Intracellular zinc-dependent TAS2R8 gene expression through CTCF activation

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ABSTRACT

Taste-2 receptors (TAS2Rs), which belong to the G-protein coupled receptor (GPCR) family, are receptors for bitter taste perception. The aim of this study was to investigate whether zinc deficiency affects the expression of *TAS2R* genes. The promoter activity of the *TAS2R7*, *TAS2R8*, and *TAS2R42* genes were determined in Ca9-22 oral squamous cell carcinoma cells cultured in the presence or absence of zinc. Luciferase reporter assays showed that zinc deprivation inhibited *TAS2R8* promoter activity, but not the promoter activity of the other two genes. Treatment of the cells with *N,N,N',N'*-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN), an intracellular chelator of Zn²⁺, in the presence of 10% fetal bovine serum reduced *TAS2R8* promoter activity. Truncation/deletion mutants of *TAS2R8* promoter-luciferase constructs showed that the region from nucleotide –1152 to nucleotide –925 was critical for intracellular zinc dependency and contained a CCCTC-binding factor (CTCF) binding motif. A chromatin immunoprecipitation (ChIP) assay showed that CTCF bound specifically to this region, a binding abrogated by zinc deficiency, suggesting that CTCF plays a critical role in zinc-dependent bitter taste perception through *TAS2R8*.

INTRODUCTION

Basic taste signals are categorized into five groups, salt, sour, sweet, bitter, and umami, with these sensory systems evolving to allow nutrition and avoid potentially noxious and/or poisonous chemicals (Chandrashekar *et al.* 2006). In humans, taste is an important sensory system for enjoyment of food and drink. Taste perceptions are associated with different receptor molecules, including sodium channels for salt, polycystic kidney disease 2-like 1 (PKD2L1) for sour, and taste receptors (TASRs) for sweet, bitter and umami (Martin and Dupré 2016). TASRs can be categorized into two groups, TAS1R and

TAS2R, which belong to the G-protein coupled receptor (GPCR) family. TAS1R and TAS2R each has isotypes, with various monomers and heterodimers functioning as different types of taste receptors. For example, the heterodimers TAS1R1/TAS1R3 and TAS1R2/TAS1R3 act as taste receptors for umami and sweet, respectively (Li *et al.* 2002; Nelson *et al.* 2002), and all TAS2R monomers, comprised of ~30 subtypes, are utilized for bitter taste perception (Martin and Dupré 2016). A comparison of *TAS2R8* and *TAS2R39* showed that they differ in recognizing specific bitter tastes, with each TAS2R subtype having high ligand specificity (Ueno *et al.* 2011).

Because zinc is an important co-factor for matrix metalloproteinases (MMPs) and zinc finger proteins, its deprivation for long periods of time reduces the

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activity of these proteins. Zinc finger is a protein motif that stabilizes protein folding by coordination with one or more Zn^{2+} ions. The nuclear protein CCCTC-binding factor (CTCF), an 11-zinc finger protein, functions as an insulator (Ong and Corces 2014). Insulators mediate intra- and inter-chromosomal interactions and play a role in allowing or preventing three-dimensional folding between enhancers and promoters in distal regions, thereby inducing or repressing gene transcription. CTCF null mice are embryonically lethal (Moore *et al.* 2012), suggesting that CTCF-target molecules have played important roles in morphogenesis during evolution. CTCF promotes alternative splicing by RNA polymerase II through a process of exon inclusion (Shukla *et al.* 2011) and also contributes to genomic imprinting (Fedoriw *et al.* 2004). In breast cancer, a CTCF binding motif was found in the 3' flanking region of the *MMP7* gene, and a single nucleotide polymorphism (SNP) in this motif was shown to be associated with reduced breast cancer susceptibility and *MMP7* promoter activity (Beeghly-Fadiel *et al.* 2008). CTCF bound to several molecules, including transcription factor II-I (TFII-I) (Peña-Hermández *et al.* 2015), the TATA-binding protein associated factor 3 (TAF3) (Liu *et al.* 2011), cohesin (Phillips-Cremins *et al.* 2013), and Smad2 (Van Bortle *et al.* 2015), was shown to function as a transcriptional activator. Thus, CTCF functions as an architectural protein in response to intracellular signal transduction.

