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The presence and response to Zn of ZnT family mRNAs in human dental pulp

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Zinc (Zn) is distributed throughout the body and within cells by saturable processes mediated by the transport proteins of the ZnT (SLC30) and ZIP (SLC39) families. The two families function in opposite directions. ZnT transporters mediate cellular zinc efflux or intracellular sequestration. Zn is found in human tooth enamel and dentine at levels that have been related to environmental exposures, such as pollution, disease, and dietary intake. The mechanism by which Zn in the odontoblast is deposited in the hard tissue of the tooth, however, is unknown but is important in determining the physical properties, and hence resilience, of enamel and in the context of the use of tooth zinc level as a biomarker of exposure. We hypothesised that zinc efflux mediated by members of the ZnT family of 10 transporters is a key step in this process and is regulated by zinc availability through effects on mRNA levels. Thus, we determined the profile of ZnT transporter mRNA in a human active-secretory odontoblast-like cell model under conditions of high- and low-extracellular Zn concentration and determined if the same transporter mRNAs were present in human dental pulp. ZnT1, ZnT5 and ZnT9 mRNAs were detected by RT-PCR in both the secretory odontoblast cells and human dental pulp. ZnT2, ZnT3 and ZnT10 mRNAs were not detected, and ZnT4 mRNA was detected in secretory odontoblasts only, which may be indicative of a specialised zinc efflux function during the active secretory phase of tooth development. ZnT1 mRNA was significantly increased in response to extracellular Zn exposure (60 μ M) after 24 h. The presence of Zn transporter mRNAs in secretory odontoblasts and dental pulp indicates that the corresponding transport proteins function to deposit zinc in the dental hard tissues. The responsiveness of ZnT1 in odontoblasts to zinc availability is concordant with this being a process that is regulated to maintain cellular Zn homeostasis and that is a mediator of the relationship between environmental Zn exposure and dental Zn deposition. These findings have likely relevance to human dental health through effects of Zn transporter expression level on the hard tissue properties.

Significance to metallomics:

This study, for the first time, demonstrates that Zn efflux transporter mRNAs of the ZnT family are present in both human dental pulp and secretory odontoblast-like cells and that exposure to zinc at high concentration increases ZnT1 mRNA in secretory odontoblast-like cells. These findings are concordant with a role for Zn transporters in the regulated incorporation of Zn in the dental hard tissues and, hence, in influencing oral health.

Introduction

Zn is a trace element with essential functions in protein structure, gene regulation and cell signalling (1). Zn homeostasis maintains human body Zn at adequate levels. Total body Zn is, mostly, stored in skeletal muscle and bones, but the stored Zn is not sufficient to replenish Zn levels under Zn deficiency. Thus, daily Zn intake from the diet needs to meet the body Zn requirements (2). Zn homeostasis is regulated by Zn transporter proteins. (3) These proteins are classified into Zn Transporter

(ZnT/SLC30) and Zrt/Irt-related proteins (ZIP/SLC39). ZnTs, which consist of 10 identified proteins in humans, act to reduce cytoplasmic Zn concentrations by Zn influx from cytoplasm into the organelles and transporting Zn from cytoplasm into the extracellular environment (4). Conversely, the ZIP transporter family, consisting of 14 identified proteins in humans, maintains cytoplasmic Zn at adequate levels by transporting Zn from the extracellular compartment into the cytoplasm across the plasma membrane and /or through efflux from organelles into the cytoplasm. (5) Zn transporters have been shown experimentally (both in vivo and in vitro) to be regulated in some tissues by the level of Zn exposure or by dietary Zn intake, concordant with maintenance of cellular and systemic Zn homeostasis. (1, 5-12) This analysis has not been exhaustive across family members and tissues, and there remains a large body of work to undertake to generate a full profile across tissue, transporters, and regulatory responses.

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See DOI: 10.1039/x0xx00000x

In rats, dietary Zn supplementation increased the expression of intestinal ZnT1 at both mRNA and protein level,(12) while ZnT1 expression in pancreatic acinar cells decreased under low dietary Zn exposure and has been associated with decreased pancreatic Zn secretion (9).

