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The effects of transformation and ZnT-1 silencing on zinc homeostasis in cultured cells☆

Kavitha Sankavaram, Hedley C. Freake*

Department of Nutritional Sciences, University of Connecticut, CT 06269-4017, USA

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Abstract

We have previously demonstrated that reducing the availability of zinc with the extracellular chelator diethylenetriaminepentaacetic acid (DTPA) promotes efflux of ^{65}Zn from rat primary hepatocytes and pituitary cells, but increases retention of label in rat hepatoma (H4IIE) and anterior pituitary tumor (GH3) cell lines. To further understand this differential response between primary cells and the corresponding cancer cell lines, we investigated the effects of immortalizing primary cells on their zinc homeostasis. Rat primary hepatocytes were electroporated with the SV40 large T-antigen-coding plasmid pSV3-neo and selected for neomycin resistance. This resulted in cell division of the normally quiescent hepatocytes. When these cells were prelabeled with ^{65}Zn, DTPA decreased efflux of ^{65}Zn, similarly to hepatoma cells and differently from primary hepatocytes. This homeostatic change may be required to account for the greater zinc requirements of dividing cells and be mediated by alterations in the activity of zinc transporter ZnT-1, which is responsible for zinc efflux. To further understand the mechanism of DTPA-induced zinc retention, we down-regulated the expression of ZnT-1 in rat hepatoma cells using vector-based short hairpin RNA interference. Expression of ZnT-1 protein was reduced to approximately 50%. Down-regulation of ZnT-1 resulted in greater retention of ^{65}Zn in control cells. However, DTPA increased rather than decreased efflux of label from knockdown cells, suggesting that functional ZnT-1 is required for the decreased efflux in response to DTPA. We conclude that ZnT-1 expression is crucial for maintaining zinc homeostasis, in particular, for the enhanced retention of zinc in transformed cells when subjected to zinc deprivation.

Keywords: Zinc; ZnT-1; shRNA; Transformation; Zinc retention; ^{65}Zn; Hepatocytes, Cultured cells

1. Introduction

Zinc is an essential nutrient for all organisms and plays a variety of roles in the biochemical processes of cells. It acts as a catalytic and structural factor in more than 300 different metalloenzymes including transcription factors, hormones, neuropeptides and receptors [1]. It is very well established that zinc is crucial for cell growth, for cell division and for the activities of a variety of enzyme systems in the body [2,3]. Due to the pleiotropic effects of zinc involving a wide range of cellular physiology, fluctuations in zinc concentrations as a result of zinc deficiency or increased supply can cause disturbances in cellular homeostasis [4]. Consistently, cellular zinc shortage induced by zinc-deficient medium or cell-permeable zinc chelators has been shown to result in dysfunction as well as induction of apoptosis in various cell types [5,6].

Studies have demonstrated a variety of relationships between zinc and cancer [7–9]. Zinc induces apoptosis in many mammalian cell types, including prostate epithelial cells, neuronal and glial cells, ovarian and esophageal epithelial cells, choriocarcinoma cells and others. In contrast, zinc exhibits antiapoptotic effects in breast cells, lung epithelial cells, renal cells, mononuclear cells, pancreatic acinar cells, Hela cells and others [9]. Prasad et al. (2010), examining the effects of dietary zinc supplementation in the development of prostate cancer using a TRAMP mouse, found a significant increase in tumor weights when dietary zinc was deficient or elevated compared to normal zinc intake levels. This suggested that an optimal dietary zinc intake may play a protective role against prostate cancer [10]. While concentration-dependent biphasic effects either inducing or inhibiting apoptosis have been observed, no common relationship between different types of cancers and zinc metabolism has been established. Thus, little is known about the mechanisms by which zinc exerts its action on cancer cells. In particular, it is not known whether zinc may directly act on tumor cells or if its *in vivo* action is related to a modulation of the immune effectiveness or to the zinc-dependent regulation of the production of other anticancer substances [7,11].

Zinc concentrations in malignant and nonmalignant tissues vary substantially [12–15], indicating differences in their homeostatic mechanisms. Given the role of zinc in cellular growth, understanding the differential zinc homeostasis in malignant and normal cells may better define the mechanisms underlying cancer development. Previously, we have reported the effects of diethylenetriaminepentaacetic acid (DTPA)-induced zinc deficiency in both primary cells and

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* Corresponding author. Tel.: +1 860 486 1704; fax: +1 860 486 3674.
E-mail address: hedley.freake@uconn.edu (H.C. Freake).

