Effects of zinc on retinoic acid-induced growth inhibition in human hepatocarcinoma HepG2 cells

by

DEANNA IBBITSON

B.Sc., The University of British Columbia, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

January 2012

Abstract

Retinoic acid (RA), a bioactive metabolite of vitamin A, inhibits growth in a variety of cancer cells including liver cancer. It is thought that this function of RA is achieved by modulating gene expression through complexing with retinoic acid receptors (RARs) and retinoid X receptors (RXRs), two groups of zinc-finger proteins. Zinc deficiency has been shown to affect gene expression and to impair DNA binding ability of zinc-finger proteins. The hypotheses of my thesis research were: 1) sufficient cellular zinc level is important for the effectiveness of RA-induced growth inhibition in hepatocarcinoma HepG2 cells; and 2) the influence of zinc on RA-induced growth inhibition is through modulating expression or function of RARs and RXRs, which in turn affects the expression of their target genes, CYP26a1 and RARβ. The overall objective was to examine the effects of zinc on RA-induced growth inhibition in HepG2 and the possible mechanisms involved.

Zinc manipulation was achieved by culturing HepG2 cells for 6 d in low-zinc media supplemented with 0, 5, and 10 μmol/L zinc to mimic low-, adequate-, and high-zinc conditions. Growth in low-zinc media for 6 d reduced total cellular zinc and the labile intracellular pool of zinc by 29 and 86%, respectively. Treating the cells with 35 μM of RA for 12 h following zinc manipulation significantly reduced cell proliferation in all zinc-treatment groups compared to their corresponding RA control, with the greatest reduction in the high-zinc group. Cell cycle analysis showed that the proportion of cells in the S-phase was reduced by RA treatment at 24 and 72 h at all zinc levels, with the greatest reduction in cells cultured in high-zinc medium. Following growth in low-, adequate- and

high-zinc medium, RA treatment elevated the abundance of RARβ and Cyp26a1 mRNA equally in all zinc-treatment groups compared to their correspondent RA controls. Growth in low zinc medium increased mRNA abundance of RXRα while RARα, RARβ, RARγ, RXRβ and RARγ were not affected. In conclusion, these results showed that increasing zinc appeared to sensitize HepG2 cells to RA-induced growth inhibition, but had no effect on RA-induced gene expression of CYP26a1 and RARβ.

Preface

This thesis was prepared in accordance to University of British Columbia Faculty of Graduate Studies requirements. I was responsible for performing all experiments. The research design, interpretation of the results, and preparation of this thesis were accomplished with the assistance and guidance of Dr. Zhaoming Xu.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADH	alcohol dehydrogenase
APL	acute promyelocytic leukemia
AP-1	activator protein 1
BrdU	5-bromo-2'-deoxyuridine
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CRABPII	cellular retinoic acid-binding protein II
d	day
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EMSA	electromobility shift assay
FABP	fatty acid binding protein
G1	gap 1
G1/S	gap 1/synthesis
G2/M	gap 2/mitosis
h	hour
hZIP1	human ZRT, IRT-like protein 1
IGFI	insulin-like growth factor I
IU	international units
LIPZ	labile intracellular pool of zinc
MAP	mitogen-activated protein
min	minute
mRNA	messenger RNA
NFκB	nuclear factor κB
Pol II	DNA polymerase II
PCR	polymerase chain reaction
PTC	pseudotumor cerebri
RA	retinoic acid
RAL	retinal
RARE	retinoic acid response element
RARα	retinoic acid receptor α
RARβ	retinoic acid receptor β
RARγ	retinoic acid receptor γ
RAS	retinoic acid syndrome
RBP	retinol binding protein
RDA	recommended dietary allowance
RE	retinyl ester
RNA	ribonucleic acid
ROL	retinol
RXRE	retinoid X response element

RXRα	retinoid X receptor α
RXRβ	retinoid X receptor β
RXRγ	retinoid X receptor γ
S-phase	synthesis phase
TGF-β	transforming growth factor-β
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine
ZIP	ZRT, IRT-like protein
ZIP4	ZRT, IRT-like protein 4
ZIP14	ZRT, IRT-like protein 14
ZnT	zinc transporter
ZnT5	zinc transporter 5

Acknowledgements

I would like to thank everybody at UBC who helped me throughout the process of completing my thesis. Specifically, I would like to thank my advisory committee members: Dr. Christine Scaman and Dr. Tim Green, my supervisor: Dr. Zhaoming Xu, my lab mates: Alice Lin, Melinda Bakker, Wendy Hempstock, Jonathan Lee, Sylvia Lymburner, and Markus Purtzki, and the Lund lab. Special thanks goes to Helen Chan who spent countless hours washing dishes, autoclaving, filling pipette boxes and assisting with experiments, always with a smile on her face. Last, but not least, thank you to Daniel Ahkong and my parents for their moral and emotional support.

My thesis research project was supported by the FNH Vitamin Research Fund (Z. Xu).

Introduction

Zinc and vitamin A are both essential nutrients for humans with numerous overlapping physiological roles such as immune function and fetal development (Vallee and Falchuk, 1993; Mark *et al.*, 2006). Both nutrients are involved in growth regulation and cellular differentiation (Blomhoff and Blomhoff, 2006; Li *et al*., 2007). There is evidence that zinc is required for the function of vitamin A at the whole body level, including the release of vitamin A from the liver into the blood, as well as vitamin A metabolism (Smith, 1980; Boron *et al*., 1988). However, information on the interactions between the two nutrients at the cellular level is much more limited.

Vitamin A, in the form of retinoic acid (RA), is used clinically as a chemotherapeutic agent for numerous types of cancer. Growth suppression following RA treatment has been observed in numerous types of cancerous cells including breast cancer, liver cancer, prostate cancer, non-small-cell lung cancer, thyroid cancer and myeloid leukemia cells (Bohnsack and Hirschi, 2004, Nakanishi *et al.*, 2008). This function of RA is thought to be due to its regulatory role in the expression of growth-regulating genes (Schug *et al.*, 2007). RA exerts its regulatory function in gene expression by forming a complex with members of two families of zinc-finger nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). This RA-receptor complex binds to a specific DNA region, the retinoic acid response element (RARE), to initiate the expression of the target genes (Soprano and Soprano, 2002).

Aberrations in intracellular zinc content have been observed in many types of tumors (Franklin and Costello, 2009). For example, zinc content in hepatocarcinoma is markedly reduced in comparison to non-cancerous liver cells, however the consequences of this altered zinc status on cell growth and response to treatment are not well understood (Gurusamy and Davidson, 2007). Although zinc has been shown to affect gene expression, enzyme function and the ability of zinc-containing nuclear receptors to bind to DNA, it is not clear whether intracellular zinc levels are important for the function of RA at the cellular level (Satre *et al.*, 2001; Dieck *et al.*, 2003; Rana *et al.*, 2008).

Due to the important role of vitamin A in many physiological processes and in anti-cancer treatment, it is important to understand the effects of other essential nutrients, including zinc, on the function of vitamin A.

Chapter 1. Literature Review, Hypothesis, and Objectives

1.1. Vitamin A Nutrition and Function

Vitamin A refers to a family of molecules derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional terminal group at the terminus of the acyclic portion (Blomhoff and Blomhoff, 2006). The two major isomers of vitamin A are: retinol (ROL) and retinal (RAL). RAL can be metabolized to retinoic acid (RA), which is also biologically active. Vitamin A is stored in the liver as retinyl esters (RE). Foods of animal origin contain ROL or RE. Foods of plant origin contain carotenoids, which can be converted to vitamin A within the intestinal cell during digestion. Within the intestinal cell, carotenoids are converted to RAL and then ROL. Upon release from hepatic stellate cells, RE is hydrolyzed by hydrolases to release ROL, which is then transported through the blood to target tissues. The ROL released can be metabolized to RA via RAL within hepatic and extrahepatic cells (Blomhoff and Blomhoff, 2006).

The dietary reference intakes for vitamin A for adults are: 700 μg/d (2333 IU/d) for females and 900 μg/d (3000 IU/d) for males (Health Canada, 2010). Common food sources of pre-formed vitamin A include animal sources such as liver, kidney, fatty fish, dairy products and eggs. Plant foods containing vitamin A precursors (carotenoids) include green leafy and orange and yellow vegetables and orange colored plants (Weber and Gruve, 2011).

Vitamin A is important for most forms of life and is required for many biological processes. The most well-known and understood function of vitamin A is its role in night vision, as it is needed for the formation of the photosensitive visual pigment of the retina (Rando, 1990; Darlow and Graham, 2007). Vitamin A is also important in many other processes including immune defense and the regulation of growth and development (Semba, 1998; Clagette-Dame and Deluca, 2002). Vitamin A, as ROL, maintains the integrity of skin and mucosal cells, the first defense against infection (McCullough *et al.*, 1999). RA is involved in the development and differentiation of white blood cells (Semba, 1998). RA plays a key role in embryonic development and tissue remodeling in the adult and it plays essential roles in maintaining the integrity of epithelial cells of the respiratory tract (Darlow and Graham, 2007; Osanai *et al.*, 2010; Donato *et al.*, 2007). During fetal development, RA is required for limb development and formation of the heart, eyes and ears (Clagette-Dame and Deluca, 2002). In addition to its role in normal growth and development, RA has important implications in cancer development and treatment. Vitamin A deficiency is thought to increase the risk of some cancers, and RA is administered therapeutically in cancer patients (Osanai *et al.*, 2010).

RA exerts its effects on growth and development by regulating gene expression. To induce gene transcription, RA binds to two families of nuclear receptors: the RARs and RXRs. The metabolites all-*trans*-RA and 9-*cis*-RA are high affinity ligands for RARs, whereas only 9-*cis*-RA has been shown to bind to RXRs (Soprano and Soprano, 2002). RARs and RXRs are two families of zinc-finger nuclear receptors (Mark *et al.*, 2006). Each family

includes three members: the RAR-α, -β, and –γ and RXR-α, -β, and -γ. RXRs form homodimers and bind to the retinoid X response elements (RXREs) within gene promoters, whereas RARs form heterodimers with RXRs and bind to RAREs in order to induce gene transcription (Mark *et al.*, 2006). In the absence of RA, RXR/RXR heterodimer complexes bind to the RARE as well as a corepressor complex, including those with histone deacetylase activity. Activation of transcription occurs when RA binds to RARs, altering the RAR/RXR interactions with corepressor proteins while increasing binding affinity to coactivator proteins, including those with histone acetyltransferase activity (Tang and Gudas, 2011; Figure 1.3).

Being fat soluble, vitamin A requires numerous proteins for its absorption, transport through the blood, intracellular transport, and metabolism (Blomhoff and Blomhoff, 2006). Transcriptional activities of RA are mediated by a small soluble protein termed cellular RA-binding protein II (CRABPII; Donato *et al.*, 2007). CRABPII shuttles RA from the cytoplasm into the nucleus and facilitates the binding of RA to RAR (Sessler and Noy, 2005). Further, CRABPII has been shown to play a critical role in sensitizing tumors to the growth suppressive effects of RA (Manor *et al*., 2003).

A large number of genes are proven or putative direct targets of RA and numerous other genes are regulated by an indirect manner (Ross *et al.*, 2011). A PCR array study in mouse liver shows that genes affected by RA include those involved in angiogenesis, cell differentiation, cell proliferation, cell migration and adhesion, hypoxic adaptation and

apoptosis (Mamoon *et al.*, 2008). Many of these genes, including those involved in cell proliferation, differentiation and apoptosis, are induced after 24 h of RA treatment, indicating that they are indirect targets of RA signaling. The expression of nuclear receptor, RARβ, is induced more than three fold after only three hours, indicating a more direct response to RA.

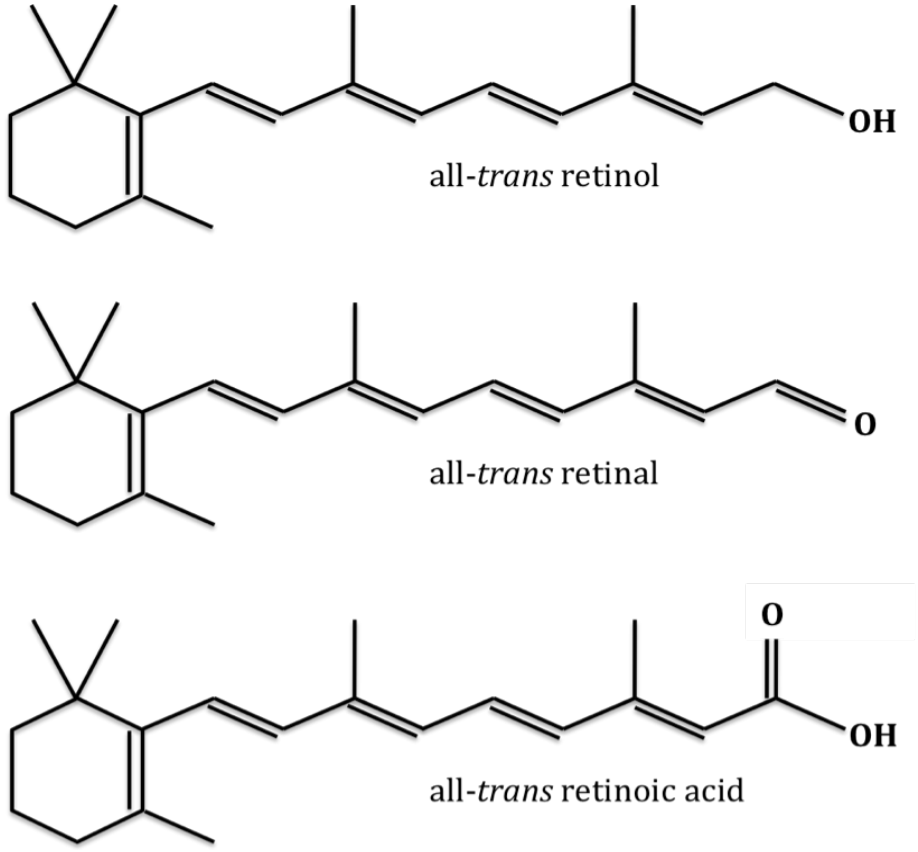

Figure 1.1: Structure of all-*trans* retinol, all-*trans* retinal, and all-*trans* retinoic acid.

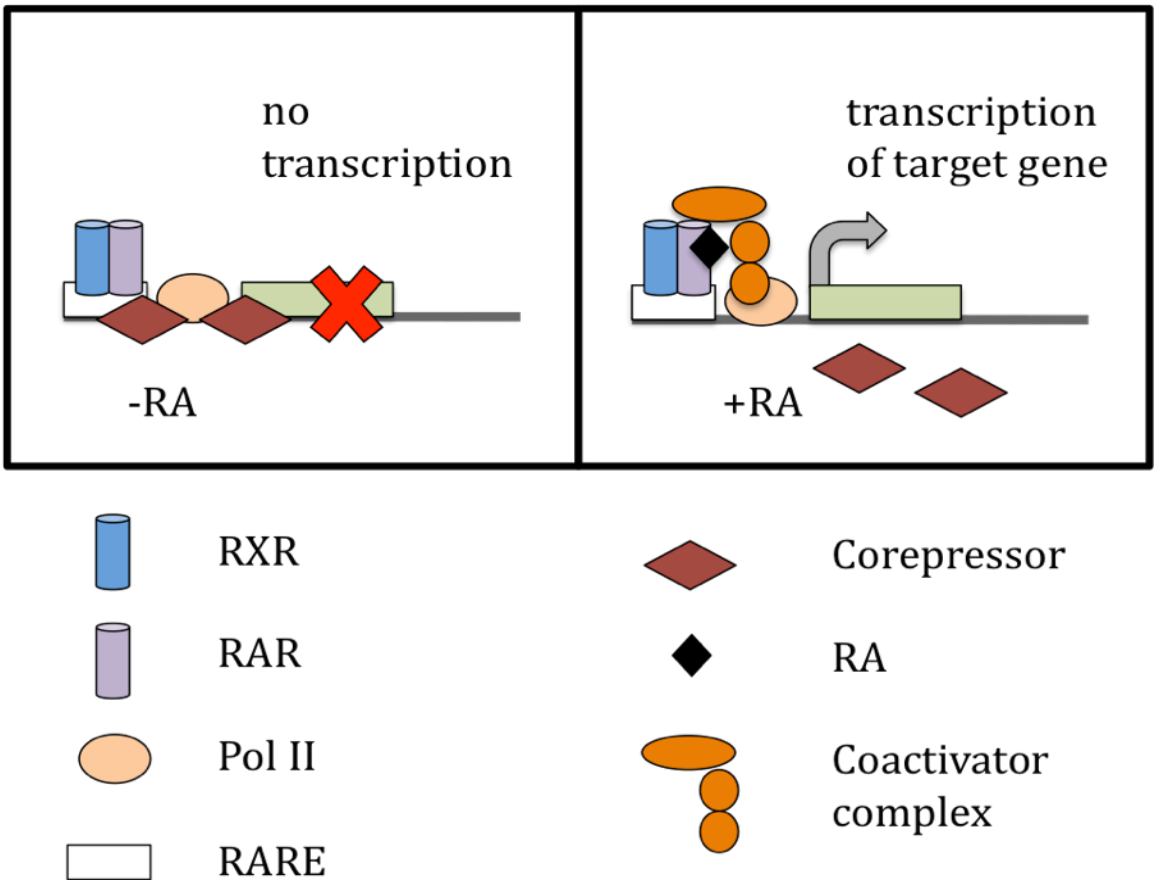

Figure 1.2: Mechanism of retinoic acid-induced gene transcription. Abbreviation: Pol II, DNA polymerase II.

1.2. Zinc Nutrition and Function

Zinc is an essential trace element for humans and is required for the catalytic and structural role of more than 300 enzymes, which control processes including DNA synthesis, normal growth, brain development, bone formation and wound healing (Sandstead, 2003; Stephanidou *et al.*, 2006). For example, zinc is required for the activity of multiple enzymes required for DNA replication and transcription including DNA and RNA polymerases, thymidine kinase, and ornithine decarboxylase, all of which are involved in mitosis (Mocchegiani *et al.,* 2000). Zinc also provides structural stabilization to a multitude of proteins, including zinc-finger transcription factors, which regulate many cellular processes (Satre *et al.*, 2001; Rana *et al.*, 2008). Some of the zinc-finger proteins are involved in DNA repair, apoptosis, cell cycle progression, and cell proliferation and differentiation. Therefore, adequate zinc status is essential for normal development and maintenance of the human body.

Zinc is found mainly in foods of animal origin, but is also present in plants (Shah, 2011). The recommended dietary allowance (RDA) for zinc is 11 mg for men and 9 mg for women (IOM, 2000). Rich dietary sources of zinc include beef and lamb, which contain 4.1 and 3.3 mg/100g tissue, respectively (McAfee *et al.,* 2010). Some seafood, especially oysters, and whole grains are also good sources of zinc (Simpson *et al,* 2011). However, the bioavailability of zinc varies widely (Simpson *et al,* 2011*).* Bioavailability from unrefined cereal grains and legumes is low due to the inhibition of absorption by phytate (inositol hexaphosphate). The phosphate groups in phytates form strong bonds with divalent

cations including zinc and because the human gastrointestinal tract lacks significant phytase activity, zinc bound to phytate is not available for absorption (Hess and Brown, 2009).

While severe zinc deficiency is rare in human populations, mild to moderate deficiency is extremely prevalent, with about two billion people worldwide ingesting inadequate amounts of zinc (Brown *et al.*, 2001; Prasad, 2003; Song *et al.*, 2009). Because of its wide-ranging functions, inadequate zinc intake may have severe consequences. The classical zinc deficiency symptoms include impaired growth, wound healing, and reproductive function in men, as well as increased severity of a variety of infections (Bohnsack and Hirschi, 2004; Hess *et al.*, 2009). Zinc deficiency also impairs developmental neurogenesis and evidence suggests that zinc deficiency may also impair neuronal differentiation (Levenson and Morris, 2011; Corniola *et al.*, 2008). The deficiency symptoms of zinc are believed to occur as a result of the role of zinc in enzymes and transcription factors (Levenson and Morris, 2011). In addition to impaired growth and development, it has been proposed that zinc deficiency could increase the risk of some types of cancers, due to the important role that zinc plays in the protection from oxidation and damage to DNA (Ho, 2004).

