AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Zinc is an essential mineral that is integral to many proteins and transcription factors that regulate key cellular functions. The normal prostate accumulates high concentrations of zinc; however, malignant tissues have significantly lower zinc levels. This unique relationship between zinc and the prostate has sparked interest in the role of zinc in protecting against prostate cancer development. The purpose of this dissertation was to examine the molecular changes that occur in the prostate in response to alterations in cellular zinc. For our approach, we examined the effects of zinc deficiency and supplementation in normal and prostate cancer cells, respectively.

Zinc supplementation decreased cell growth and viability in both prostate cancer (PC-3) and benign prostate hyperplasia (BPH-1) cells, however BPH-1 cells were more sensitive to zinc compared to PC-3 cells. Differential responses to zinc were also observed for Bcl-2:BAX expression and NFκB expression and activity. These findings suggest that zinc may differentially affect regulators of apoptosis in BPH-1 and PC-3 cells, and that zinc may control cellular proliferation in prostate cancer and hyperplasia.

Secondly, we examined molecular changes in the normal prostate cells with zinc deficiency both *in vitro* and *in vivo*. Zinc deficiency increased single strand DNA breaks in normal prostate epithelial cells (PrEC). Importantly, zinc deficiency also up-regulated gene and nuclear protein expression of p53, but no change in p53 DNA binding activity or gene expression of the downstream p53 targets BAX and p21 was observed. These studies indicate that zinc deficiency increased DNA damage and impaired activity of critical zinc dependent transcription factors responsible for mediating the DNA damage response. *In vivo*, microarray analysis revealed that dietary zinc deficiency caused differential regulation of genes involved in prostate cancer and cell signaling. Interestingly, dietary zinc deficiency decreased prostate zinc in wild-type (WT) but not Cu/Zn SOD over-expressing (SOD^{+++}) animals, suggesting that over-expression of Cu/Zn SOD may protect against zinc loss. Differential expression of zinc transporters may explain this effect, as expression of zip1 and zip3 were significantly lower in SOD^{+++} animals. Together these studies suggest that zinc may play a critical role in the maintenance of a healthy prostate.

The Role of Zinc in the Molecular and Cellular Events in the Prostate

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Michelle Yan, Author

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Chapter 1

Introduction

General Introduction

Cancer is the second leading cause of mortality in the United States, accounting for almost 23% of deaths in the U.S. (1). Risk factors for cancer include age, race and family history. Although many of these risk factors are unmodifiable, there has been increasing interest in the effects of modifiable risk factors such as diet and lifestyle on cancer prevention. It is estimated that 50-75% of cancer deaths could be prevented by changes in diet and lifestyle (2). Variations in cancer rates between countries, as well as migration studies that typically show cancer rates of migrants approach those of the country to which they migrated, strongly suggest that diet, lifestyle and environmental factors play a significant role in cancer development (3, 4). In particular, the identification of dietary agents and their protective mechanisms remains an area of intense investigation. Carcinogenesis is a complex multi-step process in which normal cells accumulate multiple cellular changes allowing for uncontrolled growth and proliferation, eventually leading to metastasis. Dietary compounds may work at multiple points in the carcinogenesis pathway, and likely possess potent protective as well as anti-cancer properties. Prostate cancer is the most commonly diagnosed cancer in American men (excluding skin cancer), and is the second leading cause of cancer related deaths (1). Epidemiological studies indicate that diet and lifestyle could play a significant role in prostate cancer risk; and several dietary agents are under investigation for protective properties against prostate cancer. This dissertation focuses on the role of zinc in protecting against prostate cancer development.

Diseases of the prostate

The human prostate can be separated into four major zones, the peripheral zone, central zone, transition zone and the anterior fibromuscular stroma. Within the prostate, two main types of diseases can occur: benign prostate hyperplasia (BPH) and prostate cancer. The risk for prostate cancer increases with age, and it is estimated that 1 in 6 men in the U.S. will be diagnosed with prostate cancer in his lifetime. Risk factors also include family history, race (African American men are more likely to be diagnosed with and die from prostate cancer compared to Caucasian Americans) and chronic inflammation (5, 6). Similar to other types of cancer, diet and lifestyle represent important modifiable risk factors for prostate cancer development.

BPH is a significant disease among aging men, affecting 43% of men over 60 years of age (7). BPH is most commonly associated with symptoms of the lower urinary tract and obstruction of the bladder outlet (7, 8) resulting in impaired urine flow and bladder emptying. BPH typically occurs in the transition zone of the prostate and is associated with changes in the make-up of the prostatic tissue, including increased stroma to epithelium ratio and alterations in the interaction between stroma and epithelia (8, 9). Increased smooth muscle in the stroma can disturb the function of the urethra, which runs through the prostate, and contribute to the symptoms of BPH (9). A review by Shah et al reported that stroma cells release a variety of growth factors which can modulate cell growth (10). Altered expression levels of apoptosis regulators, such as Bcl-2, have also been reported with BPH (11). Therefore, it is believed that an imbalance between cell proliferation and apoptosis contributes to the development of BPH.

Prostate cancer is believed to develop from premalignant lesions called prostatic intraepithelial neoplasia (PIN), which may precede cancer development by ten or more years (12, 13). Similar to other types of cancer, the transformation of normal prostate cells to malignant cells partly involves the accumulation of multiple mutations in tumor suppressor and oncogenes involved in regulating cellular

proliferation, apoptosis and DNA repair. Prostate cancer is characterized by high heterogeneity within the tumors; thus finding genetic links and biomarkers for prostate cancer development has been a challenge (5, 14). In both BPH and prostate cancer, the exact causes, and molecular and genetic events that lead up to the resulting pathologies remain unclear. However, both can be partly characterized by uncontrolled cell proliferation and impaired apoptosis.

Diet and prostate cancer

Migration studies strongly suggest diet and lifestyle play important roles in cancer development (4, 15). The ability of various dietary components to influence cancer susceptibility has been under investigation for many years. Relative to many other cancers, prostate cancer is a "slow progressing" cancer, since the progression time from PIN to prostate cancer can span many years. Thus, there is a large window of opportunity for dietary compounds to exert their protective effects. Despite numerous epidemiological studies linking diet to prostate cancer, the complexities intrinsic in analyzing dietary intakes and the long term nature of cancer development has made it difficult to determine specific associations between diet and prostate cancer. Nevertheless, immense interest remains in regards to the role of diet in prostate cancer prevention and several previous studies have found interactions with both macronutrient and micronutrient intake with prostate cancer risk.

High intakes of macronutrients such as fat and protein have been suggested to alter risk for prostate cancer (16-19). Studies have suggested a positive association between fat intake (especially saturated fat and n-6 polyunsaturated fats) and prostate cancer risk (20-23). Although the mechanism by which dietary fat contributes to prostate cancer development remains unclear, it is thought to involve changes in growth hormones, such as insulin and insulin like growth factor 1 (IGF-1), and products of lipid metabolism which may affect cellular proliferation and inflammation (17). Several studies, including one using data from the Prostate Cancer Prevention Trial, a randomized placebo control trial testing the effectiveness of finasteride in

prostate cancer, have suggested that high BMI is associated with increased risk for high grade prostate cancer (17, 24). Overall, high caloric consumption may also increase risk for prostate cancer, while caloric restriction may be protective against many cancers including prostate cancer. High intake of red meat (particularly processed red meat), milk and diary products have also been suggested to increase risk for prostate cancer (17, 25). The carcinogenic compounds created during cooking of red meat, and those present in processed meat products may contribute cancer development (17, 25).

It has been hypothesized that several micronutrients have protective effects against prostate cancer development. Therefore, many studies have focused on the effect of vitamin and mineral supplementation in reducing risk for prostate cancer. Although some studies have shown no correlation between prostate cancer risk and use of multi-vitamin supplements, intakes of specific vitamins and minerals have been reported to alter risk for prostate cancer (26, 27). For example vitamin D appears to be protective against prostate cancer. The active form of vitamin D (1,25-dihydroxyvitamin D3) functions as a hormone by binding to the vitamin D receptor (VDR) and influencing cellular differentiation and proliferation. Studies have shown that vitamin D can inhibit proliferation, increase differentiation and induce apoptosis in prostate cancer cell lines *in vitro* (28, 29). Although there are some inconsistent epidemiological reports regarding vitamin D and prostate cancer, several studies have supported an inverse relationship between vitamin D and prostate cancer risk (20, 21, 30). Low serum vitamin D levels have been correlated with increased risk for prostate cancer in men over 57 years of age (31). Additionally, recent clinical trials involving high doses of vitamin D in combination with docetaxel (a chemotherapeutic drug) in men with androgen-independent prostate cancer suggest that vitamin D may improve survival (32, 33). Together these studies indicate that vitamin D may be one vitamin with a promising role in the protection against prostate cancer.

Antioxidant vitamins and minerals have also been suggested to be protective against prostate cancer. A case-control study by Kristal et al (26) found men who

used individual supplements for zinc, vitamin C and vitamin E were at decreased risk for prostate cancer. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) also found decreased prostate cancer risk and mortality in male smokers who received alpha-tocopherol supplements (34). Thus, antioxidant vitamins and minerals may be protective against prostate cancer. Epidemiological data are often inconsistent, and studies have also reported no correlation between prostate cancer risk and intake of these and other dietary compounds (27, 35-38). The complexity of collecting accurate dietary intake records and the long-term nature of cancer development, make these studies difficult to conduct and likely contributes to the conflicting findings. Regardless, the ability of diet to modify prostate cancer risk remains a promising line of research. An excellent example of the potential of dietary compounds to protect against prostate cancer risk is illustrated in the trials with selenium.

The Nutritional Prevention of Cancer Trial was a double-blind, randomized, placebo controlled trial aimed at examining the link between selenium supplementation and skin cancer. Selenium supplementation of 200 μg/d or placebo was given for an average of 4.5 years to 1312 participants. Findings showed that selenium supplementation resulted in a 50% reduction in total cancer mortality, a 37% reduction in total cancer incidence, and a 65% decrease in prostate cancer incidence (39, 40). Other studies have also supported a link between Se and prostate cancer risk (41). These findings sparked a large cancer prevention trial, the selenium and vitamin E cancer prevention trial (SELECT), aimed at examining the potential of vitamin E and selenium supplementation to reduce prostate cancer risk (42). In addition to epidemiological studies, research has also shown that selenium can inhibit proliferation of normal and malignant prostate cells, inhibit tumor growth and increase apoptosis (43). Selenium has been shown to disrupt androgen receptor (AR) signaling by altering AR mRNA stability, protein degradation and nuclear translocation (44). Additionally, selenoproteins such as glutathione peroxidase and selenoprotein P function as antioxidants protecting cells from DNA damage.

Many other natural compounds and phytochemicals derived from foods have been proposed to have chemopreventive effects on prostate cancer. Compounds in foods such as lycopene, found in tomato products, have been negatively associated with prostate cancer risk (25). Foods such as soy products and tea are also thought to be protective (25). Multiple mechanisms can be involved in the anticancer effects of dietary agents. Antioxidant vitamins and minerals may work to protect against oxidative damage to DNA, preventing cancer initiation. Dietary compounds such as soy, omega-3 fatty acids, and fruits and vegetables may alter hormone levels, inflammatory processes, and signaling events controlling proliferation, differentiation, apoptosis and angiogenesis. Thus, dietary compounds likely exert their effects at all stages of cancer development.

Despite numerous studies examining the relationship between diet and cancer, the efficacy and mechanism of action for many dietary agents purported to possess anticancer properties is still unclear. Many nutrients (such as folate, vitamin E, vitamin C, vitamin B12 and zinc) play direct and indirect roles in protecting DNA integrity; therefore nutrient deficiencies have been linked with increasing DNA damage and risk for cancer development (45, 46). There is still much to learn about the impact of diet on prostate cancer risk. More studies are required to determine the efficacy of dietary agents in the prevention of cancer, and their mechanisms of action. The essential mineral zinc plays diverse roles in the body, including antioxidant defense, immune regulation, DNA repair and control of gene transcription, and is another candidate nutrient involved in maintaining prostate health.

Zinc biology & metabolism

Zinc is an essential mineral that functions in a variety of roles. The human body contains 1.5-2.5 g total zinc and most is found in skeletal muscle. The RDA for zinc is based on what is needed to replace daily loss of zinc (47). The recommended daily allowance (RDA) for adults for zinc is 11 mg/d for males and 8 mg/d for females (47). Oysters and red meat, such as beef and lamb, are especially high in zinc. Other

foods that come from animals, especially meats, are good sources of zinc. Fortified cereals are also good sources of zinc in the American diet (48). Absorption of dietary zinc can be affected by other dietary compounds. Foods such as grains, legumes, and nuts are often the major source of zinc for many populations, but these foods also have high levels of phytic acid, a chelator of zinc, which impairs absorption of zinc from these foods (47, 49). Absorption is also enhanced during times of low zinc status. This may be due to changes in expression and localization of specific zinc transport proteins, such as Zip1.

Dietary zinc is found primarily bound to proteins and nucleic acids, and is mainly absorbed in the proximal small intestine. Although it is unclear exactly how zinc enters the enterocyte, mechanisms that may be involved include; specific transport proteins (such as Zip1 and ZnT1, and the divalent metal transporter 1), transport via amino acid transporters (since zinc is mainly bound to proteins), and passive or paracellular transport when zinc concentrations are high. In the enterocyte zinc can be used directly, stored or transported out. Zinc can be transported to the liver via the portal vein (initially bound to albumin) or possibly to other tissues by zinc transport proteins. However, the regulation and function of zinc transporter proteins are still under investigation. Plasma zinc is found mostly bound to proteins such as albumin, macroglobulin and amino acids, and makes up only 0.1% of total zinc in the body. The majority of zinc is found in bone, prostate, skin, skeletal muscle and retina. Metallothionein is the primary zinc storage protein, and its expression is regulated by zinc. Zinc is mainly excreted in the feces, but can also be lost in sweat, urine and skin cells.

The Third National Health and Nutrition Examination Survey (NHANES III), data from 1998 – 1994, reports the mean intake of zinc for adults in the U.S. to be 13 mg/d, and the Continuing Survey for Food Intakes by Individuals (CFS II), data from 1994-1996, report mean intake for all individuals as 11 mg/d (47, 50). However, 12% of the adult population still does not consume the EAR for zinc, and in the aging population it is estimated that up to 50% of the elderly do not consume the Estimated

Average Requirement (EAR) for zinc. Thus, there is still a large proportion of individuals in the U.S. that could be at risk for marginal zinc deficiency; people with dietary limitations (such as people who do not eat foods rich in zinc), those with absorption disorders, and the elderly remain at risk for zinc deficiency. Bioavailability of zinc from food sources vary, ranging from 20-50% (48), and likely contributes to the risk for zinc deficiency in individuals who intake most of their zinc from non-animal sources. Additionally, since mean intake is an average of people with high and low intakes of zinc, these numbers may not accurately reflect the number of people who have low intakes of dietary zinc. Moreover, zinc deficiency remains a problem worldwide, especially in developing countries where intake of foods rich in zinc is limited, and diets are high in grains.

The lack of adequate biomarkers available to assess zinc status makes it difficult to identify those who may have marginal zinc deficiencies. Plasma zinc concentrations are not reflective of individual dietary intakes (47, 48, 51, 52). Studies report that only severe dietary deficiency, or moderate dietary deficiencies persisting for months will be reflected in plasma zinc levels (52). Functional evidence of zinc deficiency often occurs before measurable biochemical changes, suggesting current methods of assessing zinc status may not be optimal. Therefore, there is still a need for a specific and sensitive biomarker for zinc status.

Studies by Prasad et al have contributed greatly to the characterization of zinc deficiency in humans. Symptoms of zinc deficiency include growth retardation, rough dry skin, hair loss and testicular atrophy (52). Growth retardation is the classic symptom of zinc deficiency and is prevalent with severe and moderate deficiencies. Other symptoms include loss of taste perceptions, decreased visual ability, and immune dysfunction (52). Since zinc plays a central role in many biological functions, many symptoms of zinc deficiency are often non-specific (47). However, these deficiency symptoms do offer information about zinc functions in the human body. Zinc is important for proper growth and development, sense of taste and smell,

maintenance of the healthy immune system, wound healing and multiple functions involved in central nervous system.

The importance of zinc in immune function has been the subject of numerous studies involving animal and human models. Zinc deficiency has been shown to impair immune function, in part by inducing thymic atrophy, decreasing leukocytes, decreasing T cell populations (including an imbalance of T-helper1 and T-helper2), decreased thymulin (a hormone involved in T-cell function and maturation) activity and alterations in other proteins involved in mediating the immune response (53-55). Zinc deficiency has also been shown to alter expression of other proteins involved in immune function, both at the mRNA and protein level. These effects on immune function are seen even with mild and moderate zinc deficiencies. Functionally, zinc supplementation in populations at risk for zinc deficiency has also been reported to be beneficial. For example, zinc supplementation has been shown to decrease incidences of diarrhea in children, and may also decrease incidence of pneumonia and other types of infectious disease (56). Zinc supplementation at 30 mg/d for 1 year in elderly subjects also decreased incidence of colds and flu, compared to the placebo group (53). Overall, there is convincing evidence that one essential function for zinc is in immune function, and that even mild zinc deficiencies can have detrimental effects on health. Zinc also has many other functions that are essential for health, such as serving as co-factor for metalloenzymes, antioxidant functions, and involvement in cellular signaling pathways.

Zinc possesses structural, catalytic and regulatory roles (57). Zinc functions to stabilize protein structure, such as in DNA and RNA, and is also present in the catalytic site of many enzymes, such as DNA and RNA polymerases. Over 300 enzymes and numerous transcription factors and proteins require zinc (58). Zinc dependent enzymes such as, alkaline phosphatase, 5-aminolaevulinate dehydratase (involved in heme synthesis), and carboxypeptidase A, encompass a wide range of biological functions (59, 60). Importantly, zinc is involved in cellular proliferation, differentiation and metabolism (61). Zinc deficiency has been reported to alter

expression of genes which regulate carbohydrate and lipid metabolism, highlighting the role of zinc in regulation of basic metabolic processes (62, 63). The regulatory roles of zinc are most well illustrated by the function of zinc in transcription factors and proteins which regulate gene expression. Many transcription factors contain zinc finger domains that are essential for DNA recognition and binding. The role of zinc in regulating gene expression is discussed in greater detail later in this literature review.

Zinc is tightly regulated in the human body, and is found primarily bound to proteins. Free zinc is limited and concentrations of free zinc are tightly controlled. The genetic disorder, acrodermatitis enteropathica, is characterized by severe symptoms of zinc deficiency, and is now known to be due to mutations in the Zip4 gene (49, 64). Zip4 encodes a zinc transporter which is highly expressed in the small intestine, kidney and visceral yolk sac, and is highly regulated by zinc (65). These findings indicate that specific proteins are involved in the regulation of zinc homeostasis, and that their dysfunction can result in severe zinc imbalances. Metallothionein (MT) is the most well described regulator of zinc (66, 67). This cysteine rich protein functions in cellular zinc trafficking, regulation of zinc homeostasis, detoxification of heavy metals such as cadmium and mercury, and has antioxidant properties. Regulation of MT expression is partly controlled by zinc, and can also be induced by other heavy metals. MT expression is regulated by the metal responsive transcription factor (MTF-1), which binds to metal responsive elements (MRE) in the promoter region of the MT gene. Details regarding the regulation of MT expression by MTF-1 and the antioxidant properties of MT are discussed in more detail in the next section. MT functions as a zinc storage protein and may be involved in delivery of zinc to zinc containing proteins. However, an essential function for MT has not been discovered (68). Of the 4 main isoforms that have been characterized, only MT1 and MT2 are extensively expressed and inducible by metals, while MT3 is expressed mainly in the brain and may be involved in neuronal function (68, 69). These data imply that regulation of zinc occurs differently in different tissues, and may reflect diverse zinc requirements throughout the body.

The regulation and trafficking of zinc within the cell is also regulated by families of zinc transporter proteins. There are two main families of zinc transporter proteins. Members of the ZnT (SLC30) family function in zinc efflux, transporting zinc from cytoplasm into intracellular compartments or out of the cell through the plasma membrane (70). Members of the Zip (SLC39) family of zinc transporters function to transport zinc into the cell, or from intracellular compartments into the cytosol (71). In all, 24 zinc transporters (10 ZnT and 14 Zip) have been described to date (reviewed in (70-72)). The zinc efflux protein, ZnT1, can be directly regulated by zinc (its promoter region contains metal responsive elements) and plays key roles in zinc homeostasis (73, 74). However, the responsiveness of ZnT1 to zinc is not the same in all organs; the most responsive organs are the liver and kidney (75, 76). ZnT1 knock-outs are embryonic lethal, highlighting the essential role of ZnT1 in zinc homeostasis (74). While some zinc transporters have been found to be expressed throughout the body, such as ZnT1 and Zip1, others are most highly expressed in specific tissues. For example, ZnT3 is highly expressed in the brain, where it necessary for proper neuronal activity (70). The regulation and expression of the different zinc transporters and their responsiveness to zinc appears to be highly variable (72). Therefore, specific zinc transporters may be involved in maintaining zinc homeostasis in different tissues. The specific mechanisms by which zinc transporters function to maintain cellular zinc homeostasis, regulate zinc trafficking, and distribute zinc to where it is needed are still being elucidated.

Antioxidant properties of zinc

Zinc has several antioxidant properties (77, 78). Firstly, zinc is an essential component of the antioxidant enzyme Cu/Zn superoxide dismutase (Cu/Zn SOD), which catalyzes the dismutation of superoxide anion to less reactive hydrogen peroxide and molecular oxygen. Superoxide dismutase is part of the first line of defense against reactive oxygen species.

Zinc can also function to stabilize sulfhydryl groups on proteins and protect them from oxidation. For example, the enzyme δ-aminolevulinate, which is involved in heme biosynthesis, binds 8 moles of zinc per mole of enzyme (79). Studies by Gibbs et al (79) show that zinc protects this enzyme from oxygen inactivation by preventing the oxidation of one of its thiol groups. Zinc likely plays an important role in maintaining and stabilizing the reduced thiol configuration required for enzyme activity. Gibbs suggests three possible mechanisms by which zinc protects sulfhydryl groups: 1. zinc binds directly to thiol groups, 2. zinc is chelated in close proximity to thiol groups, leading to deactivation of the group by steric hindrance and 3. zinc elicits a conformational change that alters the thiol group reactivity (79). Zinc is a non-redox active metal and can compete with redox-active metals for binding sites on proteins, cell membranes and DNA. Redox-active metals such as copper and iron can generate hydroxyl radicals through the Fenton reaction (77). Hydroxyl radicals can cause damage to nearby structures. Zinc can compete with copper and iron and displace them from binding sites on proteins, membranes and DNA, preventing hydroxyl radical formation near these structures (77, 80).

Lastly, zinc also regulates the antioxidant protein metallothionein. Metallothionein is a low molecular weight protein containing 20 cysteine residues which primarily bind zinc, but can also bind copper and other heavy metals to prevent metal toxicity (68). Studies have suggested that the metal-thiolate clusters on MT, which are easily oxidized, function to protect against oxidative damage. Metallothionein expression is regulated, in part, by the transcription factor MTF-1 (81). MTF-1 contains six zinc finger domains, and upon zinc binding, is translocated to the nucleus, where it binds to the multiple metal response elements in the promoter region of MT to induce transcription of MT. MT expression can also be induced by other cellular stressors including oxidative stress (82).

Regulation of gene expression by zinc

The role of zinc in transcriptional regulation was first described for the *Xenopus* transcription factor TFIIIA, which was discovered to contain multiple zinc binding domains involved in DNA binding (83). Subsequently, several classes of zinc binding domains (zinc fingers) have been identified which are involved in protein-protein and protein-DNA recognition and interactions (58). Zinc plays an important role in the regulation of gene expression by its presence in many zinc-containing binding domains in transcription factors and proteins. It is estimated that genes which encode proteins that contain zinc binding domains make up 1% of the human genome (58). Numerous zinc containing transcription factors have been identified including steroid hormone receptors, GATA-1, retinoid x receptor and Sp1 (58, 84-86). The following highlights examples of two transcription factors which likely play an important role in cancer development that require zinc for proper function.

The tumor suppressor protein p53 is known as the "guardian of the genome", and plays an essential role in mediating the response to cellular stress and DNA damage (87). Mutations in p53 are common in human cancers. Many of these mutations occur in the DNA binding domain of p53, and impair proper function of the protein (88, 89). The p53 protein contains zinc in its DNA binding domain, and loss of zinc from p53 has been reported to induce conformational changes that impair DNA binding activity (89-91). Loss of zinc from p53 could occur through mutations in the zinc finger domains, altering structural characteristics needed to bind zinc. Alternatively, decreased zinc availability due to zinc deficiency could limit zinc for incorporation into zinc-dependent proteins such as p53. Previous studies have shown that zinc deficiency can alter protein expression as well as binding activity of p53 (92-98).

NFκB is a zinc associated transcription factor that regulates the transcription of an assortment of genes, such as those involved in cell growth, apoptosis, and angiogenesis, in response to a variety of stimuli such as growth factors and cellular stress (99-101). Constitutively activated NFκB has been observed in many human

cancers including prostate cancer (100). NFκB is typically composed of a heterodimer of p65 and p50, but homodimers of p50 also exist. The p65 subunit has been found to be over-expressed in prostate tumors compared to benign tissues, and NFκB binding activity has been positively correlated with tumor grade (102, 103). Inhibition of NFκB activation by zinc has been observed in prostate cancer cells *in vitro* (104). Additionally, inhibition of NFκB has been reported to reduce PC-3 cell tumorgenicity, and decrease expression of angiogenic proteins VEGF, IL-8 and MMP-9 (105). Over-expression and activation of NFκB is a hallmark of prostate cancer development. Zinc may be one way to modulate NFκB expression and activity, and ultimately decrease prostate cancer risk.

Zinc regulated genes are not limited to transcription factors. Gene array studies have reported differential expression of genes involved in a wide variety of functions due to changes in zinc status (such as zinc deficiency) (62, 106-111). In particular, genes involved in metal homeostasis, stress response, DNA repair, apoptosis, cell growth/proliferation and transcription were found to be regulated by zinc (93, 108, 110-112). However, specific genes which were differentially expressed varied with cell and tissue type; suggesting zinc may regulate gene expression differently depending on tissue type. Overall, it is evident that zinc plays a vital function in regulating gene expression through its structural and catalytic role in a range of enzymes and transcription factors.

Zinc deficiency and cancer risk

Zinc deficiency is a problem worldwide, especially in developing countries where micronutrient deficiencies are common (113-116). Plasma zinc concentrations are tightly regulated and only those with severe zinc deficiencies can be identified by changes in plasma zinc levels. The lack of adequate biomarkers of zinc status makes it impossible to accurately identify those who are marginally zinc deficient. Therefore, it is possible that many people, even in the United States, are marginally zinc deficient, but cannot be identified. This makes it even more difficult to determine the

relationship between zinc status and cancer risk. However, several lines of evidence have correlated dietary zinc deficiency with increased cancer risk.

