2003

The Effect of Copper Deficiency and Toxicity on the Tumor Suppressor Protein P53

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THE EFFECT OF COPPER DEFICIENCY AND TOXICITY ON THE TUMOR SUPPRESSOR PROTEIN p53

BY

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A Thesis submitted to
The Department of Nutrition, Food and Exercise Sciences
In partial fulfillment of the
Requirement for the degree of
Master of Science

Degree Awarded:
Spring Semester, 2003

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The Office of Graduate Studies has verified and approved the above named committee members.

This work is dedicated to my mother, and my husband

ACKNOWLEDGEMENT

During the course of this thesis, I have been privileged to receive the advice and assistance of many people. I thank them all, but mention only a few here. First and foremost, I would like to express my appreciation and acknowledge my mentor, Dr. Cathy Levenson, for all her help, and support in teaching me how to be a real scientist, and never giving up on me. She sparked a deep interest in me for science, she has a way of making things seem simple and possible. The best part was learning experimental techniques straight from her as she motivated me to do more than I could have ever thought. I am deeply indebted to her for teaching me the proper research attitude and endless knowledge. I would like to also thank other members of my committee, Dr. Jodee Dorsey, for her unconditional support during my graduate studies and her enthusiastic teaching. And Dr. Nancy Greenbaum, for all her endorsements and valuable time. Special thanks go out to my indispensable co-workers and friends in the lab, Cheryl Pye for all the valuable techniques she taught me and her patience when she had to explain things over and over again. I am grateful to Jacob VanLandingham (Yacoby) for truly everything. When I came into the lab I had no laboratory experience what so ever. Jake taught me so much and spared me a lot of embarrassments! I would also like to thank Stephanie Evans for her constant encouragement and support, but mainly for being a great friend. I would like to especially recognize Charles Badland for being so very diligent and helping me generate some exquisite images for my figures. This work would have not been completed without the prompt assistance of the Cell Culture Facility, particularly, Dr. Joan Hare. Also thanks are given to Chris Gillis, Ursula Tate and all the professors that taught me during my years here at FSU. Last but not least I would like to thank my friends and family for all their support and encouragement. Especially my husband, Tarek. He helped me in many ways through these years. He listened to my ideas, praised me in my triumphs, comforted me in my failures, believed in me unconditionally, and supported me always.

I would also like to acknowledge the financial support and scholarships from the College of Human Sciences, which made my study possible.

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ABSTRACT

Transcription of the p53 gene is stimulated by DNA damage. Newly synthesized p53 is translocated to the nucleus where it binds DNA and acts as a transcription factor to regulate gene transcription, suppress the cell cycle, and halt cellular proliferation. It has been hypothesized that cell cycle arrest allows time for the induction of repair genes. If the damage is extensive and the cell cannot repair itself, then cell death via p53-mediated apoptosis occurs. This is a protective mechanism to eliminate damaged and potentially cancerous cells. Therefore, normal p53 expression and function are essential whenever there is DNA damage. However, when p53 is damaged or mutated there is decreased repair time with no resulting apoptosis, thus permitting cells with damaged DNA to proliferate. This results in the tumorgenesis. This thesis tests the hypothesis that alterations in cellular copper (Cu) alter the ability of p53 to act as a transcription factor. This will be done by an experimental approach using the transient transfection of a p53-responsive reporter gene construct. Furthermore, we will report data suggesting that Cu toxicity induces the expression of p53 that has a mutant conformation. Macroarray technology was used to identify the genes that are induced by p53, and shows that a number of p53-regulated genes are differentially expressed in the presence and absence of Cu.

In conclusion, the goal of this work is to not only understand the possible relationship between cancer and Cu deficiency or toxicity, but also to understand the mechanisms that regulate the function of this important tumor suppressor protein.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Copper

Copper Functions. Copper (Cu) is a micronutrient trace element that plays an essential role in biology, serving as a cofactor for enzymes including superoxide dismutase, which is required for free radical scavenging, cytochrome c oxidase, a component of the electron transport chain, ceruloplasmin needed for iron metabolism, lysyl oxidase, and dopamine β-hydroxylase needed to convert dopamine to norepinephrine. Cu is also needed for the modification of a wide variety of neuropeptides and neurohormones via the enzyme peptidyl α-amidating monooxygenase (PAM). Consequently, Cu transport at the cell surface and the delivery of Cu to intercellular compartments are critical events for a wide variety of biological processes (Prohaska et al., 2001). Because Cu balance is essential, Cu conservation is organ specific and liver Cu levels are very tightly controlled (Levenson & Janghabani, 1994; Levenson, 1998). Furthermore, Cu deficiency can alter dietary preferences (Rutkoski et al., 2000). When rats were fed a Cu deficient diet for 4 weeks, and then given the choice between a Cu deficient diet and a Cu adequate diet, they ate 40% less Cu deficient diet, compared to the control rats ($p=0.023$). Together these data suggest the importance of regulating Cu balance.

Copper Toxicity. Wilson's disease (WD) is an autosomal recessive disorder that results in Cu accumulation and causes cellular injury and tissue damage. While advanced WD is characterized by Cu accumulation in most organ systems, patients generally present with symptoms related to the effects of Cu accumulation in the liver and brain, including hepatitis, cirrhosis, dementia, and Parkinsonian-like motor disturbances (Strand et al., 1998; Strausak et al., 2001). The primary defect is a mutation in the Cu transporting ATP7B P-type ATPase. Accumulation of Cu in the liver of WD patients results from the disturbed export of Cu from hepatocytes to bile and decreased incorporation into ceruloplasmin. This excess Cu initiates free radical generation and subsequent oxidative changes in liver lipids. The Long Evans Cinnamon (LEC) rat is an inbred mutant strain, which has a deletion of the coding region of the Cu-

transporting ATPase gene. LEC rats spontaneously develop liver injury and hepatocarcinoma due to abnormal Cu accumulation. In both humans and LEC rats, liver damage, hepatitis and hepatocarcinoma can be prevented by a reduction in liver Cu (Obata et al., 1996).

The mechanisms responsible for the induction of hepatocyte damage in WD and LEC have been investigated. Copper can induce DNA damage in several ways: 1) by interacting with specific bases in DNA, altering base-pairing properties, 2) by interacting with DNA polymerases, diminishing the fidelity of DNA synthesis, 3) through interaction of metal ions with deoxynucleoside triphosphate substrates affecting base pairing as well as substrate availability, and 4) by interaction with phosphodiester bonds on DNA, altering the structure of DNA and the formation of DNA protein cross-links (Tkeshelashvili et al., 1991). Both Cu^{+} and Cu^{2+} are mutagenic. 10μM Cu^{+} significantly increased mutation frequency compared to controls. Cu^{+}-induced mutagenesis was more than 3-fold greater than that obtained with H_2O_2 (Tkeshelashvili et al., 1991). To understand better where Cu binds DNA, laser Raman spectroscopy has shown that Cu has a pronounced effect on DNA structure. Cu has specific binding sites for double-stranded helical DNA (dsDNA). Furthermore, the binding of Cu to DNA shows higher specificity when compared to other metal ions (Sagripanti et al., 1991).

Copper Deficiency. Menkes disease is an inherited X-linked recessive disorder that is lethal in early childhood. Symptoms include severe mental retardation, altered brain development, and neurodegeneration detected by post-mortem histological assay, indicating that copper is essential for prenatal growth and development of the central nervous system (CNS) (Rossi et al., 2001). These patients have severe Cu deficiency due to lack of MNK, a Cu-transporting ATPase involved in Cu efflux from cells. MNK is required for the efflux of Cu from the gut epithelial cells into the portal circulation. As a result, these patients absorb little Cu leading to high Cu concentrations in gut epithelial cells, kidney and cultured fibroblasts, while the liver and brain become Cu deficient (Rossi et al., 2001).

This Cu deficiency results in cell death. Brain Cu deficiency appears to cause neurodegeneration (apoptosis) triggered by mitochondrial damage due to Cu depletion during brain development (Rossi et al., 2001). The mottled/ brindled ($Mo^{br/y}$) mouse is a phenotype resulting from a mutation at the X-linked mottled locus, making it the closest animal model of Menkes' disease. Brains from $Mo^{br/y}$ mice stained with haematoxylin-eosin revealed increased numbers of cells with morphological features of apoptosis, particularly, in the CA1 region of the

hippocampus and the cortex (Rossi et al., 2001). Furthermore, there was a dramatic down-regulation of Bcl-s and Bcl-2 (anti-apoptotic proteins) in brains of $Mo^{br/y}$ mice. While the regulation of other apoptotic and anti-apoptotic proteins during Cu deficiency have yet to be studied, these data suggest that Cu plays a role in the regulation of these important cellular and molecular mechanisms.

Cu deficiency has also been linked to tumorigenesis. Rats fed a low Cu diet showed greater dimethylhydrazine (DMH)-induced colon tumors compared to rats fed a high Cu diet or an adequate Cu diet. The differences were seen in the number of rats developing tumors; 5 of 11 rats developed tumors in Cu deficient diets and 1 of 10 developed tumors in high and adequate Cu diets Additionally, the average number of tumors was greater in Cu-restricted animals (7 vs. 2), and the average tumor mass was 3 times larger in the Cu-restricted animals (DiSilvestro et al., 1992).

Liver also appears to be susceptible to Cu deficiency-induced tumorigenesis. Cu-restriction of rat hepatoma cells resulted in widespread DNA amplification and synthesis of unstable DNA leading to increased tumorigenicity (Renault and Deschatrette, 1997). Surprisingly, these alterations were even more pronounced and rapid when Cu depletion was followed by Cu repletion (Renault and Deschatrette, 1994).

The mechanisms responsible for increased tumorigenicity following Cu depletion are not understood. Because Cu plays a role in scavenging excess reactive oxygen species (ROS) and preventing oxidative damage to critical cellular components, it has been hypothesized that Cu deficiency may prevent free radical scavenging and lead to DNA damage and mutations. Indeed, Cu is a catalytic cofactor for two important antioxidant enzymes Cu, Zn superoxide dismutase (SOD) and ceruloplasmin. Cu-deficient rats had higher hepatic activity of nuclear repair enzymes than controls after treatment with aflatoxin B, suggesting greater oxidative damage to DNA as a result of Cu deficiency. Furthermore, in cattle with hypocuprosis, an endemic Cu deficiency disease, results in failure of Cu metalloenzyme activity such as Cu, Zn superoxide dismutase (Picco et al., 2001). DNA damage was measured using the comet scale in these animals, and a strong correlation was seen between decreased Cu levels and decreased comet degree 1 cells from 70.5 to 56.5 ($p<0.01$), and increase in comet degree 2 cells from 19.3 to 32.4 ($p<0.01$) (Picco et al., 2001).

