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청각유모세포에서 산화환원 항상성과 세포주기에 아연이 미치는 영향

Effects of Zinc on Redox Homeostasis and Cell Cycle in Murine Auditory Hair Cells

울 산 대 학 교 대 학 원

의 과 학 과

이 준 영

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2021년 8월

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Abstract in English

A nutrition deficiency is one of the various causes of hearing loss. Zinc is an essential element for cell proliferation, immune system, antioxidant reactions, and the maintenance of hearing ability. Our previous studies reported that the auditory brainstem response (ABR) threshold was increased in mice fed with zinc-deficient diets. Also, supplementation of zinc restored the ABR threshold shift to a normal level, and hearing loss has been restored. However, its molecular mechanism underlying the recovery of hearing loss remain to be elucidated. In the present study, we examined the detrimental effects of zinc deficiency on cell cycle progression in murine auditory cells (HEI-OC1). The treatment of HEI-OC1 cells with 0.5 μM TPEN (*N*,*N*,*N′*,*N′*-tetrakis (2-pyridylmethyl) ethylenediamine, a zinc chelator) for 24 hours inhibited cell proliferation, accumulation of intracellular reactive oxygen species (ROS), and induction of apoptosis. The inhibition of cell proliferation progression was caused by a G1/S phase arrest. Supplementation of culture medium with 5 μM $ZnCl_2$ after exposure to TPEN attenuated ROS accumulation and the cell cycle arrest caused by the zinc deficiency. Additionally, we observed an increased expression of p21 and decreased expression of cyclin E and pRb in the spiral ganglion (SG), the organ of Corti (OC), Limbus (L), and stria vascularis (SV) in the zinc-deficient mouse cochlea. These results indicated that zinc is an essential nutrient for the maintenance of redox homeostasis and proliferation via the cell cycle progression, which the dysregulation of the cell cycle contribute to hearing loss.

Keywords: Hearing loss; zinc deficiency; cell cycle arrest; mouse auditory hair cells; cochlea

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Introduction

Hearing loss is referred to as a dysfunction of the auditory system and is classified into sensorineural or conductive hearing loss. Sensorineural hearing loss (SNHL) occurs when the cochlear hair cells or nerves are damaged. Various risk factors including aging, noise exposure, ototoxic drugs, and nutrition deficiency contribute to this hearing loss [1]. The lack of supplemental nutrients such as vitamins (A, B, C, and E), magnesium, iron, and zinc (Zn^{2+}) are also known to increase the risk of hearing loss [2]. In auditory neurons, zinc is located with glutamate in both the presynaptic calyces and excitatory synapses [3]. Several studies have reported the involvement of zinc supplementation in the recovery from hearing loss. In our previous study, mice fed with a zinc deficient-diet showed an elevated auditory brain stem response (ABR) threshold, which was rescued by supplementation with a zinc-containing standard diet [4]. In addition, it has been reported that zinc supplementation recovers the hearing abilities of patients with idiopathic sudden sensorineural hearing loss (SSNHL) [5].

Zinc is an essential metal ion in various physiological processes, such as DNA synthesis, proliferation, apoptosis, and plays an antioxidant role through the maintenance of the structural integrity and function of DNA, enzymes, proteins, and transcription factors [6,7]. With regard to its antioxidant role, zinc inhibits the activation of NADPH oxidases, thereby attenuating the generation of reactive oxygen species (ROS). Also, zinc functions as a cofactor of copper/zinc superoxide dismutase-1 (Cu/Zn SOD; SOD1), the first line antioxidant enzyme in cells. The major role of SOD1 is the reduction of superoxide to the relatively less toxic

hydrogen peroxide in the cytosolic and intermembrane spaces [8]. Zinc is reported to exist in a bound form with SOD1 in rat cochlea [9]. SOD1 knockout mice show an elevated ABR threshold and cochlear hair cell loss [10].

The cell cycle consists of four phases: G1, S, G2, and M. The first of three phases (G1, S, and G2) comprise interphase in which rapid cell growth, DNA replication, and the preceding step for cell division occur. M phase refers to the mitotic phase, in which cell growth ceases and the parent cells are divided into daughter cells. The cell cycle is regulated by specific protein complexes that are composed of the cyclin proteins and cyclin-dependent kinases (CDK) i.e. cyclin D-CDK4/6, cyclin E-CDK2, and cyclin A-CDK2. Disruption of the cell cycle leading to its arrest can be triggered by oxidative stress, nutrient-deficiency, and DNA breakdown. Excessive amounts of ROS induce a cell cycle arrest and the inhibition of proliferative pathways [11]. In this regard, a zinc deficiency was reported to promote cell quiescence via an S-phase arrest in human breast epithelial cells (MCF10A), which occurred concomitantly with excessive ROS accumulation [12]. Moreover, a lack of zinc decreases viability via cell cycle arrest and increases the sensitivity to gamma irradiation in normal human prostate epithelial cells [13]. However, the effect of a zinc deficiency on hearing ability has remained to be elucidated.

In the present study, we investigated the effects of zinc deficiency on hearing loss associated with the cell cycle in mouse auditory hair cells (HEI-OC1). Chelating zinc in the culture media of HEI-OC1 cells induced a cell cycle arrest with a substantial reduction of proliferation, elevated ROS accumulation, and subsequent apoptotic cell death. Supplementation of culture medium with zinc chloride rescued these

detrimental effects. We additionally aimed to determine the immunolocalization of cell cycle arrest-related proteins in the cochlear tissues of mice fed with the zinc-deficient diet.

Materials and Methods

Materials

Cell culture medium components were purchased from Life Technologies (Grand Island, NY, USA) unless otherwise indicated. *N*,*N*,*N*′,*N*′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), and zinc chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies against the following proteins were obtained from the indicated sources: retinoblastoma-associated protein (Rb; catalog #9313) and phospho-Rb (pRb; #9308) (Cell Signaling Technology, Danvers, MA, USA); p53 (sc-53394), p21 (sc-6246), cyclin E (sc-481), cyclin-dependent kinase 4 (CDK4; sc-601), cyclin A (sc-751), cyclin-dependent kinase 6 (CDK6; sc-56282), pRb (sc-271930, for immunohistochemistry), and cyclin D1 (sc-8396) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin (A5441) was obtained from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA).

