# Two PARP13 isoforms are associated with induction of antiviral factors in oral mucosal cells

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Received May 25, 2021; Accepted November 23, 2021

DOI: 10.3892/mmr.2022.12622

**Abstract.** Innate immune systems in the oral cavity have important roles in the host defense against viral invasion of oral mucosa. Poly(ADP-ribose) polymerase 13 (PARP13), which has a strong antiviral ability, has been reported to possess two isoforms; a full-length protein, zinc-finger antiviral protein long (ZAPL), and a shorter protein (ZAPS). However, the expression and function of these two isoforms in oral mucosa remain unknown. In the present study, the expression levels of ZAPL and ZAPS induced by transfected double-stranded (ds) RNA, Poly(I:C), and dsDNA, Poly(dA:dT), in immortalized oral keratinocytes and fibroblasts (RT7 and GT1 cell lines, respectively) were investigated. Subsequently, the effects of the knockdown of ZAPL and ZAPS on transfected nucleotide-induced antiviral factors were examined. The results demonstrated constitutive expression of ZAPL and ZAPS in RT7 and GT1 cells, and their expression in both cell types was notably increased by transfection of Poly(I:C) and Poly(dA:dT) when compared with no transfection. Specific knockdown of ZAPL and ZAPS in RT7 cells decreased IFN-β and C-X-C motif chemokine ligand 10 (CXCL10) expression induced by transfected Poly(I:C) and Poly(dA:dT). On the other hand, knockdown of ZAPL and ZAPS in GT1 cells decreased the expression of CXCL10 induced by the transfected nucleotides, whereas that had no effect on IFN-β expression induced by Poly(dA:dT). Their knockdown was also associated with transfected nucleotides-induced IFN regulatory factor 3 phosphorylation in both cell types. Taken together, these results indicate that ZAPL and ZAPS, isoforms of PARP13, in oral mucosal cells participate in host defense against viral infection of oral mucosa.

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*Key words:* poly(ADP-ribose) polymerase 13, long form of zinc-finger antiviral protein, short form of zinc-finger antiviral protein, oral keratinocytes, oral fibroblasts

## Introduction

Innate immune systems in the oral cavity constitute the first line of the host defense during infection, during which oral mucosal cells recognize microbial structures and pathogen-associated molecular patterns via pattern recognition receptors to defend against microorganism invasion of oral mucosa (1-3). Retinoic acid-inducible gene I (RIG-I) is a key cytosolic receptor that responds to viral nucleic acids by activating downstream signaling for induction of inflammatory mediators (4,5). Our previous study reported that oral keratinocytes and fibroblasts, which are major oral mucosal cells, express functional RIG-I for recognizing transfected double-stranded (ds)RNA to produce antiviral cytokine IFN-β expression (6). These systems have important roles in host defense against viral invasion of oral mucosa.

Poly(ADP-ribose) polymerases (PARPs), a superfamily with ≥17 members, are known to modulate cell division, cell cycle and cell death programs triggered by DNA damage, as well as other biological functions, such as inflammatory and degenerative diseases (7-11). PARP13, a member of the PARP superfamily [zinc-finger antiviral protein (ZAP)/zinc finger CCCH-type containing, antiviral 1 (ZC3HAV1)], has four zinc finger domains at the N terminus, each of which has a cysteine-histidine repeat in a cys-cys-cys-his construction. Moreover, it was originally found in rats as a host restriction factor and was reported to prevent infection by the moloney murine leukemia virus (7). PARP13 selectively bind to specific RNA sequences and degrades target viral RNA (10,12), and can promote antiviral innate immune responses via cytosolic receptor RIG-I (13,14). PARP13 has a strong antiviral ability to suppress the replication of various viruses, including alphaviruses, filoviruses and influenza A virus (15-17), and its expression has been reported in a wide range of tissues (18). Therefore, it is considered that oral mucosal cells may express functional PARP13.

PARP13 has two isoforms, the full-length proteins ZAP and ZAPL, and a shorter protein ZAPS, arising from alternative splicing, which differ in their individual C-terminal

domains (18). ZAPL consists of 902 amino acids and has a PARP domain in the C-terminal, while ZAPS consists of 699 amino acids and does not have a PARP domain (18). Both ZAPS and ZAPL have been reported to possess the ability to reduce viral replication, such as that of Japanese encephalitis virus, human cytomegalovirus and sindbis virus (SINV) (14,19,20). Furthermore, ZAPS has been shown to have an important role as a potent stimulator of the RIG-I-mediated pathway in the innate immune response to viral infection (21,22). Recently, it has been reported that both ZAPL and ZAPS demonstrated differential IFN responses against viral RNA (23). However, the role, expression and function of these two PARP13 isoforms in oral mucosa remain unknown.

The present study investigated the expression levels of ZAPL and ZAPS induced by transfected dsRNA and dsDNA in immortalized oral keratinocytes and fibroblasts (RT7 and GT1 cell lines, respectively). Subsequently, the effects of ZAPL and ZAPS knockdown on these ligand-induced antiviral factors were examined.

## Materials and methods

*Reagents*. Poly(I:C)-LMW, Poly(dA:dT) and LyoVec, a transfection reagent, were purchased from InvivoGen. Antibodies used for immunoblotting were anti-ZC3HAV1 (cat. no. GTX120134; GeneTex, Inc.), anti-IFN regulatory factor 3 (IRF3; cat. no. 4302), anti-phosphorylated (p)-IRF3 (cat. no. 4947; both Cell Signaling Technology, Inc.), anti-GAPDH (cat. no. MAB374; MilliporeSigma) and anti-RIG-I (cat. no. sc-376845; Santa Cruz Biotechnology, Inc.). Secondary antibodies used for labeling were HRP-conjugated antibody from Cytiva (cat. no. NA931) and Alexa 488-conjugated rabbit IgG from Invitrogen (cat. no. A-11008; Thermo Fisher Scientific, Inc.). The molecular weight marker for western blotting was purchased from Invitrogen (MagicMark™ XP Western Protein Standard; cat. no. LC5602; Thermo Fisher Scientific, Inc.).

