Novel mechanism of aberrant *ZIP4* expression with zinc supplementation in oral tumorigenesis

(口腔癌における亜鉛取り込み異常に関する *ZIP4* の新規メカニズムの解明**)**

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Abstract

Zinc transporter, ZRT- and IRT-like protein 4 (ZIP4), seems related to tumor progression in some cancers. Here we found up-regulated ZIP4 in oral squamous cell carcinomas (OSCCs) compared with normal counterparts, ZIP4 expression positively correlated with tumor size, and decreased intercellular zinc and cell growth in ZIP4 knockdown cells ($P<0.05$). Furthermore, the greater cell growth after $ZnCl_2$ treatment suggested the impact of intercellular Zinc accumulation on tumor growth. Consistent with this, decreased zinc uptake by ZIP4 knockdown and a chelating agent inhibited cell proliferation. Thus, ZIP4 and intracellular zinc are essential to tumor growth, making zinc a potential therapeutic target for OSCCs.

Keywords

ZRT- and IRT-like protein 4; zinc; ZIP4 expression

Abbreviations

ZIP4, ZRT- and IRT-like protein 4; IHC, immunohistochemistry; shZIP4, ZIP4 shRNA; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine

Introduction

Zinc is an essential element in the human body that regulates numerous physiologic processes. Free zinc is important because it can easily exchange between different cellular compartments and bind to numerous proteins, leading to activation of biologic functions [1]. Zinc also is stored and released from intracellular vesicular compartments and functions as an intracellular second messenger [2, 3].

ZRT- and IRT-like protein (ZIP) transporter increase intracellular zinc by promoting uptake of zinc from the extracellular space and vesicular zinc release to the cytosol. Fourteen human ZIP transporters that are thought to facilitate zinc influx into the cytosol [4] have been reported thus far. Of them, ZIP4 plays an important function in maintaining an intracellular zinc level by the uptake of nourishment zinc from intestinal epithelium cells and release of zinc from the vesicular compartments [5]. ZIP4 also regulates the activities of the cyclic adenosine monophosphate response element-binding protein and zinc finger transcription factors [6].

ZIP4 is up-regulated in pancreatic and hepatocellular cancers and promotes the cell-cycle, cell proliferation, and invasiveness [5, 7]. Down-regulated ZIP4 increases the survival rate in pancreatic cancer xenografts [8]. In contrast, ZIP4 expression is low in prostate cancer cells, and overexpressed ZIP4 reduces cellular migratory activity [9]. Thus, ZIP4 plays pivotal functions in development and progression of several types of cancer.

We report the results of the critical analyses of the molecular subtypes of ZIP4 in oral squamous cell carcinoma (OSCC) contributing to tumor progression of OSCCs clinically here.

Materials and methods

Ethical statement

The ethical review board of Chiba University approved the protocol (protocol number, 236) for this study.

OSCC cell lines and tissue samples

We purchased Ho-1-N-1 (JCRB-0831, buccal mucosa), KOSC-2 (JCRB-0126.1, mouth floor), SAS (RBRC-RCB 1974, tongue), HSC-2 (RBRC-RCB1945, mouth), HSC-3 (JCRB-0623, tongue), HSC-4 (RBRC-RCB1902, tongue), Ho-1-u-1 (RBRC-RCB2102, mouth floor), Ca9-22 (RCB-1976, gingiva), and Sa3 (RBRC-RCB0980, upper gingiva) cell lines, derived from human OSCCs, from the JCRB cell bank (Ibaraki, Osaka, Japan) or cell bank of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). Normal oral keratinocytes (HNOKs) as previously described [9-15] used as normal control cells.

mRNA expression analysis

Total RNA extraction and cDNA generation were performed as described previously [10, 15]. As previously described, qRT-PCR (quantitative Real-Time PCR) was conducted [9-11, 16-19]. The sequences primers were used: *ZIP4*, 5'-GCTCCAGTGTGTGGGACA-3' and 5'-GCCTGTTCCGACAGTCCA-3' and universal probe #46.

