ZINC TRANSPORT AND METABOLISM IN THE EXOCRINE PANCREAS

By

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To my parents, Zhisheng Guo and Anrong Jin
my fiancée, Xin Deng

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LIST OF ABBREVIATIONS

Ab	Antibody
ADx	Adrenalectomy
AE	Acrodermatits enteropathica
AP	Affinity purified antibody
AP-1	Activator protein 1
BSA	Bovine serum albumin
ChIP	Chromatin Immunoprecipitation
CDF	Cation Diffusion Facilitator
cDNA	complementary DNA
CNH	Chromone-based nicotinyl hydrazone
CpdA	Compound A (an analog of a hydroxyphenyl aziridine precursor)
CP	Cytoplasm
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
DTPA	Diethylene triamine pentaacetic acid
EST	Expressed sequence tag
ER	Endoplasmic reticulum or estrogen receptor
GAS	Gamma interferon activation site
GC	Glucocorticoid
GFP	Green fusion protein
GI	Gastrointestinal
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element

HEK	Human embryonic kidney 293 cells
ICC	Immunocytochemistry
IF	Immunofluorescence
IHC	Immunohistochemistry
JAK2	Janus kinase 2
LZT	LIV-1 subfamily of ZIP zinc transporters
MRE	Metal response element
mRNA	Messenger RNA
MT	Metallothionein
MTF-1	Metal response element binding transcription factor-1
NF-κB	Nuclear factor-κB
PBS	Phosphate buffered saline
PKC	Protein kinase C
PNG	Peptide N-glycosidase
qPCR	Quantitaive polymerase chain reaction
rER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
siRNA	Small interfering RNA
shRNA	Small hairpin RNA
SNP	Single nucleotide polymorphism
SLC	solute carrier
STAT5	Signal transducer and activator of transcription 5
STAT5-RE	Signal transducer and activator of transcription 5 response element
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20

TGN	Trans-golgi network
TSS	Transcription start site
TMD	Transmembrane domain
TPEN	N,N,N’,N’-Tetrakis-(2-pyridylmethyl)- ethylenediamine
TRAIL	TNF-related apoptosis inducing ligand
VDAC	Voltage-dependent anion channel
ZG	Zymogen granule
ZIP	Zrt-Irt-like zinc transporter SLC39A superfamily
ZnA	Zinc adequate
ZnD	Zinc deficient
ZnT	Zinc transporter SLC30A superfamily

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ZINC TRANSPORT AND METABOLISM IN THE EXOCRINE PANCREAS

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Introduction: Zinc deficiency affects more than one billion children under 5 years old and is responsible for 400,000 deaths annually worldwide. Secretion from the exocrine pancreas is a major route of endogenous zinc loss. Thus it plays an important role in the maintenance of zinc homeostasis. The molecular mechanisms, pathways, and the transporters for pancreatic zinc secretion are still not clear. Glucocorticoid therapy increases zinc excretion, and causes a rapid depression of serum zinc. So far, no zinc transporter has been found to be regulated by glucocorticoid hormones.

Objectives: The major aims were to identify the zinc transporters in exocrine pancreas and evaluate their role in zinc secretion and to define physiological effects of zinc transporter expression in pancreatic acinar cells.

Results: Two highly expressed zinc transporters, ZnT1 and ZnT2 were identified in acinar cells, the major cell type in the exocrine pancreas. Immunofluorescence localized ZnT1 and ZnT2 to the plasma membrane and zymogen granules, respectively. Dietary zinc restriction significantly decreased the zinc concentration over 50% in both pancreatic cell cytoplasm and in zymogen granules and was correlated with decreased expression of ZnT1 and ZnT2. In contrast, with an up-regulated ZnT1 and ZnT2

observed after oral zinc administration produced an increase in pancreatic zinc content. Zinc stimulated ZnT1 expression in a dose-dependent manner in rat pancreatic AR42J cells. ZnT2 was stimulated by 100 nM dexamethasone during AR42J differentiation. In agreement, 10 mg/kg body weight dexamethasone administered to mice induced ZnT2 expression and resulted in a reduction in pancreatic zinc content. ZnT2 siRNA in AR42J cells caused an increase in cytoplasmic zinc and decreased zymogen granule zinc, which further shows that ZnT2 mediates the sequestration of zinc into zymogen granules. A crucial metal response element was found in the ZnT2 promoter that confers the responsiveness to zinc. Both glucocorticoid receptor and Stat5 signaling were required in dexamethasone induced ZnT2 expression.

Conclusions: ZnT1 controls zinc efflux directly across the apical membrane in a zinc-dependent manner, whereas ZnT2 participates in zinc sequestration into secretory granules. The two transporters appear to function closely in acinar cell zinc secretion and constitute an important component of the entero-pancreatic zinc circulation.

Significance: Understanding of the molecular mechanisms and regulation of endogenous zinc loss via pancreatic secretion is an important part of zinc homeostasis. The results of this study suggest that normal pancreatic acinar cell function is an essential physiologic component that influences body zinc retention and prevents zinc deficiency.

CHAPTER 1
INTRODUCTION

Zinc is an Essential Nutrient

Zinc is an essential micronutrient in humans. There are about 2-3 g of zinc in our bodies, which is the second largest quantity, after 4-5 g of body iron. Zinc deficiency affects more than one billion children under 5 years old and is responsible for 400,000 deaths annually worldwide (1). Zinc deficiency is a type II nutrient deficiency, fundamentally different from iron deficiency, which is type I nutrient deficiency (2). During iron deficiency, the concentration of iron in the tissues is reduced and specific defects develop, most commonly anemia. But there is no effect on growth and body weight until anemia and other complications develop. However, if dietary zinc is low in children, an immediate growth cessation occurs, while most tissue zinc contents are normal. In other words, in both iron and zinc deficiency in growing animals, the absolute amount of the nutrient within the body is less than normal. However, the reduced amount of iron is in a normal sized body, whereas with zinc, there is a restriction in body size to maintain the cellular zinc concentration. Therefore, growth retardation, delayed sexual maturation, and hypogonadism are the most common symptoms associated with severe zinc deficiency in children.

Current knowledge shows zinc has a significant biological role in catalytic, structural, and regulatory functions in hundreds of proteins (3-5). These properties explain why zinc is of such importance to maintaining normal cellular functions, and why zinc deficiency would produces a nutrient deficiency. The details of the cellular functions of zinc are reviewed in the following paragraph. Compared to iron, zinc overload is rare, and zinc is considered to be relatively nontoxic, particularly if taken orally. However, *in*

vitro studies have showed excessive zinc in the cell is toxic, causing apoptosis and necrosis (6). Therefore, the result of excessive cellular zinc is more acute and severe than the free radical damage by iron overload. Since both excessive cellular zinc and zinc deficiency cause deleterious effects, the zinc concentration in the cell, especially the labile zinc pool must be tightly regulated.

Body zinc homeostasis is achieved through balancing the zinc absorption, tissue utilization, and excretion. Dysregulation of zinc homeostasis contributes not only to systemic zinc deficiency and toxicity, but also to the pathogenesis of several diseases, including diarrhea, cancer, lower immunity, diabetes, Alzheimer's disease, acrodermatitis enteropathica, fume fever, and age-related macular degeneration (7-11).

The mechanism of zinc-mediated cytoxicity is still not very clear. Protein and nucleic acids synthesis inhibition, calcium signaling interference, and glutathione oxidation have been proposed to be the deleterious effects of elevated cellular zinc. If an extremely large amount of zinc is taken, toxicity symptoms will occur, including nausea, vomiting, epigastric pain, lethargy, and fatigue. Long term high zinc diets will also interfere with copper absorption, resulting in severe copper deficiency (12).

Cellular Functions of Zinc

Zinc is an important catalytic and structural component of more than 300 zinc metalloenzymes in more than 50 different enzyme categories. In some cases, depletion of zinc will result in a lower enzyme activity. Here are some examples of zinc enzymes: nicotinamide adenine dinucleotide dehydrogenase (NADH), RNA polymerase, DNA polymerase, alkaline phosphatase, superoxide dismutase, carbonic anhydrase, glutamic dehydrogenase, lactate dehydrogenase, and malate dehydrogenase. However, zinc has also been found to play an inhibitory role in certain enzymes. In other words, removal of

zinc from an inhibitory, zinc-specific enzymatic site results in a marked increase of enzyme activity (> 10-fold increase). These are some examples of these enzymes, caspase-3, fructose 1,6-diphosphatase, glyceraldehyde 3-phosphate dehydrogenase, aldehyde dehydrogenase, and tyrosine phosphatase. There are also pancreatic digestive enzymes associated with zinc: carboxypeptidase A1 and A2, aminopeptidase, carboxypeptidase B, amylase, chymotrypsinogen B1, lipase, trypsin 4.

Zinc Homeostasis

Cellular Zinc Homeostasis

Cellular zinc homeostasis requires tight control of zinc uptake/influx, storage, and export/efflux. Intracellular zinc compartmentation is an important source of cellular zinc storage. The vesicular zinc exchange with the cytoplasm pool plays a critical role in the cellular physiological functions of zinc. Different tissues have their own zinc metalloprotein profiles to undertake unique functions, thus the cellular zinc homeostasis control is a complex system requiring precise regulation. Zinc concentration in the plasma is about 9-15 μm , and most of the extracellular zinc carried in the plasma is bound to albumin (75-85%) (13, 14). The rest are bound to some other proteins, such as α-2 macroglobin (13). Serum zinc concentration falls with the hypoalbuminemia that accompanies aging (15). Two zinc transporter families, SLC30A and SLC39A, of a total of 24 zinc transporters so far have been found to function in this homeostasis system. Each one of them has its own unique tissue profile, expression regulation, intracellular localization, and transport activity (3, 5). Pancreatic intracellular zinc homeostasis is very sensitive to body zinc status. Homeostasis is achieved through the coordinated regulation of zinc transporters. So far, there are two zinc transporter families (Zips and

ZnTs) involved in zinc influx, efflux and intracellular compartmentalization within pancreatic acinar cells (3, 16, 17).

Zinc Metabolism and the Pancreas

Zinc homeostasis is a balance between gastrointestinal absorption, and tissue storage and secretion into the intestinal lumen through intestine cells and via the pancreatic duct. Under normal conditions, a considerable amount of zinc is released from the exocrine pancreas. The exocrine pancreas plays an important role in zinc homeostasis (18, 19). During periods of zinc deficiency, zinc output is decreased. Similarly, during excessive zinc intake, there is a marked increase in pancreatic output of zinc. Serum zinc increases with decreasing exocrine pancreatic function (20). Pancreatectomy increases serum zinc (21) and zinc deficiency decreases the pancreatic secretory response (22). Alterations in zinc metabolism have been reported in patients with pancreatic insufficiency. It was reported that a decrease in zinc output in duodenal aspirates occurs after secretion stimulation in pancreatic insufficient patients (23). The amount of zinc in pancreatic-biliary secretions is also dependent on body zinc status (19). In addition to zinc secretion, the pancreas and liver have been shown to be the most responsive organs to Zn-induced MT synthesis. Pancreatic zinc output is also found to be correlated significantly with enzyme and bicarbonate output, indicating that pancreatic zinc output could be a simple and accurate method for evaluation of the exocrine pancreatic function (24). Therefore, the adaptive mechanism of zinc metabolism in the human body will efficiently maintain zinc homeostasis under the conditions of varying dietary intake and physiological requirements. The role of bile secretion in zinc metabolism is relatively less clearer. In my dissertation, special focus

will be given to the up- and down- regulation of zinc transporters involved pancreatic zinc secretion.

Zinc is also an especially important mediator in insulin synthesis, storage and secretion in pancreatic β-cells. The 3:1 molar ratio of insulin to zinc reveals that insulin is stored as a hexamer with two zinc ions in the center (25). Therefore, zinc is secreted along with the release of insulin from β-cells. Recently, a systemic search for the genetic variants in type 2 diabetes mellitus was conducted using high-density array, which resulted in the genotyping of 392,935 single-nucleotide polymorphisms. A non-synonymous polymorphism in the zinc transporter 8, ZnT8 (SLC30A8), was identified among the four loci containing variants that confer type 2 diabetes risk (26).

A considerable amount of zinc is secreted in pancreatic-biliary secretions, and the actual amount depends on the body zinc status. Carboxypeptidase activity is lowered in zinc deficiency, and then returns to normal when given zinc supplementation (27).

Zinc Absorption

The primary site of zinc absorption is the proximal segment of the small intestine, including duodenum and proximal jejunum. There are many factors influencing zinc absorption. Dietary intake of zinc is one of the major factors, especially long-term, chronic zinc intake, which can influence the zinc absorption rate. Other dietary factors can also influence zinc absorption, such as phytate, as an inhibitor. Hence, zinc transporter expression is affected as well. Pancreatic zinc output was also found to be correlated significantly with enzyme and bicarbonate output, indicating that pancreatic zinc output could be a simple and accurate method for evaluation of the exocrine pancreatic function (24).

Zinc Excretion

The gastrointestinal tract is the primary site of maintaining zinc homeostasis; both zinc absorption and excretion are regulated in the GI tract. Endogenous zinc can be lost through many different routes. Fecal zinc loss is the primary zinc loss route, and pancreatic zinc secretion is believed to be the most abundant source of fecal zinc loss. Zinc can also be found in urinary excretions, integument loss, and in milk during lactation.

Zinc Metabolism and Pancreatic Metallothionein

The expression of metallothionein (MT) induced by zinc through the MTF-1 transcription factor has been well studied. The most responsive organs of MT induction by zinc treatment are the liver and pancreas, suggesting the importance of these organs in the zinc metabolism and homeostasis regulation. We and other groups found that the MT expression is dramatically and rapidly increased within 3 h after zinc injections or gavage. However, the induction returns to normal level 3 h after these treatment— in agreement with the finding that a new steady-state is quickly reached. MT is considered an important component of intracellular zinc homeostasis regulation. MT knock-out mice display zinc homeostasis dysregulation and higher endogenous zinc loss into the gut because of relatively lower zinc retention in the pancreas. In combination with the changes we have seen in the zinc transporter regulation, we can conclude that the zinc transporters work closely with MT in regulating the zinc secretion from the pancreas. On the other hand, MT might also modulate zinc homeostasis and metabolism in pancreatic islet cells and pancreatic endocrine neoplasms as those tissues contain matrix metalloproteinases, which require zinc in the catalytic domain (28).

Metallothionein is an important component for reducing the efficiency of zinc absorption at elevated zinc intakes (29). The transgenic mice with multiple copies of the MT-1 gene, showed 10- to 20-fold greater MT protein levels in the pancreas (30). This over-expression resulted in 300% more zinc in the pancreas. In contrast, MT levels decreased markedly when the mice were fed a Zn-deficient diet, whereas MT in other organs decreased only moderately (31, 32).

In MT knock-out (MT -/-) mice, the zinc secretion from the pancreas of subcutaneously administered ^{65}Zn is more than twice that of wild-type mice, and the absence of MT in the pancreas has been strongly implicated in causing this increase (33). The zinc concentration of the pancreas is lower in the MT knock-out mice, which indicates that less zinc is sequestered within the pancreas under steady-state conditions, resulting in a higher rate of endogenous zinc secretion from the exocrine pancreas (33, 34). MT-2 has also been found to be present in the pancreatic secretions (32), and this suggests that the MT-2 isoform, which is more resistant to degradation, may commit some pancreatic zinc to excretion (35). With adequate zinc intake, this difference in the handling of zinc in pancreas between MT knock-out and wild-type mice does not seem to be detrimental. However, during zinc restriction or deficiency, the decreased ability to limit secretion of zinc could be deleterious and may be one of the reasons why MT knock-out mice are less able to withstand zinc deficiency (36).

Zinc Deficiency

Acrodermatitis enteropathica (AE) is a rare inherited autosomal recessive disease caused by severe zinc deficiency. The typical symptoms are periorificial and acral dermatitis, alopecia, and diarrhea in infants. Two groups identified and described the SLC39A4 gene mutation in the AE patients (37, 38). There are many different types of

mutations spread over the entire gene. Most of the patients with AE have homozygous or compound heterozygous mutations. However, some of them have either no mutation in Zip4 or mutation in only one allele. The molecular basis of AE seems to be a complex genetic defect, therefore, there could be other molecular causes of dysregulation of the SLC39A4 gene transcription. There are some other symptoms associated with zinc deficiency not limited to AE, including hypogonadism, alopecia, impaired immunity, anorexia, dermatitis and impaired wound healing. Dietary zinc deficiency is unlikely in a healthy, well-nourished population. However, there are also many other conditions and diseases causing secondary zinc deficiency.

During zinc deficiency, psoriasiform dermatitis develops around the eyes, nose, and mouth, as well as on the buttocks. There are other symptoms including hair loss, low immunity, and recurrent infection, growth retardation, and diarrhea. Some other conditions related to zinc deficiency are malabsorption, diabetes mellitus, and stress (sepsis, burns, and head injury) causing acute phase response, hepatic insufficiency, diuretics, sickle cell disease, and chronic renal failure. The elderly institutionalized and homebound patients are also at risk of zinc deficiency (39).

Cell Biology of Zinc Chelation. There are many types of zinc ion chelators being used in cell biology studies (e.g. cell membrane permeable chelator, TPEN, and cell membrane non-permeable chelators, DTPA, DPA etc). These chelators show different zinc ion binding affinities, and specificities. For example, TPEN, has a high zinc ion binding specificity, but a low zinc binding affinity. Zinc chelation causes cell apoptosis, but the molecular mechanisms are not clear. A recent study shows that zinc chelation by TPEN can induce rapid depletion of the X-linked inhibitor of apoptosis, which is the

most potent member of the inhibitor of apoptosis protein. Thus, it sensitizes prostate cancer cells to TRAIL (TNF-related apoptosis inducing ligand)-mediated apoptosis (40). There is future potential for zinc chelation to be applied as a new approach to cancer therapy.

Zinc Toxicity

The upper limit of recommended dietary intake for zinc is 40 mg/day. Prolonged high zinc intake will cause copper deficiency at a level of 100 – 150 mg/day for 6 – 8 weeks. That is due to the interference of copper absorption by high concentration of zinc in the lumen of the small intestine. The induced copper deficiency causes red blood cell microcytosis, neutropenia, and impaired immunity. Quantities of 200 – 800 mg/day can cause more acute and severe symptoms, including anorexia, vomiting, and diarrhea (41). Damage to the pancreas has also been well documented in birds and chickens (42, 43). Inhaling zinc oxide fumes in industrial workers is causing metal fume fever (also called brass-founder's ague or zinc shakes), and severe neurologic damage. A recent study suggests a novel pathway, whereby zinc activates damage-sensing TRPA1 ion channels in the nociceptive somatosensory neurons (44).

Zinc Transporter Families

There are two zinc transporter families in mammalian cells, ZnT and Zip. ZnT (SLC30A) family is a group of zinc exporter, transporting zinc out of cellular cytoplasm to the extra-cellular space or intra-cellular compartments and organelles. So far, there are ten different members have been found in mouse and human, named from ZnT1 – ZnT10 (3-5). Due to the expression level and their responsiveness to the zinc status, the two members of ZnT family, ZnT1 and ZnT2, will be the major focus of this study.

The ZnT (SLC30) Family. The members of ZnT family share certain similarity in their protein structure and membrane topology. Their amino acid sequences share high similarity. For example, they are predicted to have six transmembrane domains (TMDs), and a histidine-rich loop (HX)3-6 between TMD IV and V, except for ZnT6, which has a serine-rich loop in place of histidine-rich loop. The histidine-rich loop is essential for ZnT5 and ZnT6 heterooligomeric structure (45).

The expression of most zinc transporters show high tissue specificity. ZnT1 is the first discovered mammalian zinc transporter (46). ZnT2 was first cloned from a rat kidney cDNA library by complementation and was found to be localized to acidic vesicles and to facilitate zinc sequestration in intracellular compartments (47). ZnT2 expression is limited primarily to the pancreas, mammary gland, prostate, small intestine, kidney, and placenta (48-50). When dietary zinc intake is low, ZnT2 expression decreases in some tissues e.g. pancreas (51). Previously we found ZnT1 is regulated by dietary zinc in both small intestine and liver (52). A mis-sense mutation (H54R) in ZnT2 in women produces low breast milk zinc content, and results in neonatal zinc deficiency in breast-fed infants (53). That finding suggests an important role of ZnT2 in secretory pathways for zinc in mammary gland. In human fibroblastoid cells, ZnT2 can facilitate vesicular zinc accumulation independently of AP-3, which might regulate the trafficking of ZnT family members to late endosomes and/or lysosomes (54).

The major objectives of the study on the zinc transport and metabolism in the exocrine pancreas:

1) Identifying zinc transporter(s) regulated by dietary zinc intake in the pancreas. And determining the molecular mechanism of the regulation of zinc transporter expression by dietary zinc.
2) Determining the signaling pathway involved in glucocorticoids-induced the expression of ZnT2 in the pancreatic acinar cells.
3) Localization and functional characterization of ZnT2 in the pancreatic acinar cells.
4) Preliminary study on the expression of zinc transporter in pancreatitis and pancreatic cancer.

CHAPTER 2
MATERIALS AND METHODS

Animal Experiments

CD-1 male mice, 25-30g (about 2 – 3 mo old, Charles River) were individually housed and fed a AIN76-based diet (Research Diets) formulated with egg white protein containing 0.85 mg Zn/kg diet or 30 mg Zn/kg diet for 21 days. Body weight and food consumption were measured every 7 days. Mice were killed on day 21. Blood was drawn by cardiac puncture and serum was isolated. These methods have been described previously (51). Liver and pancreata were removed, and excised pieces kept in RNA*later* (Applied Biosystems) at 4 ^{0}C. The remainder of the pancreata were kept in ice-cold phosphate-buffered saline until all the pancreata were collected. Crude nuclei, mitochondria, zymogen granules, golgi and plasma membranes and cytosol fractions were isolated as described in detail below. In some experiments, DEX phosphate was injected interperitoneally at 10mg/kg body weight. Alternatively, zinc sulfate dissolved in 0.9% NaCl was given orally (35µg zinc/g body weight or 0.9% NaCl alone) to the mice. Mice were sacrificed at 3 and 8 h after the gavage. Blood was drawn and tissues were collected for RNA isolation and zinc analysis. The procedures with mice were approved by the University of Florida Institutional Animal Care and Use Committee.

RNA Extraction and Quantitative Real-Time PCR

Real-Time PCR was performed by either one-step or two-step method. The one-step RT-PCR was directly from the total RNA samples, while the two-step RT-PCR was from reverse transcribed cDNA samples. Tissues were excised, and immediately homogenized (Polytron) with TRI reagent (Ambion). Total mRNA was isolated by TRIzol, and stored in DEPC-water. The total RNA was treated with DNase for 30min

(Ambion) before RT-PCR. TaqMan® universal PCR master mix was used as the reaction solution. 18S rRNA primers and the probe were from the TaqMan ribosomal RNA reagent kit (Applied Biosystems) were used for normalization. The RNA was reverse transcribed by reverse transcriptase using iScript reagents (Bio-Rad) for 30 min at 42°C, followed by 95 °C for 10 min and then 95 °C for 15 s and 60 °C for 1 min for a total of 40 cycles. The reaction was performed and fluorescence detected with an iCycler instrument (Bio-Rad). Standard curve and RNA sample reactions were run in triplicate with the standard curve over a 6 log range. In some experiments, total RNA was isolated using TRI reagent (Applied Biosystems), and treated with TurboDNase (Applied Biosystems) for 30 min before RT-PCR. Primer Express® version 3.0 software (Applied Biosystems) was used to design the oligonucleotide primers and TaqMan probes. cDNAs were synthesized using high capacity cDNA reverse transcription (Applied Biosystems) with a thermal cycler (MJ Research). Quantitative Real Time PCR reactions were performed and fluorescence was detected with the StepOnePlus system (Applied Biosystems). Values were normalized to 18S rRNA.

Isolation and Culture of Pancreatic Acinar Cells

The murine pancreatic acini was prepared by limited collagenase digestion using a well established procedure (55). The pancreata were removed from mice, minced and was incubated with a buffer containing 140 mM NaCl, 5 mMKCl, 1.8 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose and 0.1% bovine serum albumin (BSA). The buffer was supplemented with 10mM glucose and 0.02% soybean trypsin inhibitor and 2.5mg of collagenase. The pancreatic tissue was incubated in the solution for 60 min at 37 °C. After digestion, the acinar cells were separated by gradient centrifugation. Then the acinar cells were transferred to the collagen precoated slide

wells, and cultured in RPMI 1640medium. The cells were counted, and viability was determined.

Zymogen Granules Isolation

The zymogen granules were isolated from acinar cells on a Percoll gradient by the method described previously (56, 57). Briefly, pancreata were finely minced and homogenized in buffer containing, 0.3M sucrose 2nM MOPS, pH 6.8 and protease inhibitors (Sigma). Nuclei and cellular debris were removed by centrifugation at 750 × g for 10 min, and the supernatant was centrifuged at 1750 × g for 20 min. The resulting pellet was resuspended in the same buffer mixed with Percoll, and zymogen granules were further purified by separation with Percoll gradients at 60,000 g for 30 min. Mitochondrial and zymogen granule fractions were collected.

FluoZin-3 AM Labile Zinc Quantification

The cellular labile Zn^{2+} was detected using fluorescence microplate reader by incubating the cells with the fluorescence Zn^{2+} indicator probe, FluoZin-3 AM (Invitrogen). The experimental procedure was modified from the previous described method (58). Briefly, cells were loaded with FluoZin-3 AM for 30 min at 37ºC, and washed with PBS, and resuspended in DMEM supplemented with 0.3% BSA. Aliquots of the cell suspensions were incubated with TPEN, or zinc/pyrithione. The concentration of intracellular labile zinc was calculated from the mean fluorescence with the formula $[Zn] = Kd \times [(F - Fmin)/(Fmax - F)]$ (59). The dissociation constant of the FluoZin-3/zinc complex is 15 nM. Fmin was determined by the addition of the zinc-specific, membrane-permeant chelator TPEN, and Fmax was determined by the addition of $ZnSO_4$ and the ionophore pyrithione.

Immunoblotting and Immunohistochemistry

Protein concentrations were measured spectrophotometrically with Rc Dc reagents (Bio-Rad). Equal amounts of protein were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Whatman). Blots were stained with Ponceau S and de-stained. For western detection, blots were blocked in Tris-buffered saline (TBS-T) containing 0.1% Tween 20, 5% nonfat dry milk for 1 h (60). Affinity-purified antibody then was applied at 2.0 µg/ml for 1 h. After washing in TBS-T, diluted (1:10,000) secondary anti-rabbit IgG horseradish peroxidase-conjugated antibody (GE Healthcare) was applied. The blots were visualized by enhanced chemiluminescence by West Pico Chemiluminescent substrate (Pierce) and exposed to x-ray film for detection.

The tissue was fixed in 10% formalin in PBS buffer, and then embedded in paraffin, cut into 5µm sections. The cell cultures were mounted on poly-Lysine coated cover slips. Paraformaldehyde was used to treat the cells and was followed by permeabilization with Triton X-100. The tissue sections and permeable cells were incubated with primary antibodies and Alexa conjugate secondary antibody. Fluorescence images were obtained on an Axiovert 100 microscope.

^{65}Zn Uptake and Efflux Assay

Zinc uptake and efflux were measured using ^{65}Zn. The cells were incubated with $^{65}Zn^{2+}$ 30nCi/500ul, and then were washed and solubilized in 1%SDS, 0.2M NaOH solution. ^{65}Zn was measured with a Packard γ-ray spectrometer and the total protein was measured using the Bio-Rad *RC DC* protein assay reagent.

siRNA Mediated Knock-down

The recently established RNAi technology for gene knock-down gives us an alternative method for sequence specific post-transcriptional gene silencing. Four small

interfering RNA duplexes targeting the coding sequences of ZnT1 and ZnT2 were synthesized targeting a distinct region of each gene. The mixture of these four siRNAs were transfected into AR42J cells. After transfection and a 48 h incubation, the mRNA and protein were measured, and zinc uptake and excretion assays were done.

Culture of Rat AR42J Acinar Cells

AR42J cells (rat pancreatoma, ATCC CRL 1492) was purchased from American Type Culture Collection and were maintained at 37 °C in Ham's F-12K medium (Mediatech) with 0.1 mg/ml L-Glutamine, 15% fetal bovine serum (Mediatech) and 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B (Sigma) in a humidified atmosphere containing 5% CO_2 (61, 62). Cells at 0.5 × 10^6 /well in 6-well plates were cultured for at least 48 h before treatments. Cells were treated with 100 nM DEX phosphate (Sigma) in culture medium for 48 h for differentiation-induction. Some cultures also contained 1 µM RU486 (63) or 1 µM CpdA (64) as GR antagonist and selective agonist, respectively. Zinc chelators: DPA (dipicolinic acid), DTPA (Diethylene triamine pentaacetic acid), and ′,N′ TPEN (N,N,N -tetrakis(2-pyridylmethyl)ethylenediamine) were used separately to induce zinc deficiency in the cells (60). The cells were cultured in medium supplemented with 15% DPA-dialyzed fetal bovine serum, which was tested and confirmed to have a low concentration of zinc. The cells were cultured in the same medium with 3.5 µM $ZnSO_4$ added as a control medium. Alternatively, DTPA (50 µM) or TPEN (3.5 µM) were added to the cell culture medium, dissolved in PBS and DMSO, respectively. Control cultures contained PBS and DMSO, respectively at comparable concentrations. For siRNA experiments, SMARTpool siRNA, and non-targeting pool negative control siRNA resuspended in siRNA buffer were purchased from Dharmacon (Thermo Scientific). The cells were

seeded at 1.0 × 10^6 cells/ well in 12-well plate. Immediately after seeding, the cells were transfected with 300ng siRNA using HyperFect transfection reagent (Qiagen). Scrambled negative control siRNA (Dharmacon, Thermo Scientific)-transfected cells were considered as negative control.

Atomic Absorption Spectrophotometry

Tissue samples were weighted and digested in concentrated HNO_3 at 90^0C. The zinc concentration absorption spectrophotometry was measured using air acetylene. Zinc concentrations of subcellular fractions were measured similarly after aliquots were removed for measurement of protein (as above) for normalization.

Transfection and Luciferase Assay

HeLa and COS-7 cells were seeded at 1x10^5 cells/ well in 48-well plates. Transfection began 12 h after seeding with 15 nM (final concentration) of siRNA for mMTF-1 (Smart Pool, Dharmacon) using HiPerFect transfection reagent (Qiagen), and was carried out for 48 h. For the luciferase assays, HeLa cells were seeded on 12-well plates and transfected with 1 µg pGL3 or pGL4 plasmid and 0.001 µg pRL-SV40 plasmid (Promega), as an internal control, using Effectene reagent (Qiagen) or FuGene HD (Roche). After a 48 h incubation, the cell medium was replaced by medium with or without 100 nM DEX or 40uM $ZnSO_4$. After a 24 h incubation, cells were washed with PBS and lysed by 100 µl Passive Lysis Buffer (Promega) per well. Luciferease activities were measured with the Dual-GloTM Luciferase Assay System (Promega) in a SpectraMax M5 microplate reader by following the manufacturer's protocol. The raw values of firefly luciferase were normalized to renilla luciferase that transfected concurrently in all the assays to correct for differences in transfection efficiency. The promoter activity assays were measured in triplicate in each experiment and shown as

fold change relative to control. At least three sets of independent experiments were performed for each set of constructs.

Statistical Analysis

Results are expressed as mean ± SD from representative one of three independent experiments. The significance of variability was determined by an unpaired 2-tailed Student's *t*-test or by two-way ANOVA. $P \leq 0.05$ was accepted as statistically significant.

CHAPTER 3
ZINC REGULATES EXPRESSION OF ZINC TRANSPORTERS ZNT1 AND ZNT2 IN PANCREATIC ACINAR CELLS

Introduction

Over 90% of the pancreas is comprised of exocrine cells (65, 66). Localization studies have shown that pancreatic zinc is concentrated in the granules of acinar cells, where digestive pro-enzymes are also stored prior to exocytosis through the apical membrane for entry into the intestine. Abnormal zinc metabolism has been reported after pancreactomy (21, 66, 67). Zinc deficiency is believed to cause pancreatic acinar cells to become depleted of zinc (22, 68). Radiotracer studies have shown that the pancreas exhibits high rates of Zn turnover (69-72). The exocrine pancreas is a target organ of zinc toxicosis because of the high-rate zinc flux. Excessive dietary zinc alters acinar cell structure, and produces necrosis, causing depletion of zymogen granules and reduces digestive enzyme secretion (42, 73, 74). This sensitivity to zinc and high rate of zinc flux support the need for tight regulation of pancreatic acinar cell zinc transport.

Identifying the Abundance and Localization of Zip5, ZnT1 and ZnT2 in Acinar Cells. The transport process for zinc release is proposed to involve three different cellular events. The transport of zinc into the acinar cell takes place at the basolateral surface. This blood-to-exocrine flow of zinc is facilitated by a zinc importer, probably ZIP5, which has been shown to be expressed in pancreas acinar cells and localized to the basolateral membrane when dietary zinc is adequate. When zinc status is low, the basolateral localized ZIP5 is internalized in response to zinc deficiency (75). The intracellular compartmentalization of Zn^{2+} may occur concurrently with the formation of zymogen granules, where the zinc transporter ZnT2 was suggested to be localized and

to mediate Zinc compartmentalization (51). Eventually, stimulation of acinar cells by secretagogues triggers fusion of zymogen granules with the apical membrane and the subsequent release of the content of zymogen granules (76). The ZnT1 is the primary cellular zinc efflux transporter, and it appears to be the predominant ZnT family member at the plasma membrane. Therefore, ZnT1 may facilitate zinc efflux across the apical membrane. However, ZnT1 localizes not only to the plasma membrane, but also to the intracellular vesicles (46, 51).

Determining the Effect of Exogenous Zinc Concentration on ZnT1, ZnT2 and MT Gene Expression Levels in AR42J Cells. The exocrine pancreas is the major route of human endogenous zinc loss, and the aim of this study is to understand how zinc is released and the role of zinc transporters in this process. Therefore, it is important to fully understand the interaction between body zinc status and intracellular zinc and their regulation of ZnT1, ZnT2, and MT gene expression. Briefly, AR42J cells were cultured in F-12K medium with 15% fetal bovine serum for 2 days. The zinc content in this standard medium was determined by atomic absorption spectrophotometry. The cells were pretreated with 100 nM dexamethasone for 48 h prior to being placed in medium containing 5 μm , 10 μm , 20 μm , 40 μm , 80 μm , 120 μm , and 160 μm Zinc as $ZnSO_4$. The cells were harvested at 6 h post-treatment. Relative amounts of ZnT1, ZnT2, and MT mRNAs were quantified by the TaqMan qPCR method. Cells were also cultured in medium with 40 μm $ZnSO_4$ for a 16-hour study. The cells were harvested, as above, at 0, 2, 4, 6, 8, and 16 hour time-points post zinc treatment. ZnT1, ZnT2 and MT mRNA levels were measured as above.

Zinc Transporters Experiment in Murine Pancreatic Zymogen Granules. The zymogen granules in pancreatic acinar cells are specialized organelles, where digestive enzymes are stored. The pancreatic secretion of zinc markedly increased over baseline when CCK infusion stimulates the exocytosis of zymogen granules in human subjects (35). Thus, we speculate that, with an adequate dietary zinc intake, endogenous zinc in acinar cells is packaged via ZnT2 into a form that allows elimination with stimulated pancreatic secretions. Zinc deficiency causes a reduction in pancreatic zinc secretion and a reduction of the need for zinc packaging for export. Thus ZnT2 and perhaps other zinc transporters are related to zymogen granule formation for sequestering and providing zinc to the metalloenzymes and other constituents inside these granules.