*Cell lines*. RT7, an immortalized human oral keratinocyte cell line, was previously established by transfection of hTERT and E7, as described in a prior study (24), while GT1, a human oral fibroblast cell line, was established by transfection of hTERT, as previously reported (25). Briefly, RT7 cells were cultured in keratinocyte growth medium containing human epithelial growth factors, insulin, hydrocortisone, Transferrin, epinephrine, bovine pituitary extract and gentamicin sulfate amphotericin-B (KGM-Gold Keratinocyte growth medium bulletkit ; Lonza Group, Ltd.), and GT1 cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) containing 10% FBS (Biological Industries), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin.

Human gingival keratinocytes and fibroblasts were obtained from a gingival mucosa excised at the extraction of an impacted tooth of healthy volunteers (one male and two females, age, 19 to 24) from April 2015 to March 2018 in Hiroshima University Hospital (Hiroshima, Japan) and primary cultures were prepared as previously reported (26). Informed consent for such acquisition and use was obtained according to a protocol approved by the Ethical Committee of Hiroshima University (approval no. E-930).

*Treatment of Poly(I:C) and Poly(dA:dT)*. For the experiments, Poly(I:C) and Poly(dA:dT) were transfected by use of the cationic lipid-based transfection reagent LyoVec, according to the manufacturer's instructions. Briefly, each (1 mg/ml final concentration) was separately mixed with LyoVec and incubated at 18-22˚C (room temperature) for 15 min to allow the formation of a lipid-RNA complex. The complex was then added to cell cultures and incubated at 37˚C for 12 h [RT-quantitative (q) PCR ] or 0, 6, 12 or 24 h (western blotting).

*RNA extraction, RT-PCR and RT-qPCR*. Gene-specific oligonucleotide primers used for PCR analysis are shown in Table I. The primers were designed by using Primer3 software (bioinfo.ut.ee/primer3-0.4.0/). Total RNA was prepared from the cell lines using a RNeasy total RNA isolation kit (Qiagen GmbH) and one-step RT-PCR was performed with an RT-PCR High Plus System (Toyobo Life Sciences), according to the manufacturer's instructions. Single-stranded cDNA for RT-PCR and a qPCR template were synthesized using a First Strand cDNA Synthesis kit (Amersham; Cytiva). The RT-PCR conditions for ZAP were 1 cycle (95˚C, 15 min), 35 cycles (95˚C, 2 min; 55˚C, 30 sec; 72˚C, 1 min) and 1 cycle (72˚C, 7 min), while those for β-actin were 1 cycle (95˚C, 15 min), 25 cycles (95˚C, 2 min; 55˚C, 30 sec; 72˚C, 1 min) and 1 cycle (72˚C, 7 min). The products were analyzed on 2% agarose gels containing SYBR-Green (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR-Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.); Initial denaturation at 95˚C for 2 min, for 40 cycles (denaturation at 95˚C for 15 sec, annealing at 60˚C for 60 sec, elongation at 72˚C for 60 sec), final extension at 72˚C for 5 min. qPCR analysis was performed using a CFX Connect Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Relative quantification of mRNA levels noted for the samples was performed according to User Bulletin #2 (Applied Biosystems; Thermo Fisher Scientific, Inc.), with the results shown as the mean ± SD from three independent experiments.

*Preparation of whole cell extracts*. Cell cultures were washed with ice-cold PBS, then subjected to lysis with SDS sample buffer using a Mammalian Cell Lysis kit (Sigma-Aldrich; Merck KGaA) to yield whole cell extracts, according to the manufacturer's instructions.

*Western blotting*. Mammalian Cell Lysis kit (Sigma-Aldrich; Merck KGaA) was used to extract protein, which was quantified using a Pierce BCA Protein Assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). Proteins from each sample (50 mg) were separated on 10% SDS-polyacrylamide gels and left for 1 h at 100 V, then transferred to PVDF membranes (Amersham; Cytiva) and left for 1 h at 90 V. After blocking for 1 h at room temperature with 5% BSA (cat. no. 01863-48; Nacalai Biochemicals Reagent) in PBS, the membrane was incubated with the primary antibody (1:1,000) at 4˚C overnight. Immunoblots were labeled with an HRP-conjugated secondary antibody (1:1,000) for 1 h at room temperature and developed using an ECL Advance Western blotting Detection kit (Cytiva). Image data were analyzed with an LAS 4000 mini-imaging system (FUJIFILM Wako Pure Chemical Corporation). ImageJ version 1.47 (National Institutes of

Table I. Primer sequences for reverse transcription-quantitative PCR.

| Gene | Primer sequence |
|---|---|
| ZAPL | F: 5'-GCTGAGTTTCCAAGGGATGAT-3' |
| | R: 5'-AGTCCTCCTGAGGACGAAAGG-3' |
| ZAPS | F: 5'-GCTGAGTTTCCAAGGGATGAT-3' |
| | R: 5'-AATGGAAACTGCAGAGTAATG-3' |
| CXCL10 | F: 5'-TGCAAGCCAATTTTGTCCACGTG-3' |
| | R: 5'-GCAGCTGATTTGGTGACCATCAT-3' |
| IFN-β | F: 5'-TGCTCTGGCACAACAGGTAG-3' |
| | R: 5'-GCTGCAGCTGCTTAATCTCC-3' |
| β-actin | F: 5'-TCACCCACACTGTGCCCATCTACGA-3' |
| | R: 5'-CAGCGGAACCGCTCATTGCCAATGG-3' |

ZAPL, zinc-finger antiviral protein long; ZAPS, zinc-finger antiviral protein short; CXCL10, C-X-C motif chemokine ligand 10; F, forward; R, reverse.

Health), was used to analyze the intensities of the western blotting bands. The ratio of the band density of each target protein to that of GAPDH was calculated.