Tumor Suppressor Protein p53

p53 Structure. The human p53 gene is located on the short arm of the chromosome 17, band 13, and is about 20kb in length (reviewed in Zupanska et al., 2002). The gene contains 11 exons that code for 393 amino acids. This p53 protein has several functional domains, the amino terminal transactivation domain, a proline-rich sequence, a central DNA binding domain, a flexible linker region, an oligomerization domain, and highly basic region of C-terminus (reviewed in Morris, 2002).

p53 Functions. p53 is known as a tumor suppressor protein because it provides key regulatory elements for monitoring the genome. First, it recognizes DNA damage caused by chemical or physical processes or through other cellular stress stimuli. p53 is activated in response to DNA damage such as chemical carcinogens, metal toxicity/ deficiency, ionizing radiation, and viruses. Stress stimuli that may also activate p53 include, heat shock, hypoxia, low extracellular pH, and certain drugs (cisplatin, adrimycin, and bleomycin). These stimuli induce p53 expression, followed by post-translational modification including specific phosphorylation, acetylation, and possibly dephosphorylation events (Morris, 1994).

These modifications result in an active isoform of p53 that can interact with other regulatory proteins (such as Bax and GADD45) and stimulate p53 translocation to the nucleus where it act as a DNA binding transcription factor to regulate gene transcription, suppress the cell cycle, and halt cellular proliferation. It has been hypothesized that cell cycle arrest allows time for the induction of repair genes (Maeda et al., 2002). If the damage is extensive and the cell cannot repair itself, then cell death via p53-mediated apoptosis occurs (Shwartz and Rotter 1998). This is a protective mechanism to eliminate damaged and potential tumorgenesis.

p53 Mutations. Mutations that prevent normal p53 function result in the development of cancer. Approximately 50% of all malignant cells carry mutations or deletions of this gene, making it the single most frequently mutated gene in cancer cells (Greenblatt et al., 1994; Nigro et al., 1989). p53 mutations account for 26% of lung cancers, 21% of breast cancers, and 19% of colon cancers (Zupanska et al., 2002).

A variety of p53 mutations have been identified including point mutations (Morris, 2002), splicing abnormalities (Holmila et al., 2003; Varley et al., 1999), and null mutations (Morris, 2002). Mutations that alter the amino acid sequence of the protein can disrupt p53 folding, DNA binding, or association with other proteins. Mutations can be caused by

environmental insults such as exposure to aflotoxin (Bergsland, 2001) or can arise from germline mutations. In some cases these germline mutations, which is present from birth, are not detected until adulthood. A recent report described the identification of a p53 germ line mutation in a patient with osteosarcoma and a choriod plexus tumor. The mutation was characterized by a 7 base pair insertion in exon 5. The in-frame alteration produced amino acid substitutions that began with alanine to glycine at position 161 and a premature stop codon at position 182 (Rutherford et al, 2002). This mutated protein was unable to induce the p53-responsive genes bax, p21 or PIG3. However, transfection of the mutant protein into cell showed that it was able to induce apoptosis. Thus, it has been hypothesized that mutations in p53 may not only disrupt normal p53 function, but some mutations may result in a "gain of function" that allows p53 to act via non-classical mechanisms (Morris 2002; Rutherford et al 2002).

p53 and Copper. The DNA damage induced by Cu toxicity results in an increase in the tumor suppressor protein p53. For example, LEC rats had elevated hepatic expression of p53. This increase could be prevented by feeding a low copper diet (Obato et al., 1996). Treatment of human hepatocytes (HepG2) with toxic levels of Cu also resulted in increased p53 expression (Strand et al., 1998; Narayanan et al., 2001). Induction of p53 was followed by the translocation of p53 into the nucleus of HepG2 cells and subsequent apoptosis (Narayanan et al., 2001). Verhaegh et al. studied effects of Cu levels on human breast carcinoma cell line (MCF-7). Pyrrolidine dithiocarbamate (PDTC) is an antioxidant, that can also bind and transport external Cu into cells (Verhaegh, 1997). PDTC down regulated the specific DNA-binding activity of p53. Furthermore BCS, a non-cell permeable Cu chelator, prevented Cu import and the p53 down-regulation, while 1,10-orthophenanthroline, a cell permeable Cu chelator prompted the activity of Cu and up-regulated p53 DNA-binding activity through a DNA damage-dependent pathway (Verhaegh, 1997).

The p53 protein is a transcription factor, which binds DNA through a structurally complex domain stabilized by a zinc (Zn) atom. Zn chelation disrupts the architecture of this domain (Hainnaut et al., 1995), inducing the protein to adopt an immunological phenotype identical to that of many mutant forms of p53. Incorporation of Zn within the protein is required for folding into the wt conformation capable of specific DNA-binding (Meplan et al., 2000). Furthermore, Zn increased p53 binding to T antigen (Tag) of DNA papova virus essential for viral replication, transformation, and DNA binding (Kernohan et al., 1996). Also, depletion of

Zn ions from p53 during purification alters conformation of wtp53, generating a mutant conformation, which is inactive in a sequence-specific DNA binding assay, and would be unable to bind Tag (Kernohan et al., 1996). This proves that there are structures in DNA repair proteins sensitive to toxic metals. Zn is one of them because of its Zn finger structures in DNA-binding motif: with in these structures Zn is complexed to four cysteines and/or histidines, creating structures that allow DNA-protein interaction as well as protein-protein interactions (Hartwig et al., 2002). It has been shown that Cu displaces Zn in Fpg Zn finger protein, decreasing its binding capacity and enzymatic activity (Hartwig et al., 2002; O'Connor et al., 1993). Cu also decreased the activity of xeroderma pigmentosum (XPA), which contains a single Zn finger motif, which is part of minimal DNA-binding domain. Furthermore, Cu decreased activity of PARP, which is involved in directing repair enzymes to sites of DNA damage (Hartwig et al., 2002).

On the other hand Zn treatment of neuronal cells, decreased Cu-induced apoptosis, decreased p53 translocation to the nucleus and at 700 μM Zn decreased p53 expression by 50% (VanLandingham et al., 2002). Furthermore, exposure of cultured cells to Zn chelator TPEN, induced wtp53 to accumulate in an immunologically "mutant" form with decreased DNA-binding activity (Verhaegh et al., 1998). Removal of TPEN from cultured medium allowed p53 to refold into the immunologically wt form, followed by transient increase in DNA binding capacity. These results indicate that modulation of intracellular Zn induces conformational changes in p53 and its binding activity, suggesting that metalloregulation may play a key role in controlling p53 structure and function.

The down-stream genes that are activated by the Cu-induction of p53 have not been fully explored. However, it is known that CD95 expression (which is dependent on p53) is very low and evenly distributed in normal liver cells compared with WD livers. WD liver had areas of high CD95 expression on hepatocyte membranes (Strand et al., 1998). In vitro studies confirmed these results. HepG2 cells treated with copper (II) chloride revealed fragmentation of apoptotic bodies and increased expression of CD95 detected by FACS analysis compared to control HepG2 cells that were not treated with Cu. To see the role of p53 in CD95 system, HepG2 cells transfected with p53 gene had the same expression of CD95 as those induced by Cu, indicating that induction of CD95 by Cu is mediated by p53 (Strand et al., 1998).

Less is known about the regulation of p53 in Cu deficiency. However, in Menkes disease, cancer develops possibly through a mechanism involving p53 (Mercer, 1998). Treating cells with a Cu chelator tetraethylenepentamine (TEPA) increased p53 mRNA, nuclear p53, and decreased cytosolic p53 compared to controls (Narayanan et al., 2001). Immunocytochemical localization reinforced that in TEPA-treated cells p53 was in the nucleus compared to the control where p53 was spread throughout the cell. However, there was no alteration in cellular proliferation and no evidence of apoptosis in TEPA-treated cells suggesting that Cu deficiency-induced p53 was inactive (Narayanan et al., 2001).

Because both Cu toxicity and Cu deficiency have been linked to the development of cancer, this work was designed to study the function of p53 under conditions of altered cellular Cu status. First, the role of Cu in the ability of p53 to act as a DNA binding transcription factor was tested using reporter gene downstream from tandem p53 binding sites. Second, p53 responsive genes were identified in Cu loaded and copper deficient cells by oligonucleotide array. And finally, the presence and relative abundance of mutant p53 was measured in Cu treated cells that normally express wild type p53.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture

Cell Maintenance. Human hepatoma cells (HepG2) were grown in Minimum Essential Media (α-MEM, Sigma Chemical, St.Louis, MO) with 10% calf serum (Cosmic Calf Serum, Hyclone Laboratories, Logan, UT) and an antibiotic-antimyotic solution (Sigma Chemicals, St.Louis, MO) containing penicillin (1 X 10^5 U/L) in T75 flasks. Flasks were housed in a humidified incubator containing 5% CO_2, and 95% air at 37°C. Cells were plated at 80-90% confluency into 6-well plates for appropriate treatment.

Copper Treatment. HepG2 cells were treated with media containing the copper (Cu) chelator tetraethylenepentamine (TEPA) at 50 μM for 48 hours (n=5; 5 wells of 6-well plates). We have previously shown this treatment to induce Cu deficiency in HepG2 cells without disrupting cellular zinc status (Narayanan et al. 2001; Levenson et al. 1999). Cells were also treated with 200 μM Cu as copper sulfate (n=5) for a period of 6-18 hours. Untreated cells served as controls (n=5).

Transfection

All cells were transfected with a reporter gene construct containing the luciferase gene downstream from a TATA box promoter element and 15 tandem repeats of a known p53-binding site. This construct was designed specifically for testing p53 *cis*-acting elements in mammalian cells and is supplied with positive control plasmid (wild-type p53 cDNA) (Stratagene, LA Jolla, CA). Co-transfection with the β-galactosidase (β-Gal) gene permitted normalization for transfection efficiency (Levenson et al., 1994). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), a proprietary formulation that is suitable for transfection of nucleic acids into mammalian cells.