Within the cell, 40% of zinc is located in the nucleus, 50% in the cytoplasm, organelles and specialized vesicles and the remainder in the cell membrane (Stephanidou *et al.*, 2006). While 90% of the total cellular zinc is tightly bound to proteins, the remaining

more dynamic pool of zinc is known as the labile intracellular pool of zinc (LIPZ). LIPZ consists of free ionic zinc within the fM - pM range and zinc bound to low molecular weight ligands such as histidine, cysteine, aspartate, glutamate, citrate, and metallothionein (Franklin and Costello, 2009). The LIPZ provides metabolically available zinc for processes such as cellular signaling, second messenger metabolism, and the function of enzymes (Kambe *et al.*, 2004).

At the cellular and molecular levels, either zinc deficiency or excess may have detrimental and even cytotoxic effects, therefore zinc homeostasis must be tightly maintained to ensure normal cellular functions (Kambe *et al.*, 2004). Zinc homeostasis is achieved through a combination of zinc importers, zinc exporters, and metallothionein. Two families of zinc transporters are the ZRT, IRT-like protein (ZIP) family, which consists of 14 members and controls the influx of zinc into the cytosol, and the zinc transporter (ZnT) family, which consists of 10 members and is responsible for transport zinc out of the cytosol (Cousins *et al.*, 2006). Zinc homeostasis is also maintained by a metal-binding protein called thionein, which binds to zinc to form metallothionein. The synthesis of thionein can be induced by zinc and metallothionein acts as a reservoir and buffer of labile zinc.

1.2.1. Zinc-Finger Proteins

Numerous proteins contain loop structures stabilized by zinc ions. This type of loop is known as zinc-finger and the protein is known as a zinc-finger protein. Zinc-finger

proteins are involved in a variety of physiological functions, including hormone secretion, immune defense, and DNA repair (Ho and Ames, 2002; Dieck *et al.*, 2003; Song *et al.*, 2009). Between 3-10% of human genes are thought to encode zinc-binding proteins and nearly half of eukaryotic transcription factors bind zinc (Loh, 2010).

The presence of zinc in zinc-fingers is essential to their function. Zinc-fingers are critical to proper folding of the proteins they are found in (Klug and Schwabe, 1995). Zinc-fingers are essential for the DNA-binding activities of the transcription factors, as their unique structure allows for recognition and binding of specific sequences in double stranded DNA (Park *et al.*, 2011). In addition to binding to DNA, zinc-fingers have also been shown to mediate protein-protein interactions (Gamsjaeger *et al.*, 2006).

1.3. Interactions Between Zinc and Vitamin A

There has been much evidence of an interaction between zinc and vitamin A (Christian and West, 1998). In rats, zinc deficiency has been shown to impair the mobilization of retinol from the liver, whereas zinc repletion restores plasma vitamin A levels (Brown *et al.*, 1976; Duncan and Hurley, 1978). In human studies, zinc deficiency limits the ability of cirrhotic patients to respond to vitamin A and combined vitamin A and zinc deficiencies are often found in these patients (Russel, 1980). In studies attempting to restore night vision in Nepalese pregnant women, when zinc supplements were given along with vitamin A supplements, the women were four times more likely to have restored night

vision than with vitamin A supplementation alone, if they were zinc deficient at baseline (Christian *et al.*, 2001).

The mechanisms involved in zinc-vitamin A interactions are presently unclear. One theory for the interaction between zinc and vitamin A is that zinc deficiency impairs the synthesis of retinol binding protein (RBP), which in turn inhibits the release of ROL from the liver into the blood (Smith, 1980). However, in HepG2 cells, culture in low zinc media has been shown to increase the expression of RBP (Satre *et al.*, 2001). It has also been suggested that zinc deficiency may interfere with the metabolism of vitamin A by affecting the activity of enzymes involved. In rats, zinc deficiency decreases the activity of alcohol dehydrogenase (ADH), a zinc-dependent enzyme that catalyzes the conversion of ROL to RAL, whereas the activity of retinal oxidase, an enzyme that converts RAL to RA, increases (Boron *et al.*, 1988).

It is clear that zinc is required for vitamin A function at the whole-body level, but *in vitro* evidence of a zinc-vitamin A interaction is sparse, resulting in limited information on a zinc-vitamin A interaction at the cellular level. Interestingly, both zinc and vitamin A are required for neuronal differentiation (Levenson and Morris, 2011). Recently, one study assessing the effects of zinc status on RA-induced differentiation of human neuronal cells found that zinc deficiency impairs RA-induced differentiation *in-vitro* in a zinc concentration-dependent manner (0.4 – 2.5 μM zinc), but the reasons for this dependence are unknown (Gower-Winters, 2008).

1.4. The Role of Vitamin A in Cancer

Vitamin A also plays key roles in the development, progression, prevention, and treatment of cancer. Early studies on animals have shown an association between vitamin A deficiency and carcinogenesis when it was discovered that vitamin A is required for normal epithelial cell morphology in rats (Walbach and Howe, 1925). More recently, it has been shown that vitamin A treatment can reduce the incidence of second primary tumors in patients with prior lung, head and neck, and liver cancer (Dragnev, *et al.,* 2000). Epidemiological studies show an association between vitamin A and risk of some cancers. For example, high intake or high circulating vitamin A (as ROL) levels correlates with a decreased risk of developing breast cancer, but an increased risk of prostate and gastric cancer (Fulan *et al.,* 2011; Miyazaki *et al.*, 2011; Mondul *et al.,* 2011) In addition, RA displays distinct anti-carcinogenic activities and is currently used in treatment or is being tested in clinical trials as a preventative and therapeutic agent in several types of cancer, including breast cancer, liver cancer, prostate cancer, non-small-cell lung cancer, thyroid cancer, and myeloid leukemias (Bohnsack and Hirschi, 2004, Nakanishi *et al.*, 2008; Donato *et al.*, 2007).

RA can exert its effects by directly or indirectly influencing the expression of genes involved in cellular growth (Alisi *et al.*, 2003). Most often, treatment with RA causes inhibition of cell growth in many RA-sensitive tumor cells, with signaling through RARα (Fitzgerald *et al.*, 1997). In these cases, transcriptional activation of RAR may trigger differentiation, apoptosis, and cell-cycle arrest, depending on the type of cancer (Schug *et*

al., 2007). For example, H157 human squamous cell carcinoma cells respond to RA treatment by inhibition of cell proliferation (Sun *et al.*, 2000). In MCF7 breast cancer cells and other estrogen receptor-positive breast cancer cell lines, RA treatment inhibits growth and induces apoptosis (Liu *et al.*, 1997; Estner *et al.*, 1998).

Numerous proteins and signaling cascades are thought to be involved in the effect of RA on growth. For example, in HepG2 cells, treatment with RA induces growth arrest and differentiation, which is thought to be due to the influence of RA on the activities of cyclin-CDK complexes involved in the regulation of G1/S transition and S-phase progression (Alisi *et al.*, 2003). RA has been shown to cause growth inhibition in liver and pancreatic cancer cells mediated through transforming growth factor-ß (TGF-ß; Salbert *et al.*, 1993; Singh *et al.*, 2007). The mitogen-activated protein (MAP) kinase pathway can also be activated by RA in NB-4 acute promyelocytic leukemia and MCF-7 breast carcinoma cell lines (Alsayed *et al.*, 2001). Also, In NB-4 cells, RA-induced apoptosis is thought to be mediated by the membrane-bound tumor-selective death ligand, TRAIL (Altucci *et al.*, 2001).

All-*trans* RA, or tretinoin, is approved to induce cytodifferentiation and decrease proliferation of acute promyelocytic leukemia (APL; Thatcher and Isoherranen, 2009). RA is also utilized in the treatment of head and neck carcinoma, and non-small-cell lung cancer (Bohnsack and Hirschi, 2004) and in combination with other drugs in the treatment of some solid tumors. However, clinical applications of RA in these cases can show limited

effects due to RA resistance (Arce *et al.*, 2005; Hua *et al.*, 2009). RA treatment in APL induces complete remission in about 90% of patients, however this remission is short-lived due to the rapid emergence of resistance (Tallman *et al.*, 2002).

Another concern regarding the use of RA as a cancer treatment is the potential toxicity. RA treatment is generally well tolerated, however, in about 2-27% of patients, adverse complications may result. The set of symptoms, termed retinoic acid syndrome (RAS), include fever, weight gain, elevated white blood cells, respiratory distress, hypotension and acute renal failure (Patatanian and Thompson, 2008). In addition, dose-related central nervous system toxicity can develop, especially in the pediatric population. This toxicity, termed pseudotumor cerebri (PTC), is characterized by neurologic and ocular symptoms, as well as increased intracranial pressure (Vanier *et al,* 2003). It has been reported that death from RAS occurs in about 2% of patients treated with RA (Larson and Tallman, 2003).

Though it is clear that RA plays an important role in gene expression and regulation of cancer cell growth, very little is known about the downstream pathways of RA-mediated signaling. The mechanisms whereby RA regulates biological processes remain to be fully understood (Thatcher and Isoherranen, 2009). Thus, investigations into the genetic mechanisms and signaling pathways behind the growth effects of RA in many cancer cell lines are subjects of ongoing research (Nakanishi *et al.*, 2008). Information on direct target genes involved in the anti-proliferative activities of RA is scarce and little is known about

the involvement of different isoforms of the RARs and RXRs (Donato *et al.*, 2007; Hua *et al.*, 2009).

1.4.1. Effect of Retinoic Acid on the Growth of HepG2

Hepatocellular carcinomas (liver cancers) are a relatively common cancer, being the fifth most common but the third leading cause of cancer mortality worldwide (Altekruse *et al.*, 2009; Alison *et al.*, 2011). RA has been shown to decrease growth of hepatocellular carcinomas through decreasing cell proliferation and induction of differentiation. Falsca *et al.* (1999) observed that treatment of HepG2 (a hepatocellular carcinoma cell line) with 5 μM of RA for 12 days resulted in 80% growth inhibition as well as progression to a more differentiated phenotype. Alisi *et al*. (2003) found in HepG2 that RA regulates proteins involved in G1/S transition in the cell cycle, particularly by altering the binding of cyclin-CDK complexes to p21 and p27 cell cycle inhibitors. Further, Nakanishi *et al.* (2008) identified entire regulatory cascades induced by RA involved in growth arrest in HepG2 cells, including the MAP kinase pathway, which is suspected to be directly regulated by RARβ and RARα.

Although treatment of HepG2 with RA results in decreased growth, it appears to be relatively less sensitive than other cell lines, requiring longer treatment duration or higher concentrations of RA. For example, Nakanishi *et al.* (2008) observed that concentrations of less than 50 μM of RA were not effective in decreasing cell number in HepG2 within 72 h of treatment. Arce *et al.* (2005) found HepG2 cells to be much more resistant to RA

treatment than Hep3B cells, another hepatocarcinoma cell line. They found a 50% decrease in viability in Hep3B following 72 h of RA treatment at 25 µM whereas in HepG2, no reduction in viability was seen at this time point until treatment reached 166 µM. Alisi *et al.* (2003) observed that a treatment of 5 µM RA for 2 weeks results in growth inhibition and a more differentiated phenotype. As a comparison, a reduction in growth was observed at RA treatment concentrations as low as 0.001 µM in MCF-7 and T-47D breast cancer cell lines after 7 days (Liu *et al.*, 1997).

1.4.2. Role of Retinoic Acid Receptor β in Cancer

Although the exact genes involved in RA-induced growth inhibition are unclear, there is much evidence that RARß, a member of the retinoic acid receptor family, is important for this process. In several in vitro systems, the retinoid anti-proliferative effect requires RARß and the inducibility of RARß predicts responsiveness to RA (Altucci *et al.*, 2001; Fabricius *et al.*, 2011). Sun *et al.* (2000) demonstrated that if RARß expression is blocked, cell responsiveness to RA is reduced. Because RARß is RA-inducible, it is a classical direct target of RA (Ross *et al.*, 2011). Strong evidence suggests that RARß acts as a tumor suppressor as it is frequently lost or epigenetically silenced in various cancers and its expression correlates inversely with tumor grade (Ross *et al.*, 2011).

1.4.3. CYP26a1 in Cancer

Cellular exposure to RA is regulated by controlled synthesis and metabolism by RA metabolizing enzymes, including CYP26a1. CYP26a1 is a member of the cytochrome p450

superfamily of enzymes and is responsible for metabolizing RA to 4-OH RA, its primary oxidation product (Thatcher *et al.*, 2010). It is thought that resistance due to rapid clearance of RA after continuous oral administration is due to the induction of CYP26a1 in tissues such as the liver (Thatcher and Isoherranen, 2009). Ozpolat *et al.* (2005) observed that RA treatment induced mRNA expression of CYP26a1 in human intestinal (Caco-2), endothelial (HUVEC), APL (NB-4), and liver (HepG2) cell lines in a dose-dependent manner. This theory is supported by the observation that overexpression of CYP26a1 in various cell lines leads to increased resistance to RA treatment (Osanai and Petkovich, 2005).

1.5. Role of Zinc in Cancer

Zinc appears to play an important role in the development and progression of cancer, but a common relationship of zinc with cancer development and progression has not yet been identified (Franklin and Costello, 2009). It has been suggested that low dietary zinc intakes may be a risk factor for cancer development. For example, in humans, zinc deficiency is associated with an increased risk of developing esophageal squamous cell carcinoma (Abnet *et al.*, 2005). The exact mechanisms by which zinc deficiency increases the risk of cancer are still unclear (Song *et al.*, 2009). It has been proposed that zinc deficiency may increase the risk of cancer by increasing oxidative stress and impairing DNA repair mechanisms (Ho, 2004). This has been demonstrated by the observations that zinc deficiency results in an increased sensitivity to oxidative stress and causes oxidative DNA damage (Taylor *et al.*, 1988; Oteiza *et al.*, 2000), and cells grown in zinc deficient media show increased oxidant production (Ho and Ames, 2002). Zinc also plays a role in the

regulation of DNA replication and transcription through zinc-finger proteins (Ho, 2004). These proteins may be impaired by zinc deficiency. For example, p53, a zinc-finger finger protein that has been shown to be impaired by inadequate zinc, modulates cell cycle progression, apoptosis, DNA repair, proliferation and differentiation (Ho, 2004).

Further, it has been observed that intracellular zinc levels are altered in many types of cancer. For example, reports have shown that zinc levels are markedly increased in breast cancer tissue as compared with non-cancerous tissue (Rizk and Sky-Peck, 1984). In pancreatic cancer cells, expression of the ZIP4 zinc importer is up-regulated in comparison to non-cancerous tissue resulting in an increased zinc levels in tumor cells and tumor growth (Logsdon *et al.*, 2003). Forced up-regulation of this transporter in cell culture studies results in a corresponding increase in intracellular zinc and increased cell proliferation in pancreatic cancer cells (Li *et al.*, 2007). In contrast, cellular zinc levels are reduced in prostate cancer and liver cancer. Normal prostate cells have high levels of zinc, however, malignant cells have lost the ability to accumulate zinc due to a down regulation of hZIP1, a major zinc uptake transporter in prostate cells (Costello and Franklin, 2006; Franklin *et al.*, 2007).

1.5.1. Zinc in Hepatocarcinoma

Several reports indicate that intracellular zinc is significantly lower in hepatocellular cancer tissue compared to normal hepatic cells (Gurusami and Davidson, 2007). For example, Ebara *et al*. (2000) observed 55% lower zinc levels in hepatomas compared to

surrounding normal liver tissue in sixteen patients. Tashiro *et al.* (2003) observed that cancerous liver tissue contains less than half of the zinc in non-cancerous liver tissue. The mechanisms and functional consequences of this decrease in zinc status in liver cancer are not clear. However, one possible explanation for the decreased zinc in cancerous liver tissue is decreased uptake of zinc due to the absence of the zinc importer, ZIP14. Protein expression of ZIP14 is not detected in HepG2 cells and microarray studies show that mRNA expression of this transporter is down-regulated (Liu *et al.*, 2007; Franklin *et al.*, 2007).

1.6. Molecular Effects of Zinc Deficiency

Zinc deficiency has been demonstrated to cause changes in gene expression. PCR array analyses indicate that alterations in cellular zinc status affect hundreds of target genes, in a tissue and cell-type specific manner (Cousins *et al.*, 2003; Kindermann *et al.*, 2004; Haase *et al.*, 2007). Low zinc status has been shown to affect gene transcription in the liver, including gene products that participate in growth and lipid metabolism (Dieck *et al.*, 2003). For example, in rat liver, zinc deficiency results in the up-regulation of insulin-like growth factor I (IGFI) and fatty acid binding protein (FABP) mRNA, and down-regulation of Ras-related protein, among many others (Dieck *et al.*, 2003). Wong *et al.* (2007) found that growth of HepG2 cells in medium depleted of zinc resulted in a 40% decrease in p21 protein expression and a 70% decrease in p21 mRNA. Reaves *et al.* (2000) reported that growth of HepG2 cells for 6.5 days in medium depleted of zinc resulted in a two fold increase in p53 mRNA compared to cells grown in media with adequate levels of zinc (4μM).

These observations demonstrate the importance of zinc in cellular processes including growth regulation; however, the mechanisms behind the effects of zinc deficiency on gene expression remain elusive. It has been suggested that the changes may be partially due to the role of zinc as an essential component in many transcription factors by stabilizing zinc-fingers (Dieck *et al.*, 2003). It is also thought that zinc may affect mRNA stability. For example, it was observed that Zinc Transporter 5 (ZnT5) mRNA is stabilized by increased zinc availability in the human intestinal Caco-2 cell line (Jackson *et al.*, 2008).

The importance of zinc for the structure and function of zinc finger proteins is demonstrated by the observation that zinc deficiency can impair the function of zinc fingers. For example, when wildtype p53, a zinc-finger protein, is exposed to zinc chelators, it adopts a mutant conformation and its ability to bind DNA is reduced, whereas addition of extracellular zinc at concentration within the physiological range results in renaturation and reactivation of the wild-type protein (Hainaut and Milner, 1993). Ho and Ames (2002) cultured rat gliomal C6 cells in zinc deficient or zinc adequate medium for five days. Using an electromobility shift assay (EMSA) they found that the DNA binding abilities of p53, nuclear factor κβ (NFκβ) and activator protein 1 (AP-1), all of which are zinc finger proteins, were impaired in the cells grown in zinc-deficient media.

The nuclear receptors, RARs and RXRs are also zinc-finger proteins. These nuclear receptors have several conserved regions in their amino acid sequence, one being the DNA -binding domain. This domain contains two zinc-finger motifs, critical to the binding of the

receptors to the RARE (Soprano and Soprano, 2002). Currently, little research has been conducted to evaluate the effects of zinc nutritional status on vitamin A function, particularly its role for the vitamin A nuclear receptors. I am unaware of any published research to date that zinc deficiency affects the binding efficiency or function of RARs and RXRs.

Table 1.1: Examples of genes affected by zinc status in the liver

Gene name (cell type)	**Zinc treatment (method of depletion)**	**Effect on gene expression (protein or mRNA)**	**Reference**
P21 (HepG2)	depletion (medium zinc removed with Chelex-100 resin)	decrease (protein and mRNA)	Wong *et al*., 2007
P53 (liver, HepG2)	depletion (medium zinc removed with Chelex 100 resin) supplementation (16 µM)	increase decrease	Reaves *et al*., 2000
IGF1 (rat liver)	depletion (rats fed zinc-deficient diet)	Increase (mRNA)	Dieck *et al*., 2003
FABP (rat liver)	depletion (rats fed zinc-deficient diet)	Increase (mRNA)	Dieck *et al*., 2003
Ras-related protein (rat liver)	depletion (rats fed zinc-deficient diet)	decrease (mRNA)	Dieck *et al*., 2003

1.7. Summary

In summary, vitamin A and zinc are both essential nutrients and are important for many cell processes, including cell growth and development. Zinc deficiency has effects on gene expression, which is thought to be partially due to the role of zinc as a constituent of many proteins, including enzymes and zinc-finger transcription factors. Vitamin A, as RA, exerts its function through binding to zinc-finger transcription factors in order to regulate gene expression, including genes involved in cell proliferation and growth. It has been shown that zinc is important for RA function *in vivo*, however, evidence of interaction between the two nutrients at the cellular level is limited.

1.8. Hypothesis

The hypotheses of my thesis research project were:

1) Sufficient cellular zinc level is important for the effectiveness of RA-induced growth inhibition in hepatocarcinoma HepG2 cells; and
2) Influence of zinc on RA-induced growth inhibition is through modulating the expression or function of RARs and RXRs, which in turn affects the expression of their target genes such as CYP26a1 and RARβ.

