Original Paper

Protective Effect of Znt7 on High Glucose-Induced Epithelial-to-Mesenchymal Transition in Renal Tubular Epithelial Cells

Xiuli Zhang^{a,b,c} Xu Lian^d Dan Liang^e Lianzhi Zhang^a Shengquan Liu^e
Lina Yang^f Zhi-Hong Chi^a Harvest F. Gu^g

^aDepartment of Pathophysiology, China Medical University, Shenyang, Liaoning, ^bDepartment of Nephrology, Benxi Center Hospital, Benxi, Liaoning, ^cKey Laboratory of Medical Cell Biology, Chinese Ministry of Education, China Medical University, Shenyang, Liaoning, ^dDepartment of Endocrinology, The Affiliated Hospital of Mudanjiang Medical University, Mudanjiang, Heilongjiang, ^eTroops of 95988 Unit, Changchun, Jilin, ^fDepartment of Geriatrics, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning, China, ^gDepartment of Clinical Science, Intervention and Technology, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden

Key Words

Epithelial-to-mesenchymal transition • High glucose • Renal tubular epithelial cells • SiRNA • The MAPK/ERK pathway • The TGF- β /Smad pathway • Zinc transporter 7

Abstract

Background/Aims: Evidence from our and other groups has demonstrated that zinc transporter 7 in SLC30 family (ZnT7) inhibited epithelial-to-mesenchymal transition (EMT) and apoptosis in rat peritoneal mesothelial cells (RPMCs) under high glucose (HG) concentration. In the present study, we investigated the effect of ZnT7 on EMT of renal tubular epithelial cells (RTECs) in an *in vitro* model of diabetic nephropathy (DN). **Methods:** A dual-fluorescent staining protocol was used for detection of ZnT7 in a normal rat kidney tubular epithelial cell line (NRK-52E cells). EMT was induced with HG (30 mM). NRK-52E cells were transfected with plasmids codifying for hZnT7-EGFP and interfering RNA for determination of the effect of ZnT7 over-expression and silencing, respectively. Expression of ZnT7, activation of the MAPK/ERK and TGF- β /Smad pathways were analyzed with by means of Western blot. **Results:** ZnT7 was localized in the perinuclear region and Golgi apparatus. In HG-induced EMT of NRK-52E cells, ZnT7 was up-regulated. Over-expression of ZnT7 led to inhibition of HG-induced EMT, while knock-down of ZnT7 increased EMT. Furthermore, knock-down of ZnT7 and increased HG-induced EMT was accompanied by activation of the MAPK/ERK and TGF- β /Smad pathways. **Conclusion:** The present study provides evidence that ZnT7 has a protective effect over EMT

Prof. Zhi-Hong Chi
and Prof. Harvest F. Gu

Department of Pathophysiology, China Medical University, No.77, Puhe Road, Liaoning (China); Department of Clinical Science, Intervention and Technology, Karolinska University Hospital, Karolinska Institutet, Stockholm, 14186 (Sweden); E-Mail chizhihong2015@126.com, harvest.gu@ki.se

of RTECs in DN and suggests that the inhibition of HG-induced EMT may be achieved through the MAPK/ERK and TGF- β /Smad pathways. Thereby, ZnT7 could be a potential target for translation medicine and prevention program in DN.

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Introduction

Zinc (Zn) is an essential element in nucleic acid metabolism, cell replication, tissue repair and growth, while zinc homeostasis in the body is mainly regulated by zinc transporter systems, including zinc transporters of SLC30 family [1, 2]. In this family, ZnT7 is the 7th member and expressed in brain, heart, lung, liver, kidneys, spleen, small intestine and peritoneum [3, 4]. Moreover, ZnT7 is presented in the face of the Golgi apparatus and functionally transports cytoplasmic zinc into the Golgi network [4, 5]. The trans-Golgi network (TGN) is part of the secretory pathway of eukaryotic cells which is distinct from the Golgi stack and important in the later stages of protein secretion. Trans-Golgi network 38 (TGN38) is an integral membrane protein and predominantly localized to the TGN in NRK cells [6]. Interestingly, a few reports have implicated that ZnT7 may be involved in the regulation of insulin and glucose metabolism. In 2010, Huang L et al. reported that ZnT7 was co-expressed with insulin in the pancreatic islets [7]. Two years later, her group demonstrated that insulin secretion was decreased while insulin resistance was increased in ZnT7 KO mice, which resulted in glucose intolerance [8]. Later on, we analyzed all ZnT members in SLC30 family and observed for the first time that ZnT7, under the condition of high glucose (HG), inhibited the epithelial-to-mesenchymal transition (EMT) in rat peritoneal mesothelial cells (RPMC) [9]. We further demonstrated that ZnT7 might inhibit HG-induced apoptosis by activation of the PI3K/Akt pathway [10]. Very recently, Tuncay et al. provided evidence that ZnT7 up-regulation was observed in hyperglycemia, and alteration of ZnT7 activity could be involved in the pathogenesis of cardiac dysfunction in diabetes [11].

