Identification of Signaling Pathways Mediating Cell Cycle Arrest and Apoptosis Induced by *Porphyromonas gingivalis* in Human Trophoblasts

Hiroaki Inaba,[a] Masae Kuboniwa,[b] Hideyuki Sugita,[a] Richard J. Lamont,[c] and Atsuo Amano[b]

Department of Oral Frontier Biology, Center for Frontier Oral Science,[a] and Department of Preventive Dentistry,[b] Osaka University Graduate School of Dentistry, Suita-Osaka, Japan, and Center for Oral Health and Systemic Disease, School of Dentistry, University of Louisville, Louisville, Kentucky, USA[c]

Epidemiological and interventional studies of humans have revealed a close association between periodontal diseases and preterm delivery of low-birth-weight infants. *Porphyromonas gingivalis*, a periodontal pathogen, can translocate to gestational tissues following oral-hematogenous spread. We previously reported that *P. gingivalis* invades extravillous trophoblast cells (HTR-8) derived from the human placenta and inhibits proliferation through induction of arrest in the G_1 phase of the cell cycle. The purpose of the present study was to identify signaling pathways mediating cellular impairment caused by *P. gingivalis*. Following *P. gingivalis* infection, the expression of Fas was induced and p53 accumulated, responses consistent with response to DNA damage. Ataxia telangiectasia- and Rad3-related kinase (ATR), an essential regulator of DNA damage checkpoints, was shown to be activated together with its downstream signaling molecule Chk2, while the p53 degradation-related protein MDM2 was not induced. The inhibition of ATR prevented both G_1 arrest and apoptosis caused by *P. gingivalis* in HTR-8 cells. In addition, small interfering RNA (siRNA) knockdown of p53 abrogated both G_1 arrest and apoptosis. The regulation of apoptosis was associated with Ets1 activation. HTR-8 cells infected with *P. gingivalis* exhibited activation of Ets1, and knockdown of Ets1 with siRNA diminished both G_1 arrest and apoptosis. These results suggest that *P. gingivalis* activates cellular DNA damage signaling pathways that lead to G_1 arrest and apoptosis in trophoblasts.

Preterm birth is defined as delivery before 37 weeks of gestation (15) and generally results in low-birth-weight infants. There are various risk factors for preterm delivery of low-birth-weight infants (PTLBW), many of which involve increased systemic inflammation (5, 16). Bacterial infection is one of the major causes of PTLBW, either ascending from the urogenital tract or occurring transplacentally following bacteremia. Up to 80% of preterm deliveries at less than 30 weeks of gestation have possible infection, and these infections usually precede the development of pregnancy complications. The pathogenesis can result directly from bacterial invasion of fetoplacental tissue, causing tissue damage and expulsion of the infected fetus. Alternatively, adverse outcomes can result from infection-induced disruption of normal immune and inflammatory status (5, 15, 43).

Various epidemiological studies have shown a link between periodontal diseases and PTLBW (59). *Porphyromonas gingivalis*, a major periodontal pathogen, was detected in the amniotic fluid of pregnant women with a diagnosis of threatened premature labor (30) as well as in placentas of those with preeclampsia (4). In addition, *P. gingivalis* antigens were detected in placental tissues, including syncytiotrophoblasts, chorionic trophoblasts, decidual cells, and amniotic epithelial cells, as well as vascular cells, which were obtained from women with chorioamnionitis at fewer than 37 weeks of gestation (25). In rodent and rabbit animal models, *P. gingivalis* was also found to invade both maternal and fetal tissues and result in chorioamnionitis and placentitis. Moreover, *P. gingivalis* was found to achieve transplacental passage in animal models (6, 10, 32). *In vitro* studies showed that *P. gingivalis* invaded placental trophoblasts and induced G_1 arrest and apoptosis through pathways involving extracellular signal-regulated kinase 1/2 (ERK1/2) and signaling through cyclins and retinoblastoma protein (21). In addition, invasion by *P. gingivalis* induced MEK-

p38 mitogen-activated protein kinase (MAPK) pathways and modulated cytokine expression by trophoblast cell lines (44).

Cell cycle arrest and apoptosis are known to be triggered by DNA damage (45), following which DNA double-strand breaks (DSBs) and single-strand breaks (SSBs) induce the activation of ataxia telangiectasia- and Rad3-related proteins (ATR), as well as ataxia telangiectasia-mutated kinases (ATM) (12, 45). ATM and ATR share many biochemical and functional similarities, including sequence homology, phosphorylation sites, and downstream targets (12, 45). The overlap between the target sets includes substrates that promote cell cycle arrest, DNA repair, and apoptosis via p53. Moreover, most substrates, such as checkpoint kinases (Chk1 and Chk2) and p53, are phosphorylated by both kinases (12, 45). However, ATR is essential for the viability of replicating human and mouse cells, whereas ATM is not. ATM functions distinctively in response to rare occurrences of DSBs (12). Viral infection can affect the activation of ATR and/or ATM. ATR activated by human immunodeficiency virus type 1 (46) and ATM by herpes simplex virus and human cytomegalovirus (33, 52). Both kinases have been reported to be activated in cells infected with Epstein-Barr virus, human polyomavirus JC virus, and minute

virus of canines ([11], [33], [34], [40]). DNA damage-dependent signaling is reported less frequently following bacterial infection. However, *Helicobacter pylori* can induce DSBs in epithelial and mesenchymal cells, and the resulting DNA discontinuities trigger a damage-signaling and repair response involving the ATM-dependent recruitment of repair factors, such as p53-binding protein (53BP1) and mediator of DNA damage checkpoint protein 1 (MDC1), and histone H2A variant X (H2AX) phosphorylation ([56]).

The mechanisms by which *P. gingivalis* induces G_1 arrest and apoptosis in trophoblasts are not fully understood. In this study, we report that invasive *P. gingivalis* activates ERK1/2-Ets1 and the cellular DNA damage signaling pathways that act through an ATR/Chk2/p53-dependent pathway to cause G_1 arrest and apoptosis.

MATERIALS AND METHODS

Bacterial and cell cultures. The bacterial strains used were *P. gingivalis* ATCC 33277 and related mutants, including a long fimbria-null (*fimA*) mutant (KDP150) ([53]) and Rgp- and Kgp-null (*rgpA rgpB kgp*-deficient) triple mutant (KDP136) ([51]). Bacterial cells were grown in Trypticase soy broth supplemented with yeast extract (1 mg/ml), menadione (1 μg/ml), and hemin (5 μg/ml), as described previously ([21]). KDP136 was fimbriated as described previously ([24]). Briefly, the supernatant from fimbria-null KDP150 culture was filtrated through a 0.2-μm-pore-size filter (Asahi Glass Co., Ltd., Tokyo, Japan), and membrane vesicle-depleted supernatants (VDS) containing soluble gingipains were obtained by centrifugation at $100,000 \times g$ for 50 min. The gingipain-null mutant (KDP136) was inoculated into fresh culture medium containing 30% VDS. The HTR-8/SVneo trophoblast cell line was provided by Charles Graham (Kingston, Ontario, Canada). HTR-8 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum at 37°C in 5% CO_2.