A case-control study examining 60 patients with cancer of the gut and 30 control subjects showed that cancer patients had significantly lower serum zinc levels compared to controls (117, 118). Zinc deficiency has also been observed in patients newly diagnosed with head and neck cancers (118), and the risk for development of esophageal cancer was lower in subjects who had higher levels of zinc intake (119). These studies suggest zinc status is compromised in cancer patients.

Fong et al have shown a correlation between dietary zinc deficiency and esophageal cancer. Utilizing a rat model of esophageal cancer induced by various doses of N-nitrosomethylbenzylamine (NMBA), the study indicated that dietary zinc deficiency increases incidence and shortens induction time of esophageal tumors in rats (120). Dietary zinc deficiency also resulted in increased esophageal cell proliferation (121). Furthermore, when rats on zinc deficient diets were given a single dose of NMBA, and then switched to a zinc adequate diet, a decrease in esophageal tumorigenesis and an increase in apoptosis was observed (122). Together these data demonstrated that zinc deficiency resulted in increased cell proliferation and increased tumor incidence while zinc replenishment lead to increased apoptosis and decreased tumor incidence in the esophagus.

Furthermore, when a non-tumorigenic dose of NMBA was given, rats on a zinc deficient diet had greater tumor incidence and multiplicity while rats on a zinc adequate diet did not develop tumors. These zinc deficient animals also had increased and sustained proliferation of esophageal epithelial cells, increased expression of p53, and mutations in Ha-*ras* oncogene and p53 tumor suppressor gene (123). Thus, zinc deficiency may increase susceptibility and/or enhance the carcinogenic potential of some cancer-causing compounds.

The phenomena of increased cell proliferation with zinc deficiency in NMBA-induced esophageal tumors was also observed in mice (124). A study found that zinc deficiency in mice with p53 deficiency (p53 -/-) had unrestrained cell proliferation in

the esophagus and forestomach even without treatment with the carcinogen NMBA. After NMBA treatment zinc deficient p53 -/- animals had rapid rates of tumor induction and progression, which was accompanied by increased cell proliferation and decreased apoptosis compared to zinc adequate p53 -/- animals (125). Mutations in the tumor suppressor protein p53 that render the protein non-functional are common in human cancers (87), and these studies indicate that zinc deficiency can further exacerbate the carcinogenic effect of NMBA when combined with loss of p53. Together these studies support the hypothesis that dietary zinc deficiency can lead to increased cancer risk, and that one possible mechanism likely involves increased cell proliferation and decreased apoptosis associated with zinc deficiency. Additionally, it appears that zinc deficient cells have increased susceptibly to NMBA, and that in combination with non-functional p53, zinc deficiency increases risk for cancer development in the esophagus.

In vitro cell culture and *in vivo* animal studies have revealed that zinc deficiency leads to increased oxidative stress and DNA damage (93, 126, 127). In rat glioma cells, zinc deficiency has also been shown to up-regulate expression of the tumor suppressor protein, p53. However, the DNA binding abilities of p53, NFκB and AP-1 transcripition factors were impaired with zinc deficiency (92).

Zinc is also a cofactor for replicative enzymes such as RNA and DNA polymerases (128). Several DNA repair proteins, such as XPA and RPA, which are involved in nucleotide excision repair, contain zinc finger domains (129-131). Consequently, zinc deficiency may alter cancer risk partly through modifying expression and activities of critical transcription factors involved in regulating cell cycle progression, apoptosis and DNA damage response as well as affect the function of zinc-containing enzymes directly involved in DNA repair.

Zinc and its role in the prostate

The prostate contains one of the highest levels of zinc, where concentrations of zinc can be 2-5 fold higher than in other soft tissues (132). The major zinc-

accumulating region of the prostate is the secretory epithelial cells of the lateral lobe of the peripheral zone. The zinc concentrations in this region can be 5-15 times greater than in other tissues. Interestingly, the peripheral zone is also the main region in which prostate cancer develops (133). Prostate cancer cells have markedly decreased zinc concentrations, typically 60-70% lower compared to normal prostate tissue in this region (133). These observations have lead to obvious questions regarding the functions of zinc in the prostate.

Zinc has been postulated to be a specific inhibitor of mitochondrial aconitase (m-aconitase), inhibiting the first step of the Kreb's cycle in which citrate is oxidized to cis-aconitate (134). This leads to an accumulation of citrate, which is secreted at high levels in prostatic fluid. Cancerous prostate cells lose their ability to accumulate citrate due to low zinc levels. Thus, prostate cancer cells can be characterized by low zinc and citrate concentrations (132). Evidence suggests that the inability to accumulate high levels of zinc and the resulting alterations in citrate metabolism occur early on in prostate carcinogenesis. Thus, low zinc concentrations in prostate cancer cells may be one condition which provides an environment which supports uncontrolled proliferation and cancer progression.

Several other functions for zinc in the prostate have been investigated. For instance, a role for zinc in protecting against prostate cancer development continues to be examined. Zinc has been shown to induce apoptosis in androgen-independent prostate cancer cells (PC-3) and human prostate epithelial cells obtained from prostate cancer patients (135, 136). Cell cycle arrest at G2/M, apoptosis and increased $p21^{waf1/cip1/sdi1}$ mRNA levels were observed in PC-3 and androgen-dependent prostate cancer cells (LNCaP) treated with zinc (137). Zinc treatment in nude mice inoculated with PC-3 cells demonstrated that zinc increased zinc accumulation, citrate production and inhibited growth of PC-3 xenografts (138). Physiological zinc concentrations can also inhibit NFκB activation in PC-3 cells, which have constitutively activated NFκB (104). Blockage of NFκB activity by zinc was followed by inhibition of angiogenesis, invasion and metastasis of prostate tumors (105).

Zinc status and prostate cancer

Several studies have examined the correlation between zinc status, dietary zinc intake and prostate cancer risk. Epidemiological studies have been inconsistent as to whether zinc is protective against prostate cancer. Several studies suggest a positive correlation between zinc and prostate cancer (35, 139). One of these studies showed that men who took zinc supplementation at 100 mg/d had a greater relative risk for prostate cancer compared to men who did not use supplements. It is important to note that 100 mg/d is a very high dose for zinc supplementation (35). The tolerable upper limit for zinc intake is only 40 mg/d (47). Studies showing positive and no correlations between zinc and prostate cancer development have also been reported (19, 26, 36, 140). Studies by Kristal et al suggest that men who had high intakes of zinc had decreased risk for prostate cancer and BPH (26, 141). Despite inconclusive epidemiological evidence, experimental data strongly suggests that zinc plays an essential role in normal prostate function (133).

Zinc is part of several enzymes and transcription factors that regulate cell proliferation, apoptosis and DNA repair that are essential for protecting cells against cancer development. Zinc deficiency could limit the availability of zinc for proteins involved in these essential functions, which could lead to increased risk for cancer development. The fact that the normal prostate contains high levels of zinc, but cancerous prostate cells lose their ability to accumulate zinc, and that this occurs early during prostate cancer development, indicate that zinc is an important for normal prostate function. Loss of zinc could disrupt mechanisms which protect against prostate carcinogenesis. Thus, zinc is likely a key dietary compound that can modulate prostate cancer risk.

The molecular and cellular changes that occur in the prostate due to zinc deficiency are still largely unknown. It has been shown that zinc deficiency leads to increased oxidative stress and DNA damage. Lack of zinc results in increased oxidative stress, altered redox status, and impaired function of essential signaling

pathways involving p53, NFκB and AP-1. Yet, it is still uncertain which proteins and signaling pathways are affected with zinc deficiency in the prostate and how these changes could increase risk for prostate cancer.

Regulation of zinc in the prostate

Regulation of zinc in the body is tightly controlled, and examining changes in zinc regulation during prostate carcinogenesis may reveal important mechanisms responsible for prostate cancer development. The most well studied regulator of zinc homeostasis is metallothionein (MT); MT is regulated mainly though the metal-responsive transcription factor 1 (MTF-1) (reviewed in Haq and Andrews) (66, 67). Yet little is known about how zinc is differentially regulated in different tissues, and particularly in the prostate. The expression of MT in the prostate has been reported to be altered with prostate cancer development (142-144). A significant down-regulation of MT 1L and MT 2A (two isoforms of MT 1) gene expression was observed in prostate samples from men with Gleason grade 4/5 cancer compared to prostate samples from benign prostate hyperplasia (143) . Studies in prostate cells in culture have shown altered expression of MT in response to zinc supplementation or deficiency (145, 146). Wei at al (144) observed a decrease in MT1/2 protein expression in prostate cancer cells in culture and malignant prostate cancer tissue compared to normal prostate cells and tissue. In this study, Wei at al also observed a correlation between lower endogenous cellular zinc and MT1/2 expression in androgen-independent prostate cancer cells compared to normal HPR-1 prostate cells. These studies strongly suggest that MT expression is specific for cell type and is correlated with cellular zinc content.

Of the known zinc transporters, Zip1 has been one of the best studied in terms of its expression in the prostate (147-151). Studies by Costello et al suggest Zip1 functions to increase intracellular zinc concentrations (152). Zinc treatment resulted in increased expression of Zip1 and accumulation of cellular zinc in androgen-dependent LNCaP and androgen-independent PC-3 cells, and appeared to be

hormonally regulated (147). Over-expression of Zip1 in the tumorigenic prostate cell line RWPE2 lead to an elevation of intracellular zinc, growth suppression and increased apoptosis (153). Down-regulation of Zip1 gene and protein expression has been observed in prostate adenocarcinoma and prostatic intraepithelial neoplasia (PIN) compared to normal and benign hyperplasia (149). The down-regulation of an important zinc transporter which functions to carry zinc into the cell may be one mechanism in which prostate cells lose the ability to accumulate zinc during cancer development. In addition, Zip1 mRNA levels may not regulated by zinc in the prostate (153). It has been shown in other cell types that it is Zip1 protein expression and cellular localization which are responsive to zinc (154, 155). Therefore, protein expression, stability and localization likely play an important part in the response of Zip1 to changes in zinc status.

To date, only Zip 1 expression has been reported to be regulated by zinc in the prostate. However, differential expression of Zip1, Zip2, Zip3 and ZnT4 has been associated with progression to prostate cancer (149, 156-159). Recent studies suggest that the response to changes in zinc status by zinc transporters occurs, in part, post-transcriptionally, through modifications in protein expression and localization or mRNA stability (160-162). Future studies examining protein expression and localization patterns would clarify mechanisms by which prostate zinc concentrations are altered in the response to changes in dietary zinc.

Dissertation Specific Aims and Hypothesis

Zinc is an essential mineral that is integral to many proteins and transcription factors that regulate key cellular functions such as the response to oxidative stress, DNA replication, DNA damage repair, cell cycle progression and apoptosis. Zinc is unique in that it has antioxidant, anti-proliferative and anti-inflammatory properties. However, the role of zinc in the prostate has yet to be fully characterized. It is well established that cancerous prostate cells show marked decreases in zinc

concentrations. Low zinc concentrations in prostate cancer cells could create an environment that fosters uncontrolled proliferation, cell survival and cancer progression. While dietary zinc deficiency may increase risk for prostate cancer by limiting the availability of zinc for critical proteins and signaling molecules that protect against DNA damage and cancer development.

The goal of these studies is to examine the role of zinc in maintaining a healthy prostate. Firstly, we will determine how zinc treatment in prostate cancer and benign prostate hyperplasia cells influence cell growth and survival, and examine mechanisms which are involved in the response to zinc in these cells. Secondly, we will examine the molecular and cellular changes that occur in the prostate with zinc deficiency and identifying possible mechanisms which may increase risk for prostate cancer using *in vitro* and *in vivo* models. We hypothesize that zinc will impair survival of prostate hyperplasia and cancer cells, and that zinc deficiency in normal prostate cells will increase DNA damage and alter expression and activity of genes that protect against cancer development. We propose that adequate zinc status is essential for maintaining a healthy prostate. We expect that zinc will be essential for protecting against prostate cancer development and that zinc will have anti-proliferative functions in BPH and prostate cancer cells.

Aim #1: Can zinc treatment impair growth and survival of prostate hyperplasia and prostate cancer cells *in vitro*, and what are the mechanisms by which zinc exerts its anti-proliferative effects in these two cell types? *Working Hypothesis*: Since previous epidemiological data suggest that zinc supplementation and high zinc intake can be protective against prostate cancer and benign prostate hyperplasia, respectively, zinc will impair growth and viability in prostate hyperplasia and prostate cancer cells. Zinc will induce growth arrest, decrease cellular viability and induce apoptosis in these cells.

Aim #2: Does zinc deficiency lead to increased DNA damage in prostate epithelial cells *in vitro*? What molecular and cellular changes occur during zinc deficiency that may lead to loss of DNA integrity? *Working Hypothesis*: Because zinc is an important component of proteins involved in DNA damage response and signaling, zinc deficiency will impair the function of these responses resulting in increased DNA damage.

Aim #3: What molecular and cellular changes occurring during zinc deficiency leads to loss of DNA integrity and increased cancer risk in prostate epithelial cells *in vivo*? Using a transgenic mouse model, we will also examine how will over-expression of the antioxidant enzyme Cu/Zn SOD influence these changes? *Working Hypothesis*: Similar to normal human prostate cells in culture, dietary zinc deficiency will also alter the expression and function of proteins involved in DNA damage response in mouse prostate. Over-expression of Cu/Zn SOD may modify some of the changes observed in wild-type animals due to dietary zinc deficiency.

Chapter 2

Differential response to zinc-induced apoptosis in benign prostate hyperplasia cells (BPH-1) and prostate cancer cells (PC-3).

Michelle Yan, Karin Hardin and Emily Ho

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Abstract

Zinc concentrations in the prostate are uniquely high, but are dramatically decreased with prostate cancer. Studies have suggested that increasing zinc in the prostate may be a potential therapeutic strategy. The goal of this study was to evaluate the anti-proliferative effects of zinc in prostate cancer cells (PC-3) and non-cancerous benign prostate hyperplasia cells (BPH-1), and to define possible mechanisms. PC-3 and BPH-1 cells were treated with zinc (0 – 250 μM) for 24 and 48 hours. Cell growth and viability were examined by evaluating total cell number and MTT assay. Apoptosis was assessed by Annexin V staining, caspase activation and protein expression of BAX and Bcl-2. Zinc induced changes in NFκB (p65) nuclear protein expression, binding activity, and nuclear p53 protein expression were analyzed. Zinc treatment decreased cell numbers and viability in both cell lines. BPH-1 cells were more sensitive to the anti-proliferative effects of zinc compared to PC-3 cells. Zinc induced apoptosis in BPH-1 cells as evidenced by increased Annexin V staining, caspase activation, and decreased Bcl-2:BAX expression. Zinc did not induce apoptosis at equivalent doses in PC3 cells, and increased Bcl-2:BAX expression in PC-3 cells. Zinc decreased p65 binding activity in both cells lines, but only BPH-1 cells had significantly decreased nuclear p65 protein. Zinc also decreased nuclear p53 protein expression in BPH-1 cells. This study suggests that the differential response to zinc in PC-3 and BPH-1 cells may be important for the role of zinc in the prevention of prostate cancer or hyperplasia.

Introduction

Prostate cancer is the second leading cause of cancer related deaths in American men (163). Benign prostate hyperplasia (BPH) is another disease of the prostate that primarily results in symptoms in the lower urinary tract, and can significantly impair quality of life (7). Together, prostate cancer and BPH make up a considerable portion of health concerns for men in the U.S. Recently, increased attention has been given to the contribution of dietary and lifestyle factors to prostate cancer risk. Several studies have shown an association between a variety of dietary compounds and decreased risk for BPH and prostate cancer (39, 141, 164-167). Zinc is an essential mineral that plays multiple roles in the human body. Low zinc status has been observed in cancer patients, suggesting a possible link between zinc and cancer development (117-119, 168). Although, data examining correlations between zinc and prostate cancer has been inconsistent, a study by Kristal et al suggested use of zinc supplements decreased risk for prostate cancer (26). Furthermore, a recent study by the same group found an inverse correlation between zinc intake and BPH risk. (141). Thus, the function of zinc in the prostate and its possible protective role against BPH and prostate cancer have been of increasing interest.

The prostate contains uniquely high zinc levels compared to other soft tissues in the body. Although zinc concentrations are relatively high throughout the prostate, zinc accumulation mainly occurs in the peripheral zone. Zinc concentrations in this region can be 3-10 times greater then in other soft tissues (133). Prostate cancer typically arises from the high zinc accumulating peripheral zone of the prostate, while BPH is thought to originate in the transition zone (9, 133). Importantly, prostate cancer cells lose the ability to accumulate zinc, resulting in significantly decreased zinc concentrations in malignant prostate cells. It is thought that this transformation occurs early on during prostate carcinogenesis, since malignant prostate cells have always been observed to have low zinc (133). It has been hypothesized that increasing cellular zinc levels and reestablishing normal zinc concentrations could impair

prostate cancer cell growth. Previous studies have shown that the treatment of androgen-dependent LNCaP cells and androgen-independent PC-3 cells with zinc resulted an inhibition of cell growth, cell cycle arrest, increased p21 mRNA levels and increased apoptosis (137). Furthermore, it has been shown that zinc induced apoptosis is only observed in cells in which there was intracellular zinc accumulation (135). In vivo, zinc supplementation also increased Bcl2-associated X protein (BAX):B-cell CLL/lymphoma 2 (Bcl-2) expression and inhibited tumor growth in nude mice inoculated with PC-3 cells (138). These studies indicate that zinc may have a protective effect by inhibiting prostate tumor cell growth, and inducing apoptosis. Although the role of zinc in limiting cell growth prior to malignancy is not clear, it has become apparent that zinc plays an essential role in prostate health and that there is a strong inverse correlation between intracellular zinc and uncontrolled cell proliferation in the prostate. Therefore, many studies have been focused on examining the regulation and specific functions of zinc in the prostate. Whether zinc supplementation could benefit both prostate cancer and benign prostate hyperplasia cells is unknown. Unlike prostate cancer cells, zinc concentrations in BPH vary, and have been reported to be equal to or higher then in normal prostate cells (133). Thus, the role of zinc and its association with the development of non cancerous BPH maybe be regulated though different mechanisms compared to prostate cancer. The goal of this study was to examine the differences in the response to zinc between prostate cancer and BPH cells. Furthermore, possible mechanisms leading to zinc-induced apoptosis in each cell type were explored, including caspase induction, Bcl-2:BAX expression, NFκB activation and p53 expression.

Methods

Cell culture

Human androgen dependent PC-3 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 with glutamine plus 10% FBS. Human benign

prostate hyperplasia cells (BPH-1) (Kind gift from Dr. Simon Hayward, Vanderbilt University, Nashville, Tennessee) were cultured in RPMI 1640 with glutamine plus 5% FBS and 1% Penicillin/Streptomycin. Cells were maintained in 5% CO_2 at 37^{o}C.

Cell growth and viability

Equal numbers of PC-3 and BPH-1 cells were seeded and allowed to reach 70% confluency before being treated with 0-250 μM zinc (as zinc chloride dissolved in deionized water) for 24 or 48 hours. To evaluate cell growth, total cells were counted using Beckman Coulter Z1 Coulter Particle Counter (Fullerton, CA). PC-3 and BPH-1 cells were seeded in 24-well plates at 1x 10^5 cells per well and allowed to attach overnight. Cells were then treated with 0-250 μM zinc for 24 hours. Cellular viability was determined by detection of formazan product from methylthiazolyldiphenyl-tetrazolium bromide (MTT). MTT assay was performed as described by Mosmann (169). Production of formazan product was detected at 580 nm using Molecular Devices SpectraMax (Molecular Devices, Sunnyvale, CA).

*Apoptosi*s

Annexin V staining and caspase activation were determined as indicators of apoptosis. Annexin V staining was conducted using the Nexin Kit from Guava Technologies (Hayward, CA) as directed by manufacturer. Briefly, floating and adherent cells were collected, washed and stained with Annexin V- phycoerythrin (Annexin V- PE) and 7-amino-actinomycin-D (7-AAD). Annexin V-PE binds with high affinity to phosphatidylserine, which is externalized during the early stage of apoptosis. The 7-AAD dye is an indicator of membrane structural integrity and is excluded from healthy live cells, but permeates late stage apoptotic and necrotic cells. Percentage of cells staining for each dye was quantified using the Guava Personal Cell Analyzer (PCA) (Hayward, CA). The Guava Multi Caspase Kit (Hayward, CA) was used to determine caspase activation. Cells were harvested and exposed to sulforhodamine-valyl-alanyl-aspartyl-fluoromethyl-ketone (SR-FAD-FMK), a cell permeable

fluorochrome which covalently binds to multiple activated caspases, and also stained with 7-AAD. Percentage of cells staining for each of these dyes was quantified using the Guava PCA (Hayward, CA).

Western blot and protein isolation

After zinc treatment, floating and adherent cells were collected for protein isolation. Whole cell lysates were obtained using IP lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium pyrophosphate, 1 mM beta-glycerolphosphate, 1 mM sodium orthovandate, and 1mg/L leupeptin) plus protease inhibitor (Complete, Mini; Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN). Nuclear and cytoplasmic fractionation was conducted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Total protein concentrations were determined using DC Protein Assay (Bio-Rad, Hercules, CA). Standard Western blot procedure was preformed using 20 μg protein loaded and separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membrane (BioRad Laboratories, Hercules, CA). Antibody dilutions were as follows: BAX 1:100, Bcl-2 1:1000, p53 1:1000 (Santa Cruz Biotechologies, Santa Cruz, CA), and β-actin 1:4000 (Sigma-Aldrich, St. Louis, MO). Secondary antibodies raised against the corresponding host conjugated to horseradish peroxidase were used. Chemiluminescence was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) on AlphaInnotech photodocumentation system. Quantification of signal intensity was determined using Image J 1.37v (NIH, Bethesda, MD) software. Triplicate samples for each treatment group were analyzed.

NFκB Binding Activity

The binding activity of NFκB subunit p65 in nuclear extracts was assessed using the TransAM™ NFκB p65 Kit (Active Motif, Carlsbad, CA) as directed by the manufacturer. TransAM™ is an ELISA based method which detects p65 binding to

NFκB consensus sequence from nuclear cell extracts. Nuclear extracts from PC-3 and BPH-1 cells treated with zinc were isolated as described above.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.01 (San Diego, CA). GraphPad Prisim was used to natural log transform data for total cell number (as percent of control) and relative viability for PC-3 and BPH-1 cells. Then linear regression lines (and equations) were determined for each data set. These data were used to calculate the EC 50 for cell growth and viability. One-way ANOVA with Tukey's post test was used to determine statistical differences between control and treatment groups for analyses except for the MTT assay. For MTT assay, 2-way ANOVA with Bonferroni post-tests was conducted to determine differences in relative viability across cell type and zinc treatment. P-value <0.05 was considered significant.

Results

Cell Growth and Viability

BPH-1 cells were more sensitive than PC-3 cells to growth inhibitory effects of zinc. Zinc concentrations ≥ 75 μM resulted in a significant decrease in total cells in BPH-1 cells, whereas higher concentrations of zinc (150 μM) were required to significantly decrease total cells in PC-3 cells (Figure 2.1A). The natural log of total cell number (as percent of control) was plotted against zinc treatment, and linear regression analysis was conducted to determine the EC 50 for cell growth (Figure 2.1B). The calculated EC 50 for cell growth was 141.1 μM and 55.1 μM for PC-3 and BPH-1 cells, respectively. Differences in cell viability between PC-3 and BPH-1 cells were also observed at zinc treatments ≥ 100 μM. Cell viability in BPH-1 cells decreased significantly at ≥ 100 μM zinc, but higher concentrations (250 μM) were required to significantly decrease viability in PC-3 cells (Figure 2.1C). The calculated EC50 for

cell viability was 190.8 μM and 95.9 μM for PC-3 and BPH-1 cells, respectively. Despite different responses to zinc, there were still significant increases in cellular zinc in both cell lines after ≥ 100 μM zinc treatment. Cellular zinc concentrations after 100 μM zinc treatment were 0.310 ± 0.007 and 0.297 ± 0.006 μg zinc/million cells for PC-3 and BPH-1 cells, respectively. Zinc treatment in androgen-dependent LNCaP cells also decreased cellular viability, however greater than 250 μM zinc was needed to reduce viability by 50% (data not shown).

Apoptosis

To further explore the mechanism of the zinc-induced decrease in cell number and viability in BPH-1 cells, we examined two markers of apoptosis, phosphatidylserine externalization and caspase activation. Zinc treatment at ≥100 μM resulted in apoptosis as evidenced by increased Annexin V staining (indicated by positive Annexin V and negative 7-AAD labeling). Increased Annexin V staining was more dramatic after 48 hours zinc treatment versus to 24 hours; however both time points showed a significant increase in Annexin V staining (Figure 2.2). A significant increase in caspase activation was observed at 24 hours with 150 μM zinc, and at 48 hours with 100 μM zinc. At both time points and treatment concentrations there was a significant increase in 7-AAD positive cells (representing late apoptotic and/or necrotic cells) (Figure 2.3). Caspase activation occurs early during apoptosis signaling, prior to loss of membrane integrity. It is possible that we did not observe stronger increases in caspase activation because it occurred earlier than 24 h, and the time points chosen did not represent the peak increases in caspase activation.

To explore possible mechanisms contributing to zinc-induced apoptosis, protein expression of BAX and Bcl-2 was examined. There was a significant decrease in Bcl-2:BAX ratio with ≥ 75 μM zinc treatment ($p < 0.05$) in BPH-1 cells after 48h (Figure 2.4). In contrast, 48 hours zinc treatment resulted in a trend of increasing Bcl-2:BAX ratio with increasing zinc treatment which reached significance at 150 μM zinc in PC-3 cells (Figure 2.5).

NFκB expression and activity

NFκB is a well characterized redox-sensitive transcription factor that controls immune function as well as cell survival and apoptosis. Previous studies have shown that zinc effectively inhibits NFκB and promotes apoptosis, thus NFκB binding activity and nuclear p65 protein expression were examined. After 48 hours, 150 μM zinc decreased nuclear p65 protein expression and NFκB binding activity in BPH-1 cells (Figure 2.6A-C). Although there was no change in nuclear p65 protein expression with zinc treatment in PC-3 cells, there was a significant decrease in NFκB binding activity at ≥ 125 μM zinc (Figure 2.7A-C).

p53 expression

Since p53 is another transcription factor involved in regulation of apoptosis, we examined the response of p53 due to zinc in BPH-1 cells only, given that PC-3 cells do not express p53. There was a significant decrease in nuclear p53 expression in BPH-1 cells treated with 150 μM zinc for 48 h (Figure 2.8).