Immunoblotting analysis

Protein extraction and immunoblotting analysis were conducted as described previously [16-19]. The antibodies were anti-ZIP4 (LifeSpan BioSciences, Seattle, WA, USA), anti-GAPDH (sc-32233; mouse, 6C5 or sc-25778; rabbit, FL-335), anti-p21 (sc-397; rabbit,

C-19), anti-cyclin D1 (sc-20044; mouse, DCS-6), anti-cyclin E (sc-377100; mouse, E-4) (Santa Cruz Biotechnology; SCB, Santa Cruz CA, USA), anti-CDK2 (#2546; rabbit, 78B2), anti-CDK4 (#12790; rabbit, D9GE3), and anti-CDK6 (#13331; rabbit, D4S8S), anti-$p27^{KIP1}$ (#3698; mouse, SX53G8.5), (Cell Signaling Technology, Danvers, MA, USA).

IHC

As previously described, IHC and IHC scoring system were performed [20-24]. In order to decide a cut-off points of the ZIP4 IHC scores of the clinical parameters, we evaluated the OSCCs IHC scores from 100 samples by Receiver Operating Characteristic curve (ROC) analysis by EZR software [25]. Cases with a score over the cutoff point were defined as ZIP4-positive.

Transfection with shRNA plasmid

ZIP4 shRNA (shZIP4) or control shRNA (shMock) vectors were purchased from Santa Cruz Biotechnology. The two OSCC-derived cell lines (Ca9-22 and Sa3) were transfected with shZIP4 or shMock vectors using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Cell growth

As previously described, we analyzed cell growth in the shZIP4 and shMock cells to evaluate the effect of the ZIP4 knockdown on cell growth [10, 15].

Zinc staining

To evaluate zinc uptake, we stained shZIP4 and shMock cells for zinc. Briefly, these cells were seeded in 6-cm dishes at a density of 1×10^4 viable cells after serum starvation for 24 hours. Before the assay, shZIP4 and shMock cells were treated with the membrane-permeable zinc chelator, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, 4 μM), at 37 °C for 1 hour to remove intracellular zinc [8, 26]. After washing with PBS, the cells were grown in the culture medium with 5 μM of $ZnCl_2$ for 96 hours. The cells were incubated in the zinc-deficient medium containing 25 μM of Zinquin, a zinc-specific fluorescent dye (Dojindo Laboratories, Kamimasuki, Kumamoto, Japan) at 37 °C for 30 minutes, and photographed using a digital Nikon Eclipse TE2000-U (Nikon, Minatoku, Tokyo, Japan) [27-31].

Cell-cycle analysis

To synchronize cells of the G0/G1 or G2/M transition, as previously described, cell-cycle analysis was conducted [16-19, 32, 33].

Zinc treatment and cell growth

To investigate the effect of zinc on cell proliferation, we performed cell growth assays for shZIP4 and shMock cells after treatment with $ZnCl_2$. Briefly, the cells were seeded in 6-cm dishes at a density of 1×10^4 viable cells after serum starvation for 24 hours. The cells were treated with TPEN (4 μM) at 37 °C for 1 hour. After washing with PBS (phosphate bufferes saline) to remove excess TPEN, the cells were incubated with DMEM in the presence of 0- to 10 μM of $ZnCl_2$ for the indicated time.

Statistical analysis

The statistical significance was determined using the Mann-Whitney U-test. Relationships between the ZIP4 IHC scores and clinicopathological profiles were analysed using the Mann-Whitney U-test and Student's t-test.

Results

Evaluation of ZIP4 mRNA and protein expression in OSCC-derived cell lines and primary OSCCs

To investigate the status of ZIP4 expression, we performed qRT-PCR and immunoblotting analyses using nine OSCC-derived cell lines and HNOKs. *ZIP4* mRNA was up-regulated significantly in all cell lines compared with the counterpart (Fig. 1A, $P<0.05$). ZIP4 protein expression also significantly increased in all cell lines compared with the counterpart (Fig. 1B, $P<0.05$). Strong ZIP4 immunoreactivity was detected in the cell membrane and cytoplasm of primary OSCCs; however, normal oral tissues showed almost weak immunoreactivity (Fig. 1C-1). The ZIP4 IHC scores in OSCCs and adjacent normal oral tissues ranged from 46 to 196 (median, 117.50) and 3 to 78 (median, 27.50). We showed representative IHC data for ZIP4 protein in normal oral tissue and primary OSCCs (Fig. 1C-2, -3). We used ROC curve analysis in order to determine the optimal cutoff point from the IHC scores (Fig. 1D). This data showed that the area under the curve (AUC) was 0.7115 (95% confidence interval, 0.5541-0.869) and the cutoff value was 117.0. ZIP4-positive OSCCs were associated significantly with the primary tumor size among the clinical parameters (Fig. 1E, $P<0.05$).