Although zinc deprivation disrupts taste perception, the association of zinc deprivation with TAS2R expression has not been determined. Although transcription factors involving *TAS2R* gene regulation by *in silico* analysis using cardiac gene expression data, were investigated, regulation of promoter activity of the bitter taste *TAS2R7*, *TAS2R8*, *TAS2R42* genes is not analyzed in-depth due to its low expression level in the heart (Foster *et al.* 2015). Because they are major molecules expressed in the taste buds (Hevezi *et al.* 2009), we focused to examine their involvement in bitter taste disorder associated with zinc deficiency. This study reports that *TAS2R8* expression is upregulated by CTCF finger protein.

MATERIALS AND METHODS

Reagents. Dulbecco's Eagle Medium (DMEM) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); fetal bovine serum (FBS) was from Hyclone (South Logan, UT, USA); and the

dual-luciferase[®] reporter assay system was from Promega (Madison, WI, USA). The intracellular membrane-permeable ion chelator *N,N,N',N'*-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) and the insoluble formazan form of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Dojindo (Kumamoto, Japan). Xfect transfection reagent, PrimeSTAR GXL and In-Fusion[®] HD Cloning Kit were from Takara (Tokyo, Japan).

Antibodies. Anti-CTCF rabbit monoclonal antibody (mAb) was purchased from abcam (Cambridge, UK) and mouse IgG1 (isotype control) was from MBL (Nagoya, Japan).

Cells and cell culture. HEK293 cells, which were derived from human embryonic kidney, were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan), and human gingival squamous carcinoma (Ca9-22) cells were the kind gift of Dr. Kimiharu Hirose (Ohu University School of Dentistry, Koriyama, Japan). Both cell lines were grown in DMEM (high glucose) supplemented with 10% fetal bovine serum (FBS) and subcultured by treatment with 0.25% trypsin/0.02% EDTA. After reaching confluency, the cells were washed twice with Mg^{2+} and Ca^{2+} -free phosphate buffered saline (PBS(-)) and maintained in serum-free DMEM (zinc-deprived medium) or in serum-free DMEM supplemented with 15.3 μM ZnSO_4 to yield a final concentration of 100 $\mu\text{g/dL}$, the serum concentration of zinc in healthy males (zinc-adequate medium) (Buxaderas and Farre-Rovira 1985), whose dose was confirmed to be within previous reports (Deters *et al.* 2003; Sharif *et al.* 2012; Takeda *et al.* 2018). To chelate intracellular zinc, TPEN, a cell-permeable Zn^{2+} chelator (Treves *et al.* 1994; Kolenko *et al.* 2001), was added to a final concentration of 20 μM ; as a vehicle control, ethanol was added to a final concentration <0.1%.

Cloning, vector construction, transfection. Genome DNA was extracted from CA9-22 cells using a Wizard genomic DNA purification kit (Promega). The DNA sequences of TAS promoter regions were amplified by polymerase chain reaction (PCR) using PrimeSTAR GXL with the primer sets shown in Table 1 and cloned into pGL4.20 vector (Promega) using an In-Fusion[®] HD Cloning Kit with the primer sets shown in Table 2. Mutant expression vector was constructed similarly using the primer sets in Table 3.

Table 1 PCR primer sets for amplifying gene promoter region

Genes	Sequences
TAS2R7	F: 5'- TAG CAA ACT ACT GAA TAC ATC TTT TCT ATC -3'
	R: 5'- TTC TTA GAT TTT GAT GTA GTT TTC TTT ACC -3'
TAS2R8	F: 5'- CAT TTT CTC TTA TAT GCT ATT GGA AGT CAT -3'
	R: 5'- GTT TGT AGA GAG AAC AAT CTG ATT TCA AAT -3'
TAS2R42	F: 5'- CTC CAG AGA CAA AAA AAT CCA AGT TTT TAA -3'
	R: 5'- CTC CAG AGA CAA AAA AAT CCA AGT TTT TAA -3'

F, forward; R, reverse.