Discussion

Overall, these data suggest that zinc supplementation may play an important role in the prevention of prostate cancer and hyperplasia. In this study, BPH-1 cells appeared to be more sensitive to zinc compared to PC-3 cells. In BPH-1 cells, zinc decreased cell number and viability, which was associated with increased apoptosis, decreased Bcl-2:BAX expression and decreased NFκB (p65) activity and nuclear p65 protein expression. Conversely, in PC-3 cells, higher concentrations of zinc were required to decrease total cell number and viability. Additionally, zinc induced Bcl-2:BAX protein expression, but did not alter nuclear p65 protein expression. This study demonstrates that zinc exerts anti-proliferative effects in both non-cancerous,

prostate hyperplasia and prostate cancer cells and suggests that the effects of zinc may be more potent prior to malignancy.

Studies indicate that the loss of zinc from prostate cells alters their metabolic function, resulting in increased m-aconitase activity, oxidation of citrate and increased production of ATP (170). Studies by Costello et al suggest that the loss of zinc and citrate is an important metabolic change that occurs with prostate cancer development; creating an optimal environment for uncontrolled proliferation, impaired apoptosis, cancer progression and metastasis (132, 134, 171). Therefore, reestablishing high zinc levels in cancer cells may restore normal cellular conditions and possibly control proliferation and induce apoptosis. Zinc treatment in prostate cancer cells in culture has been reported to inhibit cell growth, induce apoptosis and cause cell cycle arrest through the loss of mitochondrial membrane potential, release of cytochrome c and alterations in p21 expression (135, 137) . Zinc has also been shown to decrease growth and induce morphological changes and DNA fragmentation characteristic of apoptosis in BPH-1 cells (136, 172). Our studies confirm these findings, and additionally show differential response to zinc supplementation in cancerous PC-3 cells versus non-cancerous BPH-1 cells. Moreover, we find some of the differential response to zinc may be attributed to differential control of apoptotic regulators.

BAX and Bcl-2 are members of the Bcl-2 protein family which function to regulate apoptosis (173). Although the precise mechanism by which Bcl-2 family proteins control apoptosis are still unclear, examining the Bcl-2:BAX protein expression ratio can be one indication of overall cell survival state (174). Bcl-2:BAX expression was decreased in BPH-1 cells. In contrast in PC-3 cells, zinc increased Bcl-2:BAX expression, despite an observed decrease in cell number and viability at higher zinc concentrations. The increase in Bcl-2:BAX appeared to be predominately due to increased Bcl-2, in combination with no change in BAX expression.

Regulation of Bcl-2 expression can occur transcriptionally or through post-translational modifications (173, 175). Over-expression of Bcl-2 has been reported in certain types of cancers, including prostate cancer, and has been associated with

chemoresistance, increased cell survival and impaired apoptosis (176-179). The development of BPH is thought to be due partly to imbalances in cellular proliferation and apoptosis in the stroma and epithelia of the prostate (180). Increased expression of Bcl-2, as well as altered expression of other regulators of apoptosis is thought to be one mechanism by which apoptosis is impaired in BPH (11, 181, 182). The decrease in Bcl-2 in BPH-1 cells suggests that zinc may attenuate Bcl-2 expression, and ultimately decrease cell survival in benign prostate hyperplasia.

In cancer cells, high expression of Bcl-2 may create a pro-survival environment. Previously, zinc has been shown to decrease Bcl-2 expression in normal human prostate epithelial cells (136). However, in nude mice, PC-3 cell induced tumors had a small increase in Bcl-2 and significantly increased BAX expression with zinc treatment (138). Thus, the effect of zinc on Bcl-2 expression, and possibly other members of the Bcl-2 family, may depend on the state of the cell. Despite decreased cell number and viability observed with high zinc treatment in PC-3 cells, there was an observed increase in Bcl-2:BAX expression. It is possible that zinc treatment resulted in alterations in signaling molecules which are involved in post-translational modifications of Bcl-2, or that there were changes in expression of other Bcl-2 family proteins. Future studies examining these two factors would be useful in determining the role of zinc in the prostate and its effect on regulators of apoptosis.

The tumor suppressor protein p53 is known as the "guardian of the genome" and is responsible for the transcriptional regulation of multiple downstream targets; two of which are BAX and Bcl-2 (87, 183, 184). PC-3 cells have one copy of p53 which is mutated resulting in a truncated protein which can not be detected by immuncytochemistry (185, 186). BPH-1 cells do not contain mutations in the p53 gene, but do express high basal levels of p53 (187). To further examine the possible mechanistic differences in the response to zinc between PC-3 and BPH-1 cells, we examined nuclear p53 expression in BPH-1 cells only, since PC-3 cells do not express p53. Zinc resulted in a dose dependent decrease in nuclear p53 expression which only reached significance at 150 μM. In contrast, other studies have shown increased

nuclear p53 protein expression with zinc supplementation in normal human aortic endothelial and bronchial epithelial cell lines (95, 98). It is possible that the response of p53 to zinc may depend on cell type and state, and the response in hyperplasia cells of the prostate is different compared to normal cells originating from different organs. Since the change in nuclear p53 expression was not significant at concentrations of zinc which were associated with changes in BAX and Bcl-2 protein expression, it is likely that the response was primarily due to alterations in other regulators of apoptosis. NFκB is an important transcription factor that also regulates cell survival and apoptosis. Due to the effect of zinc on NFκB expression and activity observed in this study, and previous studies establishing a link between NFκB activation and prostate cancer, it is possible that zinc is a strong modulator of cell growth and survival through NFκB pathway. In addition, other signaling molecules, such as those that control phosphorylation states of Bcl-2 family of proteins, and other transcription factors, like AP-1, may be responsible for the effect of zinc treatment in prostate cells.

An important area of future work is to delineate the mechanisms by which zinc response differs in various prostate epithelial cells. One possible candidate is differential regulation of zinc flux in the cell. The ZnT and Zip family of zinc transporters work together to regulate cellular and subcellular zinc levels. Differential gene and protein expression of zinc transporters in normal, BPH and prostate cancer cells and tissues have been reported. Down-regulation of zip1 and ZnT1 gene expression has been observed in prostate cancer tissues compared to prostate hyperplasia tissue (146, 149). Henshall et al observed decreased protein expression of ZnT4 (by immunohistochemistry) in prostate cancer tissues compared to benign prostate hyperplasia tissues, suggesting decreasing expression of ZnT4 with progression to prostate cancer (158). In addition, normal and BPH tissues express ZIP2 and ZIP3 protein, while these proteins are undetectable in malignant tissues of the prostate. These data suggest that expression of various zinc transporters are uniquely altered during BPH and prostate carcinogenesis. An important future study

would be to characterize changes in prostate zinc levels with zinc supplementation and possible differences in the regulation of zinc transporter expression in the prostate.

Conclusion

Overall, this study showed that BPH-1 cells were more sensitive to zinc-induced changes in cell viability, apoptosis markers, and NFκB expression compared to PC-3 cells. Although changes in cellular zinc were identical in BPH-1 and PC-3 cells, it is possible that differential regulation of apoptotic pathways or zinc transporters in these cells could account for the differential response. Regardless, proper zinc status is likely important for the prevention of prostate cancer but may also be beneficial for those with benign prostate hyperplasia. The effectiveness of zinc against prostate cancer is likely dependent on when it is given, and may be more effective as a chemoprevention strategy rather than a chemotherapeutic agent.

Acknowledgements

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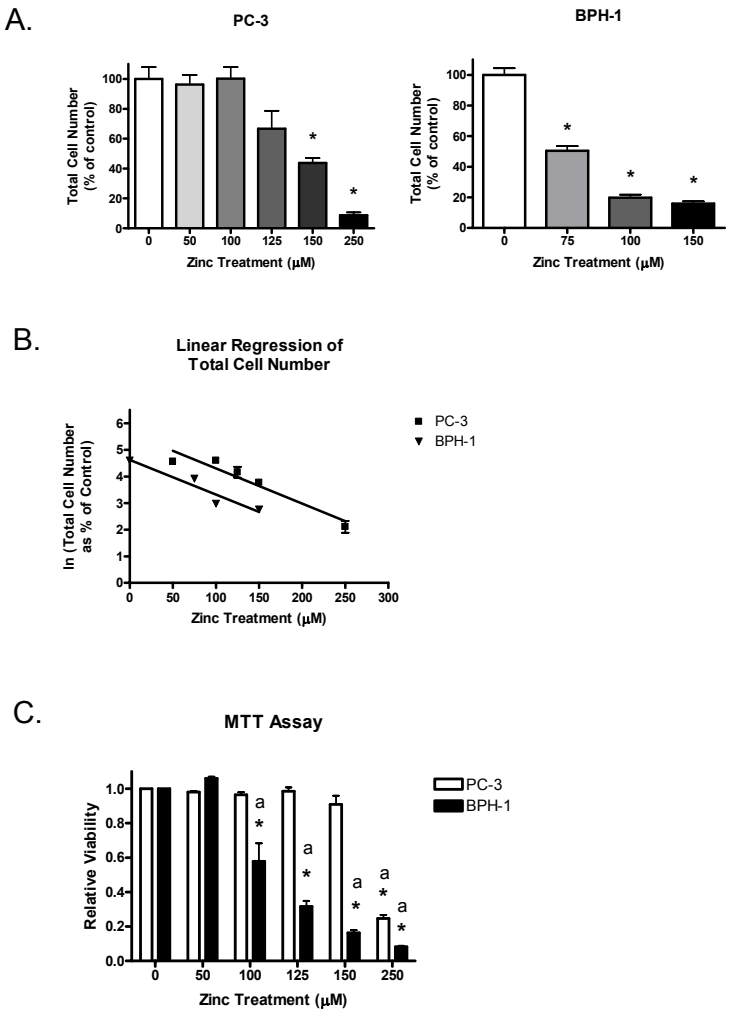

Figure 2.1. BPH-1 cells were more sensitive to anti-proliferative effects of zinc compared to PC-3 cells. Cells were treated with zinc for 48 hours, total cell numbers were determined using Beckman Coulter Z1 Coulter Particle Counter and plotted as percent of control ± SEM. (A.) Higher concentrations of zinc (150 μM) were required to significantly decrease total cell number in PC-3 cells compared to BPH-1 cells (75 μM). (B.) Linear regression lines were determined for PC-3 and BPH-1 cells, and data is shown as percent of total cells as a function of zinc treatment. (C.) PC-3 and BPH-1 cells were treated with zinc for 24 hours and viability was determined by MTT assay. Higher concentrations of zinc were required to induce a significant decrease in cell viability in PC-3 compared to BPH-1 cells. * $p < 0.05$ versus control treatment (no zinc) within a cell type. A indicates significant difference between PC-3 and BPH-1 cells within treatment.

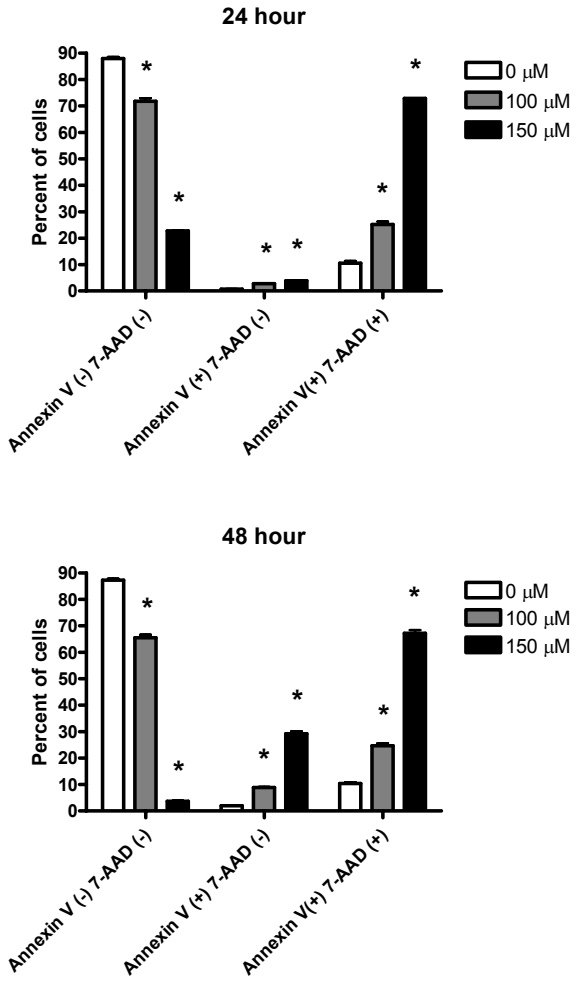

Figure 2.2. Zinc increased Annexin V staining, a marker of apoptosis, in BPH-1 cells. Cells were stained with Annexin V and 7-AAD (an indicator of membrane integrity). Annexin V (-) 7-AAD (-) represent viable cells, Annexin V (+) 7-AAD (-) represent early apoptotic cells, and Annexin V (+) 7-AAD (-) represent late apoptotic/necrotic cells. Both concentrations of zinc significantly decreased percentage of viable cells and increased apoptotic and necrotic cells. Statistical analysis was conducted using one-way ANOVA to compare zinc treatments with control (0 μM zinc) within each staining pattern (Annexin V (-) 7-AAD (-), Annexin V (+) 7-AAD (-), and Annexin V (+) 7-AAD (+)) for each time point. * indicates $p < 0.001$. Triplicate samples were analyzed and results plotted as mean ± SEM.

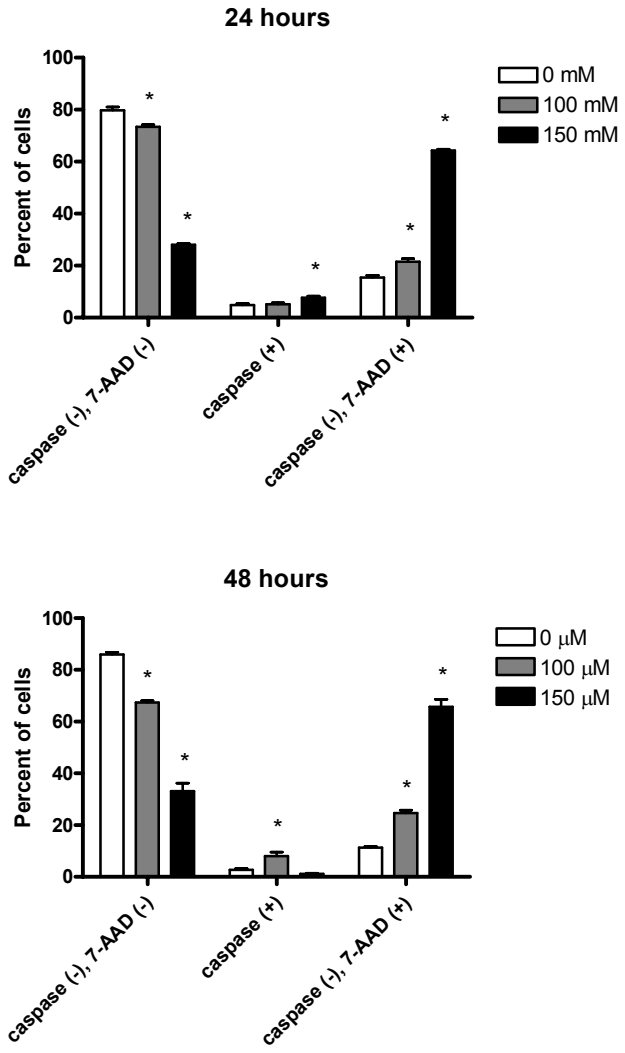

Figure 2.3. Zinc induced caspase activation, decreased cell viability and increased cell death in BPH-1 cells. Cells were analyzed for caspase activation using the Guava Multi Caspase Kit. Cells were exposed to the SR-VAD-FMK fluorochrome (which covalently binds to activated caspases) and the cell impermeant dye 7-AAD (an indicator of cell membrane integrity). Caspase (-) 7-AAD (-) represent viable cells, Caspase (+) represent early and late apoptotic cells, and caspase (-), 7-AAD (+) represent late apoptotic/necrotic cells. Cultures treated with zinc have reduced cell viability, increased caspase activation, and an increased population of apoptotic & necrotic cells. Statistical analysis was conducted using one-way ANOVA, *$p < 0.05$ compared to 0 μM zinc within each treatment for each staining pattern for each time point. Triplicate samples were analyzed and results plotted as mean ± SEM.

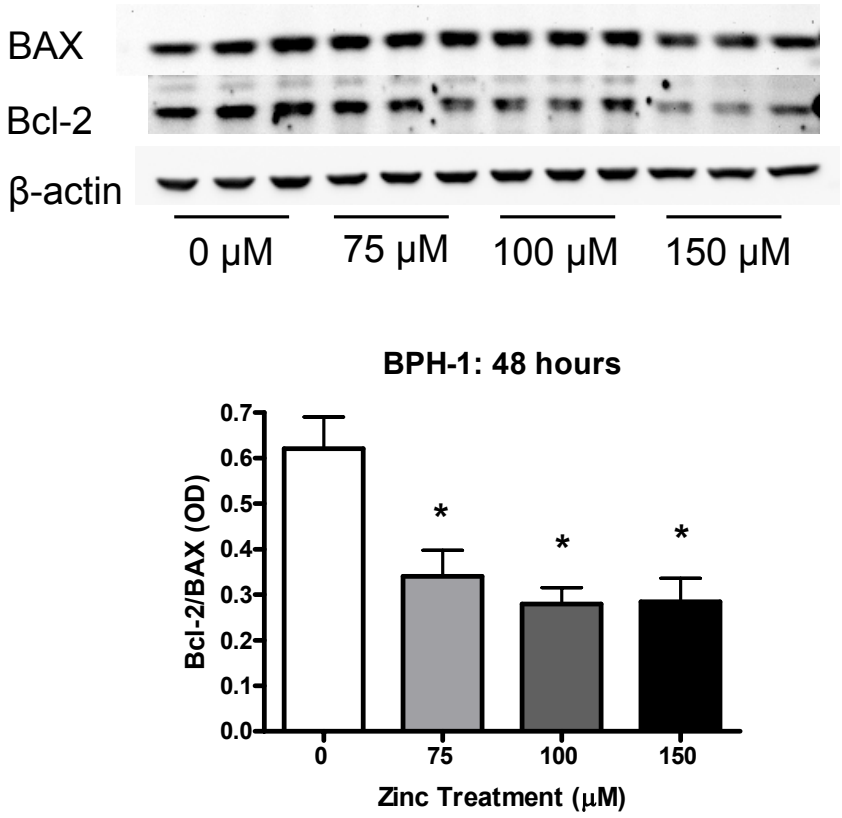

Figure 2.4. Zinc caused a significant decrease in Bcl-2:BAX ratio after 48 hours in BPH-1 cells. BAX and Bcl-2 protein expression was determined by Western blotting. B-actin was used as the loading control. Triplicate samples were analyzed. Densitometry was conducted to quantify protein expression and plotted as OD (normalized to actin) versus zinc treatment as mean ± SEM. Statistical analysis was conducted using one-way ANOVA, comparing each treatment to 0 μM zinc treatment. Triplicate samples were analyzed. BAX expression was significantly decreased at ≥100 μM zinc. Bcl-2 expression was significantly decreased at ≥75 μM zinc. The Bcl-2:BAX ratio was significantly decreased at ≥75 μM zinc. * indicate $p < 0.05$.

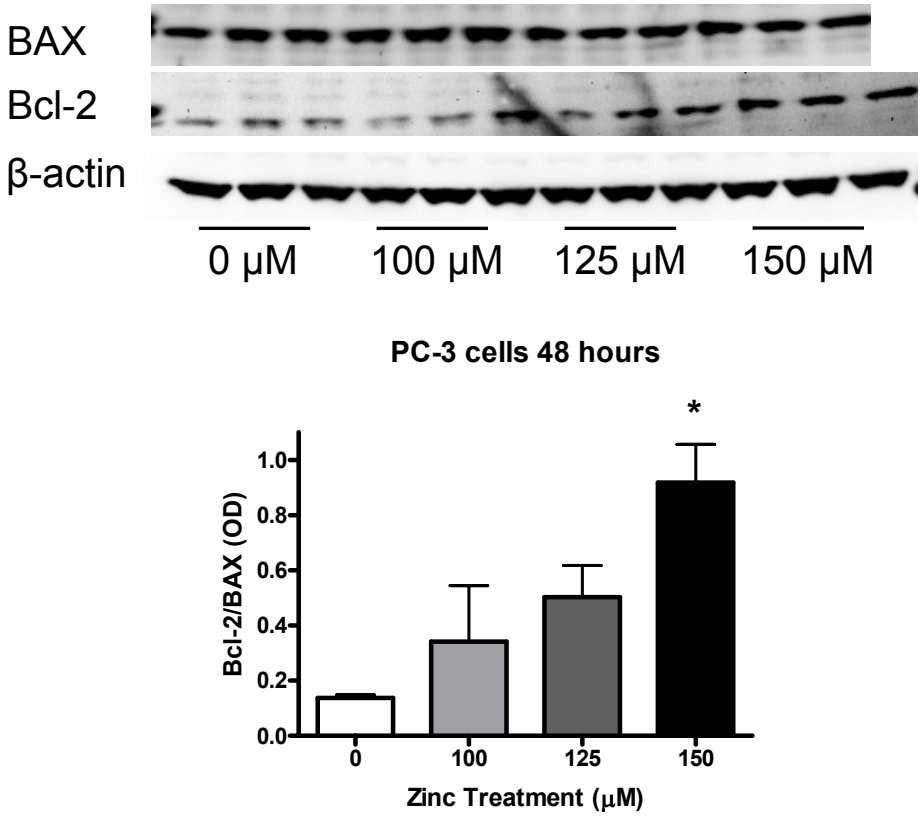

Figure 2.5. Zinc induced a significant increase in Bcl-2:BAX ratio after 48 hours in PC-3 cells. BAX and Bcl-2 protein expression was determined by Western blotting. B-actin was used as the loading control. Triplicate samples were analyzed. Densitometry was conducted to quantify protein expression and plotted as OD versus zinc treatment as mean ± SE. Statistical analysis was conducted using one-way ANOVA, comparing each treatment to 0 μM zinc treatment. Triplicate samples were analyzed. There was no significant change in BAX or Bcl-2 alone. There was a significant increase in Bcl-2:BAX expression at 150 μM zinc. * indicate $p < 0.05$.

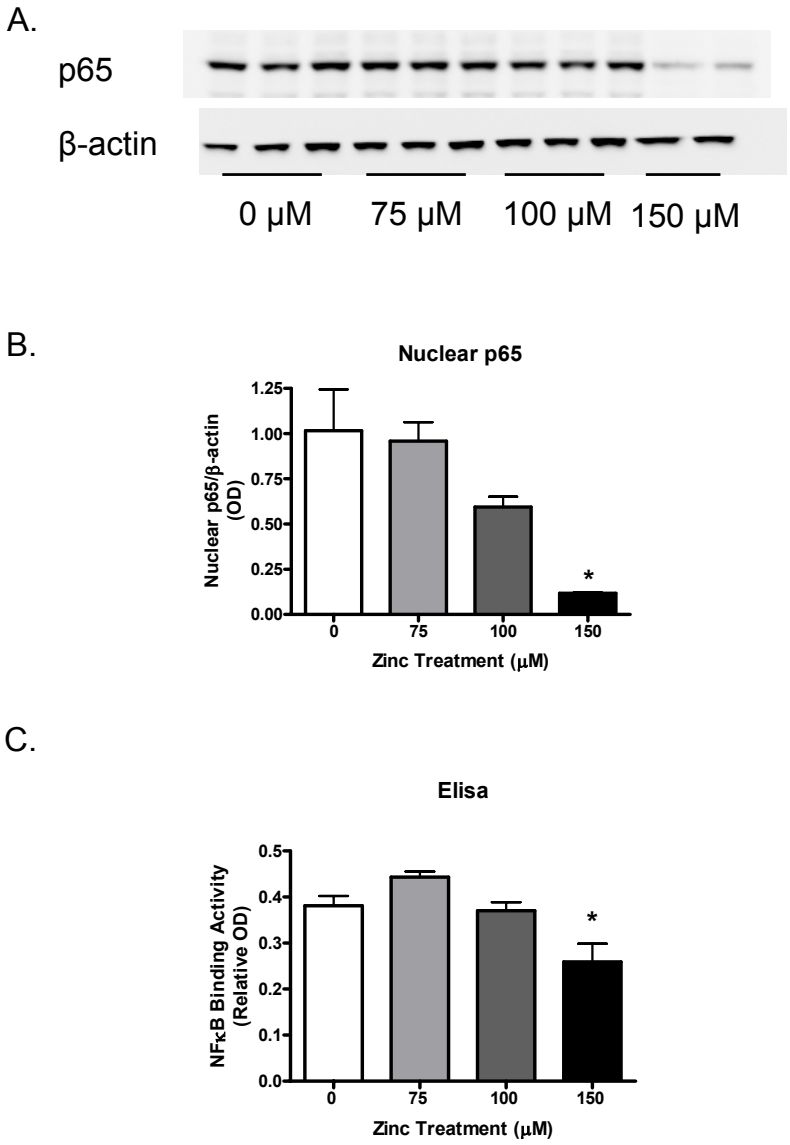

Figure 2.6. Zinc decreased nuclear p65 expression and binding activity in BPH-1 cells. BPH-1 cells were treated with zinc for 48 hours. Nuclear protein expression of p65 was determined by Western blotting (A.), quantified by densitometry analysis, and plotted as mean ± SE (B). Beta-actin was used as the loading control. 150 μM zinc induced a significant decrease in p65 nuclear protein expression after 48 hours ($p < 0.05$). (C.) Accordingly, 150 μM zinc also decreased p65 binding activity as determined by Elisa ($p < 0.05$).

