Gingipains from the periodontal pathogen *Porphyromonas gingivalis* play a significant role in regulation of Angiopoietin 1 and Angiopoietin 2 in human aortic smooth muscle cells

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Abstract:

Angiopoietin 1 (Angpt1) and angiopoietin 2 (Angpt2) are the ligands of Tyrosine kinase (Tie) receptors, and they play important roles in vessel formation and the development of inflammatory diseases, such as atherosclerosis. *Porphyromonas gingivalis* is a gram-negative periodontal bacterium that is thought to contribute to the progression of cardiovascular disease. The aim of this study was to investigate the role of *P. gingivalis* infection in the modulation of Angpt1 and Angpt2 in human aortic smooth muscle cells (AoSMCs). We exposed AoSMCs to wild type (W50 and 381), gingipain mutant (E8 and K1A), and fimbriae mutant (DPG-3 and KRX-178) *P. gingivalis* and to different concentrations of tumor necrosis factor (TNF). The atherosclerosis risk factor TNF was used as a positive control in this study. We found that *P. gingivalis* (wild type, K1A, DPG3 and KRX178) and TNF up-regulated the expression of Angpt2 and its transcription factor ETS1, respectively, in AoSMCs. In contrast, Angpt1 was inhibited by *P. gingivalis* and TNF. However, the RgpA/B mutant E8 had no effect on the expression of Angpt1, Angpt2, or ETS1 in AoSMCs. The results also showed that ETS1 is critical for *P. gingivalis* induction of Angpt2. Exposure to Angpt2 protein enhanced the migration of AoSMCs but had no effect on proliferation. This study demonstrates that gingipains are crucial to the ability of *P. gingivalis* to markedly increase the ratio of expressed Angpt2/Angpt1 in AoSMCs, which determines the regulatory role of angiopoietins in angiogenesis and their involvement in the development of atherosclerosis. These findings further support the association between periodontitis and cardiovascular disease.

Introduction

Cardiovascular atherosclerotic disease is a major cause of global morbidity and mortality. The pathological characteristics of atherosclerosis include inflammation, proteolysis, and arterial remodeling processes, such as apoptosis and angiogenesis.[1] Angiogenesis is a prominent feature of atherosclerosis and refers to the growth of new blood vessels, following an organized genetic program of vascular sprouting, vessel assembly and organotypic maturation.[2] Tie receptors, together with their corresponding angiopoietins, have been identified as the signaling system that plays a particularly central role in vascular remodeling and angiogenesis.[3]

There are 4 forms of angiopoietin (Angpt1, Angpt2, Angpt3, and Angpt4) that are produced by many different types of cells.[4, 5] Through the activation of the Tie2 receptor signaling pathway, angiopoietins regulate vascular maturation, stability and remodeling.[6] Angpt1 and Angpt2 have opposing effects in the regulation of inflammatory responses. Angpt1 is an anti-inflammatory regulator, whereas, Angpt2 functions as a pro-inflammatory regulator. Transgenically overexpressed Angpt1 in mice results in reduced vessel leakiness in response to permeability-inducing inflammatory agents.[7] Mice without Angpt2 fail to elicit a rapid inflammatory response and to produce molecules that lead to leukocyte adherence during stimulation by an inflammatory mediator, such as TNF.[8] In addition to binding to Tie2, an endothelial-specific receptor, Angpt2 is also able to bind to and activate integrins in Tie-receptor-negative fibroblasts and breast cancer cells.[9, 10] These

findings triggered a new-found interest in analyzing the function of angiopoietins in non-ECs.

P. gingivalis, a gram-negative oral anaerobe, has been identified as one of the main pathogens in the progression of periodontitis and is detected in up to 85% of disease sites.[11] By interacting with other periodontal pathogens, such as *Filifactor alocis* (*F. alocis*), a gram-positive, obligate anaerobic rod bacterium, *P. gingivalis* plays an important role in infection-induced periodontal disease.[12, 13] Many epidemiological studies have shown that severe forms of periodontitis are associated with other inflammatory diseases, such as rheumatoid arthritis and cardiovascular disease.[14, 15] The DNA of *P. gingivalis* has been found in coronary stenotic artery plaques of myocardial infarction patients.[16, 17] In animal models, *P. gingivalis* infection directly induces and accelerates the formation of coronary and aortic atherosclerosis in pigs and mice.[18, 19]

P. gingivalis produces a number of different virulence factors, such as lipopolysaccharides (LPS), fimbriae, capsule, hemagglutinins and proteases (gingipains). Gingipains are cysteine proteinases, and they have been divided into two groups: arginine gingipains (Rgp), which include RgpA and RgpB, and lysine gingipain (Kgp).[20] The gingipains have been shown to support biofilm formation, facilitate *P. gingivalis* invasion, and regulate the defensive response processes of host cells.[21] We and others have demonstrated that gingipains modulate the expression of several cytokines in multiple cell types, including endothelial cells, gingival fibroblasts,

T-cells, and monocytes.[22-25] In addition to gingipains, fimbriae are also important for *P. gingivalis* adhesion to host cells. Many studies have shown that *P. gingivalis* requires fimbriae to invade endothelial cells[26] and fibroblasts[27], and to induce the inflammatory responses.[28, 29]

Interestingly, we have previously shown, using microarray techniques, that *P. gingivalis* ATCC 33277 down-regulates the gene expression of Angpt1 while simultaneously up-regulating the gene expression of Angpt2 in AoSMCs.[30] Smooth muscle cells (SMCs) are the main components of the vascular wall, and dysfunction in these cells is directly or indirectly associated with the development of atherosclerosis.[31] To further investigate the role of different virulence factors of *P. gingivalis* in the modulation of angiopoietins, we infected AoSMCs with wild type strains (ATCC 33277, W50, 381), gingipain mutants (K1A, E8), or fimbriae mutants (DPG3, KRX178) of *P. gingivalis.*

The aim of this study was to clarify the effects of *P. gingivalis* in regulating the expression of angiopoietins in AoSMCs.

Materials and methods

Culture of AoSMCs. Human primary AoSMCs (Invitrogen, Stockholm, Sweden) were cultured in 231 smooth muscle cell culture medium containing essential growth supplements (Gibco, Carlsbad, CA). The cells were cultured in 75 cm^2 explant culture flasks (TPP, Trasadingen, Switzerland) and placed in a cell culture incubator at 37°C

with 5% CO_2 and 95% air until confluent. In this study, cells from passage 5 to 10 were used.