The expression of ZnT1 and ZnT5 at mRNA and protein level decreased significantly in the placenta of Zn-restricted and Zn-supplemented mice (11). In human ileum, a reduction in ZnT1 expression at mRNA and protein level after Zn supplementation was reported, and ZnT1 mRNA as well as ZnT5 protein expression decreased in Caco-2 cells exposed to a high extracellular Zn concentration (10).

Sensitivity of ZIP1, ZIP2, ZIP4, and ZIP5 to dietary Zn has also been reported (1, 5, 9). Zn-restricted and Zn-supplemented mice showed a significant decrease in placental *Zip1* mRNA compared with the Zn-adequate group (11). Conversely, the expression of ZIP2 in human monocytic/macrophage THP-1 cells was increased under Zn-deficient conditions (13) ZIP4 mRNA in human intestinal ileum biopsies was un-changed in response to dietary Zn supplementation, while levels of ZIP4 protein were decreased in the Zn-supplemented group (10). In mice, the expression of ZIP4 in the enterocyte was rapidly down-regulated after Zn loading (9). The expression of ZIP5 was also reported to be lower under deficient Zn intake (1).

During tooth development, odontoblast cells secrete dentine matrix protein and are also responsible for dentine mineralisation. Once the formation of primary dentine is complete, odontoblast cells localise principally within dental pulp, a soft connective tissue that acts as a microcirculatory system (14) (15). Zn content in human bones and teeth accounts for ~30% of total body zinc {Kambe, 2015 #19}. The Zn content in human teeth, deposited within enamel and/or dentine, has been studied for decades. The Zn content of enamel influences the physical properties (16), having likely implications for enamel resilience and dental health, and has been used as a proxy to identify environmental exposure to Zn from pollution, disease, and/or dietary intake. Zn and its transport is also important for normal tooth development. Individuals with Ehlers-Danlos syndrome (EDS), a group of genetic disorders that manifest as abnormalities in tooth development, exhibit a lack of ZIP13 expression. A study of siblings with EDS revealed that SLC39A13 dysfunction caused this skeletal and connective tissue disease (17) while features of the syndrome were replicated in *Zip13-KO* mice (18). The mechanism of Zn deposition within the dental hard tissue is unknown but, for these reasons, is important to elucidate.

We hypothesised that Zn efflux from the odontoblast mediated by some or all of the 10 members of the ZnT family and regulated by the availability of Zn is a key step in its deposition in the dental hard tissues. Thus, the objectives of this study were to elucidate expression of Zn transporters in human pulp tissue, removed from deciduous teeth, and to measure changes in Zn transporter mRNA in response to extracellular Zn exposure in an odontoblast like-cell during primary dentine formation.

Materials and methods

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DOI: 10.1039/C8MT00343B

Differentiation of human odontoblast-like cells and confirmation of a secretory phenotype

The HDP-hTERT cell line was supplied by Professor Takashi Takata (Department of Oral and Maxillofacial Pathobiology, Hiroshima University, Hiroshima, Japan). These were differentiated into secretory odontoblast-like cells using mineral-induced growth medium, as previously described (19). Briefly, the cells were seeded at a density of 5×10^4 cells/well on round glass coverslip in 24-well plates with α -MEM supplemented with 10% (v/v) FBS (Sigma Aldrich), and incubated at 37°C in a humidified chamber with 95% air and 5% CO₂. After 24 hours' incubation, wells were randomly divided into 'undifferentiated' and 'differentiated' groups, and each group of 12 wells were maintained in standard α -MEM supplemented with 10% (v/v) FBS medium or medium supplemented with 50 μ g/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β -Glycero-phosphate (Cayman) and 0.1 μ mol/L dexamethasone (Sigma Aldrich) for up to 14 days. Dentine-sialophosphoprotein (DSPP) protein labelling using immunofluorescence and Alizarin Red staining were performed to confirm successful differentiation.