Bacterial adhesion and invasion. HTR-8 cells were infected with bacteria at a multiplicity of infection (MOI) of 100 or 200 for 2 h and washed with phosphate-buffered saline (PBS). For total adhesion and invasion levels, cells were lysed with sterile distilled water for 15 min, and dilutions of the lysate plated and cultured anaerobically for CFU on blood agar supplemented with hemin and menadione. For invasion assay, extracellular bacteria were killed with metronidazole (200 μg/ml) and gentamicin (300 μg/ml) for 1 h ([27]). Cells were lysed, and the CFU of invasive organisms were enumerated. Invasion was calculated from CFU recovered intracellularly as a percentage of total bacteria inoculated.

Western immunoblotting. HTR-8 cells were solubilized in cell lysis and extraction reagent (Sigma-Aldrich) containing a protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) and gingipain-specific inhibitors (KYT-1 and KYT-36; Peptide Institute, Osaka, Japan). Immunoblotting was performed as previously described ([21]). Blots were probed at 4°C overnight with the following primary antibodies: anti-Fas, 1:1,000; anti-p53, 1:1,000; anti-phospho-ATR (Ser428), 1:1,000; anti-ATR, 1:1,000; anti-phospho-ATM (Ser1981), 1:1,000; anti-phospho-Chk1, 1:1,000; anti-phospho-Chk2, 1:1,000; anti-phospho-p53 (Ser15), 1:1,000; anti-phospho-p53 (Ser20), 1:1,000; anti-phospho-MDM2 (Ser166), 1:1,000; anti-phospho-ERK1/2, 1:1,000; anti-ERK1/2, 1:1,000; anti-p16, 1:1,000; anti-p21, 1:1,000 (Cell Signaling Technology, Beverly, MA); anti-ATM, 1:1,000 (Signalway Antibody, Pearland, TX); anti-Chk1, 1:1,000; anti-Chk-2, 1:1,000 (EnoGene, New York, NY); anti-Ets1, 1:500; anti-Ets2, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-MDM2, 1:1,000 (NOVUS Biologicals, Littleton, CO). Proteins and phosphorylated proteins were detected using the Pierce ECL substrate (Thermo Scientific). Blots were stripped and probed with anti-β-actin antibody (Cell Signaling Technology) as a loading control. Densitometric analysis of bands was performed using ImageJ software.

RNA interference. The following stealth small interfering RNA (siRNA) duplexes were purchased from Sigma Genosys: human Ets1, 5′-

GGAGAUGGCUGGGAAUUCAAACUUU-3′ ([22]) and 5′-GCUGACCU CAAUAAGGACA-3′ ([54]); human p53, 5′-GCAUGAACCGGAGGCCC AUTT-3′ ([50]) and 5′-CUACUUCCUGAAAACAACGTT-3′ ([49]); and control siRNA, 5′-CAUGUCAUGUGUCACAUCUCTT-3′. The siRNAs were introduced into HTR-8 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h of transfection, the medium was replaced, and cells were incubated for a further 24 h prior to challenge with *P. gingivalis*.

Flow cytometry. (i) Cell cycle analysis. Infected or control HTR-8 cells were trypsinized, washed with cold PBS, and then fixed in 70% ethanol at −20°C overnight. Ethanol-fixed cells were washed with PBS and incubated in 1 ml of 0.1 mg/ml RNase A solution at 37°C for 30 min. The cells were stained with 50 mg/ml propidium iodide (Sigma-Aldrich). Cell cycle analysis of 30,000 cells per sample was carried out with excitation at 488 nm in a flow cytometer (FACScan; Becton, Dickinson, San Jose, CA). Data were analyzed with Cell-Quest software (BD Biosciences) and Mod-Fit LT 3.0 (Verity Software, La Jolla, CA).

(ii) Apoptosis. For annexin V staining, cells were harvested and stained with an annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit (BioVision, Palo Alto, CA) according to the manufacturer's protocol, and flow-cytometric analysis was performed. Chemicals were obtained from Sigma-Aldrich. ATM/ATR inhibitor (Calbiochem, Darmstadt, Germany), 2.5 μg/ml in dimethyl sulfoxide (DMSO), was preincubated with HTR-8 cells for 2 h prior to addition of bacteria.

Caspase 3 activity. Caspase 3 activity was measured using a caspase 3/CPP32 colorimetric assay kit (BioVision, Mountain View, CA) according to the manufacturer's instructions on a microplate reader at 405 nm (Bio-Rad model 680).

RESULTS

***P. gingivalis* infection induces a cellular DNA damage response.** Fas (CD95) is a death domain containing a receptor that activates the extrinsic apoptotic pathway ([7]). p53 is involved in mediating key cellular processes, such as DNA repair, cell cycle arrest, senescence, and apoptosis ([58]). We first examined the effect of *P. gingivalis* on expression of Fas and p53 by HTR-8/SVneo trophoblasts (referred to here as HTR-8 cells). Following *P. gingivalis* infection at an MOI of 200, Fas expression was induced at 48 h, and the accumulation of p53 also reached its peak at 48 h ([Fig. 1]), while *P. gingivalis* triggered no induction of Fas expression at MOIs of 10 and 100. Indeed, G_1 cell arrest and apoptosis were not observed following *P. gingivalis* infection at MOIs of 10 and 100, a result which has also been observed at an MOI of 200 ([21]). p53 accumulates in response to DNA damage, and the resulting increase of p53 function induces either cell growth arrest or apoptosis ([5], [31]). Therefore, we next examined the activation status of DNA damage response proteins up to 48 h after infection in HTR-8 cells ([Fig. 2]). ATR and ATM, which activate downstream signaling molecules such as Chk1, Chk2, and p53, are essential regulators of DNA damage checkpoints in mammalian cells ([45]). Levels of the Ser428-phosphorylated form of ATR and the total amount of ATR were increased by *P. gingivalis* from 12 to 48 h after infection ([Fig. 2]). On the other hand, phosphorylation of ATM (Ser1981) and expression of total ATM were not observed. In the presence of DSBs, activated ATR is known to phosphorylate on Chk2, and Ser15 and Ser20 phosphorylate on p53 ([20], [23], [41]). Furthermore, ATR can also activate Chk1, which indirectly phosphorylates p53 at Ser20 ([63]). *P. gingivalis* was found to induce Thr68 phosphorylation of Chk2 (Thr68) and p53 (Ser15), whereas Chk1 (Ser296) and p53 (Ser20) did not show an increase in phosphorylation levels. MDM2 possesses the E3 ubiquitin ligase and binds to the p53 N-terminal transcriptional activation
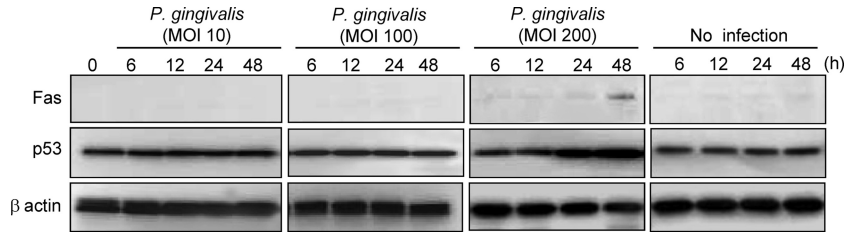
FIG 1 Fas and p53 expression is upregulated in HTR-8 cells infected with *P. gingivalis*. HTR-8 cells were infected with *P. gingivalis* at MOIs of 10, 100, and 200 for the indicated times. Cell lysates were immunoblotted with Fas and total p53 antibodies. β-Actin was included as a loading control. Data are representative of three independent experiments.

domain, leading to degradation of p53 via the 26S proteasome (36). Neither total nor phosphorylated MDM2 was increased in *P. gingivalis*-infected cells. These results indicate that p53 accumulated in response to damage by *P. gingivalis* via the ATR-Chk2 pathway and by the lack of induction of MDM2.