*Immunocytochemistry*. Cells were seeded into two-well chamber slides (Matsunami Glass Ind., Ltd.) and fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min. blocking for 30 min at room temperature with 1% BSA (Nacalai Biochemicals Reagent) in PBS, and incubation overnight at 4˚C with the primary antibody (1:400) in PBS containing 5% BSA. Next, cells were washed and incubated with diluted Alexa Fluor secondary antibody (1:400) at room temperature for 1 h. Vectashield anti-fade medium containing DAPI (Vector Laboratories, Inc.) was used to mount the cells. Fluorescent and phase contrast images were acquired with a BZ-9000 microscope (Keyence Corporation).

*Small interfering RNA (siRNA)*. A stealth siRNA for ZAPL and ZAPS was designed and purchased from Japan Bio Services Co., Ltd. The siRNA sequences were as follows: ZAPL, 5'-CAUGAAACUCAUGAAAACATT-3' and 5'-UGUUUUCAUGAGUUUCAUGTA-3'; and ZAPS, 5'-CCUGUUUUCCUGAAAAGUUTT-3' and 5'-AACUUUUCAGGAAAACAGGCT-3'. Negative control siRNA (2.5 mg/well) (Stealth RNAi™ siRNA Negative Control) was purchased from Invitrogen. RT7 and GT1 cells were transiently transfected with various combinations of the siRNAs (2.5 mg/well) using Lipofectamine® 2000 transfection reagent (6 ml/well) (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h in 6-well plates (Cellstar, Greiner Bio-one), according to the manufacturer's recommendations. Thereafter, complex of Poly(I:C) or Poly(dA:dT) and transfection reagent, Lyovec was added to cell cultures and incubated at 37˚C for 12 h (RT-qPCR ) or 6 h (western blotting).

*Statistical analysis*. SPSS software (version 24; IBM Corp., Japan, Inc.) was employed for the statistical analysis. Statistical analysis was performed using Student's t-test (paired or unpaired) and one-way ANOVA followed by Dunnett's multiple comparison test and the values are presented as the mean ± SD of three independent experiments.

## Results

*Expression of two isoforms of PARP13 in oral mucosal cells*. To investigate the effects of PARP13 on innate antiviral response in oral mucosal cells, it was first examined whether oral keratinocytes and fibroblasts expressed PARP13. Immunocytochemistry analysis showed the expression of PARP13 in the cytoplasm of both RT7 and GT1 cells, indicating that it was functional in these cells (Fig. 1A). PARP13 has two isoforms (ZAPL, full length protein; ZAPS, shorter protein) arising from alternative splicing (13). The RT-PCR results identified that RT7 and GT1 cells, as well as primary oral keratinocytes and fibroblasts, constitutively expressed ZAPL and ZAPS mRNA (Fig. 1B). However, since primary cells show limited proliferative activity (24,27), RT7 and GT1 cells, immortalized human oral keratinocytes and fibroblasts, respectively, were used in the following experiments.

*ZAPL and ZAPS expression induced by transfected dsRNA and dsDNA in oral mucosal cells*. Transfected nucleotides have been reported to increase cytosolic nucleic acid sensor expression (28,29). Next, the effects of transfected mimic dsRNA, Poly(I:C), and dsDNA, Poly(dA:dT), on ZAPL and ZAPS expression in RT7 and GT1 cells were examined. Poly(I:C) alone without the transfection reagent increased the mRNA expression levels of ZAPL and ZAPS in GT1 cells, but not in RT7 cells, whereas Poly(dA:dT) alone without the transfection reagent did not affect those isoforms mRNA expressions in either cell line. On the other hand, transfected Poly(I:C) and Poly(dA:dT) significantly increased the mRNA expression levels of ZAPL and ZAPS in both cell lines as compared with each nucleotide alone (Fig. 2). Western blotting results also demonstrated a high constitutive level of ZAPL protein expression with a molecular size of 100 kDa, indicating 0 h without nucleotides, in both cell lines, whereas a low constitutive level of expression of ZAPS protein with a molecular size of 78 kDa, indicating 0 h without nucleotides, was shown in both cell lines. Furthermore, transfected Poly(I:C) and Poly(dA:dT) enhanced the expression levels of ZAPL and ZAPS proteins, similar to their mRNA expression results, in both cell lines (Fig. 3).

*Knockdown of both ZAPL and ZAPS using specific siRNA*. To examine the effects of ZAPL and ZAPS on transfected dsRNA and dsDNA-induced antiviral factor, siRNA-mediated knockdown of each was performed and their expression was subsequently examined. Each specific siRNA decreased the mRNA expression levels of ZAPL or ZAPS (Fig. 4A). Furthermore, knockdown of ZAPL or ZAPS protein expression using each specific siRNA was also confirmed following stimulation after transfection with Poly(I:C) and Poly(dA:dT) (Figs. 4B, S1 and S2).

*Effects of knockdown of ZAPL and ZAPS on transfected dsRNA and dsDNA-induced antiviral factor expression*. In