Preliminary experiments were conducted to determine optimal conditions for transfection. These studies determined the amount of positive control that resulted in increased p53 expression without resulting in complete apoptosis. Preliminary experiments were also conducted to determine the duration of the transfection process, the amount of Cu, the period of Cu treatment, the amount of Lipofectamine to be used, and tolerance of the cells to serum free media during the transfection process. From these preliminary transfection studies, it was determined that HepG2 cells would be plated in 6-well plates and allowed to attach for 18-24 hours. After attachment, cells were transfected and treated with Cu. Transfection was accomplished by the addition of plasmid DNA (diluted to an appropriate concentration with Tris-EDTA buffer, pH 8.0) in 250 μL of serum free media (Table 2-1). Lipofectamine was mixed separately in serum free media (10 μL/ 250 μL media per well) then added to the plasmid DNA mixture and incubated for 20 min at room temperature. Because transfection is highest in serum free media, growth media was removed from cells and replaced with 1.5 μL of serum-free media. The plasmid DNA-Lipofectamine mixture was then added to the cells (final transfection volume was 2 mL). To maintain cell survival, 50μL fetal bovine was added to each well after approximately 6 hours. After 18 hours additional media containing serum (250μL) was added to the cells to a final volume of 2.5mL. At the end of the treatment period, cell lysates were collected for analysis of Luciferase activity, β-Gal, and total cellular protein. Transfection experiments were replicated in two separate batches of HepG2 cells (n=3 dishes/ condition) for a total 6 dishes per condition.

Table 2-1. Transfection of HepG2 cells

DNA Tube	LUC (1.25μg/dish)	β-Gal (1.25μg/dish)	Cu (200 μM)	TEPA (50 μM)	p53 (2.5μg/dish)
1	2.8 μL	1.33 μL			
2	2.8 μL	1.33 μL			
3	2.8 μL	1.33 μL			100 μL
4	2.8 μL	1.33 μL			100 μL
5	2.8 μL	1.33 μL	1 μL		
6	2.8 μL	1.33 μL	1 μL		
7	2.8 μL	1.33 μL		1 μL	
8	2.8 μL	1.33 μL		1 μL	

Luciferase activity. The luciferase assay kit provides rapid, sensitive, and quantitative measurement of the activity of the reporter enzyme American firefly (*photinus pysalis*). It is highly sensitive and as few as 1 x 10^{-20} moles of luciferase can be detected with a luminometer. The reaction that occurs can be described as follows:

Luciferase substrate (luciferin) + ATP + O_2 → light (560nm) + oxyluciferin + AMP + Ppi

To collect cells, media was removed from each well, 300 μL of 1X cell lysis buffer was added and incubated at room temperature for 15 min. Wells were scraped lysates and then transferred to separate 1.5mL Eppendorf tubes and centrifuged at 12, 000 rpm, for 5 min. The supernatant was transferred to new 1.5 mL tubes. Cell lysates were then immediately assayed for Luciferase activity and the remainder of the cell lysates were stored at -80°C for later β-Gal and protein assay. To each polystyrene sample tube 100 μL of luciferase substrate-assay buffer mixture and 20 μL of cell lysate were mixed and Luminescence was immediately measured using an Optocomp 1 luminometer (Hamden, CT).

β-Galactosidase Measurement. The β-Gal gene functions as a measure of transfection efficiency because the protein product is very stable, resistant to proteolytic degradation in cellular lysates, and easily assayed. To assay for β-Gal, 20 μL of cell lysate, and 130 μL of 1X chlorophenol red-β-D-galactopyranoside (CPRG) substrate was mixed in a final volume of 150 μL. After incubation for 72 h in a 37°C water bath the reaction was terminated by adding 80 μL stop solution. Samples were then read at 570nm in a Beckman DU 640 spectrophotometer.

Protein Assay. Total cellular protein content in the lysates was determined by Lowry, and used as a control for variations in cell number. A standard curve was produced using duplicates of bovine serum albumin (BSA) at 5, 10, 20, 25, 30, and 50 μg. 5 μL of cell lysate was added to separate tubes. All tubes (samples and standards) were brought to a final volume of 130 μL with deionized water. Lowry reagent (1 mL) was added and samples were incubated for 10 min at room temperature. Folin reagent (100 μL diluted 1:1 with water) was added and incubated for 30 min at room temperature. Absorbance was determined by spectrophotometry at 500nm. Sample protein concentrations were determined by linear regression. The r^2 of the standard regression line was ≥0.99.

Macroarray Technology

Separate T75 flasks of HepG2 cells were treated with 50 μM TEPA (48 hours), or 200 μM Cu (18 hours) as previously described. Control cells were untreated. Total cellular RNA was isolated using Trizol extraction method (Gibco/ BRL, Gaithersburg, MD). Concentrations were determined using spectrophotometry, and purity was confirmed by A_{260}/A_{280}.

Labeling cDNA probes. In sterile 0.5 mL microcentrifuge tubes 5 μL of total cellular RNA (2μg /μL) for each treatment condition was added to 5 μL pre-prepared primer mix (Panomics, Redwood City, CA) and briefly centrifuged. The mixtures were then heated at 70°C for 2 min, after which they were incubated for 1-2 min at 42°C. Biotin-dUTP (2 μL) and 1 μL of reverse transcriptase were added to a final volume of 20 μL. This mixture was incubated at 42°C for 2 min after which time the cDNA mix was transferred to the RNA sample mix, and incubated at 42°C for 2 h. Following incubation, 3 μL of 10X denaturing solution was added to the sample and incubated for 20 min at 68°C. Finally that 33 μL of 2X neutralizing buffer was added and incubated at 72°C for 10 min.

Hybridization. Nylon membranes spotted with 148 p53-responsive cDNAs (Transignal TM, Panomics, Redwood City, CA) were prehybridized in 3 mL of prewarmed hybridization buffer supplied by the manufacturer at 42°C for 2 hours. The biotin-labeled cDNAs were added to the bottle and hybridized at 42°C overnight. The next day hybridization buffer with the labeled cDNAs was decanted and saved. To wash the membranes, 60 mL of prewarmed hybridization wash was added and incubated at 42°C for 20 min while shaking, and this was repeated twice. Then 60 mL of prewarmed hybridization wash II was added and incubated at 42°C for 20 min while shaking. This was also repeated twice.

Detection. After hybridization, the membranes were washed with 1X blocking buffer for 15 min with gentle shaking. Streptavidin-HRP conjugate was added and incubated for another 15 min, followed by three washes with 1X wash buffer (20 mL). Membranes were placed on a plastic sheet and 3 mL of substrate solution were added onto the membranes and overlaid with a second plastic sheet. Expressed mRNAs were detected by immediately exposing Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 10sec, 30 sec, and 2 min.

Films were then used to identify p53-responsive genes expression in HepG2 cells for each condition. First, spot intensities on all films were quantified using densitometry (Quantity

One Quantification Program) created by Protein and DNA Imaging, (PDI, Boston, MA). As internal controls, the array includes the sequences for GAPDH and ubiquitin. These genes are not expected to be regulated by Cu thus permitting normalization of the films. Genes with a 1.5-fold increase or decrease in spot intensity compared to the Cu-adequate/deficient condition were identified as potentially regulated by Cu.

Immunocytochemistry

Immunocytochemistry was used to determine the localization and relative abundance of p53 in its wild type and mutant conformation. HepG2 cells were plated on glass cover slips at approximately 50% confluence in 6-well plates. After 24h cells were treated with 200 μM Cu for 18h. Cells were then rinsed with 1 mL phosphate buffered saline (PBS, pH 7.4) containing calcium and magnesium (PBS+), and fixed for 10 min with 4% paraformaldehyde (PFA) in PBS+ at room temperature. The cells were then rinsed 3X in PBS+. Care was taken between washes not to dry out the cells. Cells were permeabilized with 0.2% Triton X-100 for 5 min, followed by 3 washes using PBS without calcium and magnesium (PBS-). 10 mg/mL bovine serum albumin (BSA) was added for 15 min to prevent non-specific antibody (Ab) binding. BSA was removed and a mouse anti-human p53 monoclonal Ab, described in table 2-2 (1:250 in BSA) was added. The samples were then incubated at room temperature in a humid chamber overnight with gentle shaking. The following day, the primary Ab was washed off 3X with PBS-. BSA was again applied for 15 min. A secondary goat anti-mouse IgG labeled with Cy3 (1:200) was added and incubated for approximately 2 hours at 37°C. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 1:300; Sigma Chemicals) in distilled water in the dark for 15min at room temperature. After washing 3X with PBS-, coverslips were mounted onto microscope slides using commercially prepared anti-fade mounting medium (Gel-Mount, Biomedia). Cells from two separate experiments were examined for the presence of p53 in the wild type and mutant conformations using a Nikon microscope equipped with epifluorescence. Exposure times for all photographs were held constant to permit comparison of intensities.

Table 2-2. Antibody Properties for Immunocytochemistry in HepG2 cells

Antigen	Primary Ab	Secondary Ab	Ab number	Company
p53 Mutant	Monoclonal mouse anti-human IgG (1:200)	Cy3 conjugated goat anti-mouse IgG (1: 250)	p53 (Ab-3)	Oncogene, SanDiego, CA
p53 Wild type	Monoclonal mouse anti-human IgG (1:200)	Cy3 conjugated goat anti-mouse IgG (1: 250)	p53 (Ab-4)	Oncogene, SanDiego, CA
Nuclear DNA	DAPI (1:300)			Sigma chemicals

CHAPTER 3
RESULTS

Copper Deficiency

Effect of Copper Deficiency on p53 Transcriptional Activity. Luciferase activity was used to monitor the ability of p53 to act as a DNA-binding transcription factor. The addition of TEPA to cells prior to transfection with the reporter gene resulted in only a modest increase in luciferase activity that did not reach statistical significance (Figure 3-1). However, co-transfection with the reporter gene and the p53 expression construct resulted in a 5.5 fold increase in luciferase activity ($p \leq 0.001$). The addition of TEPA to co-transfected cells did not alter luciferase activity, suggesting that Cu deficiency does not alter p53 activity.

Genes Regulated by Cu Deficiency. Copper deficiency resulted in differential expression of 27 known p53 responsive genes that met the standard 1.5-fold cut off (Table 3-1.). The mRNA abundance for 9 genes was increased 1.7-15.8 fold by TEPA treatment. Using the same selection criterion, 18 mRNAs were decreased below control levels (Table 3-1).