HEI-OC1 cell culture and TPEN/$ZnCl_2$ treatment

The HEI-OC1 mouse auditory cell line was kindly provided by Dr. Federico Kalinec (Department of Cell and Molecular Biology, House Ear Institute, Los Angeles, CA, USA). HEI-OC1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 10% CO_2 atmosphere at 33^{o}C. For experiments involving TPEN exposure, cells were seeded into 60-mm culture dishes at ~70% confluence and incubated for 24 h under standard conditions. Cells were then incubated in a serum-free medium for 3 h and

then treated with different concentrations of TPEN or $ZnCl_2$ for 24 h. In $ZnCl_2$ supplement studies, serum-starved cells were incubated with 0.5 μM TPEN for 24 h. Next, the culture medium containing TPEN was discarded, and cells were treated with 5 μM $ZnCl_2$ for further 24 h.

Cytotoxicity Assay

Cell viability was measured using a colorimetric D-Plus^{TM} CCK cell viability assay kit (Dongin LS, Seoul, Korea) according to the manufacturer's instructions. Cells were seeded into 96-well plates at a density of 5×10^3 cells/well and grown for 24 h under standard conditions. Following serum starvation, cells were exposed to different concentrations of TPEN (0-8 μM), or $ZnCl_2$ (0-100 μM) for 24 h. The amount of formazan dye product was determined by measuring the absorbance at 450 nm using a microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA). The absorbance value in untreated control cells was taken as 100% viability. The relative viability percentages in TPEN- or $ZnCl_2$-treated cells were thereby calculated.

Cell count assay

Cells were seeded into 60-mm plates at a density of 3×10^5 cells/well and were grown for 24 h under normal culture conditions. Following serum starvation for 3 h, cells were treated with 0.5 μM TPEN for a further 48 h. To examine the supplemental effect of zinc, cells were treated with TPEN for 24 h, followed by replacement with culture media containing 5 μM $ZnCl_2$ and a 24 h incubation. Cells were then detached from the plates to be stained with trypan blue for counting. Untreated- and

24-h TPEN treated-cells were used as controls. The number of cells was counted using a hemocytometer and three independent experiments were performed.

Immunoblotting

For immunoblotting experiments, cells were washed twice with ice-cold PBS, lysed with RIPA buffer (Sigma-Aldrich) supplemented with complete protease inhibitor cocktail, and centrifuged at 13,000 g at 4^{o}C for 20 min. The protein concentrations were measured using the BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA). Total soluble proteins (20 μg) were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare Biosciences, Uppsala, Sweden). The membranes were blocked with 5% skim milk in TBS-T (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) for 1 h and then probed with the appropriate primary antibodies overnight at 4^{o}C. Dilution factor of each primary antibody was as followed: 1:1000 for Rb, pRb, p53, p21, and CDK4; 1:500 for cyclin D1 and cyclin E; 1:200 for cyclin A and CDK6. After washing three times with TBS-T, membranes were treated with the appropriate HRP-conjugated secondary antibodies. The membranes were developed using enhanced chemiluminescence (ECL; Dongin LS) and the immunoreactive bands were detected using an Image-Quant LAS 500 biomolecular imager (GE Healthcare Biosciences). To normalize protein loading, membranes were reprobed with β-actin polyclonal antibody (1:5000 dilution).

Measurement of intracellular ROS production

The intracellular ROS levels were detected using a fluorescent dye, 5-(and-6)-

chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes, Inc., Eugene, OR, USA). Briefly, cells were grown in 96-well black plates (4×10^3 cells/well) and incubated with 0.5 μM TPEN, 5 μM $ZnCl_2$, or both as described above. Cells were then washed twice with Hank's balanced salt solution (HBSS) and incubated with 5 μM CM-H_2DCFDA for 20 min at 33ºC in the dark. After washing once with HBSS, the levels of DCF fluorescence were immediately measured using a Perkin-Elmer VICTOR 3 luminescence spectrometer (Perkin Elmer, Waltham, MA, USA) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. The values were converted to fold changes for comparison with the untreated control.

Detection of apoptosis by Annexin V/PI staining

Annexin V/PI staining was performed using an Annexin V-FITC Apoptosis staining/Detection kit (Abcam, Cambridge, MA, USA) in accordance with the manufacturer's instructions. Briefly, cells grown on a glass coverslip in 35-mm culture dishes were treated as described above. Cells were then washed twice in PBS, and Annexin-V binding buffer containing Annexin V-FITC and propidium iodide (PI) was then added. Cells were next incubated in the dark for 5 min, washed once with binding buffer, fixed with 2% paraformaldehyde, and washed once with PBS. Finally, cells were observed under the appropriate filters of a fluorescence microscope (Olympus IX71). Annexin-V stained cells were visualized using green fluorescence (ex/em ~495/~515 nm) and PI-stained cells using red fluorescence (ex/em~495/~635 nm). The percentage of Annexin-V or PI-stained cells was determined by counting ~1,500 cells in 3 randomly chosen fields per coverslip.

Flow cytometry analysis of the cell cycle

For flow cytometry analysis of the cell cycle, cells treated with TPEN or TPEN plus $ZnCl_2$ and removed from the culture dishes by trypsinization. Cells were then collected by centrifugation, washed twice in ice-cold PBS, and fixed in 70% ethanol overnight at -20°C. After centrifugation, cells were resuspended in PI/RNase Staining Buffer (BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 15 min at 37°C. The stained cells were analyzed using the BD FACSCantoTM^{II} Flow cytometer (BD Biosciences).

Animal experiments

All animal experimental protocols were in compliance with the guidelines of the National Institutes of Health and the Declaration of Helsinki. The protocols were approved by the Committee on Use and Care of Animals of the University of Ulsan (IACUC No. 2016-13-271; date of approval, March 10, 2016). Animal care was performed under the supervision of the Laboratory Animal Unit of the Asan Institute for Life Sciences, Seoul, Korea. Male CBA mice of 4 weeks of age (Central Laboratory Animal Inc., Seoul, Korea) were fed with a zinc-deficient diet (0.5~1 mg zinc/kg of food; Dyets, Bethlehem, PA). The control group received a normal diet (50 mg zinc/kg of food; Teklad, Madison, WI). After 8 weeks, the mice were heavily anesthetized by an injection of Soleil (80 mg/kg) and xylazine (2 mg/kg), and both cochleae were removed from each mouse. Each cochlea was immersed in fixative (4% formalin and 1% glutaraldehyde in PBS, pH 7.4) for 48 h at 4°C. Cochleae were decalcified in 5.5% EDTA (Microdec; Microm Microtech, Francheville, France) in PBS for 5–7 days, dehydrated, and embedded in paraffin.