ATR is associated with *P. gingivalis*-mediated G_1 arrest and apoptosis. DNA damage response is a signal transduction pathway that coordinates cell cycle transitions and apoptosis (12). Therefore, we investigated whether ATR functions in the cell cycle transition and apoptosis responses of HTR-8 cells to *P. gingivalis*.

Pretreatment with the ATM/ATR inhibitor prevented G_1 arrest by *P. gingivalis* in HTR-8 cells (Fig. 3A). In addition, ATR inhibition abrogated the induction of apoptosis (Fig. 3B) and caspase 3 activity (Fig. 3C). These results suggest that G_1 arrest and apoptosis by *P. gingivalis* in HTR-8 cells are regulated by the ATR-Chk2 pathway.

Activation of p53 by *P. gingivalis* controls G_1 arrest and apoptosis. To evaluate the effects of p53 activation on cell cycle arrest and apoptosis caused by *P. gingivalis*, we silenced p53 expression using two siRNA duplexes. Expression of p53 was signif-
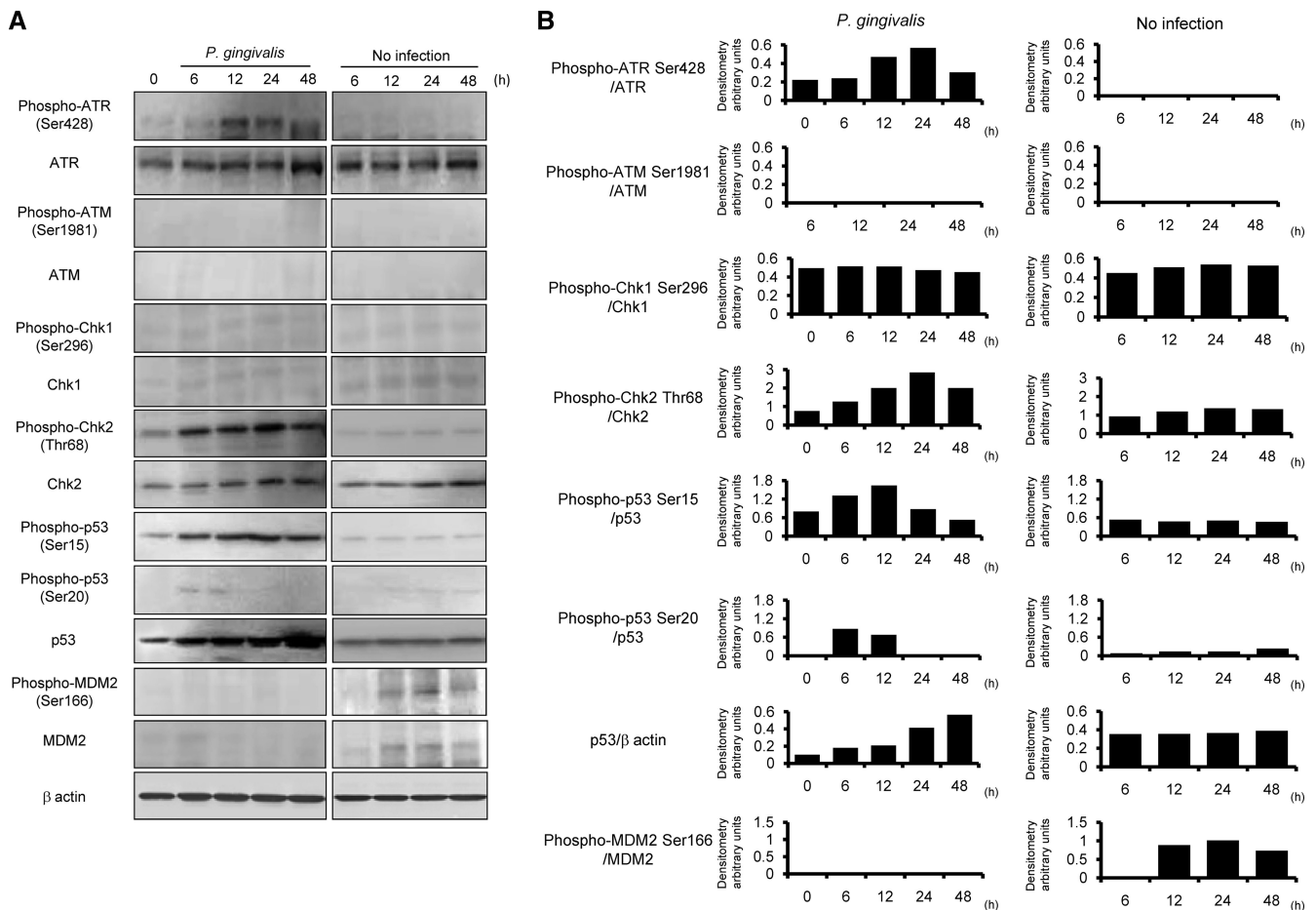

FIG 2 Activation of DNA damage-responsive proteins in HTR-8 cells infected with *P. gingivalis*. (A) HTR-8 cells were infected with *P. gingivalis* at an MOI of 200 for the indicated times. Lysates of infected and noninfected cells were immunoblotted with antibodies to DNA damage-responsive proteins. (B) Densitometric analysis of blots showing the phosphorylation and total proteins, expressed in arbitrary units. β-Actin was included as a loading control. Data are representative of three independent experiments.
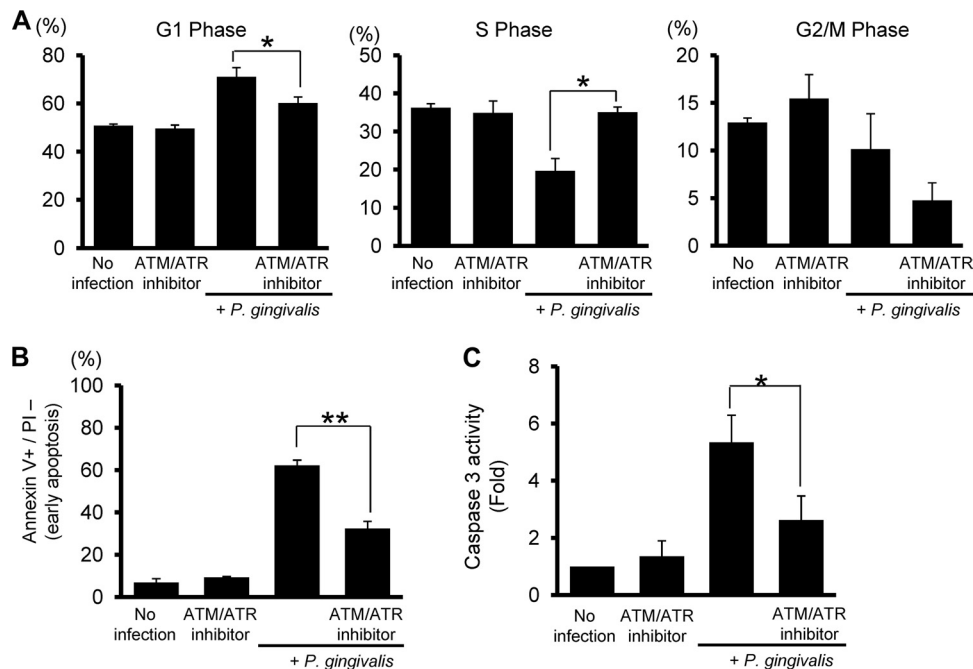
FIG 3 Effects of an ATM and ATR inhibitor on G_1 arrest and apoptosis. HTR-8 cells were infected with *P. gingivalis* at an MOI of 200 for 48 h. An inhibitor was added for 2 h prior to infection. (A) DNA content was analyzed by flow cytometry, and cell cycle profiles were obtained with ModFit software. Results are means ± standard deviations (SD) of cell cycle distribution from three independent experiments with flow cytometry parameters set to exclude cell debris and aggregates. *, $P < 0.05$ compared with infected cells without inhibitor (*t* test). (B) Cells were stained with annexin V and PI and analyzed by flow cytometry. Mean percentage ± SD of apoptotic (annexin V+/PI−) cells. Error bars indicate standard deviation ($n = 3$). *, $P < 0.01$ compared with infected cells without inhibitor. (C) Cells were treated with inhibitor were infected with *P. gingivalis* for 48 h as described for panel B, and caspase 3 activity was measured. Fold changes were calculated relative to infected cells without inhibitor (*t* test). * and **, $P < 0.05$ and $P < 0.01$ (Student's *t* test) compared with *P. gingivalis*-infected cells with inhibitor.