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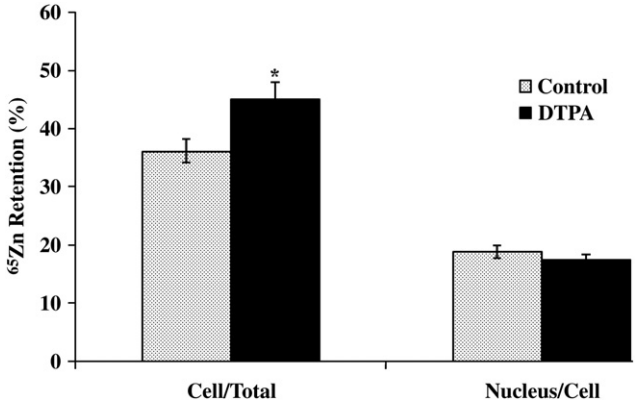

Fig. 1. Effects of DTPA on ^{65}Zn retention in virally transformed rat primary hepatocytes. Primary rat hepatocytes were immortalized by transfection with pSV3-neo. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without 50 μM DTPA for 48 h to measure retention. "Cell/total" is the total cellular ^{65}Zn recovered from cellular fractions expressed as a percentage of the total radioactivity. "Nucleus/cell" is the ^{65}Zn retention of crude nuclei expressed as a percentage of that recovered from the cells. Values are the means±S.D. of three experiments, each including at least six replicates of each treatment. *Significant effects of DTPA, $P \leq .005$.

established cancer cell lines [16]. Consistent with its chelating function, DTPA increases efflux of ^{65}Zn from prelabeled primary rat hepatocytes and pituitary cells. However, it paradoxically reduces efflux from H4IIE (rat hepatoma) and GH3 (rat pituitary tumor) cells. Furthermore, this differential response to DTPA in primary cells and established cell lines was not due to changes in mRNA or protein expression of the zinc efflux transporter ZnT-1. We hypothesized that this differential response between malignant and nonmalignant cells might be due to rapid growth requirements of cancer cells and be mediated by alterations in the functional capacity, rather than the expression, of ZnT-1. Therefore, in this study, we aimed to define the effects of transforming primary hepatocytes on zinc homeostasis and the extent to which reducing ZnT-1 expression affects zinc homeostasis in hepatoma cells.

2. Materials and methods

2.1. Cell line and culture conditions

The rat hepatoma H4IIE cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1 mM Na pyruvate, 0.1 mM nonessential amino acids, penicillin (50,000 IU/L) and streptomycin (50 mg/L). Cells were kept in a humidified CO_2 incubator (37°C, 5% CO_2). Prior to experimentation, cells were counted using trypan blue exclusion and seeded into 25-cm^2 flasks at a density of 5×10^6 cells in 5-ml medium. Cells were allowed to attach overnight before treatment.

2.2. Isolation of primary hepatocytes and gene transfer using electroporation

All animal procedures were approved by the University of Connecticut Animal Care and Use Committee. Mature Sprague–Dawley rats (Harlan Laboratories, Madison, WI, USA) weighing 200–300 g were used for isolation of primary rat hepatocytes using collagenase perfusion of livers as described previously [16,17]. Isolated hepatocytes (1×10^7) suspended in Opti-MEM serum-free medium (Invitrogen, Carlsbad, CA, USA) were subjected to electroporation in the presence of 100 μg of the plasmid pSV3-neo (ATCC) at 200 V and a capacitance of 500 μF using a Gene-Pulser System (Bio-Rad Laboratories, Hercules, CA, USA). The plasmid pSV3-neo contains the early region of the simian virus-40 large T antigen (SV-40Tag) and a selective marker for G418 resistance. After electroporation, 10^6 cells were plated on to 10-cm Primaria Tissue Culture Plates (Benton Dickinson, Franklin Lakes, NJ, USA) in William's E medium containing L-glutamine and Na pyruvate (Invitrogen) supplemented with sodium bicarbonate (26 mM), Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 23 mM], dexamethasone (10 nM), human recombinant insulin (1 nM, Invitrogen), penicillin (50,000 IU/L) and streptomycin (50 mg/L), pH 7.2. Cells were maintained in a humidified incubator at 37°C in 5% CO_2. Twenty-four hours later, the medium was changed to MEM supplemented with 10% FBS and 1 mM Na pyruvate, 0.1 mM nonessential amino acids and 100 μg/ml Geneticin (G418) (Sigma, St. Louis, MO, USA) for selection. Transformed hepatocytes were allowed to grow for 8 weeks before using for ^{65}Zn retention experiments.