Diabetes is characterized by high plasma glucose levels resulting from defective insulin secretion, insulin resistance or both [12]. The patients with diabetes often develop diabetic nephropathy (DN), which is a micro-vascular complication and causes the progressive renal fibrosis, eventually reducing functional renal mass [13]. In DN, the fibrosis develops in the glomerulus and tubulointerstitium of kidneys. In general, the renal fibrosis in DN is considered as irreversible [14, 15] although the pathogenesis of DN is not fully understood. Recent studies have demonstrated that HG could induce the complex progress of proinflammatory and profibrotic stimuli EMT of tubular epithelial cells in kidneys [16, 17]. Furthermore, the MAPK/ERK and TGF- β /Smad signaling pathways have been reported to play a vital role in the EMT [18, 19]. HG could up-regulate the expression of TGF- β 1, which is a strong inducer of EMT in the renal tubular epithelial cells [20]. Taking together the information from the studies of ZnT7 described above, we have a hypothesis that ZnT7 may have the protective effect on EMT of RTEC, which acts as an important mechanism in DN.

In the present study, we first detected the localization of ZnT7 in a normal rat kidney tubular epithelial cell line (NRK-52E cells). We then analyzed ZnT7 activities in the cells under HG conditions. We further investigated the possible role of ZnT7 in the TGF- β /Smad and MAPK/ERK pathways. The aims of this study were to test our hypothesis and to accumulate the information for the application of ZnT7 as a potential target for translation medicine and prevention program in DN.

Materials and Methods

Cell culture

Normal rat kidney tubular epithelial cell line (NRK-52E) was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; low glucose;

Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum, 4 mM L-glutamine, and 1% penicillin/streptomycin. The cells were cultured at a density of 5×10^3 cells/well in 6-well culture plates. Near confluent NRK-52E cells were then transferred to serum free DMEM medium for overnight starvation prior to each experiment.

Assessment of cell viability

Cell viability was measured by quantitative colorimetric assay with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in 96-well plates (Fig. 1). Briefly, at the indicated time after treatment, 10 μ l MTT (final concentration, 500 μ g/mL) was added to the medium and incubated at 37 °C for 3 h. The MTT solution was removed and 100 μ l dimethyl sulfoxide (DMSO) was added to dissolve the colored formazan crystals for 15 min. The absorbance at 490 nm of each aliquot was measured using a Sunrise RC micro plate reader (TECAN, Switzerland).

Fig. 1. MTT measurement of the cell viabilities.

Transfection and RNA interference

We cloned hZnT7 into enhanced green fluorescence protein (EGFP) vector and sub-cloned hZnT7 or hZnT7-EGFP into pcDNA3.1/myc-hisA vector. The presence of the hZnT7 RNA in the cells was confirmed by RT-PCR. ZnT7 siRNA (Stealth RNAi) was prepared by Invitrogen (USA). The sequence of siRNA targeting a specific sequence in ZnT7 mRNA was 5'-GCCAUAGUCACGAAGCCAATT-3'. The scrambled siRNA sequence was 5'-UUGGCUUCGUGACUAUGGCTT-3'. The target sequences of ZnT7 siRNA and control ZnT7 siRNA were searched with BLAST in the GenBank database (accession ID: NM_133496). All transfection experiments were accomplished in independent triplicates in accordance with the manufacture's protocol for Lipofectamine 2000 and described previously (5). Cells were allowed to recover in DMEM for 24 h after transfection.