icantly reduced in the knockdown cells in a concentration-dependent manner (Fig. 4A). At 48 h after transfection, p53 knockdown HTR-8 cells were infected with *P. gingivalis* and then assayed immediately (time zero) and at 24 and 48 h after bacterial infection. In cells transfected with the nontarget siRNA, *P. gingivalis* infection enhanced the expressions of all signaling molecules tested at 24 and 48 h. Conversely, no induction of p21 or Fas was observed in the p53 knockdown cells (Fig. 4B and C). p53 activation reportedly increases Fas expression at the cell surface by transport from the Golgi complex (7). Thus, Fas induction by *P. gingivalis* can be considered due to the p53-dependent response to DNA damage. In addition, G_1 arrest that is dependent on p53 requires p21 activation (61). Following infection with *P. gingivalis*, the induction of p21 was suppressed by p53 knockdown (Fig. 4B and C). Moreover, G_1 arrest was negligible, and the cell cycle accurately progressed to S phase in the p53 knockdown cells infected with the pathogen (Fig. 4D). Additionally, p53 knockdown abrogated the induction of apoptosis (Fig. 4E) and caspase 3 activity (Fig. 4F). To avoid elicitation of off-targeting, a second siRNA specific to another sequence was provided in the same target mRNA. Figure S1 in the supplemental material shows that cells with alternative p53 siRNA silencing also showed significantly reduced arrest in G_1 phase and progressed into S phase, as well as showing resistance to the apoptotic effect of *P. gingivalis*. These results suggest that *P. gingivalis* diverts p53 signaling events, which causes cell cycle disturbance and ultimately apoptosis.

Activation of Ets1 by *P. gingivalis* controls G_1 arrest and apoptosis. *P. gingivalis* was previously shown to elevate the levels

of p16 in HTR-8 cells (21), and p53 knockdown did not suppress p16 induction by the organisms (Fig. 4B). Upregulation of p16 is mediated by Ets1 and Ets2 proteins through the proximal Ets-binding site in the p16 promoter (19). Thus, we next examined the effect of *P. gingivalis* on Ets1 and Ets2 levels. Infection of HTR-8 cells with *P. gingivalis* resulted in a significant increase in Ets1 levels compared to noninfected cells, whereas little expression of Ets2 was observed in infected or control cells (Fig. 5). Ets1 also induces the expression of apoptotic genes, and p16 mediates cell cycle arrest at G_1 (19, 39). In addition, p21 is controlled by the expression of p16 in hepatoma cells (19). To evaluate the role of Ets1 activation in *P. gingivalis*-induced cell cycle arrest and apoptosis, we silenced Ets1 expression by using two sets of siRNA duplexes (Fig. 6A). Both siRNA constructs reduced expression of Ets1 at the transcriptional level. Immunoblotting showed that the levels of p53, p16, p21, and Fas were decreased in Ets1 knockdown cells following *P. gingivalis* infection. In contrast, the phosphorylation of ATR and Chk2 was not affected by Ets1 knockdown (Fig. 6B and C). Moreover, knockdown of Ets1 significantly suppressed G_1 cycle arrest (Fig. 6D) and diminished the ability of *P. gingivalis* to induce apoptosis and increased the activity of caspase 3 at 48 h (Fig. 6E and F). The use of other Ets1 siRNA sequences resulted in the same effects on cell cycle and apoptosis (see Fig. S2 in the supplemental material).