Table 2 In-Fusion primer sets for cloning the amplified promoter region of TAS2R8 into pGL4.20 vector

Genes (Region, size)	Sequences
TAS2R7 (-3285 – +1, 3.3 kb)	F: 5'- ATC AAA ATC TAA GAA ATG GAA GAT GCC AAA -3'
	R: 5'- TTC AGT AGT TTG CTA AGG CCA GAG AAA TGT-3'
TAS2R8 (-2080 – +1, 2.1 kb)	F: 5'- GTT CTC TCT ACA AAC ATG GAA GAT GCC AAA -3'
	R: 5'- ATA TAA GAG AAA ATG AGG TAC CGG CCA GTT -3'
TAS2R8 (-1572 – +1, 1.6 kb)	F: 5'- CCT TGA CAG AGA GAG GAG GCA CAG TTA TGT -3'
	R: 5'- CTC TCT CTG TCA AGG TAC CGG CCA GTT A -3'
TAS2R42 (-4873 – +1, 4.9 kb)	F: 5'- TTT TTG TCT CTG GAG ATG GAA GAT GCC AAA -3'
	R: 5'- TTC CAA TAA GAC ATT TCC TCG AGG CTA GCG -3'

F, forward; R, reverse.

Table 3 In-Fusion primer sets for cloning the deletion mutant of TAS2R8 promoter into pGL4.20 vector

Deleted region	Sequences
Δ-1572 – -1065	F: 5'- AAG TTT ATT ATT TTG AGA CAG AGT CTC ACT -3'
	R: 5'- CAA AAT AAT AAA CTT TCA TTC TTG AGA TTA -3'
Δ-1064 – -558	F: 5'- TTA TAT GTT AAA TAT CTA CAA TCT AAT TGT -3'
	R: 5'- ATA TTT AAC ATA TAA TAA TTT TTT TTT TTT -3'
Δ-557 – +1	F: 5'- TAA ATG GAA GAT GCC AAA AAC ATT AAG AAG -3'
	R: 5'- GGC ATC TTC CAT TTA GTC AGA GGA TAA TAA -3'
Δ-1572 – -1446	F: 5'- AAG TTA GAT TCC CTG GAA TGT TCT CTC TAG -3'
	R: 5'- CAG GGA ATC TAA CTT TCA TTC TTG AGA TTA -3'
Δ-1465 – -1344	F: 5'- GTG ATG GCA ATT TAT AGA TAA CTA GGT TTC -3'
	R: 5'- ATA AAT TGC CAT CAC CAG CTA GAG TAC T-3'
Δ-1366 – -1229	F: 5'- TTT TTA AAT ACA AGA AGC CCT AGG GTA TTG -3'
	R: 5'- TCT TGT ATT TAA AAA GAA AAT GAT GCA GAG -3'
Δ-1250 – -1115	F: 5'- AAA TCC TGC ATG TGT ATT AGT CCT ATT CCT -3'
	R: 5'- ACA CAT GCA GGA TTT TTT CTA TTT CTT ACA -3'
Δ-1146 – -1068	F: 5'- ACC CTT ATT ATT ATT TTG AGA CAG AGT CTC -3'
	R: 5'- AAT AAT AAT AAG GGT AAG GGA AAG TAC AAC-3'
Δ-1086 – -925	F: 5'- TGG AAA TAC TGG TAT GCC CTA TCT AGA AAT -3'
	R: 5'- ATA CCA GTA TTT CCA AGG AAT AGG ACT AAT -3'
Δ-941 – -808	F: 5'- TTA TTA TAC AAG AGA CAG TGA TTC AGG AAA -3'
	R: 5'- TCT CTT GTA TAA TAA TAA CTA CTT GGG AGA -3'
Δ-836 – -682	F: 5'- ATA ATG TAA AAT TTA AAG CAA ATT CTT AGT -3'
	R: 5'- TAA ATT TTA CAT TAT TCT AGT TCC AAT CAA -3'
Δ-716 – -572	F: 5'- TCT TTA CCT GTG TTA AAT ATC TAC AAT CTA -3'
	R: 5'- TAA CAC AGG TAA AGA TTT GTT ATC TTT AGT -3'

F, forward; R, reverse.

Table 4 RT-qPCR primer sets

Genes	Sequences
<i>TAS2R8</i>	F: 5'-CCA CCT GCC TTA ATG TCT TC-3'
	R: 5'-CTA TAA GGC TGA CCA ACA AGG-3'
<i>GAPDH</i>	F: 5'-GCA CCG TCA AGG CTG AGA AC-3'
	R: 5'-TGG TGA AGA CGC CAG TGG A-3'

F, forward; R, reverse.