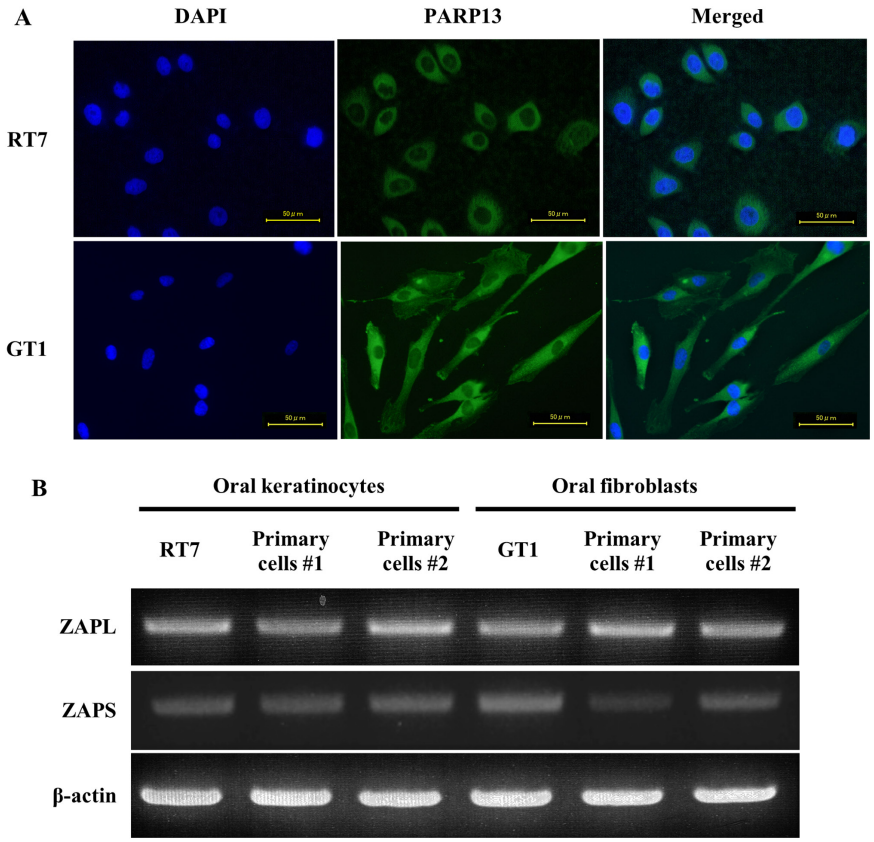


Figure 1. Expression of two PARP13 isoforms in oral keratinocytes and fibroblasts. (A) Localization of PARP13 expression in RT7 and GT1 cells. Cells were stained with anti-ZC3HAV1 (PARP13) and Alexa Fluor® 488 conjugated rabbit IgG, and nuclei were counter-stained with DAPI (blue). Green staining indicates that PARP13 was observed in the cytoplasm of the cells. Each experiment was performed ≥3 times, with representative results shown. Scale bar, 50 $\mu$m. (B) mRNA expression level of two PARP13 isoforms in RT7 cells, primary oral keratinocytes, GT1 cells and primary oral fibroblasts cell lines. Total RNA was isolated from each cell line after culturing to confluence, then reverse transcription PCR assays of ZAPL, ZAPS and β-actin were performed. ZAPL, long form of zinc-finger antiviral protein; ZAPS, short form of zinc-finger antiviral protein; PARP13, poly(ADP-ribose) polymerase 13.

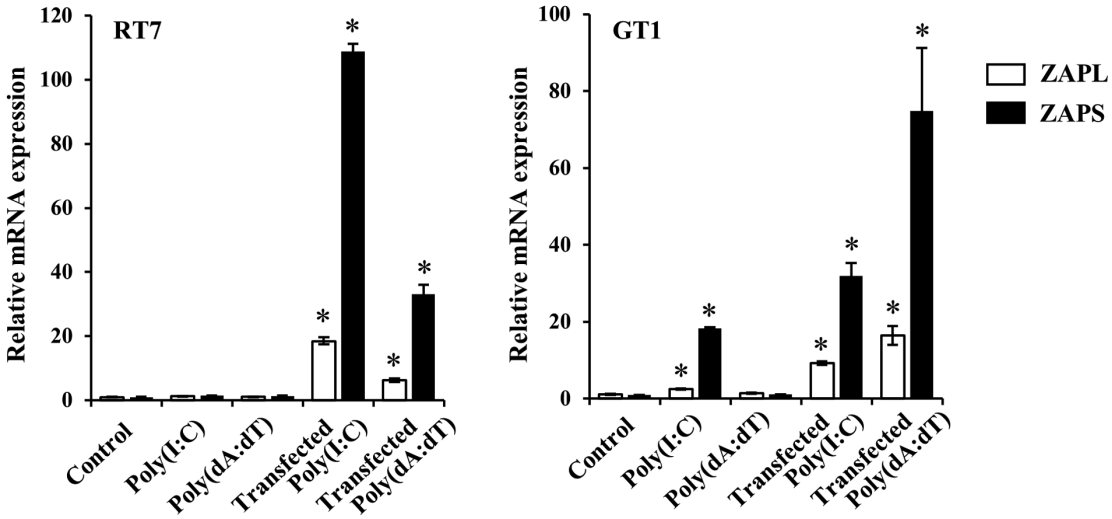


Figure 2. Effects of double-stranded nucleotides on ZAPL and ZAPS mRNA expression. The cells were exposed to Poly(I:C) alone, Poly(dA:dT) alone, transfected Poly(I:C) or transfected Poly(dA:dT) (1 $\mu$g/ml) for 12 h. Data are shown as the mean ± SD of three independent experiments. *P<0.05 vs. control cells (Student's t-test). ZAPL, long form of zinc-finger antiviral protein; ZAPS, short form of zinc-finger antiviral protein.

our previous study, transfected dsRNA and dsDNA increased the expression of antiviral cytokines and chemokines, IFN-β and CXCL10 (6,30). Therefore, the current study examined the effects knockdown of ZAPL and ZAPS on transfected dsRNA and dsDNA-induced IFN-β and CXCL10. Specific knockdown of ZAPL and ZAPS in RT7 cells caused decreased IFN-β and CXCL10 expression levels induced by transfected Poly(I:C) and Poly(dA:dT) (Fig. 5). On the other hand, knockdown of