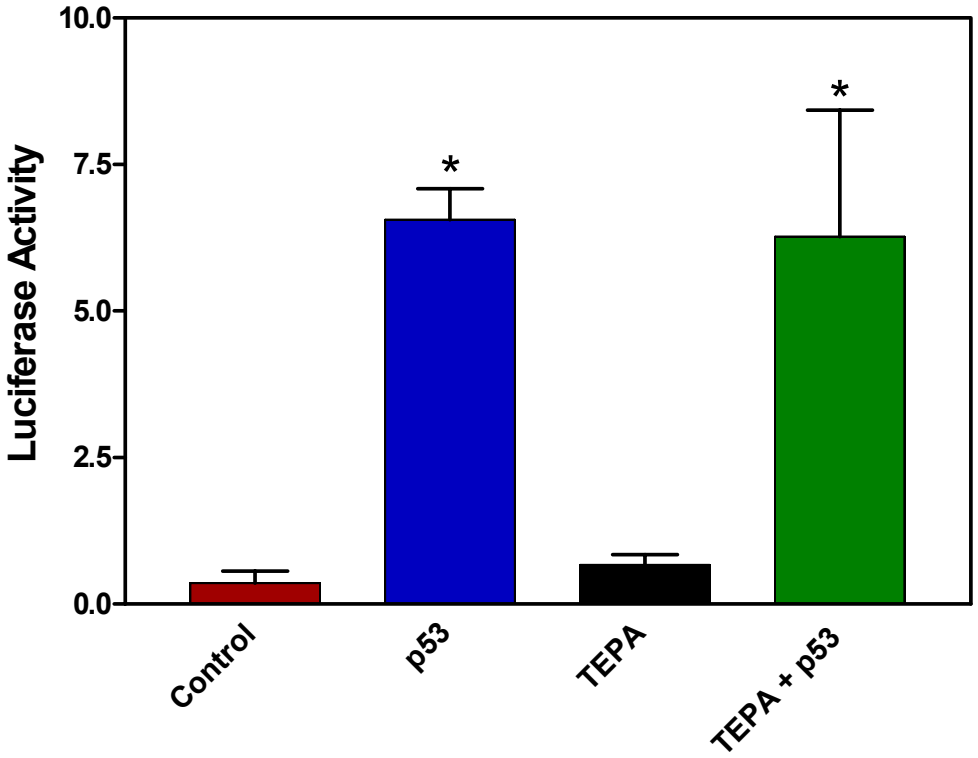

Figure 3-1. Affect of Cu deficiency on the ability p53 to activate a luciferase reporter gene with tandem p53 binding sites. Cu deficiency was induced in HepG2 cells with 50μM TEPA and then co-transfected with the reporter gene and the p53 expression vector. Control cells were transfected with reporter gene only. Bars represent mean ± SD luciferase activity (n=6 in two separate experiments). * Significantly different from control at $p \leq 0.05$.

Table 3-1. TEPA-induced alteration in the expression of p53-responsive genes.

Gene	Increased Expression	Gene Product	Function
15-LO	15.8	15 Lipooxygenase gene	Metabolism of linoleic and archedonic acid
AFP	14.1	Alpha-fetoprotein	Cell proliferation/ growth
4F2AHC	10.2	Antigen heavy chain	
β-Actin	8.4	Cell structure gene	Cell structure
VEGF	7.2	Heparin-binding vascular endothelial growth factor	Vasculization and tumorgenesis
RB1	7.1	Retinoblastoma Susceptibility	Tumor suppressor
PAI 1	5.6	Serine/ cysteine proteinase inhibitor	Metastasis suppressor genes
PTPA	3.1	Protein Phosphotase 2A	Regulator of protein phosphotase
α-Actin	1.7	α-Actin	Cell structure
Gene	**Decreased Expression**	**Gene Product**	**Function**
IGF BP6	0.07	Insulin-like growth factor binding protein 6	Inhibits cell growth
PTHLH	0.13	Parathyroid hormone-like protein	Proto-oncogene
RGS 14	0.13	Regulator of G-protein signaling 14	Growth and cell survival
tyrkin	0.19	Tyrosine Kinase	Inhibit apoptosis
Pro.Ox	0.20	p53 induced protein	Unknown
TP53 INP1	0.23	Tumor protein p53 inducible nuclear protein 1	Unknown
Hsp 70	0.26	Heat shock protein 70	Anti-apoptotic
p85	0.26	Cell cycle gene	Pro-apoptotic
TGF - α	0.31	Transforming growth factor, alpha 1	Cell proliferation
REPRIMO	0.37	Candidate mediator of the p52-dependant G2 arrest	Cell cycle arrest at G2
WIG 1	0.43	p53 target zinc finger protein	Transcription factor?
P2RXL1	0.45	Purinergic receptor P2X-like 1	Receptor protein
Bax	0.53	Pro-apoptotic gene	Pro-apoptotic
PUMA/ BBC3	0.59	Bcl-2 binding component	Pro-apoptotic
p73	0.67	Tumor protein 73	Tumor suppressor
PRG1	0.67	Cell death gene	Pro-apoptotic
TGF - β	0.67	Transforming growth factor, beta 1	Growth
Stat 3	0.67	Signal transducer & activator of transcription 3	Transcription factor

Copper Toxicity

Effect of Cu Toxicity on p53 Transcriptional Activity. Cu treatment did not significantly alter luciferase activity compared to untreated control cells (Figure 3-2). However, when p53-transfected cells were treated with Cu, luciferase activity was reduced to control levels, suggesting that Cu toxicity severely impairs the ability of p53 to act as a DNA-binding transcription factor (Figure 3-2).

Genes Regulated by Cu Toxicity. There were a total of 14 genes that were differentially regulated by Cu toxicity (Table 3-2). Of these, 5 were up-regulated between 1.5 and 3.2-fold above control. Nine genes were down-regulated after 18 h of Cu treatment (Table 3-2.).

p53 Conformation. Examination of HepG2 nuclear morphology by DAPI staining suggested that Cu-treatment results in apoptosis. Figure 3-3 shows that Cu treatment resulted in nuclear shrinkage, and apparent nuclear blebbing.

Immunocytochemistry using antibodies specific for the wild-type and mutant conformations of p53 showed that the abundance of p53 (both wild-type and mutant) was very low in untreated control HepG2 cells (Figure 3-3.). As expected from previous work, Cu treatment increased p53 that was in the wild type conformation. This protein was seen in both the cytoplasm and nucleus of Cu-treated cells. Figure 3-3 shows that in addition to wild-type p53, Cu treatment also resulted in a significant amount of p53 that was in a mutant conformation. While it is clear that both wild type and mutant p53 is formed in the presence of Cu, figure 3-4 suggests that Cu-treatment may result in the formation of more wild type than mutant p53. Furthermore, in cells immunostained with both antibodies (anti-wild type and anti-mutant) the staining intensity was visibly higher than staining in cells labeled for wild type or mutant p53 alone. This not only confirms the presence of significant amounts of both proteins (mutant and wild-type) in Cu nuclei of Cu-treated cells (Figure 3-4.), but also indicates the specificity of the antibodies for the different conformations.

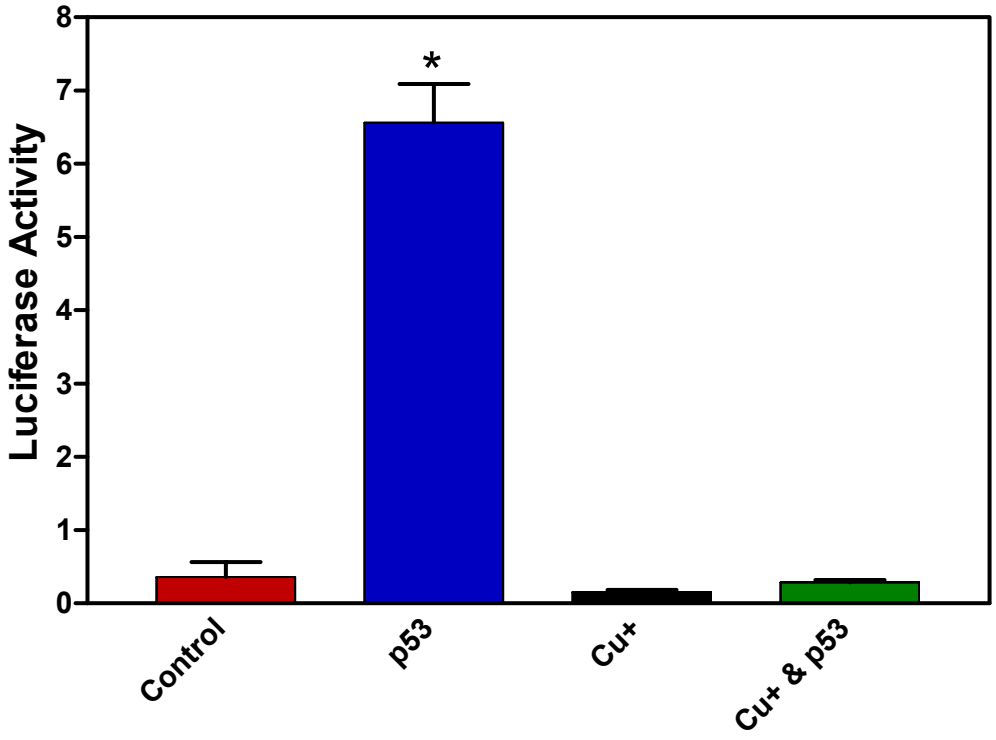

Figure 3-2. Affect of Cu toxicity on the ability p53 to activate a luciferase reporter gene with tandem p53 binding sites. Cu toxicity was induced in HepG2 cells with 200 μM Cu and then co-transfected with the reporter gene and the p53 expression vector. Control cells were transfected with reporter gene only. Bars represent mean ± SD luciferase activity (n=6 in two separate experiments).
* Significantly different from control at $p \leq 0.05$.

Table 3-2. Copper-induced alterations in the expression of p53-responsive genes.

Gene	Increased Expression	Gene Product	Function
15-LO	3.2	15 Lipooxygenase gene	Metabolism of linoleic and arachidonic acid
α-Actin	2.4	α-Actin	Cell structure
RB1	1.9	Retinoblastoma susceptibility-1	Tumor suppressor
Gene	**Decreased Expression**	**Gene Product**	**Function**
PIG 8	0.02	Etoposide-induced protein	Apoptosis
GPX	0.09	Glutathione Peroxidase	Antioxidant
MAP 4	0.15	Microtubule-associated protein	Anti-apoptosis
Ker 15	0.34	Keratin 15	Unknown
LRDD/ PIDD	0.40	Leucine-rich & death domain containing	Regulates cellular growth/ pro-apoptotic
TGF - β	0.41	Transforming growth factor, beta 1	Pro-apoptotic
fos	0.52	c-fos	Proto-oncogene
CDKN/ p14 ARF	0.56	Cyclin-dependant kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	Tumor suppressor
LATS 2	0.67	Large tumor suppressor 2	Tumor suppressor