Immunohistochemistry

Immunohistochemistry was performed using a Vectastain ABC-HRP, peroxidase (Mouse or rabbit IgG) kit (Vector Laboratories, lnc., Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, cochlear paraffin sections (5-μm thick) were deparaffinized and rehydrated, heated in a microwave oven for 1.5 min in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval and then treated with 3% H_2O_2/methanol to quench endogenous peroxidase activity. The sections were incubated with normal horse serum for 20 min and then with primary antibodies (1:200 dilution for p21, Cyclin E, and pRb) overnight at 4°C, followed by biotinylated secondary antibodies (mouse or rabbit) in PBS for 30 min. The sections were next incubated with VECTASTAIN ABC Reagent for 30 min and the immunostaining of each protein was visualized with an ImmPACT™ DAB Peroxidase Substrate kit (Vector Laboratories, lnc.). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted in mounting solution. Images of the sections were observed with an upright microscope (Nikon Eclipse *C*i, Tokyo, Japan).

Statistical analysis

All data were expressed as a means ± standard error (SE) of three independent experiments. The Student's *t*-test or one-way or two-way analysis of variance (ANOVA) was used to evaluate differences between groups. Tukey test was used as a Post-hoc analysis to evaluate which of the groups has significant differences. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of TPEN on the viability, proliferation, ROS generation, and apoptotic response in HEI-OC1 cells

To examine the effects of a zinc deficiency in murine auditory cells, HEI-OC1 cells were treated with 0.5-8 μM of TPEN, a specific zinc chelator, for 24 hours. As shown in Figure 1A, a CCK-8 assay revealed that TPEN treatment decreased cell viability in a dose-dependent manner. To next examine the effects of a zinc deficiency on cell proliferation and ROS generation, the same cells were treated with 0.5 μM TPEN for 24 and 48 hours. The rate of proliferation in these TPEN-treated cells was significantly lower at 72 hours (Fig. 1B). Exposure to 0.5 μM TPEN for 24 hours triggered an increase in intracellular ROS accumulation by ~1.4-fold (Fig. 1C). Finally, apoptosis/necrosis was evaluated in the cells treated with 0.5 μM TPEN for 24 hours, assessed by Annexin-V/PI staining. The percentage of cells positive for Annexin-V and PI staining was increased by ~5 and ~1.5% in TPEN-treated cells, respectively (Fig. 1D), indicating that the cytotoxic effects of 0.5 μM TPEN over a 24-hour incubation were minimal. Hence, our subsequent experiments were performed using this concentration and incubation time.

Effects of $ZnCl_2$ on viability and ROS generation in HEI-OC1 cells

To examine the potential cytotoxic effects of $ZnCl_2$ on HEI-OC1 cells, the cells were treated with 5-100 μM of $ZnCl_2$ for 24 hours, followed by a CCK-8 assay. There was no significant change in cell viability at a 5 μM $ZnCl_2$ exposure level but a dramatic decrease was evident at relatively high concentrations (40 and 100 μM),

compared with the untreated control (Fig. 2A). At 5 μM $ZnCl_2$, the intracellular ROS level was almost equivalent to the untreated control (Fig. 2B). Its increase was proportional to the increase in zinc concentration and cytotoxicity (data not shown). Since 5 μM $ZnCl_2$ did not affect cell viability or the ROS level in HEI-OC1 cells, this concentration was used in our subsequent experiments.

Effects of $ZnCl_2$ supplementation on TPEN-induced proliferation and ROS generation

To examine the supplementation effect of zinc on TPEN-induced cytotoxicity, HEI-OC1 cells were exposed to 0.5 μM TPEN for 24 hours, followed by treatment with 5 μM $ZnCl_2$ for a further 24 hours. A cell counting assay was then used to measure proliferation. Supplementation with $ZnCl_2$ restored the number of cells by ~1.3-fold, which was reduced by TPEN treatment (Fig. 3A). Concomitantly, the intracellular ROS levels triggered by TPEN were markedly decreased by ~1.37-fold in $ZnCl_2$-supplemented cells (Fig. 3B), indicating that zinc is essential for cell proliferation and the regulation of intracellular ROS.

$ZnCl_2$ supplementation can restore the cell cycle in TPEN-treated HEI-OC1 cells

To determine whether the decreased cell proliferation mediated by zinc deficiency is related to the cell cycle pathway, the distribution of the cell cycle phases was examined using PI-based flow cytometric analysis. As shown in Figures 4A and B, cells in the subG1 phase were not significantly changed under any of the experimental conditions. The distribution of the G_0/G1 phase in TPEN-treated cells

was significantly increased by ~ 13% compared with the untreated control, whilst TPEN plus $ZnCl_2$ treatment produced a significant decrease in the distribution of G_0/G1 phase compared with TPEN-treated cells. The distribution of the S and G2/M phases in the TPEN-treated cells was significantly decreased and there was a slight but significant decrease in the TPEN-treated cells in the S phase but no marked difference between the TPEN plus $ZnCl_2$-treated and control cells. Notably however, the G2/M phase distribution was significantly increased in TPEN plus $ZnCl_2$-treated cells, compared with TPEN-treated cells alone. This result indicated that a zinc deficiency caused a cell cycle arrest at G_0/G1.

We next examined the expression of cell cycle-related proteins in the TPEN- and TPEN plus $ZnCl_2$-treated cells. p53 and p21 expression were markedly increased by both treatments but by less in the TPEN plus $ZnCl_2$-treated cells. The cyclins, CDKs, and pRb were significantly downregulated in TPEN-treated cells but zinc supplementation almost restored these proteins to their normal levels (Fig. 4C). Taken together, these results indicated that the observed proliferation reduction in HEI-OC1 cells mediated by a zinc deficiency was likely due to a cell cycle arrest.