Establishment of ZIP4 knockdown cells

shZIP4 or shMock vectors were used for the transfections into two OSCC cell lines (Ca9-22 and Sa3) because up-regulation of ZIP4 was frequently observed in OSCC-derived cells (Fig. 1A, B). qRT-PCR and immunoblotting analyses showed that the ZIP4 mRNA and protein expression levels in shZIP4 cells were significantly lower than that in shMock cells (Fig. 2A, B, respectively; $P<0.05$).

Cell growth of ZIP4 knockdown cells

To investigate the effect of ZIP4 knockdown on cell proliferation, we performed a cell growth assay that showed that the cell growth in shZIP4 cells were lower than that in shMock cells (Fig. 2C, $P<0.05$).

Accumulation of zinc in ZIP4 knockdown cells

Zinc fluorescent imaging was performed to assess the accumulation of intracellular zinc in shZIP4 and shMock cells. Cytosolic fluorescence was observed in the shMock cells after treatment with $ZnCl_2$ (5 μM) for 96 hours, whereas fainter fluorescence was observed in shZIP4 cells (Fig. 2D).

Cell-cycle analysis of shZIP4 cells

We assessed the cell-cycle distribution by flow cytometers (Accuri C6) because the cell growth of the ZIP4 knockdown cells decreased. The number of shZIP4 cells of the G1 phase was significantly greater than in shMock cells (Fig. 2E, $P<0.05$). These results indicated that shZIP4 inhibited cell proliferation by cell-cycle arrest of the G1 phase. When we assessed the expression levels of the cyclin-dependent kinase inhibitors (CDKIs) ($p21^{Cip1}$ and $p27^{Kip1}$), cyclins, and CDKs, the CDKIs were up-regulated, and cyclin D1, cyclin E, CDK2, CDK4, and CDK6 were down-regulated in the shZIP4 cells (Fig. 2F).

Cell growth after zinc treatment in a dose-dependent manner

Before the cell proliferation assay, we treated the cells with TPEN to remove intercellular zinc. The cells then were cultured in the medium with the indicated concentrations of $ZnCl_2$

(0-10 μM) for 96 hours. The optimal concentration was determined to be 5 μM of $ZnCl_2$, because there was no cell growth in the 10-μM $ZnCl_2$ treatment (Fig. 3A). There was no significant difference in cell growth between the shMock and shZIP4 cells without $ZnCl_2$ treatment (Fig. 3B). The cell growth of the shMock cells was significantly greater than that of the shZIP4 cells after $ZnCl_2$ treatment (Fig. 3C). The data indicated that intracellular zinc plays a critical role in cell growth.

Cellullar growth after TPEN treatment

To examine the effect of TPEN on the cell growth in the shMock and shZIP4 cells, the cells were treated with TPEN (4 μM) for 1 hour before the cell proliferation assays (0 or 5 μM $ZnCl_2$; 96 hours). After treatment with TPEN, the number of cultured cells decreased dramatically in the zinc depletion medium (FBS-, TPEN+, $ZnCl_2$-) compared with control cultures (Fig. 4, $P<0.05$). However, the cell growth increased significantly in the 5-μM $ZnCl_2$ medium (FBS-, TPEN+, $ZnCl_2$+). The data indicated that depletion of intercellular and extracellular zinc led to inhibited cell growth.

Discussion

The present study provided the first evidence that ZIP4 up-regulation occurs in OSCCs and is positively correlated with tumor size. ZIP4 knockdown experiments also showed that ZIP4 controlled cell proliferation by arresting cell-cycle progression of the G1/S phase, suggesting that ZIP4 plays an important role in tumor size in human OSCCs. Depletion of intracellular zinc using TPEN, a chelating agent, showed similar results to the ZIP4 knockdown experiment (Fig. 5).