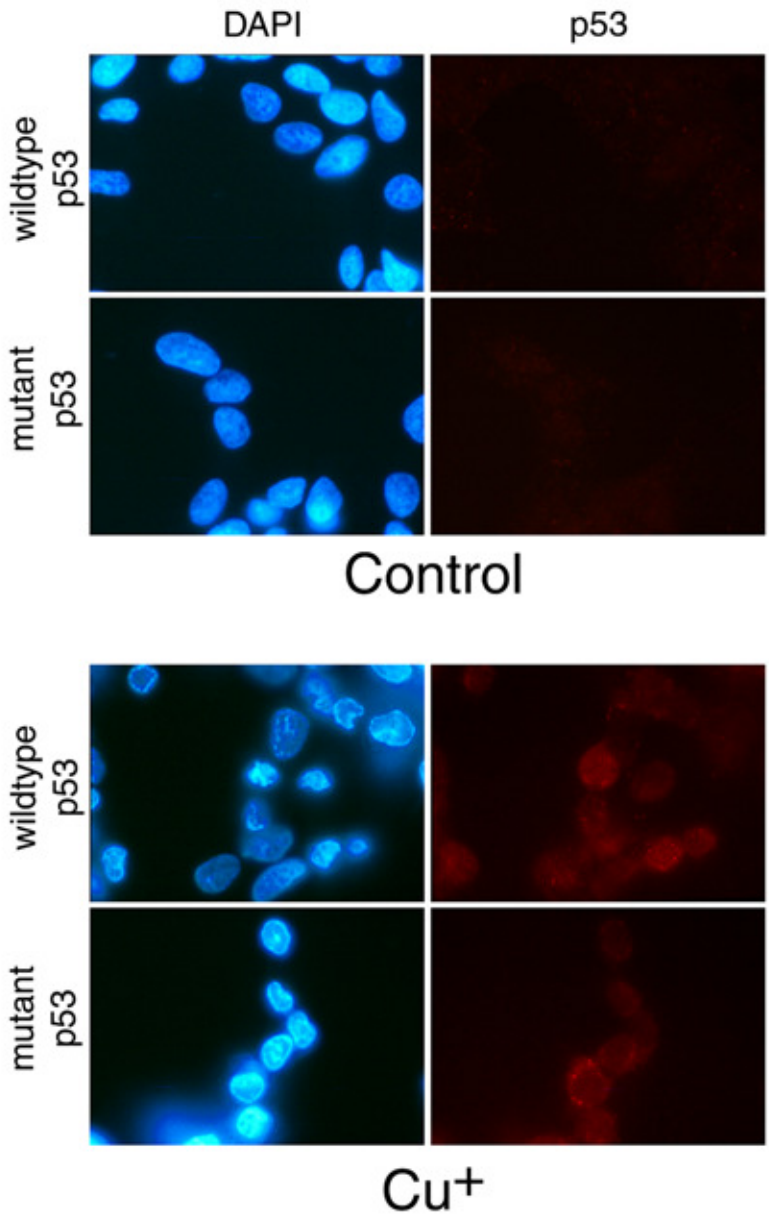

Figure 3-3. Immunocytochemical localization of p53 in control and Cu-treated (200 μM for 18 hours) HepG2 cells. Photomicrographs are representative of images from n=6 dishes at 100 X magnification. Cells were fixed with 3.7% formaldehyde and stained with 4',6-diamidino-2-phenylidole (DAPI).

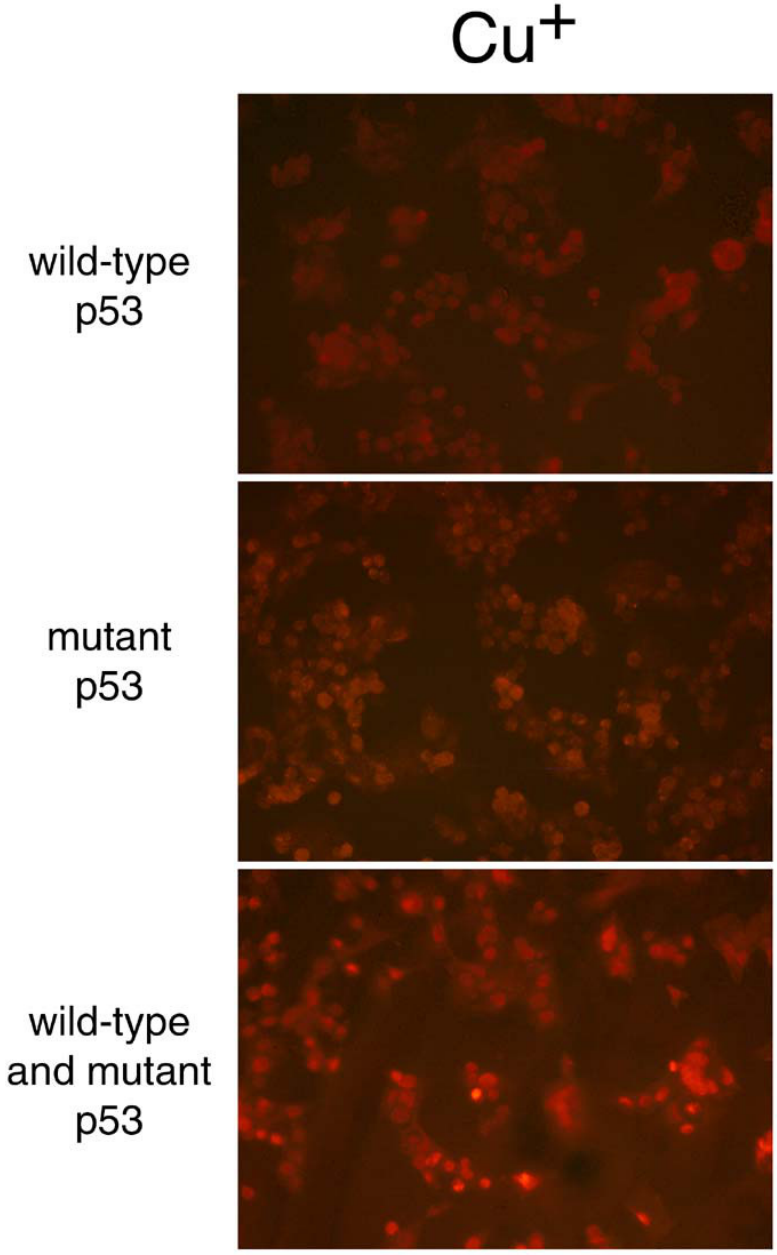

Figure 3-4. Immunocytochemical localization of wild-type (wt) and mutant (mt) p53 in Cu-treated (200 μM for 18 hours) HepG2 cells. Photomicrographs are representative of images from dishes at a 20X magnification.

CHAPTER 4
DISCUSSION

The tumor suppressor protein p53 acts as a DNA binding transcription factor to regulate a multitude of genes involved in the regulation of the cell cycle and apoptosis (Morris et al., 2002; Decraene et al., 2001). There is evidence that both Cu deficiency and Cu toxicity contribute to the development of cancer (DiSilvestro et al., 1992; Obata et al., 1996; Tkechelashvili et al., 1991). Furthermore, previous data have shown that Cu regulates p53 expression in both hepatocytes (Narayanan et al., 2001; Obata et al., 1996) and neurons (VanLandingham et al., 2002). Thus, the work described here examined the transcriptional activity of p53 under conditions of both Cu deficiency and Cu toxicity in the human hepatoma cell line, HepG2. Additionally, oligonucleotide arrays were used to determine the expression of p53-responsive genes in HepG2 cells and the effect of Cu alterations.

Cu Deficiency

Copper Deficiency and Cancer. A low Cu diet has been shown to increase the susceptibility of rats to colon cancer causing agents (DiSilvestro et al., 1992). In humans, a Cu restricted diet caused an increase in fecal free radicals and induced cytotoxicity (Davis et al., 2003). This increase in free radicals has been linked to the etiology of cancer because free radicals can lead to DNA damage, protein destruction, and eventually tumor growth (Davis et al., 2003). In vitro, Cu deficiency has been linked to the development of cancer in a variety of cell lines. Part of this may be due to increased oxidative stress in Cu deficient cells. This may be due to a reduction in the cytosolic form of superoxide dismutase (Cu, Zn-SOD) which requires 2 atoms of Cu for its catalytic activity. Cu,Zn-SOD activity was reduced in Cu-deficient neuroblastoma cells (Rossi et al., 2001), erythrocytes (Sukalski et al., 1997), and HL-60 cells (Johnson and Thomas, 1999). Cu-deficiency has lead to oxidative DNA damage in Jurkat T-

lymphocytes (Pan et al., 2000). Perhaps Cu deficiency weakens the antioxidant defense system of these cells making them more susceptible to DNA damage.

The finding that Cu deficiency leads to DNA damage has also been noted in a variety of models. DNA damage was seen in Cu-deficient cattle compared to normo-cupremia (Picco et al., 2001). Several studies have shown alterations in which DNA damage can lead to tumorigenesis in Cu-deficient cell lines (Renault et al. 1998; Renault and Deschatrette, 1994,;1997) or tumor growth (DiSilvestro et al., 1992). Interestingly, Renault et al (1998) reported that while the Cu deficient cells were committed to cell death, they had partially inhibited apoptotic mechanisms. This is consistent with previous data from our lab where Cu deficient HepG2 cells had induced p53 mRNA, marking a commitment to cell death, but no apoptosis (Narayanan et al., 2000).

Effect of Copper Deficiency on p53 Activity. It has previously been shown that the chelator TEPA reduces intracellular Cu levels without disrupting zinc (Narayanan et al., 2000). Under these conditions, p53 mRNA is induced, and p53 protein, although apparently expressed at lower levels than would be expected based on the mRNA, is translocated to the nucleus. However, as explained in the previous paragraph, there is no evidence of apoptosis (Narayanan et al., 2000). Dietary restriction has also been shown to increase p53 without inducing apoptosis (Higami et al., 2000).

To test whether Cu deficiency inhibits the activity of p53, a reporter gene with multiple p53 binding sites was used to test the activity of p53 with and without TEPA treatment. TEPA-treatment resulted in an approximately 2-fold increase in the mean luciferase activity. While this did not reach statistical significance, it certainly suggests a trend toward an increase. The lack of a significant increase can be interpreted in several ways. First, this may suggest that there is only minimal induction of p53 following Cu treatment. This would be consistent with previous work showing an approximately 2-fold induction of p53 mRNA in LEC rat liver with Cu toxicity (Obata et al., 1996). Furthermore, it is consistent with the degree of p53 mRNA induction after treatment of HepG2 cells with Cu, approximately 2-fold (Narayanan et al., 2000). However, these data could also suggest that p53 is synthesized, but that its activity is inhibited by Cu deficiency. This possibility is made less likely by the fact that TEPA treatment had no effect on the reporter gene activity in cells co-transfected with the p53 gene and suggests that once synthesized, p53 is able to function normally under conditions of Cu deficiency.

Downstream genes regulated by p53 in Cu deficiency.

The next phase of this work then moved to an attempt to characterize whether p53-responsive genes that are known to play a role in apoptosis are regulated in TEPA-treated cells. We used an oligonucleotide array to test the expression of a group of known p53-responsive genes under Cu deficient (TEPA-treated) and Cu adequate conditions. A variety of genes were differentially expressed. The major variations are discussed in the following sections.