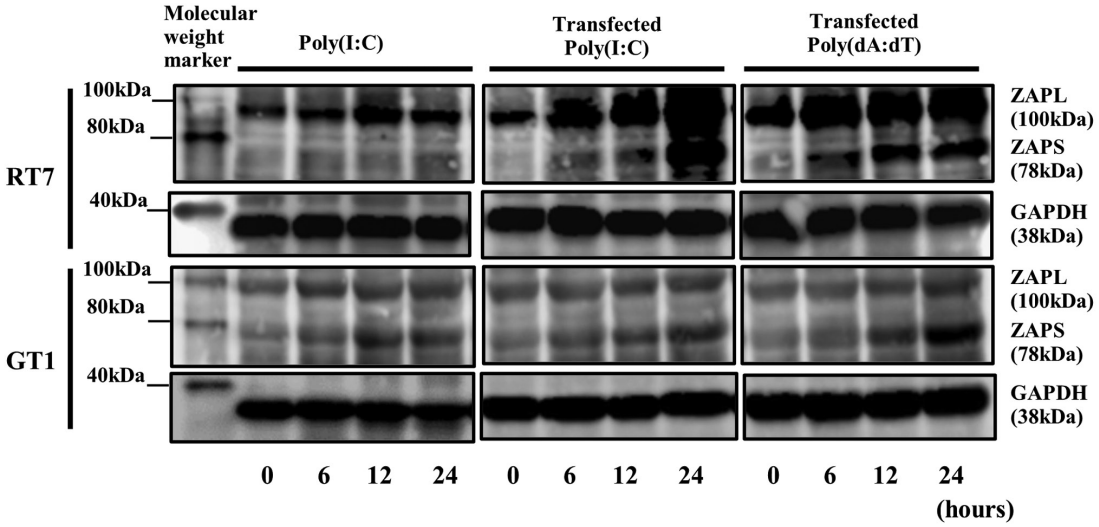


Figure 3. Effects of double-stranded nucleotides on ZAPL and ZAPS proteins. The cells were exposed to Poly(I:C) alone, transfected Poly(I:C) or transfected Poly(dA:dT) (1 $\mu$g/ml) for the 0 ,6, 12, 24 h, after which cell extracts were subjected to SDS-PAGE. ZAPL and ZAPS were examined via western blot analysis with antibodies against anti-ZC3HAV1 (PARP13) and GAPDH. These experiments were performed ≥3 times, with representative results shown. ZAPL, long form of zinc-finger antiviral protein; ZAPS, short form of zinc-finger antiviral protein; PARP13, poly(ADP-ribose) polymerase 13.

ZAPL and ZAPS in GT1 cells decreased transfected Poly(I:C) and Poly(dA:dT)-induced CXCL10 expression. Although transfected Poly(I:C)-induced IFN-β expression was also inhibited by knockdown of ZAPL and ZAPS, knockdown of each in GT1 cells had no effects on transfected Poly(dA:dT)-induced IFN-β expression (Fig. 5).

*Effects of the knockdown of ZAPL and ZAPS on transfected dsRNA and dsDNA-induced IRF3 activation*. IRF3 is a key transcriptional factor involved in the signaling pathway response to viral infection (31). Our previous study revealed that transfected Poly(I:C)-induced IFN-β was associated with IRF3 activation in RT7 and GT1 cells (6). Therefore, the current study examined the effects of knockdown of ZAPL and ZAPS on transfected Poly(I:C) and Poly(dA:dT)-induced IRF3 phosphorylation in both cell types. The results demonstrated that knockdown of ZAPL and ZAPS decreased transfected Poly(I:C) and Poly(dA:dT)-induced IRF3 phosphorylation in RT7 cells (Fig. 6). Furthermore, knockdown of both in GT1 cells decreased transfected Poly(I:C)-induced IRF3 phosphorylation, though it did not affect transfected Poly(dA:dT)-induced IRF3 phosphorylation (Fig. 6). These findings indicate that these two PARP isoforms in oral keratinocytes and fibroblasts are associated with activation of IRF3 signaling to increase antiviral factor expression.

## Discussion

The PARP superfamily is known to have important roles in several different biological and pathological processes, and some members directly regulate the replication of certain viruses (7-9). Notably, PARP13 (ZAP/ZC3HAV1) has been reported to inhibit the replication of a wide variety of viruses, including several RNA viruses, such as murine leukemia virus, SINV, HIV and Epstein-Barr virus, as well as the RNA intermediate of the hepatitis B DNA virus (7,16,32-36). PARP13 has two isoforms, full-length PARP13.1 (ZAPL) and the C-terminal truncated isoform PARP13.2 (ZAPS), both of which are expressed in a wide range of tissues, including those of the lung, colon and salivary gland; though ZAPS has a broader expression pattern compared with ZAPL in some human tissues, such as those of the kidney and liver (18). ZAPS expression has also been observed in immune cells, including human monocytes, as well as in non-immune cells, such as human embryonic kidney and human fetal lung cells (21,34). In the present study, PARP13 protein was found in cytoplasm of oral keratinocytes and fibroblasts, with constitutive mRNA expressions of both ZAPL and ZAPS also noted in both types of cells, indicating that these two isoforms have functions for defense against cytosolic viral nucleotide invasion.

Hayakawa *et al* (21) reported that ZAPL and ZAPS are constitutively expressed in human embryonic kidney cells and $CD14^+$ monocytes. In their experiments, ZAPS mRNA was shown to be markedly induced by stimulation with a 5'-triphosphate modification (3pRNA), essential for RIG-I recognition and activation in both cell types, whereas the level of ZAPL mRNA expression induced by 3pRNA was very low (21). In the present study, high levels of ZAPL protein expression were noted, whereas the level of constitutive expression of ZAPS protein was low (indicating proteins at 0 h) in oral keratinocytes and fibroblasts. Furthermore, ZAPL and ZAPS expression levels were induced by transfection of those nucleotides in both RT7 and GT1 cells. Compared with ZAPS, ZAPL is more active against alphaviruses, such as SINV and Semliki Forest virus, and carries signatures of positive selection (18), whereas ZAPS is upregulated to a greater level than ZAPL by viral infection (21,37). These two PARP13 isoforms have also been reported to be important components of cellular response to stress, and are associated with cell survival and apoptosis (34,38). Therefore, it is considered that ZAPL functions in cellular homeostasis and antiviral defense, whereas ZAPS mainly acts as an intrinsic antiviral factor in oral mucosa.