ZIP4 knockdown cells not only had less intracellular zinc (Fig. 2D) but also low cell growth activity (Fig. 2C). Zinc deficiency is related to diverse disorders, such as an impaired immune response; retardation of growth, wound healing, and skeletal development [5, 34]; and decreased activity of deoxythymidine kinase associated with DNA synthesis. A previous study reported that zinc was required for accumulation and maintenance of a protein that mediated cellular entry into the cell-cycle of the S phase [35]. Our data also showed cell-cycle arrest of the G1/S phase (Fig. 2E) in shZIP4 cells, suggesting that zinc, the target of ZIP4, plays an essential role in cell proliferation. The concentration of intracellular free zinc is strictly regulated, which is known as zinc homeostasis [36]. However, the mechanisms responsible for regulating zinc homeostasis are not well established. Since previous studies have indicated that zinc transport and metabolism are related to progression of cancer [37, 38], our TPEN treatment might be a new therapeutic tool for several types of cancers, including OSCCs. Further studies are warranted to elucidate the zinc network and the effects of zinc on storage and release on target protein regulation in OSCCs.

Previous studies have reported that liver cancers have high ZIP4 expression and low zinc levels in the tumor tissues; however, prostate cancers have low ZIP4 expression and low zinc levels in the tumor tissues [7, 39-41]. This apparent difference between those studies

might be due to different tissue and organ sites, which have different mechanisms of zinc uptake. Therefore, further studies are needed to study the ZIP4 pathway in the cancer microenvironment.

In conclusion, the present study explored the molecular function of ZIP4 in oral cancer. Our data indicated that overexpression of ZIP4 might directly affect tumor growth in OSCCs and that ZIP4 is a potential biomarker of aggressive tumor progression and a potential therapeutic target for OSCCs. In addition, zinc depletion by ZIP4 knockdown and TPEN treatment showed dramatic inhibitory effects on cell growth in oral cancer. Thus, the zinc metabolism plays an active part as a new target for cancer progression.

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Author contributions

SI, AK, YS, NK, TT, and KU designed the research; SI, AK, NK, TT, MS, and HT performed the experiments; SI, AK, DN, and KU analyzed the data; SI, AK, NK, TT, TS, MI, and KU wrote the paper.

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Legends

Fig. 1. Evaluation of ZIP4 expression in OSCC-derived cell lines and primary OSCCs. (A) Quantification of ZIP4 mRNA expression in OSCC-derived cell lines by qRT-PCR analysis. The data are expressed as the mean ± SEM of triplicate results (*P<0.05, Student's t-test). (B) Immunoblotting analysis of ZIP4 protein in OSCC-derived cell lines and HNOKs. Densitometric ZIP4 protein data are normalized to the GAPDH protein levels. The values are expressed as a percentage of the HNOKs. (C-1) ZIP4 status in primary OSCCs (n=100) and normal counterparts by the IHC (*P<0.05, Student's t-test). Representative IHC data of normal oral tissue (C-2) and OSCC (C-3). (D, E) ROC curve analysis shows that the optimal cutoff point is 117.0 (AUC, 0.7115; 95% CI, 0.554-0.869; sensitivity, 73.1%; specificity, 66.7%). ZIP4 protein expression in primary OSCCs differs significantly (P=0.0209, Student's t-test) between T1+T2 and T3+T4.

Fig. 2. Phenotypes of ZIP4 knockdown cells. (A, B) Expression of ZIP4 mRNA and protein in shZIP4 and shMock cells (*P<0.05, Student's t-test). (C) Cell growth assay of shZIP4 and shMock cells. The data are expressed as the mean ± SEM of triplicate results (*P<0.05, Student's t-test). (D) To monitor intracellular zinc, the cells are stained with Zinquin ester, a zinc-specific fluorescence dye, after treatment with 5μM $ZnCl_2$ for 96 hours. The accumulation of intracellular zinc in the shZIP4 cells is significantly lower than that in the shMock cells. (E) Flow cytometric analysis is performed to investigate cell-cycle progression in the shZIP4 and shMock cells after synchronization at the G2/M phase using nocodazole. (F) Immunoblotting analysis shows up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$ and down-regulation of cyclin D1, cyclin E, CDK2, CDK4, and CDK6 in the shZIP4cells

