

ABSTRACT

Title of Dissertation: MODULATION OF P21 HUMAN TUMOR SUPPRESSOR GENE EXPRESSION BY ZINC STATUS IN HUMAN HEPATOBLASTOMA AND NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

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The transcriptional regulation of *p21* gene in both human hepatoblastoma (HepG2) cells and normal human bronchial epithelial (NHBE) cells in response to different zinc status has been examined. In both zinc deficient (ZD) and zinc marginal deficient (ZD0.4) HepG2 cells, the *p21* mRNA and nuclear protein levels as well as *p21* promoter activity were repressed as compared to that of zinc normal (ZN) control cells. However, they were not altered in zinc adequate (ZA) and zinc supplement (ZS) cells as compared to ZN cells. Moreover, transfection of a construct consisted of a constitutive promoter fused with a full length *p21* coding sequence in ZD cells, normalized *p21* protein expression to that of the ZN cells, but failed to correct cell growth reduction. Similar transfection in ZN cells overexpressed *p21* and repressed cell growth. Thus, the present data indicate that in zinc-deficient HepG2 cells, the *p21* transcriptional process is depressed. However, depressed *p21* transcriptional process is not responsible for repressed cell growth in zinc-deficient HepG2 cells.

In NHBE cells, the nuclear p21 protein level and mRNA abundance as well as promoter activity in ZS cells, but not in ZD cells, were markedly elevated to almost 2-fold when compared to ZN control cells. G2/M blockage in ZS cells was coupled with enhanced p21 gene expression. In ZS cells, the abrogation of p21 protein induction by the transfection of p21 siRNA was shown to alleviate the G2/M blockage. A similar gene knock-down approach was used to establish if the p21 upregulation in ZS cells was p53 dependent. Abolishment of the increase in p53 protein in ZS cells with transfection of p53 siRNA normalized the elevated p21 protein to a similar level as in ZN control cells, which demonstrated that the p21 induction is p53-dependent. Furthermore, the normalization of p53 protein by siRNA in ZS cells alleviated cell growth depression and G2/M blockage, which demonstrated that p53 was involved in the high zinc status induced G2/M blockage and growth depression. Thus, high zinc status in NHBE cells upregulated p53 expression which in turn elevated p21 that eventually induced G2/M blockage.

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BY ZINC STATUS IN HUMAN HEPATOBLASTOMA AND NORMAL HUMAN
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By

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Chapter I: Introduction

Zinc is a structural component of a wide variety of functional proteins, enzymes, and transcriptional factors which is involved in a wide range of physiological processes such as growth, development, and functioning of the endocrine, immune, and nervous systems. Both epidemiological and experimental studies have reported positive association between dietary zinc deficiency and cancer diseases. Cell proliferation is induced by active cyclin/cdk (cyclin-dependence kinase) complexes. Cell cycle progression is impaired by the inhibition of cyclin/cdk complexes by cyclin kinase inhibitors (CKIs). p21 *WAF1/Cip1*, a member of the Cip/Kip family of CKIs, is capable of inhibiting the activity of cyclin/cdk complexes. DNA damage induces p21 overexpression, which leads to growth arrest at the G1 and G2 phase or S-phase. Paradoxically, p21 stabilizes cyclin D1-cdk4/cdk6 assembly into active complexes, which modulate cell cycle positively. The role p21 as an assembly factor for cyclin D1-cdk4/cdk6 is completely opposite to its function as a cdk inhibitor. Repression of p21 depresses cyclin D1-cdk4/cdk6 complex formation and results in impaired cell cycle progression. Both p53-dependent and -independent mechanisms are involved in transcriptional modulation of p21 expression. Two conserved p53-binding sites are located in the p21 promoter. After DNA damage, at least one of these is required for p53 responsiveness. The p53-independent activation of p21 transcription involves a variety of transcription factors which are induced by a number of different signaling pathways.

In these studies, we have elected to induce zinc deficiency by using zinc depleted media (for HepG2 cells) and zinc-free basal BEGM medium (for Normal Human

Bronchial Epithelial cells) to examine the influence of zinc status on gene expression. Cells were cultured for one passage in a basal medium depleted of zinc to induce severe zinc-deficient (ZD) cells or in the basal medium supplemented with 0.4, 4.0, 16, or 32 μ M zinc to represent mild zinc deficiency (ZD0.4), the amount of zinc in most normal media (ZN), the normal human plasma zinc level (zinc-adequate, ZA), or the high end of plasma zinc attainable by oral supplementation (ZS), respectively..

The transcriptional regulation of p21 gene in both human hepatoblastoma (HepG2) cells and normal human bronchial epithelial (NHBE) cells in response to different zinc status has been examined. These cells were selected because our preliminary data have shown marked reduction and enhancement in p21 mRNA expression, as well as depressed G1/S and G2/M cell cycle progression, in zinc-deficient HepG2 cells and zinc-supplement NHBE cells, respectively. Thus, the effect of zinc on cellular proliferation was hypothesized to act through the manipulation of p21 pathway. The following approaches have been applied to test the hypothesis. Firstly, the effect of zinc status on p21 mRNA and protein levels was determined by RNase protection assay and Western blotting, respectively. Secondly, to determine if the p21 promoter was involved in this regulation, p21 promoter activity in response to different zinc status was measured by transient transfection of a p21-promoter-luciferase construct into cells. Moreover, the influence of zinc status on the modulation of p21 downstream components such as cdk-2 that tightly regulates the cell-cycle progression was examined. Furthermore, the zinc-effect on p21 transcription was studied for its relationship with p53. Finally, the gain of function approach using a p21 overexpressing plasmid and the loss of function approach involving siRNA technology were employed to investigate the physiological role of p21

related to cell cycle and growth proliferation in response to different zinc status. Experiments were designed to generate data to aid in the understanding of the influence of zinc status on cellular growth and proliferation as well as on the regulation of p21 signal transduction pathway.

Chapter II Literature Review

1 Zinc Biology

1.1 Zinc history and physiological function

The role of zinc as an essential trace mineral for humans was identified in 1960s (Prasad et al., 1984). Before that, the physiological function of zinc in humans was debatable. In 1934, the first in vivo study was performed to show that zinc deficiency caused poor growth and appetite loss in rats. In another in vivo study, swine fed the zinc inadequate diet showed serious side effects including parakeratosis. A few years later, detrimental effects of zinc deficiency in humans were also demonstrated to include stunted growth, poor reproductive development and suppressed immune function. Since then, detailed physiological function of zinc has been clearly elucidated. Zinc is an essential component of the catalytic site or sites of at least one enzyme in every enzyme classification (Salgueiro et al, 2000). The functions of numerous proteins in the body are supported by zinc. Among these proteins are the metalloenzymes, which are involved in a variety of metabolic processes. Zinc finger motif is an ubiquitous structure of most transcriptional factors. The configuration of these "zinc fingers," which directs their binding to DNA, is determined by the single zinc atom. The binding of these zinc fingers to corresponding sites on DNA initiates the transcription process and gene expression. Similar motifs have been identified in nuclear hormonal receptors; including those for estrogen, testosterone and vitamin D. Table 1 lists some typical functions of zinc (Table 1).

Table 1: Zinc functions in the organism

Immune response onset and regulation
Antioxidant
Enzymatic cofactor
Spermatogenesis and steroidgenesis
Vitamin A metabolism
Insulin storage and release
Energetic metabolism
Proteins synthesis
Stabilization of macromolecules
Regulaiton of the DNA transcription
Cellular division

Source: Salgueiro et al., 2000

1.2 Sources of zinc

Dietary zinc is mostly associated with the protein fraction of foods. Specifically, zinc is found in food, complexed with amino acids that are part of peptides and proteins as well as with nucleic acids. Table 2 shows zinc content of typical foods. The zinc content of these foods varies widely. Seafood and red meats are the two which contain plenty of zinc. Most vegetables also contain zinc, although in much lesser extent compared to seafood and red meats. Most U.S. mixed diets supply between 8.6 to 14 mg/day of zinc; a relatively high amount due to the presence of zinc in animal protein (Sandstead and Smith 1996).

1.3 Recommend Daily Allowance

Different laboratory and clinical tests, together with the results of many zinc

Table 2: Zinc content of food

| | Mg of zinc/100g of food |
|--------------------|-------------------------|
| Meat | 3.2 |
| Liver | 5.1 |
| Egg | 1.35 |
| Milk | 0.38 |
| Refined Cereals | 0.50 |
| Whole wheat | 1.00 |
| Whole corn | 2.50 |
| Whole rye | 1.30 |
| Carobs | 2.65 |
| Onion | 1.40 |
| Peanut | 2.00-3.00 |
| Chocolate | 1.00-2.00 |
| Fish and Shellfish | 1.50 |

Source: Salgueiro et al., 2000

Table 3: Recommended dietary allowance

| | |
|--|----------|
| Infants | 3mg/day |
| Children 1-3 years old | 3mg/day |
| Children 4-8 years old | 5mg/day |
| Males | 11mg/day |
| Females | 8mg/day |
| Pregnant | 11mg/day |
| Lactating women 1 st semester | 12mg/day |
| Lactating women 2 nd semester | 12mg/day |

Source: Dietary Reference Intake, Food and Nutrition Book, 2001

supplementation trials, have provided the evidence of zinc deficiency in several populations. The laboratory methods proposed to determine zinc status are: serum and plasma zinc concentrations, leukocyte and neutrophil zinc concentrations, the activity of selected enzymes such as 5'nucleotidase and lately, the erythrocyte metallothionein concentration. The major problem has been to find a reliable laboratory index of zinc status to estimate the individual zinc nutritional status, at the onset of zinc deficiency or zinc toxicity signs. Table 2 shows some of the zinc status indicators proposed (Grider and Young 1996). The lack of a reliable method to determine the zinc nutritional status also reflects the problem to estimate the RDA for zinc. Table 3 shows the recommendations proposed by the National Research Council. Nevertheless, more research is needed because of the difficulty in the application and interpretation of the usual methods to estimate the RDA, when trace metals are evaluated.

1.3 Digestion, Absorption, Transport, Uptake, and Storage

1.3.1 Digestion

Before absorption of zinc is possible, zinc needs to be hydrolyzed from amino acids. Zinc is believed to be released from food during the digestive process in the stomach and small intestine. Proteases and nucleases in the stomach and small intestine play important roles. Hydrochloric acid also favors the digestion of protein to liberate the zinc from foods (Prasad 1984).

1.3.2 Absorption

Active and passive transport are two main mechanisms of zinc absorption in the mammalian small intestine. Active transport is saturable at high concentrations of the

zinc metal in the intestine lumen, and its efficiency increases during low intake periods. On the contrary, passive transport is a diffusion mechanism, which is inalterable during low intake periods and its efficiency is proportional to luminal zinc concentration (Cousins et al., 1986). Zinc absorption can be facilitated by activators. These are picolinic acid secreted by the pancreas, vitamin B6 that increases picolinic acid secretion, as well as citrate and aminoacids such as glycine, histidine, lysine, cystein and methionine (Sandstead et al., 1996; Evans et al., 1981). On the other hand, zinc absorption can also be impeded by inhibitors. These are phytic and oxalic acids, tannins, fiber, selenium, iron and calcium (Prasad 1984). Iron and calcium are debatable as inhibitors, but they appear to only interfere with zinc absorption at higher concentrations (Sandstead and smith 1996; Solomons and Jacob 1981; Wood et al., 1997). Zinc absorption efficiency is only between 15 to 40% of the amount of zinc ingested, the rest are excreted with the feces. There is also an endogenous zinc secretion into the intestinal lumen. This endogenous zinc comes from pancreatic, biliar and intestinal secretions as well as from desquamated mucosal cells (Sian et al., 1996; Berger and Schneeman 1986). Once in the intestine, both dietary and endogenous zinc are under the same homeostatic regulation, and they might be reabsorbed at distal segments of the intestine, or finally excreted with the feces. Homeostatic regulation of zinc is very important at the intestinal level. In order to maintain zinc homeostasis, during low zinc intake periods, there is a marked decrease of endogenous zinc secretion instead of an increase in zinc absorption (Sian et al., 1996). A more recent understanding is that the intestinal metallothionein is one of the proteins involved in this secretion process (Davis et al., 1998). Once zinc is absorbed, it may form

an intestinal pool bound to the intestinal metallothionein or it may be transported from the enterocyte to the blood.

1.3.3 Transport

Zinc transport in blood is mainly carried out by being complexed with albumin. Therefore, in conditions such as pregnancy and malnutrition, with low plasma albumin concentrations, zinc absorption decreases appreciably (Whitney et al., 1993). Other plasma components, such as α_2 -macroglobulin, transferrin, cystein and histidine, may also bind the metal.

1.3.4 Metabolism

Most of plasma zinc is bound to hepatic metallothionein and stored in liver. Methallothioneins are cytosolic proteins with high cystein content and they are the main proteins involved in zinc metabolism. These metalloproteins can bind seven atoms of zinc per molecule of protein, but they can also bind copper with higher affinity. Metallothionein isoforms 1 and 2 are highly expressed in liver, intestine, kidney and pancreas and their expression may be induced by many factors such as zinc rich diets, IL-1 (Interleukin-1), IL-6, glucocorticoids, and stress, etc. Their biochemical roles are associated with heavy metal detoxification, scavenging of free radicals, and redistribution of body zinc in response to acute infection or stress. Hepatic metallothionein mainly appears to act as a zinc reservoir that may convey protection against zinc deficiency (Blain et al., 1998; Kelly et al., 1986). Intestinal metallothionein appears to act as a negative regulator of zinc absorption, and may participate in the intestinal mucosa-lumen zinc efflux.

A family of mammalian zinc transporters that plays an important role in the regulation of zinc metabolism at the intracellular level has been described. Zinc transporters such as ZnT-1 (Zinc Transporter), ZnT-2, Zn-T3 and ZnT-4 are zinc specific, while the divalent cation transporter-1 (DCT-1) is not zinc specific. The general structure of these zinc specific zinc transporters consist of 6 transmembrane domains, an intracellular histidine rich region and the amino and carboxyl terminus. ZnT-1 is widely expressed in the organism, and it functions as a zinc exporter. The direct regulation of the ZnT-1 gene by zinc was demonstrated in the mouse intestine and liver, independent of the level of metallothionein. ZnT-2 is expressed in intestine, kidneys and testes. It functions as a zinc exporter, but it has the capacity to compartmentalize zinc into intracellular vesicles. ZnT-3 expression is restricted to the brain, suggesting an important role for zinc in the central nervous system, and it is also expressed in testes. ZnT-3 transporter activity is associated with vesicular packaging of zinc, and it could be involved in spermatogenesis and is related to insulin secretion. ZnT-4 is expressed in the mammary gland and the brain. A single point mutation in ZnT-4 gene resulting in a premature termination of the protein causes the lethal mouse syndrome. This syndrome is characterized by a marked decrease of zinc transport from the mammary gland to the milk (Davis et al., 1995; McMahon and Cousins 1998; Palmiter et al., 1996).

1.4 Zinc deficiency

1.4.1 Immune system

Zinc is known to play a central and unique role in the immune system. Association of zinc deficiency and susceptibility to a variety of pathogens has been

widely reported (Palmiter et al., 1996; McMahon and Cousins 1998; Barone et al., 1998; Gyorffy et al., 1992). Clinical and experimental evidence showed that severe zinc deficiency causes thymus gland deterioration (McMahon and Cousins 1998; Chandra and Au 1980). This symptom may be explained in part by a raise of blood glucocorticoid concentration which reduces the number of circulating lymphocytes (Mocchegiani et al., 1995; Shankar and Prasad et al., 1998; Good et al., 1980). Adverse effects of zinc deficiency on specific cells of the immune system include: reduction in the number of lymphocytes in both central and peripheral lymphoid tissue; depression of the T and B lymphocyte function; and the decrease of proliferative responses to mitogens (Shankar and Prasad 1998; Prasad 1985; Prasad 1983; Shi et al., 1998). With regard to soluble mediators of immunity, zinc deficiency reduces thymulin activity and production or biological activity of multiple cytokines such as: IL-1, IL-2, IL3, IL4, IL-6, INF- γ , INF- α and TNF- α . Mild zinc deficiency also shows an imbalance of Th-1 cells and Th-2 cells function (Shankar and Prasad 1998; Lesourd et al., 1998; Saha et al., 1995). On the other hand, an adequate zinc status has been found to contribute to the preservation of the CD4:CD8 ratio, which prevents human immunodeficiency virus (HIV) infection for the transition to overt acquired immune deficiency syndrome (AIDS) (Sazawal et al., 1996; Shankar and Prasad 1998). Furthermore, zinc supplementation was widely reported to be able to reverse the immune defect symptoms caused by zinc deficiency (Shanka and Prasad 1998).

1.4.2 Anorexia nervosa

Zinc deficiency has been reported to be involved in the pathogenesis of anorexia nervosa. Animal studies showed that rats fed the zinc-deficient diet consumed 50% less

of total food intake compared with the rats fed the zinc-adequate diet. When these rats were forced fed, they became seriously ill and in some cases they died. However, upon zinc repletion, the rats recovered from the anorexia nervosa symptoms (Lee et al., 1998; Browning et al., 1998; Oner et al., 1984). The possible mechanism underlying anorexia nervosa onset was proposed to be related to a reduction in the synthesis of certain neurotransmitters by zinc deficiency. One of these neurotransmitter factors called neuropeptide Y (NPY), a potent appetite stimulant in the hypothalamus, was synthesized less in response to zinc deficiency (Browning et al., 1998). However, Lee et al. (1998) proposed that zinc deficiency may impair the processing of the NPY, which results in NPY with lower affinity for its receptors causing a decreased food intake (Lee et al., 1998).

1.4.3 Reproduction

Zinc is necessary for the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), gonadal differentiation, testicular growth, formation and maturation of spermatozoa, testicular steroidogenesis and fertilization (Om and Chung 1996; Madding et al., 1986). Zinc supplementation has been established to be beneficial in sterility and impotence in men and infertility in women. However, the mechanisms underlying these functions involved numerous zinc-dependent enzymes and the complexity of the complete pathway is not fully understood. Om and Chung (1996) reported that in zinc-deficient male rats, LH and testosterone concentrations were reduced, the hepatic steroid metabolism was altered and sex steroid hormones levels were modified (Om and Chung 1996). They concluded that zinc deficiency contributes to the

pathogenesis of male reproductive dysfunction such as infertility, hypogonadism and feminization (Om and Chung).

1.4.4 Pregnancy

Zinc deficiency has detrimental effects on many stages of pregnancy because many reproductive and developmental hormones are dependent on zinc for proper function. Several reports showed zinc deficiency to be associated with skeletal malformations, spontaneous abortion, prematurity or prolonged gestation and complication during delivery such as excessive loss of blood (Goldenberg et al., 1995; Hurley et al., 1983). The main teratogenic effects of zinc deficiency are related to neural tube defects in children of mothers with altered zinc status.

1.4.5 Lactation and infancy

Factors responsible for zinc deficiency in premature infants include high fecal losses of zinc in the newborn, low body stores of zinc at birth, increased zinc requirements during rapid growth, and also in a rare circumstance, a defect in transfer of zinc from maternal serum to breast milk (Prasad 1996). Zinc deficiency may be a common transient disorder in premature infants characterized by skin lesions resembling acrodermatitis enteropathica, diarrhea, and growth stunting and low weight for age (Piela et al., 1998; Folwaczny 1997). Potential benefits of maternal zinc supplementation to infants include enhancement of either quantity or quality of human milk and optimization of infant growth, development, and immune function. Favier (1992) found that supplementation of the food of breast-feeding mother with 15 mg zinc resulted in higher weight gain in their babies than in babies of control mothers (Favier 1992). Clinical manifestations of zinc deficiency are listed in Table 4.

Table 4: Zinc deficiency consequences

| | |
|-------------------------------|---------------------|
| Growth retardation | Oligospermia |
| Hypogonadism | Weight loss |
| Intercurrent infections | Anorexia |
| Altered immune response | Diarrhea |
| Increased absorption risk | Alopecia |
| Complications during delivery | Mental lethargy |
| Premature pregnancy | Skin changes |
| Neural tube defects of fetus | Taste abnormalities |
| Delayed wound healing | Emotional disorders |
| Abnormal dark adaptation | Hyperammonaemia |

Source: Salgueiro et al., 2000

1.5 Zinc toxicity

High zinc concentration is well known to interfere with copper metabolism. Among patients with Wilson's disease, zinc is the treatment of choice because high concentrations of zinc significantly limits the amount of copper absorbed, slowing the progression of the disease. Patients with acrodermatitis enteropathica need high doses of zinc to prevent zinc deficiency, but most of them develop copper deficiency (Grider and Young 1996). The hypothetical mechanism for zinc-copper interaction is related to their similar chemical and biochemical properties. Some authors proposed that the direct interference of zinc and copper occurs at the intestinal level. However, others suggest that high dietary zinc induces the increase of intestinal metallothionein which prevents copper absorption by binding to them with higher affinity (CastilloDuran et al., 1990; Sandstead 1995). Acute toxicity studies showed that although multiple organs are exposed to zinc toxicity, the pancreas is the most affected, but the reason is not clear (Kelly et al., 1986;

Sutomo et al., 1992). The signs and symptoms that an acute oral zinc dose may provoke are: tachycardia, vascular shock, dyspeptic nausea, vomiting, diarrhea as well as pancreatic and hepatic parenchymal damage (Kelly et al., 1986). Nevertheless, the amounts of zinc that provoke toxic effects are much higher than those contained in regular diets, therefore, zinc toxicity is scarce.

2. Zinc Deficiency and Cancer

Apart from the well established role of zinc in growth and development, reproduction, and immune function, available evidence indicates that zinc also exerts positive effect on genetic stability and function. Zinc is not only a component of more than 300 transcription factors that regulate expression of other genes. Enzymes including copper/zinc superoxide dismutase (CuZnSOD) and several proteins involved in DNA repair also require zinc as functional units (Prasad 1998; Prasad and Kucuk 2002; Prasad 2003). Therefore, zinc may be implicated in cancer prevention by protecting genetic components from oxidation and damage.