1.9. Overall Objectives and Specific Aims

The overall objective of my thesis research project was to examine the effects of zinc on RA-induced growth inhibition in hepatocarcinoma HepG2 cells and the possible mechanisms involved. The specific aims were:

1) To assess the effects of zinc on RA-induced inhibition of cell proliferation in hepatocarcinoma HepG2 cells.

2) To determine the effects of zinc on the expression of the nuclear receptors (RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ).

3) To determine the effects of zinc on the RA-induced expression of CYP26a1 and RARβ, downstream targets of RA signaling.

Chapter 2. Effects of Zinc on Retinoic Acid-Induced Growth Inhibition in Human Hepatocarcinoma HepG2 Cells

2.1. Introduction

Vitamin A has many functions within the human body including well-understood roles in night vision, as well as regulating growth and development (Mark *et al.*, 2006; Soprano and Soprano, 2007). Research in more recent years has uncovered additional roles of vitamin A, which involves the action of retinoic acid (RA), a bioactive metabolite of vitamin A, on regulating gene expression (Schug *et al.*, 2007). The circulating form of vitamin A, retinol, is converted to RA within the cell and then RA is transported into the nucleus where it binds to nuclear receptors including RARs. RARs form heterodimers with RXRs and this complex binds to the retinoic acid response element (RARE) within the target gene (Mark *et al.*, 2006). Binding of RA causes the release of corepressors and the recruitment of coactivators which causes a subsequent activation of transcription (Soprano and Soprano, 2002). It has been shown that the expression of more than 500 genes with numerous physiological roles is regulated by RA (Mamoon *et al.*, 2008).

One function of great interest is the role of RA in the inhibition of cell growth in promyelocytic leukemia and many solid tumor cell lines including breast, pancreatic and liver cancers (Salbert *et al.,* 1993; Liu *et al.,* 1997; Singh *et al.,* 2007; Thatcher and Isoherranen, 2009). In hepatocarcinoma HepG2 cells, RA treatment arrests the cell cycle at

G1/S transition, therefore, slowing cell proliferation and ultimately cell growth (Nakanishi *et al.*, 2008).

Although RA is successful in inducing remission in promyelocytic leukemia, it has numerous side effects and prolonged treatment results in resistance (Arce *et al.*, 2005; Hua *et al.*, 2009). It is believed that the resistance is mediated by the induction of CYP26a1, a RA-metabolizing enzyme, which is markedly induced by RA in the liver (Ozpolat *et al.,* 2005). In addition, HepG2 cells are less sensitive to RA treatment than other hepatocarcinoma cells such as Hep3B, requiring 85% higher concentration of RA to induce a decrease in cell viability (Arce *et al.*, 2005). A RA treatment of 5×10^{-6} M for two weeks is required to reduce the growth of HepG2 cells, whereas only 1×10^{-9} M of RA is needed to reduce the growth of MCF7 and T47D breast cancer cells (Liu *et al,* 1997; Alisi *et al.,* 2007). Therefore, finding means to increase the sensitivity of cancerous cells towards RA treatment will increase the effectiveness of RA as an anticancer agent.

The regulatory role of RA in cell proliferation is mediated via gene expression through binding to RAR and RXR nuclear receptors (Mark *et al.,* 2006). These nuclear receptors are zinc-finger proteins and zinc is an essential structural component required for their binding to the RARE (Soprano and Soprano, 2002). It has been shown that zinc deficiency impairs the DNA-binding ability of other zinc-finger proteins, such as p53 and AP-1 (Ho and Ames, 2002). However, the relationship between zinc status and the structure and function of RAR and RXR is presently not known.

Based on the available evidence, I hypothesized that sufficient cellular zinc level is important for the effectiveness of RA-induced growth inhibition in hepatocarcinoma HepG2 cells; and the influence of zinc on RA-induced growth inhibition is through modulating the expression or function of RARs and RXRs, which in turn affects the expression of their target genes such as CYP26a1 and RARβ. The objectives were: 1) To assess the effects of zinc on RA-induced inhibition of cell proliferation in hepatocarcinoma HepG2 cells, 2) To determine the effects of zinc on the expression of the nuclear receptors (RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ), and 3) to determine the effects of zinc on the RA-induced expression of Cyp26a1 and RARβ, two downstream targets of RA signaling.

2.2. Materials and Methods

2.2.1. Cell Culture System

HepG2, a hepatocarcinoma cell line (ATCC, Manassas, VA), was cultured in Minimum Essential Medium (MEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), sodium pyruvate (110 mg/L), sodium bicarbonate (1.5 g/L) and penicillin/streptomycin (5,000 U/L). Cells were grown at 37°C in an atmosphere containing 5% CO_2.

2.2.2. Preparation of Chelex 100-Treated FBS

In the culture system described above, FBS is the source of zinc in the medium. To remove zinc, Chelex-100 resin (BioRad, Hercules, CA) was mixed with FBS at 100 g/L for 24 h in an ice bath at 4°C with gentle stirring. The Chelex-100-FBS mixture was then

centrifuged at 1,000 rpm for 15 min at 4°C to remove the majority of the resin followed by immediate filtration using a Steritop TM filter (0.22 µM; Millipore, Billeria, MA) to remove the remaining Chelex-100 resin and to sterilize the FBS. The Chelex-100-treated FBS was then stored at -20°C until needed.

2.2.3. Zinc Manipulation

To mimic low, adequate, and high levels of zinc, cells were grown in their corresponding media supplemented with 10% Chelex-100-treated FBS plus 0, 5, or 10 µM of Zn^{2+} as $ZnSO_4$, respectively. The concentration of zinc in normal interstitial fluid is 2-5 µM (Franklin and Costello, 2009) and the level of zinc in normal medium supplemented with 10% FBS is 4 µM (Reaves *et al.*, 2000). Zinc concentrations in the media supplemented with 10% FBS and Chelex-100- treated FBS was reported previously in our lab to be 4.7 µM and 0.15 µM, respectively (Tsukada, 2003). Chelex-100 is also capable of removing a number of divalent cations. These divalent cations were added back after Chelex-100 treatment to restore their pre-Chelex-100 treatment concentrations (Appendix 1; table A.1.; Tsukada, 2003).

2.2.4. Quantification of the Labile Intracellular Pool of Zinc (LIPZ)

HepG2 cells were grown in 10 cm Petri dishes at an initial density of 300,000 cells per dish. To determine the duration required for depleting intracellular zinc, the cells were grown in MEM medium supplemented with 10% Chelex-100-treated FBS for 3 or 6 days (n=3). Cells in the control group were grown in MEM medium supplemented with regular

FBS for 6 days (n=3). To determine the intracellular zinc levels of cells grown in low, adequate, and high zinc concentrations, cell were grown for 6 d in MEM medium supplemented with 10% Chelex-100-treated FBS plus 0, 5, or 10 µM zinc, respectively (n=3).

At the end of each culture period, cells were rinsed once with warm PBS (37°C) and trypsinized with 2 ml of warm 0.25% Trypsin-EDTA. After the cells were detached, the trypsin was neutralized with an equal volume of the respective media. The cell suspension was then transferred to a 15 mL Falcon tube, centrifuged at 300 X g for 5 min and the supernatant aspirated. The cell pellet was re-suspended in 3 mL cold PBS (4°C) using a 26 G 5/8 needle to separate clumped cells, and counted using a particle counter (Z1 Particle Counter, Beckman Coulter, Fullerton, CA) with a cut-off point set at 8 µm. For each replicate, 2.2 million cells were transferred to a microcentrifuge tube and centrifuged at 300 X g for 5 min. Following aspiration of the supernatant, cells were re-suspended in 400 µL Hanks buffered saline solution (HBSS; Gibco, Grand Island, NY) and transferred in 180 µL aliquots to a black 96-well plate at 1 million cells per well (2 technical replicates). Cells were then incubated in the dark with 20 µL of Zinquin working solution (Sigma, St. Louis, MO) to obtain a final concentration of 25 µM for 30 min at 37°C with gentle shaking using a thermomixer (Thermomixer R, Eppendorf, Hauppauger, New York). A Zinquin working solution was prepared by diluting the Zinquin stock solution (5 mM Zinquin in DMSO) with HBSS to a concentration of 250 µM. The abundance of the LIPZ was assessed by determining the intensity of the Zinquin-dependent fluorescence (excitation: 365 nm;

emission: 475 nm; auto-cutoff point: 455 nm) using a microplate reader (Spectra, Molecular Devices, Sunnyvale, CA). The background, which was the fluorescence intensity in the absence of cells (HBSS and Zinquin only), was subtracted from the fluorescence intensity obtained from the samples.

2.2.5. Quantification of Total Cellular Zinc

HepG2 cells were cultured, harvested and counted as described above. After re-suspension in cold PBS, cell suspension (1 mL) was transferred to a microcentrifuge tube followed by centrifugation at 300 X g for 5 min and aspiration of the supernatant. The pellet was dissolved in 100 µL of concentrated nitric acid and allowed to lyse overnight at room temperature. After complete lysis, double deionized water was added to a final volume of 1 ml. Zinc concentration was determined by flame atomic absorption spectrophotometry as described by Thompson *et al.* (2002).

2.2.6. Assessment of Cell Viability and Proliferation

To assess cell proliferation in cells treated with RA alone, HepG2 cells were seeded into 96-well plates at an initial density of 7,000 cells and allowed to grow for 2 d. Cells were then treated with *all-trans* RA (Sigma Aldrich, Oakville, ON) at 0 (DMSO only; Sigma Aldrich, Oakville, ON), 15, 25 or 35 µM (n=6) for 12 h. RA treatments were performed in low light conditions and culture dishes containing RA were protected from light in the cell culture incubator by aluminum foil. Cell proliferation following RA treatment was assessed using the 5-Bromo-2′-deoxy-uridine (BrdU) colorimetric assay (Roche, Indianapolis, IN)

according to the manufacturer's instructions using a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA, USA) at 405 nm with a reference wavelength of 490 nm. BrdU is incorporated into newly synthesized DNA, therefore only cells undergoing active DNA synthesis were labeled with BrdU (Terry and White, 2006).

To assess cell proliferation following treatment with a combination of zinc and RA, HepG2 cells were seeded into 10 cm plates at an initial density of 300,000 cells. Cells were allowed to attach overnight. Subsequently, the cells were cultured in Chelex-100-treated medium supplemented with 0 (low), 5 (adequate) or 10 µM (high) of zinc ($ZnSO_4$; day 0). On day 4, cells were transferred into 96-well plates at a density of 7,000 cells per well, and on day 6, cells were treated with RA at 0 (DMSO only) or 35 mM (n=6) for 12 h. Cell proliferation was assessed using a BrdU assay as described above.

To assess cell viability following zinc and RA treatments, cells were seeded, cultured, and treated with zinc and RA as described above with changes as follows. HepG2 cells were transferred to 96-well plates after 5 d, and on day 6, cells were treated with RA at 0 (DMSO only) or 35 µM (n=12) for 72h. Cell viability was measured using the colorimetric Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Sigma-Aldrich, Oakville, ON) according to the manufacture's instruction. Briefly, at the end of the treatment period, medium was removed, and 100 µL fresh medium was added per well plus 10 µL MTT (5 mg/mL in PBS) solution. The plates were incubated at 37°C for 2 h. After removing the media, 100 µL 2-Propanol was added per well followed by incubation for 15 min at 37°C

with gentle shaking using a thermomixer The plates were read in a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, California) at 560 nm with a reference wavelength of 650 nm.

2.2.7. Cell Cycle Analysis

HepG2 cells were cultured into 10 cm culture dishes in regular MEM at an initial seeding density of 150,000 cells and allowed to attach overnight. Medium was changed to MEM supplemented with Chelex-100-treated FBS plus 0 (low), 5 (adequate), or 10 µM (high) of Zn as $ZnSO_4$. The medium was changed once more after 3 days and after 6 days. Cells were treated with RA at 35 µM for 0, 24, or 72 h. At the end of each treatment period, the cells were analyzed for cell cycle using flow cytometry according to the established protocol (Terry and White, 2006). The main steps of the assay were as follows.

BrdU treatment and cell fixation: Briefly, dishes were treated with 1 µM BrdU (Sigma-Aldrich, Oakville, ON) for 20 min at 37°C. Cells were trypsinized as described above and counted using a particle counter. The cells were fixed by adding a solution of 0.8 ml cold PBS and 1.2 ml 100% ethanol (-20°C) per 2 X 10^6 while vortexing and left to fix overnight at 4°C in the dark.

Staining with PI and BrdU antibody incubation: Briefly, fixed cells were incubated with 1.5 ml 2N HCl/ 2 X 10^6 cells at 37°C for 20 min. Subsequently, the cells were rinsed with 6 ml of 0.1 M sodium borate while vortexing, and then centrifuged at 350 X g for 4

min at 20°C. The supernatant was aspirated and 6 ml of cold PBTB (4°C; PBS + 0.5% Tween-20 +0.5% BSA) was added while vortexing and the solution was centrifuged at 350 X g for 4 min and 20°C. Following aspiration of the supernatant, the cells were incubated with a 1:100 dilution of anti-BrdU monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBT (PBS + 0.5% Tween-20) for 60 min at room temperature, in the dark, then rinsed with PBTB. The cells were then incubated with FITC-conjugated goat anti-mouse antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) in PBTG (PBTB + 1.0% goat serum) for 45 min at room temperature in the dark, followed by rinse with PBTB. The cells were then stained with 10 µg/mL propidium iodine (PI; Sigma-Aldrich, Oakville, ON) in PBTB overnight. Thirty minutes prior to analysis, RNAse A (1mg/ml in water; Sigma-Aldrich, Oakville, ON) was added to each tube.

FACS analysis: The samples (10,000 cells/treatment group) were analyzed using flow cytometry (CellQuest, Becton-Dickinson, Franklin Lakes, NJ) at excitation of 488 nm. BrdU (FITC, green fluorescence) was measured using a logarithmic amplifier with a 530 nm short-pass filter and linear DNA content (PI, red fluorescence) was measured using a 610 nm long-pass filter. The percentage of cells in the G1, S and G2/M phases were determined using FlowJo 8.7 analysis software.

2.2.8. RNA Extraction and Real Time Quantitative PCR

HepG2 cells were cultured in 6 cm culture dishes at a seeding density of 150,000 cells in regular MEM and allowed to attach overnight. The culture medium was changed to

low-, adequate- or high-zinc medium. Medium was changed once more after 3 days. After 6 days, dishes were treated with RA at 0 (DMSO only) or 10 μM (n=4) for 6 h for assessing the expression of RARβ, and at 35 μM for 48 h for assessing the expression of Cyp26a1.

Total cellular RNA was isolated using TriZol (Invitrogen, Burlington, ON) in accordance with the manufacturer's directions, with the following modifications. Briefly, 1mL of TriZol was added per plate, and scraped using a cell scraper. At the precipitation step, samples were incubated in equal volumes of 2-propanol and high salt solution (0.8 M sodium citrate, 1.2 M sodium chloride) for 10 min at -80^{o}C. The final RNA product was dissolved in 30 μL of DEPC-treated ddH_2O and stored at -80^{o}C until analysis. The integrity of RNA was assessed by electrophoretic separation of total RNA on a 0.8% agarose gel containing GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA) at 80 V for 45 minutes followed by visualization under UV light. RNA purity was assessed by determining the OD_{260}/OD_{280} ratio using a spectrophotometer (Nanodrop ND-1000, Wilmington, DE, USA). Samples with an OD_{260}/OD_{280} or ≥1.7 were used for the synthesis of cDNA.

Total RNA (5 μg) was used to generate cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen, Burlington, ON) according to the manufacturers' directions. Briefly, 5 μg RNA dissolved in DEPC-treated water was mixed with 1 μL of 50 μM Oligo-DT and 1 μL of 100 mM dNTP mix and DEPC-treated water to a total reaction volume of 10 μL and incubated at 65°C for 5 min. Then 10 μL of master mix was added to each reaction (1X RT buffer, 5 mM MgCl2, 10 mM DTT, 1 μL RNase OUT and 1 μL Superscript III RT) and the

reaction was carried out at 50°C for 50 min and then 85°C for 20 min. The product (50 ng) was amplified using RT^2 Real-Time™ SYBR Green/ROX PCR 2X master mix (SABiosciences, Frederick, MD, USA) with 500 nM each reverse and forward primers (Table A.2) to a final volume of 20 µL with DEPC-treated water in a thermocycler (7500 Real-Time PCR System, Applied Biosytems, Foster City, CA, USA). The abundance of the mRNA of the target gene was normalized on the fluorescence of the corresponding cyclophilin control.

2.2.9. Protein Extraction and Western Blot Analysis

HepG2 cells were cultured into 10 cm culture dishes at an initial seeding density of 250,000 cells in MEM plus 10% FBS and allowed to attach overnight. Medium was changed to MEM plus 10% Chelex 100-treated FBS plus 0 µM zinc (low), 5 µM zinc (adequate) or 10 µM zinc (high; n=4). Medium was changed once more after 3 days. After 6 days, the cells were harvested and counted, as described above, except that the cells were re-suspended in 1.5 mL of cold PBS twice following centrifugation at 1000 rpm for 5 m at 4°C to rinse the cells. The cell suspension was then transferred to a microcentrifuge tube and centrifuged 14 X g for 5 min at 4^0C. After aspiration of the supernatant, whole cell radioimmunoprecipitation assay buffer (RIPA: 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% triton X-100, 1 mM phenylmethylsulphonyl-fluoride (PMSF), 10% Protease Inhibitor Cocktail) was added at 30 µL/10^6 cells. Samples were incubated on ice for 30 min and then centrifuged for 10 min at 14 X g. The total protein was quantified using the BioRad DC method (BioRad, Hercules, CA, USA) and the samples were stored at -80°C.

Protein samples (35 μg/lane for RXRα and 50 μg/lane for RARα) were size-fractioned by 10% SDS-PAGE at 175 V for 60 min and transferred to a PVDF membrane (Millipore, Billerica, MA) for 3 h at a constant current of 200 mA. The membrane was subsequently blocked with 2.5% skim milk powder in TTBS buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 h, incubated with an appropriate primary antibody as listed in Appendix 4 (Santa Cruz Biotechnology, Santa Cruz, CA), washed with TTBS for 10 min 3 times. Finally, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% skim milk powder in TTBS for 45 min at dilutions listed in Table A.3. Membranes were stripped and re-probed with a β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to confirm equal loading of protein. Briefly, the membrane to be stripped was incubated at room temperature for 5 min twice in stripping buffer (pH 2.2; 0.2M glycine, 3.5mM SDS, 1% Tween-20), and then rinsed for 10 min twice in PBS and 5 min twice in TTBS. Presence of the target protein was visualized using an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA) and chemiluminescence film (Kodak BioMax Light film, Sigma-Aldrich, St. Louis, MO, USA). Images of the bands were captured by a digital camera and the band intensity was measured using a software (Kodak ID v. 3.6.5, Scientific Imaging Systems, New Haven, CT, USA).

2.2.10. Statistical Analyses

The results were analyzed using one-way ANOVA followed by Tukey's Honesty Test ($p < 0.05$). Statistical analyses were performed using GraphPad Prism (5.0 for Mac OS X; GraphPad Software, San Diego, CA, USA).

2.3. Results

2.3.1. RA-Mediated Reduction in Cell Proliferation

In the absence of zinc manipulation, no reduction in cell proliferation was observed in HepG2 cells treated with 0, 15, and 25 µM RA for 12 h (Figure 2.1). At 35 µM RA treatment, a 44% reduction in cell proliferation was observed.

2.3.2. Treatment Duration-Dependent Reduction in Total Cellular Zinc Level and the Abundance of LIPZ

In HepG2 cells, the abundance of LIPZ was reduced by 74 and 86% after being cultured in the low zinc medium for 3 and 6 d, respectively, compared to the control (Figure 2.2A). Total cellular zinc level was significantly decreased by 29% after 6 d of culture (Figure 2.2B). These results suggested that treating the HepG2 cells with low zinc for a total of 6 d was sufficient to reduce the abundance of LIPZ as well as the total cellular zinc levels in the cells.