Table 5 PCR primer sets for ChIP assays for the *TAS2R8* gene promoter

Target region	Sequences
-1595 – -1346	F: 5'-GTA TAA TCT CAA GAA TGA AAG TTT GAC AGA-3'
	R: 5'-TAC TGC TGG AAA CAA AAT AGA AAA GAA AAT-3'
-1152 – -925	F: 5'-CTT ACC CCA AAA CAA ATA AAT CTA ATT TAA-3'
	R: 5'-CAA ACA CAT AAA AAA GTA ACT ACT TGG GAG-3'

F, forward; R, reverse.

Promoter activity. Cells were transfected with plasmid using Xfect transfection reagent according to the manufacturer's instructions. After transfection, the cells were cultured in serum-free medium without zinc for 24 h (HEK293 cells) or 7 days (Ca9-22 cells). The cells were also co-transfected with CMV-driven *Renilla* luciferase (pGL4.75, Promega) vector to correct for transfection efficiency. At the end of incubation, cells were subjected to evaluation of luciferase activity.

Reverse-transcription quantitative PCR (RT-qPCR). Total RNA was extracted according to the acid-guanidinium-thiocyanate-phenol-chloroform (AGPC) method and reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit. *TAS2R8* gene expression level was quantified by SYBR® Premix Ex Taq™ II. Primer sequence that was used listed in Table 4.

Chromatin immunoprecipitation (ChIP) assays. Specific CTCFs that bound to the promoter region of *TAS2R8* was determined by ChIP assays as described, with some modifications (Agata *et al.* 2001; Maeda *et al.* 2015). Briefly, cells were suspended in 1% formaldehyde and sonicated. Glycine was added to stop cross-linking reactions, and the lysates were washed with PBS supplemented with 2% BSA and 0.05% NaN₃, and resuspended in a solution containing 10 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS). Antibodies were added and the solutions were incubated overnight at 4°C. Using magnetic agarose (ThermoFisher), the protein-DNA complex was purified and the DNA was extracted by the phenol-chloroform meth-

od. Immunoprecipitated DNA was amplified by PCR with PrimeSTAR GXL DNA Polymerase and the primer sets in Table 5. The PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized ethidium bromide staining.

Statistics. Triplicate samples were included in each experiment, and each was performed at least three times. Results were reported as mean ± SE and compared by two-tailed Student's *t*-tests. For comparison among more than 2 groups, ANOVA with Bonferroni correction was used. P values less than 0.05 indicated statistical significance.

RESULTS

Zinc depletion represses TAS2R8 promoter activity

The present study assessed the effects of zinc depletion on the bitter taste receptor, *TAS2R8*, as well as on the receptors *TAS2R7* and *TAS2R42*. Cells were transfected with promoter constructs of *TAS2R7* (nucleotide (nt) -3285 to nt +1, 3.3 kb), *TAS2R8* (nt -2080 to nt +1, 2.1 kb), and *TAS2R42* (nt -4873 to nt +1, 1.6 kb) and the effects of zinc deprivation on promoter activity were determined. Incubation of HEK293 cells in zinc-deprived medium for 24 h after transfection had no effect on promoter activity, nor did zinc supplementation (Fig. 1A), suggesting that incubation for 24 h in serum-free condition may not be sufficient for intracellular zinc deprivation. Because HEK293 cells are unable to survive longer in serum-free culture, as apoptosis is induced in normal cells by zinc deprivation (Treves *et al.* 1994; Chai *et al.* 1999; Kolenko *et al.* 2001), the effect of zinc deprivation was further tested in Ca9-22 oral

Fig. 1 *TAS2R8* promoter activity depends on zinc. Luciferase reporter vectors were constructed with promoter sequence of *TAS2R7* (nt -3285 to nt +1), *TAS2R8* (nt -2080 to nt +1), and *TAS2R42* (nt -4873 to nt +1). **(A)** HEK293 cells and Ca9-22 cells were preincubated in serum-free, zinc deprived medium for 24 h and 7 days, respectively. The cells were transfected with each promoter-luciferase vector and further incubated in the presence (black columns) or absence (empty columns) of 15.3 μ M ZnSO_4 for 24 h. **(B)** Ca9-22 cells were transfected with *TAS2R8* promoter-luciferase construct and incubated with or without 20 μ M TPEN in 10% FBS containing adequate zinc concentration. The cells were subsequently lysed and their luciferase activity assayed. **(C)** Ca9-22 cells were incubated in the presence or absence of 10% FBS for 12 h. Total RNA was extracted, reverse-transcribed, and subjected to qPCR. *** P < 0.001; NS, not significant.

