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Zinc transporter expression profiles in the rat prostate following alterations in dietary zinc

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Abstract Zinc plays important roles in numerous cellular activities and physiological functions. Intracellular zinc levels are strictly maintained by zinc homeostatic mechanisms. Zinc concentrations in the prostate are the highest of all soft tissues and could be important for prostate health. However, the mechanisms by which the prostate maintains high zinc levels are still unclear. In addition, the response of the prostate to alterations in dietary zinc is unknown. The current study explored cellular zinc levels and zinc transporter expression profiles in the lobes of the prostate during dietary marginal zinc depletion. Rats were given either zinc-adequate (ZA, 30 mg Zn/kg) or marginal zinc-deficient (MZD, 5 mg Zn/kg) diet for 9 weeks. In addition, a subgroup of the MZD rats was supplemented with phytase (1,500 unit/kg diet) to improve zinc bioavailability. We found that both zinc concentrations and ZnT2 expression in the prostate dorsolateral lobes were substantially higher than in the ventral lobes ($P < 0.05$). Marginal zinc depletion significantly decreased ZnT2 expression in the dorsolateral lobes ($P < 0.05$), and phytase supplementation had a trend to increase ZnT2 expression. In addition, of all measured zinc transporters, only ZnT2 mRNA abundance was significantly correlated to the zinc concentrations in the dorsolateral lobe. No correlations were found between zinc transporter expression and zinc concentrations in the ventral lobes. These results indicate that ZnT2 may play a significant role in the maintenance of zinc homeostasis in the prostate.

Keywords Zinc transporter · ZnT2 · Prostate · Marginal zinc deficiency

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Introduction

Zinc is an essential trace mineral and is ubiquitously distributed in hundreds of proteins in mammalian cells. Since zinc is widely involved in the normal functions of many proteins, zinc is required for a variety of biological activities such as growth and development, immune response, wound healing, neurological function and reproduction. Intracellular zinc levels are strictly controlled by zinc homeostatic mechanisms that maintain stable zinc supply and appropriate zinc distribution in cells. The activities of zinc uptake, efflux and cellular compartmentalization rely on the functions of zinc transporters. Two zinc

transporter gene families have been identified in mammals: the solute-linked carrier 30A (SLC30A, ZnT) family and the SLC39A (Zip) family of metal ion transporters. The ZnT family of transporters functions in zinc efflux from the cytoplasm to either the extracellular space and/or intracellular organelles. The ZIP family of protein functions in zinc uptake from the extracellular matrix and/or intracellular organelles into the cytoplasm (Lichten and Cousins 2009). These zinc transporters are expressed in a tissue-specific manner, and respond differentially to dietary zinc levels and physiological conditions. Therefore, a loss of function or dysregulation of certain zinc transporters would result in an impairment of zinc homeostasis and predispose the body to zinc-imbalance-related diseases, such as cancer, asthma, diabetes, and Alzheimer's disease (Devirgiliis et al. 2007). For example, mutations of Zip4 gene have been identified as the cause of human acrodermatitis enteropathica, an autosomal recessively inherited disease (Kury et al. 2002), and polymorphisms in ZnT8 have been associated with type 2 diabetes (Sladek et al. 2007).

The maintenance of zinc homeostasis in the prostate may be even more critical than the other organs. The prostate contains the highest zinc concentration of any other soft tissues in the body. The prostate epithelial cells have a characteristic high aerobic glycolytic activity, low respiration (Muntzing et al. 1975; Nyden and Williams-Ashman 1953) and high citrate secretion. Zinc may be required for maintaining these properties by reducing the activity of mitochondrial aconitase and inhibiting the terminal oxidation through the electron transport chain (Costello et al. 1997, 2004). Moreover, as the prostate becomes malignant, prostate zinc concentration decreases by 75–90% (Costello and Franklin 2006). Therefore, it has been proposed that maintaining the high zinc content is essential for prostate health, and loss of ability to accumulate high zinc levels may contribute to the prostate malignancies (Costello et al. 1999; Franklin et al. 2005; Huang et al. 2006; Iguchi et al. 2004; Rishi et al. 2003). However, little is known regarding mechanisms by which the normal prostate maintains high levels of zinc. Therefore, the objectives of the current study were to examine the mechanisms by which the prostate modulates zinc concentrations and profile zinc transporter expression during dietary marginal zinc depletion.