2.3.3. Dose-Dependent Reduction in Total Cellular Zinc Level and the Abundance of LIPZ

LIPZ abundance doubled in HepG2 cells cultured for 6 d in high-zinc medium compared to low- and adequate-zinc media (Figure 2.3A); however, there was no significant difference in LIPZ abundance between the low- and adequate-zinc groups (Figure 2.3A). Total cellular zinc concentration was doubled in the adequate- and high-zinc groups compared to the low-zinc group, but the levels were the same between the adequate- and high-zinc groups (Figure 2.3B). These results suggested that the abundance of LIPZ and total cellular zinc level, responded to zinc treatment in HepG2.

2.3.4. Zinc Appeared to Sensitize HepG2 Cells to RA-Induced Reduction in Cell Proliferation

In HepG2, in the absence of RA treatment, cells grown in high-zinc media exhibited lower cell proliferation as measured by BrdU incorporation compared to cells grown in low- or adequate-zinc media. RA suppressed cell proliferation at all levels of zinc treatment compared to their corresponding RA controls. The greatest suppression was in the adequate zinc group (50%), compared to the low zinc group (35%) in response to RA treatment. In the high-zinc group, suppression of cell proliferation was only 30% compared to the RA control. However overall, treatment with high-zinc and 35 μM RA resulted in the lowest cell proliferation (Figure 2.4).

Cell cycle analysis was performed on HepG2 cells following 6 d of growth in low-, adequate-, and high-zinc media and 0, 24, and 72 h of treatment with 35 μM RA. No clear

trend in the proportion of cells in the G2/M phase was observed following zinc and RA treatment. RA increased the proportion of cells in the G1 phase after 24 and 72 h following growth in adequate- and high-zinc media (Table 2.1; Figure 2.5). In contrast, cells grown in low-zinc responded to RA treatment with a decrease in the percentage of cells in the G1 phase after 24 h and no change after 72 h. In cells grown in the low-zinc medium, the percentage of cells in G1 phase was 67, 63 and 68% in 0, 24, and 72 h RA treatments, respectively. When cells were grown in the adequate-zinc medium, the proportion of cells in the G1 phase was 57, 69 and 68%. In cells grown in the high-zinc medium, the proportion of cells in G1 was 58, 68, and 70%.

In the absence of RA, zinc appeared to slightly increase the proportion of cells in the S-phase with 19, 20 and 22% of cells in the S-phase in the low-, adequate-, and high-zinc groups, respectively (Figure 2.5A). In contrast, when RA was present, the proportion of cells in the S-phase was decreased with increasing zinc concentration. After treating the cells with 35 µM of RA for 24 h the proportion of cells in the S-phase was reduced from 16% in cells grown in the low-zinc media to 13% in the adequate-zinc media to 9% in the high-zinc media (Figure 2.5B). At 72 h of treatment, the proportion of cells in the S-phase was reduced from 9 to 8 to 6% (Figure 2.5C). These results suggested that RA reduced the proportion of cells in the S-phase at 24 and 72 h of treatment and this effect was dependent on the level of media zinc.

2.3.5. Cell Viability Was Unaffected by RA and Zinc

The cell viability was affected by neither zinc nor RA treatment (Figure 2.6).

2.3.6. Growth of HepG2 in the Low-Zinc Medium Increased mRNA Abundance, But Not Protein Levels of the RXRα Nuclear Receptor

Abundance of mRNA of RARα, RARβ, RARγ, RXRβ and RXRγ was generally unaffected by zinc treatment in HepG2 cells. However, abundance of RXRα mRNA was increased by 2-fold in cells grown in the low-zinc medium compared to those grown in adequate-zinc medium (Figure 2.7). Western blot analysis revealed no changes in abundance of RXRα or RARα protein levels among zinc treatment groups (Figure 2.8).

2.3.7. Zinc Treatment Did Not Affect the RA-Induced Increase in mRNA Abundance of Downstream Targets, RARβ and CYP26a1

In HepG2 cells, treatment of RA for 6 h at 10 μM caused 3 fold increase in RARβ mRNA expression in all zinc treatment groups compared to their RA control (DMSO only; data not shown). There was no difference in induction of RARβ by RA among the zinc treatment groups (Figure 2.9)

Treatment of RA for 24 h at 35 μM following zinc treatment caused an approximate 6-fold increase in CYP26a1 mRNA in all zinc treatment groups compared to the RA control (DMSO only; data not shown). However, there was no difference in induction of CYP26a1

among the zinc treatment groups (Figure 2.10). This data suggests that zinc status did not affect RA-induced expression of the downstream targets: RARβ and CYP26a1.

2.4. Discussion

2.4.1. Growth in Medium Depleted of Zinc was Effective in Reducing Cellular Zinc in HepG2

My hypothesis was that zinc status is important for RA function, particularly the growth suppressive effects in HepG2 hepatocarcinoma cells. In order to test this, it was first necessary to manipulate cellular zinc. Since a change in medium zinc does not necessarily induce a change in cellular zinc, to confirm the effects of zinc treatment on cellular zinc levels, a time course was performed to determine the length of time necessary to deplete cellular zinc and a dosage test to confirm a difference among the zinc treatments.

Cellular zinc status was assessed by measuring both total cellular zinc level and the abundance of the LIPZ. Total cellular zinc is a measure of the amount of zinc present in the entire cell, much of which is tightly bound to proteins. LIPZ is a dynamic pool of zinc loosely bound to low molecular weight ligands and a small amount of free ionic zinc (in the fM-pM range), with the entire pool making up less than 10% of the total cellular zinc (Franklin and Costello, 2009). This labile pool provides a supply of zinc for cellular signaling, second messenger metabolism, and the function of enzymes (Kambe *et al.*, 2004). Total cellular

zinc therefore represents mostly zinc incorporated into proteins, and LIPZ represents zinc readily available to the cell.

It was observed that growth of HepG2 cells in the low-zinc medium significantly reduced the level of total cellular zinc and the abundance of the LIPZ after 6 d and that cellular zinc is significantly different between low-, adequate-, and high-zinc treatments. Similarly, one other study by Reaves *et al.* (2000) found that growth of HepG2 in a low-zinc media (0.4 μM) also significantly depleted total cellular zinc levels after 6.5 d. These observations confirm that growth in media depleted of zinc was a successful method of depleting cellular zinc in HepG2 cells and that cellular zinc levels were influenced by media zinc levels in HepG2 hepatocarcinoma cells.

An alternate method of zinc depletion involves incubation with N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane permeable zinc chelator, (Kolenko *et al.*, 2001). However, this method was not chosen in my research as TPEN exhibits high zinc-binding affinity, which could remove the entire labile pool of zinc as well as a fraction of immobile macromolecular-bound zinc. This would not be achievable under normal pathological conditions and could ultimately result in cell death (Franklin and Costello, 2009). In addition, excess TPEN is not zinc-specific and could remove the cellular pools of Mn and Fe (Franklin and Costello, 2009). Reaves *et al.* (2000) concluded that culturing cells in low-zinc medium to deplete cellular zinc more closely resembles physiologic conditions than exposing the cells directly to a metal chelator.

2.4.2. Zinc Appeared to Sensitize HepG2 Cells to RA-Induced Reduction in Cell Proliferation

I hypothesized that zinc status would effect the growth suppressant function of RA in HepG2 cells. To determine if the altered zinc status in HepG2 cells had an effect on RA-induced inhibition of cell proliferation, cells were treated with RA following growth in low-, adequate-, and high-zinc media. The level of reduction of cell proliferation following RA treatment, as measured by the BrdU assay, appeared to be dependent on the level of media zinc in HepG2 cells. Cells grown in high-zinc media exhibited lower cell proliferation than those grown in low- or adequate-zinc medium.

Further investigation into the effect of RA on cell cycle progression following a similar treatment regime revealed a positive correlation between media zinc and the percentage of cells in the S-phase of the cell cycle following RA treatment. RA treatment also caused an increase in the percentage of cells in the G1-phase compared to control following growth in adequate- and high-zinc media, but not in low-zinc media. These observations suggest that zinc may be important to the growth-suppressant function of RA in HepG2 cells, particularly the role of RA in the inhibition of cell cycle progression from G1- to S-phase.

In another study, a correlation between media zinc and RA function was observed when testing the effects of zinc deficiency on RA-induced differentiation of neuronal cells (Gower-Winters, 2008). However, to my knowledge, this is the only other study attempting

to assess the effect of zinc status on RA function. The authors did not uncover a mechanism but hypothesized that the impaired RA function during growth in media containing low levels of zinc is due to impaired RAR/RXR nuclear receptor function.

An inconsistency between the BrdU assay and cell cycle analysis was observed in cells grown in high-zinc medium and treated with 0 µM RA. In this group, the BrdU assay showed a decrease in cell proliferation compared to low- and adequate-zinc treatments, however cell cycle analysis showed a slight increase in BrdU incorporation in this treatment group. One possible explanation for this inconsistency is that high-zinc conditions may induce apoptosis, which would affect the BrdU assay results by decreasing the cell number per well. This would not be detected in cell cycle analysis with flow cytometry, as this method measures the percentage of live cells stained with BrdU (dead cells are not included in the analysis). However, I am unaware of any published data showing observations of increased apoptosis in HepG2 cells due to zinc supplementation.

Although the BrdU assay and cell cycle analysis appeared to show a sensitizing effect of zinc on RA-induced inhibition of cell proliferation, it is unclear whether this relationship is significant to produce a clinically relevant change. RA treatment following growth in low-, adequate-, or high-zinc, resulted in no change in cell viability between RA-treated and un-treated cells in all levels of media zinc after 72 h, indicating that the changes observed in cell proliferation may not be significant enough to induce a change in viability within this time frame.

2.4.3. Growth of HepG2 in Low Zinc Media Resulted in Increased mRNA Abundance, But Not Protein Levels of the RXRα Nuclear Receptor

The effects of zinc status on RAR and RXR nuclear receptor expression was investigated as a possible mechanism for the change in sensitivity of HepG2 cells to RA following zinc manipulation. RAR/RXR nuclear receptors are critical for RA function (Soprano and Soprano, 2002). Zinc deficiency has been shown to have wide-ranging effects on gene expression (Dieck *et al.,* 2003), but it was not known if zinc status affects the expression of RAR/RXR receptors in HepG2 cells.

The only receptor observed to be affected by zinc status was RXRα, which exhibited an increase in mRNA abundance, but not protein level, following growth in low-zinc medium compared to adequate-zinc. It is not known if this receptor is involved in the growth regulation pathway. However, if this receptor were involved, the increase in mRNA abundance might indicate a compensatory mechanism for decreased function, similar to the response of p53, another zinc-finger, to zinc deficiency. Low zinc results in an increased mRNA expression of p53, whereas its DNA binding ability is reduced (Ho and Ames, 2002). However, it is thought that the increase in p53 expression may be a response to DNA damage resulting from low intracellular zinc (Ho *et al.*, 2004). The level of nuclear receptor mRNA is not necessarily reflective of the function of this protein. Therefore, it is possible that the DNA-binding ability of the nuclear receptors, RARs and RXRs, is impaired by low zinc.

An inconsistency between mRNA and protein level in RXRα could possibly be due to improper protein folding. Zinc-fingers are essential for proper protein folding (Klug and Schwabe, 1995). An effect of zinc depletion on improper folding of zinc-finger proteins is demonstrated by p53, which adopts a mutant conformation following induction of zinc-deficiency (Hainaut and Milner, 1993). Misfolded proteins are degraded via the ubiquitin-proteasome system (Eisele and Wolf, 2008).

2.4.4. Zinc Treatment Did Not Affect the RA-Induced Increase in mRNA Abundance of Downstream Targets, RARβ and CYP26a1

To determine if the function of RXR/RAR nuclear receptors was affected by zinc status, the mRNA expression of two well established RA-inducible genes, CYP26a1 and RARβ (Ozpolat *et al.*, 2005; Altucci *et al.*, 2007), was measured. It has been observed that zinc deficiency causes impaired DNA binding of some zinc-finger proteins, including p53, NFkB, and AP-1 in rat gliomal cells grown in medium supplemented with Chelex-100 treated FBS (Ho and Ames, 2002). Since RAR and RXR nuclear receptors contain zinc-fingers, then theoretically, low zinc could lead to impaired DNA-binding of these receptors and ultimately, impaired expression of downstream genes.

A difference among zinc groups in RA-induced expression of CYP26a1 or RARβ following growth in low-, adequate-, and high-zinc media was not observed. This implied that the function of the RAR/RXR receptors was not affected by zinc.

It is likely that some other mechanism is responsible for the observation that zinc sensitized HepG2 cells to RA-induced reduction in cell proliferation. Firstly, since zinc has wide-ranging effects on gene expression, low zinc could alter the expression of genes involved in the RA-induced inhibition of cell proliferation. For example, p21 is a key regulator of the cell cycle. This protein binds to and inhibits the activity of cyclin-CDK2 complexes, therefore inhibiting cell cycle progression and slowing growth (Maddika *et al.*, 2007). Wong *et al.* (2007) found that growth of HepG2 cells in medium depleted of zinc resulted in a 40% decrease in p21 protein expression and a 70% decrease in p21 mRNA. Therefore, if low zinc decreases the expression of p21, less of this protein would be available to participate in RA-induced inhibition of cell proliferation.

Secondly, if zinc deficiency impairs zinc-finger binding, this may also apply to protein-protein interactions, as zinc-fingers are thought to mediate protein-protein binding (Gamsjaeger *et al.*, 2006). Cell cycle regulation involves numerous protein-protein interactions. For example, p21 is a zinc-finger protein, and the region required for binding to cyclins is within the zinc-finger domain of the protein (The UniProt Consortium, 2011). Impaired protein-protein interactions among cell cycle regulating proteins could potentially result in altered cell cycle progression.

Third, the expression of p21 is controlled by the zinc-finger protein, p53 (Hawkes and Alkan, 2011). Zinc deficiency has been shown to impair DNA binding ability of p53 (Ho and Ames, 2002). Recently it was discovered that RA induces hypomethylation in the

promoter region of cell cycle regulatory genes, including p21, via down-regulation of DNA methyltransferases. This hypomethylation facilitates the binding of p53 to the p21 promoter, resulting in up-regulation of p21 and subsequent blockage in the G1-phase and cell cycle arrest (Lim *et al.,* 2011). Therefore, it is possible that impaired DNA-binding ability of p53 could lead to impaired up-regulation of p21 and interference with the inhibition of cell proliferation.

2.4.5. Summary

In summary, my thesis research revealed that growth in zinc-deficient medium is sufficient to reduce intracellular zinc levels in HepG2 cells. When HepG2 cells grown in low-, adequate-, or high-zinc medium were subsequently treated with RA, it appeared that zinc sensitized HepG2 cells to RA-induced inhibition of cell proliferation. However, the mechanism behind this interaction remains unclear. Zinc deficiency increased expression of one nuclear receptor, RXRα, however it is unknown if this protein is involved in RA-induced inhibition of cell proliferation. Further, the function of the nuclear receptors, measured as RA-induced expression of two downstream targets, RARβ and CYP26a1, did not appear to be affected by zinc status.

Table 2.1: The effect of retinoic acid on proportion of cells in the G1- and S-phases following zinc treatment.*

Retinoic Acid Treatment Duration (h)	Proportion of cells in the S, G1, and G2/M phases of the cell cycle (%)		
	Low	Adequate	High
0	S= 19 G1=67 G2/M=12	S= 20 G1=57 G2/M=21	S= 22 G1=58 G2/M=17
24	S= 16 G1=63 G2/M=16	S= 13 G1=69 G2/M=14	S= 9 G1=68 G2/M=18
72	S= 9 G1=68 G2/M=20	S= 8 G1=71 G2/M=17	S= 6 G1=70 G2/M=20

*Data from the cell cycle analysis shown in Figure 2.6 was used in this analysis.

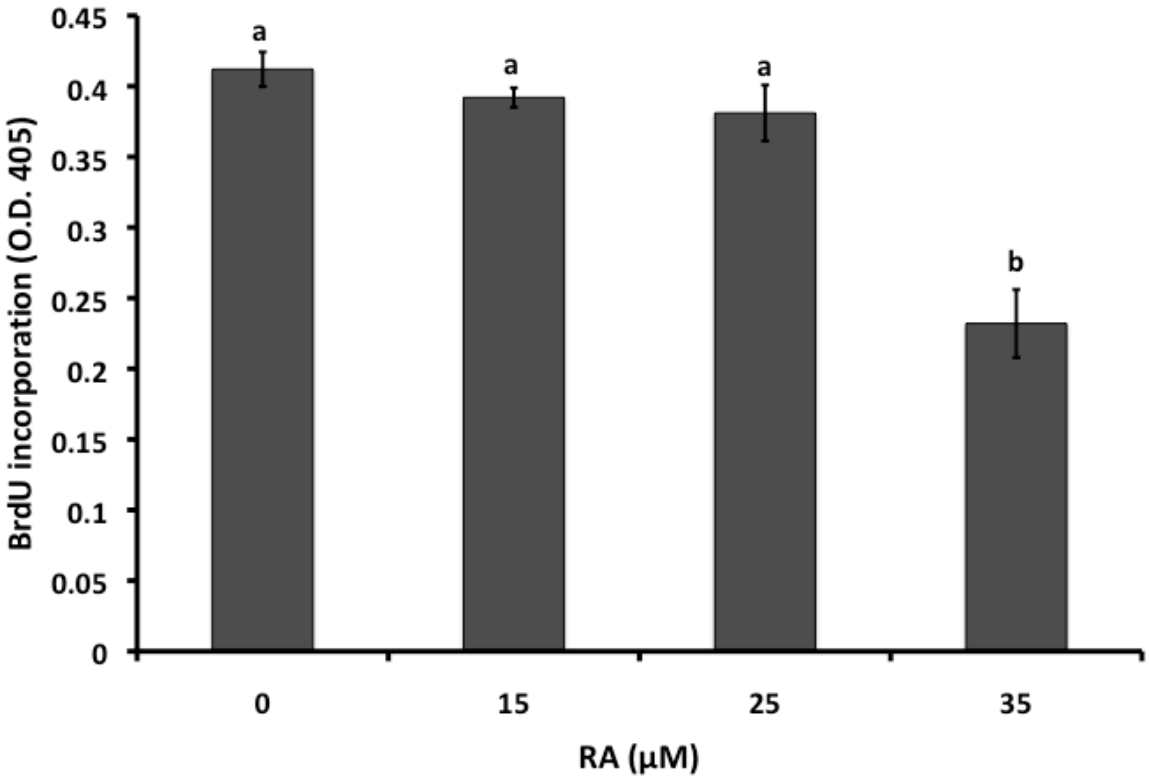

Figure 2.1: Cell proliferation of HepG2 cells treated with retinoic acid. HepG2 cells were cultured in medium supplemented with 0, 15, 25 and 35 µM retinoic acid (RA) for 12 h. Cell proliferation was quantified using the BrdU incorporation assay with absorbance as a measure of BrdU incorporation. Values represent mean ± SEM (n=6; the experiment was repeated once independently). Means with different letters are significantly different (*p<0.05).*