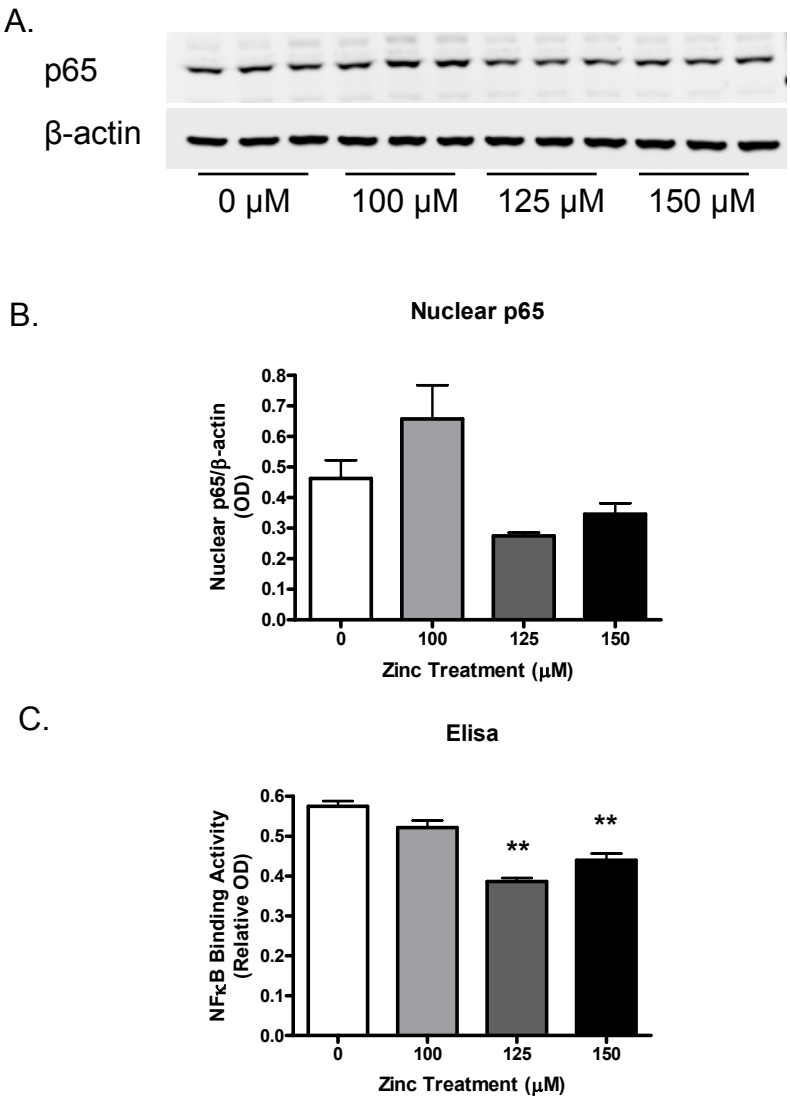

Figure 2.7. Zinc did not change nuclear p65 expression in PC-3 cells. PC-3 cells were treated with zinc for 48 hours. Nuclear protein expression of p65 was determined by Western blotting, quantified by densitometry analysis, and plotted as mean ± SE. Beta-actin was used as the loading control. Zinc concentrations of ≥ 125 μM resulted in increased p65 binding activity as determined by Elisa ($p < 0.01$).

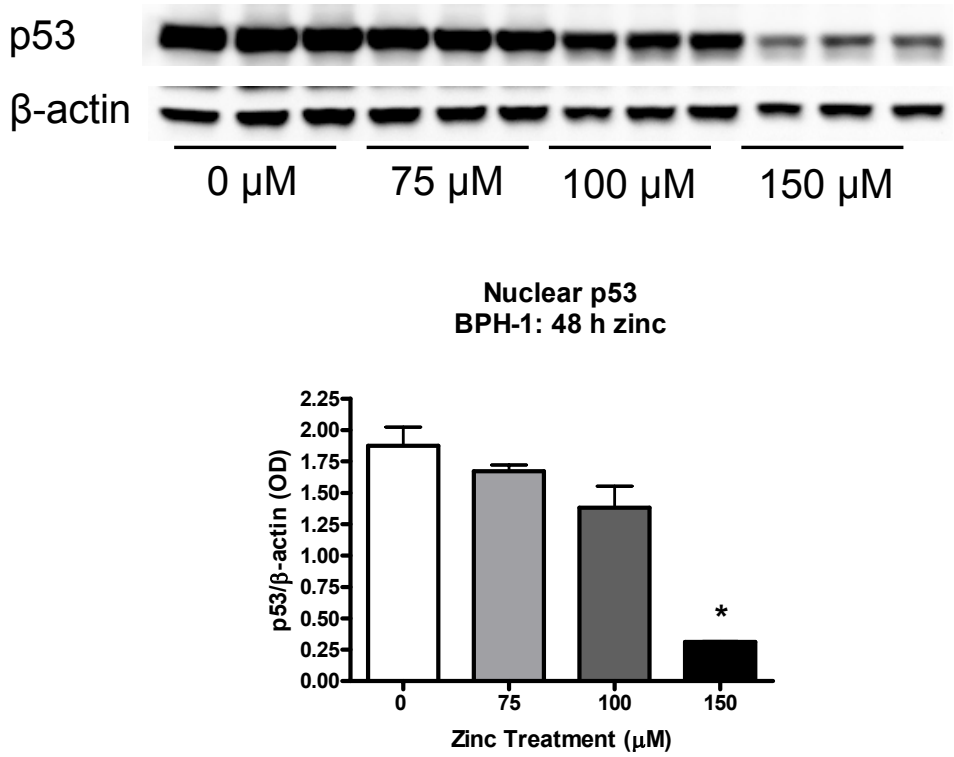

Figure 2.8. Zinc reduces nuclear p53 expression in BPH-1 cells. BPH-1 cells were treated with 0 – 150 μM zinc for 48 h and then harvested for analysis. Nuclear p53 expression was determined by Western blotting, quantified by densitometry analysis, and plotted as mean ± SE. Triplicate samples were analyzed. One-way ANOVA was conducted with Tukey's multiple comparison testing. * indicates $p < 0.001$ compared to 0 μM zinc treatment.

Chapter 3

Zinc deficiency alters DNA damage response genes in normal human prostate epithelial cells

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Abstract

Zinc is an essential trace element for human health and is a critical component of many proteins and transcription factors involved in DNA damage response and repair. The prostate is known to accumulate high levels of zinc, but levels are markedly decreased with cancer development. We hypothesized that zinc plays a critical role in maintaining DNA integrity in the prostate, and zinc deficiency would lead to increased DNA damage and altered DNA damage response mechanisms. To test this hypothesis, the goal of this study was to determine the effects of zinc deficiency on DNA damage and DNA repair mechanisms by examining changes in global gene expression and transcription factor binding abilities in normal prostate epithelial cells (PrEC). Increased single-strand DNA breaks (Comet assay) were observed in PrEC grown in zinc deficient media (ZnDF) compared to cells grown in zinc adequate media (ZnAD) for 7 d. Using Affymetrix HG-U133A gene chips, differential expression of genes involved in cell cycle, apoptosis, transcription and DNA damage response and repair were identified with low cellular zinc. Among genes involved in DNA damage response and repair, TP73, MRE11A, XRCC4 and BRCA2 were down-regulated and TP53 was up-regulated. Additionally, western blotting showed increased nuclear p53 protein expression with zinc deficiency. Despite increased p53 gene and nuclear protein expression, there was no significant change in p53 binding activity. Zinc deficiency also induced an increase in binding activity of transcription factors involved in regulating cell proliferation and apoptosis. Thus, zinc deficiency may compromise DNA integrity in the prostate by impairing the function of zinc-containing proteins.

Introduction

Zinc is an essential mineral that is integral to many proteins and transcription factors that regulate key cellular functions such as the response to oxidative stress, DNA replication, DNA damage repair, cell cycle progression and apoptosis. In particular, several proteins involved in DNA damage signaling and repair, replicative enzymes, such as DNA and RNA polymerases, and transcription factors, such as p53, require zinc for proper function (89, 129, 130). Consequently, zinc deficiency could disrupt the function of both signaling molecules and proteins directly involved in DNA replication and repair. Limited availability of cellular zinc due to zinc deficiency could result in loss of activity of these zinc-dependent proteins involved in the maintenance of DNA integrity, and may contribute to the development of cancer. *In vitro* and *in vivo* studies have revealed that zinc deficiency leads to increased oxidative stress and DNA damage (93, 126, 127). Zinc deficiency has also been shown to up-regulate expression of the tumor suppressor protein, p53, but impair the DNA binding abilities of p53, NFκB and AP-1 transcription factors in rat glioma C6 cells (92). These studies suggest that a decrease in cellular zinc alone causes DNA damage and impairs DNA damage response mechanisms resulting in a loss of DNA integrity and potential for increased cancer risk.

The prostate contains the highest concentrations of zinc compared to other soft tissues in the body (132). Interestingly, as prostate cells develop cancer, zinc concentrations decrease 60-70% (133). Moreover, the main region of zinc accumulation in the prostate, the peripheral zone, is also the main region of prostate cancer development (133). Although the connections between prostate zinc concentrations and prostate cancer have been well established, the precise function of zinc in the prostate remains unknown. We hypothesized that zinc deficiency would disrupt the function of critical zinc dependent proteins that maintain DNA integrity in prostate cells, ultimately resulting in increased DNA damage.

To further understand the role of zinc in maintaining DNA integrity in the prostate, we used genomic approaches to examine the molecular changes that occur with zinc deficiency. The goal of this study was to investigate the impact of zinc deficiency on DNA damage and subsequent alterations in gene expression and protein function by examining global gene expression changes and alterations in transcription factor binding activities using microarray and transcription factor array technologies in normal prostate epithelial cells. These studies will offer insight into the function of zinc in the maintenance of DNA integrity, as well as help identify zinc-regulated genes and zinc-dependent transcription factors, in the prostate.

Methods

Cell culture

Clonetics® normal human prostate epithelial cells, PrEC, were purchased from Cambrex (East Rutherford, NJ) and maintained in Prostate Epithelial Cell Medium (PrEGM™) (Cambrex). Custom made zinc deficient media was purchased from Cambrex. Zinc-adequate media were prepared by adding 0.864 g/L (3 μmol/L) zinc sulfate heptahydrate to zinc-deficient media, which was equivalent to zinc concentration in normal PrEGM media (3 μmol/L zinc). Zinc concentrations in normal, zinc-free (ZnDF) and zinc-free plus 3 μmol/L zinc (ZnAD) media were 0.027, 0.006 and 0.026 ppm, respectively. PrEC cells were seeded at 3000 cells/cm^2 in T75 flasks and allowed to attach overnight. The cells were maintained in zinc deficient (ZnDF) or zinc adequate (ZnAD) media for 7 d, with fresh media replaced every third day. Following the 7 d treatment period, cells were harvested by trypsinization and cell pellets were stored at -80^{o}C for further analysis.

Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES)

Zinc levels in ZnDF and ZnAD cells and media were determined using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES). Cell pellets containing 5 million cells, or 1 mL media, were vortexed with 1 mL 70% ultrapure nitric acid

(EMD Omnitrace, Gibbstown, NJ) overnight. Following incubation, samples were diluted with chelex-treated nanopure water to a 7% acid solution, centrifuged and analyzed by the Prodigy High Dispersion ICP-OES instrument (Teledyne Leeman Labs, Hudson, NH) against known standards.

Comet assay

Detection of single strand DNA breaks were determined by alkali single-cell gel electrophoresis as described by Singh (188). Cells were suspended in 0.5% agarose and applied to microscope slides. Cells were subsequently lysed in Comet lysis buffer (Trevigen, Gaithersburg, MD) for 1 h, subjected to alkali buffer for 20 min, and underwent electrophoresis. Nuclear material was stained with Sybr-green (Molecular Probes, Eugene, OR). Fifty cells from four independent samples were blindly scored on a scale of 0-4 for tail migration intensity.

RNA isolation and microarray

Total RNA was isolated from ZnDF and ZnAD PrEC cells using the Qiagen RNeasy Mini Kit (Valencia, CA). RNA integrity was determined using OD260/280 ratios and agarose gel electrophoresis with ethidium bromide staining. Global alterations in RNA transcripts from ZnDF and ZnAD cells were determined using the Affymetrix human genome HG-U133A GeneChip (Affymetrix, Santa Clara, CA). Three replicates per treatment group were performed. For microarray analysis, RNA samples were sent to the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University, Corvallis, OR for RNA integrity screening, probe synthesis, hybridization and scanning according to the GeneChip® Expression Analysis Technical Manual (701021 Rev. 5).

Array data analysis

GeneSifter software (VizX Labs, Seattle, WA) was used for all analysis of microarray data. Array data was normalized to the all median signal intensity value for each

experiment (each gene chip) and signal values were log base 2 transformed. Genes that did not have present calls for all three replicates in at least one treatment group were omitted. Fold change was calculated and genes with ≥2 or ≤-2 fold change in the ZnDF group compared to ZnAD group, and p value < 0.05 after t-test was applied, were identified as differentially expressed by zinc deficiency. Genesifter grouped differentially expressed genes according to gene function using Gene Ontology and KEGG databases.

Real-time quantitative PCR (qPCR)

P53, Calpain 6, tyrosine 3-monooxygenase, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NFKB2), Ribosomal P2, myeloid cell leukemia sequence 1 (BCL-2 related), metallothionein 1 (MT1), cyclin-dependent kinase inhibitor 1A (p21), BCL2-associated X protein (BAX), Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) (Mdm2), growth arrest and DNA-damage-inducible, alpha (GADD45) and insulin-like growth factor binding protein 3 (IGF-BP3) were analyzed by qPCR. Five μg total RNA was reverse transcribed to cDNA using SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Primer sequences and annealing temperatures are listed in Table 3.1. Presence of double-stranded PCR product was monitored using DyNAmo SYBR® Green qPCR Kit (New England Biolabs, Ipswich, MA) using the Chromo4 Real Time PCR detections system (MJ Research, Waltham, MA). Melting curve analysis and agarose gel electrophoresis with ethidium bromide staining was conducted to ensure single PCR product of correct amplicon length. Each sample was run in triplicate. Normalized intensity was determined using the standard curve method. Fold change for each gene was assessed after normalization of intensity value to beta-actin (ACTB).

Western blot

Nuclear extracts were isolated from ZnDF and ZnAD PrEC cells using NE-PER™ Nulcear Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Standard Western blot procedure was preformed using 20 μg nuclear extract loaded and separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membrane (BioRad Laboratories, Hercules, CA). Antibody dilutions were as follows: p53 1:1000 (p53 (DO-1), Santa Cruz Biotechnologies, Santa Cruz, CA), β-actin 1:4000 (A5441, Sigma-Aldrich, St. Louis, MO), and secondary antibody concentrations were 1:20,000 (goat anti-mouse IgG-HRP, Santa Cruz). Detection was by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) with image analysis on an AlphaInnotech photodocumentation system. Quantification of signal intensity was determined using Image J 1.37v (NIH, Bethesda, MD) software. Triplicate samples for each treatment group were analyzed.

Transcription Factor Array

Transcription factor binding activity was analyzed using Panomics Protein/DNA Array I (Panomics, Fremont, CA). Nuclear extracts were prepared as described previously and hybridized to the Protein/DNA Array I membrane as directed by the manufacturer. The membrane is spotted with 54 different consensus-binding sequences, each corresponding to a different transcription factor or family of transcription factors. Chemiluminescence detection of consensus sequence binding was obtained with Hyperfilm^{TM} ECL. Quantification of signal intensity was determined using Image J software. Triplicate Arrays were conducted per treatment group. Fold change was calculated as the ratio of the signal intensity as ZnDF/ZnAD for each spot on the membrane.

Electrophoretic mobility shift assay (EMSA)

EMSA for p53 was analyzed with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NB), using the p53 IRDye™ 700 Infrared Dye Labeled

Oligonucleotides as directed by manufacturer. Briefly, five µg nuclear extract, 50 fmol p53 oligo IRDye™ 700 Infrared Dye, 1 µg poly (dI·dC), 2 µL 10 x binding buffer (1 mol/L Tris, 1 mol/L KCl, 1 mol/L DTT, and 500 mmol/L EDTA), 2 µL 25mmol/L DTT plus 2.5% Tween-20, and 2 µL loading dye was incubated at room temperature in a total reaction volume of 20 µL for 30 min. For specific competitor reaction, the sample was incubated for 10 min with 5 pmol of unlabeled p53 oligo before addition of 50 fmol of labeled probe. Reaction mixture was separated on a 6 % acrylamide gel at 150 V for 2 hours, and then imaged and quantified using the Odyssey Infrared Imaging System.

Statistics

Statistical analysis of microarray data was performed using GeneSifter software as described above. Western blot and EMSA were analyzed by unpaired t-test using GraphPad Prism Version 4.01 (San Diego, CA). For Transcription Factor Array T-ratio was calculated using the mean fold change, and was compared to one, representing no change in transcription factor binding ability. The critical two-tailed p-value was determined using T-distribution table with alpha equals 0.05. Values in the text are means +/- SEM.

Results

Zinc concentrations and DNA damage. PrEC cells grown in ZnDF media for 7 days had significantly lower zinc levels compared to PrEC cells grown in ZnAD media. Zinc contents in ZnAD and ZnDF cells were 0.01291 ±0.00138 and 0.00314 ±0.00054 µg/million cells, respectively. This represents a statistically significant 75% decrease in zinc concentration which confirmed the loss of cellular zinc ($p < 0.001$). No effects on cell growth and confluency were observed in ZnDF cells (data not shown). Comet scores in ZnDF cells (1.38 ±0.065) were more than twice that in ZnAD cells (0.640 ±0.028; $p < 0.05$), indicating a significant increase in single-strand DNA breaks with loss of cellular zinc.

Microarray and qPCR. A total of 286 of ~22,000 genes represented on the Affymetrix HG-U133A gene chip were significantly differentially expressed at least 2-fold with zinc deficiency. Of these genes, 146 genes were down-regulated (Appendix Table 1) by zinc deficiency and 140 genes were up-regulated by zinc deficiency (Appendix Table 2). Further analysis using Genesifter showed that differentially expressed genes were involved in a variety of biological processes, such as cellular metabolism, regulation of physiological processes, transport, cell organization and biogenesis, cell growth, cell homeostasis and response to extracellular stimuli (Figure 3.1). Microarray data revealed none of the well characterized genes known to be involved in regulating zinc homeostasis were differentially expressed. Transcription of metallothionein, which is known to be largely controlled by zinc levels (67), was not differentially expressed. Quantitative PCR analysis of showed that MT-1 expression tended to be lower in ZnDF than ZnAD cells ($p=0.06$; Table 3.2).

To gain a better understanding of the relationship between zinc deficiency and the increased DNA damage observed in these cells, we focused on down-regulated (Table 3.3) and up-regulated (Table 3.4) genes associated with DNA damage response and repair, cell cycle regulation, and apoptosis that were identified using gene ontology and KEGG pathway databases**.** There were 18 genes involved in cell cycle and proliferation, 9 involved in apoptosis and cell death and 6 involved in DNA damage response and DNA repair. Genes involved in cell cycle included tumor protein p53, cyclin T1 and cell division cycle 25A (CDC25A) which were up-regulated; and adenomatosis polyposis coli, breast cancer 2, early onset (BRCA2), growth factor independent 1B, meiotic recombination 11 homolog A (MRE11A), and tumor protein p73 were down-regulated. Genes involved in apoptosis and cell death included tumor protein p53, adrenergic, alpha-1-A, receptor (ADRA1A), BCL2-associated athanogene 5, and nucleolar protein 3 which were up-regulated; and complement component 7, FOS-like antigen 2, Harakiri, BCL2-interacting protein, tumor protein p73 and

myeloid cell leukemia sequence 1 which were down-regulated. Genes such as p53, p73, BRCA2 and MRE11A have overlapping functions as they are also involved in DNA damage response and repair. Additionally, the gene, X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4) was also down-regulated. XRCC4 is also involved in DNA repair.

Additionally, several genes which encode for transcription factors were also differentially expressed due to zinc deficiency. NFκB2, Fos-like antigen 2, and TP73 were down-regulated while p53, metal responsive element binding factor 2, (MTF2), and Kruppel-like factor 6 (KLF6) were up-regulated with zinc deficiency. Furthermore, genes directly involved in transcription such as the AT rich interactive domain 1A (SWI-like), which encodes a protein that is part of the chromatin remodeling complex (SNF/SWI), and Polymerase (RNA) II (DNA) directed polypeptide A which encodes for the protein which makes up the largest subunit of RNA polymerase II, were also up-regulated with zinc deficiency. Overall, several key genes that regulate cell cycle progression, apoptosis, DNA damage response and repair, as well as genes which function in transcription and as transcriptional regulators were influenced by zinc deficiency.

Gene expression changes of p53, Calpain 6, tyrosine 3-monooxygenase, NFκB2 and myeloid cell leukemia sequence 1 (BCL-2 related) were confirmed by qPCR. ACTB was chosen to normalize copy numbers before assessment of fold change since microarray and qPCR analysis of ACTB showed no change in mRNA expression with zinc deficiency in PrEC cells. The direction and magnitude of the fold changes found with qPCR for these genes were similar to microarray results, with the exception of ribosomal P2 (p53 and Calpain 6, $p < 0.05$; tyrosine 3-monooxygenase, $p = 0.08$; NFκB2, $p = 0.06$; myeloid cell leukemia sequence 1 (BCL-2 related) and ribosomal P2, non-significant) (Table 3.2).

Nuclear p53 protein expression. Western blot analysis revealed a significant increase in nuclear p53 expression in ZnDF PrEC compared to ZnAD PrEC cells. After normalization to beta-actin the mean OD for ZnDF cells (0.8606 ± 0.09322) were significantly higher than that for ZnAD cells (0.4386 ± 0.04055, $p < 0.05$) (Figure 3.3 A). Importantly, the expression of targets downstream of p53 including p21, BAX, GADD45, Mdm2 and IGF-BP3 showed no significant change in expression with zinc deficiency by qPCR analysis (Table 3.2).

Transcription factor binding activity. The DNA binding ability of 54 different transcription factors were simultaneously assessed using Transcription Factor Array technology. The consensus sequences on the array were spotted in duplicate as well in two different dilutions. Table 3.5 summarizes transcription factors which showed at least 2- fold statistically significant change in binding activity, and representative membranes hybridized with nuclear extracts from ZnDF and ZnAD cells are depicted in Figure 3.2. The transcription factor with the highest change in binding activity was retinoid X receptor (RXR), which had a 4.5 fold increase in binding activity due to zinc deficiency. Other transcription factors with increased activity due to zinc deficiency included Sp1, vitamin D receptor, POU domain, class 4, transcription factor 1 (Brn-3), signal transducer and activator of transcription 4 (Stat4), cAMP responsive element binding protein (CREB), forkhead box H1 (FAST-1), MAD, mothers against decapentaplegic homolog 3/4 (Smad3/4), and thyroid hormone receptor. Additionally, binding to the serum inducible element responsive factor (SIE), heat shock transcription factor (HSE) and GATA binding protein (globin transcription factor) (GATA) consensus sequences on the Array were also increased with zinc deficiency. Importantly, Transcription Factor Array data did not indicate a change in p53 binding activity with zinc deficiency. Further analysis of p53 binding activity by EMSA confirmed there was no significant increase in p53 binding activity in ZnDF (integrated intensity 34.27 ± 1.178) compared to ZnAD cells (41.07 ±7.958) (Figure

3.3 B) despite increased gene and nuclear protein expression, indicating possible p53 dysfunction with zinc deficiency.

Discussion

The precise function of zinc in the prostate, and the effects of zinc deficiency on the molecular and cellular processes in the prostate are relatively unknown. This study demonstrated that decreased cellular zinc in normal prostate epithelial cells resulted in increased single strand DNA breaks and differential expression of genes involved in cell cycle progression, apoptosis, transcription and DNA damage response and repair. RNA transcript and nuclear protein expression of p53 was up-regulated with decreased cellular zinc, but the DNA binding ability of p53 was compromised resulting in impaired signaling of downstream p53 targets responsible for mediating the DNA damage response. These data were consistent with our hypothesis that zinc plays an essential function in maintaining DNA integrity in the prostate. To our knowledge this is the first report to examine DNA integrity and global gene expression changes due to low cellular zinc in a normal prostate epithelial cell line. Overall, these data suggest that an important function of zinc is to protect cellular DNA and adds to the body of literature that suggests that loss of zinc may play an important role in the development of prostate cancer.

Prostate epithelial cells have a unique ability to accumulate high levels of zinc and have the highest concentration of zinc compared to other soft tissues in the body. It has been hypothesized that zinc accumulation is required for the inhibition of m-aconitase so citrate can accumulate for secretion in prostatic fluid (170, 189). Moreover, during malignancy the ability of the prostate cells to accumulate zinc is lost (190). The mechanisms leading to loss of zinc during prostate cancer progression is unclear, but has been linked to aberrant expression of the zinc transporters Zip1,2 and 3 in cancer cells (149, 156). A loss of cellular zinc through dietary zinc deficiency may exacerbate or accelerate this process leading to malignancy. Likewise, alterations

in a variety of genes, mostly involved in signal transduction, stress response and metabolism have also been observed other cell and tissue types (106, 110, 191). Ho et al. (93) have reported zinc deficiency in IMR 90 cells, a primary human lung fibroblast cell line, resulted in altered expression of genes involved in stress response, cell signaling, protein degradation, and DNA damage/repair. Studies of zinc deficiency in rats have also shown differential expression of genes involved in growth and metabolism, stress response and transcription/translation (62, 109). Together these data support the diverse biological role of zinc, and indicate regulation of genes involved in DNA damage/repair and transcription by zinc.

Unexpectedly, none of the well described genes involved in regulating zinc homeostasis, such as metallothionein and zinc transporters were differentially expressed during zinc deficiency in prostate cells. Metallothionein functions to regulate zinc levels, acts as an antioxidant (due to multiple thiol groups on the molecule), and serves to protect against heavy metal toxicity. Zinc, other heavy metals, and oxidative stress can induce metallothionein expression. Transcription of metallothionein is partly controlled through interactions between the metal responsive transcription factor 1 (MTF-1) and metal response elements (MRE) located in the promoter region (67, 81, 82). However, the presence of other promoter elements also control metallothionein expression due to cadmium overload, or oxidative stress (66). Increased expression of metallothionein and MTF-1 with zinc treatment has been reported in several different prostate cancer cell lines (145, 146). Decreased MT-1 expression due to zinc deficiency has been observed in other cell and tissue types (62, 93). In most cell types, zinc is often sequestered through binding to MT, keeping concentration of free zinc at fairly low concentrations. In contrast, in the prostate, there is considerable free zinc available in the cell much of it bound to citrate. Thus, the control, regulation and synthesis of MT may be different in prostate cells compared to other cell types. Indeed, variable and low MT expression intensity and localization patterns in normal human prostate samples and normal RWPE prostate

epithelial cells have been observed (192). Thus, it is possible there are unique mechanisms to control zinc homeostasis in the prostate and could account for the lack of change in typical zinc-sensitive genes. In this study, zinc deficiency in normal prostate epithelial cells resulted in a non-significant decrease in MT-1 gene expression, a 1.6 fold increased binding of metal response factors to MRE and no change in zinc transporter expression. These data highlight the unique metabolic responses to zinc depletion that are specific to the prostate and are an important area of future research.

In addition to the direct increases in DNA damage, our data suggests that zinc deficiency in the prostate impaired p53 binding activity, leading to an inability to signal downstream targets responsible for carrying out essential mechanisms involved in DNA damage signaling and repair. Increased gene and/or nuclear p53 protein expression due to zinc deficiency has also been reported in other cell types both in vitro and in vivo (92, 93, 95, 97, 98). Studies have shown that removal of zinc from the DNA binding region of p53 results in a non-functional protein that has lost its site specific DNA binding activity (89, 90). Increases in p53 expression without concomitant increase in downstream targets has also been seen in other cell types such as aortic endothelial cells (30,31). *In vivo* studies by Fong et al have shown dietary zinc deficiency results in increased esophageal cell proliferation, increased expression of p53, mutations in p53 and the Ha-*ras* oncogene as well as increased tumor development in rats (120, 121, 123). However, these effects were modified with zinc replenishment (122). These data further support the essential role of zinc in DNA damage response/repair mechanisms, and protection against cancer development.