In addition, since the prostate lobes differ markedly in zinc concentration, function and embryological origin (Costello and Franklin 1998; Iguchi et al. 2002), we compared the zinc transporter expression between dorsolateral and ventral lobes. Finally, since phytase supplement may improve overall zinc status in rats (McClung et al. 2006; Rimbach and Pallauf 1993), we added phytase to the marginally zinc-deficient diet to examine whether phytase supplementation to a low-zinc diet could increase zinc levels and upregulate zinc transporter expression in the prostate.

The current study is one of the first to quantitatively evaluate the correlations between zinc transporter expressions and zinc concentration in the prostate with dietary zinc depletion. The results from this study provide insights into the mechanisms by which the prostate regulates cellular zinc levels and provide directions for future studies establishing connections between defects in zinc transporter regulation and the development of prostate diseases.

Materials and methods

Animals

Male Sprague-Dawley rats ($n = 24$, 4 weeks old, 125–150 g) from Charles River Laboratories, Inc. (Wilmington, MA), were housed individually in polycarbonate cages and acclimated for 2 weeks to the temperature- and humidity-controlled environment with a 12-h-dark: light cycle. Rats were fed a zinc-adequate (ZA, 30 mg Zn/kg) or marginal zinc-deficient (MZD, 5–6 mg Zn/kg) diet for 9 weeks. To further examine the effects of phytase on zinc status and DNA integrity in the prostate, 1,500 phytase units/kg was added to the MZD diets and fed to the rats (MZD + P). Diets were based on modified AIN-93 M diets (Reeves 1997) formulated with egg white and with zinc provided as zinc carbonate (Research Diets Inc). All diet also contained 0.3% phytic acid (Naphytate, Sigma–Aldrich). Deionized water was provided as drinking water. Rats were killed following anesthesia with isoflurane overdose (1–5%; Henry Schein). 24-h dietary intakes and body weights were measured weekly. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at USARIEM and Oregon State University,

and animals were maintained in accordance with IACUC's guidelines for the care and use of laboratory animals.

Tissue and blood collection

Blood samples were collected by cardiac puncture into trace element-free vials containing EDTA. Plasma was separated immediately and frozen at −80°C until analysis. For the dissection of rat prostates, the genitourinary complex including the prostate, bladder, seminal vesicles and coagulant glands were removed as a unit. Then the ventral and dorsolateral prostate lobes were detached from the urinary bladder. The urinary bladder and seminal vesicles with coagulation glands were removed. The two ventral lobes were separated from each other and removed from the dorsolateral section. Samples of rat prostate dorsolateral and ventral lobes were immediately either snap frozen at −80°C or stored in RNA stabilization solution (RNAlater, Ambion) until further analysis.

Zinc analysis

Plasma ($n = 16$), ventral lobe ($n = 12$) and dorsolateral lobe ($n = 7$) zinc concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Teledyne Leeman Labs) with small modification of a previous described method (Verbanac et al. 1997). Samples were digested in 69–70% OmniTrace nitric acid (VWR) overnight. Following digestion, samples were diluted 10 times with water treated with chelex 100 resin (Bio-Rad Laboratories) and analyzed by ICP-OES against known standards (Bruno et al. 2007).

Quantitative real-time PCR analysis (qRT-PCR)

The mRNA abundance of zinc transporters in rat prostates (ZnT1-4 and Zip1-4) were measured by qRT-PCR. RNA was extracted from prostates using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). First-strand cDNA was reverse transcribed from the isolated RNA using Superscript First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen Corporation). Primers and annealing temperatures of each transporter gene are listed in Table 1. Primers were designed by online software Primer3 (v.0.4.0), and the specificity was verified by melting curve and DNA gel. Real time PCR reactions were performed using DyNAmo HS SYBR Green qPCR kit (New England Biolabs). Gene copies were determined using the standard curve which was generated from serial dilutions of purified plasmid DNA that encoded for the gene of interest. Data represent averaged copy number normalized to the 18s housekeeping gene.

Western analysis of ZnT2 protein

The frozen portions of prostate were placed in the standard immunopreciptation assay lysis buffer, homogenized, centrifuged (15,000 rpm, 5 min, 4°C), and the supernatant was collected for analysis. Protein concentrations were determined using DC Protein Assay (Bio-Rad Laboratories). Proteins (30 μg/lane) were separated by SDS–PAGE on a 4–12% bis–Tris gel (Invitrogen Corporation) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Equal protein loading were confirmed with β-actin levels. After blocking the membrane, antigens were probed using rabbit anti-ZnT2 (1:1000 dilution with 5% milk in phosphate buffered saline/0.05% Tween 20 (PBST), provided by Dr. Kelleher, Penn State University) Bound antibodies were detected using goat anti-rabbit IgG-HRP (1:30,000 dilution with 5% milk in PBST Santa Cruz Biotechnology), and developed with SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL). Images were acquired using an Alpha Innotech photodocumentation system (Alpha Innotech, Hayward, CA) and analyzed using Image J 1.37v software (NIH, Bethesda, MD).

