR1, and NF-κB2 [219]. Overall, gain-of-function *TP53* mutations can produce alterations in DNA contact, protein conformation, and p53 protein levels [220].

Numerous strategies have investigated restoring normal p53 function to malignant cells. Regarding loss-of-function mutations, attempts have been made to use metallo-chaperones and small molecules to reactivate p53 [221–223]. Additionally, Zn^{2+} administration has been utilized to restore the active conformation of misfolded p53 proteins [224]. Importantly, Zn^{2+} administration in combination with other chemotherapeutics can enhance the antitumor effects [215, 225, 226]. For mutations related to p73 inhibition, small peptides have been used to disrupt the mtp53/p73 complex [227].

4.5 Summary

Zn atoms support critical catalytic and structural roles, and the dysregulation of Zn homeostasis can contribute to carcinogenesis through various pathways. Intracellular Zn levels are often reduced in human cancers; as such, chemotherapeutic strategies aimed at increasing intracellular Zn^{2+} levels through supplementation with or without the use of Zn-specific ionophores have been developed. In contrast, some tumors utilize increased Zn levels to promote proliferation. Increased expression and activity of catalytic Zn^{2+}-dependent proteins eliciting tumorigenic proliferation and metastasis have been targeted with small-molecule inhibitors. While many Zn finger transcription factors are associated with carcinogenesis, directed chemotherapeutic strategies are limited. Available evidence thus far suggests that transcription factor p53 is a potential chemotherapeutic target, as increasing intracellular Zn levels may restore proper protein folding. In summary, Zn is involved in a vast number of biological processes relevant to carcinogenesis, which in turn can potentially be exploited by targeted therapeutic strategies.

Disclaimer The study was reviewed by the National Center for Environmental Assessment of the US Environmental Protection Agency. Approval does not signify that the contents necessarily reflect the view and policies of the US Environmental Protection Agency.

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Chapter 5
Arsenic Carcinogenesis

J. Christopher States

Abstract Arsenic is a Class I carcinogen causing cancer of the skin, lungs, bladder, liver, kidney, and probably prostate and ovary. Exposure can be by ingestion of contaminated drinking water or food, or by inhalation of fumes from burning coal. Arsenic does not induce point mutations like a classic DNA-damaging mutagen. The carcinogenic mechanism is unclear, but evidence exists supporting DNA repair inhibition, stem cell expansion, reactive oxygen generation, aneuploidy, and epigenetic dysregulation. The lack of UV signature mutation spectra in arsenic-induced skin cancers argues against DNA repair inhibition as a mechanism. Recent studies on epigenetic dysregulation point toward differential gene expression consistent with a role in arsenic carcinogenesis. Limited animal models for arsenic carcinogenesis and limited studies conducted in human cancers caused by arsenic exposure limit the ability to elucidate mechanisms. Research focused on tumors from people suffering from arsenicosis is needed for a clearer understanding of molecular events underlying arsenic-induced carcinogenesis.

Keywords Arsenic • Carcinogenesis • Aneuploidy • Epigenetics

5.1 Introduction

Arsenic is the 20th most common element in the earth's crust. Inhalation exposure can be occupational as in smelter work, or via exposure to burning hard coal. Historically, arsenicals were used as medicinals. In current times the most common exposure is via arsenic-contaminated drinking water. Most contamination is naturally occurring and is dependent on the local geology of the aquifer [1]. Currently, it is estimated that 140 million people are chronically exposed to arsenic via contaminated drinking water worldwide. The largest population impacted is in the Ganges River delta region comprising Bangladesh and the state of West Bengal in

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India where more than 70 million people are suffering from the effects of chronic arsenic exposure.

The International Agency for Research on Cancer (IARC) has determined arsenic to be a Class I human carcinogen [2]. Indications that arsenic ingestion was carcinogenic originally came from observations of patients treated with various arsenicals. Hutchinson recognized the association of skin cancers subsequent to arsenical use in the 19th century [3–5]. In the mid-twentieth century, arsenic contamination of wine led to arsenicism and skin cancer [6, 7]. Chronic exposure to arsenic via inhalation causes lung cancer, and ingestion causes cancer of the skin, lung, bladder, liver, and kidney. Arsenic also may cause cancer of the prostate and ovary. Dose-response relationships have been demonstrated between arsenic ingested in drinking water and lung, skin, bladder, and kidney cancers [8–10]. Long-term impact on incidence of lung cancer in people exposed in utero or during early childhood also has been demonstrated [11, 12]. Although it is clear that chronic arsenic exposure causes cancer, and there has been abundant research over the past two decades, no clear unifying mechanism for arsenic-induced carcinogenesis has been shown.

Arsenic is referred to as a "non-genotoxic" carcinogen because it does not induce point mutations in in vitro test systems. Several hypotheses for mode of action have been proposed, and there are substantial data supporting each of these. Hypotheses include inhibition of DNA repair/co-mutagenesis, alteration of cell proliferation/stem cell expansion, aneuploidy/clastogenesis, and alteration of epigenetics. Each of these hypotheses will be discussed in the following sections.

Arsenic can exist in multiple valence states. Environmental arsenic is most commonly present as As^{5+} or As^{3+} [1]. In groundwater, As^{3+} dominates in the form of arsenite. Arsenite is readily converted to arsenate (As^{5+}) upon exposure to oxygen in air. Arsenite is metabolized by arsenic 3 methyltransferase (AS3MT) to a series of trivalent and pentavalent mono- and dimethylated species [13]. Generally, the trivalent species are more toxic than the pentavalent species. Monomethylarsonous acid is the most toxic metabolite. These trivalent arsenicals all bind sulfhydryl groups on proteins and other biological molecules. It is likely that the mechanisms of arsenic toxicity and carcinogenicity are a result of these interactions with sulfhydryls on key proteins [14]. The challenge has been to determine which molecules are the targets for interactions leading to cancer.

5.2 Cancer Epidemiology

5.2.1 Skin Cancers

Skin is a major target organ for arsenic-induced pathology. A variety of nonmalignant lesions are commonly observed and are pathognomic for arsenicosis. These skin abnormalities include hyperpigmentation, raindrop lesions, and hyperkeratosis [15]. The hyperkeratoses are premalignant lesions that most commonly appear on

the palmar and plantar regions. However, they also have been reported on the trunk and in body areas not exposed to sunlight. Arsenic-induced hyperkeratoses generally are raised pigmented lesions, and individuals often exhibit multiple lesions. These premalignant lesions often give rise to both basal and squamous cell carcinomas.

Both squamous cell carcinoma and basal cell carcinoma are linked to chronic arsenic ingestion. The earliest reports suggesting a causal link were related to ingestion of arsenic-containing medicinals and are summarized by Hutchinson in 1911 [5]. These reports were followed by reports of skin and lung cancers following chronic exposure to arsenical pesticides in German wine workers [6, 7] and respiratory cancers and skin lesions in patients treated with Fowler's solution [16, 17].

Chronic exposure to arsenic from drinking water also is linked to skin cancer. Skin cancer as well as cancers of the bladder, kidney, and lung in both males and females and cancers of the prostate and liver in males were linked to arsenic in artesian well water in Taiwan [10]. Ecological studies showed dose response for squamous cell carcinoma and basal cell carcinoma [18] but no increase in malignant melanoma [19]. Chronic arsenicosis caused by chronic consumption of drinking water in West Bengal, India and Bangladesh came about as an unintended consequence of tube wells installed by the World Health Organization to combat gastrointestinal disease caused by microbial contamination of surface water used for drinking [20–22]. Similar to the situation in Taiwan, individuals suffering from chronic arsenicosis in the Ganges delta region often develop basal and squamous cell carcinoma [23, 24]. Bowen's disease which is squamous cell carcinoma in situ also is common [25]. It is curious that although the premalignant hyperkeratoses are pigmented, they do not give rise to malignant melanoma.

Although arsenic in drinking water is regulated by the USEPA for public supplies, private wells are not regulated. In many parts of the USA, groundwater tapped by private wells has elevated arsenic levels. Even relatively low exposures to arsenic in drinking water are linked to skin cancer in the USA [26].

5.2.2 Lung Cancer

Lung cancer induced by arsenic exposure was first linked to inhalation by workers in arsenical pesticide manufacture and copper smelting [27]. Later epidemiological studies showed that ingestion of arsenic-contaminated water also could induce lung cancer [28] and also nonmalignant pulmonary disease [29–33]. Later studies of a cohort exposed to high arsenic levels in drinking water for a defined period in Antofagasta, Chile, showed that early life exposure could induce both lung cancer and nonmalignant pulmonary disease in adulthood [34]. Thus, regardless of the route of exposure, the lung is a target tissue for arsenic-induced pathology, and the latency period can be quite long.

5.2.3 Urinary Bladder and Kidney Cancer

Studies in Taiwan, Argentina, and Chile both linked increased incidence of death from urinary bladder and kidney cancers with high arsenic exposures [35–37]. Kidney cancers included renal cell carcinoma as well as transitional cell carcinoma of the renal pelvis [38]. The latter is also found in the urinary bladder [35]. Cohort and case control studies reported increased odds ratios for bladder and kidney cancers that showed dose response [11, 38]. These cancers appeared after long exposure periods of up to 40 years [11]. Cohort studies showed correlation with higher MMA^{v} in urine [39, 40].

5.2.4 Liver and Prostate Cancers

Evidence from mortality and population studies suggests a link between chronic arsenic exposure and both liver and prostate cancers [8, 18, 41–43]. However, the studies of liver cancer are mainly from Taiwan where hepatitis virus infection is endemic [44]. Thus, a clear causal effect for arsenic exposure is confounded. Curiously, although both hepatitis virus infection and arsenic exposure cause hepatitis, arsenic exposure reduces hepatitis and cirrhosis in people chronically infected with hepatitis virus [45]. Liver cancer may require exposure to very high levels of arsenic in drinking water [46]. There also may be a differential susceptibility to arsenic-induced liver cancer by gender [47]. Thus, the particular population under study may not show an increase in liver cancer with arsenic exposure [48].

There is some evidence of a dose-response relationship for prostate cancer in the studies from Taiwan [8]. However, the studies from South America are inconsistent [36]. An ecological study found an association of prostate cancer with arsenic in drinking water in Illinois [49]. Increased mortality from prostate cancer was associated with arsenic exposure in the Strong Heart Study population [50]. Thus, although suggestive, clear causal relationships have not yet been established for liver and prostate cancers in humans.

5.3 Arsenic Carcinogenesis in Laboratory Animals

Induction of cancer in adult laboratory animals by inorganic arsenic exposure has eluded investigators. A laboratory model of arsenic carcinogenesis by in utero exposure of mice to arsenic via drinking water was developed in the Waalkes laboratory [51]. The model originally showed a decreased latency and increased multiplicity of tumors in C3H mice exposed in utero to high levels of arsenic in drinking water [52]. The model has since been refined and improved. Lower exposures and alternate mouse strains also result in increased carcinogenesis [53]. The introduction of “whole

life" exposure starting two weeks prior to conception and continuing on into adulthood in the exposed pups has dramatically improved the model [54]. The reader is referred to a recent thorough review of the development of this model [55].

5.4 Cell Proliferation/Stem Cells

In vitro studies of the impact of arsenite exposure on cell cycle kinetics have yielded conflicting results. Under different conditions, arsenite stimulates cell proliferation [56–58], slows proliferation [56, 59, 60], or even induces cell cycle arrest [61–63]. It appears that both concentration and cell line are important in determining the impact of arsenic on cell cycle [64]. Mitotic arrest by arsenite is dependent on functional spindle checkpoint [65]. Thus, some of the cell line specificity in cell cycle effect may be related to specific mutations present.

Expansion of stem cell populations has been observed both in vitro [66] and in vivo [67]. This expansion may increase the target cell population for carcinogenesis. Alternatively, the increase in stem cells may be a consequence of decreased maturation and terminal differentiation thus contributing directly to carcinogenesis. The reader is referred to a recent review of arsenic targeting of stem cells as part of the mechanism of carcinogenesis [68].

5.5 DNA Repair/Co-mutagenesis

Overexposure to the ultraviolet (UV) component of sunlight is well known to cause skin cancer [69]. Overexposure to sunlight causes all three major forms of skin cancer: squamous cell carcinoma, basal cell carcinoma, and malignant melanoma. The mechanism of action is the induction of photodimers in the DNA leading to mutagenesis. Sunlight-induced SCC is driven by mutations resulting from photodimers in *TP53* [70]. Thus, it was reasonable to investigate the potential of arsenic to induce mutations. Early studies showed that arsenic by itself did not cause mutations in standard mutagenesis assays such as *HPRT* [71]. However, arsenic exposure was able to increase mutagenesis by UV [72] and by MNNG [73]. Studies by another group included some intriguing results indicating that the order of exposure was important, in that arsenic exposure after the MNNG exposure did not increase the mutant fraction as did coexposure or preexposure [74] and that posttreatment with arsenite altered the UV mutational spectrum [75]. In vivo studies with UV and arsenic administered to mice via drinking water suggested that the arsenic exposure could enhance the yield of UV-induced skin tumors. These data were interpreted as arsenic being a co-mutagenic and likely to be inhibiting DNA repair. However, *TP53* mutations in arsenic-induced skin cancers either are not present [76, 77] or did not result from photodimer damage [78].

It was several years before a potential mechanism for arsenic inhibition of DNA repair began to appear. XPA plays an essential role in nucleotide excision repair [79], the DNA repair pathway responsible for removal of UV-induced photoproducts. Hartwig's group showed that arsenite could displace the zinc from the zinc finger of XPA [80]. However, earlier reports from this group indicated that XPA binding of damaged DNA was not impaired by arsenite [81, 82]. Thus, it is not clear that arsenite targeting of XPA plays a role in inhibition of DNA repair.

Poly(ADP-ribose) polymerase 1 (PARP1) plays a key role in signaling DNA damage [83]. PARP1 plays a key role in base excision repair, the DNA repair system responsible for removal of oxidative DNA damage and of alkylation adducts [84]. Hartwig's group showed that PARP could be inactivated by very low concentrations of arsenite [85]. A few years later, Liu's group showed that arsenite could displace the zinc from PARP1 zinc finger [86]. This group also published evidence suggesting that arsenite inhibits PARP1 in repair of oxidative damage [87] and UV photoproducts [88]. The cellular experiments were performed with concentrations of arsenite well above in vivo levels of individuals suffering from arsenic exposure via drinking water [56]. The extreme sensitivity of PARP1 to arsenite suggests that arsenite targeting of PARP1 may be a mechanism of DNA repair inhibition by arsenic. However, the situation is likely much more complicated. Chronic incubation of human keratinocytes with 100 nM arsenite suppresses global protein poly(ADP-ribosyl)ation but increases poly(ADP-ribosyl)ation of TP53 and PARP1 levels [89].

Thus, it would appear that inhibition of DNA repair by arsenite may be a mechanism whereby arsenic exposure could increase the mutagenic potential of sunlight and cause skin cancer. However, there is a clear difference in the spectrum of cancers caused by sunlight and chronic arsenic exposure. First, sunlight, but not arsenic, causes malignant melanoma. Chronic arsenic exposure causes only squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). Furthermore, chronic arsenic exposure causes skin cancers in areas of the body not overexposed to sunlight. The premalignant lesions induced by the two agents also differ. Sunlight causes actinic keratosis which gives rise only to squamous cell carcinoma. Chronic arsenic exposure induces hyperkeratosis, which can give rise to both SCC and BCC [15]. Molecular epidemiology studies of sunlight-induced SCC and actinic keratosis show that mutation of *TP53* is an early event and is present in the vast majority of lesions [70]. In contrast, careful study of *TP53* mutation status in arsenic-induced skin cancers showed that *TP53* mutation was rare [77]. Thus, *TP53* mutation is not likely to be driving arsenic-induced skin cancer.

Another confounder of the inhibition of DNA repair hypothesis is that the arsenite concentrations used in vitro were above physiologic concentrations (2 μM vs. 1–100 nM). Thus, it is possible that other more sensitive targets are disrupted by arsenite in persons consuming water with moderate to high levels of arsenic. These more sensitive targets may play a role in other pathways that contribute to carcinogenesis. Thus, an alternative interpretation of the rodent studies of UV-induced skin cancer is that these alternate pathways, similar to those affected by tumor promoters like phorbol esters, are the targets for arsenic carcinogenesis.

5.6 Aneuploidy/Clastogenesis

It is well established that arsenic and its methylated metabolites can cause both numerical and structural chromosomal abnormalities [61, 90–92]. Aneuploidy, or numerical imbalances, causes imbalances in expression of components of multiprotein complexes leading to malfunction and disruption of cellular homeostasis [93]. Clastogenesis, or chromosome breakage, can lead to structural abnormalities such as chromosomal translocations and gene fusions [94] contributing to cancer. Thus, chromosomal abnormalities induced by arsenic exposure are clearly a likely mechanism of carcinogenesis.

In vitro, arsenite exposure delays mitosis and induces aneuploidy in diploid human fibroblasts [95] and peripheral blood lymphocytes [96]. Increases in the frequency of micronuclei have been reported in lymphocytes and urothelial and buccal cells of people chronically exposed to arsenic in drinking water [97]. Micronuclei containing centromeres indicating aneuploidogenic events and without centromeres indicating clastogenic events occur. Activity of TP53 and induction of CDKN1A appear to play a deciding role in whether the arsenite effect is aneuploidogenic or clastogenic [91].

5.7 Epigenetics

Epigenetic effects of arsenic exposure also have been documented. These effects can be changes in DNA methylation patterns, changes in histone acetylation or methylation, and changes in miRNA expression profiles. There is a large body of literature linking epigenetic alterations with carcinogenesis.

5.7.1 DNA Methylation

DNA methylation in promoter regions of RNA polymerase II transcribed genes generally is associated with gene silencing. Altered DNA methylation as a mechanism of arsenic carcinogenesis was proposed in 1997 [98]. These authors reported dose-dependent increases in genome-wide DNA methylation and in specific hypermethylation of CpG sites in the *TP53* promoter in human lung adenocarcinoma A549 cells treated with sodium arsenite or sodium arsenate, but not dimethylarsenic acid. Since then, there have been reports of both hypermethylation [99, 100] and hypomethylation [101–103] of key genes by arsenite exposure using in vitro and in vivo experimental systems. Chronically exposing human keratinocytes to low-level arsenic caused decreases in S-adenosylmethionine (major methyl donor) and DNA methyltransferase gene expression (DNMT1 and DNMT3A) and decreased 5-methylcytosine content in DNA [104] supporting the hypothesis that chronic arsenic exposure induces DNA hypomethylation.

Studies using peripheral blood lymphocytes also have investigated arsenic exposure-related changes in DNA methylation. Hypermethylation of p16 gene (*INK4A*) was reported in DNA from whole blood leukocytes of persons exposed to high arsenic in drinking water (>0.05 mg/L, average = 0.6 mg/L) in Bameng, Inner Mongolia regardless of the presence of arsenicosis [105]. DNA repair genes *ERCC1* and *ERCC2* were shown to be hypermethylated in lymphocytes isolated from endemic arsenicosis patients in China [106]. However, a study conducted in West Bengal, India, showed that *ERCC2* was hypomethylated [107]. Likewise, *LINE-1* methylation was decreased in whole blood DNA from arsenic exposed children in West Bengal whose lymphocytes showed evidence of genotoxic stress indicated by increased micronucleus formation [108]. DNA methylation in exfoliated urothelial cells from patients in Chihuahua, Mexico showed arsenic-associated changes in 22 of 49 genes examined [109]. The genes showing differential methylation also are identified as differentially methylated in urinary bladder cancers in The Cancer Genome Atlas repository. This correlation supports the hypothesis that epigenetic changes induced by arsenic exposure are driving the carcinogenesis.

The impact of arsenic exposure on 5-hydroxymethylcytosine in DNA has been examined recently in a Bangladeshi cohort [110]. 5-Hydroxymethylcytosine is thought to lead to demethylation and gene activation. Quantitation of 5-hydroxymethylcytosine in leukocyte DNA showed no association with arsenic exposure overall, whereas an earlier study saw a trend toward in inverse correlation in an American cohort [111]. However, when parsed by gender, it became apparent that 5-hydroxymethylcytosine had a positive association with arsenic exposure in males, but a negative association in females. This gender-specific epigenetic response to arsenic exposure has not yet been linked to carcinogenesis. However, these results correlate with an earlier report that early prenatal arsenic exposure appears to decrease DNA methylation in mononuclear cells from cord blood in boys, but not girls [110].

Most intriguing is a recent paper showing that a relatively short (48 h) incubation with physiologically relevant arsenite concentrations induces long-lasting DNA methylation and gene expression changes in human keratinocytes (HaCaT) [112]. These authors show that gene-specific hypermethylation occurs in spite of genome-wide hypomethylation and that these patterns persist. They specifically examined DNA methyltransferase and mismatch repair genes and found that the hypermethylation and corresponding decreased gene expression occurs in a gene selective manner.

A study of urothelial carcinomas found significant associations between cumulative arsenic exposure, and DNA methylation levels of 28 patients were observed in nine CpG sites of nine genes [113]. The small size of this study weakens any conclusions. Hypermethylation of death-associated protein kinase (*DAPK*) gene I in urothelial cancers arising in arsenic-contaminated areas in Taiwan compared to tumors from patients from noncontaminated areas links gene-specific hypermethylation with chronic arsenic exposure [114].

5.7.2 Histone Modification

DNA is packaged into chromatin, and transcriptional activity is modulated by changes in chromatin structure. The fundamental organizational unit of chromatin is the nucleosome in which 165 bp of DNA is wrapped around an octamer of the four core histones, two each of H2A, H2B, H3, and H4. Posttranslational modifications (PTMs) of histones govern chromatin structure and hence modulate gene expression. Histone PTMs include acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination. Most research determining role of histone PTMs in gene expression has focused on acetylation of lysine residues and methylation of lysine and arginine residues in histone H3 and H4 tails. Arsenic exposure has been shown to affect these histone PTMs in vitro and in vivo. The reader is directed to recent reviews [115, 116] for broad discussion. Here we will highlight two examples of arsenic-exposure-induced changes in histone PTMs potentially related to carcinogenesis.

Canonical histone mRNAs are not polyadenylated but rather have a stem-loop structure in the 3′-untranslated region (3′-UTR) that is bound by stem-loop binding protein (SLBP) that directs the 3′-end maturation of histone mRNAs. An interesting secondary effect of arsenic-induced epigenetic changes is the observation that epigenetic silencing of SLBP expression results in polyadenylation of histone H3.1 mRNA that accumulates [117]. The authors showed that overexpression of histone H3.1 in BEAS-2B cells results in increased ability to form colonies in soft agar consistent with a potential role in carcinogenesis.

Cell-type specificity in global H3K9Ac and H3K9me3 levels was observed in peripheral blood lymphocytes from women exposed to arsenic through drinking water in the Argentinean Andes [118]. CD4+ and CD8+ were sorted and H3K9Ac and H3K9me3 levels measured. The authors reported arsenic dose-dependent decrease in global H3K9me3 in CD4+ cells, but not CD8+ cells. In contrast to an earlier report of an inverse correlation of global H3K9Ac levels with arsenic exposure in peripheral blood mononuclear cells (PBMC) in Bangladeshi women [119], the Argentinean study saw no difference in H3K9Ac levels in CD4+ or CD8+ T-cells. PBMC include many other cell types besides CD4+ and CD8+ T-cells. It remains unclear whether the lack of correlation in findings between these two studies is due to a genetic basis or cell-type specificity of response.

A common finding has been discordance between global effects and gene-specific effects. For instance, global H3K9 hypoacetylation was induced by arsenite exposure in HEK293 and UROtsa cells [120]. However, in the same experiments, H3K9 hyperacetylation in the FOS gene was observed. Similar discordance between global and gene-specific changes in histone PTM levels has been observed for other PTMs as well indicating that in order to understand the mechanisms of PTM modification in relation to carcinogenesis, it is important to understand gene-specific effects.

5.7.3 miRNA

A third class of epigenetic elements is microRNA (miRNA). These are small (21–22 nucleotide) RNAs that are posttranscriptional regulators of gene expression. Most often, miRNAs act by binding to target mRNAs and suppress translation and/or promote mRNA degradation. Each miRNA may have thousands of target mRNAs, and each mRNA may have several miRNAs targeting it. Expression of miRNAs is cell-type specific, and the expression responds to environmental and developmental stimuli. Arsenic exposure and its impact on miRNA expression have been examined in a variety of in vitro and in vivo systems and related to a wide variety of arsenic-induced disease endpoints. We will present a few examples related to carcinogenesis.

Differential miRNA profiling was performed in HaCaT cells exposed to 0.5 μM sodium arsenite for 4 weeks [121]. Among the 30 miRNAs expressed at higher levels in arsenic-exposed cells were miR-21, miR-200a, and miR-141, all with known roles in carcinogenesis. In contrast, in arsenic-transformed human prostate epithelial cells, suppression of specific miRNA expression was correlated with increased *KRAS* expression and transformation of the cells [122]. Furthermore, restoration of miR-143 expression by lentiviral transduction reverted the cancer stem cell phenotype [123].

Profiling of miRNAs in cord blood revealed a panel of 12 miRNAs with expression positively correlating with urinary arsenic levels in the mothers [124]. Pathway analysis of the predicted targets of these miRNAs showed that they are involved in signaling pathways associated with cancer and diabetes mellitus.

5.8 Conclusions

Clearly, arsenic exposure is carcinogenic to humans. There has been a great deal of research supporting a variety of potential carcinogenic mechanisms. Unfortunately, only a handful of studies have been performed using tumors from patients with arsenicosis. These studies using skin cancers have suggested that mutagenesis is not a driving force for arsenic carcinogenesis. A number of studies have been done in peripheral blood lymphocytes from arsenic-exposed people. These studies have pointed to epigenetic and aneuploidogenic mechanisms of carcinogenesis. Most of the published research has been performed using in vitro cell culture systems and used physiologically irrelevant concentrations of arsenic species that were much too high. These studies point to a myriad of effects that if operational at physiological exposures might play a role in carcinogenesis. More recent in vitro studies have used relevant concentrations (0.1–0.5 μM arsenite). The results of these latter studies point mostly toward epigenetic mechanisms, i.e., alterations in histone modifications, DNA methylation, and microRNA expression. Noticeably absent are studies of epigenetic alterations in arsenic-induced human tumors.

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Chapter 6
Cadmium Carcinogenesis and Mechanistic Insights

Anuradha Mudipalli

Abstract The heavy metal cadmium is ubiquitous in the environment. Occupational exposures to cadmium have long been linked to cancers of various organs. Emerging epidemiological data, although often limited by study deficits, provide convincing evidence of lung, kidney, prostate, and breast cancers after cadmium exposure. Experimental evidences from animal models and in vitro cell culture systems aid in discerning the molecular pathways of these cancers and provide biological plausibility for cadmium carcinogenesis. The International Agency for Research on Cancer declared cadmium as group I carcinogen with sufficient evidence for cancer in humans. This chapter discusses the molecular pathways of cadmium carcinogenesis for specific organs followed by a brief discussion on general molecular pathways of cadmium-induced carcinogenesis. Finally, conclusions are drawn on the existing database in order to identify common and unique molecular pathways of these cancers and to infer biological plausibility.

Keywords Cadmium exposure • Cancer • Organ-specific cancer mechanisms • General molecular mechanisms of carcinogenesis • Conclusions and perspectives

Abbreviations

A549	Human lung epithelial carcinoma cells A549 cells
AR	Androgen receptor (AR)
Bax	bcl-2-like protein 4
Bcl-2	B-cell lymphoma2

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Bcl-xL	B-cell lymphoma-extra-large
BER	Base excision repair (BER)
CCR4	C-C chemokine receptor type 4 (CCR4)
CD44	Cell surface glycoprotein44
CHO	Chinese hamster Ovary cells
CK8	Cytokeratin 8
DNMT3b	DNA methyl transferase3b
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross-complementing 1
FOXO	Forkhead box class O
FOXO3a	Forkhead box class O transcription factor a
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRP30	G-protein coupled receptor
GSK3α/β	Glycogen synthase kinase-3α/
HER2	Human Epidermal growth factor receptor
hMSH2	Human mismatch repair protein homologue2
hoGG1	The human 8-oxoguanine DNA N-glycosylase 1
IARC	International Agency for Research on Cancer
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
Ki67	Antigen KI-67
NHANES	National health and nutrition examination survey
MMR	Mismatch repair
MMS	Methyl methane sulfonate
MNU	N-methyl -N-nitrosourea
MT	Metallothionein
NER	Nucleotide excision pathway (NER)
NKX3.1	Homeobox protein NKX3.1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2’
OCT4	Octamer-binding transcription factor 4
P53	Tumor suppressor protein p53
PI3K	Phosphoinositide-3-kinase
PSA	prostate-specific antigen
PSCA	Prostate stem cell antigen
RSK2	Ribosomal s6 kinase 2
ROS	Reactive oxygen species (ROS)
S100P	S100 calcium-binding protein P
SCID mice	The severe combined immunodeficiency mice
SPCA	Secretory pathway Ca-ATPase
TNF-α	Tumor necrosis factor-alpha
XIAP	X-linked inhibitor of apoptosis protein
XPA	Xeroderma Pigmentosum group A protein
XPC	Xeroderma pigmentosum, complementation group C protein
XRCC1	X-ray repair cross-complementing protein 1

6.1 Introduction

The ubiquitous, transition heavy metal cadmium (Cd), originally discovered in 1817, has widespread industrial and agricultural applications. It exists both in organic and inorganic forms in nature. The concentration of cadmium in earth crust is estimated to be around 0.1–0.2 ppm [1]. The solubility of cadmium compounds varies widely, and this property, by virtue of differences in absorption, distribution, and bioaccumulation, contributes to their bioaccumulation. Environmental exposure to cadmium occurs through diverse routes (oral, dermal, and inhalation). Occupational exposures to cadmium happen through industrial processes such as pigment and dye industry, nickel and cadmium battery processing, and metal soldering such as in the jewelry industry. Additional exposures take place via agricultural applications such as pesticide spraying and fertilization, diet (consumption of rice, fruits, and vegetables from contaminated soils), and involuntary exposures through smoking [1]. Cadmium accumulates and stays in the human body over a prolonged period of time (20–30 years) [1]. The adverse health effects to cadmium exposure are mostly attributed to its ionic form rather than as a salt. The toxic insult and resultant pathophysiological changes due to cadmium exposure have been observed for several organ systems including the bone, liver, kidney, lung, bladder, and reproductive organs. In addition, environmental and occupational epidemiological studies carried out in the USA and Europe showed positive association of cadmium exposure to cancers of various types such as the lung, bladder, kidney, prostate, pancreas, ovary, and endometrium. The 2014 National Health and Nutrition Examination Survey (NHANES) study showed a positive association between cadmium exposure and cancer mortality of the kinds studied, more specifically to prostate cancer mortality in men. Such exposure-related positive association to cancer(s) was also demonstrated by experimental animal studies with great mechanistic detail. The International Agency for Research on Cancer (IARC) has classified cadmium as a group I carcinogen, with sufficient evidence for carcinogenicity in humans [1].

