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Zinc transporter mRNA expression in the RWPE-1 human prostate epithelial cell line

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Abstract The human prostate gland undergoes a prominent alteration in Zn^{+2} homeostasis during the development of prostate cancer. The goal of the present study was to determine if the immortalized human prostate cell line (RWPE-1) could serve as a model system to study the role of zinc in prostate cancer. The study examined the expression of mRNA for 19 members of the zinc transporter gene family in normal prostate tissue, the prostate RWPE-1 cell line, and the LNCaP, DU−145 and PC−3 prostate cancer cell lines. The study demonstrated that the expression of the 19 zinc transporters was similar between the RWPE-1 cell line and the in situ prostate gland. Of the 19 zinc transporters, only 5 had levels that were different between the RWPE-1 cells and the tissue samples; all five being increased (ZnT-6, Zip-1, Zip-3A, Zip-10, and Zip-14). The response of the 19 transporters was also determined when the cell lines were exposed to 75 μM Zn^{+2} for 24 h. It was shown for the RWPE-1 cells that only 5 transporters responded to Zn^{+2} with mRNA for ZnT-1 and ZnT-2 being increased while mRNA for ZnT-7, Zip-7 and Zip-10 transporters were decreased. It was shown for the LNCaP, DU-145 and PC-3 cells that Zn^{+2} had no effect on the mRNA levels of all 19 transporters except for an induction of ZnT-1 in PC-3 cells. Overall, the study suggests that the RWPE-1 cells could be a valuable model for the study of the zinc transporter gene family in the prostate.

Keywords Prostate · Zinc · Cancer · RWPE-1

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Introduction

The human prostate gland undergoes a prominent alteration in Zn^{+2} homeostasis during the development of prostate cancer. The normal prostate gland has a greatly elevated concentration of Zn^{+2} compared to any other organ or cell in the human body, and in prostate cancer, this level of Zn^{+2} is reduced dramatically (Naide 2002). In contrast to the prostate, most cells have low concentrations of intracellular zinc, and the zinc that is present is sequestered by binding to proteins such as MT (Vallee and Falchuk 1993). However, in the case of the prostate gland

there is a considerable amount of free zinc in the cytosol, much of it bound to citrate (Kavanagh 1983; Larue and Morfin 1984a, b). It has been proposed that the high levels of zinc in the prostate stimulates citrate production within the glandular secretory epithelium by inhibition of mitochondrial m-aconitase (Costello et al. 1997, 2004). As a result of this inability to metabolize citrate, citrate is found in prostate secretions at a concentration of about 20 mM, and cellular levels are about 100 times greater within the acinar cells as that normally seen in any other soft tissue of the body (Partin and Rodriguez 2002). Thus, the normal prostate epithelial cell has a highly elevated concentration of Zn^{+2} compared to other cell types of the human body. A consistent finding in prostate cancer is that Zn^{+2} homeostasis appears to undergo a drastic alteration. The total cellular content of Zn^{+2} is dramatically reduced; no longer is zinc secreted, nor is there any production of citrate, and mitochondrial zinc is significantly reduced (Costello et al. 1997). No prostate tumors have been found that maintain zinc and citrate levels, and this alteration is also a very early event in prostate tumorigenesis (Habib et al. 1979). It has been suggested that this reduction in zinc and decreased production of citrate is the most consistent and persistent biochemical characteristic of prostate cancer (Costello et al. 2004).

Cells and tissues control the levels of Zn^{+2} within cells and between subcellular compartments through the deployment of a large number of transmembrane transporter proteins. Many of the genes for these transporters have been identified, functionally characterized and compose two large families based on sequence criteria as well as transport function (Eide et al. 2006; Liuzzi and Cousins et al. 2004). The ZnT Zn^{+2} transporter class also know as SLC30A (Solute-Linked Carrier 30A) or CDF (Cation Diffusion Facilitator) is comprised of members that exhibit six transmembrane (TM) domains that function to decrease cytosolic Zn^{+2} by plasma membrane efflux or the transport of Zn^{+2} into membrane bound subcellular compartments. The Zip class of Zn^{+2} transporters consists of members that exhibit eight TM domains and increase cytosolic Zn^{+2} by either uptake through the plasma membrane or the transport out of membrane bound organelles. The balance of the activities of these two classes of transporters regulates the level of Zn^{+2} within cells as well as within various subcellular compartments. Furthermore, tissue specific regulation of zinc transport is thought to occur through tissue specific expression of many of these transporters. Two prominent examples are Zip-4, the mutation of which is the molecular alteration causing acrodermatitis enteropathica [a defect in intestinal uptake of zinc (Wang et al. 2002)] and ZnT-4 the molecular alteration in the lethal milk mutant mouse [a defect of zinc transport into breast milk (Huang et al. 1997)]. The complement of zinc transporters that enable the prostate gland to accumulate zinc is just beginning to be defined.

Whereas many of the prostate transporters have been identified, it remains to be determined what members define the high zinc accumulation phenotype. This is mainly due to the lack of information on the subcellular distribution and transport function of several members of these families. Nevertheless, a comparison of transporter expression between normal and tumorigenic prostate cells may help define critical zinc transport determinants essential to prostate physiology.