Bacteria culture and preparation. *F. alocis* ATCC 35896 (CCUG-Culture Collection, University of Göteborg), *P. gingivalis* ATCC 33277 (American Type Culture Collection, Manassas, VA), and W50 (wild type) and its isogenic mutant strains E8 (Rgp mutant strain), K1A (Kgp mutant), and 381 (wild type), were grown in fastidious anaerobe broth (29.7 g/l, pH 7.2). The *P. gingivalis* 381 corresponding fimbriae mutant strains DPG3 (major fimbriae mutant) and KRX178 (minor fimbriae mutant) were grown in fastidious anaerobe broth supplemented with 1 µg/ml erythromycin. The different *P. gingivalis* strains were grown in an anaerobic chamber (80% N_2, 10% CO_2, and 10% H_2, at 37°C) (Concept 400 Anaerobic Workstation; Ruskinn Technology Ltd., Leeds, United Kingdom).

After 72 hours of culturing, bacteria were harvested by centrifugation for 10 min at 10000 rpm and then washed and resuspended in Krebs-Ringer-Glucose (KRG) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose and 1.1 mM $CaCl_2$, PH 7.3).

The concentration of *P. gingivalis* was determined by counting CFU (Colony-forming unit) of different dilutions of bacteria on blood agar after 5 to 7 days. The optical density (OD) at 600 nm of bacteria suspension was measured with a spectrophotometer (BioPhotometer plus) (Eppendorf AG, Hamburg, Germany) and was correlated with the concentration (CFU/ml) of the bacteria.

Gingipain quantification. The activity of arginine and lysine gingipains from different strains of *P. gingivalis* were quantified using arginine and lysine substrates (Peptanova, Sandhausen, Germany). The arginine gingipain substrate peptide sequence was Boc-Phe-Ser-Arg-AMC (t-Butyloxycarbonyl- L-phenylalanyl- L-seryl- L-arginine- 4-methylcoumaryl-7-amide) and the lysine gingipain substrate peptide sequence was Z-His-Glu-Lys-AMC (Benzyloxycarbonyl- L-histidyl- L-glutamyl- L-lysine- 4-methylcoumaryl-7-amide). 10^6 CFU of different strains of washed *P. gingivalis* were incubated with either of the substrates at a final concentration of 100μM for one hour at 37°C, and the enzyme activity was registered in a fluorescence microplate reader (Fluostar Optima, Ortenberg, Germany) at excitation/emission wavelength settings of 380/ 460 nm.

Stimulation of AoSMCs with *P. gingivalis,* LPS and TNF. AoSMCs were seeded at a density of 150,000 cells per well in 6-well plates coated with Type I collagen (Gibco, Carlsbad, CA), Thereafter, cells were serum starved for 24 hour using DMEM medium (Gibco, Carlsbad, CA) containing 0.5% FBS (Sigma, St. Louis, MO), 2 mM L-glutamin and antibiotics (Gibco, Carlsbad, CA). After being washed and resuspended in fresh DMEM medium, AoSMCs were challenged with different strains of *P. gingivalis* at a concentration of 10 MOI for 2, 8, 16, 24 or 48 hours. Because *F. alocis* was found to co-infect with *P. gingivalis*, AoSMCs infected with *F. alocis* for 24 hours was served as a control in this study. For AoSMCs stimulated with the fimbriae mutants DPG3 and KRX178, AoSMCs treated with 1 ug/ml of erythromycin served as control. To determine the role of arginine gingipains, *P. gingivalis*, ATCC

33277, W50, and K1A were incubated with 1mM of Leupeptin (Roche Diagnostics Corporation, USA), which is an arginine gingipain inhibitor, for 1 h prior stimulation of the AoSMCs. AoSMCs were also stimulated with 10 ng/ml or 50 ng/ml of TNF (Sigma-Aldrich, St. Louis, MO) or 10 ng/ml or 100 ng/ml of *P. gingivalis* LPS (InvivoGen, Toulouse, France) for 24 or 48 hours.

Knockdown of ETS1 in AoSMCs. Knockdown of ETS1 was performed by using human ETS1 siRNA (Perkin-Elmer Applied Biosystems, Foster City, CA) in 150,000 cells/well 6-well plates. A volume of 4 µl of Lipofectamine 2000 (Life Technologies, Carlsbad, CA) was added to 250 µl of Opti-MEM medium (Life Technologies, Carlsbad, CA) for 5 min, which was then mixed with 250µl of Opti-MEM medium containing 50 pmol of ETS1 siRNA (VHS40614) (Life Technologies, Carlsbad, CA) or 50 pmol non-targeting siRNA (Life Technologies, Carlsbad, CA) as a control. After 20 min at room temperature, the transfection mixture was added to each cell culture well containing 500 ml of Opti-MEM medium and cultures were incubated for 6 h. A volume of 1ml of antibiotic-free growth medium was then added and cultures were incubated for another 18 h. The cells were then starved for 24 h and treated with *P. gingivalis* W50 for 24 h.

Quantitative real-time PCR. Isolation of RNA from AoSMCs was carried out using a Genejet RNA isolation kit (Fermentas, Sweden). cDNA were synthesized using equal amounts of RNA and High Capacity cDNA Reverse Transcription Kits (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's

protocol. The primer sequences for Angpt1, Angpt2, ETS1, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Eurofins, Ebersberg, Germany) used in this study are listed in table 1. Quantitative real-time PCR for SYBR Green (Fermentas, Sweden) was performed with an ABI Prism 7900HT Sequence Analyzer. Relative quantification of gene expression was determined using the ΔΔCt method and normalized by the Ct value of GAPDH.

Western blot assay. Proteins were extracted from AoSMCs that had been stimulated or not stimulated with *F. alocis,* different strains of *P. gingivalis* or TNF for 24 h or 48 h, using RIPA Buffer (Sigma-Aldrich, St. Louis, MO) mixed with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation at 10,000 rpm for 10 min, the total protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, USA). An equal amount of each sample (30 µg) was electrophoresed on precast SDS–PAGE gels and transferred onto PVDF membrane (Bio-Rad, Hercules). After blocking in 3% BSA (Sigma-Aldrich, St. Louis, MO) for 1 h, membranes were probed overnight at 4°C with goat polyclonal anti-Angpt2 (R&D systems, UK) at the concentration of 1µg/ml. Rabbit polyclonal anti-GAPDH at a 1:15000 (Santa Cruz Biotechnology, Dallas, Texas) dilution was used as a loading control. For Angpt2, blots were incubated with anti-goat IgG (R&D systems, UK) at a concentration of 1:3000 for 2 h. For GAPDH, blots were incubated with anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, Texas) at a concentration of 1:15000 for 2 h. The blots were visualized using Luminata Forte Western HRP substrate (Millipore, Darmstadt, Germany) and a ChemiDoc MP imager (Bio-Rad, Hercules).