Herein, mouse pancreatic acinar zymogen granules were isolated from the animals fed with high-zinc and zinc-deficient diets. Zinc contents were measured by atomic absorption spectrophotometry. Granule membrane fractions were used to detect ZnT/Zip proteins associated with the membrane by immunoblotting. As described above, amylase is a loading control for zymogen granules. Amylase is a zymogen granule content protein, which is used as a marker for organelle enrichment using an antibody against mouse amylase (77). The zinc concentrations in the zymogen granules were normalized to zymogen granule protein content.

Determining the Effect of Low Exogenous Zinc on ZnT1, ZnT2 and MT gene Expression Levels in AR42J cells. Similar to the rationale of the zinc treatment experiments, zinc-deficient status causes the reduction of zinc output from pancreas secretions. Zinc deficiency also decreases the pancreatic secretory response. Dipicolinic acid (Sigma) was used to chelate zinc from fetal bovine serum by over-night

dialysis (78). Picolinic acid is a metabolite of tryptophan and is considered a zinc chelator (79). Dipicolinic acid is a membrane-impermeable zinc-selective chelator. It appears to be relatively well tolerated in patients with pancreatic insufficiency (80). It has been demonstrated that adding picolinic acid to standard diets increased the amount of zinc actually absorbed. So, it is a more physiological zinc-binding ligand than TPEN, which is a cell-permeable zinc chelator. TPEN causes apoptosis at low concentrations. In this study, the F-12K medium with 15% dipicolinic acid dialysed FBS produced a medium with a zinc concentration as low as 0.45uM. ZnT1, ZnT2, and MT mRNA levels were measured by the TaqMan qPCR method. The protein level changes were measured through immunoblotting and the localization of the transporters was also shown by immunocytochemistry.

Results

Mice fed a zinc-deficient diet developed signs of zinc restriction in 3 weeks, as shown by dramatically decreased ZnT1, ZnT2 and MT expression (Fig.3-1), as well as a depressed serum zinc concentration (Fig. 3-2). However, high zinc diet did not alter the expression of ZnT1, ZnT2, or MT. In order to understand how zinc affects the pancreas, the zinc concentrations in subcellular fractions were measured. Zinc restriction resulted in pancreatic cytoplasm and zymogen granules having less than half the amount of zinc found in mice fed the zinc-adequate diet ($p < 0.01$). Interestingly, zinc concentrations of the mitochondria (data not shown) and crude nuclear fractions were not reduced by zinc restriction (Fig. 3-2). By contrast, the cytoplasmic and nuclear fractions from the liver did not change significantly (Fig. 3-2). Previously we showed that, of the 15 zinc transporter gene transcripts surveyed, only two, ZnT1 and ZnT2 mRNAs were differentially regulated by zinc restriction (51). The relative transcript levels of MT, ZnT1, and ZnT2 in

pancreatic RNA from the mice used in the present studies are shown in Fig. 3-1. These responses show the sensitivity of these zinc transporter genes to the dietary zinc intake level. Zip5 had previously been found in the pancreas, and its protein levels at the plasma membrane are regulated by zinc status (75, 81, 82). The immunoblotting results confirmed the disappearance of Zip5 during zinc deficiency (Fig. 3-3). Western blotting clearly showed the decrease of ZnT1 in the plasma membrane-enriched fraction during zinc restriction (Fig. 3-4, Fig. 3-5). The greater abundance of ZnT1 in the plasma membrane is consistent with the zinc efflux function of this transporter (46). Of particular interest is that ZnT2 was exclusively detected in the isolated zymogen granule fraction and showed a reduction in response to dietary zinc restriction (Fig. 3-4, Fig. 3-5). This novel finding of ZnT2 localized to isolated zymogen granules was confirmed by immunofluorescence confocal microscopy (Fig. 3-7). Consistent with secretory vesicle trafficking, the presence of some ZnT1 in the zymogen fraction is to be expected. Also of note is that the two marker proteins for plasma membranes and zymogen granules, Na^+/K^+ ATPase and amylase, respectively, were unaffected by zinc restriction.

When 35μg zinc/g body weight was given orally, pancreatic zinc content increased (Fig. 3-8, Fig. 3-9), and there were transient elevations in pancreatic MT, ZnT1, and ZnT2 mRNAs (Fig. 3-10). These results showed both ZnT1 and ZnT2 were very sensitive to elevated zinc intake, and suggested crucial roles in endogenous zinc excretion and homeostasis. None from the Zip family was found to have changed at the level of mRNA expression (Fig. 3-11).

Rat AR42J pancreatic acinar cells were used as a model to further understand the regulation of MT, ZnT1 and ZnT2 n pancreatic acinar cells. Various concentrations

of zinc were treated to the cells, causing a dose-dependent up-regulation of ZnT1 mRNA expression (Fig 3-12). The highest ZnT1 mRNA level was found at 160μM zinc, a much higher concentration than the concentration causing zinc toxicity to other types of cells. In contrast to ZnT1, the expression of ZnT2 did not response to supplemental zinc in the medium in the same cells (Fig 3-12).

The MT mRNA was significantly up-regulated by supplemental zinc, and the responsiveness was shown to be dose-dependent (Fig. 3-13). This result, as well as previous published findings, indicated a high MT-synthesizing capacity in the pancreas when there is an excess of zinc. By expressing a high level of MT, the pancreatic acinar cells can retain and tolerate a great amount of zinc. This transient accumulated zinc in the pancreas can be secreted gradually through pancreatic secretions. High zinc secretion and low retention were observed in MT knock-out mice.

Because the metal-responsive transcription factor MTF-1 regulates both *MT* and *ZnT1* genes, it was not surprising to see a similar response with the *ZnT1* gene (Fig. 3-12, Fig. 3-13). However, this is not the case of ZnT2 expression in AR42J cells treated with zinc. No zinc-induced mRNA expression increase was found (Fig. 3-12, Fig. 3-13). The mechanism is not clear here. It is speculated that hypermethylation of the ZnT2 promoter might cause the gene silencing. This will be discussed more in detail later.

To better understand the temporal regulation of MT and ZnT1 by zinc through MTF-1 activation, MT and ZnT1 mRNA abundance were measured in a 48 h time course with 40μm zinc. Both MT and ZnT1 mRNA levels were regulated in a time-dependent manner (Fig. 3-14). MT mRNA reached the peak level around 60-fold at 18 h and gradually decreased (Fig. 3-14). In contrast, ZnT1 showed a rapid increase at 6 h

for a 3-fold elevation, and the mRNA abundance kept at 2- to 3-fold higher than control throughout the rest of the 16 h (Fig. 3-14). ZnT1 protein level changes were confirmed by western blotting results (Fig. 3-15, Fig. 3-16). Although both genes are transcriptionally regulated through zinc-coupled MTF-1 activation, the expression patterns were found to be different. Again, we did not observe a transcriptional activation of the ZnT2 gene in this experiment (Fig. 3-14).

AR42J cells were also used in a cell culture model of zinc deficiency. Cells were cultured in a low zinc medium. Three different types of zinc chelators were tested in this series of experiments: DPA, DTPA, and TPEN. All three zinc chelators successfully induced zinc deficiency in AR42J cells in the culture medium, as confirmed by MT mRNA level as a sensitive cellular zinc indicator gene (Fig. 3-17, Fig. 3-18). Both ZnT1 and ZnT2 gene expression were found to be decreased after 24 h in low zinc culture medium at mRNA level (Fig. 3-17, 3-18) and protein level (Fig. 3-19, 3-20).

A mZnT1 expression plasmid was transfected into HEK 293 cells for an over expression study. twenty four, thirty six and forty eight hours after the transfection, cells were harvested and mZnT1 production was measured by western blotting with a polyclonal antibody raised against a ZnT1 peptide in a rabbit. A 60kDa band was detected at 36 h and 48 h after transfection, but not 24 h (Fig. 3-21). This 60kDa band matched the expected molecular weight of mouse ZnT1. Immunofluorescence microscopy images showed that, under membrane-permeable conditions, high fluorescence signals were observed to be located not only to the plasma membrane, but also intracellular compartments (Fig. 3-22).

There are many studies that have shown a difference regarding the size of ZnT1 on western blotting, and the reason is not clear. To validate and confirm the correct bands for ZnT1, total IgG and affinity purified antibodies were compared in western blotting. Soluble protein fractions and membrane protein fractions of AR42J cells were revolved on PAGE gels. The background was much less intense, and two clear bands of ZnT1 at about 36kDa and 20kDa were shown on western blotting by using the affinity purified antibody (Fig. 3-24). Both of these two bands could be competed out by pre-incubating primary antibody with the peptide, to which the polyclonal antibodies were raised against. Another weak band around 60kDa was also observed in ZnT1 membrane fractions, but not seen in soluble fraction. None of these three bands (60, 36, and 20kDa) were changed by peptide N-glycosidase digestion, suggesting that there is no N-glycosylation present in ZnT1 protein. Suprisingly, N-glycosylation modification was found in human ZnT1 protein with the total membrane protein sample prepared from HEK 293 cells, as shown in Fig. 3-24. The same glycosylation was also found in other zinc transporter members (38, 83, 84). However, the significance and functionality of this post-translational modification is not clear.

The multiple band sizes of ZnT1 on western blot make it difficult to interpret the results. The calculated molecular weight of ZnT1 is about 60kDa. However, an all western analysis of ZnT1 from the lab, 36kDa band is a predominant one, present in the total membrane fraction. In some experiments, when the protein samples of plasma membrane and ZG membrane were prepared through a gradient centrifugation, this 36kDa ZnT1 band could still be found in both factions, even more intense band in ZG membrane. I suspect this 36kDa band is a short form of ZnT1 present in secretory

vesicles in pancreatic acinar cells. When secretory vesicles are fusing to the plasma membrane to release their content, the membrane of these vesicles becomes a part of plasma membrane. Thus, presence of some 36kDa ZnT1 in the plasma membrane is to be expected. However, the 60kDa band in ZnT1 western blot might have a distinct localization. Based on the calculated molecular weight, the 60kDa band would likely be the full length ZnT1 protein.

The calculated molecular weight of ZnT2 protein is 48kDa; However, band size of ZnT2 in western blotting has not been well documented. Herein, we tested our ZnT2 polyclonal antibody on total soluble and membrane protein samples from AR42J cells. By comparing total IgG and affinity purified antibody, a distinct 42kDa band was found with much less background signal when probing with affinity purified antibody than total IgG antibody (Fig. 3-25). A peptide adsorption experiment showed a successful elimination of the 42kDa band, suggesting this band is a specific target of our polyclonal antibody binding (Fig. 3-25). This 42kDa band could not be altered by peptide N-glycosidase treatment to the total membrane protein samples (Fig. 3-24).

Discussion

Zinc transporter expression in the pancreas is of interest because pancreatic secretions constitute an important component of mammalian zinc homeostasis (22, 68, 72, 85-89). ZnT1 and ZnT2 are expressed in the pancreas (51), and they are associated with isolated plasma membrane and zymogen granules, respectively. In this study, we focus on regulation of the zinc transporter ZnT2 and have identified the role it may play in an endogenous zinc secretory pathway within pancreatic acinar cells. We propose that zinc output from acinar cells follows two distinct pathways: cell-to-ductal zinc efflux via the apical membrane, which is zinc-dependent and involves primarily ZnT1 for

cellular efflux; and zinc that is released along with digestive proenzymes from zymogen granules, where zinc is transported into the granules by ZnT2. These respective functions for ZnT1 and ZnT2 are in agreement with current proposed roles in zinc transport in other cell types (90).

Zinc in stimulated pancreatic sections has been shown to be associated with high-molecular-weight proteins, and is closely associated with the enzyme activities of zinc-containing enzymes, especially carboxypeptidase A and carboxypeptidase B (22, 68, 85). Since zymogen granules are the storage sites of digestive enzyme precursors (91), our finding that ZnT2 influences zinc incorporation into zymogen granules suggests this transporter may provide zinc for incorporation into digestive pro-metalloenzymes, or maintain a zinc-rich environment for the holo-metalloenzymes for full activity upon release. The reduction in activity observed during dietary zinc restriction is in agreement with our finding that transcript abundance for nine pancreatic digestive enzymes is not influenced by the dietary zinc restriction model used in the present experiments (data not shown). Acute zinc toxicity has been shown in avian species, mice, and the pig to be detrimental to normal pancreatic exocrine function and produces pancreatic atrophy (42, 73, 74). This sensitivity suggests that secretory pathways of zinc loss, are essential for preventing pancreatic enzyme release, necrosis, and atrophy. These signs of zinc toxicity are similar to the autodigestion of pancreatitis which is traced to abnormal calcium signaling within zymogen granules (92).

The secretory zymogen granules have an acidic intra-granule pH (93), which is in line with the notion that ZnT2 favors acidic vesicles for its maximal transport activity (47). It is relevant that ZnT8, expressed almost exclusively in the pancreatic Beta cell,

facilitates zinc transport required for proinsulin aggregation, using a process that involves proton exchange (94, 95).

The zinc and MT content of the pancreas are among the highest among tissues under normal conditions of dietary zinc intake. Such high expression suggests MT has an important role in regulating zinc metabolism and function in the pancreatic acinar cells. MT declines almost completely with a zinc deficient diet, whereas in other organs, it decreases only moderately (96). Pancreatic zinc secretion in $MT^{-/-}$ mice is much higher than in wild-type mice, and they are more vulnerable to damage caused by zinc deficiency(33, 97). MT is also found in pancreatic secretions, however, through a route that does not involve granule secretory pathways (32). In that regard, MT-bound zinc in pancreatic secretions could provide an endogenous zinc source for zinc re-absorption. Studies that could reflect on the glucocorticoid responsive expression of ZnT2 and a role in pancreatic zinc secretion are limited. It has been reported that adrenal insufficiency increases serum zinc concentrations, while administration of glucocorticoids and ACTH and the excess control production in Cushing's Syndrome decrease these concentrations (reviewed in (98)). Hypozincemia associated with glucocorticoid action has been related to induced synthesis of MT in rodents (99). Radiotracer kinetic studies with Zinc-69m given intravenously to humans revealed that carbohydrate-active steroids (glucocorticoids) may alter rate constants of the fecal excretion of zinc (98).

The pancreatic acinar AR42J cell model has been widely used to characterize effects of glucocorticoid hormones on secretory activity of the exocrine pancreas (62, 100). Dexamethasone treatment of AR42J cells induces a highly differentiated

phenotype. It has been reported that RU486 can reduce GR concentrations in AR42J cells by 50% with concomitant changes in GR regulated gene expression (101). That ZnT2 regulation is controlled by GR was established by demonstrating inhibition through RU486. This strongly suggests that the signaling pathway involves GR dimerization, rather than the anti-inflammatory pathway involving assembly of the NF-kB · GR dimer complex. GR involvement in pancreatic tissue organization and the differentiation of acinar cells, as well as enzyme and zymogen granule production, is compatible with a role in ZnT2 regulation. Furthermore, the control zinc provides via MTF-1 responsive ZnT2 expression is consistent with a role in endogenous zinc secretion. That control, combined with the DEX responsiveness, makes ZnT2 analogous to MT in terms of a teleological basis for dual regulation. To our knowledge ZnT2 may represent the first member of either the ZnT or Zip gene families to be glucocorticoid-regulated. The next chapter will focus on this topic.

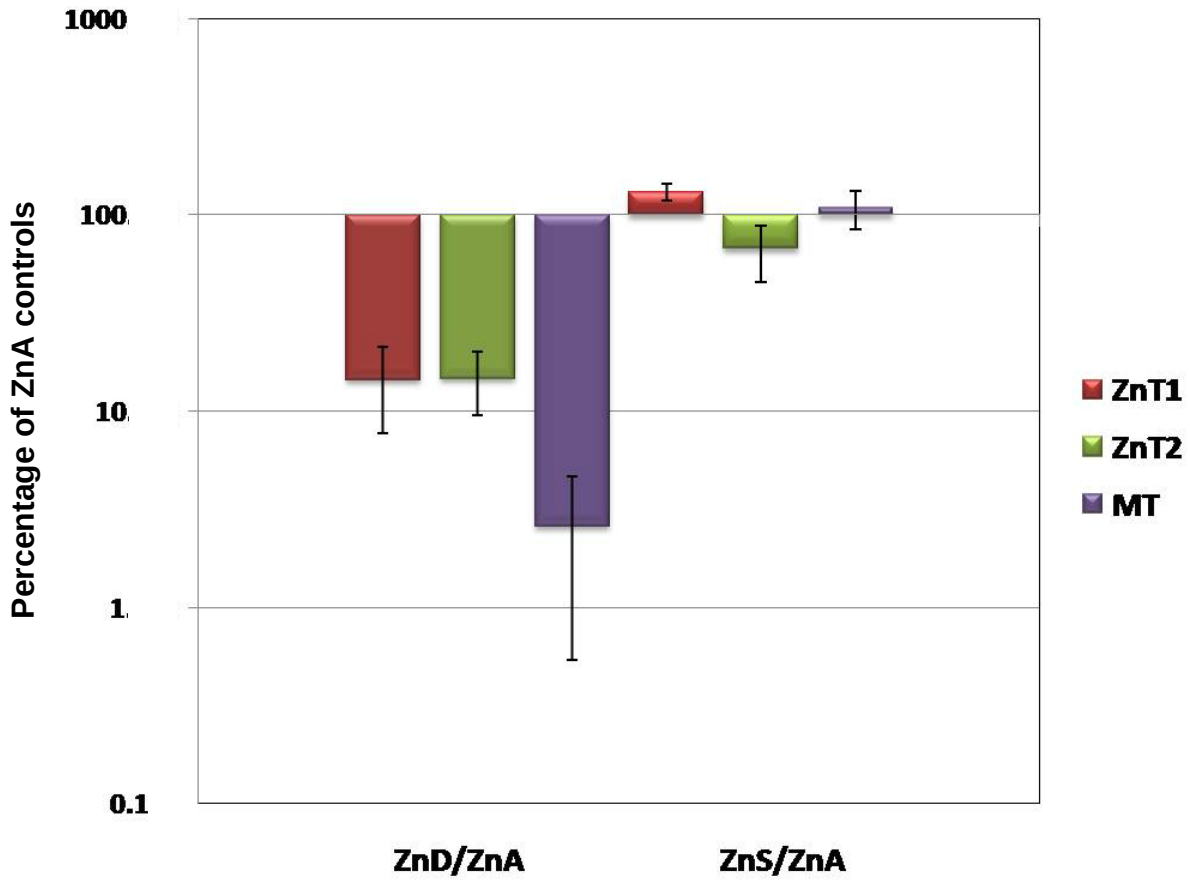

Figure 3-1. ZnT1, ZnT2 and MT mRNA expression in mouse pancreas after 3 wks of dietary zinc restriction (ZnD) or zinc supplementation (ZnS). qPCR was used to measure the relative mRNA abundance change to zinc restriction and supplementation diet, and the results were expressed as the percentage of ZnA control. Y-axis is in log scale. ZnA is zinc adequate control (n = 3-4).

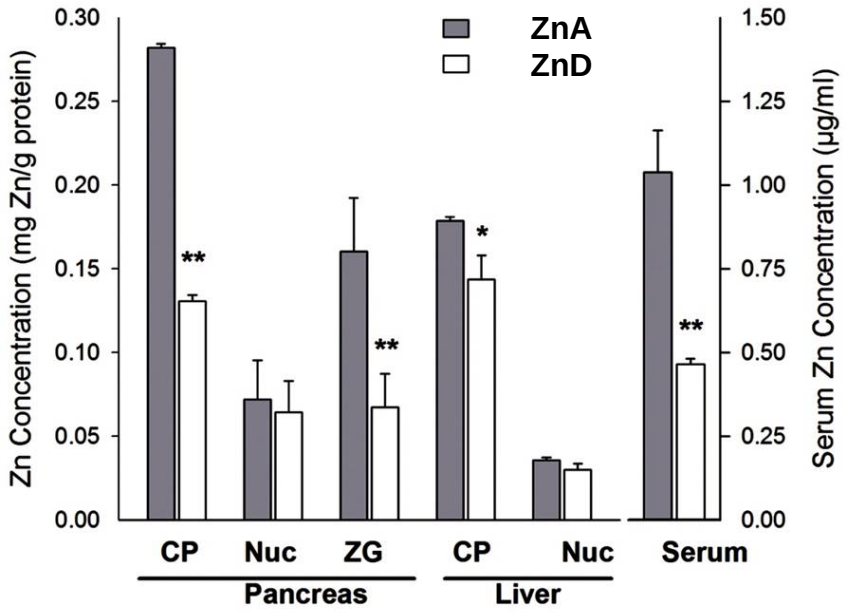

Figure 3-2. Zinc concentrations in serum, pancreas and liver from mice fed with zinc adequate or deficient diet. CD-1 mice were fed zinc adequate or deficient diets for 3 wks. Blood was drawn and serum was isolated. The pancreas and liver were collected and homogenized for cell fractionation. Cytoplasm, nucleus, and zymogen granule compartments were isolated and the zinc concentrations were measured by atomic absorption spectrophotometry and normalized against the protein concentration. The values are expressed as the mean of mg Zn/g protein ± standard deviation (n=3).

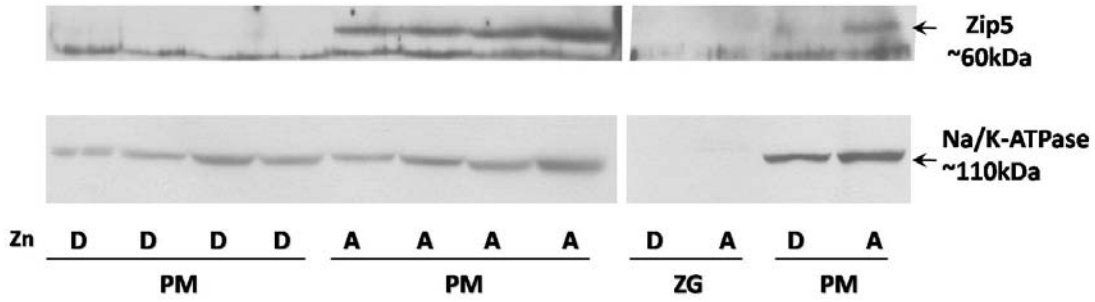

Figure 3-3. Zip5 expression in mouse pancreas after 3 wks dietary zinc restriction (ZnD) or a zinc adequate diet. Mouse pancreas was removed and fractionated, plasma membrane (PM) protein and zymogen granule (ZG) were obtained. Western analysis showed Zip5 protein abundance in plasma membrane fraction. No zip5 was detected in ZG fractions. Na^+/K^+-ATPase is the loading control. D, is for zinc deficiency; A, is for zinc adequate. A non-specific band (slightly lower than the Zip5 band) is detected in the plasma membrane (PM) and resistant to peptide competition.

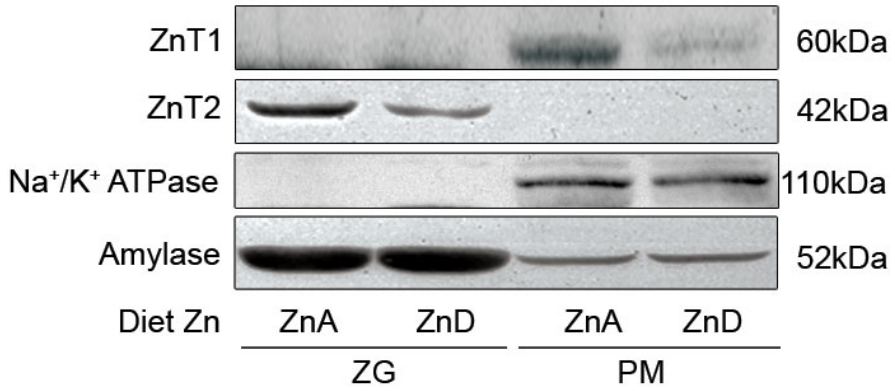

Figure 3-4. ZnT1 and ZnT2 western blotting in mouse pancreas after 3 wks dietary zinc restriction (ZnD). Mouse pancreas was removed and fractionated, plasma membrane enriched fraction (PM) and zymogen granule (ZG) were purified. Western blots here showed the protein abundance of ZnT1 and ZnT2 in plasma membrane, and zymogen granule. Na^+/K^+ ATPase is the plasma membrane loading control. Amylase is the zymogen granule loading control. ZnA is dietary zinc adequate control.

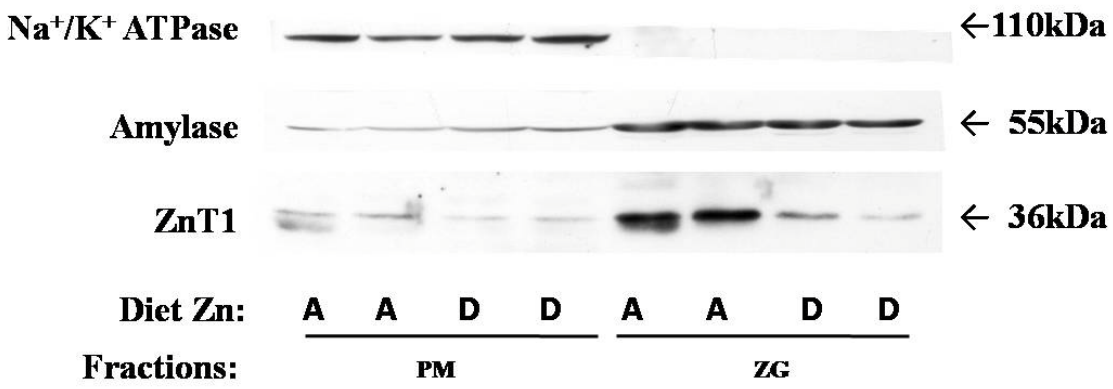

Figure 3-5. ZnT1 western blotting in mouse pancreas after 3 wks dietary zinc restriction (ZnD). Mouse pancreas was removed and fractionated, plasma membrane enriched fraction (PM) and zymogen granule (ZG) were purified. Western blots here showed the protein abundance of ZnT1 in plasma membrane, and zymogen granule. Na^+/K^+ ATPase is the plasma membrane loading control. Amylase is the zymogen granule loading control. ZnA is dietary zinc adequate control.

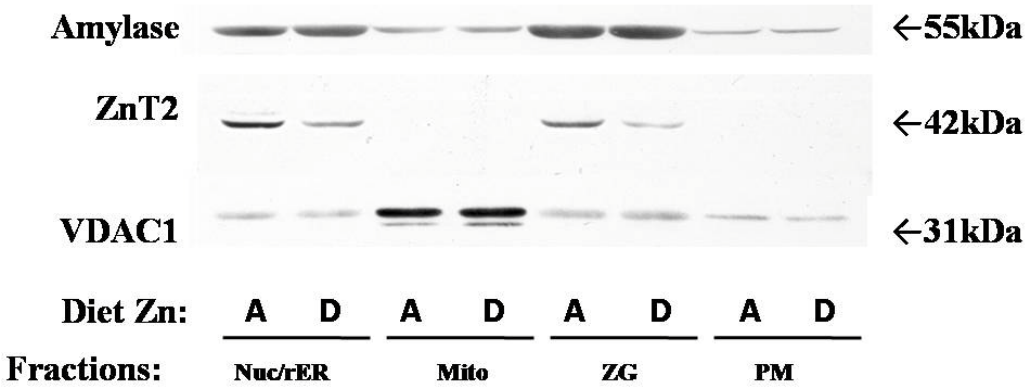

Figure 3-6. ZnT1 western blotting in mouse pancreas after 3 wks dietary zinc restriction (ZnD). Mouse pancreas was removed and fractionated, crude nuclear fraction (Nuc) with rough ER (rER), mitochondria (Mito), plasma membrane enriched fraction (PM) and zymogen granule (ZG) were purified. Western blots here showed the protein abundance of ZnT2 in each fraction. Amylase is the zymogen granule loading control. VDAC1 is the loading control for mitochondria. A, is dietary zinc adequate control.

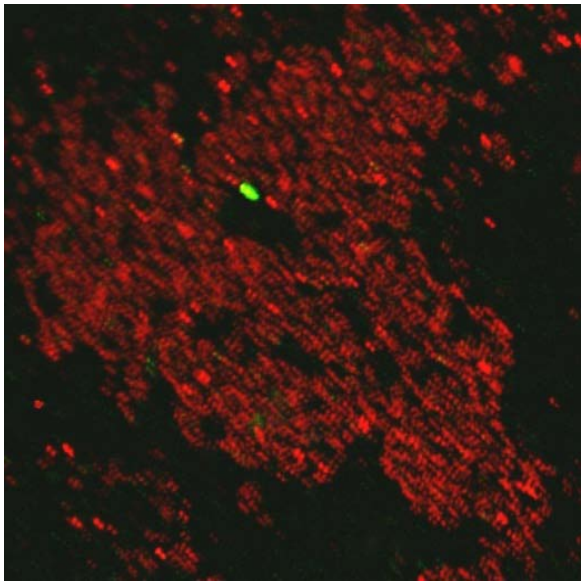

Figure 3-7. Confocal immunofluorescence analysis of ZnT2 in purified zymogen granules isolated from mouse pancreas.

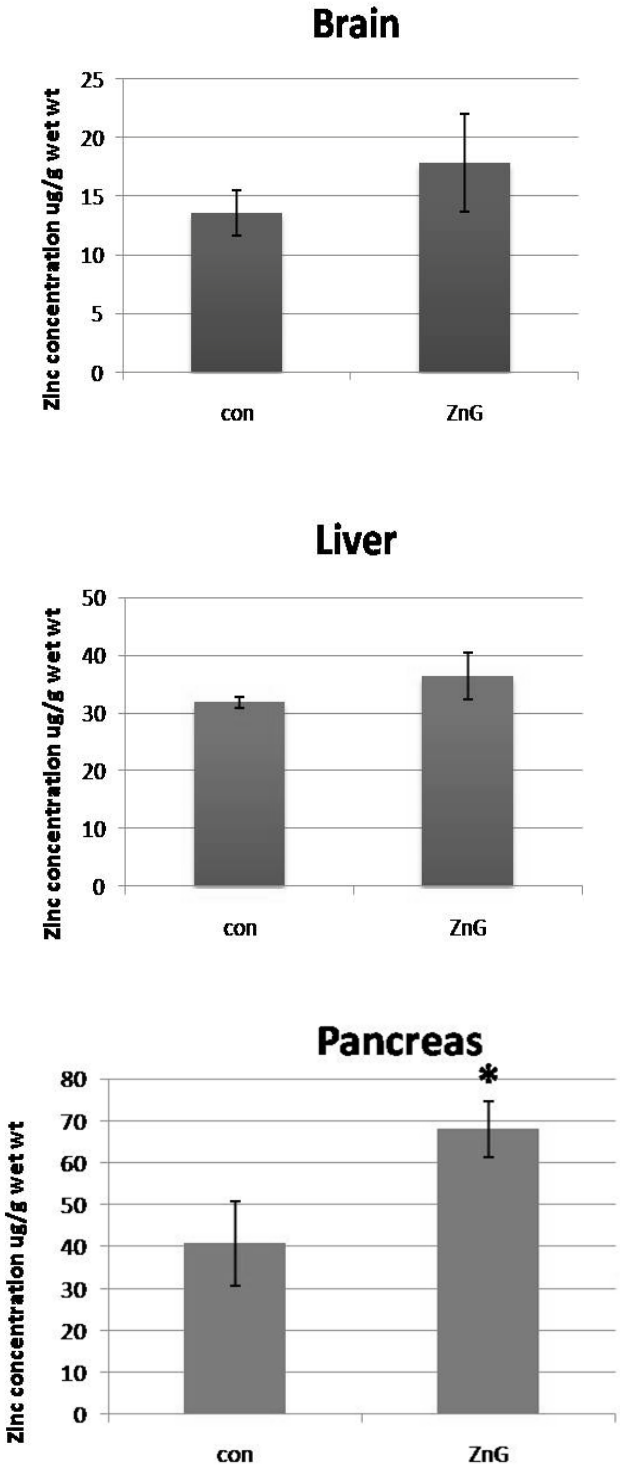

Figure 3-8. Zinc concentrations in the brain, pancreas and liver of mouse 3 h after a zinc gavage (ZnG). Saline was given as control (con). Tissue zinc concentrations were measured by atomic absorption spectrophotometry and normalized against wet wight (n = 3).

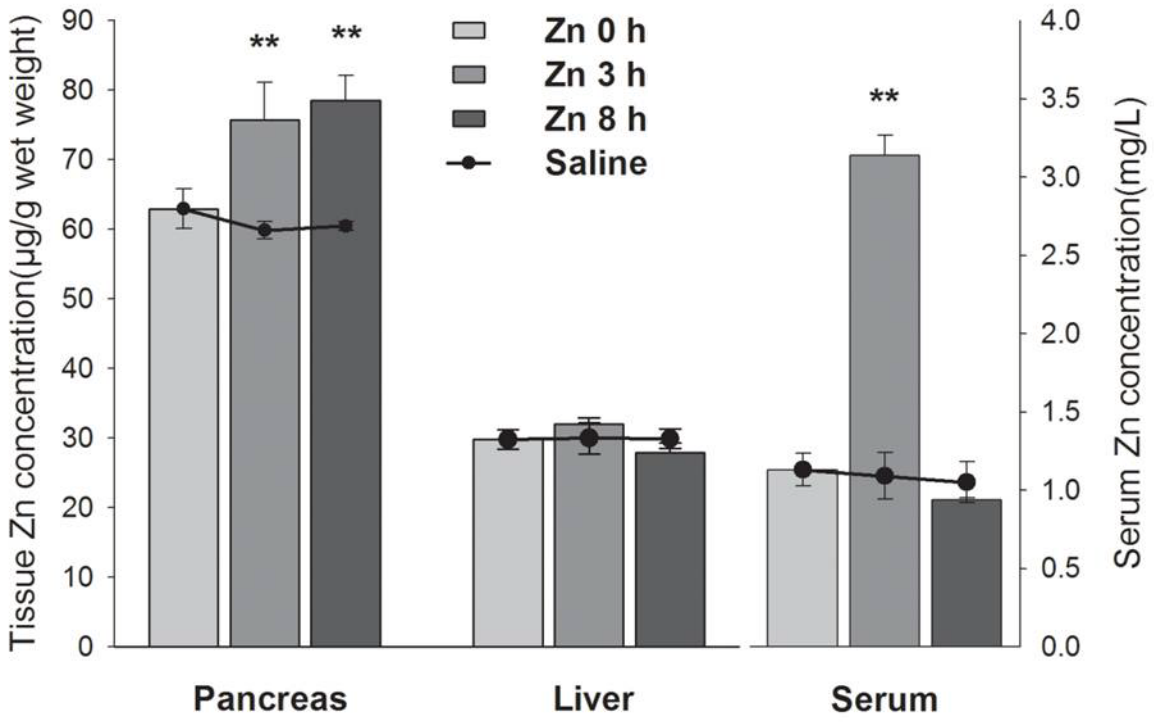

Figure 3-9. Zinc concentrations in the pancreas, liver and serum in mouse 3h and 8h after zinc gavage. Saline was given as control. The pancreas, liver and serum were collected, and tissue samples were digested in nitric acid. Zinc concentrations were measured by atomic absorption spectrophotometry, normalized against tissue wet weight. The values from mice given zinc by gavage (Zn) are expressed as bars, and the saline controls are expressed as lines (n=3-4).