2.3. Transfection of H4IIE cells using short hairpin RNA

Short hairpin RNA (shRNA) lentiviral plasmid (pLKO.1-puro, Sigma) targeting ZnT-1 (TRCN0000079575) or nontarget shRNA control vector (SHC002) was transfected into H4IIE cells using Fugene HD transfection reagent (Roche, Indianapolis, IN, USA) per the manufacturer's protocol. After 24 h, fresh H4IIE medium was added, and the transfected cells were selected initially with 2 μg/ml puromycin for 1 week and then with 4 μ/ml puromycin (P8833, Sigma).

2.4. Expression of ZnT-1 mRNA and protein in shRNA-transfected H4IIE cells

Total RNA was extracted from shRNA-transfected H4IIE cells using Trizol reagent (Invitrogen). ZnT-1 mRNA was quantified using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) (OneStep RT-PCR Kit, Qiagen, Valencia, CA, USA). The primers for ZnT-1 (forward, 5′-GTTTTCCTGATCCCTGCAA-3′ and reverse, 5′-GGACTTAAGCGATCGCATCC-3′) and glyceraldehyde phosphate dehydrogenase (GAPDH; forward, 5′-CATGCCAGTGAGCTTCCCGTT-3′ and reverse, 5′-CTTCTGCGGTCATCTGAGGTG-3′) were designed using Primer 3 software (http://frodo.wi.mit.edu/). Amplification was carried out at an annealing temperature of 58°C for 35 cycles using a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) thermal cycler. The amplified DNA was visualized by ethidium bromide staining following agarose gel electrophoresis and detected on a Bio-Rad Chemidoc XRS system.

Cellular proteins were isolated using ice-cold RIPA lysis buffer (Teknova, Hollister, CA, USA) containing protease inhibitor cocktail (Sigma), and protein concentrations were determined using a Bio-Rad DC Assay Kit (Bio-Rad Laboratories). Samples (20 μg of protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred on to a nitrocellulose membrane (Pierce, Rockford, IL, USA) [16]. After blocking for 1 h [0.5% nonfat dry milk in TBS/T (0.1% Tween-20 in Tris-buffered saline)], the membranes were washed three times for 5 min with TBS/T and incubated overnight with anti-ZnT-1 (generously provided by Drs. J. Luizzi and R.J. Cousins, University of Florida) and anti-actin (Sigma) primary antibodies in blocking buffer. The membrane was rinsed three times for 5 min each with TBS/T, incubated with horseradish-peroxidase-coupled secondary antibody (Sigma) for 1 h and washed for 3×5 min with TBS/T [16]. The signal was visualized using enhanced chemiluminescent Western blotting substrate reagents (PI 32109, Fisher Scientific, Pittsburgh, PA, USA) and was detected on a Bio-Rad Chemidoc XRS system. Both amplified DNA and the protein signals were quantified using Quantity One software (Bio-Rad).

2.5. Cellular retention of ^{65}Zn

Radioactive ^{65}Zn ($ZnCl_2$; 62.5 MBq/mg) was purchased from Perkin Elmer (Shelton, CT, USA). Cells were incubated with 1000 Bq of ^{65}Zn for 48 h. Radioactive medium was removed, and the cell monolayers were rinsed with Hank's buffered salt solution and then incubated with fresh medium in the presence or absence of 50 μM DTPA (Sigma) for 48 h. After the treatment, medium was collected, and cells were detached with trypsin-EDTA, washed and centrifuged at 250*g* for 5 min. The cell pellets were then homogenized in STMT buffer (0.25 M sucrose/20 mM Tris–HCl/1.1 mM $MgCl_2$/1% Triton ×100, pH 7.8) using a Teflon glass homogenizer and centrifuged at 1500*g* for 10 min to obtain the crude nuclear and cytosolic fractions. The ^{65}Zn contents of media, cytosol and nuclei were quantified by gamma spectroscopy (Cobra II System, Packard, Meriden, CT, USA).