Gingipains trigger a signal transduction cascade. *P. gingivalis* expresses several virulence factors, including fimbriae as well as gingipain enzymes comprised of arginine-X [Arg-gingipain A and B (RgpA and RgpB)]- and lysine-X [Lys-gingipain (Kgp)]-specific
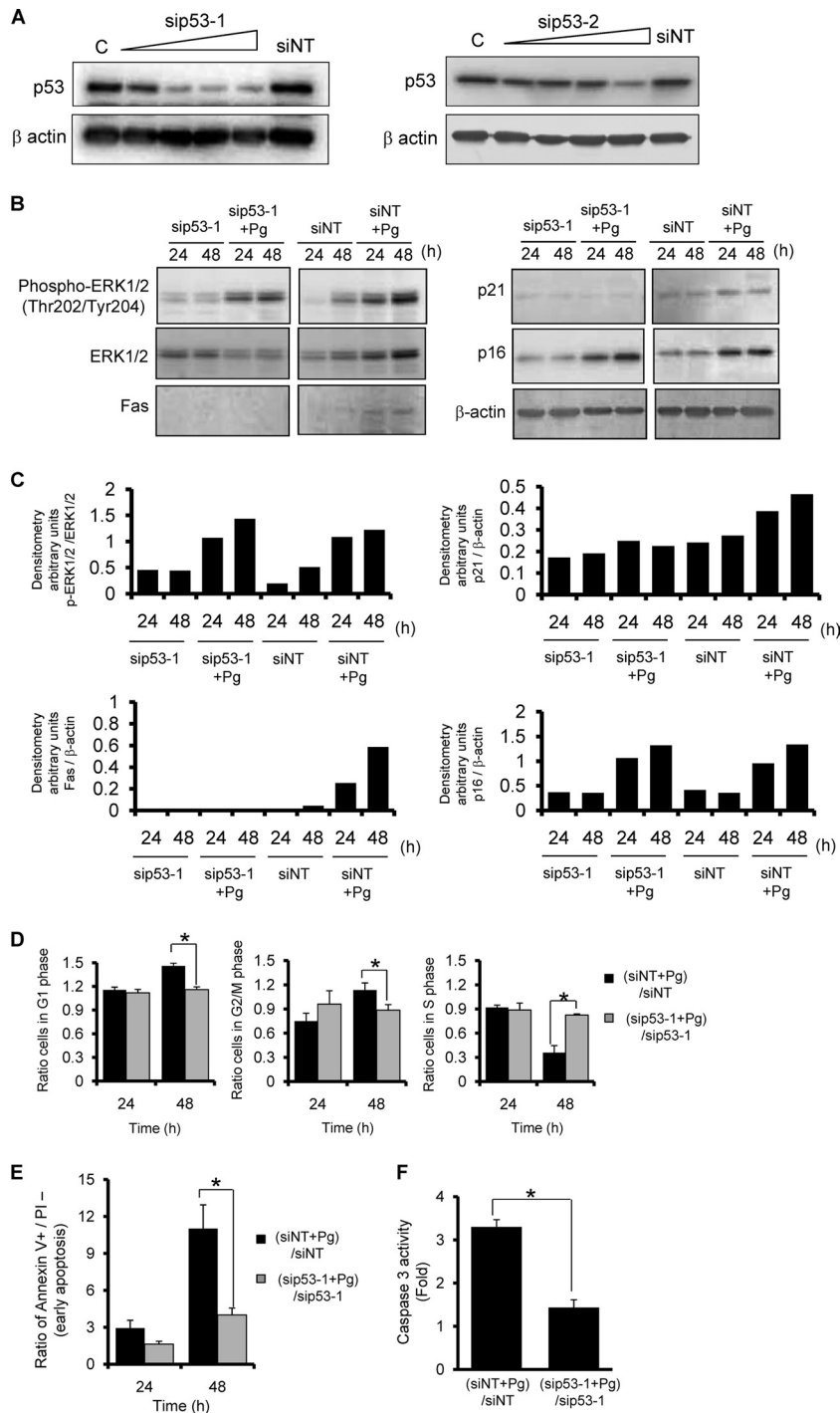
FIG 4 siRNA knockdown of p53 suppresses *P. gingivalis*-mediated G_1 arrest and apoptosis. HTR-8 cells were transfected with siRNA for p53 (sip53-1 and -2) or with a nontarget control (siNT). (A) Cells were lysed and immunoblotted with anti-p53 or β-actin antibodies at 48 h after transfection. (B) siRNA knockdown cells were infected with *P. gingivalis* at an MOI of 200 for 24 or 48 h. Expression profiles of signaling molecules were examined by immunoblotting. β-Actin was included as a loading control. (C) Densitometric analysis of blots showing the phosphorylation and total proteins, expressed in arbitrary units. Data are representative of three independent experiments. (D) siRNA knockdown cells were infected with *P. gingivalis* as described for panel B, and then cell cycle distribution was determined using flow cytometry. The ratio of cells in each cell cycle phase is expressed relative to that of noninfected controls. (E) siRNA knockdown cells were infected as described for panel B, followed by staining with annexin V and propidium iodide (PI), and analyzed by flow cytometry. Fold change was calculated as described for panel C. (F) siRNA knockdown cells were infected with *P. gingivalis* for 48 h as described for panel D, and then caspase 3 activity was measured. The fold change was calculated as described for panel D. Error bars indicate standard deviations ($n = 3$). *, $P < 0.01$ (Student's *t* test) compared with the ratio of *P. gingivalis*-infected NT-siRNA control to NT-siRNA control.
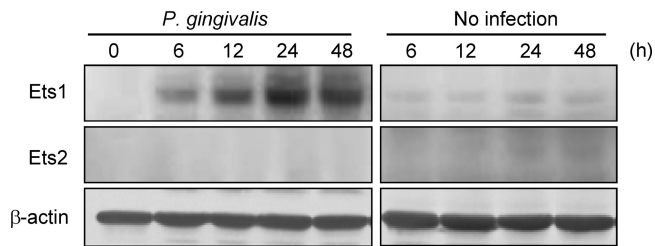
FIG 5 Ets1 expression is upregulated in HTR-8 cells infected with *P. gingivalis*. HTR-8 cells were infected with *P. gingivalis* at an MOI of 200 for the indicated times. Cell lysates were immunoblotted with anti-Ets1 or Ets2 antibodies. β-Actin was included as a loading control. Data are representative of three independent experiments.

cysteine proteinases (18). We sought to investigate the role of these virulence factors in the initiation of signal transduction. Rgp processes precursor fimbria proteins (38), and thus, Rgp-null mutants lack mature FimA fimbriae on their surfaces. As the fimbriae are required for adherence to HTR-8 cells (data not shown), we utilized bacterial culture supernatants containing soluble Rgp to express mature fimbriae on the surface of Rgp-null mutants as previously described (24). The Kgp- and Rgp-null mutant (KDP136) was treated with exogenous gingipains as described in Materials and Methods, and the treated KDP136 mutant was shown to possess mature fimbriae (see Fig. S3A in the supplemental material). Gingipain activities of treated KDP136 were confirmed to be negligible (see Fig. S3B). Furthermore, the adhesion and invasion abilities did not differ significantly between the wild type and treated KDP136 (Fig. 7A). Conversely, treated KDP136 failed to cause a progressive increase in G_1-phase cells or a corresponding decrease in S- and G_2/M-phase cells (Fig. 7B). The wild-type strain caused rounding and detachment of some cells. In contrast, treated KDP136 did not induce these changes, though slightly deformed cell shapes were observed (Fig. 7C). We also examined the involvement of gingipains in apoptosis. Early apoptosis was reduced in response to treated KDP136, whereas necrotic responses were similar between the parental and treated KDP136 strains (Fig. 7D). In addition, treated KDP136 significantly decreased the levels of caspase 3 induction compared to the parental strain (Fig. 7E). Finally, we examined the effect of gingipain on the expression and phosphorylation status of ATR, ATM, Chk1, Chk2, p53, and MDM2 and on levels of Ets1, Fas, p16, and p21 in HTR-8 cells. Notably, the amount and phosphorylation of MDM2, which are suppressed by the wild-type strain (Fig. 2), were increased in response to treated KDP136 (Fig. 7F). For the other molecules tested, the effects of treated KDP136 were similar to those of the wild type. It is possible that the activation of ATR-p53 and ERK-Ets1 pathways may be directly affected by loss of gingipain activity. To clarify the effect of gingipains on those pathways, expression of Ets1, p16, Chk2, and p21 mRNAs was determined using real-time PCR (see Table S1 in the supplemental material). Wild-type and treated KDP136 elevated levels of Chk2 and p16 mRNA to the same degree, although the increase in Ets1 and p21 mRNA expression following wild-type infection was higher than that following infection with treated KDP136. These results suggest that *P. gingivalis* gingipains may specifically degrade MDM2 protein and modulate the activity of multiple signaling pathways, resulting in both cell cycle arrest and cell death in HTR-8 cells.