This laboratory has been exploring the use of the immortalized human prostate cell line, RWPE-1, as a potential model for studying the alterations that Cd^{+2} might elicit on Zn^{+2} homeostasis in the prostate epithelial cell. In a previous study it was shown that the RWPE-1 cell line has the same profile of metallothionein isoform 1 and 2 (MT-1/2) expression as the normal human prostate gland (Albrecht et al. 2007). It was also shown that MT-1/2 protein, a major Zn^{+2}-binding protein of the cell, is induced upon exposure of the RWPE-1 cells to either Cd^{+2} or Zn^{+2}. The present study expands this analysis to include the expression of mRNA for selected members of the Zn^{+2} transport protein family in this cell line. The first goal was to compare expression of selected Zn^{+2} transporters between the RWPE-1 cell line, the in situ prostate gland, and commonly used prostate cancer cell lines. The second goal was to determine if Zn^{+2} was able to induce accumulation of Zn^{+2} transporter mRNAs in these cell lines.

Materials and methods

Cell culture

The RWPE-1, LNCaP, DU-145, and PC-3 cell lines were obtained from the American Type Culture Collection (ATCC). The RWPE-1 cells were grown

in keratinocyte serum free medium (K-SFM) containing 50 μg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor as described previously (Albrecht et al. 2007). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were fed fresh growth medium every 3 days and at confluence the cells were subcultured at a 1:4 ratio using trypsin-EDTA (0.05%, 0.02%). The LNCaP, DU-145, and PC-3 cell lines were grown in Dulbecco's Modified Eagles' medium (DMEM) supplemented with 10% (v/v) fetal calf serum, and routinely passaged at a 1:4 ratio upon attaining confluence. The cells were used in experimental protocols at confluent density 24 h after being fed with fresh growth medium. To determine the effect of Zn^{+2} on transporter mRNA expression and intracellular Zn^{+2} levels, the cells were exposed to 75 μM Zn^{+2} for 24 h.

Primer design and amplification standards

The measurement of individual zinc transporter specific mRNA expression was assessed with real-time RT-PCR utilizing either primers previously reported in the literature (Beck et al. 2004; Devergnas et al. 2004) or those designed with primer design software (Oligo 6.61, Molecular Biology Insights Inc Cascade, CO). The primer sequences are shown in Table 1. The reference sequence for each gene transcript (RefSeq RNA) was obtained from the NCBI website (http://www.ncbi.nlm.nih.gov). In each case, primer pairs were designed such that each primer pair flanked at least one exon–intron boundary. Each primer pair was used to initially amplify a PCR product from prostate cell lines or tissues, testing a range of primer annealing temperatures by utilizing the gradient function of the iCycler PCR machine. PCR product size was verified on 2% agarose gels and correlated to its melting point temperature (T_m) which was measured by post-run melt curve analysis. Two transporters failed to amplify at all annealing temperatures tested (ZnT-8 and ZIP-12). The amplification of each isoform was then scaled up, and the DNA product purified with the Geneclean Turbo kit (Q-BioGene, Irvine, CA) and quantified by spectrophotometry. Each gene amplimer was then diluted for use in producing standard curves for each transporter gene.

PCR reaction conditions and data analysis

Total RNA was purified from cultures of each prostate cell line (RWPE-1, DU-145, LNCaP, and PC-3) and RNA from human prostate tissue was used from a previous study (Garrett et al. 1999). All RNA exhibited similar expression levels of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and metallothionein-2A, indicating that very little degradation had occurred in storage from the previous study. Purified RNA (1 μg) was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules CA) in a total volume of 20 μl. Real-time PCR was performed utilizing the SYBR Green kit (Bio-Rad Laboratories) with 2 μl of cDNA, 0.2 μM primers in a total volume of 20 μl in an iCycler iQ real-time detection system (Bio-Rad Laboratories). Amplification was monitored by SYBR Green fluorescence and compared to that of a standard curve generated from each gene specific amplimer. Correct amplification was monitored by post-run melt curve analysis. Cycling parameters consisted of denaturation at 95°C for 30 s, annealing (temperature specified in Table 1) for 45 s, and extension at 72°C for 30 s. Annealing temperatures were chosen that gave greater than 90% amplification efficiency, and correct amplification was monitored by post-run melt curve analysis. The level of expression of each transporter was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) assessed by the same assay. Levels of expression are calculated as the number of transporter gene transcripts per transcript of G3PDH × 1000. Since G3PDH is a highly expressed gene (estimated to be between 1,000 and 10,000 transcripts per cell) the final ratio is multiplied by 1000 to bring the final ratio closer to 1.0. The limit of detection for the assay is estimated to be 0.00001 gene transcripts per transcript of G3PDH (or 0.01in the final reported number). This represents about 300 transcripts per reaction tube, a level that was often near the overall false positive contamination level for the assay. It is also estimated that this level of expression is about the level of one transcript per 10 cells based on estimates of RNA yield. Since cell numbers were not actually counted, this limit-of-detection benchmark, expressed in transcripts per cell, is only semi-quantitative.