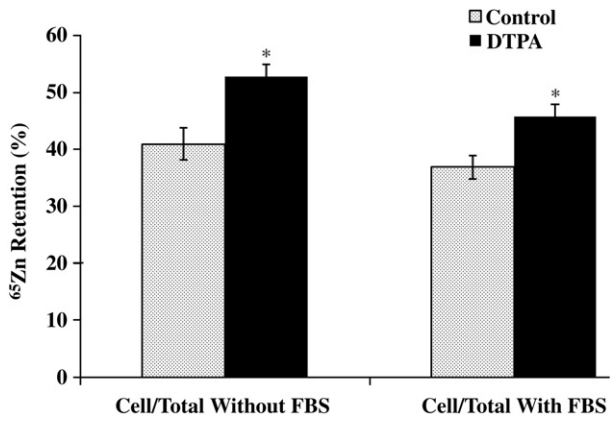

Fig. 2. Effects of DTPA on ^{65}Zn retention in virally transformed rat primary hepatocytes in the presence and absence of serum. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without 50 μM DTPA for 48 h to measure retention in the presence or absence of serum. Values are the means±S.D. of three experiments, each including at least six replicates of each treatment. *Significant effects of DTPA, $P \leq .005$.

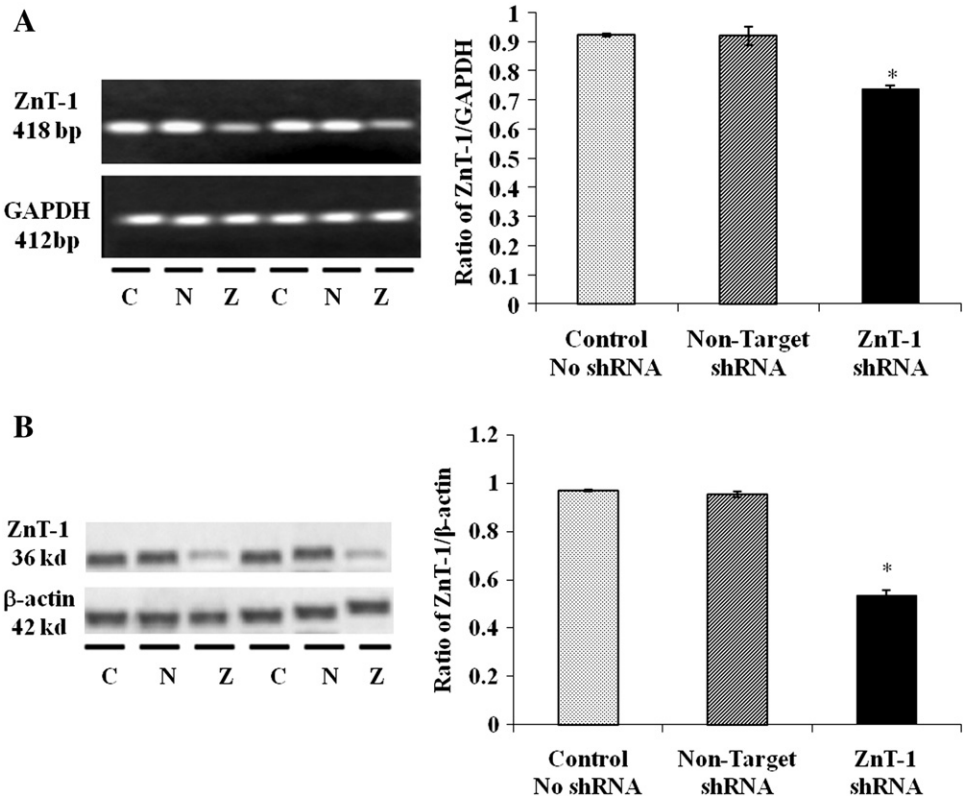

Fig. 3. Effect of shRNA transfection on ZnT-1 expression in H4IIE cells. Cells were transfected with or without ZnT-1 shRNA or nontarget shRNA. Cells were allowed to grow for at least two passages and selected for puromycin resistance prior to extraction of RNA and protein. (A) ZnT-1 and GAPDH mRNA concentrations were measured by RT-PCR with ethidium bromide staining of products separated by agarose gel electrophoresis. (B) ZnT-1 and β-actin proteins were measured by Western blot analysis following SDS-PAGE separation of proteins. Representative lanes and quantified bar graphs are shown. C=nontransfected cells, N=cells transfected with nontargeting shRNA, Z=cells transfected with ZnT-1 shRNA. Experiments were repeated at least four times with three replicates in each group. Values are the means±S.D. *Significant effect of ZnT-1 shRNA transfection, $P \leq .005$.