Immunofluorescence analysis

For single labeling with α -SMA or E-cadherin, the cells were preincubated with normal donkey serum (NDS) (1:20) for 1 h and then incubated with mouse α -SMA antibody (1:50) overnight. The sections were incubated with Texas Red-conjugated donkey anti-mouse IgG (1:50) for 2 h after several rinses. The cells were mounted with an anti-fading mounting medium and examined using a macro zoom fluorescence microscope (MVX10, Olympus, Japan). Images were taken using a microscope equipped with a digital color camera (DP71, Olympus, Japan). For double labeling, the cells were preincubated with NDS for 1 h and then incubated overnight in a monoclonal anti-trans-Golgi network (TGN38) antibody and ZnT7 (1:50). The cells were incubated for 2 h with a mixture of secondary antibodies, FITC-conjugated donkey anti-rabbit IgG (1:50, for labeling ZnT7) and Texas Red-conjugated donkey anti-mouse IgG (1:50, for labeling TGN38). The sections were mounted with an anti-fading mounting medium and examined using a confocal laser scanning microscope (SP2, Leica, Germany). Excitation filters for FITC (488 nm) and Texas-Red (568 nm) were used. Images were processed using an Adobe Photoshop program.

Western Blot

NRK-52E cells were lysed in RIPA buffer (ThermoScientific, Waltham, MA, USA). The protein concentration was determined by DC Protein Assays (BioRad, Hercules, CA, USA) in accordance with manufacturers' instructions. The used primary antibodies were summarized in Table 1. These separated proteins in SDS-PAGE were electrotransferred to Hybond-polyvinylidene fluoride (PVDF) membrane. The soaked PVDF membrane was then incubated in TBST containing primary antibodies overnight at 4°C,

Table 1. The primary antibodies used in the experiments

Antibody	ID number	Manufacturer	Concentration
rabbit monoclonal anti-ZnT7	#SAB1303313	Sigma-Aldrich, St. Louis, MO, USA	1:400
rabbit polyclonal anti-vimentin	#SAB4503081	Sigma-Aldrich, St. Louis, MO, USA	1:250
mouse monoclonal anti- α -SMA	#SAB5500002	Sigma-Aldrich, St. Louis, MO, USA	1:400
mouse monoclonal anti- β -actin	#BS6007MH	Bioworld Technology, Inc., St. Louis Park, MN, USA	1:400
rabbit monoclonal anti-E-cadherin	#SC-71008	Santa Cruz Biotechnology, Inc. USA	1:400
rabbit polyclonal anti-phospho p38	#SAB450449	Sigma-Aldrich, St. Louis, MO, USA	1:1000
rabbit polyclonal anti-total JNK	#SAB4200176	Sigma-Aldrich, St. Louis, MO, USA	1:1000
rabbit polyclonal anti-phospho JNK	#SAB4504449	Sigma-Aldrich, St. Louis, MO, USA	1:1000
rabbit polyclonal anti-total ERK	#SAB1305560	Sigma-Aldrich, St. Louis, MO, USA	1:800
rabbit polyclonal anti-phospho ERK1/2	#SAB1306604	Sigma-Aldrich, St. Louis, MO, USA	1:800
rabbit monoclonal anti-phospho Smad2	#SAB4300251	Sigma-Aldrich, St. Louis, MO, USA	1:800
rabbit monoclonal anti-phospho Smad3	#SAB4504210	Sigma-Aldrich, St. Louis, MO, USA	1:800

washed with TBST buffer and incubated at room temperature for 2 hours in TBST containing horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit IgG Ab (Santa Cruz Inc, USA). The membranes were incubated in ECL (Pierce, Thermo Co. Ltd, USA) reagent for HRP (30s) and exposure to autoradiography film for visualization of the bands. The relative amounts of various proteins were analyzed. The results were quantified by Quantity One Software.

Zinc analysis

To analyze the distribution and concentration of zinc in the cells, N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ) fluorescence staining was performed as described previously [21]. TSQ is a one-step staining procedure and by the stoichiometric formation of zinc. Therefore, this method can be used for histochemically visualizing and quantitatively estimating the reactive zinc in tissues. The cultured NRK-52E cells were immersed in a solution of 4.5 μ M TSQ (Molecular Probes, Eugene, OR, USA) in 140 mM sodium barbital and 140 mM sodium acetate buffer (pH 10.5) for 1 min. TSQ binding was imaged with a fluorescence microscope.