2.1 Zinc deficiency and oxidative stress

Increased oxidant stress with low cellular zinc has been extensively reported. Increased oxidant production has been demonstrated in cells cultured in zinc-deficient media (Ho and Ames 2002). In an animal study, zinc-deficient rats showed increased oxidative protein and DNA damage (Taylor et al., 1988; Olin et al., 1993; Oteiza et al., 1996). Moreover, zinc-deficient rats are more susceptible to oxidative injury. In addition, hyperoxic lung damage, carbon tetrachloride toxicity and lipid peroxidation have been

demonstrated in zinc-deficient rats when compared with zinc-adequate rats (Taylor et al., 1988; Oteiza et al., 1996; Bray et al., 1986; Burke et al., 1987). In another approach, zinc supplementation has been shown to be beneficial against oxidant damage and the progression of ROS-induced diseases. For example, zinc supplementation has been established to protect against chemically induced models of Type 1 diabetes in CD1 mice (Ho et al., 2001; Mocchegiani et al., 2000). In another case, addition of zinc has been shown to protect human fibroblasts from UV-induced DNA damage and apoptosis (Leccia et al., 1999). Thus, the antioxidant role of zinc could be an important mechanism in maintaining DNA integrity in the cell and therefore preventing cancer development.

2.2 Mechanism of zinc as an antioxidant

Oxidative stress has been widely reported as an important contributor in several chronic degenerative diseases including cancer. Zinc deficiency can induce oxidative stress which may explain the mechanism by which zinc deficiency increases the risk for cancer development (Taylor et al., 1988). CuZnSOD is an important defense against reactive oxygen species, which are generated under both normal physiological metabolism and certain adverse conditions. The fact that zinc is an essential structure component of CuZnSOD and enables it to function properly in the removal of ROS, is one of the reasons why zinc is such an important antioxidant element (Prasad 1998; Prasad and Kucuk 2002; Prasad 2003). The second possible mechanism for zinc's antioxidant effect is that zinc can prevent hydroxyl radical formation by transition metals, such as iron or copper. Hydroxyl radicals ($\text{HO}\cdot$) are powerful oxidizing species that can damage biological molecules, such as nuclei acid, significantly. $\text{HO}\cdot$ species are formed

by Fenton reaction when a single electron is transferred to H₂O₂ from transition metals such as iron and copper. Zinc can prevent this reaction by acting as competitor for binding sites for pro-oxidant transition metals such as iron and copper (Kelly et al., 1986; Washabaugh and Collins 1986; Conte et al., 1996; Hesketh 1983; Fu et al., 1996). Although the magnificent role of zinc in maintaining DNA integrity and therefore preventing oxidative stress induced diseases, the pathology and disease development associated with oxidative stress may not be due simple to increases in oxidative damage (Datta et al., 2000). ROS may act as signaling molecules that trigger distinct pathways to induce pathology of disease. Thus, the role of zinc deficiency in the development of chronic disease, such as diabetes and cancer, may be far more complex than simply causing oxidative damage.

2.3 Zinc and DNA repair

Besides an antioxidant role of zinc in maintaining DNA integrity, zinc also plays a critical role in the regulation of transcription and replication of DNA through zinc finger proteins. Zinc finger motif is an ubiquitous structure of most enzymes and transcriptional factors. Zinc atoms have specific structural roles in this motif. The configuration of these "zinc fingers," which directs their binding to DNA, is determined by the single zinc atom. In fact, many DNA repair enzymes are zinc finger proteins and therefore require zinc to function properly. The well-known tumor suppressor protein p53 is a zinc finger protein. p53 plays an important role in modulating cell cycle progression, apoptosis, DNA repair and cell proliferation/differentiation. p53 induces G1 arrest to allow the cell to repair damaged DNA before cellular division (Lane 1992). In fact, over

50% of human malignancies contain a mutation in p53 (Hollstein et al., 1991). The majority of these mutations are found in the region of the gene that distorts the DNA-binding ability (Bedwal and Bahuguna 1994; Pavletich et al., 1993). This binding region also contains the zinc-binding domain. Increases in p53 protein expression accompanied with low intracellular zinc were demonstrated in several different cell lines (Ames 2001; Ho et al., 2003). Although the authors explained that the increases of p53 protein expression in response to low intracellular zinc is probably a mechanism to repair damaged DNA due to oxidative stress, p53 is likely dysfunctional to bind and activate downstream genes due to conformation alteration resulted from zinc deficiency. In fact, a marked decrease in the ability of p53 to bind to downstream DNA targets has been found in zinc deficiency (Ho and Ames 2002). Researchers have found that the removal of zinc, either by chemical chelation or by culturing in zinc-deficient media, alters the expression of p53 (Fanzo et al., 2001; Meplan et al., 2000). Direct chemical chelation also appears to reversibly alter p53 conformation, with the loss of DNA-binding activity (Hainaut and Hollstein 2000). In another case, low cellular zinc also increases the expression of apyrimidic endonuclease (APE) (Ho and Ames 2002). APE is an important endonuclease involved in base excision repair (Fritz 2000). In fact, APE levels appear to be elevated in a number of cancers (Thomson et al., 2000; Puglisi et al., 2002). Thus, the increased expression of APE in low cellular zinc cell line may probably be a response to DNA damage induced by low zinc (Ho and Ames 2002).

2.4 Zinc deficiency, cell signaling and apoptosis

Apoptosis is a process that cells undergo leading to programmed cell death. The induction of apoptosis is an important mechanism for the cell to “commit suicide” in response to DNA damage. Association of zinc deficiency and increased apoptosis in various cells and tissues has been reported. In one example, zinc deficiency markedly reduced lymphocyte numbers in both the thymus and marrow of zinc-deficient mice (Fraker et al., 1987). Increases in apoptosis are critical changes in lymphopoiesis and myelopoiesis in T and B cells with zinc deficiency (King and Fraker 2002). Zinc depletion has also been associated with caspase activation and increases in apoptosis in airway epithelial cells, lung and hepatocytes (Hennig et al., 1999; Truong-Tan et al., 2001; Nakatani et al., 2000). The possible mechanisms of zinc deficiency induced apoptosis include loss of zinc in critical proteins involved in the apoptotic cascade or undirected effects due to increased oxidative stress or a response to DNA damage. ROS are also known as inducers of apoptosis.

2.5 Zinc deficiency and its relevance to cancer

The possible association of zinc deficiency and cancer has been established in humans, animals and cell culture systems. Lower zinc status has been observed in patients with cancer (Federico et al., 2001; Prasad et al., 1998). Zinc deficiency induced DNA damage and chromosome breaks have been reported in animals fed with a zinc-deficient diet. In rats, dietary zinc deficiency causes an increased susceptibility to tumor development when exposed to carcinogenic compounds (Fong et al., 1996; Fong and Magee 1999). In the same animal model, zinc deficiency has been suggested to be a potential factor of esophageal tumors (Newberne et al., 1997). In vitro studies have also

shown that zinc deficiency can lead to increased oxidative damage to DNA of testicular cells (Oteiza et al. 1996). In the case of prostate cancer, zinc deficiency appears to play an important role, although the precise mechanism is unknown. Normal human prostate accumulates the highest levels of zinc. In contrast, a marked decrease in zinc content is associated with prostate cancer cell lines (Dhar et al., 1973; Platz and Helzlsouer 2001). A recent finding by Huang et al. (2006) demonstrated that tumorigenic prostate epithelial cells accumulated less intracellular zinc than non-tumorigenic prostate epithelial cells. Moreover, ZIP1 protein expression was lower in tumorigenic prostate epithelial cells. When ZIP1 gene was overexpressed in tumorigenic prostate epithelial cells, the cells not only demonstrated increase in intracellular zinc content but also showed suppressed tumor cell growth (Huang et al., 2006). Several studies have also shown that high cellular zinc levels inhibit prostate cancer cell growth (Costello et al., 1998; Feng et al., 2000; Uzzo et al., 2002). Although zinc supplementation has the potential to target multiple points of the carcinogenesis initiation, there exists controversy toward the efficacy of zinc supplements to prevent prostate cancer. An epidemiologic study showed an increase in risk for prostate cancer with high zinc supplement use (Leitzmann et al., 2003). Increased risk was seen in subjects with very high dose of over 100 mg/day zinc supplement or very long term usage of more than 10 years.

3. p53

3.1 History

The open reading frame of p53 is 393 amino acids long, with the central region (consisting of amino acids from about 100 to 300) containing the DNA-binding domain. This core is flanked by a C-terminal end mediating oligomerization and an N-terminal end containing a strong transcription activation signal. Initially, p53 was mistakenly described as an oncogene (Eliyahu et al., 1984) because the cloned p53 was a mutant form of wild type p53. The mutant form p53 used in transfection experiments contains a point mutation which activates their transforming properties. Further studies indicated that the wild type p53 protein has emerged as a key tumor suppressor protein at the crossroads of cellular stress response pathways. During their life, normal cells are constantly exposed to various endogenous and exogenous stresses that alter their normal behavior. Genetic insults that can lead to mutations are particularly harmful, as the cell's transmission to daughters cells can lead to neoplasia. The p53 protein expression was found at very low levels in normal unstressed cells. After stress, different pathways lead to post-translational modification of the protein and its stabilization. This accumulation activates the transcription of a wide range of p53 downstream genes involved in various activities, including cell-cycle arrest, DNA repair, senescence, and apoptosis depending on the cellular context, the extent of damage or other unknown parameters (Bode et al., 2004). Table 5 lists some examples of p53 downstream genes which are well studied.

Table 5 some examples of p53 downstream genes and its consensus binding sequences

| Gene Name | Binding Sequence | Gene Name | Binding Sequence |
|------------------------|-----------------------|----------------------------------|----------------------|
| Apoptosis and survival | | Cell-cycle arrest and DNA repair | |
| Bax | tcACAAGTTaAGACAAGCCT | EGFR | GAGCTAGaCgGGGCAgcCCC |
| IGFBP3 | AAACAAGCCacAACATGCTT | Gadd45 | GAACATGTCTAAGCATGCTg |
| | GGGCAAGaCCtGcCAAGCCT | p21/WAF1 | GAACATGTCCcAACATGTTg |
| p53AIP1 | tctCTTGCCCGGGCTTGTCg | | GAAgAAGaCTGGGCATGTCT |
| KILLER/DR5 | GGGCATGTCCGGGCAAGaCg | p53R2 | TGACATGCCcAGGCATGTCT |
| TGF-alpha | GGGCAgGCCctGcCTAGTCT | 14-3-3-sigma | GtAgcAtTagAGACATGTCC |
| | AGcCAAGTCTtGGCAAGCgg | BTG2/TIS21/PC3 | AgtCcgGgCaAGcCcgagCa |
| EE-lalpha | GGGCAgaCCCGAGCATGCCc | TGF-beta | CAtCTTGCCcAGACTTGTCT |
| | AAACATGaTTAGGgAcaTCT | | AGcCATGCCcGGGCAAGaaC |
| | | PCNA | GAACAAGTCCGGGCATaTgT |
| NOS3/ECNOS | GAGCcTcCCaGGGCTTG TTC | | AcAtATGCCcGGACTTGTCT |
| DKK1 | AGcCAAGCTTGAACcAGTTC | | |
| LRDD | AGGCcTGCCTGGACATGTCT | | |
| Cathepsin D | AAGCTgGgCCGGGCTgaCCC | | |
| | AAcCTTGgTTcAAgAgGCTT | | |
| BDKRB2 | GGAagTGCCcAGGaggcTga | | |
| P2XM | GAACAAGggCGAGCTTGTCT | | |
| | | | |

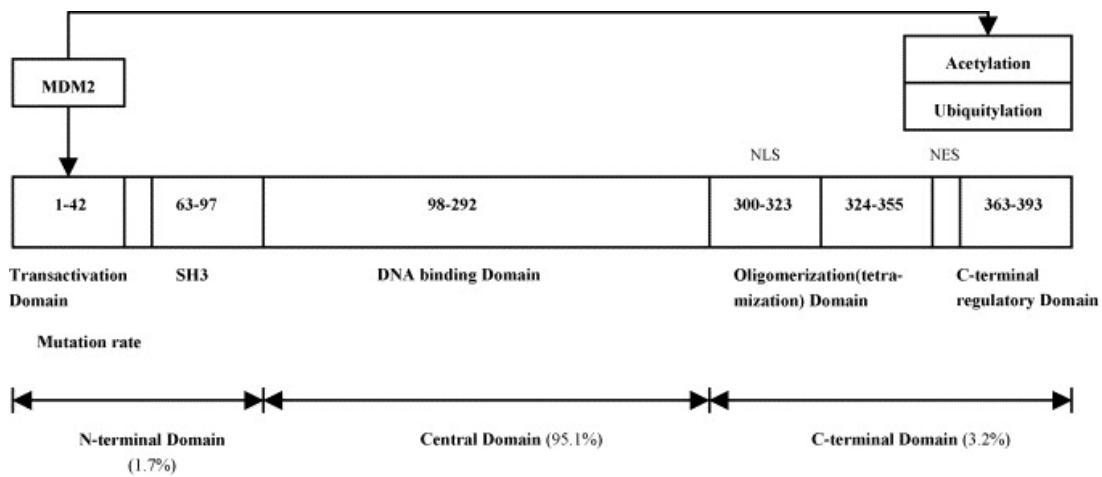
Source: Shu et al., 2006

3.2 p53 structure and functional domain

The human p53 protein consists of 393 amino acids; a complete three-dimensional structure for entire p53 protein is not available. The full-length protein is difficult to crystallize or to be used in NMR studies due to the size (212 kDa) of its full-length tetrameric form. However, the analyses on the p53 functional domains have greatly increased the understanding and knowledge we have about the structure of this protein and how it relates to the functions of p53. The p53 protein is commonly divided into three functional domains (Fig. 1). The acidic amino-terminal domain is required for transcriptional activation. MDM2 interacts with the N-terminal transactivation domain of p53 to inhibit p53 transcriptional activity. Most of the interactions between p53 and its downstream genes take place in the central core sequence-specific DNA binding domain

of p53 (Veprintsev et al., 2006; Ho et al., 2006). The carboxy-terminal end contains the tetramerization domain, which contains a nuclear export signal and nuclear localization signals, and the C-terminal regulatory domain. More than 20,000 mutations in the TP53 gene have accrued in IARC (International Agency for Research on Cancer) TP53 mutation database. TP53 predominantly shows missense mutations, in which the encoded protein contains amino acid substitutions. The missense mutation not only abrogates the tumor suppressive function, but also leads to the gain of oncogenic function by changing the repertoire of genes whose expression are controlled by this transcription factor (Dittmer et al., 1993; Hsiao et al., 1994).

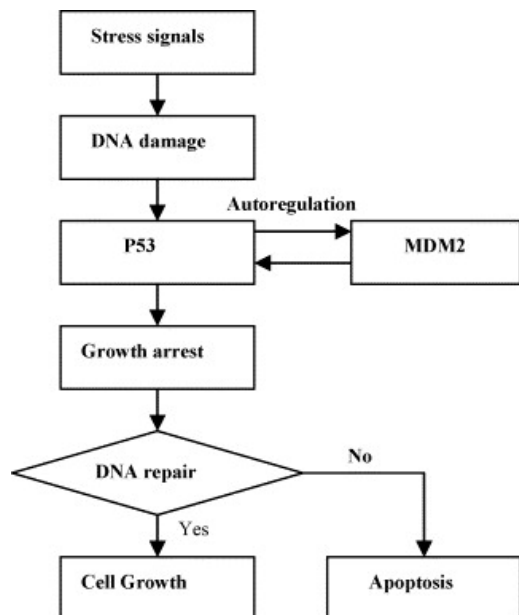
Figure 1 The human p53 structure



Source: Shu et al., 2006

3.3 p53 pathway

The p53 pathway is composed of p53 gene and a network of its downstream genes that are targeted to respond to a variety of intrinsic and extrinsic stress signals, which impact upon cellular homeostatic mechanisms that monitor DNA replication, chromosome segregation and cell division (Shu et al., 2006). Having a short half-life, p53 is normally maintained at low levels in unstressed mammalian cells. When the cell is confronted with stress, however, p53 ubiquitylation is suppressed and p53 is stabilized and accumulates in the nucleus, where it forms a homotetrameric complex. Only tetrameric p53 seems to be fully active as a transcriptional activator or repressor of p53 downstream genes that contain p53 sequence-specific DNA binding sites. The widely studied p53 downstream responses include cell cycle arrest, DNA repair, and apoptosis (Figure 2).



Source: Shu et al. 2006

Figure 2. p53 is a key molecular node in the cellular stress pathways

3.3.1 Growth arrest

p21^{WAF1/CIP1} is known to be a p53-downstream gene, and has been suggested to mediate p53-induced growth arrest triggered by DNA damage. The p21 protein is a cycline-dependent kinase inhibitor of cyclin dependent kinase 2 (CDK2). After CDK2 kinase activity is inhibited by p21, pRB is dephosphorylated and associated with transcriptional factor E2F, which finally leads to cell cycle arrest at G1. As long as pRb is bound to E2F, the cell is prevented from entering into S phase. This G1 arrest affords the cell time to repair the DNA damage. Should repair be unsuccessful, p53 levels drop and CDK-cyclin protein kinase activity resumes, which leads to entry into S phase. In the event that the DNA is not repair, p53 triggers apoptosis (Kim and Zhao 2005).

3.3.2 DNA repair

The p53 tumor suppressor gene plays an important role in the maintenance of genomic stability, and DNA repair is a major factor in maintaining genomic stability (Avkin et al., 2006; Nowak et al., 2002; Liu et al., 2005). Therefore, p53 mediated DNA repair may play an essential role in the maintenance of genomic stability. p53 has been shown to be involved in various types of DNA repair, including nucleotide excision repair (NER), base excision repair (BER), nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Smith et al., 2002; Bertrand et al., 2004) (Table 2).

p53-dependent transcriptional activity is important for the regulation of NER by p53 (Adimoolam et al., 2002). p53 binds to and modulates the activities of the NER-associated helicases XPB, XPD (Wang et al., 1997), regulates the expression of the

DDB2 and XPC (Hwang et al., 1999; Adimoolam and Ford 2002; Rubbi and Milner 2003), and serves as a chromatin accessibility factor for NER of DNA damage (Wang et al., 1997). BER is mediated by at least two pathways: a ‘short-patch-repair’ pathway, which involves the repair of a single nucleotide, and a ‘long-patch-repair’ pathway, which involves repairing 2–15 nucleotides (Hoeijmakers 2001). In mammalian cells, most DNA double-strand breaks (DSBs) are repaired by NHEJ (Lieber et al., 2003). NHEJ is carried out by the combined action of different proteins, including DNA-PK (DNA-dependent protein kinase), XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells-4) and DNA ligase IV (LIG4). However, other studies indicated that the p53-mediated apoptotic response is intact, even in the absence of DNA-PK (Sengupta and Harris 2005). p53 can physically bind to RAD51 and RAD54, major components of HR machinery, and controls the level of HR (Sengupta et al., 2003). Mutation in the WTp53 hotspot codon 273 reduces the capacity of p53 protein to bind with RAD51-DNA complexes (Susse et al., 2000).

3.3.3 *Apoptosis*

The role of p53 in apoptosis has been studied extensively and linked to its tumor suppressor activity (Yee et al., 2005). In response to cellular stress, p53 transactivates or transrepresses many different downstream genes to trigger apoptotic responses. The p53-mediated transactivation of apoptosis-related genes includes proapoptotic Bcl-2 family members, apoptotic protease activating factor-1 and Fas/CD95, death receptor 4. In addition to the mechanism involving p53-dependent transactivation of apoptotic genes, transcription independent mechanisms have also been suggested in p53-mediated

apoptosis (Haupt et al., 2003). p53 can also directly bind to and inhibit the Bcl-XL and Bcl2 proteins, leading to the release of cytochrome C (Mihara et al., 2003) and the initiation of the caspase cascade. There is strong evidence suggesting PUMA as a critical component of p53-mediated apoptosis, and in other cell types, NOXA seems to be equally significant (Villunger et al., 2003).

4. p21

4.1 History

Using a subtractive hybridization approach, El-Deiry et al. (1995) identified a gene they called WAF1 (for wildtype p53-activated fragment 1), whose induction was associated with wildtype but not mutant p53 gene expression in a human brain tumor cell line. El-Deiry et al. (1995) found that the sequence, structure, and activation by p53 was conserved in rodents. Transfection of WAF1 cDNA suppressed the growth of human brain, lung, and colon tumor cells in culture. Using a yeast enhancer trap, they identified a p53-binding site 2.4 kb upstream of WAF1 coding sequences. The WAF1 promoter, including this p53-binding site, conferred p53-dependent inducibility upon a heterologous reporter gene. WAF1 is commonly named as p21 indicating its protein size of 21kD.

4.2 p53 dependent regulation of p21 gene function

The tumor suppressor protein p53, a nuclear protein, plays an essential role in the regulation of cell cycle, specifically in the transition from G0 to G1. It is found in very low levels in normal cells. However, in a variety of transformed cell lines, it is expressed

in high amounts, and is believed to contribute to transformation and malignancy (Wiman 1997). Mutants of p53 that frequently occur in a number of different human cancers fail to bind the consensus DNA binding site, and hence cause the loss of tumor suppressor activity (Wiman 1997). Sequence comparison of the rat, mouse, and human p21 promoter sequences revealed conservation of two p53-responsive elements in these promoters (El-Deiry et al., 1995). Therefore, p53 is a major regulator of p21 transcriptional activity through the p53-dependent pathway. After gamma-irradiation induced DNA damage, p53 transactivates p21 expression through the binding to at least one of p53-responsive elements (El-Deiry et al., 1995). Irradiation of human cell lines also leads to p53-dependent cell cycle arrest at G1 (Dulic et al., 1994; Namba et al., 1995). Mouse embryonic fibroblasts which are deficient in both copy of p21 alleles exhibit impaired ability to undergo G1 arrest in response to DNA damage (Deng et al., 1995; Brugarolas et al., 1995). A study using CHIP assay indicated that the activation domains, the proline-rich domain, and the C-terminal basic domain in p53 are necessary for acetylation of histones on the proximal p21 promoter and for interaction with p300/CREB-binding protein (Liu, et al., 2003). In this study, the down-regulation of p300/CBP by short interference RNA (siRNA), markedly decreases the ability of p53 to induce endogenous p21, and therefore demonstrated the importance of p300/CBP in p53-dependent regulation of p21 expression. In addition, another study has demonstrated that p53 cooperates with the transcription factor Sp1 in the activation of the p21 promoter, whereas histone deacetylase 1 (HDAC1) counteracts p53-induced transcription of the p21 gene (Lagger et al., 2003). To localize the tumor suppression function within the structure of p21, Zakut and Givol (1995) used vectors constructed with systematic truncations of

p21 and tested their efficiency in suppressing tumor cell growth. They demonstrated that the N-terminal half of the p21 molecule shows better tumor cell growth suppression than the entire p21 molecular, whereas the C-terminal half of p21 did not show this effect. After DNA damage, many cells appear to enter a sustained arrest in the G2 phase of the cell cycle. Bunz et al. (1998) demonstrated that this arrest could be sustained only when p53 was present in the cell and capable of transcriptionally activating the cyclin-dependent inhibitor p21. After disruption of either p53 or the p21 gene, gamma-radiated cells progressed into mitosis and exhibited a G2 DNA content only because of a failure of cytokinesis. Thus, p53 and p21 appear to be essential for maintaining the G2 Checkpoint in human cells.