Bax. The most significant finding from the array study is that expression of the pro-apoptotic gene Bax is decreased following TEPA treatment. Normally, an increase in Bax is not only correlated with apoptosis, but also with increases in p53 gene expression (De Lujan Alvarez et al., 2002; Yin et al., 1997; McCurrah et al., 1997 and others). After p53 induces Bax, Bax gets translocated to mitochondria, where it induces release of cytochrome c. This, in turn, induces the series of caspases that lead to apoptosis (Oh et al., 2003).

Treating cells with EGCG, a polyphenolic compound found in tea, showed significant increases in p53, Bax and apoptosis (Kuo et al., 2003). Furthermore, severe hypoxia led to increased cell death by increasing Bax (Bossenmeyer Pourie et al., 2002). Several chemotherapeutic agents, such as cisplatin, have been shown to decrease cell proliferation, increase cell accumulation at G1 phase, and up-regulate both p53 and Bax (Munehis et al., 2002). This was also true in HepG2 cells treated with Resveratrol (Kuo et al., 200), and Aloe-Emodin treatment of hepatocellular carcinoma cells (Kuo et al., 2002).

In contrast, low Bax levels make cells resistant to apoptosis (Oliver et al., 2002; McCurrach et al., 1997) and susceptible to cancer. Mutations in Bax are seen in pancreatic cancer (Ku et al., 2002), and UICC III tumors with increased Bax have a better prognosis (Schelwiess et al., 2002). Thus, the finding that TEPA-treated cells have reduced Bax expression may explain why these cells are not undergoing apoptosis. While these data may help to explain the survival of HepG2 cells during Cu deficiency, the mechanism responsible for the down-regulation of Bax in TEPA treated cells is not known. Down regulation of Bax could represent a p53-independent mechanism of regulation. Thus, the effect of Cu deficiency on other regulators of Bax expression needs to be explored.

Alpha-fetoprotein. After birth, transcription of alpha-fetoprotein (AFP) is turned off in most adult cells, except in the liver where it is found in low amounts (Spivak et al., 1987). Its

expression can be reactivated during liver regeneration or in certain carcinoma cells of hepatic origin (Spivak et al., 1987) where it appears to play a role in proliferation (Wu et al., 2002). AFP has previously been shown to be up-regulated in Cu deficiency. Both p53 and AFP are expressed in germ cells (Wu et al., 2002), in hepatocarcinoma (Fu et al., 2003), in liver cancers (Cheng et al., 2000), in HepG2 (wtp53), HuH-7 cells (mtp53) but not in Hep3B cells where there is a deletion of p53 (Arima et al., 2002). AFP regulates growth and proliferation in HeLa cells by increasing cAMP concentration, and this regulation was not seen when AFP was blocked (Li et al., 2002).

The finding that AFP is up-regulated in TEPA-treated HepG2 cells is consistent with previous data showing that Cu deficiency in pancreatic cells is marked by increased AFP expression (Debeva et al., 1995). It is also interesting to note that AFP is a Cu-binding protein (Anderson et al., 1987; Lau et al., 1989) and may play a role in intracellular Cu transport (Lau et al, 1989). If this is true, an increase in AFP during Cu restriction may protect cells from deficiency. There is also evidence to suggest that treatment with AFP can increase expression of both mtp53 and mtp21 (Li et al., 2002). Thus, the finding that AFP increased in TEPA-treated HepG2 cells suggests that future studies should include examination of mtp53 and its effects in this cell type.

Vascular Endothelial Growth Factor. The finding that the vascular endothelial growth factor, VEGF, is up-regulated by TEPA in HepG2 cells is consistent with the bulk of the literature supporting an association between increased p53 expression and increased VEGF expression (Lee et al., 2002; Liu et al., 2002). In esophageal squamous carcinoma both these genes were increased, while p53(-) and VEGF(-) cells had decreased levels of both genes (Liu et al., 2002). Furthermore, inhibition of p53 degradation by hypoxia increased expression of VEGF (Chio et al., 2003). VEGF has also been implicated as an angiogenesis-related marker (Qin et al., 2002) and is over expressed in gliomas (Chinot, 1995). Increased NO-generation in tumor cells may select mtp53 and contribute to tumor angiogenesis by up-regulating VEGF (Weiming et al., 2002). This increase in VEGF in response to increase NO production, leads to increased vascularization (blood vessel formation to replace damaged vessels), but also increased tumor growth and invasiveness. VEGF was also increased in response to DNA damage induced by Riddelliine, which led to cell cycle arrest in S phase and an increase in p53 expression(Nyska et al., 2002). It was proposed that VEGF increased because Riddelliine increases endothelial

damage, thrombi are released as a result, which leads to VEGF up-regulation. But only a certain threshold of VEGF is needed to induce apoptosis in these cells. In contrast, in two animal models of breast cancer Cu-deficiency has been shown to decrease expression of VEGF and other growth factors (Pan et al., 2002). Furthermore, some studies found no association between VEGF and p53 expression. For example, in hypoxia VEGF increased regardless of p53 expression (Horiuchi et al., 2002).

Plasminogen Activator 1. TEPA treatment increased Plasminogen Activator 1 (PAI 1). While the significance of this regulation is not fully understood, p53 suppresses tumor metastasis by up-regulating metastasis suppressor genes such as PAI1 and masprin (Zou et al., 2000).

Phosphotyrosyl Phosphatase Activator. Phosphotyrosyl Phosphatase Activator (PTPA) has been shown to be a regulator of protein phosphotase 2A (Cayla et al., 1990), a major Ser/Thr phosphatase implicated in cell cycle control, response to signaling, cell differentiation, and cell transformation (Mumby et al., 1993). It has several p53 binding sites on its promoter region (Janssens et al., 2000). Binding of p53 to the PTPA promoter appears to down-regulate PTPA in a dose-dependent manner. Furthermore, HepG2 exposed to UV light accumulated p53 and down-regulated PTPA (Janssens et al., 2000), while four mtp53 cells failed to repress PTPA promoter activity. In a p53-negative cell line (Saos-2 cells) the PTPA activity increased much more than in p53 positive cells lines (U2OS and HepG2 cells). The finding that PTPA is up-regulated in p53(+) HepG2 cells after Cu deficiency is curious and suggests that Cu may regulate PTPA independently of p53.

Insulin-Like Growth Factor Binding Protein-6. Insulin-Like Growth Factor Binding Protein-6 (IGF BP6) has been shown to inhibit cell growth through IGF-dependent and independent mechanisms (Sueoka et al., 2000). Infection of NSCLC cells lines with IGFBP6 decreased cell number by activating programmed cell death, while injection of IGFBP6 into NSCLC cells reduced cell size by 45% (Sueoka et al., 2000). Furthermore, retinoids (RA) inhibit cell growth thru IGFBP6 as their mediator, human bronchial epithelial cells (HBE) treated with RA showed an increase in IGFBP6 mRNA and protein levels (Sueoka et al., 2000). In the current work, IGFBP6 was down-regulated in TEPA treated cells. This is consistent with the fact that TEPA-treated cells were not undergoing apoptosis.

Regulator of G Protein Signaling (RGS 14). Several p53 binding sites have been found on RGS14 promoter region indicating this gene can be regulated by p53 (Buckbinder et al.,

1997). Through RGS14 regulation, p53 may cause cellular sensitivity to growth and/or survival, using G protein-coupled receptor pathways. Treatment of RKO colon carcinoma cells with the anticancer and DNA-damaging agent doxocubicin leads to induction of RGS14. RKO-E6 cells with defective p53 signaling as a result of human papilloma-virus were unable to induce RGS14. Mutant p53 is also capable of inducing RGS14 expression (Buckbinder et al., 1997). While this gene is down-regulated in TEPA treated cells, there is evidence that RGS14 expression can be regulated by both p53 dependent and independent pathways based on cell type, culture condition, and tissue type.

Heat Shock Protein 70 (Hsp70). The heat shock family of proteins, of which Hsp 70 is a member, are characterized as chaperone proteins. They are responsible for the localization of regulatory proteins, and the mitogen-activated signal cascade (Hembrecht et al., 2000). Moreover, Hsp70 assists in folding of proteins, recovery from stress by repairing proteins and promoting cell survival (Jolly et al., 2000). It appears that both mt and wtp53 can interact with Hsp70 (Henkler et al., 1995). Increases in Hsp70 in p53 positive cells inhibited UV-induced apoptosis in HepG2 cells, while p53 deficient cells with increased Hsp70 showed no inhibition in UV-induced apoptosis (Chen et al., 1999). Cells with p53 antisense, showed increases in Hsp70 with no apoptosis (Chen et al., 1999). Hsp70 expression in WEH1-S cells resulted in increased tumorigenesis and increased resistance to apoptosis (Jaattela et al., 1995). It appears that Hsp70 prevents apoptosis upstream of caspase 3 and downstream of cytochrome c, making it a strong suppressor of apoptosis (Chun-Ying et al., 2000; Mosser et al., 2000). Furthermore, Hsp70 prevents cytochrome c/dATP mediated caspase activation and suppresses apoptosis by directly associating with Apaf-1 (Beere et al., 2000).

Decreases in Hsp 70 expression increased apoptosis in a caspase-dependent pathway in breast cancer cells and hsp70 -/- cells had increased cell death (Nylandsted et al., 2000). Hsp 70 anti-sense treatment inhibits the expression of Hsp70, which in turn inhibits cell proliferation and induces apoptosis in tumor cells suggesting that Hsp70 is required for tumor cells to proliferate and survive under normal conditions (Wei et al., 1995). Furthermore, Hsp 70 negative cells with mtp53 still died (Nylansted et al., 2000).

Despite the fact that most data suggest a protective role for Hsp 70, there is evidence showing that Hsp 70 can have both anti-apoptotic and pro-apoptotic effects. (Sapozhnikov et al.,

2002; Levenson, unpublished data). This novel function would be consistent with the down regulation seen in HepG2 cells treated with TEPA and warrants further investigation.

p85. Evidence in the literature indicates that p85 may be involved in cancer etiology since it has been shown to be up-regulated in human lung cancers (Eymin et al., 2002). p85 also plays a role in apoptosis, since in macrophages treated with Wortmannin (inhibitor of phosphatidyllinositol kinase) showed increases in apoptosis as well as up-regulation of p85 (Chen et al., 2002). p85 acts as a signal transducer in the cellular response to oxidative stress mediating cell death regulated by p53 (Yin et al., 1998). Disruption of p85 impairs cellular apoptosis response (Yin et al., 1998). This is consistent with the finding that TEPA treatment decreases p85 expression. Furthermore, increased expression of p53 (200%) and decreased p85 expression (90%) in L929 cells treated with hyaluronidase (Chang et al., 1998) also parallels results shown in TEPA treated HepG2 cells.