Densitometric analysis was performed using Image Lab software (Bio-Rad, Hercules).

Enzyme-linked immunosorbent assay (ELISA). The supernatants from AoSMCs that were challenged with the *P. gingivalis* strain of ATCC33277, W50, E8, or K1A for 24 h or 48 h were collected and centrifugated at 1500×g for 5 min at 4°C. Thereafter, the supernatants were stored at −80°C until use. TNF was analyzed by ELISA (Biolegent, San Diego, CA) according to the manufacturer's instructions.

Proliferation assay. To investigate proliferation responses, serum-starved AoSMCs were incubated with different concentrations of recombinant human Angpt2 for 24 h, The medium was then replaced with medium containing 0.5% FBS for 24 h. The proliferation responses were monitored using an MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. MTT (Sigma-Aldrich, St. Louis, MO) was dissolved in the cell culture DMEM medium at a concentration of 500 μg/ml. After different stimulations, the supernatant was aspirated and cells were washed twice with PBS. Then, 1 ml of MTT medium was added to each well of the plate. After 2h incubation at 37°C, the medium was removed and MTT was extracted from viable cells by adding 1 ml DMSO. Measurements of OD absorbance at 540 nm were then performed in a microtiter plate reader (SpectraMax 340, Microplate Reader; Molecular Devices Corp., Sunnyvale, CA).

Wound healing assay. Studies of the regulation of AoSMCs migration by ANGPT2 were preformed using a wound-healing assay. AoSMCs were seeded into 6-well culture plates and starved for 24 h in DMEM medium containing 0.5% FBS. The

resulting single cell layer was then carefully wounded using a 100 µl pipette tip. Cells were washed twice with DPBS to remove cellular debris and then 2 ml of DMEM medium containing 0.5% FBS was added to each well. AoSMCs were then treated with 10 ng/ml, 100 ng/ml, or 500 ng/ml of Angpt2 or DPBS as control. Wounds were photographed immediately (0 h) and 18 h after wounding with an Olympus inverted CKX41 phase-contrast microscope. Migration was evaluated by measuring the reduction in the area of the wound after migration of the cells into the cell-free zone with the NIH software package Image J (ImageJ 1.32j; NIH, Bethesda, MD).

Statistical Analysis. Data are expressed as the average ± standard error of the mean (SEM), with $p < 0.05$ considered to be significant. Student's t-test was used for statistical comparisons of two groups and One-way ANOVA with Bonferroni or Dunnett post-tests were used for calculating the statistical significance between differences in data groups obtained from the real-time qPCR, western blot, proliferation, and wound healing experiments that have more than two groups. Statistical analysis was performed using GraphPad Prism software.

Results

Gingipain activity of *P. gingivalis.* In this study, we used gingipain substrates and a fluorescence assay to validate the activity of gingipains in different *P. gingivalis* strains. As expected, E8 showed only Kgp activity and K1A showed only Rgp activity. There was no clear difference in gingipain activity between the *P. gingivalis* strains ATCC 33277, W50, 381, DPG3 and KRX178. (Fig. S1A and S1B)

235 ***P. gingivalis* and its gingipains regulate Angpt1 and Angpt2 production in**
236 **AoSMCs.** mRNA was collected from samples of each different group and assessed by
237 quantitative real-time PCR for Angpt1 and Angpt2. The results were normalized
238 against GAPDH. For Angpt1, the gene expression level was significantly inhibited by
239 wild type *P. gingivalis* ATCC 33277 and W50 after 24 h compared to unstimulated
240 cells that served as negative control. In contrast, the Rgp-deficient strain E8 had
241 almost no effect on Angpt1 gene expression in AoSMCs. The Kgp-deficient strain
242 K1A also significantly inhibited the expression of Angpt1 after 24 h, but to a lesser
243 extent than the wild type strains (Fig. 1A). Leupeptin only sparsely reversed the
244 inhibitory effect of wild type *P. gingivalis* on Angpt1, but it completely neutralized
245 the inhibition of K1A (Fig. S2A).

246 The gene expression of Angpt2 was markedly increased by Wild type *P. gingivalis*
247 ATCC 33277 after 16 h and 24 h and by W50 after 16 h, 24 h, and 48 h, whereas the
248 Rgp-deficient strain E8 had no effect on the gene expression of Angpt2. K1A also
249 increased the expression of Angpt2; however, the results were not significant (Fig.
250 1B). Inhibition of Rgp with Leupeptin effectively antagonized the stimulatory effect
251 of wild type and K1A *P. gingivalis* on Angpt2 (Fig. S2B). We also infected AoSMCs
252 with *F. alocis*, a different bacterial species often found to co-infect with *P. gingivalis.*
253 However, no significant change was observed in Angpt1and Angpt2 (Fig. S3) gene
254 expression compared to control samples after 24 h, which indicates changes to
255 Angpt1/2 are a result of *P. gingivalis* specifically.

Here, we focused on Angpt2 protein expression because the gene expression of Angpt2 was markedly induced by *P. gingivalis*. AoSMCs were infected with *F. alocis* or different strains of *P. gingivalis* at a concentration of 10 MOI for 24 h and 48 h. The *P. gingivalis* ATCC 33277 strain significantly increased the Angpt2 protein expression in AoSMCs at 24 h (Fig. S4A and S4B) after infection, whereas both ATCC 33277 and W50 significantly increased Angpt2 protein level at 48 h (Fig. 1C and 1D) after infection. As expected, the western blot results for 48 h incubations with the bacteria followed the same trend that was observed for Angpt2 gene expression.

Fimbriae and LPS are not involved in *P. gingivalis*-mediated regulation of Angpt1 and Angpt2 production in AoSMCs. We further investigated the role of fimbriae of *P. gingivalis* in modulating Angpt1 and Angpt2 expression in AoSMCs. The wild type strain *P. gingivalis* 381 and its corresponding major fimbriae mutant DPG3 and minor fimbriae mutant KRX178 significantly down-regulated the gene expression of Angpt1 in AoSMCs after 24h (Fig. 2A). All three of these strains of *P. gingivalis* significantly up-regulated the gene expression of Angpt2 in AoSMCs after 24 h of incubation (Fig. 2B). We next assessed whether changes to Angpt1/2 were a result of LPS, considering that previous research has demonstrated that the quantity of LPS produced and released differs between various strains.[25] Stimulation of AoSMCs with *P. gingivalis* LPS for 24 h or 48 h resulted in no significant changes in Angpt1 or Angpt2 expression level (Fig. S5A and S5B).