4.3 p53 independent regulation of p21

Apart from p53-dependent regulation, a variety of factors have been found to activate p21 transcription in a p53-independent pathway. Several of these factors are discussed below. Sp1 is a member of a multigene family that binds DNA through C-terminal zinc-finger motifs (Kennett et al., 1997). Six Sp1 binding sites, that span the region between -119 bp and transcription start site of p21 promoter, play a major role in the regulation of p21 transcription. Sp1 transcriptional factor mediates induction of the p21 gene through Sp1-1 and Sp1-2 sites in response to phorbol ester and okadaic acid in human leukemic cells undergoing differentiation toward macrophages (Biggs et al., 1996). The tumor-suppressor protein BRCA1 transactivates p21 in a p53-independent fashion via the region from -143 to -93 bp, which contains the Sp1-1 and Sp1-2 sites (Somasundaram et al., 1997). Many other agents such as transforming growth factor- β ,

Ca²⁺ and butyrate have also been shown to require Sp1 site to induce p21 transcription (Datto et al., 1995; Prowse et al., 1997; Nakano et al., 1997). Interaction of Sp1 with Smad proteins mediates induction of the p21 promoter by transforming growth factor- β . Calcium induces p21 promoter through only Sp3 site in keratinocytes. In butyrate treated cells, the binding of Sp1 to Sp1 site of p21 promoter is involved in the induction of p21 (Lee et al., 1998). The transcriptional coactivators p300/CBP can cooperate with Sp1 and/or Sp3 to induce expression from the p21 promoter. Through the regulation of p300 transcriptional coactivator activity, nerve growth factor (NGF) is able to induce neuronal differentiation and p21 expression in PC12 cells (Yan et al., 1997; Billon et al., 1996). Many other transcriptional factors such as AP2, E2Fs, STATs, C/EBP α , C/EBP β , and the homeobox transcription factor *gax* can also induce p21 transcription in response to different signals. Some of these are discussed below. The p21 promoter is transactivated via an activator protein 2 (AP2) consensus binding site located between -103 and -95 bp. AP2 induced p21 transcription and growth arrest after treatment of cells with phorbol ester (Zeng et al., 1997). E2F1 and E2F3 strongly transactivate p21 transcription through cis-acting elements located between nucleotides -119 to +16 of the p21 promoter (Gartel and Tyner, 1999; Hiyama et al., 1998). EGF and IFN- γ have been shown to induce cell growth arrest and upregulation of the p21 promoter by STAT1 via three STAT binding sites, which are located at -690, -2590, and -4233 bp in the p21 promoter (Chin et al., 1996). Overexpression of *gax* in vascular smooth muscle cells and fibroblasts induced cell growth arrest via p53-independent upregulation of p21 by unknown mechanism (Chinery et al., 1997). Hikasa et al. (2003) found p21 downregulation in conjunction with c-fos regulation in the lymphocytes of patients with rheumatoid arthritis. Phosphorylation

of STAT1 was also decreased in rheumatoid arthritis lymphocytes. Hikasa et al. (2003) determined that c-fos overexpression led to reduced phosphorylation and dimerization of STAT1, which in turn downregulated p21 gene expression. They concluded that this regulatory pathway may enhance the proliferation of lymphocytes in rheumatoid arthritis patients. By electrophoretic mobility shift assays, Nakatani et al. (2003) found that EWS-FLI1 interacted with the ETS consensus sequence within the promoter region of the p21(WAF1) gene. Reporter gene assays indicated that the binding of EWS-FLI1 to at least 2 ETS-binding sites negatively regulated p21(WAF1) promoter activity. EWS-FLI1 also suppressed p21(WAF1) induction by interacting with p300 and inhibiting its histone acetyltransferase activity. Asada et al. (2004) determined that p21 interacts directly with BRAP in vitro and in vivo, and the interaction requires the C-terminal portion of BRAP and the nuclear localization signal of p21. When cotransfected with BRAP, p21 was expressed in the cytoplasm. Monocyte differentiation of promyelomonocytic cell lines was associated with upregulation of BRAP expression concomitantly with upregulation and cytoplasmic relocalization of p21. Asada et al. (2004) concluded that BRAP plays a role in the cytoplasmic translocation of p21 during monocyte differentiation. Carreira et al. (2005) showed that MITF can act as a novel antiproliferative transcription factor able to induce a G1 cell cycle arrest that is dependent on MITF-mediated activation of the p21 cyclin-dependent kinase inhibitor gene. Moreover, cooperation between MITF and the retinoblastoma protein RB1 potentiates the ability of MITF to activate transcription. Carreira et al. (2005) suggested that MITF-mediated activation of p21 expression and the consequent hypophosphorylation of RB1 contribute to cell cycle exit and activation of the differentiation program. Rangarajan et al. (2001) found that Notch1 activation induced

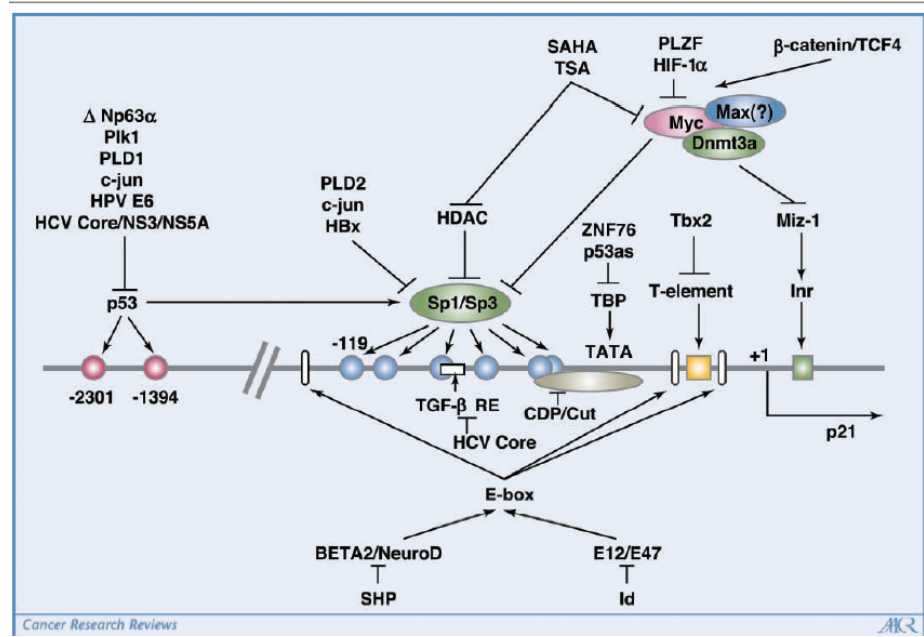
p21 in differentiating mouse keratinocytes, and the induction was associated with the targeting of the p21 promoter. Mammucari et al. (2005) showed that Notch1 also activated p21 through a calcineurin-dependent mechanism by acting on the p21 TATA box-proximal region. Notch signaling through the calcineurin/NFAT pathway also involved calcipressin and Hes1. Zhang et al. (2007) noted that hematopoietic stem cells are resistant to human immunodeficiency virus (HIV)-1 infection, despite of the presence of CD4 and CXCR4, which are involved in viral entry. They found that small interfering RNA-mediated knockdown of p21, but not other CDK inhibitors, altered HIV-1 infection, enhanced HIV-1 replication, and increased HIV-1 integration prior to changes in cell cycling and without altering CD4 and CXCR4 surface expression. Coimmunoprecipitation and Western blot analyses showed that p21 interacted with the HIV-1 integration machinery and appeared to inhibit the ability of HIV-1 to integrate into cellular DNA. Silencing of p21 had no effect on expression of other mediators of resistance to HIV-1 infection. Zhang et al. (2007) concluded that p21 is an endogenous cellular component in stem cells that provides a molecular barrier to HIV-1 infection. Figure 3 shows p21 proximal and distal p21 promoter with p53-dependent and – independent binding elements.

4.4 The consequence of p21 repression is not always promoting cell proliferation

In the majority of cases, the repression of p21 released its inhibition of cyclin/cdk2 complexes activity and resulted in enhanced cell cycle progression. In this context, p21 acts as an inhibitor of cyclin/cdk2 complexes. However, p21 can function as an assembly factor for cyclin D-cdk4/cdk6 and modulate the complex activity in a

concentration dependent manner. At low and intermediate concentrations, p21 stabilizes cyclin D-cdk4/cdk6 assembly into active complexes and modulate cell cycle positively (LaBaer et al., 1997). p21 repression in this context understandably impairs cell cycle progression by reducing cyclin D-cdk4/cdk6 formation and this has been demonstrated in several reports. In smooth muscle cells, Sp1 repression of p21 transcription mediated by Sp1 binding sites in the proximal promoter, reduces formation of cyclin D-cdk4-p21 complex, whose integrity is essential for G1/S transition, and subsequently inhibited cell growth (Kavurma & Khachigian 2003). Moreover, in endothelial cells, the Notch pathway mediated p21 repression, decreased cyclin D-cdk4 formation and nuclear targeting, which lead to a reduced retinoblastoma protein phosphorylation as well as a depressed S-phase entry and cell cycle progression (Nosedá et al., 2004). Thus, p21 repression may not always lead to growth promotion. Furthermore, treatment of LoVo colon cancer cells with anticancer camptothecin represses p21 expression and sensitizes the tumor cells to apoptosis. Similarly, chemical inhibitors, such a triptolide (Chang et al., 2001) and mithramycin A (Koutsodontis & Kardassis, 2004), which repress p21, have been shown to induce tumor cell death when used together with chemotherapeutic drugs.

Figure 3. p21 promoter showing p53 dependent and independent regulation



Gartel and Radhakrishnan 2005

CHAPTER III

Zinc deficiency depresses p21 gene expression: Inhibition of cell cycle progression is independent of the decrease in p21 protein level in HepG2 cells

ABSTRACT

The influence of zinc status on p21 gene expression was examined in human hepatoblastoma (HepG2) cells. Cells were cultured for one passage in a basal medium depleted of zinc to induce severe zinc-deficient (ZD) cells or in the basal medium supplemented with 0.4, 4.0, 16, or 32 μ M zinc to represent mild zinc deficiency (ZD0.4), the amount of zinc in most normal media (ZN), the normal human plasma zinc level (zinc-adequate, ZA), or the high end of plasma zinc attainable by oral supplementation (ZS), respectively. In ZD and ZD0.4 cells, the nuclear p21 protein level, mRNA abundance and promoter activity were reduced to 40%, 70%, and 65%, respectively, of ZN cells. However, p21 protein and mRNA levels as well as p21 promoter activity were not altered in ZA and ZS cells as compared to ZN cells. Moreover, the amounts of acetylated histone 4 associated with the proximal and distal p21 promoter regions, as a measure of p21 promoter accessibility, were decreased in ZD (73 and 64 %, respectively) and ZD0.4 (82 and 77 %, respectively) cells than in ZN cells (100 and 100 %, respectively). Thus, multiple lines of evidence indicate that the transcriptional process of p21 is down-regulated by depressed zinc status in HepG2 cells. Furthermore, the transfection of 5Cg of PCMV-p21 plasmid, which constitutively expressed p21, was able to normalize the reduction in p21 protein level and cyclin D1- cdk4 complex activity but not the inhibition of cell growth and G1/S cell cycle progression in ZD cells.

INTRODUCTION

Cell proliferation is induced by active cyclin/cdk (cyclin-dependence kinase) complexes. Cell cycle progression is impaired by the inhibition of cyclin/cdk complexes by cyclin kinase inhibitors (CKIs). p21^{WAF1/Cip1}, a member of the Cip/Kip family of CKIs (El-Deiry et al., 1993; Xiong et al., 1993), is capable of inhibiting the activity of cyclin/cdk complexes. DNA damage induces p21 overexpression, which leads to growth arrest at the G1 and G2 phase (Niculescu et al., 1998) or S-phase (Ogryzko et al., 1997). Cellular p21 level is modulated by various mechanisms: transcriptional regulation; epigenetic silencing; mRNA stability; as well as ubiquitin-dependent and -independent protein degradation. p21 is a short-lived protein and proteasome inhibition leads to an increase in p21 protein levels and in p21 half-life (Maki and Howley 1997; Sheaff et al., 2000). Basal turnover of p21 controlled by the proteasome does not appear to require ubiquitination (Bendjennat et al., 2003), although a role for N-terminal ubiquitination of p21 has been proposed (Bloom et al., 1991). Thus, p21 expression may be regulated posttranslationally by both ubiquitin-dependent and -independent proteasome-mediated degradation (Maki and Howley 1997; Smirnova 2000). Moreover, p21 protein levels have been reported to be regulated by posttranslational modifications such as phosphorylation (Li et al., 2002).

As for the transcriptional modulation of p21 expression, both p53-dependent and -independent mechanisms are involved. Two conserved p53-binding sites are located in the p21 promoter. After DNA damage, at least one of these is required for p53 responsiveness (El-Deiry et al., 1995). The p53-independent activation of p21

transcription involves a variety of transcription factors, including Sp1, Sp3, Ap2, STATs, C/EBP α , C/EBP β , and the bHLH proteins BETA2 and MyoD, which are induced by a number of different signaling pathways (Gartel and Tyner 1999). Six conserved Sp1 binding sites 126 are located in the proximal promoter of the human p21 gene. Within the p21 promoter, the Sp1 site between -87 and -72 from the start site appeared to be essential for the activation of the p21 promoter by HDAC inhibitors (Huang et al., 2000).

Paradoxically, p21 stabilizes cyclin D1-cdk4/cdk6 assembly into active complexes, which modulate cell cycle positively (LaBaer et al., 1997). The role p21 as an assembly factor for cyclin D1-cdk4/cdk6 is completely opposite to its function as a cdk inhibitor. Repression of p21 depresses cyclin D1-cdk4/cdk6 complex formation and results in impaired cell cycle progression. In smooth muscle cells, Sp1 repression of p21 transcription mediated by Sp1 binding sites in the proximal p21 promoter, reduced formation of cyclin D1-cdk4/cdk6-p21 complex, whose integrity is essential for G1/S transition, and subsequently inhibited cell growth (Kavurma and Khachigian 2003; Kavurma and Khachigian 2004). Moreover, in endothelial cells, the Notch pathway mediated p21 repression, decreased cyclin D1-cdk4 formation and nuclear targeting, which lead to a reduced retinoblastoma protein phosphorylation as well as a depressed S-phase entry and cell cycle progression (Noseda et al., 2004). Thus, p21 repression may also lead to depressed cell cycle progression.

Reductions of zinc status may promote apoptosis in certain cell type (Fraker and Telford 1997) and depressed G1/S cell cycle progression in HepG2 cells (Cui et al.,

2002). In studies using membrane permeable chelator to induce severe zinc deficiency, zinc has been shown to play a structural role in p53 and is essential for its DNA binding activity as well as its stability. However, membrane permeable chelators may induce unknown side effects as well as extremely severe deficiency not achievable by dietary depletion. Thus, we have elected to induce zinc deficiency by using low zinc or zinc depleted media, without culturing cells with membrane permeable chelators, to examine the influence of zinc status on gene expression. Cells were cultured for one passage in a basal medium depleted of zinc to induce severe zinc-deficient (ZD) cells or in the basal medium supplemented with 0.4, 4.0, 16, or 32 μM zinc to represent mild zinc deficiency (ZD0.4), the amount of zinc in most normal media (ZN), the normal human plasma zinc level (zinc-adequate, ZA), or the high end of plasma zinc attainable by oral supplementation (ZS), respectively. Reductions in cellular zinc are at the same magnitude as observed in tissue zinc seen in animals fed zinc-deficient diets and are much less severe than the use of membrane permeable chelators. Compared with ZN cells, both p53 mRNA and nuclear protein abundance were first reported to be increased 2-fold in ZD cells and normalized by culturing ZD cells in ZA medium in the last 24 hours in HepG2 cells by Reaves et al., (Reaves et al., 2000). By using a low zinc growth medium (ZD), nuclear p53 protein levels were increased about 5-fold and 2-fold in ZD cells than that in ZN cells of the normal human bronchial endothelial (NHBE) and normal human aortic endothelial (HAECs) cells, respectively. p53 mRNA level was increased 2.5-fold in ZD cells than that of ZN NHBE cells and it was too low to be quantitated in HAECs cells (Fanzo et al., 2002; Fanzo et al., 2001). Our first observation that p21 mRNA was not up regulated in the presence of p53 nuclear accumulation was seen in the ZD NHBE cells.

Moreover, in HAECs, although there was no significant difference for p21 mRNA abundance between ZD and ZN cells, there was a trend of 20% reduction in ZD cells.

The present study was designed to test the hypothesis that zinc deficiency would repress the expression of p21 mRNA and nuclear protein, as well as the p21 promoter accessibility and activity in HepG2 cells. In addition, the hypothesis that in zinc-deficient HepG2 cells, the impaired cell growth and G1/S cell cycle progression is independent of the depressed p21 expression was also examined. The reasons for using HepG2 hepatoblastoma cells for the current study are as follow: a). Normal human hepatocytes do not divide and the cost for their usage would be financially impractical; b). HepG2 hepatoblastoma cells will grow for many passages in serum-containing medium of various zinc status to provide sufficient quantities of cells for needed assays; c). HepG2 cells are known to have normal p21 and p53 genes as well as they have been used for studying p21, p53, and signal transduction pathways; d). HepG2 cells response well in the serum-containing culture system with different zinc status and they do not require the expensive serum-free medium required for normal human cells in primary culture; and e). The zinc depleted medium for HepG2 cell culture is lower in zinc than those used for HAECs and NHBE cells and can induce a more severe zinc-deficient state.

MATERIALS AND METHODS

Cell Culture and Treatment -- The human hepatoblastoma cell line, HepG2, was purchased from the American Type Culture Collection (Manassas, VA). Chelex100 ion exchange resin (Bio-Rad, Hercules, CA) was used to deplete zinc from FBS before FBS

was added to DMEM (Reaves et al., 2000). The resin was first neutralized to physiological pH with 0.25 M HEPES, pH 7.4, and then mixed with FBS in a 1:4 ratio at 4°C for 2 h. The Chelex100 resin was separated from FBS by centrifugation, followed by filtration through a 0.4- μ m filter for sterilization and removal of residual Chelex100 resin. The zinc concentration in this chelexed FBS was < 1.0 μ M, as determined by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). The basal medium consisted of DMEM with 10% chelexed FBS, containing <0.1 μ M zinc, was termed the zinc-deficient (ZD) medium. For the other treatment groups, zinc was added to the media in the form of ZnSO₄ so the only difference between these media was the zinc concentration. The ZD0.4 medium was prepared by addition of 0.4 μ M ZnSO₄ to ZD medium. Previous studies have shown that addition of at least 0.4 μ M ZnSO₄ to ZD medium is required to prevent marked depression of HepG2 cell growth observed in severe zinc deficiency. The zinc normal (ZN) and the zinc-adequate (ZA) media were prepared by adding 4.0 and 16.0 μ M ZnSO₄ to ZD medium to mimic the zinc level observed in normal culture medium or in human plasma, respectively. The zinc-supplemented (ZS) medium consisted of the ZD medium plus 32 μ M ZnSO₄ was used to represent plasma zinc level attainable by oral zinc supplementation in humans. The HepG2 cells were cultured overnight in ZN medium before being changed to their respective media. Cells were then cultured in ZD, ZD0.4, ZN, ZA, or ZS media for the time period as indicated. In experiments with the rescue treatment, cells were cultured in ZD medium for 4 days and then switched over to ZS medium for the last 2 days of culture prior to being harvested as ZD+32 μ M cells.

Determination of Cell number and Viability as well as Cellular Zinc Levels -- Cells and media were collected from tissue culture plates. Cell number was measured by using a hemocytometer. Cell viability was assessed by the traditional Trypan Blue assay. In addition, nonviable cell counts were also determined by the NucleoCounter using the NucleoCassette Kit and NucleoView software (ChemoMetec A/S, Allerød, Denmark), according to the manufacturer protocol. The system features an integrated fluorescence microscope designed to detect signals from a fluorescent dye, propidium iodide, which intercalate to DNA in the nuclei of nonviable cells. Moreover, cell pellets, resulting from centrifugation at 200 x g for 2 min at 4 °C, were washed twice with phosphate-buffered saline (PBS), resuspended in 1.5 mL PBS, and sonicated. Cellular zinc content was measured by flame atomic absorption spectrophotometry (Model 5000, Perkin Elmer, Norwalk, CT) by using standard curves of 0.05-1.0 ppm generated with certified zinc reference solutions (Fisher Scientific, Fair Lawn, NJ) as previously described (Reaves et al., 2000). Furthermore, the certified zinc solutions were compared to Bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. Cellular zinc was calculated as ng per million cells and presented as % of ZN cells.