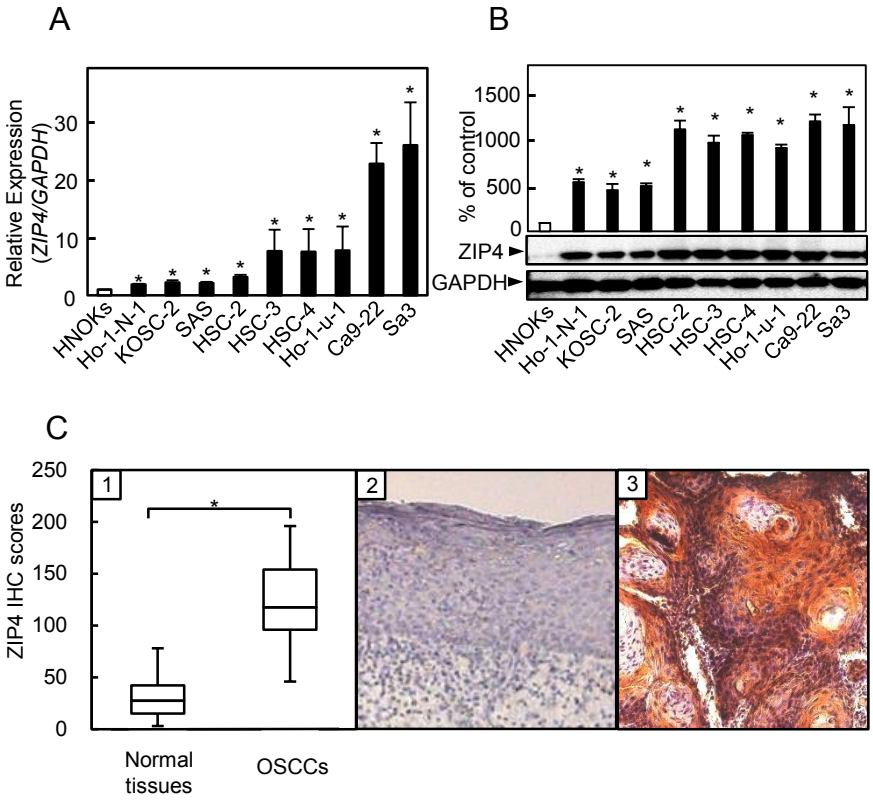
compared with the shMock cells.

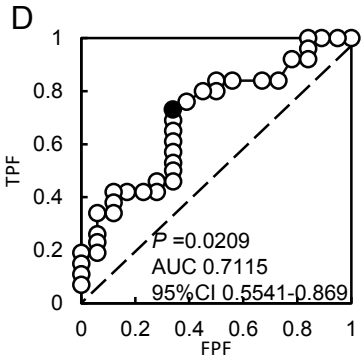
Fig. 3. Effect of zinc on cell growth. (A) Determination of the optimal concentration for cell growth. The shMock and shZIP4 cells were cultured in the medium with the indicated concentrations of $ZnCl_2$ (0-10 μM) for 96 hours. In shMock cells, 5 μM $ZnCl_2$ is the most effective concentration for cell growth. (B, C) Cell growth with and without zinc. There is no difference of cell growth between shMock and shZIP4 cells without $ZnCl_2$ treatment. The cell growth of the shMock cells is significantly higher than that of the shZIP4 cells in the 5-μM $ZnCl_2$ medium. The data are expressed as the mean ± SEM of triplicate results (*$P<0.05$).