Table 1 PCR primers

Transporter	Primer sequence	Annealing temperature (°C)	Product size (bp)	Reference
ZnT-1	CCTGGGCTTCTTCTCTAGATTG	58	634	Beck (2004)
	TTGTCTTGGAAAGGTTGTTCTG			
ZnT-2	ATGTGATCCTGGTGTTGATG	56	68	Lab designed
	CAGCAGATCACGAACAGC			
ZnT-3	GAGTCTCTTCACAGAGCCCTCA	65	126	Beck (2004)
	CTGGTGCAGCACAAAGGCCATT			
ZnT-4	GGAGAACTTGTAGGTGGATAC	48	491	Beck (2004)
	TATTAGCACACCAACACTC			
ZnT-5	GGAGGCATGAATGCTAACATGAGG	60	83	Devergnas (2004)
	GTGGATACGATCACACCAATGCTG			
ZnT-6	TTGGCTGGATCAGTGCATGTA	53	204	Lab designed
	AGGCATTGGGATTACGTGA			
ZnT-7	TTTCTTCCTGTGCCTGAACCTCTC	60	87	Devergnas (2004)
	GAGTCGGAAATCAAGCCTAAGCAG			
ZnT-8	TAAAGCTGCCAAGATGTATGC	56	356	Lab designed
	GCTTAGAGGGAGGCTTCGAT			
ZnT-10	GTCCCAAAAGGAGTCAACAT	57	104	Lab designed
	TTTCCACTTACAAGTTCCC			
Zip-1	TACAAGGAGCAGTCAGGGCCGTCA	60	617	Beck (2004)
	CTAGATTTGGATGAAGAGCAGGCCAGT			
Zip-2	GACACTCCAGTGTTGACCACC	56	261	Lab designed
	AACACCAGCAGAAATACAGC			
Zip-3a	GAGAAGGCCCATCGCTCGAAA	56	160	Lab designed
	CGGCCAGCGGGTAGTCGGT			
Zip-3b	TTTCTGGCCACGTGCT	56	180	Lab designed
	TTCACTTCCTACTTTCCGTCA			
Zip-4	TGTTAAATACGCTGGCGGACC	62	148	Lab designed
	AGGCGGGCGACGTACCTGG			
Zip-5	GGGGCTGTCAGTGCTCGGAG	66	138	Lab designed
	TCCGGATCCAAGTTGCGTGTT			
Zip-6	TTGGCTGGATCAGTGCATGTA	60	175	Lab designed
	AGGCATTGGGATTACGTGA			
Zip-7	GGGGCTCCAGGCATCAAGCA	58	120	Lab designed
	CGACTCCACGGGGATAAGGAA			
Zip-8	ATTTCTCTGGCAGATATGTT	55	70	Lab designed
	CGGTTTTTCTTCCAGTTAC			
Zip-10	ACTGCCACATGAATTAGGAG	50	147	Lab designed
	ATGTTATTGGCATACTGACC			
Zip-12	CTGATGGGATTAATTGGAG	50	145	Lab designed
	TTCTGTTGGAGGCTGTCATA			
Zip-14	CATCAGCACCTCGGTGGC	55	88	Lab designed
	GATGCTCATCCCAGCGTTGA			
G3PDH	TCCTCTGACTTCAACAGCGACAC	68	126	Lab designed
	CACCCTGTTGCTGTAGCCAAATTC			

Zinc metal analysis

Confluent cells were exposed to 75-μM zinc for 1 day. The samples were then harvested in 0.1% SDS and frozen until analysis. Protein concentration was determined using the BCA assay (Pierce Chemical). Samples were prepared for metal analysis by heating at 100°C in 10 ml of concentrated nitric acid until the solution was colorless and then evaporated further to 1 ml. The samples were then diluted to 12% nitric acid and the zinc content was measured by inductively coupled plasma atomic emission spectrometry (ICP). To enable a comparison to tissue samples, the protein content was converted to g weight of cell pellet by determining the amount of protein liberated in harvest buffer per weight of cell pellet for each cell line. Cellular zinc accumulation is thus reported as μg zinc per g cell pellet. Frozen prostate tissue was obtained from a previous study (Garrett et al. 1999) and prepared for zinc metal analysis by first incubating in 10 ml of nitric acid overnight followed by heating as described above for cell culture samples. The final concentration of zinc is reported as μg zinc per gram of tissue.

Statistical analysis

All experiments were performed in triplicate. The values are shown as the mean ± the standard error of measurement (mean ± SEM). Statistical analyses were performed using Systat and Sigmastat software using separate variance *t*-tests. Unless otherwise stated, the level of significance was 0.05.

Results

Zinc transporter mRNA expression in prostate tissues and cell lines

The present study analyzed the expression of mRNA for the ZnT-1, ZnT-2, ZnT-3, ZnT-4, ZnT-5, ZnT-6, ZnT-7, ZnT-8, ZnT-10, Zip-1, Zip-2, Zip-3A, Zip-3B, Zip-4, Zip-5, Zip-6, Zip-7, Zip-8, Zip-10, Zip-12 and Zip-14 genes in normal human prostate tissue, the immortalized RWPE-1 prostate cell line, and the PC-3, DU-145, and LNCaP prostate cancer cell lines. The presence of mRNA for each transporter was found in at least one of the samples except for ZnT-3, ZnT-10, Zip-2, Zip-4 and Zip-5, which were absent in all the samples analyzed in this study. In addition, despite several attempts at primer design, no amplification product could be obtained for the ZnT-8 and Zip-12 mRNAs under control conditions.