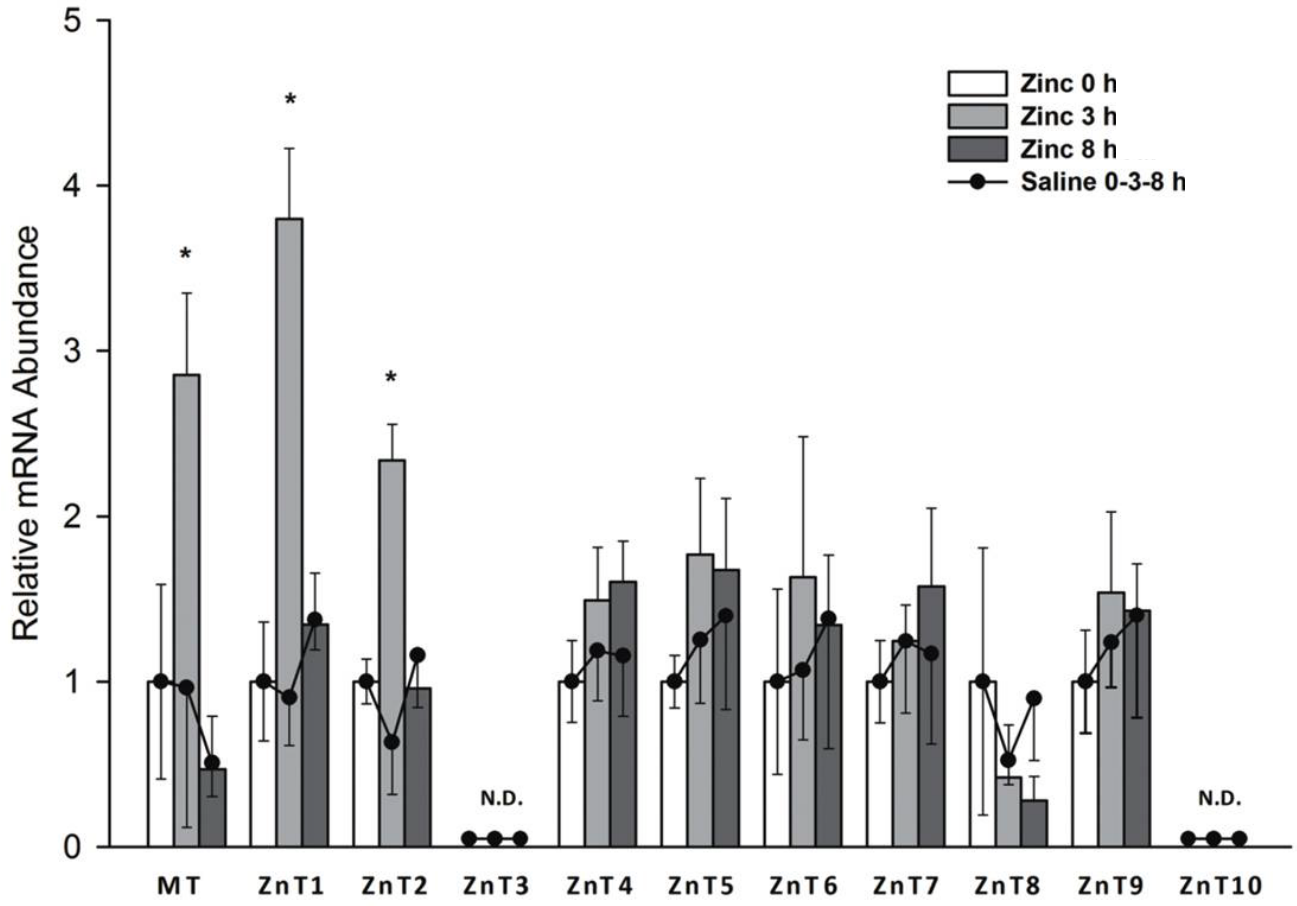

Figure 3-10. ZnT zinc transporter family mRNA expression in the pancreas from mice 3 h and 8 h after zinc gavage of 35 mg zinc / g bwt. Saline was given as control (n = 3-4).

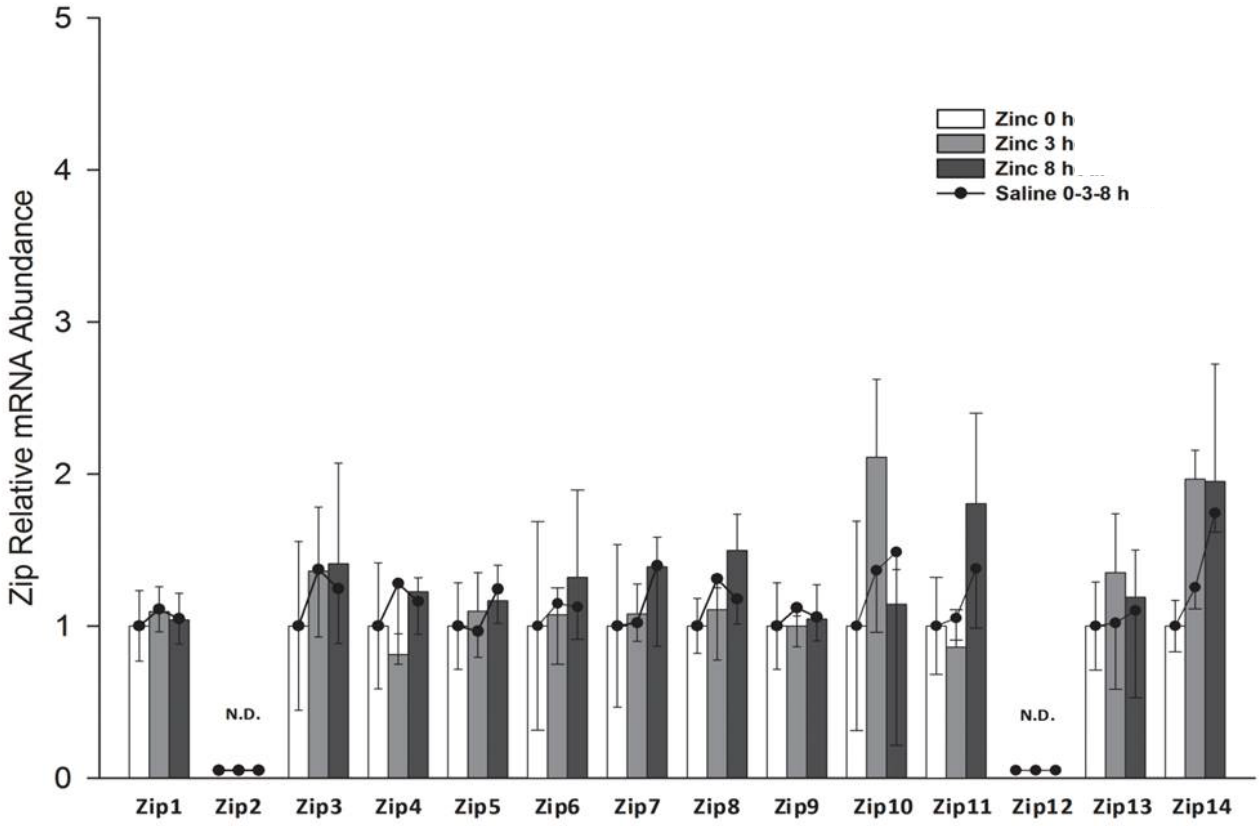

Figure 3-11. Zip zinc transporter family mRNA expression in the pancreas from mice 3 h and 8 h after zinc gavage of 35 mg zinc / g bwt. Saline was given as control (n = 3-4).

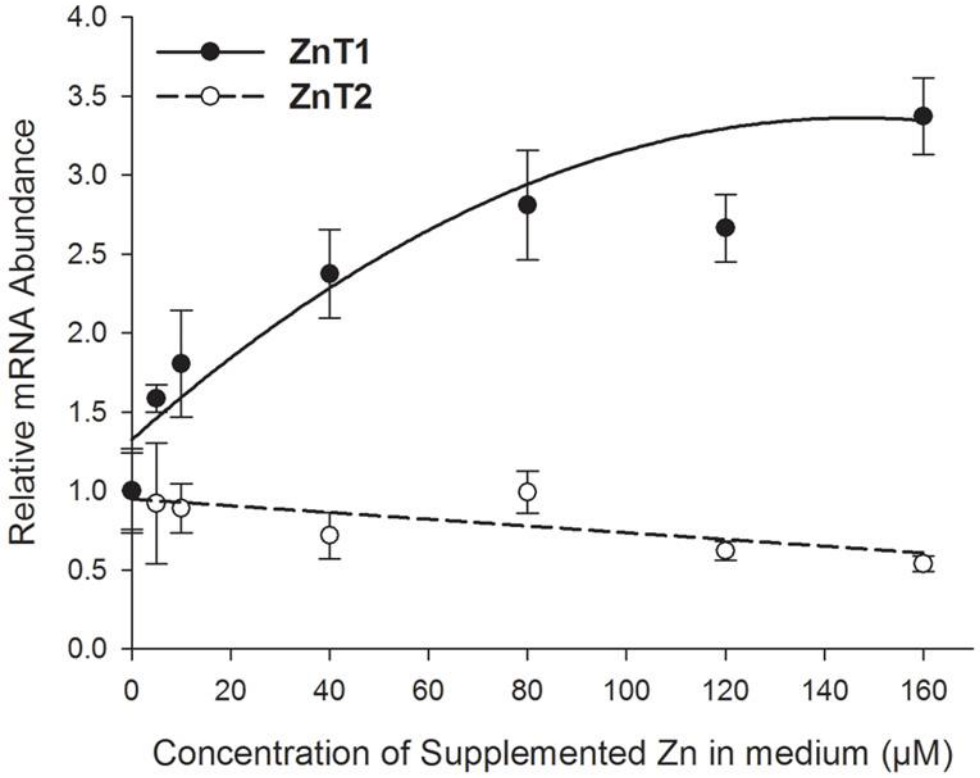

Figure 3-12. Zinc influences ZnT1 mRNA levels in a dose-dependant manner in AR42J cells. The cells were pretreated with dexamethasone (100 nM) for 48 h. Different concentrations of $ZnSO_4$ (0 μM — 160 μM) were then added to the culture medium for 6 h. qPCR was used to measure ZnT1 and ZnT2 mRNA levels after zinc treatment. A fitted correlation line is shown (n = 3).

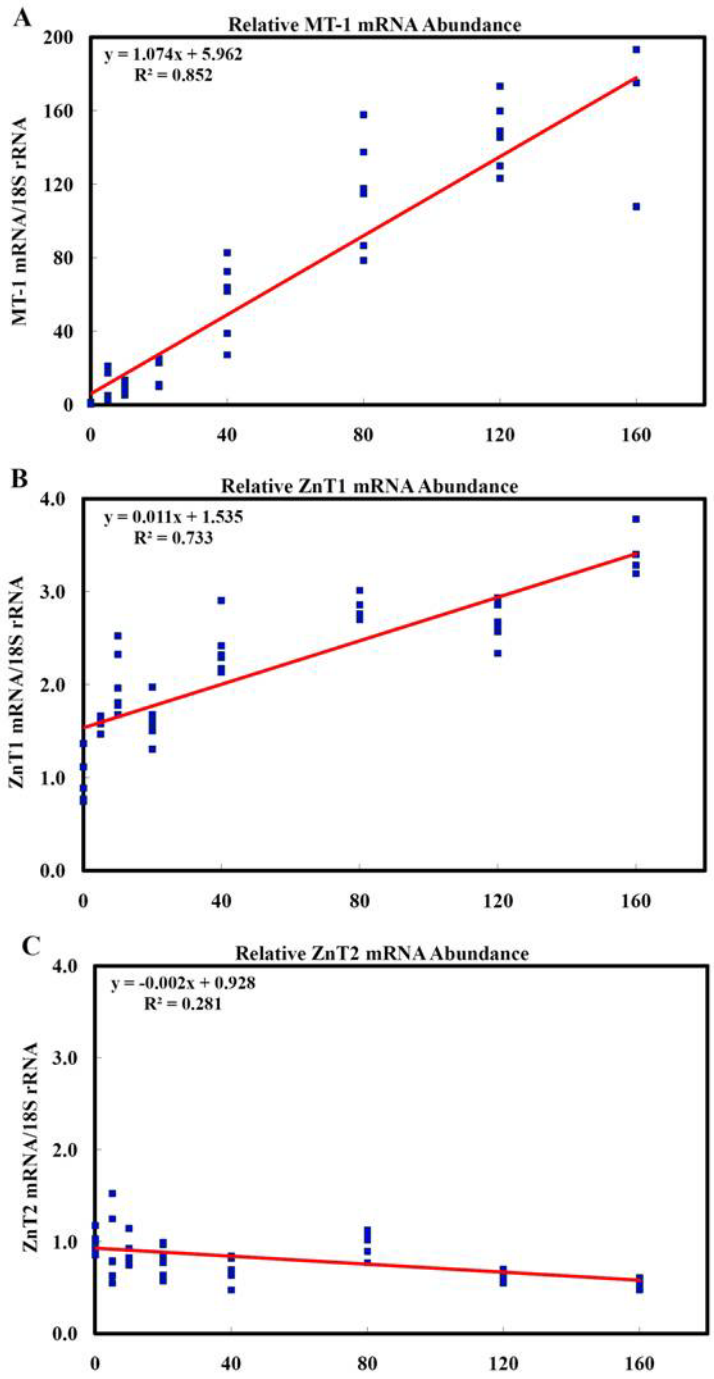

Figure 3-13. Zinc influences MT1, and ZnT1 mRNA levels in a dose-dependant manner in AR42J cells. The cells were pretreated with dexamethasone (100 nM) for 48 h. Different concentrations of $ZnSO_4$ (0 μM — 160 μM) were then added to the culture medium for 6 h. qPCR was used to measure MT (A), ZnT1 (B), and ZnT2 (C) mRNA levels after zinc treatment. Fitted linear correlation and Pearson's correlation coefficient (R) are shown (n = 3).

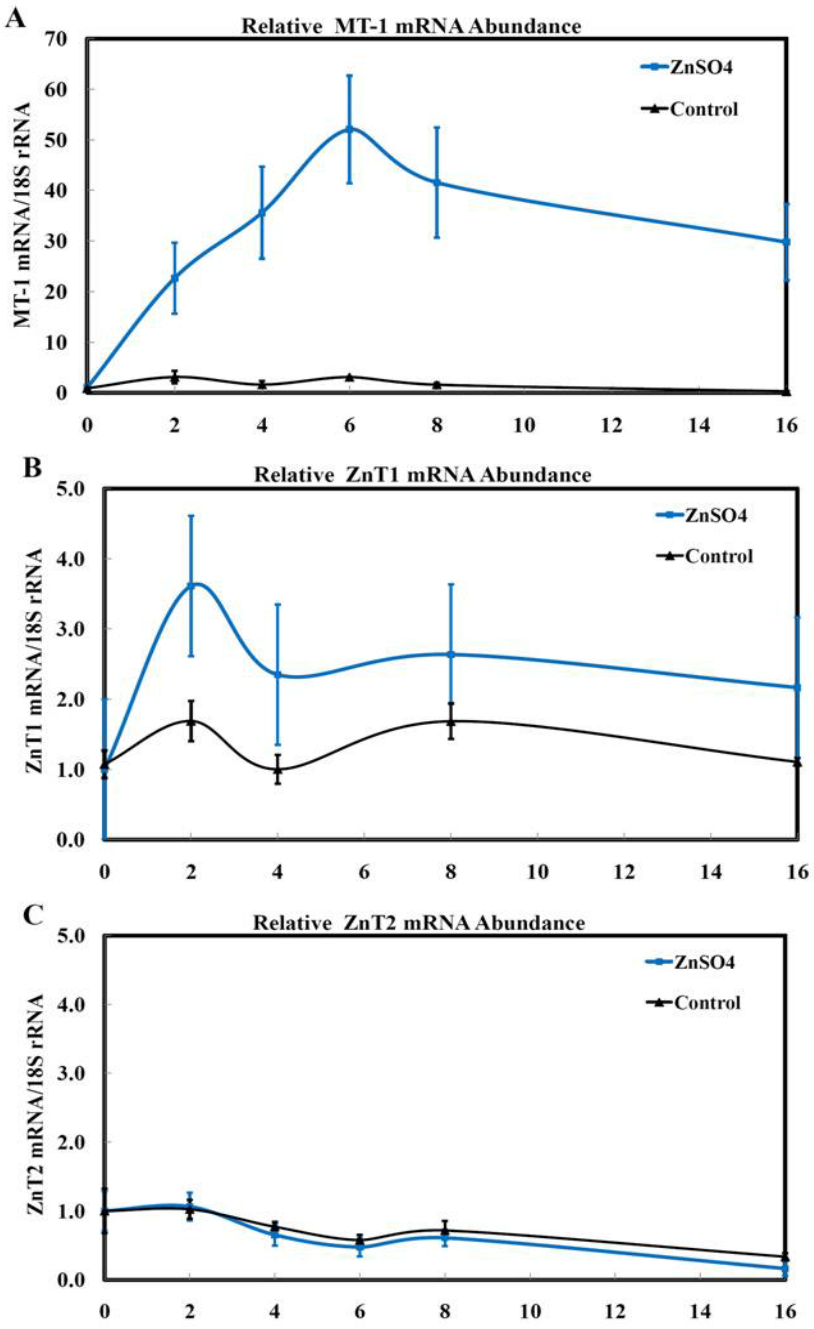

Figure 3-14. Zinc influences MT and ZnT1 mRNA levels in a time-dependant manner AR42J cells. The cells were pretreated with dexamethasone (100 nM) for 48 h. $ZnSO_4$ (40 μM) was then added to the culture medium for 16 h. The cells were collected at various time points post-treatment. The qPCR was used to measure MT (A), ZnT1 (B), and ZnT2 (C) mRNA levels after zinc treatment (n = 3).

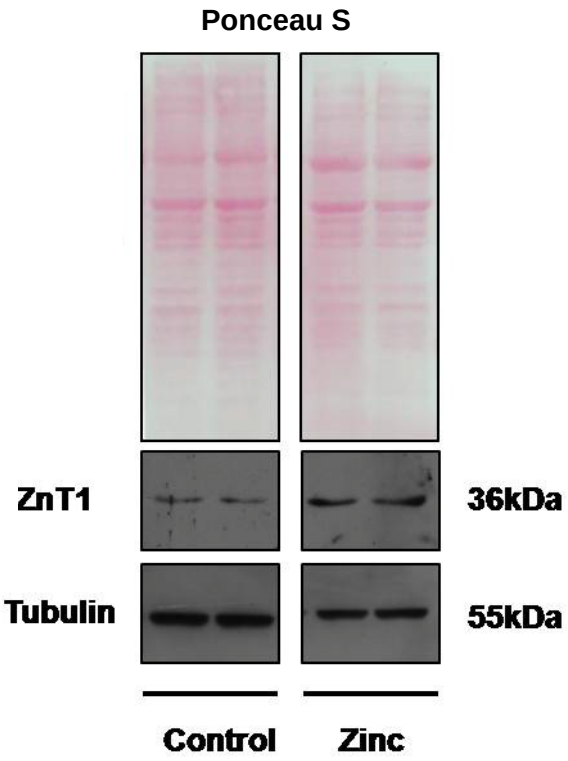

Figure 3-15. Influence of zinc on ZnT1 in AR42J cells. The cells were incubated with 40μm zinc for 24 h. Total cell lysate was prepared, and the ZnT1 abundance was measured by western analysis.

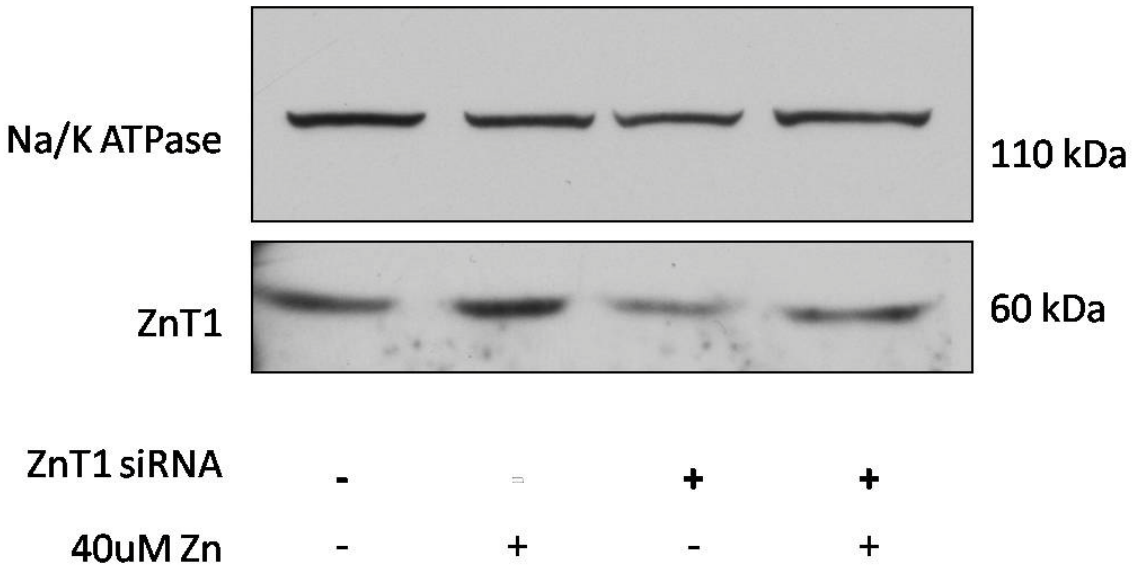

Figure 3-16. Influence of zinc and ZnT1 siRNA on ZnT1 expression. AR42J cells were transfected with ZnT1 siRNA for 48 h and treated with zinc for 24 h, and then harvested. Total membrane proteins were purified and ZnT1 was measured by western analysis. Na^+/K^+-ATPase was used as a loading control.

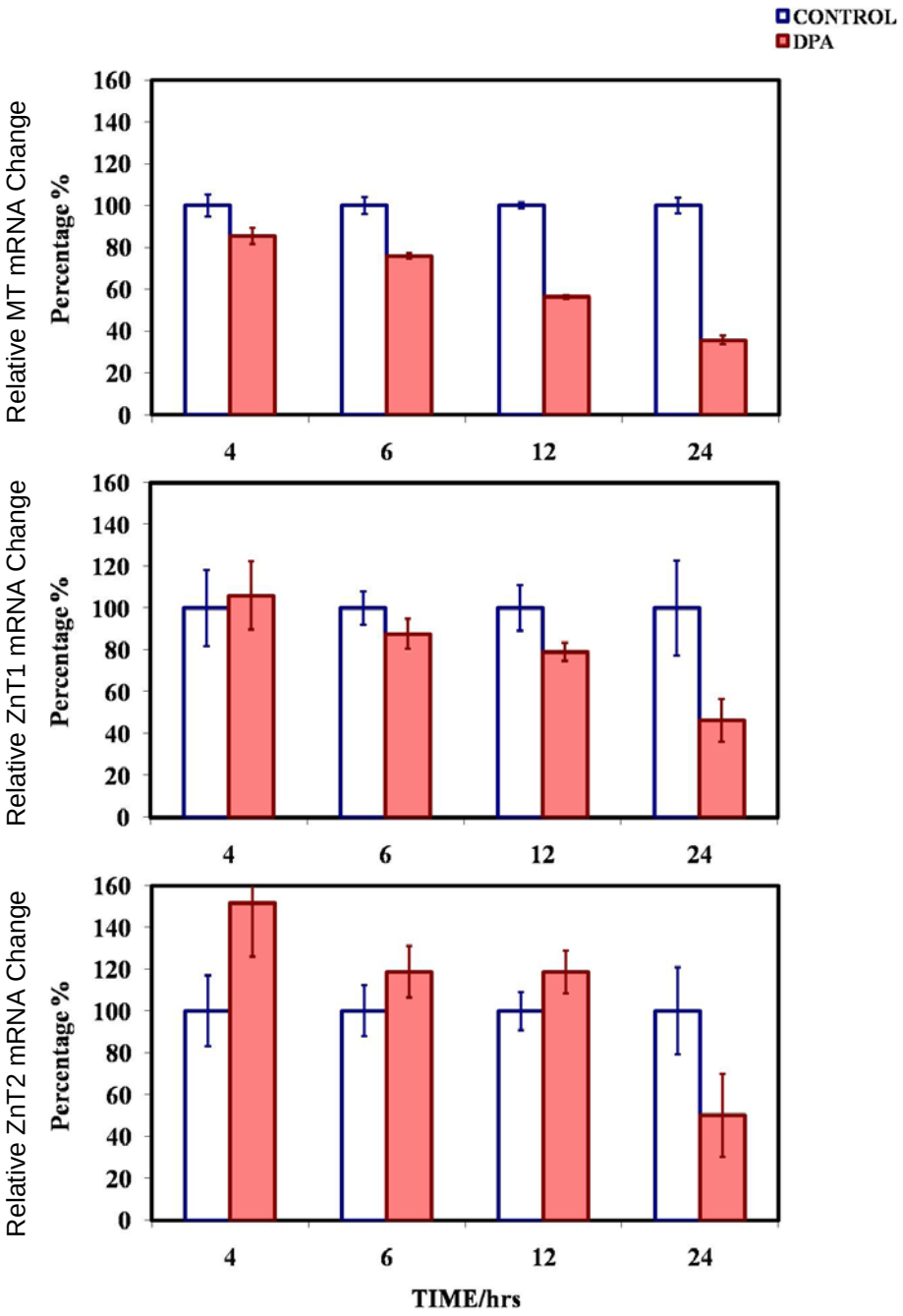

Figure 3-17. MT, ZnT1 and ZnT2 mRNA abundance in AR42J cells incubated with low zinc medium. 15% DPA-dialyzed FBS was used in low zinc medium. The DEX pretreated cells were incubated in the low zinc medium for 4 h, 6 h, 12 h, and 24 h. qPCR was used to measure the mRNA levels, the values are expressed as the percentages of relative changes to control (n=3).

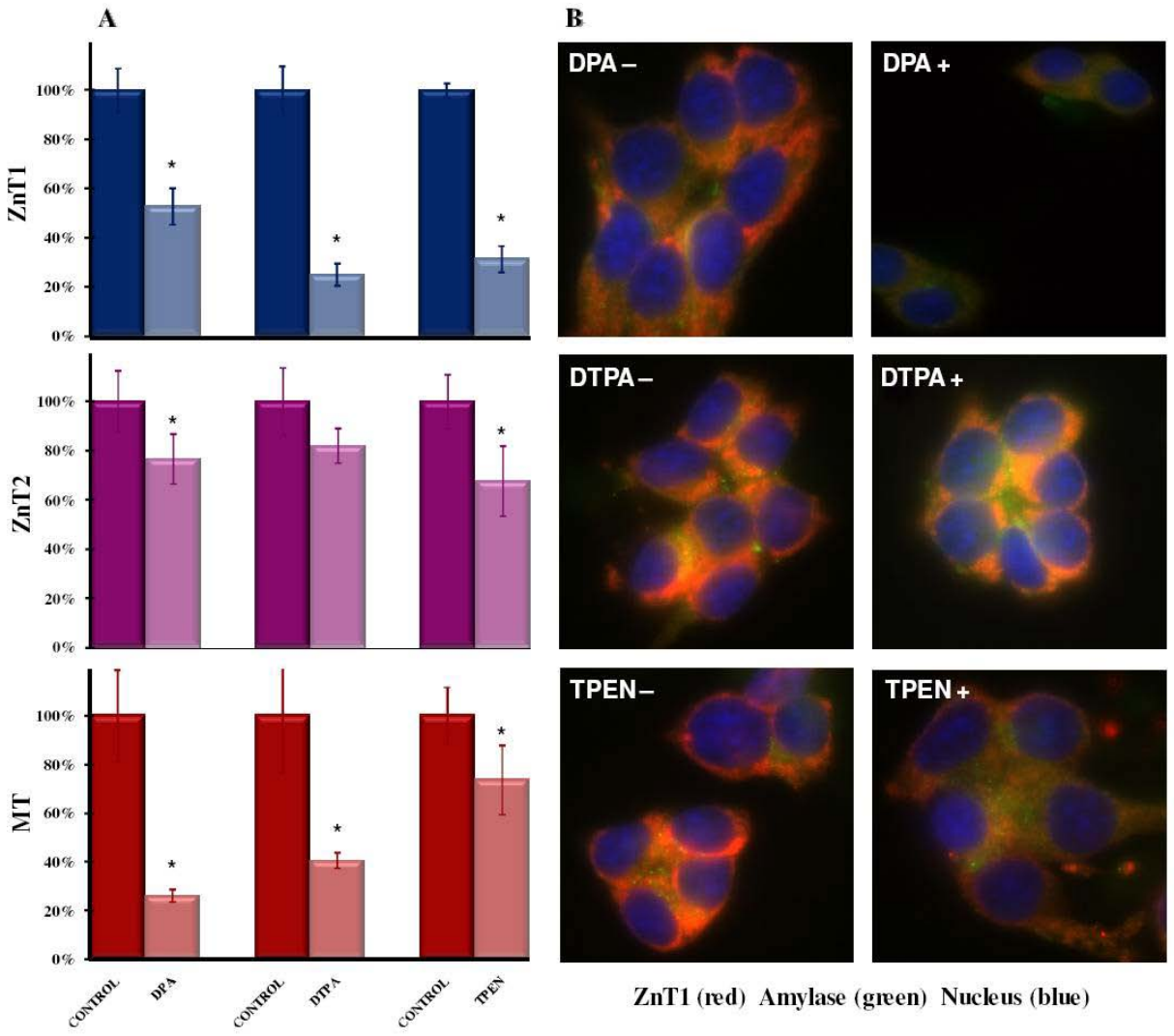

Figure 3-18. Zinc chelators influences ZnT1, ZnT2 and MT mRNA levels and their protein expression. (A) AR42J cells were pretreated with dexamethasone (100 nM) for 48 h. Three different zinc chelating reagents were used, by adding DTPA (50 μM), TPEN (4 μM) to the medium or using medium with 15% DPA-dialyzed FBS . Cells were treated for 6 h. qPCR was used to measure the mRNA levels of ZnT1, ZnT2, and MT after treatment (n = 3). * $p<0.05$. (B) Immunocytochemistry analysis of changes in ZnT1 (red) in AR42J cells responsiveness to DTPA, TPEN and DPA. Amylase (green) is also shown as a zymogen granule marker; DAPI staining shows the nuclei (blue).

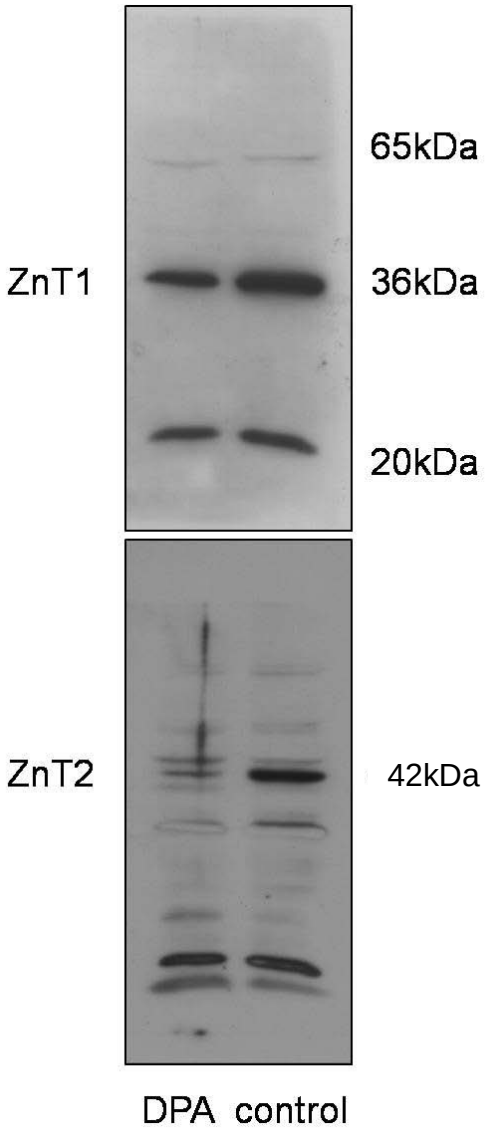

Figure 3-19. Low zinc medium influences ZnT1 and ZnT2 expression in AR42J cells. The low zinc medium contained 10% DPA-dialyzed FBS, and normal medium was used as control. The cells were incubated with this low zinc medium for 24 h and protein abundance was measured by western analysis.

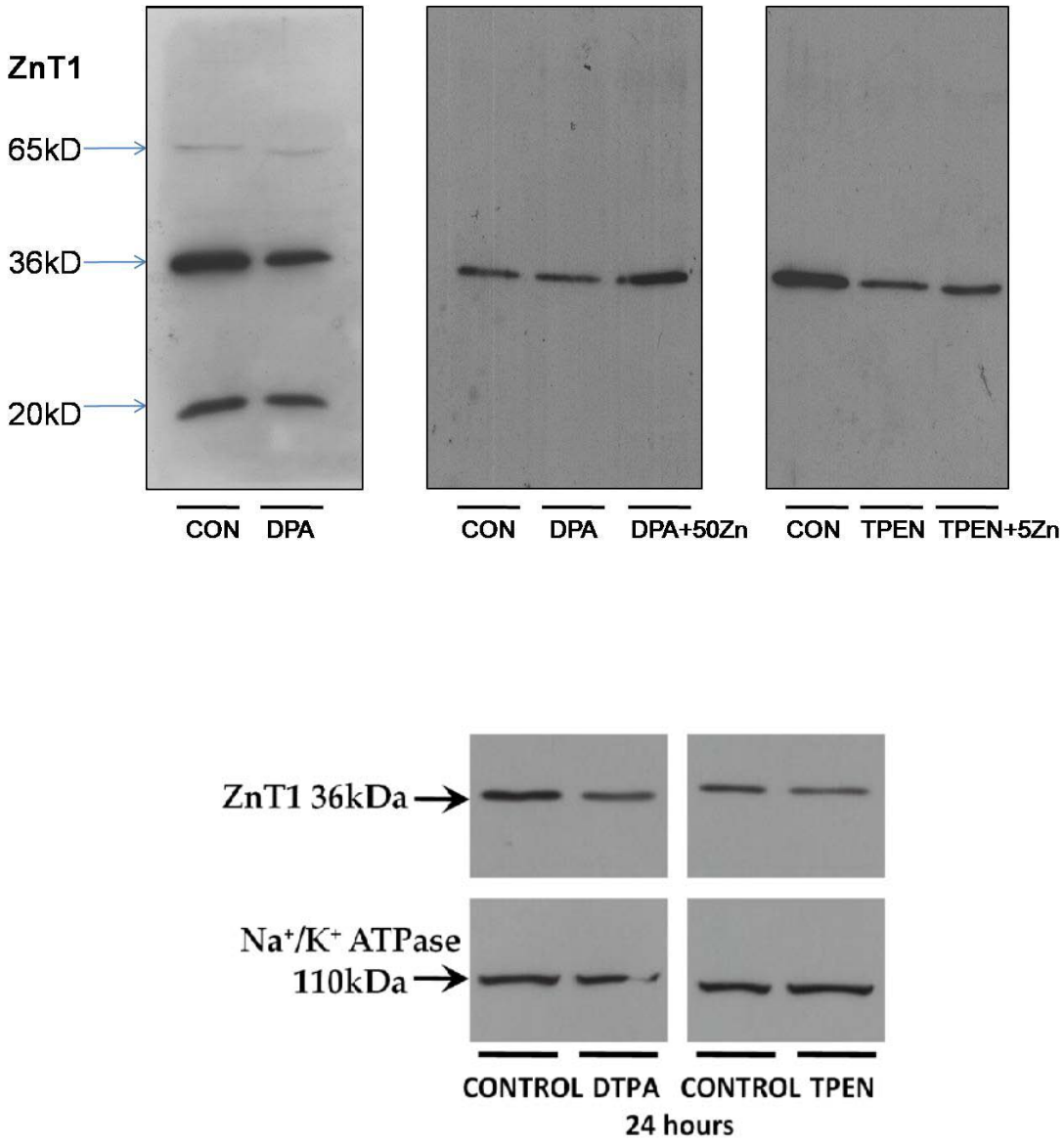

Figure 3-20. Zinc chelating reagents influence ZnT1 expression in AR42J cells. Zinc chelating reagents -DPA, DTPA and TPEN- were used to obtain zinc deficiency in AR42J cells. The cells were incubated with low zinc medium or control medium for 24 h and the protein abundance were measured by western analysis. Na^+/K^+-ATPase is the loading control.