Statistical analysis

Statistical analysis was performed with the use of PRISM (version 4.0; GraphPad Software). Significantly differences between means were analyzed by one-way ANOVA followed by Bonferroni post hoc test when appropriate. Pearson correlation coefficients were calculated between zinc transporter expression and zinc concentrations. Logarithm data transformations were taken when uneven variations were detected. Differences were considered statistically significant at $P < 0.05$. All data are reported as means ± SEM (standard error of the mean) unless otherwise indicated.

Table 1 Primers of genes analyzed by qRT-PCR

Gene name	Primers	Sequence (5′ → 3′)	Annealing temperature	Amplicon size (bp)
ZnT1	Forward	CACGCTAGTGGCTAACACCA	60	296
	Reverse	AGGAAAACACGGGTTCACAC		
ZnT2	Forward	TGCTCGTGTACCTGGCTGTA	60	138
	Reverse	TCCATGTCCAGACTGATGGA		
ZnT3	Forward	GCAGAGTATGCACCACTGGA	60	202
	Reverse	CAAGGGCGCAGATAGAGAAG		
ZnT4	Forward	CCTTTGGATTTCATCGCCTA	60	144
	Reverse	GTTCTCTGCACAGCCTCGTA		
Zip1	Forward	AAGCCTAGTGAGCTGCTTCG	58	148
	Reverse	ATGGCCAGGATGAACTCTTG		
Zip2	Forward	TTCAGAAATTCGTGGTGCAG	58	140
	Reverse	GCGACTCCAAAAGGAAGACA		
Zip3	Forward	CGTCTTCCTGGCTACATGCT	58	148
	Reverse	TCCACGAACACAGTGAGGAA		
Zip4	Forward	ATGAGCTGCCTCACGAACTT	58	130
	Reverse	CTGCTAGAGCCACGTAGAGG		
18s	Forward	GGACCAGAGCGAAAGCATTTGC	60	115
	Reverse	CGCCAGTCGGCATCGTTTATG		

Results

There was no change in food intake among dietary groups (data not shown, $P > 0.05$). At the end of the study, the body weights in the MZD group (312.8 ± 5.3 g) were significantly lower than in the ZA group (368.7 ± 6.8 g, $P < 0.001$). Phytase supplementation significantly increased body weights in rats fed the MZD diet (346.2 ± 5.5 g, $P < 0.05$).

Zinc concentrations in the MZD group decreased 44% in plasma and 68% in the prostate dorsolateral lobes compared to the ZA rats (Table 2, $P < 0.05$). In contrast, zinc concentrations in prostate ventral lobes were not affected by marginal zinc depletion, indicating that prostate dorsolateral lobes were more responsive to marginal zinc depletion. In addition, phytase supplementation significantly increased plasma and dorsolateral lobe zinc concentrations (Table 2, $P < 0.05$), indicating that phytase facilitated improved zinc status in the rats fed the MZD diet.

Prostate dorsolateral lobes contain higher zinc concentrations than the ventral lobes, which were also observed in the current study. Zinc transporter mRNA expression profiles of the ZA rats were compared between the dorsolateral and ventral lobes. The mRNA levels of ZnT3, Zip2 and Zip4 in both lobes were very low in comparison with the other

Table 2 Zinc concentrations in the plasma and prostates of the rats fed the MZD, MZD +P or ZA diet

	MZD	MZD + P	ZA	*P*-value
Plasma (mg/l)	0.56 ± 0.03[a]	0.90 ± 0.06[b]	1.15 ± 0.05[c]	<0.0001
Prostate				
Ventral lobe (mg/kg)	11.92 ± 0.80[a]	14.39 ± 0.91[a]	12.49 ± 0.70[a]	0.09
Dorsolateral lobe (mg/kg)	66.06 ± 1.48[a]	169.82 ± 1.58[b]	204.17 ± 1.26[b]	<0.0001

Values are means ± SEM. Main effects were determined by one-way ANOVA with Bonferroni's post hoc test. Means without a common letter differ, $P < 0.05$

transporters (Fig. 1). No significant differences in ZnT1, ZnT4, Zip1 and Zip3 mRNA levels were detected between the dorsolateral and ventral lobes. However, ZnT2 gene expression levels were markedly different between the dorsolateral and ventral lobes. The mRNA abundance of ZnT2 in the dorsolateral lobes was about 1,000 times higher than that in the ventral lobes ($P < 0.05$), indicating that ZnT2 may play an important role in accumulating very high levels of zinc in rat prostate dorsolateral lobes.