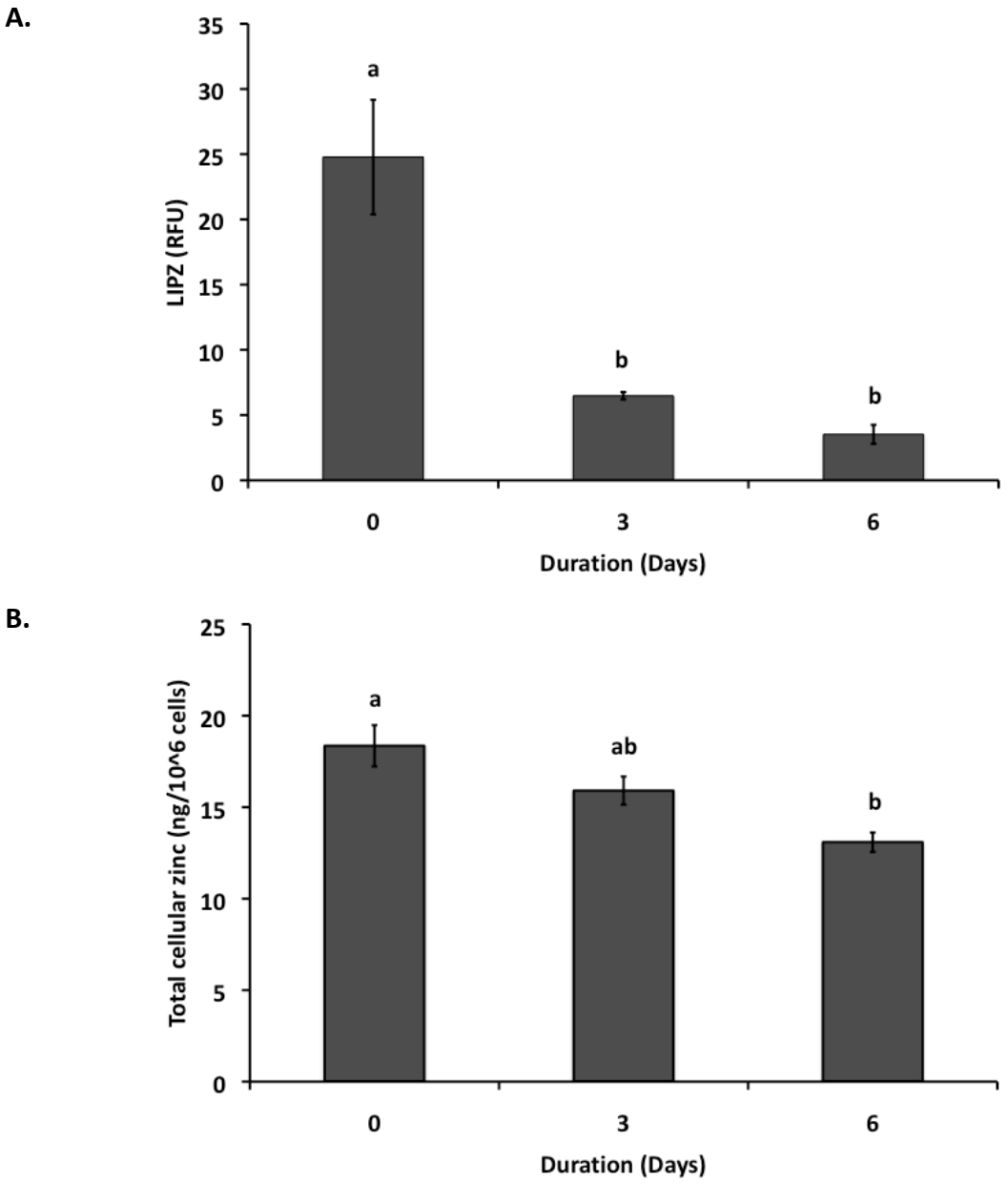

Figure 2.2: Time-dependent reduction in total cellular zinc level and the abundance of the labile intracellular pool of zinc. HepG2 cells were grown in media supplemented with Chelex-100 treated FBS for 0, 3 and 6 days. (A) Labile intracellular pools of zinc (LIPZ) as measured by Zinquin fluorescent assay. (B) Total cellular zinc as measured by atomic absorption spectrophotometry. Values represent mean ± SEM (n=3; the experiment was repeated once independently). Means with different letters are significantly different (*p<0.05).*

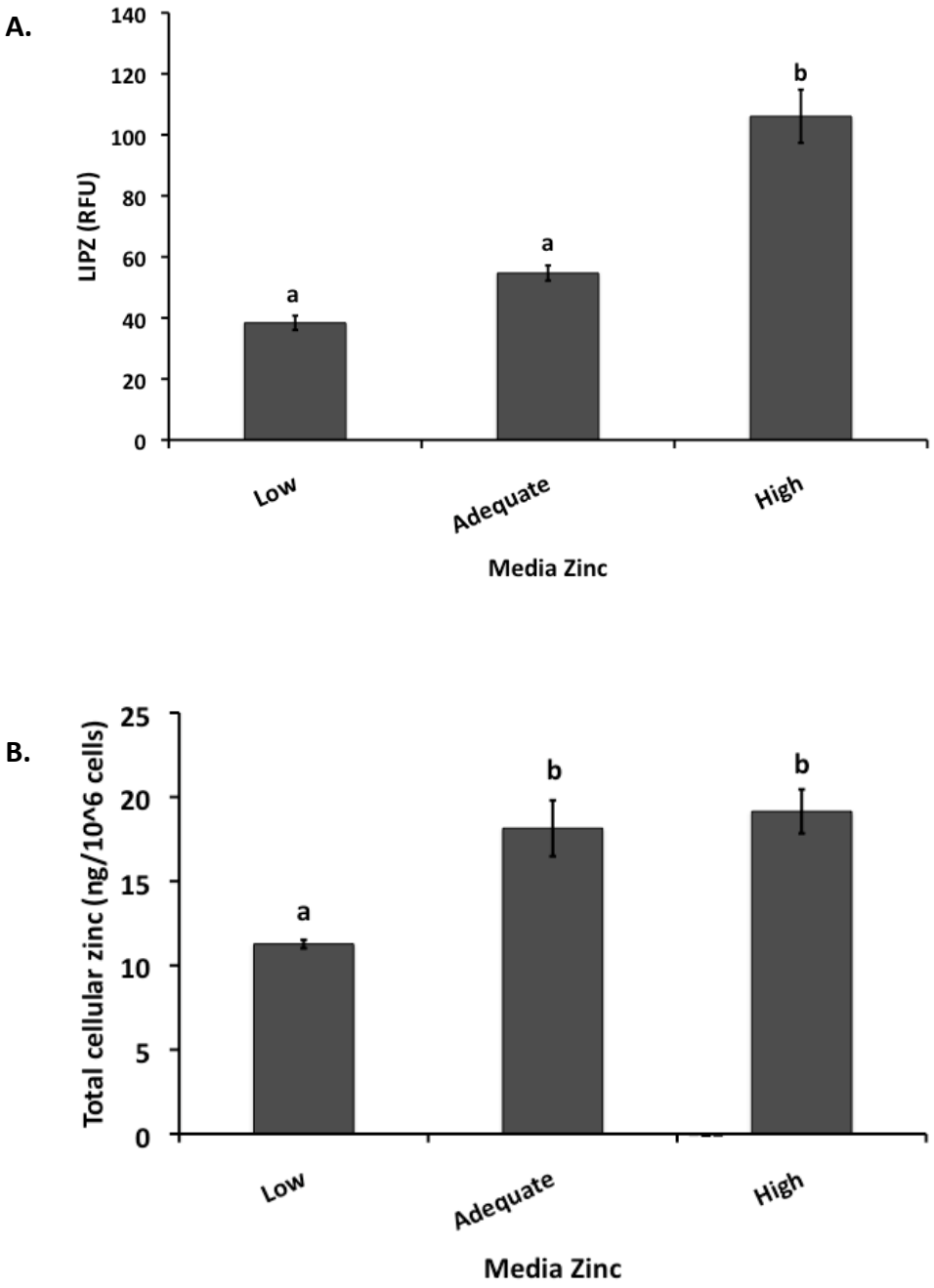

Figure 2.3: Dose-dependent reduction in total cellular zinc level and the abundance of the labile intracellular pool of zinc. HepG2 cells were grown in media containing Chelex-100-treated FBS and 0 (Low), 5 (Adequate), and 10 (High) µM of Zn ($ZnSO_4$) for 6 days. (A) Total cellular zinc as measured by atomic absorption spectrophotometry. (B) Labile intracellular pools of zinc (LIPZ) as measured by the Zinquin fluorescent assay. Values represent mean ± SEM (n=3; the experiment was repeated once). Means with different letters are significantly different (*$p<0.05$).*

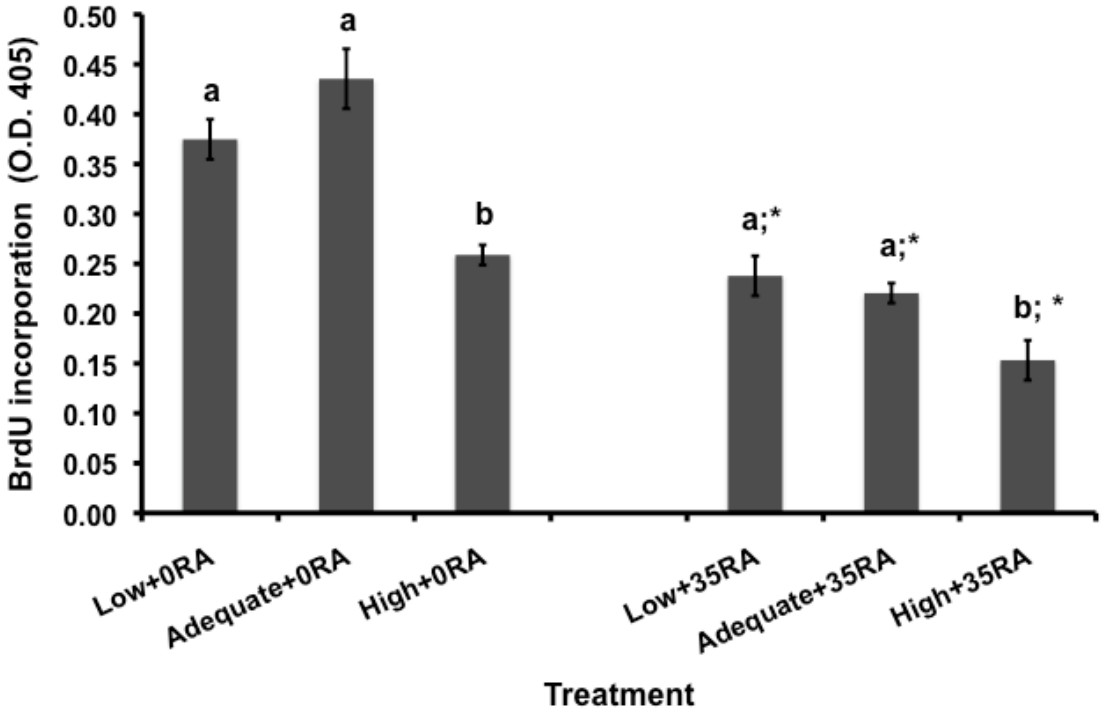

Figure 2.4: The effect of retinoic acid on the proliferation of HepG2 cells following growth in low-, adequate-, and high-zinc medium. HepG2 cells were grown in media containing Chelex 100-treated FBS plus 0 (Low), 5 (Adequate), and 10 (High) µM Zn ($ZnSO_4$) for 6 days followed by 0 or 35 µM retinoic acid (RA) for 12 h. Cell proliferation was quantified using the BrdU colorimetric assay with absorbance as a measure of BrdU incorporation. Values represent mean ± SEM (n=6). Means with different letters are significantly different. Asterisks represent a significant difference between the RA group and its respective control (*p<0.05)*.

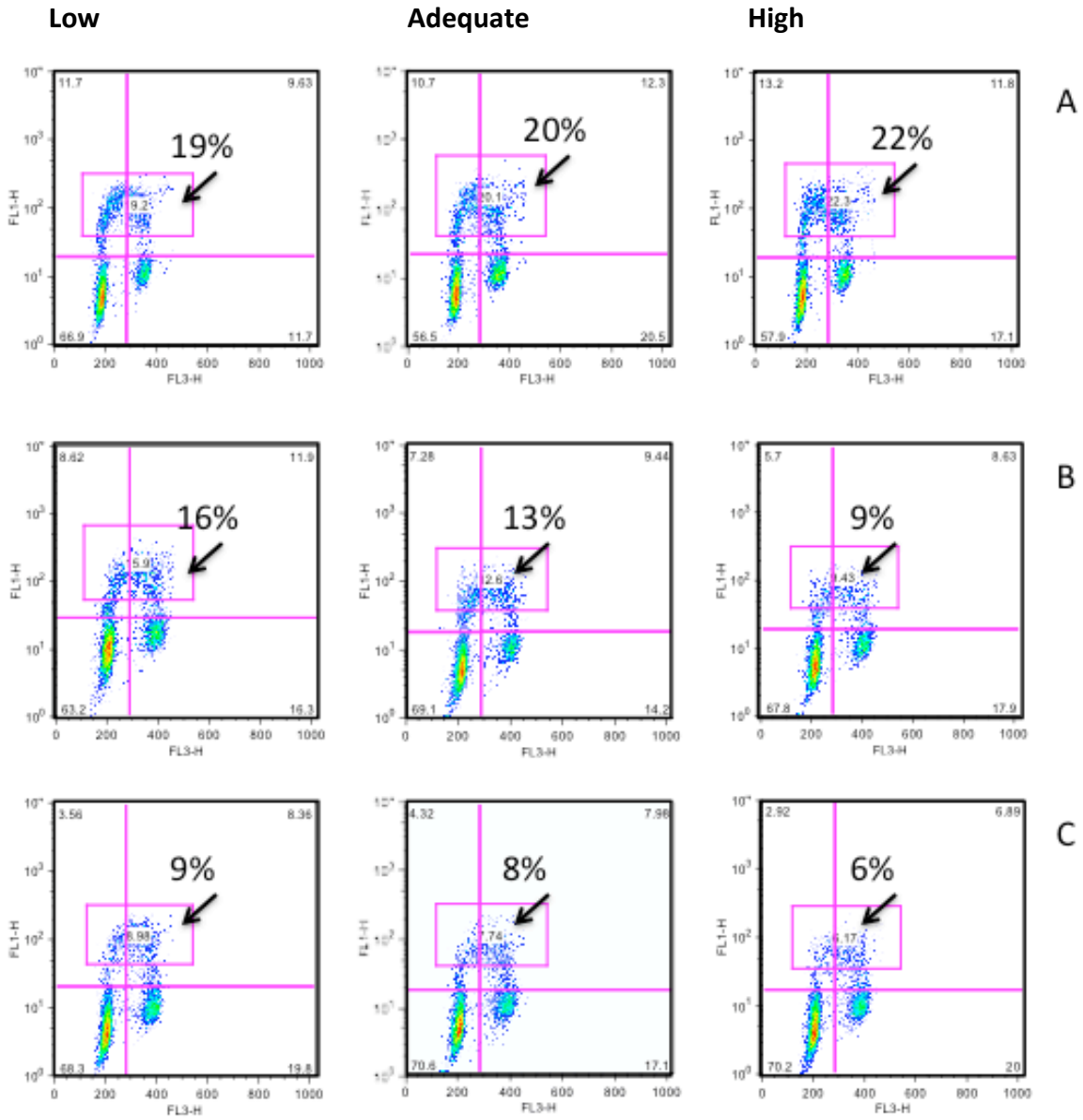

Figure 2.5: The effect of RA on the percentage of cells in the S-Phase following growth in low-, adequate-, and high-zinc medium. HepG2 cells were grown in media containing Chelex-100-treated FBS plus 0 (Low), 5 (Adequate), and 10 (High) µM of Zn ($ZnSO_4$) for 6 d followed by 35 µM of RA for 0 (A), 24 (B) and 72 (C) h. Cell cycle analysis was performed by BrdU incorporation for determination cells within S-phase and PI staining for total DNA content using flow cytometry. Cells in the S-phase, G1/G0 and G2/M phases are located within the small box, lower left quadrant, and lower right quadrant, respectively.

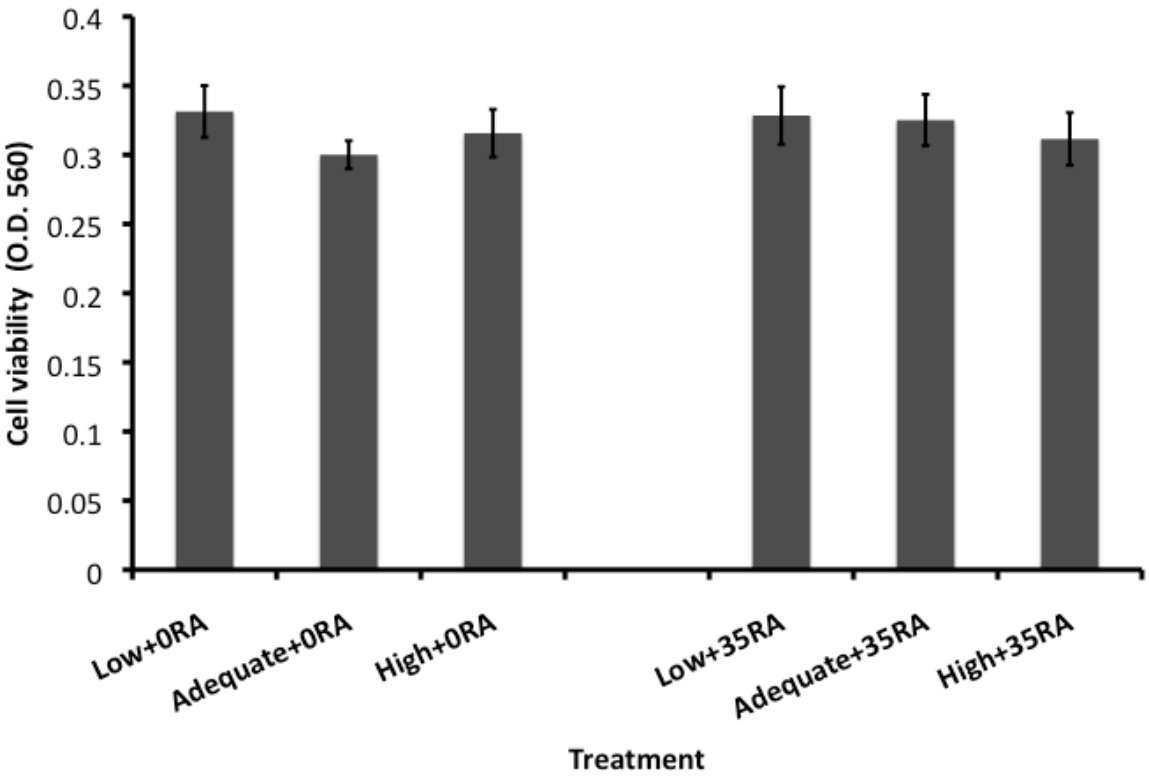

Figure 2.6: The effect of retinoic acid on viability of HepG2 cells following growth in low-, adequate-, and high-zinc medium. HepG2 cells were grown in media containing Chelex-100-treated FBS plus 0 (Low), 5 (Adequate), and 10 (High) µM of Zn ($ZnSO_4$) for 6 d followed by 0 or 35 µM of retinoic acid (RA) for 72 h. Cell viability was quantified using the MTT colorimetric assay with absorbance as a measure of cell viability. Values represent mean ± SEM (n=12). No significant difference among the treatment groups was identified (*p< 0.05*)

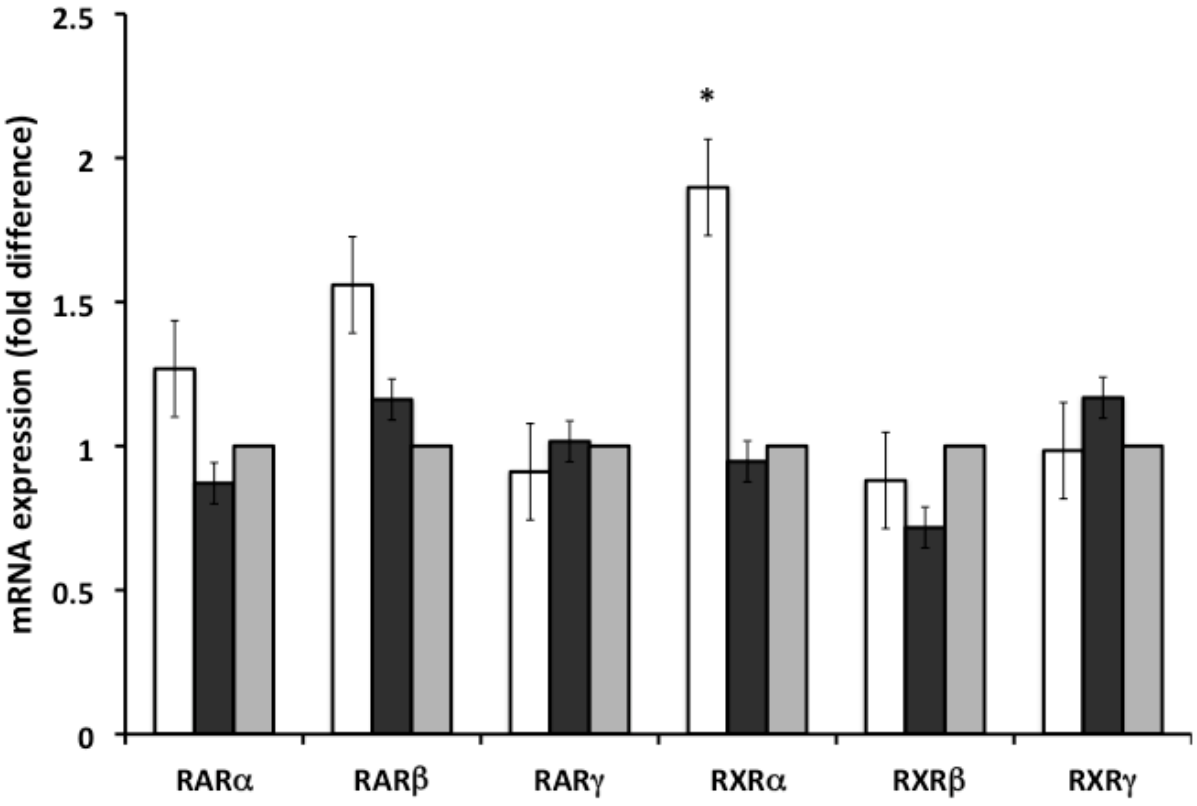

Figure 2.7: The relative expression of retinoic acid receptor (RAR) and retinoid X receptor (RXR) nuclear receptor mRNA in HepG2 following growth in low- and high- zinc media. HepG2 cells were grown in media containing Chelex-100-treated FBS plus 0 (Low; open bars), 10 (High; black bars) and 5 (Adequate; control; grey bars) µM of Zn ($ZnSO_4$) for 6 d. Values represent mean fold difference in comparison to adequate-zinc group ± SEM (n=4; the experiment was repeated once independently). The asterisk indicates a statistically significant difference in ΔΔCT value from adequate zinc treatment.