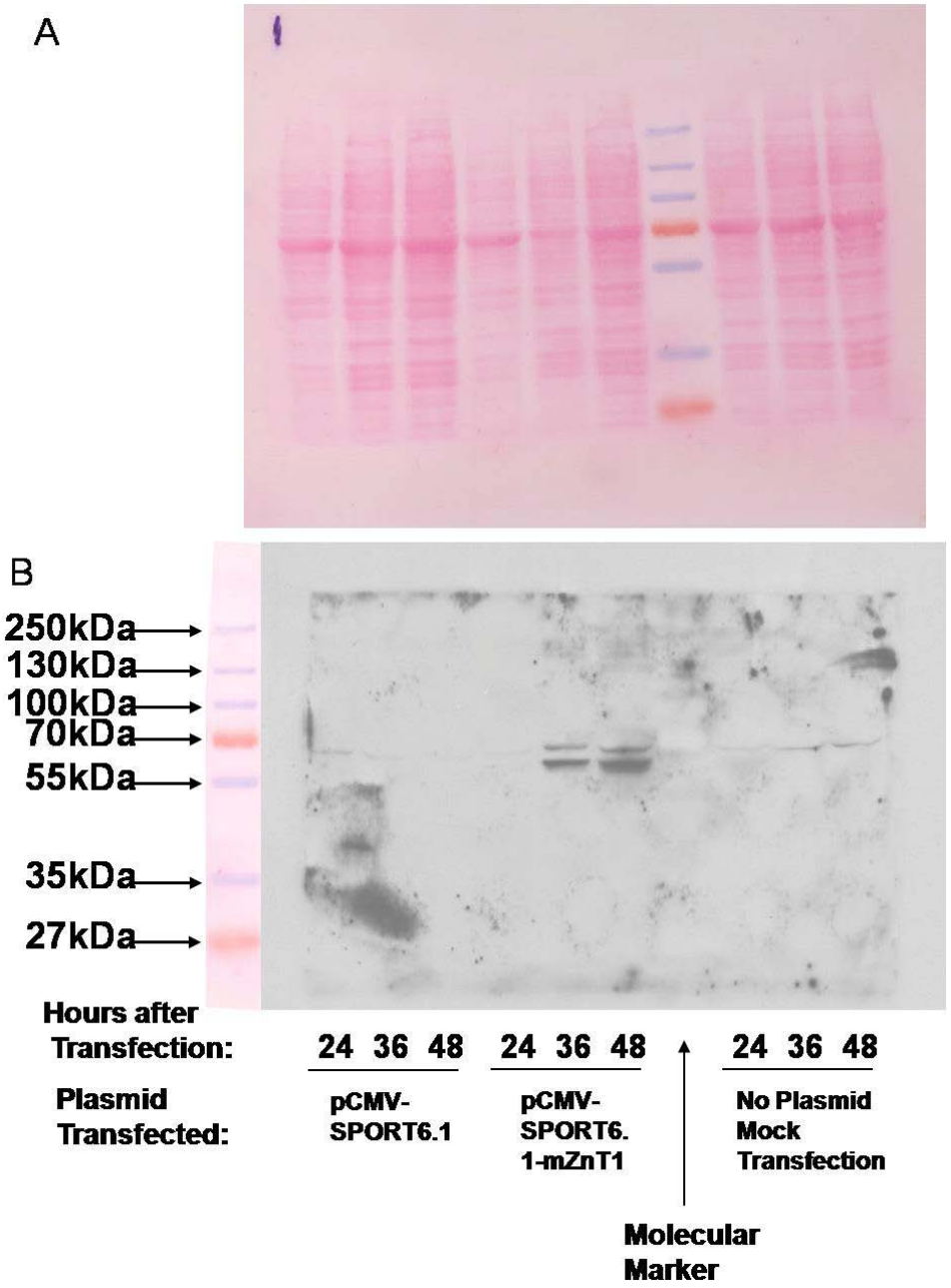

Figure 3-21. Western analysis of mZnT1 over-expression in HEK 293 cells. pCMV-SPORT6.1-mZnT1 was transfected into HEK 293 cells. pCMV-SPORT6.1 was the vector control. Cells were harvest at 24 h, 36 h, and 48 h after the transfection and mZnT1 protein abundance were measured by western analysis. A) Ponceau S red staining of the membrane. B) Western blotting of mZnT1.

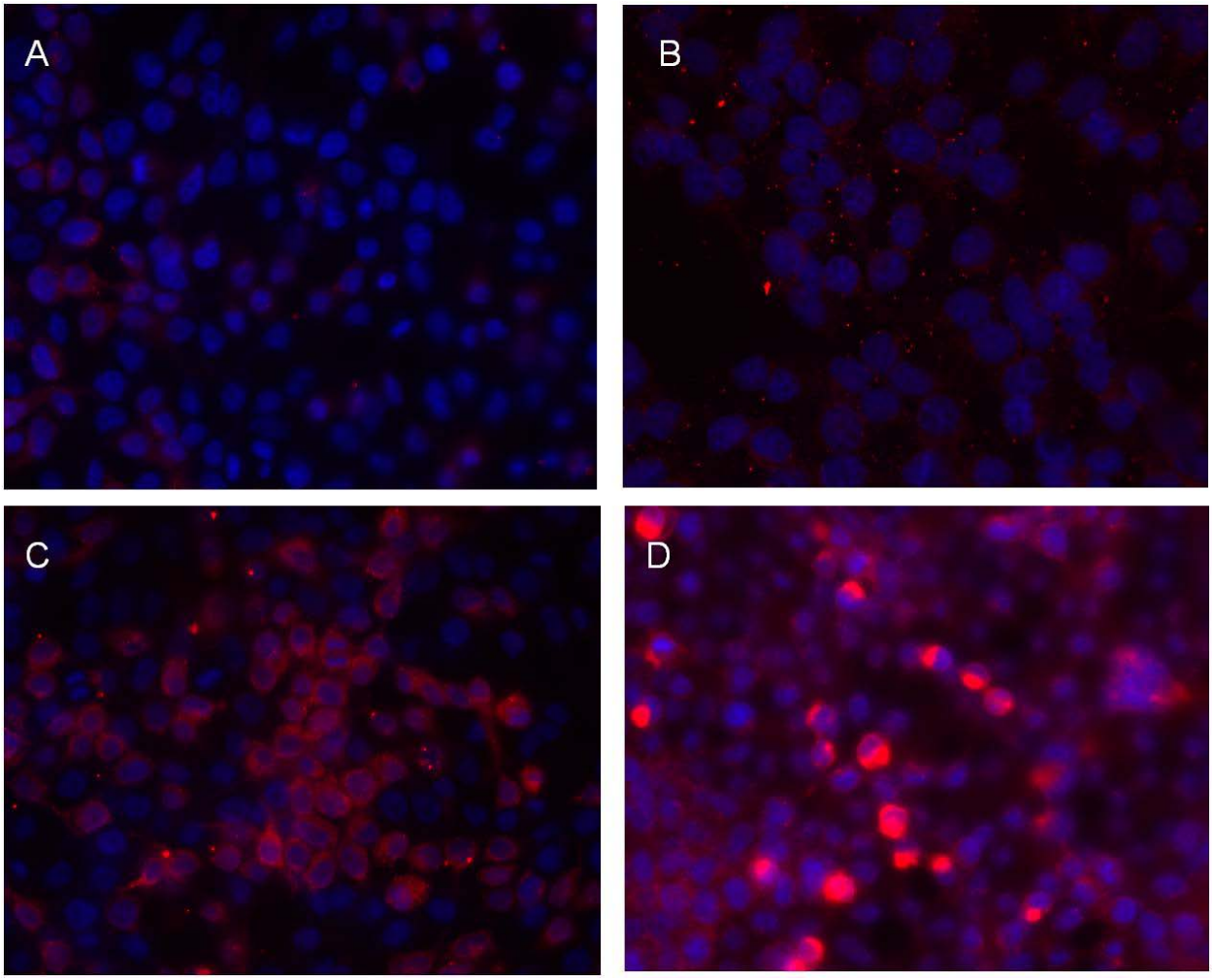

Figure 3-22. IF analysis of mZnT1 over-expression in HEK 293 cells. 48 h after the transfection, as described in Fig. 3-21, cells were washed and treated for immunefluorescnece staining. DAPI was used to stain nuclei. A) Cells were transfected with control vector and stained without permeabilization. B) Cells were tranfected with control vector and stained after permeabilization. C) Cells were transfected with mZnT1 and stained without permeabilization. D) Cells were tranfected with ZnT1 and stained after permeabilization.

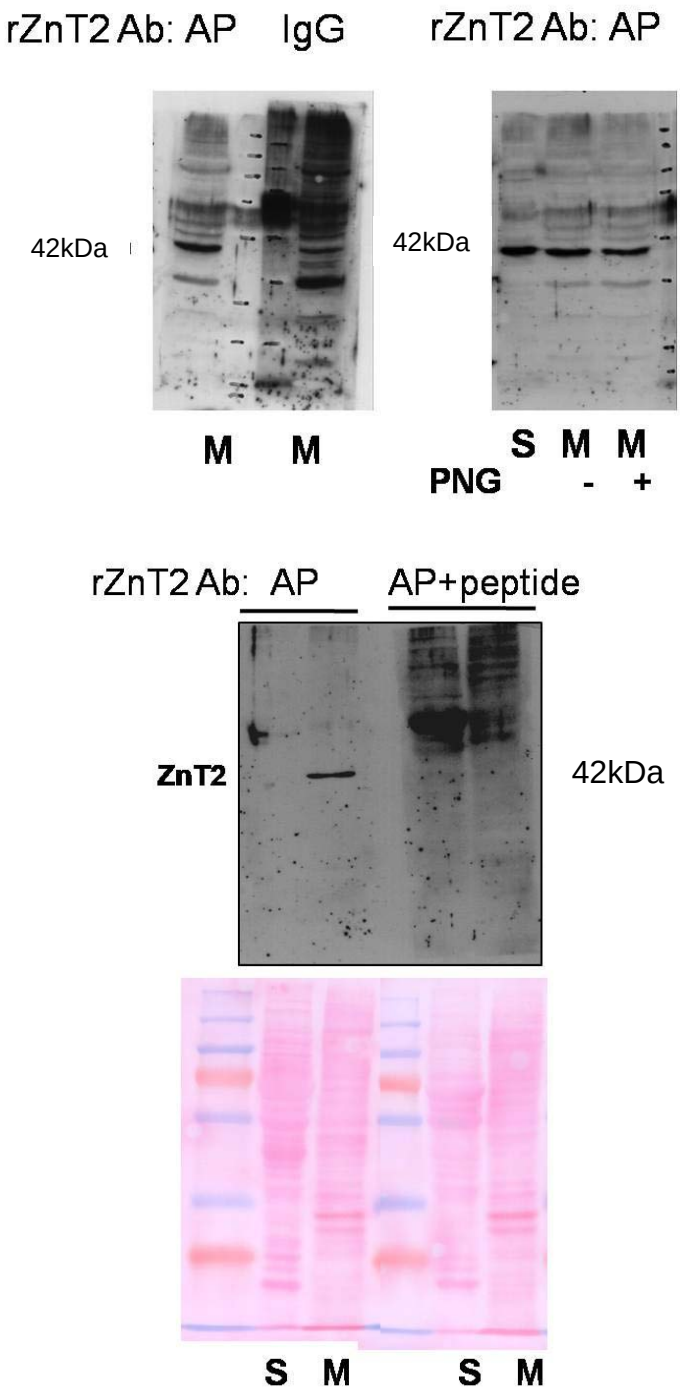

Figure 3-23. Western analysis of ZnT2. AR42J cell cytoplasm soluble protein (S) and total membrane protein (M) or total membrane protein digested with PNG F (PNG +) were probed with total IgG, affinity purified IgG antibody against ZnT2 or affinity purified IgG antibody pre-incubated with peptide (C). Ponceau red staining of the membrane (D).

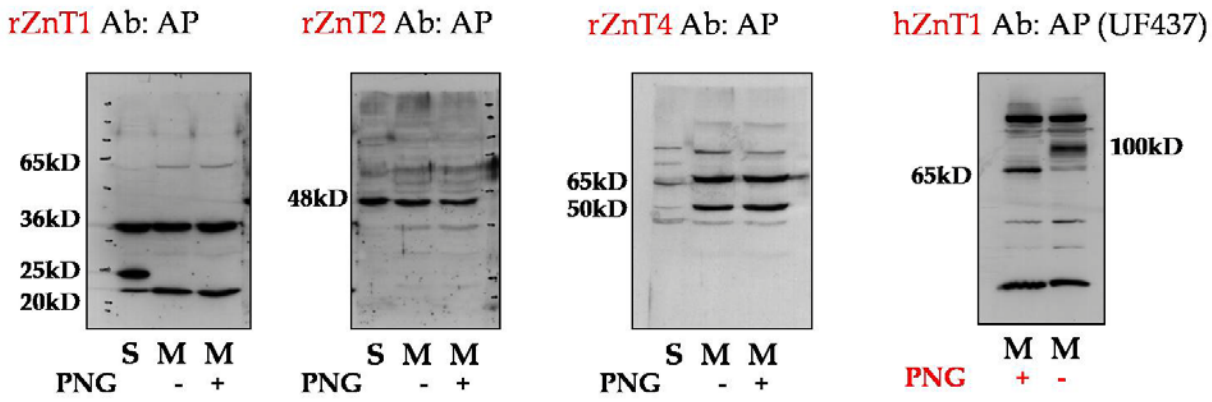

Figure 3-24. Western analysis of N-glycosation in rat ZnT1, ZnT2, ZnT4 in AR42J cell membrane fraction, and human ZnT1 in HEK 293 cell fraction by PNGase F digestion. AR42J and HEK 293 cell total membrane protein were prepared and digested with PNGase F. Western analysis were performed to check the protein migration on SDS-PAGE.

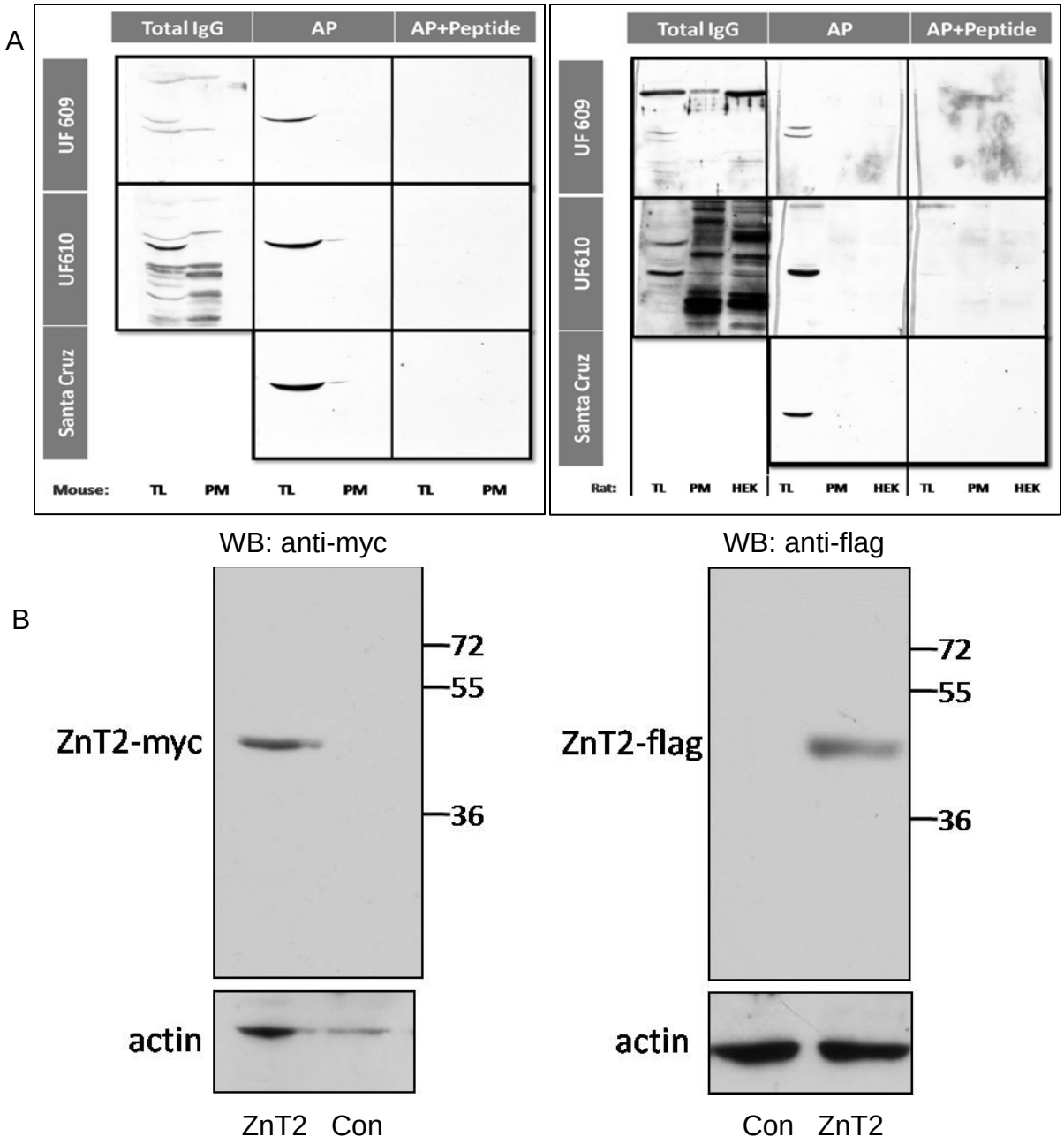

Figure 3-25. Western analysis of ZnT2 in mouse and rat pancreas total lysate (TL), plasma membrane (PM) protein samples by incubating with total IgG, affinity purified IgG, or affinity purified IgG antibody pre-incubated with peptide. Antibodies are polyclonal antibodies raised in rabbit (UF609, UF610) or raised in donkey (Santa Cruz) against ZnT2 (A). B) Western blotting of HEK cells transfected with pCMV-3tag-ZnT2-myc or pCMV-3tag-ZnT2-flag and measured by western analysis using anti-myc or anti-flag monoclonal antibodies.

CHAPTER 4
GLUCOCORTICOIDS REGULATE ZNT2 EXPRESSION IN PANCREATIC ACINAR CELLS

Introduction

Glucocorticoids and the Physiology of the Exocrine Pancreas. Numerous earlier studies have related the glucocorticoids with the physiology of the exocrine pancreas. It has been clearly established that adrenalectomy (ADx) causes a marked depletion in the number of the zymogen granules in acinar cells (102, 103) and a profound decrease in protein secretion both in the basal state(104) and under CCK stimulation (105). This situation can be reversed by hydrocortisone administration (104-106). Additionally, it has been reported that the administration of glucocorticoids to suckling rats caused hypertrophy of the pancreas(107-111) and led to early maturation of the pancreatic acini in neonatal rats (112). Glucocorticoid hormones have been thought to play a modulating role in the development of the pancreas (110, 111, 113, 114). Modulation of the balance in the exocrine/endocrine differentiation has been suggested by *in vitro* studies of rat pancreatic explants treated with corticosterone, showing decreased insulin secretion and islet mass, while exocrine enzyme contents and acinar mass were enhanced (114, 115).

Actions of glucocorticoids on secretory activity of the exocrine pancreas have not led to uniform conclusions. In this sense, *in vitro* studies with AR42J cells have demonstrated an increase in CCK receptors and differentiation in the presence of dexamethasone (62, 100, 116). By contrast, *in vivo* studies have pointed to a dual effect (both stimulatory and inhibitory) on secretagogue-stimulated pancreatic secretion that depends on the dose of glucocorticoids employed (104, 105, 117, 118). Morisset et al. and Otsuki et al. reported that administration of hydrocortisone to rats increased the

enzyme content and the size of the pancreas (109, 119). Conversely, Beaudoin et al. have shown that ADx caused a marked depletion of zymogen granules in the rat pancreas seven days after the surgery (120). Similar results have been reported by several other investigators. However, in a short-term study of endogenous glucocorticoid depletion, zymogen granules of ADx rats were preserved based on ultrastructure(121). Short-term effects may be related to the susceptibility of acinar cells to apoptosis during acute pancreatitis, whereas long-term effects may be related to the acinar cell differentiation and enzyme synthesis.

Glucocorticoids promote acinar cell differentiation, showing that the increase in acinar cell area is a direct consequence of glucocorticoid-mediated stimulation of differentiation and not acinar cell proliferation. Gesina et al. showed that *in vitro* treatment of the embryonic rat pancreas with DEX did not affect the number of precursor cells, but decreased the number of differentiated beta cells and increased the differentiated acinar cell area(122). Their conclusion was further supported by the finding of decreased proliferation of amylase-expressing cells upon DEX treatment, a result suggesting that glucocorticoids could also control the proliferation of already differentiated acinar cells and thereby prevent their overgrowth. The differentiation process from precursor to differentiated endocrine or exocrine cells is altered, suggesting that just the precursor cells, not the differentiated beta cells, are potential targets for glucocorticoids. Whether this *in vitro* situation also applies *in vivo* has yet to be fully investigated. In support of this idea, rats undernourished during their prenatal life and thereby exposed to increased corticosterone levels in utero show increased pancreatic weight at adult age.

Determining the Effect of Dexamethasone Induced Acinar Secretory Maturation on ZnT1, ZnT2 and MT Gene Expression in AR42J Cells. Since there are no lines of murine pancreatic acinar cells, the rat pancreatic acinar cell line, AR42J (ATCC CRL 1492) in place of a murine model, was used. AR42J cells were derived from a carcinoma of an azaserine-treated rat and have features of pluripotency of the common precursor cells of the pancreas. Dexamethasone converts pluripotent pancreatic acinar AR42J cells into the exocrine cell phenotype. Intracellular and secreted amylase contents are markedly increased by dexamethasone (62). Dexamethasone stimulates secretory granules formation along with extensive re-organization of the ER. Expression of cholecystokinin (CCK) receptors and responsiveness of AR42J cells to CCK stimulation are also found to be up-regulated by dexamethasone (100). The advantage of using cultured AR42J cells is that they are the only pancreatic acinar cell type studied in the culture. Also, we could manipulate the concentration of exogenous zinc in the culture medium and eliminate the effects of other factors that can influence the zinc transport *in vivo*, i.e. cytokines, hormones, cholecystokinin and growth factors, etc.

It is unclear if processing of zinc in immortalized culture cells will accurately reflect *in vivo* acinar cells responses. Presumably AR42J cells maintain the same responsiveness and regulation as acinar cells *in vivo*. Therefore, to understand whether ZnT1 and ZnT2 are involved in the exocrine secretory granules maturation, AR42J cells were treated with 100nM dexamethasone for 48 h and harvested at 0, 3, 6, 12, 24, and 48 h post-treatment. ZnT1, ZnT2 and MT mRNA were measured by the TaqMan qPCR method.

Potential Responsiveness of pGL3 and pGL4 Luciferase Reporter Vector to Steroid Hormones. The pGL3-basic luciferase reporter vector has been widely used in the study of GRE responsiveness to glucocorticoid hormones. A recent article in BioTechniques reported both the pGL3-Basic and pGL4 were induced by glucocorticoid in transient transfection of primary oviduct tubular gland cells, which contain GRs (123). My results agreed with their findings on the effects of the pGL3-Basic control vector, but not the pGL4 vector, on dexamethasone treatment. Therefore, the pGL4 vector should be a better plasmid when considering the reporter system for glucocorticoid regulation.

Results

ZnT2 Expression is Up-regulated during Glucocorticoid-stimulated Pancreatic Acinar Cell Differentiation. To further understand the regulation of ZnT1 and ZnT2 expression in pancreatic acinar cells, rat AR42J pancreatic acinar cells were used as a model. Dexamethasone (DEX) was added to the AR42J cell cultures for 48 hour to stimulate cell differentiation. An increase in amylase mRNA, a signature of acinar differentiation and secretory enzyme production, was observed following the addition of DEX (Fig. 4-1). A strong up-regulation of ZnT2 mRNA was found, upon stimulation with DEX (Fig. 4-1, 4-2, 4-4). Because ZnT2 was known to be localized to intracellular vesicles and may transport cellular zinc into these vesicles, increased expression of the *ZnT2* gene during dexamethasone-induced zymogen granule formation may indicate ZnT2 localization to zymogen granules and sequestration of cytoplasmic Zinc into the these granules. Surprisingly however, no change of ZnT1 expression was observed in response to DEX suggestive of selective GC regulation of these two zinc transporter genes (Fig. 4-1, 4-2), and no interaction between zinc and DEX in regulating ZnT1 and ZnT2 expression (Fig 4-2). The synergic effects on the MT

gene, which were documented before (99), were also confirmed in this experiment (Fig. 4-3).

To examine if ZnT2 is regulated by GC hormones *in vivo*, mice were given DEX by injection. As expected from previous work (99), serum zinc level decreased (by 20%) and liver zinc content increased (by 30%). Surprisingly, pancreatic zinc content decreased significantly (Fig. 4-5, 4-6). ZnT2 mRNA expression was significantly upregulated nearly 2-fold by 8 h after the injection and a high expression level was maintained at 16 h (Fig. 4-7). In contrast, MT mRNA as a positive control was increased 7.0-fold at 8 h and decreased to the normal level by 16 h after the DEX injection (Fig. 4-7). These results are similar to what the data suggested in DEX treated AR42J cells (Fig. 4-1). Differences in half-lives of these two mRNAs are apparent. Pancreatic ZnT1 mRNA levels were not changed in response to DEX (data not shown).

ZnT2 mRNA was effectively knocked down in AR42J cells by using ZnT2 siRNA. With either the presence or absence of DEX, ZnT2 mRNA was knocked down by about 70% at 48 h after ZnT2 siRNA transfection (Fig. 4-8). These results show that there was no interaction between the two independent events, i.e. transcription initiation by GR and ZnT2 mRNA degradation by siRNA. Western blotting confirmed the knock down of ZnT2 at the protein level (Fig. 4-8). Effects of ZnT2 siRNA knockdown on intracellular zinc concentrations were accessed following transfection. MT is regulated by MTF-1, therefore, knocking down ZnT2 in acinar cells should produce a transient zinc accumulation in the cytoplasm and activation of MT gene expression via zinc-induced MTF-1 translocation to the nucleus. Significant elevation of MT mRNA was found to reach a peak around 24 h post-transfection(Fig. 4-11). ZnT1 expression exhibited a

comparable response (Fig. 4-10). In accord with the induction of MT, an increase in cytoplasmic (^{65}Zn) zinc accumulation from the medium was observed through ZnT2 knockdown with siRNA (Fig. 4-9). Cytoplasmic ^{65}Zn was increased by 36% with ZnT2 siRNA, which supports the hypothesis that ZnT2 transports cytoplasmic zinc into zymogen granules. Further support for this role of ZnT2 is that ^{65}Zn in the zymogen granules was decreased by 15% (Fig. 4-9). However, no change in ^{65}Zn content was found in the crude nuclear fraction, suggesting specificity in cellular accumulation. It was hypothesized that overexpression of ZnT2 would enhance zinc loss via the zymogen granules. Since this could not be measured directly in AR42J cells, HeLa cells were transfected with a ZnT2 cDNA vector or empty vector (Fig. 3-25) and the cells were allowed to accumulate ^{65}Zn. Efflux of ^{65}Zn from preloaded cells was greater in the overexpressing cells (Fig. 4-12). This finding is congruent with the ZnT2 transport function in zymogen granules.

To understand the mechanism of ZnT2 regulation by DEX, the GC antagonist RU486 and a newly-discovered GR modulator CpdA, were exploited to study the association of ZnT2 gene transcription with signaling via the GR. CpdA having a higher binding affinity competes with DEX for binding to GR, and induces GR release from chaperones, and nuclear translocation and transrepression of NF-κB-driven gene expression. CpdA exhibits no transactivation potential on GRE-driven gene transcription (64) and was not able to activate ZnT2 gene expression. However, when AR42J cells were treated with DEX and CpdA at the same time, the hormonal analogy could still initiate expression of MT and ZnT2 (Fig. 4-13), presumably via transactivation of GR. In contrast, presence of the GR antagonist RU486, prevented CpdA and DEX from

stimulating the up-regulation of MT and ZnT2 (Fig. 4-13). These differing results with the two antagonists indicate DEX stimulated ZnT2 expression via transactivation of GRE-driven gene expression, but associated with NF-κB activation. MT, a well-characterized glucocorticoid-regulated gene was used as positive control in these experiments (99, 124).

The responsiveness of ZnT2 expression to DEX in both AR42J cells and pancreas of the intact mouse led to an analysis of GRE in the upstream promoter region. Two half GRE site were found but no full GRE. While realizing that the non-canonical half sites may impart GC regulation for some genes (125, 126), the involvement of STAT5 and GR in transductive pathways for regulation of other GC-controlled genes (127-129) was examined. Two STAT5-RE's were identified in the ZnT2 promoter (130) (Fig. 4-14). Consequently, we used a chromo-based nicotinoyl hydrazone (CNH), a STAT5-specific inhibitor (131) and the Janus kinase 2 inhibitor (AG490) to examine STAT5 involvement in ZnT2 activation by DEX (Fig. 4-15). As shown in Fig. 4-16, 4-17, 4-18, the inhibition of STAT5, particularly in combination with Jak2 inhibition, completely blocked DEX induction of ZnT2. Notably, the DEX-induced increase in MT expression was not inhibited by either AG490 or the STAT5 inhibitor (Fig. 4-16, 4-17, 4-18).

An MRE sequence was identified in the downstream of TSS in mouse ZnT2 promoter, and it is highly conserved across species (Fig. 4-19). A role for the transcription factor MTF-1 was shown through transfection with its vector containing human MTF-1 cDNA (Fig. 4-20). Markedly enhanced luciferase was observed with hMTF-1 transfection (Fig. 4-20). Zinc doubled promoter activity under these conditions.

The contribution of the MRE sequence to ZnT2 regulation was supported through mutation of this sequence in the ZnT2 promoter (Fig. 4-20). Cells transfected with the mutated ZnT2 promoter responded to DEX, but did not respond to zinc (Fig. 4-20, 4-21).

The pGL3-basic and pGL4 vectors were tested for dexamethasone responsiveness. Agreeing with another group's finding (123), there is a 1.5- to 2.0-fold increase in luciferase activities upon dexamethasone treatment (Fig. 4-22). But the increase was not seen in pGL4 vector. This re-engineered vector eliminated many potential transcription factor binding sites in its backbone sequences (Fig. 4-22).

Discussion

Zinc transporter expression in the pancreas is important because pancreatic secretions constitute an important component of mammalian zinc homeostasis (22, 68, 72, 85-89). ZnT1 and ZnT2 are expressed in the pancreas (51), and ZnT1 and ZnT2 are associated with isolated plasma membrane and zymogen granules, respectively. In this chapter, special focus is given to the regulation of the zinc transporter ZnT2 and the role it may play in an endogenous zinc secretory pathway from pancreatic acinar cells. It is proposed that zinc output from acinar cells follows two distinct pathways. One of these is cell-to-ductal zinc efflux via the apical membrane, which is zinc-dependent and primarily involves ZnT1 for cellular efflux. The other is that zinc is released along with digestive proenzymes from zymogen granules, where zinc is transported into the granules by ZnT2. These respective functions for ZnT1 and ZnT2 agree with current proposed roles for zinc transport in other cell types (90).

Zinc in stimulated pancreatic sections has been shown to be associated with high-molecular-weight proteins, and is closely linked to the activities of zinc-containing enzymes, especially carboxypeptidase A and carboxypeptidase B (22, 68, 85). Since

zymogen granules are the storage sites of digestive enzyme precursors (91), our finding that ZnT2 influences zinc incorporation into zymogen granules suggests that this transporter may provide zinc to maintain full activity of digestive pro-metalloenzymes. The reduction in activity observed during dietary zinc restriction is in agreement with our finding that transcript abundance for nine pancreatic digestive enzymes is not influenced by the dietary zinc restriction model used in the present experiments (data not shown). High zinc consumption has been shown in avian species, mice, and pigs to be detrimental to normal pancreatic exocrine function and to produce organ damage (42, 73, 74). This sensitivity suggests secretory pathways of zinc loss are essential for preventing pancreatic enzyme release, cell necrosis and organ atrophy. These signs of zinc toxicity are similar to the autodigestion of pancreatitis, which are due to abnormal calcium signaling within zymogen granules (92).

In the current studies we have identified ZnT2 as a component for zinc transport into the secretory zymogen granules. These granules have an acidic intra-granule pH (93), which is in line with the notion that ZnT2 favors acidic vesicles for its maximal transport activity. Also, ZnT8, which is expressed almost exclusively in the pancreatic β-cells, facilitates zinc transport required for proinsulin aggregation using a process that involves proton exchange (94, 95).

The zinc and MT contents of the pancreas are the highest among tissues under normal conditions of dietary zinc intake. Such high expression suggests MT has an important role in regulating zinc metabolism and function in the pancreatic acinar cells. MT declines almost completely with a zinc-deficient diet, whereas in other organs, it decreases only moderately (33, 97). Pancreatic zinc secretion in $MT^{-/-}$ mice is much

higher than in wild-type mice, and they are more vulnerable to damage caused by zinc deficiency (33). MT is also found in pancreatic secretions, although it is through a route that does not involve granule secretory pathways (32). In that regard, MT-bound zinc in pancreatic secretions could provide a source of endogenous zinc for reabsorption. Studies that could reflect on the glucocorticoid responsive expression of ZnT2 and its role in pancreatic zinc secretion are limited. It has been reported that adrenal insufficiency increases serum zinc concentrations, while administration of glucocorticoids, and ACTH, and the excess corisol production in Cushing's Syndrome decrease these concentrations (reviewed in (98)). Hypozincemia associated with glucocorticoid action has been related to induced synthesis of MT in rodents (99). Radiotracer kinetic studies with Zinc-69m given intravenously to humans reveal that carbohydrate-active steroids (glucocorticoids) may alter rate constants of the fecal excretion of zinc (98).

The pancreatic acinar AR42J cell model has been widely used to characterize effects of glucocorticoid hormones on secretory activity of the exocrine pancreas (62, 100). Dexamethasone treatment of AR42J cells induces a highly differentiated phenotype. It has been reported that RU486 can reduce GR concentrations in AR42J cells by 50% with concomitant changes in GR regulated gene expression (101). It was established that ZnT2 regulation is controlled by GR by demonstrating inhibition through RU486. This strongly suggests that the signaling pathway involves GR dimerization rather than the anti-inflammatory pathway involving assembly of the NF-kB/GR dimer complex. GR involvement in pancreatic tissue organization, the differentiation of acinar cells, and enzyme and zymogen granule production is compatible with its role in ZnT2

regulation. Furthermore, the control by zinc via MTF-1 responsive ZnT2 expression, is consistent with a role in endogenous zinc secretion. That control, combined with the DEX responsiveness, makes ZnT2 analogous to MT in terms of a teleological basis for dual regulation. To our knowledge ZnT2 may represent the first member of either the ZnT or Zip gene families to be glucocorticoid regulated.

The MRE mutation and deletion results confirm the importance of the functional MRE element in the ZnT2promoter. The crucial base level transcription is dependent on this MRE sequence in ZnT2 promoter, suggesting that the active MRE in the ZnT2 promoter is the dominant transcription element. As shown in Fig. 4-14, there is another potential transcription start site in the downstream of this MRE sequence. This is based on the cDNA library sequences in the database. 4 different transcript variants of mouse ZnT2 were found in the database. In spite of the presence of inaccurate sequences in this database, another potential transcription start site needs to be considered for the future study.

Amylase and ZnT2 induction by DEX seem to be regulated through distinctly different mechanisms. The kinetics of dexamethasone induction of amylase gene expression are slow. In the case of the amylase gene, significant effects were not observed in the first 6 h of treatment, and maximal effects were not observed until 24-48 h. However, it does not seem to be the case for ZnT2. ZnT2 mRNA levels increased within 3 h and reached the maximal level at 12 h, gradually decreasing to the baseline level 48 h after the treatment. It has some similarities to MT induction by dexamethasone, in spite of the short half-life of MT mRNA. The inhibition of protein synthesis blocked the ability of dexamethasone to increase amylase gene expression

(132). Both cycloheximide and puromycin, agents with different mechanisms of action, blocked the induction of dexamethasone on the amylase gene (132). There are unknown newly synthesized factors by dexamethasone, which are involved in amylase transcription activation. The possibility of an unknown secondary regulatory factor involved in ZnT2 transcription should be tested by cycloheximide, puromycin, and/or actinomycin D treatments in the future.