DISCUSSION

Placental growth occurs in the first trimester and involves coordinated events in the trophoblasts and mesenchyme. Progenitor cytotrophoblasts differentiate into villous intermediate cells, and these cells are programmed to fuse with the syncytium or into extravillous migratory cells that transform the maternal vascular supply. The villous trophoblast bilayer is the primary barrier between maternal and fetal tissues. The syncytium is a structural component of maternal-facing layer and a barrier to pathogens and the maternal cells (2). Extravillous trophoblast (EVT) invasion is an essential step in placental formation and is important for fetal growth and well-being (26). HTR-8 cells were established from first-trimester EVTs (17), while HTR-8/SVneo cells are the immortalized derivative of HTR-8 cells and exhibit a normal EVT phenotype and genotype (17, 29). *P. gingivalis* can invade trophoblasts *in vitro* and cause apoptosis and cell cycle arrest at G_1 (21). In addition, *P. gingivalis* has been detected in the amniotic fluid and placenta in preterm labor, as a result of hematogenous spread (4, 30). A role for *P. gingivalis* in pregnancy complications is also supported by animal models, indicating bacterial invasion of both maternal and fetal tissues (6, 10, 32). Trophoblasts differentially regulate the expression of over 2,000 genes following *P. gingivalis* infection (44), which may impact many signaling pathways, including those involved in control of cell cycle and apoptosis. Thus, we examined the effect of *P. gingivalis* infection on the signaling pathways which regulate cell cycle and apoptosis. *P. gingivalis* infection was shown to alter expression profiles of DNA damage response proteins, which was accompanied by G_1 cell cycle arrest and apoptosis. This is the first comprehensive examination of the signaling pathways of DNA damage response induced by *P. gingivalis* infection. The involvement of p53, Ets1, and ATR in cellular responses was also shown by siRNA knockdown and chemical inhibition. Furthermore, gingipain proteolytic activity was found to play an essential role in the induction of G_1 arrest and apoptosis, due to the lack of induction of MDM2. Based on these findings, we propose that *P. gingivalis* initiates the signaling cascade shown in Fig. 8.

The Ras-dependent ERK1/2 MAPK pathway plays a central role in control of cell proliferation, and sustained activation of ERK1/2 is necessary for G_1-to-S-phase progression and positive regulation of the cell cycle. However, excessive activation of ERK1/2 can instead lead to cell cycle arrest and apoptosis (21, 48). Downstream of Ras are the effector kinases Raf/MEK/ERK1/2 (13), while Ets1 and p16 are the downstream mediators of ERK1/2 (19, 61). Moreover, p16 has an antiproliferative function through the G_1 phase of the cell cycle in response to Ets1 and ERK signaling activity cycles (19, 61). Hence, activation of this pathway is consistent with our finding of the G_1 arrest and apoptosis mediated by *P. gingivalis*. Chk2 was phosphorylated at threonine 68 when cells were damaged by ionizing radiation (IR), UV irradiation, hydroxyurea (HU) (37), arsenic trioxide (23), and cisplatin (41), and activation was shown to be mediated by ATM (20, 37) and ATR (23, 37, 41). In addition, Chk2 phosphorylation parallels p53 phosphorylation on Ser15 (37). In the present study, DNA damage response induced by *P. gingivalis* infection led to ATR but not ATM activation (Fig. 2 and 7F). Moreover, Chk2 was phosphorylated at threonine 68 (Fig. 2 and 7F). Interestingly, ATR-Chk2 pathways were activated by *P. gingivalis* invasion with and without gingipain proteases. Accordingly, ATR likely phosphorylates and