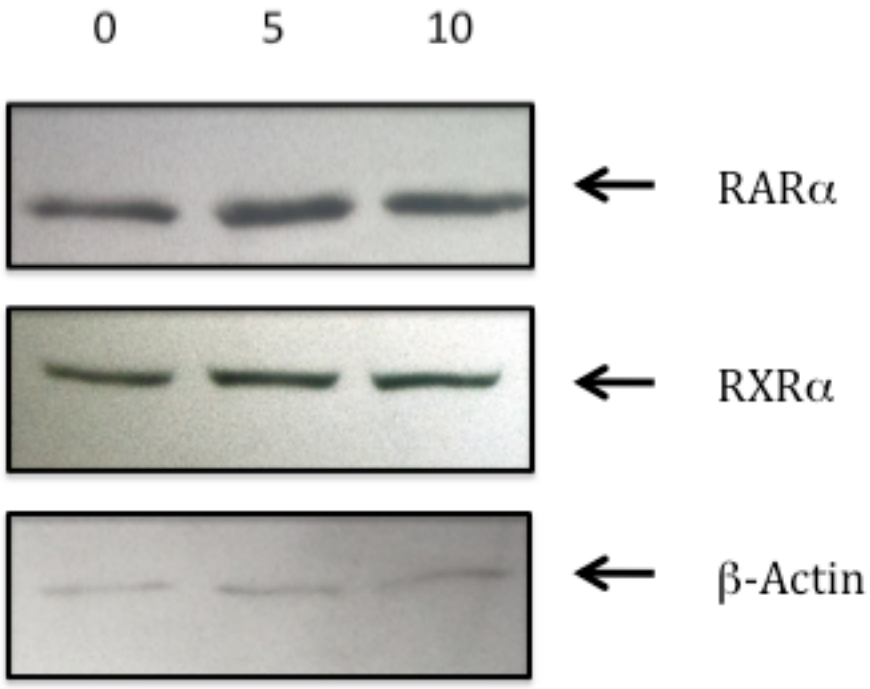

Figure 2.8: Western blot of retinoid X receptor-α (RXRα) and retinoic acid receptor-α (RARα) following growth in low-, adequate-, and high-zinc medium. HepG2 cells were grown in media supplemented with Chelex 100-treated FBS plus 0 (Low), 5 (Adequate) or 10 (High) μM of Zn ($ZnSO_4$) for 6 d. Protein levels were assessed using Western blot analysis. Following probing with anti-RARα antibody, the membrane was stripped and re-probed with β-actin antibody to ensure equal protein loading. The same protein samples were used for RARα and RXRα Western blot analysis.

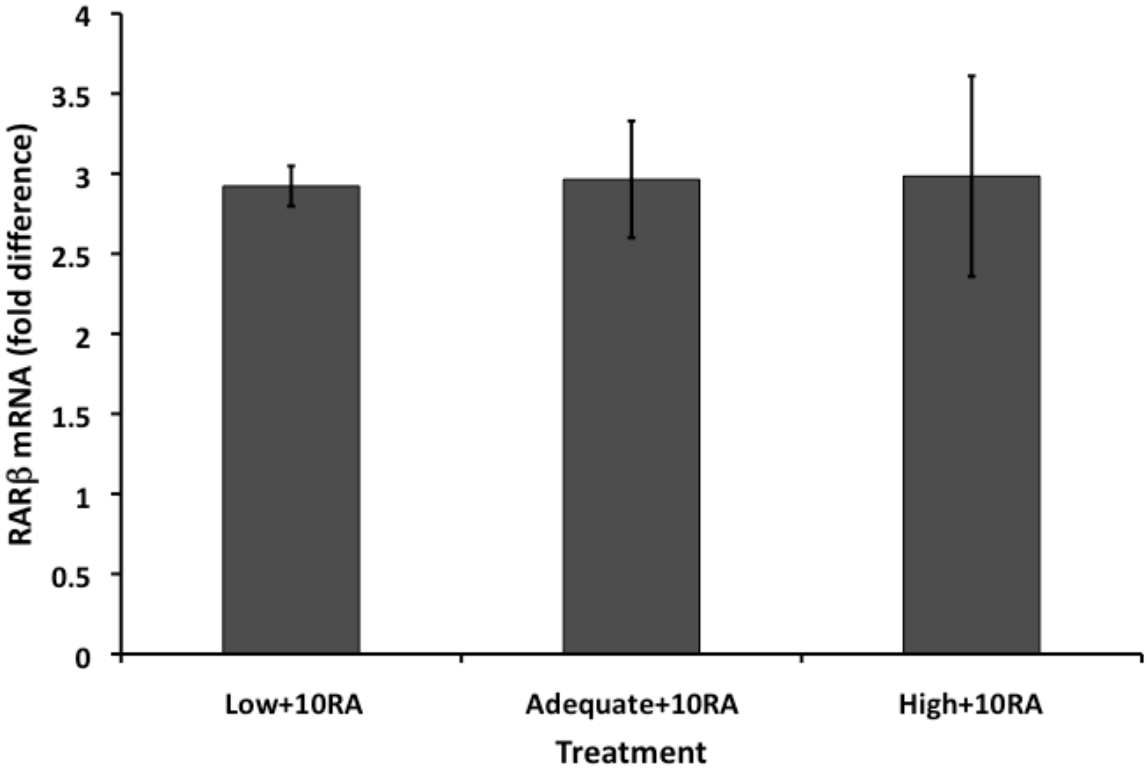

Figure 2.9: The relative expression of retinoic acid receptor-β (RARβ) mRNA in retinoic acid-treated HepG2 cells following growth in low-, adequate-, and high-zinc medium. HepG2 cells were grown in media containing Chelex 100-treated FBS plus 0 (Low), 5 (Adequate) or 10 (High) μM of Zn ($ZnSO_4$) for 6 d and subsequently treated with 0 or 10 μM retinoic acid (RA) for 6 h. Values represent mean fold difference in comparison to control (adequate zinc treatment + 0 μM RA) ± SEM (n=4; the experiment was repeated once). No statistically significant difference in ΔΔCT value was observed when compared to control.

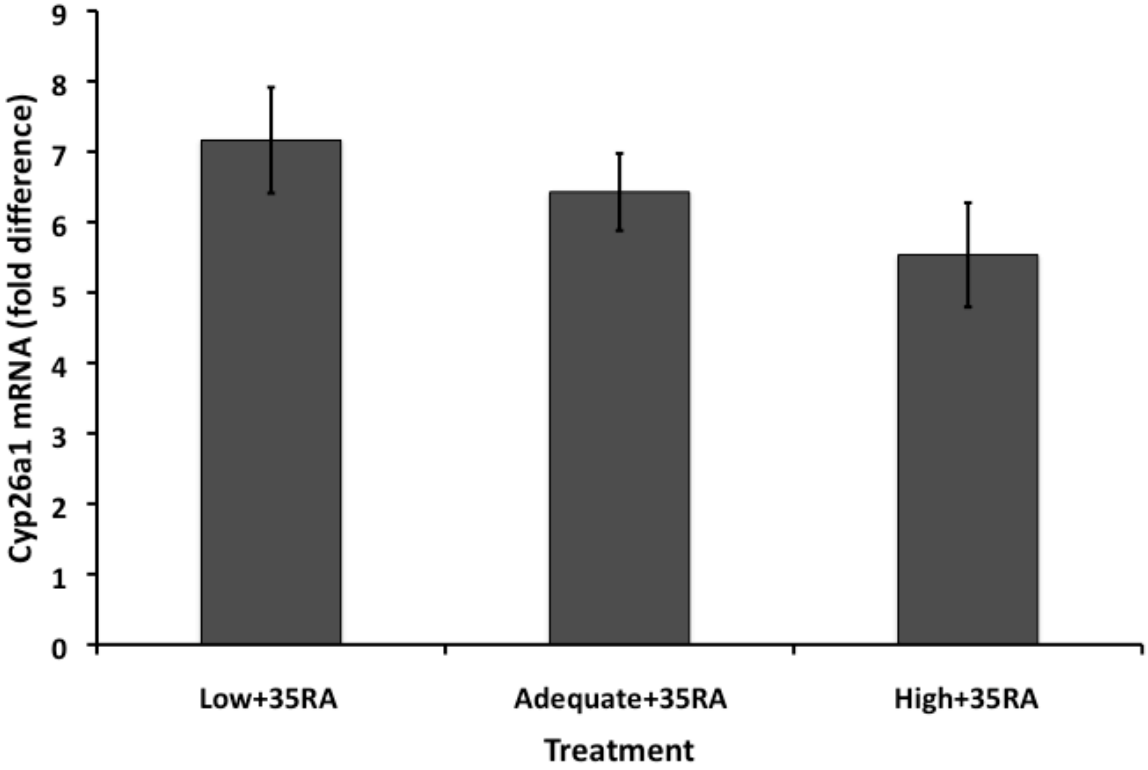

Figure 2.10: The relative expression of CYP26a1 mRNA in retinoic acid-treated HepG2 cells following growth in low-, adequate-, and high-zinc medium. HepG2 cells were grown in media containing Chelex 100-treated FBS plus 0 (Low), 5 (Adequate) or 10 (High) µM of Zn ($ZnSO_4$) for 6 d and subsequently treated with 0 or 35 µM of retinoic acid (RA) for 24 hours. Values represent mean fold difference in comparison to control (adequate zinc treatment + 0 µM RA) ± SEM (n=4; the experiment was repeated once). No statistically significant difference in ΔΔCT value was observed when compared to control.

Chapter 3. General Discussion, Limitations, and Future Directions

3.1. General Discussion

Initial experiments were performed in AML12, a murine liver cell line, in order to compare zinc levels between HepG2 and a non-cancerous liver cells and whether a difference in zinc level is correlated with sensitivity to RA treatment. During these initial experiments I noted that increased cellular zinc was correlated with an increased sensitivity to RA treatment. For example, the total cellular zinc levels in HepG2 were much lower than those observed in AML12 (about 75% less zinc; Figure A.1). Interestingly, lower concentrations of RA were required to inhibit growth in AML12 than HepG2. A reduction in cell proliferation was seen at as low as 15 μM of RA treatment in AML12, whereas reduction in cell proliferation was not observed until 35μM of treatment in HepG2 cells (Figure A.2; Figure 2.1). In addition, cell viability was reduced in AML12 after 72 h of RA treatment, whereas no reduction was seen in HepG2 (Figure A.3, Figure 2.6). This relationship between zinc level and sensitivity to RA treatment was further supported by the observation that growth in high-zinc medium sensitized HepG2 hepatocarcinoma cells to RA-induced reduction in cell proliferation.

One possibility for an interaction between zinc and vitamin A at the cellular level is that zinc deficiency impairs the DNA-binding ability of RAR/RXR receptors, as an effect of zinc on the binding ability of other zinc-finger proteins, including p53 and AP-1, has been

observed (Ho and Ames, 2002). However, my observation that zinc level did not affect the RA-induced expression of the downstream targets, RARβ and CYP26a1, fails to support this hypothesis. This suggests that other mechanisms are likely present. These mechanisms could include the influence of zinc on the expression or function of other proteins involved in regulation of the cell cycle, such as p21, a cell cycle regulator.

The results of my thesis point to the possibility that zinc may be important in the growth-suppressant function of RA in HepG2. This observation brings to light the possibility that a similar phenomenon could be present in other tumor and cell types. In one study, zinc deficiency impaired the RA-induced differentiation of neuronal cells (Gower-Winters, 2008). If it is the case that zinc deficiency impairs the function of RA in other cancer cell types, then it would be important to ensure that the patients' zinc levels are adequate for optimum RA function.

However, in order for zinc manipulation to be a viable method of increasing the sensitivity of cancer cells to RA treatment, it would be necessary for the sensitivity of non-cancerous cells to be unaffected, or decreased, by zinc. In my comparisons between the effects of zinc treatment on RA-induced reduction in cell proliferation between HepG2 and AML12 cells, I observed that, similar to HepG2, high zinc treatment resulted in a greater reduction in cell proliferation following RA treatment in AML12 cells (figure A.4). This observation suggests that zinc treatment may not be an appropriate method of increasing the sensitivity of liver cancer to RA treatment, however, because AML12 is a murine cell

line, confirmation of a similar response in a non-cancerous human liver cell line is necessary.

Currently, RA is used clinically in the treatment of acute promyelocytic leukemia (APL), head and neck carcinoma, and non-small-cell lung cancer (Bohnsack and Hirschi, 2004). In APL, treatment induces differentiation and often results in complete remission (McNamara *et al.*, 2010). However, these results are not long lasting, as prolonged treatment leads to the emergence of RA resistance (Tallman *et al.,* 2002). In addition, in about 2-27% of patients, RA treatment can have severe side effects, including acute renal failure and central nervous system toxicity, with about 2% of treatments resulting in death (Vanier *et al,* 2003; Patatanian and Thompson, 2008). In the case of APL, it is understood that in 33% of cases, resistance is brought on by an acquired mutation, however, in the remaining 67%, the mechanisms of resistance are unknown (McNamara *et al.,* 2010).

It has been proposed that a major contributing factor to acquired RA resistance is the induction of CYP26a1 (a P450 enzyme) in tissues such as the liver (Thatcher and Isoherranen, 2009). In cases of resistance, instead of increasing RA dosage, P450 inhibitors are co-administered to prevent the metabolism and clearance of RA (Sun and Lotan*,* 2002). These inhibitors are referred to as RA metabolism blocking agents (RAMBAs; Verfaille *et al.,* 2008). In one case study, co-administration of fluconazole (a P450 inhibitor) led to a 70% reduction in RA dosage (Vanier *et al.*, 2003).

An attractive alternative to administering additional drugs would be manipulation of nutrient intake, such as zinc. According to my results, zinc status did not affect RA-induced CYP26a1 expression in HepG2 liver cells. Therefore, zinc manipulation does not appear to be a viable option for controlling RA metabolism through CYP26a1 expression.

Because inadequate zinc is so widely prevalent in the developed and under-developed world (Prasad, 2003; Song *et al.*, 2009), it is important to understand the implications of zinc on the function of other nutrients. Since vitamin A has many crucial functions within the body and RA is used as a cancer treatment (Bohnsack and Hirschi, 2004; Mark *et al.*, 2006), an understanding of the implications of zinc status on vitamin A function is important, especially if zinc manipulation can alter the sensitivity of cancer cells to RA treatment.

3.2. Limitations

There are several limitations apparent in my thesis research. The first limitation is the use of an *in vitro* system. Cell systems do not replicate the complexity of whole body systems, therefore it is difficult to extrapolate the results of this project.

The second limitation is the method of zinc depletion. In order to mimic low-zinc conditions, zinc was removed by treating the FBS with Chelex-100 resin. The filtration steps required during preparation of the low zinc FBS may have removed small peptides which could potentially be important in cellular processes. Also, it is possible that zinc within the

FBS bound to low molecular weight proteins was removed by the Chelex-100 resin. This zinc was subsequently replaced by free zinc. Free zinc and zinc-bound to proteins may have different availability to the cell.

Thirdly, the treatment durations were relatively short-term, with a maximum of 72 h. This is likely not reflective of a clinical treatment situation, where treatment durations are weeks to months (Huang *et al.*, 1988).

Finally, the cell cycle analysis experiment was performed only once. Although each treatment consists of the analysis of 10,000 individual cells, a repeat of the experiment would strengthen the observation.

3.3. Future Directions

I examined the gene expression of the RAR and RXR receptors in response to zinc manipulation. However, the level of these receptors does not necessarily indicate a change in function. Therefore, it would be valuable to investigate the effects of zinc status on the DNA-binding ability of these zinc-finger proteins using protein-DNA binding assays such as the electromobility shift assay (EMSA), which is used to detect protein complexes with nucleic acids (Hellman and Fried, 2007). This process was described by Ho and Ames (2002). I had attempted to perform an EMSA, however could not obtain useable results due to technical challenges. Alternatively, a new method of assessing protein-DNA binding, negative-ion electrosparay ionization mass spectrometry, is described by Park *et al.* (2011).

Since zinc has wide ranging effects on gene expression, it is plausible that other proteins involved in the metabolism, transport and function of vitamin A may be affected by zinc status. Therefore it would be interesting to investigate the effect of zinc status on other vitamin A related proteins, including transport proteins such as CRABPII, which is necessary for the transport of RA into the nucleus (Donato *et al.,* 2007).

RA as a cancer treatment has toxic side effects (Fabricius *et al.*, 2011). It may be beneficial to research the effects of zinc on RA-induced growth suppression in other cancerous and non-cancerous cell types including human hepatic and extrahepatic cells, as well as whole body animal models. Increasing the sensitivity of cancerous cells or decreasing the sensitivity of non-cancerous cells to the effects of RA during cancer treatment could be an attractive method of attenuating the toxic effects of this treatment regime or could perhaps postpone the onset of resistance.

BIBLIOGRAPHY

Abnet, C.C., Lai, B., Qiao, Y-L., Vogt, S., Luo, X-M., Taylor, P.R., Dong, Z-W., Mark, S.D., & Dawsey, S.M. (2005). Zinc concentration in esophageal biopsy specimens measured by x-ray fluorescence and esophageal cancer risk. *Journal of the National Cancer Institute,* 97, 301-306.

Alisi, A., Leoni, S., Piacentani, H. & Conti Devirgiliis, L. (2003). Retinoic acid modulates the cell cycle in fetal rat hepatocytes and HepG2 cells by regulating cyclin-cdk activities. *Liver International*, 23, 179-186.

Alison, M.R., Nicholson, L.J., & Lin, W.R. (2011). Chronic inflammation and hepatocellular carcinoma. *Recent Results in Cancer Research,* 185, 135-148.

Alsayed, Y., Uddin, S., Mahmud, N., Lekmine, F., Kalvakolanu, D.V., Minucci, S., Bokoch, G., & Platanias, L.C. (2001). Activation of Rac1 and p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. *Journal of Biological Chemistry,* 276, 4012-4019.

Altekruse, S.F., McGlynn, K.A., & Reichman, M.E. (2009). Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975-2005. *Journal of Clinical Oncology,* 27, 1485-1491.

Altucci, L., Rossin, A., Raffelsberger, W., Reitmar, A., Chomienne, C. & Gronemeyer, H. (2001). Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. *Nature Medicine*, 7, 680-686.

Arce, F., Gatjens-Boniche, O., Vargas, E., Valverde, B., & Diaz, C. (2005). Apoptotic events induced by naturally occurring retinoids ATRA and 13-cis retinoic acid on human hepatoma cell lines Hep3B and HepG2. *Cancer Letters,* 229, 271-281.

Blomhoff, R. & Blomhoff, H.K. (2006). Overview of retinoid metabolism and function. *Journal of Neurobiology*, 66, 606-.630

Bohnsack, B.L. & Hirschi, K.K. (2004). Nutrient regulation of cell cycle progression. *Annual Review of Nutrition,* 24, 433-53.

Boron, B., Hupert, J., Barch, D.H., Fox, C.C., Friedman, H., Layden, T.J., & Mobarhan, S. (1988). Effect of zinc deficiency on hepatic enzymes regulating vitamin A status. *Journal of Nutrition*, 118, 995-1001.