For DSPP protein labelling, cells were fixed using 100% (V/V) methanol for 5 min at room temperature, before washing in five stage cycles using PBS for 2 min. Cells were incubated with a primary DSPP antibody (Santa Cruz; 1:50 dilution) at 4°C overnight. Cells were then washed in three stage cycles using PBS for 2 min and incubated with a secondary anti-IgG -FITC conjugate antibody (Santa Cruz; 1:100 dilution) at 4°C for 2 h. After further PBS wash steps, cover slips containing stained cells were mounted on glass slides, cell side down, in Vectashield (Vector laboratories). The stained cells were examined using an Olympus BX61 microscope (Olympus Corporation) at 10 X magnification with fluorescence filter Alexa Fluor 488 and 594. Representative images were captured using a microscope-mounted Olympus XM10 monochrome camera and analysed using ImageJ software (Java-based image processing program—National Institute of Health (USA)).

For Alizarin Red staining, the medium was removed and cells were washed using PBS. Cells were fixed in cold 70% (V/V) ethanol for 1 h at room temperature. Fixed cells were washed once using sterile distilled water and stained with Alizarin Red dye (40 mM at pH 4.2; Sigma Aldrich) for 20 minutes. The dye was then discarded, and stained cells were washed twice using sterile distilled water to remove excess stain. Representative photographs were taken for each group using a digital camera.

Treatment with ZnCl₂, cell viability assay and RNA extraction

HDP-hTERT cells were seeded at 5000 cells/well into a 96-well plate with α -MEM supplemented with 10% (v/v) FBS (Sigma Aldrich) overnight. Cells were treated with different concentrations of extracellular Zn by addition of 10, 20, 40, 60,

Table 1 Primer sequences for human ZnTs, ZIPs, GAPDH, and 18S used for RT-qPCR and PCR

Product	Accession number ^a	Primer sequences	Product Size (bp)	Anneal temp. (°C)
ZnT1	NM_021194.2	735GCAACTCCAACGGGCTGAAA ⁷⁵⁴ 883ACGCATGTTAAGTTGTCAGCC ⁸⁶²	149	60
ZnT2	NM_032513.3	743ATCGGCGACTTTATGCAGAGC ⁷⁶³ 848TGGAGAAGACGAAGGTGCAGA ⁸²⁸	106	60
ZnT3	NM_003459.4	655ACTGGCATCCTCCTGTACCT ⁶⁷⁴ 771GGCCATTAACAGGTTGGCAC ⁷⁵²	117	60
ZnT4	NM_013309.4	760GACCTAAGCGCCATCATACTC ⁷⁸⁰ 864AGCTGACAAAACCTCTAAGCG ⁸⁴⁴	105	60
ZnT5	NM_022902.4	748AGCAAAGACAAGGGGAGCTG ⁷⁶⁷ 895GGCAATGGCTGTGTAAGCA ⁸⁷⁶	148	60
ZnT6	NM_001193515.1	276CCTGGCTGTATTTGCTCCA ²⁹⁵ 381TCTCCCGTGTGTATCTCGG ³⁶²	106	60
ZnT7	NM_133496.4	367GCTTAGGCTTGATTCCGACT ³⁸⁷ 504CCAGAACTCCGCTCTAACATAC ⁴⁸²	138	60
ZnT8	NM_173851.1	175ACT GGG CAG TGA GTT CAA CA ¹⁹⁵ 339CTG TTG GAG TTC CAC ACT TTC T ³¹⁷	144	56
ZnT9	NM_006345.3	1130TGGAGTCATGGGATTGCTTCAT ¹¹⁵¹ 1230CAAGAAGTGTGCTCCTCAGA ¹²⁰⁹	101	60
ZnT10	NM_018713.2	836TTCGCAAACGTAGCAGGTGA ⁸⁵⁵ 983TGATGACCACAACCACGGAC ⁹⁶⁴	148	60
GAPDH	NM_001289746.1	185TGAAGTCCGGAGTCAACGGATTTG ²⁰⁸ 312CATGTAACCATGTAGTTGAGGTC ²⁸⁹	128	60
18S	NM_022551.2	340GGACCTGGCTGTATTTCCA ³²¹ 226GAGGATGAGGTGGAACTGT ²⁴⁵	115	60

The end-point PCR carried out using BioMix™ Red (Bioline), included an initial activation step at 94°C for 5 minutes and extra extension at 72°C for 10 minutes at the end of the PCR cycle, while RT-qPCR was performed using SensiFAST SYBR mix No ROX (Bioline) with initial activation step at 95°C for 2 mins. Cycle parameters (denaturation, annealing-extension and number of cycles) for PCR are 60s; 60s; 60s x30 cycles and for RT-qPCR, 5s; 15s x 40 cycles. ^a) Numbered according to GenBank™

80, 100, and 200 μM ZnCl_2 to the growth medium. Cells were incubated for either 4 or 24 h. Cells without Zn treatment were used as controls. Following incubation, viability was assessed by AlamarBlue® (Invitrogen) at a volume of 10% (V/V) of the total well. AlamarBlue was added 1 hour before the end of the incubation period. Absorbance values were measured using a BioTek Synergy HT plate reader at 570 and 600 nm. Cell viability was calculated following the manufacture's protocol.