Many other transcription factors, in addition to p53, contain zinc finger DNA-binding motifs (193). Several studies have indicated that loss of zinc from zinc-dependent enzymes, or mutations in the zinc-finger domain can result in loss of protein function (79, 89, 130, 194). In this study, 4 of the 16 transcription factors which showed significant increases in DNA binding activity with zinc deficiency were

zinc ion binding. In addition to p53, Sp1, RXR, and GATA also contain zinc finger motifs in their DNA binding region (85, 86). A hierarchy may exist of proteins/transcription factors in which proteins acquire or lose zinc depending on the tissue type and extent of zinc deficiency. Determining this hierarchy could reveal novel biomarkers for zinc deficiency, as well as identify specific mechanisms by which zinc deficiency may increase prostate cancer risk.

Several other genes involved in DNA damage response were also affected with zinc deficiency including down-regulation of tumor protein p73, XRCC1, MRE11A and BRCA2. Recent studies suggest that p73 may also play a role in regulating p53 transcription (195). The BRCA2 gene, which is involved in repair of double-strand DNA breaks through homologous recombination,(196) and mutations in BRCA2 have been associated with increased risk of developing breast and prostate cancer (197-200). Finally, protein phosphatase 1A was also up-regulated. Protein phosphatase 1A has been shown to be involved in activation of p53, possibly via its dephosphorylation activity (201).

In conclusion, low cellular zinc levels in PrEC cells resulted in DNA damage and altered expression of genes involved in cell cycle, apoptosis, DNA damage and repair and transcription. Interestingly, zinc deficiency increased both the transcript and nuclear p53 protein expression, but did not significantly up-regulate the DNA binding activity of p53. In addition, there was no change in gene expression of the p53 downstream targets, p21, BAX, and Mdm2. Although epidemiological studies are often inconsistent (19, 26, 35, 140, 202), there is experimental evidence which supports the protective role of zinc against prostate cancer (133). This study confirms the role of zinc in protecting DNA integrity and demonstrates that zinc deficiency may compromise DNA integrity by impairing the function of zinc-dependent proteins involved in the DNA damage response. These data suggest that zinc deficiency may impair cellular mechanisms which respond to and repair DNA damage that could result in an accumulation of DNA mutations and increased cancer risk.

Acknowledgements

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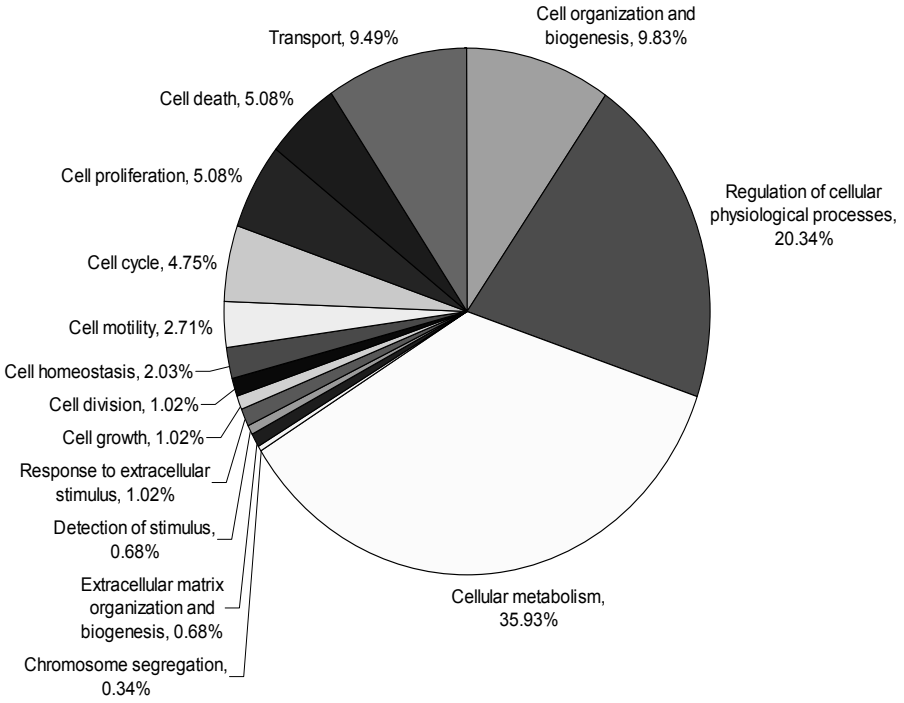

Figure 3.1. Zinc deficiency in PrEC cells differentially expressed genes involved in a variety of biological functions. Microarray analysis of ZnDF and ZnAD PrEC cells was performed using the Affymetrix HG-U133A gene chip. Genesifter was used to analyze microarray data. The pie chart represents ontology distribution of differentially expressed genes involved in different cellular physiological processes.

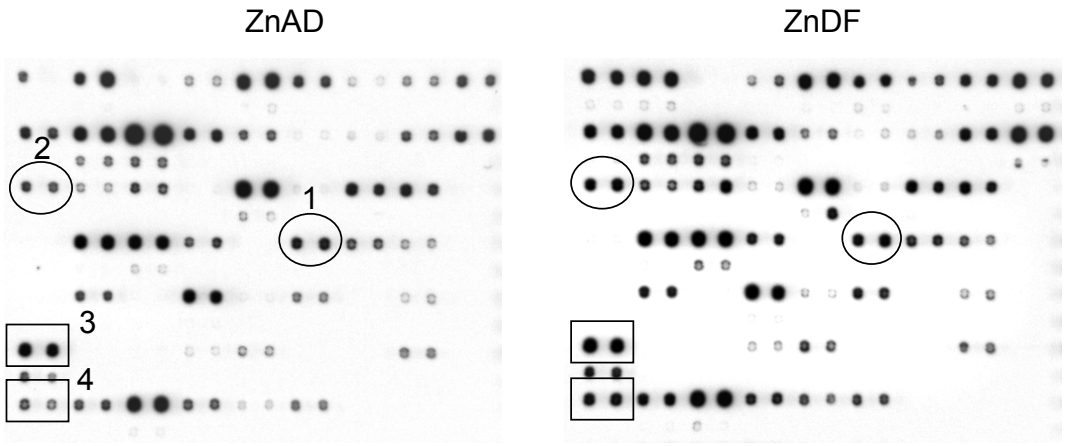

Figure 3.2. Representative membranes from Panomics Protein/DNA Array I analysis of ZnAD and ZnDF PrEC cells. For a complete list of transcription factors with a statistically significant change in binding activity greater than 2-fold refer to Table 4. Each spot on the membrane represents the consensus sequence for a specific transcription factor or family of transcription factors. Several transcription factors with increased binding activity with ZnDF are noted (1. p53, 2. GATA binding protein, and 3. Sp1 and 4. Thyroid hormone receptor (TR)).

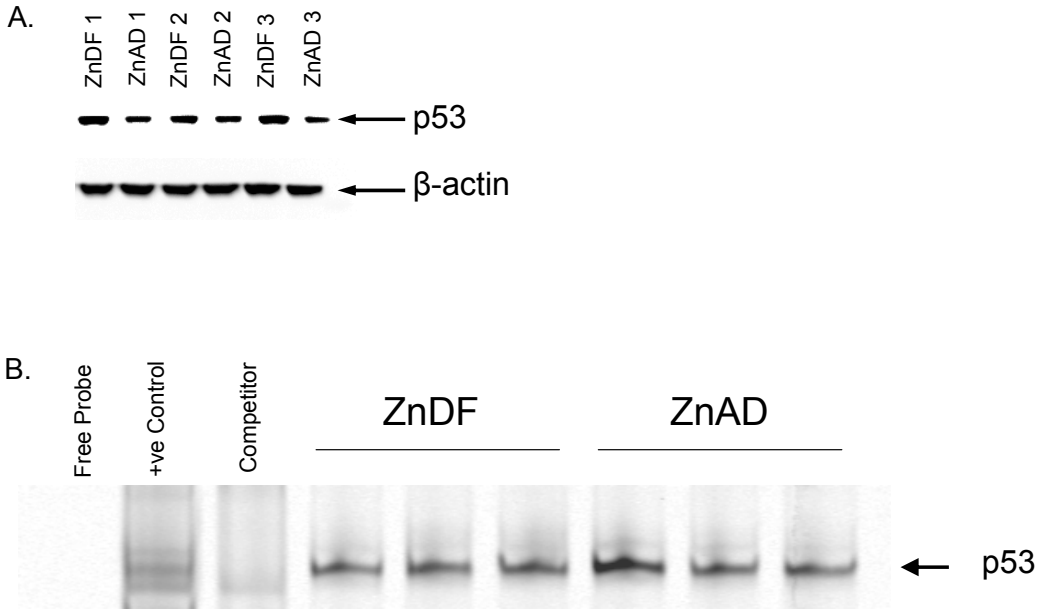

Figure 3.3. Western blot and electromobility shift assay for p53 from nuclear proteins obtained from PrEC cells treated with ZnAD or ZnDF media for 7 d. A. Western blot for nuclear p53 protein. Triplicate samples, representing three independent experiments, for each treatment were analyzed. B. Electromobility shift assay of p53 using nuclear extracts from ZnAD and ZnDF PrEC cells. Positive control represents HeLa extracts and specific competitor reaction (competitor) represents HeLa extracts pre-incubated with unlabeled p53 oligonucleotides. Three samples per treatment group were analyzed.

Table 3.1. Primer sequences and annealing temperature used for qPCR analysis.

Gene		Primer Sequence	Annealing Temp
beta-actin	fwd	TCTTCCAGCCTTCCTTCCTGGGCATG	56°C
	rev	GCTCAGGAGGAGCAATGATCTTGATC	
metallothionein 1	fwd	TCCTGCAAGTGCAAAGAGTG	58 °C
	rev	CAGCTGCACTTCTCCGATG	
ribosomal P2	fwd	ACCGGCTCAACAAGGTTATC	52°C
	rev	TCTTTCTTCTCCTCTGCTGCAG	
TP53	fwd	ATCTACAAGCAGTCACAGCACATGAC	52°C
	rev	TTGTAGTGGATGGTGGTACAGTCAGA	
tyrosine 3 monooxygenase	fwd	GATACGACGAAATGGTGGAGTC	52°C
	rev	CTCAGTCTCAACCATTTGCCGA	
Calpain 6	fwd	GCTTTGAGCTCTTCAAGGTGGAGATG	66°C
	rev	ATGTCCAGAGTCAGTTCCCTGAGCTG	
NFKB2	fwd	TCGTACAACCCAGGTCTGGATGGTA	66°C
	rev	TCTTGACAGTGGGATAGGTCTTTCGG	
Myeloid cell leukemia sequence 1 (BCL2-related)	fwd	CTGGAGATTATCTCTCGGTACC	56°C
	rev	AGTTTCCGAAGCATGCCTTGGA	
BAX	fwd	CTTCAGGGTTTCATCCAGGATCGAG	56°C
	rev	CATCCTCTGCAGCTCCATGTTACTG	
GADD45	fwd	GCTGGTGACGAATCCACATTCA	56°C
	rev	CATTCAGATGCCATCACCGTTCAG	
Mdm2	fwd	GCTTCTCTGTGAAAGAGCACAGGA	56°C
	rev	CAAGGTCCTTTTGATCACTCCCACC	
IGF-BP3 (203)	fwd	GGCCATGACTGAGGAAAGGA	56°C
	rev	CCTGACTTTGCCAGACCTTCTT	
p21 (204)	fwd	CAGACCAGCATGACAGATTTC	56°C
	rev	GCGGATTAGGGCTTCCTCTT	

Table 3.2. Quantitative PCR analysis of PrEC cells treated with ZnAD or ZnDF media for 7 d. Quantitative PCR analysis was conducted on a selection of genes which were found to be differentially expressed by microarray analysis and genes which are downstream targets of p53.

Gene[1]	qPCR Fold Change [2]	Genesifter Fold Change	Gene	qPCR Fold Change[3]
p53	1.91 ± 0.03	4.83	p21	1.45 ± 0.231
Calpain 6	2.24 ± 0.121	3.49	BAX	1.13 ± 0.250
tyrosine 3 monooxygenase	2.18 ± 0.380	2.48	GADD45	1.11 ± 0.110
cyclin T1	1.43 ± 0.315	3.84	MDM2	1.50 ± 0.448
NFKB2	-1.54 ± 0.091	-2.6	IGF-BP3	0.992 ± 0.053
Ribosomal P2	1.74 ± 0.435	-2.24	MT1	0.5767 ± 0.07219
Myeloid cell leukemia sequence 1 (BCL2-related)	-1.23 ± 0.087	-2.41		

1. Genes which were significantly differentially expressed with microarray analysis.
2. Mean fold change ± SEM, calculated from qPCR analysis, representing 3 biological replicates. Intensity values were normalized to β-actin, and fold change was calculated as ZnDF/ZnAD (using normalized intensity values).
3. Downstream targets of p53 which were analyzed by qPCR.

Table 3.3. A list of selected genes which were down-regulated at least 2-fold in PrEC cells treated with ZnDF versus ZnAD media for 7 d.

Fold Change	Gene Identifier	Gene Name (Down-regulated genes)
Cell cycle		
- 2.44	S67788	Adenomatosis polyposis coli
- 4.47	NM_000059	Breast cancer 2, early onset
- 2.77	NM_004188	Growth factor independent 1B
- 4.94	AK026910	Ciliary rootlet coiled-coil, rootletin
- 2.05	BC005241	Meiotic recombination (S. cerevisiae) 11 homolog A
- 2.36	NM_005427	Tumor protein p73
Cell Proliferation		
- 4.76	U01134	Fms-related tyrosine kinase 1
- 2.77	NM_004188	Growth factor independent 1B
- 2.04	NM_017409	Homeo box C10
- 4.78	NM_000590	Interleukin 9
- 4.38	NM_002309	Leukemia inhibitory factor (cholinergic differentiation factor)
Apoptosis/Cell Death		
- 3.68	NM_000587	Complement component 7
- 2.65	NM_005253	FOS-like antigen 2
- 5.9	U76376	Harakiri, BCL2-interacting protein (contains only BH3 domain)
- 2.36	NM_005427	Tumor protein p73
- 2.41	H71805	Myeloid cell leukemia sequence 1 (BCL2-related)
DNA damage response and DNA repair		
- 2.36	NM_005427	Tumor protein p73
- 2.05	BC005241	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
- 2.51	AB017445	X-ray repair complementing defective repair in Chinese hamster cells 4
- 4.47	NM_000059	Breast cancer 2, early onset
Transcription		
- 2.04	NM_017409	Homeo box C10
- 2.18	AL136823	Thyroid hormone receptor coactivating protein
- 2.18	NM_006914	RAR-related orphan receptor B
- 2.21	AF061192	Ectodermal dysplasia 1, anhidrotic
- 2.29	NM_000280	Paired box gene 6 (aniridia, keratitis)
- 2.36	NM_005427	Tumor protein p73
- 2.39	BC001161	Zinc finger protein 174
- 2.6	U09609	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2

Table 3.3 (Continued)

Fold Change	Gene Identifier	Gene Name (Down-regulated genes)
- 2.65	NM_005253	FOS-like antigen 2
- 2.77	NM_004188	Growth factor independent 1B
- 2.78	AL117663	Transcription factor 3
- 4.47	NM_000059	Breast cancer 2, early onset
- 4.92	U11701	LIM homeobox protein 2
- 5.47	NM_002196	Insulinoma-associated 1

Table 3.4. A list of selected genes which were up-regulated at least 2-fold in PrEC cells treated with ZnDF versus ZnAD media for 7 d.

Fold Change	Gene Identifier	Gene Name (Up-regulated genes)
Cell Cycle		
4.83	K03199	Tumor protein p53 (Li-Fraumeni syndrome)
3.84	NM_001240	Cyclin T1
2.48	AI343459	Cell division cycle 25A
Cell Proliferation		
4.83	K03199	Tumor protein p53 (Li-Fraumeni syndrome)
3.74	NM_000618	Insulin-like growth factor 1 (somatomedin C)
3.68	U02569	Adrenergic, alpha-1A-, receptor
2.48	AI343459	Cell division cycle 25A
Apoptosis/Cell Death		
4.83	K03199	Tumor protein p53 (Li-Fraumeni syndrome)
3.68	U02569	Adrenergic, alpha-1A-, receptor
3.62	AA457021	BCL2-associated athanogene 5
2.26	AF043244	Nucleolar protein 3 (apoptosis repressor with CARD domain)
DNA damage response and DNA repair		
4.83	K03199	Tumor protein p53 (Li-Fraumeni syndrome)
4.59	NM_005692	ATP-binding cassette, sub-family F (GCN20), member 2
Transcription		
7.35	X79990	Core-binding factor, runt domain, alpha subunit 2; translocated to, 1; cyclin D-related
5.23	AW589975	Sin3A-associated protein, 30kDa
4.83	K03199	Tumor protein p53 (Li-Fraumeni syndrome)
4.59	NM_005692	ATP-binding cassette, sub-family F (GCN20), member 2
3.84	NM_001240	Cyclin T1
3.09	NM_003153	Signal transducer and activator of transcription 6, interleukin-4 induced
2.74	AF072814	Metal response element binding transcription factor 2
2.7	NM_002919	Regulatory factor X, 3 (influences HLA class II expression)
2.63	BE675435	Kruppel-like factor 6
2.37	NM_002135	Nuclear receptor subfamily 4, group A, member 1
2.32	NM_003441	Zinc finger protein 141 (clone pHZ-44)
2.18	AF231056	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily f, member 1

Table 3.4 (Continued)

Fold Change	Gene Identifier	Gene Name (Up-regulated genes)
2	NM_000937	Polymerase (RNA) II (DNA directed) polypeptide A (220kD)

Table 3.5. Transcription factors that showed a greater than 2-fold increase in binding activity which was statistically significant in ZnDF versus ZnAD PrEC cells.

Mean Ratio[1]	T-Ratio[2]	Transcription Factor	Description
2.015	9.47	SIERF	serum inducible element responsive factor
2.027	5.12	VDR	VDR: vitamin D receptor
2.158	3.45	Brn-3	POU4F1: POU domain, class 4, transcription factor 1
2.243	6.87	Sp1	SP1: Sp1 transcription factor
2.265	4.29	TR(2)	thyroid hormone receptor
2.270	3.72	Stat4	signal transducer and activator of transcription 4
2.362	2.61	c-Myb	MYB: v-myb myeloblastosis viral oncogene homolog
2.364	4.46	CREB	CREB1: cAMP responsive element binding protein
2.808	3.38	FAST-1	FOXH1: forkhead box H1
2.887	2.73	Ets-1/PEA3	ETS-domain transcription factor pea3
2.906	4.58	TR(1)	thyroid hormone receptor
3.246	2.59	CDP	CCAAT displacement protein
3.486	2.85	Smad3/4	MADH3/4: MAD, mothers against decapentaplegic homolog ¾
4.086	3.93	HSF	heat shock transcription factor
4.187	4.96	GATA	GATA: GATA binding protein (globin transcription factor)
4.451	3.52	RXR	RXR: retinoid X receptor

[1.] Ratio calculated as intensity of ZnDF/ZnAD.
[2.] A T-Ratio value of ≥ 2.571 indicates the mean fold change is statistically different than 1 at alpha equals 0.05 level.

Chapter 4

In vivo analysis of global gene expression changes in the prostate using a mouse model of dietary zinc deficiency.

Michelle Yan, Emily Colgate, Karin Hardin and Emily Ho

Formatted for submission

Abstract

Zinc is an integral component of multiple enzymes and transcription factors which function to maintain DNA integrity. Zinc deficiency has been associated with increased DNA damage, increased oxidative stress and impaired activity of transcription factors which function to regulate essential cellular functions. The goal of this study was to examine gene expression changes in the prostate, using an *in vivo* model of dietary zinc deficiency to identify zinc regulated genes which may play a role in protecting DNA integrity and cancer development. Secondly, we sought to determine how the over-expression of a zinc containing antioxidant enzyme, Cu/Zn SOD, would effect these gene expression changes. Wild-type (WT) and Cu/Zn SOD over-expressing animals were fed zinc adequate or zinc deficient diet for 6 weeks and changes in global gene expression in the prostate was assessed using Affymetrix microarray GeneChip. WT animals fed zinc deficient diet showed decreased prostate cellular zinc, but no change in zinc concentrations were observed in the prostates of Cu/Zn SOD over-expressing animals. In WT animals, zinc regulated expression of genes involved in a variety of biological functions. Pathway analysis revealed a significant portion of genes involved in Wnt signaling and prostate cancer were differentially expressed; many of these genes have been shown to influence androgen receptor activity. These data suggest that zinc may regulate expression of genes involved in androgen receptor signaling. Furthermore, it appeared that over-expression of Cu/Zn SOD protected against the loss of zinc from the prostate associated with dietary zinc deficiency. Decreased expression of zip1 and zip3 in Cu/Zn SOD over-expressing animals compared to WT was observed, and may be one explanation for the differential response to dietary zinc deficiency between these two genotypes.

Introduction

Zinc is an essential nutrient that is an integral part of many enzymes and transcription factors. Many of these proteins are involved in regulation of antioxidant defense mechanisms, DNA damage response and maintenance of DNA integrity. The prostate contains exceptionally high concentrations of zinc which is markedly decreased with cancer development. Due to this unique relationship between the prostate and zinc, the regulation and mechanistic role of zinc in the prostate has been of interest.

Zinc deficiency is associated with impaired activity of important transcription factors which regulate cell proliferation, differentiation and apoptosis; many of which are zinc containing proteins (92, 118, 119). Decreased zinc status has been reported in cancer patients, and zinc deficiency has been shown to increase cell proliferation, DNA damage, and tumor formation (93, 121, 124). Previous studies in our lab have also shown increased DNA damage and impaired p53 activity in a cultured cell model of normal human prostate epithelial cells (205). Together these data strongly suggest a role for zinc in the protection against DNA damage as well as in signaling events involved in cancer development.

Zinc also possesses antioxidant properties, partly through its regulation of and function in antioxidant proteins such as metallothionein and copper zinc superoxide dismutase (Cu/Zn SOD). Increased oxidative stress has been associated with decreased zinc status (92, 93, 126, 206). Together, dietary zinc deficiency could impair both antioxidant defense and DNA damage response mechanisms in the prostate and increase the risk for cancer development.

The goal of this study was two fold; first, we wanted to identify zinc regulated genes involved in protecting DNA integrity in the prostate using an *in vivo* model, and second, we wanted to determine how over-expression of the zinc containing antioxidant enzyme, Cu/Zn SOD, would effect these changes. Over-expression of Cu/Zn SOD has been shown to decrease growth of human breast cancer cells and glioma cells (207, 208). However, the effect of over-expression of Cu/Zn SOD in the

prostate has yet to be examined. We hypothesize that dietary zinc deficiency will alter expression of genes involved in protecting against cancer development, and that the pattern of gene expression changes will differ with over-expression of Cu/Zn SOD. These studies will further our understanding of zinc and cancer development in the prostate, and pinpoint mechanisms which may be involved.

Methods

Animals and diet.

TgHS transgenic male mice that over-express Cu/Zn SOD and their wild-type littermates were obtained from Dr. Tammy Bray (Oregon State University, Corvallis, OR). The animal protocol was approved by Oregon State University's Institutional Laboratory Animal Care and Use Committee. TgHS mice ubiquitously over-express Cu/Zn SOD via multiple inserts of the human SOD1 gene (7 copies of the transgene, made of the entire human SOD1 sequence, inserted into chromosome 3). These mice have Cu/ZnSOD expression 3- to 6-fold higher depending on tissue type (209, 210). Cu/Zn SOD over-expressing (SOD^{+++}) animals and their wild type (WT) littermates were fed either zinc deficient (ZnDF) or zinc adequate (ZnAD) diet forming 4 treatment groups: ZnDF WT (n=9), ZnAD WT (n=8), ZnDF SOD^{+++} (n=8) and ZnAD SOD^{+++} (n=9). Modified AIN-93G rodent diet contained zinc in the form of zinc carbonate. Zinc adequate diet contained 50 mg/kg zinc and zinc deficient diet contained <0.5 mg/kg zinc (Dyets, Bethlehem, PA). Diet formulations are presented in Table 4.1. Animals began treatment diets at 5 weeks of age and were maintained on treatment diets for 6 weeks. At the end of 6 weeks, animals were sacrificed; prostates, liver, pancreas and blood were collected for subsequent analysis.

Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES).

Zinc content in livers and prostates were determined using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES). Zinc concentrations in the liver and prostate from 3 animals per treatment group were analyzed. Snap frozen livers

were homogenized in 5 times volume lysis buffer per gram tissue using an OMNI homogenizer (OMNI International, Marietta, GA). Lysis buffer consisted of 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium phyrophosphate, 1 mM beta-glycerolphosphate, 1 mM sodium orthovandate, 1mg/L leupeptin, and freshly added protease inhibitor (Complete, Mini; Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN). To determine zinc concentrations in the prostate, the remaining homogenate the RNA isolation was further processed to isolate total protein. Due to limited number of animals in this experiment, this method was used to determine zinc concentration in the prostate. The prostate homogenate (in RLT buffer from RNeasy Mini Kit) was mixed with 2 M sodium acetate (pH 4.0), buffer-saturated phenol, and 49:1 chloroform/isoamyl alcohol, mixing between each addition. Solution was incubated at 4^{o}C for 15 min., centrifuged at 10,000 x g at 4^{o}C for 20 min, and the upper aqueous layer was discarded. To the bottom organic layer, 100% ethanol was added, mixed by inversion and incubated at room temperature for 2-3 min. Finally the DNA was pelleted by centrifugation at 2,000 x g at 4^{o}C for 5 min. The remaining supernatant contained the protein fraction. The protein was precipitated with isopropanol, and then washed 3 times with 0.3M guanidine HCl. For each wash, the protein pellet was incubated at 15-30^{o}C for 20 min then centrifuged at 7,500 x g at 4^{o}C for 5 min. After the final wash the protein pellet was vortexed with 100% ethanol, incubated at room temperature for 20 min., and then centrifuged at 7,500 x g at 4^{o}C for 5 min to recover the protein pellet. Lastly, the protein pellet was dissolved in 1% SDS. Prostate zinc concentrations were normalized to total protein in the sample. 100 μL of liver homogenate or prostate protein solution was shaken overnight with 1 mL 70% ultrapure nitric acid (EMD Omnitrace, Gibbstown, NJ). Following incubation, samples were diluted with chelex-treated nanopure water to a 7% acid solution, centrifuged and analyzed by the Prodigy High Dispersion ICP-OES instrument (Teledyne Leeman Labs, Hudson, NH) against known standards.

RNA isolation.

Prostates from 3 animals per group were placed in RNA*later*®-ice (Ambion, Austin, TX) directly after harvesting and subsequently stored at -80^{o}C until analysis as directed by the manufacturer. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) as directed by the manufacturer. RNA was isolated from snap frozen livers from 3 animals per group using TRIzol® reagent (Invitrogen, Carlsbad, CA) as directed by the manufacturer. RNA quantity and integrity was determined using OD 260/280 ratios and agarose gel electrophoresis with ethidium bromide staining. Presence of organic or protein contamination in RNA was assessed with OD 230 reading using NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). If necessary, RNA was clean up was conducted using RNeasy Mini Kit as directed by manufacturer.