Epigenetic modification has been proposed in regulating zinc transporter expression. CpG island have be identified in the promoter region of ZnT2 gene (Fig. 4-23). Whether this CpG is functional in regulating gene expression needs further investigation.

Computer-based modeling (133, 134) of human ZnT2 structure showed tightly arranged six transmembrane domains along with a C-terminal cytosolic domain (Fig. 4-24). There are four potential zinc-binding sites in the structure of ZnT2, and one of these is in that C-terminal intracellular domain. The other three sites are closed to the tunnel space embedded in six-transmembrane helix, suggesting important function of these zinc binding sites in transport of zinc ion.

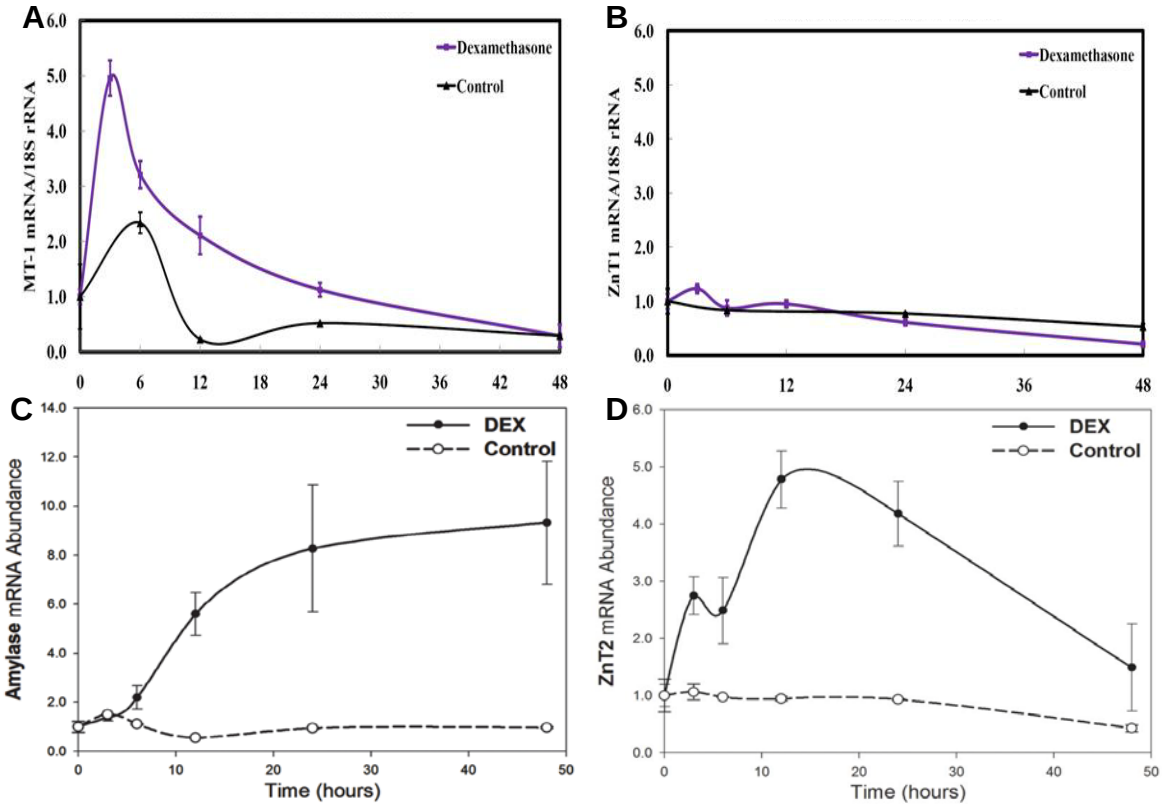

Figure 4-1. Dexamethasone regulates MT, and ZnT2 mRNA levels in AR42J cells. The cells were treated with Dexamethasone (100 nM) for 48 h. Cells were collected at various time points post-treatment. The qPCR was used to measure MT (A), ZnT1 (B), amylase (C), and ZnT2 (D) mRNA levels after the treatment. (n = 3).

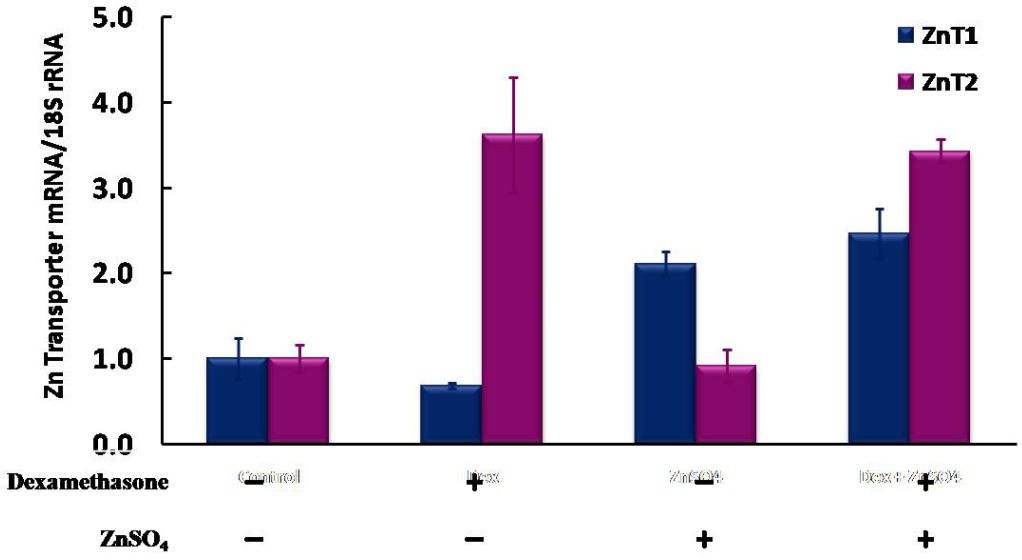

Figure 4-2. The responsiveness of ZnT1 and ZnT2 expression to Zinc and/or dexamethasone in AR42J cells. $ZnSO_4$ (40 μM) and dexamethasone (100 nM) were added to AR42J cell culture medium for 6 h. The qPCR measurement of ZnT1 and ZnT2 demonstrates mRNA levels change after treatment with dexamethasone and/or $ZnSO_4$. The values are expressed as mean ± standard deviation (n = 3).

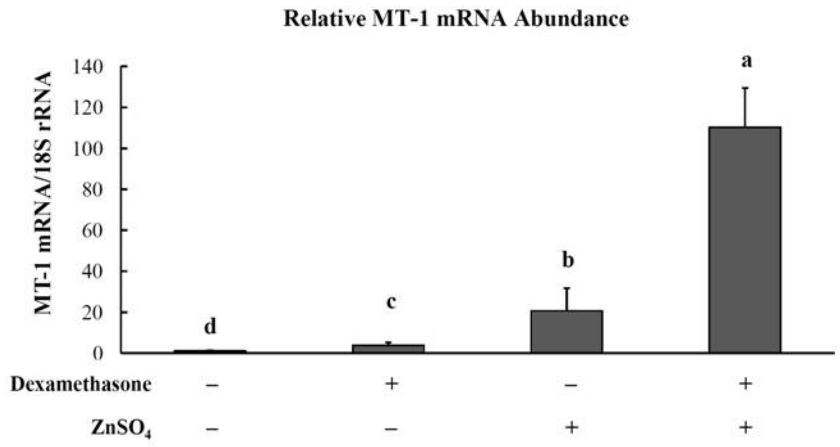

Figure 4-3. The responsiveness of MT-1, and ZnT1 expression to zinc and/or dexamethasone in AR42J cells. The cells were treated with zinc (40 μM) and/or dexamethasone (100 nM) for 6 h. qPCR was used to measure mRNA levels of MT (A), and ZnT1 (B) after treatments. Each bar value is the mean + SD (n = 3).

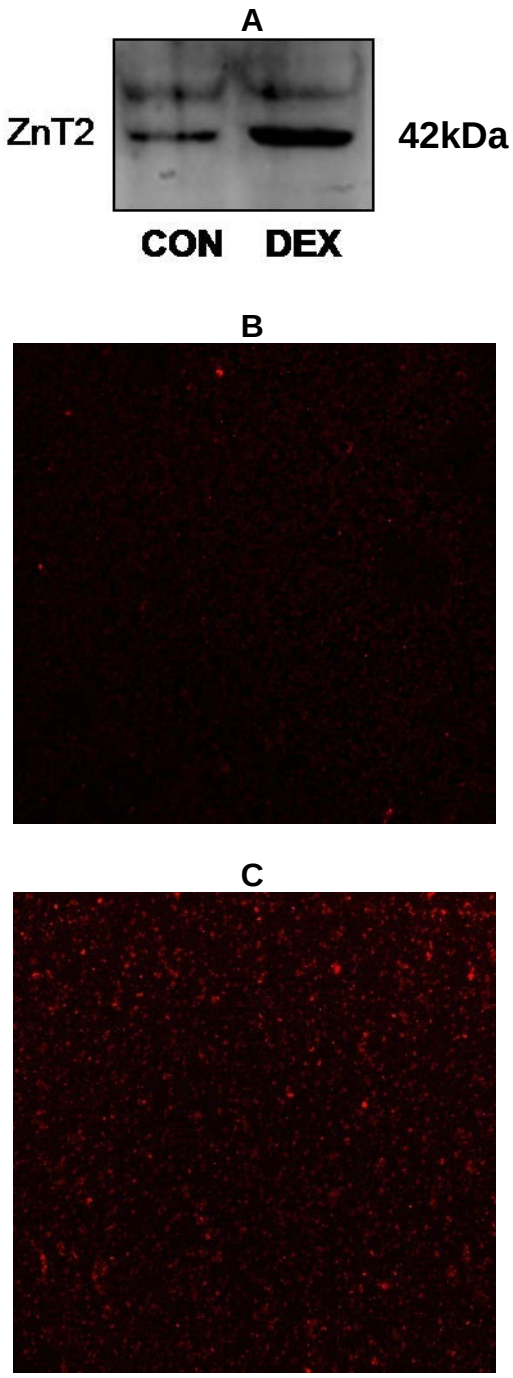

Figure 4-4. Dexamethasone influences ZnT2 expression in pancreatic acinar cells. (A) AR42J cells were treated with 100nM dexamethasone for 24 h. ZnT2 protein abundance was measured by western analysis. (B,C) Mice were given dexamethasone via i.p. injection and ZG were purified from the pancreas 24 h after injection. ZnT2 were shown by IF in purified. (B) saline control; (C) dexamethasone injection.

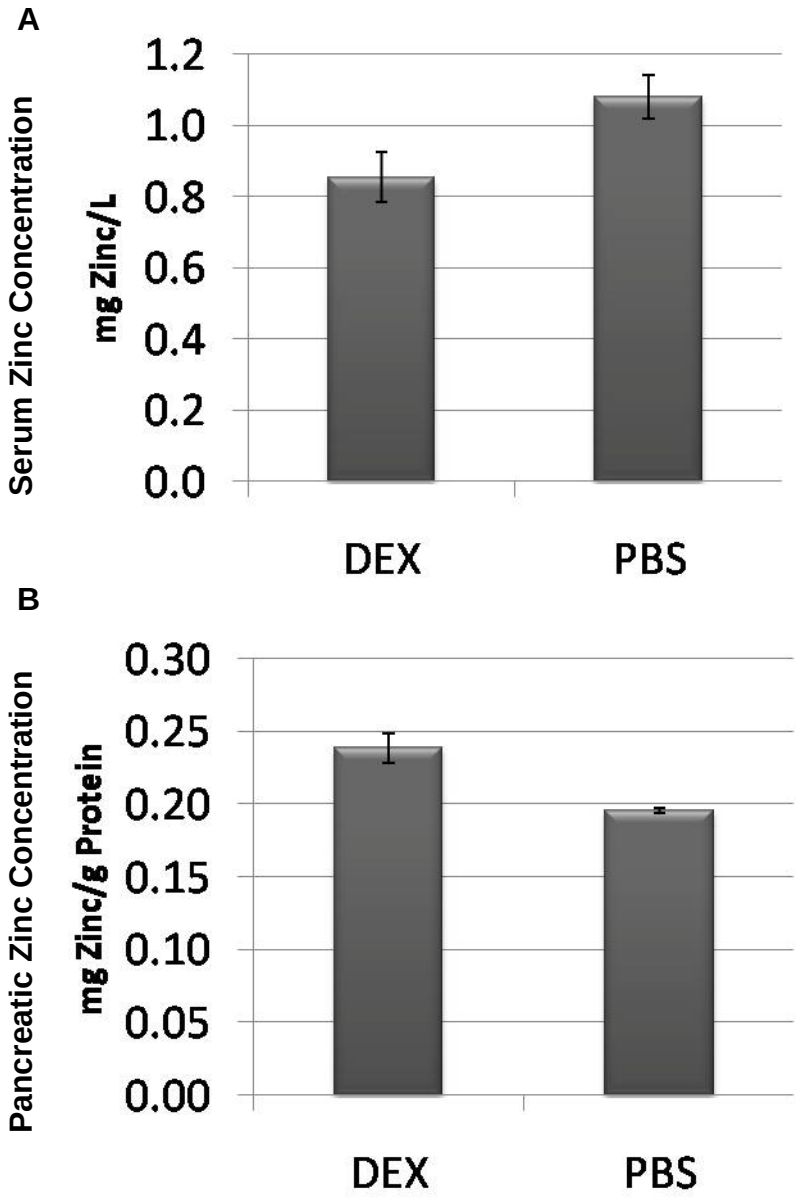

Figure 4-5. Zinc concentration in the pancreas and serum in mouse 3 h after dexamethasone (DEX) i.p. injection. PBS was given as control. Serum (A) and pancreas (B) were collected and pancreatic cytoplasmic soluble fraction was isolated. Zinc concentrations were measured by atomic absorption spectrophotometry.

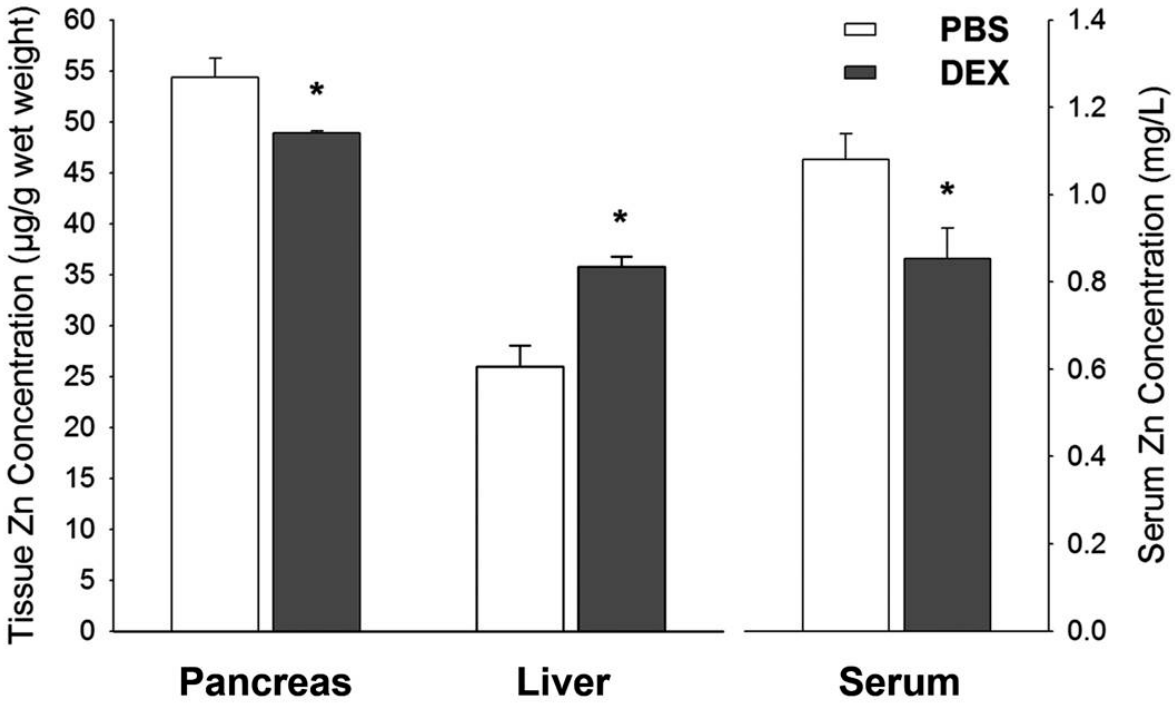

Figure 4-6. Dexamethasone influences zinc concentration in the pancreas, liver, and serum in mice 8 h after dexamethasone (DEX) i.p. injection. PBS was given as control. The pancreas, liver, and serum were collected and the pancreas and liver were weighted and digested with nitric acid. Zinc concentrations were measured by atomic absorption spectrophotometry and normalized against the weight..

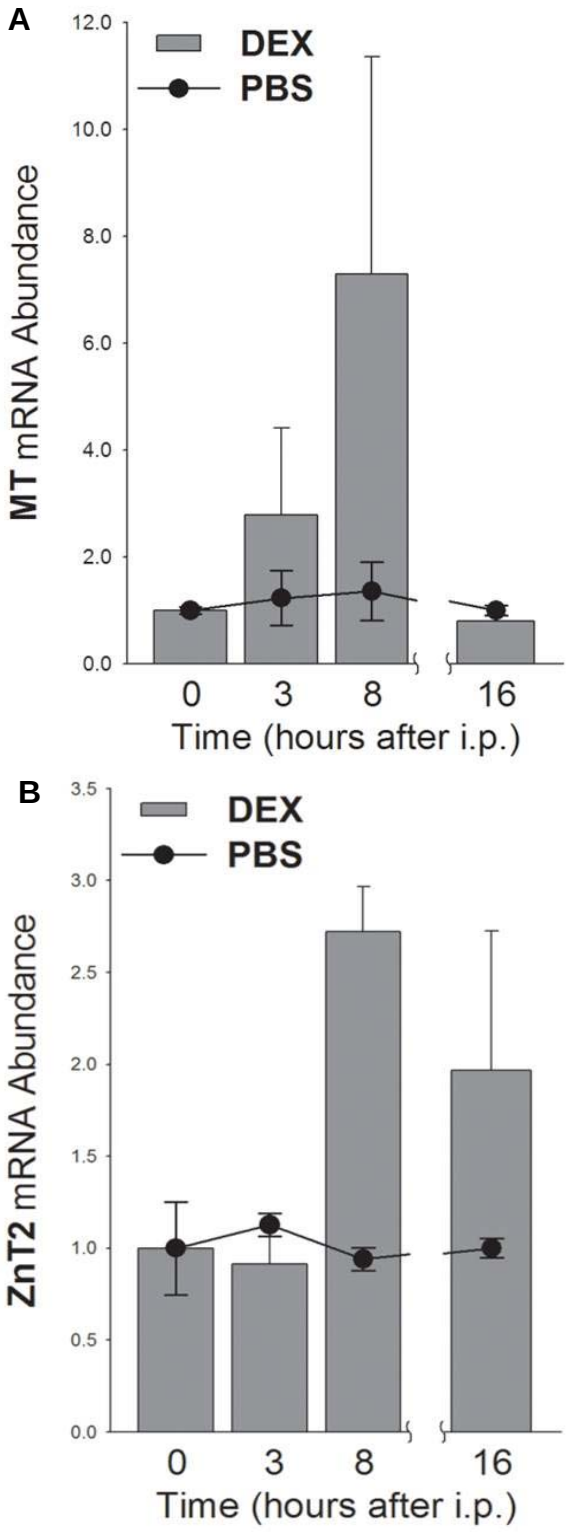

Figure 4-7. CD-1 mice were injected i.p. with either dexamethasone or PBS and killed 3, 8, or 16 h thereafter. MT (A) and ZnT2 (B) mRNA were measured by qPCR. (n = 3-4)

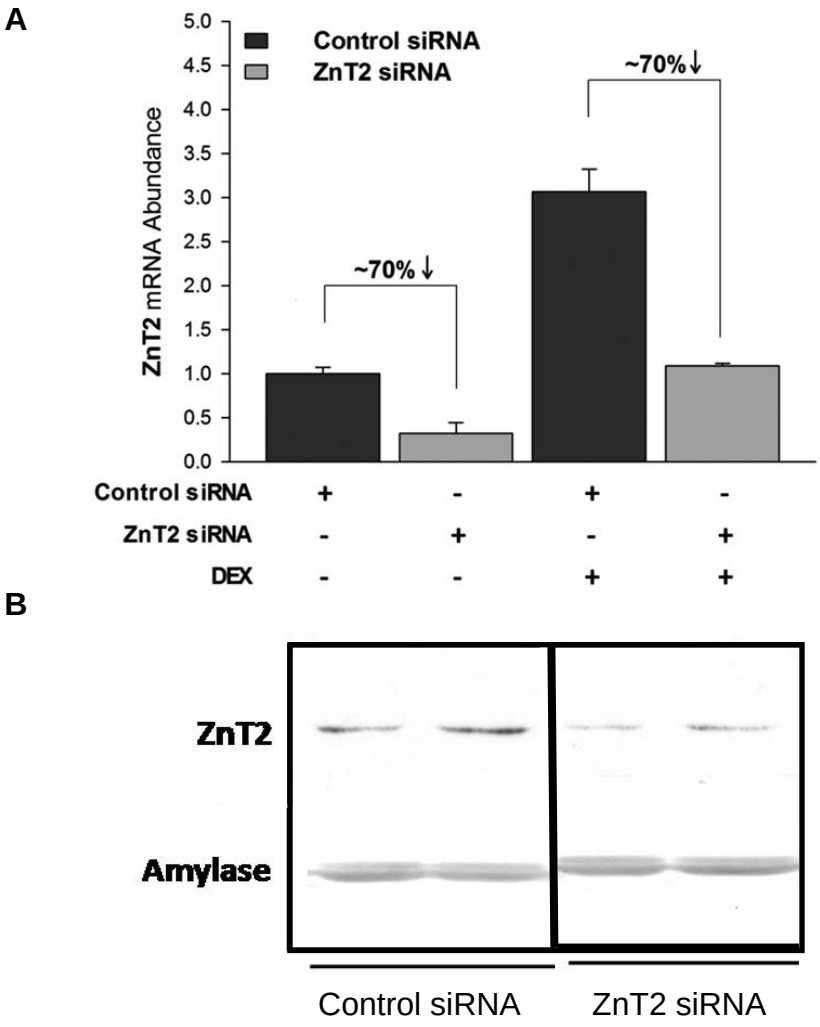

Figure 4-8. ZnT2 knock-down in AR42J cells by ZnT2 siRNA. AR42J cells were transfected with ZnT2 siRNA for 48 h, and/or treated with DEX for 48 h. Scrambled siRNA was used as control siRNA. The cells were harvested at 48 h after the transfection and ZnT2 mRNA expression was measured by qPCR (A). ZG were isolated and ZnT2 protein levels were detected by western blotting (B). Amylase was ZG loading control.

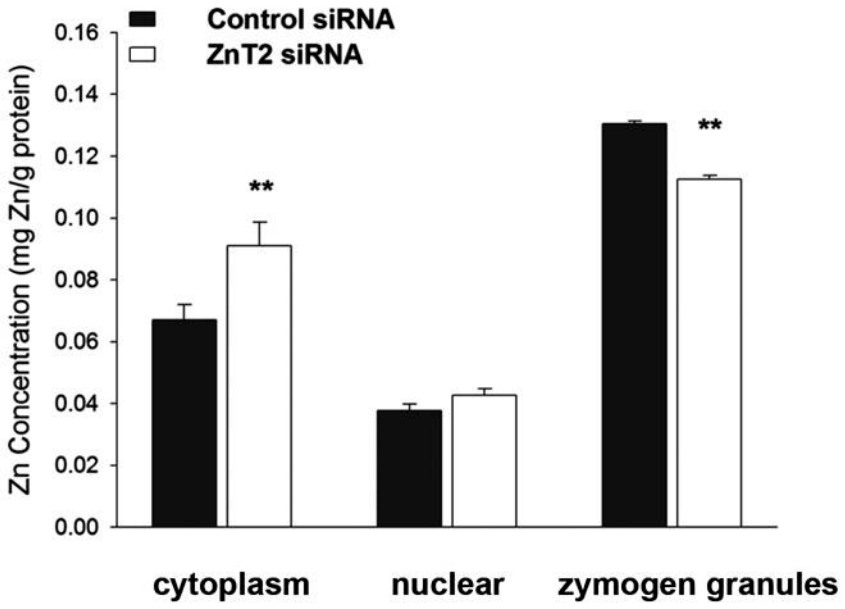

Figure 4-9. Zinc concentrations in cytoplasm and ZG fractions after ZnT2 knock-down by ZnT2 siRNA in AR42J cells. The cells were transfected with ZnT2 siRNA for 48 h. Scramble siRNA was used as control siRNA. The cells were preloaded with ^{65}Zn over night. Then cells were homogenized, cytoplasm and ZG fractions were purified. ^{65}Zn were counted with a Packard γ-ray spectrometer and normalized to total protein content. Zinc concentration was calculated based on the specific activity.

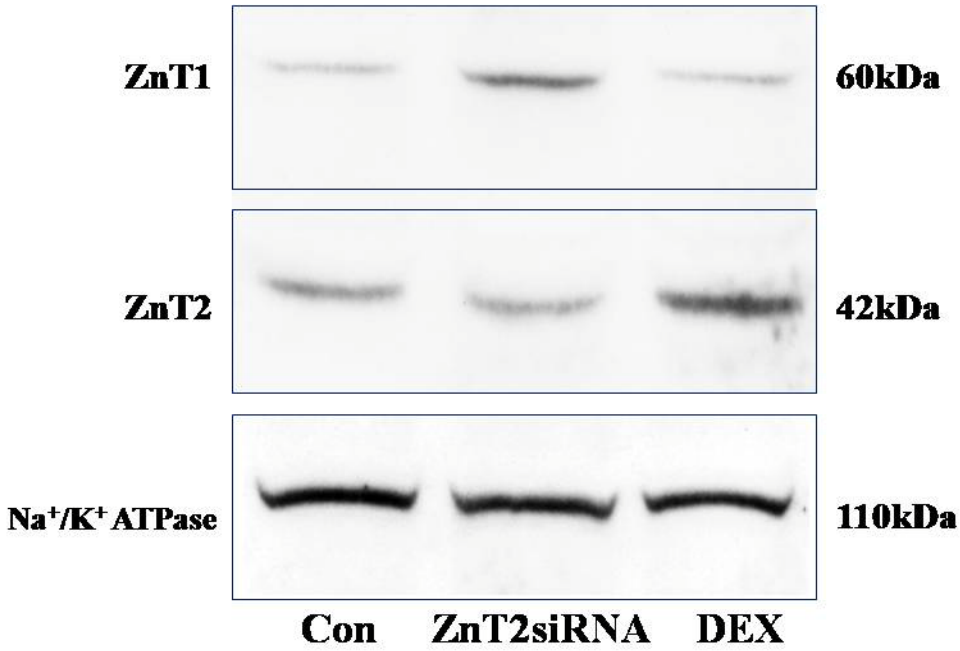

Figure 4-10. ZnT2 siRNA-knockdown and dexamethasone influences ZnT1 and ZnT2 expression in AR42J cells. The cells were transfected with ZnT2 siRNA or treated with dexamethasone for 48 h and total membrane protein was purified. ZnT1 and ZnT2 protein abundance were measured by western analysis. Na^+/K^+ ATPase was shown as total membrane loading control.

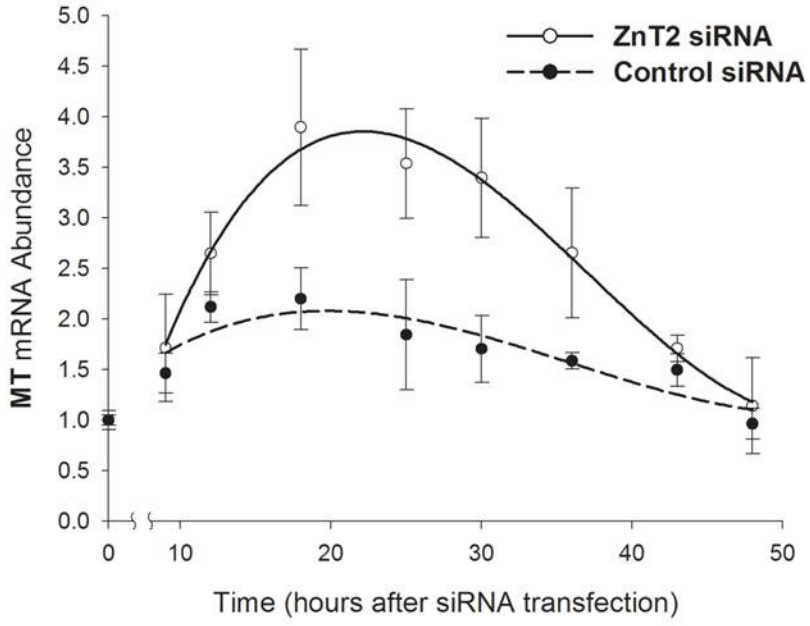

Figure 4-11. ZnT2 siRNA-knockdown influences MT mRNA in AR42J cells. The cells were transfected with ZnT2 siRNA for 48 h and were harvested at various time points post-transfection. Total RNA were isolated and MT mRNA was measured by qPCR. Scrambled siRNA transfection was the control.

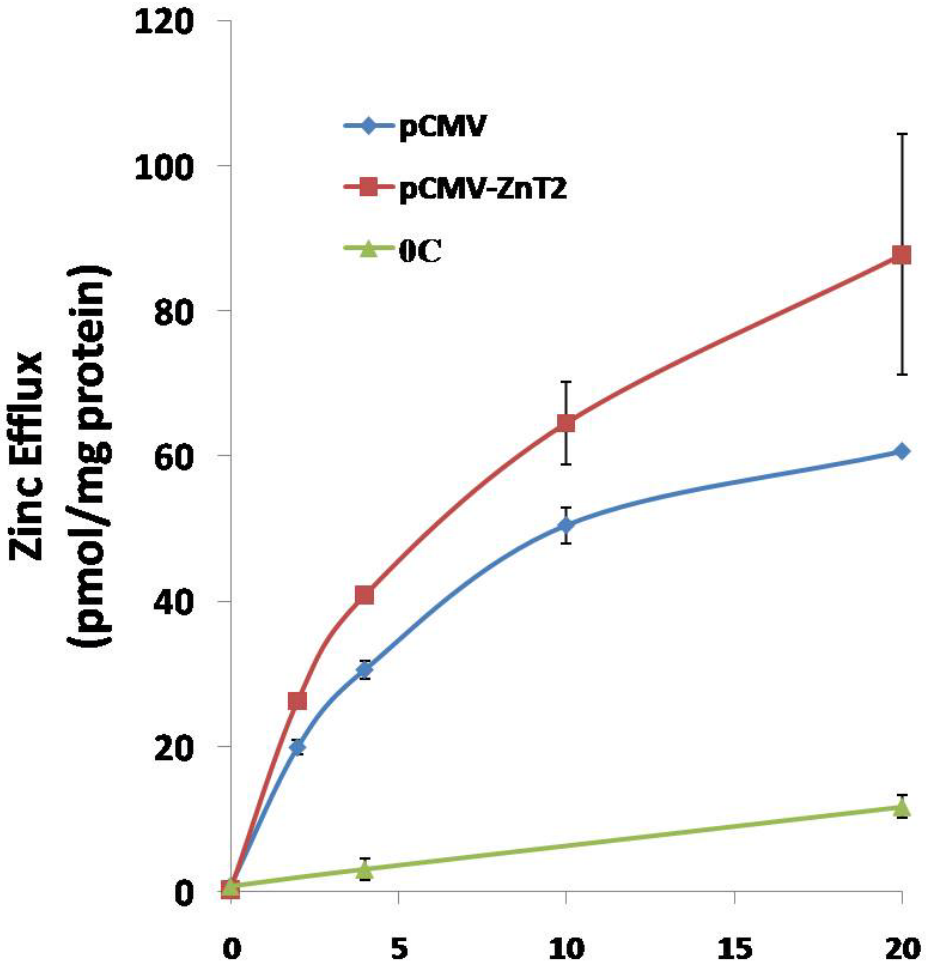

Figure 4-12. ZnT2 overexpression increases zinc efflux in HeLa cells. The cells were transfected with pCMV-3tag-ZnT2 vector or a pCMV-3tag control vector for 48 h and loaded with ^{65}Zn. Zinc efflux into the medium was calculated from the specific activity of ^{65}Zn in the fresh medium and expressed on a cell protein basis. Values are means ± SD (n = 3).

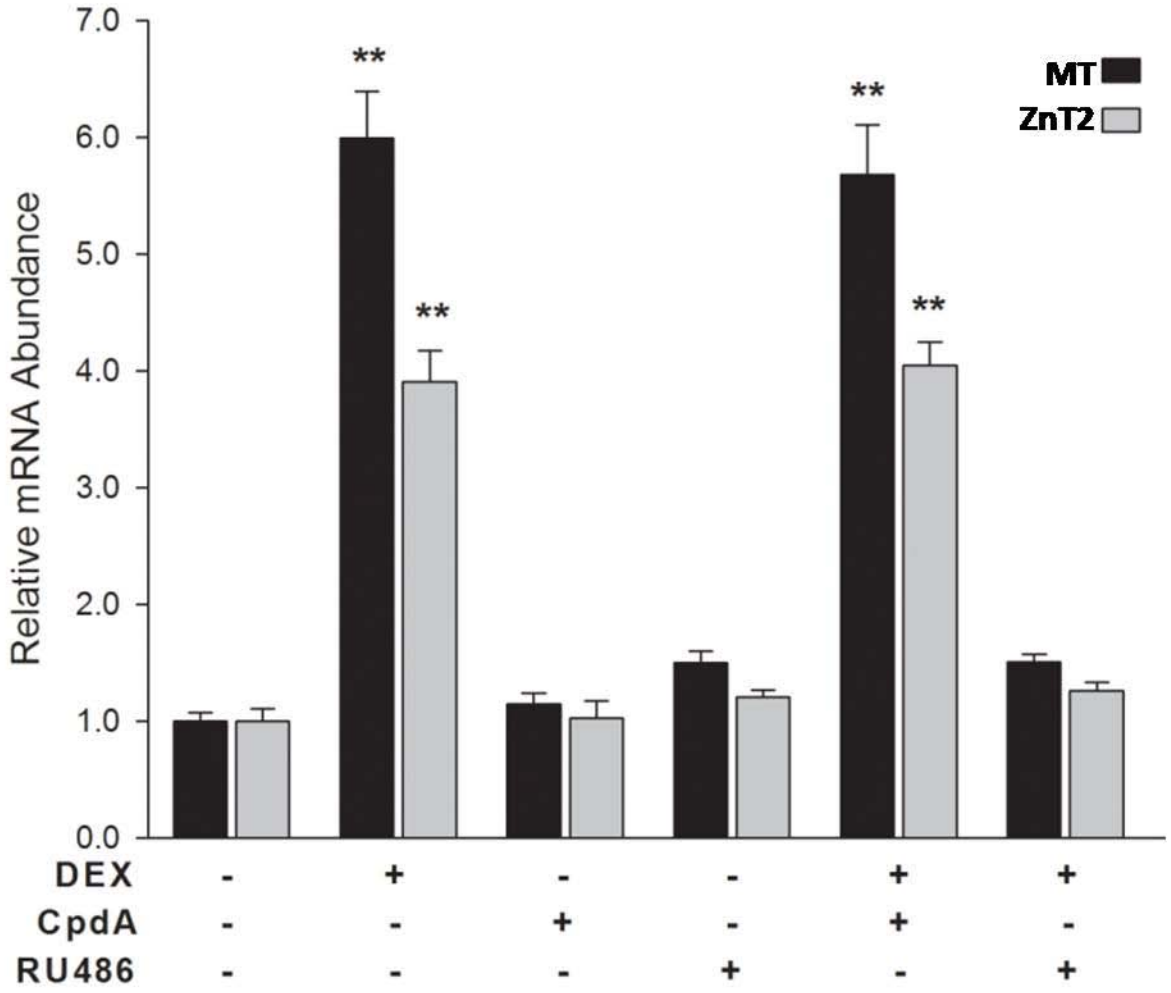

Figure 4-13. The effects of dexamethasone and glucocorticoid modulators, RU486 and CpdA, on the MT and ZnT2 mRNA expression in AR42J cells. The cells were treated for 12 h. Total RNA was isolated, MT and ZnT2 mRNA expression levels were measured by qPCR (n=3).