Comparison of zinc transporter mRNA expression between RWPE-1 cells and normal prostate tissue

The first goal of the present study was to determine if the RWPE-1 cell line recapitulated the expression of the mRNAs for the zinc transporters found in situ in the human prostate gland. As noted above, neither the RWPE-1 cell line or normal prostate tissue expressed the ZnT-3, ZnT-10, Zip-2, Zip-4 and Zip-5 transporter mRNAs. In addition, Zip3B was also not detected in the RWPE-1 cell line or normal prostate tissue. The analysis demonstrated that the RWPE-1 cell line and the four independently processed normal prostate samples expressed mRNAs for the ZnT-1, ZnT-2, ZnT-4, ZnT-5, ZnT-6, ZnT-7, Zip-1, Zip-3A, Zip-6, Zip-7, Zip-8, Zip-10 and Zip-14 genes (Table 2). It was also demonstrated that the expression of the individual transporter mRNAs were highly variable among the four tissue samples. However, the pattern of expression within the normal samples also suggested that there was no predictable pattern to the variability in expression for the individual transporters. That is, a tissue sample elevated for the expression of one transport mRNA compared to the other samples, would have a correspondingly low expression when compared of another transporter gene with high expression. This provides evidence that the variability in expression was not due to differences in sample preparation, such as, the degradation of total RNA due to time of removal to time of total RNA preparation.

The levels of expression of mRNAs for the ZnT-1, ZnT-4, ZnT-5, ZnT-7, Zip-6, Zip-7 and Zip-8 genes were similar between the RWPE-1 cells and the four independently processed tissue samples, with the values of expression of the RWPE-1 cells either falling within the variable range of expression of the 4 tissue samples or not statistically significantly

Table 2 Expression of zinc transporter mRNA in RWPE-1 cells and normal prostate tissue[a]

	ZnT-1	ZnT-2	ZnT-4	ZnT-5	ZnT-6
RWPE-1	8.34 ± 0.48	0.13 ± 0.04	0.20 ± 0.03	12.81 ± 0.85	0.57 ± 0.04[c]
T1[b]	4.29 ± 0.53	0.22 ± 0.03	0.45 ± 0.10	14.80 ± 1.51	0.19 ± 0.02
T2[b]	1.41 ± 0.18	4809 ± 8.18	0.13 ± 0.02	1.43 ± 0.16	0.10 ± 0.01
T3[b]	2.48 ± 0.18	72549 ± 4456	0.99 ± 0.22	11.96 ± 0.56	0.18 ± 0.03
T4[b]	4.88 ± 1.01	10.51 ± 2.1	8.07 ± 3.38	18.58 ± 2.65	0.33 ± 0.04
	ZnT-7	Zip-1	Zip-3A	Zip-3B	Zip-6
RWPE-1	18.78 ± 1.14	1.96 ± 0.39[c]	1.16 ± 0.11[c]	ND	0.85 ± 0.09
T1[b]	14.46 ± 1.06	0.10 ± 0.01	0.13 ± 0.01	ND	1.18 ± 0.12
T2[b]	10.45 ± 0.06	0.11 ± 0.01	0.12 ± 0.03	ND	0.48 ± 0.07
T3[b]	12.70 ± 1.28	0.11 ± 0.03	0.37 ± 0.01	ND	1.18 ± 0.04
T4[b]	51.46 ± 9.86	0.29 ± 0.07	0.55 ± 0.10	ND	2.41 ± 0.57
	Zip-7	Zip-8	Zip-10	Zip-14	
RWPE-1	1.42 ± 0.29	0.66 ± 0.04	4.05 ± 0.03[c]	1.06 ± 0.04[c]	
T1[b]	ND	3.45 ± 0.34	2.26 ± 0.22	0.03 ± 0.01	
T2[b]	0.28 ± 0.01	0.24 ± 0.04	0.28 ± 0.02	0.13 ± 0.01	
T3[b]	0.11 ± 0.01	5.69 ± 0.93	0.63 ± 0.02	0.31 ± 0.02	
T4[b]	3.44 ± 1.42	0.41 ± 0.09	0.10 ± 0.02	0.05 ± 0.02	

[a] The level of each zinc transporter mRNA was determined by quantitative real-time PCR, normalized to the level of mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and reported as transporter mRNA transcripts per 1000 G3PDH

[b] Normal human prostate tissues specimens, T1, T2, T3, and T4

[c] Significantly elevated compared to four tissue samples at $P < 0.05$

ND, Not detected

elevated or decreased from all four tissue samples (Table 2). The expression of the mRNAs for the ZnT-6, Zip-1, Zip-3A, Zip-10 and Zip-14 genes were elevated in the RWPE-1 cell line compared to the four tissue samples (Table 2). It is noteworthy that the expression of mRNA for the ZnT-2 gene showed exceptional variability among the four normal tissue samples, with expression varying from a low of 0.22 to a high of 72,549 copies of mRNA per 1000 G3PDH. Expression of ZnT-2 mRNA in the RWPE-1 cells was 0.13 copies. Overall, there was good agreement between the expression of the zinc transporter mRNAs between the RWPE-1 cell line and those present in tissue from areas of the normal prostate. However, the variability of mRNA expression in the normal samples should be taken into account when making the comparisons between the level of expression in the cell line and the tissue preparations.