Fig 4. Cell growth after TPEN treatment. The cells in the 6-cm dishes (1×10^4 cells/dish) were treated with 4 μM of TPEN for 1 hour at 37°C, washed with PBS, and incubated in the culture medium with 5 μM $ZnCl_2$ for 96 hours. The data are expressed as the mean ± SEM of triplicate results (*$P<0.05$). After treatment with TPEN, the number of cultured cells decreases dramatically in the zinc-depleted medium (FBS-, TPEN+, $ZnCl_2$-) compared with control cultures, whereas a significant increase in cell growth is seen in the 5-μM $ZnCl_2$ medium (FBS-, TPEN+, $ZnCl_2$+). These data indicate that depletion of intercellular and extracellular zinc led to inhibited cell growth.

Fig 5. The effect of zinc in ZIP4 and TPEN treatment. ZIP4-positive OSCC overexpression causes increased levels of intracellular zinc and cell growth compared with ZIP4-negative OSCC cells. ShZIP4 cells decrease zinc uptake and cell growth. After chelation for zinc with TPEN, the cell growth decreases.

Figure 1





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	No.patients (%)			
	total	**ZIP4 (-)**	**ZIP4 (+)**	***P* value**
T1＋T2	37	9 (24%)	28 (76%)	**0.0209**
T3＋T4	63	4 (6%)	59 (94%)	