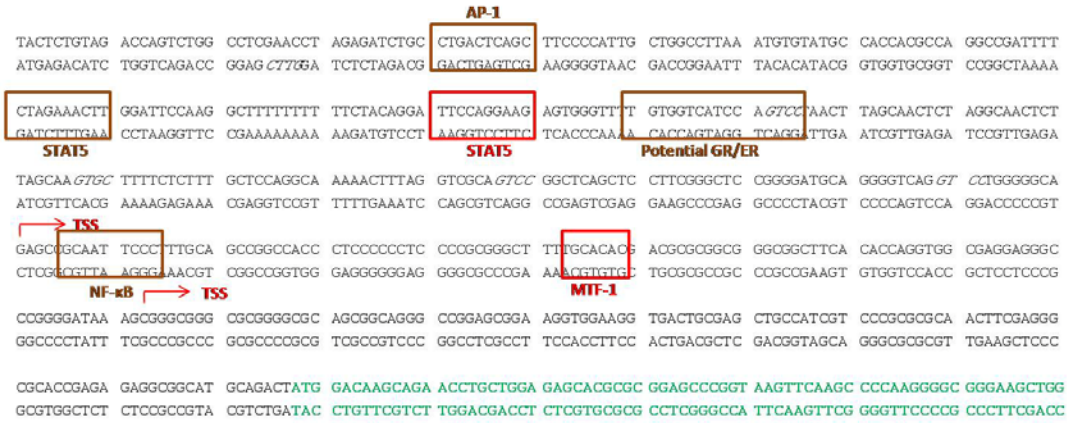

Figure 4-14. Transcription factor binding site analysis in the proximal region of mouse ZnT2 promoter. The sequences of activator protein 1 (AP-1), signal transducer and activator of transcription 5 (STAT5), glucocorticoid receptor (GR), estrogen receptor (ER), Nuclear factor-kappaB (NF-kB), and Metal-responsive transcription factor-1 (MTF-1) are shown in the box. Two transcription start site (TSS) are shown as arrows. The ZnT2 translated sequence is shown in green.

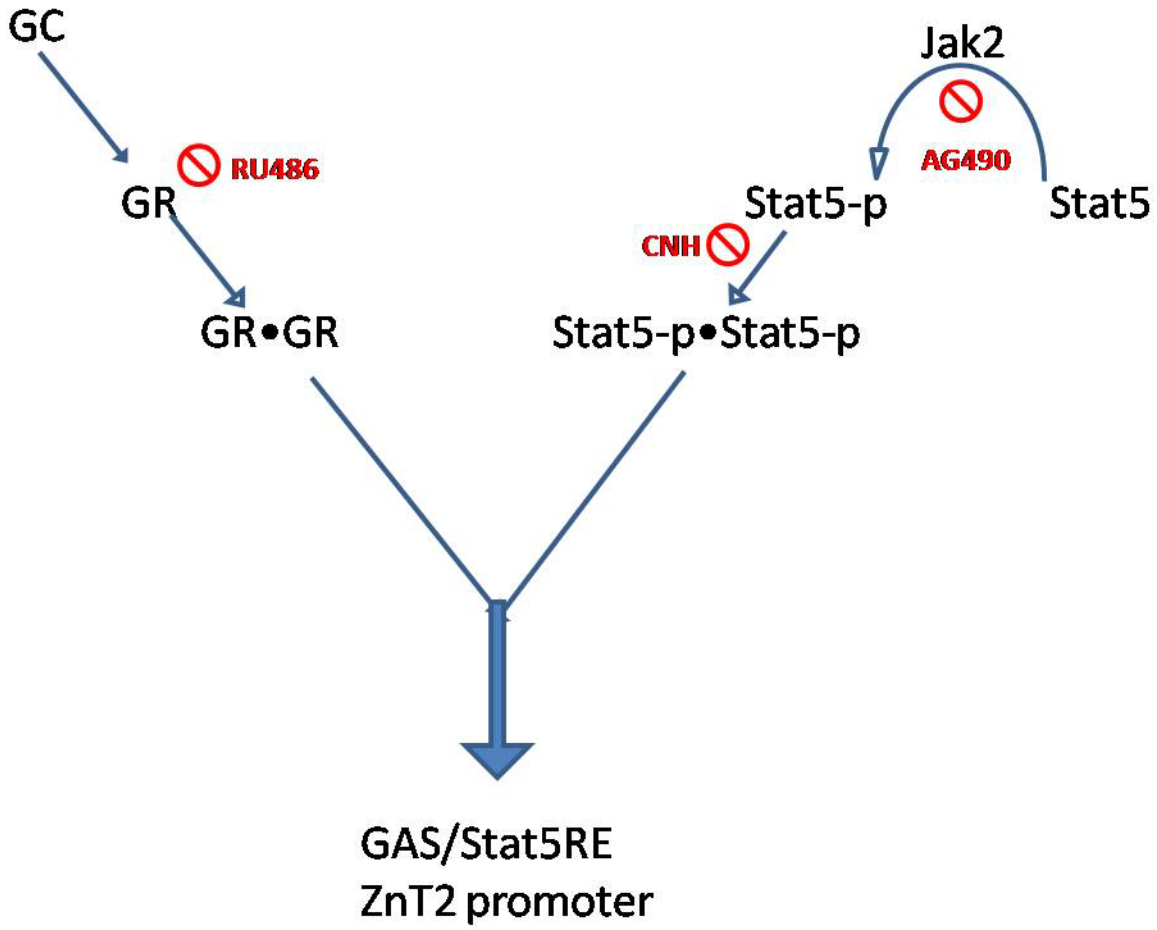

Figure 4-15. Possible GR and Stat5 synergistic signaling pathway. Upon the glucocorticoids (GC) binding to glucocorticoid receptors (GR), GR form active homo-dimmer and translocate into nucleus. Upon activation, Stat5 SH2 domain is phosphorylated by Jak2, and the phosphorylated Stat5 also form homo- or hetero-dimmer in nucleaus. GR and Stat5 interact and bind to the GAS/Stat5 RE elements in the promoter with other transcription activators for transcription activation.GR Antagonist RU486, Jak2 inhibitor AG490, and Stat5 inhibitor CNH are shown in the diagram.

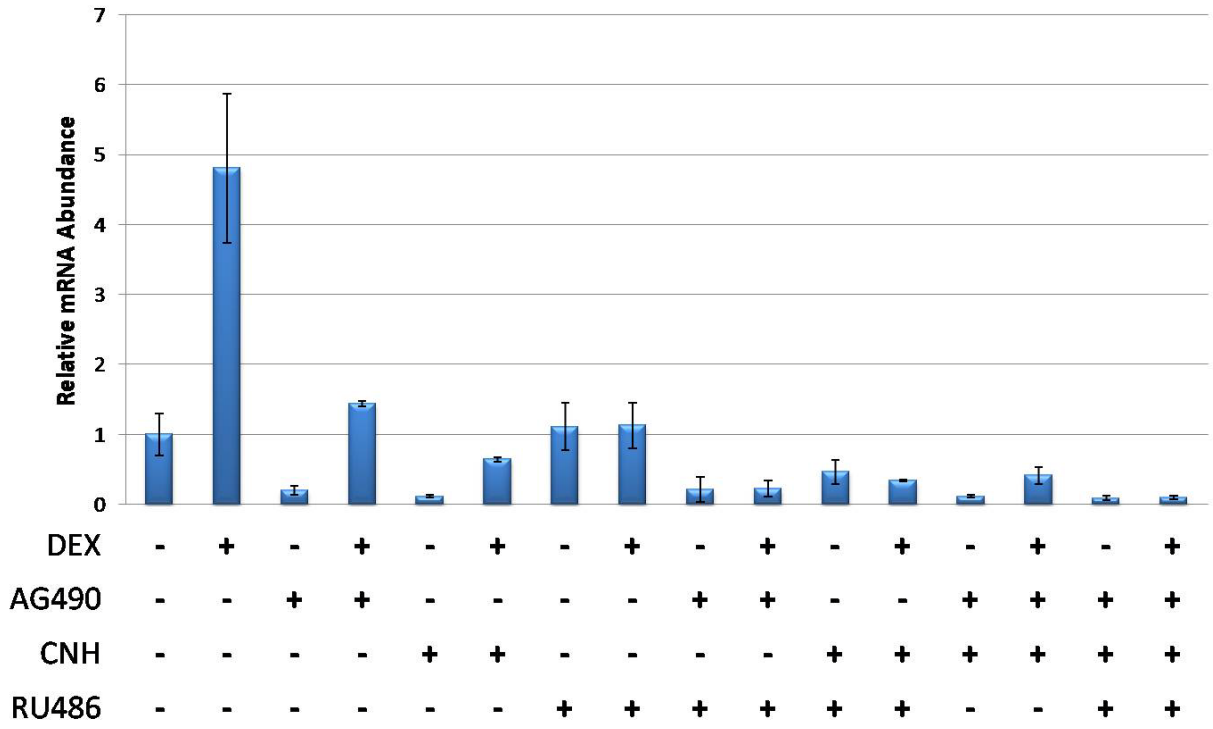

Figure 4-16. The effects of dexamethasone and glucocorticoid antagonist RU486, Stat5 inhibitor CNH, and Jak2 inhibitor AG490, on the MT mRNA expressions in AR42J cells. The cells were treated with 100nM DEX and/or 50uM AG490, 400uM CNH, and 1uM RU486 for 12 h, and cells were harvested. Total RNA were isolated, MT and ZnT2 mRNA expression levels were measured by qPCR (n=3).

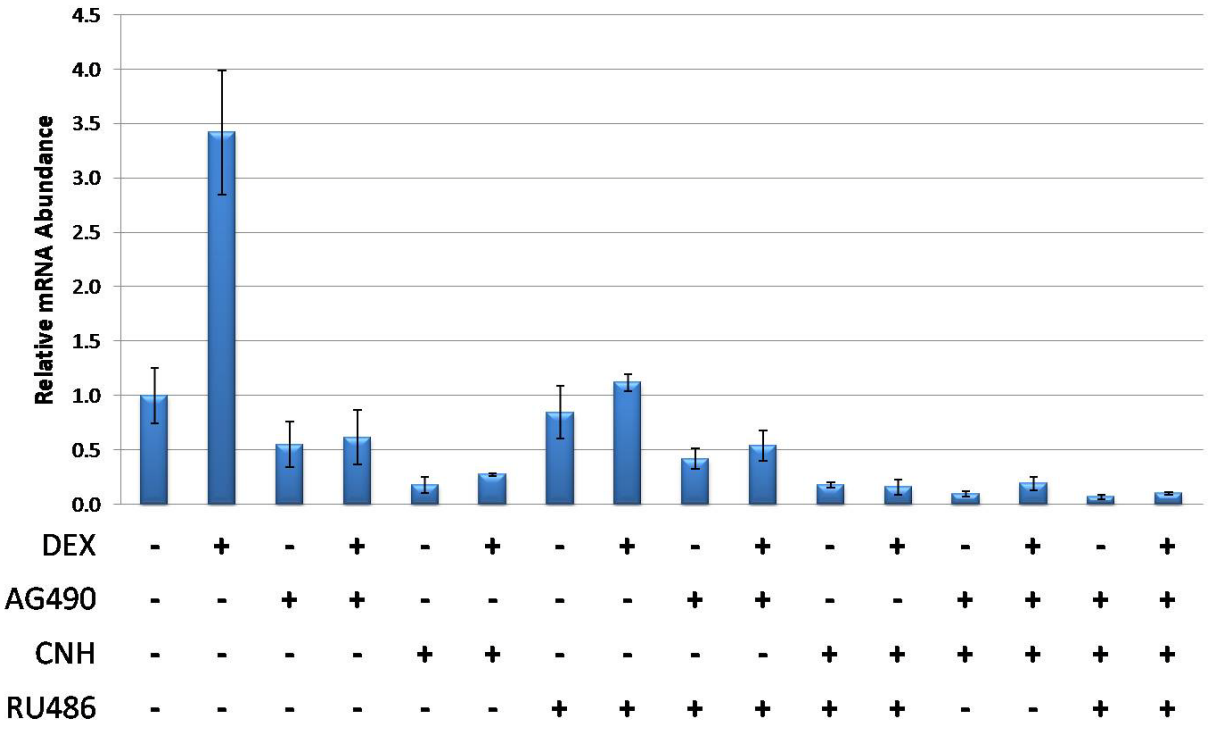

Figure 4-17. The effects of dexamethasone and glucocorticoid antagonist RU486, Stat5 inhibitor CNH, and Jak2 inhibitor AG490, on the ZnT2 mRNA expressions in AR42J cells. The cells were treated with 100nM DEX and/or 50uM AG490, 400uM CNH, and 1uM RU486 for 12 h, and cells were harvested. Total RNA were isolated, MT and ZnT2 mRNA expression levels were measured by qPCR (n=3).

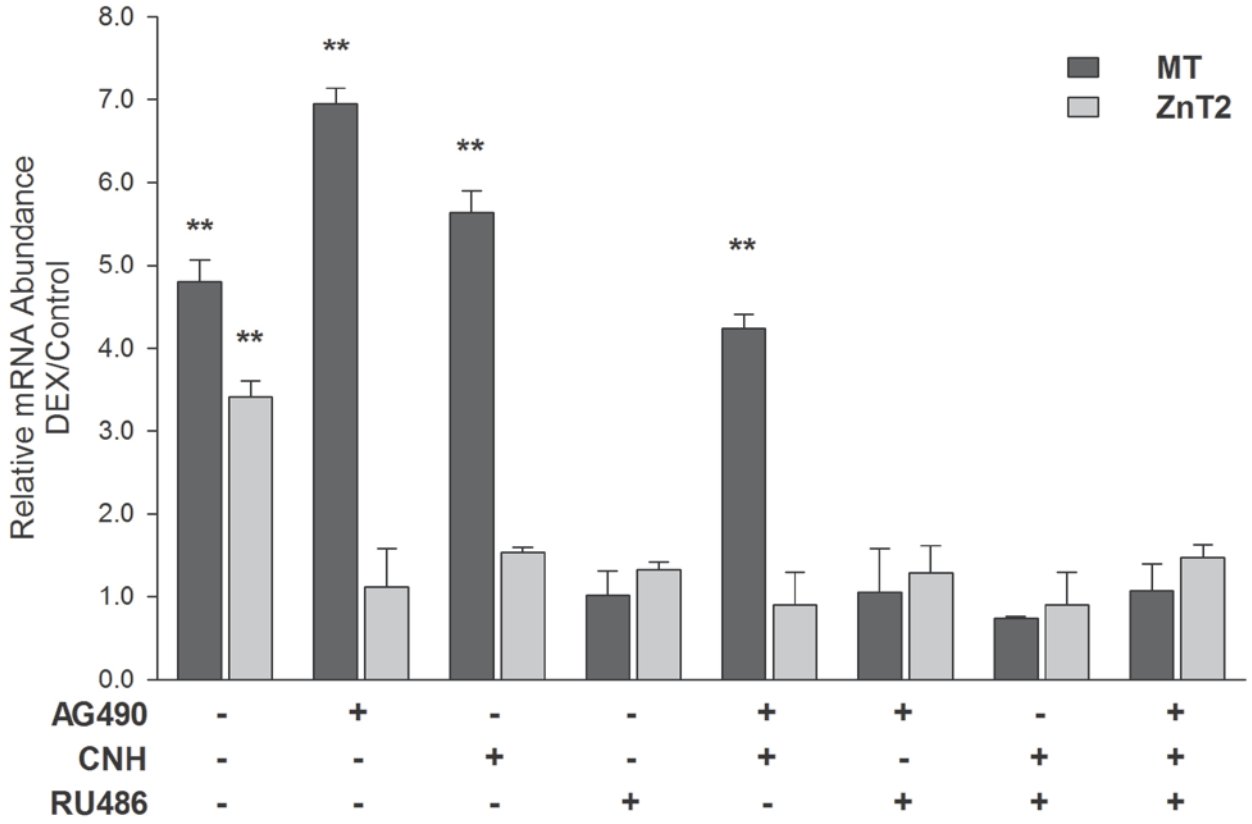

Figure 4-18. The effects of dexamethasone and glucocorticoid antagonist RU486, Stat5 inhibitor CNH, and Jak2 inhibitor AG490, on the MT and ZnT2 mRNA expressions in AR42J cells. The cells were treated with 100nM DEX and/or 50uM AG490, 400uM CNH, and 1uM RU486 for 12 h, and cells were harvested. Total RNA were isolated, MT and ZnT2 mRNA expression levels were measured by qPCR (n=3).

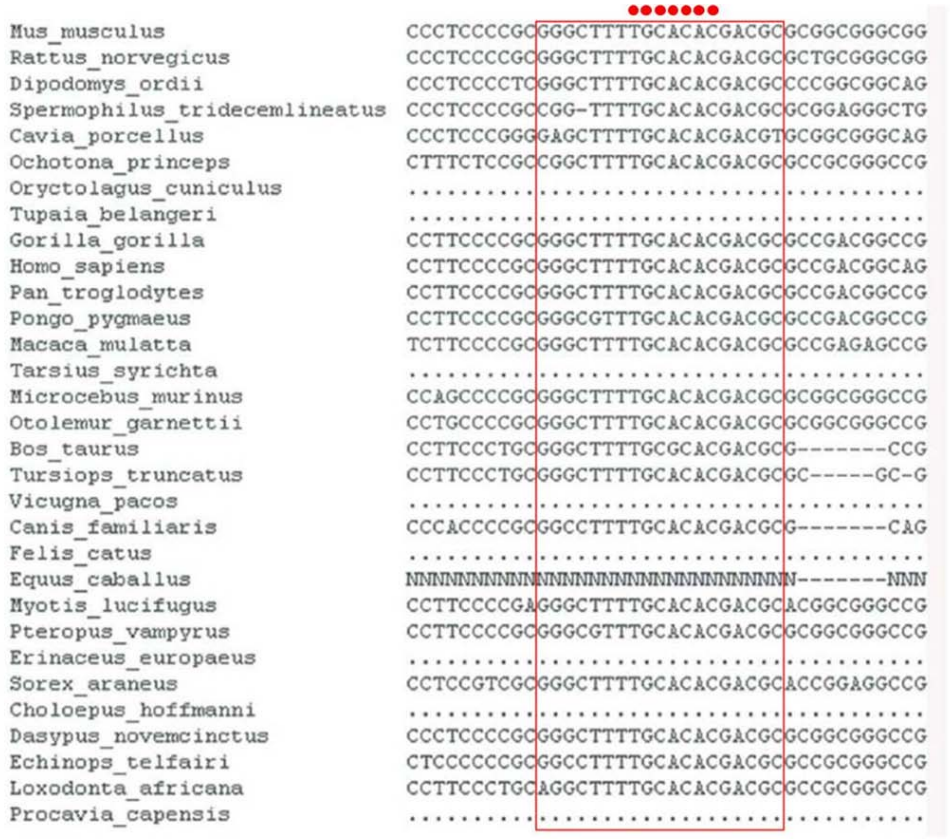

Figure 4-19. Conserved MRE sequences in the promoter sequence alignment of multiple species. The red dots marked the MRE consensus sequence.

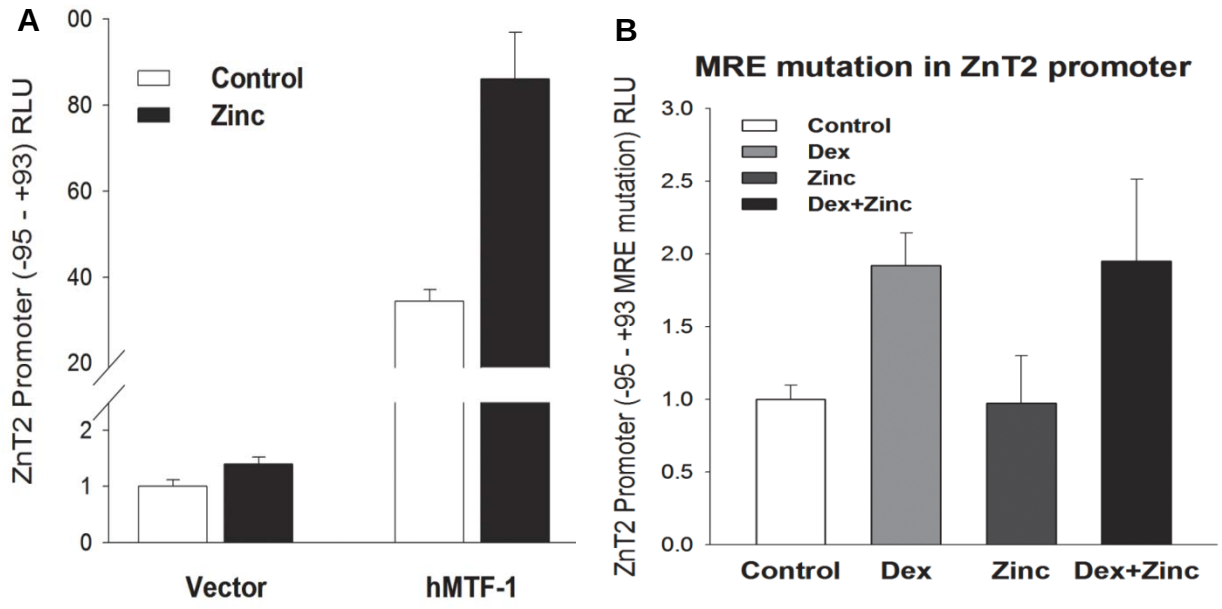

Figure 4-20. ZnT2 promoter activity in transfected HEK 293 and HeLa cells in response to zinc. HeLa cells were transfected with murine ZnT2 promoter constructs over the range (−95 - +93) ligated into pGL3-Basic vector. (A) HEK 293 cells were co-transfected with an human MTF-1 expression vector and the −95 - +93 ZnT2 promoter construct. (B) The Site Directed Mutagenesis was used to change the MRE consensus sequence into a restriction site. HeLa cells were transfected with mutant −95 - +93 ZnT2 promoter construct. The cells were treated with 100nM DEX or 100uM zinc for 24 h. Luciferase activity was measured 48 h after transfection.

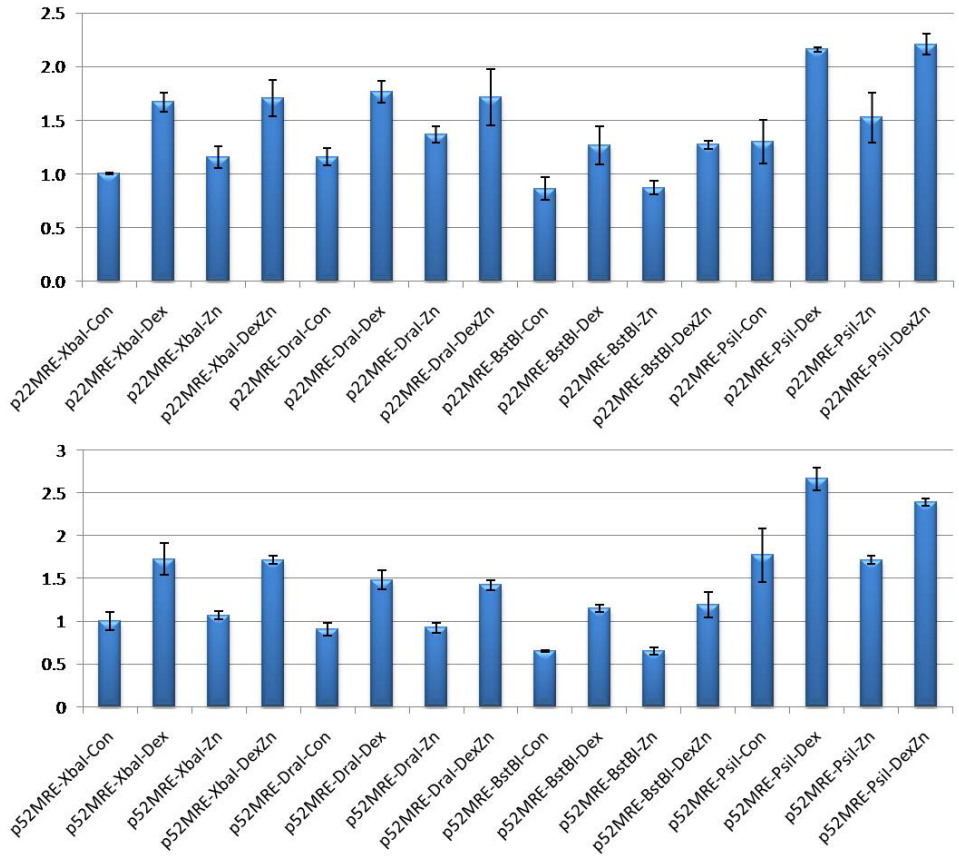

Figure 4-21. ZnT2 promoter activity in transfected HeLa cells in response to zinc. HeLa cells were transfected with mutant ZnT2 promoter constructs over the range p52(−95 - +93) or p22 (−815 - +93) ligated into pGL3-Basic vector. The Site Directed Mutagenesis was used to change the MRE consensus sequence to a XbaI, DraI, BstBI or PsiI site. The cells were treated with 100nM DEX or 100uM zinc for 24 h. Luciferase activity was measured 48 h after transfection.

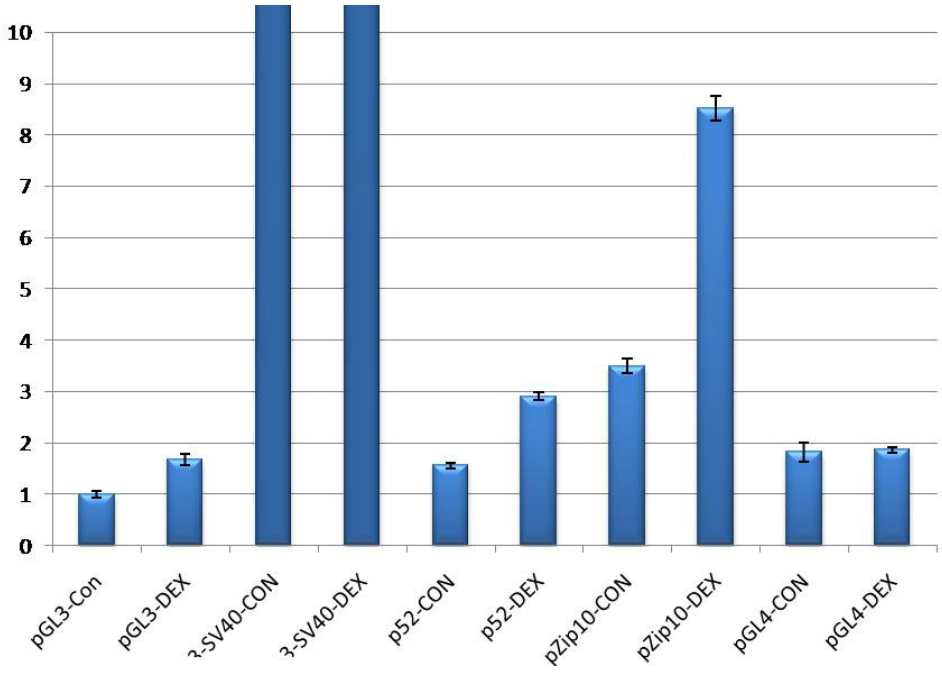

Figure 4-22. pGL3-Basic and pGL4 vector luciferase activities under DEX treatment in HeLa cells. SV-40 promoter construct was used as a positive control. p52 construct is the −95 - +93 ZnT2 promoter construct in pGL3-Basic vector. pZip10 is the Zip10 promoter construct in pGL3-Basic vector.

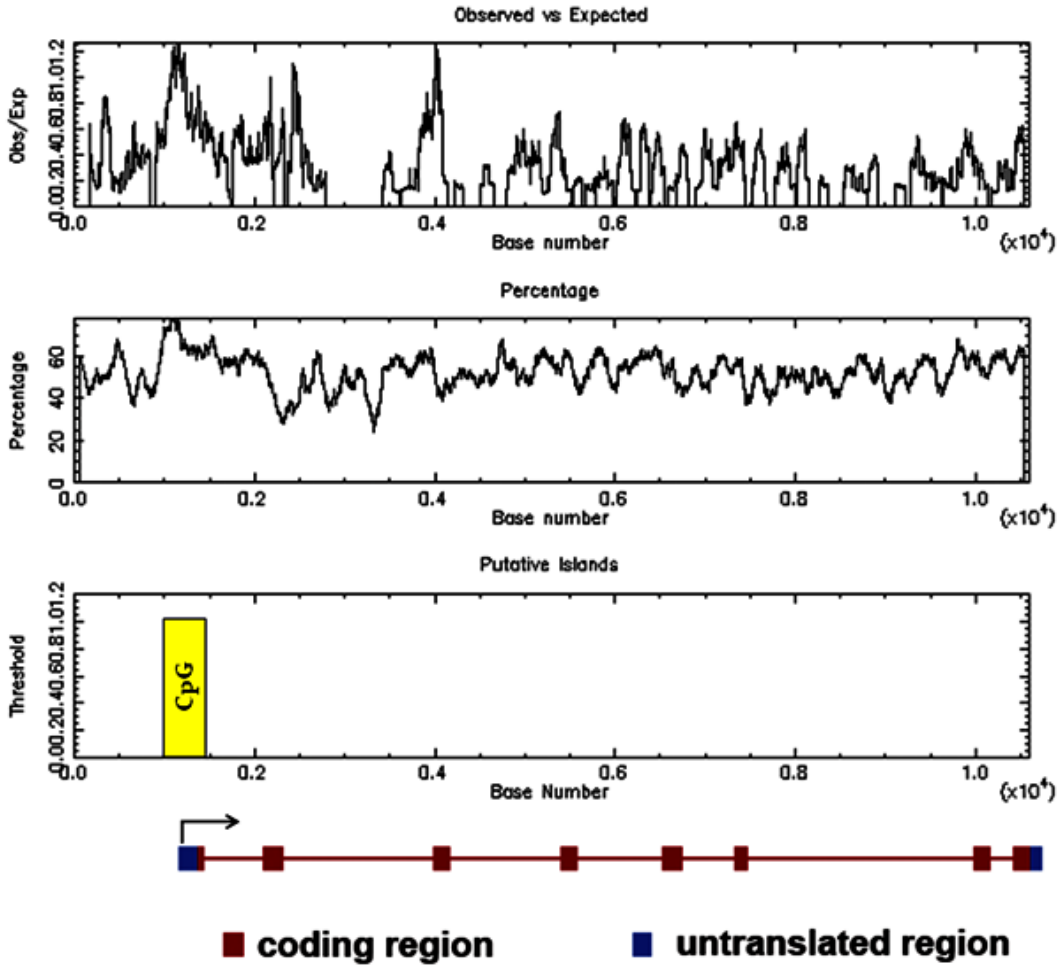

Figure 4-23. CpG island analysis in ZnT2 gene.

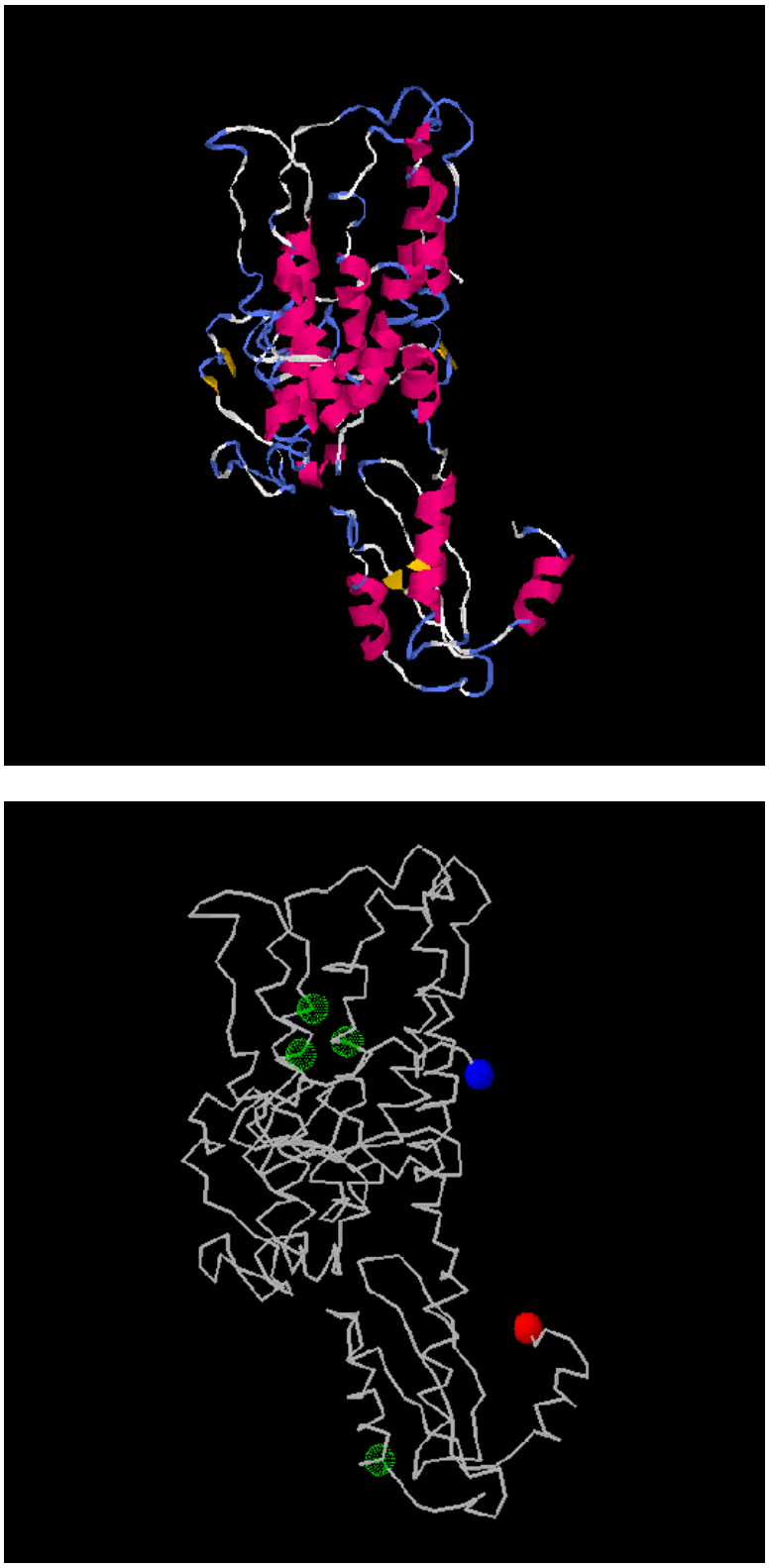

Figure 4-24. Predicted human ZnT2 protein structure (A) and zinc binding sites (B).