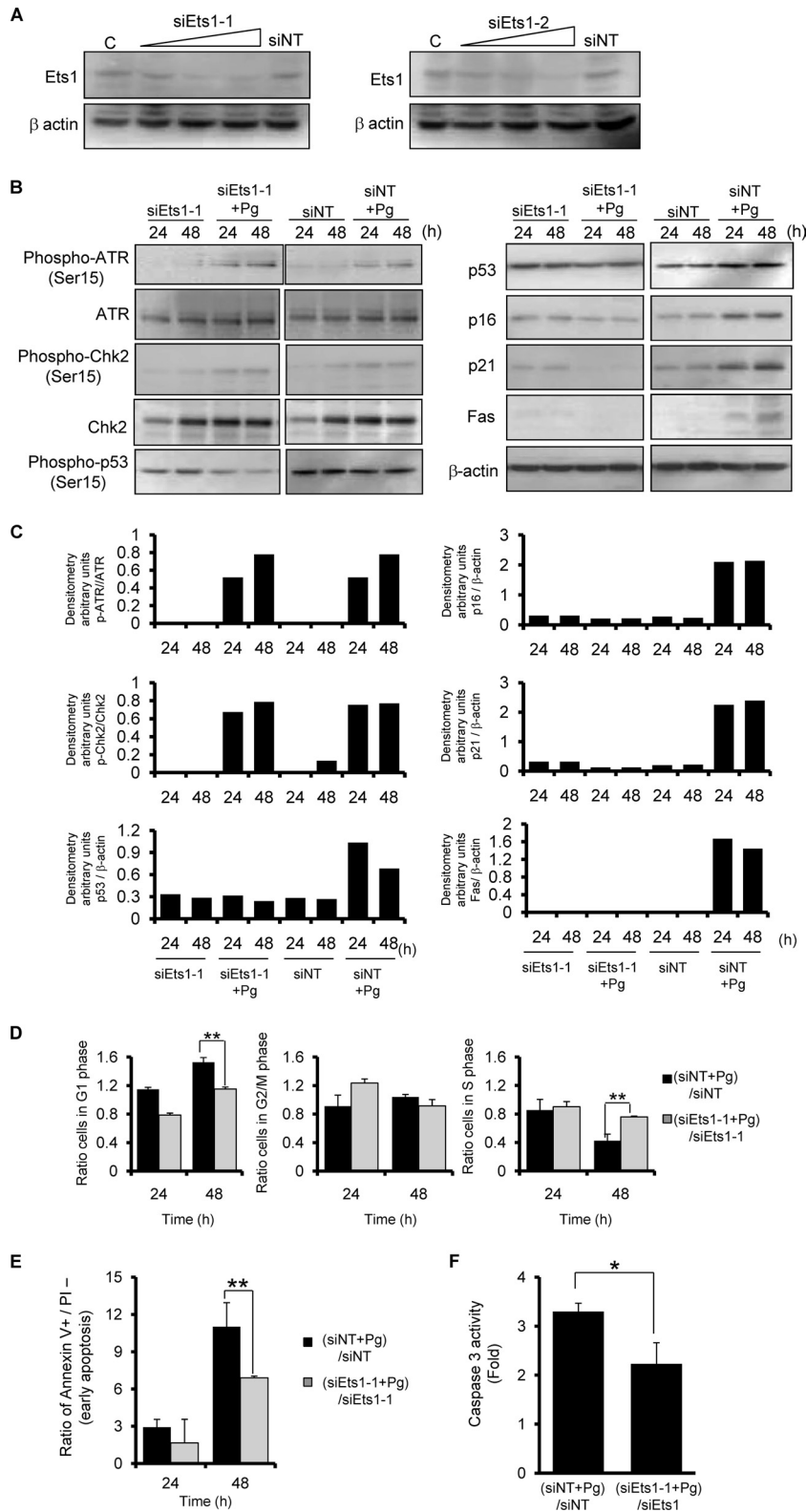
FIG 6 siRNA knockdown of Ets1 suppresses *P. gingivalis*-mediated G_1 arrest and apoptosis. HTR-8 cells were transfected with siRNA for Ets1 (sipEts1-1 and -2) or with a nontarget control (siNT). (A) Immunoblot with anti-Ets1 or β-actin antibodies at 48 h after transfection and with β-actin as a loading control. (B) siRNA knockdown cells were infected with *P. gingivalis* at an MOI of 200 for 24 and 48 h. Expression profiles of signaling molecules were examined by immunoblotting. β-Actin was included as a loading control. (C) Densitometric analysis of blots of phosphorylated and total proteins, expressed in arbitrary units. Data are representative of three independent experiments. (D) siRNA knockdown cells were infected with *P. gingivalis* as described for panel B, and the cell cycle distribution was determined using flow cytometry. (E) siRNA knockdown cells were infected as described for panel B, followed by staining with annexin V and propidium iodide (PI), and analyzed using flow cytometry. (F) siRNA knockdown cells were infected with *P. gingivalis* for 48 h as described for panel B, and caspase 3 activity was measured. Fold changes were calculated as described for panel D. Error bars indicate standard deviations ($n = 3$). * and **, $P < 0.05$ and $P < 0.01$ (Student's *t* test) compared with the ratio of *P. gingivalis*-infected NT-siRNA control to NT-siRNA control.
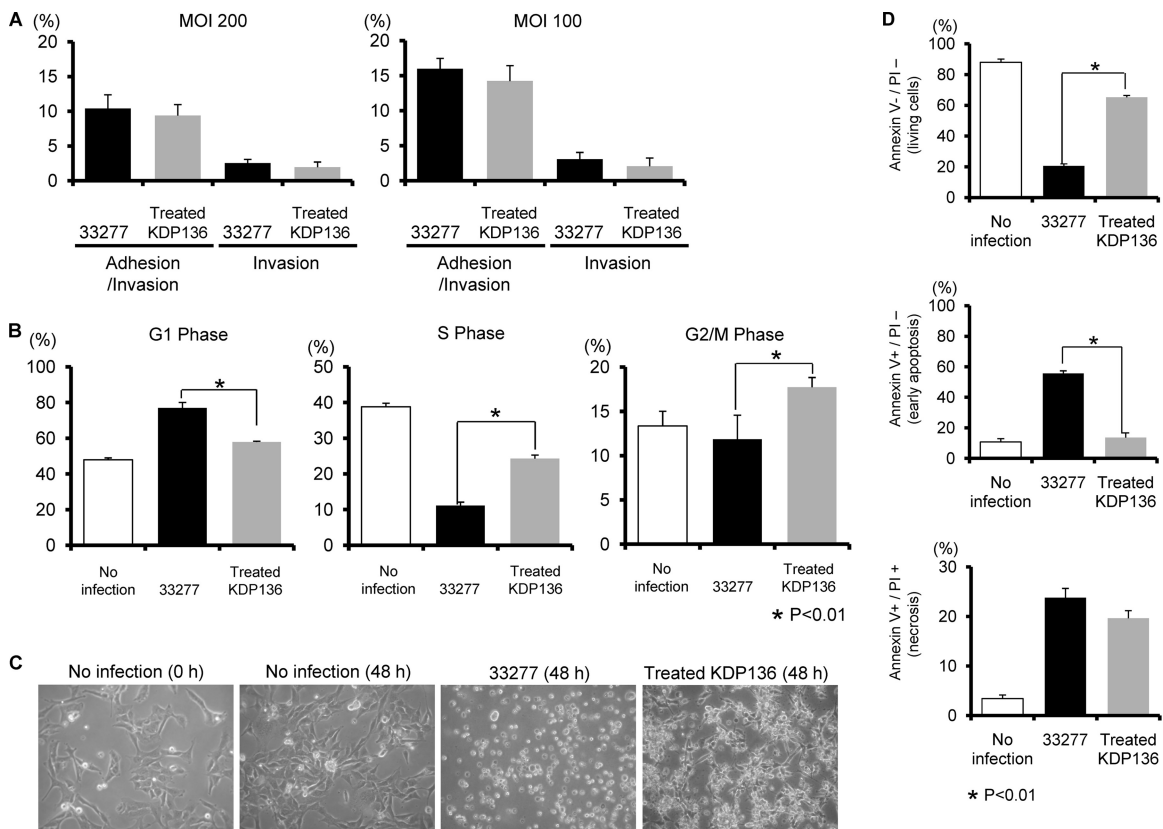
FIG 7 HTR-8 cells infected with a *P. gingivalis* fimbriated gingipain-null mutant strain do not show G$_1$ arrest and apoptosis. (A) Antibiotic protection invasion assay with *P. gingivalis* ATCC 33277 (wild type) and fimbriated KDP136 (fimbriated gingipain-null mutant strain). HTR-8 cells were infected with *P. gingivalis* strains at an MOI of 100 or 200 for 2 h. Numbers of adherent and/or intracellular bacteria were determined by viable counting of the cell lysates and are expressed as percentage of input bacterial cell number. Data are means ± SD of three independent experiments and were analyzed with a *t* test. (B) Cell cycle distribution was determined using flow cytometry. The fold change in numbers of cells in each phase of the cell cycle was calculated in comparison with noninfected cells. Data are means ± SD of three independent experiments and analyzed with a *t* test. (C) Light microscopy showing morphology of HTR-8 cells infected with *P. gingivalis* ATCC 33277 and fimbriated KDP136 at an MOI of 200 for 48 h. (D) Cells were stained with annexin V and PI and analyzed using flow cytometry. The fold change was calculated as described for panel B. The mean percentage ± SD of apoptotic (annexin V+/PI−) cells from three independent experiments was determined and analyzed with a *t* test. (E) Fold change in caspase 3 activity. Data are means ± SD from three independent experiments analyzed with a *t* test. (F) Cells were infected with *P. gingivalis* fimbriated KDP136 at an MOI of 200 for the indicated times. Lysates of infected and noninfected cells were subjected to immunoblotting to analyze expression profiles of DNA damage-responsive proteins, MDM2 phosphorylated at Ser166, MDM2, Ets1, Fas, p16, and p21. β-Actin was included as a loading control. Data are representative of three independent experiments.