Comparison of zinc transporter mRNA expression between RWPE-1 cells and prostate cancer cell lines

An analysis of zinc transporter mRNA expression between the RWPE-1 cells and the prostate cancer cell lines demonstrated that there was no significant difference in the expression of the mRNAs for the ZnT-2, ZnT-5, ZnT-6, ZnT-7, Zip-3A, and Zip-7 genes (Table 3). This observation was also true when the 3 prostate cancer cell lines were compared to one another (Table 3). The expression of ZnT-1 and Zip10 genes were elevated in RWPE-1 cells compared to the three prostate cancer cell lines. The expression of ZnT-4 in LNCaP cells was shown to be elevated compared to RWPE-1, PC-3 and DU-145 cells. Zip-1 mRNA was elevated in the DU-145 cells compared to the other three cell lines. Zip-6 mRNA was elevated in the LNCaP and DU-145 cells

compared to the RWPE-1 and PC-3 cell lines, but not significantly different between the LNCaP and DU-145 cells. The expression of Zip-8 mRNA was significantly elevated in the 3 cancer cell lines compared to the RWPE-1 cells and also significantly elevated in the LNCaP and DU-145 cells compared to PC-3 cells. The expression of Zip-14 was higher in DU-145 cells compared to the other 3 cell lines.

Effect of zinc exposure on zinc transporter mRNA expression

The RWPE-1, LNCaP, DU-145 and PC-3 cells were exposed to 75 μM Zn^{+2} for 24 h and total RNA was prepared for the analysis of the zinc transporter mRNAs. It was demonstrated that Zn^{+2} exposure had no effect on mRNA expression for the majority of individual transporters and cell lines that were analyzed in this study (Table 4). The only exceptions were for the RWPE-1 cells where Zn^{+2} increased ZnT-1 and ZnT-2 expression and decreased the expression of the ZnT-7, Zip-7 and Zip-10 genes (Table 4). Zinc exposure also induced the expression of ZnT-1 in PC-3 cells but at a magnitude considerably less than that of RWPE-1 (Table 4).

Levels of zinc in the prostate tissues and the RWPE-1, LNCaP and PC-3 cell lines

The level of zinc in the 4 prostate specimens used for the preparation of total RNA was determined using ICP. The zinc content of the 4 samples was between 25 and 78 μg zinc/g tissue (Fig. 1). The RWPE-1 cell line contained similar levels of zinc to that of the prostate tissues. Prostate tissue sample T1 appeared to contain lower levels of zinc compared to the other three tissue samples as well as the RWPE-1 cell line. The zinc content of the RWPE-1, LNCaP and PC-3 cell lines was determined under basal conditions and after 24 h of exposure to 75 μM zinc. It was demonstrated that the zinc content of the RWPE cells was significantly elevated compared to the LNCaP and PC-3 cell lines under both basal conditions and when the cells were exposed to Zn^{+2} (Fig. 2). It was also demonstrated that Zn^{+2} exposure

Table 3 Expression of zinc transporter mRNA in RWPE-1 and three prostate cancer cell lines[a]

	ZnT-1	ZnT-2	ZnT-4	ZnT-5	ZnT-6
RWPE-1	8.34 ± 0.48[b]	0.13 ± 0.04	0.20 ± 0.03	12.81 ± 0.85	0.57 ± 0.06
LNCaP	5.61 ± 0.49	0.11 ± 0.02	3.13 ± 0.32[b]	13.15 ± 0.71	0.50 ± 0.11
DU-145	3.22 ± 0.13	0.14 ± 0.03	0.13 ± 0.03	7.74 ± 0.33	1.42 ± 0.33
PC-3	3.17 ± 0.06	0.14 ± 0.04	0.15 ± 0.01	9.89 ± 0.64	1.42 ± 0.21
	ZnT-7	Zip-1	Zip-3A	Zip-3B	Zip-6
RWPE-1	18.78 ± 1.41	1.96 ± 0.39	1.16 ± 0.11	ND	0.85 ± 0.09
LNCaP	29.11 ± 2.71	2.40 ± 0.22	1.71 ± 0.18	0.22 ± 0.01	3.65 ± 0.41[c]
DU-145	18.41 ± 1.78	10.52 ± 0.79[b]	0.78 ± 0.05	0.16 ± 0.03	4.78 ± 0.33[c]
PC-3	36.58 ± 6.99	3.87 ± 0.30	1.18 ± 0.12	0.03 ± 0.01	0.74 ± 0.05
	Zip-7	Zip-8	Zip-10	Zip-14	
RWPE-1	1.42 ± 0.29	0.66 ± 0.04	4.05 ± 0.03[b]	1.06 ± 0.04	
LNCaP	1.01 ± 0.38	13.05 ± 0.38[bc]	2.45 ± 0.39	2.36 ± 0.37	
DU-145	1.75 ± 0.58	16.45 ± 0.58[bc]	2.60 ± 0.07	6.74 ± 0.70[b]	
PC-3	3.52 ± 0.62	3.88 ± 0.62[b]	2.72 ± 0.08	2.45 ± 0.59	

[a] The level of each zinc transporter mRNA was determined by quantitative real-time PCR, normalized to the level of mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and reported as transporter mRNA transcripts per 1000 G3PDH

[b] Significantly different compared to all three cell lines: $P < 0.05$

[c] Ssignificantly elevated compared to RWPE-1 and PC-3 cells

ND, Not detected

significantly increased the level of Zn^{+2} in RWPE-1 cells, but not in LNCaP or PC-3 cells.