carcinoma cells, which can be maintained longer under serum-free conditions. Culture of Ca9-22 cells for 7 days after transfection under serum-free conditions reduced promoter activity of *TAS2R8*, but not of *TAS2R7* and *TAS2R42* genes (Fig. 1B). Although incubation of transient transfectants for 7 days is generally considered long, *Renilla* luciferase activity was maintained at about 4–6 million relative luminescence units (RLU) (background, 1–2 thousand RLU), a level high enough to detect changes in activity, including changes in response to zinc deprivation.

Fig. 2 Zinc supplementation increases *TAS2R8* promoter activity in serum-deprived Ca9-22 cells. Ca9-22 cells were transfected with reporter vector and cultured in serum-free medium without zinc for 7 days (day zero for luciferase assay). The cells were further cultured with or without 15.3 μ M ZnSO_4 for the indicated time. The cells were subsequently lysed and their luciferase activity assayed. *** P < 0.001.

We also confirmed whether intrinsic expression *TAS2R8* was affected by TPEN treatment. Expectedly, *TAS2R8* mRNA expression was inhibited by Zn^{2+} deprivation (Fig. 1C).

To assess whether the reduction in *TAS2R8* promoter activity was due to zinc deprivation, the effects of TPEN, an intracellular zinc chelator, were assessed. Culture of Ca9-22 cells transfected with a *TAS2R8* promoter construct in DMEM containing 10% FBS (adequate zinc concentration) and TPEN for 24 h significantly reduced *TAS2R8* promoter activity. To assess whether zinc supplementation restored promoter activity, cells cultured without serum for 7 days were treated with zinc sulfate, increasing promoter activity within 3 h and for at least 24 h (Fig. 2). These findings strongly suggested that intracellular zinc deprivation reduced *TAS2R8* promoter activity.

Zinc-responding maximal region of the TAS2R8 promoter is at nt -1572 to nt -1344 and from nt -1146 to nt -925

To determine the region of the *TAS2R8* promoter associated with the response to intracellular zinc, truncation and deletion mutants of the *TAS2R8* promoter were constructed (Fig. 3A). Truncation of 0.5 kb at the 5'-end of the *TAS2R8* promoter (1.6 kb) did not significantly affect promoter activity. However, the

Fig. 3 Identification of the zinc dependent region of *TAS2R8* promoter. Ca9-22 cells were transfected with truncated or deleted mutants of *TAS2R8* promoter-luciferase reporter constructs, and the cells were cultured for 24 h in the presence of 10% FBS (A) or for 7 days in serum-free medium with or without 15.3 μ M ZnSO₄ (B). At the end of incubation, the cells were lysed and their luciferase activity assayed. The region in which the promoter activity was not changed by the addition of zinc was regarded as zinc dependent. *** $P < 0.001$.

0.5 kb-deletion mutant of the promoter construct (from nt -1572 to nt -1065 and from nt -1064 to -558 regions) showed about 30% promoter activity in the presence of 10% FBS. Interestingly, the deletion mutant from nt -557 to nt +1 showed significantly higher promoter activity. Because both regions, nt -1572 to nt -1065 and nt -1064 to nt -558, are responsible for *TAS2R8* promoter activity in the presence of 10% FBS, their zinc dependency required confirmation. After transfection with the promoter construct, cells were cultured for 7 days in serum-free medium with or without 15.3 μ M ZnSO₄. As expected, deletion of these regions not only markedly reduced promoter activities, but these activities were not affected by the addition of zinc, strongly suggesting that these regions were responsible for zinc dependency. To identify more specific regions responsible for the zinc dependency of

TAS2R8 promoter, we tested the regions from nt -1572 to nt -558 and from nt -1146 to nt -925, finding that the regions nt -1572 to nt -1344 and from nt -1146 to nt -925, were responsible (Fig. 3).