Statistical Analysis

Statistical analysis was performed using SPSS (Version 18, IBM Corporation, Armonk, NY, USA). Data were expressed as means $\pm$ standard error (SEM). Variance was homogenous for use of standard ANOVA methodology. After statistical significance was established by ANOVA, individual comparisons were made using Tukey's multiple comparison test. The level of significance was defined at $P < 0.05$.

Results

Intracellular Zn^{2+} levels in HG-stimulated NRK-52E cells

We evaluated free intracellular Zn^{2+} levels in normal and HG-stimulated NRK-52E cells at concentration of 30 mM for 12 h, 24 h, 48 h, and 72 h, respectively. TSQ fluorescence staining showed that the intracellular Zn^{2+} levels in HG-stimulated NRK-52E cells after 24 h were significantly decreased compared with non-stimulated NRK-52E cells (Fig. 2 a-f).

ZnT7 expression levels in HG-stimulated NRK-52E cells

We examined ZnT7 expression levels with Western blot under non-stimulated and HG conditions and demonstrated that ZnT7 expression was increased in HG-stimulated NRK-52E cells after 48 h (Fig. 3a and b). To determine whether ZnT7 protected HG-induced EMT, we analyzed the effects of HG on EMT by modulating ZnT7 expression in the cells with both siRNA knock-down and over-expression techniques. ZnT7 expression at 48 h by using siRNA knock-down was reduced by approximately 65% (Fig. 3c and d), while the highest expression level after transfection of hZnT7-EGFP was found at 48 h (Fig. 3e and f).

Fig. 2. Intracellular Zn²⁺ levels in HG-stimulated NRK-52E cells. Stimulation of high glucose (HG at 30 mM) in NRK-52E cells was performed for 12 h, 24 h, 48 h, and 72 h (n=10), respectively (Fig. 2a-e). Relative TSQ fluoreescaping scores indicating free intracellular Zn²⁺ levels were analyzed (n=10) and summarized in Fig. 2f. Scale bars=10 μ m. *P<0.05 vs. control group; **P<0.001 vs. control group.

Fig. 3. ZnT7 expression levels in HG-stimulated NRK-52E cells. After NRK-52E cells were treated with 30 mM HG for 24 h, 48 h, 72 h and 96 h, relative ZnT7 protein expression levels were assessed with Western blot. The amount of protein loaded is 20 μ g (Fig. 3a and b). ZnT7 expression was increased in HG-stimulated NRK-52E cells after 48 h. However, ZnT7 expression at 48 h was decreased by using siRNA knock-down (Fig. 3c and d), while the highest ZnT7 expression was observed after transfection of hZnT7-EGFP (Fig. 3e and f). Each value represents the means $\pm$ SEM (n=10). The observed molecular weights of ZnT7-EGFP, hZnT7, ZnT7 and GAPDH were 85, 65, 42 and 36 Da, respectively. All experiments were performed at least three times. *P<0.05 vs. control group; **P<0.001 vs. control group.

Dual immunofluorescence of ZnT7 and TGN38

To observe the subcellular distribution of ZnT7 in the NRK-52E cells, double labeling of ZnT7 and TGN38 (the trans-Golgi marker) was used. ZnT7 was mainly localized in the perinuclear region, which was consistent with its Golgi apparatus localization (Fig. 4a). TGN38 was also found mainly residing in the perinuclear region of the cell (Fig. 4b). The

Fig. 4. Dual immunofluorescence of ZnT7 and TGN38. The intensity of ZnT7 staining was exclusively located in perinuclear region of the cells (Fig. 4a), while the intensity of TGN38 staining was located in perinuclear region of the cells (Fig. 4b). Fig. 4c shown the stained nucleus by DAPI. Merged images indicated a co-localization of ZnT7 and TGN38 (Fig. 4d). Scale bars=10 μ m.

nucleus was stained by DAPI and observed under a fluorescence microscope as described (Fig. 4c). After merging the ZnT7 and TGN38-labeled images, ZnT7 and TGN38 immunostaining overlapped in the in the perinuclear region of the cell (Fig. 4d). This result suggested that ZnT7 subcellular localization in Golgi apparatus.