Discussion

These studies demonstrate that the RWPE-1 cell line could be a valuable model for the study of the alterations of zinc homeostasis that occur during the development of prostate cancer. This is especially true since the RWPE-1 cell line has been highly characterized and shown to undergo malignant transformation by both Cd^{+2} and As^{+3}. The RWPE-1 cell line was established from adult human prostate epithelial cells that were immortalized using HPV18 (Bello et al. 1997). In response to androgen treatment, the RWPE-1 cells showed growth stimulation, prostate specific antigen (PSA) and androgen receptor (AR) expression. The RWPE-1 cells do not form colonies in soft agar, are not tumorigenic in nude mice and are non-invasive in an in vitro invasion assay. The RWPE-1 cells display acinar differentiation when placed in the environment provided by Matrigel (Webber et al. 1997). The RWPE-1 cells have also been directly malignantly transformed by both Cd^{+2} and As^{+3} with the resultant tumor heterotransplants shown to display histological and biochemical features consistent with prostate cancer, including the development of androgen resistance (Achanzar et al. 2001, 2002; Benbrahim-Tallaa et al. 2005a, b).

The initial goal of this study was to determine if the RWPE-1 cell line could serve as a model system for the study of selected members of the zinc transporter gene family. The results from the analysis of this large number of transporters demonstrated that the RWPE-1 cells had an expression profile of zinc transporters similar to that found in total RNA isolated from 4 independent tissue specimens from the human prostate gland. Extending the comparison from an expression profile to the relative level of expression between the RWPE-1 cell line and the in situ tissue preparations was rendered difficult due to the wide variability of individual expression levels for the transporters among the 4 preparations. However, out of the 19 transporters analyzed for mRNA expression, the RWPE-1 cells had expression levels of 14 of the transporters, five of which were significantly elevated in the RWPE-1 cell line compared to the tissue samples. The variability of expression of one transporter, ZnT-2, in the tissue samples demonstrated such a high degree of variability that any comparison would not be significant. The most likely explanation for the variability in expression among the 4 tissue samples is the local geography of the prostate gland chosen for use and the differing proportions of stromal and epithelial components in each of the specimens. The fact that the patterns of expression are random among the specimens argues against errors introduced during specimen preparation, such as total RNA degradation as a function of the time specimen processing. There was a much greater variability of zinc transporter expression than in the variability of zinc content among the tissues samples. The zinc content of three of the 4 tissue specimens used for the preparation of total RNA had similar levels of total zinc. Tissue sample T1 had 35–40% the level of zinc compared to the other three tissue samples. It is possible that local geography of the prostate gland chosen for use also may have influenced the level of tissue zinc. The lower zinc content of this sample may also be related to transporter gene expression since this sample had the lowest level of ZnT-2, no expression of Zip-7 and the highest level of Zip-10. For the cultured cells it was shown that the RWPE-1 cell line had higher levels of basal zinc, and accumulated more zinc upon zinc exposure, than the LNCaP and PC-3 cell lines. This is in agreement with in situ observations that zinc content is elevated in the normal prostate gland and reduced in prostate cancer (Naide 2002). Overall, these findings suggest that the RWPE-1 cells could be a valuable model for the study of the zinc transporter gene family in the prostate and there alteration by environmental toxicants.

The second goal of this study was to determine if a short-term exposure to Zn^{+2} would induce the expression of any of the zinc transporter genes. The cells were exposed to 75 μM Zn^{+2} for 24 h because this level of exposure elicited no loss of cell viability for any of the 4 cell lines. For the RWPE-1 cells, only 5 of the 19 zinc transporter genes displayed an altered mRNA level following exposure to Zn^{+2}. The ZnT-1 and ZnT-2 genes showed a significant increase in mRNA expression following Zn^{+2} exposure, where as, mRNA for the ZnT-7, Zip-7 and Zip-10 genes was repressed following exposure to Zn^{+2}. For the prostate cancer cell lines (LNCaP, DU-145, PC-3), only

Table 4 Effect of zinc exposure on the expression of zinc transporter mRNA in the RWPE-1 and prostate cancer cell lines[a]