2.6. Cell viability assay

The cell titer blue reagent (Promega, Madison, WI, USA) was utilized to define treatment effects on cell viability and growth. Cells were seeded into a 96-well plate at a density of 4000 cells/well. After overnight attachment, medium was removed, and fresh medium was added to the wells with or without 50 μM DTPA. After 48 or 72 h, medium was removed, and cells were incubated at 37C for 1 h with fresh medium and 20 μl of cell titer blue reagent. Viability was measured spectrophotometrically using a

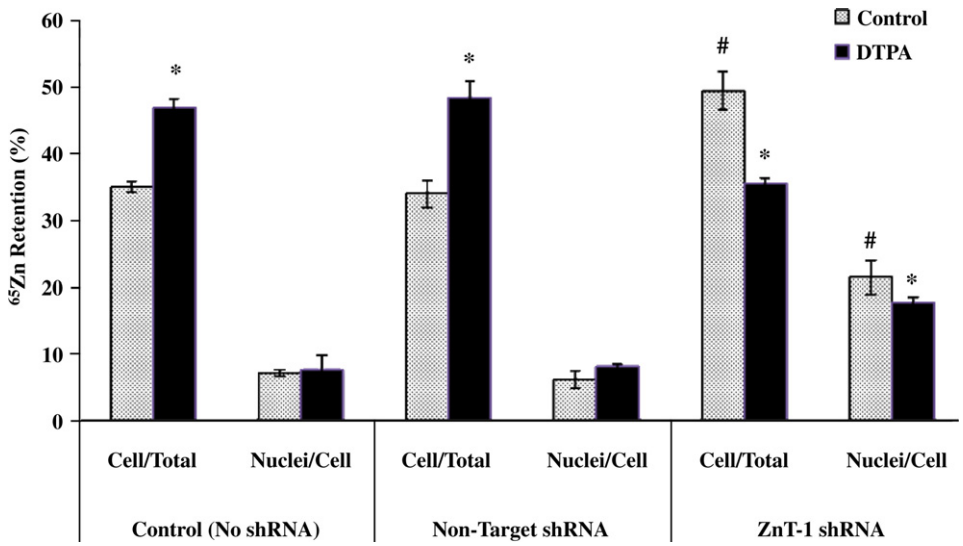

Fig. 4. Effects of shRNA transfection and DTPA on ^{65}Zn retention in H4IIE cells. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without 50 μM DTPA for 48 h to measure retention. Values are the means ± S.D. of three experiments, each including at least six replicates of each treatment. *Significant effects of DTPA, #significant effects of ZnT-1 shRNA transfection, $P \leq .005$.

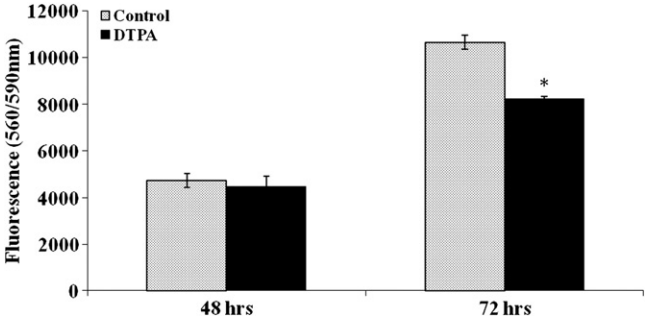

Fig. 5. Effects of DTPA on cell viability in virally transformed rat primary hepatocytes. Transformed hepatocytes were seeded into a 96-well plate at a density of 4000 cells/well. After overnight attachment, medium was removed, and fresh medium with or without 50 μM DTPA was added. After 48 or 72 h of treatment, medium was removed, and cells were incubated with cell titer blue reagent. Values are the means±S.D. of six replicates of each treatment. *Significant effect of DTPA, $P \leq .005$.

fluorescence microplate system (Molecular Devices M2, CA, USA) at an excitation of 560 nm and emission of 590 nm.

2.7. Statistical analyses

One- or two-way analysis of variance with Bonferroni's post hoc test (SPSS version 13.0) was used for all analyses. An α level of $P<.05$ was considered statistically significant. All treatments were performed with three to six replicates, and each experiment was performed at least twice. Data used are means±S.D.

3. Results

3.1. DTPA increases ^{65}Zn retention in virally transformed rat primary hepatocytes

We first sought to determine whether DTPA-mediated increases in ^{65}Zn retention of cancer cells were associated with their rapid growth requirements. For this purpose, rat primary hepatocytes were transformed using the plasmid pSV3neo, which was deemed successful by their continuous growth in the presence of neomycin. Nontransfected cells died after 2 weeks. Stably transfected primary hepatocytes continued to proliferate actively in culture after 1 month of G418 selection. At this stage, we observed a doubling time of ~48 h.