Expression of p21, cyclin E, and pRb in the mouse cochlea

To next elucidate whether a zinc deficiency could contribute to a cell cycle arrest *in vivo*, we immunohistochemically examined cell cycle-related protein expression in the cochlea tissues from mice fed with a normal or zinc-deficient diet. As shown in Figure 5A, p21 was more intensely expressed in the organ of Corti (OC), limbus (L), stria vascularis (SV), and spiral ganglion (SG) of zinc deficient-diet mice compared with the normal diet mice. In contrast, cyclin E and pRb were decreased in these

regions in the zinc-deficient cochlea. Quantification analysis revealed a ~2.5-fold increase in p21 expression and ~0.5- and ~0.7-fold decreases in cyclin E and pRb expression in the zinc-deficient mice (Fig. 5B). These results indicated that a Zn deficiency was closely correlated with changes in the cochlea cell cycle-related protein expression levels, thereby contributing to hearing loss.

Discussion

Zinc is an essential element in various physiological processes, and it has been reported that the symptoms of a zinc-deficiency include growth inhibition, dizziness, tinnitus, and hearing loss. As an example of this, the levels of zinc are reported to be decreased in the serum of tinnitus patients [14,15]. Moreover, a zinc deficiency in C6 rat glioma cells impairs the binding affinity of p53 leading to increased oxidative stress [16]. In our prior study, we observed that the ABR thresholds levels are increased in mice receiving a zinc-deficient-diet and this leads to a temporal hearing loss [4]. However, this prior study did not elucidate the molecular mechanism associated with zinc deficient-induced hearing loss. In our present study, we observed that a zinc deficiency suppresses proliferation and enhances intracellular ROS accumulation in mouse auditory hair cells (HEI-OC1), leading to cell cycle arrest in the G1/S phase. Zinc supplementation rescued these detrimental effects, indicating that the proper level of this metal in auditory cells is important for maintaining hearing ability.

TPEN has high affinities for several metal ions, including zinc, iron, and manganese. Due to its highest affinity for zinc and cell membrane permeability, it is commonly used as an intracellular chelator to induce zinc deficiency in various types of cells. It has been reported that high concentrations of TPEN induce apoptosis in various cell lines [17,18]. In the present study, increases in the concentrations of TPEN were proportional to the decreases in HEI-OC1 cell viability (Fig. 1A). A crucial regulatory role of zinc is as a cofactor of SOD1, a critical antioxidant enzyme. SOD1 suppresses ROS production and attenuates cellular apoptosis [19,20]. SOD1

null transgenic mice suffer hearing loss at an early age via the degeneration of spiral ganglion cells [21]. Zinc is present in the form of Cu/Zn SOD1 in the rat cochlea [9]. Hence, a zinc deficiency in the cochlea may lead to the inactivation of SOD1, which substantially increases the ROS generated during noise exposure with consequent ROS-triggered apoptosis. In this regard, It has been reported that a SOD1deficiencymediates the accumulation of superoxide radicals resulting in impaired proliferation, cell cycle arrest, and apoptosis in fibroblasts [22], and oxidative stress-induced cell death in *Schizosaccharomyces pombe* [23]. Also, the transgenic expression of zinc deficient human SOD1 in *Drosophila melanogaster* results in dysfunctional mitochondrial activity, which decreases the production of ATP [24]. Consistent with these findings, treatment of HEI-OC1 cells with 0.5 μM TPEN for 24 hours in the present experiments reduced proliferation, increased intracellular ROS production, and caused a slight induction of apoptosis (Fig. 1B-D). The same concentration of TPEN we used herein has been reported to cause apoptosis in prostate cancer (PCa) cells and human adenocarcinoma (A549) cells [16,25]. Taken together, the evidence to date indicates that a zinc deficiency impairs redox homeostasis in mouse auditory cells, leading to the accumulation of ROS, reduction of cell proliferation, and the onset of apoptosis.

Zinc supplementation has been reported to restore the hearing abilities of patients with sudden sensorineural hearing loss [5]. In animal models, the decreased ABR level due to a zinc-deficient diet was found to be rescued by a normal diet [4]. However, high concentrations of zinc have been reported to cause cytotoxicity in various cells. For example, human melanoma cells exposed to above a 30 μM

concentration of zinc sulfate ($ZnSO_4$) underwent apoptosis [26]. In addition, undifferentiated rat pheochromocytoma (PC12) cells treated with 100 μM $ZnCl_2$ showed decreased cell viability and increased ROS accumulation (by two-fold) compared with untreated control cells [27]. In contrast, cell viability in various cell lines was not significantly affected by low concentrations of $ZnCl_2$, i.e. below 10 μM [28]. Consistent with these findings, we found in the present experiments that HEI-OC1 cell viability was not significantly decreased, and the levels of intracellular ROS accumulation were not significantly increased, by exposure to 5 μM $ZnCl_2$ (Fig. 2). Supplementation of 5 μM $ZnCl_2$ into the culture medium of TPEN-treated cells restored normal proliferation and attenuated the accumulation of ROS triggered by TPEN (Fig. 3), suggesting that zinc may play an essential role in cell proliferation and redox homeostasis in the acoustic system.

Cell cycle arrest and a proliferation block typically occur to enable repair during the recovery from damage. This is characterized by the excessive accumulation of ROS, accompanied by increased levels of p53 and p21 proteins [29,30]. In particular, elevated p21 expression has been reported to be closely correlated with both a reduction of cell proliferation and induction of cell cycle arrest and apoptosis in β-cells [31]. Also, a zinc-deficiency was reported previously to induce caspase-dependent apoptosis in human neuronal precursor (NT-2) cells [32]. In addition, mice fed with a zinc-deficient diet showed increased ROS production in the testicular tissue, concomitantly with the elevated expression of phospho-p53, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) protein [33]. A zinc deficiency also causes cell cycle arrest in a human malignant lymphoblastoid cell line (HUT-78)