Transforming Growth Factors. The transforming growth factors, TGF-α. and TGF-β both play roles in cellular proliferation and apoptosis. However, their exact mechanism of regulation by p53 is not fully understood. TGF-α has been shown to have proliferative function in response to increased p53 (Inoe et al., 2002, Tan et al., 1994; Wu et al., 2002). It has been hypothesized that the proliferative response needed to replace damaged cells. While TGF-α has been shown to be activated by wtp53, it can also be increased by mtp53 (Shin et al., 1993). Furthermore, TGF-α activation increased p53, Bax and apoptosis, but did not affect cell cycle (Teramoto et al., 1998). Activation of TGF-β induced apopotosis via similar p53-dependent mechanisms (Yonish-Rouach et al., 1993; Loyer et al., 1996), but did not have an effect on the cellular growth rate (Teramoto et al., 1998).

In contrast to these data, other work has shown p53 had no effect on TGF-α (Lennartsson et al., 1999). Furthermore, dietary restriction increased p53 expression that resulted in no increases in TGF-α, TGF-β or apoptosis (Higami et al., 2000). This is consistent with the findings of the current study that show that TEPA treatment resulted in down-regulation of TGF-α, and the expression of TGF-β was very close to the cut-off for down-regulation. It is interesting to note that mtp53 can decrease both TGF-β and TGF-α (Kiss et al., 1997). While the luciferase data reported here do not indicate that there is mtp53 in the presence of TEPA, the findings of Kiss et al., suggest that future studies should examine TEPA-treated cells for the presence of mtp53.

REPRIMO. REPRIMO leads to cell cycle arrest at the G2 phase (Ye at al., 2002; Taylor et al., 2001; Ohki et al., 2000). REPRIMO induction was only possible in p53+/+ cells and not in p53 deficient cells (Ohki et al., 2000). Induction of p53 and REPRIMO in MEF cells decreased cell proliferation, induced complete cell cycle arrest and eventually programmed cell death (Ohki et al., 2000).

In TEPA-treated HepG2 cells REPRIMO expression is decreased. This is consistent with the fact that TEPA-treated cells are not undergoing apoptosis. However, it is not clear why this p53-stimulated gene is down-regulated. Possibilities include 1) the disruption of REPRIMO transcription by Cu deficiency, and 2) p53-independent mechanisms of REPRIMO regulation during Cu deficiency.

WIG-1. WIG-1 is a p53 target zinc finger protein, and appears to be induced by p53 and by DNA damage (Helborg et al., 2001). A p53 binding motif has been identified on the WIG1 promoter, and is very responsive to p53 induction (Margareta et al., 2002). Not all of WIG1 functions have been identified; those identified include inhibition of tumor cell growth and inhibition of colony formation (Helborg et al., 2001). Wig1 down-regulation expression in TEPA-treated HepG2 cells correlates well with other data showing that over-expression promotes apoptosis (Tomasevic et al., 1999)

Copper Toxicity

Effect of Cu toxicity on p53 activity. Luciferase data show that treatment of HepG2 cells with 200 μM Cu inhibits p53-mediated expression of the reporter gene used to monitor p53 activity. Previous work (Narayanan et al., 2001) has shown that Cu induces p53 expression. Thus, the reporter gene data are not the result of reduced p53 expression. Rather, it appears that Cu interferes with the ability of p53 to act as a DNA binding transcription factor. (Figure 3-2).

There are a number of possible explanations for these results. First, it is known that the phosphorylation state of p53 regulates its function. Phosphorylation increases the activity of p53, even in the absence of increases in protein expression (Choi et al 2002). Thus, it would be interesting to explore the ability of Cu to prevent the phosphorylation of p53 in hepatocytes using phosphorylation specific antibodies. Second, it appears that a reduction in p53 acetylation is necessary for the ability of p53 to bind to DNA (Chao et al, 2000). The role of Cu on acetylation also has not been tested, but poses an interesting avenue for future research.

The third possibility is that Cu causes p53 mutations in HepG2 cells. Human mutations in p53 have been shown to drastically alter its function. For example, mutant p53 (delta 62-91), which lacks all five PXXP (where P represents proline and X represents any amino acid), can induce cell cycle arrest but not apoptosis, while mutant p53 (gln22-ser23/delta 62-69), which contains double point mutation in the activation domains as well as deletion of the proline rich domain, has no activity. It is particularly interesting to note that mutations such as these have very different effects on the ability of p53 to regulate the expression of specific genes. For example, deletions of the proline-rich region reduced the ability of p53 to induce a variety of genes including p21, p85, and PIG3. However, induction of BAX, PIG2, PIG7, and PIG8 was unaffected (Zhu et al., 1999).

In vitro treatment of p53 cDNA with cupric chloride resulted in dose dependent mutations a majority of which were point mutations (Yu et al, 2002). Data from WD patients confirms that Cu can cause mutations in p53. WD patients had a higher frequency of G:C to T:A transversions at codon 249 ($P < 0.001$), C:G to A:T transversions, and C:G to T:A transitions at codon 250 ($P < 0.001$ and $P < 0.005$, respectively) even in non-cancerous tissue. Together these data suggest that the alterations in luciferase activity reported here may be the result of Cu-induced mutations in the p53 gene.

Another type of mutant that may be formed as a result of copper treatment is a conformational mutant. p53 depends on the coordination of a Zn atom for its wild-type conformation (Hartwig et al., 2002; Verhaegh et al., 1998; Hainaut et al., 1995). A second type of mutation that may occur in the presence of Cu is a conformational mutant. The presence of zinc permits folding of p53 into a conformation that is able to bind specific DNA sequences and regulate gene expression (Kernohan et al., 1996; Verhaegh et al., 1998; Meplan et al., 2000). Removal of Zn from p53 would induce a conformational change inconsistent with DNA binding activity. It has been shown that high Cu can bind to recombinant p53 and disrupt it ability to bind to DNA in normal, wild type fashion (Hainaut et al., 1995). The ability of Cu to alter the DNA-binding ability of p53 does not simply result from oxidative damage to the p53 protein as antioxidants did not prevent the action of Cu (Hainaut et al., 1995). This suggests that Cu may be displacing Zn from p53 (VanLandingham et al., 2002; Hartwig et al., 2002; O'Connor et al., 1993). While Cu-p53 has not been crystallized, it is likely that the conformation of Cu-bound

p53 would differ from wild type p53 with Zn. Thus, Cu toxicity may result in the formation of a conformational mutant in HepG2 cells.

Immunocytochemisty using antibodies for mutant (mt) and wild-type (wt) p53 supports the hypothesis that Cu causes mutations in p53. In fact, in Cu-treated HepG2 cells both forms of p53 were present. Unfortunately the currently available antibodies to p53 do not permit measurement of mt and wt p53 by immunocytochemistry in the same cell. Development of a set of antibodies that could be used in this manner (double label fluorescent immunocytochemistry) would be helpful in understanding the mechanism that is responsible for the production of mt p53 in Cu-treated HepG2 cells. The presence of both wt and mt p53 in the same cell would indicate that the mutation was conformational, not genomic. If the mutation was genomic, we would expect to find only mt p53 in that cell. Despite this limitation, our data suggest that the mutation caused by Cu is largely conformational. Examination of figure 3-4 shows that every cell nucleus is stained with wt or mt p53, suggesting the presence of both conformations in each cell.

Downstream genes regulated by p53 in Cu toxicity.

15-Lipoxygenase. The finding that the 15-lipoxygenase gene (15-LO) is up-regulated during Cu toxicity further supports the hypothesis that Cu causes a mutation in p53. Using a temperature sensitive mtp53 (mutant when cells were grown at 39° C, and wild type at 32° C) it was shown that expression of the15-LO gene is up-regulated by mtp53 (Kelavkar and Badr, 1999). 15-LO is responsible for the oxidative metabolism of linoleic and arachidonic acid to a variety of metabolites including 12-*S*-hydroxyoctadecadienoic acid (HODE), lipoxins (LXs), and 15-*S*-hydroxyeicosatetraenoic acid (HETE), which are involved in anti-inflammatory mechanisms (Kelavkar et al., 2000). 15-LO gene expression has been identified in human liver and a variety of normal and cancerous cells including prostate, kidney, brain, spleen and lung (Kelavkar et al 2000). To our knowledge this report represents the first finding of 15-LO expression in human hepatoma cells.

The biological significance of 15-LO in cancer cells is not known (Kelavkar et al 2000). Two reports suggest that HODE (the main metabolite of linoleic acid) inhibits cellular proliferation (Liu et al., 1995) and induces apoptosis (Shureigi et al, 2000). However, by far the weight of the literature suggests that HODE induces cellular proliferation (Ikawa et al., 1999;

Kamitani et al., 1998; Reddy et al, 1997; and others). It appears that at low (normal) levels 15-LO plays an anti-inflammatory role in tissue repair. However, when induced by mtp53 15-LO levels are abnormally elevated inducing cellular proliferation (Kelavkar et al., 2000). This suggests that while Cu toxicity clearly induces apoptosis in HepG2 cells, in vivo the formation of Cu-induced mtp53 would cause the overexpression of 15-LO, the subsequent proliferation of hepatocytes, and the development of cancer. It is also interesting to note that 15-LO was also induced in TEPA-treated cells. This suggests the presence of mtp53 not only in Cu toxicity, but in Cu deficiency as well. Thus, the possible role of both Cu toxicity and Cu deficiency in abnormal cellular proliferation needs to be explored.

Alpha-actin. Actin filaments are essential components of the cytoskeleton and are responsible for integrity of cell and membrane integrity. During apoptosis changes in the cytoskeletal filaments result in nuclear and cytoplasmic blebbing and the formation of apoptotic bodies (Zitterbart and Veselska, 2001). These morphological changes are clearly seen in Cu-mediated apoptosis. Thus, the differential regulation of actin by copper may be part of the process of apoptosis.

Retinoblastoma-1. While p53 guards against instability and oncogene expression by inducing both arrest of the cell cycle and apoptosis, the retinoblastoma protein regulates apoptosis during development, and its loss results in deregulation of growth and apoptosis (Hickman et al., 2002). pRB, the gene product of RB-1, is a tumor suppressor protein that plays an important role in cell cycle and apoptosis by governing the passage of cells through G1 phase-restriction point, promoting terminal differentiation and preventing cell cycle re-entry (Lai et al., 2003). Thus loss of RB function can lead to cancer. In addition to the well known role of RB mutations in retinoblastoma, loss of Rb protein expression has been correlated with lymph node metastasis, and Rb-/p53+ cells had decreased survival when compared to Rb+/p53- cells (Dosaka-Akita et al., 1996). The role of pRB is performed through interactions with p53 and MDM2. p53 can control the phosphorylation status of pRB and regulate its activity, while both p53 and pRB can interact with the oncogene MDM2. Furthermore, there is evidence that pRB impairs certain functions of MDM2 during the process of forming trimeric complex with p53; pRB overcomes the ability of MDM2 to inhibit p53-mediated apoptosis (Martin et al., 1995). In prostate adenocarcinoma RB1 was methylated in 6% of the cancers (Konishi et al., 2002).