Nuclear and Cytoplasmic Extract Preparation -- The NE-PER Nuclear and Cytoplasmic Extraction Reagents and the Halt Protease Inhibitor Cocktail Kits (Pierce Biotechnology, Rockford, IL) were used for nuclear and cytoplasmic extracts preparation according to the manufacturer's instructions, which are based on the method of Smirnova et al. (Smirnova 2000). Nuclear and cytoplasmic extracts were then stored in aliquots at -80 °C. Protein concentrations were determined by using the BCA Protein Assay Reagent

kit (Pierce). Contaminations of nuclear extracts by cytoplasmic proteins or contamination of cytoplasmic extracts by nuclear proteins, detected by Western blot analysis of Hsp90 or Oct-1, respectively, were routinely found to be less than 5% in our lab.

Western Blot Analysis -- Forty μg of nuclear and cytoplasmic protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by using a mini-transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10mM phosphate buffer pH7.3, 137mM NaCl, 2.7mM KCL, and 0.1% Tween 20) for 1 h at room temperature, prior to incubation with 1 $\mu\text{g}/\text{ml}$ of a rabbit anti-p21 polyclonal antibody (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA), in PBS-T containing 5% nonfat milk at 4 °C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the Western Blot Luminol Reagent (Santa Cruz Biotechnology) and exposed to film. The optical densities of the protein bands were quantified by the Alpha Innotech Imaging System (San Leandro, CA).

RNase Protection Assays (RPA) -- Total cellular RNA was isolated from HepG2 cells, using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to manufacturer's instructions. The integrity of the RNA was verified by RNA gel electrophoresis. The mRNA abundance of p21 was measured by the non-radioactive RNase Protection Assay System (BD Biosciences, San Diego, CA), according to manufacturer's instructions. The

human GAPDH probe was used as internal reference for normalization. Biotin-labeled riboprobes were synthesized using non-Rad In Vitro Transcription kit with T7 RNA polymerase (BD Biosciences) according to manufacturer's instructions.

p21 Promoter Activity -- The influence of zinc status on the human p21 gene promoter activity was studied by transient transfection of a p21-promoter-luciferase gene into Hep G2 cells. This approach was used to provide further evidence that the transcription process is depressed by a reduction in p21 gene promoter activity in zinc-deficient Hep G2 cells.

Preparation of Luciferase Construct. The p21 promoter construct was a kind gift provided by Dr. Lieberman (Yin et al., 2004), which consisted of a 2,337-bp fragment of the human p21 promoter (El-Deiry et al., 1995), containing two p53-binding consensus sites, and with HindIII and XhoI sites added on the ends. It was isolated and inserted into the plasmid pGL3-basic (Promega, Madison, WI) to generate construct pGL3-p21-Luci plasmid. This pGL3-p21- Luci plasmid was transformed into *E. coli* DH5Q competent cells (Invitrogen) by standard protocol for mass production. The plasmid was prepared by using Wizard PureFection Plasmid DNA purification system from Promega.

Transient Transfection and Luciferase Assay -- Hep G2 cells were transfected by using Tfx-20 reagent according to the protocol provided by the manufacturer (Promega). Hep G2 cells, in DMEM with 10% FBS without antibiotics, were seeded at a density of 2×10^5 cells/well in 24-well plates and cultured for 4 days in DMEM containing 10%

Chelexed FBS plus 0, 0.4, 4.0, 16.0, or 32.0 μ M zinc. Just before transfection, the medium was removed. Transfections were performed in triplicate with 500 ng of the plasmid DNA containing the wild-type p21 promoter luciferase reporter construct and 10 ng of an internal control plasmid, pRL-SV40 (Promega). One hour after transfection, the transfection medium was changed to the respective medium and cultured for 2 more days. Luciferase activity was measured in the Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA) by using the Dual-Luciferase reporter Kit according to recommendations by the manufacturer (Promega). Changes in firefly luciferase activity was calculated and plotted after normalization with changes in renilla luciferase activity in the same sample.

p21 Promoter Accessibility -- The Chromatin Immunoprecipitation (ChIP) Assay of Lagger et al. (Laggar et al., 2003), was performed using the ChIP Assay Kit (Upstate Biotechnology) according to the manufacturer's recommended protocol for the determination of the association of acetylated histone with the proximal and distal region of the p21 promoter. This approach was used to verify our contention that the depression of the p21 transcription process by zinc deficiency is partially due to a depressed p21 promoter accessibility of the proximal and distal (with p53 responsive element) regions of the p21 promoter (**Fig. 5A**). Formaldehyde-crosslinked chromatin was prepared from Hep G2 cells and immunoprecipitations was performed using the ChIP Assay Kit. One tenth of the DNA from each immunoprecipitation was used in each PCR reaction. All PCRs were performed on a Perkin Elmer GeneAmp PCR System 2400 with Applied Biosystems' Golden Taq polymerase. PCR primers were the same as those published by other researchers (Laggar et al., 2003), which amplified the proximal (5'-GGT GTC TAG

GTG CTC CAG GT-3' and 5'-GCA CTC TCC AGG AGG ACA CA-3') and distal (5'-GGT CTG CTA CTG TGT CCT CC-3' and 5'-CAT CTG AAC AGA AAT CCC AC-3') regions of p21 promoter. Primers for GAPDH promoter (5T-AAA AGC GGG GAG AAA GTA GG-3T; 5T-CTA GCC TCC CGG GTT TCT CT-3') were used as control. PCR condition: 95 °C for 5 min, once; 95 °C for 1min, 50°C for 1min, 72°C for 2 min, 35 cycles; and 72°C for 7 min, once. The amplified DNA was separated on 2.0 % agarose gel and visualized with ethidium bromide. The optical densities of the DNA bands were quantified by scanning densitometry (Alpha Innotech) and normalized to that of ZN cells.

Transient Transfection of p21 Overexpression Plasmid PCMV-p21 -- HepG2

cells were cultured in media with respective zinc concentration (ZD = <0.1 μM; ZN = 4 μM) for 4 days. Thereafter, cells were transiently transfected in serum free DMEM media by using Tfx-20 with graded amounts of PCMV-p21 plasmids (1, 3, 5, 7 and 10 μg). PCMV plasmid without carrying p21 gene (PCMV-control) was used as control. ZD and ZN cells transfected with PCMV-control are indicated as ZD+0 and ZN+0. The PCMV-p21 plasmid was a kind gift provided by Dr. Ariga (Ono et al., 2000), which consisted of a constitutive promoter followed by the full length p21 coding sequence. Both PCMV-p21 and PCMV320 control plasmids were individually transformed into *E. coli* DH5α competent cells (Invitrogen) by standard protocol for mass production. The plasmids were prepared by using Wizard PureFection Plasmid DNA purification system from Promega. After transfection, cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to protein expression analysis by western-blot.

Cdk4 Immunoprecipitation Kinase Assay – Cdk4 binds with cyclin D1 to form an active complex that can phosphorylate Rb protein at Ser⁷⁹⁵ (Pietenpol and Stewart 2002; Xiong et al., 1993). The established method of Ding et al. (Ding et al., 2005) that measures the phosphorylation of Rb at Ser⁷⁹⁵ was used. Cells were lysed by using RIPA lysis buffer according to manufacturer protocol (Santa Cruz). Total cell lysate was immunoprecipitated overnight at 4 °C with: mAb anti-cdk4, mAb anti-cyclin D1, or mouse IgG. Immunoprecipitates were then washed twice with the Rb kinase buffer [50 mM Hepes (pH 7.5), 1 mM EGTA, 10 mM KCl, 10 mM MgCl₂, and 1 mM dithiothreitol), resuspended in the Rb kinase buffer with the addition of 10 mM ATP and 0.5 µg of recombinant Rb protein (QED Bioscience, Inc., San Diego), and incubated with gentle shaking for 30 min at 30°C. The kinase assay was stopped by the addition of SDS sample buffer. The reactants were subjected to 12% SDS-PAGE, immunoblotted with rabbit anti-phosphospecific Rb [pSer⁷⁹⁵] IgG, stripped, and reprobed with rabbit anti-Rb IgG.

Cell cycle analysis -- DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS) by using a FACScalibur cytometer (Becton Dickinson, San Jose, CA). HepG2 cells were cultured in respective zinc media for one passage, trypsinized, washed in PBS (Ca²⁺, Mg²⁺ free), and fixed in 70% ethanol. Cells were then stained with propidium iodide. Flow cytometry and FACS analysis (FACScanner; Becton Dickson) were used to quantify the distribution of DNA fluorescence and intensity. Flow cytometric data files were collected and analyzed by using the CELLQuest program (Becton Dickinson). A total of 10,000 cell events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow

green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

Statistical Analysis -- The data were analyzed with one-way ANOVA by using SPSS 14.0 Windows software (SPSS Inc., Chicago, IL). The means were further analyzed by least significant differences. Values were expressed as means \pm SE, with $P < 0.05$ being considered significant.

RESULTS

Zinc-deficient medium depletes cellular zinc.

HepG2 cells were cultured for one passage in the zinc depleted and supplemented media. Growth as measured by cell number was significantly depressed in ZD and ZD0.4 cells to 63 % and 65% of ZN cells (**Fig. 4A**). In addition, no differences in cell number were observed among the ZN, ZA, ZS and ZD+32 μ M cells. Cell viability was very high, at the optimal level, and no difference was detected among all treatments (98% and 99% or higher as determined by the NucleoCounter method and the Trypan Blue Exclusion assay, respectively). Cellular zinc levels were expressed per million cells to correct for any differences in cell numbers between plates. In ZD and ZD0.4 cells, cellular zinc concentration was reduced to 43 % and 50 %, respectively, of ZN controls (**Fig. 4B**). Moreover, cellular zinc levels in ZA, ZS and ZD+32 μ M cells were 52%, 77 % and 85 %, respectively, higher than ZN cells. Furthermore, cellular zinc levels in ZS and ZD+32 μ M cells were higher than ZA. Thus, the supplementation of ZD cells with ZS medium (ZD+32 μ M), for the last 2 days of culture, was found to normalize cell growth and

cellular zinc level to that of the ZN cells. This rescue approach demonstrated the specificity of zinc treatment and the speed of normalization of cell growth and cellular zinc level by zinc replenishment.

Zinc depletion decreases nuclear and cytoplasmic p21 protein as well p21 mRNA levels.

Nuclear p21 protein levels in the ZD and ZD0.4 cells were significantly reduced to 40 % and 43%, respectively, of ZN cells (**Fig. 5A**). Cytoplasmic p21 protein levels in ZD and ZD0.4 cells were similarly reduced (**Fig. 5B**). The p21 mRNA levels in ZD and ZD0.4 cells were almost 30 % lower than in ZN cells (**Fig. 6**). In contrast, no significant differences were detected in nuclear and cytoplasmic p21 protein levels as well as p21 mRNA abundance among the ZN, ZA, ZS, and ZD+32 μ M cells. Most importantly, the rescue approach demonstrated the specificity of zinc treatment and the rapid normalization of both p21 protein and mRNA levels by zinc replenishment.

p21 promoter activity is decreased in zinc depleted HepG2 cells.

Transient transfection of a p21-promoter-luciferase reporter gene into HepG2 cells indicated that the p21 promoter activity of ZD and ZD0.4 cells was significantly reduced to 64% and 66% of ZN cells, respectively (**Fig. 7**). In contrast, no differences were observed among the ZA, ZS, ZD+32 μ M, and ZN cells. Thus, the supplementation of ZD cells with ZS medium (ZD+32 μ M), for the last 2 days of culture, was found to normalize the p21 promoter activity to that of the ZN cells. This rescue approach demonstrated the specificity of zinc treatment and the speed of normalization of p21 promoter activity by zinc replenishment.

Zinc depletion reduces the p21 promoter accessibility

To test whether cellular zinc status affects local histone acetylation pattern on the p21 promoter, we performed ChIP experiments with chromatin isolated from HepG2 cells. After cross-linking with formaldehyde, chromatin was sonicated and immunoprecipitated with antibodies against the acetylated form of histone H4. In addition, ChIP assays were performed in parallel without antibodies to control the specificity of the reaction. Moreover, primers specific for the human GAPDH gene were used as control. Furthermore, PCRs were performed with standards consisting of fixed amounts of genomic DNA as template to assure that amplification reactions were within the linear range. As shown in **Fig. 8**, the amounts of acetylated histone H4 associated with the proximal and distal p21 promoter regions were significantly decreased in ZD ($73 \pm 5\%$; $64 \pm 5\%$, respectively) and ZD0.4 ($82 \pm 1\%$; $77 \pm 6\%$, respectively) cells than in ZN ($100 \pm 3\%$; $100 \pm 3\%$, respectively) cells. Although there appeared to be a trend for the amounts of acetylated histone H4 associated with the proximal or distal p21 promoter regions to be lower in ZD than ZD0.4 cells, the differences were not significant. No significant changes in acetylation at the GAPDH locus were observed among the treatments. In the absence of specific antibodies, no specific PCR products were amplified. Our data demonstrate that, in zinc-deficient HepG2 cells, the reductions in p21 protein and mRNA expression correlate well with the depressed p21 promoter activity and accessibility.

Transfection of the PCMV-p21 plasmid, at 5 >g/plate, normalizes p21 protein level in ZD cells to that of ZN cells

The method of Ariga et. al., (Ono et al., 2000) to overexpress p21, by transient transfection of the PCMV-p21 plasmid, was used to normalize the decrease in p21 protein level induced by zinc deficiency. By transfecting graded amounts of PCMV-p21 plasmid (1, 3, 5, 7, 10 microgram per plate), which constitutively expressed p21, the amount of PCMV-p21 at 5 µg per transfection was established to be the right dosage capable of restoring the p21 expression level in the PCMV-p21 transfected ZD cells 427 (ZD+5) back to the level of ZN control cells (ZN+0) (Fig 6). In addition, ZN cells transfected with 5 µg of PCMV-p21 (ZN+5) demonstrated a 48% increase in p21 protein level than ZN control cells (ZN+0).

Normalization of p21 protein level in ZD-PCMV-p21 cells restores the cyclin D1-cdk4 complex activity to the level of ZN cells.

The role of p21 as an assembly factor for the formation of active cyclin D1-cdk4 complex, which modulate cell cycle positively, has been reported in several cell models (Kavurma and Khachigian 2003; Kavurma and Khachigian 2004; Nosedá et al., 2004). Thus, studies were designed to examine whether the repressed p21 is responsible for the reduction in cell growth observed in zinc-deficient HepG2 cells. Firstly, the cyclin D1-cdk4 complex activity was determined by the standard non radioactive method of Ding et. al. (Ding et al., 2005). The experimental design involved the usage of ZN and ZD cells transfected with PCMV-p21 or PCMV-control plasmids. As a measure of cyclin D1-cdk4 complex activity, the level of phosphorylation at Ser⁷⁹⁵ of a recombinant truncated Rb protein (Pietenpol and Stewart 2002, Swanton 2004) was determined and was found to be significantly lower by only 20% in ZD than ZN cells (**Fig. 10**). Most interestingly, after

the transfection of 5 μ g PCMV-p21, the cyclin D1-cdk4 complex activity was normalized in ZD cells and remained not altered in ZN cells as compared to ZN cells transfected with control plasmid (**Fig 10**). In addition, as a control to assess the quality of the lysate, equivalent amount of the lysate from both ZD and ZN cells transfected with PCMV-p21 or PCMV-control plasmids were electrophoresed by SDS-PAGE and immunoblotted with mAb anti-GAPDH (**Fig. 10C**) and was found to be optimal among all treatments. Thus, the results indicated that the small reduction in cyclin D1-cdk4 complex activity appeared to be the result of the decreased p21 protein level in ZD cells.

Inhibition of cell growth and G1/S cell cycle progression in zinc-deficient HepG2 cells are independent of the decrease in p21 protein level.

Cell growth and cell cycle progression analysis were also determined in the same batch of cells used for the measurement of cyclin D1-cdk4 complex activity after the rescue of p21 level in ZD cells. The result indicated that cell growth remained repressed in ZD PCMV-p21 cells, with normalized p21 expression, as compared to ZD-PCMV-control cells. (**Fig. 11**). Most importantly, in the ZN-PCMV-p21 cells, transfected with the same amount of PCMV-p21 plasmid, the marked increase in p21 expression was accompanied by a 30% reduction in cell growth, which demonstrated the growth inhibitory effect of p21 expression at high level. In addition, consistent with our previous report (Cui et al., 2002), cell cycle progression was impaired at the G1/S phase in ZD cells (**Fig. 12**). However, the impaired G1/S cell cycle progression remained unchanged in the ZD-PCMV-p21 cells, with normalized p21 level and cyclin D1-cdk4 complex activity (**Fig. 12**). Moreover, the G1/S progression was mildly impaired in the ZN-

PCMV-p21 cells, with markedly elevated p21 protein level, which indicated that the overexpressed p21 is functionally effective in inhibiting cell cycle progression and growth. Thus, these findings suggest that, in zinc-deficient HepG2 cells, the decreased p21 protein level is not responsible for the associated inhibition of cell growth and cell cycle progression.

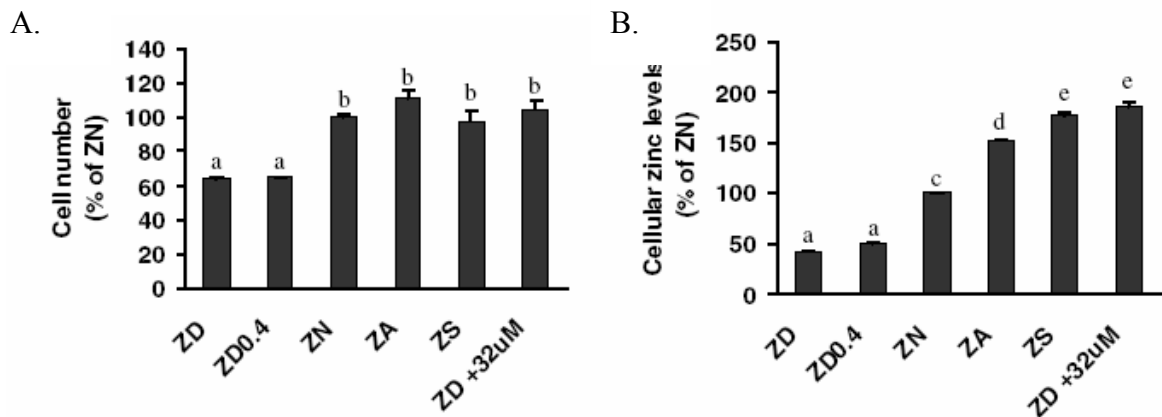
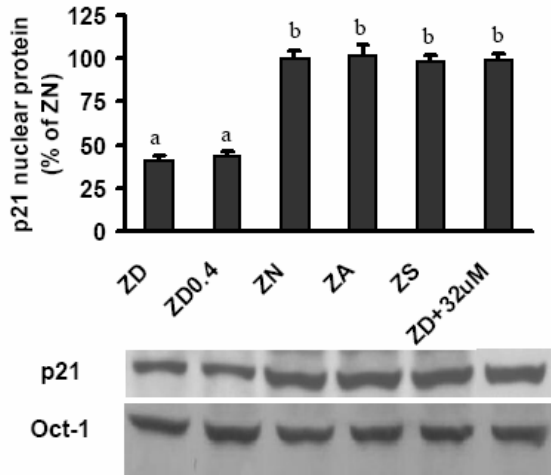


Figure 4. Zinc-deficient medium depletes cellular zinc.

Fig. 4A: HepG2 cells were cultured for one passage in a basal medium depleted of zinc to induce severe zinc-deficient (ZD) cells or in the basal medium supplemented with 0.4, 4.0, 16, or 32 μM zinc to represent mild zinc deficiency (ZD0.4), the amount of zinc in most normal media (ZN), the normal human plasma zinc level (zinc-adequate, ZA), or the high end of plasma zinc attainable by oral supplementation (ZS), respectively. For the rescue treatment, cells were cultured in ZD medium for 4 days and then switched over to ZS medium for the last 2 days of culture prior to being harvested as ZD+32 μM cells. Cells were counted by using hemacytometer. Cell number was expressed as a percentage of ZN controls. Values are means \pm SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

Fig. 4B: Cellular zinc was measured by atomic absorption spectrophotometry. Cellular zinc levels were expressed as a percentage of ZN controls. Values are means \pm SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$.

A.



B.

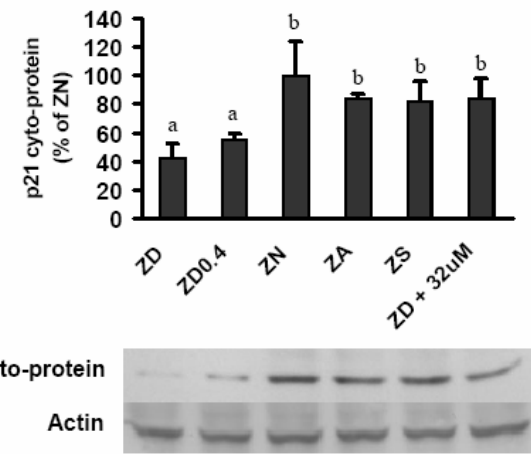


Figure 5. Low intracellular zinc status reduces p21 protein level.