For RNA extraction, HDP-hTERT cells were seeded at 7.5×10^5 cells into a T25 flask with growth medium overnight. Following incubation, cells were cultured using mineral-induced growth medium for 7d. The medium was replaced every 2 d. On the seventh day, the medium was removed and cells were washed using Dulbecco's PBS (Sigma Aldrich). The cells were then treated with extracellular Zn by adding ZnCl_2 into α -MEM medium at a concentration of 10 μM and 60 μM for 4 and 24 h incubation period. Cells, cultured using α -MEM medium only, were used as control.

Extraction of mRNA from human dental pulp

To measure Zn transporter mRNA in pulp removed from deciduous teeth, ethics was obtained from the Committee of The Medical Research Ethics of the Faculty of Medicine University of Indonesia (526/UN2.F1/ETIK/2014). Written informed consent was obtained from all participants. Dental pulps were removed from five deciduous teeth collected from

different children. Total RNA was extracted using Trizol reagent (ThermoFisher) and converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed using SensiFAST SYBR mix No ROX (Bioline) in a DNA Engine Opticon® 2 system (MJ Research). A standard curve was produced for each pair of primers using a cDNA template from human brain RNA (Ambion RNA tissue panel), RNA extracted from Caco-2 intestinal cells and/or SH-SY5Y neuroblastoma cells, and the efficiency of primers was determined.

RT-qPCR

Primers listed in Table 1 were designed to measure the ZnT family Zn transporter mRNAs in human dental pulp and differentiated HDP-hTERT cells. Primer specificity and product identity was confirmed in HDP-hTERT, Caco-2, SH-SY5Y cells or RNA extracted from testes by end-point PCR analysed by agarose gel electrophoresis and sequencing of products.

Results

Presence and regulation of ZnT transporters in secretory odontoblast-like cells

HDP-hTERT cells are immortalised human dental pulp cells (19). To replicate odontoblasts at the secretory stage, cells were grown in mineral-induced growth medium for 3, 7, and 14 days.

PAPER

For comparison, control, HDP-hTERT cells were grown in α -MEM supplemented with 10% (v/v) FBS.

Immunofluorescence was performed using anti-DSPP antibody after differentiation time points (3, 7 and 14 days). DSPP is a specific protein known to be expressed by odontoblast cells during the secretory stage. DSPP protein detected in cells grown in mineral-induced growth medium as early as 3 days post treatment, as presented in Figure 1A.

The capacity of the differentiated cells for secretion was determined by assessing the mineralisation activity after the 14-day time point using Alizarin Red. This assay stains mineral nodule formation in differentiated cells, which is compared with control in Figure 1B.