CTCF is responsible for zinc-dependent TAS2R8 promoter activity

Using *in silico* analysis and the open-access databases JASPR (Fornes *et al.* 2020) and melina II (Okumura *et al.* 2007), we searched for transcription factor binding motifs in these two zinc dependent regions. Zinc finger transcription factor binding motif(s) are frequently present in the regions from nt -1572 to nt -1344 and from nt -1146 to nt -925 (Fig. 3B) but are absent from other region. The *in silico* analyses showed candidate binding motifs for CTCF (from nt -1535 to nt -1526 and from nt -997 to nt -983), EGR1 (from nt -1570 to nt

–1557, from nt –998 to nt –985, and from nt –995 to nt –982), ESRRB (from nt –1538 to nt –1528 and from nt –768 to nt –758), and PUT3 (from nt –1442 to nt –1435, from nt –1440 to nt –1433, and from nt –985 to nt –978). Among them, we focused contribution of CTCF as further study, because role of CTCF is not clear in the taste bud. Mutations were introduced into one or both CTCF binding motifs, ccaccttgag to TTaTAAATaA at nt –1535 to nt –1526 and caacctctgcctcgcc to TaaTAtctATAtcATT at nt –997 to nt –983 of the *TAS2R8* promoter and promoter activity was assessed in the presence of 10%FBS. Although each mutant reduced promoter activity 50%, co-mutations in both regions dramatically decreased *TAS2R8* promoter activity in Ca9-22 cells (Fig. 4A). Furthermore, we confirmed the role of CTCF on *TAS2R8* promoter activity on the short exposure of 20 μ M TPEN for 3 h to HEK293 cells. *TAS2R8* promoter activity was confirmed to be reduced to 69.3% by the TPEN treatment (Fig. 4A), although this reduction was not obviously in HEK293 by zinc-deprived medium treatment for 24 h as shown in Fig. 1A. Noteworthy, the effect of the mutation for CTCF binding motifs on *TAS2R8* promoter activity was 73.5% reduction in the absence of TPEN. On the contrary, in the presence of TPEN, the reduction rate by the mutations decreased to 46.0%, suggesting that transcriptional activation of the *TAS2R8* gene by CTCF is zinc-dependent. ChIP assay using Ca9-22 cells showed that CTCF bound to the CTCF-binding motifs of *TAS2R8* promoter regions at nt –1535 to nt –1526 and nt –997 to nt –983, with zinc-dependency being greater at the nt –997 to nt –983 region than at the nt –1535 to nt –1526 region (Fig. 4B).

DISCUSSION

Zinc plays an important role in taste perception (Catalanotto *et al.* 1975; Henkin *et al.* 1975; Solomons *et al.* 1976) and has been clinically administered to patients, including those on peritoneal dialysis (Atkin-Thor *et al.* 1978) and those with alcoholic cirrhosis (Weismann *et al.* 1979), to improve taste functions. To date, however, the mechanism by which zinc alters taste perception is not fully understood. This study focused on the *TAS2R8* promoter to assess the role of zinc in bitter taste perception. Deprivation of zinc from cells in culture by replacement with serum-free medium resulted in a marked reduction in *TAS2R8* promoter activity, but not in the promoter activity of two other bitter taste receptors, *TAS2R7* and *TAS2R42*. Moreover, we found

Fig. 4 CTCF binds *TAS2R8* promoter. Based on JASPR analysis, mutations were introduced into possible CTCF-binding regions. **(A)** Ca9-22 cells were transfected with promoter constructs (wild type and mutant), incubated for an additional 24 h in the presence of 10% FBS (a zinc-containing condition). They were lysed to be obtained to luciferase assay. HEK293 cells were transfected with promoter constructs (wild type and mutant) were treated with or without 15.6 μ M TPEN in the presence of 10% FBS for 3 h. They were lysed to be obtained to luciferase assay. *** $P < 0.001$. **(B)** Cells were incubated in serum-free condition for 7 days, further incubated in the presence or absence of zinc, and subjected to ChIP assays. Input, whole DNA as the reference.

that zinc dependent *TAS2R8* promoter activity was driven by CTCF, an 11-zinc finger insulator protein. To our knowledge, this is the first study to investigate the association between CTCF and bitter taste perception through *TAS2R8*.