Nuclear and cytoplasmic proteins (40 μ g) prepared from Hep G2 cells were separated on SDS-PAGE, Western blotted, and probed with antibody against p21 (C-19). The nuclear or cytoplasmic protein blots were also probed with antibody against Oct-1 (H-65) or actin (I-19), respectively. The optical densities of the protein bands were quantified by the Alpha Innotech Imaging System (San Leandro, CA). The nuclear (**Figure 5A**) or cytoplasmic (**Figure 5B**) p21 protein band was normalized to the corresponding Oct-1 or actin value, respectively, and expressed as a percentage of ZN cells. Values shown in bar graphs represent means \pm SEM from 3 separate experiments. Data were analyzed by one way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

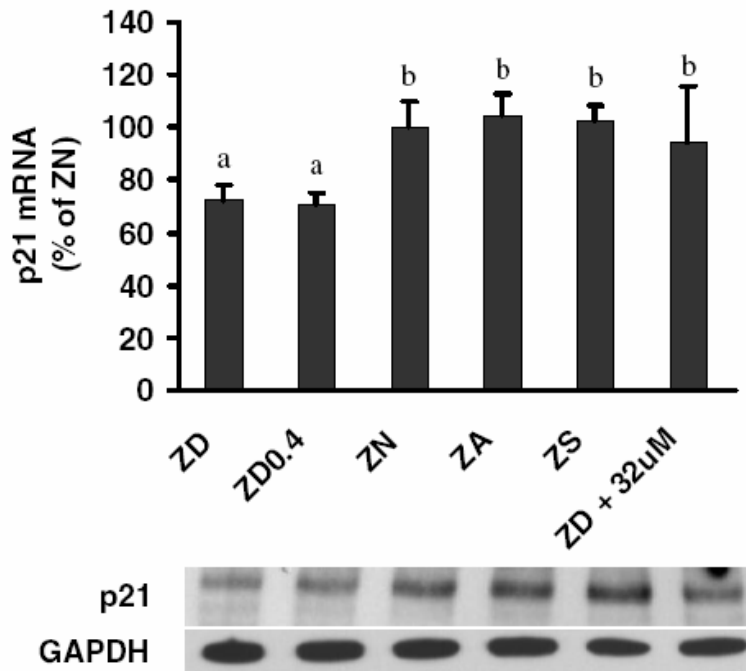


Figure 6. Low intracellular zinc status reduces p21 mRNA level.

Total RNA was isolated from cells by using RNeasy kit (Qiagen, Valencia, CA). The abundance of p21 mRNA was measured by using a nonradioactive RPA kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. The RPA probe set, which included p21 and GAPDH probe, was from the same manufacturer. The optical densities of the protected p21 bands were quantified by the Alpha Innotech imaging system (San Leandro, CA) and then normalized to that of GAPDH. Values shown in bar graphs represent means \pm SEM from 3 separate experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

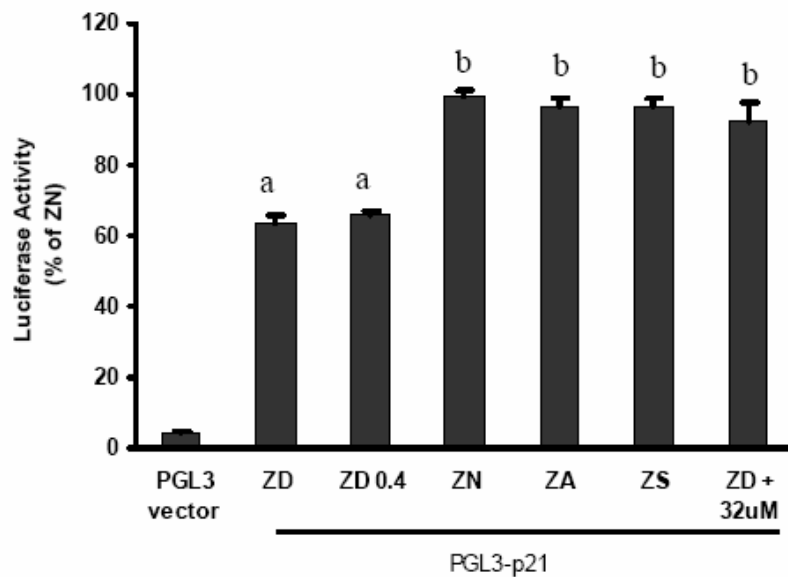


Figure 7. Low intracellular zinc status depresses *p21* promoter activity.

HepG2 cells were cultured in media with respective zinc concentration (ZD = $<0.1 \mu\text{M}$; ZD 0.4 = $0.4 \mu\text{M}$; ZN = $4 \mu\text{M}$; ZA = $16 \mu\text{M}$; and ZS = $32 \mu\text{M}$ zinc supplemented to the basal medium) for 4 days. Thereafter, cells were transiently transfected in serum free DMEM media by using Tfx-20 with 500 ng of luciferase reporter construct (PGL3-p21) containing the *p21* promoter, together with 10 ng pRL-SV40 as internal control. PGL3 basal vector without carrying any promoter was used as the vector control. After transfection, cells were cultured in corresponding media for 2 more days. For the rescue treatment, cells were cultured in ZD medium for 4 days, transfected and then switched over to ZS medium for the last 2 days of culture prior to being harvested as ZD+32 μM cells. Cells extracts were then assayed by Dual-Luciferase reporter system and signals were measured by a Luminometer. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

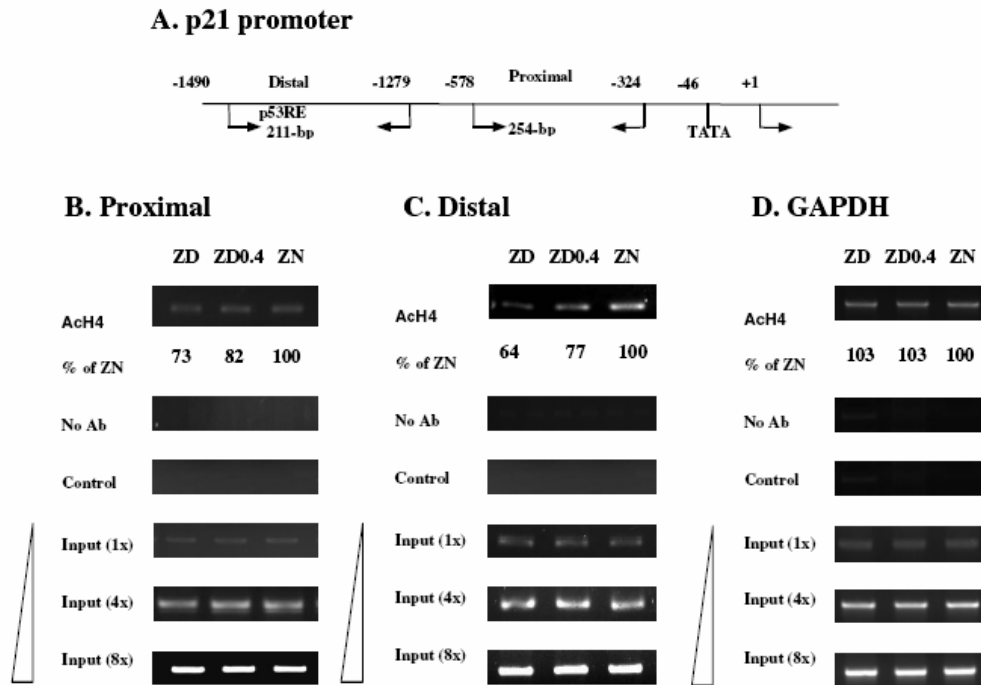


Figure 8. Acetylation of histones on the proximal and distal *p21* promoter is depressed in zinc-deficient HepG2 cells.

(A) Relative positions of PCR primers for the amplification of proximal or distal *p21* promoter fragments are shown in a schematic drawing. (B) Chromatin immunoprecipitation (ChIP) analysis of the *p21* proximal promoter was performed with antibodies to acetylated histone H4 and unspecific antibodies (control) by using chromatin isolated from HepG2 cells. (C) Same as for panel B, but with ChIP analysis for the *p21* distal promoter. (D) Primers for the human GAPDH gene were used as a control. The data shown are representative of three independent experiments. AcH4, acetylated histone H4; No Ab, without antibody.

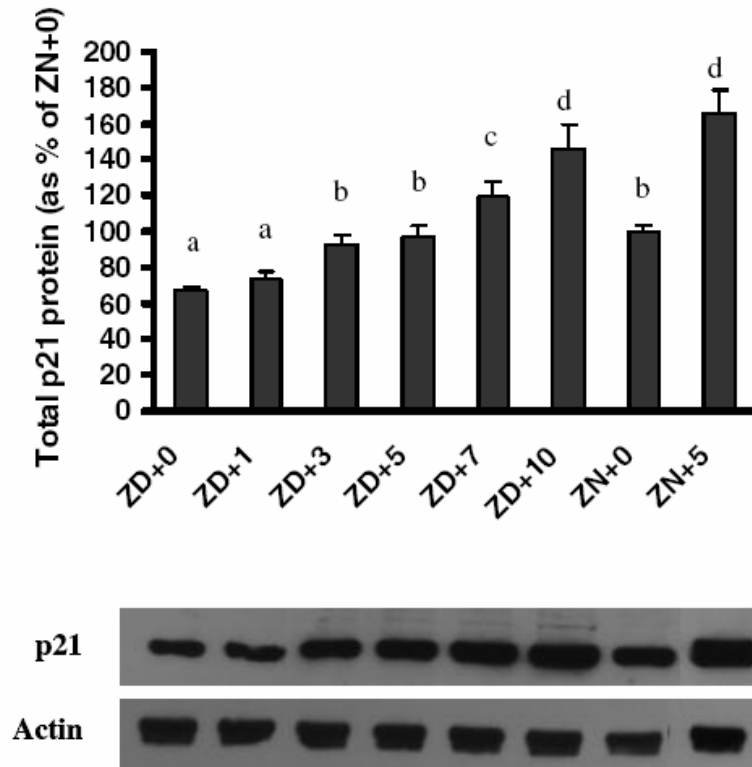


Figure 9. Transfection of p21 expression plasmid PCMV-p21, at 5 μ g/plate, restores the p21 protein level in ZD cells back to that of ZN cells.

HepG2 cells were cultured in media with respective zinc concentration (ZD = $<0.1 \mu\text{M}$; ZN = $4 \mu\text{M}$) for 4 days. Thereafter, cells were transiently transfected in serum free DMEM media by using Tfx-20 with graded amounts of PCMV-p21 plasmids (1, 3, 5, 7 and 10 μg). PCMV plasmid without carrying p21 gene was used as control (PCMV801 control). ZD or ZN cells transfected with PCMV-control plasmid are indicated as ZD+0 or ZN+0 cells, respectively. After transfection, cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to protein expression analysis by western-blot. Values are means \pm SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$);

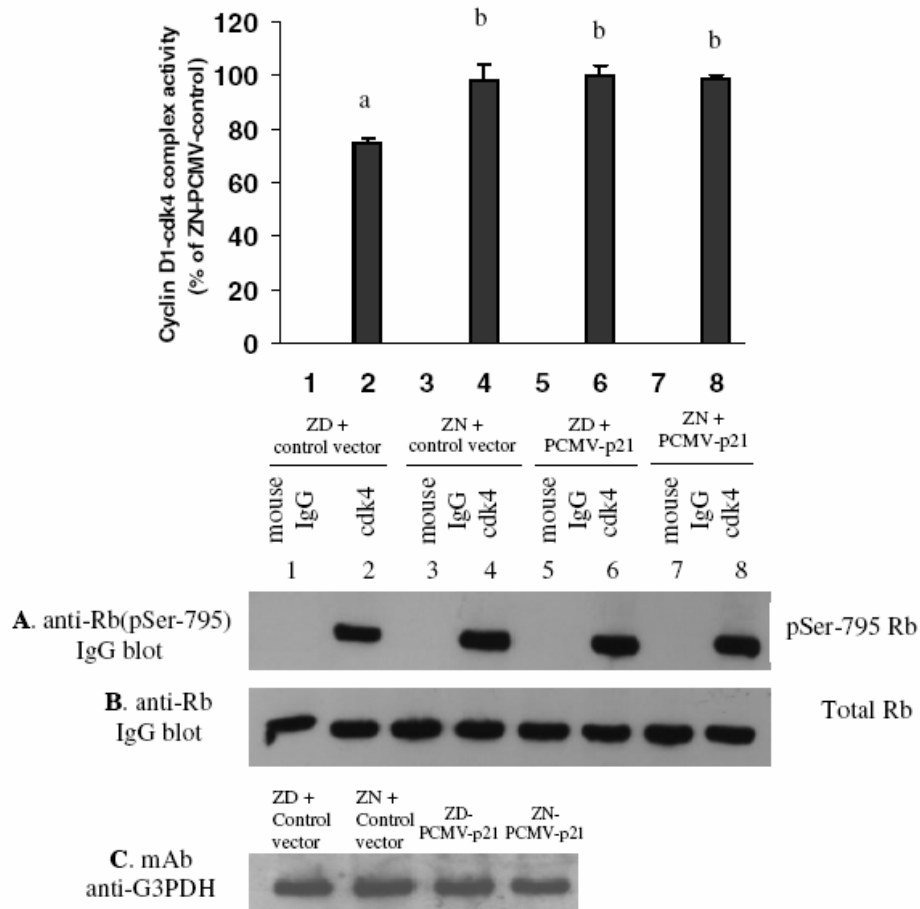


Figure 10. Transfection of p21 expression plasmid PCMV-p21, at 5 μ g/plate, restores the cyclin D1-cdk4 complex activity in ZD cells back to that of ZN cells. Equivalent amount of lysate (900 μ g) was immunoprecipitated (IP) with mAb anti-cdk4 or mouse IgG, washed, the immunoprecipitated protein subjected to the cdk4 kinase assay, followed by 12% SDS-PAGE and immunoblotting with anti-Rb [pSer⁷⁹⁵] IgG (A), stripped, and reprobed with anti-total Rb IgG (B). C, an equivalent amount of lysate (100 Cg) was subjected to SDS-PAGE and immunoblotted with mAb anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a control for the quality 816 of the lysate. Values are means \pm SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

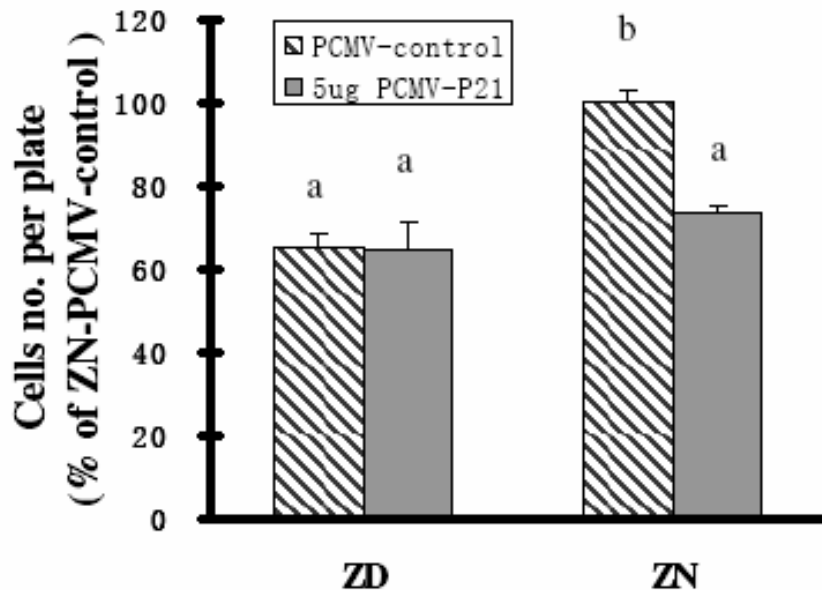


Figure 11. Zinc deficiency induced inhibition of cell growth remains unchanged after normalization of p21 protein level.

HepG2 cells were cultured in media with respective zinc concentration (ZD = $<0.1 \mu\text{M}$; ZN = $4 \mu\text{M}$) for 4 days. Thereafter, cells were transiently transfected in serum free DMEM media by using Tfx-20 with either $5 \mu\text{g}$ PCMV-p21 or control plasmid per 100 mm plate. After transfection, the cells were cultured in corresponding media for 2 more days. Cell numbers were counted and expressed as % of ZN control. Values are means \pm SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

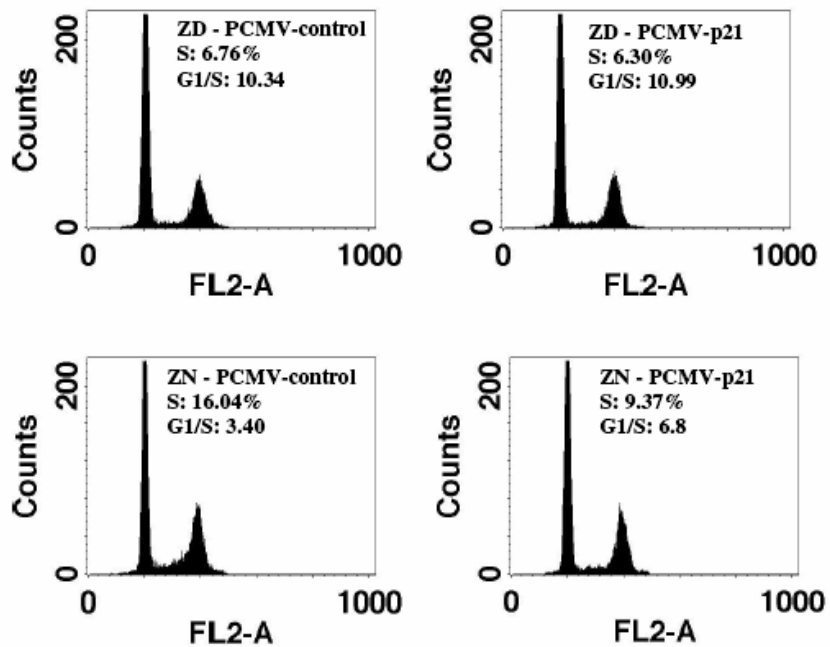


Figure 12. Zinc deficiency induced inhibition of G1/S cell cycle progression remains unchanged after normalization of p21 protein level.

In cell cycle analyses, DNA content of Hep G2 cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in respective zinc concentration (ZD = <math><0.1 \mu\text{M}</math>; ZN =

DISCUSSION

In previous studies (Cui et al., 2002), we have reported reductions in cell growth and cellular zinc status in zinc-deficient HepG2 cells. In addition, cellular zinc status was highly responsive to the zinc content of the culture medium among treatments. Moreover, cell cycle analysis indicated that progression from G1 to S phase was depressed in ZD cells. Furthermore, no DNA fragments, were detected in the region M1 of flow cytometric examinations for all treatments, which indicate the presence of very little or no apoptotic cells. In view of available data indicating that p21 repression and overexpression may lead to depressed cell cycle progression, we have designed the present study to examine the influence of zinc status on the transcriptional process of the p21 gene. In the present study, similar reductions in cell growth for ZD cells and cellular zinc status among zinc deficient cells were observed as in our previous studies. Thus, the changes in cell growth in ZD cells and cellular zinc status among treatments were similarly replicated in the present studies.

In the present report, we have presented multiple lines of evidence for the first time that the transcriptional process and expression of p21 are depressed in zinc-depleted HepG2 cell. The observed reductions of 60% in nuclear p21 protein and almost 30% in p21 mRNA in zinc-depleted cells as compare to control ZN cells suggest that the transcriptional process might be compromised in zinc-depleted cells. In view of a similar magnitude of reduction for cytoplasmic p21 protein was also observed in zinc depleted cells, the observed reduction in nuclear p21 protein appears unlikely to have resulted from a decreased transport into the nucleus or an enhanced turnover in the nucleus. To

explain for the larger magnitude of reduction in protein level than mRNA abundance in zinc-deficient cells, an impaired translational process or enhanced degradation of p21 protein or both may possibly have contributed to the higher magnitude of reduction in p21 protein level in zinc-deficient HepG2 cells. Nevertheless, in the present study we have elected to concentrate our efforts on the transcriptional process by determining the influence of zinc status on the p21 promoter activity and accessibility. In the present study, the p21 promoter activity was measured by transient transfection of a p21 promoter-reporter gene into HepG2 cells. Marked reductions of 36% and 34% in p21 promoter activity in ZD and ZD0.4 cells, respectively, as compared to ZN cells indicate that the transcription process was compromised by zinc deficiency. In addition, the supplementation of ZD cells with ZS medium, for the last day of culture, was able to normalize the p21 promoter activity to that of ZN cells. This approach substantiates the specificity of zinc supplementation and the speed for normalization of p21 promoter activity by zinc replenishment. Moreover, the similar magnitude of reduction for p21 mRNA level to almost 30% lower in zinc-deficient cells than controls as compared to the 34 to 36% reduction for p21 promoter activity, may suggest that the depressed promoter activity is mainly responsible for the reduced mRNA abundance.

DNA exists in the form of highly organized chromatin in the eukaryotic nucleus. The nucleosome is the basic unit with DNA wrapped around an octamer of histones, comprising of a pair of H2A, H2B, H3, and H4. To activate gene transcription, the highly dense chromatin structure must be disrupted by chromatin remodeling complexes to enhance the accessibility of the promoter of a gene to transcription factors. Histone

modifying enzymes and ATP-dependent remodeling complexes are two major groups of chromatin modifying complexes capable of disrupting chromatin structure. Among the well-characterized histone modifying enzymes, histone acetyltransferase is one that acetylates the histone tails, reduces the interaction between DNA and histone, enhances the accessibility of DNA to transcriptional factors, and promotes transcriptional activation (Smirnova 2000). The chromatin immunoprecipitation (ChIP) assay, which employs antibodies against epigenetic protein markers such as acetylated histones, has been used to determine the chromatin state of individual genes.

Recent ChIP experiments performed by Lagger et al. (Lagger et al., 2003) demonstrated that, upon induction by actinomycin D, p53 can replace HDAC at the p21 promoter, and the resultant increase in histone acetylation on the p21 promoter correlates well with the activation of the p21 gene. Similar ChIP studies by Liu et al. (Liu et al., 2003) reported that the amount of acetylated histones on the p21 promoter, especially the proximal promoter, directly correlate with the extent of p21 expression.