Cadmium is designated as an indirect carcinogen and is recognized as a non-mutagenic, non-genotoxic chemical. A large body of existing scientific studies, the majority of them utilizing in vitro and rodent animal models, suggest that the cadmium-induced carcinogenic process is complex and involves multiple molecular pathways. This book chapter provides the current state of our understanding on cadmium-induced perturbations in the molecular pathways of carcinogenesis for specific organ systems such as the lung, bladder and kidney, prostate, breast, and pancreas with an effort to identify common and unique genes in these cadmium-induced cancers (Fig. 6.1 and Table 6.1). Additionally, an illustration of the general mechanisms and pathways underlying carcinogenic processes after metal exposures is provided in Fig. 6.2. The chapter will conclude with a perspective on how the available scientific evidence provides support for the positive association between cadmium exposure and cancer(s) from the mechanistic standpoint. The chapter also discusses the limitations in the database to answer the remaining questions on the modes of cadmium-induced cancer and the association to cancer from the environmental exposure to cadmium.

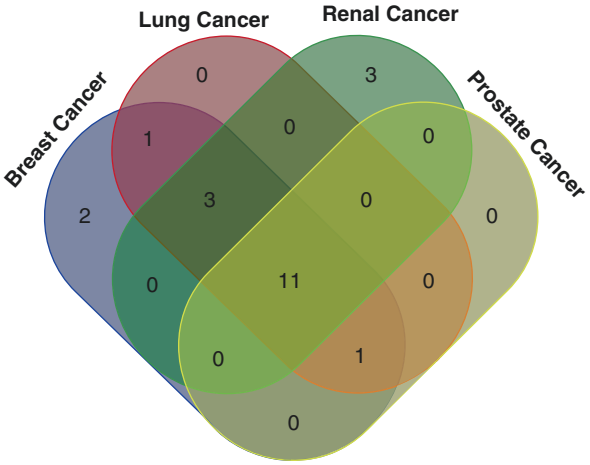

Fig. 6.1 Venn diagram showing number of common genes altered in organ specific cancers induced by cadmium

Table 6.1 Common set of altered genes for different cadmium-induced cancers

Type of cancer	Common genes/ pathways	Genes/pathways
Breast	11	PCNA, AP1, MAP kinases, C-Jun/c-Fos, Caspases, DNA damage repair, NF-kB, P53, MT, Cytokines, NRF2
Lung		
Prostate		
Renal		
Breast	3	E-cadherin, MMP, β-catenin
Lung		
Renal		
Breast	1	Hsp
Lung		
Prostate		
Breast	1	EGFR
Lung		
Breast	2	EGF-R, PgR
Renal	3	m-TOR, Kim-1, Wnt/Notch-1

PCNA proliferating cell nuclear antigen, *AP-1* activator protein1, *MAP kinases* mitogen activated protein kinase, *c-jun* cellular putative transforming virus of avian sarcoma virus 17, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells protein, *p53* tumor suppressor p53, *NRF2* nuclear factor erythroid 2-related factor 2, *Hsp* heat shock protein, *EGF* epidermal growth factor receptor, *PgR* progesterone receptor, *m-TOR* mammalian target of rapamycin, *Kim1* kidney injury molecule 1, *Wnt/Notch-1* Wnt1 wingless-type MMTV integration site 1 protein, *Notch1* a protein of transmembrane proteins with repeated extracellular EGF domains and the notch (or DSL) domains

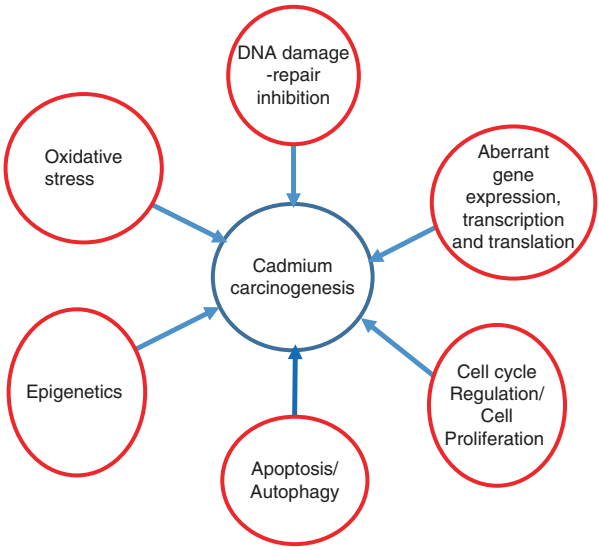

Fig. 6.2 General mechanisms of cadmium-induced carcinogenesis

6.2 Lung Cancer

The classification of cadmium as a human carcinogen arises from evidence of lung and renal cancer in humans reported at occupational and environmental levels [1–4]. Chronic and repeated exposure of human bronchial epithelial cells (16BHE) to cadmium chloride resulted in malignant transformation and tumor formation (poorly differentiated squamous cell carcinoma) in nude mice [5]. This is consistent with earlier observations of a malignant transformation of human cell lines [6] and initiation of pulmonary adenomas in rodents [7].

The process of cadmium-mediated pulmonary carcinogenesis appears to be influenced by perturbations in genome stability, DNA damage repair, apoptosis and autophagy, EGF receptor, and related downstream signaling mechanisms.

6.2.1 DNA Repair

Cadmium is not directly mutagenic and is not a direct DNA-damaging agent. However, alterations in several DNA repair genes were reported [8]. When human bronchial epithelial cells were exposed to a log range of cadmium concentrations, transcriptional and translational changes in several DNA repair genes as well as frameshift mutations in human mismatch repair protein homologue2 (hMSH2),

excision repair cross-complementing 1 (ERCC1), the human 8-oxoguanine DNA N-glycosylase 1 (hoGG1), and X-ray repair cross-complementing protein 1' (XRCC1) were observed suggesting pro-mutagenic lesions, DNA damage, and genomic instability [8]. In addition, these observations lend support to the earlier observation of increased apoptosis, upregulation of p53, and Bax expressions in rat lung primary cells exposed to concentrations of cadmium in the range of 1–10 μM [9].

6.2.2 Apoptosis/Autophagy

Autophagy is a highly conserved catabolic process that targets cellular contents to lysosomal degradation [10] and has emerged often as a regulator of cellular invasion, metastasis, and migration in the process of carcinogenesis by virtue of regulating cell growth and differentiation control [11]. Recent in vitro evidence of cadmium-induced autophagy was reported from the increased accumulation of LC3-II, a light chain of the microtubule-associated protein 1 (eukaryotic analog of autophagy-related protein 8) in JB-6 mouse epidermal cells exposed to micromolar concentrations of cadmium [12]. In some cell lines, cadmium seems to regulate apoptosis or autophagy by the localization of enzymes. In HL60 "non-small lung cells," it was demonstrated that the acquisition of cadmium resistance is through the interruption of GSK3α/β (glycogen synthase kinase-3α/β) phosphorylation and the change in the intracellular localization of p-SerGSK3 regulation of apoptosis and autophagy [13]. Similarly it is suggested that cadmium toxicity is regulated by the transcriptional regulation, stabilization, and subcellular redistribution of multidrug resistance protein, MRP1, via the posttranslational modification of GSK3αβ [14]. Cadmium-transformed cells acquire autophagy deficiency which led to overexpression of constitutive nucleoprin 62 (p62) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Additionally, this upregulated the antioxidant protein catalase superoxide dismutase (SOD) and the antiapoptotic proteins B-cell lymphoma2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL). These changes resulted in decreased reactive oxygen species (ROS) generation and apoptotic resistance which led to increased cell survival, proliferation, and tumorigenesis indicating the importance of apoptosis in the process of carcinogenesis [15].

6.2.3 EGF Receptor (EGFR) and Cell Cycle Regulatory Genes in Cadmium-Induced Lung Cancers

Mutations in epidermal growth factor receptor (EGFR) are well documented and linked to several types of cancers (including environmentally related cancers), the most notable of which is lung cancer [16]. To date, about eight mutations in EGFR have been implicated in lung cancer. Functional alterations in EGFR and the subsequent downstream cellular signaling cascade are linked to cadmium-mediated

cancers of other types which are detailed in the text on the organ-specific cancers. It is reported that cadmium chloride, when administered intraperitoneally in mice at a dose of (5 mg/kg), increased inflammation in lung cells along with an increase in several pro-inflammatory markers such as interleukin-6 (IL-6) and cyclooxygenase-2 (Cox2) indicating toxicity [16]. In addition, several cell cycle regulatory proteins were also expressed at higher levels in the treated group than in the controls. In human lung epithelial carcinoma cells (A549 cells), the same author group demonstrated that cadmium treatment at a lower dose (2.5 μM) resulted in the proliferation of the transformed lung cells, while a higher dose (5 μM) resulted in cytotoxicity and cell death [17]. Cadmium at low doses resulted in EGF receptor activation, subsequently altering the downstream signaling events in cell cycle regulatory molecules such as the cell proliferative markers KI-67 (Ki67) and proliferating cell nuclear antigen (PCNA) proteins, resulting in enhanced production of several pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α). Cadmium treatment also resulted in more cells in S-phase of the cell cycle [17]. EGFR signaling affects cyclin D1 expression as demonstrated by transcriptional regulation of cyclin D1 by EGFR cross signaling [18]. When the A549 cells treated with cadmium were additionally treated with Gefitinib (EGFR tyrosine kinase inhibitor), both cyclin D1 and Cox2 expressions were blocked indicating these signaling alterations by cadmium occur via EGFR pathway [17].

6.3 Renal Cancer

6.3.1 Cadmium Uptake by Kidneys

Approximately 50% of cadmium found in the human body is found in the liver and kidney due to their high metallothionein (MT) (a high-affinity metal-binding protein) concentration [19]. Cadmium absorbed into the blood stream is transported to the gatekeeper tissue, the liver, for detoxification process that involves complexation of cadmium with metallothionein (MT) and export of cadmium–metallothionein complex (Cd-MT) back into the blood stream for removal by glomerular filtration. Although the Cd-MT complex is considered a major detoxification step, it has also been recognized that cadmium is loosely bound to albumin, peptides (glutathione), and amino acid, cysteine, suggesting multiple mechanisms to enter proximal tubules [20, 21]. There is accumulating evidence that a variety of metal transporter proteins, such as ZIP8 (zinc ion transporter protein 8), and ion channels also participate in the uptake of cadmium by proximal tubules [21]. Irrespective of the method of uptake, chronic accumulation and prolonged retention of cadmium by proximal tubule epithelial cells is considered as the initial step for the nephrotoxicity of cadmium [22–25]. Cadmium-induced proximal tubule dysfunction and subsequent renal cancer have been reported [26–28]. The experimental data from cell culture and animal models suggest diverse molecular mechanisms are involved in cadmium renal carcinogenesis.

6.3.2 Apoptosis, Necrosis, and Autophagy in Proximal Tubule Epithelial Cells

The experimental evidence to date from rodent studies indicates that the accumulation of cadmium in proximal tubule epithelial cells and subsequent nephrotoxic effects appear to involve apoptosis, necrosis, and autophagy mechanisms. It should also be noted that in a majority of the studies described below, either acute or chronic exposure to cadmium caused a small percentage of cell death. The early studies observed proximal tube epithelial injury to be mediated by apoptosis, but not necrosis [29–32], while other studies noted that not all proximal cells were apoptotic and some of these apoptotic cells were proliferative in response to apoptotic injury [33]. It has also been observed that not all proximal epithelial cells become apoptotic, as kim-1-mediated tissue repair was found activated before apoptosis in some cells [34, 35]. Using an acute intraperitoneal exposure route of exposure in rats, activation of autophagic response in proximal tubule was reported [36] well before any evidence of apoptosis or tubule dysfunction was observed.

6.3.3 Cell Survival Response

As discussed above, cadmium-induced cell death or apoptotic events simultaneously trigger cell survival signals in many instances. Cell culture studies using various cell types showed activation of phosphoinositide-3-kinase (PI3K) and its downstream serine/threonine kinase, Akt/protein kinase B [37–42]. The role of forkhead box class O (FOXO) subfamily of transcription factor was also studied in relation to cell survival signaling [43]. Utilizing a cell culture system of renal proximal cell line HK-2 [41] with a combination of diverse enzyme inhibitors has elegantly demonstrated that cadmium-induced phosphorylation of FOXO3a (forkhead box class O transcription factor a) was inactivated, which played a significant role in determining the cell fate toward survival or apoptosis. Further, this process involved regulation of a complex set of downstream signaling molecules such as m-TOR (mammalian target of rapamycin), RSK2 (ribosomal s6 kinase2), and glycogen synthase kinase-3α (GSK-3α) in protecting HK2 cells from metal-induced cellular death/damage.

6.3.4 Notch1, Wnt/β-Catenin, and E-Cadherin-Mediated Cell Signaling in Cadmium-Exposed Renal Proximal Cells and Carcinogenesis

Cadmium-induced disruption of cadherins and cell–cell communications are considered as a primary mechanism of toxicity and cancer in epithelial cells [44, 45]. Cadherins are important to maintain epithelial polarity and epithelial barrier

function, and cadmium-mediated disruption of cadherin affected these functions [46]. Consistent with the observations made in vitro, a pronounced change, specifically in N-cadherin localization patterns, was also reported in vivo in segments of the nephron [47, 48]. Sub-chronic nephrotoxicity of cadmium in rats also caused significant damage and fragmentation of DNA, depolymerization of microtubules, and loss of cadherin in the subapical domain of the proximal tubules [49, 50]. In cadmium-treated cells, the proximal tubule Na+-K+ ATPase located at the basolateral surface of the epithelia, which plays an important role in sodium and fluid absorption, delocalizes to apical surfaces (labeled extensively in apical surfaces) affecting renal absorption [50]. Another sub-chronic cadmium exposure study investigated the potential role for Wnt signaling associated with perturbations in β-catenin and cadherins in mouse renal fibrosis. In this study, the authors report upregulation of cell proliferation and survival genes (c-Myc, cyclin D1, and the multidrug transporter P-glycoprotein Abcb1b) that are known to promote malignancy [51].

Recent in vitro studies investigated the role for other molecular mechanisms implicated in cadmium-induced cell damage, such as the Notch1 pathway. The Notch1pathway is an evolutionarily conserved signaling pathway involved in diverse biological processes ranging from cell fate, differentiation, proliferation, and death [52]. Cadmium-induced activation of Notch1 signaling pathway and its effect and interaction on other signaling pathways were studied using HK-2 human renal proximal tubule epithelial cells [53]. These studies suggested that cadmium-induced activation of Notch1 signaling led to activation of p53 and PI3K/Akt signaling pathways and together resulted in the expression of “snail”; a repressor of E-cadherin is another mechanism for cellular damage and decreased cell–cell adhesion and thus affecting cell–cell communication and invasion.

6.4 Prostate Cancer

A meta-analysis of 14 studies reported significantly higher levels of cadmium in the prostate tissue of prostate cancer patients than those in healthy controls [54]. Although, several epidemiological studies observed a positive association between cadmium levels and incidence of prostate cancer, such association was not demonstrated conclusively at environmental exposure levels [54]. Animal toxicology studies have shown cadmium may be linked to prostate cancers, with supportive mechanistic data [55–58].

A majority of animal studies investigating the relationship between cadmium exposure and prostate cancer used the rat as a model due to similarities that exist between the conditions of dysplastic changes in rat prostate to the intraepithelial prostatic neoplasia observed in humans [55–58]. SV40 immortalized, non-tumorigenic human prostate epithelial cells transformed with repeated exposure to cadmium exhibited anchorage-independent tumor growth in soft agar and formed poorly differentiated adenocarcinomas in severe combined immunodeficiency

(SCID) mice. These tumors exhibited enhanced expression of prostate- and tumor-specific markers such as prostate-specific antigen (PSA), androgen receptor (AR), prostate stem cell antigen (PSCA), the homeobox protein NKX3.1(NKX3.1), and cytokeratin 8 (CK8) suggesting direct relationship between cadmium and prostate cancer [59].

6.4.1 Cell Transformation Studies

In cadmium-transformed prostate cells, genes relating to cell cycle, cell growth and differentiation, DNA repair and apoptosis, and potentially involved in prostate carcinogenesis were differentially expressed [60, 61]. Specifically, a more comprehensive comparative gene expression analysis on cadmium-transformed prostate epithelial cells indicated downregulation of several proapoptotic genes [62]. Later studies that investigated the role of signaling pathways in cadmium-mediated apoptotic resistance observed overexpression of Bcl-2 due to inhibition of JNK signal transduction pathways (decreased phosphorylation of JNK1/2 and JNK kinase activity) to be responsible for the lowered transcript and protein levels of Bax [63].

A number of studies that exposed diverse cell lines (normal prostate cells RWPE-1,cadmium-transformed cell line CTPE, the primary adenocarcinoma cell line 22Rv1 and CWR-R1 cells, LNcaP, PC-3, and DU145 metastatic cancer cell lines) to a broad range of cadmium concentrations (sub-micromolar to micromolar) for 24 h observed dose- and cell line-dependent effects such as apoptosis; inhibition of cell proliferation, correlating with accumulation of functional p53; and overexpression of p21 in p53 wild-type cells [63]. Recently, El-Atta et al. reported that RWPE-1 cells were more resistant to arsenic-induced caspase 3/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression compared to cadmium exposure [64]. Both arsenic and cadmium were more resistant compared to chromium at low and moderate concentrations. At the highest concentration, RWPE-1 cells were the most resistant to cadmium-induced caspase 3/GAPDH expression when compared to arsenic and chromium, confirming the earlier reports that in prostatic cells, cadmium is both pro- and antiapoptotic depending on the concentration.

Rats treated with cadmium chloride in drinking water for 18 months exhibited a negative association for cell proliferation and apoptosis in the prostate tissue as evident by the differential expression of PCNA immune-reactive nuclei, bcl-2, and the numerical density of epithelial cells in the dysplastic prostatic acini compared to normal acini of treated rats and control animals. Also, the percentage of apoptotic nuclei in the ventral dysplastic acini was significantly decreased compared to that of normal acini [65]. This decrease in the apoptosis in the rat dysplastic prostatic hyperplasia seems to be different, as it does not occur in humans and it is likely mediated by enhanced bcl-2 expression.

In a 2-year study, where rats were exposed to cadmium in drinking water, preneoplastic morphology in the ventral prostate using immune histochemical approaches was investigated [66]. In this study there is a significant increase in the expression of

PCNA and of Bcl-2 in prostate L1 lobe due to cadmium exposure. In the same study, the authors also inquired on the potential role of Zn supplementation in drinking water on cadmium-induced neoplastic changes in prostate and found no protective effects.

The cadmium-induced dysplastic prostatic lesions demonstrated alterations in the expression of immune-reactive lysophosphatidic acid receptor (LPAR), a phosphatidic acid receptor [67]. This gene had been widely implicated in the cellular processes such as differentiation, angiogenesis, tumor invasion, etc. In these lesions, immune-reactive p53 expression was higher, while volume fraction of Bcl-2 (VFBcl-2) was unaltered, and enhanced expression of PCNA in the prostatic lobe1(LIPCNA), marker of proliferation, was observed. A significant correlation between LILPA1 and immune-reactive ubiquitin (LIUB1) observed in these lesions suggest potential involvement of ubiquitin pathway in mediating this pathology [68].

Pathway analysis of differentially expressed genes from transcriptomics data on normal prostate epithelial cells to low-dose cadmium (2.5 μM, 4–32 h) identified a greater representation of genes related to TNF family with known role in oncogenic potential and immunomodulatory functions [69]. The role for TNF in cadmium-mediated apoptosis is further supported by downregulation of X-linked inhibitor of apoptosis protein (XIAP), in cadmium-treated prostate cells [70]. This downregulation appears to occur at posttranscriptional level via NF-ΚB-independent, proteasome-mediated mechanism leading to TNF-α sensitive apoptosis.

In summary, as discussed above, cadmium-induced prostate carcinogenesis involved perturbations in cell cycle regulation, apoptosis or apoptotic resistance, and a potential for these events toward the progression of tumorigenic process.

6.5 Breast Cancer

A number of epidemiological studies that evaluated the associations between the dietary cadmium intake, the urinary cadmium level, and the breast cancer risk did not find a strong correlation. In some studies, a significant association between the dietary cadmium intake and postmenopausal breast cancer risk is reported [71]. In comparison, a systematic meta-analysis of studies found a relationship which is not statistically significant [72]. However, epidemiological studies that evaluated the association between the urinary cadmium level and breast cancer risk concluded that women with higher urinary cadmium levels showed an increased risk for breast cancer with different grades [73–76]. A few other studies did show that the concentration of cadmium found in the breast cancer tissues was higher than in the adjacent normal or benign breast tissue, suggesting that cadmium exposure may contribute to breast tumorigenesis [77–79].

A recent meta-analysis of several observational studies showed positive associations between breast cancer and environmental levels of cadmium [80]. In a study with rural Bangladeshi women, elevated levels of cadmium were found to interfere with transfer of zinc to the fetus and subsequent low concentration of zinc in breast milk [81]. A few studies observed consistently higher accumulation of cadmium in

cancerous breast tissue [77–79]. The breast density in the breast cancer patients was closely associated with cadmium exposure with stronger association shown between nulliparous women, current or former smokers [82]. A hospital-based case–control study of 585 cases and 1170 controls adjusted for age and other confounders reported positive association between creatinine-corrected urinary cadmium with greater risk of breast cancer both in ER (estrogen receptor) positive and human epidermal growth factor receptor 2 (HER2) positive patients [76].

While there is some inconsistency in the association between exposure to environmental cadmium and breast cancer incidence from population studies, there is a wealth of accumulated scientific evidence from studies using cell culture and animal models. In vivo animal studies of cadmium exposure in mice demonstrate the possible mechanistic pathway that cadmium might interfere with mammary physiology [83]. The reduced expression of secretory pathway Ca-ATPase (SPCA) by cadmium in the lactating mouse mammary gland led to decreased levels of β-casein in milk [83]. This and other studies suggest that cadmium may compete with transport channels for essential metals like iron, zinc, and calcium accumulating in the mammary gland, affecting these physiological demands of the gland [83–85].

The next section will provide an overview on our current understanding of cellular and molecular data supporting a defined role for cadmium-induced breast carcinogenesis.

6.5.1 Estrogen and Estrogen Receptor

Estrogen is produced by female ovaries and plays a significant functional role both in the normal and neoplastic breast epithelium. The deregulation of estrogenic pathways can lead to molecular perturbations at the level of gene transcriptional and/or translational activity and associated downstream signaling pathways contributing to the development of cancer. It is clearly understood that overexposure to estrogen and overexpression of estrogen receptors can contribute to breast cancer [86].

In vitro estrogenic potency of cadmium had been tested in many breast cancer cells [87–99] such as MCF-7, T-47D, ZR-751, and SKBR3 with varied expression of estrogen receptors (ERs) and G protein-coupled receptor (GRP30). All these experiments have demonstrated that cadmium interacts and activates both kinds (endogenous and exogenous) of estrogen receptors, alters the expression and function of several downstream target genes, leads to the activation of several intracellular signaling cascades similar to estrogen, and contributes to translational regulation of estrogen-dependent proteins [87–99]. Aptly, based on these observations, cadmium is designated as a metalloestrogen [87].

One of the earliest pieces of evidence for the estrogenic potential of cadmium was the cadmium-induced estrogenic response through estrogen response element (ERE) in MCF-7 breast cancer cells [88]. These changes included the expression of certain estrogen-dependent genes as well as a 5.6-fold increase in the cell growth compared to estrogen-treated controls. A series of experiments revealed that cadmium in nanomolar range activated estrogen receptor-α (ERα) and blocked binding of estradiol to

ERα in a noncompetitive manner [89]. This study further demonstrated the ability of cadmium to bind ERα and its various mutant forms. The study also showed that cadmium-induced estrogen activity was blocked by antiestrogen compounds (ICI-164,384) and ER alpha-binding mutants (C381, C447, E523, H524, and D538) which are specific to cadmium-binding sites in the ligand-binding domain of ERα (cystein38, 44, glutamic acid 523, histidine 524, and aspartic acid 538). Estrogen mimicking activity of cadmium was also demonstrated in T47D breast adenoma cells transfected with triple estrogen response element (ERα,β and estradiol) luciferase construct in combination with antiestrogen compounds (ICI and genistein) [90].

Cadmium-induced proliferation in MCF-7 cells appears mediated by increased activity of ERK1/2 and AKT phosphorylation. In the experiment, these activities were blocked by antiestrogen compound (ICI182780) confirming that cadmium-mediated cell growth was facilitated by binding to ERα and the resulting signaling cascade [91]. Cadmium stimulated the growth of three ER-positive breast cancer cells (MCF-7, T4D, and ZR-75-1) to a lesser degree than estrogen. Detailed molecular characterization of this proliferative activity demonstrated involvement of a complex interaction of a cellular signaling cascade such as the interaction between c-jun and ERα, mobilization of the transcription factor complex to cyclin D and c-myc promoter, and resulting alterations of cell proliferation and cell survival [92].

The estrogenic activity of cadmium was found to involve induction of heat shock proteins Hsp22 [93] and Hsp27 [94] in estrogen receptor-positive human breast cancer cell line MCF-7, but not in ER-negative breast cancer cell line MDA-MB-231. It was also found that in addition to heat shock proteins, downregulation of cytochrome oxidase subunits II and IV in MDA-MB-231 breast cancer cells led to differential expression of p38 and accumulation of downstream proteins [95]. Cadmium induces Hsp22 in a manner similar to estrogen, which was blocked by antiestrogen compound ICI182780. Such induction was not observed in ER-negative breast cancer cell line MDA-MB-231 [93]. Cadmium strongly induced the activity of hTERT (the subunit that is principally involved in telomerase activity) transcription in an estrogen receptor-dependent manner [96].

A comparative transcriptomics analysis in five clonal cell lines of the human estrogen-positive breast cancer cell line MCF7, namely, MCF-7-cd4, MCF-7-cd6, MCF-7cd7, MCF-7cd8, and MCF-7cd12, indicated cadmium-induced differential gene expression profiles across the cell lines. A recent hierarchical gene clustering analysis demonstrated gene signatures associated with breast cancer were distinct and unique, from the control cells suggesting their potential role in breast cancer progression [97].

The estrogenic activity of cadmium is mediated through both nuclear and membranous estrogen receptor-mediated signaling, and this is concentration-dependent [98]. When T47D breast cancer cells were exposed to pico-molar concentrations of cadmium, it led to induced expression of progesterone receptor (PgR) and/or ps2 (two indices of responses mediated through nuclear ER); however, at nanomolar concentrations, it resulted in increased phosphorylation of ERK1and ERK2. However, cadmium at 1–3 μM induced activity and phosphorylation of ERK1/2 kinases in MCF-7 cells appear to be mediated by EGFR and Src kinase, demonstrating that the membranous receptor is important in eliciting these responses [99].

Animal studies also observed estrogenic activity of cadmium, for example, a single i.p. injection of cadmium (5 μg/kg) in ovariectomized SD (Sprague Dawley) rats, resulting in enhanced mammary epithelial density, formation of alveolar buds and side chain branches, and production of casein, whey acidic protein, progesterone receptor, and complement component C3 [100]. Pubertal administration of cadmium resulted in less developed mammary glands in treated adult rats than controls. In control animals, where mammary gland was well developed, alveolar development was comparable to the first day of pregnancy [101]. Similar to mammary gland, estrogenic effects of cadmium exposure were also reported in uterus and/or endometrium in rats and mice [100–103].

Two studies by [104, 105] looked at the role of GPR30 (membranous G protein-coupled receptor) that can mediate estrogen (E2)-induced non-genomic signaling and its role in cadmium-induced signaling process in ERα- and β-negative SKBR3 breast cancer cells [104]. While the former study demonstrated that GPR30 antagonizes the growth of ERα-positive breast cancer cells, the latter study demonstrated that cadmium-induced breast cancer cell proliferation is mediated by GPR30 activation, similar to estrogen by eliciting rapid activation of erk1/2, ribosomal S6 kinase, elk, etc. [105].

In MCF-7 cells treated with 1–3 μM cadmium, both ERK1/2 kinase activity and phosphorylation were increased. Blockage of cadmium entry into the cells by manganese did not alter these responses, while inhibition of EGFR and the Src kinases completely abolished the ERK1/2 activity demonstrating that the membranous receptor is important in eliciting these responses. Either silencing or inhibition of ERα did inhibit cadmium-induced ERK activity, suggesting that both EGFR and ER are important in cadmium-mediated estrogenic responses and downstream signaling cascades [99].