Figure 2

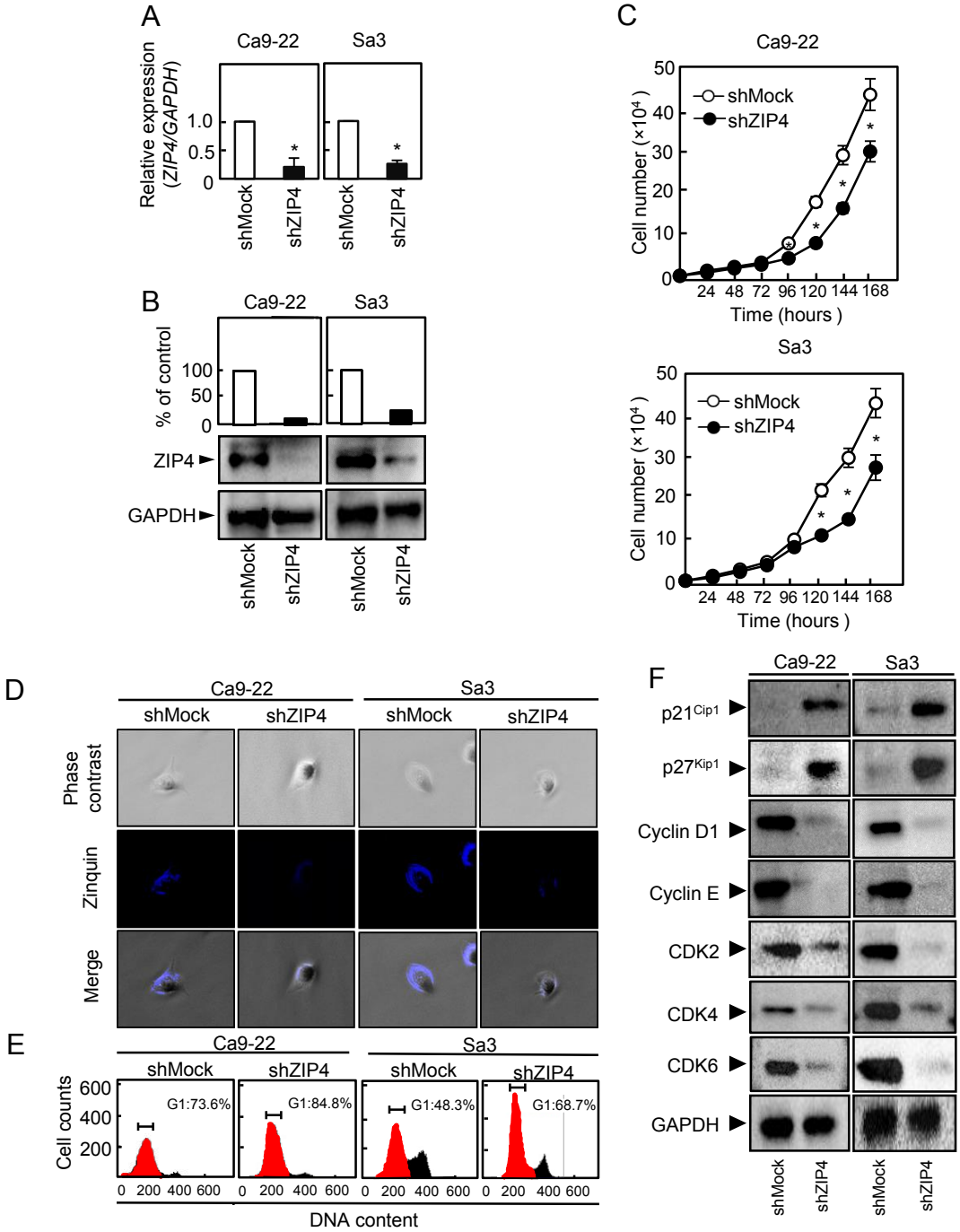

A
Ca9-22
Sa3
Relative expression (ZIP4/GAPDH)
1.0
0.5
0
shMock
shZIP4
B
% of control
100
50
ZIP4
GAPDH
C
Cell number (×10⁴)
shMock
shZIP4
Time (hours)
24 48 72 96 120 144 168
D
Phase contrast
Zinquin
Merge
E
Cell counts
G1:73.6%
G1:84.8%
G1:48.3%
G1:68.7%
DNA content
F
p21Cip1
p27Kip1
Cyclin D1
Cyclin E
CDK2
CDK4
CDK6
GAPDH

Figure 3

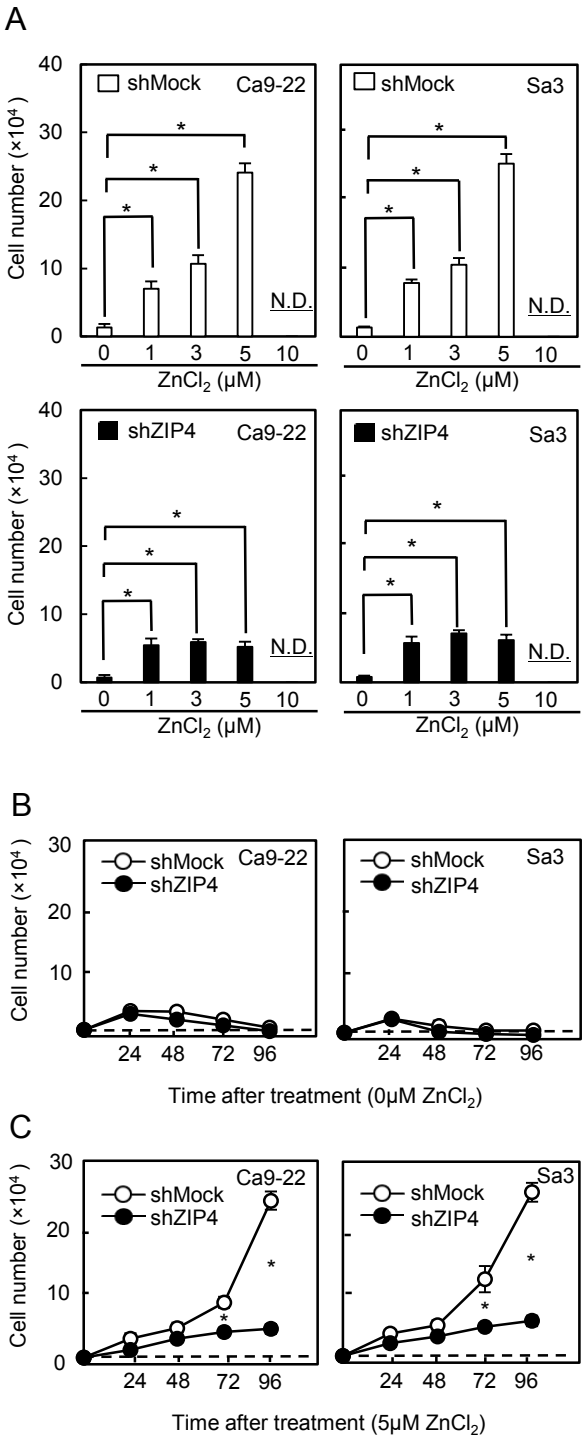
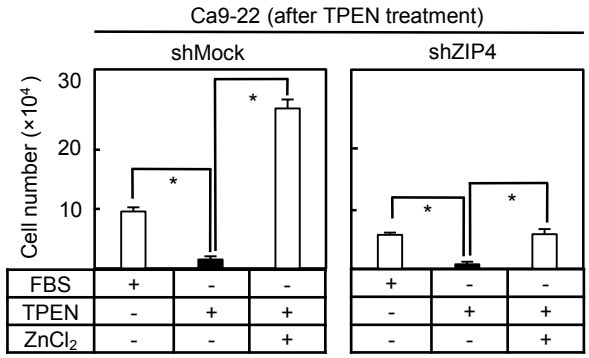