In our ChIP study, we have selected a region proximal to the TATA box as the proximal p21 promoter region. The proximal promoter is the region to which the basal transcription machinery would bind. In addition, we have selected a region containing the first downstream p53 responsive element as the distal p21 promoter region. Both promoter regions are the same as those used by Lagger et al. (Lagger et al., 2003) and are similar to those used by Liu et al. (Liu et al., 2003). Data from our ChIP experiments indicate that in ZD and ZD0.4 cells, the amounts of acetylated histone H4 on the

proximal region of the p21 promoter were decreased to 73% and 82% of ZN cells, respectively. Similarly, the amounts of acetylated histone H4 on the distal region of the p21 promoter were markedly reduced in ZD and ZD0.4 cells to 64 % and 77% of ZN cells, respectively. These reductions in acetylated histone H4 on the proximal and distal regions of the p21 promoter suggest that the p21 promoter in zinc-depleted cells may be less accessible to the transcription machinery. Thus, the present ChIP data provide another line of evidence, which supports the promoter activity data, indicating that the transcriptional process is depressed in zinc depleted cells. Furthermore, the magnitude of reductions in histone acetylation on the p21 promoter correlates well the magnitude of depressed p21 promoter activity and p21 mRNA abundance in zinc depleted cells.

p21 has been commonly reported as a negative cell cycle regulator by acting as an inhibitor of cyclin/cdk. In this context, a repression of p21 enhances cell cycle progression. However, p21 may also function as positive cell cycle regulator by functioning as an assembly factor for cyclin D1-cdk4 complex (LaBaer et al., 1997). In this context, repression of p21 impairs cell cycle progression. Recently, impairment of cell proliferation induced by a repressed p21 expression has been demonstrated in several cell models including smooth muscle cells (Kavurma and Khachigian 2003; Kavurma and Khachigian 2004), endothelial cells (Nosedá et al., 2004) and LoVo colon cancer cells (Arango et al., 2003). Therefore, the ability of the depressed p21 level, induced by zinc deficiency, in down-regulating cyclin D1-cdk4 activity, which in turn contributing to the cell growth depression was investigated. The result indicated that cyclin D1-cdk complex kinase activity was significantly lower by 20% in ZD cells as compared to ZN

cells. By restoring the p21 protein level in ZD cells back to the level of ZN cells, with transient transfection of PCMV-p21 plasmid that constitutively expressed p21, the cyclin D1-cdk4 complex activity in ZD-PCMV-p21 cells was normalized to the same level as in ZN cells. Thus, the decrease in p21 protein level in ZD cells appeared to be responsible for the small reduction in cyclin D1-cdk4 complex activity. However, data from both cell growth and cell cycle analysis indicated that impaired cell growth and G1/S cell cycle progression remained unaltered in ZD-PCMV-p21 cells, with restored p21 protein level and cyclin D-cdk4 complex activity. Thus, in zinc-deficient HepG2 cells, the decrease in p21 level appeared not to be responsible for the inhibition of cell growth and G1/S progression. Therefore, in zinc-deficient HepG2 cells, other p21 unrelated effects of zinc deficiency may be responsible for the inhibition of cell cycle progression and growth. Recently, MacDonald (MacDonald 2000) reviewed past reports on the role of zinc in growth and cell proliferation. However, the exact mechanism responsible for the inhibition of cell growth induced by zinc deficiency has not been fully established. The author postulated that metalloenzymes (Chesters and Boyne 1991; Wu and Wu 1987) such as DNA polymerase, involved in DNA synthesis, may be responsible for the reduction in DNA synthesis and cell growth.

In summary, the present data suggest that the reduction in acetylated histone 4 on the p21 promoter resulted in a depressed p21 promoter accessibility, which contributed to the decrease in p21 promoter activity and the down-regulation of p21 mRNA and protein expression in zinc-depleted HepG2 cells. Moreover, the decreased level of p21 protein appeared not to be responsible for the impaired cell growth and G1/S cell cycle

progression in zinc-depleted HepG2 cells. Recent studies in our laboratory (Alshatwi et al., 2006) have established in zinc-deficient HepG2 cells a marked reduction in the level of nuclear p300 protein, which is known to be the main coactivator recruited by p53 to responsive elements of a target gene. Apart from the scaffolding role of p300, its function in chromatin remodeling has been identified through the intrinsic histone acetyltransferase (HAT) activity of p300 (Kalkhoven 2004). Thus, the marked reduction in nuclear p300, accompanied by an expected reduction in its intrinsic HAT activity, may have contributed to the observed decrease amounts of acetylated histone H4 associated with the proximal and distal promoter regions of the p21 gene in the zinc-deficient cells. Our future studies will be designed to establish the influence of cellular zinc status on the interactions and functions of transcription factors, including p300, on the p21 promoter.

CHAPTER IV

Zinc induced G2/M blockage is p53 and p21 dependent in normal human bronchial epithelial cells

Abstract

The involvement of p53 and p21 signal pathway in the G2/M cell cycle progression of zinc supplemented normal human bronchial epithelial (NHBE) cells was examined using the siRNA approach. Cells were cultured for one passage in different concentration of zinc: $<0.4 \mu\text{M}$ (ZD) as zinc-deficient; $4 \mu\text{M}$ as normal zinc level in culture medium; $16 \mu\text{M}$ (ZA) as normal human plasma zinc level; and $32 \mu\text{M}$ (ZS) as the high end of plasma zinc attainable by oral supplementation. The nuclear p21 protein level and mRNA abundance as well as promoter activity in ZS cells, but not in ZD cells, were markedly elevated to almost 2-fold when compared to ZN control cells. G2/M blockage in ZS cells was coupled with the observation of elevated p21 gene expression. The positive linkage of p21 elevation and G2/M blockage in ZS cells was demonstrated by gene knock-down approach. In ZS cells, the abrogation of p21 protein induction by the transfection of p21 siRNA was shown to alleviate the G2/M blockage. Because p53 protein level was also found to be elevated to a magnitude similar to that of p21 protein in ZS cells, a similar gene knock-down approach was used to establish if the p21 upregulation in ZS cells was p53 dependent. Abolishment of the increase in p53 protein in ZS cells with transfection of p53 siRNA treatment normalized the elevated p21 protein to a similar level as in ZN control cells, which demonstrated that the p21 induction is p53-dependent. Furthermore, the normalization of p53 protein by siRNA in ZS cells

alleviated cell growth depression and G2/M blockage, which demonstrated that p53 was involved in the high zinc status induced G2/M blockage and growth depression. Thus, high zinc status in NHBE cells upregulates p53 expression which in turn elevates p21 that eventually induces G2/M blockage.

Introduction

The accuracy of genomic DNA replication is ensured by two major checkpoint controls at G1-S and G2/M transition during cell division. Therefore, deregulation of these checkpoint controls would cause genomic instability, which has been implicated in carcinogenesis (Elledge et al., 1996). The tumor suppressor gene, p53, has been found to be mutated in a large fraction of human cancers and is a major gatekeeper of cell cycle division (Levine et al., 1997). p53 protects mammals from carcinogenesis by inducing apoptosis, DNA repair and cell cycle arrest in response to a variety of stresses (Ko and Prives 1996; Levine, 1997; Agarwal et al., 1998). In response to ionizing or UV radiation or other chemical stresses, p53 has been found to mediate cell cycle arrest mainly at G1-S transition through the immediate downstream target, p21/waf1 (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). However, circumstantial evidences have emerged to support that p53 is also involved in the regulation of G2/M transition through the modulation of p21 expression.

Involvement of p53 in G2/M transition in response to DNA damage stress has been elucidated by two major approaches. The first approach was to investigate the effect of eliminating p53 on the G2/M checkpoint. When p53 protein was inactivated by the human papilloma virus E6 protein (HPV-E6), the number of IMR-90 normal fibroblasts

entering mitosis after exposure to ionizing radiation was much higher, suggesting that p53 is required for G2 arrest (Thompson et al., 1997). In another case, expression of a truncated form of p53 that formed inactive tetramers with endogenous wild type p53 shortened the G2 delay in IMR-90 fibroblasts (Thompson et al., 1997). Overexpression of SV40 large T antigen in IMR-90 fibroblasts shortened the G2 delay stimulated by exposure to ionizing radiation, which was explained by inactivation of p53 protein bound by large T antigen (Chang et al., 1997). The p53 null cell line, human colorectal tumor cell line HCT116, more clearly revealed the role of p53 played in the G2 checkpoint (Bunz et al., 1998). After exposure of HCT116 to ionizing radiation, although the cells initially arrested at G2/M, the arrest was not stable and cells eventually entered mitosis. This indicates that p53 is not required for the initial arrest of HCT116 cells in G2 but is essential for the long-term maintenance of the arrest. The second approach was to examine the effect of overexpressing p53 on the G2/M transition. Agarwal et al. (1995) have used a tetracycline-inducible system in p53-null human fibroblasts to demonstrate the role of p53 in G2 arrest. They firstly synchronized all p53-null human fibroblasts population to the beginning of S phase with mimosine. Thereafter, mimosine was removed and p53 was induced with tetracycline. They found that up to 60% of cells became arrested at G2/M and the level of phosphorylated histone H1b, which is normally highest during mitosis, was very low in these arrested cells (Agarwal et al., 1995; Taylor et al., 1999). These results indicate that p53 is involved in G2 arrest and that the arrest is not due to an aborted attempt at mitosis, followed by arrest in G1.

Successful G2/M transition is governed by cyclin dependent kinase Cdc2 (Nurse 1990). Cdc2 binds to Cyclin B to form a complex which is activated by Cdc25 at the onset of mitosis (Draetta and Eckstein 1997). The mechanism by which p53 regulates the G2/M transition is proposed to mediate its immediate downstream transcriptional target, p21, which is a Cdc2 inhibitor (Winters et al., 1998). The involvement of p21 in G2 checkpoint has been widely documented. Induction of p21 using a tetracycline-regulated system caused a number of different cell lines, including human Hela cervical carcinoma cells, Saos-2 and U2OS osteosarcoma cells, RKO colorectal carcinoma cells, H1299 lung carcinoma cells, and Rat 1 fibroblast cells, to arrest at G2 (Bates et al., 1998; Medema et al., 1998; Niculescu et al., 1998). Similar to HCT116 colorectal tumor cells lacking p53, the cells lacking p21 also did not arrest stably in G2 after exposure to ionizing radiation (Bunz et al., 1998). This failure to arrest was associated with levels of Cdc2 kinase activity that is higher than those observed in cells with p21. Additional evidence linking p21 to the G2/M transition comes from its restored abundance when the cells enter G2 (Dulic et al., 1998).

Metal ions are vital for many biological processes, such as transcription, respiration and growth. However, excess accumulation of essential metals such as zinc, copper, cadmium, and mercury can be detrimental. Although, zinc is not redox-active under physiological concentrations and is less toxic than most metal ions, at high concentration, zinc toxicity is associated with reduced iron absorption, impaired immune function (Whittaker et al., 1998) and neuronal death (Chen et al., 2003; Koh et al., 1994). In our previous study, we found that G2/M arrest coupled with depressed growth were

observed in Normal Human Bronchial Epithelial (NHBE) cells cultured in zinc-supplement media (unpublished data). Signal cascade of Gadd45 and p53 as well as p38 were found to be associated with this G2/M arrest (unpublished data). To further uncover the detailed molecular mechanism, this study was designed to investigate the cyclin inhibitor, p21, for its possible role in zinc-supplement induced G2/M arrest in NHBE cells. This study has provided evidence to show that in response to the adverse effect of zinc supplementation, the enhanced expression of p53 may be triggered to suppress G2/M transition through the upregulation of the Cdc2/CyclinB inhibitor, p21. NHBE cells have been selected for this study because they are more representative of the cell population during lung tissue transformation and are considered to be progenitor cells for human bronchial cancer.

Methods and Materials

Cell culture -- NHBE cells were purchased from Cambrex Bio Science (Walkersville, MD). Cells were cultured with bronchial epithelial cells growth medium (BEGM) supplemented with 0.5 µg/ml epinephrine, 10 µg/ml transferrin, 5 µg/ml insulin, 0.1 ng/ml retinoic acid, 52 µg/ml bovine pituitary extract, 0.5 µg/ml hydrocortisone, 0.5 pg/ml human recombinant epidermal growth factor, and 6.5 ng/ml triiodothyronine (as growth supplements) without antibiotics, and incubated at 37°C in a 5% CO₂ incubator. Endotoxin-free medium was used (<0.005 endotoxin units/ml). Seeding density was around 3,500 cells per cm² of tissue culture dish. The medium was changed every 2 days. The cells reaching 80% confluence after culturing for 6 days were counted as one passage. The cells were subcultured at a ratio of 1: 8 with trypsin-EDTA after reaching confluency.

Cells at passage 3 were used for zinc treatment. Zinc treatments were carried out by adding zinc as a form of ZnSO₄ to the zinc-free BEGM baseline media to make the zinc-normal (ZN) medium that contained 4 μM of ZnSO₄, the zinc-adequate medium (ZA) that contained 16 μM of ZnSO₄, and the zinc-supplemented medium (ZS) that contained 32 μM ZnSO₄. The zinc-free basal BEGM medium, which contained trace amount of detectable zinc ion (<0.4 μM) as measured by flame atomic absorption spectrophotometry, was used as the zinc-depleted medium (ZD). The ZN medium was used as a comparison to standard culture media and was used as the control group for experiments. The ZA treatment was used as a representative of human plasma zinc levels, and the ZS group was used to represent plasma zinc levels attainable by oral supplementation in humans. After NHBE cells were subcultured into one of the four corresponding groups, the cells were cultured overnight in ZN media before changing to their respective medium. Cells were then cultured in ZD, ZN, ZA and ZS for 6 days. The cell number was determined by using a hemocytometer, and cell viability was assessed by trypan blue dye exclusion. Cell morphology was evaluated by using a phase-contrast microscope (Olympus, Tokyo, Japan).

Cellular zinc and DNA determination – After reaching 80% confluence, cells were harvested by trypsinizing with trypsin-EDTA for 5 min in 37°C incubator. Cell suspensions were then centrifuged at 500 x g for 5 min at room temperature. Cell pellets were washed with phosphate-buffered saline (PBS) buffer, centrifuged again and then resuspended into 1.5ml PBS and sonicated for two 30 second intervals. Thereafter, an aliquot of the sonicated cell suspension was used to measure cellular zinc content by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). Zinc standard

solutions (Fisher, Pittsburgh, PA) ranging from 0.05 ppm to 1.0 ppm were used to generate a linear standard curve. The zinc content of the cells was determined based on these zinc reference solutions. In addition, the certified zinc solutions were compared to bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. From the same sample, a small aliquot of the sonicated cell suspension was used to measure cellular DNA content using diphenylamine (Williams et al., 1986). Data were expressed as cellular zinc per microgram of DNA because of the linear relationship between cellular DNA and cell number we previously established (Williams et al., 1986).

RNase Protection Assay -- Total cellular RNA was isolated from HepG2 cells, using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to manufacturer's instructions. The integrity of the RNA was verified by RNA gel electrophoresis. The mRNA abundance of p21 was measured by the non-radioactive RNase Protection Assay System (BD Biosciences, San Diego, CA), according to manufacturer's instructions. The human GAPDH probe was used as internal reference for normalization. Biotin-labeled riboprobes were synthesized using non-Rad In Vitro Transcription kit with T7 RNA polymerase (BD Biosciences) according to manufacturer's instructions.

Nuclear and Cytoplasmic Extract Preparation -- The NE-PER Nuclear and Cytoplasmic Extraction Reagents and the Halt Protease Inhibitor Cocktail Kits (Pierce Biotechnology, Rockford, IL) were used for nuclear and cytoplasmic extracts preparation according to the manufacturer's instructions, which are based on the method of Smirnova et al. (Smirnova 2000). Nuclear and cytoplasmic extracts were then stored in aliquots at -80°C. Protein concentrations were determined by using the BCA Protein Assay Reagent

kit (Pierce). Contaminations of nuclear extracts by cytoplasmic proteins or contamination of cytoplasmic extracts by nuclear proteins, detected by Western blot analysis of Hsp90 or Oct-1, respectively, were routinely found to be less than 5% in our lab.

Western Blot Analysis -- Forty μg of nuclear and cytoplasmic protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by using a mini-transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10mM phosphate buffer pH7.3, 137mM NaCl, 2.7mM KCL, and 0.1% Tween 20) for 1 h at room temperature, prior to incubation with 1 $\mu\text{g}/\text{ml}$ of a rabbit anti-p21 polyclonal antibody (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA), in PBS-T containing 5% nonfat milk at 4 °C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the Western Blot Luminol Reagent (Santa Cruz Biotechnology) and exposed to film. The optical densities of the protein bands were quantified by the Alpha Innotech Imaging System (San Leandro, CA).

p21 Promoter Activity - The influence of zinc status on the human p21 gene promoter activity was studied by transient transfection of a p21-promoter-luciferase gene into Hep G2 cells. This approach was used to provide further evidence that the transcription process is depressed by a reduction in p21 gene promoter activity in zinc-deficient Hep G2 cells.

Preparation of Luciferase Construct. The p21 promoter construct was a kind gift provided by Dr. Lieberman (Yin et al), which consisted of a 2,337-bp fragment of the

human p21 promoter (base pairs -2258 to -4594) (El-deiry 1995), containing two p53-binding consensus sites, and with HindIII and XhoI sites added on the ends. It was isolated and inserted into the plasmid pGL3-basic (Promega, Madison, WI) to generate construct pGL3-p21-Luci plasmid. The pGL3-p21-Luci plasmid was transfected into *E. coli* DH5 α competent cells (Invitrogen) by standard protocol for mass production. The plasmid was prepared by using Wizard PureFectin Plasmid DNA purification system from Promega.

Transient Transfection and Luciferase Assay - Hep G2 cells were transfected by using Tfx-20 reagent according to the protocol provided by the manufacturer (Promega). Hep G2 cells, in DMEM with 10% FBS without antibiotics, were seeded at a density of 2×10^5 cells/well in 24-well plates and cultured for 4 days in DMEM containing 10% Chelexed FBS plus 0, 0.4, 4.0, 16.0, or 32.0 μ M zinc. Just before transfection, the medium was removed. Transfections were performed in triplicate with 500 ng of the plasmid DNA containing the wild-type p21 promoter luciferase reporter construct and 10 ng of an internal control plasmid, pRL-SV40 (Promega). One hour after transfection, the transfection medium was changed to their respective media and cultured for 2 more days. Luciferase activity was measured in the Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA) by using the Dual-Luciferase reporter Kit according to recommendations by the manufacturer (Promega). Changes in firefly luciferase activity was calculated and plotted after normalization with changes in renilla luciferase activity in the same sample.

Cell Cycle Analysis -- DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS). NHBE cells were cultured in ZD, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca $^{2+}$, Mg $^{2+}$ free), and fixed in 70% cold ethanol.

Cells were stored at 4°C. For staining, cells were collected by centrifugation, and pellets were suspended in 1.0 ml propidium iodide staining solution (50 mg per ml propidium iodide, 100 U per ml RNase in PBS), and incubated at room temperature for 1 h. Staining was quantitated with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). The data files were analysed by using the CELLQuestPro software program (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

siRNA transfection – siRNA duplex against p21 (sc-29427) and p53 (SC-29435) were purchased from Santa Cruz Biotechnology company. A control siRNA specific targeting to the Luciferase DNA sequence was used as a negative control. For cell transfection with siRNA, NHBE cells cultured on culture dishes at 60% confluency, and siRNA were introduced into the cells using RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Aliquots of 600pmol of siRNA-p21 were diluted with Opti-MEM medium (Invitrogen, Carlsbad, CA), and another solution containing lipofectamine transfection reagent was also diluted with Opti-MEM medium. These two mixtures were combined together at room temperature with gentle vortex and incubated for 20 mins followed by an addition of 10 ml corresponding medium for the treatment groups. The mixture was then overlaid onto the 60% confluent NHBE cells. After 48 hours, cells were harvested for cell cycle and western blot analyses.

Statistical Analysis -- Each experiment was repeated at least three times with each experiment yielding essentially identical results. Data were expressed as mean \pm SEM. Statistical comparisons were carried out by one-way analysis of variance (ANOVA). Means were examined by the Least Significant Difference post hoc analysis (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Result

Zinc supplementation reduces cell growth of Normal Human Bronchial Epithelial (NHBE) Cells.

In vitro content of cellular zinc in ZD cells, as measured by flame atomic absorption spectrophotometry, was 70% lower than ZN control cells (Fig. 13A). Moreover, cellular zinc levels in ZA and ZS cells were 118% and 435%, respectively, higher than ZN cells. Growth as measured by cell number was significantly depressed in ZD, ZA and ZS cells to 85%, 90 % and 70%, respectively, of ZN cells (Fig. 13B).

Zinc supplementation suppresses G2/M progression

A significant delay in G2/M cell cycle progression was observed in ZS NHBE cells (19.8%) when compared to ZN cells (12.9) (Fig.14). The finding confirmed our previous report of G2/M blockage in ZS NHBE cells (Shih et al., 2006). The G2/M suppression was smaller in magnitude in ZA cells, with 15.9% of cells in G2/M (Fig.14). In contrast, no change was observed in ZD cells, with 11.9% cells in G2/M, when compared to ZN cells (Fig.14). This result indicates that the impaired G2/M progression in zinc supplemented NHBE cells is responsible for depressed cell growth.

Zinc supplementation upregulates p21 protein and mRNA levels as well as p21 promoter activity

p21 mRNA levels in the ZA and ZS cells were drastically upregulated to almost 200% of ZN cells (Fig. 15). Consistent with the pattern of p21 mRNA expression, nuclear p21 protein levels in ZA and ZS cells were similarly upregulated (Fig. 16). In contrast, no significant differences were detected in nuclear p21 protein levels as well as p21 mRNA abundance between the ZD and ZN cells (Fig. 15 & 16). Transient transfection of a p21-promoter-luciferase reporter gene into NHBE cells indicated that the p21 promoter activity of ZS cells was significantly induced to 150% and 200% of ZN cells, respectively. In contrast, no difference was observed between ZD and ZN cells (Fig. 17).

Transfection of the p21 siRNA, at 200 pmole/plate, normalizes the induced p21 protein level in ZS cells to that of ZN control cells

By transfecting graded amounts of p21 siRNA (600, 400, and 200 pmole per plate), the amount of p21 siRNA at 200 pmole per transfection was able to abrogate the elevated p21 protein level in ZS cells and normalize it to that of ZN control cells (Fig. 18). In addition, ZN cells transfected with 200 pmole of p21 siRNA showed a near 20% decrease in p21 protein level than ZN control cells. The same batch of cells with normalized p21 protein level in ZS cells were used for G2/M cell cycle analysis to determine if G2/M arrest was altered or not.