CHAPTER 5
ZINC TRANSPORTERS IN PANCREATIC ACINAR CELLS UNDER STRESS CONDITIONS

Introduction

The primary disease of the pancreas is pancreatitis, a condition in which there is an inflammation localized to the pancreas (92). A disturbance in zinc metabolism has been documented in patients with pancreatitis. Both urinary and serum zinc levels are increased in chronic pancreatitis, along with a deterioration of exocrine pancreatic function (20). Stimulation with cholecystokinin and secretin increased secretion of zinc in healthy persons but not in pancreatitis patients (135). Serum zinc levels in advanced chronic pancreatitis patients were higher when compared to patients with normal exocrine pancreatic function (20). However, diabetes, a complication due to chronic pancreatitis, has generally been associated with decreased serum zinc concentration (136). By contrast, acute pancreatitis presents a different scenario due to the acute pharse response and inflammation, where lower serum zinc levels and an increased liver zinc content are observed (137, 138), which could be explained by Zip14 activated through IL-6/Stat3 signaling in the liver (139). In alcoholic pancreatitis, the patients have a higher risk of acute zinc deficiency (140, 141). Altered mineral metabolism in the pancreas may contribute to the pathophysiology of mice with acute pancreatitis (142), suggesting that zinc supplementation could be therapeutic in pancreatitis (142). In the early stages of acute pancreatitis, the pancreatic concentration of zinc is significantly decreased. Zinc could increase both MT and glutathione levels in the pancreas and combat oxidative damage in cerulein-induced acute pancreatitis (143). MT is present in the exocrine and endocrine cells of patients with chronic pancreatitis and chronic pancreatitis with concomitant diabetes. Increased expression of MT, particularly in

acinar cells, protects against cerulein-induced acute pancreatitis in MT overexpressing transgenic mice (144). It is believed that MT can reduce the oxidative damage that occurs during acute pancreatitis. MT induction in pancreatic islets and specifically β-cells is regulated by cytokines and DEX, and PKC activation might play a role (145). Because of the relationship of zinc to pathophysiologic events in the pancreas, in this chapter of the dissertation, the zinc transporter expression was studied in relation to the supramaximal cerulein model of pancreatitis, as well as the *in vitro* alcohol-related acinar cell damage.

Methods and Results

Supramaximal cerulein decreased ZnT1 and ZnT2 expression in AR42J pancreatic acinar cells. A supraphysiological dose of cerulein, a synthetic analogue of CCK, was added to AR42J cells cultured at low (0.5uM), normal (5uM), and high (50uM) zinc levels to induce acute inflammation. I found that at these various zinc conditions, both ZnT1 and ZnT2 mRNA expression levels were reduced during cerulein-induced pancreatitic damage (Fig. 5-1). However, MT mRNA were greatly induced by cerulein. These results might indicate that MT, as cellular stress response, was induced due to cerulein-induced acinar cell damage. Cellular zinc also mobilizes and interacts with MT, which at high cellular levels could lead to lower zinc-MTF coupled transcription activation in the promoter regions of ZnT1 gene.

The question whether CCK could regulate MT and zinc transporters. To pursue this issue, AR42J cells were treated with different concentrations of CCK. MT mRNA expression was not altered by CCK simulation (data not shown). However, both ZnT1 and ZnT2 mRNA were decreased in a dose-dependent manner by CCK in AR42J cells. Most notablely, ZnT1 mRNA decreased to only half the levels of the control samples

(Fig. 5-2, ZnT2 mRNA is not shown here). Previously, we found that CCK mRNA in the small intestine were up-regulated during zinc deficiency in rats (146). These present results suggest a repression of ZnT1 and ZnT2 in pancreatic acinar cells by CCK signaling pathways and suggest a dynamic regulation of zinc homeostasis in the pancreas through CCK signaling and cross-talk between the small intestine and pancreas.

Long-term excess alcohol consumption could induce alcohol-related pancreatic damage, chronic pancreatitis, and zinc deficiency. However, the mechanism is unknown. In this study, AR42J cells were treated with 300mM ethanol for 12h and 24h. Cells were harvested and relative mRNA abundance of MT was found to be elevated during ethanol stimulation. We found a progressive decrease in ZnT2 mRNA abundance, which was inversely correlated with MT levels (Fig. 5-4). These results indicate a lowered zinc secretion from secretory pathways and zinc accumulation could occur during the first 24h of alcohol-related cell damage in pancreatic acinar cells. However, the long-term effects of excess alcohol consumption on the pancreatic zinc homeostasis need to be further investigated.

Discussion

Supramaximal cerulein is widely used to induce pancreatic acinar cell damage and pancreatitis. In this series of experiments, AR42J cells were treated with cerulein, and ZnT1 and ZnT2 expressions were found to be decreased. These results might indicate that MT as a cellular stress response gene is induced during cerulein-induced acinar cell damage. High levels of metallothionein, which could lead to a lower zinc-MTF coupled transcription activity in the promoter regions of ZnT1 and ZnT2 genes. This could potentially result in a higher cellular zinc level as a defense response to oxidative

stress induced by the supramaximal dose of cerulein. Further *in vivo* evidence is needed to fully understand how zinc transporters and intracellular zinc homeostasis are changed during pancreatitis and the relationship between this interaction and disease progression and prognosis.

This chapter provided a preliminary result of the zinc transporter regulation in acinar cells under stress conditions. Inconsistent results made it difficult to interpret the data. Further investigation is needed to fully understand the role of zinc homeostasis and function of zinc transporter in the progression of pancreatitis. Especially when these experiments were designed, the role of STAT5 signaling in ZnT2 regulation was not known. Future experiments will need to consider the STAT5 signaling pathway in study design.

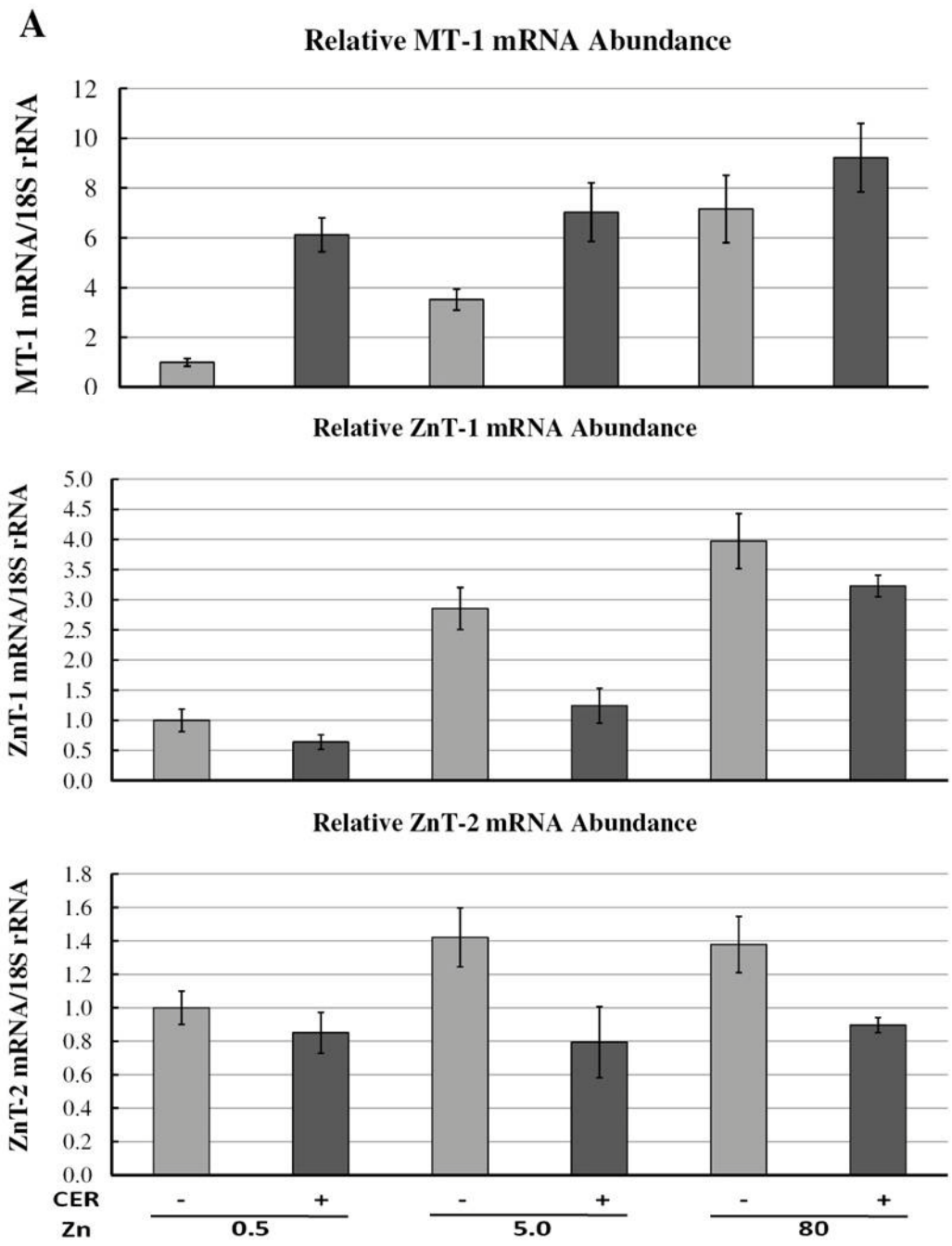

Figure 5-1. MT and ZnT1/2 mRNA expression in AR42J cells stimulated with cerulein. AR42J cells were cultured in low (0.5uM), normal (5.0uM), and high (80uM) zinc medium and stimulated with cerulein for 6 h. After stimulation, cells were harvested, and total RNAs were isolated. Relative mRNA abundance was measure by qPCR.

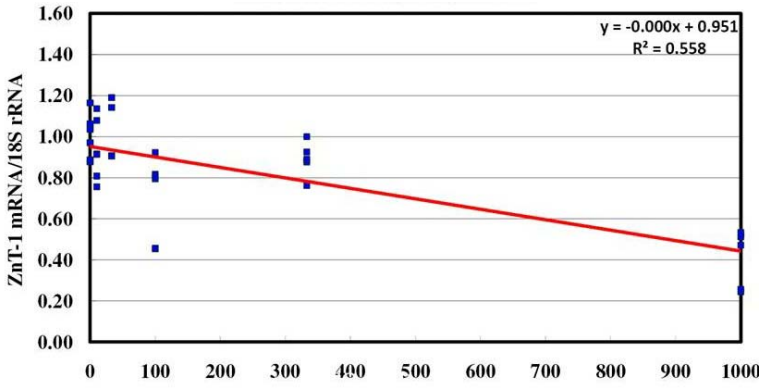

Figure 5-2. Supreamaximal CCK8 stimulation decreased the expression of ZnT1 in AR42J cells. AR42J cells were stimulated with CCK8 at various concentration. After stimulation, cells were harvested, and total RNAs were isolated. Relative mRNA abundance was measure by qPCR.

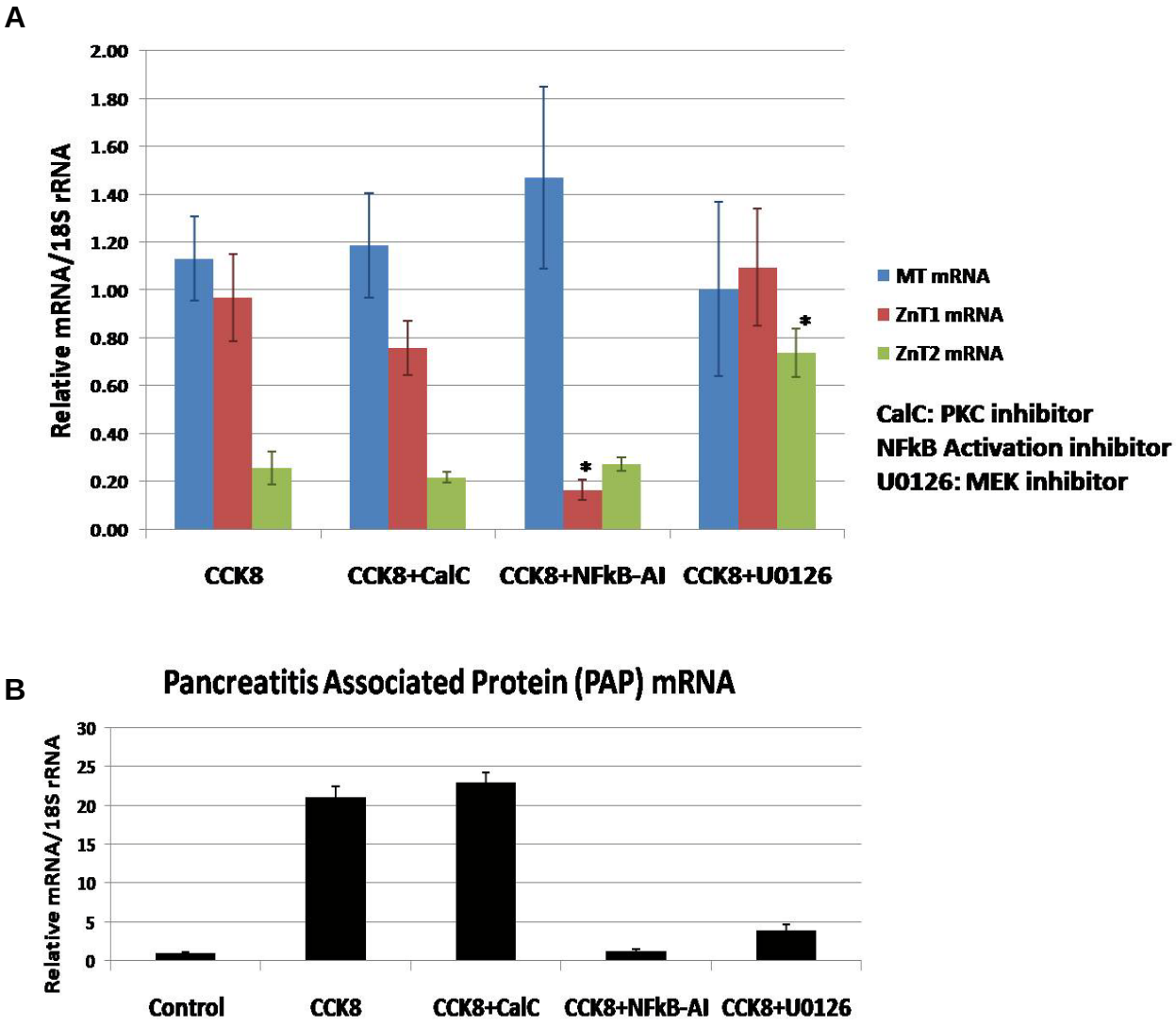

Figure 5-3. MT, and ZnT1/2 mRNA expressions in AR42J cells stimulated with supreamaximal CCK8. AR42J cells were stimulated with CCK8 or together with PKC inhibitor (CalC), NF-KB activation inhibitor (NFkB-AI), MEK inhibitor (U0126). Relative mRNA abundance was measure by qPCR. A) MT, and ZnT1/2 mRNA expression level. B) Pancreatitis associated protein (PAP) mRNA expression.

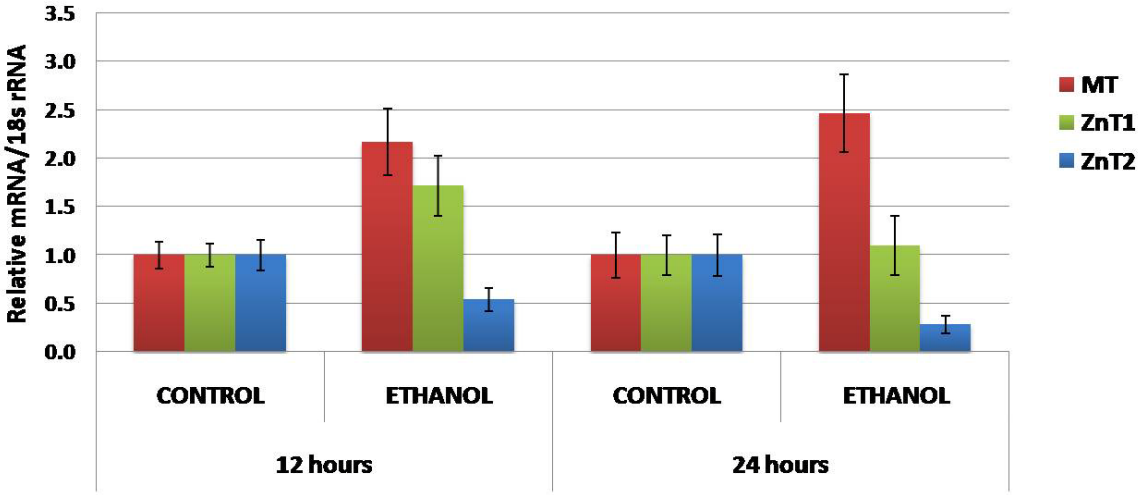

Figure 5-4. MT and ZnT1/2 mRNA expressions in AR42J cells stimulated with ethanol. AR42J cells were stimulated with ethanol for 12 h and 24 h. After stimulation, cells were harvested, and total RNAs were isolated. Relative mRNA abundance was measure by qPCR.

CHAPTER 6
ZINC TRANSPORTERS IN HUMAN PANCREATIC CANCER

Introduction

Pancreatic ductal adenocarcinoma is the fourth-leading cause of cancer deaths in the United States with an average expected survival of less than 6 moths (147). Recently the study by Dr. Min Li and his group from Baylor College of Medicine found aberrant expression of Zip4 was involved in pancreatic cancer progression (148, 149). To further understand the cellular zinc homeostasis changes in pancreatic cancer progression, qPCR was utilized to create an mRNA expression profile of human ZnT and Zip genes from a normal human pancreas.

Methods

Adult human pancreas total RNA, isolated by a modified guanidinium thiocyanate method, was obtained from a commercial source (Cell Application, San Diego, CA). Total RNA was. qPCR primers and the TaqMan probes were designed by using PRIMER EXPRESS V.3.0 (Applied Biosystems). The 18S rRNA level was used for normalization. ZnT and Zip transcript levels in a human reference RNA (Stratagene) were measured and considered as reference average levels. ZnT1 and ZnT2 were found to have higher transcript level amounts among ZnT transporters in the adult human pancreas on a relative basis, whereas Zip5 is the most abundant transcript of the SLC39A Zip family.

Results

Aberrant expression of zinc transporter in human pancreatic cancer. Recent findings suggest the aberrant expression of zinc transporter 4 , Zip4, contributes to human pancreatic cancer pathogenesis and progression (149). To further understand

how zinc transporter genes are regulated in pancreatic cancer, we compared the zinc transporter mRNA expression panel in the normal human and carcinoma pancreas. First, qPCR results confirmed the elevated pancreatic adenocarcinoma marker genes CD56 and Palladin expression. However, we noted that acinar cell markers of digestive enzyme gene expression, such as amylase, elastase, chymotrypsinogen, and trypsinogen, were nearly completely lost. These results indicate that pancreatic adenocarcinoma progression transforms the normal acinar cells into a duct-cell like cell type. ZIP4 expression levels were found to be increased more than 100-fold, as we expected (Fig. 6-1). Surprisingly, as one of the major and most abundant zinc transporter in the normal pancreas, Zip5 expression decreased more than 100-fold in RNA from pancreatic cancer. The most abundant ZnT transporters in normal pancreas are ZnT2 and ZnT8, and are predominantly found in acinar cells and islets, respectively. Both ZnT2 and ZnT8 expressions were found to decrease dramatically in pancreatic adenocarcinoma (Fig. 6-2). Also of note is the 50% reduction in MT transcript (MT1) in RNA from pancreatic carcinoma (Fig. 6-3).

The caution of interpreting these results is that total RNA from whole human pancreas tissue includes 85% from the exocrine pancreas. Another limitation is the use of the commercial human reference RNA. These RNAs are derived from a combination of different types of human cultured cell lines. It is comprised of total RNA isolated from various cultures of human cell lines representing different human tissues. The cell lines were chosen to ensure a broad coverage of human genes on human arrays and other type of assays. The cell lines include adenocarcinoma, mammary gland; hepatoblastoma, liver; adenocarcinoma, cervix; embryonal carcinoma, testis;

glioblastoma, brain; melanoma; liposarcoma; histiocytic lymphoma; macrophase; histocyte; lymphoblastic leukemia and plasmacytoma; myoloma and B lymphocyte. This is a good representation of most genes, but these cell lines may not be representative of certain highly tissue-specific or exclusively expressed genes, e.g. ZnT2, which has very low mRNA levels in human reference RNA.

Discussion

The initial aim of this experiment was to investigate the zinc transporter expression in acinar cell carcinoma. However, according to our cancer marker gene expression results, the pancreatic ductal adenocarcinoma marker genes CD56 and palladin are positive and all the acinar cell marker genes are negative, which indicated that the human pancreatic cancer sample was pancreatic ductal adenocarcinoma. Nonetheless, the full panel of zinc transporter gene expression results demonstrated a dramatic potential change of cellular zinc transport capability in cancer progression. Zip4 expression is dramatically increased in pancreatic cancer. This result is in agreement with the previous finding of aberrant expression of Zip4 in pancreatic cancer progression (149). The turn-off of Zip5 expression observed in pancreatic cancer might suggest a complete change in zinc uptake behavior and kinetics and the possibility of a cell type change is not excluded in this experiment. The abnormally high expression of Zip4 may be important in providing zinc for cancer cell metastasis. shRNA mediated silencing of ZIP4 resulted in a dramatic decrease in tumorgenesis, including decreased volume, weight, and differentiation grade, and significantly increased the survival rate of the subjects (148). This molecular mechanism has been proposed to be associated with cyclin D1 activity (148).

The only decreased ZnT transporters in pancreatic cancer tissue were ZnT2 and ZnT8. These zinc transporters are cell type-specific and are expressed at very high levels in acinar and beta cells, respectively, in the pancreas. However, this qPCR panel screening result showed a significant decrease in these two transcripts. Hence, it is curious that the transporter genes, which appear to be the major component of zinc metabolism in the pancreas, are down regulated in pancreatic cancer. Future studies are needed to fully understand the role of zinc transporters in pancreatic cancer progression.

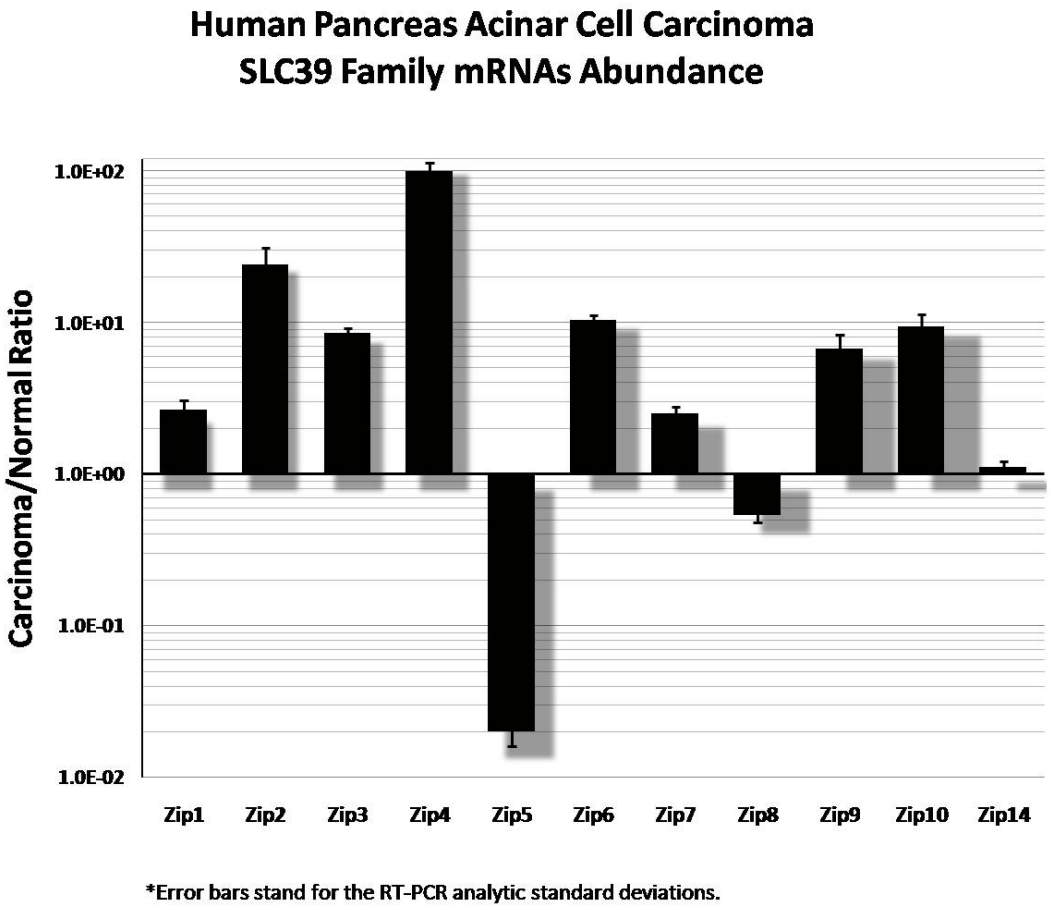

Figure 6-1. mRNA abundance panel of Zip family in human pancreatic cancer.

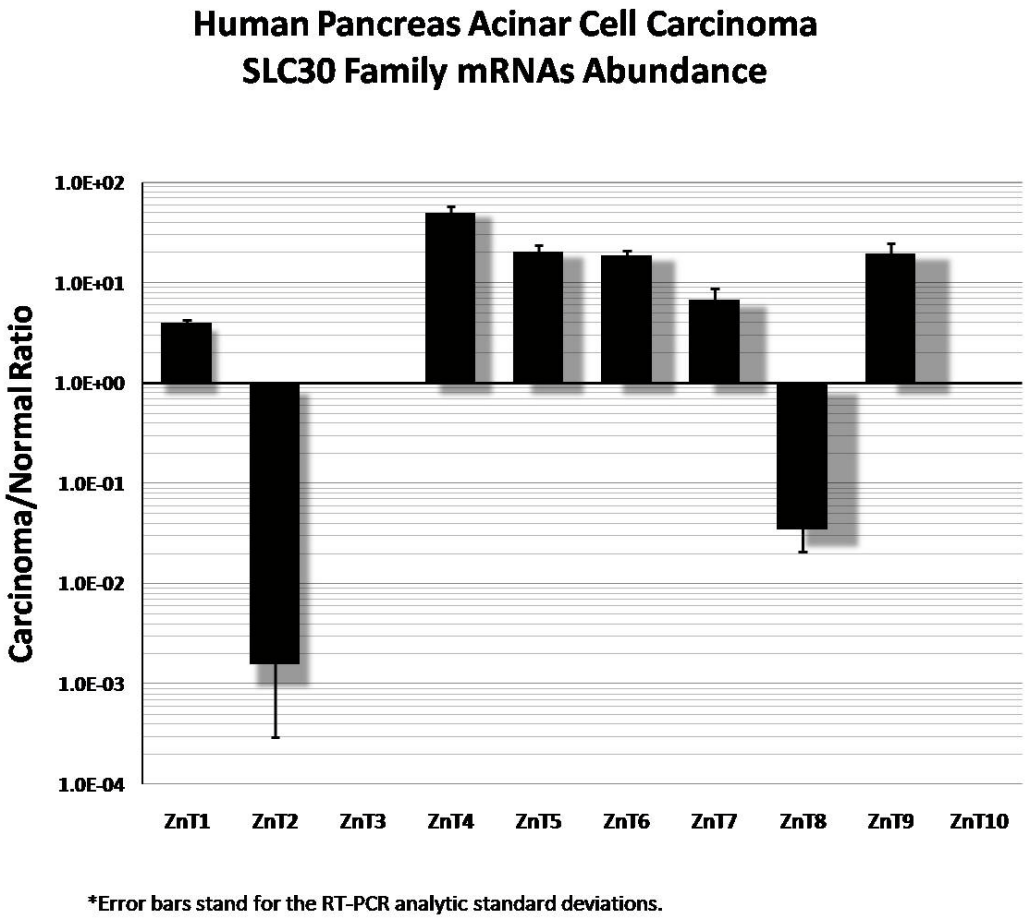

Figure 6-2. mRNA abundance panel of ZnT family in human pancreatic cancer.

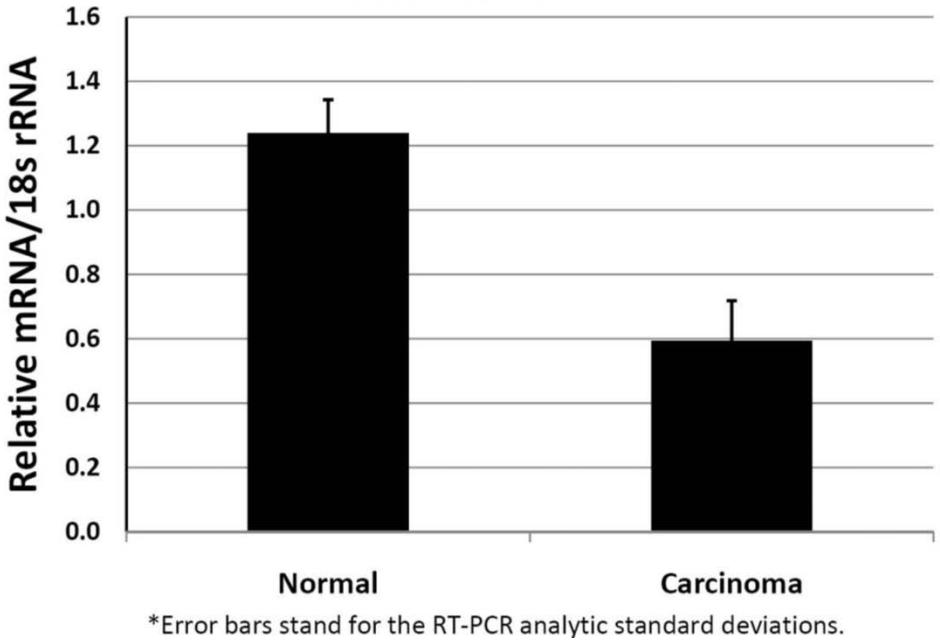

Figure 6-3. MT mRNA abundance in normal human pancreas and pancreatic cancer.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Mice fed a zinc-restriction diet developed covert signs of zinc deficiency in 3 weeks, as shown by dramatically decreased ZnT1, ZnT2 and MT expression in pancreas, as well as a depressed serum zinc concentration. Zinc deficiency could develop in a similar manner in human. Zinc restriction resulted in pancreatic cytoplasm and zymogen granules having less than half the amount of zinc found in mice fed the zinc-adequate diet. These responses show the sensitivity of these zinc transporter genes to the dietary zinc intake level. Western blotting clearly showed the decrease of ZnT1 in the plasma membrane-enriched fraction during zinc restriction. Of particular interest is that ZnT2 was exclusively detected in the isolated zymogen granule fraction and showed a reduction in response to dietary zinc restriction. This novel finding of ZnT2 localized to isolated zymogen granules was confirmed by immunofluorescence confocal microscopy. When 35μg zinc/g body weight was given orally, pancreatic zinc content increased, and there were transient elevations in pancreatic MT, ZnT1, and ZnT2 mRNAs.

Various concentrations of zinc were treated to the AR42J cells, causing a dose-dependent up-regulation of ZnT1 mRNA expression. The MT mRNA was significantly up-regulated by supplemental zinc, and the responsiveness was shown to be dose-dependent. Both MT and ZnT1 mRNA levels were regulated in a time-dependent manner. All three zinc chelators successfully induced zinc deficiency in AR42J cells in the culture medium, as confirmed by MT mRNA level as a sensitive cellular zinc indicator gene. Both ZnT1 and ZnT2 gene expression were found to be decreased after

24 h in low zinc culture medium at mRNA level and protein level. N-glycosylation modification was found in human ZnT1 protein with the total membrane protein sample prepared from HEK 293 cells.

Dexamethasone (DEX) was added to the AR42J cell cultures for 48 hour to stimulate cell differentiation. An increase in amylase mRNA, a signature of acinar differentiation and secretory enzyme production, was observed following the addition of DEX. A strong up-regulation of ZnT2 mRNA was found, upon stimulation with DEX. To examine whether ZnT2 is regulated by GC hormones *in vivo*, mice were given DEX by injection. The pancreatic zinc content decreased significantly. ZnT2 mRNA expression was significantly upregulated nearly 2-fold by 8 h after the injection and a high expression level was maintained at 16 h.

ZnT2 mRNA was effectively knocked down in AR42J cells by using ZnT2 siRNA, with either the presence or absence of DEX. Knocking down ZnT2 in acinar cells should produce a transient zinc accumulation in the cytoplasm and activation of MT gene expression via zinc-induced MTF-1 translocation to the nucleus. Significant elevation of MT mRNA was found to reach a peak around 24 h post-transfection. In accord with the induction of MT, an increase in cytoplasmic (^{65}Zn) zinc accumulation from the medium was observed through ZnT2 knockdown with siRNA. Cytoplasmic ^{65}Zn was increased by 36% with ZnT2 siRNA, which supports the hypothesis that ZnT2 transports cytoplasmic zinc into zymogen granules. Further support for this role of ZnT2 is that ^{65}Zn in the zymogen granules was decreased by 15%. HeLa cells were transfected with a ZnT2 cDNA vector or empty vector and the cells were allowed to accumulate ^{65}Zn.

Efflux of ^{65}Zn from preloaded cells was greater in the overexpressing cells. This finding is congruent with the ZnT2 transport function in zymogen granules.

To understand the mechanism of ZnT2 regulation by DEX, the GC antagonist RU486 and a newly-discovered GR modulator CpdA, were exploited to study the association of ZnT2 gene transcription with signaling via the GR. CpdA exhibits no transactivation potential on GRE-driven gene transcription (64) and was not able to activate ZnT2 gene expression. However, when AR42J cells were treated with DEX and CpdA at the same time, the hormonal analogy could still initiate expression of MT and ZnT2 , presumably via transactivation of GR. In contrast, presence of the GR antagonist RU486, prevented CpdA and DEX from stimulating the up-regulation of MT and ZnT2. These differing results with the two antagonists indicate DEX stimulated ZnT2 expression via transactivation of GRE-driven gene expression, but associated with NF-κB activation. Two half GRE site were found but no full GRE. While realizing that the non-canonical half sites may impart GC regulation for some genes, two STAT5-RE's were identified in the ZnT2 promoter (130). Consequently, we used a chromo-based nicotinoyl hydrazone (CNH), a STAT5-specific inhibitor (131) and the Janus kinase 2 inhibitor (AG490) to examine STAT5 involvement in ZnT2 activation by DEX. The inhibition of STAT5, particularly in combination with Jak2 inhibition, completely blocked DEX induction of ZnT2. Notably, the DEX-induced increase in MT expression was not inhibited by either AG490 or the STAT5 inhibitor.

An MRE sequence was identified in the downstream of TSS in mouse ZnT2 promoter, and it is highly conserved across species. Markedly enhanced luciferase was observed with hMTF-1 transfection. Zinc doubled promoter activity under these

conditions. The contribution of the MRE sequence to ZnT2 regulation was supported through mutation of this sequence in the ZnT2 promoter.

A supraphysiological dose of cerulein was added to AR42J cells cultured at low (0.5uM), normal (5uM), and high (50uM) zinc levels to induce acute inflammation, at these various zinc conditions, both ZnT1 and ZnT2 mRNA expression levels were reduced during cerulein-induced pancreatitic damage. AR42J cells were treated with different concentrations of CCK. MT mRNA expression was not altered by CCK simulation. However, both ZnT1 and ZnT2 mRNA were decreased in a dose-dependent manner by CCK in AR42J cells. AR42J cells were treated with 300mM ethanol for 12h and 24h. Cells were harvested and relative mRNA abundance of MT was found to be elevated during ethanol stimulation. We found a progressive decrease in ZnT2 mRNA abundance, which was inversely correlated with MT levels.