As discussed above, there is a clear biological basis for the estrogenic effects of cadmium. This suggests the potential for cadmium exposure to cause developmental and functional alterations to the mammary gland. Although epidemiological studies point to a positive association between breast cancer and cadmium exposure, additional studies in a larger cohort with better study design and exposure characterization and relevant biomarkers for early detection may shed more light on the estrogenic role of cadmium.

6.5.2 E-Cadherin, β-Catenin, and Associated Signaling Events

Selective higher accumulation of cadmium was reported in breast tumors compared to normal and benign mammary gland tissues [106]. Transformation of normal breast cells to a metastatic phenotype on repeated cadmium exposure was also reported. This observation led to further studies on the cadmium-induced disruption of cell-to-cell communication which potentially influences cell–cell adhesion and metastatic cell type [107]. One of the earliest reports of cadmium-mediated disruption of cell–cell communication suggested a role for an adhesion molecule, cadherin, in renal proximal tubule cells [44, 46, 108].

Cadherins are a class of calcium-dependent cell adhesion molecules localized at epithelial junctions [44]. These molecules have both intra- and extracellular domains with extracellular regions for calcium-binding sites, and the intracellular domains are bound to β-catenin. The β-catenin is linked to α-catenin which is bound to actin cytoskeleton [109]. The β-catenin controls nuclear Wnt signaling pathway [44, 109, 110]. Upon release from the junctional complexes to the cytosol, β-catenin can either get degraded by the proteasome or translocate to the nucleus, binding to transcription factors such as T-cell factor-lymphocyte enhancer factor [44, 110]. Cadherins are primarily two types, namely, E-cadherin and N-cadherin. Cadmium had been demonstrated to disrupt both E- and N-cadherin junctions in many epithelial cell types and vascular endothelial cadherin (VE cadherin) junctions in vascular endothelial cells [108]. It is clearly understood that disruption of E-cadherin leads to release of β-catenin and its subsequent translocation to nuclease functions as transcription factor and activates diverse cell proliferation and metastatic signals through NF-κB, c-myc, matrilysin, and c-Jun [111–116].

Initial observation of the relationship between cadmium and cleavage of E-cadherin in T47D breast cancer cell line exposed to cadmium was made by Park et al. [117]. In a presenilin 1 (PS1) gamma secretase-dependent manner as studies using mutant PS1 and secretase inhibitors (DAPT), calcium chelator and antioxidants, cadmium-inhibited cell motility and invasiveness of T47D breast cancer cells. Both short-term and repetitive exposures to low levels of cadmium have been found to initiate of E-cadherin protein degradation via ubiquitination pathway and translocation of β-catenin and transcriptional activation of TCF-4 and downstream genes such as c-jun and cyclin D1 in diverse breast cancer cell lines [118]. These studies suggest that cadmium-induced cell proliferation and metastatic phenotype may include disruption of E-cadherin-mediated signaling pathways and cell-to-cell communication.

6.6 Pancreatic Cancer

Chronic exposure of pancreatic cells to cadmium (micromolar concentrations) induced malignant phenotypes such as increased expression of matrix metalloproteases-9, increased invasiveness, and anchorage-independent growth as well as increased expression genes involved in cell growth and metastasis genes, CD44, CCR4, OCT4, and S100P [119]. This observation along with reported positive association for pancreatic cancer in men in the recent meta-analysis of epidemiological studies suggests a possible relationship and is worth investigating further with relevant models.

6.7 General Molecular Pathways of Cadmium-Mediated Carcinogenesis

The common pathways implicated in cadmium-mediated carcinogenic process are illustrated in Fig. 6.2 and are detailed below.

6.7.1 Oxidative Stress

Induction of oxidative stress is hallmark of cadmium toxicity. Induced expression of anti-oxidative response genes–glutathione peroxidase and reductase as an early response mechanism to mitigate toxicity and protection against cadmium-induced carcinogenesis has been observed both in cell culture and animal studies. This enhanced glutathione-related activity is demonstrated to occur in two ways. One is induction of gamma-glutamylcysteine synthetase resulting in enhanced glutathione synthesis and secondly by enhancing the induction of genes encoding glutathione S-transferase [120]. This detoxification mechanism, however, can be overridden sometimes by excessive cadmium exposure, resulting in toxicity and carcinogenesis.

6.7.2 DNA Damage Repair Pathways

Several lines of evidence point to the fact that cadmium is not a direct genotoxicant, rather cadmium is recognized as a weak co-genotoxicant. In mammalian cells and bacterial mutagenic tests, cadmium was not identified as genotoxic. In in vitro test systems, a very high concentration of cadmium (in micromolar range) exhibited some genotoxicity, and the observed genotoxic effects were hypothesized to be the result of reactive oxygen species induced by high doses of cadmium [121, 122]. These studies further confirmed that the genotoxicity was elicited by cadmium-induced generation of 8-OH guanine, and this was inhibited when the cells were treated with both the scavengers and modulators of free radicals.

Cadmium had been demonstrated in several studies [123–125] to inhibit DNA damage repair. In cadmium-exposed alveolar epithelial cells, a significant decrease in the activity of formamido-pyrimidine DNA glycosylase was reported [123]. This enzyme is responsible for the removal of DNA adducts such as 8-OH guanine and 8-OH adenine [123]. In a panel study of workers exposed to cadmium and other metals, cobalt and lead, blood mononuclear cells exhibited an inverse association for DNA strand breaks and the repair capability of 8-oxoguanine (8-oxo-G) [124]. This may be due to cadmium exposure-related depletion of cellular glutathione and subsequent reduced repair of 8-OH guanine or increased formation of 8-oxoguanine [125]. The lesions caused by these adducts can become mutagenic if unrepaired.

To maintain the genome integrity, the cell is equipped with diverse DNA repair pathways, namely, base excision repair (BER), nucleotide excision pathway (NER) and mismatch repair (MMR), DNA strand cross-link repair, homologous recombination, and nonhomologous end-joining pathways. Cadmium has been found to interfere with at least three DNA repair pathways—BER, NER, and MMR. To assess the effect of cadmium on DNA repair capacity in vitro, [126] used a focused microarray that can assess in parallel multiple DNA repair genes. This study reported that cadmium chloride differentially inhibited base and nuclear excision repair pathways. In addition, differential sensitivity of glycosylases toward uracil excision by cadmium was also observed.

The tumor suppressor gene, p53, is known to influence BER activity by direct physical interactions with hOGG1, APE1, and Pol b protein constituents [127] and also indirectly by affecting the transcriptional regulation of a DNA glycosylase protein hOGG1, human apurinic/apyrimidinic endonuclease1(APE1) [127]. Cadmium is known to interfere with p53 function and oxoguanine glycosylase (OGG) activity [127]. To further understand the relationship between cadmium-induced perturbations in p53 function and its effect on BER constituents, a series of experiments with cell extracts and isolated DNA repair proteins were carried out [127]. These experiments showed that cadmium is capable of interfering with the BER repair pathway by different mechanisms that include impairing the activity of DNA repair proteins hOGG1 and APE1 [127]. hoGG1, but not APE1 (another key enzyme in the BER pathway), was inactivated by increased oxidation and recruited to stress granules in cells treated with sublethal treatment of cadmium [128].

Studies using comet assay demonstrated that low (nano- and micromolar range) concentrations of cadmium inhibit DNA damage repair of NER pathway induced by UV radiation, methyl methanesulfonate (MMS), and N-methyl-N-nitrosourea (MNU) [129, 130]. Additional direct evidence for cadmium-induced inhibition of NER has been reported from studies, where the genotoxic potential of soluble and particulate cadmium was investigated using immortalized human lung epithelial cells. In these studies, inhibition of benzopyrene diol epoxide (BPDE)-induced bulky DNA adducts and UVC-induced photo lesions was observed in a dose-dependent manner at noncytotoxic concentrations of cadmium that correlated with the nuclear uptake of cadmium [131]. In the studies using only the soluble form of cadmium (cadmium chloride), it found decreased levels of the principle NER initiator protein, XPC leading to disturbance in the disassembly of xeroderma pigmentosum group A protein (XPA) and xeroderma pigmentosum, complementation group C (XPC) proteins were reported. These studies further support the earlier observations that cadmium treatment results in conformational changes in the DNA-binding zinc finger domain of the tumor suppressor p53, which is a transcription factor for XPC [132, 133].

The process of cell division is equipped with DNA polymerase proofreading or by post-replication mismatch repair (MMR) to correct base pair mismatches that may occur during normal cell duplication event. Accumulating evidence points to potential involvement of MMR in cadmium-induced carcinogenesis. Earlier studies using yeast system demonstrated that chronic low-level exposure to cadmium inhibits repair of small misalignments and base–base mismatches [134]. Using human cell culture systems, these authors also showed cadmium-induced inhibition of at least one step of mismatch removal, leaving 20–50% of DNA unrepaired [135]. Later studies using model systems and in vitro culture studies demonstrated a direct binding of cadmium to specific proteins (MSH2–MSH6 heterodimer) resulting in blockage of DNA-binding and ATPase activities [136–139].

These observations provide experimental evidence that cadmium is capable of targeting DNA repair systems, resulting in diminished removal of endogenous and exogenous DNA damage resulting in increased frequency of genomic alterations. Current experimental evidence indicates that cadmium interferes with all major DNA repair pathways, and this appears to also involve direct interactions of cadmium with the DNA repair proteins.

6.7.3 Aberrant Gene Expression, Transcription, and Translation

Cadmium-induced aberrant gene expression appears to be one of the mechanisms implicated in carcinogenesis. Gene expression data derived from both in vitro and in vivo studies using high-throughput expression systems indicated alteration of gene expression in a variety of genes such as early response genes, stress-related genes, and transcriptional and translational genes. Primary classes of stress response genes (genes that collectively work in combating stress) that are affected or elicited by cadmium exposure include those that are involved in oxidative stress, induction, and operation of metallothionein (MTs) [140–143] genes involved in glutathione synthesis and homeostasis and heat shock responsive genes [140]. The aberrant gene expression in the aforementioned gene types has been extensively covered under cadmium-induced organ-specific cancers that are described earlier in this chapter.

6.7.4 Transcriptional and Translational Alterations

The AP-1 transcription factor (constituted of c-fos and c-jun) present in the promoter regions of several genes involved in cell growth, cell cycle control, apoptosis, and autophagy had been found to be altered by the induction of c-fos and c-jun in cadmium-transformed prostate cell lines [144]. Both cadmium resistance and toxicity involves altered DNA-binding activity and transcription of specificity protein1(Sp-1) and hypoxia inducible factor-1(HIF-1), two transcription factors, as well as expression of downstream genes associated with apoptosis and cell cycle regulation [145, 146]. The role for transcription factors NF-kB and NRf-2 in the expression of genes such as heme oxygenase, c-fos, and c-myc and the cell signaling cascade of MAP kinases under the regulation of these transcription factors by means of upstream and downstream regulation has been implicated in cadmium-induced oncogenic activation and apoptotic regulation [15, 147, 148].

Accumulated experimental data from studies in cadmium-transformed cell lines and analysis of tumor samples obtained from animal models suggest that in addition to transcription factors, discussed above, several translational factors also appear to play a defined role in cadmium-mediated carcinogenesis. The initial observation of a role for translational initiation factor (TIF3) and translational elongation factor 1-delta (TIF1δ) came from the observation of their overexpression in the observed anchorage-independent growth and tumorigenesis for cadmium chloride-transformed BALB/cT3 cells [149–151]. In this study the authors demonstrated the role for these translation factors by combination transfection studies using c-DNAs and antisense mRNA. Malignant transformation of NIHT3 cells exposed to cadmium appeared to be mediated by overexpression of TIF3 and TEF1δ. These cells also exhibited anchorage-independent growth and tumor formation in nude mouse and this phenomenon got nullified when the cells were transfected with antisense

mRNA for these translational factors [152]. Overall, the existing data, though limited and observed primarily in in vitro studies, suggests that exposure to cadmium results in alterations in the expression and/or function of several transcriptional and translational factors involved in the oncogenic pathway.

6.7.5 Cell Proliferation and Cycle Regulation

A normal eukaryotic cell is equipped with a built-in network of pathways called cell cycle check points. Their primary goal is to accomplish two critical steps: to ensure normal propagation of cell division and to delay the cell cycle into the next phase by repairing any DNA damage. Elicit responses, such as apoptosis, senescence, autophagy, etc., eliminate surviving damaged cells, thereby ensuing genomic stability. Studies using micromolar concentrations of cadmium have demonstrated interference with several cell cycle check points. The earliest study was the observation of cadmium-induced inhibition of cell cycle arrest at both G1 and G2/M phases in gamma-irradiated MCF-7 cells. This study also observed cadmium-induced conformational changes to p53 zinc finger domain and impairment in function [132]. Such interference with cell cycle regulation by cadmium was observed in benzo[a]pyrene diol epoxide-treated fibroblasts, where the metal disrupted the expression of cyclin-dependent kinase inhibitor P^{21WAF1}. However, at low concentrations, p53 was not affected and these effects were attenuated at concentrations >40 μM [153].

Cadmium also had been shown to interfere with cell cycle in the absence of exogenous DNA-damaging agents. Studies on various cell lines that have constitutionally expressed p53 have shown that cadmium-induced G1 and G2/M arrest involves activation of p53 leading to expression and/or function of several downstream genes [61, 154–157]. As noted above, cadmium is capable of disrupting p53 conformation and subsequently inhibit its function. Studies carried out using p53-deficient cells helped to understand the role of other compensatory cycle regulatory pathways operate in cadmium-induced carcinogenesis. p53-inactivated kidney proximal tubule cell line exposed to micromolar concentrations of cadmium resulted in cell cycle arrest in the G2/M phase mediated by cdc2 phosphorylation, facilitating these cells from premalignant to malignant state [158]. Together this evidence points out cadmium-mediated genomic instability as one of the mechanisms of carcinogenesis mediated by this metal.

6.7.6 Cadmium: Apoptosis and Autophagy in Carcinogenesis

The fine balance of apoptosis and apoptotic resistance is pivotal to cellular and tissue homeostasis and integrity. Avoidance of apoptosis in fact is considered a hallmark of cancer [159]. The early observation of cadmium-induced apoptosis in the carcinogenic process comes from studies of cadmium-transformed prostate

epithelial cells [63, 160–162]. On the other hand, cadmium was also found to inhibit genotoxin-induced apoptosis at higher concentrations. In CHO (Chinese hamster ovary) cells, cadmium at concentrations between 5 and 20 μM inhibited apoptosis induced by hexavalent chromium, which was mediated through caspase 3 activities [161, 162]. Cadmium also had been shown to inhibit apoptosis induced by other metal and nonmetal toxins [61, 158]. In macrophages, cadmium induced apoptosis in a calcium-dependent manner trough oxidative stress [159]. The multifaceted role of cadmium in apoptosis was also demonstrated where cadmium had been shown to induce apoptosis by both caspase-dependent and caspase-independent mechanisms [160–162]. Cadmium plays a pivotal role in the carcinogenic process through pro- and antiapoptotic pathways by eliciting distinct genes in a concentration-dependent manner.

Several studies report cadmium exposure-related autophagy in a variety of cell lines such as PC12 cells, MES-13mesengial cells, epidermal skin cells, and vascular endothelial cells [12, 163–165]. Low levels of cadmium in several instances had been shown to inhibit apoptosis by the deprivation of serum basic fibroblast growth factor and induce autophagy [165]. Similarly, in some instances, low levels of cadmium elevate intracellular calcium, in turn activating ERK kinase leading to autophagy [163]. The mechanistic details on cadmium exposure culminating in autophagy had been reported in tumorigenesis of lung and renal cancers that are detailed earlier in this chapter.

6.7.7 Epigenetics

Alterations in epigenetic events disturb cellular homeostasis and influence cellular transcription and translation [166]. Cadmium, depending on the dose and duration, is reported to have influenced both DNA hypo- and hypermethylation [167]. While acute exposures have been linked to inhibition of DNA methyltransferase activity that led to global DNA hypomethylation, long-term exposures result in DNA methyltransferase activity and DNA hypermethylation [107, 168]. Life-long exposure to cadmium in women is evidenced by low-level urinary cadmium, inversely associated with a global DNA methylation marker, long interspersed nuclear element1 (LINE-1) methylation, and expression of DNA methyltransferase3b (DNMT3b) which is involved in de novo CpG methylation. This change is of particular concern as such change has been implicated as a frequent epigenetic change in malignancies [169–171]. Compared to DNA methylation, the influence of cadmium on histone modifications has been studied in a very limited number of studies [172, 173, 174]. A number of studies have implicated alterations in miRNA expression under the influence of cadmium. Micro-RNAs, such as mir138, 15b, and mir-372, have been implicated in cadmium-induced tumorigenic process [166]. In summary, cadmium is involved in the epigenetic regulation of all the three pathways, namely, DNA methylation, histone modifications, and micro-RNA regulation. Dysregulation of each of these pathways had been demonstrated in the carcinogenic process of several types of cancer including that of cadmium.

6.8 Conclusions and Perspectives

There is a growing body of epidemiological studies providing a positive association between exposure to cadmium and cancers of different organs, specifically the breast, renal, lung, and prostate. This association, although more robust in the occupational exposure settings, is increasingly evident in a limited number of studies of environmentally relevant levels, suggesting causative role for cadmium in carcinogenic effects at these levels as well. If one analyzes this data in the context of huge body of experimental data collected over two to three decades using defined cell culture and laboratory animal model systems, potential trends supporting biological plausibility are apparent. Although the initial experimental data were questioned for the high exposure concentrations used, recent data from studies with sub-micromolar up to 10 μM concentrations of cadmium provided convincing evidence of positive association of cadmium and carcinogenesis.

The experimental data discussed in this chapter sums up to a pattern of certain common and unique mechanisms perturbed or initiated toward carcinogenic effects of cadmium and eventual malignancy in organs investigated. However, these molecular changes seem to be dependent upon cadmium concentration, route of exposure, the timing of exposure, and the experimental model. The common and specific genes altered in the major types of cancer induced by cadmium are captured in Fig. 6.1 and Table 6.1. A recent published study on the network analysis of cadmium-induced gene pathways in cancer also reported some of the genes (as identified in the present analysis) as common genetic alterations in cadmium-induced cancer process [175].

Recent IARC-led workshops of international experts identified key events characteristic of human carcinogens as evidenced by systematic review of mechanistic data [176]. These workshops identified ten key characteristic properties of human carcinogens which include:

1. Is electrophilic or can be metabolically activated to electrophiles
2. Is the chemical genotoxic
3. Alters DNA repair or causes genomic instability
4. Induces epigenetic alterations
5. Induces oxidative stress
6. Induces chronic inflammation
7. Is immune suppressive
8. Modulates receptor-mediated effects
9. Causes immortalization
10. Alters cell proliferation, cell death, or nutrient supply

The general carcinogenesis process as influenced by cadmium falls into the following common mechanisms: oxidative stress, aberrant gene expression, inhibition of DNA repair, modulation of receptor-mediated effects, cell death/apoptosis, cell proliferation and autophagy, and epigenetic (Fig. 6.2). In line with the analyses of Smith et al. [176] on the mechanistic cancer hallmarks, these data largely support the potential hazard by this metal as a human carcinogen. A careful review and analysis of the present data set for this chapter indicates that the molecular players

mediating these cellular processes and associated downstream molecular pathways are influenced by cadmium. Further, these processes and pathways are dependent upon cadmium concentration, route and timing of exposure, and the selected experimental model.

The interaction among these key players and pathways should be understood to translate this information toward developing potential biomarkers for prognosis and prevention. To comprehend the resulting complex interplay and spatiotemporal dynamics of these events in the causation of cancer(s) by cadmium, both at occupational and environmental levels of exposure, it needs further active research. As in the case of any environmental public health areas of research, understanding the role of cadmium in the etiology of cancer requires more realistic human exposure scenarios including experimental studies using low-dose and chronic exposure regimens.

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Chapter 7
Molecular Mechanisms of Chromium-Induced Carcinogenesis

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Abstract Hexavalent chromium (Cr(VI)) has been utilized for industrial applications for over 200 years. Due to its frequent use, workers in over 80 different industries are exposed to Cr(VI). Epidemiological studies indicate particulate Cr(VI) compounds are the most potent carcinogens, resulting in sinusoid and lung tumors following inhalation. Although Cr(VI) is well established as a human lung carcinogen, the mechanism of carcinogenesis remains unknown. Here, we examine the results of Cr(VI)-induced tumor, *in vivo*, cell culture, and in vitro studies in the context of three major models of carcinogenesis: multistage carcinogenesis, genomic instability, and epigenetic modification. A wealth of data support the conclusion that genomic instability is a driving mechanism of Cr(VI)-induced carcinogenesis. However, recent studies suggest epigenetic modifications also play a crucial role in its carcinogenic mechanism. Therefore, we propose a mechanism of Cr(VI)-induced carcinogenesis that involves both genomic instability and epigenetic modification.

Keywords Chromium (VI) • Chromate • Genomic instability • Epigenetic • Chemical carcinogenesis • Metal carcinogenesis

Abbreviations

AC	Adenocarcinoma
Asc	Ascorbate
BER	Base excision repair
CLR	Crosslink repair
Cr	Chromium
Cr(III)	Trivalent chromium

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Cr(VI)	Hexavalent chromium
DNA	Deoxyribonucleic acid
DSB	DNA double-strand break
H3K4me3	Trimethylated histone 3 at lysine 4
H3K9me2	Dimethylated histone 3 at lysine 9
HR	Homologous recombination
IF	Immunofluorescence
miRNA	MicroRNA
MMR	Mismatch repair
NHEJ	Nonhomologous end joining
NSCC	Non-small cell carcinoma
SCC	Small cell carcinoma
SqCC	Squamous cell carcinoma
WB	Western blot

7.1 Chromium: History, Production, and Uses

Chromium (Cr) is a naturally occurring element found in the Earth's crust. While Cr exists in oxidation states ranging from Cr(−2) to Cr(6+), only trivalent and hexavalent Cr are stable enough to occur in the environment. Cr(III) is found readily in nature, while Cr(VI) is mainly produced through industrial activities [1]. To extract chromium, trivalent chromite ore is roasted with soda ash and sometimes lime in a furnace and purified to produce soluble sodium chromate. If lime is utilized in the extraction process, insoluble calcium chromate is also produced. Other chromate compounds can then be produced from sodium chromate or calcium chromate through reactions with acids or soluble forms of zinc, lead, strontium, or other metals [2].

Cr is a lustrous, hard metal that resists tarnishing and has a high melting point. Due to these properties, chromium is utilized to produce stainless steels, iron alloys, and nonferrous alloys [3, 4]. In fact, Cr is the component of stainless steel that makes it "stainless." Worldwide, approximately 80% of the Cr mined is used for metallurgical applications, ranging from chrome plating to the production of chrome alloy hip replacements. Cr salts display a wide variety of bright colors, earning the metal its name derived from the Greek word "chroma," meaning color. The bright colors of the Cr salts have led to its use in a variety of other applications, such as pigment production, leather tanning, and wood preservatives [3, 4].

7.2 Cr Exposure and Evidence of Carcinogenesis

Over 80 occupations have been identified in which workers experience Cr exposure [2]. The majority of workplace exposures to Cr occur during welding and other types of "hot work" on stainless steel. Workers are also commonly exposed during

chromate metal and pigment production, ferrochrome alloy production, and while operating chrome plating baths [2, 5]. Cases of Cr-induced cancers within the respiratory tract of chromate workers have been documented for over 125 years. The first epidemiological study on chromate workers in the United States in 1948 found 21.8% of chromate worker deaths were attributed to respiratory cancers. This outcome was 16 times higher than the ratio of respiratory cancers in the control population [6]. Since then numerous epidemiological studies have shown an increased occurrence of lung cancer in Cr-exposed workers [7–11]. Ishikawa et al. [12] estimated the lung cancer morbidity rate for ex-chromate workers to be 21.6 times higher than that of the general population. There is a strong relation between lung cancer risk and cumulative Cr(VI) exposure [7, 9, 10, 13]. Valence state and solubility also affect the carcinogenic potential of Cr. Machle and Gregorius [6] found no incidence of lung cancer in plants where workers only handled Cr(III) compounds, suggesting these compounds are not carcinogenic. This weight of evidence resulted in the determination of Cr(VI) compounds as Group 1 carcinogens, defined as "carcinogenic to humans" (IARC 1990).

7.3 The Role of Physicochemical Properties in Cr-Induced Carcinogenesis

The physicochemical properties of Cr play an important role in the mechanism of Cr(VI)-induced carcinogenesis. Epidemiological studies suggest insoluble chromate is more carcinogenic than soluble chromate, evidenced by a reduction in lung cancer rates in chromate workers after lime was removed from the production process, thus eliminating the production of the insoluble calcium chromate [8]. In addition, several studies demonstrate particulate Cr(VI) induces tumor formation when administered by intrabronchial pellet implantation in rat lungs [14–16]. However, soluble Cr(VI) administered by intrabronchial implantation did not increase tumor formation [16]. Patierno et al. [17] supported these findings in C3H/10T1/2 cells, demonstrating particulate Cr(VI)-induced neoplastic transformation while soluble Cr(VI) did not.

As described above, Cr(VI) exposure has been associated with an increased risk of lung cancer, but Cr(III) is considered noncarcinogenic. Animal studies also support the valence state of Cr that plays an important role in its carcinogenic potential. No tumor formation occurred when rats were exposed to Cr(III) compounds, while Cr(VI) exposure increased the incidence of lung tumors [15, 16]. This difference is largely due to the poor cellular absorption of Cr(III). Cr(III) readily binds to ligands forming a hexacoordinate complex that is not easily taken up by the cell [18]. Cr(VI) closely resembles the structure of sulfate and phosphate ions, allowing its cellular uptake via the anion transport system. Thus, when Cr(VI) particle dissolves extracellularly, the chromate anion enters the cell by facilitated transport [19, 20]. The chromate anion is rapidly reduced by ascorbate, glutathione, cysteine, and NADPH [21–26]. The combined activity of these reducing molecules reduces >95% of

Cr(VI) *in vivo* [27]. The reduction of the chromate anion results in the formation of Cr^{5+}, Cr^{4+}, and Cr^{3+} species as well as reactive oxygen species [28, 29]. These Cr intermediate species bind to the DNA phospholipid backbone, resulting in the formation of bulky adducts and, subsequently, DNA damage [30–32].

Although the lung is the primary target organ of inhaled Cr(VI), the stomach and intestines may also be affected by ingested Cr(VI). Upon ingestion, Cr(VI) is reduced extracellularly by saliva and gastric fluids due to their low pH. Gastric juice alone is capable of reducing approximately 70% of Cr(VI) after 30 min [33]. This environment provides a protective mechanism against Cr(VI)-induced genotoxicity (DeFlora 2000). Recent studies demonstrate the intracellular presence of Cr in various organs following ingestion, suggesting some Cr(VI) may escape detoxification and be absorbed [34, 35]. However, epidemiological evidence of Cr-induced health effects following ingestion is very limited, with only weak correlations found between oral Cr exposure and human health effects [36–38]. Only one animal study has shown the carcinogenicity of chronic Cr(VI) exposure via drinking water. This study exposed F344/N rats and B6C3F1 mice to high levels (up to 180 mg/L) of Cr(VI) for 2 years. Tumors in the oral cavity and small intestines only developed at the highest doses of Cr(VI) [39]. The environmental relevance of this study is controversial, however, due to the high doses used to induce carcinogenesis and the high reducing properties of the human digestive tract. Since the inhalation of Cr(VI) provides much stronger evidence of carcinogenicity than oral exposure, the majority of the research on the mechanism of Cr(VI)-induced carcinogenesis has been focused on this exposure route.

7.4 Characteristics of Cr-Induced Lung Tumors

Cr particles lodge at bifurcation points of the bronchi, and these are the primary sites of Cr-induced tumor formation [40]. Kondo et al. [41] showed Cr deposited in the bronchial stroma, not the epithelium, and accumulated at higher levels in the bronchioles and subpleural regions of the lung. The majority of Cr-induced tumors were characterized as squamous cell carcinomas, with a small percentage of small cell carcinomas and adenocarcinomas reported [42–46]. There is a strong correlation between cumulative Cr exposure and lung cancer, with the lung cancer mortality rate increasing with the length of exposure (Davies 1984; [7], [9, 13]). For example, Davies [7] showed a significant increase in mortality in chromate workers employed for over 1 year, but not in workers employed for less. In addition, Cr accumulation significantly increased with the progression to malignancy [41].