	75 μM[b] Zn^{+2}	ZnT-1	ZnT-2	ZnT-4	ZnT-5	ZnT-6
RWPE-1	–	8.34 ± 0.48	0.13 ± 0.04	0.20 ± 0.03	12.81 ± 0.85	0.17 ± 0.06
	+	136.94 ± 0.05[c]	1.38 ± 0.19[c]	0.26 ± 0.02	7.03 ± 1.23	0.32 ± 0.05
LNCaP	–	5.61 ± 0.49	0.11 ± 0.02	3.13 ± 0.32	13.15 ± 0.71	0.50 ± 0.10
	+	5.45 ± 0.79	0.07 ± 0.02	2.41 ± 0.03	11.00 ± 2.02	0.65 ± 0.05
DU-145	–	3.22 ± 0.13	0.14 ± 0.03	0.13 ± 0.03	7.74 ± 0.33	1.42 ± 0.33
	+	4.33 ± 0.17	0.10 ± 0.03	0.11 ± 0.05	6.79 ± 1.27	1.58 ± 0.23
PC-3	–	3.17 ± 0.06	0.14 ± 0.04	0.15 ± 0.01	9.89 ± 0.64	1.42 ± 0.21
	+	9.15 ± 0.50[c]	0.08 ± 0.04	0.16 ± 0.02	11.23 ± 0.95	1.13 ± 0.17
	75 μM Zn^{+2}	ZnT-7	Zip-1	Zip-3A	Zip-3B	Zip-6
RWPE-1	–	18.78 ± 1.41	1.96 ± 0.39	1.16 ± 0.13	ND	0.85 ± 0.09
	+	8.71 ± 0.46[c]	1.36 ± 0.13	0.49 ± 0.01	ND	0.36 ± 0.01
LNCap	–	29.11 ± 2.71	2.40 ± 0.22	1.71 ± 0.18	0.22 ± 0.01	3.65 ± 0.41
	+	22.47 ± 0.16	2.08 ± 0.21	2.16 ± 0.03	0.35 ± 0.07	2.58 ± 0.17
DU-145	–	18.41 ± 1.78	10.52 ± 0.79	0.28 ± 0.05	0.16 ± 0.03	4.78 ± 0.33
	+	23.13 ± 1.84	9.77 ± 0.25	0.95 ± 0.15	0.24 ± 0.01	5.21 ± 0.30
PC-3	–	36.58 ± 6.99	3.87 ± 0.30	1.18 ± 0.30	0.03 ± 0.01	0.74 ± 0.05
	+	39.71 ± 1.93	2.77 ± 0.15	1.15 ± 0.14	0.05 ± 0.01	0.88 ± 0.04
	75 μM Zn^{+2}	Zip-7	Zip-8	Zip-10	Zip-14	
RWPE-1	–	1.42 ± 0.29	0.66 ± 0.04	4.05 ± 0.03	1.06 ± 0.04	
	+	0.29 ± 0.08[c]	0.76 ± 0.04	0.83 ± 0.03[c]	0.58 ± 0.13	
LNCaP	–	1.01 ± 0.38	13.05 ± 0.38	2.45 ± 0.39	2.36 ± 0.37	
	+	1.49 ± 0.49	12.80 ± 0.25	3.40 ± 0.36	1.80 ± 0.12	
DU-145	–	1.75 ± 0.58	16.45 ± 0.58	2.60 ± 0.07	6.74 ± 0.70	
	+	3.05 ± 0.44	16.81 ± 0.63	2.34 ± 0.85	5.69 ± 0.08	
PC-3	–	3.56 ± 0.62	3.88 ± 0.62	2.72 ± 0.08	2.45 ± 0.59	
	+	3.31 ± 0.22	3.62 ± 0.14	2.20 ± 0.32	2.07 ± 0.50	

[a] The level of each zinc transporter mRNA was determined by quantitative real-time PCR, normalized to the level of mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and reported as transporter mRNA transcripts per 1000 G3PDH

[b] Zinc exposure was for 24 h

[c] Significantly different compared to non-treated group $P < 0.05$

ND, Not detected

ZnT-1 showed an alteration in expression, being induced only in PC-3, albeit much smaller in magnitude than that of RWPE-1. The finding on an increase in ZnT-1 mRNA in RWPE-1 cells following Zn^{+2} exposure is in agreement with previous studies in other types of cells that show that regulation of the gene is under the control of the MTF-1 transcription factor (Langmade et al. 2000). However, the finding that Zn^{+2} treatment had no effect on ZnT-1 transporter expression in LNCaP is not in agreement with the only other study using prostate cancer cells (Hasumi et al. 2003). In this study, treatment with 100 μM Zn^{+2} for 96 h was shown to induce ZnT-1 mRNA in both LNCaP and PC-3 cells. The only difference between the two studies is that in the present study, Zn^{+2} exposure was at a lower concentration, of a shorter duration, and resulted in no loss of cell viability; which is in contrast to the previous study where there was a significant loss of cell viability. ZnT-2 was also shown to be induced by Zn^{+2} in RWPE-1 cells. This protein had been shown to confer zinc resistance by facilitating vesicular sequestration and is known to be expressed in the rat lateral and dorsal prostate (Pamiter et al. 1996; Iguchi

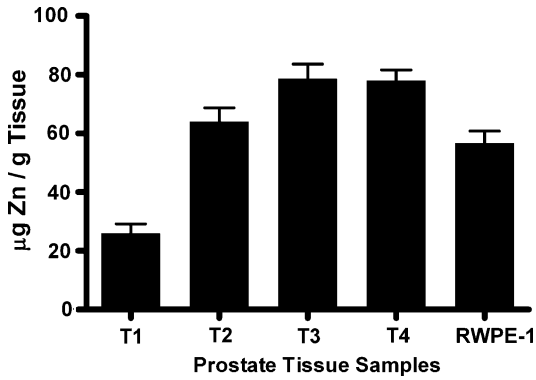

Fig. 1 Zinc content of prostate tissue samples: Four normal human prostate tissue specimens, T1, T2, T3, and T4 as well as the RWPE-1 Cell line were analyzed for zinc content. Values are reported as µg zinc per g of wet tissue or per g of cell pellet in the case of RWPE-1