277 **TNF regulates Angpt1 and Angpt2 production in AoSMCs.** AoSMCs were
278 stimulated with different concentration of TNF for 24 h and 48 h. Gene expression of
279 Angpt1 was significantly reduced (1.64-fold and 1.87-fold) after stimulation for 24 h
280 with 10 ng/ml and 50 ng/ml TNF, respectively (Fig. 3A). The levels of Angpt2
281 mRNA were significantly increased (8.30-fold and 9.67-fold) after stimulation for 24
282 h with 10 ng/ml and 50 ng/ml TNF, respectively. Similar to our results for the gene
283 expression of Angpt1, we did not observe a significant change for Angpt2 mRNA
284 expression levels after 48 h (Fig. 3B). With respect to protein expression, TNF
285 stimulation induced Angpt2 after 24 h (Fig. S4C and S4D) and 48 h (Fig. 3C and 3D),
286 with a significant up-regulation induced by stimulation with 50 ng/ml for 48 h.

287 The TNF ELISA kit detects human TNF levels from 7.8 pg/ml to 500 pg/ml.
288 However, the TNF levels in the supernatants from AoSMCs treated with the *P.*
289 *gingivalis* strains of ATCC33277, W50, E8, or K1A were undetectable (data not
290 shown).

291 ***P. gingivalis* and TNF up-regulate ETS1 in AoSMCs.** The wild type strains *P.*
292 *gingivalis*, ATCC 33277 and W50, significantly increased ETS1 expression in
293 AoSMCs after 16 and 24 h (Fig. 4A). Compared with W50, the Rgp and Kgp mutant
294 strains E8 and K1A, respectively, had no effect on ETS1 gene expression. Inhibition
295 of Rgp with Leupeptin, significantly antagonized the stimulatory effect of W50 (Fig.
296 S2C). The wild type strain 381 and its fimbriae mutants, DPG3 and KRX178,
297 significantly increased ETS1 expression in AoSMCs after 24 h (Fig. 4B). TNF also

298 significantly increased the expression of ETS1 in AoSMCs after 24h when applied at
299 a concentration of 10 ng/ml and 50 ng/ml (Fig. 4C). Compared with wild type *P.*
300 *gingivalis* and TNF, *F. alocis* showed no effect on ETS1 gene expression after 24 h
301 (Fig. S3). To ensure that the up-regulation of ETS1 by *P. gingivalis* infection was
302 correlated with the up-regulation of Angpt2 in AoSMCs, we performed an ETS1
303 siRNA knock down experiment. We found that the ETS1 siRNA significantly
304 reduced the up-regulation of ETS1 in AoSMCs that were treated with *P. gingivalis*
305 W50 (Fig. 5A). This result confirms our finding by showing that the up-regulation of
306 Angpt2 at the mRNA (Fig. 5B) and protein levels (Fig. 5C and 5D) by W50 is
307 significantly reduced in ETS1 siRNA knock down AoSMCs.

308 **Regulation of AoSMCs proliferation and migration by Angpt2.** To investigate
309 how Angpt2 regulates the cellular function of AoSMCs, we studied the proliferation
310 and migration of AoSMCs after stimulation with Angpt2 protein. The results of MTT
311 assays showed that Angpt2 has no effect on AoSMCs proliferation (Fig. 6A).
312 However, Angpt2 protein dose-dependently increased the migration of AoSMCs in
313 cells analyzed with the scratched assay. After 18 h, cells stimulated with 100 ng/ml or
314 500 ng/ml of Angpt2 protein covered almost the entire scratched area (Fig. 6B and 6C).

315 ## Discussion

316 Considerable evidence has indicated that periodontal infection is a mild but
317 significant risk factor for developing cardiovascular disease. The
318 periodontopathogenic bacteria *P. gingivalis* is considered to be directly or indirectly

involved in the development of atherosclerosis and cardiovascular disease. We have previously reported that *P. gingivalis* causes platelet aggregation, sensitizes platelets to epinephrine, suppresses the inflammatory responses of immunological cells and modifies LDL.[32-34]

This study indicates that wild type *P. gingivalis* decreases the gene expression level of Angpt1 but increases the gene and protein expression of Angpt2 in AoSMCs. For Angpt1, we did not observe significant differences at the protein level. This might be due to the fluctuations in results observed in Angpt1 gene expression in AoSMCs infected by *P. gingivalis* at early time points. Endothelial cells, which are stored in the endothelial-specific Weibel-Palade bodies (WPBs), are the main source of Angpt2. Certain stimuli, such as hypoxia, thrombin, or phorbol-12-myristate-13-acetate (PMA), are know to induce the rapid release of Angpt2 from WPBs.[35, 36] We found that AoSMCs are also able to produce Angpt2. However, although unstimulated AoSMCs produce Angpt2 at a low level, stimulation with wild type *P. gingivalis* dramatically increases the gene expression of Angpt2 in AoSMCs. Elevated levels of Angpt2 have been suggested to be a marker of cardiovascular disease, oral squamous cell carcinoma and lung cancer.[37-39] These results are also relevant because a high angiopoietin-2/angiopoietin-1 ratio is associated with pathogenesis in hemangiomas, atherosclerosis, and hemorrhagic endometrium.[40, 41]

The RgpA/B mutant E8 was unable to alter the expression of Angpt1 or Angpt2 in AoSMCs. The Kgp mutant K1A down-regulated the expression of Angpt1 and up-regulated the expression of Angpt2 in AoSMCs, but compared to the wild type,

341 K1A had only small effects on the regulation of these angiopoietins. These results
342 indicate that gingipains, especially Rgp, play important roles in *P. gingivalis*-induced
343 modulation of angiopoietins in AoSMCs. In support of this idea, inhibition of Rgp with
344 Leupeptin antagonized the up-regulation of Angpt2 and the down-regulation of Angpt1
345 that was induced by *P. gingivalis* infection. The infection of AoSMCs with *F. alocis*,
346 which has a low cysteine protease activity, [42] had no effect on the expression of Angpt1,
347 Angpt2, or ETS1. Gingipains are cell surface trypsin-like cysteine proteases that are
348 produced by *P. gingivalis*. Both Rgp and Kgp are secreted by *P. gingivalis* and are
349 indispensable for the ability of the bacterium to obtain nutrients from the
350 environment.[43] Studies have shown that the RgpA/B mutant markedly decreases
351 hemagglutinating activity, whereas the Kgp mutant only slightly affects
352 hemagglutinating activity.[44, 45] In contrast to RgpB, which only has a catalytic domain,
353 RgpA and Kgp, contain non-covalent complexes that are composed of separate
354 catalytic and adhesion/ hemagglutinin domains.[46] These results show that RgpA and
355 Kgp are important to the ability of *P. gingivalis* to acquire hemagglutinins from the host
356 cells through proteolytic processing. Moreover, gingipains have the ability to impair
357 host immune response function through degradation and inactivation of
358 immunoglobulins, such as IgG, IgA, and secretory IgA,[47] and proinflammatory
359 cytokines, such as interleukin 6 (IL-6), interleukin 8 (CXCL8), and interleukin-1β
360 (IL-1β).[48] The results of this study suggest that gingipains, especially Rgp, are
361 responsible for *P. gingivalis*-mediated regulation of Angpt1 and Angpt2 in AoSMCs.