Molecular studies of Cr-induced tumors show little evidence of mutations in key oncogenes or tumor suppressor genes. However, these tumors do exhibit genomic instability and epigenetic alterations (Table 7.1). For example, no mutations were found in the K-ras or H-ras oncogenes in tumors from ex-chromate workers [42]. While p53 point mutations were detected in 20% of chromate-induced tumors, this level was lower than that detected in non-chromate

Table 7.1 Characterization of Cr-induced tumors

Study population	Cr-exposed tumor class	Summary of findings	Reference
20 lung tumors from 19 ex-chromate workers	85% SqCC 10% SCC 5% AC	p53 point mutations in 20% of tumors Fewer p53 mutations in exposed tissue than controls No association between p53 mutations and length of exposure	[46]
Four dysplastic lesions from three ex-chromate workers	100% SqCC	p53 protein overexpression in 75% of lesions	[47]
19 lung tumors from ex-chromate workers; 26 lung SqCC from non-exposed individuals	84.2% SqCC 10.5% AC 5.3% SCC	Significantly higher detection of cyclin D1 expression in Cr-exposed tumors No difference in Bcl2 and p53 expression between Cr-exposed and non-exposed tumors	[45]
38 lung tumors from 32 ex-chromate workers	92.1% SqCC 5.3% AC 2.6% SCC	No point mutations found in critical positions of the Ki-ras or Ha-ras oncogenes	[42]
38 lung tumors from 28 ex-chromate workers; 26 lung SqCC from 26 non-exposed individuals	92.1% SqCC 5.3% AC 2.6% SCC	78.9% Cr-exposed tumors exhibit microsatellite instability Higher frequency of microsatellite instability in Cr-exposed than non-exposed tumors. Loss of heterozygosity comparable between Cr-exposed and non-exposed tumors	[44]
35 lung tumors from 26 ex-chromate workers; 26 lung tumors from non-exposed individuals	100% SqCC	Cr-exposed tumors displayed: • Microsatellite instability (78%) • Significantly higher repression rate of hMLH1 and hMLH2 protein • Methylation of hMLH1 promoter (62.5%) • Strong correlation between inactivation of hMLH1 and microsatellite instability	[48]
30 lung tumors from 23 ex-chromate workers; 38 lung SqCC from non-exposed individuals	90% SqCC 6.7% AC 3.3% SCC	Methylation of p16 gene occurred more frequently in Cr-exposed tumors than non-exposed but the difference was not significant	[49]
31 lung tumors from 31 ex-chromate workers	90.3% SqCC 6.45% AC 3.23% SCC	Surfactant protein B gene variants detected in 61.3% Cr-exposed tumors	[43]
67 lung tumors from 67 ex-chromate workers	74.6% NSCC 25.4% SCC	Survivin expression inhibited in SCC but not NSCC Survival time not related to p53 or survivin expression	[50]
36 lung tumors from ex-chromate workers; 25 lung tumors from non-exposed individuals	Not described	Aberrant methylation of tumor suppressor genes more frequent in Cr-exposed tumors (95%) than non-exposed (52%) Aberrant methylation occurred at multiple loci in 48% of Cr-exposed tumors Methylation of APC (86%), hMLH1 (28%), and MGMT (20%) detected in Cr-exposed tumors Methylation of hMLH1 and APC higher in Cr-exposed tumors than non-exposed Methylation of MGMT gene same in Cr-exposed and non-exposed tumors	[51]

tumors [46]. Two studies reported no difference in Bcl2 or p53 expression between chromate-exposed and non-exposed tumors [45, 50]. Although a third study found p53 overexpressed in three of four dysplastic lesions from ex-chromate workers, dysplasia is considered a precancerous condition, and p53 may be overexpressed in order to prevent the progression to a malignant tumor [47]. In addition, this study did not compare p53 levels in lesion from chromate workers with those from non-chromate workers. Therefore, it is unknown if the observed p53 overexpression was specific to chromate exposure or a characteristic of dysplastic lesions in general.

Hirose et al. [44] showed loss of heterozygosity was comparable between chromate-exposed and non-exposed tumors, but Cr-exposed tumors exhibited a significantly higher frequency of microsatellite instability. A second study found microsatellite instability in 78% of Cr-exposed tumors and demonstrated a strong correlation between hMLH1 inactivation and microsatellite instability in these tumors [48]. Methylation of the hMLH1 gene promoter was detected in 62.5% of chromate-exposed tumors. hMLH1 and hMLH2 protein levels were both lower in Cr-exposed tumors than in non-exposed. [48]. Ali et al. [51] supported these findings, showing aberrant methylation of tumor suppressor genes that occurred at a higher frequency in chromate-exposed tumors, often occurring at multiple loci. Increased methylation of APC, hMLH1, and MGMT were detected in these tumors. Kondo et al. [49] found methylation of p16 occurred more frequently in chromate-exposed tumors, although the difference was not significant. Gene expression changes in cyclin D1 and survivin were also detected in Cr-exposed tumors. Cyclin D1 expression was significantly elevated in Cr-exposed tumors [45]. Survivin expression was inhibited in small cell carcinomas but not in non-small cell carcinomas, which include the subtype squamous cell carcinoma and make up the majority of the tumors analyzed [50]. A third study investigated the status of surfactant protein B in Cr-exposed tumors. Pulmonary surfactant protects the lungs from injuries and infections caused by inhaled particles by improving mucociliary transport and facilitating their removal [52]. Ewis et al. [43] detected surfactant protein B gene variants in 61.3% of chromate-exposed tumors. The presence of surfactant protein B variants correlated with the development of squamous cell carcinoma in chromate workers.

7.5 Potential Mechanisms of Cr(VI)-Induced Carcinogenesis

The mechanism of Cr(VI)-induced carcinogenesis is currently unknown. Over the past century, three models of carcinogenesis have become the major focus of research efforts. These models include multistage carcinogenesis, genomic instability, and epigenetic modification. We will review the data on chromium in the context of each of these models to support or refute each as a potential model of chromium-induced carcinogenesis.

7.5.1 Multistage Carcinogenesis

Multistage carcinogenesis is described as a progressive process consisting of initiation, promotion, and progression to malignancy. The initiation step is defined as the acquisition of an irreversible, heritable genetic mutation [53]. As this model of carcinogenesis requires the acquisition of a genetic mutation, we will consider whether Cr(VI) induces base mutations.

One study investigated the mutagenic potential of Cr(VI) in animals (Table 7.2). Cheng et al. [54] exposed Big Blue transgenic mice to soluble Cr(VI) via intratracheal installation and measured mutagenesis using a LacI mutagenesis assay. Deposition analysis demonstrated that 5% of Cr(VI) was retained in the mouse lung. This study showed a dose- and time-dependent increase in Cr-induced mutation frequency, with a significant increase in mutation frequency occurring after 2 weeks of exposure and at doses above 3 mg/kg.

The results of cellular mutagenesis studies are not consistent (Table 7.2). Snow et al. [58] showed Cr(VI) induced a twofold decrease in replication fidelity in NR9064 *E. coli*, resulting in mutagenesis. However, Quievryn et al. [23] found Cr–DNA adducts were not mutagenic in MBL50 *E. coli*. The difference in these results could possibly be due to differences in the *E. coli* strains utilized in each study. Snow et al. [58] used a mismatch repair-deficient *E. coli*, which would be more sensitive to replication errors.

Two cell culture studies investigated the mutagenic potential of Cr(VI) [17, 56]. Both studies demonstrated Cr(VI) does not induce mutation to ouabain resistance, indicating Cr(VI) does not induce base substitutions. Patierno et al. [17] then showed soluble Cr(VI)-induced mutation to 6-thioguanine while particulate Cr(VI) did not. In contrast, Klein et al. [56] found both soluble and particulate Cr(VI)-induced mutation to 6-thioguanine, reaching 3–3.5 times the level of background mutagenesis. However, the effect is not concentration dependent. The Cr(VI)-induced resistance to 6-thioguanine suggests Cr(VI) can induce insertions, deletions, and frameshift mutations. Differences between these two studies may be due to the cell lines utilized. Klein et al. [56] utilized G12 cells, which are transgenic and contain a gpt reporter gene, while Patierno et al. [17] used fibroblasts that utilize the endogenous hprt gene to detect mutations. Differences in mutation frequency could result from differences in Cr(VI)-induced mutations in exogenous versus endogenous genes.

Four studies performed by one research group utilized a shuttle vector mutagenesis assay to investigate the mutagenic potential of Cr(VI). All of these studies reacted DNA to Cr(VI) and a Cr(VI)-reducing agent extracellularly and then exposed human fibroblasts to the resulting Cr–DNA reaction products. A concentration-dependent increase in mutagenesis was detected in each study [23, 24, 26, 55]. Additionally, Cr–DNA binding was required for mutagenesis [23, 24, 55]. Zhitkovich et al. [55] demonstrated ternary cysteine–Cr(III)–DNA adducts were four to five times more mutagenic than binary Cr(III)-DNA adducts. Likewise, Quievryn et al. [23] found ascorbate-Cr(III)-DNA adducts to be more mutagenic than Cr(III)-DNA adducts, accounting for over 90% of Cr-induced mutagenesis. While one study found single base substitutions at G/C pairs to be the predominant

Table 7.2 Cr(VI)-induced mutations

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Calcium chromate 1–20 μM Lead chromate 10–50 μM 5–24 h	CHO cells; 10T1/2 mouse embryo fibroblasts	6-Thioguanine resistance; ouabain resistance	Soluble Cr(VI)-induced mutation to 6-thioguanine but not to ouabain resistance Particulate Cr(VI) did not induce mutation to 6-thioguanine or ouabain resistance	[17]
$^{51}CrCl_3$ 0.4–50 μM 0–30 min	M13mp20 single-stranded DNA; NR9064 MMR-deficient *E. coli*	Mutagenesis assay in MMR-deficient *E. coli*	Twofold decrease in replication fidelity, resulting in mutagenesis	[58]
Potassium chromate 1.7–6.75 mg/kg 4 weeks intratracheal instillation	C57BL/6 Big Blue transgenic mice	LacI mutagenesis assay	Dose- and time-dependent increase in mutation frequency in mouse lung Significant increase in mutation frequency seen after 2 weeks of exposure and at doses above 3 mg/kg	[54]
$Na_2{}^{51}CrO_4$ or potassium chromate 25–200 μM (100 mM cysteine) 1 h	ϕX174 and pSP189 DNA; HF/SV human fibroblasts	Shuttle vector mutagenesis assay	Cr(VI) reduction by cysteine increased mutagenesis Blocking Cr(III) and DNA binding eliminated Cr-induced mutagenesis Cysteine–Cr(III)–DNA adducts were 4–5× more mutagenic than binary Cr(III)-DNA adducts Single base substitutions at G/C pairs were the predominant form of Cr-induced mutations	[55]
Potassium chromate 5–50 μM 2 h Barium chromate 0.05–0.25 μg/cm^2 24 h	G12 gpt$^+$ cells	6-Thioguanine resistance ouabain resistance	Soluble and particulate Cr(VI)-induced mutation to 6-thioguanine resistance, up to 3–3.5× the background mutagenesis level Peaks in mutagenesis levels were followed by a decline at higher Cr(VI) concentrations No mutagenesis was detected by ouabain resistance for either Cr(VI) compound	[56]

Table 7.2 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium chromate 25–200 μM (2 mM cysteine) 1 h	pSP189 DNA; human fibroblasts	Shuttle vector mutagenesis assay	Cr(III)–DNA adducts induced mutagenesis and inhibited replication	[26]
Potassium chromate 10–200 μM (1 mM ascorbate) 30–120 min	pSP189 DNA; HF/SV human fibroblasts; MBL50 *E. coli*	Shuttle vector mutagenesis assay	Concentration-dependent increase in mutagenesis Disruption of Cr–DNA binding abolished mutagenesis Asc–Cr(III)–DNA crosslinks were more mutagenic than Cr(III)–DNA adducts and account for >90% of Cr-induced mutagenesis Cr(VI) induced included an equal number of deletions and G/C point mutations Cr–DNA adducts not mutagenic in *E. coli* cells	[23]
Potassium chromate 10–200 μM (0.2 mM ascorbate) 30 min	pSP189 DNA; HF/SV fibroblasts	Shuttle vector mutagenesis assay	Concentration-dependent increase in mutagenic DNA lesions required Cr–DNA binding	[24]
Potassium chromate 0–220 μM (0.25–2 mM ascorbate) 3 h	CHO; V79	hprt mutagenesis assay	Cr(VI) non-mutagenic in control CHO and V79 cells (contain 15 μM Asc) Raising ascorbate levels to 0.45 mM induced a 3.8-fold increase in Hprt mutants Preloading cells with 1.4 mM Asc induced a linear increase in Hprt mutants	[57]

form of Cr-induced mutation, the other showed Cr induced an equal number of deletions and G/C point mutations [23, 55].

Reynolds et al. [57] demonstrated a crucial role of Cr(VI) reduction in its mutagenesis. In this study, Chinese hamster ovary cells (CHO) and Chinese hamster lung (V79) cells were treated with Cr(VI), with and without the addition of ascorbate. Untreated, CHO and V79 cells contain low levels of ascorbate (15 μM). No Cr(VI)-induced mutagenesis was detected by the hprt mutagenesis assay when no ascorbate was added to the cells. However, a 3.8-fold increase in mutagenesis was detected once intracellular ascorbate levels were raised to 450 μM. Preloading the cells with 1400 μM ascorbate induced a concentration-dependent increase in mutagenesis [57]. These results suggest intracellular levels of Cr(VI)-reducing agents directly affect the mutagenic potential of Cr(VI).

Human lung tissue, the target site of Cr-induced carcinogenesis, contains much lower levels of ascorbate than required to induce mutagenesis in the aforementioned

studies. Ascorbate levels in adult lung tissue range from 0.045 to 0.065 mg/g which is approximately 256 μM [59]. Therefore, the ascorbate level required to induce mutagenesis in cellular and *in vitro* studies is two to five times higher than the level detected in human lung tissue. The results of animal mutagenesis studies may also not be relevant to human lung mutagenesis. While humans obtain ascorbate from food, rodents generate it in their liver, providing a fundamental difference in the availability of this reduction agent. In addition, while these studies have assessed the role of ascorbate in Cr-induced mutagenesis, they do not take into account other Cr-reducing agents such as glutathione or NADPH. The lack of mutations seen in Cr-induced tumors [42, 46] suggests Cr reduction in human lung tissue does not occur at levels necessary to induce mutagenesis. The initiation of multistage carcinogenesis usually involves the mutation of a key oncogene or tumor suppressor gene. There is no evidence that Cr induces mutations in such genes. Additionally, any small frameshift mutations induced by Cr would occur randomly throughout the genome and have a low chance of occurring in one of these genes. Therefore, this model of carcinogenesis does not appear to be a good fit for Cr-induced carcinogenesis.

7.5.2 Genomic Instability

The second model of carcinogenesis, genomic instability, is described as an increased occurrence of genomic alterations. Accumulation of genomic alterations can induce amplification of genetic sequences, insertions or deletions resulting in mutations, deletion, or rearrangement of chromosome segments and the gain or loss of entire chromosomes. Due to these major and frequent genetic alterations and the resulting misregulation of crucial signaling pathways, genomic instability is considered a major driving force of carcinogenesis [60]. Genomic instability is subdivided into microsatellite instability and chromosome instability.

Microsatellites are extensive repetitions of nucleotide motifs of up to six base pairs [61]. Microsatellite instability manifests as a change in the number of repeated DNA nucleotide motifs within the microsatellites [62]. Two studies documented microsatellite instability in 79% of tumors from ex-chromate workers [44, 48]. No animal or cell culture studies have been conducted to support the observations that Cr(VI) exposure induces microsatellite instability.

Microsatellite instability usually arises due to the incorporation of incorrect base pairs during replication. Mismatch repair corrects such replication-associated errors, keeping the spontaneous mutation rate low [61]. Microsatellite instability frequently arises when inactivation of a mismatch repair gene results in deficient mismatch repair. Takahashi et al. [48] showed the hMLH1 gene promoter methylated in 63% of tumors from ex-chromate workers. A recent study supported this result, finding increased methylation of hMLH1 in tumors from ex-chromate workers, compared to non-exposed individuals [51]. Inactivation of hMLH1 expression strongly correlated with microsatellite instability [48].

Cell culture studies have utilized siRNAs and mismatch repair-deficient cell lines to investigate the role of mismatch repair in Cr-induced carcinogenesis (Table 7.3).

Table 7.3 Cr(VI)-induced genomic instability: the role of mismatch repair

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium chromate 5–30 μM 3 h	A549; HCT116 (MLH1−/−); HCT116 + ch3 (MLH1+); DLD1 (MSH6−/−); DLD1 + ch2 (MSH6+)	Clonogenic survival assay; Annexin V/PI analysis with flow cytometry; WB for caspases 2 and 7, PARP, p53, p53ser15; IF for γH2AX, cyclin B1, BrdU incorporation	MMR-deficient cells are less sensitive to Cr(VI) than control cells MMR status had no effect on the formation or removal of Cr–DNA adducts MLH1: • Is required for Cr(VI)-dependent activation of caspases 2 and 7 and the apoptotic cleavage of PARP • Inhibited DNA replication of Cr-modified templates • Deficiency suppressed γH2AX foci formation Cr-induced apoptosis is primarily p53 independent Cr-induced γH2AX foci formation occurs in G2 and not S-phase	[63]
Potassium chromate 0.5–5 μM (0.25–2 mM ascorbate) 3 h	IMR90 fibroblasts expressing MSH2 or MLH1 shRNA	Micronucleus assay; IF for γH2AX foci	Depletion of either MLH1 or MSH: • Almost eliminated γH2AX foci formation in Cr(VI)- and Cr(VI)/Asc-exposed cells • Decreased micronuclei formation in Cr(VI)- and Cr(VI)/Asc-exposed cells	[57]

(continued)

Table 7.3 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium chromate 0–10 μmol/L (0.5–2 mM ascorbate) 3 h	IMR90; H460; HCT116 (MLH1−/−); DLD1 (MSH6−/−)	DNA-protein pulldown; WB for MLH1, MSH2, MSH3, and MSH6; IF for MSH6, MSH3, γH2AX, 53BP1, cyclin B1	MSH2 bound to Cr–DNA adducts MSH2–MSH6 and MSH2–MSH3 complexes: • Recognized Cr–DNA adducts • Were required for Cr-induced cytotoxicity MSH6 showed stronger colocalization with γH2AX and 53BP1 than MSH3 Cr(VI)-induced MSH6 and MSH3 foci when replication was inhibited, before cells entered G2 MSH6 or MSH3 knock down suppressed: • γH2AX foci formation • Micronuclei formation	[64]
Potassium chromate 5–30 μM 3 h	IMR90; HTC116 (MLH1−/−); HCT116 + ch3 (MLH1+)	Clonogenic survival assay; IF for 53BP1, γH2AX and RAD51;	MMR proteins are required for WRN retention in the nucleoplasm after Cr(VI) exposure MMR-deficiency inhibited RAD51 foci formation	[65]

Mismatch repair-deficient cells were less sensitive to Cr(VI) exposure, suggesting mismatch repair induces cell death after Cr(VI) exposure. In fact, MLH1 was required for cleavage of PARP and the activation of caspases 2 and 7 following Cr(VI) exposure [63]. This outcome is not surprising as mismatch repair has been previously shown to induce apoptosis after exposure to chemical carcinogens [66]. Reynolds et al. [64] showed MSH2 binds to Cr–DNA adducts. In addition, MSH2–MSH6 and MSH2–MSH3 complexes recognize Cr–DNA adducts and are required for Cr-induced cytotoxicity [64]. However, Peterson-Roth et al. [63] showed mismatch repair status had no effect on the formation or removal of Cr–DNA adducts. While mismatch repair proteins have the potential to recognize DNA adducts [67],

excision repair is thought to be primarily responsible for their detection and removal. The activity of excision repair may explain why mismatch repair status had no impact on the removal of Cr–DNA adducts in this study.

Active mismatch repair was also necessary for DNA replication inhibition, micronuclei formation, and DNA double-strand break (DSB) formation following Cr exposure [57, 63–65]. Reynolds et al. [64] demonstrated Cr(VI) stalled replication and induced MSH6 and MSH3 foci, suggesting that mismatch repair was active. Active mismatch repair was also required for Cr(VI)-induced micronuclei formation [57, 64].

Interestingly, mismatch repair status appears to play a role in DSB formation. Although an indirect measure, γH2AX foci are commonly utilized as an indicator of DNA double-strand breaks. MSH6, MSH3, or MLH1 deficiency all inhibited Cr(VI)-induced γH2AX foci formation, suggesting an inhibition of DSB [57, 63, 64]. MLH1 was required for nuclear localization of WRN helicase and RAD51 foci formation after Cr(VI) exposure, indicating a role in the response of the high fidelity DSB repair pathway, homologous recombination [65]. These data suggest active mismatch repair is initially active following Cr exposure as it is required for Cr-induced DSB formation, a key step in the mechanism of Cr(VI)-induced carcinogenesis. The inhibition of mismatch repair demonstrated by Cr-induced tumors may be a later step in the carcinogenic mechanism that facilitates cell survival and microsatellite instability.

The second form of genomic instability, chromosome instability, is a hallmark of human tumors. Chromosome instability can be numerical or structural in nature. Numerical chromosome instability is described as an alteration in the number of chromosomes due to the gain or loss of entire chromosomes. Structural chromosome instability involves physical changes in the genome structure, often resulting from chromosome breaks, translocations, or unequal exchange of material between two chromosome regions [68].

Numerical chromosome instability has not been investigated *in vivo* or in chromate-induced tumors. Cell culture studies show Cr(VI) induces aneuploidy (Table 7.4). Cr(VI)-induced numerical chromosome instability appears to be related to the length of exposure. Two studies investigated the ability of Cr(VI) to induce aneuploidy from 24 to 120 h, showing no effect after 24 h exposure but a concentration- and time-dependent increase in aneuploidy starting at 48 h exposure [72, 74]. Two different studies documented Cr(VI)-induced aneuploidy after only 30 h of exposure, the earliest recorded timepoint of Cr(VI)-induced aneuploidy [69, 70]. While the majority of the studies describing Cr(VI)-induced aneuploidy were conducted in human lung cells, Wise et al. [76] showed chronic Cr(VI) exposure also induced this effect in human urothelial cells.

Cr(VI)-induced numerical chromosome instability was characterized by hypodiploidy, hyperdiploidy, polyploidy, and tetraploidy [70–72, 74]. Seone et al. (2002) documented the occurrence of lagging chromosomes and the formation of kinetochore-positive micronuclei, indicating these were formed following mitotic catastrophe. Holmes et al. [72] supports this finding, showing evidence of Cr(VI)-induced abnormal mitotic figures, including disorganized anaphase and mitotic

Table 7.4 Cr(VI)-induced numerical chromosome instability

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium dichromate 0.25–1 μM 30 h	MRC-5 human lung fibroblasts	Chromosome counting	Cr(VI)-induced aneuploidy	[69]
Potassium dichromate 1–4 μM 30 h	MRC-5 human lung fibroblasts	Chromosome counting; anaphase–telophase assay; CRST-stained micronuclei analysis	Cr(VI)-induced aneuploidy, characterized by hypodiploidy and lagging chromosomes and the formation of kinetochore-positive micronuclei	[70]
Lead chromate 0.1–1 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	Chromosome analysis for centromere spreading, premature anaphase, and premature centromere division; WB for Mad2	Chronic Cr(VI) exposure induced: • Spindle assembly checkpoint bypass • Decreased Mad2 protein l • Formation of tetraploid cells Tetraploid cells survived to form colonies	[71]
Lead chromate 0.1–1 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	Chromosome counting; IF for centrosomes proteins; mitotic stage analysis	Concentration- and time-dependent increase in aneuploidy (starting at 48 h exposure) Aneuploid cells survived to form colonies Centrosome amplification and aberrant mitosis occurred after 96–120 h Cr(VI) exposure	[72]
Potassium dichromate 1 μM Continuous exposure up to 41 passages	BEAS-2B; BALB/c-nu/nu mice	Cytogenetic analysis with GTG-banding, microsatellite analysis; gene expression analysis	Cr(VI)-induced aneuploidy and structural chromosome instability. Cr(VI)-aneuploid cloned cells exhibited: • Altered morphology • Karyotype drift • No microsatellite instability Cr(VI)-aneuploid cloned cells induced tumors in nude mice	[73]
Zinc chromate 0.1–0.2 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	Chromosome analysis for centromere spreading, premature anaphase, and premature centromere division; IF for centrosomes, microtubule, and centrin analysis	Concentration- and time-dependent increase in Cr(VI)-induced aneuploidy starting at 72 h exposure Cr(VI)-induced concentration and time-dependent increase in spindle assembly checkpoint bypass starting at 96 h exposure Cr(VI) induced time-dependent increase in centrosome amplification and centriole defects	[74]

Table 7.4 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Zinc chromate 0.1–0.2 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	IF for centrosomes, microtubule, centrin, and C-Nap1 analysis	Cr(VI)-induced numerical CIN correlated with centrosome amplification Chronic Cr(VI) induced: • Centrosomes with supernumerary centrioles • Premature centriole disengagement • Premature centrosome separation in interphase	[75]
Sodium chromate 1–5 μM 24 h and 120 h	HUC; hTUC1-38 hTERT-immortalized human urothelial cells	Chromosome counting	Chronic Cr(VI) exposure induces a concentration-dependent increase in aneuploidy hTERT status did not affect levels of Cr-induced aneuploidy	[76]

catastrophe. As one would expect, when Cr(VI)-treated cells exhibiting aneuploidy were expanded into clonal cell lines, the cells exhibited altered morphology and karyotype drift, but no microsatellite instability [73]. In addition, Cr(VI)-aneuploid clonal cells exhibited signs of transformation, forming colonies [71, 72] and inducing tumors when implanted into nude mice [73].

Mitosis is tightly regulated by the spindle assembly checkpoint to ensure proper centrosome formation, anchoring of the mitotic spindle to the chromosomal kinetochores, chromosome separation, and cytokinesis. Numerical chromosome instability can occur if any of these steps fails or if the spindle assembly checkpoint is disrupted [77]. Two studies showed chronic Cr(VI) exposure induced centromere spreading, premature anaphase, and premature centromere division, all indicators of spindle assembly checkpoint bypass (Table 7.4) [71, 74]. Wise et al. [71] found chronic Cr(VI) exposure decreased Mad2, a key component of the spindle assembly checkpoint. Holmes et al. [74] investigated the effect of chronic Cr(VI) on the centrosomes, as supernumerary centrosomes has also been shown to induce numerical chromosome instability. This study found a time-dependent increase in centrosome amplification. After 120 h Cr(VI) exposure, up to 46% of mitotic cells exhibited centrosome amplification, with some cells containing as many as 14 centrosomes. Martino et al. [75] demonstrated Cr(VI)-induced centrosome amplification correlated with numerical chromosome instability. All of the known mechanisms for centrosome amplification involve alterations in the number of centrioles. Holmes et al. [74] described a Cr(VI)-induced increase in mitotic cells containing an abnormal centriole number. Martino et al. [75] also showed chronic Cr(VI) exposure induced centrosomes with supernumerary centrioles. Both studies also found Cr(VI) exposure can result in the formation of cells with a normal number of centrioles, but extra centrosomes. This outcome can occur if the

centrioles disengage prematurely (between S-phase and mitosis). Chronic Cr(VI) exposure induced premature centriole disengagement (in S, G2, and mitotic cells) [75]. In addition, chronic Cr(VI) exposure induced premature centrosome separation, suggesting the protein linker that normally holds duplicated centrosomes together until the G2/M transition may be either severed prematurely or not formed properly [75].

Structural chromosome instability has been investigated in chromate workers, using Giemsa staining and fluorescence in situ hybridization (FISH) technique to examine chromosome aberrations and translocations, respectively. Maeng et al. [78] showed an increase in chromosome aberrations in Cr-exposed workers compared to age-matched controls, although the increase was not significant. However, blood Cr concentrations were statistically correlated with both chromatid exchanges and chromosome aberrations. These results were supported by two other studies, which show modest increases in overall chromosome aberrations in Cr-exposed workers compared to controls, but positively correlate chromosome aberrations with the level of Cr in the blood [79, 80]. In addition, the frequency of translocations, insertions, and acentric fragments was significantly higher in Cr-exposed workers [78].

Cell culture studies provide a wealth of data that indicate Cr(VI) induces structural chromosome instability (Table 7.5). Both particulate and soluble Cr(VI) induce a concentration-dependent increase in chromosome aberrations [81, 82, 86, 88, 89, 91]. While Qin et al. [91] showed particulate Cr(VI) induced a time-dependent increase in chromosome aberrations, it is not clear whether this time-related effect holds for soluble Cr(VI). Wise et al. [76] found sodium chromate induced more chromosome aberrations after 120 h exposure than 24 h exposure. However, Holmes et al. [87] showed a decrease in chromosome aberrations after chronic (up to 72 h) exposure to sodium chromate. Two differences between these studies may account for the difference in their results. First, Holmes et al. [87] examined Cr-induced chromosome aberrations after 24, 48, and 72 h exposure, while Wise et al. [76] examined this endpoint after 24 and 120 h exposure. It is possible the increase in chromosome instability is not induced by soluble Cr(VI) until after 72 h exposure and would not have been detected in the Holmes et al. [87] study. A second explanation may be that the two studies utilized different cell lines. Wise et al. [76] utilized human urothelial cells while Holmes et al. [87] employed human lung fibroblasts. Xie et al. [92] demonstrated Cr(VI) can induce different levels of clastogenesis in different cell lines.

There are also differences in the clastogenic potential of the different particulate Cr(VI) compounds. Zinc chromate induced the highest level of chromosome aberrations, followed by barium chromate and lead chromate [85, 90]. For particulate Cr(VI), clastogenesis depends on the extracellular dissolution of the Cr particle rather than its internalization [20]. The chromate anion has been shown to be the proximate clastogenic species [84]. Reynolds et al. [57] showed preloading cells with ascorbate increased the yield of micronuclei 6.6-fold. These micronuclei were negative for kinetochore CREST staining, suggesting they resulted from chromosomal breaks. This outcome suggests the reduction of intracellular Cr(VI) is involved in Cr(VI)-induced clastogenesis.