By *in silico* screening, Foster *et al.* (2015) showed that *TAS2R7*, *TAS2R8*, and *TAS2R42* did not ubiquitously express in human tissues, suggesting that these expressions were relatively specific to taste bud. In their report, transcription factor binding motifs in the promoter region of *TAS* genes were screened by JASPR. *TAS2R7*, *TAS2R8*, and *TAS2R42*

were excluded due to their low expression levels in the heart (Foster *et al.* 2015), although these gene expressions were major in the taste bud (Hevezi *et al.* 2009). Our *in silico* analysis showed the absence of CTCF binding motifs from the promoter sequences of *TAS2R7* and *TAS2R42*, perhaps explaining why the promoter activities of *TAS2R7* and *TAS2R42* were not affected by zinc deprivation. A zinc-deficient diet was found to reduce *TAS2R40* and *TAS2R107* expression on rat tongue but had no effect on the expression of *TAS2R105*, *TAS2R118*, and *TAS2R121* mRNAs (Sekine *et al.* 2012). Although that study did not assess *TAS2R8*, *TAS2R7* or *TAS2R42* expression, our JASPR analysis showed that CTCF or a CTCF-like motif was present in the *TAS2R40* and *TAS2R107* promoters, which were reported to be zinc-dependent. These findings strongly suggested that CTCF plays a critical role in the zinc dependency of bitter taste perception.

DNA methylation is a type of epigenetic transcriptional regulation that down-regulates gene expression, with CpG islands being typical target sequences (Soozangar *et al.* 2018). A linear regression analysis at 6707 CTCF binding sites showed an association between methylation and CTCF occupancy at 1677 (41%) sites (Wang *et al.* 2012). However, DNA methylation may not be involved in the regulation of *TAS2R8* gene expression, as we were unable to find typical CpG islands within 2.1 kb of the *TAS2R8* promoter. Rather, *TAS2R8* gene expression may be regulated by a CTCF-associated balance between promoter enhancing and blockage; *e.g.*, the nt -1572 to nt -1344 and nt -1146 to nt -925 regions may be responsible for enhancing promoter activity whereas the region from nt -557 to +1 may be responsible for enhancer blocking as an insulator function or by interacting with as yet unidentified repressor(s). Although further analysis is required to understand the critical role of CTCF in *TAS2R8* gene expression, the exon skip function of CTCF (Shukla *et al.* 2011) is likely uninvolved because the *TAS2R8* gene contains no introns (Andres-Barquin and Conte 2004).

SNPs have been reported in *TAS2Rs* (Kim *et al.* 2005), suggesting inter-individual differences in bitter taste perception. Interestingly, an SNP of the *TAS2R8* gene was reported associated with glioma susceptibility (Lee and Song 2015). In contrast, constitutive expression of *TAS2R8* reduced the expression of vascular endothelial growth factor (VEGF) and glucose transporter-1, as well as cancer stem cell markers such as DLK1, CD133, Notch1 and Sox2 in neuroblastoma (Seo *et al.* 2017). Thus, bit-

ter taste receptors in the tissues function differently from taste bud.

Radiation therapy for head and neck cancer causes taste disorder as a side effect. These patients are frequently treated with zinc sulfate to rescue the disorder. An evaluation of 169 patients with head and neck cancer who experienced taste alteration assessed the effects of zinc sulfate (Halyard *et al.* 2007), finding that taste alterations included the absence of bitter (8%), salty (5%), sour (4%), and sweet (5%) taste, as well as the absence of any taste (16%) and the presence of a metallic taste (10%), suggesting that zinc sulfate did not affect the time to taste recovery. UV-irradiation of cutaneous squamous cell carcinoma resulted in a high mutational frequency in the CTCF binding motif (Mueller *et al.* 2019), suggesting that DNA damage-based taste disorder and lack of a response to zinc may be caused by a mutation in the CTCF binding motif.

In conclusion, this study found that the expression of *TAS2R8*, a bitter taste receptor, was driven by CTCF. Because this regulation was zinc dependent, CTCF might play an important role in the positive effect of zinc in patients with taste perception disorder.

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CONFLICT OF INTEREST

None of the authors has any conflicts of interest associated with this manuscript.

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