362 In contrast to gingipains, fimbriae and LPS are not involved in *P. gingivalis*-mediated

regulation of Angpt1and Angpt2 production in AoSMCs. *P. gingivalis* 381, DPG3, and KRX178 all down-regulate Angpt1 and up-regulate Angpt2 and ETS1 expression. In our previous study,[25] we found that the amount of LPS that was produced and released differed between various bacterial strains. Consequently, we studied how LPS produced by *P. gingivalis* affects Angpt1 and Angpt2 expression in AoSMCs. The results showed that LPS from *P. gingivalis* does not modulate the expression of Angpt1 or Angpt2 in AoSMCs. However, different acylated and phosphorylated isoforms of LPS have been reported to display different bioactivity,[49] which makes it difficult to compare outcomes when researchers use different isoforms of LPS.

TNF is a key regulator in the pathogenesis and progression of periodontitis and atherosclerosis. This cytokine plays crucial roles in the initiation and maintenance of immune responses to *P. gingivalis* infection.[50-52] TNF can induce the migration, proliferation and apoptosis of vascular smooth muscle cells.[53] Studies have shown that there is an association between plasma levels of Angpt2 and TNF in endotoxemia and sepsis patients.[54, 55] To study the role of TNF in the modulation of Angpt1 and Angpt2, we exposed AoSMCs to various doses of TNF. We found that TNF inhibited the expression of Angpt1 but increased the expression of Angpt2 in AoSMCs. These effects are similar to those obtained in AoSMCs that were stimulated with *P. gingivalis*. However, TNF was not detected in the supernatants of AoSMCs that were stimulated with *P. gingivalis,* which indicates that *P. gingivalis*-mediated regulation of Angpt1 and Angpt2 in AoSMCs is independent of TNF.

Because we found that *P. gingivalis* and TNF dramatically increase the expression of

the Angpt2 gene in AoSMCs, we further examined the gene expression of ETS1, the transcription factor of Angpt2.[56, 57] Wild type, fimbriae mutant of *P. gingivalis,* and TNF increased the expression of the ETS1 gene, respectively, which was then correlated with the effects on Angpt2. However, the RgpA/B mutant E8 was unable to up-regulate ETS1 expression, which supports a role for Rgp in *P. gingivalis*-mediated regulation of Angpt2 in AoSMCs. After knock down of ETS1 in AoSMCs, the up-regulation of Angpt2 by *P. gingivalis* W50 was inhibited, which reveals that ETS1 is critical for the induction of Angpt2.

To understand the regulatory role of Angpt2 in AoSMCs, we treated AoSMCs with recombinant human Angpt2. Angpt2 did not induce proliferation of AoSMCs, but it significantly induced the migration of the cells in a dose-dependent manner. During the progression of atherosclerosis, smooth muscle cells change from a contractile phenotype to a synthetic phenotype, and the migration of vascular smooth muscle cells in the intima layer marks a key event in the disease pathogenesis.[58] Several reports have shown that high levels of Angpt2 are associated with atherosclerosis and coronary heart disease.[59, 60]

In summary, we found that *P. gingivalis* infection induces comparable effects on the expression of Angpt1, Angpt2, and ETS1 in AoSMCs, and gingipains are crucial for this regulation. However, the cardiovascular risk factor TNF is not involved. In combination with observed cellular effects, our findings suggest that Angpt2 plays a role in the association between periodontitis and atherosclerosis.

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Acknowledgements

Infection and Immunity

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Figure legends

439 **Fig. 1.** *P. gingivalis* and its gingipains regulate Angpt1 and Angpt2 expression in
440 AoSMCs. Quantitative real-time PCR results demonstrate relative transcription levels
441 for Angpt1 (A) and Angpt2 (B) in AoSMCs stimulated with 10 MOI of wild type *P.*
442 *gingivalis* (ATCC 33077 and W50), Rgp mutant (E8), and Kgp mutant (K1A) for 2 h,
443 8 h, 16 h, 24 h, and 48 h. All results were normalized to the gene expression level of the
444 housekeeping gene GAPDH. Representative western blot showing Angpt2 protein
445 expression levels of AoSMCs exposed to different *P. gingivalis* strains and *F. alocis* at
446 10 MOI for 48 h (C). Quantification of Angpt2 protein expression levels by
447 densitometry is shown in (D) for cells incubated for 48 h. Angpt2 density signals were
448 normalized to GAPDH signal values. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$. A, B,
449 n=4-7; C-D, n=3.

450 **Fig. 2.** Fimbriae is not involved in *P. gingivalis*-mediated regulation of Angpt1 and
451 Angpt2 production in AoSMCs. Quantitative real-time PCR results demonstrate
452 relative transcription levels for Angpt1 (A) and Angpt2 (B) in AoSMCs stimulated with
453 10 MOI of wild type *P. gingivalis* 381, major fimbriae mutant (DPG3), minor fimbriae
454 mutant (KRX178), or medium containing 1 µg/ml of erythromycin (Control+EM) as a
455 control for fimbriae mutants for 24 h. All results were normalized to the gene
456 expression level of the housekeeping gene GAPDH. Statistically significant differences
457 for the wild type *P. gingivalis* strain 381 are shown compared to the negative control.
458 For fimbriae mutants, statistically significant differences are shown compared to group
459 Control+EM. *, $p<0.05$; **, $p<0.005$. A, n=7; B, n=10.

Fig. 3. TNF regulates Angpt1 and Angpt2 expression in AoSMCs. Quantitative real-time PCR results demonstrate relative transcription levels for Angpt1 (A) and Angpt2 (B) of AoSMCs stimulated with 10 ng/ml or 50 ng/ml of TNF for 24 h or 48 h. All results were normalized to the gene expression level of the housekeeping gene GAPDH. Representative western blot showing Angpt2 protein expression levels in AoSMCs exposed to 10 ng/ml or 50 ng/ml of TNF for 48 h (C). Quantification of Angpt2 protein expression levels by densitometry is shown in (D). Angpt2 density signals were normalized to GAPDH signal values. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$. A, B, n=4; C-D, n=3.