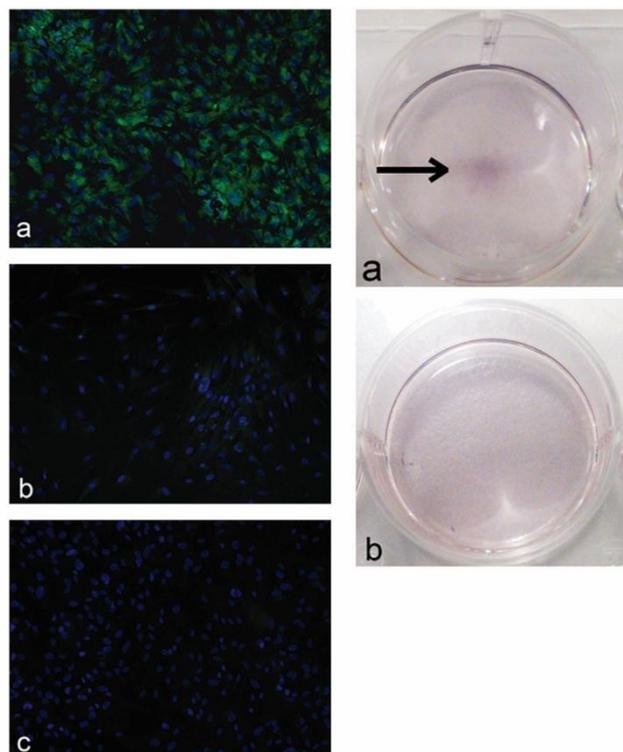


Figure 1 Differentiation of HDP-hTERT cells into secretory odontoblast-like cells. Cells were seeded at a density of 5×10^4 cells/well in 24-well plates and grown in the presence of 50 $\mu\text{g}/\text{mL}$ ascorbic acid (Sigma Aldrich), 10 mmol/L β -Glycero-phosphate (Cayman), and 0.1 $\mu\text{mol}/\text{L}$ dexamethasone (Sigma Aldrich) as mineral-induced medium or growth medium for 3, 7, 14 days. (A) After 3 days, cells were prepared for immunostaining using DSPP-antibody (Santa Cruz; 1:50 dilution) and anti-IgG-FITC conjugate (Santa Cruz; 1:100 dilution) as secondary antibody. Positive staining for DSPP protein is shown in green. Blue staining represents DAPI nuclear DNA stain. The figures show: a) cells grown in the presence of mineral-induced medium treated with DSPP and secondary antibody; b) cells grown in standard culture media treated with DSPP and secondary antibody; c) cells grown in the presence of mineral-induced mediums treated with secondary antibody only (control positive); (B) After 14 days, cells were prepared for nodule mineralisation using Alizarin Red staining, where it was identified by darker staining (arrow) in cells grown in the presence of mineral-induced medium (a), but none in cells grown in standard culture medium (b).

The differentiated HDP-hTERT cells were screened for ZnT mRNAs using end-point PCR. Caco-2 cells (intestinal model) and SH-SY5Y cells (neuronal) were used as a positive control to ensure primers and conditions were optimal for successful PCR. For ZnT8 RNA from testes (Ambion Human Tissue Panel) was used as the positive control. ZnT1, 4, 5 and 9 were detected (Table 2).

Table 2 Zn transporters expressed in human dental pulp

Zn transporters	Expression in human pulp
ZnT1	✓
ZnT2	X
ZnT3	X
ZnT4	X
ZnT5	✓
ZnT9	✓
ZnT10	X

All primers were assayed for their annealing qualities with RNA from with brain, Caco-2 or SH-SY5Y cells. Only those transporters identified with a ✓, gave a positive result with RNA extracted from all human pulp tissue sample

Products of the correct size and sequence were generated from the positive control cell lines and tissue RNA using primers for ZnT2, 3, 6, 7, 8 and 10. Thus we conclude that secretory odontoblasts express ZnT1, 4, 5, and 9 but not ZnT2, 3, 6, 7, 8 and 10.

To test the hypothesis that ZnTs in secretory odontoblasts show altered expression in response to changes in Zn availability, we first determined the maximum Zn concentration at which cell viability was maintained. Thus, HDP-hTERT cells were treated with different concentrations of extracellular Zn (as ZnCl_2) for up to 24 hours after differentiation for 3 days. Viability was assayed using Alamar Blue at 30 min, 2, 4 and 24 h time points. The analysis revealed that cells were viable up to a maximum concentration of 60 μM ZnCl_2 at all time points (Figure 2). For measurement of ZnT mRNAs, differentiated HDP-hTERT cells were exposed to extracellular ZnCl_2 at a concentration of 10 μM (approximate physiological level of serum Zn) and 60 μM concentration (supra-physiological level) for either 4 or 24 hours.