Zinc transporter expression in pancreatic cancer was studied. Recent findings suggest the aberrant expression of zinc transporter 4 , Zip4, contributes to human pancreatic cancer pathogenesis and progression (149). As one of the major and most abundant zinc transporter in the normal pancreas, Zip5 expression decreased more than 100-fold in RNA from pancreatic cancer. The most abundant ZnT transporters in normal pancreas are ZnT2 and ZnT8, and are predominantly found in acinar cells and islets, respectively. Both ZnT2 and ZnT8 expressions were found to decrease dramatically in pancreatic adenocarcinoma. Also of note is the 50% reduction in MT transcript (MT1) in RNA from pancreatic carcinoma.

Future Directions

Zinc- and glucocorticoid-regulated ZnT2 expression in the pancreas is a key finding of this study. The transcription factors, MTF-1, glucocorticoid receptor, and

Stat5, have been found to be associated with this regulation. As a predominant zinc transporter involved in secretory pathways, ZnT2 is highly expressed in many of the other secretory glands and tissues, primarily, the mammary glands, prostate, testes, kidneys, adipose, placenta, small intestine, and adrenal glands. The functions and regulation of each transporter influence zinc secretion in these exocrine organs.

Many new technologies and methods are emerging in the nutritional sciences field. Gene knock-out and tissue specific conditional knock-out techniques increasingly provide powerful models to study the functionality of genes including the zinc transporters. Further study would be beneficial concerning nutrient-gene interactions in specific gene-deficient settings.

Epigenetic modification has been proposed in regulating zinc transporter expression. CpG islands can be identified in many of the zinc transporter gene promoters, including ZnT2 and ZnT5. A recent report by Dr. Coneyworth suggested that the methylation of the human ZnT5 promoter resulted in reduced expression, which could be associated with age-related decline in zinc status. The epigenetic effects of aging and cancer are being studied, and more and more findings are suggesting the interrelationship between epigenetic effects on zinc status and altered gene expression related to zinc metabolism. For example, zinc deficiency can decrease DNA methylation status, as in global DNA hypomethylation in rat liver. DNA hypermethylation status can alter the zinc metabolism. Hypermethylation in the promoter of the MT gene alters the MTF-1 binding affinity to the multiple MREs in the promoter, thus changing MT gene transcription. There are other MTF-1 regulated zinc transporter genes that have not been investigated concerning whether the methylation status can change the promoter

activity. The consensus sequence of MRE is TGCRCNC, and all of the three Cs in this sequence can be a potential methylated ^{m}CpG site as follow:

TG^{m}CGCNC

TGCR^{m}CGC

TGCRCN^{m}CG

Methylation status changes dramatically in aging and cancer cells consequently. It is important to investigate how the methylation status changes in the course of aging and cancer progression and alters the zinc transporter gene expression. This in turn might affect cellular zinc metabolism, and consequently either promotes or inhibits aging and cancer progression.

Cellular zinc homeostasis dysregulation has been associated with several types of cancer. For example, in pancreatic cancer, an aberrant expression of Zip4 increases cellular zinc concentration, which is associated with cancer progression (149). Another example is found in the LIV-1 subfamily, which is involved in invasive behavior of breast cancer (150). The future question for investigation is how intracellular zinc influences cancer progression and what therapeutic strategies can be developed to target those involved zinc transporters in cancer treatment.

MT plays an important role in zinc homeostasis, particularly in the pancreas, where it not only protects against zinc deficiency, but it also prevents any toxic effects of zinc on the pancreas (32). MT-1 is a well-studied metallothionein protein. However, there are other forms of metallothionein that are not so well studied. For example, it has been suggested that MT-2 is found in the pancreatic secretions (32), however the molecular mechanism is still not well understood. Another interesting observation is that

MT-3 overexpressing transgenic mice die from pancreatic necrosis. It would be of interest to study the function of MT-3 in the pancreas. MT-4 is another novel metallothionein protein, which is not well studied either. Genome wide transcript analysis from the Genomics Institute of Novartis Research Foundation suggests a highly tissue specific expression is found only in the epiderm and stomach, and MT-4's function and regulation in these tissues need further investigation.

microRNA's function in gene regulation has been unknown until recent. Plant microRNAs have been found to be important in sensing and responding to the mineral content of the environment (151, 152). In humans, miRNA-584 was found to mediate post-transcriptional regulation of the lactoferrin receptor and was involved in regulation of nutrient metabolism in newborns (153). Several genes in mineral metabolism have been found to be translationally regulated. Therefore, the roles of microRNAs in micronutrient-gene regulation need to be further explored.

Another future direction of study could be the development of new molecular sensors that can reveal the amount of zinc in cells (154). Zinc dysregulation is becoming more and more important in the progression of a number of diseases. Zinc imaging methods will continue to emerge as a powerful tools, not only in disease prevention, but also in the diagnosis, treatment of many diseases, including diarrhea, cancer, lower immunity, diabetes, Alzheimer's disease, acrodermatitis enteropathica, fume fever, and age-related macular degeneration.

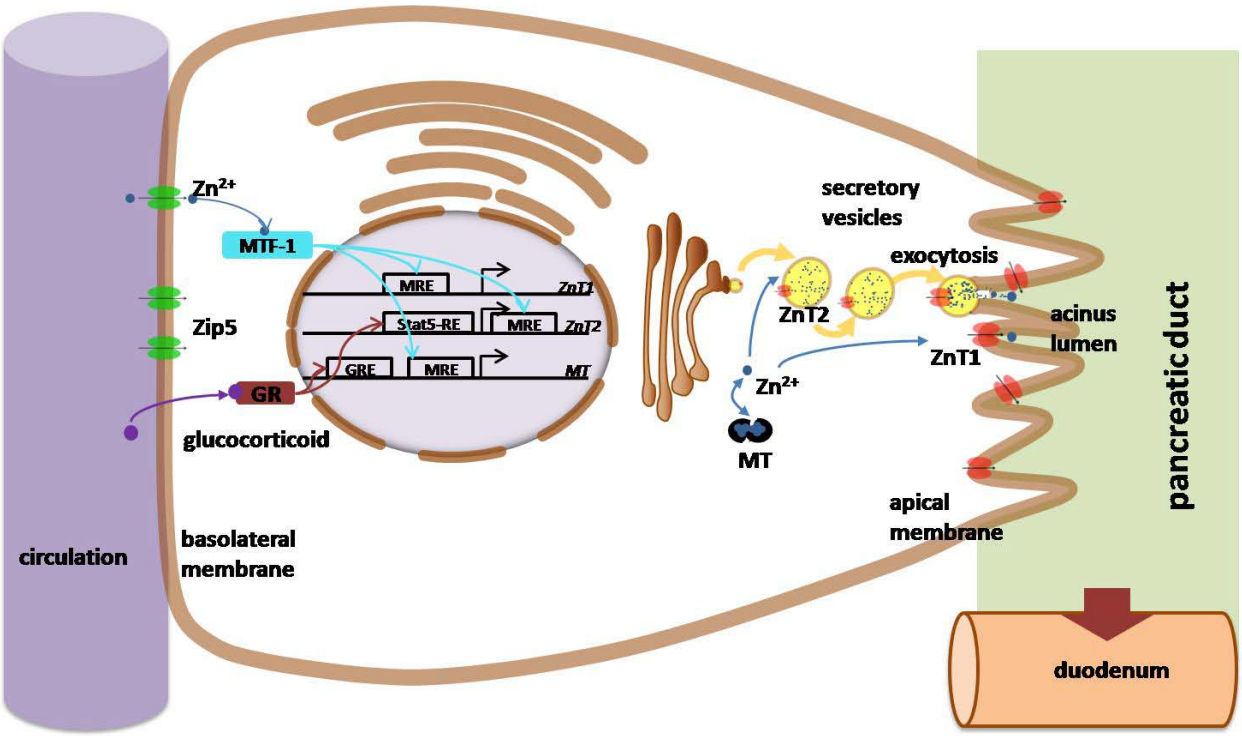

Figure 7-1. Hypothetical schematic model of zinc transport and secretion in pancreatic acinar cells. Zinc influx is influenced by ZIP5 located at the basolateral plasma membrane. Zinc secretion at the apical plasma membrane is regulated through two different pathways. Zinc is transported into lumen through ZnT1 localized on apical plasma membrane. Cytosolic zinc is sequestrated into zymogen granules by ZnT2 and is released during regulated exocytosis. Expression of ZnT2 is mediated by MTF-1, depending on the intracellular zinc level. MT and ZnT2 expression is regulated through the glucocorticoid receptor (GR) through an essential interaction of the GR with STAT5. ZnT2 expression is more likely to be associated with secretory stimulation of digestive enzymes.

LIST OF REFERENCES

1. Black RE*, et al.* (2008) Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* 371(9608):243-260.

2. Garrow JS, Ralph A, James WPT (2000) *Human nutrition and dietetics* (Churchill Livingstone, Edinburgh ; New York) 10th Ed pp xii, 900 p.

3. Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. *Annu Rev Nutr* 29:153-176.

4. Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. *J Biol Chem* 281(34):24085-24089.

5. Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. *Annu Rev Nutr* 24:151-172.

6. Iitaka M*, et al.* (2001) Induction of apoptosis and necrosis by zinc in human thyroid cancer cell lines. *J Endocrinol* 169(2):417-424.

7. Cummings JE, Kovacic JP (2009) The ubiquitous role of zinc in health and disease. *J Vet Emerg Crit Care (San Antonio)* 19(3):215-240.

8. Dong J, Robertson JD, Markesbery WR, Lovell MA (2008) Serum zinc in the progression of Alzheimer's disease. *J Alzheimers Dis* 15(3):443-450.

9. Maret W, Krezel A (2007) Cellular zinc and redox buffering capacity of metallothionein/thionein in health and disease. *Mol Med* 13(7-8):371-375.

10. Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. *Nat Rev Neurosci* 6(6):449-462.

11. Fischer Walker C, Black RE (2004) Zinc and the risk for infectious disease. *Annu Rev Nutr* 24:255-275.

12. Cousins RJ (1985) Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol Rev* 65(2):238-309.

13. Foote JW, Delves HT (1984) Albumin bound and alpha 2-macroglobulin bound zinc concentrations in the sera of healthy adults. *J Clin Pathol* 37(9):1050-1054.

14. Cousins RJ (1986) Toward a molecular understanding of zinc metabolism. *Clin Physiol Biochem* 4(1):20-30.

15. Brown KH*, et al.* (2004) International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr Bull* 25(1 Suppl 2):S99-203.

16. Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. *Annual Review of Nutrition* 24:151-172.

17. Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. *Journal of Biological Chemistry* 281(34):24085-24089.

18. McClain CJ (1990) The pancreas and zinc homeostasis. *J Lab Clin Med* 116(3):275-276.

19. Lee DY, Prasad AS, Hydrick-Adair C, Brewer G, Johnson PE (1993) Homeostasis of zinc in marginal human zinc deficiency: role of absorption and endogenous excretion of zinc. *J Lab Clin Med* 122(5):549-556.

20. Kondo T*, et al.* (1989) Urinary and serum zinc levels in chronic pancreatitis. *Pancreas* 4(1):79-82.

21. Smith ML, Fariss BL, Jennings PB (1986) Serum zinc levels in sheep with experimental pancreatic abnormalities. *Pancreas* 1(1):20-23.

22. Sullivan JF, Burch RE, Quigley JH, Magee DF (1974) Zinc deficiency and decreased pancreatic secretory response. *Am J Physiol* 227(1):105-108.

23. Adler M*, et al.* (1980) The pancreatic secretion of zinc in man and rat. *Gastroenterol Clin Biol* 4(6-7):441-449.

24. Dominguez-Munoz JE, Martinez SM, Leodolter A, Malfertheiner P (2004) Quantification of pancreatic zinc output as pancreatic function test: making the secretin-caerulein test applicable to clinical practice. *Pancreatology* 4(1):57-62.

25. Andersson T, Berggren PO, Flatt PR (1980) Subcellular distribution of zinc in islet beta-cell fractions. *Horm Metab Res* 12(6):275-276.

26. Sladek R*, et al.* (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 445(7130):881-885.

27. Mills CF, Quarterman J, Williams RB, Dalgarno AC (1967) The effects of zinc deficiency on pancreatic carboxypeptidase activity and protein digestion and absorption in the rat. *Biochem J* 102(3):712-718.

28. Tomita T (2000) Metallothionein in pancreatic endocrine neoplasms. *Mod Pathol* 13(4):389-395.

29. Davis SR, Khoo C, Cousins RJ (1996) Metallothionein null and metallothionein transgenic mice exhibit altered intestinal processing of zinc. *Faseb Journal* 10(3):1112-1112.

30. Dalton T, Fu K, Palmiter RD, Andrews GK (1996) Transgenic mice that overexpress metallothionein-I resist dietary zinc deficiency. *J Nutr* 126(4):825-833.

31. Andrews GK, Kage K, Palmiter-Thomas P, Sarras MP, Jr. (1990) Metal ions induce expression of metallothionein in pancreatic exocrine and endocrine cells. *Pancreas* 5(5):548-554.

32. De Lisle RC, Sarras MP, Jr., Hidalgo J, Andrews GK (1996) Metallothionein is a component of exocrine pancreas secretion: implications for zinc homeostasis. *Am J Physiol* 271(4 Pt 1):C1103-1110.

33. Rofe AM, Winters N, Hinskens B, Philcox JC, Coyle P (1999) The role of the pancreas in intestinal zinc secretion in metallothionein-null mice. *Pancreas* 19(1):69-75.

34. Liu J, Liu Y, Michalska AE, Choo KH, Klaassen CD (1996) Distribution and retention of cadmium in metallothionein I and II null mice. *Toxicol Appl Pharmacol* 136(2):260-268.

35. Lee HH*, et al.* (1990) Pancreaticobiliary secretion of zinc and copper in normal persons and patients with Wilson's disease. *J Lab Clin Med* 116(3):283-288.

36. Kelly EJ, Quaife CJ, Froelick GJ, Palmiter RD (1996) Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. *J Nutr* 126(7):1782-1790.

37. Kury S*, et al.* (2002) Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. *Nat Genet* 31(3):239-240.

38. Wang K, Zhou B, Kuo YM, Zemansky J, Gitschier J (2002) A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. *Am J Hum Genet* 71(1):66-73.

39. Guigoz Y, Lauque S, Vellas BJ (2002) Identifying the elderly at risk for malnutrition - The Mini Nutritional Assessment. *Clinics in Geriatric Medicine* 18(4):737-+.

40. Makhov P*, et al.* (2008) Zinc chelation induces rapid depletion of the X-linked inhibitor of apoptosis and sensitizes prostate cancer cells to TRAIL-mediated apoptosis. *Cell Death and Differentiation* 15(11):1745-1751.

41. Porter R, Kaplan JL, Homeier BP (2008) *The Merck manual of patient symptoms* (Merck Research Laboratories, Whitehouse Station, N.J.) pp xx, 651 p.

42. Lu J, Combs GF, Jr. (1988) Effect of excess dietary zinc on pancreatic exocrine function in the chick. *J Nutr* 118(6):681-689.

43. Graham TW, Holmberg CA, Keen CL, Thurmond MC, Clegg MS (1988) A pathologic and toxicologic evaluation of veal calves fed large amounts of zinc. *Vet Pathol* 25(6):484-491.

44. Hu H, Bandell M, Petrus MJ, Zhu MX, Patapoutian A (2009) Zinc activates damage-sensing TRPA1 ion channels. *Nat Chem Biol* 5(3):183-190.

45. Kambe T, Suzuki T, Nagao M, Yamaguchi-Iwai Y (2006) Sequence similarity and functional relationship among eukaryotic ZIP and CDF transporters. *Genomics Proteomics Bioinformatics* 4(1):1-9.

46. Palmiter RD, Findley SD (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. *EMBO J* 14(4):639-649.

47. Palmiter RD, Cole TB, Findley SD (1996) ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. *EMBO J* 15(8):1784-1791.

48. Iguchi K*, et al.* (2002) High-level expression of zinc transporter-2 in the rat lateral and dorsal prostate. *J Androl* 23(6):819-824.

49. Liuzzi JP, Blanchard RK, Cousins RJ (2001) Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. *J Nutr* 131(1):46-52.

50. Kelleher SL, Lonnerdal B (2002) Zinc transporters in the rat mammary gland respond to marginal zinc and vitamin A intakes during lactation. *J Nutr* 132(11):3280-3285.

51. Liuzzi JP, Bobo JA, Lichten LA, Samuelson DA, Cousins RJ (2004) Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis. *Proc Natl Acad Sci U S A* 101(40):14355-14360.

52. Liuzzi JP, Bobo JA, Cui L, McMahon RJ, Cousins RJ (2003) Zinc transporters 1, 2 and 4 are differentially expressed and localized in rats during pregnancy and lactation. *J Nutr* 133(2):342-351.

53. Chowanadisai W, Lonnerdal B, Kelleher SL (2006) Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency. *J Biol Chem* 281(51):39699-39707.

54. Falcon-Perez JM, Dell'Angelica EC (2007) Zinc transporter 2 (SLC30A2) can suppress the vesicular zinc defect of adaptor protein 3-depleted fibroblasts by promoting zinc accumulation in lysosomes. *Exp Cell Res* 313(7):1473-1483.

55. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP (1985) An improved method for isolation of mouse pancreatic islets. *Transplantation* 40(4):437-438.

56. Fuller CM, Eckhardt L, Schulz I (1989) Ionic and osmotic dependence of secretion from permeabilised acini of the rat pancreas. *Pflugers Arch* 413(4):385-394.

57. Rindler MJ (2006) Isolation of zymogen granules from rat pancreas. *Curr Protoc Cell Biol* Chapter 3:Unit 3 18.

58. Haase H, Hebel S, Engelhardt G, Rink L (2006) Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells. *Anal Biochem* 352(2):222-230.

59. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 260(6):3440-3450.

60. Liuzzi JP, Guo L, Chang SM, Cousins RJ (2009) Kruppel-like factor 4 regulates adaptive expression of the zinc transporter Zip4 in mouse small intestine. *Am J Physiol Gastrointest Liver Physiol* 296(3):G517-523.

61. Ulrich AB, Schmied BM, Standop J, Schneider MB, Pour PM (2002) Pancreatic cell lines: a review. *Pancreas* 24(2):111-120.

62. Logsdon CD, Moessner J, Williams JA, Goldfine ID (1985) Glucocorticoids increase amylase mRNA levels, secretory organelles, and secretion in pancreatic acinar AR42J cells. *J Cell Biol* 100(4):1200-1208.

63. Bardon S, Vignon F, Chalbos D, Rochefort H (1985) RU486, a progestin and glucocorticoid antagonist, inhibits the growth of breast cancer cells via the progesterone receptor. *J Clin Endocrinol Metab* 60(4):692-697.

64. De Bosscher K*, et al.* (2005) A fully dissociated compound of plant origin for inflammatory gene repression. *Proc Natl Acad Sci U S A* 102(44):15827-15832.

65. Palade G (1975) Intracellular Aspects of the Process of Protein Synthesis. *Science* 189(4206):867.

66. Matsuno S, Miyashita E, Ejiri T, Sato T (1982) Zinc and magnesium output in pancreatic juice after pancreaticoduodenectomy. *Tohoku J Exp Med* 136(1):11-22.

67. Kato K, Isaji S, Kawarada Y, Hibasami H, Nakashima K (1997) Effect of zinc administration on pancreatic regeneration after 80% pancreatectomy. *Pancreas* 14(2):158-165.

68. Reinstein NH, Lonnerdal B, Keen CL, Schneeman BO, Hurley LS (1987) The effect of varying dietary zinc levels on the concentration and localization of zinc in rat bile-pancreatic fluid. *J Nutr* 117(6):1060-1066.

69. Sheline GE, Chaikoff IL, Jones HB, Montgomery ML (1943) Studies on the metabolism of zinc with the aid of its radioactive isotope II. The distribution of administered radioactive zinc in the tissues of mice and dogs. *Journal of Biological Chemistry* 149(1):139-151.

70. Montgomery ML, Sheline GE, Chaikoff IL (1941) Elimination of sodium in pancreatic juice as measured by radioactive sodium. *American Journal of Physiology* 131(3):0578-0583.

71. Birnstingl M, Stone B, Richards V (1956) Excretion of Radioactive Zinc Zn-65 in Bile, Pancreatic and Duodenal Secretions of the Dog. *American Journal of Physiology* 186(3):377-379.

72. McIsaac RJ (1955) The distribution of zinc-65 in the rat pancreas. *Endocrinology* 57(5):571-579.

73. Kazacos EA, Van Vleet JF (1989) Sequential ultrastructural changes of the pancreas in zinc toxicosis in ducklings. *Am J Pathol* 134(3):581-595.

74. Gabrielson KL, Remillard RL, Huso DL (1996) Zinc toxicity with pancreatic acinar necrosis in piglets receiving total parenteral nutrition. *Vet Pathol* 33(6):692-696.

75. Dufner-Beattie J, Kuo YM, Gitschier J, Andrews GK (2004) The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5. *J Biol Chem* 279(47):49082-49090.

76. Nadin CY, Rogers J, Tomlinson S, Edwardson JM (1989) A specific interaction in vitro between pancreatic zymogen granules and plasma membranes: stimulation by G-protein activators but not by Ca2+. *J Cell Biol* 109(6 Pt 1):2801-2808.

77. Antonin W, Wagner M, Riedel D, Brose N, Jahn R (2002) Loss of the zymogen granule protein syncollin affects pancreatic protein synthesis and transport but not secretion. *Mol Cell Biol* 22(5):1545-1554.

78. Martin SJ, Mazdai G, Strain JJ, Cotter TG, Hannigan BM (1991) Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. *Clin Exp Immunol* 83(2):338-343.

79. Krieger IE (1981) Acrodermatitis enteropathica and the relation to pellagra. *Med Hypotheses* 7(4):539-547.

80. Boosalis MG, Evans GW, McClain CJ (1983) Impaired handling of orally administered zinc in pancreatic insufficiency. *Am J Clin Nutr* 37(2):268-271.

81. Weaver BP, Dufner-Beattie J, Kambe T, Andrews GK (2007) Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zip5). *Biol Chem* 388(12):1301-1312.

82. Wang F, Kim BE, Petris MJ, Eide DJ (2004) The mammalian Zip5 protein is a zinc transporter that localizes to the basolateral surface of polarized cells. *J Biol Chem* 279(49):51433-51441.

83. Besecker B*, et al.* (2008) The human zinc transporter SLC39A8 (Zip8) is critical in zinc-mediated cytoprotection in lung epithelia. *Am J Physiol Lung Cell Mol Physiol* 294(6):L1127-1136.

84. Wollscheid B*, et al.* (2009) Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nat Biotechnol* 27(4):378-386.

85. Sullivan JF*, et al.* (1981) The zinc content of bile an pancreatic juice in zinc-deficient swine. *Proc Soc Exp Biol Med* 166(1):39-43.

86. Sheline GE, Chaikoff, I. L., Jones, H. B., & Montgomery, M. L. (1943) *J. Biol. Chem.* (149):139-151.

87. Montgomery ML, Sheline, G. E., & Chaikoff, I. L. (1943) *J. Exp. MEd.* (78):151-159.

88. Birnstingl M, Richards V, Stone B (1956) Excretion of radioactive zinc (Zn65) in bile, pancreatic and duodenal secretions of the dog. *Am J Physiol* 186(3):377-379.

89. Pekas JC (1971) Pancreatic incorporation of 65Zn and histidine-14C into secreted proteins of the pig. *Am J Physiol* 220(3):799-803.

90. Lichten LA, Liuzzi JP, Cousins RJ (2009) Interleukin-1beta contributes via nitric oxide to the upregulation and functional activity of the zinc transporter Zip14 (Slc39a14) in murine hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 296(4):G860-867.

91. Gorelick F, Jamieson F.S. (2006) Structure-Function Relations in the Pancreatic Acinar Cell. *Phsiology of the Gastrointestinal Tract*, ed Johnson L.R. B, K.E. (Elservier Academic Press, Burlington, MA), 4th Ed Vol 2, pp 1313-1335.

92. Raraty M*, et al.* (2000) Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. *Proc Natl Acad Sci U S A* 97(24):13126-13131.

93. De Lisle RC, Williams JA (1987) Zymogen granule acidity is not required for stimulated pancreatic protein secretion. *Am J Physiol* 253(6 Pt 1):G711-719.

94. Chimienti F, Devergnas S, Favier A, Seve M (2004) Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. *Diabetes* 53(9):2330-2337.

95. Wenzlau JM, Hutton JC, Davidson HW (2008) New antigenic targets in type 1 diabetes. *Curr Opin Endocrinol Diabetes Obes* 15(4):315-320.

96. Moore JB, Blanchard RK, McCormack WT, Cousins RJ (2001) cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. *J Nutr* 131(12):3189-3196.

97. Liu J*, et al.* (1999) Metallothionein-I/II knockout mice are sensitive to acetaminophen-induced hepatotoxicity. *J Pharmacol Exp Ther* 289(1):580-586.

98. Henkin RI, Foster DM, Aamodt RL, Berman M (1984) Zinc metabolism in adrenal cortical insufficiency: effects of carbohydrate-active steroids. *Metabolism* 33(6):491-501.

99. Etzel KR, Shapiro SG, Cousins RJ (1979) Regulation of liver metallothionein and plasma zinc by the glucocorticoid dexamethasone. *Biochem Biophys Res Commun* 89(4):1120-1126.

100. Logsdon CD (1986) Glucocorticoids increase cholecystokinin receptors and amylase secretion in pancreatic acinar AR42J cells. *J Biol Chem* 261(5):2096-2101.

101. Kaiser A, Stier U, Riecken EO, Rosewicz S (1996) Glucocorticoid receptor concentration modulates glucocorticoid-regulated gene expression in rat pancreatic AR42J cells. *Digestion* 57(3):149-160.

102. Grossman A (1984) An overview of pancreatic exocrine secretion. *Comp Biochem Physiol B* 78(1):1-13.

103. Lerner A, Lee PC, Lebenthal E (1984) Chemical adrenalectomy by aminoglutethimide and the pancreas in suckling rats. *Am J Physiol* 247(4 Pt 1):G346-351.

104. Manso M, De Dios I, Garcia A, Calvo JJ, Lopez MA (1988) Influence of glucocorticoids on the secretion of pancreatic juice in the rat. *Arch Int Physiol Biochim* 96(2):81-87.

105. Manso MA, De Dios I, Garcia AM, Calvo JJ (1988) Response of exocrine pancreatic secretion to CCK in adrenalectomized rats. *Peptides* 9(3):493-496.

106. Gullo L, Priori P, Labo G (1982) Influence of adrenal cortex on exocrine pancreatic function. *Gastroenterology* 83(1 Pt 1):92-96.

107. Bencosme SA, Lazarus SS (1956) The pancreas of cortisone-treated rabbits; pathogenic study. *AMA Arch Pathol* 62(4):285-295.

108. Wellmann KF, Volk BW (1976) Fine structure of pancreas in cortisone-treated guinea pigs and rabbits. *Arch Pathol Lab Med* 100(6):334-338.

109. Morisset J, Jolicoeur L, Genik P, Lord A (1981) Interaction of hydrocortisone and caerulein on pancreatic size and composition in the rat. *Am J Physiol* 241(1):G37-42.

110. Puccio F, Chariot J, Lehy T (1988) Influence of hydrocortisone on the development of pancreas in suckling rats. Ultrastructural morphometric and biochemical studies. *Biol Neonate* 54(1):35-44.

111. Puccio F, Lehy T (1988) Influence of hydrocortisone on the development of the gastric mucosa in suckling rats. Inhibitory effect on gastrin cell population. *Digestion* 40(3):157-167.

112. Leung YK, Jirapinyo P, Lebenthal E, Lee PC (1987) Effect of hydrocortisone on the maturation of cholecystokinin (CCK) binding and CCK stimulated amylase release in pancreatic acini of neonatal rats. *Pancreas* 2(1):73-78.

113. Majumdar AP, Nielsen H (1985) Influence of glucocorticoids on prenatal development of the gut and pancreas in rats. *Scand J Gastroenterol* 20(1):65-71.

114. Rall L, Pictet R, Githens S, Rutter WJ (1977) Glucocorticoids modulate the in vitro development of the embryonic rat pancreas. *J Cell Biol* 75(2 Pt 1):398-409.

115. McEvoy RC, Hegre OD, Lazarow A (1976) Foetal rat pancreas in organ culture. Effect of corticosterone concentrations on the acinar and islet cell components. *Differentiation* 6(1):17-26.

116. Christophe J (1994) Pancreatic tumoral cell line AR42J: an amphicrine model. *Am J Physiol* 266(6 Pt 1):G963-971.

117. Otsuki M, Okabayashi Y, Ohki A, Suehiro I, Baba S (1987) Dual effects of hydrocortisone on exocrine rat pancreas. *Gastroenterology* 93(6):1398-1403.

118. Gartemann H, Zeitz M, Emde C, Stelzer M, Riecken EO (1985) Three-day continuous drainage of pancreatic juice in the cortisone-treated conscious rat: analysis of enzyme activities and ultrastructural changes. *Cell Tissue Res* 242(1):191-196.

119. Otsuki M*, et al.* (1989) Hydrocortisone treatment increases the sensitivity and responsiveness to cholecystokinin in rat pancreas. *Am J Physiol* 257(3 Pt 1):G364-370.

120. Beaudoin AR*, et al.* (1986) Steroids and the secretory function of the exocrine pancreas. *Endocrinology* 119(5):2106-2117.

121. Kimura K*, et al.* (1998) Endogenous glucocorticoids decrease the acinar cell sensitivity to apoptosis during cerulein pancreatitis in rats. *Gastroenterology* 114(2):372-381.

122. Gesina E*, et al.* (2004) Dissecting the role of glucocorticoids on pancreas development. *Diabetes* 53(9):2322-2329.

123. Dougherty DC, Sanders MM (2005) Comparison of the responsiveness of the pGL3 and pGL4 luciferase reporter vectors to steroid hormones. *Biotechniques* 39(2):203-207.

124. Failla ML, Cousins RJ (1978) Zinc accumulation and metabolism in primary cultures of adult rat liver cells. Regulation by glucocorticoids. *Biochim Biophys Acta* 543(3):293-304.

125. Ma P, Liu Y, Reddy KP, Chan WK, Lam TJ (2004) Characterization of the seabass pancreatic alpha-amylase gene and promoter. *Gen Comp Endocrinol* 137(1):78-88.

126. Merkulov VM, Merkulova TI (2009) Structural variants of glucocorticoid receptor binding sites and different versions of positive glucocorticoid responsive elements: Analysis of GR-TRRD database. *J Steroid Biochem Mol Biol* 115(1-2):1-8.

127. Gross KL, Cidlowski JA (2008) Tissue-specific glucocorticoid action: a family affair. *Trends Endocrinol Metab* 19(9):331-339.

128. Stoecklin E, Wissler M, Moriggl R, Groner B (1997) Specific DNA binding of Stat5, but not of glucocorticoid receptor, is required for their functional cooperation in the regulation of gene transcription. *Mol Cell Biol* 17(11):6708-6716.

129. Lechner J*, et al.* (1997) Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of beta-casein gene transcription. *J Biol Chem* 272(33):20954-20960.

130. Qian L, Lopez V, Seo YA, Kelleher SL (2009) Prolactin regulates ZNT2 expression through the JAK2/STAT5 signaling pathway in mammary cells. *Am J Physiol Cell Physiol* 297(2):C369-377.

131. Muller J, Sperl B, Reindl W, Kiessling A, Berg T (2008) Discovery of chromone-based inhibitors of the transcription factor STAT5. *Chembiochem* 9(5):723-727.

132. Logsdon CD, Perot KJ, McDonald AR (1987) Mechanism of glucocorticoid-induced increase in pancreatic amylase gene transcription. *J Biol Chem* 262(32):15765-15769.

133. Zhang Y (2009) I-TASSER: fully automated protein structure prediction in CASP8. *Proteins* 77 Suppl 9:100-113.

134. Zhang Y (2008) I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40.

135. Gjorup I*, et al.* (1991) Pancreatic secretion of zinc and copper in normal subjects and in patients with chronic pancreatitis. *Digestion* 49(3):161-166.

136. Quilliot D*, et al.* (2001) Evidence that diabetes mellitus favors impaired metabolism of zinc, copper, and selenium in chronic pancreatitis. *Pancreas* 22(3):299-306.

137. Bojarski K, Dabrowski A, Wallner G, Maciejewski R (2002) Shift of zinc in serum and tissues in course of experimental acute pancreatitis. *Ann Univ Mariae Curie Sklodowska Med* 57(2):74-78.

138. Ferahman M*, et al.* (2003) Zinc and copper status in acute pancreatitis: an experimental study. *Biol Trace Elem Res* 91(1):89-94.

139. Liuzzi JP*, et al.* (2005) Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. *Proc Natl Acad Sci U S A* 102(19):6843-6848.

140. Williams RB, Russel RM, Dutta SK, Giovetti AC (1979) Alcoholic pancreatitis: patients at high risk of acute zinc deficiency. *Am J Med* 66(5):889-893.

141. Nagaoka Y*, et al.* (2002) [Malabsorption syndrome due to chronic pancreatitis resulting in severe zinc deficiency]. *Nippon Naika Gakkai Zasshi* 91(1):453-455.

142. Song MK, Adham NF (1989) Role of zinc in treatment of experimental acute pancreatitis in mice. *Dig Dis Sci* 34(12):1905-1910.

143. Wang ZH*, et al.* (1996) Increased pancreatic metallothionein and glutathione levels: protecting against cerulein- and taurocholate-induced acute pancreatitis in rats. *Pancreas* 13(2):173-183.

144. Fu K, Tomita T, Sarras MP, Jr., De Lisle RC, Andrews GK (1998) Metallothionein protects against cerulein-induced acute pancreatitis: analysis using transgenic mice. *Pancreas* 17(3):238-246.

145. Laychock SG, Duzen J, Simpkins CO (2000) Metallothionein induction in islets of Langerhans and insulinoma cells. *Mol Cell Endocrinol* 165(1-2):179-187.

146. Blanchard RK, Cousins RJ (2000) Regulation of intestinal gene expression by dietary zinc: induction of uroguanylin mRNA by zinc deficiency. *J Nutr* 130(5S Suppl):1393S-1398S.

147. Jemal A*, et al.* (2003) Cancer statistics, 2003. *CA Cancer J Clin* 53(1):5-26.

148. Li M*, et al.* (2009) Down-regulation of ZIP4 by RNA interference inhibits pancreatic cancer growth and increases the survival of nude mice with pancreatic cancer xenografts. *Clin Cancer Res* 15(19):5993-6001.

149. Li M*, et al.* (2007) Aberrant expression of zinc transporter ZIP4 (SLC39A4) significantly contributes to human pancreatic cancer pathogenesis and progression. *Proc Natl Acad Sci U S A* 104(47):18636-18641.

150. Taylor KM (2008) A distinct role in breast cancer for two LIV-1 family zinc transporters. *Biochem Soc Trans* 36(Pt 6):1247-1251.

151. Ding YF, Zhu C (2009) The role of microRNAs in copper and cadmium homeostasis. *Biochem Biophys Res Commun* 386(1):6-10.

152. Chiou TJ (2007) The role of microRNAs in sensing nutrient stress. *Plant Cell Environ* 30(3):323-332.

153. Liao Y, Lonnerdal B (2009) miR-584 mediates post-transcriptional expression of lactoferrin receptor in Caco-2 cells and in mouse small intestine during the perinatal period. *Int J Biochem Cell Biol*.

154. Vinkenborg JL*, et al.* (2009) Genetically encoded FRET sensors to monitor intracellular Zn2+ homeostasis. *Nat Methods* 6(10):737-740.

BIOGRAPHICAL SKETCH

Liang was born in 1981 in Hami, Xinjiang province, China. He received his Bachelor of Science in biological sciences at Fudan University (Shanghai, China) in 2004. He joined the graduate program in the Food Science and Human Nutrition Department at University of Florida in 2005. In 2009, the Nutritional Sciences doctoral program was developed and implemented by Dr. Robert J. Cousins. Liang completed his dissertation under Dr. Robert J. Cousins' supervision, and became the first Ph.D. graduate in the Nutritional Sciences doctoral program at the University of Florida.

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