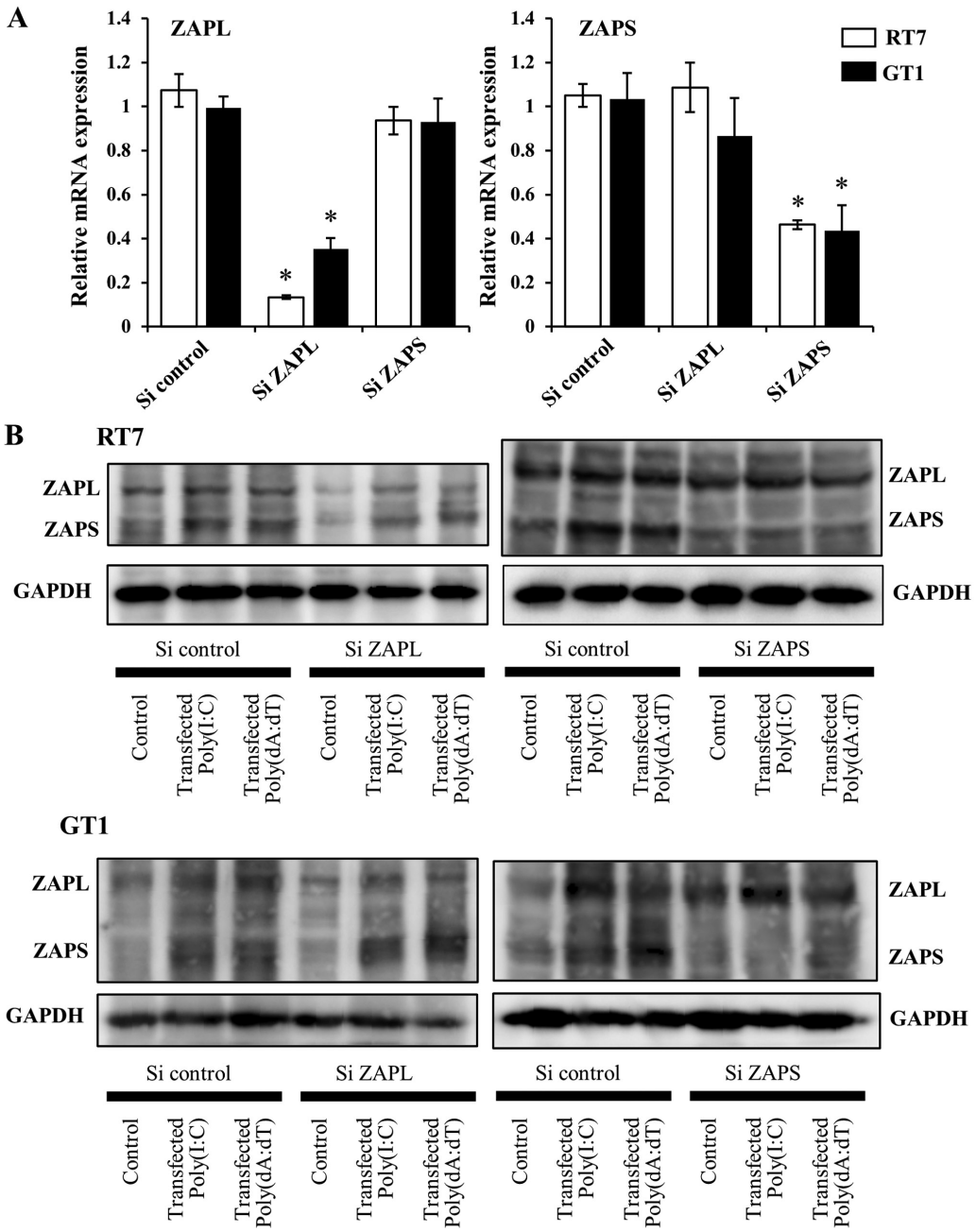


Figure 4. Knockdown of ZAPL and ZAPS using specific siRNA. (A) Knockdown of ZAPL and ZAPS mRNA expression using siRNA. Cells were transfected with siRNA specific for ZAPL or ZAPS for 48 h. Gene mRNA expression levels are shown relative to β-actin. Values are shown as fold increase as compared with Si control-treated cells and presented as the mean ± SD of three independent experiments. *P<0.05 vs. Si control (Student's t-test). (B) Knockdown of ZAPL and ZAPS protein expression by siRNA. Cells were transfected with siRNA specific for ZAPL or ZAPS for 48 h. The cells were exposed to transfected Poly(I:C) and Poly(dA:dT) (1 $\mu$g/ml) for the indicated times, after which cell extracts were subjected to SDS-PAGE. ZAPL and ZAPS were examined via western blot analysis with antibodies against anti-ZC3HAV1 (PARP13) and GAPDH. These experiments were performed ≥3 times, with representative results shown. ZAPL, long form of zinc-finger antiviral protein; ZAPS, short form of zinc-finger antiviral protein; PARP13, poly(ADP-ribose) polymerase 13; Si/siRNA, small interfering RNA.

IRF3 has been shown essential for induction of antiviral response. Various type of viral infection trigger IRF3 phosphorylation, and result in induction of antiviral cytokine, such as IFN-β (31). Our previous study revealed that transfected Poly(I:C) increased IRF3 activation in oral keratinocytes and fibroblasts (6). On the other hand, ZAPS in 239T cells was shown to promote oligomerization and ATPase activity of RIG-I, thereby increasing downstream RIG-I signaling via IRF3 signaling, when stimulated with 3pRNA (21). Although IRF3 selectively binds the ZAPL promoter following viral infection, it is unclear whether the effect of ZAPL is associated with IRF3 activation (39). In the present study, knockdown of ZAPL and ZAPS in both cell types decreased transfected Poly(I:C)-induced IRF3 activation. While transfected Poly(dA:dT)-induced IRF3 activation in RT7 cells was shown to be decreased by knockdown of ZAPL and ZAPS, knockdown of these in GT1 cells did not affect transfected Poly(dA:dT)-induced IRF3 phosphorylation. Taken together, these results indicate that ZAPL and ZAPS in oral mucosal cells are important for activated antiviral response against viral infection of oral mucosa.