Brown E.D., Chan W., & Smith Jr J.C. (1976). Vitamin A metabolism during the repletion of zinc deficient rats. *Journal of Nutrition,* 106, 563.

Brown, K.H., Wuehler, S.E., & Peerson, J.M. (2001). The importance of zinc in human nutrition and estimation of the global prevalence of zinc deficiency. *Food and Nutrition Bulletin,* 22, 113-125.

Christian, P., Khatry, S.K., Yamini, S., Stallings, R., LeClerq, S.C., Shrestha, S.R., Pradhan, E.K., & West, Jr. K.P. (2001). Zinc supplementation might potentiate the effect of vitamin A in restoring night vision in pregnant Nepalese women. *American Journal of Clinical Nutrition*, 73, 1045-32.

Christian, P. & West, Jr. K.P. Interactions between zinc and vitamin A: An update. (1998). *American Journal of Clinical Nutrition*, 68, 435S-441S.

Clagett-Dame, M., & Deluca, H.F. (2002). The role of vitamin A in mammalian reproduction. *Annual Review of Nutrition,* 22, 347-381.

Corniola, R.S., Tassabehji, N.M., Hare, J., Girdhari, S., Levenson, C.W. (2008). Zinc deficiency impairs neuronal precursor cell proliferation and induces apoptosis via p53-mediated mechanisms. *Brain Research,* 1237, 52-61.

Costello, L.C., & Franklin, R.B. (2006). The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. *Molecular Cancer,* 5, 17.

Cousins, R.J., Blanchard, R.K., Popp, M.P., Liu, L., Cao, J., Moore, J.B., & Green, C.L. (2003). A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. *Proceedings of the National Academy of Sciences USA*, 100, 6952-6957.

Cousins, R.J., Liuzzi, J.P., & Lichten, L.A. (2006). Mammalian zinc transport, trafficking, and signals. *Journal of Biological Chemistry,* 281, 24085-4089.

Darlow, B.A & Graham, P.J. (2007). Vitamin A supplementation to prevent mortality and short and long-term morbidity in very low birthweight infants. *Cochrane Database of Sytematic Reviews,* 4, CD000501.

Dieck, H., Doring, F., Roth, H.P. & Daniel, H. (2003). Changes in rat hepatic gene expression in response to zinc deficiency as assessed by DNA arrays. *Journal of Nutrition*,133, 1004-1010.

Donato, L.J., Suh, J.H., & Noy, N. (2007). Suppression of mammary carcinoma cell growth by retinoic acid: the cell cycle control gene Btg2 is a direct target for retinoic acid receptor signaling. *Cancer Research,* 67, 609-615.

Dragnev, K.H, Rigas, J.R., & Dmitrovsky, E. (2000). The retinoids and cancer prevention mechanisms. *The Oncologist,* 5, 361-368.

Duncan, Jr & Hurley, L.S. (1978). An interaction between zinc and vitamin A in pregnant and fetal rats. *Journal of Nutrition,* 108, 1431-1438.

Ebara, M., Fukuda, H., Hatano, R., Saisho, H., Nagato, Y., & Suzuki, H. (2000). Relationship between copper, zinc, and metallothionein in hepatocellular carcinoma and its surrounding liver parenchyma. *Journal of Hepatology,* 33, 415-422.

Ebara, M., Fukuda, H., Hatano, R., Saisho, H., Nagato, Y., & Suzuki, H. (2000). Relationship between copper, zinc, and metallothionein in hepatocellular carcinoma and its surrounding liver parenchyma. *Journal of Hepatology,* 33, 415-422.

Eisele, F. & Wolf, D.H. (2008). Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. *FEBS Letters,* 582, 4143-4146.

Estner, E., Muller, C., Koshizuka, K., Williamson, E.A., Park, D., Asou, H., Shintaku, P., Said, J.W., Heber, D., & Koeffler, H.P. (1998). Ligands for peroxisome proliferator-activated receptor and retinoic acid receptor inhibit and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proceedings of the National Acadamy of Sciences,* 95, 8806-8811.

Fabricius, E., Kruse-Boitschenko, U., Schneeweiss, U., Wildner, G., Hoffmeister, B., & Raguse, J. (2011). Model examination of chemoprevention with retinoids in squamous cell carcinomas of the head and neck region and suitable biomarkers for chemoprvention. *International Journal of Oncology,* 39, 1083-1097.

Falsca, L., Marcellini, P., Ara, C., Rufu, A., & Devirgiliis, L.C. (1999). Growth inhibition and induction of specific hepatic phenotype expression by retinoic acid in HepG2 cells. *Anticancer Research,* 19, 3283-3292.

Fitzgerald P., Teng M., Chandraratna R.A., Heyman R.A., & Allegretto E.A. (1997). Retinoic acid receptor alpha expression correlates with retinoid-induced growth inhibition of human breast cancer cells regardless of estrogen receptor status. *Cancer Research,* 57, 2642-50.

Franklin, R.B., Levy, B.A., Zou, J., Hanna, N., Desouki, M.M., Bagasra, O., Johnson, L.A. & Costello, L.C. (2007). ZIP14 zinc transporter downregulation and zinc depletion in the development and progression of hepatocellular cancer. *Journal of Gastrointestinal Cancer,* doi: 10.1007/s12029-011-9269.

Franklin, R.B., & Costello, L.C. (2007). Zinc is an antitumor agent in prostate cancer and in other cancers. *Archives of Biochemistry and Biophysics,* 463, 211-217.

Franklin, R.B. & Costello, L.C. (2009). The important role of the apoptotic effects of zinc in the development of cancers. *Journal of Cellular Biochemistry,* 106, 750-757.

Fulan, H., Changxing, J., Baina, W.Y. Wencui, Z., Chunquing, L., Fan, W., Dandan, L., Dianjun, S., Tong, W., Da, P., & Yashuang, Z. (2011). Retinol, vitamins A, C, and E and breast cancer risk: a meta-analysis and meta-regression. *Cancer Causes Control,* 22, 1383-1396.

Gamsjaeger, R., Liew, C.K., Loughlin, F.E., Crossley, M. & Mackay, J.P. (2006). Sticky fingers: zinc-fingers as protein-recognition motifs. *Trends in Biochemical Sciences,* 32, 63-70.

Gower-Winters S.D. (2008). Zinc deficiency impairs retinoic acid-induced differentiation of human neurons. Florida State University (Thesis). http://etd.lib.fsu.edu/ theses/available/etd-04142008-142329/ (accessed 12 Mar, 2008).

Gurusamy, K., & Davidson, B.R. (2007). Trace element concentration in metastatic liver disease – A systematic review. *Journal of Trace Elements in Medicine and Biology,* 21, 169-177.

Haase, H., Mazzatti, D.J., White, A., Ibs, K.H., Engelhardt, G., & Silke, H. (2007). Differential gene expression after zinc supplementation and deprivation in human leukocyte subsets. *Molecular Medicine*, 13, 362-370.

Hainaut, P. & Milner, J. (1993). A structural role for metal ions in the "wild-type" conformation of the tumor suppressor protein p53. *Cancer Research,* 53, 1739-1742.

Hawkes, C.W. & Alkan, Z. (2011). Delayed cell cycle progression from SEPW1 depletion is p53- and p21-dependent in MCF-7 breast cancer cells. *Biochemical and Biophysical Research Communications,* doi: 10.1016/j.bbrc.2011.08.032.

Health Canada. Reference Values for Vitamins. *Food and Nutrition.* November 2010. http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/ref_vitam_tbl-eng.php (accessed 30 Oct, 2011).

Hellman, L.M. & Fried, M.G. (2007). Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nature Protocols,* 2, 1849-1861.

Hess, S.Y. & Brown, K.H. (2009). Impact of zinc fortification on zinc nutrition. *Food and Nutrition Bulletin,* 30, S79-S107.

Hess, S.Y., Lonnerdal, B., Hotz, C., Rivera, J.A., & Brown, K. (2009). Recent advances in knowledge of zinc nutrition and human health. *Food and Nutrition Bulletin,* 30, S5-S11.

Ho, E. & Ames, B.N. (2002). Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkB, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *PNAS,* 99, 16770-16775.

Ho, E. (2004). Zinc deficiency, DNA damage and cancer risk. *Nutritional Biochemistry,* 15, 572-578.

Hua, S., Kittler, R. & White, K.P. (2009). Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. *Cell,* 137, 1259-1271.

Huang, M.E., Ye, Y.C., Chen, S.R., Chai, J.R., Lu, J.X., Zhoa, L., Gu, L.J., & Wang, Z.Y. (1988). Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood,* 72, 567-572.

Institute of Medicine. Zinc. In: Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington, DC: National Academy Press; 2000. P. 442-501.

Jackson, K.A., Valentine, R.A., Coneyworth, L.J, Mathers, J.C. & Ford, D. (2008). Mechanisms of mammalian zinc-regulated gene expression. *Biochemical Society Transactions,* 36, 1262-1266.

Kambe, T., Yamaguchi-Iwai, Y., Sasaki, R., & Nagao, M. (2004). Overview of mammalian zinc transporters. *Cellular and Molecular Life Sciences,* 61, 49-68.

Kindermann, B., Doring F., Pfaffl, M., and Daniel, H. (2004). Identification of genes responsive to intracellular zinc depletion in the human colon adenocarcinoma cell line HT-29. *Journal of Nutrition*, 134, 57-62.

Klug, A., & Schwabe, J.W. (1995). Zinc fingers. *FASEB Journal,* 9, 597-604.

Kolenko, V.M., Uzzo, R.G., Dulin, N., Hauzman, E., Bukowski, R., & Finke, J.H. (2001). Mechanism of apoptosis induced by zinc deficiency in peripheral blood T lymphocytes. *Apoptosis,* 6, 419-429.

Larson, R.S. & Tallman, M.S. (2003). Retinoic acid syndrome: manifestations, pathogenesis, and treatment. *Best Practice & Research Clinical Haematology,* 16, 453-461.

Levenson, C.W. & Morris, D. (2011). Zinc and neurogenesis: making new neurons from development to adulthood. *Advances in Nutrition,* 2, 96-100.

Li, M., Zhang, Y., Liu, Z., Bharadwaj, U., Wang, H., Wang, X., Zhang, S., Liuzzi, J., Chang, S., Cousins, R.J., Fisher, W.E., Brunicardi, F.C., Logsdon, C.D., & Chen, C. (2007). Aberrant expression of zinc transporter ZIP4 (SLC39A4) significantly contributes to human pancreatic cancer pathogenesis and progression. *PNAS,* 104, 18636-18641.

Lim, J.S., Park, S.H., & Jang, K.L. (2011). All-trans retinoic acid induces sellular senescence by up-regulating levels of p16 and p21 via promotor hypomethylation. *Biochemical and Biophysical Research Communications,* 412, 500-505.

Liu, Y., Lee, M., Wang, H., Li, Y., Hashimoto, Y., Klaus, M., Reed, C., & Zhang, X. (1997). Retinoic acid receptor ß mediates the growth inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Molecular and Cellular Biology,* 16, 1138-1149.

Liu, Y., Zhu, X., Zhu, J., Liao, S., Tang, Q., Liu, K., Guan, X., Zhang, J., & Feng, Z. (2007). Identification of a differential expression of genes in hepatocellular carcinoma by suppression subtractive hybridization combined cDNA microarray. *Oncology Reports,* 18, 943-951.

Logsdon, C.D., Simeone, D.M., Binkley, C., Arumugam, T., Greenson, J.K., Giordano, T.J., Misek, D.E., & Hanash, S. (2003). Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer Research,* 63, 2649-2657.

Loh, S.N. (2010). The missing zinc: p53 misfolding and cancer. *Metallomics,* 2, 442-449.

Maddika, S., Ande, S.R., Panigrahi, S., Paranjothy, T., Weglarczyk, K., Zuse, A., Eshraghi, M., Manda, K.D., Wiechec, E., & Los, M. (2007). Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. *Drug Resistance Updates,* 10, 13-29.

Mamoon, A., Ventura-Holman, T., Maher, J.F., & Subauste, J.S. (2008). Retinoic acid responsive genes in the murine hepatocyte cell line AML 12. *Gene*, 408, 95-103.

Manor, D., Shmidt, E.N., Budhu, A., Flesken-Nikitin, A., Zgola, M., Page, R., Nikitin, A.Y., & Noy, N. (2003). Mammary carcinoma suppression by cellular retinoic acid binding protein-II. *Cancer Research,* 63:4426-4433.

Mark, M, Ghyselinck, N.B., & Chambon, P. (2006). Function of retinoid nuclear receptors: Lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annual Review of Pharmacology and Toxicology,* 46, 451-480.

McAfee, A.J, McSorley, E.M., Cuskelly, G.J., Moss, B.W., Wallace, J.M.W., Bonham, M.P., & Fearon, A.M. (2010). Red meat consumption: An overview of the risks and benefits. *Meat Science,* 84, 1-13.

McCullough, F.S.W., Northrop-Clewes, C.A., & Thurnham, D.I. (1999). The effect of vitamin A on epithelial integrity. *Proceedings of the Nutrition Society,* 58, 289-293.

McNamara, S., Nichol, J.N., Wang, H., & Miller, W.H. (2010). Targeting PKCd-mediated topoisomerase IIb overexpression subverts the differentiation block in a retinoic acid-resistant APL cell line. *Leukemia,* 24, 729-739.

Miyazaki, M., Doi, Y., Ikeda, F., Ninomiya, T., Hata, J. Uchida, K., Shirota, T., Matsumoto, T., Iida, M., & Kiyohara, Y. (2011). Dietary vitamin A intake and incidence of gastric cancer in a general Japanese population: the Hisayama study. *Gastric Cancer,* doi: 10.1007/s10120-011-0092-7

Mocchegiani, E., Muzziolie, M., & Giacconi, R. (2000). Zinc and immunoresistance to infection in ageing: new biological tools. *Trends in Pharmacological Sciences,* 22, 112-113.

Mondul, A.M., Watters, J.L., Mannisto, S. Weinstein, S.J., Snyder, K., Virtamo, J., & Albanes, D. (2011). Serum retinol and risk of prostate cancer. *American Journal of Epidemiology,* 173, 813-821.

Nakanishi, M., Tomaru, Y., Miura, H., Hayashizaki. Y., & Suzuki, M. (2008). Identification of transcriptional regulatory cascades in retinoic acid-induced growth arrest of HepG2 cells. *Nucleic Acids Research,* 36, 3443-3454.

Osanai, M. & Petkovich, M. (2005). Expression of the retinoic acid-metabolizing enzyme CYP26Q1 limits programmed cell death. *Molecular Pharmacology,* 67, 1808-1817.

Osanai, M., Sawada, N., & Lee, G-H. (2010). Oncogenic and cell survival properties of the retinoic acid metabolizing enzyme, Cyp26a1. *Oncogene,* 29, 1135-1144.

Oteiza, P.I., Olin, K.L., Fraga, C.G., & Keen, C.L. (2000). Zinc deficiency induces oxidative stress and AP-1 activation in 3T3 cells. *Free Radical Biology and Medicine,* 213, 85-91.

Ozpolat, B., Mehta, K., & Lopez-Berestein, G. (2005). Regulation of a highly specific retinoic acid-4-hydroxylase (CYP26A1) enzyme and all-trans-retinoic acid metabolism in human intestinal, liver, endothelial, and acute promyelocytic leukemic cells. *Leukemia & Lymphoma,* 46, 1497-1506.

Park, S., Jo, K., & Oh, H.B. (2011) Zinc-finger motif noncovalent interactions with double-stranded DNA characterized by negative-ion electrospray ionization mass spectrometry. *Analyst,* 135, 3739-3746.

Patatanian, E. & Thompson, D.F. (2008). Retinoic acid syndrome: a review. *Journal of Clinical Pharmacy and Therapeutics,* 33, 331-338.

Prasad, A.S. (2003). Zinc deficiency. *British Medical Journal,* 326, 409-410.

Rana, U., Kothinti, R., Meeusen, J., Tabatabai, N.M., Krezoski, S. & Petering, D.H. (2008). Zinc binding ligands and cellular zinc trafficking: Apo-metallothionein, glutathione, TPEN, proteomic zinc, and zn-Sp1. *Journal of Inorganic Biochemistry,* 102, 489-499.

Rando, R.R. (1990). The chemistry of vitamin A and vision. *Angewandte Chemie International Edition in English,* 29, 461-480

Reaves, S.K., Fanzo, J.C., Arima, K., Wu, J.Y., Wang, Y.R., & Lei, K.Y. (2000). Expression of p53 tumor suppressor gene is up-regulated by depletion of intacellular zinc in HepG2 cells. *Journal of Nutrition,* 130, 1688-1694.

Rizk, S.L.,& Sky-Peck, H.H. Comparison between concentrations of trace elements in normal and neoplastia human breast tissue. (1984). *Cancer Research,* 44, 5390-5394.

Ross, A.C., Cifelli, C.J., Zolfaghari, R., & Li, N-Q. (2011) Multiple cytochrome P-450 genes are concomitantly regulated by vitamin A under steady-state conditions and by retinoic acid during hepatic first-pass metabolism. *Physiological Genomics,* 43, 57-67.

Russell, R. Vitamin A and zinc metabolism in alcoholism. (1980). *American Journal of Clinical Nutrition*, 33, 2741.

Sandstead, H.H. (2003). Zinc is essential for brain development and function. *The Journal of Trace Elements in Experimental Medicine,* 16, 165-173.

Salbert, G., Fanul, A., Piedrafita, F.J., Lu, X.P., Kim, S.J., Tran, P., & Pfahl, M. (1993). Retinoic acid receptors and retinoid x receptor-a down-regulate the transforming growth factor-b1 by antagonizing AP-1 acitivity. *Molecular Endocrinology,* 7, 1347-1356.

Satre, M.A., Jessen, K.A., Clegg, M.S. & Keen, C.L. (2001). Retinol binding protein expression is induced in HepG2 cells by zinc deficiency. *FEBS Lett*ers, 491, 266-271.

Schug, T.T., Berry, D.C., Shaw, N.S., Travis, S.N. & Noy, N. (2007). Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell*, 129, 723-733.

Semba, R.D. (1998). The role of vitamin A and related retinoids in immune function. *Nutrition Reviews,* 56, 538-548.

Sessler, R.J. & Noy, N. (2005). A ligand-activated nuclear localization signal in cellular retinoic acid binding protein-II. *Molecular Cell,* 18, 343-53.

Shah, D. (2011). Magnitude of zinc deficiency and efficacy of zinc. *Indian Journal of Pediatrics,* doi: 10.1007/s12098-011-0556-0.

Simpson, J.L, Bailey, L.B., Pietrzik, K., Shane, B., & Holzgreve, W. (2011). Micronutrients and women of reproductive potential: required dietary intake and consequences of dietary deficiency or excess. Part II – vitamin D, vitamin A, iron, zinc, iodine, essential fatty acids. *The Journal fo Maternal-Fetal and Neonatal Medicine,* 24, 1-24.

Singh, B., Murphy, R.F., Ding, X.Z., Roginsky, A.B., Bell, R.H. & Adrian, T.H. (2007). On the role of transforming growth factor beta in the growth inhibitory effects of retinoic acid in human pancreatic cancer cells. Molecular Cancer, doi: 10.1186/1476-4598-6-82

Smith, J.C. (1980). The vitamin A-zinc connection: A review. *Annals of the New York Acadamy of Sciences,* 355(1 Micronutrient Interactions: Vitamins, Minerals, and Hazardous Elements), 62-75.

Song, Y., Leonard, S.W., Traber, M.G. & Ho, E. (2009). Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. *The Journal of Nutrition,* 139, 1626-1631.

Soprano, K.J. & Soprano, D.R. (2002). Retinoic acid receptors and cancer. Journal of Nutrition, 132, 3809-3813.

Stefanidou, M., Maravelias, C., Dona, A., & Spiliopoulou, C. (2006). Zinc: a multipurpose trace element. *Archives of Toxicology,* 80, 1-9.

Sun, S-Y., Wan, H., Yue, P., Hong, W.K. & Lotan, R. (2000). Evidence that retinoic acid receptor b induction by retinoids is important for tumor cell growth inhibition. *The Journal of Biological Chemistry,* 275, 17149-17153.

Sun, S.Y. & Lotan, R. (2002). Retinoids and their receptors in cancer development and chemoprevention. *Crit Reviews in Oncology/Hematology,* 41, 41-55.