Transformed cells were then used for ^{65}Zn retention experiments. DTPA treatment increased retention of ^{65}Zn in prelabeled virally transformed rat hepatocytes by 30% (Fig. 1). It did not affect the proportion of radioactivity in the nuclear fraction (Fig. 1). ^{65}Zn retention tended to increase in these cells in the absence of serum ($P<.08$), but DTPA consistently increased zinc retention (Fig. 2). Overall, transformation of hepatocytes increases retention of ^{65}Zn in the presence of DTPA, an effect similar to our previous work in cancer cell lines derived from other tissues in comparison to their corresponding primary cells [16].

3.2. ZnT-1 shRNA effectively reduces ZnT-1 expression in H4IIE cells

To better define how DTPA affects zinc flux, we conducted ZnT-1 knockdown studies since cellular zinc efflux is regulated by the plasma membrane localized zinc transporter ZnT-1. We utilized shRNA to reduce ZnT-1expression using a lentiviral plasmid pLKO.1-puro that specifically targets ZnT-1. Transfection of ZnT-1 shRNA into H4IIE cells reduced ZnT-1 mRNA and protein expression by 30% and 50%, respectively (Fig. 3). ZnT-1 expression was unaffected in cells transfected with the control shRNA. Also, no differences in the mRNA expression of GAPDH or protein expression of β-actin were observed in shRNA-transfected cells.

3.3. Reduction of ZnT-1 expression in H4IIE cells enhances DTPA-mediated ^{65}Zn efflux

Cells transfected with active or control shRNA were prelabeled with ^{65}Zn for 48 h and then treated in the presence or absence of DTPA for an additional 48 h. In the absence of DTPA, the reduction in ZnT-1 expression occurred concomitantly with a greater retention of ^{65}Zn (Fig. 4). DTPA treatment decreased zinc retention in ZnT-1 silenced cells, whereas it increased retention in control and nontarget shRNA groups (Fig. 4), suggesting that ZnT1 is dysfunctional in H4IIE cells. Likewise, knockdown of ZnT-1 increased the proportion of cellular ^{65}Zn associated with the nuclear fraction compared to control and nontarget shRNA groups. However, DTPA did not affect ^{65}Zn recovery in the nuclear fraction in any of the cells.

3.4. DTPA treatment time dependently decreases cell viability in virally transformed hepatocytes

Diethylenetriaminepentaacetic acid increases retention of ^{65}Zn in transformed primary hepatocytes (Fig. 1). We therefore examined if these changes in zinc flux would affect cell viability. Vector-transformed hepatocytes were cultured in serum-supplemented MEM and treated for 48 or 72 h with or without 50 μM DTPA. The 48-h DTPA treatment did not affect cell number of immortalized hepatocytes (Fig. 5). Nonetheless, cell number was decreased by about 20% when cells were deprived of zinc for 72 h. Thus, long-term exposure to DTPA decreased cell viability in transformed hepatocytes.

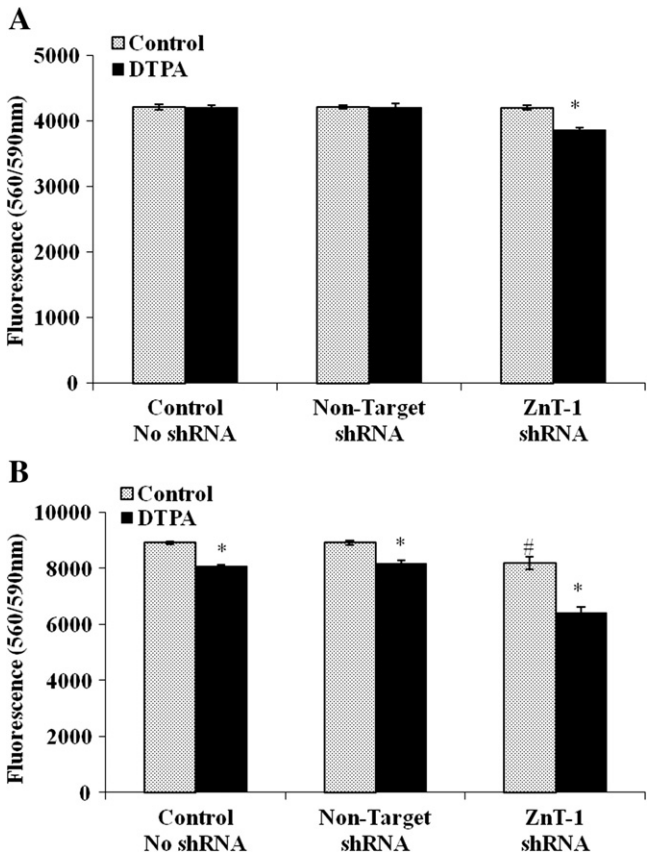

Fig. 6. Effects of shRNA and DTPA on cell viability in H4IIE cells. H4IIE cells were seeded into a 96-well plate at a density of 4000 cells/well. After overnight attachment, medium was removed, and fresh medium with or without 50 μM DTPA was added. After (A) 48 h or (B) 72 h of treatment, medium was removed, and cells were incubated with cell titer blue reagent. Values are the mean±S.D. of six replicates of each treatment. *Significant effects of DTPA, #significant effect of ZnT-1 shRNA transfection, $P \leq .005$.