ZnT7 expression levels and Zn^{2+} in the perinuclear region

To evaluate the effect of ZnT7 on Zn^{2+} distribution, we employed TSQ fluorescence staining to visualize Zn^{2+} distribution in the perinuclear region of HG-stimulated NRK-52E cells. The images of Zn^{2+} distribution in the perinuclear region of non-HG-stimulated and HG-stimulated NRK-52E cells with either siRNA knock-down or over-expression of ZnT7 were represented in Fig. 5a-f, respectively. Zn fluorescence scores of each condition in the perinuclear region were summarized in Fig. 5g. A dispersed pattern of Zn staining was seen in the perinuclear region of the cells of HG, ZnT7 RNAi and HG/ZnT7 RNAi groups (Fig. 5b, d and f). Notably, a strong granular Zn staining was found in the perinuclear region of hZnT7 and HG/hZnT7 groups (Fig. 5c and e). The highest score of Zn fluorescence staining was detected in hZnT7 group. The fluorescent intensity in the HG/ZnT7 RNAi group was weaker than in any other group (Fig. 5f and g).

Effect of ZnT7 on HG-induced EMT

We further evaluate the effect of ZnT7 on HG-induced EMT of NRK-52E cells. After transfection of ZnT7 siRNA or hZnT-7-pcDNA3.1/*myc*-HisA, the NRK-52E cells in these two groups were treated with HG for 48 h. First, the cell viability was studied by MTT analysis and no significant difference was detected between cells transfected with hZnT7-EGFP or ZnT7 siRNA. However, after treatment with HG for 48 h, the cell viability in hZnT-7-pcDNA3.1/*myc*-HisA/HG group was higher than that in the group with HG alone, while the ZnT7/siRNA/HG group was lower than that of HG group (See Fig. 1). Next, HG treatment in the NRK-52E cells for 48 h led to cell morphological changes to a fibroblast-like shape (Fig. 5). Meanwhile, α -SMA and vimentin (the specific markers for myofibroblasts) were found to be up-regulated (Fig. 6a1-4 and b1-4), while E-cadherin (a Ca^{2+} -dependent cell adhesion molecule that plays a vital role in the maintenance of renal epithelial polarity) was down-regulated (Fig. 6c1-4). ZnT7siRNA-transfected cells showed a markedly increased expression of α -SMA and vimentin during HG-induced EMT, compared with cells just treated with HG (Fig. 6a4, b4 and c4). Whereas this HG-induced EMT can be attenuated by over-expressing ZnT7 in NRK-52E cells (Fig. 6a3, b3 and c3). These results suggest that ZnT7 expression is associated with HG-induced EMT in a way that knock down of ZnT7 increases EMT event. In contrast, over-expressing ZnT7 in NRK-52E cells significantly decreased HG-induced EMT, as evidenced by the reduced up-regulation of α -SMA and vimentin, and ameliorated expression of E-cadherin (Fig. 6d-g).

Fig. 5. ZnT7 expression levels and free Zn²⁺ distribution in NRK-52E cells. NRK-52E cells treated with HG, hZnT7, ZnT7 RNAi, HG/hZnT7 and HG/ZnT7 RNAi, respectively (Fig. 5b-f). Relative Zn fluorescing score was analyzed in each group (n=10) and summarized in Fig. 5g. Scale bars=20 μ m. *P<0.05 vs. control, **P<0.001 vs. control; ##P<0.001 vs. HG and #P<0.05 vs. HG.

Fig. 6. Effects of Transfection and RNA interference of ZnT7 on EMT and Effects of over-expression ZnT7 on expression of EMT markers. Immunofluorescence analyses of EMT markers in NRK-52E cells, including α -SMA (Fig. 6. a1-a4), vimentin (b1-b4) and E-cadherin (c1-c4). Cultures were untreated (a1-c1), exposed to HG (a2-c2), with HG+transfection of ZnT7 (a3-c3) or exposed to HG+transfection of ZnT7 siRNA (a4-c4). Results were representatives of two experiments. Magnification was $\times 200$. Scale bars=20 μ m. NRK-52E cell treatments were then classified into six groups. Western blotting analyses of EMT marker expressions in NRK-52E cells. The corresponding protein levels were assessed using densitometry and expressed in relative intensities (Fig. 6d-g). Data were expressed as mean $\pm$ SEM from at least three experiments. **P<0.001 vs. control; ##P<0.001 vs. HG; #P<0.05 vs. HG.