Tallman, M.S., Andersen, J.W., Schiffer, C.A., Appelbaum, F.R., Feusner, J.H., Woods, W.G., Ogden, A., Weinstein, H., Shepherd, L., Willman, C., Bloomfield, C.D., Rowe, J.M. & Wiernik, P.H. (2002). All-*trans* retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup Protocol. *Blood,* 100, 4298-4302.

Tang, X.H. & Gudas, L.J. (2011). Retinoids, retinoic acid receptors, and cancer. *Annual Reviews of Pathology: Mechanisms of Disease,* 6, 345-64.

Tashiro, H., Kawamoto, T., Okubo, T., & Koide, O. (2003). Variation in the distribution of trace elements in hepatoma. *Biological Trace Element Research,* 95, 49-63.

Taylor, C.G., Bettger, W.J., & Bray, T.M. (1988). Effect of dietary zinc or copper deficiency on the primary free radical defense system in rats. *Journal of Nutrition,* 118, 613-621.

Terry, N.H.A. &White, R.A. (2006). Flow cytometry after bromodeoxyuridine labeling to measure S and G2+M phase durations plus doubling times in vitro and in vivo. *Nature Protocols,* 1, 859- 869.

Thatcher, J.E. & Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opinions in Drug Metabolism and Toxicology,* 5, 875-886.

Thatcher, J.E., Zelter, A., & Isoherranen, N. (2010). The relative importance of CYP26A1 in hepatic clearance of all-trans retinoic acid. *Biochemical Pharmacology,* 80, 903-912.

The UniProt Consortium. (2011). The Universal Protein Resource (UniProt). *Nucleic Acids Research,* 39: D214-D219. UniProt ID: P38936. http://www.uniprot.org/uniprot/ P38936 (Accessed 12 Sept, 2011)

Thompson, K.H., Tsukada, Y., Xu, Z., Battell, M., McNeill, J.H., & Orvig, C. (2002). Influence of chelation and oxidation state on vanadium bioavailability, and their effects on tissue concentrations of zinc, copper, and iron. *Biological Trace Element Research,* 86, 31-44.

Tsukada, Y.A. (2003). Modulating effects of zinc on the efficacy of tamoxifen in human breast cancer cells. The University of British Columbia (Thesis).

Vallee, B.L. & Falchuk, K.H. (1993). The biochemical basis of zinc physiology. *Physiological Reviews***,** 73, 79 -118.

Vanier, K.L, Mattiussi, A.J., & Johnston, D.L. (2003). Interaction of all-trans-retinoic acid with fluconazole in acute promyelocytic leukemia. *Journal of Pediatric Hematology/Oncology,* 25, 403-404.

Verfaille, C.J., Borgers, M., & van Steensel, M.A. (2008). Retinoic acid metabolism blocking agents (RAMBAs): a new paradigm in the treatment of hyperkeratotic disorders. *Journal der Deutschen Dermatologischen Gesellschaft*, 6, 355-64

Walbach, S.B. & Howe, P.R. (1925). Tissue changes following deprivation of fat-soluble A vitamin. *The Journal of Experimental Medicine,* 52, 753-777.

Weber, D. & Gruve, T. (2011). The contribution of b-carotene to vitamin A supply of humans. *Molecular Nutrition and Food Research,* 55, 1-8.

Wong, S.H.K, Zhao, Y., Schoene, N.W., Han, C-T., Shih, R.S.M, & Lei, K.Y. (2007). Zinc deficiency depresses p21 gene expression: inhibition of cell cycle progression is independent of the decrease in p21 protein level in HepG2 cells. *American Journal of Physiology: Cell Physiology,* 292, C2175-C2184.

Appendices

Appendix 1

Table A.1: Mineral supplements added to Chelex-100 treated medium.

Mineral	**Weight (mg/L medium)**
$Ca(CH_3COO)_2.H_20$	50.06
KH_2PO_4	3.845
$MgSO_4$	10.94
$MnCO_3$	0.0027
MoO_3	0.0009
$CuCO_3.H20$	0.0275
$FeC_6H_5O_7$	0.0175

Appendix 2

Table A.2: Primer Sequences for real time quantitative PCR

Gene	Primer	Reference
RARα	Forward: 5'-CTG TGA GAA ACG ACC GAA AC-3' Reverse: 5'-TTG TCC CCA GAG GTC AAT GTC-3'	Wei et al., 2007
RARβ	Forward: 5'GGA ACG CAT TCG GAA GGC T-3' Reverse: 5'-TTC CCA GCC CCG AAT CAT-3'	Wei et al., 2007
RARγ	Forward: 5'-ACC AGG AAT CGC TGC CAG TA-3' Reverse: 5'TGG GCT TTG CTG ACC TTG G-3'	Wei et al., 2007
RXRα	Forward: 5'-GAC AAG CGG CAG CGG AAC-3' Reverse: 5'-CTC TCC ACC GGC ATG TCC T-3'	Wei et al., 2007
RXRβ	Forward: 5'-GGG GTG CGA AAA GAA ATG-3' Reverse: 5'-CGG GGT TTG TTG TTC TCC-3'	Wei et al., 2007
RXRγ	Forward: 5'-GAG GAC GAT AAG GAA GGA C-3' Reverse: 5'AAG CGA CTT CTG ATA GCG-3'	Wei et al., 2007
CYP26a1	Forward: 5'-TGG TAC TGC AGC GGA GGA-3' Reverse:5'-GCG TCT TGT AGA TGA AGC CGT A-3'	Deng et al., 2003
Cyclophilin	Forward: 5'-ACG GCG AGC CCT TGG Reverse: 5'-TTT CTG CTG TCT TTG GGA CCT-3'	Deng et al., 2003

Appendix 3

Table A.3: Western Blot antibody dilutions and incubation times

Protein	**Primary Ab (dilution)**	**Primary Ab incubation**	**Secondary Ab (dilution)**	**Secondary Ab incubation**
RARα	Rabbit polyclonal (1:400)	Overnight at 4 °C in 2.5% skim milk powder in TTBS	HRP conjugated goat anti-rabbit (1:10,000)	45 min at room temperature in 1% skim milk in TTBS
RXRα	Rabbit polyclonal (1:400)	1 hour at room temperature in 2.5% skim milk powder in TTBS	HRP conjugated goat anti-rabbit (1:10,000)	45 min at room temperature in 1% skim milk in TTBS
Actin	Mouse monoclonal (1:400)	Overnight at 4 °C in 2.5% skim milk powder in TTBS	HRP conjugated goat anti-mouse (1:10,000)	45 min at room temperature in 1% skim milk in TTBS

Appendix 4

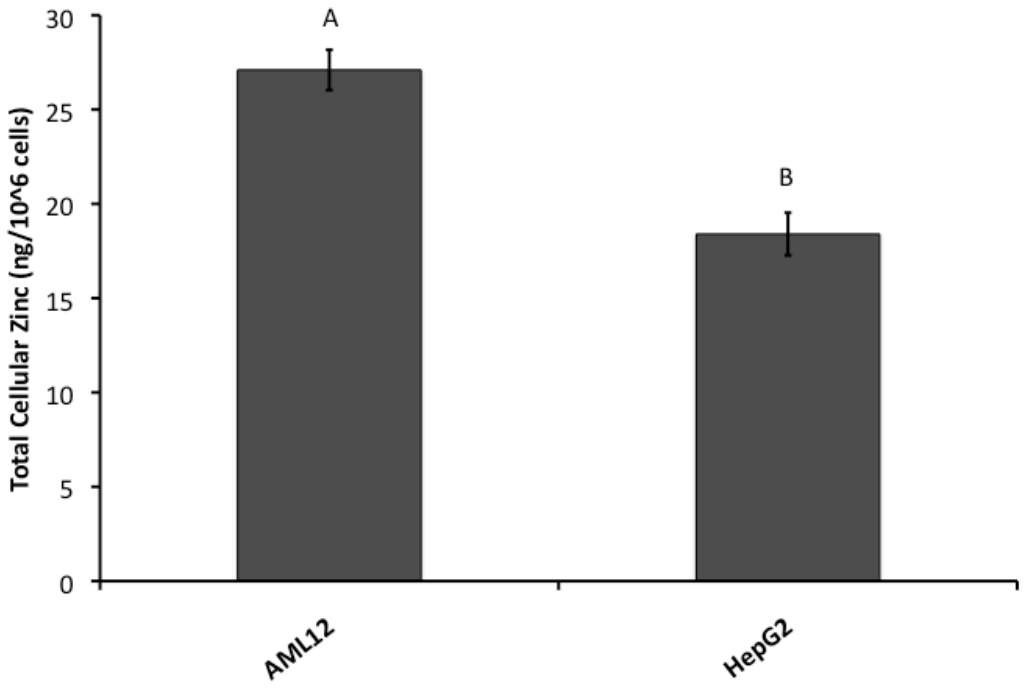

Figure A.1: Total cellular zinc levels in HepG2 and AML12 cells. HepG2 and AML12 cells were cultured into 10 cm plates at initial seeding densities of 300,000 and 200,000 cells respectively, for 6 d. HepG2 were cultured in MEM and AML12 were cultured in F12/DMEM 1:1 (Gibco, Grand Island, NY) containing 10% FBS, sodium pyruvate (110 mg/L), sodium bicarbonate (1.2 g/L), insulin-transferin-selenium (Invitrogen; 1%), Hepes buffer (15 mM), penicillin/streptomycin (5,000 U/L), and dexamethasone (40 ng/ml) At the end of the culture period, cells were harvested and total cellular zinc was measured by atomic absorption spectrophotometry. Values represent mean ± SEM (n=3; the experiment was repeated once). Asterisk indicates a significant difference between AML12 and HepG2 cells ($p<0.05$).

Appendix 5

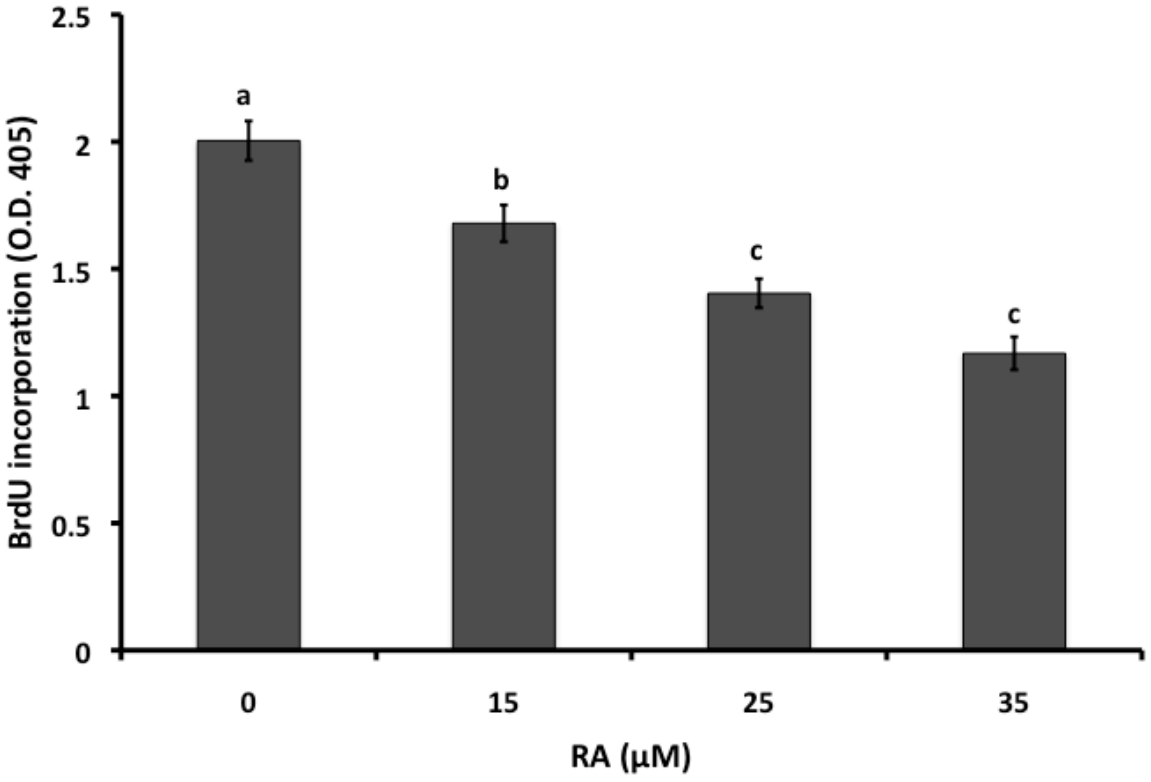

Figure A.2: Cell proliferation of AML12 cells treated with retinoic acid. AML12 cells were cultured into 96-well plates at an initial seeding density of 5000 cells/well in F12/DMEM 1:1 medium and allowed to grow for 2 d. Cells were then treated with *all-trans* retinoic acid (RA) at 0 (DMSO only), 15, 25 and 35 µM for 12 h. Cell proliferation was quantified using the BrdU colorimetric assay with absorbance as a measure of BrdU incorporation. Values represent mean ± SEM (n=6;the experiment was repeated once). Means with different letters are significantly different (*p<0.05).*

Appendix 6

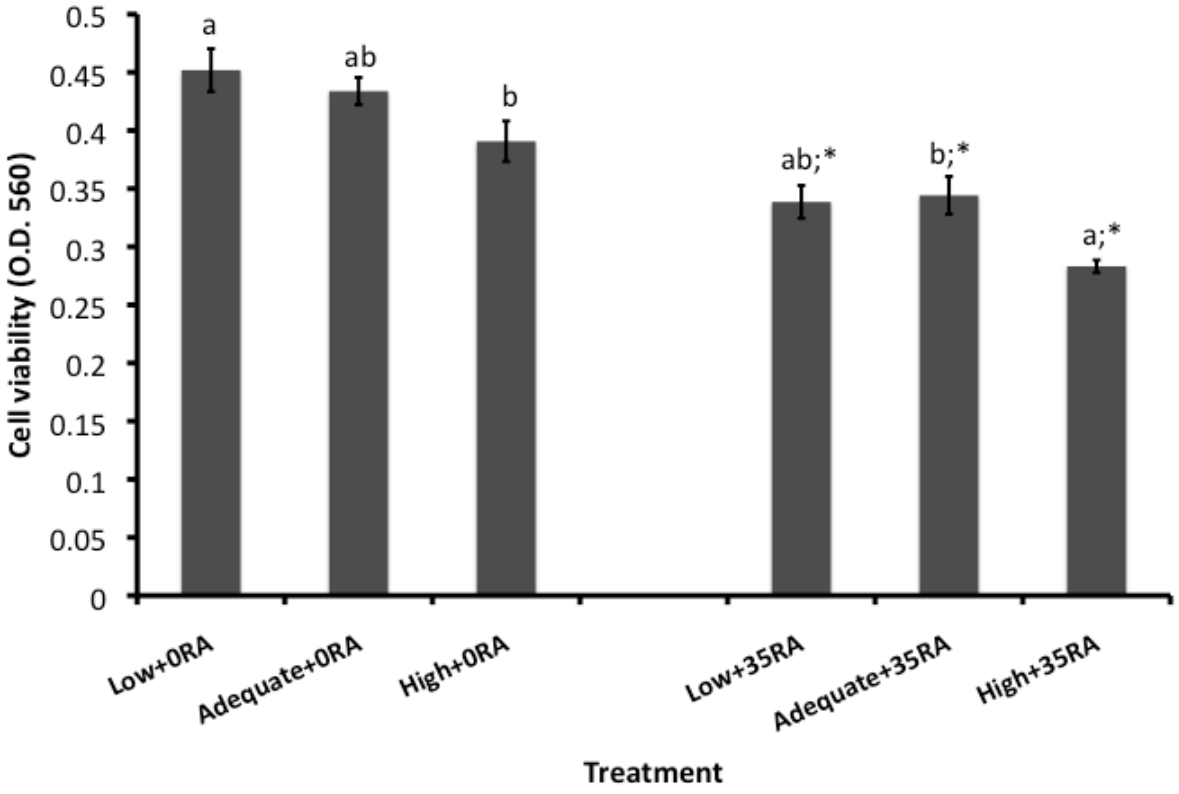

Figure A.3: The effect of retinoic acid on viability of AML12 cells following growth in low-, adequate-, and high-zinc medium. AML12 cells were cultured into 10 cm plates at an initial density of 200,000 cells in F12/DMEM 1:1 medium. Cells were allowed to attach overnight. Subsequently, the cells were cultured in medium containing Chelex 100-treated FBS plus 0 (Low), 5 (Adequate), and 10 (High) µM zinc. On day 5, cells were transferred into 96-well plates at a density of 5,000 cells per well, and on day 6, cells were treated with *all-trans* retinoic acid (RA) at 0 (DMSO only) or 35 µM for 72 h. Cell viability was quantified using the MTT colorimetric assay with absorbance as a measure of cell viability. Values represent mean ± SEM (n=12). Means with different letters are significantly different. Asterisks represent a significant difference between RA-treatment and its respective control. (*p<0.05).*

Appendix 7

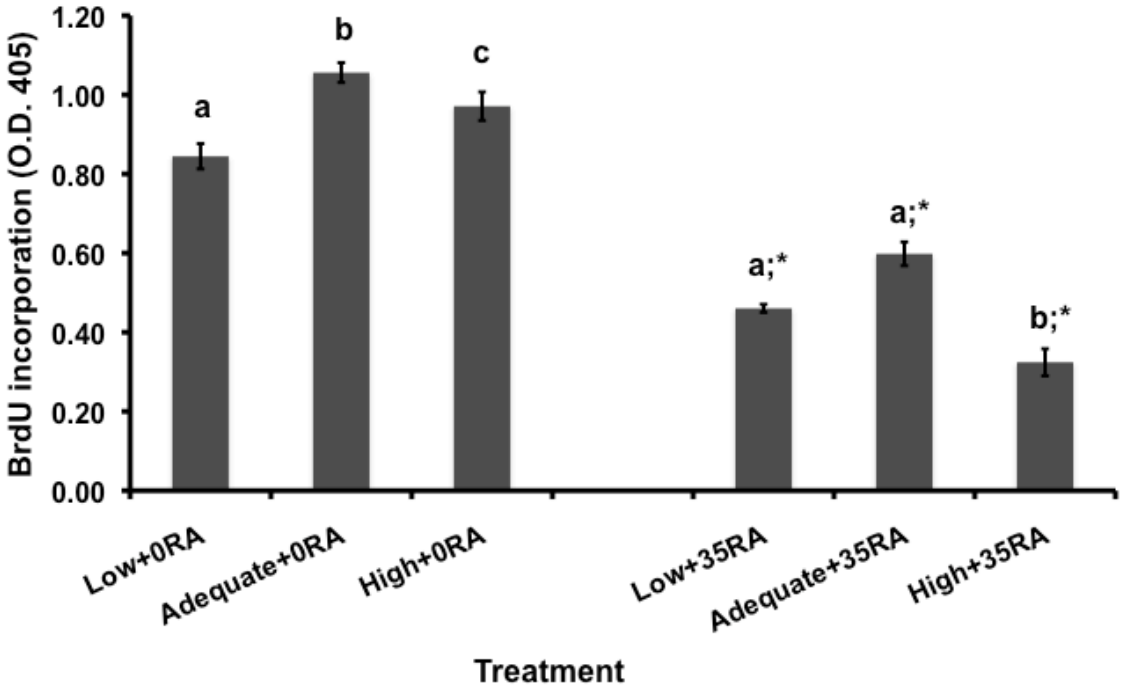

Figure A.4: The effect of RA on the proliferation of AML12 cells following growth in low-, adequate-, and high-zinc medium. AML12 cells were cultured into 10 cm plates at an initial density of 200,000 cells in F12/DMEM 1:1 medium. Cells were allowed to attach overnight. Subsequently, the cells were cultured in medium containing Chelex 100-treated FBS plus 0 (Low), 5 (Adequate), and 10 (High) µM zinc. On day 4, cells were transferred into 96-well plates at a density of 5,000 cells per well, and on day 6, cells were treated with *all-trans* retinoic acid (RA) at 0 (DMSO only) or 35 µM for 12 h. Cell proliferation was quantified using the BrdU colorimetric assay with absorbance as a measure of BrdU incorporation. Values represent mean ± SEM (n=6). Means with different letters are significantly different. Asterisks represent a significant difference between RA-treatment and its respective control. (*p<0.05).*