through the decreased expression of DNA-synthesis enzyme deoxythymidine kinase (TK) gene in the G1 phase [34], and cell cycle quiescence in S-phase in human breast epithelial cells (MCF10A), which was reversed by zinc supplementation [12]. Consistent with these findings, we here observed that TPEN treatment caused a G1/S phase arrest in HEI-OC1 cells and that subsequent zinc supplementation led to cell cycle reentry (Fig. 4A, B). Different types of cyclin-Cdk complexes are involved in each cell cycle phase. For example, in the G1 phase, cyclin D binds to Cdk4/6 and cyclin E binds to Cdk2, which is followed by the transcription of S phase target genes via phosphorylation of the Rb. In S phase, the cyclin A-Cdk2 complex plays a functional role, whereas the cyclin B-Cdk1 complex regulates the G2 phase [35]. Exposure to DNA-damaging agents such as diaminocyclohexane-trans-diacetato-dichloro-platinum(IV) (DAP) activates a p53-p21 pathway, leading to arrest in G1 phase and the inhibition of Cdk4 and Cdk2 activation [36]. The p21 protein is a well-known Cdk inhibitor that regulates the cell cycle via the suppression of cyclin E-Cdk2 or cyclin A-Cdk2 complex activity [37]. In our present study, the treatment of cells with TPEN upregulated p21 protein expression, and decreased the levels of the cyclins, CDKs, and pRb protein. The zinc deficiency-mediated changes in these protein expressions were restored to almost normal levels via zinc supplementation (Figure 4C). Consistent with the prior findings of *in vitro* cell experiments, the present study showed an increase in p21 immunoreactivity and decrease in cyclin E and pRb immunoreactivities in the OC, SV, L, and SG regions of the mice cochlea in the animals on a zinc deficient-diet, compared with normal-diet counterparts (Fig. 5). As shown previously, pRb knockout mice in the inner ear result in a hearing loss since hair cell death in the organ of Corti and also no response in an ABR test [38].

Since OC, SV, L, and SG are indispensable organs for hearing ability, we postulate that dysregulation of cell cycle-related proteins in these regions may contribute to hearing loss. Also, it is worth to investigate whether zinc deficiency affects other inner ear structures, including vestibular organ. Taken together, a zinc-deficiency induces G1/S arrest in mouse auditory hair cells which can be recovered by zinc supplementation (Fig. 6). Our findings suggest that zinc is an important nutrient in hearing ability via its involvement in cell cycle mechanisms. However, mammalian cochlea hair cells lack the capacity for regeneration since it was exited the cell cycle at the embryonic stage. Many studies have approached regenerating the auditory hair cells or proliferating the supporting cells to transdifferentiate into new hair cells via cell cycle reentry [39]. The mice co-deficient *p21* and *p19* triggered proliferation of auditory hair cells via reentry the S phase but it followed by apoptosis [40]. However, the coactivation of *Notch1* and cell cycle activator *Myc* in adult mouse inner ear induce proliferation of supporting cells and regeneration of hair cell-like cells [41]. In this regard, it is worth to examine the relation of *Notch1* and *Myc* expression and zinc-mediated cell cycle in zinc deficient-diet mice.

Conclusion

In conclusion, a zinc deficiency triggers oxidative stress-induced cell cycle arrest in mouse auditory hair cells. Supplementation with zinc reduces intracellular ROS accumulation and restores the cell cycle. Our findings provide new insights into the molecular mechanisms underlying the effects of a zinc deficiency and at the same time provide new perspectives for designing therapeutic approaches to nutrient-related hearing loss.

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Abstract in Korean

영양소 결핍은 난청을 유발하는 다양한 원인들 중 하나이다. 특히 아연은 세포의 증식, 면역체계, 산화환원 반응, 그리고 청력 유지에 필수적인 요소이다. 선행 연구결과 아연결핍 식이 마우스에서 ABR (Auditory brainstem response) threshold가 증가되었으며 일시적인 난청이 나타났고 정상식이를 통해 아연을 보충해주면 ABR threshold가 정상에 가깝게 회복되며 난청 역시 회복되었다. 하지만 현재 아연결핍이 난청을 유발하는 분자생물학적 기전은 밝혀지지 않았다. 본 연구는 마우스 청각 유모세포에서 아연결핍이 세포 주기에 미치는 영향을 조사하였다. 마우스 청각 유모세포에 0.5 μM TPEN (*N*,*N*,*N′*,*N′*-tetrakis (2-pyridylmethyl) ethylenediamine, 아연 킬레이터)을 24시간 동안 처리하면 세포증식이 억제되고 활성산소종이 증가했으며, 세포 사멸사가 유도되었다. FACS (Fluorescence-activated Cell Sorting) 측정결과 이러한 세포 증식의 억제는 G1/S phase의 억제로 인해 발생하였다. TPEN을 처리한 세포에 5 μM $ZnCl_2$를 넣은 세포 배양 배지를 넣어주면 아연 결핍에 의한 활성산소종의 축적과 세포주기 정지가 약화되었다. 추가적으로 아연 결핍 식이 마우스 내이에서 세포주기 연관 단백질의 발현을 조사한 결과 spiral ganglion, organ of corti, limbus, 그리고 stria vascularis에서 p21 단백질의 발현이 증가하였고, cyclin E와 pRb의 발현이 감소한 것을 확인하였다. 이러한 결과들은 아연이 산화환원 항상성 유지와 세포주기를 통한 세포 증식에 필수적인 영양소이며, 세포주기의 손상이 청력손실을 유발할 것으로 추측할 수 있다.