Effect of ZnT7 on the TGF- β /Smad Pathway

To understand whether ZnT7 mediated its effects on EMT in the NRK-52E cells through the TGF- β /Smad pathway, we analyzed p-Smad2/3 levels under HG conditions by Western blot. Under the controlled conditions, ZnT7 over-expression or ZnT7 RNAi did not enhance p-Smad2/3 levels (Fig. 7a-d). The levels of p-Smad2/3 were significantly increased in HG group compared with the control group, which robustly enhanced in HG/ZnT7 RNAi group (Fig. 7a-d). Conversely, over-expression of ZnT7 significantly inhibited HG-induced levels of p-Smad2/3 (Fig. 7a-d). To confirm the involvement of TGF- β /Smad signaling in the protective effect of ZnT7 on HG-induced EMT in NRK-52E cells, 10 μ M SB43152 (the inhibitor of TGF- β receptor I) was added to the medium of NRK-52E cells for 1 h [22]. The cells were subsequently exposed to HG for 48 h after the treatment with ZnT7 RNAi. The expected results showed that HG or HG/ZnT7 RNAi group increased the expression of p-Smad2/3 and HG-induced EMT in the NRK-52E cells, i.e. the up-regulations of α -SMA and vimentin were reduced, while the ameliorated expression of epithelial protein E-cadherin increased (Fig. 7e-j). However, the co-treatment with SB43152 effectively inhibited Smad2 and Smad3 phosphorylation and HG-induced EMT (Fig. 7e-j).

Fig. 7. Effect of ZnT7 on HG-induced TGF- β /Smad signaling pathway in NRK-52E cells and its protection of HG-induced EMT. Relative p-Smad2/3 and t-Smad2/3 expression levels were calculated and normalized to the loading control with Western blotting analyses (Fig. 7a and c). The corresponding protein levels were then assessed using densitometry and expressed in relative intensities (Fig. 7b and d). All the results were obtained from three experiments. ** $P < 0.001$ vs. control; ## $P < 0.001$, # $P < 0.05$ vs. HG. Furthermore, the cells were incubated with or without 10 μ M SB43152 for 1 h and subsequently exposed to HG for 48 h after ZnT7 RNAi for 24 h. The protein expressions of EMT markers, t-Smad2/3 and p-Smad2/3 were analyzed with Western blot (Fig. 7e-j). Quantitative analysis was performed by measuring the fluorescence intensity compared with the control. Data were expressed as mean $\pm$ SEM of at least three experiments. ** $P < 0.01$, vs. control, ## $P < 0.001$, vs. HG, # $P < 0.05$, vs. HG, ▲ $P < 0.05$, vs. HG/ZnT7 RNAi, ▲▲ $P < 0.001$, vs. HG/ZnT7 RNAi.

Effect of ZnT7 on the MAPK Pathway

The MAPK signaling pathway plays a role in EMT of the various kinds of epithelial cells and includes JNK, p38 MAPK and ERK [23-25]. We observed a robust increase of the phospho-p38, and phospho-ERK in the HG-treated NRK-52E cells compared with the control group (Fig. 8a and c). Conversely, over-expression ZnT7 significantly inhibited HG-induced phosphorylation of ERK (Fig. 8c and f) but no effect on phosphorylation of p38 MAPK was found (Fig. 8a and d). Moreover, there was no significant difference of phosphorylation of JNK in the HG, HG/ hZnT7 or HG/ZnT7 RNAi compared with the control group, respectively (Fig. 8b and e). These results suggested that ZnT7 could be involved in HG-induced EMT through the regulation of the ERK pathway. Therefore, we further studied the ERK signaling pathway. The cells were incubated with or without 10 μ M of ERK inhibitor PD98059 for 1 h [23] and then exposed to HG for 24 h after ZnT7 RNAi. As expected, when compared with the cells treated with HG alone, the co-treatment of PD98059 with HG significantly decreased the expression of ERK phosphorylation (Fig. 9a and b). Once again, we found that ZnT7 RNAi significantly increased HG-induced EMT in NRK-52E cells. However, pretreatment of these cells with PD98059 decreased the HG-induced EMT (Fig. 9a, c-e).

Fig. 8. Effect of ZnT7 on HG-induced MAPK signaling pathways. Relative p38, JNK, ERK, p-p38, p-JNK and p-ERK protein expressions were calculated with Western blot and normalized compared with the loading control (Fig. 8a-c). The corresponding protein levels were assessed using densitometry

and expressed in relative intensities (Fig. 8d-f). Data were expressed as mean $\pm$ SEM of at least three experiments. ** P <0.001 vs. control; ## P <0.001, # P <0.05 vs. HG.