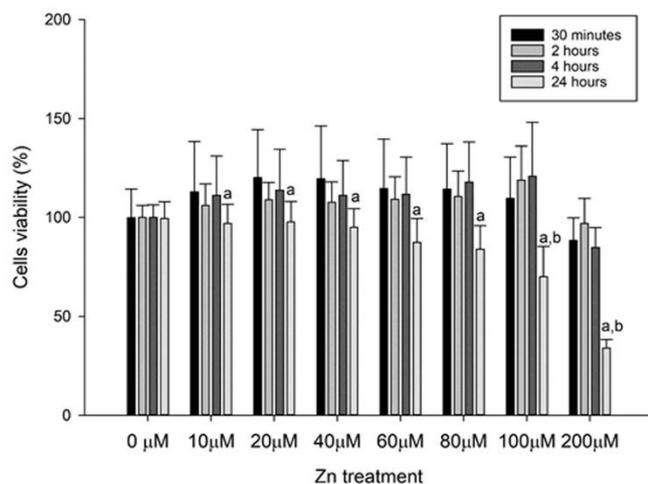


Figure 2 Cell viability of HDP-hTERT cells after extracellular $ZnCl_2$ exposure at different concentration and times. Data are expressed as normalised data to cells grown in basal medium as 100% cell viability. Mean values (\pm SEM) ($n=6$) are presented for each experiment. The differences between times and concentration were significant analysed using two-way Repeated Measurement ANOVA ($p=0.001$). Bonferonni Post-Hoc Test was performed to analyse the differences between times at the same concentration, a) $p<0.05$; and the differences between 10 μM $ZnCl_2$ and other concentrations, b) $p<0.05$.

ZnT1 was the only ZnT mRNA affected by treatment with extracellular Zn (at both 10 μM or 60 μM ; Figure 3). ZnT1 mRNA showed a significant increase after 24-hour exposure.

ZnT mRNA in human dental pulp

To validate the finding that ZnTs are expressed in odontoblasts in vivo (and exclude the possibility that their expression in HPD hTERT cells is an artefact of immortalisation), the mRNA expression profile of seven members of the ZnT family was measured in human dental pulp from five children. The analysis could not be extended to include all 10 family members, due to scarcity of material.

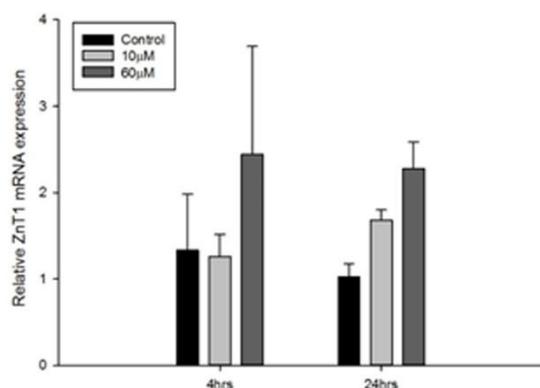


Figure 3 mRNA expression of ZnT1 in response to extracellular $ZnCl_2$ treatment. HDP-hTERT cells were grown with mineral-induced medium for 7 days. The cells were then treated with basal medium (as control), 10 μM and 60 μM $ZnCl_2$. RNA was extracted after 4 and 24 hours, and the expression levels of ZnT1, relative to the level at the control

condition, were measured by RT-qPCR using GAPDH and 18S as a reference gene and calculated using $2^{-\Delta\Delta Ct}$ method.⁽²⁰⁾ Negative control RT-qPCR reactions were analysed and identical prepared to those yielding the products, but in omission of Moloney murine leukaemia virus reverse transcriptase. Standard curves generated using serial dilution of cDNA from Caco-2 cells were used to ensure the efficiency of primers. To control the quality between plates, Caco-2 cell cDNA was used as a calibrator. Data is presented in histogram as geometric mean (\pm SEM) between two reference genes from three independent experiments ($n=3$). The mRNA expression level of ZnT1 were significantly different, ANOVA ($p<0.05$). The differences between concentrations and times were analysed using Bonferonni as post Hoc test, (*), $p<0.05$.

Thus, to compare the overall profile between active, secretory odontoblast-like cells and human dental pulp we selected ZnT 1, 4, 5 and 9 as transporters expressed in the odontoblast-like cells and ZnT 2, 3 and 10 to confirm negative results.

Concordant with the findings in HDP-hTERT cells, ZnT1, ZnT5 and ZnT9 were detected by end-point RT-PCR in the human dental pulp samples, and ZnT2, ZnT3 and ZnT10 were not detected. In contrast with the findings in HDP-hTERT cells ZnT4 mRNA was not detected in human dental pulp, which may reflect a specialised role for ZnT4 in actively secreting odontoblasts in the developing tooth only.