3.5. Knockdown of ZnT-1 and DTPA treatment decreases cell viability in shRNA-transfected H4IIE cells

While DTPA increased zinc retention in control and nontarget shRNA groups, ZnT-1 knockdown reverses this DTPA-mediated enhancement of retention in hepatoma cells. Therefore, we examined whether these manipulations would affect cell proliferation. DTPA treatment for 48 h did not affect the viability of control cells as well as cells transfected with nontargeting shRNA (Fig. 6). However, in ZnT-1 knockdown cells, DTPA treatment decreased viability at 48 h. Treatment with the same dose of DTPA for 72 h significantly decreased cell viability in all groups relative to their respective controls (Fig. 6). In addition, at 72 h, ZnT-1 knockdown with or without DTPA treatment resulted in a significant decrease in cell number compared to other control groups.

4. Discussion

Zinc is a critical cofactor for many cellular metalloproteins, including transcription factors, oxidation–reduction regulators, metalloproteases and other proteins [1]. Disruptions in zinc homeostasis have been reported to accompany many pathological conditions including cancer [18–20]. However, no common relationship between zinc and cancer development has been identified. In an attempt to understand zinc homeostasis in malignant and nonmalignant cells, we previously investigated the effects of DTPA-induced zinc deprivation in cancer cell lines and their corresponding primary cells. While DTPA increased efflux of radioactive zinc in primary cells obtained from rat liver and anterior pituitary, it reduced efflux of zinc in established cancer cell lines from the corresponding tissues [16]. Since cellular zinc efflux is regulated by the SLC30A (cation diffusion facilitator) gene family, we investigated the role of the only identified plasma membrane zinc efflux transporter ZnT-1 [8,21] in this differential response. However, no changes in the expression of ZnT-1 with DTPA treatment were observed [16]. To further understand the differential response between primary and cancer cell lines and to probe the mechanism underlying these effects, we performed two independent studies. First, we virally transformed rat primary hepatocytes to establish a new immortalized cell line for examining the effects of DTPA, since we hypothesized that cancer cells retain more zinc under conditions of zinc deprivation as a homeostatic mechanism to meet their increased growth demands. Secondly, we reduced the expression of the ZnT-1 transporter by using stable shRNA transfection in rat hepatoma cells to examine the effects of DTPA under altered expression of ZnT-1. This was based on the hypothesis that differences in homeostatic response between primary and cancer cells are mediated by alterations in the functional capacity of this zinc transporter.

To achieve our first objective, we established a new cell line by transfection of hepatocytes with an immortalizing SV-40Tag containing plasmid pSV3neo. For successful *in vitro* immortalization, overexpression of cell cycle stimulating genes is required. Besides, mature hepatocytes have a low proliferation capacity; and therefore, strong stimulation of cell cycle progression is necessary for immortalization. In the majority of *in vitro* immortalizations of primary liver cells, the gene encoding simian virus-40 large T antigen (SV40T), an inhibitor of the cell cycle inhibitors p53 and the retinoblastoma protein, has been used [22–25]. The neo gene of the pSV3neo plasmid confers resistance to the antibiotic G418. We therefore used G418 to select for cells that harbor the pSV3neo plasmid. Successful transformation was demonstrated by continuous growth of cells in the presence of neomycin.

We then utilized these virally transformed hepatocytes for ^{65}Zn retention experiments. Transformed hepatocytes exhibited a decrease in efflux of ^{65}Zn in the presence of DTPA, similar to cancer cell lines (GH3, H4IIE and MCF-7) but different from primary cells (hepatocytes and pituitary cells) [16]. Primary hepatocytes were routinely cultured in the absence of serum, whereas the media for both H4IIE cells and the transformed hepatocytes included 10% FBS. To check whether this difference in behavior between primary and transformed hepatocytes was influenced by these different culture conditions, we treated with DTPA in the presence and absence of serum. We observed that ^{65}Zn retention in the presence of DTPA was enhanced whether or not serum was included in the media, eliminating FBS as an explanation of the different behavior of the cells.