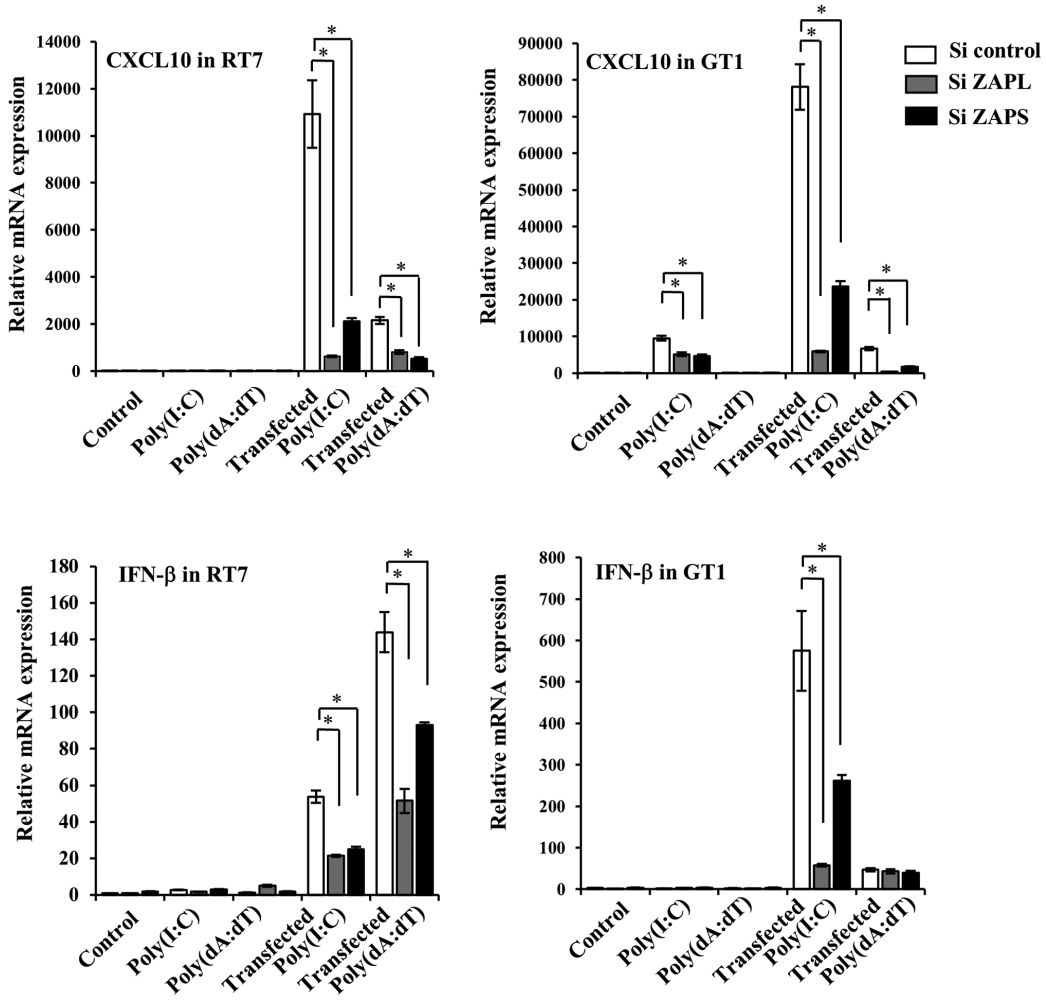


Figure 5. Effects of siRNAs for ZAPL and ZAPS on double-stranded nucleotide-induced CXCL10 and IFN-β. Cells were transfected with siRNA specific for ZAPL or ZAPS for 48 h, then exposed to Poly(I:C), Poly(dA:dT), transfected Poly(I:C) or transfected Poly(dA:dT) (1 $\mu$g/ml) for 12 h. Data are shown as the mean ± SD of three independent experiments. *P<0.05 vs. Si control (Dunnett's multiple comparison test). ZAPL, long form of zinc-finger antiviral protein; ZAPS, short form of zinc-finger antiviral protein; Si/siRNA, small interfering RNA; CXCL10, C-X-C motif chemokine ligand 10.

IFN-β plays an important role in antiviral immunity by directly inhibiting viral replication in infected cells (40,41). However, the response of ZAPS for induction of IFN-β was different in two prior studies. For instance, Hayakawa *et al* (21) revealed that knockdown of ZAPS decreased 3pRNA-induced IFN-β expression and activation of IRF3 in 239T cells. Conversely, Schwerk *et al* (23) reported that knockdown of ZAPS increased IFN-β expression induced by polyU/UC RNA, whereas knockdown of ZAPL did not have an effect on IFN-β expression in Hu7 or 239T cells. This authors also showed that IRF3 phosphorylation was not affected by ZAPL knockdown in those cell types (23). In the present study, knockdown of ZAPL and ZAPS decreased Poly(I:C)-induced IFN-β in RT7 and GT1 cells, while knockdown of those decreased transfected Poly(dA:dT)-induced IFN-β expression in RT7 cells but not GT1 cells. These results are in accordance with the notion of nucleotide-induced IRF3 phosphorylation in those cell types. The differential regulation of IFN-β via ZAP and ZAPS shown previously and also in the present report may be associated with differences related to the intracellular signaling pathways, including IRF3, among the examined cell types. Therefore, a future study is required to investigate the contributions of ZAPL and ZAPS to the regulation of IFN-β expression induced by various nucleotides in assorted cell types.