We used RT-qPCR to compare the quantities of the different ZnT mRNAs between the 5 individuals in the sample. Data, shown as Figure 4, reveal inter-individual differences. For example, the highest level of all 3 mRNAs was measured in sample Z0961; sample Z1662 had the next-highest level of ZnT1 mRNA but the lowest level of ZnT5 mRNA, whereas sample Z2364 had the lowest level of ZnT1 mRNA but next-highest level of ZnT5 mRNA.

Discussion

We report the expression of ZnT1, ZnT5, and ZnT9 at the mRNA level in human dental pulp. To our knowledge, ZnT transporter mRNA in native human dental pulp and across the different

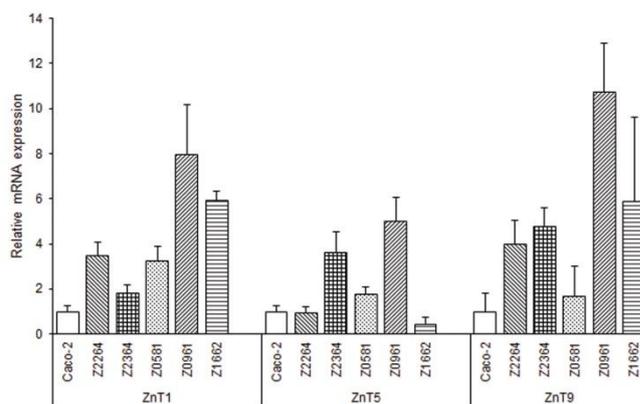


Figure 4. The profile of ZnT mRNAs in human dental pulp. RNA extracted from 5 samples was converted to cDNA and quantified by qPCR using primers (Table 1). RT-qPCRs were run in triplicate. Relative concentrations of target and reference genes were calculated from Ct value using the Pfaffli method⁽²⁰⁾. Standard curves were generated using serial

dilution of cDNA from human brain (Ambion RNA tissue panel) and Caco-2 cells were used to calibrate across plates. Data for each mRNA are presented relative to the quantity measured in Caco-2 cells.

family members has not been previously reported. We also show that these same ZnT mRNAs are present in human dental pulp cells immortalised using h-TERT (HDP-hTERT cells), which grown under the conditions we applied, have an odontoblastic secretory capacity that replicates the function of odontoblasts during active dentine formation (19). These findings are concordant with our hypothesis that Zn efflux from the odontoblast mediated by members of the ZnT family is a key step in its deposition in the dental hard tissues.

We found also that exposure of differentiated HDP-hTERT cells to an elevated extracellular Zn concentration of 60 μM induced an increase in ZnT1 mRNA after 24 h. ZnT1 has previously been shown to be responsive to Zn exposure in mineralised tissues, including bone, as well as human and mouse dental pulp cells (21) (22). In differentiated HDP cells, up-regulation of ZnT1 by exposure to extracellular Zn at concentrations of 40 μM and 80 μM for 7 days was measured (21). Thus, our observation is generally concordant with published findings, but reveals that the response occurs after a relatively shorter exposure. Induction of ZnT1 by Zn has been reported widely and has been observed in multiple tissues. The mechanism has been very well-characterised and is known to be through transcriptional activation as a result of the zinc-activated transcription factor MTF1 acting through ZRE motifs in the promoter region (23).

It is well-established that ZnT1 is the principal exporter of Zn from the cytosol across the plasma membrane (24). Thus, our findings mark out ZnT1 as a likely key determinant of tooth Zn content through secretion of Zn into the matrix during the process of mineralisation in the developing tooth. The positive direction of response of the mRNA to increased Zn availability would augment, rather than apply homeostatic control to, increased deposition of Zn in the hard tissue matrix at higher levels of Zn exposure during tooth development. ZnT knockout mouse models of all of the transporters we detected (25-27) show phenotypic effects that demonstrate ZnTs are not functionally redundant. Thus, ZnTs 1, 5 and 9 in the differentiated odontoblast are likely to play key functional roles in general cellular processes. Unless other homeostatic mechanisms, thus far undetected, come into play, this positive feedback loop is likely to be a critical determinant of the Zn content, and hence enamel resilience, of the mature tooth.