Microarray array hybridization and data analysis.

Global alterations in prostate RNA transcripts were determined using the Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). The RNA isolated (above) from 3 animals per treatment group were sent to the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University, Corvallis, OR for RNA integrity screening, probe synthesis, hybridization and scanning according to the GeneChip® Expression Analysis Technical Manual (701021 Rev. 5).

Microarray data was analyzed using Genesifter software (VizX Labs, Seattle, WA). Since only WT animals had significantly lower prostate zinc concentrations, microarray data was initially analyzed separately as two data sets, comparing ZnDF and ZnAD for each genotype separately. Array data was normalized to the all median signal intensity value for each experiment (each gene chip) and signal values were log base 2 transformed. Genes that did not have present calls for all three replicates in at least one treatment group were omitted. Fold change was calculated and genes with $\geq$2 or $\leq$-2 fold change in the ZnDF group compared to ZnAD group, and p value <

0.05 after t-test was applied, were identified as differentially expressed by zinc deficiency. We also examined the effect of over-expression of Cu/Zn SOD on gene expression by comparing ZnAD Cu/Zn SOD over-expressing animals to ZnAD WT animals using the same criteria. Genesifter software grouped differentially expressed genes according to gene function using Gene Ontology and KEGG databases. Genesifter was also used to conduct z-score analysis to further examine ontology and pathway changes due to dietary zinc deficiency in WT animals. Z-scores were calculated by comparing the number of genes in a certain pathway or ontology that are on the list of differentially expressed genes, with the total number of genes in the pathway or ontology term that are represented on the GeneChip. Significant z-scores represent ontology terms or genes belonging to certain pathways which are over-represented in the list of differentially expressed genes (211). Z-scores greater than 2 represent ontology terms and pathways which were significantly over-represented in the list of differentially expressed genes. Ontology terms which represented <5 or >100 genes were not included in the gene list. This was done to eliminate ontology terms which were very specific or too broad.

Quantitative real-time PCR (qPCR).

One to five μg total RNA was reverse transcribed to cDNA using SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Custom primers for each gene were designed using Oligo Analysis & Plotting Tools and purchased from Operon Biotechnologies (Huntsville AL). Primer sequences for each gene are shown in Table 4.2. Presence of newly synthesized double-stranded PCR product was monitored using DyNAmo SYBR® Green qPCR Kit (New England Biolabs, Ipswich, MA) on the Chromo4 Real Time PCR detection system (MJ Research, Waltham, MA). Melting curve analysis and agarose gel electrophoresis with ethidium bromide staining was conducted to ensure single PCR product of correct amplicon length. Each sample was run in triplicate. Copy numbers were determined using the standard curve method. Normalized intensity was determined for each sample by dividing the

copy number for each gene by the copy number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold change was determined by dividing the normalized intensity for ZnDF samples by the normalized intensity for ZnAD samples. Tissues from three animals per treatment group were analyzed by qPCR.

Nuclear and cytoplasmic fractionation.

Snap frozen prostates (3 animals per treatment group) were ground to a fine powder in liquid nitrogen using a mortar and pestle. Nuclear and cytoplasmic fractions were separated using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Briefly, 6.5 mL hypotonic buffer was added per gram of tissue and homogenized using OMNI homogenizer (OMNI International, Marietta, GA). Homogenate was incubated on ice for 10 min, centrifuged at 850 x g at 4^{o}C for 5 min, and the cytosolic supernatant collected. Next 1 mL of lysis buffer was added to the nuclei pellet, homogenized using a tight fighting dounce homogenizer, incubated on ice for 30 min with vortexing every 10 min, and then centrifuged at 4^{o}C for 30 min. The nuclear extract supernatant was aliquoted and stored at -80^{o}C until analysis.

Western blotting.

Nuclear and cytosolic protein extracts were isolated as described above. Standard Western blot procedure was preformed. Twenty μg nuclear of extract (3 animals per treatment group) were loaded, separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membrane (BioRad Laboratories, Hercules, CA). Primary antibody dilutions were as follows: p53 1:1000 (DO-1, Santa Cruz), and β-actin 1:4000 (A5441, Sigma-Aldrich, St. Louis, MO). Primary antibody were diluted in 2% BSA in DPBS and incubated overnight at 4^{o}C. Secondary antibodies were goat-anti-rabbit IgG-HRP (1:10,000) and goat-anti-mouse IgG-HRP (1:20,000) (Santa Cruz). Chemiluminescence was detected with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) with image analysis on AlphaInnotech

photodocumentation system. Quantification of signal intensity was determined using Image J 1.37v (NIH, Bethesda, MD) software.

Statistics.

Statistical analyses were conducted using GraphPad Prism 4.01. For analyses comparing WT and Cu/Zn SOD over-expressing animals, 2-way ANOVA with Bonferroni posttest was used. Prostate zinc concentrations were first natural log transformed (due to unequal variances), then subjected to 2-way ANOVA with Bonferroni posttest. Statistical analysis of microarray data was conducted using Genesifter software as described above, and subsequent statistical analyses of qPCR data was conducted using 1-way ANOVA with two-tailed t-test, when only WT animals were analyzed, otherwise 2-way ANOVA with Bonferroni posttest was used. In all analyzes $p < 0.05$ was considered statistically significant.

Results

Animal growth, food intake and zinc status.

There were no significant differences in average weight gain and food intake between treatment groups (Figure 4.1). Prostate SOD1 (Cu/Zn SOD) expression, as determined by Western blot, was 2-fold greater in Cu/Zn SOD over-expressing animals compared to WT controls (data not shown). Liver zinc concentrations were not significantly different between treatment groups. Prostate zinc concentrations in WT animals decreased 91% with dietary zinc deficiency (55.3 ± 4.8 μg zinc/g protein for ZnDF versus 615.7 ± 188.0 μg zinc/g protein for ZnAD) (Figure 4.2). However, there was no significant difference in prostate zinc levels among Cu/Zn SOD over-expressing animals (297.1 ± 75.0 μg zinc/g protein and 321.0 ± 72.9 μg zinc/g protein for ZnDF and ZnAD animals, respectively) (Figure 4.2). Values are expressed as mean ± SEM.

Changes in global gene expression.

Microarray analysis revealed differential gene expression with either zinc deficiency or genotype alone. Array data showed that 808 genes were differentially expressed, at least 2-fold, due to over-expression of Cu/Zn SOD (374 up-regulated and 434 down-regulated). With zinc deficiency, a total of 442 genes were differentially expressed at least 2-fold (224 down-regulated and 218 up-regulated) (WT animals only). There were 74 genes which were common in these two gene lists (Figure 4.3 A-B). When we examined the effects of both Cu/Zn SOD and dietary zinc deficiency, a total of 460 genes were differentially expressed in Cu/Zn SOD over-expressing animals due to dietary zinc deficiency (220 down-regulated and 240 up-regulated). Gene expression profiles with zinc deficiency differed significantly between wild-type and Cu/ZnSOD over-expressing animals. Only 15 genes were commonly differentially expressed in both WT and Cu/Zn SOD over-expressing animals due to dietary zinc deficiency; these are presented in Table 4.3 and shown in Figure 4.3 C. Many of these genes have unknown function (8 of 15), while the rest are mainly involved in transport and cellular signaling. Given that only WT animals had decreased prostate zinc, additional analysis focused on WT animals.

Z-score analysis of ontology terms involved in biological processes showed that a significant portion of genes involved in metabolic processes, regulation of transcription, protein modification, cell-cell signaling, and transport were differentially expressed with zinc deficiency in WT animals; these are listed in Table 4.4. KEGG pathway analysis showed that genes classified under Wnt signaling pathway had the highest z-score (3.63), indicating that a significant portion of genes involved in Wnt signaling were altered with zinc deficiency. There were 7 genes in the Wnt signaling pathway which were differentially expressed; these are presented in Table 4.5. Table 4.6 is a full list of pathways which were significantly affected with zinc deficiency, which include calcium signaling pathway, various cancer pathways (including prostate cancer) and neurodegerative disorder pathways. Genes categorized in the prostate cancer pathway, which can be found in Table 4.5, include: transcription factor 7-like

2, T-cell specific, HMG-box, CREB binding protein, and lymphoid enhancer binding factor 1, which were up-regulated, and heat shock protein 90kDa alpha (cytosolic), class A member 1, which was down-regulated.

To examine the role of zinc in DNA damage response mechanisms in the prostate, we focused on differentially expressed genes which were specifically involved in cell cycle, apoptosis, DNA damage repair and transcription; these genes are listed in Table 4.7. Of the genes involved in these functions, 8 were involved in apoptosis, 8 involved in cell cycle, and 25 in transcription. However, only 1 gene involved in DNA repair (transcript receptor potential cation channel, subfamily C, member 2) was differentially expressed. The transcript receptor potential cation channel, subfamily C, member 2 gene product is also involved in calcium ion transport. Microarray data showed no change in p53 gene expression due to dietary zinc deficiency in WT animals. However, there was a 2.81 fold up-regulation of p53 in Cu/Zn SOD over-expressing animals with zinc deficiency. Z-score analysis (in WT animals) of the biological process terms; cell cycle, cell death, and response to stress, showed that a significant portion of genes involved in these functions were not significantly altered with dietary zinc deficiency. However, these genes are still of interest since they are zinc-regulated in the prostate and may serve as key markers of zinc deficiency in the prostate and may be directly involved in cancer development.

Additionally, because we were examining the impact of dietary zinc deficiency in the mouse prostate, we examined genes which were differentially expressed and classified as zinc ion binding. In all there were 29 genes which were classified under the molecular function of zinc ion binding. Interestingly, the well known zinc regulated gene, metallothionein was not differentially expressed. Microarray analysis also did not show differentially expression of zinc transporters. Further confirmation of zinc transporter expression was confirmed by qPCR. Zinc ion binding genes which were differentially expressed are listed in Table 4.7. Most of the zinc ion binding genes were categorized under the ontology terms metabolic processes, and regulation of transcription.

Quantitative real-time PCR confirmation.

A small subset of genes which were found to be differentially expressed by microarray analysis was also analyzed by qPCR. Rho GTPase activating protein 25, ataxin3, carboxylesterase 2, interleukin enhancer binding factor 3, transformed mouse 3T3 cell double minute 4, ninjurin 1, and signal transducing adaptor molecule (SH3 domain and ITAM motif) 2 were analyzed by qPCR. Quantitative PCR showed changes in expression for these genes followed a similar pattern as microarray results, and is shown in Table 4.8.

Nuclear protein expression for p53.

There was no significant change in prostate nuclear p53 protein expression in WT animals, and a trend for increased expression of nuclear p53 in Cu/Zn SOD over-expressing animals (optical density normalized to β-actin equals 1.120 ± 0.339 and 0.589 ± 0.211 for ZnDF and ZnAD, respectively) with zinc deficiency (Figure 4.4).

Zinc homeostasis genes.

To further delineate possible mechanisms by which zinc metabolism may differ between WT and Cu/Zn SOD over-expressing animals, we examined metallothionein 1 (MT1) and zinc transporter expression in the prostate and liver using qPCR. Both the liver (data not shown) and the prostate (Table 4.8) showed no change in metallothionenin (MT1) expression. Expression of the zinc transporters; ZnT1, ZnT6, ZnT7, zip1, zip3 and zip 6 were also examined. There was a significant decrease in ZnT1 expression in the liver with dietary zinc deficiency in WT but not Cu/Zn SOD over-expressing animals ($p < 0.05$) (Figure 4.5). Zinc deficiency did not alter the expression of any of the zinc transporters tested in the prostate from both WT and Cu/Zn SOD over-expressing animals. However, expression zip family of zinc transporters differed between WT and Cu/Zn SOD over-expressing animals. Zip1 expression was significantly lower in Cu/Zn SOD over-expressing animals in both the

liver and the prostate ($p < 0.05$) (Figure 4.6). Cu/Zn SOD over-expressing animals also had significantly lower expression of zip3 in the liver ($p < 0.05$) (data not shown).

Discusssion

The goal of this study was to examine the effect of dietary zinc deficiency in the prostate using an *in vivo* model. This study examined the potential of using dietary zinc deficiency in a mouse model to study molecular changes in the prostate, and to determine if over-expression of Cu/Zn SOD abrogated the effect of dietary zinc deficiency. Dietary zinc deficiency significantly lowered zinc concentrations in the prostate of WT animals, but not Cu/Zn SOD over-expressing animals. This observation was surprising and suggests a possible protective effect of Cu/Zn SOD over-expression against decreased cellular zinc levels in the prostate in times of limited zinc. It is possible that over-expression of Cu/Zn SOD contributed to the mechanisms which helped the prostate retain zinc, despite dietary zinc deficiency.

Previously, we have shown that zinc deficiency *in vitro*, altered expression of genes involved in a wide range of biological functions normal human prostate epithelial cells (205). Similar findings were observed in this *in vivo* study. These results highlight the essential role of zinc in many aspects of cellular function.

One goal for this study was to identify zinc-regulated genes involved in cancer development in the prostate. A significant portion of genes classified in the Wnt signaling pathway and prostate cancer pathway were differentially expressed due to zinc deficiency. Mutations in genes involved in Wnt signaling have been linked with accumulation of β-catenin, constitutive activation of Wnt signaling and cancer development (212, 213). Moreover, genes involved in Wnt signaling, such as CREB binding protein and MAP kinases, have also been reported to regulate androgen receptor expression and activity. (213-215). The androgen receptor contains zinc finger binding domains; therefore the possibility for direct or indirect regulation of the androgen receptor by zinc is not improbable. However, further studies are needed to

determine the association between androgen receptor activity in the prostate and dietary zinc deficiency.

Our original hypothesis was that zinc deficiency would increase the risk for prostate cancer, partly through altered expression of genes involved in the DNA damage response. Several genes involved in the regulation of cell cycle, apoptosis and DNA damage response were found to be differentially expressed. Microarray data also revealed no change in p53 gene expression in WT animals, but was up-regulated in Cu/Zn SOD over-expressing animals. Correspondingly, there was no change in nuclear p53 protein expression in WT animals, but a trend for increased expression in Cu/Zn SOD over-expressing animals. These findings were unexpected since zinc deficiency has been reported to alter p53 nuclear protein expression in multiple cell types (93-95, 97, 98). Additionally, a previous study conducted in our lab showed zinc deficiency resulted in increased gene and nuclear protein expression of p53 in normal human prostate epithelial cells in culture. In the current study, it appeared that the response of p53 to dietary zinc deficiency differed between the two genotypes. It is possible that over-expression of Cu/Zn SOD partly protected the normal p53 response by helping to maintain prostate zinc levels.

The prostate contains the highest concentration of zinc of all the soft tissues in the body. However, the mechanisms regulating cellular zinc in the prostate and its response to dietary zinc levels remain unclear. In most tissues, the regulation of zinc is tightly controlled, and is primarily regulated by metallothionein (MT). Studies in prostate cells in culture have shown altered expression of MT in response to zinc supplementation or deficiency (145, 146), and decreased MT1/2 protein expression in prostate cancer cells have been observed and correlated with lower endogenous cellular zinc (144). In rodents, MT is primarily expressed in the dorsolateral lobe of the prostate, with very little expressed in the ventral lobe (216-218). Our microarray and qPCR data confirmed there was no change in the expression of MT 1 in the prostate due to dietary zinc deficiency in WT and Cu/Zn SOD over-expressing animals. However, since MT 1 gene expression was analyzed from whole prostate

samples, region specific changes in MT expression could not be detected. Future studies examining changes in expression and localization patterns of MT with dietary zinc deficiency may help determine whether dietary zinc deficiency leads to a pattern of MT expression in specific regions that is similar to that observed with prostate cancer. These data would help elucidate the potential mechanisms by which dietary zinc deficiency contributes to prostate cancer development.

The regulation and movement of zinc within the cell is also regulated by zinc transporter proteins. There are two main families of zinc transporter proteins. Members of the ZnT (SLC30) family function in zinc efflux (70), while members of the zip (SLC39) family of zinc transporters function to transport zinc into the cell (71).

Of the zinc transporters analyzed, only liver ZnT1 mRNA expression was altered (down-regulated) with dietary zinc deficiency in WT animals. ZnT1 functions to decrease intracellular zinc by transporting zinc out of the cytoplasm, and is ubiquitously expressed in a variety of tissues (70, 73). Studies in animal models have reported changes in ZnT1 mRNA expression in response to dietary zinc deficiency in the intestine, kidney, visceral yolk sac, and in the brain (74-76, 219), but not in the liver (75, 76). Few reports have examined the expression of ZnT1 in the prostate. Iguchi et al showed that ZnT2 but not ZnT1 mRNA expression correlated with zinc concentrations in the lateral and dorsal regions (the zinc accumulating regions) of the rat prostate (220). Our studies suggest that dietary zinc deficiency in the mouse model does influence ZnT1 expression in the liver, but not in the prostate.

The zip1 transporter has been well studied in terms of its expression in the prostate (147-151). Zinc treatment in PC-3 and LNCaP cells resulted in increased expression of zip1 and accumulation of cellular zinc which appeared to be hormonally regulated (147). The down-regulation of an important zinc transporter which functions to carry zinc into the cell may be one mechanism by which prostate cells lose the ability to accumulate zinc. However, similar to results found in this study, it has been suggested that mRNA levels of zip1 are not regulated by zinc in the prostate (153); and in other cell types it is zip1 protein expression cellular localization which

respond to zinc (154, 155). Recent studies suggest that zinc transporters are regulated, in part, post-transcriptionally, through modifications in protein expression and localization or mRNA stability (160-162). Thus, it is possible that regulation of zip1 does not occur at the mRNA level. Future studies should focus on examining protein expression and localization patterns of these transporters in response to dietary zinc deficiency. Ultimately, these studies would help determine how dietary zinc deficiency modulates prostate zinc concentrations, and could identify important mechanisms by which prostate lose zinc during cancer development.

The expression of zip1 and zip3 was lower in Cu/Zn SOD over-expressing animals compared to WT. It is also possible that zip1 and zip3 serve as important transporters for delivering zinc to zinc containing proteins, such as Cu/Zn SOD, and may help to preserve cellular zinc levels even in times of low zinc conditions.

In summary, dietary zinc deficiency altered the expression of a number of genes in the prostate which function to maintain normal cellular function. This study identified genes involved in the Wnt signaling pathway and prostate cancer pathway which were regulated by zinc in the prostate. In addition to being critical genes involved in cancer development, these genes have also been reported to be involved in androgen receptor signaling. Due to the critical role that the androgen receptor plays in normal prostate function, alterations in androgen receptor activity due to zinc deficiency may be yet another mechanism by which zinc deficiency may increase risk for prostate cancer.

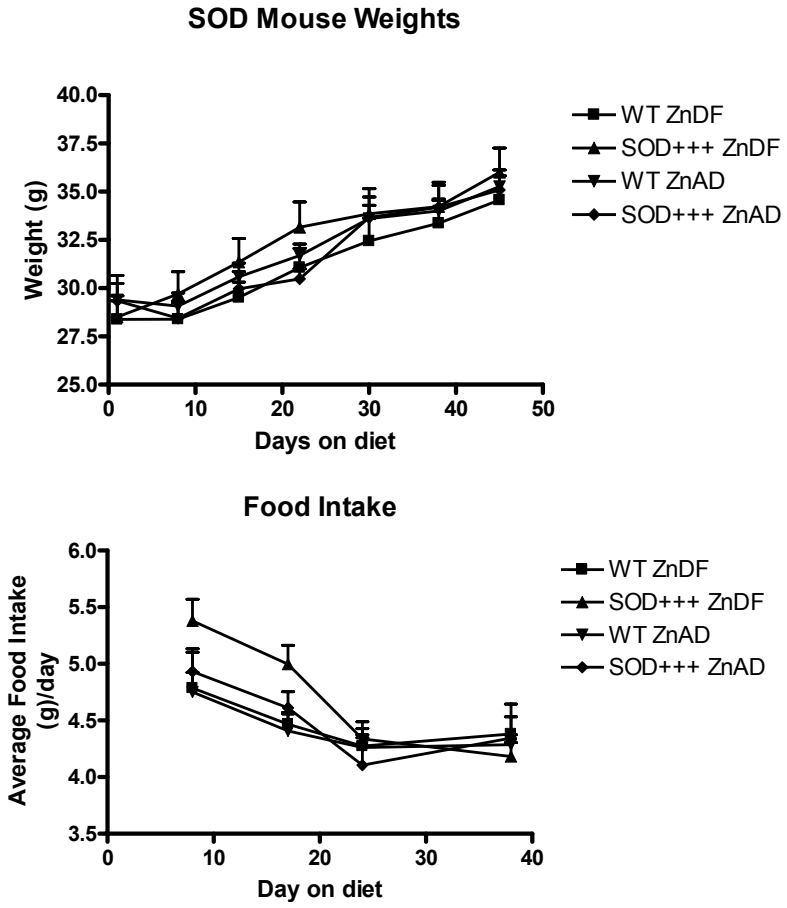

Figure 4.1. Food intake and weights of animals during the study period. There was no significant difference in animal weights between the WT and SOD over-expressing animals fed zinc deficient (ZnDF) and zinc adequate (ZnAD) diets. There was no significant difference in food intake between WT and SOD over-expressing animals fed ZnDF and ZnAD diets. ZnDF WT (n=9), ZnAD WT (n=8), ZnDF SOD^{+++} (n=8), and ZnAD SOD^{+++} (n=9).

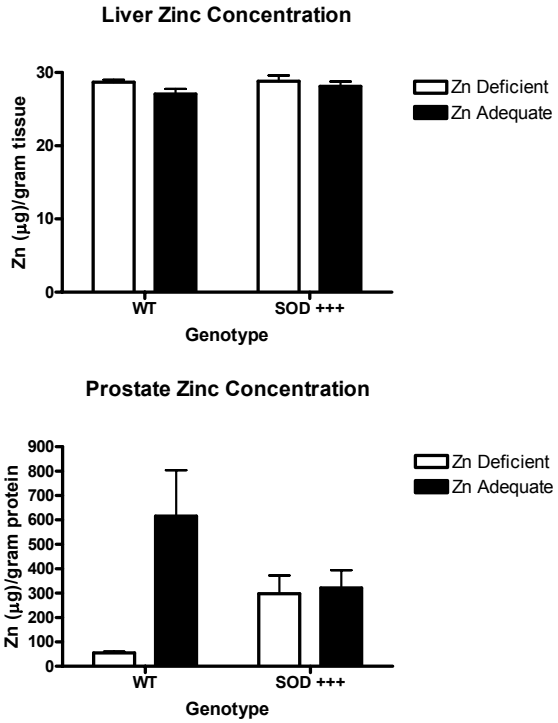

Figure 4.2. Zinc concentrations in liver and prostate analyzed by ICP-OES. Zinc concentrations in liver did not change with dietary zinc deficiency. WT animals fed zinc deficient (ZnDF) diet had significantly lower prostate zinc compared to WT animals fed zinc adequate (ZnAD) diet (* $p < 0.001$). There was no difference in prostate zinc concentrations among SOD over-expressing animals fed ZnDF and ZnAD diet. Two-way ANOVA with Bonferroni post-tests were conducted, data represent mean ±SEM. Data represents 3 animals per treatment group.

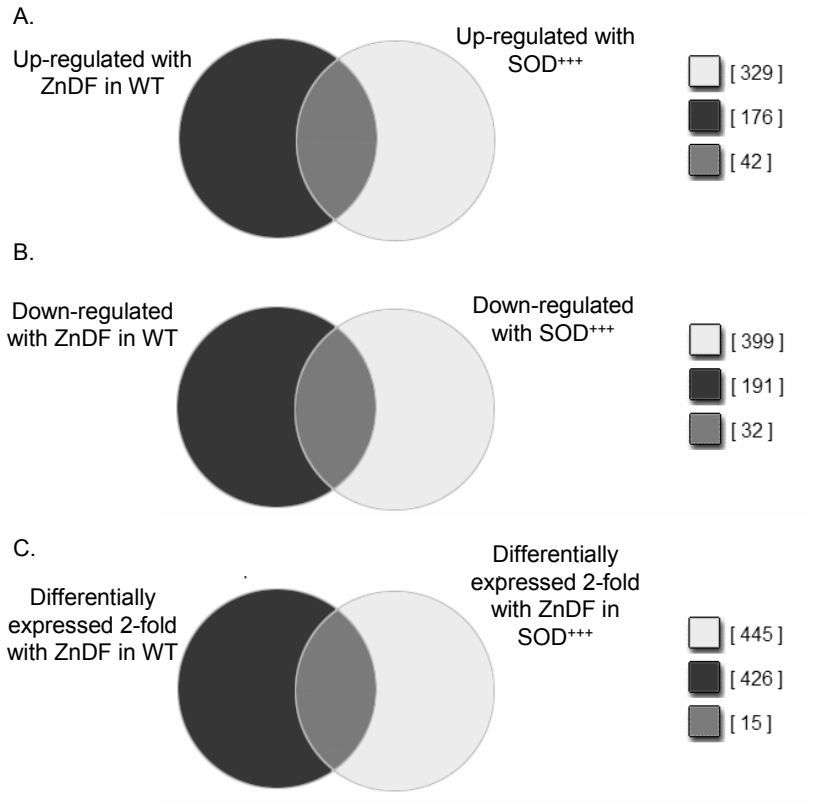

Figure 4.3. Venn diagrams showing number of genes differentially expressed in each comparison, and number of genes common between comparison groups. A-B. Comparison of genes differentially expressed (at least 2-fold) due to zinc deficiency (WT animals) and genes differentially expressed due to Cu/Zn SOD over-expression (ZnAD animals). A. shows up-regulated genes and B. shows down-regulated genes. C. Comparison of genes differentially expressed 2-fold due to dietary zinc deficiency in WT and Cu/Zn SOD over-expressing animals.