중심단어: 청력 손실, 아연 결핍, 세포 주기 정지, 마우스 청각 유모 세포, 내이

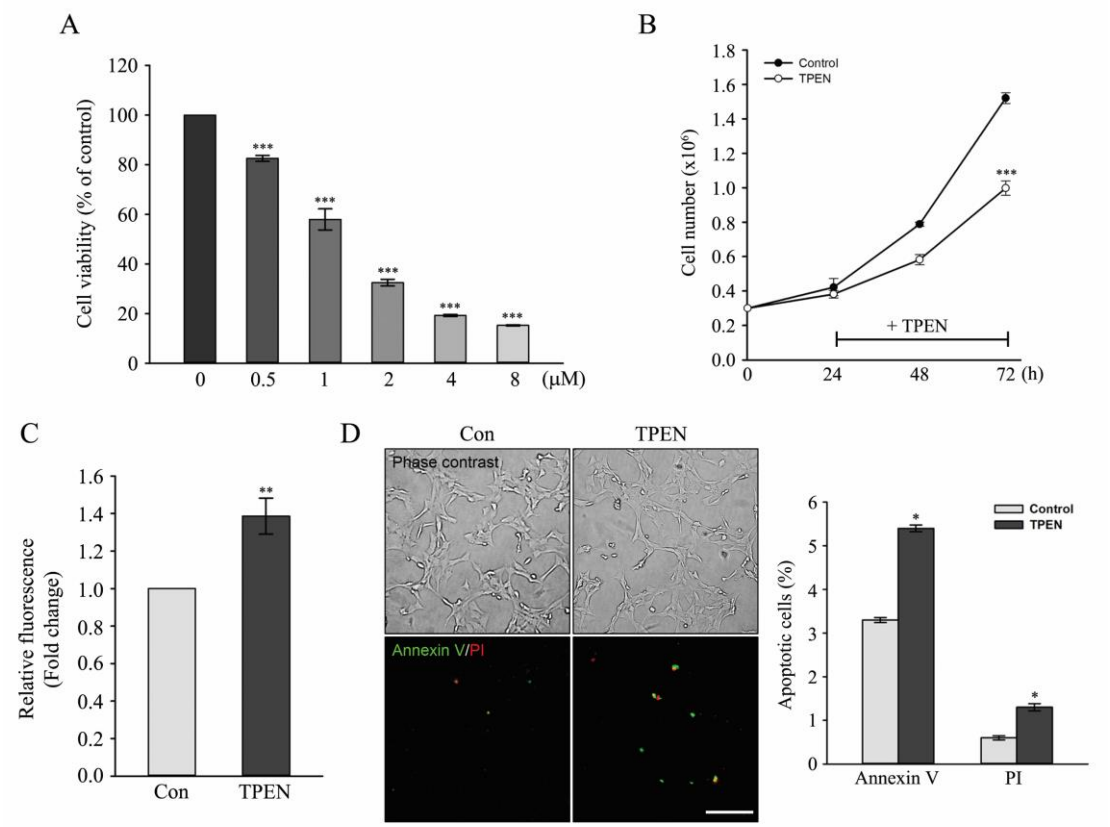

Fig. 1. Effects of TPEN on viability, proliferation, ROS accumulation, and apoptosis in HEI-OC1 cells. (A) Cells were treated with 0-8 μM TPEN for 24 h and viability was measured using a CCK-8 assay. Values are the means ± SE of three independent experiments, expressed as a percentage of the untreated control values; $^{***}P < 0.001$ compared with the untreated control. (B) Cells were treated with 0.5 μM TPEN and cell proliferation was assayed. Values are the means ± SE of three independent experiments; $^{***}P < 0.001$ compared with the untreated control. (C) Cells were treated as described above and the ROS levels were measured using CM-H_2DCFDA. Data are presented as fold changes relative to the untreated control, expressed as a mean ± SE of four independent experiments; $^{**}P < 0.01$ compared with the untreated control. (D) Annexin-V/PI staining was used to observe apoptosis/necrosis in the cells. Fluorescence images of Annexin-V (green) cells indicated "early-apoptosis" and positive staining with PI (red) was suggestive of "late-apoptosis or necrosis". Scale bar, 100 μm; Original magnification, 100×. Quantification of the stained cells is indicated in the graphs. Values are the mean ± SE; $^{*}P < 0.01$ compared with the untreated control.

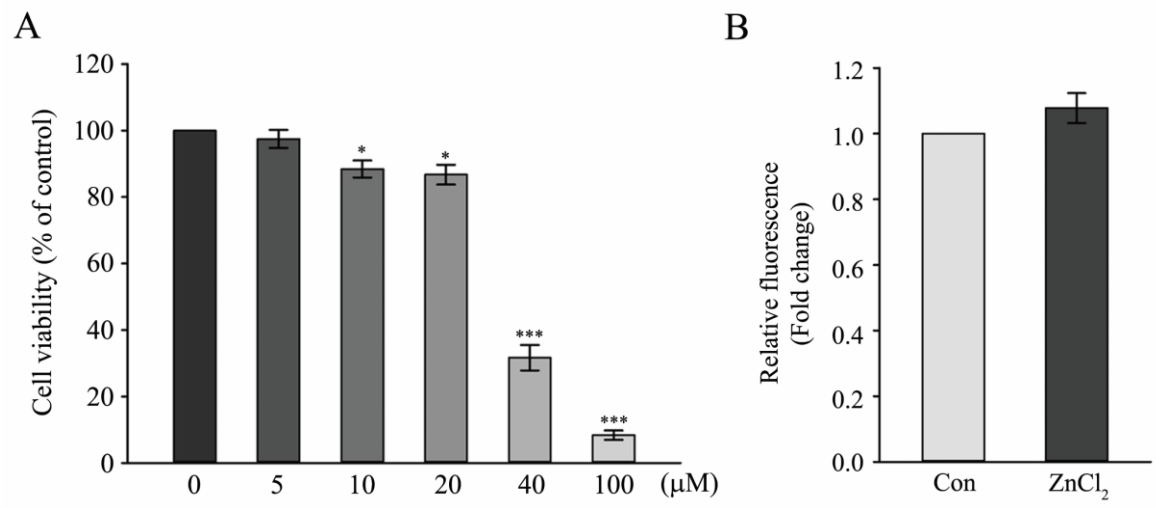

Fig. 2. Effects of $ZnCl_2$ on viability and ROS generation in HEI-OC1 cells. (A) Cells were treated with 0-100 μM $ZnCl_2$ for 24 h. Cell viability was then measured using a CCK-8 assay. Values are the means ± SE of four independent experiments, expressed as a percentage of the untreated control values; $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the untreated control. (B) Cells were treated with 5 μM $ZnCl_2$ for 24 h. The accumulation of intracellular ROS was then determined using CM-H_2DCFDA. Data are the fold changes relative to the untreated control, expressed as the mean ± SE of four independent experiments.