Normalization of p21 protein level in ZS cells with p21 siRNA alleviates the G2/M blockage and restores depressed cell growth

After knocking down the induced protein level of p21 in ZS cells to a similar level as in ZN cells with p21 siRNA, the cells were subjected to cell cycle analysis to examine the association of p21 induction with G2/M arrest in ZS cells. As shown in Figure 19, the normalization of p21 protein level in ZS cells alleviated the G2/M blockage from 20% and normalized it back to 12% which was similar to the % of ZN control cells in G2/M phase. As for ZN cells transfected with same amount of p21 siRNA, the percentage of cells in G2/M was slightly reduced. The cell growth as measured by cell numbers per plate indicated that, in ZS+siP21 cells, the normalization of p21 protein level by using p21 siRNA restored the originally depressed growth of ZS cells back to the same level of ZN control cells (Figure 20).

Upregulation of p21 in ZS cells is dependent on p53 expression

p53 was widely reported as an immediate upstream regulator of p21 for its role played in G1/S cell cycle regulation (Eldeiry et al., 1993). In addition, p53 has also been widely reported to be the major regulator of p21 for another role in G2/M cell cycle checkpoint (Reviewed in Taylor and Stark 2001). To investigate whether in ZS cells, the upregulation of p21 and its involvement in G2/M arrest were modulated by p53, the gene knock-down approach with p53 siRNA was applied. Firstly, p53 protein level was found to be induced to more than 2 folds higher in ZS cells than ZN cells (Figure 21). By transfecting graded amounts of p53 siRNA (600, 400, and 200 pmole per plate), the amount of p53 siRNA at 200 pmole per transfection was found to be able to abrogate the amount of elevated p53 protein level in ZS cells and normalize it to that of ZN control cells. The same batch of cells with normalized p53 protein level in ZS cells were used for

p21 protein level measurement. Interestingly, in ZS-si-p53 cells with normalized p53 level, the originally elevated p21 protein level was found to be abolished and reduced to the level almost identical to that of ZN control cells (Figure 22). In addition, ZN cells transfected with the same amount of p53 siRNA showed a near 40% decrease in p21 protein level than ZN control cells. Next, the ZS cells, with normalized level of p53 protein, were used for cell growth determination.

Normalization of p53 protein level in ZS cells with p53 siRNA alleviates the G2/M blockage and restores depressed cell growth

p53 is a well known tumor suppressor gene. In response to various stresses, upregulation of p53 induces immediate downstream target, p21, which arrests cell cycles at different phases for DNA repair (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). To establish that G2/M blockage is caused by elevated p53 expression, ZS-si-p53 cells with normalized p53 protein level were subjected to cell cycle analysis by flow cytometry. The result indicated that (Figure 23), with normalized p53 protein in ZS cells, the normalization of p53 protein level in ZS cells alleviated the G2/M blockage from 22.5% and normalized it back to 14 % which was similar to the % of ZN control cells in G2/M phase. As for ZN cells transfected with same amount of p53 siRNA, the percentage of cells in G2/M was slightly reduced. Furthermore, the original growth repression was abolished and cell growth was restored back to almost the same level of ZN control cells (Figure 24). In addition, in ZN cells transfected with the same amount of p53 siRNA, a near 40% higher cell growth than ZN control cells was observed.

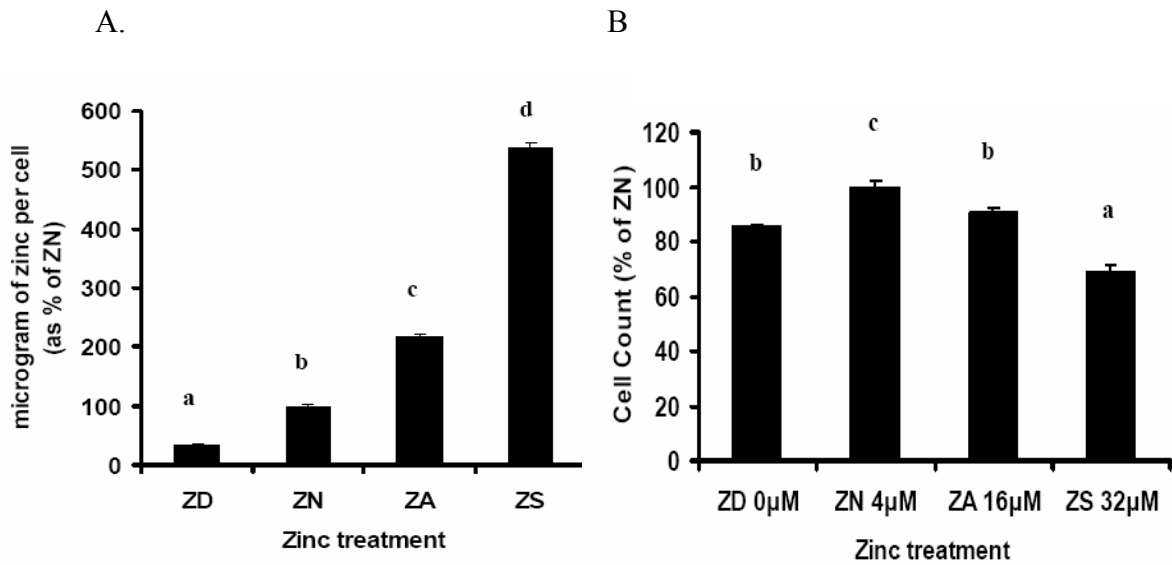


Figure 13 Zinc-supplemented medium depresses NHBE cells growth

Fig. 13: NHBE cells were cultured for one passage in basal medium depleted of zinc to induce zinc-deficient (ZD) cells or in the basal medium supplemented with 4.0, 16, or 32 μ M zinc to represent the amount of zinc in most normal media (ZN), the normal human plasma zinc level (zinc-adequate, ZA), or the high end of plasma zinc attainable by oral supplementation (ZS), respectively. Cellular zinc was measured by atomic absorption spectrophotometry. Cellular zinc levels were expressed as a percentage of ZN controls. Values are means \pm SEM from 3 experiments. Different letters indicate significant different means, $P < 0.05$.

Fig. 13: Cell numbers were counted by using hemacytometer. Cell number was counted count as per plate and expressed as a percentage of ZN controls. Values are means \pm SEM from 3 experiments. Different letters indicate significant different means, $P < 0.05$.

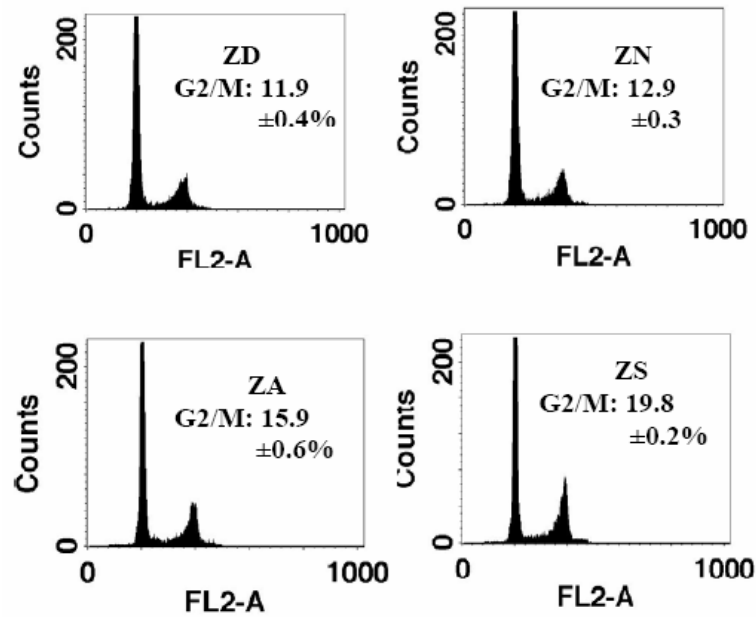


Figure 14 High intracellular zinc status induces G2/M blockage

Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in ZD, ZN, ZA, and ZS media for one passage. Washed cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow green and the DNA QC particle kit were used for verification of instrument performance. Histograms are representative of three independent experiments. The proportions of cells in G0/G1, S phase and the G2/M ratios are indicated for each treatment group. Similar results were obtained from experiments with cells from another subject.

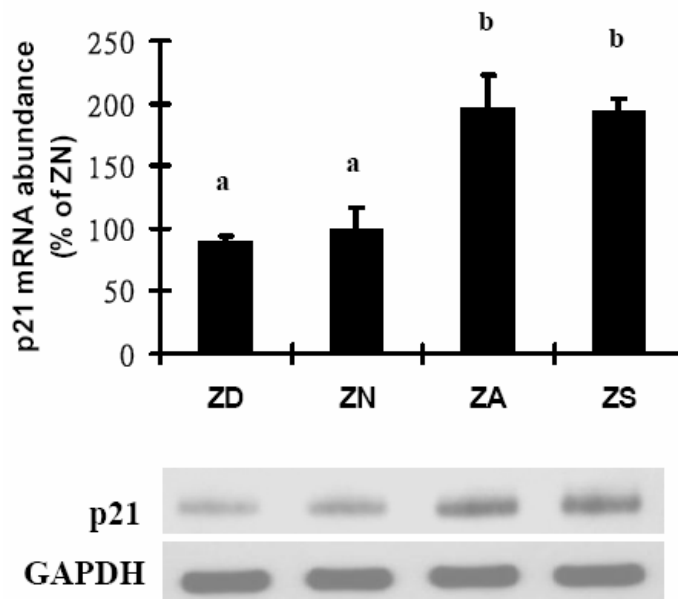


Figure 15 High intracellular zinc status induces p21 mRNA level

Total RNA was isolated from cells by using RNeasy Kit (Qiagen, Valencia, CA). The abundance of p21 mRNA was measured by using a nonradioactive RPA kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. The RPA probe set, which included p21 and GAPDH probe, was from the same manufacturer. The optical densities of the protected p21 bands were quantified by the Alpha Innotech imaging system (San Leandro, CA) and then normalized to that of GAPDH. Values shown in bar graphs represent means \pm SEM from 3 separate experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

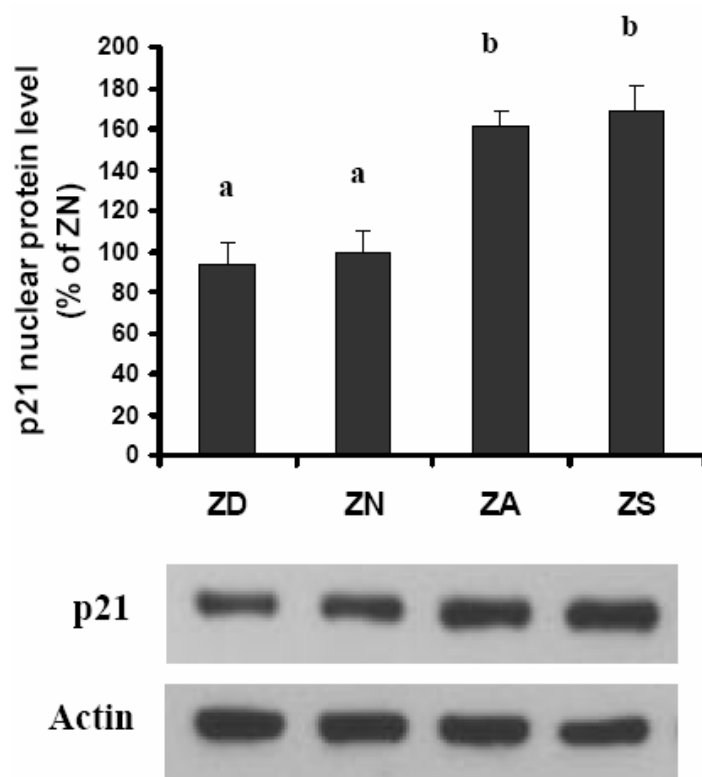


Figure 16 High intracellular zinc status induces p21 protein level

Nuclear proteins (40 μ g) prepared from NHBE cells were separated on SDS-PAGE, Western blotted, and probed with antibody p21 (C-19). The nuclear protein p21 band was normalized to the corresponding housekeeping gene, Oct-1, and expressed as a percentage of ZN cells. Values shown in bar graphs represent means \pm SEM from 3 separate experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

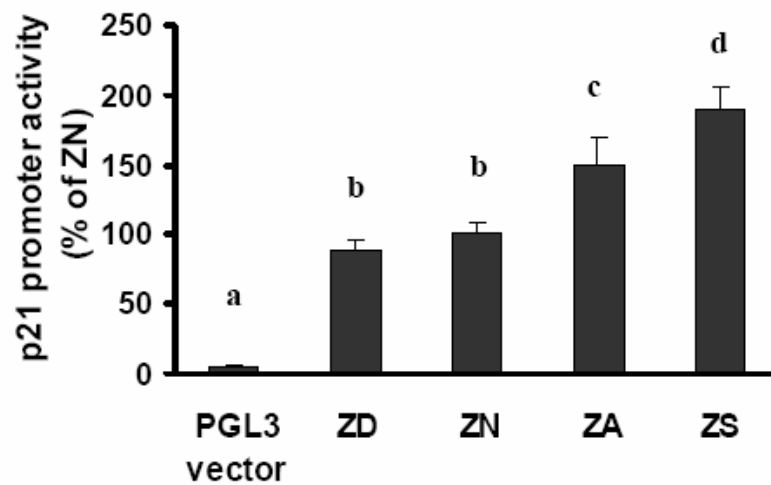


Figure 17. High intracellular zinc status enhances p21 promoter activity

NHBE cells were cultured in media with respective zinc concentration (ZD = 0 μ M; ZN = 4 μ M; ZA = 16 μ M; and ZS = 32 μ M zinc supplemented to the basal medium) for 4 days. Thereafter, cells were transiently transfected with 500 ng of luciferase reporter construct (PGL3-p21) containing the *p21* promoter, together with 10 ng pRL-SV40 as internal control. PGL3 basal vector without carrying any promoter was used as the vector control. After transfection, the cells were cultured in corresponding media for 2 more days. Cells extracts were then assayed by Dual-Luciferase reporter system and signals were measured by a Luminometer. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

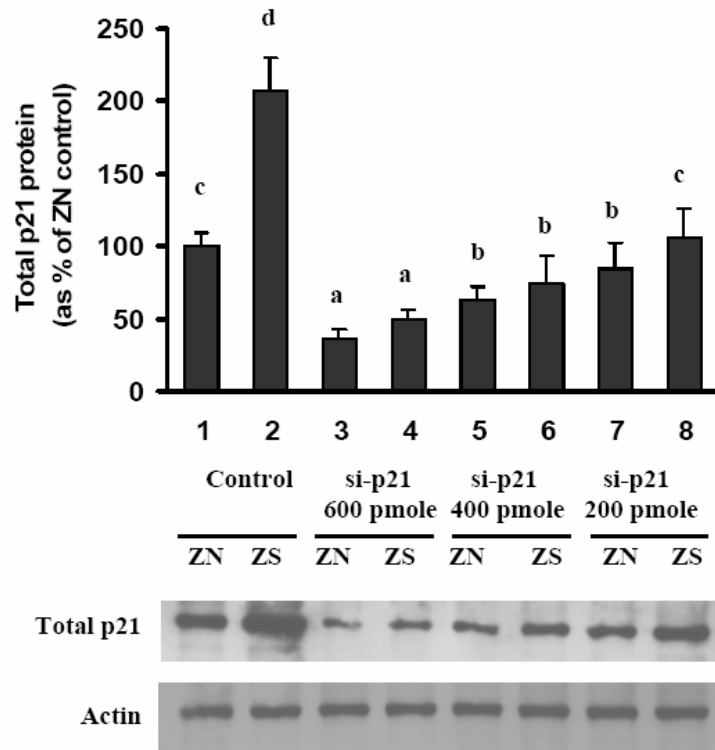


Figure 18. Transfection of p21 siRNA, at 200 pmole/plate, abolishes the induced p21 protein level in zinc supplemented NHBE cells.

NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μ M and ZS = 32 μ M) for 4 days. Thereafter, cells were transiently transfected with graded amounts of p21 siRNA (600, 400 and 200 pmole/plate). After transfection, the cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to protein expression analysis by western-blot. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

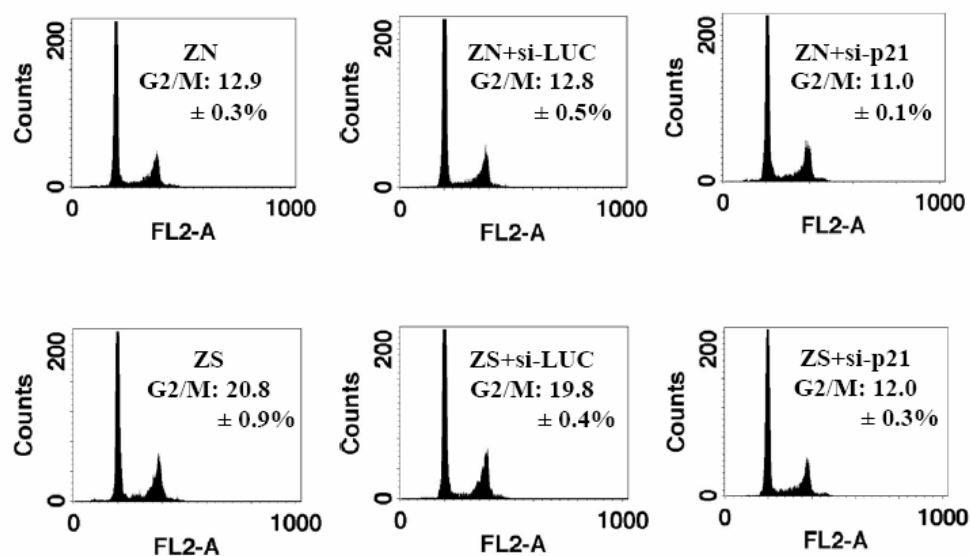


Figure 19. G2/M blockage in zinc supplemented NHBE cells is released after normalization of p21 protein level.

In cell cycle analyses, DNA content of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in respective zinc concentration (ZD and ZN) for 4 days. Thereafter, cells were transiently transfected with 200 pmole p21 siRNA per plate. After transfection, cells were cultured in corresponding media for 2 more days. Washed cells were fixed in ethanol and stained for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. A total of 10,000 cells events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow green and DNA QC particle kit were used for verification of instrument performance. Histograms are representative of 3 independent experiments.

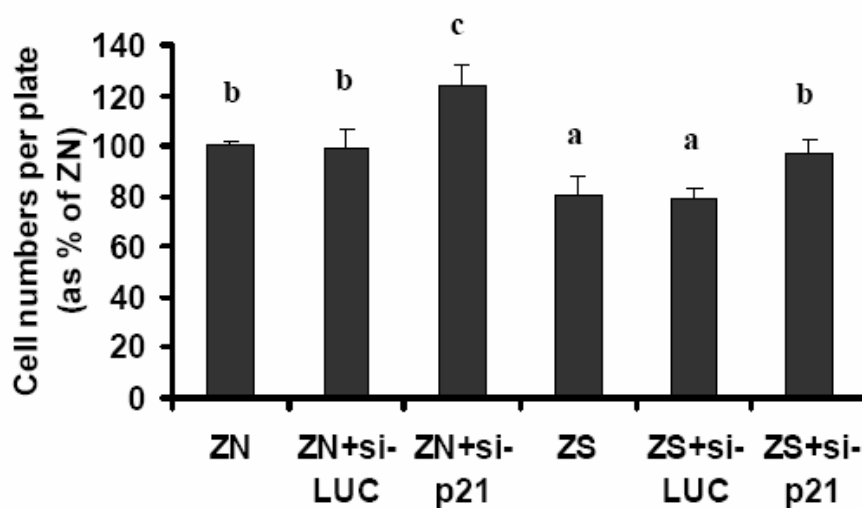


Figure 20. Cell growth depression in zinc supplemented NHBE cells is abolished after abrogation of induced p21 protein level

NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μ M and ZS = 32 μ M) for 4 days. Thereafter, cells were transiently transfected with either p21 siRNA at concentration of 200 pmole/plate or Luc siRNA as a negative control. After transfection, the cells were cultured in corresponding media for 2 more days. Total cell numbers were counted using hemacytometer and was expressed as % of ZN control. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

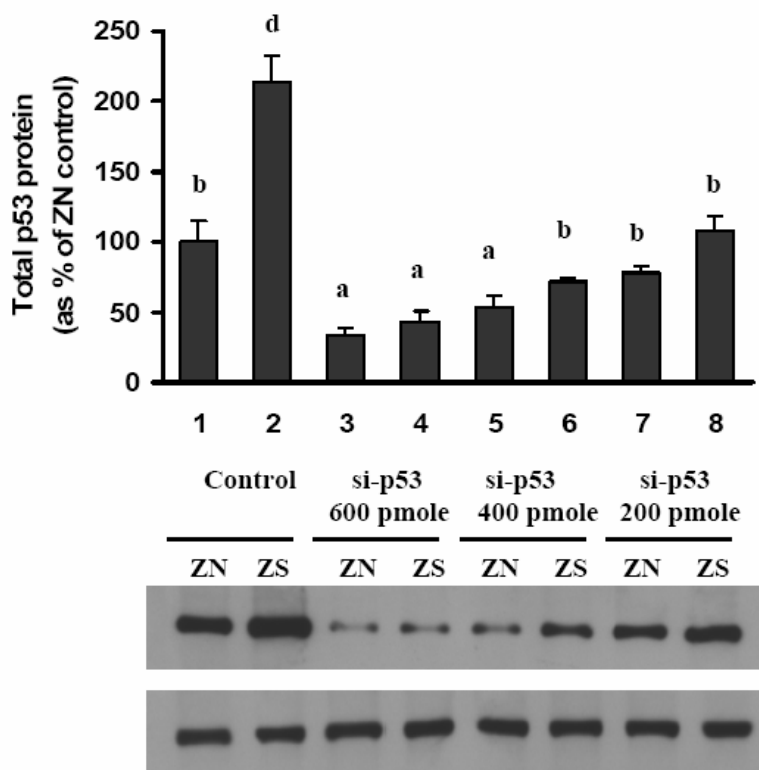


Figure 21. Transfection of p53 siRNA, at 200 pmole/plate, abolishes the induced p53 protein level in zinc supplemented NHBE cells.

NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μ M and ZS = 32 μ M) for 4 days. Thereafter, cells were transiently transfected with graded amounts of p53 siRNA (600, 400 and 200 pmole/plate). After transfection, the cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to protein expression analysis by western-blot. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

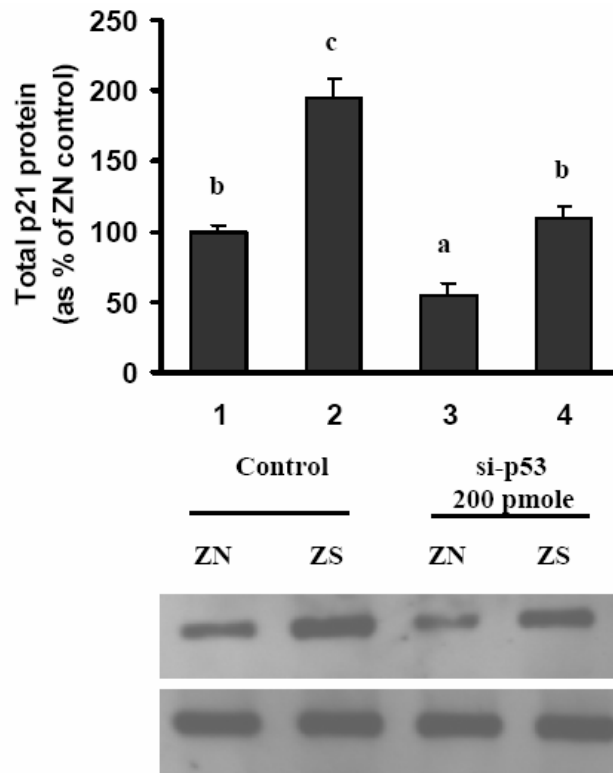


Figure 22. Zinc supplemented NHBE cells with normalized p53 protein level abrogate the elevated p21 level.

NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μ M and ZS = 32 μ M) for 4 days. Thereafter, cells were transiently transfected with p53 siRNA at concentration of 200 pmole/plate. After transfection, the cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to p21 protein expression analysis by western-blot. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

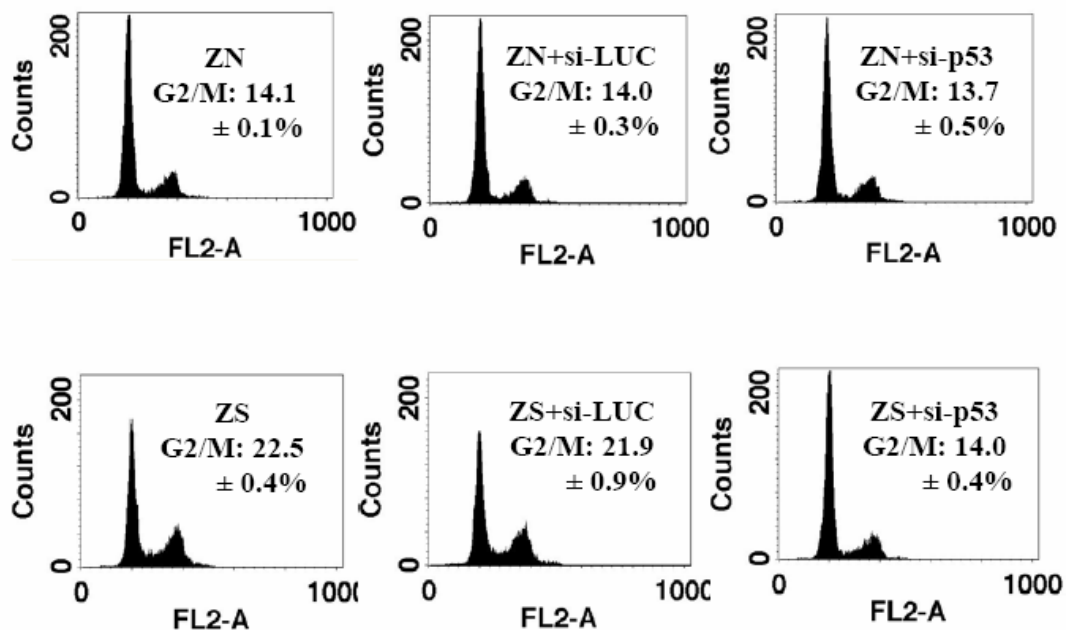


Figure 23. G2/M blockage in zinc supplemented NHBE cells is released after normalization of p53 protein level.

In cell cycle analyses, DNA content of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in respective zinc concentration (ZD and ZN) for 4 days. Thereafter, cells were transiently transfected with 200 pmole p53 siRNA per plate. After transfection, cells were cultured in corresponding media for 2 more days. Washed cells were fixed in ethanol and stained for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. A total of 10,000 cells events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow green and DNA QC particle kit were used for verification of instrument performance. Histograms are representative of 3 independent experiments.

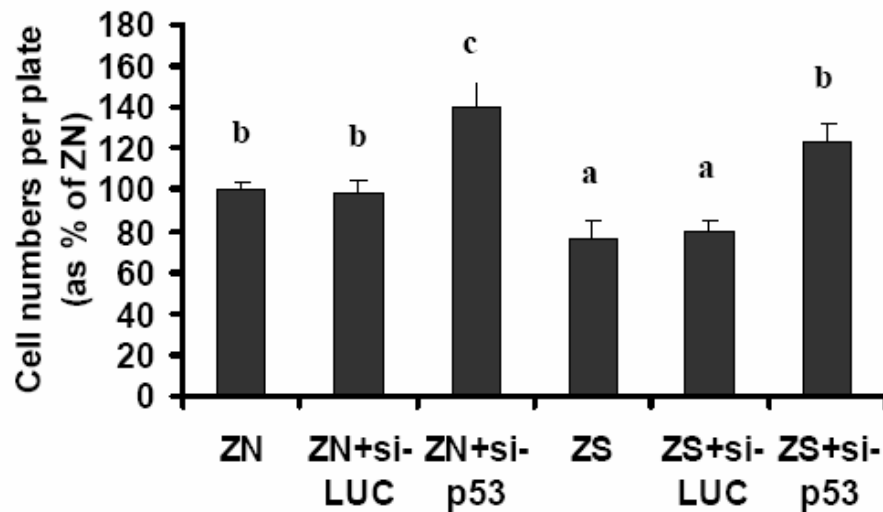


Figure 24. Cell growth depression in zinc supplemented NHBE cells is abolished after abrogation of induced p53 protein level

NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μ M and ZS = 32 μ M) for 4 days. Thereafter, cells were transiently transfected with either p53 siRNA at concentration of 200 pmole/plate or Luc siRNA as a negative control. After transfection, the cells were cultured in corresponding media for 2 more days. Total cell numbers were counted using hemacytometer and was expressed as % of ZN control. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

DISCUSSION

In a previous study (Shih et al., 2006), we have reported that in ZS NHBE cells, the enhancement of cellular zinc status depressed G2 to M phase progression, which resulted in cell growth reduction. Moreover, Gadd45 induction in ZS NHBE cells was found to be partially responsible for the depressed G2/M progression. In view of available evidence indicating that p21, which is a major cell regulator at G1 and is also involved in G2/M regulation (Dulic et al., 1998), we have designed the present study to examine if p21 and p53 expression are affected and whether they are also involved in the G2/M arrest in zinc supplemented NHBE cells.

In the present report, we have presented multiple lines of evidence for the first time to substantiate that the transcriptional process and expression of p21 are upregulated in zinc-supplemented NHBE cells in primary culture. The observed induction of almost 2-fold higher nuclear p21 protein and p21 mRNA in ZA and ZS cells than control ZN cells (**Fig. 15 & 16**) indicates that the transcriptional process is enhanced in zinc-supplemented cells. The p21 promoter activity was measured to address the question whether induced expression of mRNA and protein were caused by an upregulation of promoter activity. A similar pattern of induction of p21 promoter activity, as in p21 mRNA and protein levels, was observed in ZA and especially ZS cells as compared to ZN cells (**Fig. 17**). These findings indicate that an enhanced promoter activity, instead of another mechanism such as mRNA stability, may be mainly responsible for the upregulation of p21 gene and protein expression.

Available evidences indicate that p21 is involved in G2/M transition. For example, in human fibroblasts, p21 mRNA abundance shows bimodal periodicity with peaks in G1 and G2/M (Li et al., 1994) and that nuclear p21 protein reaccumulates at the onset of mitosis (Dulich et al., 1998). On top of this, inducible p21 expression was demonstrated to be responsible for cell cycle arrest at both G1 and G2 (Carol et al., 1998). By synchronizing the cell population at S phase, p21 was found to induce G2 arrest in human fibroblasts cells (Smits et al., 2000). With circumstantial evidences suggesting that p21 may also be involved in G2/M checkpoint, we have designed the present study to investigate if the enhanced p21 level in ZS cells was associated with the G2/M arrest. A gene knock-down approach was employed to examine if G2/M blockage would be alleviated after abrogation of the enhanced p21 expression in ZS cells. By transfecting graded amounts of p21 siRNA, the transfection of siRNA at 200 pmole/plate was found to abolish the elevated level of p21 protein in ZS cells to that of ZN cells (**Fig. 18**). The ZS-si-p21 cells with normalized level of p21 protein were subjected to cell cycle analysis. The result indicated that, after the normalization of p21 protein level in ZS cells, the original G2/M blockage was alleviated to the same level in ZN control cells (**Fig.19**). While for ZN cells transfected with same amount p21 siRNA, G2/M cell cycle transition was slightly reduced as compared with ZN control cells. Moreover, alleviated G2/M blockage in ZS cells with normalized p21 protein level was also accompanied by a normalized cell growth. The original growth repression in ZS cells was abolished and cell growth was restored to a similar level as in ZN controls cells (**Fig. 20**). Thus, the elevation of p21 expression in zinc supplemented NHBE cells is largely if not entirely responsible for the observed G2/M blockage as well as growth repression induced by

high zinc status in NHBE cells. In fact, three mechanisms have been proposed for how upregulation of p21 participates in inhibiting Cdc2 activity to cause G2 arrest. First, p21 directly binds to cdc2/cyclin B1 complexes and inhibits Cdc2 activity (Boulaire et al., 2000). p21 was found in Cyclin B1 immunoprecipitates in cells overexpressing p21 (Medema et al., 1998; Taylor et al., 1999b). However, in some studies, it was not found in Cyclin B1 immunoprecipitates (Bates et al., 1998; Dulic et al., 1998). It was suggested that binding to Cdc2/Cyclin B1 may be only observed when there are very high levels of p21 (Taylor and Stark 2001). A second mechanism for inhibiting Cdc2 has been suggested by a report showing that p21 may inhibit Cdk2, which is an activator of Cdc2, and resulted in the loss of Cdc2 activity (Guadagno and Newport, 1996). A third mechanism of Cdc2 inhibition by p21 was suggested by the report of Smits et al. (2000) which showed that overexpression of p21 reduced the phosphorylation of Cdc2 on threonine 161 and the Cdc2 activity. By adding recombinant CAK protein, which phosphorylated Cdc2 on threonine 161, the immunoprecipitated Cdc2 activity was restored (Smits et al., 2000).

Next, we investigated if the elevation of p21 expression in ZS cells was dependent on p53. Although p53-independent regulation of p21 expression has been widely reported, p53 is still well recognized as a major immediate upstream regulator of p21 expression (Eldeiry et al., 1993). In particular, in the modulation of G2/M progression, p53 has been proposed to employ p21 in the regulation of Cdc2/Cyclin B complex activity, which is a cell cycle regulator of G2 to M transition (Reviewed in Taylor and Stark 2001). In ZS cells, p53 was found to be induced to almost 2-fold higher than ZN control cells (**Fig 21**).

The magnitude of elevation of p53 is similar to that of p21 protein. Therefore, a similar gene knock-down approach used to examine if p21 protein induction would be dependent on p53 expression. By testing graded amounts of p53 siRNA, the transfection of 200 pmole of siRNA per plate was found to abolish the elevated level of p53 protein in ZS cells and normalized it to that of ZN cells (**Fig. 22**). The ZS-p53-siRNA cells, with normalized level of p53 protein, were subjected to p21 protein measurement as well as cell growth determination. Our results indicated that in ZS-siRNA cells, after the normalization of p53 protein level, the original 2-folds elevated p21 protein was abolished and the protein level was normalized to a level as in ZN control cells (**Fig. 22**). The ZN cells transfected with same amount of p53 siRNA showed 50% less p21 protein than that of ZN control cells, indicating the efficacy of p53 knock-down by using p53 siRNA. Next, the ZS-si-p53 cells with normalized level of p53 protein were subjected to cell cycle analysis to examine if G2/M arrest would be alleviated. The result indicated that, after the normalization of p53 protein level as in ZS cells, the original G2/M blockage was alleviated to the same level in ZN control cells (**Fig. 23**). However, for ZN cells transfected with same amount p53 siRNA, the G2/M cell cycle transition was only slightly reduced as compared with ZN control cells. Furthermore, with the normalization of p53 protein in ZS-si-p53 cells, original growth repression was abolished and cell growth was restored back to 20% higher than ZN control cells (**Fig. 24**). This result is consistent with previous contention that p53 functions as a negative growth regulator. In addition, ZN cells transfected with the same amount of p53 siRNA showed an almost 40% higher cell growth than ZN control cells. Thus, high intracellular zinc content

appeared to induce p53 which in turn lead to the elevation of p21 gene expression, blockage of G2/M progression and suppression of cell proliferation.

Several studies have indicated that high cellular zinc may exert adverse effect on human cell growth (Bataineh et al., 2002; Feng et al., 2000; Uzzo et al., 2002). Moreover, studies also reported that treatment with high level of zinc inhibited cancer cells proliferation by mediating G2/M arrest (Jaiswal et al 2004; Liang et al., 1999). In one report, human primary liver cells cultured in 100 μ M zinc were found to exhibit DNA fragmentation (Paramanantham et al., 1996). However, the present report is the first to show that 32 μ M of zinc concentration, which represents the high end of plasma zinc attainable by oral supplementation, can cause G2/M arrest and cell growth repression in primary cell line. In our NHBE study, the cytotoxic stress induced by zinc supplementation may be one of the stress factors that elevates p53 expression, which then suppress G2/M progression through the upregulation of cyclin dependent kinase inhibitor, p21. In fact, preliminary results in our laboratory indicated that the amount of endogenous reactive oxygen species (ROS) generated in ZS NHBE cells was higher than that in ZN cells (Shih et al., 2006). Reactive oxygen species have been implicated in DNA damage by genotoxic agents such as UV and ionizing radiation or doxorubicin. Subsequent accumulation of ROS-damaged DNA is a critical event during carcinogenesis and aging (Ames et al., 1993). DNA damage triggers a variety of signaling pathways that lead either to apoptosis or to DNA damage repair that is coupled with cell cycle regulation. One junction of such pathways is controlled by the tumor suppressor protein p53. The principal function of p53 is to promote survival or apoptosis of cells exposed to

agents that cause DNA damage, such as hypoxia, UVR, ROS or mutagens (Graeber et al., 1996; Renzing et al., 1996; Clarke et al., 1997; Griffiths et al., 1997). The p53 protein is involved in complex cellular responses to DNA damage. These responses involve DNA editing and repair followed either by normal cell division (Polyak et al., 1997) or by apoptosis (Hale et al., 1996). Cells damaged by UVR and oxidative stress often remain in the G1/G2-phase of the cell cycle for long periods (Evan and Littlewood, 1998). Therefore, the oxidative stress induced in ZS cells may trigger the upregulation of p53 signal transduction pathway that causes the G2/M arrest in our cell system.

In summary, the present data suggest that the upregulation in p53 expression in zinc supplemented NHBE cells resulted in an elevation of p21 expression, which contributed to the G2/M blockage. Moreover, the oxidative stress induced in zinc supplemented NHBE cells may be the possible trigger of p53 signal transduction pathway responsible for the G2/M blockage.

Chapter V: Summary

In HepG2 cells, nuclear and cytoplasmic p21 protein levels in the ZD and ZD0.4 cells were significantly repressed when compared to ZN control cells. The p21 mRNA levels in ZD and ZD0.4 cells were also repressed compared to ZN cells. In contrast, no significant differences were detected in nuclear and cytoplasmic p21 protein levels as well as p21 mRNA abundance among the ZN, ZA, ZS, and ZD+32 μ M cells. Most importantly, the ZD+32 μ M rescue approach demonstrated the specificity of zinc treatment and the rapid normalization of both p21 protein and mRNA levels by zinc replenishment. The p21 promoter activity of ZD and ZD0.4 cells was significantly reduced to 64% and 66% of ZN cells, respectively. In contrast, no differences were observed among the ZA, ZS, ZD+32 μ M, and ZN cells. However, supplementation of ZD cells with ZS medium (ZD+32 μ M), for the last 2 days of culture, was able to normalize the p21 promoter activity to that of the ZN cells. This rescue approach shows the specificity of zinc treatment and the speed of normalization of p21 promoter activity by zinc replenishment.

By transfecting graded amounts of PCMV-p21 plasmid (1, 3, 5, 7, 10 microgram per plate), which constitutively expressed p21, the amount of PCMV-p21 at 5 μ g per transfection was capable of restoring the p21 expression level in the PCMV-p21 transfected ZD cells (ZD+5) back to the level of ZN control cells (ZN+0). In addition, ZN cells transfected with 5 μ g of PCMV-p21 (ZN+5) demonstrated a 48% increase in p21 protein level than ZN control cells (ZN+0). Most interestingly, after the transfection of 5 μ g PCMV-p21, the cyclin D1-cdk4 complex activity was normalized in ZD cells but

was not altered in ZN cells as compared to ZN cells transfected with control plasmid. As a control to assess the quality of the lysate, equivalent amount of the lysate from both ZD and ZN cells transfected with PCMV-p21 or PCMV-control plasmids were electrophoresed by SDS-PAGE and immunoblotted with mAb anti-G3PDH and were found to be optimal among all treatments. Thus, the results indicated that the small reduction in cyclin D-cdk4 complex activity appeared to be the result of the decreased p21 protein level in ZD cells.

Cell growth and cell cycle progression analyses were also determined in the same batch of cells used for the measurement of cyclin D1-cdk4 complex activity after the rescue of p21 level in ZD cells. The result demonstrated that cell growth remained repressed in ZD PCMV-p21 cells, with normalized p21 expression, as compared to ZD-PCMV-control cells. Most importantly, in the ZN-PCMV-p21 cells transfected with the same amount of PCMV-p21 plasmid, the marked increase in p21 expression was accompanied by a 30% reduction in cell growth, which illustrated the growth inhibitory effect of p21 expression at high level. Furthermore, cell cycle progression is impaired at the G1/S phase in ZD cells. However, the impaired G1/S cell cycle progression remained unchanged in the ZD-PCMV-p21 cells, with normalized p21 level and cyclin D1-cdk4 complex activity. Moreover, the G1/S progression was mildly impaired in the ZN-PCMV-p21 cells, with markedly elevated p21 protein level, which indicated that the overexpressed p21 was functionally effective in inhibiting cell cycle progression and growth. Thus, these findings suggested that, in zinc-deficient HepG2 cells, the decreased

p21 protein level was not responsible for the associated inhibition of cell growth and cell cycle progression.

In NHBE cells, p21 mRNA levels in the ZA and ZS cells was drastically upregulated to almost 200% of ZN control cells. Consistent with the pattern of p21 mRNA expression, nuclear p21 protein levels in ZA and ZS cells was similarly upregulated. In contrast, no significant differences were detected in nuclear p21 protein and p21 mRNA levels between the ZD and ZN cells. Transient transfection of a p21-promoter-luciferase reporter gene into NHBE cells established that the p21 promoter activity of ZS cells was also significantly induced to 150% and 200% of ZN cells, respectively. In contrast, no difference was detected between ZD and ZN cells.

The transfection of graded amounts of p21 siRNA (600, 400, and 200 pmole per plate) was used to establish that 200 pmole per transfection was able to abrogate the elevated p21 protein level in ZS cells and normalize it to that of ZN control cells. In addition, an almost 20% decrease in p21 protein level was observed in ZN cells transfected with 200 pmole of p21 siRNA than ZN control cells. After knocking down the induced protein level of p21 in ZS cells to a similar level as in ZN cells with p21 siRNA, the cells were subjected to cell cycle analysis to examine the association of p21 induction with G2/M arrest in ZS cells. The present findings indicated that the normalization of p21 protein level in ZS-si-p21 200 pmole cells alleviated the G2/M blockage from 20% and normalizes it back to 12%. As for ZN cells transfected with same amount of p21 siRNA, there were little or no reduction in the percentage of cells in G2/M.

In ZS-si-p21 cells, the normalization of p21 protein level by using p21 siRNA restored the originally depressed cell growth of ZS cells back to the same level of ZN control cells. To investigate whether in ZS cells, the upregulation of p21 and its involvement in G2/M arrest were modulated by p53, the gene knock-down approach with p53 siRNA was applied. Firstly, p53 protein level was induced to more than 2-fold higher in ZS cells than ZN cells. The transfection of graded amounts of p53 siRNA (600, 400, and 200 pmole per plate) was used to establish that 200 pmole of p53 siRNA at per transfection was able to abrogate the elevated p53 protein level in ZS cells and normalize it to that of ZN control cells. Interestingly, in ZS-si-p53 cells with normalized p53 level, the originally elevated p21 protein level was abolished and normalized to the level almost identical to that of ZN control cells. In addition, ZN cells transfected with the same amount of p53 siRNA demonstrated an almost 40% decrease in p21 protein level than ZN control cells. To establish that G2/M blockage was caused by the elevated p53 expression, ZS-si-p53 cells with normalized p53 protein level were subjected to cell cycle analysis by flow cytometry. The normalization of p53 protein level in ZS-si-p53 cells was found to alleviate the G2/M blockage from 22.5% and normalized it back to 14 % as in ZN control cells. As for ZN cells transfected with 200 pmole of p53 siRNA, there was little or no reduction in the percentage of cells in G2/M. In addition, the original growth repression was abolished and cell growth was restored back to almost the same level of ZN control cells. Furthermore, in ZN cells transfected with the same amount of p53 siRNA, an almost 40% higher cell growth than ZN control cells was observed, which was consistent to the established role of p53 as a negative cell cycle regulator.

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