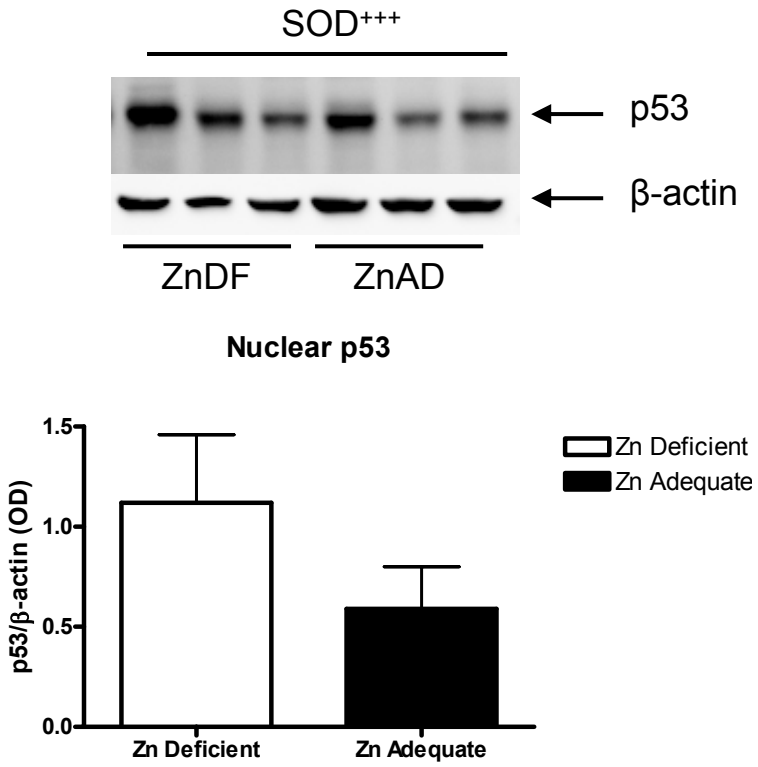

Figure 4.4. Nuclear p53 protein expression in the prostate of Cu/Zn SOD over-expressing animals. Although not statistically significant Western Blot showed a trend for increased expression of nuclear p53 with dietary zinc deficiency in Cu/Zn SOD over-expressing animals.

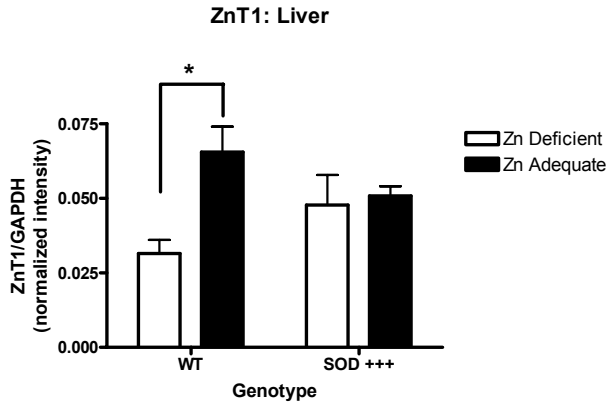

Figure 4.5. Quantitative real-time PCR analysis showed significant decrease in liver ZnT1 expression in animals fed zinc deficient (ZnDF) diet compared to animals fed zinc adequate (ZnAD) diet. This difference was only significant in WT animals. Normalized intensity represents copy number of ZnT1/copy number of GAPDH for each sample. 2-way ANOVA with Bonferroni post tests, * $p < 0.05$.

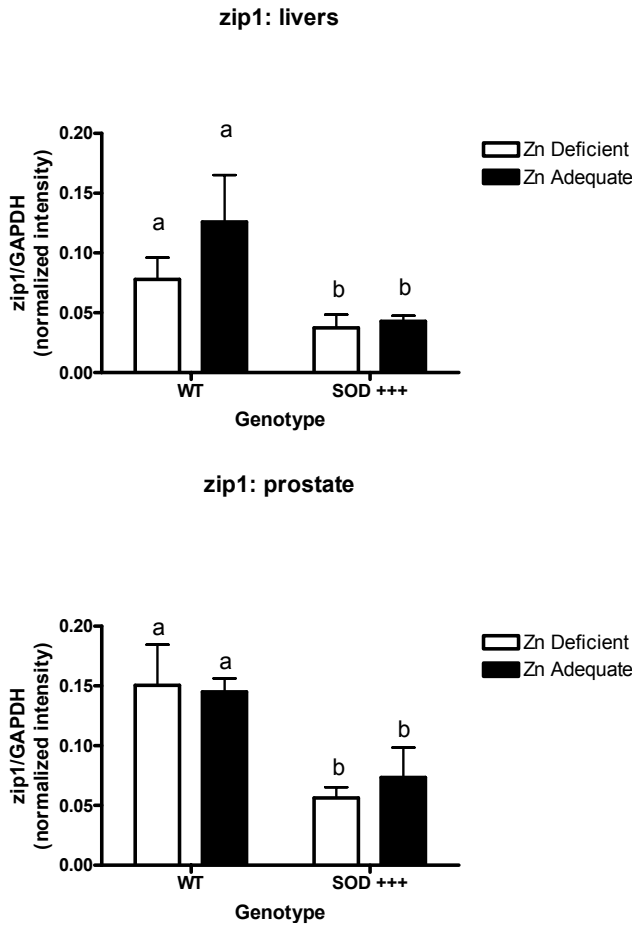

Figure 4.6. Expression of zip1 was lower in Cu/Zn SOD over-expressing animals compared to WT controls in both the prostate and liver, as determined by qPCR. Normalized intensity represents copy number of zip1/copy number of GAPDH for each sample. No significant difference with dietary treatment was observed. 2-way ANOVA with Bonferroni post test, $p < 0.05$.

Table 4.1. Composition of animal diet used for *in vivo* dietary zinc deficiency study. Data are expressed as grams per kilogram. The salt mix was prepared without zinc. The biotin/sucrose premix measured 1 mg of biotin/g. The zinc carbonate premix measured 5 mg of zinc/g.

Diet composition		
	Zinc-adequate diet	Zinc-deficient diet
Egg whites	200	200
Cornstarch	381.364	373.986
Dyetrose (maltodextrin)	127.122	124.5
Dextrose	100	120
Soybean oil	70	70
t-Butylhydroquinone	0.014	0.014
Cellulose	50	50
Salt mix no. 215264	35	35
Vitamin mix no. 310025	10	10
Lab premix no. 410750	10	10
Biotin	4	4
Choline bitartrate	2.5	2.5
Zinc carbonate premix	10	0

Table 4.2. Primer sequences used for qPCR analysis for *in vivo* dietary zinc deficiency study.

Gene	Acession #		Primer Sequence
GAPDH	NM_008084	fwd	TCCCACTCTTCCACCTTCGA
		rev	AGTTGGGATAGGGCCTCTCTTG
Zip-1 (Slc39a1)	NM_013901	fwd	GGCCATGGGTTTCTTCCTGG
		rev	GCAGCAGAGCTAGGCACAGC
Zip-3 (Slc39a3)	NM_134135	fwd	GCGTATTCCTGGCTACATGC
		rev	TGAAGGTCTCCAGGTCTATAAAGG
Zip-6 (Slc39a6)	NM_139143	fwd	AAGTGAGAAGAAGGCAGAAATCC
		rev	GGAGAAGATGTAACAGAGCATCG
Znt-1 (Slc30a1)	NM_009579	fwd	TGGATGTACAAGTAAATGGGAATCT
		rev	GTCTTCAGTACAACCCTTCCAGTTA
Znt-4 (Slc30a4)	NM_011774	fwd	TAGGTGGATACATGGCAAATAGC
		rev	AGTTCATATGGATGGTTCTCTGC
Znt-6 (Slc30a6)	NM_144798	fwd	TTAGAAGTCCTGGCTGTATTTGC
		rev	GAATAGAAAGCATCGTGAACAGG
Znt-7 (Slc30a7)	NM_023214	fwd	CGCTTTCTCTTATGGGTATGTTAGA
		rev	TCTCTCGACTCCTTCTGAGAAAATA
interleukin enhancer binding factor 3	NM_010561	fwd	GGAACAGAAGGCGGAACACATGACTA
		rev	GTTACAGTAGTGAGCTGGATG
Mdm4 transformed mouse 3T3 cell double minute 4	NM_008575	fwd	GATCCAAGCCCTCTCTATGACATG
		rev	AGTATTCTGTTGCACCGTGCTTCAGTCG
ataxin 3	NM_029705	fwd	AGAGCAGAGTGCCCTCAAAGCAGA
		rev	CTACCTTGCATACTGAGCTGAATGGC
Rho GTPase activating protein 25	NM_001037727	fwd	AGAGGGACTCCTCAGATCCAAAGA
		rev	GGCATCAGGACTACTTGCTGTGTTAC
metallothionein 1	NM_013602	fwd	CTTCTCCTCACTTACTCCGTAGCTCCA
		rev	TGTTCGTCACATCAGGCACAGC

Table 4.2 (Continued)

Gene	Acession #		Primer Sequence
ninjurin 1	NM_013610	fwd	GCTGCTCATCTTCCTGGTCAAGTATG
		rev	ACCCTTAAAGTCTCTGGGCGTTCTACTG
carboxylesterase 2	NM_145603	fwd	GTTGTTGGCATCTGAGGATTTTCACC
		rev	TGTCCCCCATGTACTCTTCCATTAGC
signal transducing adaptor molecule 2	NM_019667	fwd	ACAGAGGTTGAGACAGCAACG
		rev	CATCTTCCAAGTCCAAGAGGTC

Table 4.3. Common genes differentially expressed in both WT and Cu/Zn SOD over-expressing animals.

Gene Identifier	Gene title
AF230802	Transient receptor potential cation channel, subfamily C, member 2
AI428845	Transcribed locus
AK008641	Gastrokine 1
AK014050	RAB3C, member RAS oncogene family
AK015112	RIKEN cDNA 4930408K08 gene
AV283456	Four and a half LIM domains 4
BB100478	ESTs
BB177678	ESTs
BB277125	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 2
BB319311	ESTs
BE983452	Zinc finger and BTB domain containing 2
BG067016	Transcribed locus
BG069414	Transcribed locus, weakly similar to XP_574432.1 similar to LRRGT00194 [Rattus norvegicus]
BM243959	Rap guanine nucleotide exchange factor (GEF) 6
NM_013693	Tumor necrosis factor

Table 4.4. Biological process ontology terms which were significantly altered in WT animals. The gene list of significantly differentially expressed genes in the prostate of WT animals fed ZnAD diet compared to those feed ZnDF diet.

Ontology[1]	List[2]	Array[3]	z-score[4]
▪ cellular macromolecule metabolic process	47	2535	2.11
▪ cellular protein metabolic process	47	2494	2.24
▪ biopolymer modification	32	1458	2.69
▪ protein modification process	32	1404	2.92
▪ post-translational protein modification	27	1221	2.49
▪ phosphate metabolic process	19	757	2.64
▪ phosphorus metabolic process	19	757	2.64
▪ phosphorylation	17	636	2.76
▪ protein amino acid phosphorylation	15	573	2.5
▪ positive regulation of metabolic process	10	357	2.25
▪ cell-cell signaling	9	323	2.11
▪ positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	8	285	2.01
▪ positive regulation of transcription	8	280	2.07
▪ secretion	8	242	2.52
▪ positive regulation of transcription, DNA-dependent	8	237	2.58
▪ secretion by cell	8	194	3.22
▪ transmission of nerve impulse	7	228	2.14
▪ synaptic transmission	7	195	2.59
▪ secretory pathway	7	164	3.11
▪ regulation of biosynthetic process	5	149	2.02
▪ monosaccharide metabolic process	5	130	2.36
▪ hexose metabolic process	5	127	2.42
▪ exocytosis	5	84	3.53
▪ regulation of transport	5	79	3.71
▪ regulated secretory pathway	5	64	4.34

[1.] Biological process gene ontology term. [2.] Genes that were differentially expressed within each ontology term on the gene list. [3.] Total genes within the gene ontology term represented in the Affymetrix array. [4.] A z-score of >2 signifies a significant portion of genes were differentially expressed for that specific ontology term due to dietary zinc deficiency.

Table 4.5. Specific genes involved in Wnt signaling and prostate cancer pathways which were differentially expressed in the prostate of WT animals fed ZnAD diet compared to those feed ZnDF diet.

Wnt signaling pathway:

Gene Identifier	Gene Title
BM114349	Adenomatosis polyposis coli 2
BG487404	Transcription factor 7-like 2, T-cell specific, HMG-box
BB084315	Protein kinase C, beta 1
BB475090	CREB binding protein
BG076035	Protein phosphatase 3, catalytic subunit, beta isoform
NM_010703	Lymphoid enhancer binding factor 1
BE947490	Mitogen activated protein kinase 10

Prostate cancer:

Gene Identifier	Gene Title
BG487404	Transcription factor 7-like 2, T-cell specific, HMG-box
BB475090	CREB binding protein
NM_010703	Lymphoid enhancer binding factor 1
AK016582	Heat shock protein 90kDa alpha (cytosolic), class A member 1

Table 4.6. KEGG pathways for which a significant portion of genes were differentially expressed due to dietary zinc deficiency in WT animals. The gene list contained significantly differentially expressed genes in the prostate of WT animals fed ZnAD diet compared to those fed ZnDF diet.

KEGG Pathway	List[1]	Array[2]	z-score[3]
▪ Wnt signaling pathway	7	142	3.63
▪ Renin-angiotensin system	2	18	3.5
▪ Melanogenesis	5	97	3.17
▪ Type II diabetes mellitus	3	44	3.06
▪ Fc epsilon RI signaling pathway	4	76	2.88
▪ Endometrial cancer	3	52	2.69
▪ Taurine and hypotaurine metabolism	1	8	2.66
▪ Colorectal cancer	4	84	2.64
▪ Basal cell carcinoma	3	55	2.56
▪ Prostate cancer	4	88	2.53
▪ Thyroid cancer	2	29	2.52
▪ Glycosphingolipid biosynthesis - lactoseries	1	9	2.47
▪ Calcium signaling pathway	6	166	2.47
▪ Neurodegenerative Disorders	2	34	2.22
▪ Long-term potentiation	3	65	2.21
▪ Phosphatidylinositol signaling system	3	70	2.06

1. Genes that were differentially expressed within each pathway term on the gene list.
2. Total genes within the pathway term represented in the Affymetrix array. 3. A z-score of >2 means the number of genes differentially expressed for that specific term are over-represented.

Table 4.7. A subset of the differentially expressed genes classified by their role in apoptosis, cell cycle, transcription and zinc ion binding. These genes were differentially expressed at least 2-fold in the prostates of WT animals fed ZnAD diet compared to those fed ZnDF diet.

Ratio	Direction	Gene Identifier	Gene Name
Apoptosis:			
5.12	Up	BG082186	Expressed sequence AU024076
2.53	Up	NM_013693	Tumor necrosis factor
2.15	Up	AK013411	BCL2-like 12 (proline rich)
2.18	Down	AK020730	RIKEN cDNA A330102K23 gene
2.3	Down	AF229434	Tumor necrosis factor receptor superfamily, member 18
3.53	Down	BE197100	Eukaryotic translation elongation factor 1 alpha 2
3.95	Down	AB046693	RAB27A, member RAS oncogene family
5.32	Down	BB539404	Glutamate receptor, metabotropic 7

Ratio	Direction	Gene Identifier	Gene Name
Cell cycle:			
4.72	Up	AB049732	RIKEN cDNA B230120H23 gene
3.45	Up	BB203873	NIMA (never in mitosis gene a)-related expressed kinase 2
3.41	Up	U15443	Ros1 proto-oncogene
3.31	Up	NM_008552	MAS1 oncogene
2.6	Up	AK005799	RIKEN cDNA 1700009N14 gene
2.4	Up	AF059177	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)
2.01	Down	NM_011524	transforming, acidic coiled-coil containing protein 3
3.72	Down	BC004043	DnaJ (Hsp40) homolog, subfamily C, member 2

Ratio	Direction	Gene Identifier	Gene Name
DNA Repair:			
2.54	Up	AF230802	Transient receptor potential cation channel, subfamily C, member 2

Table 4.7 (Continued)

Ratio	Direction	Gene Identifier	Gene Name
Transcription:			
4.55	Up	BB259151	Centrosomal protein 290
3.93	Up	NM_013922	Zinc finger protein 354C
3.9	Up	BG487404	Transcription factor 7-like 2, T-cell specific, HMG-box
3.75	Up	NM_010812	forkhead box K1
3.66	Up	U76759	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein
3.65	Up	BC018510	Activating transcription factor 7 interacting protein 2
3.03	Up	AA275278	PHD finger protein 6
3.02	Up	BB475090	CREB binding protein
2.81	Up	AV345303	SRY-box containing gene 8
2.78	Up	AK020261	Pancreatic and duodenal homeobox 1
2.53	Up	NM_013693	Tumor necrosis factor
2.27	Up	BB559399	Metadherin
2.23	Up	BG229265	Kruppel-like factor 12
2.2	Up	NM_010703	Lymphoid enhancer binding factor 1
2.04	Down	AI562059	mediator complex subunit 13-like
2.18	Down	BQ180054	Zinc finger and BTB domain containing 43
2.39	Down	BB478668	Zinc finger and BTB domain containing 46
2.82	Down	NM_010636	Kruppel-like factor 12
3.05	Down	NM_010561	Interleukin enhancer binding factor 3
3.35	Down	BB238025	ZXD family zinc finger C
3.5	Down	U79738	Distal-less homeobox 3
3.56	Down	BB428001	MYST histone acetyltransferase monocytic leukemia 4
3.62	Down	AI647473	Ataxin 3
3.72	Down	BC004043	DnaJ (Hsp40) homolog, subfamily C, member 2
4.77	Down	NM_133256	GS homeobox 2

Ratio	Direction	Gene Identifier	Gene Name
Zinc Ion Binding:			
4.16	Up	BB310805	CDNA sequence BC066028

Table 4.7 (Continued)

Ratio	Direction	Gene Identifier	Gene Name
3.93	Up	NM_013922	Zinc finger protein 354C
3.45	Up	BB084315	Protein kinase C, beta 1
3.38	Up	BB474297	Membrane-associated ring finger (C3HC4) 7
3.03	Up	AA275278	PHD finger protein 6
3.02	Up	BB475090	CREB binding protein
2.83	Up	BG068505	Sciellin
2.52	Up	AF250293	Parkin
2.26	Up	BG076035	Protein phosphatase 3, catalytic subunit, beta isoform
2.23	Up	BG229265	Kruppel-like factor 12
2.18	Up	NM_138954	Ret finger protein-like 4
2.12	Up	BE305862	SH3 domain containing ring finger 1
2.12	Down	AB053181	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2
2.18	Down	BQ180054	Zinc finger and BTB domain containing 43
2.29	Down	C85810	Transformed mouse 3T3 cell double minute 4
2.3	Down	BE985117	TRAF type zinc finger domain containing 1
2.38	Down	BE983452	Zinc finger and BTB domain containing 2
2.38	Down	AV283456	Four and a half LIM domains 4
2.39	Down	BB478668	Zinc finger and BTB domain containing 46
2.42	Down	BB083438	Tripartite motif protein 9
2.68	Down	BQ031472	PHD finger protein 2
2.71	Down	AV238106	RIKEN cDNA 6530401C20 gene
2.82	Down	NM_010636	Kruppel-like factor 12
3.27	Down	AF439513	Pregnancy-associated plasma protein A
3.35	Down	BB238025	ZXD family zinc finger C
3.54	Down	BB054699	Zinc finger, DHHC domain containing 15
3.56	Down	BB428001	MYST histone acetyltransferase monocytic leukemia 4
4.27	Down	BB245038	TRH-degrading enzyme
4.38	Down	NM_007608	Carbonic anhydrase 5a, mitochondrial

Table 4.8. Quantitative real-time PCR confirmation of genes found to be differentially expressed by microarray analysis.

Gene Name	Gene	Fold Change	SEM	Microarray
Rho GTPase activating protein 25	Arhgap	1.38	0.2698	2.79
Ataxin3	Atxn	-1.32	0.1624	-3.62
Carboxylesterase 2	Ces2	1.61	0.5875	5.28
Interleukin enhancer binding factor 3	IEBF	-1.29	0.1735	-3.05
transformed mouse 3T3 cell double minute 4	Mdm4	-1.26	0.1756	-2.29
Ninjurin 1	Ninj1	-1.45	0.0868	-2.81
signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	STAM	1.30	0.2935	4.57
metallothionein 1	MT1	1.70	0.2885	< 1.5

Chapter 5

General Conclusions

Diet and lifestyle represent modifiable risk factors which have been implicated in the development of several chronic diseases such as atherosclerosis, diabetes, and cancer. In particular the role of diet in the prevention and management of chronic disease has been of keen interest. The contribution of these modifiable risk factors, such as diet, is the focus of many cancer prevention strategies. Factors such as caloric balance, activity level and nutritional deficiencies are some examples of diet and lifestyle factors which may contribute to cancer risk. Nutritional deficiencies have been proposed to increase cancer risk due to their function in crucial processes that protect the cell from damage, especially to DNA. Thus, the focus of this dissertation was to investigate the impact of zinc, an essential mineral, on DNA integrity and the development of prostate cancer.

Zinc is an essential mineral that has catalytic, structural and regulatory roles in the human body. Among the many functions of zinc, its role in enzymes and transcription factors are of particular interest for examining the association between zinc and cancer risk. Because prostate cancer cells lose cellular zinc, the relationship between zinc and prostate cancer development have been of particular interest.

Epidemiological studies have suggested that zinc intake may be inversely correlated with prostate cancer and benign prostate hyperplasia (BPH) risk. However, limited studies have examined the mechanisms by which zinc may protect against these prostatic diseases. Therefore, the goals of this dissertation were to further explore the role of zinc in the prostate by using two approaches. First, we examined the potential of zinc treatment to impair cell proliferation and induce apoptosis in prostate cancer and benign prostate hyperplasia cells. Second, we used *in vitro* and *in vivo* models to investigate molecular changes in the prostate in response to zinc deficiency. We hypothesized that zinc deficiency increases the susceptibility to

prostate cancer by increasing DNA damage and impairing DNA damage response mechanisms. In prostate cancer cells, which have lost cellular zinc, strategies to increase cellular zinc would alter the regulation of cellular proliferation and induce apoptosis.

Based on these studies we have made the following conclusions:

Zinc has anti-proliferative effects in BPH and prostate cancer cells.

In the normal prostate, elevated zinc levels function to inhibit mitochondrial aconitase, leading to accumulation of citrate and decreased ATP production. It has been hypothesized that the increase in ATP production, as a result of decreased zinc levels, creates favorable conditions for increased cell proliferation and cancer development (132). Thus, restoring zinc levels, through zinc treatment, may inhibit ATP production and re-establish conditions which limit cell proliferation and cancer development. BPH is another common disease of the prostate which is characterized by uncontrolled proliferation, thus we also examined how these cells responded to zinc treatment. We hypothesized that zinc treatment in prostate cancer and benign hyperplasia cells would impair growth and viability. We found that prostate cancer cells (PC-3) and benign prostate hyperplasia cells (BPH-1) had different responses to zinc treatment. Although zinc had anti-proliferative effects in both cell types, BPH-1 cells were more sensitive to zinc than PC-3 cells. Additionally, the mechanisms by which zinc exerted its anti-proliferative effects on these two cell types appeared to be slightly different. Specifically, zinc increased Bcl-2:BAX ratio in BPH-1 cells, but decreased Bcl-2:BAX in PC-3 cells. Additionally, zinc decreased both NFκB (p65) nuclear protein expression and binding activity in BPH-1 cells, but only decreased NFκB (p65) binding activity in PC-3 cells.

The mechanisms by which zinc inhibits proliferation and induces apoptosis in prostate cancer cells has been previously reported to be mediated by NFκB, BAX and Bcl-2 (104, 136, 138). Constitutive activation of NFκB and over-expression of Bcl-2 have been observed in prostate cancer (102, 103, 176). These studies showed that the

response of NFκB and Bcl-2 were different in BPH and PC-3 cells even though zinc had anti-proliferative effects in both cell types. It is possible that zinc targets other members of the NFκB signaling pathway (such as p50, IκBα, or IκB kinase) or alters post-translational modifications to Bcl-2 affecting apoptosis. Future studies examining these factors would help elucidate why the response to zinc is different between these two cells types.

Zinc deficiency alters gene expression, increases DNA damage and impairs p53 signaling in the normal prostate.

Using an *in vitro* cultured cell model of normal human prostate epithelial cells (PrEC), we found zinc deficiency induced DNA damage, as evidenced by increased Comet score. These findings were in line with previous reports of zinc deficiency induced increases in oxidative stress and oxidative DNA damage. Zinc deficiency was also associated with differential expression of genes involved in a range of biological functions. These results support the ubiquitous role of zinc in the human body. In particular, genes involved in cell cycle regulation, apoptosis, DNA damage response, DNA repair and transcription were differentially expressed. Importantly, gene and nuclear protein expression of the zinc-dependent tumor suppressor protein, p53, was up-regulated with zinc deficiency. However, we found no change in p53 binding activity, as well as no change in the expression of downstream p53 targets. These findings strongly suggest that zinc deficiency impairs the function of critical zinc dependent transcription factors which are responsible for mediating the DNA damage response. Moreover, the studies reinforce the concept that the combination of increased DNA damage and impaired DNA damage response mechanisms increase potential for increased mutations and risk for prostate cancer development.

To further examine molecular changes that occur with zinc deficiency in the normal prostate, we conducted an *in vivo* study using a mouse model of dietary zinc deficiency. In addition, we also explored the response to dietary zinc deficiency in mice which over-expressed the zinc dependent antioxidant enzyme Cu/Zn SOD.

Surprisingly, dietary zinc deficiency decreased prostate zinc levels in WT but not Cu/Zn SOD over-expressing mice, suggesting that over-expression of Cu/Zn SOD may be protective against loss of zinc during dietary zinc deficiency. Differences in the expression of zinc transporters may be one explanation for this observation. Expression of the zinc transporters zip1 and zip3 were down-regulated in Cu/Zn SOD over-expressing animals compared to WT. The zip family of zinc transporters function to transport zinc into the cell, or out from intracellular organelles into the cytosol (71). The mechanisms behind the down-regulation of zinc transporters and how this would protect against the loss of cellular zinc is unclear. Studies have shown that post-translational modifications to zinc transporters, in response to zinc, play an important role in protein stability and localization (160, 161). These post-translational modifications could be more important than gene expression in terms of protein activity, and would be an important area of future investigation.

Patterns of global gene expression changes were also different between the two genotypes with zinc deficiency. Although in all analysis, differential expression of genes involved in a wide variety of biological functions was observed, specific genes which were differentially expressed were quite different between genotypes. Few of the same genes were differentially expressed in both WT and Cu/Zn SOD over-expressing animals. This observation was not surprising since only WT animals had decreased prostate zinc. Unlike the *in vitro* findings, microarray analysis did not show altered expression of p53 in WT animals. However, genes involved in cell cycle regulation, apoptosis and transcription were also differentially expressed. Additionally, a significant portion of genes involved in Wnt signaling and prostate cancer pathways were altered with zinc deficiency in WT animals, indicating that zinc deficiency may alter signaling pathways involved in cancer development.

Significance and direction for future research.

These studies highlighted the importance of zinc in the maintenance of prostate health. Zinc is a ubiquitous mineral which serves multiple functions in the human

body. For example, zinc plays structural roles in DNA and RNA stability and enzymatic roles in proteins involved in DNA and RNA synthesis. Numerous transcription factors, including hormone receptors, which regulate cellular proliferation and differentiation, require zinc (61, 221). It is clear that zinc is essential in a wide range of proteins involved in maintaining proper cellular function.