Moreover, members of both pRB and p53 pathways have been found to be mutated in many human cancers (Stewart et al., 2001)

We have shown that RB-1 is expressed in HepG2 cells. To our knowledge this is the first report of RB-1 expression in any type of liver cell. We have also shown that RB-1 is up-regulated by Cu toxicity in HepG2 cells. This is consistent with the fact that in addition to the protein-protein interactions described above; RB-1 is also regulated at the transcriptional level by p53 (Lange et al., 2001). Thus, it is likely that in Cu-treated cells the induction of p53 increases RB-1 expression. This is part of the mechanism that triggers apoptosis.

PIG8 (EI24). The PIG8 gene, also known as EI24, gene is located on chromosome 11q23, a region frequently altered in human cancers (Gentile et al., 2001). The most commonly deleted chromosomal region in solid tumors, 11q23-q24, harbors both p53 and PIG8. In a population of early onset breast cancer patients, 39% had a mutation in the PIG8 gene (Gentile et al., 2001). PIG8, a p53-target gene, induces apoptosis when over-expressed and plays an important role in the prevention of many cancers (Gu et al., 2000; Gentile et al., 2001). Furthermore, M1tsp53 cells express high levels of p53 in the mutant conformation and actively proliferate when grown at 37°C. However, when the cells are shifted to 32.5°C, the tsp53 proteins assume a wt conformation, and PIG8 mRNA is markedly induced causing these cells to be rescued from p53-mediated cell death (Gu et al., 2000).

The finding that Cu-induced p53 reduced the expression of PIG8 is somewhat surprising. This may be the result of the presence of mt p53 in the cells. Alternatively, given that PIG8 is in close proximity to p53, both genes may have developed mutations as a result of Cu treatment. Another reason why this gene may have been decreased in Cu toxicity could be due to the fact that it is an early response gene (Gu et al., 2000) and the mRNA may have been degraded by the time these cells were sampled.

Glutathione Peroxidase. Glutathione peroxidase (GPX), is an enzyme that participates in the antioxidant process by converting hydrogen peroxide into water. This essential antioxidant enzyme decreased in many cancers such as anaplastic carcinoma and papillary carcinomas (Hasegawa et al., 2002). GPX, along with other antioxidant enzymes was significantly lowered in cervical cancer patients as compared to normal subjects (Nalini et al., 2002). Furthermore, GPX was decreased in prostate cancer cells (Suzuki et al., 2000). Decreased GPX provides the cells with weak protection against free radicals and reactive oxygen species (ROS), further

exacerbating DNA damage. In contrast, the administration of lycopene, a potent carotenoid and strong antioxidant, significantly decreased the formation of lipid peroxides and enhanced the activities of hepatic biotransformation enzymes such as GPX and reduced glutathione (GSH) (Bhuvaneswari et al., 2002). Treatment with ascorbic acid, also an antioxidant in citrus fruits, induced redifferentiation of human gastric cells, and increased the activities of superoxide dismutase and GPX (Zheng et al., 2002).

The finding that GPX mRNA is down-regulated in Cu-treated cells is consistent with the apoptosis observed in these cells. Given the fact that Cu is a powerful oxidant, a reduction in this enzyme during Cu toxicity would be expected to exacerbate Cu-induced DNA damage and cell death.

Microtubule-associated protein 4. The transcriptional status of p53 determines the sensitivity of cells to anti-microtubule drugs. This effect is mediated through the regulation of the microtubule-associated protein, MAP4 (Zhang et al., 1999). UV light increased the expression of wtp53 5-fold, but lead to a 5-7-fold decrease in expression of MAP4 5-7-fold. This increased the sensitivity of mouse ductal epithelial carcinoma cells to the drug vinblastine. Wtp53 induction by doxorubicin in C127 breast cancer cells repressed MAP4, and decreased microtubule polymerization (Bash-Babula et al., 2002). However, mtp53 increased MAP4 expression (Zhang et al., 1999; Chao et al., 2000).

The finding that Cu toxicity decreased MAP4 expression supports the finding that Cu induced wtp53 as well as mtp53 in these cells. If only mtp53 was expressed, an increase in MAP4 would have been expected. The finding of decreased MAP4 is also consistent with apoptosis in Cu-treated cells as over-expression of MAP4 in other cell types delayed p53-dependent apoptosis (Murphy et al., 1996).

Leucine Rich Death Domain Containing Protein. The Leucine Rich Death Domain Containing Protein (LRDD), also known as PIDD, is a novel death domain protein that participates in death receptor signaling (Lin et al., 2000). PIDD is an effector of p53-dependent apoptosis, since overexpression of PIDD inhibited cell growth in a p53-like manner by inducing apoptosis. Furthermore, anti-sense mRNA inhibition of PIDD expression attenuated p53-mediated apoptosis (Lin et al., 2000).

Despite the role of PIDD in apoptosis, we found that Cu treatment resulted in a reduction in PIDD suggesting that it is not playing a significant role in Cu-mediated cell death. The role of

mtp53 in the regulation of PIDD is not known and may account for the down-regulation of this gene.

Transforming Growth Factor-Beta. The p53-mediated up-regulation of the Transforming Growth Factor-Beta (TGF-ß) gene promotes apoptosis (Higami et al., 2002). On the other hand some literature supports the idea that TGF-ß may also act through p53-independent mechanisms. In this case, TGF-β induces p15, p27, p21 and RB hypophosphorylation (Tavassoli et al., 2002). The finding that Cu treatment reduced TGF-β mRNA abundance is consistent with a previous report that Cu (delivered as a tripeptide chelate) reduced fibroblast TGF-β secretion (McDormack et al., 2001). While the significance of this down-regulation is not known, it is interesting to note that hepatocellular carcinoma cells have been shown to have high levels of TGF-β that are correlated not to the apoptotic state of the cell, but rather to the differentiation state with increased differentiation resulting in lower levels of TGF-β (Idobe et al., 2003).

Fos. Fos is an early-immediate response gene. Its gene product, c-fos, is a transcription factor that plays a role in the regulation of a wide variety of genes. It also function as a proto-oncogene and induces the development of cancer. Under exposure of known carcinogens such as cadmium, fos expression increased (Spruill et al., 2002). Furthermore, in situations of increased stress, such UV exposure, the transcription of fos is increased while p53 is induced at post-transcriptional level (Matsumoto et al., 1994). The opposite response is seen when glycolic acid exerts an inhibitory effect on the UVB-induced skin tumor development by blocking the UVB-induced apoptosis and cytotoxicity through inhibition of c-fos and p53 (Ahn et al., 2002). In other studies, primary rat embryo fibroblast cells (REF) normally express mtp53. When shifted to 32.5° C, the protein assumes a wt-like conformation. When these cells were maintained at 37.5° C and then induced to resume proliferation upon re-addition of serum, c-fos mRNA levels increased rapidly. However when such experiments were carried out at 32.5° C induction of c-fos mRNA was less efficient (Ginsberg et al., 1991).

Our finding that fos expression is down-regulated in Cu-treated cells is consistent with an increase in RB1 expression. RB1, which is increased in Cu toxicity, can reduce the transcriptional activity of c-fos promoter (Ginsberg et al., 1991).

$p14^{ARF}$. The tumor suppressor ARF plays an important role as an inhibitor of Mdm2-mediated degradation of p53 (Menendez et al., 2003). By binding Mdm2 p14 prevents p53

degradation by Mdm2. Interestingly, both mt and wtp14Arf can bind to Mdm2 and increases levels of p53. Expression of this gene is decreased in gastric cancers (Tsujimoto et al., 2002). Furthermore, $p14^{ARF}$ was inactivated in 73% of glioblastomas (Ghumenti et al., 2003). Some studies show inverse correlation between $p14^{ARF}$ and p53 in NSCLC cells (Park et al., 2003; Vonlanthen et al., 1998). In cell lung carcinoma TP53 was mutated in at least 87% of cell lines, 14 of 27 cell lines with mutant TP53 also had inactivated $TP14^{ARF}$ whereas the remaining 13 cell lines had wild-type $TP14^{ARF}$ (Park et al., 2003). Other studies have shown that $p14^{ARF}$ is directly correlated to p53 in the same cell type (Sanchez-Cespedes et al., 1998). Mice with Rb+/- genotype survived 276 days while Rb+/-; $p14^{ARF}$ mice survived 168 days (Tsai et al., 2002).

The significance of the decrease in the expression of this gene is not known. However, it is interesting to note that loss of $p14^{ARF}$ has been shown to increase cellular proliferation (Tsai et al., 2002). Thus, this may, in part, help explain the role of Cu in proliferative cancers.

Conclusions and Future Studies:

In summary, the present work has identified a number of p53-responsive genes that are putatively regulated by Cu deficiency and toxicity. Future studies should include:

1. Confirmation of mRNA regulation by Northern analysis or other measures of mRNA abundance,
2. Examination of the protein levels and localization for each regulated gene in Cu deficiency and toxicity using immunocyotchemistry,
3. Examination of the in vivo effects of alterations of Cu on these genes and their protein products,
4. Identification of the threshold of responsiveness by doing dose response curves for individual genes,
5. Determine if TEPA treatment results in the formation of mtp53,
6. Crystallization of p53 in presence of Cu and elucidation of its 3-dimensional structure.
7. Test phosphorylation and acetylation of p53 in TEPA-treated and Cu-treated cells.

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BIOGHRAPHICAL SKETCH

Nadine Tassabehji was born in Beirut, Lebanon in 1978. During the war her family traveled to Saudi Arabia where she grew up. At the age of 15 years, she went to Malvern Girls' College in Worcestershire, UK where she did her A-levels. For her undergraduate studies, Nadine attended the American University of Beirut where she was actively involved in many activities such as the Environment club and was also member of the Student Representative Committee. Upon completion of her Bachelor degree in Dietetics and Food Technology, Nadine decided to attend graduate school at Florida State University where she did her Masters in Nutrition and worked in Dr. Cathy Levenson's lab on her thesis research project. At FSU she had the opportunity to teach HUN1201 science of nutrition course for the spring semester of 2003.

Nadine hopes to continue now with her PhD. at FSU under the guidance of Dr. Cathy Levenson. She hopes to continue being part of the research community and being involved in teaching by continuing in academia.