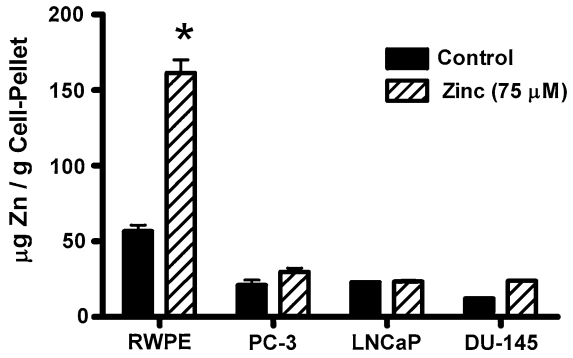

Fig. 2 Zinc content of prostate cell lines exposed to zinc: Cell lines were expose to normal or zinc containing (75 µM) medium for 24 hours and the cells were harvested for zinc analysis. Values are reported as µg zinc per g of cell pellet. * Significantly elevated compared to control $P < 0.01$

et al. 2002). ZnT-2 was also shown to be induced in the small intestine of zinc-supplemented rats (Liuzzi et al. 2001).

The finding that ZnT-7 mRNA was reduced in RWPE-1 cells by Zn^{+2} agrees with studies in other cells and tissues that Zn^{+2} can regulate gene expression of ZnT-7 (Devergnas et al. 2004; Helston et al. 2007; Kirscheke and Huang 2003). However, to the author's knowledge it does not appear that ZnT-7 has been studied in normal prostate cells and tissues or their malignant counterparts. As such, the finding that Zn^{+2} has no effect on ZnT-7 expression in prostate cancer cell lines could warrant further examination. A very similar pattern of mRNA expression was demonstrated for Zip-7 which is known to be involved in zinc homeostasis in the Golgi apparatus (Huang et al. 2005; Taylor et al. 2004). The Zip-7 gene and its expression has not been examined in normal and malignant prostate cells or tissues. Messenger RNA expression was also found for the Zip-10 family member which has been shown to be involved in zinc uptake across the brush border membrane of the kidney (Kaler and Prasad 2007). It has also been shown to be regulated by thyroid hormone in intestine and kidney (Pawan et al. 2007). The Zip-10 gene and its expression has not been examined in normal and malignant prostate cells or tissues. It is noteworthy that the down-regulation of mRNA in all 3 of these genes by Zn^{+2} occurs only in the immortal, but not tumorigenic, RWPE-1 cells and not in the prostate cancer cell lines.

The present study showed that exposure of the RWPE-1 cells or the prostate cancer cell lines to Zn^{+2} had no effect on the expression of mRNAs representing the ZnT-4, Zip-1 and Zip-6 genes in the RWPE-1 cell line or the prostate cancer cell lines. For these genes there is literature-detailing expression in either prostate cells or tissues. The expression of the ZnT-4 gene has been analyzed in human prostate tissues and its protein expression shown to decrease during the progression of early prostate disease to invasive prostate cancer (Henshall et al. 2003; Beck et al. 2004) however, in the Henshall study, mRNA expression was found to actually increase. Its expression has also been analyzed in LNCaP cells (Iguchi et al. 2004). The Zip-1 protein has been reported to be the major zinc uptake transporter for the accumulation of zinc in prostate cells and that it is down-regulated in prostate cancer (Franklin et al. 2003, 2005); another study found the expression unaltered (Beck et al. 2004). The Zip-6 transporter, also known as LIV-1, has been shown to be differentially expressed in breast cancer and associated with better patient outcome (Taylor 2000; Kasper et al. 2005; Tozlu et al. 2006). It has also been reported to be expressed in the prostate (Taylor et al. 2003).

Other transporters shown to be expressed in the prostate tissues and cell lines used in this study have limited information regarding expression or function. The ZnT-5 transporter has been shown in other systems to be responsive to zinc levels (Devergnas et al. 2004; Jackson et al. 2007). The ZnT-6 transporter in leukocytes has been shown to be altered by dietary zinc (Aydemir et al. 2006). The Zip-3A and

3B transporters have been associated with zinc deficiency during development and pregnancy (Chowanadisai et al. 2006; Dufner-Beattie et al. 2005, 2006). The Zip-8 transporter has been associated with cadmium toxicity in the testis and renal proximal tubule (Dalton et al. 2005; Wang et al. 2007). The Zip-14 transporter has been shown to mediate non-transferrin-bound iron uptake into cells and to transport zinc in the liver (Liuzzi et al. 2005, 2006). Of the 5 transporters mRNAs shown not to be expressed in the normal prostate or the 4 prostate cell lines in the present study, only ZnT-3 has been shown to be expressed in a prostate tissue or cell line (Iguchi et al. 2004). In this study, ZnT-3 was elevated in an androgen-independent subline of LNCaP cells. There was no information on the expression of ZnT-10, Zip-2, Zip-4 or Zip-5 in prostate cells of tissues.

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