Correlations between zinc concentrations and zinc transporter mRNA levels in the dorsolateral lobes of all rats in all dietary groups were analyzed by Pearson analysis. We found that only ZnT2 mRNA levels were significantly correlated to the alterations of zinc concentrations in the dorsolateral lobes ($P = 0.034$, Table 3). We evaluated the effects of variable dietary zinc intake on ZnT2 expression, and found that marginal zinc depletion significantly decreased ZnT2 mRNA levels specifically in the prostate dorsolateral lobes (Fig. 2, $P < 0.05$) and phytase supplementation partially restored ZnT2 mRNA levels. However, ZnT2 protein levels in the lysate of prostate dorsolateral lobes were not significantly altered by diet treatments (Fig. 3a, b). No effects on ZnT2 or other zinc transporters were detected in the ventral lobes. Altogether, these results indicate that ZnT2 may play an important role in maintaining dorsolateral lobe zinc homeostasis during dietary zinc depletion.

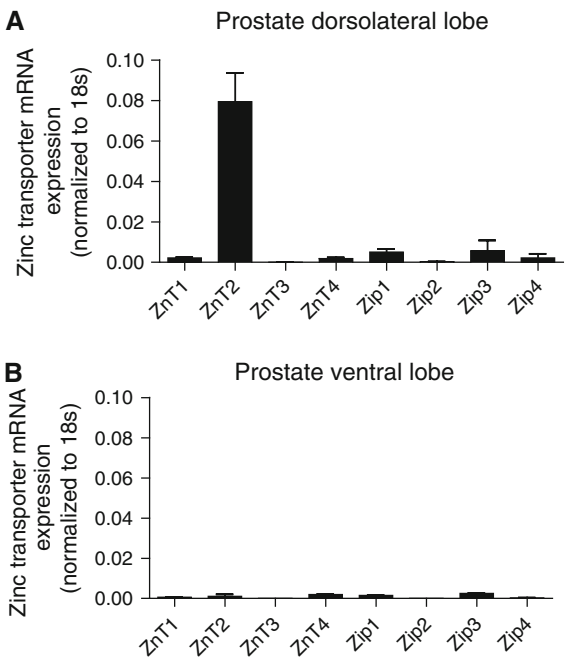

Fig. 1 Zinc transporter mRNA abundance profiles in the **a** prostate dorsolateral and **b** ventral lobes. Sprague-Dawley rats were fed a ZA diet for 9 weeks. mRNA levels were measured as described in "Material and methods". Results are transcripts copy number normalized to 18s transcripts. Values are means ± SEM ($n = 12$)

Table 3 Correlations between zinc transporter mRNA levels and zinc concentrations in the dorsolateral lobes

	Correlation coefficient	*P*-value
ZnT1	0.04	0.81
ZnT2	0.38*	0.034
ZnT3	−0.20	0.28
ZnT4	−0.18	0.32
Zip1	0.24	0.18
Zip2	0.35	0.053
Zip3	−0.12	0.50
Zip4	−0.14	0.45

Sprague–Dawley rats were fed a MZD, MZD + P or ZA diet for 9 weeks. Zinc concentrations were measured by ICP-OES and ZnT2 mRNA levels were measured by qRT-PCR as described in "Material and methods"

Pearson analysis was performed to analyze the correlation between dorsolateral prostate zinc concentrations and zinc transporter mRNA transcript levels, $n = 32$

* $P < 0.05$

Discussion

The regulation of ZnT2 may be particularly important in tissues with high zinc requirements such as the prostate glands. The current study shows that the dorsolateral lobes had higher ZnT2 mRNA levels as well as higher zinc concentrations than the ventral lobes of the prostate. ZnT2 mRNA levels in the dorsolateral lobes were also significantly correlated to dorsolateral zinc concentrations, and ZnT2 decreased with marginal zinc deficiency. These results indicate that ZnT2 may play an essential role in modulating prostate zinc homeostasis. Altogether, the current study gives insights for understanding the mechanisms in place that maintain prostate zinc homeostasis, and helps identify zinc transporters that may play roles in zinc regulation.