It remains possible that the increased zinc retention in the transformed hepatocytes in the presence of DTPA might be due to the enhanced zinc requirements for continuously growing cells. Cells require zinc to propagate, and when zinc is not accessible, cells are retained in the S-phase of the cell cycle and thus not allowed to undergo mitosis [3]. Zinc supplementation has been shown to promote DNA synthesis, whereas deficiency suppresses it [18]. Therefore, it is possible that transformed cells have developed a mechanism to restrict zinc efflux under conditions of zinc deprivation in order to preserve intracellular concentrations and continue their proliferation. This conclusion is further supported by our cell viability data. DTPA-induced zinc deprivation for 48 h did not alter cell viability compared to controls, suggesting that cells were able to continue to grow for a limited period of time. However, prolonged periods of exposure to DTPA (more than 48 h) significantly reduced the number of viable cells.

In our next study, we used an shRNA approach to knock down the expression of ZnT-1 in the H4IIE cell line to further understand its role in the mechanism of DTPA-enhanced zinc retention. We were able to knock down ZnT-1 mRNA and protein expression by ~30% and ~50%, respectively, using the shRNA plasmid. To our knowledge, this is the first study that used stable transfection to knock down expression of ZnT-1. Previously, studies have demonstrated that homozygous knockout of the SLC30A1 (ZnT-1) gene in embryonic mice is lethal, suggesting that ZnT-1 is essential for normal embryonic development [26]. Reduction of ZnT-1 expression in these studies by 50% still permitted cell growth, although a small reduction in the number of viable cells was observed after 72 h. Hepatoma cells are known to have lower levels of zinc compared to other cancer cell lines [27,28], and they may be less susceptible to zinc toxicity. It appears that these cells can survive even with partial expression of ZnT-1. Interestingly, zinc deprivation with DTPA is associated with decreased cell viability when expression of ZnT-1 is reduced. It may be worth investigating if similar effects can be observed in an *in vivo* model since some cancers like breast cancer are associated with low serum zinc levels but high zinc concentrations in the breast tissue [29,30]. Targeting ZnT-1 under these circumstances may reduce tumor growth.

Knockdown cells were also used for exploring the role of ZnT-1 in the effects of DTPA on zinc flux. Greater zinc retention was observed in ZnT-1 knockdown H4IIE cells in the absence of DTPA compared with control and nontarget shRNA groups, consistent with its role as a zinc exporter. However, in the presence of DTPA, zinc retention in ZnT-1 knockdown cells significantly decreased, whereas it increased in control and nontarget shRNA groups. This loss of zinc could underlie the reduced viability of these cells. Also, knockdown of ZnT-1 significantly increased nuclear retention of ^{65}Zn compared to control and nontarget shRNA groups in a manner unaffected by DTPA. A role for ZnT-1 relative to the nucleus has not been described previously. Were it to function in the nuclear membrane, it would be expected to transport zinc into the nucleus, in line with its role in reducing cytoplasmic zinc. Thus, reducing ZnT-1 expression might be expected to reduce rather than enhance nuclear retention of ^{65}Zn. This paradox will be explored in future studies. In any event, these results with the knockdown cells suggest that ZnT-1 expression is crucial for maintaining zinc homeostasis, in particular the enhanced retention

of zinc by transformed cells in response to the reduced availability of zinc engendered by DTPA. Although ZnT-1 has been recognized as the efflux transporter, recent studies have suggested that its function may vary depending on the intracellular zinc levels and/or may be dependent on an outwardly directed Zn^{2+} gradient [31,32]. In addition, ZnT-1 has been observed to inhibit influx of zinc through the L-type calcium channel [33,34]. Our study demonstrates that when ZnT-1 expression is reduced, there is an increased zinc accumulation, confirming that ZnT-1 is an efflux transporter. However, for DTPA to stimulate retention of zinc and reduce efflux, functional ZnT-1 is required.

In summary, we were able to transform primary hepatocytes into an established cell line. Our findings reveal that, with transformation, cellular zinc homeostasis is altered, supporting the hypothesis that cancer cells retain more zinc to meet their growth demands under conditions of zinc deprivation. We were also able to down-regulate the expression of ZnT-1 using a stable transfection method. Knockdown experiments suggest that ZnT-1 behaves as an efflux transporter and that the ability of cancer cells to retain zinc under conditions of zinc deprivation for maintaining cell growth is dependent upon full expression of ZnT-1. Since DTPA does not change expression of ZnT-1 in intact cells, presumably it must influence the functional capacity of this zinc transporter.

Acknowledgments

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