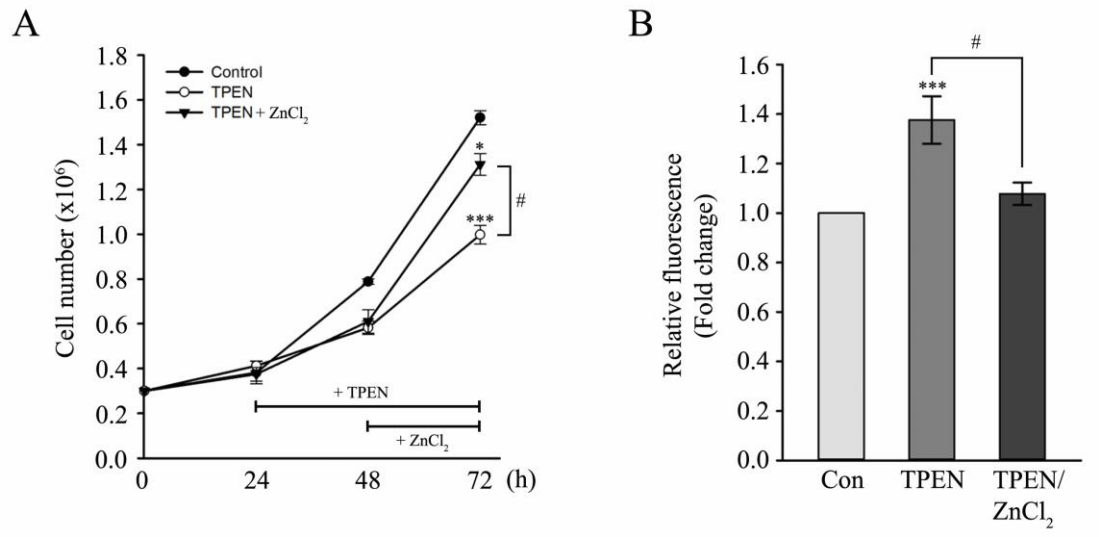

Fig. 3. Effects of $ZnCl_2$ supplementation on proliferation and ROS generation in TPEN-treated HEI-OC1 cells. (A) Cells were treated with TPEN plus $ZnCl_2$ as described in Section 2.2. Cell proliferation was measured by counting trypan blue-stained cells. Data are the mean ± SE of three independent experiments; $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the untreated control; $^{\#}P < 0.01$ TPEN only versus TPEN plus $ZnCl_2$. (B) Intracellular ROS generation was measured using DCF fluorescence intensity spectrofluorometry. Values are the mean ± SE of four independent experiments and are expressed as a fold change of the untreated control values; $^{***}P < 0.001$ compared with the untreated control; $^{\#}P < 0.01$ TPEN only versus TPEN plus $ZnCl_2$.

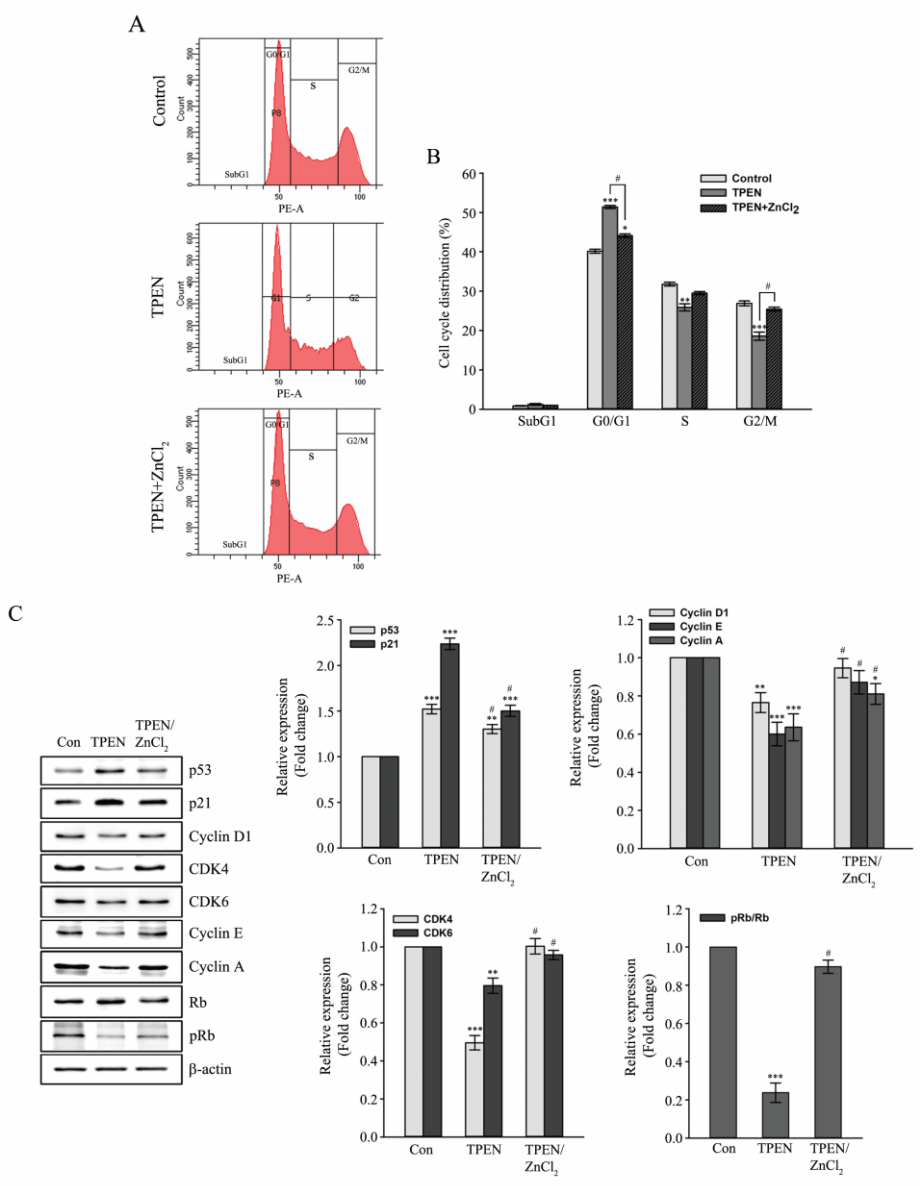

Fig. 4. Zinc supplementation restored the cell cycle in TPEN-treated HEI-OC1 cells. (A) Flow cytometry analysis of the cell cycle phase distribution of control, TPEN, or TPEN plus $ZnCl_2$-treated HEI-OC1 cells. (B) Quantitation of the control, TPEN-treated, or TPEN plus $ZnCl_2$-treated cells in the subG1, G_0/G1, S, and M2 phases. Data are the mean ± SE of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control; $^{\#}P <$ 0.01, TPEN only versus TPEN plus $ZnCl_2$. (C) Representative immunoblots of cell cycle-related proteins. Protein bands were quantified by densitometry and normalized using β-actin expression. Values are the mean ± SE of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control; $^{\#}P < 0.01$, TPEN only versus TPEN plus $ZnCl_2$.