Fig. 9. ZnT7 inhibits the ERK signaling pathway and protects the cells from HG-induced EMT. NRK-52E cells were pre-treated with or without 10 μ M PD98059 for 1 h and subsequently exposed to HG for 48 h after ZnT7 RNAi for 24 h. The protein expressions of EMT markers, t-ERK and p-ERK were assessed by Western blot analysis (Fig. 9a). Quantitative analysis was performed by measuring the fluorescence intensity compared with the control (Fig. 9b-e). Data were expressed as mean $\pm$ SEM of at least three experiments. ** P <0.001 vs. control; ## P <0.001 vs. HG; # P <0.05 vs. HG; ▲ P <0.05 vs. HG/ZnT7 RNAi; ▲▲ P <0.001 vs. HG/ZnT7 RNAi.

Discussion

In the present study, we first observed that ZnT7 not only was localized in the perinuclear region and Golgi apparatus but also up-regulated in HG-induced EMT of NRK-52E cells. We then found that ZnT7 had a protective effect on HG-induced EMT. Furthermore, we explored the cellular mechanism of ZnT7 in the MAPK/ERK and TGF- β /Smad pathways.

ZnT7 is localized on the membrane of the Golgi/endoplasmic reticulum (ER) and vesicular compartments [3-5]. The function of ZnT7 is to facilitate zinc transport from the cytoplasm into the Golgi apparatus and to regulate cellular zinc homeostasis [3]. In the present study, we used dual immunofluorescence of ZnT7 together with TGN38 and observed that ZnT7 was mainly located in the perinuclear region and Golgi apparatus of the NRK-52E cell, which is consistent with the reports from our [9, 10] and other groups [11, 26]. Furthermore, we found that ZnT7 facilitated Zn transport from the cytoplasm into the Golgi apparatus in the cells within 48 h of HG treatment. Over-expressing ZnT7 in NRK-52E cells significantly decreased HG-induced EMT, as evidenced by the reduced up-regulation of α -SMA, vimentin, and the ameliorated expression of epithelial protein E-cadherin. The levels of free zinc ions in the NRK-52E cells were significantly increased after over-expression of ZnT7.

We have previously reported that ZnT7 inhibited HG-induced EMT and apoptosis in RPMC [9, 10], and further designed the present study to understand whether ZnT7 has the similar effect but in NRK-52E cells. A previous study showed that over-expression of ZnT7 in Chinese hamster ovarian cells under high zinc conditions lead to accumulation of free Zn²⁺ in the Golgi apparatus [3]. A recent study demonstrated that hyperglycemia decreased ZnT7 activity following redistribution of cellular free Zn²⁺, which plays an important role during ER stress in cardiomyocytes [11]. In our present study, HG increased ZnT7 expression and over-expression of ZnT7 induced accumulation of free Zn²⁺ in perinuclear region and Golgi apparatus of NRK-52E cells. These results implicated that ZnT7 transport zinc into Golgi apparatus in the HG-induced EMT in NRK-52E cells, which was consistent with our previous studies in RPMCs [9, 10]. The up-regulation of ZnT7 in NRK-52E cells suggested that ZnT7 was involved in mobilizing Zn from the cytoplasm into the Golgi apparatus and subsequently indicated a need to reduce Zn efflux from the Golgi apparatus and to maintain Zn levels during HG conditions. Another previous study demonstrated that the Golgi apparatus can influence the expression of EMT-maker proteins [27]. Considering the role of Golgi apparatus is for protein synthesis and post-translational modification, we thereby considered that ZnT7 may protect NRK-52E cells from HG-induced EMT by regulating EMT gene transcription. These results from the present study thus supported our hypothesis that ZnT7 has a protective effect on EMT of NRK-52E cells. Considering that the gene therapy for ZnT7 is not an available resource yet, Zn supplementation to diabetic patients may induce ZnT7 expression. Further investigation with transgenic animal model to induce a therapeutic increase of ZnT7 expression *in vivo* has been taken into our consideration.