CXCL10 is an inflammatory chemokine that mainly recruits activated T and natural killer cells to sites of infection or inflammation, and plays a critical role in host defense towards a variety of viral infections when its expression is significantly induced (42,43). Our previous study reported that transfected dsRNA increased IFN-β expression via RIG-I-mediated IRF3 activation, after which IFN-β enhanced CXCL10 expression via the IFN-β/α receptor in oral keratinocytes and fibroblasts (6). In the present study, though knockdown of ZAPL and ZAPS did not affect IFN-β expression induced by transfected Poly(dA:dT) in GT1 cells, their knockdown decreased CXCL10 expression induced by transfected Poly(dA:dT) and Poly(I:C). Therefore, transfected-Poly(dA:dT)-induced CXCL10 induction in oral fibroblasts via ZAPL and ZAPS may be independent of IRF3/IFN-β-mediated signaling.

The present study has certain limitation. First, we examined immune responses via ZAPL and ZAPS using immortalized human oral keratinocytes and fibroblasts. Because primary cells show limited proliferative activity, they are unsuitable for long-term experiments and do not guarantee

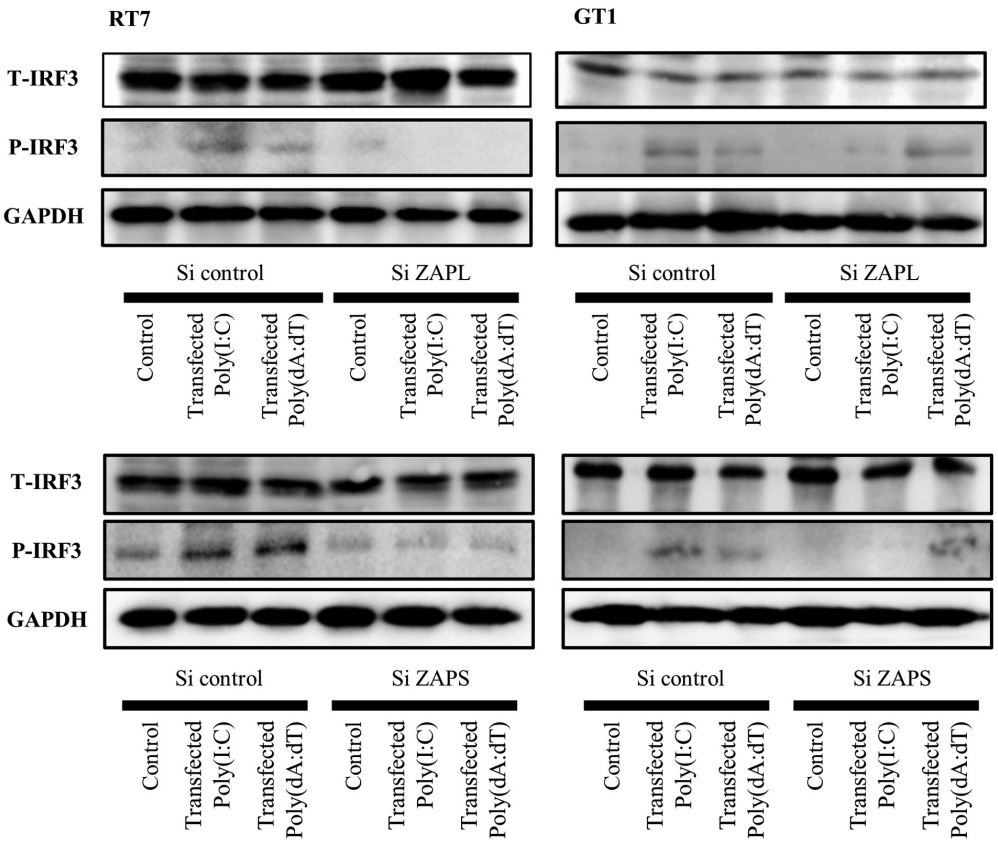


Figure 6. Effects of knockdown of ZAPL and ZAPS on transfected dsRNA and dsDNA-induced IRF3 activation. Cells were transfected with siRNAs specific for ZAPL and ZAPS for 48 h. The cells were exposed to transfected Poly(I:C) and Poly(dA:dT) (1 $\mu$g/ml) for 6 h, after which cell extracts were subjected to SDS-PAGE. Phosphorylation of IRF3 was examined via western blot analysis with antibodies against p-specific IRF3 (P-IκBa), total IRF3 (T-IRF3) and GAPDH. These experiments were performed ≥3 times, with representative results shown. P-, phosphorylated; T-, total; IRF3, IFN regulatory factor 3; ZAPL, long form of zinc-finger antiviral protein; ZAPS, short form of zinc-finger antiviral protein; Si/siRNA, small interfering RNA.

reproducibility (24,27). Secondly, transfected mimic ds nucleic acids Poly(I:C) and Poly(dA:dT) were used instead of actual viruses. Therefore, function of ZAPS and ZAPL in oral keratinocytes and fibroblast against viral invasion need to be investigated in future.

In conclusion, the present findings are the first known to demonstrate that oral keratinocytes as well as oral fibroblasts express two PARP13 isoforms, ZAPL and ZAPS. Furthermore, the expression of each was increased by transfected dsRNA and dsDNA. In addition, ZAPL and ZAPS were found to be associated with antiviral chemokine and cytokine expressions via IRF3 activation. Taken together, these results suggest that ZAPL and ZAPS in oral keratinocytes and fibroblasts may participate in host defense against viral infection of oral mucosa.

**Acknowledgements**

Not applicable.

**Funding**

This work was supported by Grant-in-Aids for Scientific Research from the Japan Society for Scientific Research (C) (grant no. 2646301) and the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 17K11840).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

KO and HK designed the study, analyzed data and wrote the manuscript. MS, SF, TN, HS and HN performed the experiments, analyzed the data and drafted the manuscript. MT contributed to the conception and design, as well as drafting of the manuscript. All authors have read and approved the final version of the manuscript. KO and HK confirm the authenticity of the raw data.

**Ethics approval and consent for participation**

Informed consent for such acquisition and use was obtained according to a protocol approved by the Ethical Committee of Hiroshima University (approval no. E-930).

**Patient consent for participation**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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