Our studies indicate that zinc deficiency can impair the function of critical proteins involved in the DNA damage response. The loss of the ability to repair the damaged DNA or induce apoptosis in cells containing DNA damage can result in the accumulation of DNA mutations, which is the first step in cancer initiation. Zinc may also affect activity of transcription factors which regulate expression of genes involved in cellular proliferation, apoptosis, and angiogenesis. Dysregulation of these transcription factors could promote cancer cell growth and contribute to metastasis. Future studies examining other zinc-regulated transcription factors known to be dysregulated during prostate cancer, such as NFκB and the androgen receptor, would help further define the role of zinc in protecting against prostate carcinogenesis.

Mechanisms responsible for regulating cellular zinc concentrations and its distribution to organs and organelles are still poorly defined. For example, the mechanisms responsible for zinc accumulation in the prostate epithelial cells, and subsequent loss during malignancy remain unclear. Additionally, the response of zinc transporters to changes in zinc status is still being elucidated in many tissues, including the prostate. However, recent reports indicate that cellular localization, activity and stabilization of these transport proteins in response to zinc may provide answers to these critical questions. These data may also identify patterns of expression and activity associated with prostate cancer. The determination of similar patterns with zinc deficiency may also further our understanding of the role of zinc in the prostate.

Overall, these studies support previous reports indicating an essential role for zinc in the prostate. Furthermore, these studies suggest that the importance of proper zinc status is likely two-fold. Loss of zinc in normal prostate cells appears to increase

DNA damage and impair DNA damage response mechanisms. In addition, low zinc levels in prostate cancer may partly contribute to uncontrolled proliferation and impaired apoptosis. Together, these data indicate that zinc may have cancer prevention properties that are involved in all stages of cancer development; initiation, promotion and progression.

The efficacy of dietary agents in cancer prevention strategies is still being researched. Due to complex modes of action that involve multiple mechanisms, detailing how dietary compounds modify cancer risk continues to be a challenge. Cancer remains a leading cause of mortality in Americans. Therefore, prevention strategies may provide key avenues by which to modulate cancer risk and development.

In conclusion, this dissertation supports a protective role for zinc in the prostate, and identifies mechanisms by which zinc exerts these effects. Although zinc has widespread distribution in the human body, the unique relationship between zinc and the prostate may indicate that the prostate is especially sensitive to changes in zinc status. Importantly, these studies suggest that low zinc levels may be unfavorable in both healthy and malignant prostate cells, and that proper zinc status is crucial for prostate health.

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APPENDIX

Table 1: Genes significantly down-regulated in PrEC cells treated with ZnDF versus ZnAD media for 7 d.

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-2	NM_005538	Inhibin, beta C	3626	INHBC
-2.01	W87901	Small nuclear ribonucleoprotein polypeptide E	6635	SNRPE
-2.02	NM_004166	Chemokine (C-C motif) ligand 15	6359	CCL15
-2.03	NM_013954	NADPH oxidase 1	27035	NOX1
-2.04	NM_017409	Homeobox C10	3226	HOXC10
-2.05	BC005241	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	4361	MRE11A
-2.07	NM_000257	Myosin, heavy chain 7, cardiac muscle, beta	4625	MYH7
-2.08	AF132811	Immunoglobulin superfamily, member 4	23705	IGSF4
-2.08	NM_005666	Complement factor H-related 2	3080	CFHR2
-2.08	AW163148	Myristoylated alanine-rich protein kinase C substrate	4082	MARCKS
-2.08	NM_000749	Golgi autoantigen, golgin subfamily a, 7	51125	GOLGA7
-2.09	AF015124	Mitogen-activated protein kinase-activated protein kinase 5	8550	MAPKAPK5
-2.13	NM_024947	Polyhomeotic homolog 3 (Drosophila)	80012	PHC3
-2.13	AK024177	Homo sapiens, clone IMAGE:3851018, mRNA	-	-
-2.15	AI345238	tb81b07.x1 NCI_CGAP_Lu26 Homo sapiens cDNA clone IMAGE:2060725 3 similar to gb:M10119 FERRITIN LIGHT CHAIN (HUMAN);, mRNA sequence.	-	-
-2.16	NM_016511	C-type lectin domain family 1, member A	51267	CLEC1A
-2.17	NM_002108	Histidine ammonia-lyase	3034	HAL
-2.18	NM_006914	RAR-related orphan receptor B	6096	RORB
-2.18	AL136823	Bromodomain containing 8	10902	BRD8
-2.19	NM_006581	Fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	10690	FUT9

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-2.21	AF061192	Ectodysplasin A	1896	EDA
-2.21	NM_006394	Regulated in glioma	-	-
-2.23	NM_007101	Sarcosine dehydrogenase	1757	SARDH
-2.24	BC005354	Tetraspanin 4	7106	TSPAN4
-2.26	AY029208	Collagen, type VI, alpha 2	1292	COL6A2
-2.26	BC002635	Colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	1438	CSF2RA
-2.27	AF113008	Ribosomal protein S20	-	-
-2.27	BC005319	Glycophorin A (MNS blood group)	2993	GYPA
-2.28	NM_016831	Period homolog 3 (Drosophila)	8863	PER3
-2.29	NM_000280	Paired box gene 6 (aniridia, keratitis)	5080	PAX6
-2.31	AA102667	zn73e05.s1 Stratagene NT2 neuronal precursor 937230 Homo sapiens cDNA clone IMAGE:563840 3 similar to contains Alu repetitive element;contains element MSR1 repetitive element ;, mRNA sequence.	-	-
-2.32	NM_000573	Complement component (3b/4b) receptor 1 (Knops blood group)	1378	CR1
-2.32	AK021640	Serologically defined colon cancer antigen 8	10806	SDCCAG8
-2.33	AJ275469	Homo sapiens partial IGVH3 gene for immunoglobulin heavy chain V region, case 2, cell E 172.	-	-
-2.35	NM_005039	Proline-rich protein BstNI subfamily 1	5542	PRB1
-2.36	NM_017805	Ras interacting protein 1	54922	RASIP1
-2.36	NM_005427	Tumor protein p73	7161	TP73
-2.38	NM_004646	Nephrosis 1, congenital, Finnish type (nephrin)	4868	NPHS1
-2.39	BC001161	Zinc finger protein 174	7727	ZNF174
-2.41	BC000603	Ribosomal protein L38	6169	RPL38

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-2.41	H71805	Myeloid cell leukemia sequence 1 (BCL2-related)	4170	MCL1
-2.43	AW188214	Myosin, heavy chain 14	79784	MYH14
-2.43	AI221950	Leucine rich repeat neuronal 3	54674	LRRN3
-2.44	S67788	Adenomatosis polyposis coli	324	APC
-2.44	AL031778	SEE ALSO	-	-
-2.45	NM_002234	Potassium voltage-gated channel, shaker-related subfamily, member 5	3741	KCNA5
-2.48	AA417256	DKFZP564C196 protein	284649	DKFZP564C196
-2.51	AB017445	X-ray repair complementing defective repair in Chinese hamster cells 4	7518	XRCC4
-2.53	NM_016566	Pparl	-	-
-2.53	NM_000873	Intercellular adhesion molecule 2	3384	ICAM2
-2.58	NM_016519	Ameloblastin (enamel matrix protein)	258	AMBN
-2.6	AA320764	Ribosomal protein S10	6204	RPS10
-2.6	U09609	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	4791	NFKB2
-2.6	BC006441	RNA polymerase I transcription factor RRN3-like	94431	LOC94431
-2.61	NM_001939	Dystrophin related protein 2	1821	DRP2
-2.65	NM_005253	FOS-like antigen 2	2355	FOSL2
-2.66	AF070588	Hypothetical protein LOC55565	55565	LOC55565
-2.66	BE877796	Collagen, type VIII, alpha 1	1295	COL8A1
-2.7	NM_022436	ATP-binding cassette, sub-family G (WHITE), member 5 (sterolin 1)	64240	ABCG5
-2.71	NM_024749	Vasohibin 2	79805	VASH2
-2.74	AW134812	Transcribed locus, strongly similar to XP_508943.1 similar to polypeptide N-acetylgalactosaminyltransferase 8; protein-UDP a	-	-
-2.74	U34249	Tripartite motif-containing 15	89870	TRIM15

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-2.75	NM_017616	ankyrin repeat domain 25	25959	-
-2.77	NM_004188	Growth factor independent 1B (potential regulator of CDKN1A, translocated in CML)	8328	GFI1B
-2.78	AL117663	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	6929	TCF3
-2.78	AI005043	WAS/WASL interacting protein family, member 1	7456	WIPF1
-2.78	NM_006498	Lectin, galactoside-binding, soluble, 2 (galectin 2)	3957	LGALS2
-2.83	AW303136	Ribosomal protein L38	6169	RPL38
-2.83	NM_024802	Hypothetical protein FLJ21369	-	-
-2.86	AA669336	Coagulation factor C homolog, cochlin (Limulus polyphemus)	1690	COCH
-2.9	AI435828	Stanniocalcin 2	8614	STC2
-2.92	U66584	Crystallin, alpha A	1409	CRYAA
-2.93	AK001727	WW and C2 domain containing 1	23286	WWC1
-2.94	NM_002962	S100 calcium binding protein A5	6276	S100A5
-2.96	AF262973	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	3811	KIR3DL1
-3.03	NM_003519	Histone cluster 1, H2bl	8340	HIST1H2BL
-3.04	U31216	Glutamate receptor, metabotropic 1	2911	GRM1
-3.06	NM_022159	EGF-TM7-latrophilin-related protein	-	-
-3.12	AK025174	Gasdermin-like	55876	GSDML
-3.12	AI126492	Chromosome 4 open reading frame 9	8602	C4orf9
-3.15	AW408194	Immunoglobulin kappa variable 1D-13	28902	IGKV1D-13
-3.17	NM_030975	Keratin associated protein 9.9	-	-
-3.17	BC005924	Pregnancy specific beta-1-glycoprotein 1	5669	PSG1
-3.18	X60502	Sialophorin (leukosialin, CD43)	6693	SPN

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-3.29	NM_006198	Purkinje cell protein 4	5121	PCP4
-3.45	NM_004072	Chemokine-like receptor 1	1240	CMKLR1
-3.46	N25429	High-mobility group 20B	10362	HMG20B
-3.49	NM_005256	Fanconi anemia, complementation group F	2188	FANCF
-3.5	NM_018196	Trimethyllysine hydroxylase, epsilon	55217	TMLHE
-3.51	AL136572	Zinc finger, MIZ-type containing 2	83637	ZMIZ2
-3.51	AL080180	3-hydroxyisobutyrate dehydrogenase	11112	HIBADH
-3.54	NM_000703	ATPase, Na+/K+ transporting, alpha 3 polypeptide	478	-
-3.55	AL049925	Pygopus homolog 1 (Drosophila)	26108	PYGO1
-3.62	D00943	Myosin, heavy chain 7, cardiac muscle, beta	4625	MYH7
-3.63	U18549	G protein-coupled receptor 6	2830	-
-3.63	NM_024111	ChaC, cation transport regulator homolog 1 (E. coli)	79094	CHAC1
-3.68	NM_000587	Complement component 7	730	C7
-3.73	D78132	Ras homolog enriched in brain	6009	RHEB
-3.77	BE962186	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	6929	TCF3
-3.79	AI950380	B-cell CLL/lymphoma 7A	605	BCL7A
-3.81	AB016898	Similar to myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	653483	LOC653483
-3.9	N92920	Chromosome 1 open reading frame 77	26097	C1orf77
-3.91	D60132	Glutamate receptor, metabotropic 5	2915	GRM5
-3.93	AB023144	Seizure related 6 homolog (mouse)-like	23544	SEZ6L
-3.99	NM_001739	Carbonic anhydrase VA, mitochondrial	763	CA5A

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-3.99	BE646396	Ribosome binding protein 1 homolog 180kDa (dog)	6238	RRBP1
-4.02	NM_000638	Vitronectin	7448	VTN
-4.05	AI191118	Peptidylprolyl isomerase A (cyclophilin A)	5478	PPIA
-4.07	AI200443	Melanoma antigen family A, 10	4109	MAGEA10
-4.11	NM_022063	Chromosome 10 open reading frame 84	63877	C10orf84
-4.21	NM_022783	DEP domain containing 6	64798	DEPDC6
-4.24	NM_020482	Four and a half LIM domains 5	9457	FHL5
-4.24	NM_001048	Somatostatin	6750	SST
-4.3	NM_021208	chromosome 9 open reading frame 27	58483	-
-4.32	NM_019098	Cyclic nucleotide gated channel beta 3	54714	CNGB3
-4.36	D88214	Myocilin, trabecular meshwork inducible glucocorticoid response	4653	MYOC
-4.37	AF113018	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	1656	DDX6
-4.38	NM_002309	Leukemia inhibitory factor (cholinergic differentiation factor)	3976	LIF
-4.45	U08092	Histamine N-methyltransferase	3176	HNMT
-4.47	NM_000059	Breast cancer 2, early onset	675	BRCA2
-4.58	L38424		-	-
-4.67	NM_014421	Dickkopf homolog 2 (Xenopus laevis)	27123	DKK2
-4.76	U01134	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	2321	FLT1
-4.78	NM_000590	Interleukin 9	3578	IL9
-4.91	AA521034	Hypothetical protein MGC61571	152100	MGC61571
-4.92	U11701	LIM homeobox 2	9355	-
-4.94	AK026910	Ciliary rootlet coiled-coil, rootletin	9696	CROCC

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-5.15	Z25425	NIMA (never in mitosis gene a)-related kinase 2	4751	NEK2
-5.15	NM_016046	Exosome component 1	51013	EXOSC1
-5.35	BE312027	Transcribed locus, weakly similar to XP_370196.1 protein MG06693.4 [Magnaporthe grisea 70-15]	-	-
-5.37	NM_016236	Alanine-glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicaciduria; serine-pyruvate aminotransferase)	-	-
-5.47	NM_002196	Insulinoma-associated 1	3642	INSM1
-5.57	AK023446	Aminoadipate-semialdehyde synthase	10157	AASS
-5.8	BC001279	Microfibrillar-associated protein 3-like	9848	MFAP3L
-5.89	BG403790	602419627F1 NIH_MGC_93 Homo sapiens cDNA clone IMAGE:4526599 5, mRNA sequence.	-	-
-5.9	U76376	Harakiri, BCL2 interacting protein (contains only BH3 domain)	8739	HRK
-5.91	J05158	Carboxypeptidase N, polypeptide 2, 83kD	1370	CPN2
-6.02	D00267	putative; Homo sapiens pseudogene for cytochrome c-like protein, clone pHGC4E1.	-	-
-6.24	BE857772	Ribosomal protein L37a	6168	RPL37A
-6.69	NM_014548	Tropomodulin 2 (neuronal)	29767	TMOD2
-6.86	BC000023	Ribosomal protein S19	6223	RPS19
-7.21	NM_004795	Klotho	9365	KL
-8.2	M69039	Complement component 1, q subcomponent binding protein	-	-
-8.81	AL049037	Syntaphilin	9751	SNPH
-10.83	X89657	Similar to a disintegrin and metalloprotease domain 3 (cyritestin)	651370	LOC651370

Table 1 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
-14.09	BC006370	Inversin	27130	INVS

1. Ratio calculated as -1/(ZnDF/ZnAD).

Table 2: Genes significantly up-regulated in PrEC cells treated with ZnDF versus ZnAD media for 7 d.

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
2	BC002327	KIAA0699 protein	-	-
2	NM_000937	Polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	5430	POLR2A
2.01	NM_002610	Pyruvate dehydrogenase kinase, isozyme 1	5163	PDK1
2.01	U18945	Cyclic nucleotide gated channel beta 1	1258	CNGB1
2.01	U63542	SCC-112 protein	23244	SCC-112
2.03	NM_000962	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	5742	PTGS1
2.03	AI813758	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	1281	COL3A1
2.04	NM_000774	Cytochrome P450, family 2, subfamily F, polypeptide 1	1572	CYP2F1
2.05	NM_013445	Glutamate decarboxylase 1 (brain, 67kDa)	2571	GAD1
2.07	NM_012198	Grancalcin, EF-hand calcium binding protein	25801	GCA
2.07	NM_002991	Chemokine (C-C motif) ligand 24	6369	CCL24
2.09	AA988241	RAB3A, member RAS oncogene family	5864	RAB3A
2.09	BF983406	Heterogeneous nuclear ribonucleoprotein H1 (H)	3187	HNRPH1
2.1	AK024677	N-acylsphingosine amidohydrolase (acid ceramidase)-like	27163	ASAHL

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
2.11	AF073745	Phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunce homolog, Drosophila)	5141	PDE4A
2.15	X72475	Immunoglobulin kappa constant	3514	IGKC
2.15	AA215854	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	3688	ITGB1
2.17	X16866	Cytochrome P450, family 2, subfamily D, polypeptide 6	1565	CYP2D6
2.18	U85978	Human chloride channel protein (p64) pseudogene, partial cds.	-	-
2.18	AF031137	Natural cytotoxicity triggering receptor 3	259197	NCR3
2.18	AF231056	AT rich interactive domain 1A (SWI-like)	8289	ARID1A
2.18	NM_017707	Development and differentiation enhancing factor-like 1	55616	DDEFL1
2.2	BE890314	601431767F1 NIH_MGC_72 Homo sapiens cDNA clone IMAGE:3916816 5, mRNA sequence.	-	-
2.21	AW237172	Jumonji domain containing 2B	23030	JMJD2B
2.21	NM_012182	Forkhead box B1	27023	FOXB1
2.23	AJ275374	in-frame rearrangement, stop codon in junction; Homo sapiens partial IGVH3 gene for immunoglobulin heavy chain V region, case 1, clone 19.	-	-

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
2.23	NM_025102	Hypothetical protein FLJ21497	-	-
2.24	AF047338	Solute carrier family 12 (potassium/chloride transporters), member 4	6560	SLC12A4
2.24	AA126789	Centrosomal protein 170kDa	9859	CEP170
2.24	NM_002290	Laminin, alpha 4	3910	LAMA4
2.25	AF038194	Cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	79577	CDC73
2.26	AF043244	Nucleolar protein 3 (apoptosis repressor with CARD domain)	8996	NOL3
2.26	U80737	Nuclear receptor coactivator 3	-	-
2.3	NM_003541	Histone cluster 1, H4i	8294	HIST1H4I
2.32	NM_003441	Zinc finger protein 141	7700	ZNF141
2.32	NM_019093	UDP glucuronosyltransferase 1 family, polypeptide A1	54658	UGT1A1
2.33	NM_000782	Cytochrome P450, family 24, subfamily A, polypeptide 1	1591	CYP24A1
2.34	U00956	SEE ALSO	-	-
2.34	NM_017414	Ubiquitin specific peptidase 18	11274	USP18
2.37	NM_002135	Nuclear receptor subfamily 4, group A, member 1	3164	NR4A1
2.4	NM_018644	Beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	27087	B3GAT1
2.42	J03208	Dihydrolipoamide branched chain transacylase E2	1629	DBT
2.43	AU158818	Transcribed locus	-	-
2.48	AI343459	Cell division cycle 25 homolog A (S. cerevisiae)	993	CDC25A

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
2.48	U28936	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	7531	YWHAE
2.48	AK024258	HBS1-like (S. cerevisiae)	10767	HBS1L
2.5	NM_000466	Peroxisome biogenesis factor 1	5189	PEX1
2.51	M76477	GM2 ganglioside activator	2760	GM2A
2.52	U94364	Glycogenin 2	8908	GYG2
2.59	NM_004951	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	1880	EBI2
2.61	NM_024687	Hypothetical protein FLJ23049	79740	FLJ23049
2.63	NM_018586	Hypothetical protein PRO1584	-	-
2.63	BE675435	Kruppel-like factor 6	1316	KLF6
2.64	NM_000672	Alcohol dehydrogenase 6 (class V)	130	ADH6
2.67	NM_003335	Ubiquitin-activating enzyme E1-like	7318	UBE1L
2.7	AF034102	Solute carrier family 29 (nucleoside transporters), member 2	3177	SLC29A2
2.7	NM_002919	Regulatory factor X, 3 (influences HLA class II expression)	5991	RFX3
2.74	NM_001609	Acyl-Coenzyme A dehydrogenase, short/branched chain	36	ACADSB
2.74	AF072814	Metal response element binding transcription factor 2	22823	MTF2
2.76	AF226990	HLA-G histocompatibility antigen, class I, G	3135	HLA-G

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
2.81	AB007923	Phosphodiesterase 4D interacting protein (myomegalin)	9659	PDE4DIP
2.83	AL049987	Small EDRK-rich factor 1A (telomeric)	8293	SERF1A
2.83	AL078636	MRNA full length insert cDNA clone EUROIMAGE 117929	-	-
2.85	NM_021651	PRO0457 protein	-	-
2.85	NM_018510	Hypothetical protein PRO1866	-	-
2.85	NM_001990	Eyes absent homolog 3 (Drosophila)	2140	EYA3
2.87	AA707199	Neurotrophic tyrosine kinase, receptor, type 2	4915	NTRK2
2.91	NM_022578	chorionic somatomammotropin hormone-like 1	1444	-
2.93	U84569	Chromosome 21 open reading frame 2	755	C21orf2
2.95	AL096842	Mitochondrial tumor suppressor 1	57509	MTUS1
2.96	AI923972	Tetratricopeptide repeat domain 22	55001	TTC22
2.96	AV686235	Mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	5648	MASP1
2.97	NM_001407	Cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila)	1951	CELSR3
3.02	NM_002149	Hippocalcin-like 1	3241	HPCAL1
3.07	NM_017975	Zwilch, kinetochore associated, homolog (Drosophila)	55055	ZWILCH
3.09	NM_003153	Signal transducer and activator of transcription 6, interleukin-4 induced	6778	STAT6

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
3.1	NM_017986	G protein-coupled receptor 172B	55065	GPR172B
3.18	AA845710	Breakpoint cluster region	613	BCR
3.25	NM_000872	5-hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase-coupled)	3363	HTR7
3.27	AB002361	KIAA0363 protein	23148	KIAA0363
3.39	AI193899	SMAD family member 6	4091	SMAD6
3.4	AW170602	Regulator of G-protein signalling 12	6002	RGS12
3.41	AB033088	Spectrin repeat containing, nuclear envelope 1	23345	SYNE1
3.44	NM_002725	Proline/arginine-rich end leucine-rich repeat protein	5549	PRELP
3.46	L08599	Cadherin 1, type 1, E-cadherin (epithelial)	999	CDH1
3.46	NM_017643	Mbt domain containing 1	54799	MBTD1
3.49	NM_014289	Calpain 6	827	CAPN6
3.5	NM_007198	Proline synthetase co-transcribed homolog (bacterial)	11212	PROSC
3.51	L37033	FK506 binding protein 8, 38kDa	23770	-
3.52	NM_005417	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	6714	SRC
3.52	H49382	Hypothetical gene supported by BC041875; BX648984	400642	LOC400642
3.62	AA457021	BCL2-associated athanogene 5	9529	BAG5
3.65	NM_024803	Tubulin, alpha-like 3	79861	TUBAL3
3.65	BE901081	601674431F1 NIH_MGC_21 Homo sapiens cDNA clone IMAGE:3957319 5, mRNA sequence.	-	-

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
3.67	AA731709	Olfactory receptor, family 2, subfamily A, member 9 pseudogene	441295	OR2A9P
3.68	U02569	Adrenergic, alpha-1A-, receptor	148	ADRA1A
3.69	AF090895	CDK5 regulatory subunit associated protein 1-like 1	54901	CDKAL1
3.72	X55503	metallothionein (MT)-like gene; H.sapiens pseudogene for metallothionein and AG/CT repetitive element.	-	-
3.72	AA218868	zr01g01.s1 Stratagene NT2 neuronal precursor 937230 Homo sapiens cDNA clone IMAGE:650256 3, mRNA sequence.	-	-
3.74	NM_000618	Insulin-like growth factor 1 (somatomedin C)	3479	IGF1
3.8	U58331	Sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	6444	SGCD
3.81	NM_018048	Mago-nashi homolog 2	55110	FLJ10292
3.83	AB028239	Homo sapiens pseudogene for necdin.	-	-
3.84	NM_001240	Cyclin T1	904	CCNT1
3.84	NM_000850	Glutathione S-transferase M4	2948	GSTM4
3.84	U97145	GDNF family receptor alpha 2	2675	GFRA2
3.86	NM_003669	inactivation escape 1	8552	-
3.86	AF130071	SEE ALSO	-	-
3.94	AK001118	Ubiquitin protein ligase E3 component n-recognin 2	23304	UBR2
4.1	NM_024884	L-2-hydroxyglutarate dehydrogenase	79944	L2HGDH
4.18	AF070670	Protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	5494	PPM1A

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
4.27	AA243659	Family with sequence similarity 49, member A	81553	FAM49A
4.28	AF022048	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	3811	KIR3DL1
4.36	NM_003969	Ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)	9040	UBE2M
4.41	AJ243936	Ring finger protein 5	6048	RNF5
4.46	NM_025120	Hypothetical protein FLJ13480	-	-
4.47	X96666	Y box binding protein 1	4904	-
4.55	NM_016134	Plasma glutamate carboxypeptidase	10404	PGCP
4.56	AL049988	Inositol 1,4,5-triphosphate receptor, type 2	3709	ITPR2
4.59	NM_005692	ATP-binding cassette, sub-family F (GCN20), member 2	10061	ABCF2
4.64	AU147166	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	5290	PIK3CA
4.66	AC002550		-	-
4.74	BE552409	FXYD domain containing ion transport regulator 5	53827	FXYD5
4.75	NM_022136	SAM domain, SH3 domain and nuclear localization signals 1	64092	SAMSN1
4.83	K03199	Tumor protein p53 (Li-Fraumeni syndrome)	7157	TP53
4.98	NM_017848	Family with sequence similarity 120C	54954	FAM120C
5.03	BF574664	Superkiller viralicidic activity 2-like 2 (S. cerevisiae)	23517	SKIV2L2
5.03	AI979087	Tetraspanin 4	7106	TSPAN4
5.08	NM_005930	CTAGE family, member 5	4253	CTAGE5
5.12	NM_015978	TNNI3 interacting kinase	51086	TNNI3K

Table 2 (Continued)

Ratio[1]	Gene Identifier	Gene Name	LocusLink	Gene ID
5.23	AW589975	Sin3A-associated protein, 30kDa	8819	SAP30
5.32	NM_000780	Cytochrome P450, family 7, subfamily A, polypeptide 1	1581	CYP7A1
6.26	AI825798	Rap2-binding protein 9	154661	RPIB9
6.34	NM_001829	Chloride channel 3	1182	CLCN3
6.44	NM_022444	Solute carrier family 13 (sodium/sulfate symporters), member 1	6561	SLC13A1
6.78	U79301	Hypothetical protein LOC728759	728759	LOC728759
7.35	X79990	Runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	862	RUNX1T1
8.02	AF196478	Annexin A10	11199	ANXA10
9.92	NM_005966	NGFI-A binding protein 1 (EGR1 binding protein 1)	4664	NAB1
11.95	NM_017774	CDK5 regulatory subunit associated protein 1-like 1	54901	CDKAL1

1. Ratio calculated as zinc deficient/zinc adequate.