Table 7.5 Cr(VI)-induced structural chromosome instability

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Lead chromate 0.1–5 μg/cm^2 24 h Sodium chromate 1–10 μM 24 h	Primary human bronchial fibroblasts	Chromosome aberration assay	Particulate and soluble Cr induced a concentration-dependent increase in chromosome aberrations	[81]
Barium chromate 0.01–0.5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Concentration-dependent increase in chromosome aberrations	[82]
Lead chromate 0.1–5 μg/cm^2 Sodium chromate 1–10 μM 24 h	Primary human lung fibroblasts; WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Cr-induced clastogenesis comparable in primary and hTERT-immortalized cells	[83]
Lead chromate 0.05–10 μg/cm^2 Sodium chromate 0.25–2.5 μM Lead glutamate 250–1000 μM 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	The Cr anion and not the lead cation was the proximate clastogenic species	[84]
Lead chromate Barium chromate 0.1–5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay; ICPMS	Barium chromate was more clastogenic than lead chromate even though intracellular Cr uptake is comparable	[85]
Lead chromate 0.1–10 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Cr clastogenesis dependent on extracellular dissolution of the Cr particles	[20]
Lead chromate 0.1–5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Concentration-dependent increase in chromosome aberrations	[86]
Lead chromate 0.1–1 μg/cm^2 Sodium chromate 0.5–2.5 μM 24–72 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Chronic exposure to particulate Cr induced persistent levels of chromosome damage, while chromosome damage decreased with chronic exposure to soluble Cr	[87]
Lead chromate 0.5–50 μg/cm^2 Sodium chromate 1–5 μM 24 h	BEP2D bronchial epithelial cells	Chromosome aberration assay	Particulate and soluble Cr(VI) induced a concentration-dependent increase in chromosome aberrations	[88]

(continued)

Table 7.5 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium chromate 0.5–5 μM (50–2000 μM ascorbate) 3 h	IMR90 human lung fibroblasts; HBE bronchial epithelial cells	Colocalization of γH2AX and 53BP1 foci in cyclin B1 expressing cells	Preloading cells with Asc increased the yield of micronuclei 6.6-fold Micronuclei were negative for anti-kinetochore CREST staining	[57]
Zinc chromate 0.1–0.5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Concentration-dependent increase in chromosome aberrations	[89]
Barium chromate Zinc chromate 0.01–0.5 μg/cm^2 Lead chromate 0.01–1 μg/cm^2 Sodium chromate 0.05–2.5 μM 24 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Zinc chromate was more clastogenic than barium chromate, lead chromate, or sodium chromate	[90]
Zinc chromate 0.1–0.2 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	Chromosome aberration assay	Concentration- and time-dependent increase in chromosome aberrations	[91]
Lead chromate 0.1–1 μg/cm^2 Sodium chromate 0.5–5 μM 24 h	WTHBF-6 human lung fibroblasts; BJhTERT human skin fibroblasts	Chromosome aberration assay	Soluble Cr induced comparable amounts of chromosome aberrations, while particulate Cr was more clastogenic to human skin cells than lung cells	[92]
Sodium chromate 1–5 μM 24 h and 120 h	HUC human urothelial cells; hTUC1-38 hTERT-immortalized human urothelial cells	Chromosome aberration assay	hTERT status did not alter the amount of Cr-induced chromosome aberrations Chronic exposure induced higher levels of Cr-induced chromosome aberrations than acute exposure	[76]

The misrepair of DNA double-strand breaks (DSBs) is a major mechanism of structural chromosome instability. DSBs are a highly detrimental form of DNA damage as incorrect rejoining of the broken DNA ends can introduce structural chromosome abnormalities such as deletions or translocations [77]. Although Cr(VI)-induced DSB formation has not been investigated *in vivo*, a multitude of cell culture studies demonstrate Cr(VI) induces DSB formation (Table 7.6). Using intracellular Cr concentrations, Wise et al. [90] showed soluble and particulate Cr(VI) compounds induced similar levels of DSBs. Interestingly, Wise et al. [76] found Cr(VI)-induced

Table 7.6 Cr(VI)-induced DNA double-strand breaks

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Sodium chromate 3–6 μM 1–3 h	Normal human dermal fibroblasts	Neutral comet assay; IF for γH2AX foci; cell cycle analysis	Concentration-dependent increase in DSBs in S-phase cells No increase in DSBs in G1 cells	[93]
Potassium chromate 10–40 μM 30 min to 24 h	HeLa cells	Neutral comet assay; IF for γH2AX foci	Cr exposure induced DSBs and γH2AX foci formation	[94]
Lead chromate 0.1–5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Neutral comet assay; WB for pATM; IF for γH2AX foci	Concentration-dependent increase in DSBs and activation of ATM and γH2AX proteins	[86]
Potassium chromate 0.5–5 μM 0.05–5 mM ascorbate 3 h	IMR90 human lung fibroblasts; human bronchial epithelial cells	IF for γH2AX and 53BP1 foci in cyclin B1 expressing cells	DNA DSBs were generated in G2 phase Preloading cells with Asc increased number of γH2AX and 53BP1-containing cells	[57]
Lead chromate 0.1–1 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	Neutral comet assay	Concentration-dependent increase in DSBs	[95]
Zinc chromate 0.1–0.5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	IF for γH2AX, flow cytometry for cell cycle, and γH2AX	Concentration-dependent increase in γH2AX foci formation in G2/M phase Chromium induced a G2 arrest	[89]

(continued)

Table 7.6 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Barium chromate Lead chromate 0.01–1 μg/cm^2 Zinc chromate 0.1–0.5 μg/cm^2 Sodium chromate 0.05–5 μM 24 h	WTHBF-6 human lung fibroblasts	IF for γH2AX	All Cr(VI) compounds induced a concentration-dependent increase in γH2AX foci formation All Cr(VI) compounds induced similar levels of DSBs at similar intracellular concentrations	[90]
Zinc chromate 0.1–0.3 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	Neutral comet assay	Concentration-dependent but no time-dependent increase in DSB formation	[91]
Potassium chromate 5–20 μM 3–6 h	H460 ascorbate-restored human lung epithelial cells; IMR90 normal human lung fibroblasts; MEFs	WB for γH2AX; IF for γH2AX, 53BP1 and H3K9me3	Concentration-dependent increase in γH2AX foci formation Cr-induced DSBs developed in G2 phase Cr-induced DSBs were located in euchromatic DNA	[96]
Sodium chromate 1–5 μM 24 and 120 h	HUC; hTERT-immortalized human urothelial cells	IF for γH2AX	Cr(VI) induced a concentration- and time-dependent increase in γH2AX foci formation hTERT status did not alter the extent of Cr-induced DNA damage	[76]

DSB formation was time dependent while Qin et al. [91] did not. Both studies examined DSB formation at the same exposure times, spanning from 24 to 120 h. However, Wise et al. [76] utilized γH2AX foci as an indicator of DSBs, while Qin et al. [91] employed the neutral Comet assay, which may explain the difference in their results. The neutral Comet assay directly measures DSBs. In contrast, γH2AX foci are an indirect indicator of DSBs and also form in response to apoptosis (Rogakou et al. 2000). Therefore, the increase in γH2AX foci observed in response to 120 h Cr(VI) exposure by Wise et al. [76] may actually be indicative of an increase in apoptosis. Additionally, these two studies utilized different cell lines, which may contribute to the observed difference in the effect of exposure time on DSB formation.

Reynolds et al. [57] found preloading lung fibroblasts with ascorbate increased the number of γH2AX and 53BP1 containing cells, indicating Cr(VI) reduction is related to DSB formation. Cr(VI)-induced DSBs developed in S and G2 cell cycle phases [57, 89, 93, 96]. Accordingly, Cr(VI) exposure induced a G2 arrest [89]. DeLoughery et al. [96] demonstrated Cr(VI)-induced DSBs are localized to euchromatic DNA. Cr(VI)-induced DSBs were repaired within 24 h [95, 97].

The two main DNA repair pathways that actively repair DSBs are nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ is considered error prone as it processes the DNA ends and ligates them back together, resulting in the loss of several nucleotides. HR repair protects against structural chromosome instability by utilizing a homologous sequence to repair the DSB and maintain high genomic fidelity. Camyre et al. [98] showed although NHEJ-deficient cells were more sensitive to Cr(VI) exposure, NHEJ activity did not protect against Cr-induced chromosome instability. Three studies show HR deficiency results in increased Cr(VI) sensitivity and Cr(VI)-induced chromosome instability (Table 7.7). Bryant et al. [97] and Stackpole et al. [99] found increases in Cr(VI)-induced chromosome aberrations in BRCA2- and RAD51C-deficient cell lines, respectively. Both of these proteins are crucial to the formation of the RAD51 nucleofilament, the defining biochemical step of HR. Tamblyn et al. [100] further demonstrated the importance of HR repair in repairing Cr(VI)-induced DSBs, using cells deficient in Mus81. Mus81 is involved in resolving Holiday junctions and completing HR repair [101]. Cr(VI) induced higher levels of γH2AX foci formation in Mus81-deficient cells, suggesting inhibition of the HR resolution step leads to an accumulation of DSBs. In addition, RAD51 foci removal was delayed in Mus81-deficient cells [100]. Together, these studies demonstrate the importance of HR in preventing Cr-induced structural chromosome instability.

Bryant et al. [97] showed acute Cr(VI) exposure (24 h) induces a concentration-dependent increase in homologous recombination. In addition, the HR signaling proteins, γH2AX, ATM, and ATR were all phosphorylated in response to Cr(VI) exposure, indicating their activation [89, 91, 93–96]. Wakeman et al. [94] demonstrated ATM was required for Cr(VI)-induced phosphorylation of SMC1, which promotes sister chromatid HR. In addition, Ha et al. [93] showed ATM was required for Cr(VI)-induced γH2AX foci formation. In direct contrast to this result, DeLoughery et al. [96] showed suppressed ATM and DNA-PK did not affect Cr-induced γH2AX foci formation, while ATR inhibition completely abolished its response. There are two possible explanations for this discrepancy. First, DeLoughery et al. [96] preloaded the cells with ascorbate, increasing the level of Cr(VI) reduction, while Ha et al. [93] did not. Increasing the level of Cr(VI) reduction would alter levels of different Cr valence states and the level of ROS within the cell. Secondly, DeLoughery et al. [96] inhibited ATM with the chemical inhibitors KU60019 and KU55933, while Ha et al. [93] used ATM-deficient fibroblasts. These chemical inhibitors have off-target effects, such as altering the cell cycle, which could impact γH2AX foci formation.

Table 7.7 Cr(VI)-induced genomic instability: the role of DNA double-strand break repair

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Sodium chromate 6 μM 1–3 h	Normal human dermal fibroblasts; ATM-null fibroblasts	Neutral comet assay; γH2A.X	ATM is required for Cr(VI)-induced γH2AX foci formation	[93]
Potassium chromate 10 μM 30 min to 8 h	HeLa cells; SV40-transformed human fibroblasts and $ATM^{-/-}$ fibroblasts	WB for pATM and SMC1	pATM was induced by Cr ATM is required for Cr-induced: • SMC1 phosphorylation	[94]
Sodium chromate 0.5–25 μM 24 h	AA8; SPD8; V3-3; VC8; VC8 + B2; UV4	IF for RAD51; pulse field gel electrophoresis; HPRT recombination assay	Cr-induced DSBs were repaired within 24 h Cr exposure induced RAD51 foci formation Concentration-dependent increase in homologous recombination activity HR-deficient and BER-deficient cells were hypersensitive to Cr	[97]
Lead chromate 0.1–10 μg/cm^2 24 h	CHO-K1; xrs-6; 2E	Clonogenic survival assay; chromosome aberration assay	NHEJ-deficient cells were more sensitive to Cr NHEJ did not protect against Cr-induced chromosome instability	[98]
Lead chromate 0.1–1 μg/cm^2 24 h	AA8; irs1SF; 1SFwt8; V79; irs3; irs3#6	Clonogenic survival assay; chromosome aberration assay	HR-deficient cells were more sensitive to Cr HR-protected cells from Cr-induced chromosome instability	[99]
Lead chromate 0.1–1 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts; ATLD2 MRE11-deficient human skin fibroblasts	Neutral comet assay; IF for γH2AX foci	Cr-induced DSBs were repaired within 24 h MRE11 and pATM foci co-localized with Cr-induced γH2AX foci Repair of Cr-induced DSBs was delayed in MRE11-deficient cells	[95]
Sodium chromate 5–10 μM 6–48 h	Wild-type and Mus81−/− mouse fibroblasts	Clonogenic survival assay; BrdU incorporation; γH2AX analysis by flow cytometry; IF for RAD51; chromosome aberration assay	Mus81-deficient cells exhibit: • Increased sensitivity to Cr(VI) • Increased γH2AX response • Higher incidence of Cr-induced chromosome aberrations RAD51 foci removal was delayed in Mus81-deficient cells	[100]

Table 7.7 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Zinc chromate 0.1–0.5 μg/cm^2 24 h	WTHBF-6 human lung fibroblasts	WB for pATM, pATR, and MRE11	Concentration-dependent increase in MRE11 and pATM levels. pATR was highest at low Cr levels	[89]
Zinc chromate 0.1–0.3 μg/cm^2 24–120 h	WTHBF-6 human lung fibroblasts	WB for MRE11, pATM, and RAD51; IF for MRE11, pATM, and RAD51	Concentration- and time-dependent increase in MRE11 and pATM foci Concentration-dependent increase in RAD51 foci at 24 h RAD51 foci formation decreased in a time-dependent manner, starting at 48 h exposure	[91]
Potassium chromate 5–20 μM (0.5–1 mM) 3–6 h	H460 human lung epithelial cells; IMR90 human lung fibroblasts; MEFs	WB for γH2AX; IF for γH2A.X, 53BP1, and H3K9me3	ATR inhibition completely abolished Cr(VI)-induced γH2AX formation Suppressed ATM and DNA-PK did not affect γH2AX formation	[96]

Several studies showed the nuclease MRE11 responds following Cr(VI) exposure, indicated by an increase in MRE11 foci [89, 91, 95]. Qin et al. [91] showed Cr(VI) induced a concentration- and time-dependent in MRE11 foci formation. Xie et al. [95] further demonstrated Cr(VI)-induced DSB repair was delayed in MRE11-deficient cells. This outcome indicates HR is utilized to repair Cr(VI)-induced DSBs. The central HR protein, RAD51, also responds to Cr(VI)-induced DSBs [91, 97]. Interestingly, while RAD51 foci formation increases after 24 h exposure, RAD51 foci levels decreased starting at 48 h exposure [91]. The RAD51 response was completely inhibited by 72 h exposure. RAD51 nuclear protein levels also decreased in a time-dependent manner [91]. Interestingly, Qin et al. [91] reported the presence of RAD51 agglomerates in the cytoplasm after chronic Cr(VI) exposure, suggesting a problem with RAD51 nuclear transport. This Cr(VI)-induced inhibition of RAD51 indicates HR repair is inhibited by chronic Cr(VI) exposure. As such, the RAD51-dependent HR pathway that results in high fidelity DNA repair would not be available to repair Cr(VI)-induced DSBs. Recent studies indicate classical NHEJ repair cannot be utilized if HR has already been initiated, and the DNA ends have been resected to form single-stranded overhangs. In this case, alternative NHEJ or single-strand annealing can be utilized to repair the DSB [102]. These two repair processes have low fidelity, resulting in high levels of structural chromosome instability.

Cr(VI)-induced tumor characterization and epidemiological and cell culture studies show Cr(VI) induces genomic instability. However, more studies need to be completed to confirm the mechanism. Mismatch repair inactivation has been indicated in Cr(VI)-induced microsatellite instability, but no studies elucidate the

mechanism of how Cr(VI) inactivates mismatch repair. Substantial work has been done to identify the mechanisms of Cr(VI)-induced numerical and structural chromosome instability. However, these mechanisms, namely, centrosome and centriole defects and RAD51 inhibition, need to be confirmed *in vivo* and in Cr(VI)-induced tumors. Considering the wealth of data showing Cr(VI) induces multiple forms of genomic instability, and new studies elucidating the mechanisms of Cr(VI) induced chromosome instability; the genomic instability model is a good fit for Cr(VI)-induced carcinogenesis.

7.5.3 Epigenetic Modification

Epigenetic modification is a third major model of carcinogenesis. Epigenetics is defined as a heritable change in gene expression that does not change the underlying DNA sequence. Epigenetic modifications involved in regulating gene expression can be grouped into four general categories: DNA methylation, covalent histone modifications (such as acetylation, methylation, and biotinylation), histone variants (i.e., γH2AX) and nucleosome repositioning, and miRNAs [103]. Epigenetic modification either induces or inhibits gene expression, often inducing altered expression of tumor suppressor genes or oncogenes. The result is the dysregulation of key cellular regulatory and growth control pathways, leading to carcinogenesis [104].

Epigenetic modifications have been detected in Cr(VI)-induced tumors. Aberrant methylation of tumor suppressor genes was detected more frequently in Cr-exposed tumors than non-exposed, occurring at multiple loci in 48% of Cr-exposed tumors [51]. Methylation of the tumor suppressor genes APC, hMLH1, and p16 was detected in tumors from ex-chromate workers [48, 49, 51]. Cell culture studies describing Cr(VI)-induced epigenetic modifications and their effects on gene transcription have been published at an increasing rate over the past 15 years. These studies showed Cr(VI) alters phosphorylation, methylation, acetylation, biotinylation, and microRNA levels (Table 7.8).

Two studies showed Cr(VI) exposure induced a concentration- and time-dependent increase in phosphorylation [105, 107]. Qian et al. [105] demonstrated Cr(VI) induced phosphorylation of tyrosine residues. Cr(VI)-induced tyrosine phosphorylation resulted from H_2O_2 and OH radical production that resulted during intracellular Cr(VI) reduction. In the second study, Vasant et al. [107] demonstrated Cr(VI) and Cr(V) species both induce phosphorylation of the hydroxyl side groups of tyrosine and serine/threonine residues [107].

Three studies investigated the effect of Cr(VI) on methylation. The first study found soluble Cr(VI) induced aberrant DNA methylation, while particulate Cr(VI) did not [56]. The other two studies examined Cr(VI)-induced methylation of histone proteins. Histone modifications alter the accessibility of chromatin and the recruitment of effector proteins. Histone methylation can induce transcriptional activation or repression depending on which residue is modified and the degree of methylation [103]. Interestingly, Cr(VI) induces histone methylation patterns that induce both

Table 7.8 Effects of Cr(VI) on epigenetic alterations

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium dichromate 300 μM 5–60 min	A549 human lung carcinoma cells	WB for phosphotyrosine expression	Cr(VI) induced time-dependent increase in tyrosine phosphorylation Tyrosine phosphorylation resulted from H_2O_2 and OH radical production	[105]
Potassium chromate 5–50 μM 2 h Barium chromate 0.05–0.25 μg/cm^2 24 h	V79-derived cells containing gpt reporter gene	Southern blot for methylation variants	Soluble Cr(VI) induced aberrant DNA methylation (detected at the gpt reporter) Particulate Cr(VI) did not induce DNA methylation changes	[56]
Potassium chromate 50 μM 1 h	Hepa-1 cells containing a pAhRDT-KLuc3 luciferase reporter	Reporter system for AHR-dependent gene expression, RT-PCR	Cr(VI) increased the level of HDAC bound to Cyp1a1 promoter chromatin, blocking the entry of p300 and the transcriptional complex	[106]
Potassium dichromate Cr(V) species 100–500 μM 2 min to 18 h	Bovine serum albumin and radiolabeled ATP	*In vitro* BSA phosphorylation	Concentration- and time-dependent increase in BSA phosphorylation induced by Cr(VI) and Cr(V) Cr(V) induced higher BSA phosphorylation levels than Cr(VI) Sites phosphorylated were the hydroxyl side groups of tyrosine and serine/threonine residues	[107]
Potassium chromate 50 μM 1.5 h	Mouse hepatoma Hepa-1c1c7 cells	DNA adduct analysis, WB for Cyp1a1, qRT-PCR	Cr(VI) crosslinked HDAC1-DNMT1 complexes to Cyp1a1 promoter chromatin Cr(VI) inhibited histone modifications including: • Phosphorylation of H3ser10 • Trimethylation of H3K4 • Acetylation marks in H3 and H4 These changes inhibit RNA polymerase II recruitment and transcription of Cyp1a1	[108]

(continued)

Table 7.8 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium chromate 0–10 μM 1 h	A549 human lung carcinoma cells	*In vitro* demethylase reaction, Northern blotting for mRNA, IF, WB	Cr(VI) increased levels of: • Di- and trimethylated H3K9 and H3K4 • Methylation in the MLH1 gene promoter • G9a methyltransferase protein and mRNA Cr(VI)-induced H3K9 dimethylation correlated with decreased MLH1 expression Cr(VI) decreased trimethylated H3K27 and H3R2	[109]
Potassium chromate 5–10 μM 24 h	A549 human lung carcinoma cells	WB, IF	Acute and chronic Cr(VI) exposure increased trimethylated H3K4 levels Acute Cr(VI) exposure increased global H3K4 trimethylation and H3K9 dimethylation	[110]
Potassium chromate 1.56–12.5 μM 24 h	16HBE human bronchial epithelial cells	WB, qPCR	Cr(VI) decreased biotinidase gene expression and protein levels in a concentration-dependent manner Chemically induced histone acetylation reversed Cr(VI)-induced inhibition of biotinidase, suggesting Cr(VI) may inhibit biotinidase expression by decreasing histone acetylation	[111]
Sodium dichromate 1 μM 6 months	BEAS-2B human lung epithelial cells	Colony formation in soft agar, qPCR, chorioallantoic membrane assay, IHC	miR-143 levels were reduced 35-fold in Cr(VI)-transformed cells miR-143 repression induced: • Upregulation of IGF-IR and IRS1 • ERK, HIP-1α, and NF-κB signaling • Angiogenic factor interleukin-8 • Increased angiogenesis	[112]

Table 7.8 (continued)

Cr(VI) treatment	Model system	Assay(s)	Summary of findings	Reference
Potassium chromate 0.3–5 μM 24 h	16HBE human bronchial epithelial cells	IF, WB	Cr(VI) • Decreased H3 and H4 acetylation • Increased HDAC2 and HDAC3 levels • Increased holocarboxylase synthetase protein levels Biotinidase protein levels and histone biotinylation increased at low Cr(VI) concentration, but effect was lost at higher concentrations	[113]
Potassium dichromate 5–20 μg/mL 24–48 h	*Drosophila melanogaster* (midgut tissue)	miRNA microarray, qPCR	Cr(VI) induced expression changes of 28 miRNAs Expression of 13 miRNAs increased in a concentration-dependent manner Expression of six miRNAs decreased in a concentration-dependent manner Targets of misregulated miRNAs are involved in DNA repair, oxidation/reduction, and stress-activated MAPK cascade	[114]
Potassium dichromate 5–20 μg/mL 24–48 h	*Drosophila melanogaster* (midgut tissue)	qRT-PCR, WB	Cr(VI) induced a concentration- and time-dependent inhibition of mus309 (*Drosophila* homologue of BLM)	[115]

transcriptional silencing and activation. Zhou et al. [110] demonstrated acute Cr(VI) exposure increased global H3K4me3 and H3K9me2, while chronic Cr(VI) only increased H3K4me3 levels. The second study supported this result, showing increased di- and trimethylation of H3K4 and H3K9 after 1 h exposure to potassium chromate [109]. Cr(VI) exposure also induced an increase in mRNA and protein levels of G9a methyltransferase, which plays a dominant role in H3K9 methylation. Sun et al. [109] further investigated the effect of Cr(VI) methylation on the mismatch repair gene, MLH1. Cr(VI) exposure increased methylation of the MLH1 gene promoter and correlated H3K9 dimethylation with decreased MLH1 gene expression. Sun et al. [109] also found Cr(VI) decreased trimethylated H3K27 and H3R2. H3R2me3 abrogates H3K4 methylation, and, thus, the Cr(VI)-induced decrease in H3R2me3 and increase in H3K4 methylation makes sense. However, it is curious that Cr(VI) induces decreased H3K27me3. Along with H3K9me3,

H3K27me3 is one of the strongest transcriptional repressors, producing a dramatic and predictable effect. It is interesting that Cr(VI) increases levels of one transcriptional repressor (H3K9me3) but decreases levels of the other (H2K27me3). Further work needs to be done to investigate the effect of Cr(VI)-induced decreased H2K27me3 on oncogene activity.

In contrast to the previously discussed studies, Schnekenburger et al. [108] showed Cr(VI) inhibited H3K4me3. However, this study was conducted in mouse hepatoma cells, while the others utilized A549 human lung carcinoma cells. The differences in the species and organ of origin of these cell lines may explain the difference in Cr(VI)-induced H3K4me3 levels. Schnekenburger et al. [108] also found reduced phosphorylation of H3Ser10 and acetylation of H3 and H4 following Cr(VI) exposure. This study went on to show Cr(VI) crosslinked HDAC1–DNMT1 complexes to the promoter region of Cyp1a1. These epigenetic modifications resulted in the inhibition of RNA polymerase II recruitment to the promoter and reduced Cyp1a1 transcription. Wei et al. [106] also showed Cr(VI) increased HDAC levels at the Cyp1a1 promoter region. This blocked entry of the transcriptional coactivator p300 and the transcriptional complex to the promoter region [106]. Cr(VI)-induced reduced acetylation of H3 and H4 was supported by Xia et al. [113]. In accordance with this result, Cr(VI) increased histone deacetylase HDAC2 and HDAC3 levels [113].

Cr(VI)-induced histone acetylation inhibition was shown to be related to another epigenetic modification, histone biotinylation [111]. Biotinidase and holocarboxylase synthetase mediate the binding of biotin to histones. Xia et al. [111] showed Cr(VI) exposure decreased biotinidase gene expression and protein levels in a concentration-dependent manner. Cr(VI)-induced biotinidase inhibition correlated with decreased histone acetylation. However, when histone acetylation levels were restored, biotinidase levels recovered, indicating biotinidase reduction is a result of Cr(VI)-induced histone acetylation inhibition [111]. Protein levels of the other mediator of histone biotinylation, holocarboxylase synthetase, increased following Cr(VI) exposure [113]. Interestingly, histone biotinylation increased in response to low Cr(VI) concentrations, but the effect was lost at higher concentrations. It is possible biotinidase is the stronger mediator of histone biotinylation, and holocarboxylase synthetase cannot keep biotinylation levels elevated once biotinidase levels are decreased by Cr(VI).

MicroRNAs (miRNAs) are short, noncoding small RNAs that repress the expression of genes by binding to and degrading target mRNAs [116]. This epigenetic factor has previously been shown to mediate mechanisms of toxicity in a wide spectrum of environmental chemicals [117]. Although miRNA levels have not been studied in Cr(VI)-induced tumors, plasma miRNA profiles have been described in chromate production workers. Of the miRNAs examined, Cr(VI) exposure induced decreased expression of miR-3940-5p, miR-3138, miR-4433-3p, and miR-2392 and increased expression of miR-590-5p [118]. After confounding factors were considered, only miR-3940-5p level was associated with blood Cr level. Plasma miR-3940-5p level was associated with micronuclei frequency at high blood Cr levels.

Levels of HR proteins, XRCC2 and BRCC3, were also associated with miR-3940-5p levels. These results suggest miRNA may be involved in the regulation of DNA repair proteins following Cr(VI) exposure.

Two studies have investigated the effect of Cr(VI) on miRNA expression in *Drosophila melanogaster* (Table 7.8). The first study utilized global expression profiling to examine the effect of Cr(VI) on a set of miRNAs, finding expression changes of 28 miRNAs. Expression levels of 13 miRNAs increased in a concentration-dependent manner, while six decreased in a concentration-dependent manner. The remaining nine miRNAs did not show a concentration-related effect [114]. The target genes of the Cr(VI)-misregulated miRNAs were aligned in three functional categories: DNA repair, oxidation/reduction, and the stress-activated MAPK cascade [114]. The second study focused on miR-314-3p, a miRNA significantly upregulated by Cr(VI) exposure [114]. This study showed Cr(VI) induced a concentration- and time-dependent decrease in a target of miR-314-3p, mus309 [115]. As mus309 is a homologue of the DNA repair protein BLM, this outcome suggests Cr(VI)-induced alterations in miRNA expression may inhibit DNA repair signaling.

To date, one study utilized cell culture to investigate Cr(VI)-altered miRNA expression [112]. This study transformed BEAS-2B human lung epithelial cells with chronic Cr(VI) and measured miRNA expression in transformed cells. Cr(VI)-induced cellular transformation was supported by growth in soft agar and the ability to induce tumors when injected into nude mice. miRNA microarray analysis of miRNA profiles between BEAS-Cr cells and the parental cell line revealed miR-143 was suppressed in BEAS-Cr cells. RT-qPCR analysis confirmed miR-143 was reduced 35-fold in the Cr(VI)-transformed cells. He et al. [112] showed the Cr(VI)-induced repression of miR-143-induced upregulation of IGF-IR and IRS1 as well as ERK, HIP-1α, and NF-κB signaling. miR-143 inhibition was responsible for increased levels of angiogenic factor interleukin-8 and resulted in increased angiogenesis.

Altogether, these data show Cr(VI) induces epigenetic modifications. Evidence from Cr(VI)-induced tumors, Cr-exposed workers, and cell culture studies indicate Cr(VI)-induced epigenetic modifications impact tumor suppressor genes. Epigenetic modifications may be the underlying mechanism of many of the observed Cr(VI)-induced changes in gene expression. Izzotti et al. [119] showed Cr(VI) induced increased expression of 56 genes in lung tissue of Sprague-Dawley rats. The affected genes were involved in Cr(VI) metabolism, apoptosis, cell cycle regulation, stress response, DNA repair, and signal transduction. A wealth of cell culture studies support Cr(VI) that alters expression of genes involved in all of these cell regulation pathways. For example, Cr(VI) upregulates and activates the MAPK pathways: JNK, p38, and ERK, which promote cell survival [120–124]. More research is needed to further elucidate the consequences of Cr(VI)-induced epigenetic modifications on gene expression. While epigenetic modification is likely an important factor in Cr(VI)-induced carcinogenesis, it does not provide a mechanism for the profound effect of Cr(VI) on clastogenesis.

7.6 Mechanism of Cr(VI)-Induced Carcinogenesis

Based on the body of literature discussed here, we suggest Cr(VI)-induced carcinogenesis does not adhere to just one model of carcinogenesis but involves both the genomic instability and epigenetic modification. Experimental evidence does not suggest a role of Cr(VI)-induced base mutations early in the carcinogenic pathway. However, such mutations undoubtedly occur as a result of Cr(VI)-induced DNA damage and the inhibition of high fidelity DNA repair. Thus, base mutations are likely to occur late in the carcinogenic pathway and would likely be seen in Cr(VI)-induced tumors.