A
shMock
Ca9-22
Sa3
shZIP4
Cell number (×10⁴)
N.D.
ZnCl2 (μM)
0 1 3 5 10
B
Time after treatment (0μM ZnCl2)
24 48 72 96
C
Time after treatment (5μM ZnCl2)

Figure 4


Ca9-22 (after TPEN treatment)
shMock
shZIP4
Cell number (×10⁴)
30
20
10
*
FBS
TPEN
ZnCl2

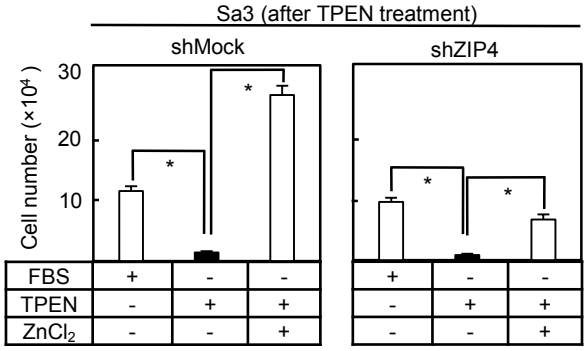
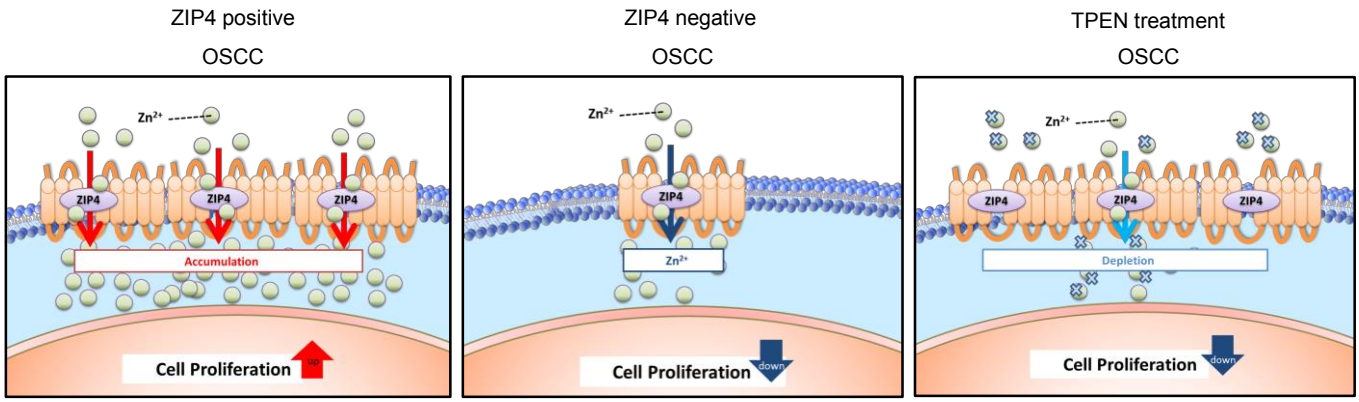

Sa3 (after TPEN treatment)
shMock
shZIP4
Cell number (×10⁴)
30
20
10
*
FBS
TPEN
ZnCl2

Figure 5


ZIP4 positive
OSCC
ZIP4 negative
OSCC
TPEN treatment
OSCC
Zn2+
ZIP4
Accumulation
Cell Proliferation
up
Zn2+
ZIP4
Zn2+
Cell Proliferation
down
Zn2+
ZIP4
Depletion
Cell Proliferation
down

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