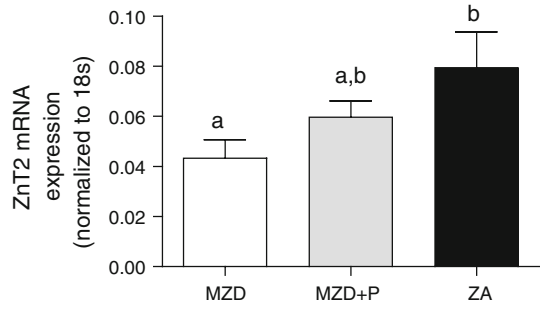

Fig. 2 Effects of dietary marginal zinc depletion on ZnT2 mRNA abundance in the prostate dorsolateral lobes. Sprague-Dawley rats were fed a MZD, ZA or MZD + P diet for 9 weeks. mRNA levels were measured as described in "Material and methods". Results are transcripts copy numbers normalized to 18s transcripts. Values are means ± SEM ($n = 12$). Difference between means is determined by one-way ANOVA followed by Bonferroni's post hoc test. Means without a common letter differ ($P < 0.05$)

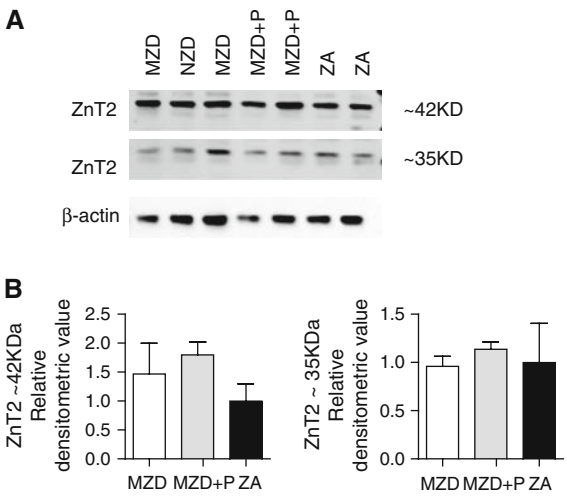

Fig. 3 Effects of dietary marginal zinc depletion on ZnT2 protein levels in the whole tissue lysate of prostate dorsolateral lobes. Sprague-Dawley rats were fed a MZD, ZA or MZD + P diet for 9 weeks. **a** ZnT2 protein levels are shown in 3 representative samples from each dietary group. ZnT2 isoforms with sizes of ~42 and ~35 kDa are shown in the inserts. **b** Relative abundance of each protein was determined by densitometry analysis

The prostate contains high levels of zinc compared to many other soft tissues, although the precise function of high cellular zinc in this tissue remains unclear. Zinc may be required for inhibition of terminal oxidation and the activity of mitochondria aconitase in the prostate. (Costello et al. 2004; Ho 2004; Muntzing et al. 1975). Therefore, a loss of zinc in the prostate may remove the inhibitory effects of zinc on mitochondrial terminal oxidation, and increase respiration rates and possibly increasing the production of free radicals by the electron transport chain. (Franklin et al. 2005). In our laboratory, we have also postulated an important function of zinc in the prostate may be maintaining DNA integrity by reducing oxidative stress and preserving DNA repair functions (Yan et al. 2008). We found that loss of cellular zinc levels in the prostate epithelial cells resulted in oxidative stress and increased DNA damage.

Since high zinc concentrations may be essential for prostate health, a failure to maintain zinc homeostasis may increase the risk of prostate cancer. Dysregulation of zinc transporters including ZnT1, ZnT3, ZnT4, Zip1, Zip2 and Zip3 have been found to be associated with the low intracellular zinc content and tumorgenicity in human prostate cancer tissues or prostate epithelial cancer cell lines (Franklin et al. 2005; Iguchi 2004; Beck et al. 2004). To date, no mutations in ZnT2 have been identified in the prostate, however, ZnT2 mutations have been identified in mammary tissues, a tissue that has similar secretory functions and high zinc requirement (Ryu et al. 2008). These previous studies provide important preliminary evidence for the potential role of specific zinc transporters in prostate cancer progression.