Figure 7.1 outlines our proposed mechanism of particulate Cr(VI)-induced carcinogenesis. Each step of this proposed mechanism is delineated with a number that corresponds to its position in Fig. 7.1. To begin, the particulate Cr(VI) particle (1) dissolves extracellularly into the chromate anion and cation (2). Intact Cr(VI) particles can enter the cell by phagocytosis (3) but have no apparent contribution toward carcinogenesis. Likewise, the cation can enter the cell through a calcium channel (4) but also does not appear to contribute to carcinogenesis. The chromate anion mimics the structure of phosphate and sulfate and enters via facilitated transport (5). The chromate anion is then reduced intracellularly, resulting in the formation of reactive oxygen species and Cr(VI)-reduction species, Cr^{5+}, Cr^{4+}, and Cr^{3+} (6). Both the reactive oxygen and the Cr-reduction products can interact with DNA, forming adducts (7,8), which lead to stalled replication forks (9). The Cr–DNA adducts can be repaired through crosslink repair (10), which primarily relies upon nucleotide excision repair and results in the formation of DSBs (11). Mismatch repair can attempt to repair Cr–DNA adducts, but fails, undergoing a series of futile repair cycles that collapse the replication fork and result in a DSB (12). Oxidative damage can also result in the formation of DSBs if base excision repair fails (13). In response to DSB formation, Cr(VI) induces a G2 arrest (14). Prolonged Cr(VI) exposure, which would occur due to the Cr(VI) particle lodging within the bifurcation sites of the lung, results in premature centriole disengagement (15) resulting in centrosome amplification (16). Spindle assembly checkpoint bypass also results from chronic Cr(VI) exposure (17). We propose both centrosome amplification and spindle assembly checkpoint bypass lead to numerical chromosome instability (18). At the same time, prolonged Cr(VI) exposure induces a deficiency in high fidelity HR repair of DSBs (19), resulting in the use of low fidelity DNA repair pathways (20) and, ultimately, structural chromosome instability (21). Both structural and numerical chromosome instability lead to neoplastic transformation and cancer (22).

We propose Cr induces epigenetic modifications that contribute to the mechanisms underlying structural and numerical chromosome instability (23,24). Methylation of the hMLH1 promoter may induce a defect in mismatch repair that induces microsatellite instability (25,26), contributing to neoplastic transformation (22). While there is some evidence that epigenetic modifications also influence signaling pathways regulating cell survival and growth, further research is needed to understand how these contribute to the mechanism of Cr(VI)-induced carcinogenesis.

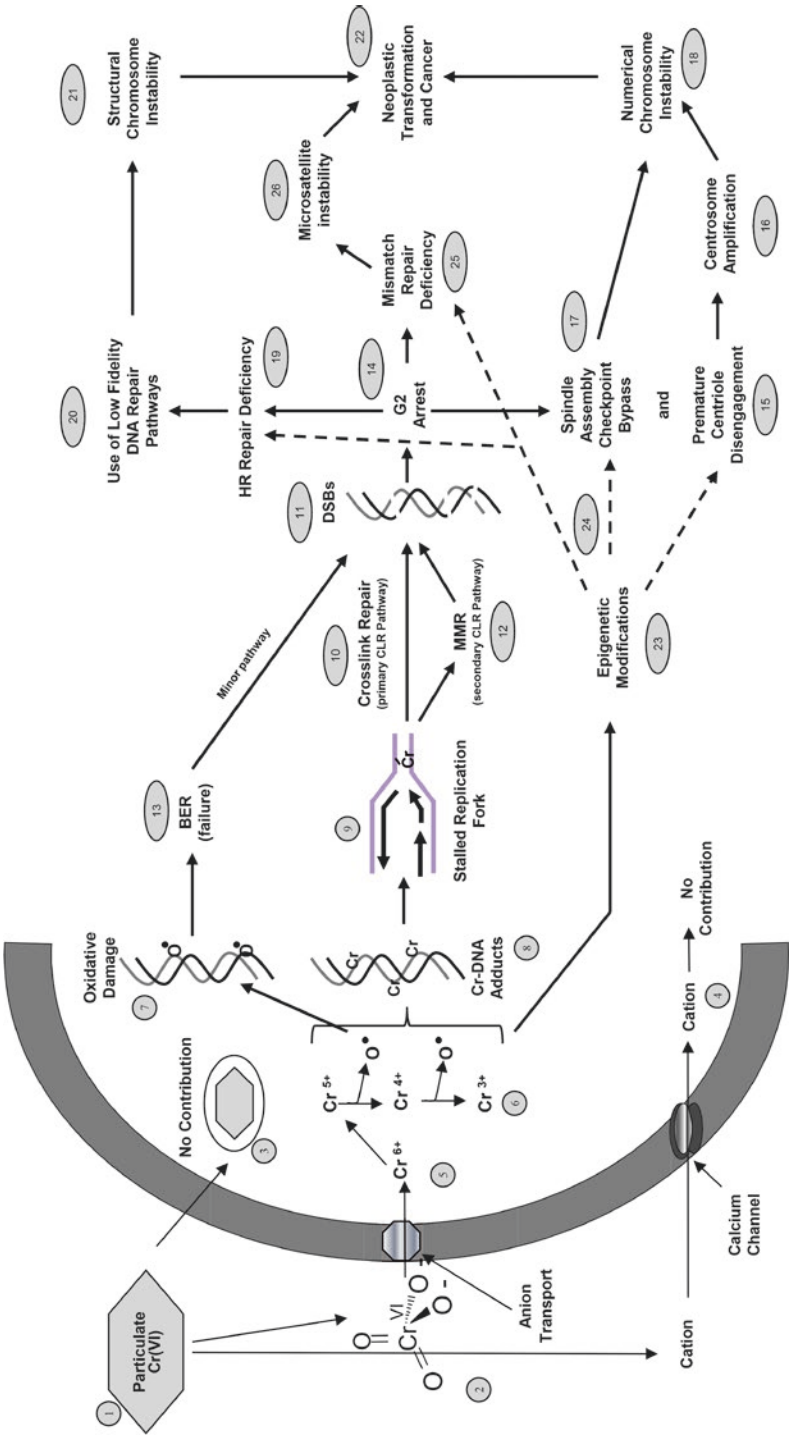

Fig. 7.1 Proposed mechanism of Cr(VI)-induced carcinogenesis. This figure outlines the molecular modifications that drive Cr(VI)-induced carcinogenesis. This process involves the formation of both numerical and structural chromosome instability, which results in neoplastic transformation. The numerals correspond with specific steps in the mechanism that are outlined in the text

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Chapter 8
Mechanisms of Nickel Carcinogenesis

Qiao Yi Chen, Jason Brocato, Freda Laulicht, and Max Costa

Abstract Nickel (Ni) is a naturally occurring metal that is widely used in an array of industries such as nickel plating, refinery, welding, as well as in the manufacturing of stainless steel, jewelry, coins, batteries, and medical devices. Despite tremendous economic values, exposure to this carcinogenic metal either through acute dermal contact or chronic inhalation in occupational settings can elicit a wide range of health problems including contact dermatitis, cardiovascular diseases, and respiratory tract cancer. Nickel-induced carcinogenesis has long been validated and studied by scientists; however despite extended studies in cell culture, animal, and epidemiology, the precise mechanism of Ni carcinogenesis is still uncertain. This chapter will seek to provide a comprehensive overview of the mechanistic roles, genetic and epigenetic alterations, in Ni carcinogenesis, as well as a review of recent advances in the area.

Keywords Nickel • Carcinogenesis • Epigenetic alterations • Mutagenicity

Abbreviations

AcCoA	Acetyl coenzyme A
BEAS-2B	Human bronchial epithelial cells
CpG	5′-C-phosphate-G-3′
DNMT	DNA methyltransferase
GPT	Glutamic-pyruvate transaminase
IARC	International Agency for Research on Cancer
JMJD1A	Jumonji domain containing 1A
JMJD3	Jumonji domain containing 3
Ni(C2H3O2)	Nickel acetate
Ni3S2	Nickel sulfide
NiSO4	Nickel (II) sulfate

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pri-miRNA	Primary miRNA
PTEN	Phosphatase and tensin homolog
RARB2	Retinoic acid receptor beta
RASSF1A	Ras association domain family 1 isoform A
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species

8.1 Introduction to Nickel

Nickel (Ni) was first isolated by Axel Fredrik Cronstedt in 1751. The silvery-white transition metal occurs naturally via forest fire, volcanic emission, and rock erosion. Ni is found in 3% of the Earth's total composition, ranking as the 24th most abundant element. Although no existing evidence indicates nutritional value of Ni in humans, it has been identified as an essential nutrient for some microorganisms, plants, and animal species [1]. Along with iron, cobalt, copper, zinc, and five other transition metals, nickel resides in the 3d orbital on the periodic table. Under normal environmental conditions, Ni mostly exists in the +2 valence state, although other oxidation states can also be found (−1, +1, +3, and +4) [1–3]. They are inherently resistant to corrosion and stable under extreme temperature fluctuations. Because of their exceptional physiochemical properties, nickel is widely used in an array of industries such as nickel plating, refinery, welding, as well as in the manufacturing of stainless steel, jewelry, coins, batteries, and medical devices. Furthermore, nickel alloy constitutes approximate 50% of materials used in the production of plane engines [4]. Despite its economic importance in the world's market, Ni is classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) and therefore poses immense environmental concerns due to its substantial commercial usage. Nickel can be found in water, air, soil, and other biological matters. The combustion of fuel, coal, and other waste materials pollutes the ambient air with nickel particulates [1, 2, 5–7]. Additionally, food-processing equipment made of stainless steel may contaminate the products with traces of Ni compounds [6, 7]. Although there are many routes of Ni exposure, inhalation is the most common form of exposure in occupational settings and has also been shown to be the primary path to Ni toxicity [8]. Despite the excessive presence of nickel in occupational settings, other than industrial workers, people living in the immediate proximity of the industrial areas and waste-receiving sites are also under the threat of Ni toxicity. Depending on the dose and length of exposure, as an immunotoxic agent and carcinogen, Ni can elicit a range of health effects such as contact dermatitis, asthma, cardiovascular disease, lung fibrosis, and respiratory tract cancer [3, 6, 9]. Despite extended studies in cell culture, animal, and epidemiology, the precise mechanism of Ni carcinogenesis is still uncertain. This chapter will seek to provide a comprehensive overview of Ni carcinogenesis as well as review of recent advances in the area.

8.2 Overview of Cancer

There are trillions of cells in the human body. Normally, new cells evolve and old/damaged cells die. However, in the case of cancer formation, cells continuously divide without stopping and eventually spread to neighboring tissues [10]. In other words, genetic alterations in cancer cells make them ignorant toward normal cell regulations such as apoptosis or programed cell death. Cell proliferation is tightly regulated by two major groups of genes: oncogenes and tumor suppressor genes [11]. Oncogenes such as Ras, Myc, and FLIP stimulate cell survival and proliferation. On the other hand, tumor suppressor genes like p53 and Rb inhibit cellular growth. The precise balance between cancer-promoting and cancer-inhibiting genes is nonetheless controlled by a sophisticated system of molecules and pathways. Dysregulation in important pathways such as cell cycle and mutation in DNA repair systems can lead to tumor initiation and eventually progression [12]. Although tumor cells can be simply defined as uncontrollable cell growth, their methods of manifestation and survival are less straightforward. Not only can cancer cells flourish by feeding off of normal surrounding cells through formation of blood vessels, they are also capable of evading immune responses. There are hundreds of different types of cancers, and none develops with the same specific set of rules. Although elucidating the mechanism of carcinogenesis is still overwhelmingly difficult, studies show that genetic susceptibility and environmental exposure are conspicuous contributors of cancer. Today, more and more substances are found to be cancer causing, and these candidates are evaluated and categorized into five different groups by the IARC: carcinogenic to humans, probably carcinogenic to humans, possibly carcinogenic to humans, carcinogenicity not classifiable, and probably not carcinogenic. There are 118 IARC-classified Group 1 substances, or identified human carcinogens, which include tobacco, radiation, processed meat, virus, etc. Most heavy metals are also considered to be carcinogens such as nickel, arsenic, chromium, cadmium, and beryllium. Due to their usefulness in many industrial processes, the opportunity for heavy metal exposure becomes increasingly threatening to public health.

8.3 Modes of Carcinogenesis

Cancer is an intricate disease that varies from person to person in development, appearance, and end point. Parallel to the disease complexity, cancer requires a multistep process for cells to undergo behavioral and metabolic changes, which will in turn prompt excessive and unnecessary growth and eventually lead to metastasis. The steps include mechanistic changes in the cells' ability to evade the immune defense, cell cycle pathways, and interaction with adjacent cells. There are two major modifications that can lead to cancer: genetic and epigenetic. Genetic changes such as mutations, translocations, copy number variations,

sister chromatid exchanges, and karyotype variations are all important factors of cancer formation. Changes in the genetic sequence can accumulate and become permanent mutations, which can be neutral, harmful, or beneficial depending on the location and/or context [13]. In fact, mutations are indispensable for the diversity among organisms. However, if these permanent DNA changes occur in essential cell cycle regulators such as tumor suppressor and promoter genes, the effects may be detrimental. Cell programming can also be disrupted through epigenetic changes, where structure rather than sequence of the DNA is altered. There are several different types of epigenetic mechanisms that can impact gene expression including DNA methylation, histone modification, and RNA-mediated silencing. The essence of the epigenetic principle lies in the fact that all cells share an identical genome; yet cells are able to demonstrate remarkably different functional and structural characteristics. Therefore, the role of epigenetic alteration on gene expression becomes ever so relevant. To date, the most common and well-understood epigenetic mechanism is DNA methylation, the addition of a methyl group to the 5-carbon position of the cytosine ring and in turn forming 5-methylcytosine [14, 15]. Cancer is generally exhibited as gene-specific hypermethylation and global hypomethylation. Hypermethylation is defined by heightened methylation in the gene promoter region, which serves to silence the expression. Hypermethylation (silencing) of tumor suppressor genes and hypomethylation (activation) of proto-oncogenes can both contribute to carcinogenesis. Posttranslational modifications of histones are regulated by histone acetyltransferases (HAT) and deacetyltransferases (HDAC), which add and take away acetyl groups, respectively. The importance of histone modifications and the correlation to cancer have been reported by multiple studies [16–18]. One other form of epigenetic change is microRNA (miRNA). These small noncoding RNAs are thought to regulate up to 30% of protein-coding genes through targeted mRNA degradation and translational repression [19]. Unsurprisingly, miRNA plays an essential role in cell proliferation and apoptosis [20, 21]. The deregulation of miRNA has also been shown in several types of cancers [22–24]. Understanding the role of genetic and epigenetic changes in cancer has provided far-reaching knowledge not only for determining the mechanism of carcinogenesis but also the discovery of potential therapeutic targets.

8.4 Environmental Exposure

The level of nickel found in the natural environment is usually low and of minor concern. Nickel is a very useful metal applicable in the production of batteries, jewelries, various alloys, nickel plating, and stainless steel [5, 20, 25, 26]. The assorted use of nickel also allows for multiple routes of exposure such as ingestion, inhalation, and dermal contact. Of all the sources of exposure, occupational inhalation from combustion of fossil fuels and nickel-related manufacturing factories is of utmost concern. Multiple studies have reported the detrimental effect of Ni on human and animal health [27–31]. Nickel refinery workers have been found to

experience very high incidences of lung, nasal, and pharyngeal cancers compared to the unexposed populations [32–34]. Figures 8.1 and 8.2 represent two separate literature searches on PubMed illustrating the growing wave of recognition and interest for nickel and its mechanistic pathways. The following sections will summarize the epidemiological, animal, and in vitro investigations in review of nickel's carcinogenic effects.

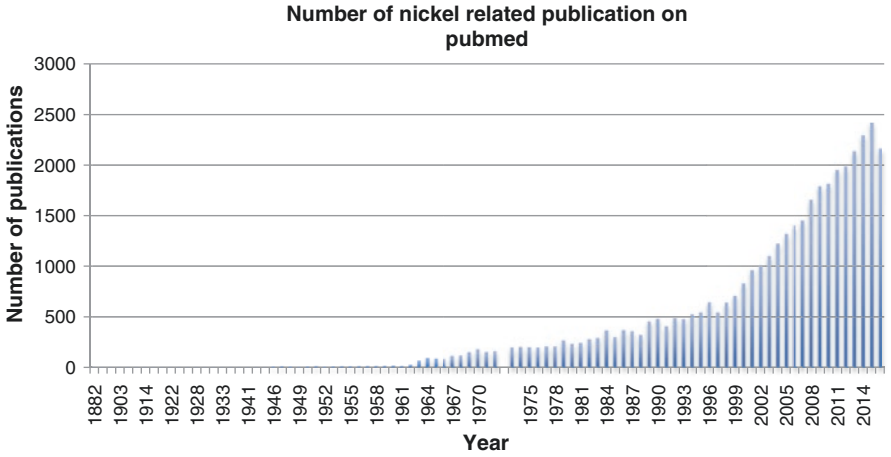

Fig. 8.1 Increasing number of PubMed publications regarding Nickel, from 1882 to 2016. The first article to report on nickel was in 1882. Since then, there has been a steady growth in the number of publications regarding nickel. By 2016, a total of 39,599 articles had been published (Data and graph generated through PubMed search)

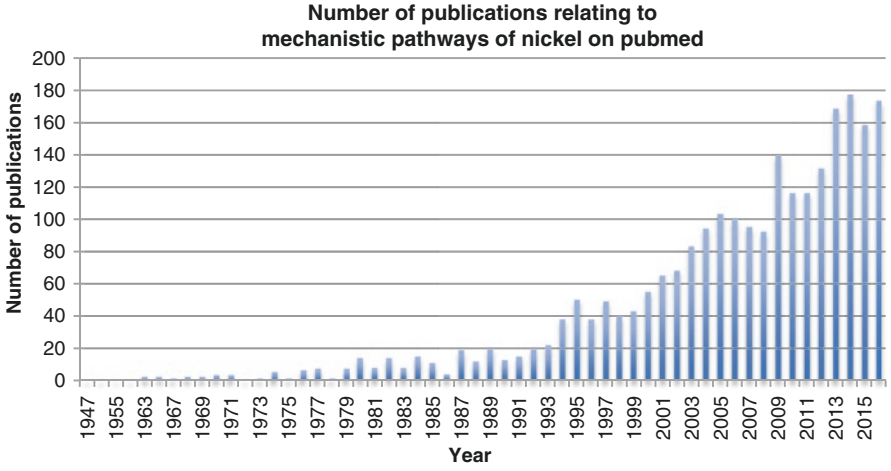

Fig. 8.2 Increasing number of PubMed publications regarding the mechanistic pathways of Nickel, from 1947 to 2016. Sixty-five years after the first publication on nickel, in 1947, the first paper exploring mechanistic pathway of nickel was published. The following rapid increase in the number of publications in this area of research is indisputable. From 1947 to 2016, 2451 papers have been published (Data and graph generated through PubMed search)

8.5 Epidemiological Investigations

Back in 1949, the National Insurance of Great Britain and Minister of Pensions categorized respiratory diseases such as lung and nose cancers, as industrial diseases due to the prominence in occupationally exposed nickel refinery workers [33]. Multiple lines of evidence have implicated nickel's role in human lung cancer. A large retrospective cohort involving 5389 men carried out by a research team led by Grimsrud reported a dose-dependent association between water-soluble Ni and lung cancer. The investigators indicated an estimated population attributable risk of 54%, suggesting that the excess number of lung cancer found in the cohort was due to occupational Ni exposure [31].

In a retrospective cohort study conducted in Sudbury, Ontario, and Canada, the researchers examined the cancer incidence and mortality rate of male nickel workers [35]. There were a total of 10,253 participants in this study, all of which worked for Xstrata Nickel between 1928 and 2001. The study concluded that cancer incidence and mortality rates significantly elevated in conjunction with increased number of working years. Although the study could not examine the specific exposure level of these workers, the results clearly indicate that the risk of nickel carcinogenesis is highly correlated with the exposure time.

In another investigation conducted in South Wales, researchers aimed to determine the risk of developing lung and nasal carcinoma of 845 men who have worked at least 5 years in the nickel refinery prior to 1944 [36]. Inhalation is the primary route of exposure in occupational settings, which explains high incidences of cancer in the lung and nasal sinuses. In this study, nasal carcinoma was found to persistently affect nickel refinery workers even after exposure to the carcinogen has been eliminated for 15–42 years. On the other hand, lung cancer incidences decreased over time. This suggests that the site of primary exposure and, in this case nasal sinus, suffers from the greatest impact.

8.6 Animal Bioassays

An in vitro study carried out by Sunderman and Donnelly back in 1965 studied the effect of nickel on metastasizing pulmonary tumors in rats [37]. The study was divided into six groups: three exposed groups and three control groups. The exposed groups were differentiated by the exposure dose and length: single dose of 80 parts per million for 30 min (Group I), single dose at 80 parts per million and additional "dithiocarb"(Group II), and 4 parts per million for 30 min for the rest of their lives (Group III). Within 3 weeks of the study, 72% of the rats in Group I died. After 1 year, the rats in Group III (chronic low-level exposure) showed 25% mortality compared to 7% in the control group. After 2 years, 88% of the rats in Group III died compared to 30% in the control group. This strain of rats is known for its rarity in developing pulmonary tumors. Over a period of 12 years, not one rat in the control group developed tumor. On the other hand, six rats in the exposed group were

found to have developed pulmonary carcinoma with metastasis [37]. The study reveals that inhaled nickel carbonyl may be especially carcinogenic.

Another study led by Ottolenghi focused on pulmonary carcinogenesis in rats after chronic inhalation of nickel sulfide [38]. 226 and 241 F344 rats were randomly placed in the exposure and control groups, respectively. In the treatment group, the animals were exposed to nickel sulfide through inhalation 5 days per week, 6 h per day for 78 weeks. The control group rats were alternatively exposed to filtered air. After another 30-week period of observation, the rats were sacrificed and examined for tumor development. Overall, incidences of lung tumor in the control and treatment groups were 1% and 14%, respectively. Furthermore, pulmonary inflammatory response was also significantly increased in the exposed group.

Although lung carcinogenesis is predominantly studied in nickel exposure studies due to high occupational exposure, nickel is in fact detrimental to any exposed parts of the body. In a study done by Damjanov et al., various concentrations of nickel subsulfide were injected in the testis of Fischer rats. Rats injected with 0.6–10 mg of nickel subsulfide showed immediate inflammatory reaction at the site of injection [39]. At doses of 5 or 10 mg, the testis displayed subtotal destruction, inability to regenerate the seminiferous tubules, and overall atrophy. The damage was remarkably specific to the site of exposure as the other testis showed no effect.

8.7 In Vitro Investigations

Previous studies have confirmed that carcinogenic metals are capable of binding to the chromatin and proteins [40], and thereby inducing chromosomal aberrations, DNA-protein cross-links, and DNA single-strand breaks. In vitro studies also illustrated nickel's ability to induce cell transformation and epigenetic changes.

Anchorage independent-growth is an important trait for carcinogenesis. Biedermann and Landolph studied the effect of three different types of nickel compounds and their induction of anchorage-independent growth in human diploid foreskin fibroblasts cells (HFC). Cells were treated for 48 h with various metals including nickel sulfide (Ni3S2), nickel acetate (Ni(C2H3O2)), and nickel (II) sulfate (NiSO4). As a result, the cells treated with all three types of nickel compounds showed dose-dependent anchorage-independent growth. As another aspect of this study, the researchers also demonstrated dose-dependent cytotoxicity due to nickel treatment [41]. Because no detectable levels of mutations were found, the researchers believe that anchorage-independent growth may be due to chromosomal breakage and/or gene amplification.

More and more emphasis is being placed on the epigenetic mechanism of nickel carcinogenesis. DNA methylation was found to inactivate the expression of a stably integrated reporter gene, gpt, near the telomeres of Chinese hamster cells [42]. It is thought that DNA methylation, and subsequently induced chromatin condensation, engulfed the nearby gpt gene, thereby inhibiting its expression. In turn, after reverse activation of gpt, DNA methylation and condensation were lost.

Both oxidative stress and DNA methylation have been implicated in a study involving C57BL/6 and p53 heterozygous mice treated with nickel sulfide. After nickel sulfide injection, malignant fibrous histiocytomas were observed in all treated mice. DNA methylation of tumor suppressor gene p53 and activation of the mitogen-activated protein kinase signaling pathways were detected in all tumors [43].

8.8 Genetic Mechanisms Underlying Nickel Carcinogenesis

Due to weak mutagenic potential found in mammalian cells, and even weaker response in prokaryotic assays, nickel compounds have generally been considered to be weakly mutagenic [9, 44–48]. The lack of mutagenic activity of nickel compounds in prokaryotic assays may be due to bacteria's inability to induce phagocytosis, a factor important for nickel toxicity. Another potential explanation for the stronger mutagenic potential found in mammalian cells is the number of DNA-associated proteins. Nickel's binding affinity for proteins is substantially higher than for DNA; thus the genotoxic effects would be greater in mammalian cells due to heightened interaction [44]. In these mammalian studies, nickel compounds have been found to induce both mutations and chromosomal aberrations.

Nickel compounds have been found to have slightly positive mutagenic effects in a number of forward mutation assays using various cell lines such as V79 and mouse lymphoma cells [3, 44, 49, 50]. Deletion mutations were detected from these studies and have been deemed as a potential mutagenic mechanism for nickel. Occurrence of mutagenesis on an autosomal gene is justifiable because allelic chromosome can compensate for the loss from large deletions. The finding that nickel sulfide induced strong mutagenic response in an autosomal gene, transfected bacteria gpt gene, supports this notion [44, 51, 52].

In addition to nickel's ability to promote mutations, other potential genetic mechanisms of nickel carcinogenesis include chromosomal aberrations, DNA-protein cross-links, and DNA base damage [53–57]. In a study conducted by Conway and Costa, anchorage-independent clones from nickel-transformed Chinese hamster cells were chosen and collected for karyotyping analysis [9]. Study results demonstrated abnormal structural and numerical chromosomal changes in all nickel-transformed cell lines [54]. Another study examined the effect of Ni (II) sulfate on cultured human lymphocytes and found an approximately twofold increment in sister chromatid exchanges [55].

8.9 Epigenetic Mechanisms

Despite some evidence of nickel's weak mutagenicity, the recent advances in understanding the mechanism of nickel carcinogenesis have shifted toward epigenetic alterations. Epigenetics is defined as the inheritable and reversible changes in gene

expression without changing the DNA sequence [58, 59]. DNA resides in a highly compact structure called the chromatin. The accessibility of the chromatin, depending on its closed or open structure, is essential for biological functions such as replication, translation, gene expression, etc. [58, 59]. Epigenetic alterations such as DNA methylation, histone modification, and small noncoding RNA are critical factors in inducing changes in the chromatin structure.

DNA methylation is correlated with repetitive sequence suppression, X-chromosome inactivation in imprinting, and long-term transcriptional silencing. Although methylation in the promoter region is linked to suppression due to interference with the binding of transcription factors, gene body methylation usually signifies activation. In mammalian cells, approximately 60–90% of 5′-cytosine-phospate-guanine-3′ (CpGs) are methylated. DNA methyltransferases (DNMTs) are responsible for transferring a methyl group from S-adenosyl methionine to the fifth carbon of cytosine. CpG sites are regions of the DNA where cytosine is followed by guanine. CpG islands contain methylated CpGs about every 15 nucleotides as opposed to the rest of the genome where CpGs occur every 80–100 nucleotides.

DNA methylation-induced gene inactivation is associated with numerous human diseases such as fragile X mental retardation and various types of cancers [42, 60–64]. In a study conducted by Costa et al., nickel was shown to directly inhibit DNA methyltransferase activity [65]. 5-Azacytidine is another known inhibitor of DNA methyltransferases, but in contrast, the inhibitory effect of nickel seems only transient [65, 66]. More specifically, after a period of recovery following nickel exposure, the methyltransferase activity will rebound slightly, while genome-wide DNA methylation levels will rise even higher than that of control cells [65]. Research suggests that the Ni-induced hypermethylation may be targeted toward tumor suppressor genes and/or senescence as part of its carcinogenesis mechanism [65, 67]. Nickel-induced promoter hypermethylation has been observed both in vitro and in vivo. In human bronchial epithelial (BEAS-2B) cells, nickel induced the silencing of E-cadherin, a gene encoding for surface adhesion glycoprotein [68]. Furthermore, Ni treatment has also been shown to silence p16 gene through hypermethylation of CpG sites and subsequently bypassing cell senescence [69]. Studies using p53 heterozygous mice treated with nickel sulfide exhibited promoter hypermethylation of the tumor suppressor p16 gene. Similar results were also shown in studies involving Wistar rats, which showed promoter hypermethylation in p16, Ras association domain family 1 isoform A (RASSF1A), and retinoic acid receptor beta (RARB2) genes [43, 58, 70]. Early studies have demonstrated that instead of active euchromatic regions, nickel selectively targets the inactive heterochromatic regions such as the long arm of chromosome X in Chinese hamster ovary cells. One study demonstrated nickel's heterochromatic-specific hypermethylation through inserting the glutamic-pyruvate transaminase (gpt) gene in two different locations of two different cell lines: G12 and G10. The gpt gene was inserted near an active euchromatic region in the G10 cell line, while in G12 cell line, the gene was close to the telomere [71]. If the gpt gene were silenced by Ni exposure, the cells would exhibit high-level resistance to 6TG. As results conclude, while Ni-induced gpt gene silencing could be observed in both G12 and G10 cells, the silencing was much more efficient in

G12 cells. Thus, the study supported previous findings indicating the importance of location for Ni-induced gene silencing. Heterochromatin is a highly compact region of the DNA characterized by late S-phase replication and minimal gene activity due to its condensed structure. The mechanism of gpt gene silencing in G12 cells demonstrates the ability of Ni to spread heterochromatinization through DNA condensation [42]. The idea of position effect variegation such as heterochromatin spreading has been well studied in drosophila [42, 72, 73].