Fig. 4. *P. gingivalis* and its gingipains and fimbriae mutants up-regulate ETS1 in AoSMCs. Quantitative real-time PCR results demonstrate relative transcription levels of ETS1 in AoSMCs stimulated with 10 MOI of wild type ATCC 33277 and W50 and the W50 corresponding gingipain mutants (E8 and K1A) for 2 h, 8 h, 16 h, 24 h, and 48 h (A), and wild type 381 and its corresponding fimbriae mutants (DPG3 and KRX178) of *P. gingivalis* for 24 h or TNF for 24 h and 48 h (C). All results were normalized to the gene expression level of the housekeeping gene GAPDH. Statistically significant differences for LPS and wild type *P. gingivalis* strain of 381 are compared to the negative control. For fimbriae mutants, statistically significant differences are compared to the group Control+EM. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$. A, n=5; B, n=4; C, n=7.

Fig. 5. *P. gingivalis* regulates Angpt2 through ETS1 in AoSMCs. AoSMCs were

treated with (KD) or without (Control) ETS1 siRNA or non-targeting siRNA (NT). The cells were then infected or not infected with *P. gingivalis* W50 for 24 h. Quantitative real-time PCR results demonstrate the relative transcription levels of ETS1 (A) and Angpt2 (B) in AoSMCs. The protein level of Angpt2 was determined by western blot (C) and the quantification of Angpt2 protein expression levels by densitometry is shown in (D). All results were normalized to the gene or protein expression level of GAPDH. Asterisks above W50 represent statistical comparisons to the negative control. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$. A, B, C, D, n=5.

Fig. 6. Angpt2 induces migration, but not proliferation, in AoSMCs. AoSMCs were treated with or without Angpt2 for 24 h. Then, the proliferation of the cells was measured using an MTT assay (A). The migration of AoSMCs after treatment with Angpt2 or DPBS (Control) was measured in a wound-healing assay. The photos were taken after wounding, at 0 h (as Control) and 18 h (B). The quantitative results were calculated by the reduction in the area of the wound after migration of the cells into the cell-free zone (C). **, $p<0.005$; ***, $p<0.0001$. A, n=4; B, C, n=6.

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695

Table 1. Primer sequences for real-time PCR

Gene	Forward primer	Reverse primer
Angpt1	CAACAGTGTCCTTCAGAAGCAGC	CCAGCTTGATATACATCTGCACAG
Angpt2	ATTCAGCGACGTGAGGATGGCA	GCACATAGCGTTGCTGATTAGTC
ETS1	GAGTCAACCCAGCCTATCCAGA	GAGCGTCTGATAGGACTCTGTG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