The primary function of ZnT2 is to transport zinc from the cytoplasm into vesicles such as endosomes or lysosomes (Jou et al. 2009; Liuzzi et al. 2003). In general, ZnT2 is not ubiquitously expressed among all tissues. Its expression is restricted to tissues with unique zinc requirements such as small intestine, kidney, retina, placenta, pancreas, mammary gland, testis, seminal vesicles, and prostate (Lichten and Cousins 2009). ZnT2 is upregulated in the small intestine during high dietary zinc intake, and during late-stage gestation and early lactation in both maternal and fetal tissues (Liuzzi et al. 2003). In mammary and prostate tissue, hormones such as prolactin also increases ZnT2 expression (Costello et al. 1999; Qian et al. 2009). Zinc transporters such as ZnT5 have alternative isoforms as a result of alternative splicing events in response to zinc and the proteins are differentially localized (Jackson et al. 2007). Recently, using in silico analysis, two distinct isoforms of ZnT2 have been identified, a ~42 kDa

protein (NM_032513) and a shorter ~35 kDa protein (NM_001004434) that result from alternative splicing of exon 3. Both isoforms appear to function as zinc transporters, but have differing subcellular localization patterns (Lopez and Kelleher 2009). In mammary tissue, the larger 42 kDa isoform localizes to secretory vesicles and predominates in expression level over the 35 kDa isoform that localizes to plasma membrane (Gyorkey et al. 1967). Thus, the long 42 kDa isoform appears to function to transport zinc into the secretory compartment for zinc efflux in a hormone-dependent manner. In the current study, we found that similar to mammary tissue, the larger isoform appears to predominate in the prostate. Thus, it is possible that a decrease in ZnT2 expression with marginal zinc deficiency in the prostate may reduce the amount of zinc transported into endosomes or secretory vesicles. In that case, zinc may be preserved for other cell compartments, such as the mitochondria and nucleus, and may be more available for activities more essential for prostate cell function and survival. Although we detected alterations in RNA expression in the prostate with zinc deficiency, we surprisingly did not see corresponding decreases in protein expression of ZnT2. However, a major limitation of this analysis was that we were restricted to the evaluation of ZnT2 in whole tissue lysates, rather than membrane or subcellular fractions. Given the importance of ZnT2 localization, further studies to examine alterations in specific subcellular fractions or use of in situ immunohistochemistry methods are necessary to confirm changes protein alterations in specific cellular locations are apparent. For example, in the mammary gland, ZnT2 expression remains constant at the basolateral membrane, and only changes at the apical membrane are detectable during lactation (Kelleher and Lonnerdal 2003). Further, it is possible that other alternative splice variants are present in the prostate that have not been identified. In the rat small intestine, ZnT2 has a lower molecular mass protein (~28 kDa) that is localized to the apical membrane. Thus, an important direction of future research would be to identify the ZnT2 localization and function in the prostate. To understand zinc homeostatic mechanisms in the prostate, studies should focus on the redistribution of subcellular zinc levels during zinc depletion and identify cellular compartments which are most resistant to zinc depletion.

Although zinc concentrations are high in the whole prostate, zinc is not uniformly distributed among all prostate lobes. The human prostates are composed of the peripheral zone (70%), the central zone (25%) and the transition zone (5%). The peripheral zone contains much higher zinc and citrate concentrations than the other zones, and 80% of prostate malignancies develop in the peripheral zone (Costello and Franklin 1998). Therefore, the differential functional and metabolic properties of prostate epithelial cells in the peripheral zone may determine their high potential for malignant transformation. It has been reported that the dorsolateral lobe of the rat prostate is embryologically homologous to the peripheral zone of the human prostate (Iguchi et al. 2002). The dorsolateral lobe also retains higher levels of zinc compared to the ventral lobe (Iguchi et al. 2002). However, the mechanisms for the uneven distribution of zinc within the prostate are unknown. A previous study done by Iguchi et al. detected higher expression of ZnT2 in the dorsal and lateral lobes of rat prostates than in ventral lobes by using semiquantitative RT-PCR. The current study quantitatively assessed that ZnT2 transporter expression was about 1,000 times higher in the dorsolateral lobes than in the ventral lobes where ZnT2 mRNA levels were very low. These findings suggest that ZnT2 may play a role in accumulating high concentration of zinc in prostate dorsolateral lobes and likely important in secretory functions. However, more studies are needed to address the mechanism and significance of this lobe-specific expression of ZnT2.

In summary, the current study suggests that ZnT2 may play an important role in regulating zinc homeostasis and maintaining high zinc levels in the prostate dorsolateral lobes. The current study also provides evidence and directions for future research to explore the possible interactions among prostate cancer, zinc status, and defects in zinc transporter families.

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