ZnT5 has also been observed at the plasma membrane. However, this may be a specialised feature exclusive to the enterocyte (28). More generally, ZnT5 has been detected at the Golgi membrane, where likely functions include delivery of Zn to secreted proteins, at least in some instances acting as a heterodimer with ZnT6 (29). ZnT5 may, therefore, not be a major determinant of the Zn content of the dental hard tissues through a direct efflux function, but this possibility cannot yet be excluded.

ZnT9 is expressed at the nuclear membrane (30). Nuclear Zn content in the odontoblast varies during tooth development and appears highest during the early stages of enamel maturation (31). The level of expression of ZnT9 is likely to play a key role in determining oscillations in odontoblast nuclear Zn content that accompany tooth development and maturation.

ZnT4 mRNA was found only in actively-secreting HDP-hTERT cells, and not in human dental pulp. ZnT4 has specialised secretory functions, including the secretion of Zn into milk in the mammary gland (32). We posit that the presence of ZnT4 in secreting HDP-hTERT cells reflects a specialised role for ZnT4 in actively secreting odontoblasts in the developing tooth only.

Levels of ZnT mRNAs measured differed between 5 samples of pulp tissue from human deciduous teeth that we studied. The small quantities of RNA we could isolate from these teeth precluded repetition of the measurements to ascertain differences that are statistically-significant. However, the variance in measurements we observed with respect to level of ZnT1 mRNA present may be related to our observation in the human odontoblast-like HDP-hTERT cell model that ZnT1 mRNA abundance is influenced by extracellular zinc concentration. Thus, it is likely individual variation could be a result of different dietary patterns or other exposures to Zn.

The variance between individuals observed for ZnT5 and ZnT9 mRNAs, which did not show a significant response to extracellular Zn in our experiments using differentiated HDP-hTERT cells, may reflect the different cellular composition of the dental pulp samples. Dental pulp is a mixed population of cells, including odontoblast cells, sub-odontoblastic and stromal fibroblast cells, as well as neuronal, immune and vascular system cells.²³ Alternatively, inter-individual variation in expression of these transporters in dental pulp may result from variables other than Zn exposure, and/or our assay using HDP-hTERT cells may not replicate fully the in vivo situation.

Our findings add new knowledge about potential influences on the Zn content of enamel, which has been shown to influence its physical properties. The specific implications of variance in ZnT expression levels for enamel resilience and, hence, dental health, is a topic that that merits further study.

Confidence in the use of tooth Zn content as an accurate biomarker of individual Zn exposure requires a full understanding of the processes that determine Zn content and their regulation, as well as exposure data. We did not have exposure data on the 5 individuals used in our study. Hence, analysis of this potential relationship was not possible. Our findings indicate that ZnT1 activity in the odontoblasts of the developing tooth is a zinc-sensitive process and likely to be one of the modifiers of zinc concentration measured as a snapshot in time. However, the contribution of other processes during tooth mineralisation that contribute to the quantity of zinc deposited in the tooth must be elucidated and mechanisms through which tooth zinc may turnover even in the mature

tooth should be investigated to obtain a full-understanding of this complex relationship.

Conclusion

This study revealed that specific ZnT family mRNAs are present in human dental pulp and in secretory odontoblast-like cells and hence may play a role in depositing Zn in the matrix during dentine mineralisation. We found evidence for inter-individual variability in the levels of these mRNAs in dental pulp, which may reflect different environmental exposures to Zn. ZnT1, a plasma membrane Zn export protein, was regulated by extracellular Zn concentration in odontoblast-like cells after relatively short periods of time through an apparent positive feedback loop that marks out ZnT1 activity as a likely key determinant of tooth Zn content, and hence, enamel properties and oral health.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was supported by Directorate General of Higher Education, Ministry of Research, Technology and Higher Education of the Republic of Indonesia as a funded doctoral programme. We wish to thank Professor Takashi Takata (Department of Oral and Maxillofacial Pathobiology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan) for the gift of hDP-HTERT cells, Dental Hospital of Universitas Indonesia and Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Indonesia for facilitating the sample collection.

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PAPER

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