Fig. 1

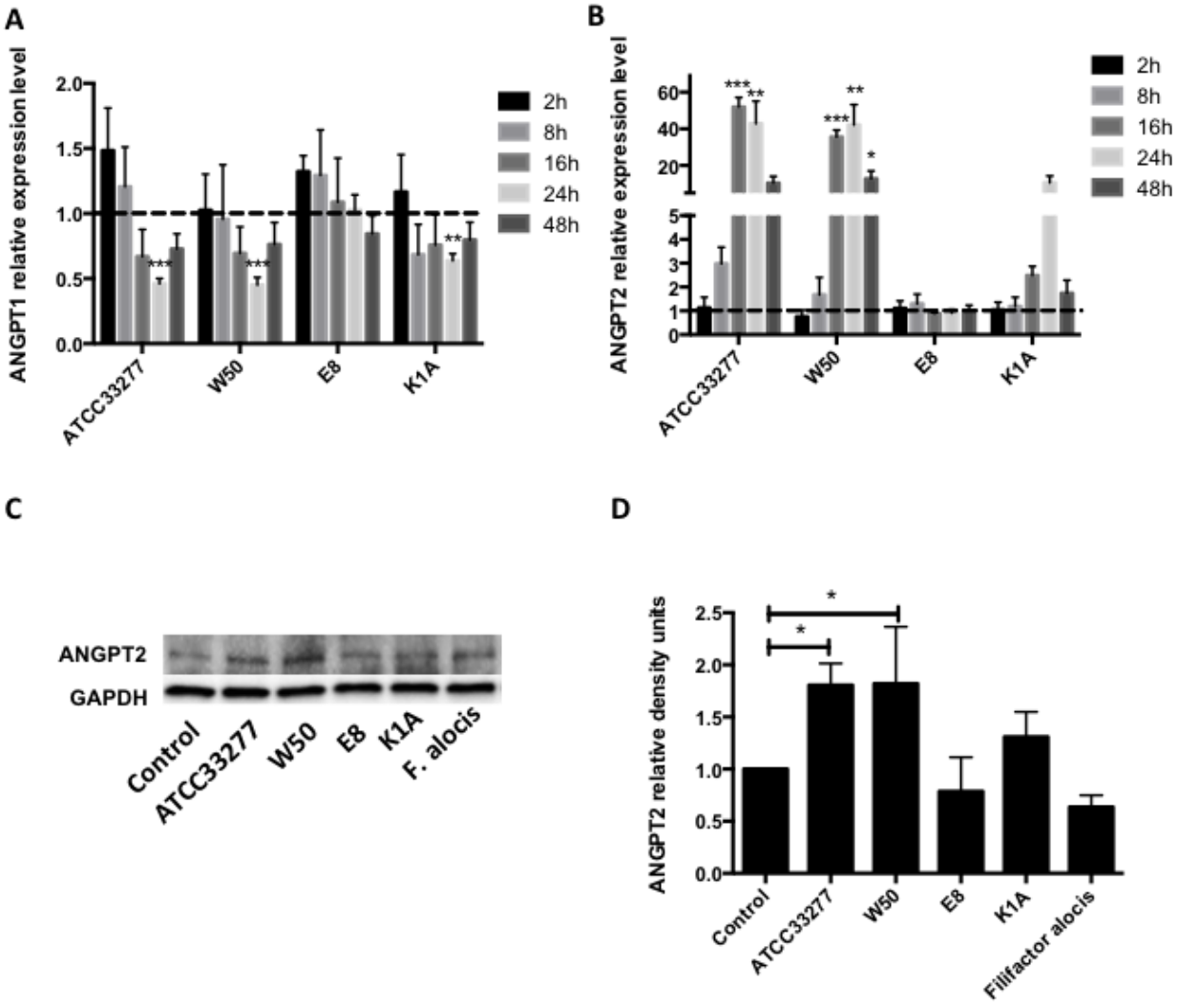

Fig. 2

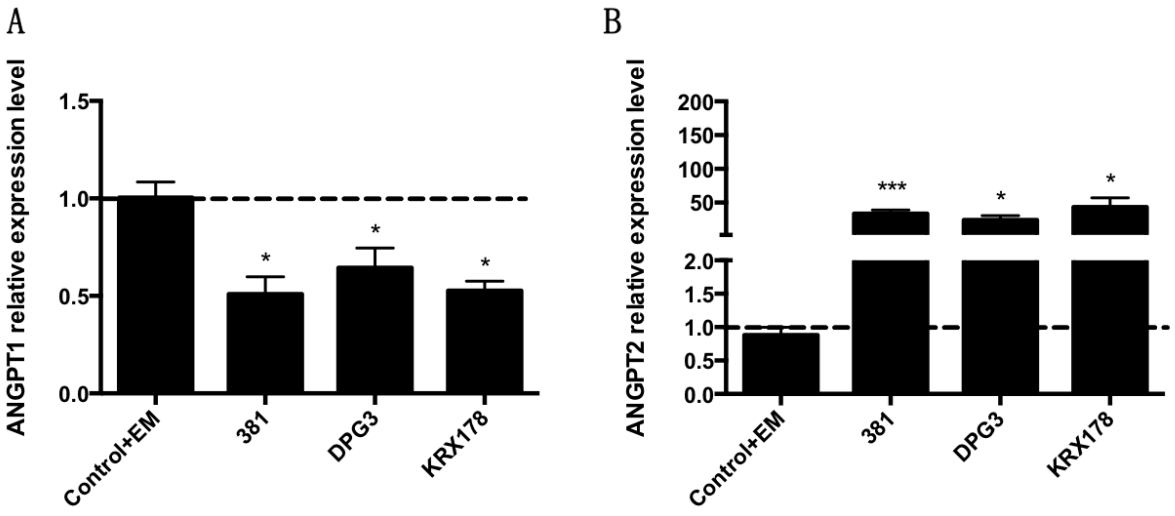

Fig. 3