Nickel has also been found to inhibit dioxygenases, a family of enzymes essential for a balanced epigenetic landscape, and requires iron, oxygen, ascorbate, and alpha-ketoglutarates as cofactors [74]. Nickel has been shown to target the iron-binding motif of dioxygenases due to its effectively higher affinity than iron. The resulting irreversible inhibition of dioxygenases led to remarkable increases in DNA methylation marks [74–77]. Previous studies have shown that nickel exposure inhibited Jumonji domain containing 1A (JMJD1A, demethylase) expression while at the same time increased H3K9me2 [77]. An even more recent study illustrated the effect of Ni on Jumonji domain containing 3 (JMJD3), another prominent demethylase. The study concluded that following nickel exposure, JMJD3 expression increased while H3K27me3 decreased. The reduction of H3K27me3 can stimulate gene activation and lead to various types of cancers such as gastric, colon, kidney, prostate, breast, and ovarian cancers [78–81].

8.9.1 Acetylation

Other than DNA methylation, nickel can also trigger gene silencing through histone modifications. Histones are alkaline proteins in which the DNA winds around and forms nucleosomes, the basis of chromatin. There are five major families of histones: H1, H2A, H2B, H3, and H4. H1 serves as the linker histone connecting nucleosomes and forming higher order structures, while the other four are essential core histones. The N-terminal tails protruding from the nucleosome beads have more than 60 different residues, each of which can be altered by posttranslational modifications such as acetylation, methylation, sumoylation, phosphorylation, biotinylation, and ubiquitination [58, 82]. Of the above posttranslational modifications, histone acetylation is one of the most extensively studied topics. Histone acetylation is a very dynamic phenomenon and is balanced by the contrasting activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC). HAT serves to transfer an acetyl group from acetyl coenzyme A (AcCoA) to an e-amino group of a lysine residue. Upon the acetylation of a lysine residue, the positive charge of the histone side chain is removed, subsequently decreasing histone's affinity to the negatively charged DNA. The loosely bound DNA will then become more accessible to transcriptional factors in the promoter region.

Ni exposure has been identified to reduce global histone acetylation levels both in vitro and in vivo [83–85]. In G12 cells, both H3 and H4 hypoacetylation were observed in the promoter of the gpt transgene [68]. Hepatoma cells exposed to $Ni2^{+}$

resulted in reduced histone acetylation levels. The reduction in histone acetylation may be due to two factors: inhibition of HAT and activation of HDAC. Since prior studies have shown that HDAC activity has no effect on Ni-induced histone hypoacetylation, the globally reduced acetylation levels must be triggered by HAT inhibition [86, 87]. In vitro HAT and HDAC assays conducted by Kang et al. confirmed this idea through demonstrating that $Ni2^{+}$ inhibited HAT activity in a dose-dependent manner while HDAC remained unaffected. The same study also confirmed the claim that Ni2+ may induce histone hypoacetylation through reactive oxygen species (ROS) generation [83, 88, 89]. The in vitro results illustrated increase in ROS production after Ni exposure as well as dose-dependent decrease in HAT activity correlated with ROS levels [86]. Because ROS are extremely reactive and can bind to the DNA, histones, and surrounding proteins, it may play an important role in Ni-induced histone hypoacetylation and carcinogenesis.

Another interesting study demonstrated that carcinogenic nickel is capable of inducing alpha-helical conformation of the histone H4 tail. Because the secondary structure is similar to the effect of lysine acetylation, this phenomenon will prevent the transfer of an acetyl group to the lysine residue. In other words, carcinogenic nickel seems to have tricked the histone acetyltransferase into believing that the transfer of an acetyl group has already been done. Furthermore, if the histone modification by enzyme 1 is able to influence the activity of enzyme 2, then the Ni-induced alpha-helical structure will influence a spectrum of enzymatic changes acting on the histone tails [89].

8.9.2 MicroRNA

MicroRNAs (miRNAs) are a family of small noncoding RNAs (18–25 nucleotides) important for many cellular processes such as metabolism, apoptosis, proliferation, and differentiation [90]. To date, more than 2000 mature miRNAs have been identified in the human genome each with unique functions in negatively regulating gene expression [58, 90, 91]. Forming mature miRNA requires a series of sophisticated micro-processing events. Primary miRNAs (pri-miRNA) stretching hundreds to thousands of nucleotides long are first transcribed in the nucleus by RNA polymerase II then capped, polyadenylated, and processed by Drosha, a type III RNase, into short (~70 nt) precursor miRNA (pre-miRNA) [92–94]. A complex formed with exportin-5 and Ran-GTP will assist in exporting the pre-miRNA into the cytosol where it is further processed by RNase III Dicer into ~18–25 nucleotide long mature miRNAs [92, 95, 96]. Following the formation of mature miRNAs, one of the strands will typically be degraded and while the other single-stranded miRNAs will exert their regulatory functions through binding to the complementary sequences of coding as well as noncoding regions of target mRNAs. Incorporation of miRNA and aragonite protein into RNA-induced silencing complex (RISC) will direct the 3′ to 5′ binding to the mRNA and elicit target mRNA inhibition and/or degradation [58, 92].

MiRNA dysregulation in cancer cells was first discovered by Calin et al. [97]. Since then, multiple studies have confirmed and indicated that miRNA profiles significantly differ between tumor and normal tissues [97–99]. Because miRNA is nonspecific and is able to bind to multiple mRNAs, dysregulation in these small noncoding RNAs may elicit tremendous effect on gene expression and ultimately carcinogenesis. MiRNA-21 is one of the most commonly upregulated miRNAs found in various types of cancers such as glioblastoma, stomach, lung, colon, ovarian, etc. [100, 101]. A new study recently reported that miR-21 expression levels found in nickel-induced human lung cancers increased in a dose-dependent manner. Clinically, patients with high nickel exposure and high miR-21 expression have significantly lower rate of survival. For example, the upregulation of miRNA-222 was observed in both Ni-transformed 16HBE cells and rat rhabdomyosarcomas [102]. MiRNA-222 targets several important cancer suppressor genes such as p57, p27, and phosphatase and tensin homolog (PTEN) and may play an important role in accelerating cell proliferation as observed in cell transformation and tumor growth. These results strongly indicate that miRNA may play important roles in Ni-induced carcinogenesis.

8.10 Conclusion

Despite the importance and practicality of using nickel in various industrial settings, there are immense repercussions for people who are exposed to this carcinogenic metal. Exposure to nickel comes in many forms ranging from acute dermal contact to chronic inhalation in occupational environments. And depending on the dose and length of exposure, nickel can elicit a spectrum of health concerns such as contact dermatitis, cardiovascular diseases, and respiratory tract cancer. Nickel has long been known to induce chromosomal aberrations and DNA damage, with recent advances in epigenetic studies, research has further improved our understanding of nickel carcinogenesis. Nickel is able to silence genes near heterochromatin regions through initiating chromatin condensation, and depending on the effected gene, the suppression of gene expression may evoke initiation and/or progression of tumor formation. Furthermore, nickel has also been found to influence the epigenetic landscape through DNA methylation, histone acetylation, and miRNA. The downregulation of important tumor suppression genes such as p16, PTEN, p57, etc. through epigenetic silencing and oncogene-specific activation may all contribute to nickel-induced cancer initiation and progression.

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Chapter 9
Application of Metallomics and Metalloproteomics for Understanding the Molecular Mechanisms of Action of Metal-Based Drugs

Yuchuan Wang, Haibo Wang, Hongyan Li, and Hongzhe Sun

Abstract Metals play a significant role in biological processes, and metal-based drugs nowadays have been commonly used for therapeutic and diagnostic purposes. However, due to severe side effects with metallodrugs and acquired drug resistance, more metallodrugs are being developed, with improved pharmacological profiles and less side effects. With the rapid development of metallomic strategies in understanding metals in complex biological systems, their successful application in the field of medicinal inorganic chemistry has led to significant progresses in understanding the mechanisms of actions of metal-based drugs. This chapter introduces the concepts and research techniques in metallomics and metalloproteomics and expatiates the fate of metal-based drugs as well as metallic nanoparticles in biological systems revealed by metallomic studies.

Keywords Metallomics • Metalloproteomics • Metal-based drugs • Cancer therapy • Nanoparticles

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Abbreviations

16HBE	Normal bronchial epithelial cells
2D-GE	Two-dimensional gel electrophoresis
A549	Human alveolar adenocarcinoma epithelial cells
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
Au NRs	Au nanorods
CBS	Colloidal bismuth subcitrate
CE	Capillary electrophoresis
ESI-MS	Electrospray ionization mass spectrometry
GE-ICP-MS	Online coupling of column-type gel electrophoresis with inductively coupled plasma-mass spectrometry
GO	Gene ontology
H. pylori	*Helicobacter pylori*
HF5	Hollow fiber flow field-flow fractionation
HSAB	Hard-soft acid-base
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-TOF-MS	Inductively coupled plasma time-of-flight mass spectrometry
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
IMAC	Immobilized metal affinity chromatography
KP1019	*Trans*-[Ru(III)$(Ind)_2Cl_4$][IndH]
LA-12	(*OC*-6-43)-bis(acetato) (1-adamantylamine) amminedichloro-platinum (IV)
LA-ICP-MS	Laser ablation-inductively coupled plasma-mass spectrometry
LoVo	Human colon carcinoma cell line
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAPK	Mitogen-activated protein kinase
MRP	Multidrug resistance protein
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification
NAMI-A	*Trans*-[Ru(III)(dmso)(Im)Cl_4][ImH]
NanoSIMS	Nanoscale secondary ion mass spectrometry
NPs	Nanoparticles
PAK	Protein kinase
pIs	Isoelectric points
PMH	Primary hepatocytes
PTA	1,3,5-Triaza-7-phosphatricyclo-[3.3.1.1]decane

RBC	Ranitidine bismuth citrate
RBP4	Retinol-binding protein 4
RPLC	Reversed-phase liquid chromatography
RPTECs	Renal proximal tubule epithelial cells
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SRIXE	Synchrotron radiation-induced X-ray emission
SR-TXM	Synchrotron radiation-beam transmission X-ray microscopy
SR-XANES	SR-X-ray absorption near edge structure
SR-XRF	Synchrotron radiation X-ray fluorescence
TEM	Transmission electron microscopy
THP-1	Human monocyte
WHO	World Health Organization
XAS	X-ray absorption spectroscopy

9.1 Introduction

Metals are integral components of life and serve as essential cofactors of many enzymes involved in various biological processes such as respiration, gene transcription, and cell proliferation [1, 2]. The introduction of metal ions into pharmaceuticals for therapeutic or diagnostic purposes constitutes the basic research issues in medicinal inorganic chemistry. Metal compounds provide an ideal platform for the rational design of drug candidates with predictable pharmacodynamics and pharmacokinetics due to the characteristic geometries, coordination numbers, and redox states that metals can offer [3–5]. The success of cisplatin and other platinum-based drugs in clinic has demonstrated great potentials of metal-based compounds in cancer therapy [6]. However, due to severe side effects accompanying traditional platinum chemotherapy and the acquired drug resistance, other classes of metal-based compounds, such as ruthenium, gold, gallium, and titanium, were developed and extensively studied in the search for a better drug with improved pharmacological profiles and less side effects [5, 7, 8]. Besides metal-based anticancer drugs, bismuth, silver, and gallium complexes have been used for decades owing to their antimicrobial properties [4, 9, 10]; the metalloid element arsenic in its inorganic form, i.e., arsenic trioxide (ATO, Trisenox®), has been successfully used in clinic for the treatment of patients suffering from acute promyelocytic leukemia (APL) [11]. The choice of metals/metalloids with different chemical properties may result in different spectrum of anticancer or antimicrobial activities, enabling better therapeutic options (Fig. 9.1)

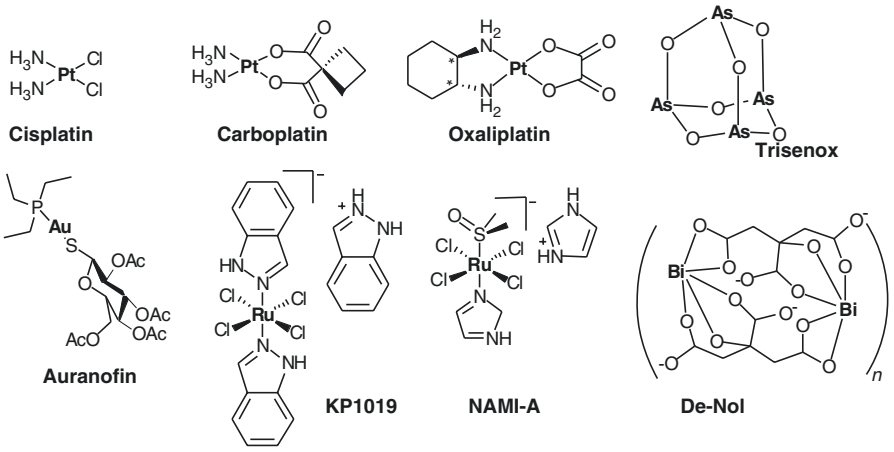

Fig. 9.1 Selected structures of Pt-, Ru-, Au-, As-, and Bi-based drugs/agents

[4, 5, 12]. Meanwhile, nanotechnology is increasingly recognized with nanoparticles being produced and utilized as widespread commercial products [13]. Despite their wide applications in healthcare and industry, it remains to be elusive whether increased in the exposure of human beings to these products leads to short- or long-term toxicity.

As the mode of action and the toxic mechanisms of many metal-based compounds have not, or only partially, been elucidated even for those established treatments, a precise knowledge of the molecular mechanisms of actions of metal-based drugs/agents including their physiological processes (e.g., cellular levels, distribution, biotransformation), molecular targets, and functional pathways is essential not only for the exploitation of the full therapeutic potential of a metallodrug but also for the design of novel metallodrug candidates with higher therapeutic efficacy but less side effects. Traditionally, the mechanism of action of a (metallo)drug is interpreted on the basis of accumulated experimental data, which were obtained from the in vitro studies of individual putative drug-targeting proteins or other biomolecules, and the real targets are often needed to differentiate from a false one by extensive research. However, considering the inherent complexity of the biological systems, such target-oriented studies are laborious to provide a holistic picture on the biological response of a (metallo)drug. An integrative "omics" approach is thus necessary for the investigation of a (metallo)drug or a biologically active substance within the system biology perspective. With the rapid development of metallomic strategies in understanding metals in complex biological systems, their successful application in the field of medicinal inorganic chemistry has led to notable progresses in understanding the mechanisms of actions of metal-based drugs/agents [14–16].

In this chapter, the concepts and research techniques in metallomics and metalloproteomics will be first introduced. The fate of Pt-, Ru-, and Bi-based drugs/agents as well as metallic nanoparticles in biological systems revealed by metallomic studies will be expatiated in the following sections.

9.2 Metallomics and Metalloproteomics: The Role in Metallodrug Research

9.2.1 Definition and Research Subjects in Metallomics

Metallome refers to "an element distribution" in a cell which was initially proposed by R.J.P. Williams in 2001 [17]. *Metallomics* is an emerging research area aimed at the entirety of metal and metalloid species within a cell or tissue, with a goal to comprehensively analyze the physiological role of metals/metalloids as well as their functions associated with various biomolecules [18, 19]. In 2004, Hiroki Haraguchi first described metallomics as "integrated biometal science" [20], after which metallomics has been receiving continuous attentions. As an interdisciplinary scientific filed, metallomics is anticipated to develop as a frontier of chemistry, complementary to genomics and proteomics (Fig. 9.2).

Metallomics covers a wide range of research subjects related to biometals, including the distribution and chemical speciation of metals and metalloids in biological systems, identification of metal-binding proteins/enzymes, characterization of metal-binding biomolecules, design of inorganic drugs for chemotherapy, and metabolisms of metals and biomolecules [20, 21]. Among them, the distribution and chemical speciation of metallome in biological samples are considered as the basic research issues in metallomics. In the field of medicinal inorganic chemistry, metallomics offers great tools for monitoring the cellular behavior of metal-based drugs/agents including their intracellular distribution, levels, biotransformation, and interference with other essential elements, which is critical for evaluation of drug efficacy and tolerance.

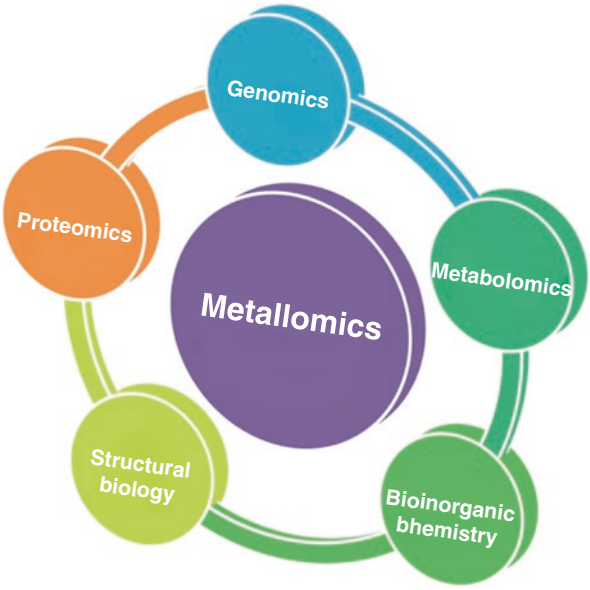

Fig. 9.2 The interdisciplinary feature of metallomics. Metallomics is an integrated biometal science that is complementary to genomics, proteomics, metabolomics, bioanalytical and bioinorganic chemistry, and structural biology of metalloproteins

9.2.2 *Metalloproteomics*

It is estimated that about a quarter to one third of all proteins and enzymes are associated with metals in biological milieu [2]. Metalloproteins are defined as proteins whose function or structural stabilization is conferred by metal cofactors, while the concept of metalloproteins should be differentiated from metal-binding proteins, which refer to proteins that bind metal ions owing to favorable thermodynamic conditions [22]. As defined by Sarkar et al., metalloproteome is the set of proteins with metal-binding capacity by being metalloproteins or possessing metal-binding sites. As a branch of metallomics, metalloproteomics aims to recognize the important relationship between biometals and proteins [23]. An interesting aspect of metalloproteomics is to identify metal-binding proteins, which would be an efficient way to reveal novel functions of proteins with metal-binding abilities.

Identification of drug-binding proteins is a critical step toward understanding the molecular mechanism of action of a drug. Different from organic compounds that often target on a single specific protein, metal-based compounds show strong inclinations to attack on multiple protein targets due to the versatile coordination number and geometry of a metal in complexation with proteins [12, 24]. It is thus important to identify the reliable druggable targets as comprehensively as possible and to unveil the complicated interactions established among the drug-targeting proteins and the large variety of biomolecules within a cell, in order to evaluate the full therapeutic potential of a metallodrug.

Conventional comparative proteomics is based on the comparison of the protein expression profiles of drug-treated and untreated samples analyzed by the well-established proteomic techniques, in which the identified up- or downregulated proteins provide clues for the cellular responses to the drug [25]. With the aid of high-throughput metal selection, detection, and characterization techniques developed in metallomics, unequivocal identification and characterization on a set of metalloproteins, metal-binding proteins, and their metal-binding motifs in a given biological sample could be successfully achieved nowadays [26–29]. Metalloproteomic study offers metal-specific and complementary information with regard to the metallodrug-associated proteins at the proteome-wide scale, which enables a holistic picture to be obtained on the molecular mechanisms of action of a metallodrug.

9.3 The Technical Platform in Metallomics and Metalloproteomics

As a promising interdisciplinary field, metallomics has manifested its potential in various research areas, such as biological chemistry, clinical chemistry, environmental chemistry, and nutrition [19, 30, 31]. Meanwhile, significant progress has been made in this field, largely attributed to the rapid development of a variety of analytical techniques.

9.3.1 Chemical Speciation of Bioactive Metallome

The physiological functions of metals and metalloids (metallome) are substantially dependent on their chemical forms and the cellular biomolecules they are associated with. In analytical chemistry, quantification and identification of the chemical forms of metallic elements is defined as "chemical speciation" [20, 32]. Quantitative determination of trace elements in biological samples can be achieved by inductively coupled plasma-mass spectrometry (ICP-MS), which is a highly sensitive and robust technique that is capable of detecting a wide range of metals and several nonmetals at low concentrations. ICP-MS has the advantage of detecting multiple elements simultaneously, making it particularly useful for monitoring metallodrug pharmacokinetics, i.e., quantification of metallodrug absorption and distribution in different cellular compartments [33, 34]. Recently, time-resolved ICP-MS has attracted much attention for elemental analysis in single cells, providing metallome information concerning cell-to-cell variations [35, 36]. By applying ICP time-of-flight mass spectrometry (ICP-TOF-MS), multiparametric analysis of over 30 receptors at the single-cell level can be achieved similar to that of cytometric analysis, providing insights into a single cell's response to therapeutics [37]. Hyphenation of laser ablation to ICP-MS (LA-ICP-MS) is a powerful tool for in situ probing of elemental distribution in biological samples with high spatial resolution (<1 μm) and is feasible for quantitative imaging [38]. It uses a high-powered laser to ablate the surface of solid samples, e.g., the surface of electrophoresis gels or biological tissues. The aerosols formed by the ablated analytes are swept into the ICP source by a continuous stream of argon, and the ions formed are analyzed by mass spectrometry [39].

Advanced nuclear analytical techniques have also been extensively applied to monitor the bioactive metallome [40]. Advances in instrumentation for the third-generation synchrotron microprobe beamlines have allowed high-resolution spatial speciation analysis by synchrotron radiation X-ray fluorescence (SR-XRF) in tissues, cellular compartments, or even individual cells. The X-ray microbeams can penetrate sample in depth with resolution up to 0.05 μm [41]. X-ray absorption spectroscopy (XAS) is a valuable tool for probing the changes in the chemical environment of metal centers, such as metal oxidation states in cells, the coordination motif of the probed metal, and the identity and number of adjacent atoms [40, 42]. As a noninvasive technique, XAS has been successfully used to follow the biotransformation of metallodrugs in biological fluids [43].

9.3.2 Metalloproteome Separation and Identification

Many well-established proteomic approaches, in particular the platform for protein separation and identification, are applicable for metalloproteomics. However, separation and identification of metal-binding proteins from a complex biological sample

remains challenging since the non-covalently bound metal-protein complexes may readily dissociate during analysis, largely owing to the experimental conditions employed for better separation efficiencies, e.g., denaturing buffer conditions in the presence of detergents and denaturants, the high voltage applied for electrophoresis [44]. Moreover, given the complexity of biological samples, it is often necessary to perform several fractionation steps to achieve better resolution [45, 46].

Two-dimensional gel electrophoresis (2D-GE) is one of the most widely used high-resolution separation techniques in proteomics, although it cannot be coupled online to detectors. It separates protein mixtures in the first dimension according to the differences in protein isoelectric points (pIs) by isoelectric focusing (IEF), followed by the second-dimensional SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) separation that separates proteins according to molecular masses. To preserve metal-protein interactions in metalloproteomic analysis, native IEF and non-denaturing PAGE separations were often employed. The latter includes native PAGE, in which SDS and reducing agents are excluded in the buffer system, and blue native (BN)-PAGE, in which coomassie blue dye provides the charges to protein complexes, as well as anodal native (AN)-PAGE that performs in a basic buffer [47]. The separated proteins are usually visualized by coomassie blue or silver staining, and the metallic elements on the unstained gels can be detected and quantified by LA-ICP-MS or SR-XRF, alongside protein identification by MS techniques after tryptic digestion of the protein spots of interest.

Liquid-based separation techniques such as size-exclusion chromatography (SEC), capillary electrophoresis (CE), ion-exchange chromatography (IEC), and reversed-phase liquid chromatography (RPLC) employ much softer experimental conditions thus favoring the metal-protein integrity during separation [44]. These separation techniques are often coupled online/off-line with a variety of mass spectrometry [48], including electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), tandem mass spectrometry (MS/MS), and ICP-MS, which allow the position, identity, and the elemental composition of a metalloprotein to be determined. Recently, continuous flow column-type gel electrophoresis coupled with ICP-MS (GE-ICP-MS) was developed and for the first time applied to complex biological samples, allowing the detection of metals and their associated cellular proteins simultaneously [28, 49]. Although liquid chromatography is inherently limited with regard to resolution, they could constitute one potential separation dimension in a multidimensional fractionation strategy [26, 50]. As demonstrated by Cvetkovic et al., improved resolution can be successfully achieved by employing multiple chromatographic steps for microbial metalloproteome separation, although sometimes substantial amounts of protein mixtures as starting materials are required (300 mg of total bacterial proteins in [26]).

Immobilized metal affinity chromatography (IMAC) is a metal-specific separation method for selective enrichment of proteins/peptides with metal-binding abilities [51]. The interactions between proteins and the immobilized metal ions are mainly governed by coordination chemistry and the hard-soft acid-base (HSAB) theory. Numerous metal ions such as Ni^{2+}, Cu^{2+}, Zn^{2+}, and Bi^{3+} being chelated on

IMAC columns have been applied for analyzing the bio-coordination of metals in cells and for exploring the roles of metal-binding proteins in metal homeostasis, metal-induced toxicities, protein phosphorylation, and so forth [52]. It is worth paying attention that IMAC is not selective to a specific class of proteins since all molecules presenting affinity for a specific metal ion have the chance to be retained on IMAC column, and proteins with occupied and buried metal-binding sites may not be captured by the immobilized metals. Thus, it is important to evaluate the possible interfering substances that would inhibit protein binding to the active site of the column.

Determining the exact mode of action of a metallodrug is not only to identify reliable druggable targets but also to understand the basis of the numerous interactions established between the drug target and a large variety of biomolecules. The typical technological platforms for generating the complex sets of data to interpret the modes of action of metallodrugs are represented by the *omics* approaches, such as proteomics and genomics that are capable of providing large amounts of qualitative and quantitative information of the proteins and genes in a biological sample, and bioinformatics can be subsequently applied to interpret the large collections of omics data by incorporating computer tools and statistical methods. However, comprehensive identification and functional annotation of proteins/genes are firstly needed for this type of analysis.

9.4 Biotransformation, Cellular Distribution, and Molecular Targets of Pt-and Ru-Based Anticancer Drugs/Agents

9.4.1 Platinum

Platinum-based drugs are nowadays essential components in cancer chemotherapy that are highly effective. Cisplatin, *cis*-diammine-dichloroplatinum(II), is the first metal-based drug to enter into clinical use worldwide for the treatment of various types of solid tumors. New-generation platinum anticancer drugs such as carboplatin, oxaliplatin, nedaplatin, and satraplatin, which exhibit clinical activity against cisplatin-resistant cancers with less side effects, were subsequently developed and approved for clinical use [6].

It is well understood that cisplatin and its analogues enter cells by passive diffusion and active transport with the aid of human copper transporter hCTR1, followed by intracellular hydrolysis and activation, and subsequent formation of intra-strand cross-linked adducts with DNA [53–55]. The in situ cellular distributions of Pt within human cancer cells after treatment with cisplatin or other Pt-based compounds were monitored by synchrotron radiation-induced X-ray emission (SRIXE) and microprobe synchrotron radiation X-ray fluorescence (SR-XRF), which showed that Pt accumulated more in the cell nucleus than in the surrounding cytoplasm, indicating that DNA is the major target of Pt-based compounds, and

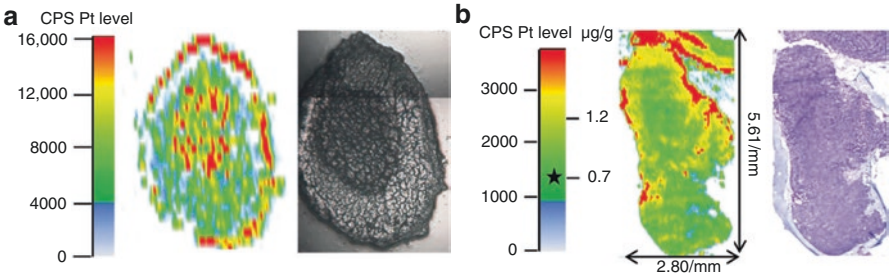

Fig. 9.3 Platinum distribution in tumor models. (**a**) LA-ICP-MS analysis of HCT116 tumor spheroids treated with satraplatin. Adapted with permission from ref. [60]. Copyright (2016) Royal Society of Chemistry. (**b**) LA-ICP-MS analysis of tumor sections from a satraplatin-treated CT-26 tumor-bearing mouse. Adapted with permission from ref. [61]. Copyright (2015) Royal Society of Chemistry

relatively stable Pt-based compounds may potentially overcome cellular toxicity induced by Pt binding to cytoplasmic proteins [56–58]. The complementary use of LA-ICP-MS and MALDI MS imaging analysis showed the differences in the penetration and distribution of cisplatin and oxaliplatin in human tumor samples. Cisplatin was found to penetrate deeply into the tumors, whereas oxaliplatin was mostly identified at the periphery of the tumor tissues. However, results obtained from ICP and MALDI MS imaging were not much coherent in the case of cisplatin [59]. Very recently, the distribution of platinum-based compounds in multicellular tumor spheroids was assessed by LA-ICP-MS with optimized setup, demonstrating the feasibility of LA-ICP-MS in studying the spatially resolved metal distribution in a 3D tumor model [60]. Predominant platinum accumulation was found at the center as well as the periphery of the spheroids, which corresponds to the necrotic core and the proliferating outermost layers of cells, respectively (Fig. 9.3a). The deep penetration of platinum into tumor spheroids indicates an increased chance of drug damaging nonproliferating tumor cells, which could be taken as a criterion for preclinical drug selection [60].