To understand the possible mechanism of the protection of ZnT7 on EMT, we analyzed the key molecules of the TGF- β /Smad and MAPK/ERK pathways by using hZnT7-EGFP and ZnT7 siRNA in NRK-52E cells. Previous studies indicated that TGF- β 1 as a stronger stimulator of EMT. Inhibition of TGF- β /Smad signaling has been considered a central mechanism to prevent earlier renal fibrosis [17]. TGF- β receptor 1 phosphorylates Smad2 and 3 and subsequently associates with the common partner Smad 4, which translocate into the nucleus and activate the expression of TGF- β -responsive genes [28, 29]. Szuster-Ciesielska et al. previously reported that Zn supplementation decreases ethanol- and acetaldehyde-induced activation of liver stellate cells and inhibit liver fibrosis changes though inhibiting TGF- β /Smad signaling [30]. Maywald et al. have very recently demonstrated that zinc supplementation increased TGF- β 1-dependent regulatory T cell induction in allogeneic reaction due to a triggered intracellular zinc signal, which in association with an increased Smad 2/3 activation [31]. Data from the present study suggested that TGF- β /

Smad signaling activation might also occur through this process in NRK-52E cells with HG treatment, and also implicated that ZnT7 protect HG-induced EMT in NRK-52E cells partly by inhibiting TGF- β /Smad signaling.

The MAPK pathway is a highly conserved pathway involved in diverse cellular functions, including cell proliferation, cell differentiation and apoptosis, while JNK, p38 MAPK and ERK in this pathway are involved in a Zn-sensing process [32, 33]. The results from the present study indicated that over-expression ZnT7 significantly inhibited HG-induced expression of phospho-ERK but not phospho-p38 MAPK. There was no difference of the expression of phospho-JNK in the HG, HG/hZnT7 or HG/ZnT7 RNAi groups. A previous study showed that MAPK signaling from the Golgi apparatus, in striking contrast to the plasma membrane, generated a graded ERK output [34]. Data from our experiments confirmed that ZnT7RNAi under HG conditions triggered a rapid activation of ERK, which then initiated downstream signaling events including enhanced HG-induced EMT. Moreover, chemical inhibition of ERK with PD98059 completely suppressed HG-stimulated EMT and increased cellular injury. Therefore, we interpreted that endogenous ZnT7 protected HG-induced EMT in NRK-52E cells through inhibiting not only the activation of TGF- β /Smad but also ERK signaling pathways.

There is growing evidence suggesting that tubulointerstitial fibrosis processes play an important role in the pathogenesis of DN [13, 14]. EMT in renal tubular epithelial cells, as a phenotypic conversion, plays a major role in renal interstitial fibrogenesis under pathologic condition [35]. Recent studies indicated that hyperglycemia could induce EMT of renal tubular epithelial cells, acting as a critical mechanism of renal tubulointerstitial fibrosis in DN [15, 16]. EMT course of tubular epithelial cells consists of four key steps: (1) loss of epithelial cell adhesion; (2) de novo α -SMA expression and actin reorganization; (3) disruption of the tubular basement membrane; and (4) enhanced cell migration and invasion [17]. Although EMT is not the only process involved in fibrosis, data from the present and other studies suggest that blocking EMT may prevent, attenuate or delay fibrosis.

Conclusion

The present study provides the first evidence that ZnT7 has a protective effect on HG-induced EMT in NRK-52E cells and suggests that the activity of ZnT7 in HG-induced EMT of the NRK-52E cells is most likely involved in the MAPK/ERK and TGF- β /Smad pathways. Considering that EMT plays an important role in the development and progression of interstitial fibrosis in DN, ZnT7 may be a potential target for therapeutic intervention research of DN. Further investigation of ZnT7 in the pathogenesis of DN has been taken into our consideration.

Abbreviations

AAS (atomic absorption spectrophotometry); DCF-DA (2, 7-dichlorofluorescein diacetate); DMEM (Dulbecco's modified Eagle's medium); EMT (Epithelial-to-mesenchymal transition); EGFP (enhanced green fluorescence protein); FITC (fluorescein isothiocyanate); NDS (normal donkey serum); NRK-52E (cells, normal rat kidney tubular epithelial cells); RTEC (renal tubular epithelial cells); PBS (phosphate-buffered saline); PI (propidium iodide); RIPA (radioimmune precipitation assay); SMA (Smooth muscle cell actin); TBS (tris-buffered saline); TGF (transforming growth factor); TGN (trans-Golgi network); ZnT7 (zinc transporter 7).

Disclosure Statement

There was no potential conflict of interest relevant to this article.

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