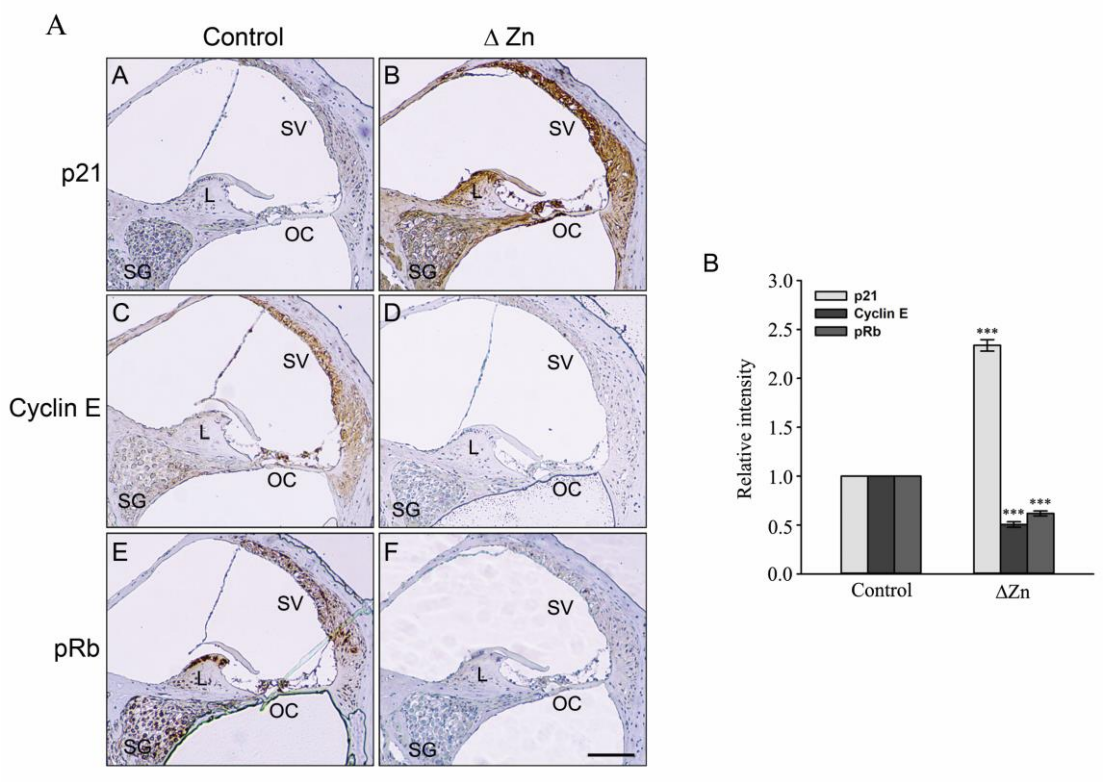

Fig. 5. Expression of p21, cyclin E, and pRb in the cochlea of normal- or Zn-deficient mice. Mouse cochlea paraffin sections were used to detect p21, Cyclin E, and pRb. (A) The expression levels of p21 (A; normal-diet, B; Zn deficient-diet), cyclin E (C; normal-diet, D; Zn deficient-diet), and pRb (E; normal-diet, F; Zn deficient-diet) are marked with a brown-colored dot generated by DAB staining and the cell nuclei are counterstained with hematoxylin. SV, stria vascularis; L, limbus; OC, organ of Corti; SG, spiral ganglion. Scale bar, 100 μm, original magnification ×100. (B) The Image J program was used to quantify the intensities of the DAB staining regions. The representative graph shown presents the fold changes relative to the control, expressed as a mean ± SE of five cochleae from each group; $^{***}P < 0.001$, compared with the control.

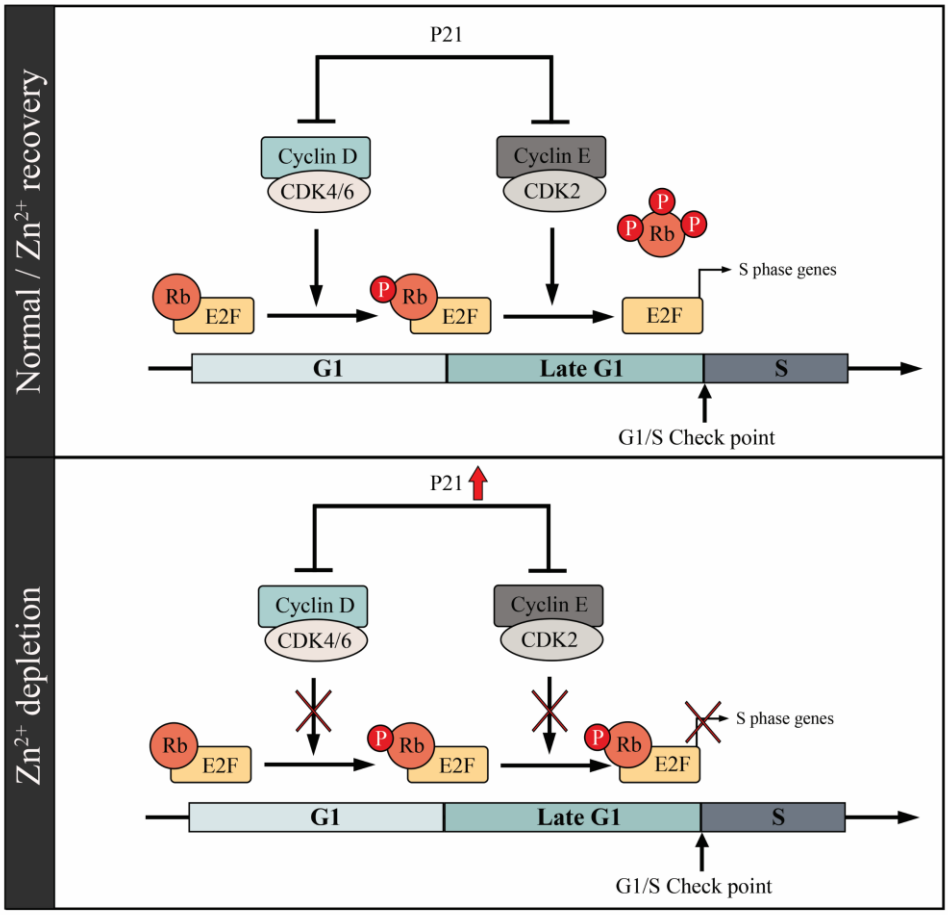

Fig. 6. Schematic diagram of the relationship of zinc to cell cycle mechanisms in mouse auditory cells. Zinc depletion can induce a G1/S phase arrest via increases in the p21 expression level. The addition of zinc can then restore the cell cycle progression.