To gain information on the in vivo distribution of platinum-based anticancer agents, quantitative LA-ICP-MS was applied to the tumor and kidney sections of platinum-treated mice bearing the preclinical CT-26 tumor model [61]. Correlated analysis of the platinum distribution in tumor samples obtained by LA-ICP-MS with the histological pictures of a consecutive H&E-stained cryosection showed that much higher amounts of platinum were found in areas of (loose) soft tissues than in the malignant parts of the tumor samples (Fig. 9.3b). As the extent of drug penetration into cancerous tissue is important in estimating the potential of antitumor drug candidates, data on average platinum uptake determined by ICP-MS may sometimes lead to biased conclusions. A combined imaging approach consisting of LA-ICP-MS, nanoscale secondary ion mass spectrometry (NanoSIMS), and transmission electron microscopy (TEM) was recently applied to the tissue samples of tumor-bearing mice upon administration of platinum(IV) compounds, showing uneven platinum distribution in the organs [62]. Further subcellular-scale imaging

with NanoSIMS and TEM revealed that platinum accumulated mainly in sulfur-rich organelles such as lysosomal in the tumor, which is in good agreement with a generally high affinity of platinum to sulfur.

Platinum(IV) compounds have been extensively explored to replace cisplatin, as they are usually more inert than their platinum(II) analogues and have the potential to be more resistant to nonspecific bio-interactions. Metal-based compounds with high oxidation states are always speculated to be reduced to active species in vivo due to the intracellular reduction atmosphere. Indeed, the intracellular reduction of Pt(IV) compounds was first observed by using XANES [63]. The proportions of Pt(II) and Pt(IV) present in A2780 ovarian cancer cells determined from the peak-height ratios of XANES spectra revealed that, after 24 h incubation, all Pt in the +IV oxidation state was fully reduced to the +II oxidation state. However, the rapid and premature reduction of Pt(IV) complexes in vivo is a major impediment to reduce the selectivity and activity of Pt(IV) prodrugs toward cancer cells. Nevertheless, by use of XANES spectroscopy, Pt(IV) complexes with dicarboxylato equatorial ligands were observed to exhibit more inert kinetic properties than their analogous with dichloride ligands in the reduced biological context, providing an important basis on the design of novel platinum(IV) prodrugs [64].

Although DNA has long been believed to be the primary target of platinum, several proteins/enzymes have recently been proposed to be involved in the antitumor activities of platinum compounds [65]. The first cisplatin-binding protein in *E. coli* cell extracts was successfully identified using 1D-SDS-PAGE off-line coupled with LA-ICP-MS [66]. The protein band corresponding to the most intense Pt peaks was found to contain outer membrane protein A, which acted as an ion channel with potential relevance for cisplatin uptake. With the aid of LA-ICP-MS, cisplatin-binding proteins in rat serum and renal proximal tubule epithelial cells (RPTECs) were also tracked [67]. A number of proteins such as transferrin, serum albumin, α-2-macroglobulin, α and β hemoglobin subunits were identified in the serum sample from an in vivo cisplatin-treated rat, which were in accordance with those reported cisplatin-binding serum proteins obtained from in vitro studies. Several proteins from RPTECs were identified to contain Pt, including core histones (H2A, H2B, H3, H4), 40S and 60S ribosomal proteins, and enzymes such as malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, enolase and elongation factor Tu, etc., which may have a connection with cisplatin-induced cell type-specific nephrotoxicity. Recently, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was applied to measure the plasma proteomic profiles of rats in response to a new platinum anticancer agent (*OC*-6-43)-bis(acetato)(1-adamantylamine) ammine dichloroplatinum (IV) (LA-12) [68]. By analyzing 72 rat plasma samples randomized according to the LA-12 administration dosages and time, the level of retinol-binding protein 4 (RBP4) was identified to be significantly correlated with LA-12 level in treated rats. RBP4 could thus serve as a serum marker for LA-12 activity. Moreover, in view of the decreased expression level of RBP4 in a number of human cancers and the importance of retinol in controlling cellular differentiation, the induction of RBP4 levels upon LA-12 treatment may indicate the restoration of retinol-induced signaling pathways in cancer cells.

To obtain a systemic view on the mechanisms of cisplatin toxicity, an integrated genomic and proteomic analysis of cisplatin-treated mouse primary hepatocytes (PMH) was performed, which enabled the identification of 19 pathways that were simultaneously altered, providing novel insights into the possible pathways of cisplatin-induced hepatotoxicity [69]. The pathway map constituted by cisplatin-regulated proteins and genes clearly illustrated that cisplatin perturbs several well-known pathways such as oxidative stress, drug metabolism, fatty acid metabolism, glycolysis and TCA cycle, and also some less known pathways such as urea cycle and inflammation metabolism. Alteration of these interconnecting pathways would inevitably lead to deleterious effects on human hepatocytes.

9.4.2 Ruthenium

Ruthenium compounds are regarded as promising alternatives to platinum compounds, as they are often identified as less toxic and generally more selective to tumors. Two Ru(III) compounds, *trans*-[Ru(III)(dmso)(Im)Cl_4][ImH] (NAMI-A, Im = imidazole, dmso = dimethylsulfoxide) and *trans*-[Ru(III)$(Ind)_2Cl_4$][IndH] (KP1019, Ind = indazole), have successfully completed phase I clinical trials. The antitumor activities of other Ru compounds with similar groups of ligands but different modes of reactivity in biological media, such as polypyridyls, arenes, and ammines, are attracting great attention as well [70, 71]. Transportation of ruthenium compounds into tumor cells via the transferrin pathways serves as an important step toward their therapeutic activities, as the transferrin receptors are often overexpressed in tumor cells [72].

The first XRF imaging of cellular distribution of Ru in single human neuroblastoma cells revealed the distinct cellular fates of KP1019 and NAMI-A [73]. Colocalization of Ru with Fe in both the cytosol and nuclear regions could be observed after treatment with KP1019. In contrast, no Ru could be visualized in cells after treatment with NAMI-A, which is in accordance with the proposed membrane-binding mechanism of action of NAMI-A. XRF analysis of analogues of KP1019 containing iodinated indazole ligands revealed the identical cellular distributions of Ru and I in single A549 cells [74], indicating the conservation of the intact Ru-N bonds in the studied complexes after a series of biological interactions. Comparative analysis of the biotransformation of NAMI-A and KP1019 under biological relevant conditions by XAS suggested that the higher cytotoxicity of KP1019 than NAMI-A is most likely due to its slower extracellular decomposition, resulting in enhanced cellular uptake through passive diffusion [75].

RAPTA (ruthenium organometallic complex based on 1,3,5-triaza-7-phosphaadamantane) complexes with a general formula of [Ru(arene)(PTA)X_2] (PTA = 1,3,5-triaza-7- phosphatricyclo-[3.3.1.1]decane, X = halogenide or biscarboxylate) are a class of organometallic ruthenium (II) compounds, which exhibit remarkable in vitro and in vivo antimetastatic effects [76]. A combined metallomics and proteomics study of RAPTA-T (T = chloride) in human ovarian cancer

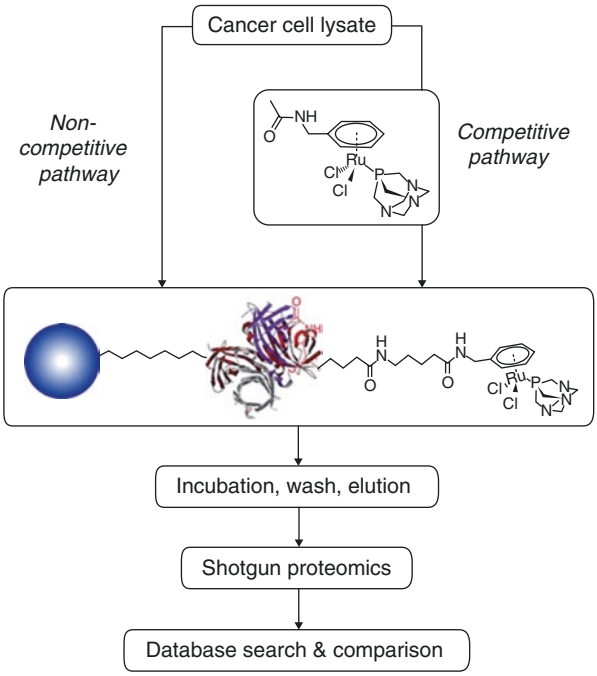

Fig. 9.4 Workflow of the metallodrug pull-down experiments. Only high-affinity RAPTA-binding proteins could bind to the modified beads in the presence of the competitive drug binder. Adapted with permission from ref. [78]. Copyright (2015) Royal Society of Chemistry

cells revealed the distinct physiological properties of RAPTA-T in comparison to cisplatin. The compound is less affected than the platinum-based drugs by the detoxification mechanisms in cisplatin-resistant cell lines, evidenced by the altered uptake, cellular distribution, and metallation of DNA of RAPTA-T in A2780cisR cells [77]. The molecular targets of RAPTA compounds were recently revealed by a metallodrug pull-down study [78]. By passing cancer cell lysates through RAPTA-analogue-immobilized beads, the high-affinity drug-binding proteins were comparatively identified in the presence of a competitive binder (Fig. 9.4). Among them a number of cancer-related proteins, including cytokines midkine, pleiotrophin, and fibroblast growth factor-binding proteins 3, are identified, which may be associated with the antimetastatic and antiproliferative activity of RAPTA compounds.

Owing to the different pharmacokinetic, the combination therapy of Pt- and Ru-based drugs is considered of great potential to circumvent drug resistance and increase the effectiveness of treatment [79, 80]. Analysis of the binding preference of NAMI-A and cisplatin in human plasma by metallomic approaches revealed that both drugs interact with essentially the same proteins such as human serum albumin precursor, macroglobulin α2, and human serotransferrin precursor, without affecting each other's metabolism [79, 81]. Cisplatin interacts with proteins much stronger, while the interactions of NAMI-A with proteins are largely reversible, which may be contributed to the significantly lower toxicity of NAMI-A [79]. Along with the demonstrated efficacy of Pt and Ru drug combination in mice experiments, metallomics data provided more mechanistic insights to support further exploration of the combination therapy in clinical studies.

9.5 Multi-targeted Bi-Based Antimicrobial Drugs

Helicobacter pylori (*H. pylori*), a transmissible human pathogen strongly related to gastrointestinal diseases, is now infecting over half of the world's population [82]. Epidemiological data in 1990, 2002, and 2008 revealed that more than 5% global cancer burden was attributable to the infections of *H. pylori* [83–85], and the bacterium was designated as class I carcinogen by the World Health Organization (WHO) [86]. Bismuth, the heaviest stable element in the periodic table with a highly variable coordination number from 3 to 10, exhibits strong affinities to thiolate sulfur and oxygen but can be well tolerated by human even at high doses [87]. Bismuth-based triple or quadruple therapies, such as colloidal bismuth subcitrate (CBS, De-Nol®) and ranitidine bismuth citrate (RBC, Tritec® or Pylorid®) combined with antibiotics, have been commonly recommended for eradicating *H. pylori* in clinic [9].

The uptake of bismuth by *H. pylori* cells can be rapidly monitored by time-resolved ICP-MS at the single-cell level [36]. Intact cells are directly introduced into ICP-MS, producing spike signals with intensities proportional to the quantity of the analyte ions in single cells. The large variation of the observed ^{209}Bi spike intensities revealed the significant cellular heterogeneity (Fig. 9.5a), suggesting the cells growing at different cell cycle stages that led to distinct cellular responses to the metallodrug. CBS-treated *H. pylori* deposited ca. 1.0×10^6 Bi atoms/cell and the uptake process took ~3 h to reach the half maximum (Fig. 9.5b). Interference of ferric ions on bismuth uptake was also observed, indicative of competitive transport pathways between Bi and Fe (Fig. 9.5c).

Albeit the clinical usage of bismuth-based antimicrobial agents for decades, surprisingly no resistance of *H. pylori* to bismuth drugs has ever been reported [88]. Accumulative studies indicated that the binding of bismuth to multiple proteins, particularly some key enzymes, might contribute to their antimicrobial effects. Bi-binding proteins in *H. pylori* have been systematically analyzed by different metalloproteomic approaches (Fig.9.6), including Bi-IMAC in combination with 2-DE [89], partial denatured 1D SDS-PAGE coupled with LA-ICP-MS [90], and

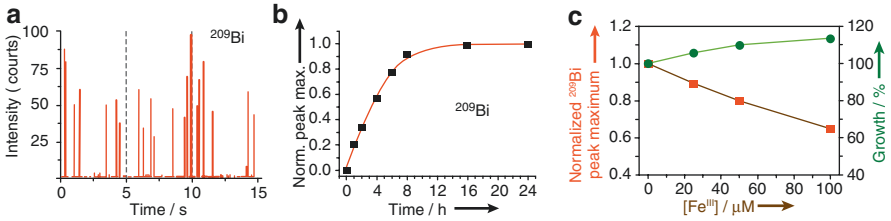

Fig. 9.5 Tracking CBS uptake in single *H. pylori* cells. (**a**) ICP-MS temporal profile of CBS-treated *H. pylori* suspension. (**b**) Normalized peak maximum of ^{209}Bi vs incubation time for CBS-treated *H. pylori*. (**c**) Protective effect of Fe(III) against CBS accumulation in *H. pylori*, demonstrated by plots of the normalized peak maximum of ^{209}Bi. Adapted with permission from ref. [36]. Copyright (2011) American Chemical Society

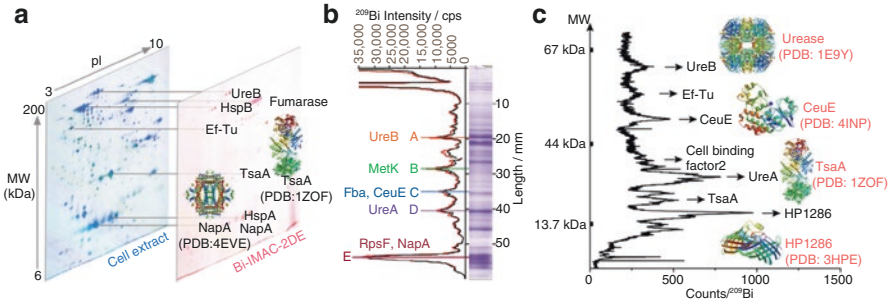

Fig. 9.6 Bi-binding proteins in *H. pylori* analyzed by different metalloproteomic approaches. (**a**) Bi-binding proteins in *H. pylori* 11637 enriched by Bi-IMAC and separated by 2-DE. (**b**) Bi-binding proteins in *H. pylori* 26695 analyzed by partial denatured SDS-PAGE coupled with LA-ICP-MS. (**c**) Profile of Bi-binding proteins in *H. pylori* 26695 analyzed by GE-ICP-MS. The identified proteins with structures deposited in PDB are illustrated in the figure. Adapted with permission from ref. [16]. Copyright (2015) Royal Society of Chemistry

the recently developed robust strategy based on online coupling of column-type gel electrophoresis with ICP-MS (GE-ICP-MS) [28, 91]. Several common but also unique Bi-binding proteins were dug out by different methods, indicating the reliability and complementarity of these strategies. Using GE-ICP-MS, the profile of bismuth-associated proteins in *H. pylori* was established for the first time, and the protein fractions corresponding to the major bismuth peaks were subsequently collected and identified by MALDI-TOF-MS. Seven Bi-binding proteins were unequivocally identified (Fig. 9.6c), including five previously reported proteins UreA, UreB, TsaA, CeuE, and Ef-Tu that related to the colonization, antioxidation, and translational process of the bacterium, as well as two newly identified proteins HP1286 and cell-binding factor 2, involving isoprenoid quinone metabolism and toll-like receptor 4 binding, respectively. The observed profile of Bi-binding proteins in *H. pylori* verified that Bi exhibited its antimicrobial activity via a multi-targeted mode of action [28].

Bi-IMAC on-column digestion coupled with high-throughput LC-MS generates rich information on Bi-protein-binding interfaces [29]. Over 300 nonredundant Bi-binding peptides from 166 proteins in *H. pylori* were identified by Bi-IMAC. Bi(III) exhibits high selectivity toward peptides rich in cysteines and histidines with the dominated motif patterns of CX_nC, CX_nH, and HX_nH (Fig. 9.7) and may broadly interfere with protein functions. Gene ontology (GO) enrichment analysis further characterized the GO categories that enriched by the identified proteins. The identified putative Bi-binding proteins are mainly involved in protein metabolic process, GTP catabolic process, and oxidation-reduction process, with the functions of RNA binding, transition metal ion binding, protein binding, and GTPase activity. Any malfunctions of the related proteins might lead to the bacterium to be eradicated. The versatile modes of action proposed in this study provide a rational basis for the high efficacy and low resistance of Bi-based antimicrobials.

Intriguingly, bismuth drugs rarely exert acute toxicity in human. A recent systematic pharmacological study offers a potential explanation for the selective

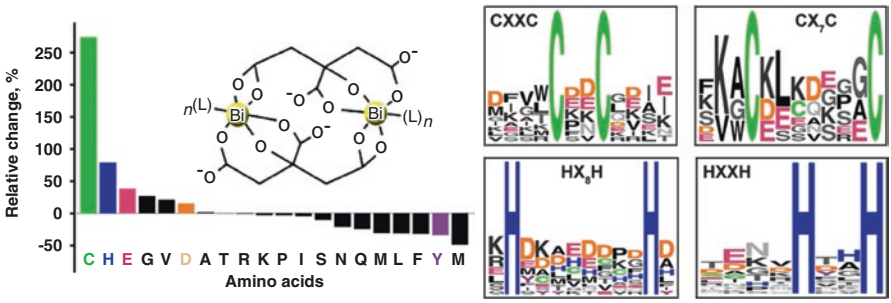

Fig. 9.7 Bi(III) exhibits cysteine and histidine-oriented binding preference in *H. pylori*. The dominated motif patterns of CXXC, CX_7C, HX_8H, and HXXH are illustrated. Adapted with permission from ref. [29]. Copyright (2015) Royal Society of Chemistry

action of bismuth on certain pathogens [92]. Quantitative analysis of bismuth metabolism in human cells revealed that over 90% of bismuth was passively absorbed, conjugated to glutathione, and transported into vesicles via multidrug resistance protein (MRP) transporter. The consumption of intracellular glutathione activates de novo biosynthesis of glutathione, which subsequently facilitates the passive uptake of bismuth and thus completes a self-propelled positive feedback loop. This mechanism robustly removes bismuth from both intra- and extracellular space, protecting cells from acute toxicity. Glutathione is thus the key for the selectivity of bismuth drugs between human and pathogens that lack of glutathione, for instance *H. pylori*.

9.6 Cytotoxicity of Metallic Nanoparticles

The number of publications dealing with the toxicity of nanoparticles (NPs) is increasing steadily. Applications of NPs in medicine as diagnostic and therapeutic agents [13, 93], the interactions of NPs with proteins and other biomolecules [94, 95], as well as the advanced analytical techniques [64, 96] developed for nanotoxicology have been well summarized and critically commented in many reviews. Herein, we will focus on the uptake, distribution, transformation, and bio-nano interactions as revealed by metallomics and metalloproteomics.

Understanding the chemical transformation during intracellular processes of NPs is vital to evaluate their toxicity. However, it is a great challenge to capture image of metallic NPs with high resolution in a single cell and to monitor the chemical transformation of intracellular NPs. By integrating synchrotron radiation-beam transmission X-ray microscopy (SR-TXM) and SR-X-ray absorption near edge structure (SR-XANES) spectroscopy, 3D spatial distribution of AgNPs inside single human monocyte (THP-1) cells and the chemical transformation of silver were captured (Fig. 9.8). It was found that the cytotoxicity of AgNPs was largely due to the chemical transformation of silver from elemental silver $(Ag^0)_n$ to Ag^+ ions and

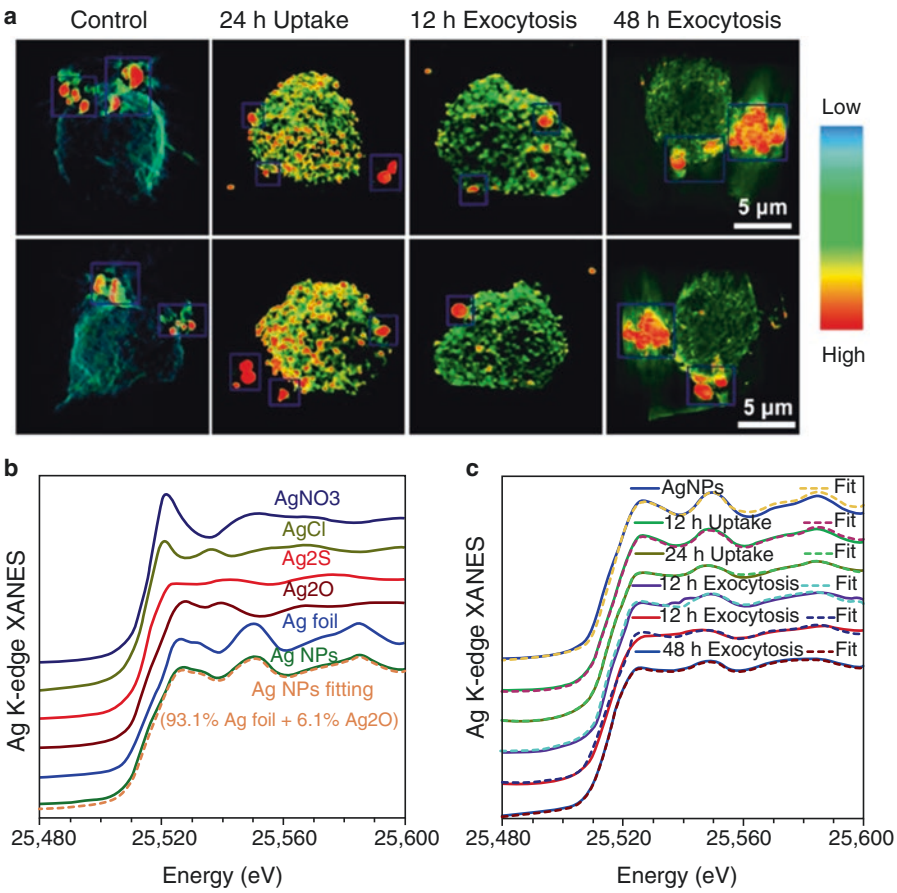

Fig. 9.8 Distribution and chemical transformation of silver species in cells. (**a**) The spatial distribution of AgNPs in a single THP-1 cell captured by SR-TXM. The highest accumulation of silver was observed at 24 h, while during exocytosis, the intracellular concentration of silver decreased with time. (**b**) Chemical species of silver in reference samples and AgNPs as determined by silver K-edge XANES. (**c**) Changes in silver chemical species of the cell samples during the cellular uptake of AgNPs and the removal of silver according to silver K-edge XANES. Results indicated that AgNPs were gradually transformed into Ag-O- and then Ag-S- forms. Adapted with permission from ref. [97]. Copyright (2015) American Chemical Society

Ag-O-, then Ag-S- species, which provides direct evidence for the long-lasting debate on whether the nanoscale or the ionic form dominates the cytotoxicity of silver nanoparticles [97]. Furthermore, the present approach together with physiological tests provides an integrated strategy capable of exploring the chemical transformation and cytotoxicity in metallic nanoparticles in general.

Apart from the in situ monitoring of the distribution and transformation of metallic nanoparticles, approaches to simultaneously separate and quantify metallic nanoparticles and free metal ions are also necessary. Full spectrum separation, characterization,

and quantification of various Ag^+ species (i.e., free Ag^+, weak and strong Ag^+ complexes) and AgNPs with different sizes were achieved by online coupling of hollow fiber flow field-flow fractionation (HF5) and minicolumn concentration with multiple detectors (including UV-vis spectrometry, dynamic light scattering, as well as ICP-MS) [98]. Among the multiple components of the hyphenated device, HF5 was employed for filtration and fractionation of AgNPs (>2 nm), while the minicolumn packed with Amberlite IR120 resin was used to trap free Ag^+ or weak Ag^+ complexes from the radical flow of HF5 together with the strong Ag^+ complexes and tiny AgNPs (<2 nm), which were further discriminated in the second run of focusing by aiming to oxidize more than 90% of tiny AgNPs to free Ag^+ and trapped in the minicolumn. The feasibility of the new method was well verified by comparison with the results obtained from transmission electron microscopy and further confirmed the satisfactory recoveries for seven silver species, including Ag^+, Ag^+-cysteine, and five AgNPs with diameters of 1.4 nm, 10 nm, 20 nm, 40 nm, and 60 nm in surface water samples. This approach has a great potential to study the fate and transformation of AgNPs and Ag^+, as well as other engineered NPs in environment and healthcare.

Considering the synchrotron radiation-based facilities are not readily accessible, LA-ICP-MS is a good alternative for elemental bioimaging in cell/tissue due to its convenience, high sensitivity, as well as spatial resolution. LA-ICP-MS was utilized for subcellular mapping of the distribution of AgNPs and AuNPs in single fibroblast cells [99]. High spatial resolution with visualization of subcellular structures was achieved after optimization of ablation/scanning parameters, i.e., scan speed, ablation frequency, and laser energy, and the results demonstrated that nanoparticles were accumulated in the perinuclear region with a dose and time dependence. The precise quantification of the number of AgNPs and AuNPs at the single-cell level was achieved with a matrix-matched calibration using nitrocellulose membranes doped with nanoparticle suspension, which can be further extended to the quantification of other metallic nanoparticles.

Systematic investigation of the cellular response of nanoparticles at metabolomics level is another useful approach to evaluate toxicity of nanoparticles. NMR, GC-MS, and CE-LC-MS are the main techniques involved in analyzing metabolic alterations upon treatment of NPs systematically. The comparative responses of human alveolar adenocarcinoma epithelial cells (A549) and normal bronchial epithelial cells (16HBE) exposed to Au nanorods (Au NRs) showed that Au NRs are translocated from the lysosome to the mitochondria in A549 cells but not in normal 16HBE cells. NMR-based metabolomic technique was also applied to analyze the metabolic changes in Au NR-induced A549 and 16HBE cells, revealing time-dependent and cell-specific metabolic response of tumor cells and normal cells to protein-coated Au NR exposure [100]. Modulation of the microenvironment by suppressing the levels of lactate as well as induction of severe oxidative stress and subsequent cell death in tumor cells compared with normal 16HBE cells might be the main factors involved in selectivity and cell-specific toxicity of the protein-coated Au NRs, demonstrating the potential of metabolomic techniques in screening anticancer nanodrug candidates and elucidating molecular mechanisms of their action.

Besides the uptake, transformation, and distribution of NPs, there is an urgent need to investigate the molecular targets and mechanisms underlying the cellular

responses that might be triggered by NPs. Conventional 2-DE combined with MALDI-TOF-MS has been extensively applied in identification of up- or down-regulated proteins in different organisms post to metallic NPs [101]; however, only limited information has been obtained due to the compromised separating resolution of 2-DE. Using MS-based shotgun proteomics and quantifying peptides with labeling of iTRAQ could be a better option to gain an insight into the alteration of protein levels induced by NPs. The toxicity of AgNPs evaluated in human colon carcinoma cell line (LoVo), an in vitro model of the human intestinal tract by iTRAQ [102] showed that some unique cellular processes are driven by the size of NPs. The 20 nm nanoparticles induced direct effects on cellular stress, including generation of reactive oxygen species and protein carbonylation. In addition, proteins involved in SUMOylation were upregulated after exposure to 20 nm AgNPs, whereas the 100 nm nanoparticles exerted indirect effects via serine/threonine protein kinase (PAK), mitogen-activated protein kinase (MAPK), and phosphatase 2A pathways. MS-based proteomics are capable of accurately identifying and quantifying proteins involved in cellular events, providing information about protein-protein interactions, which cannot be achieved by other nuclear techniques.

Despite of the extensive researches on the underlying mechanisms of nanotoxicity, there is still a paucity of comprehensive biological and toxicological information in this area. To facilitate the study of toxicity of NPs, it is necessary to develop novel techniques with non-destructiveness, high sensitivity for in situ monitoring of their bio-interactions [64]. Additionally, integrated methodologies to reveal the nanotoxicity at genome, transcriptome, proteome, metabolome, and metallome levels will allow to gain a whole scenario on their toxicological mechanisms. A good showcase study is to investigate how *Chlamydomonas reinhardtii* responses to silver contamination at proteome, transcriptome, and cellular levels by microarray and multidimensional protein identification (MudPIT) [103]. Such information is particularly important to assess potential health risks arising from the use of NPs and for rational design of novel NPs for clinical application. Besides, scientists from different disciplines of materials, chemistry, physics, biology, microbiology, and medicine can work together to tackle the crisis caused by the increasing usage of NPs [95].

9.7 Conclusion and Perspective

Metallomics and metalloproteomics in combination with chemical biology and bioinformatics approaches have significantly facilitated the in-depth understanding of the cellular fate of metallodrugs, as well as the functional connections between metallodrugs and numerous biomolecules. Such knowledge combined with clinical data would allow more effective metallodrugs with low toxicity and high therapeutic indices to be rationally designed. Many methodologies have been implemented into metallomics and metalloproteomics as described in this chapter, with *pros and cons* for each of them. In spite of remarkable progress being made, the exact mode of action and toxic mechanisms of many metal-based drugs/agents are still elusive,

largely owing to the intrinsic complex nature of the biological system. Continuous efforts are required to improve the current analytical approaches and to introduce innovative methodologies in the hope of establishing an integrated platform to unveil the role of metals in biology.

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