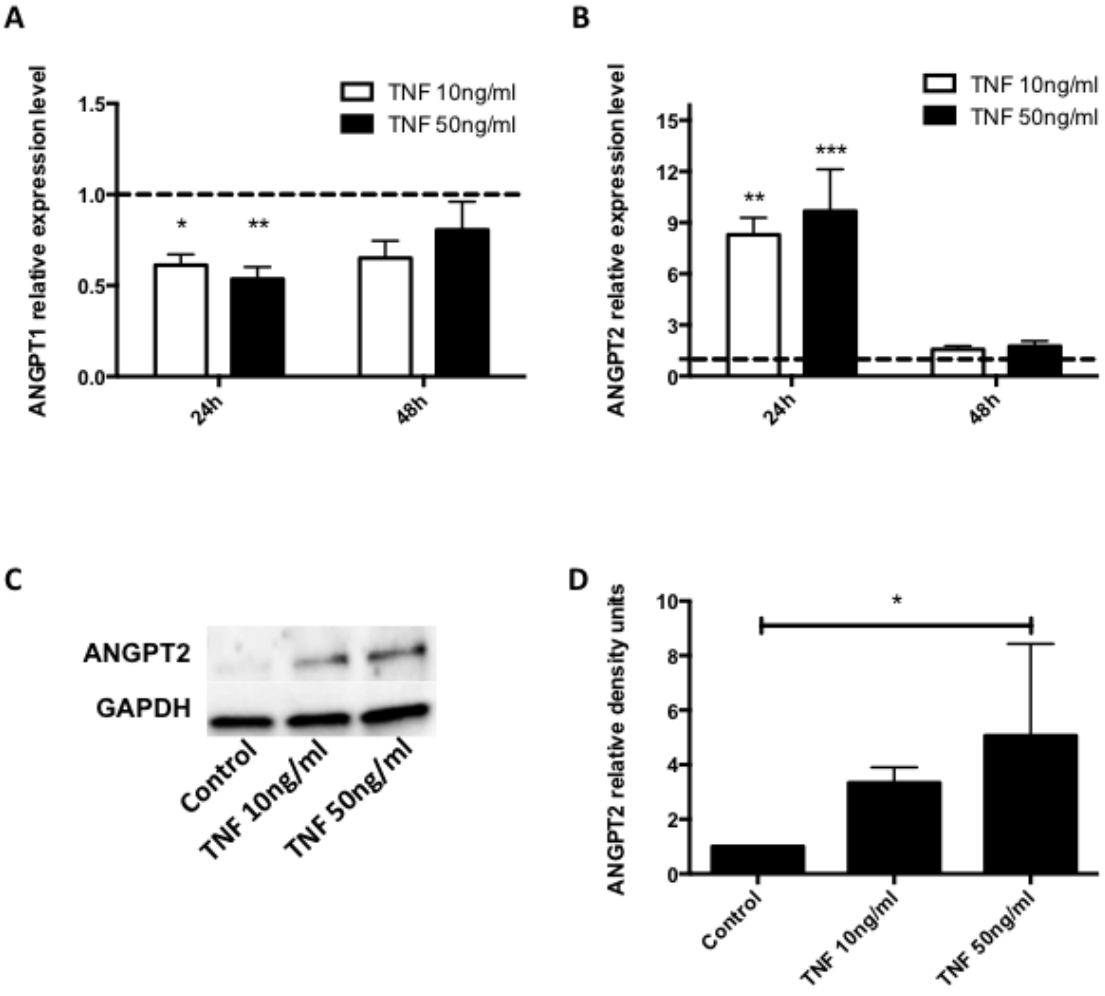

Fig. 4

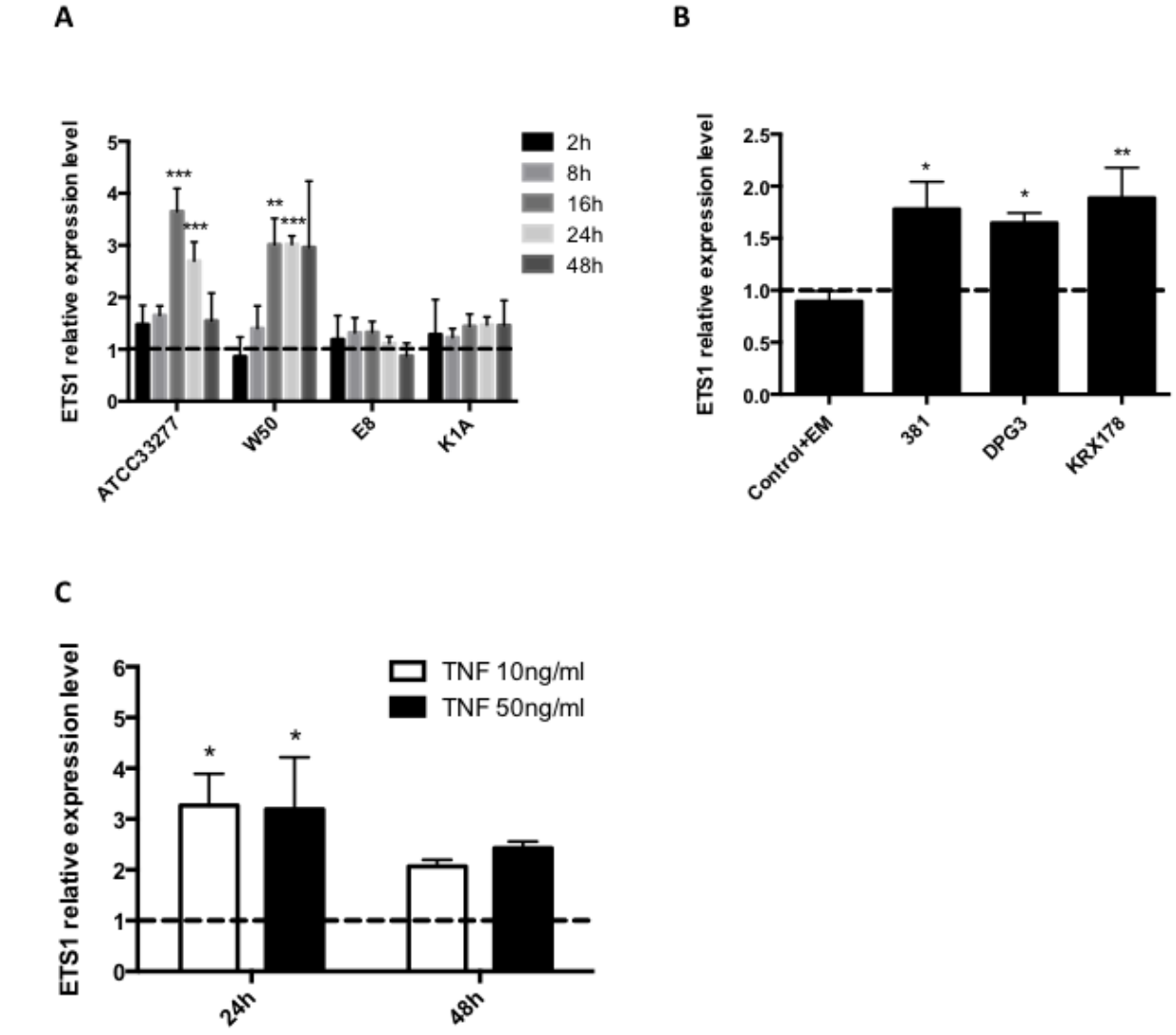

Fig. 5

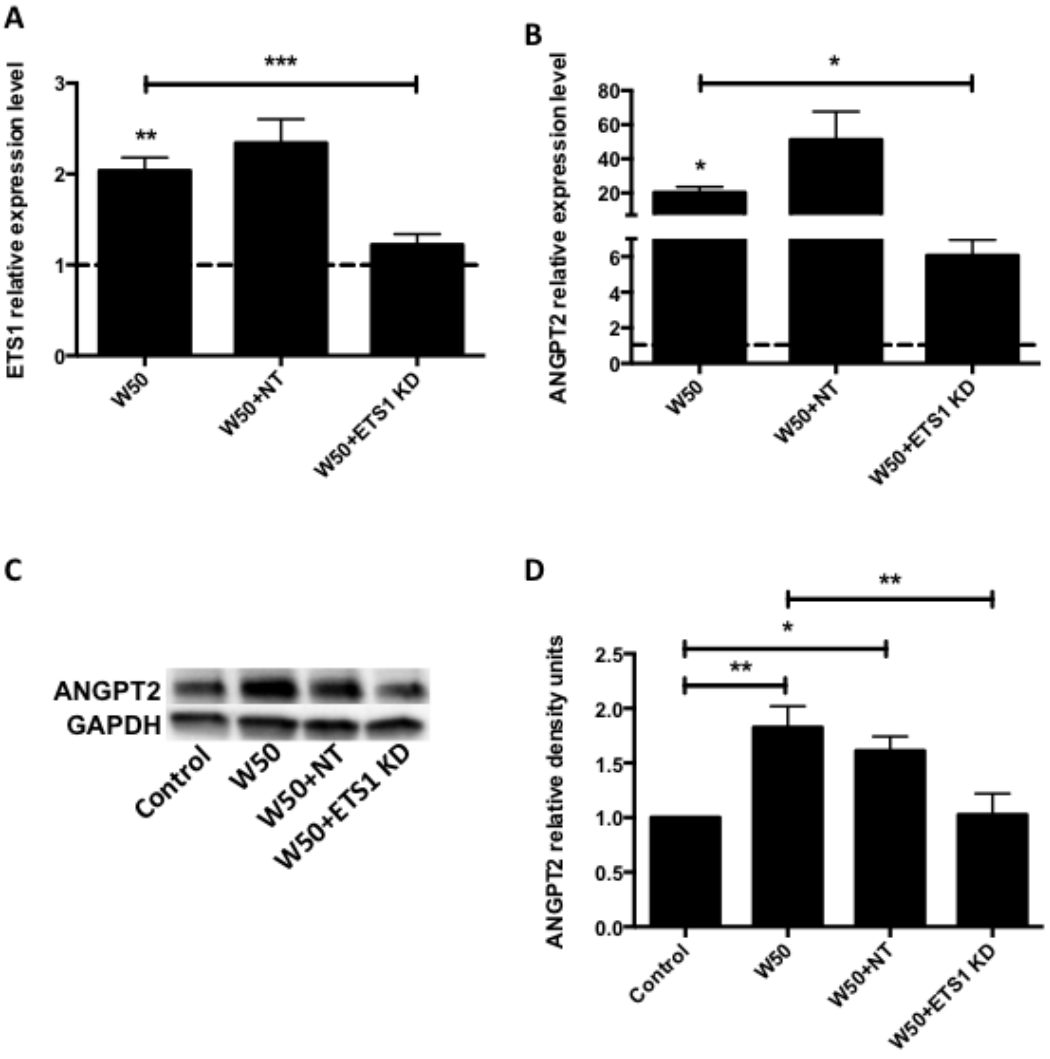

Fig.6