activates Chk2 during *P. gingivalis* invasion damage, which in turn leads to p53 phosphorylation at Ser15. Previous studies have noted that p53 phosphorylation at Ser15 is crucial for p53-dependent p21 expression (60) and does not require the presence of ATM kinase (5).

Several pathogens, such as *Salmonella enterica* (42), *Streptococcus pyogenes* (57), and *Helicobacter pylori* (55), have been reported to induce apoptosis mediated by Fas. *H. pylori* can also induce apoptosis through activation of p53 (8). However, the interaction between p53 and Fas expression in bacterial infection has yet to be characterized. The finding that DNA damage response in HTR-8 cells induced by *P. gingivalis* increased p53 accumulation and Fas activation suggests that both proteins are targets of *P. gingivalis* in an MOI-dependent manner (Fig. 1). Those findings also suggest that G$_1$ arrest or apoptosis related to p53 and Fas expression may be MOI dependent in HTR-8 cells infected with *P. gingivalis* in vivo. While the levels of *P. gingivalis* in placental tissue are unknown, an immunohistochemical study suggested that they can be quite high in cases of preterm delivery (25). Knockdown of p53 with siRNA inhibited Fas expression, G$_1$ arrest, and apoptosis (Fig. 4), suggesting that DNA damage response can induce transcriptional upregulation of FAS through p53-dependent mechanisms, as demonstrated with other cell types (14). These findings suggest that Fas induction is controlled by p53 after *P. gingivalis* infection. The p53 protein plays a central role in cellular responses to a variety of stress signals. Normally, p53 levels are kept low through activity of the negative regulator MDM2. However, those levels rapidly increase after stimulation by several stressors, which results in disruption of MDM2-p53 interaction (36, 58). Ser166 phosphorylation of MDM2 has been shown to increase its ubiquitin ligase activity and increase p53 degradation (35). Notably, a significant decrease in levels of phosphorylated and total MDM2 was observed in *P. gingivalis*-infected cells, and this decrease was dependent on the presence of gingipains. Together, these results suggest that *P. gingivalis* gingipain proteases degrade MDM2, thus contributing to p53 accumulation and apoptosis. This is the first report of such an MDM2-specific degradation response to pathogen infection.
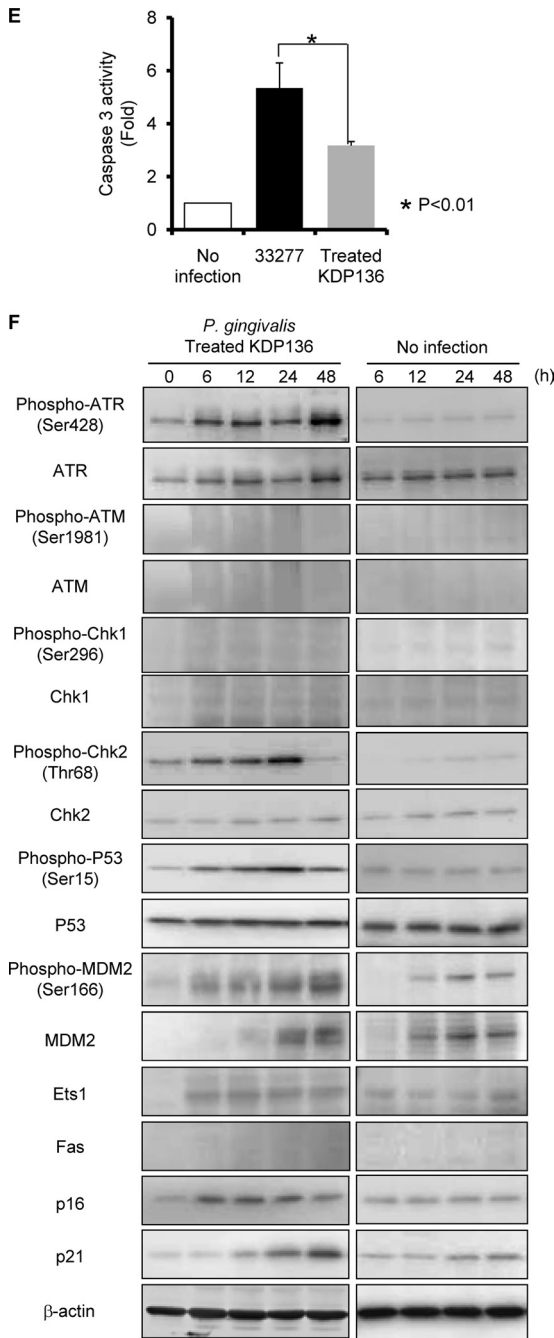
E

* P<0.01

F

FIG 7 continued

FIG 8 Proposed schematic model for cell cycle regulation and induction of apoptosis in HTR-8 cells infected with *P. gingivalis. P. gingivalis* invasion leads to activation of ATR. Upon activation, ATR phosphorylates Chk2, resulting in progressive Chk2 activation. Chk2 then phosphorylates and activates p53, inducing p21 and the gene expressing proapoptotic Fas. Phosphorylation of ERK1/2 causes Ets1 activation, which in turn activates p16 and p53.

Although neither caspase 3 activity nor Fas induction occurred in response to treated KDP136, necrotic changes were observed in response to both the wild-type and treated KDP136 strains. Recent evidence suggests that necrosis can also be programmed, though in a manner distinguishable from apoptosis (47). In addition, that report noted that under conditions where caspases are inhibited or signaling through Fas is otherwise prevented, apoptotic stimuli can induce necrosis. Our results suggest that caspase activity and/or Fas induction via the ATR/Chk2/p53 pathway require gingipains, while induction of p21 by *P. gingivalis* occurs in a gingipain-independent manner. The negative regulation of
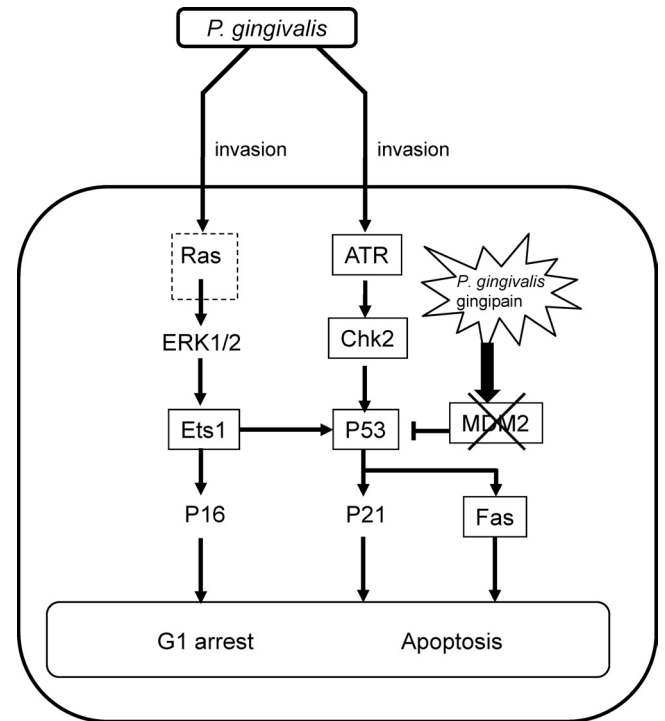
apoptosis by p21 has been attributed to interaction with procaspase 3, resulting in inhibition of its activity (1). Thus, necrosis may be induced following infection with treated KDP136. When all the data are considered, gingipains are strongly implicated as an important factor in apoptosis induction.

P. gingivalis impacts the cell cycle as well as apoptosis through the upregulation of p16 and p21 and phosphorylation of ERK1/2 (21). p53 can either cause cell cycle arrest by transactivation of p21 or induce cells to undergo apoptosis (28). In addition, Ets1 controls the regulation of p16 (19, 39). However, the interaction between p53 and Ets1 in regard to the cell cycle has yet to be investigated. We found that the levels of p16 and p21 were diminished by knockdown of p53 and Ets1, respectively, while p16 amounts were decreased in Ets1-silenced cells following *P. gingivalis* infection. Additionally, p21 was diminished by the silencing of p53 and/or Ets1. HTR-8 cells infected with *P. gingivalis* also exhibited a sustained activation of Ets1, and knockdown of Ets1 abrogated both G_1 arrest and apoptosis. Moreover, the levels of p16, p21, p53, and Fas were decreased when Ets1 amounts were reduced (Fig. 4 and 6). This impact of *P. gingivalis* suggests that p16 and p21 may be under the control of ERK1/2-Ets1 and p53 pathways. This is consistent with reports showing that the p53 gene is transcriptionally regulated by Ets1 (3), and a deficiency of Ets1 decreases p53 expression level and reduces apoptosis induced by p53 in response to UV exposure (62). Collectively, these results indicate that *P. gingivalis* diverts the ERK1/2-Ets1 and p53 signaling pathways, resulting in both cell cycle arrest and apoptosis.

In conclusion, *P. gingivalis* infection of HTR-8 cells causes cy-

topathic effects that are likely mediated by DNA damage response and MAPK activation. We propose that the activation of DNA damage response and ERK1/2 results from *P. gingivalis* invasion of trophoblasts, and that the ensuing apoptosis and cell cycle arrest are potentially synergistic mechanisms that contribute to PTLBW associated with this periodontal pathogen.

ACKNOWLEDGMENTS

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