The protease interpain A of *Prevotella intermedia* promotes human OSCC cells proliferation and migration

Szu-Min Chang[a 1], I-Hsiu Huang[b 1], Yun-Jhen Ciou[c], Wen-Rong Wang[c], Ni-Hung Wu[c], Chih-Pin Chuu[d], Jenn-Ren Hsiao[e], Jeffrey S Chang[f], Jang-Yang Chang[g, h], Jenn-Wei Chen[a, c, i *]

[a] Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan
[b] Department of Biochemistry and Microbiology, Oklahoma State University Center for Health Sciences, Tulsa, OK 74107, USA.
[c] Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan
[d] Institute of Cellular and System Medicine, National Health Research Institutes, Miaoli 35053, Taiwan
[e] Department of Otolaryngology, National Cheng Kung University Hospital, Tainan 70101, Taiwan
[f] National Institute of Cancer Research, National Health Research Institutes, Tainan 70101, Taiwan
[g] Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Miaoli 35053, Taiwan
[h] TMU Research Center of Cancer Translational Medicine, Taipei Medical University, Taipei 110301, Taiwan
[i] Center of Infectious Disease and Signaling Research, National Cheng Kung University, Tainan 70101, Taiwan

*** Correspondence:**
Jenn-Wei Chen
jc923@mail.ncku.edu.tw

[1] These authors contributed equally to this article.

Key words: *Prevotella intermedia*; OSCC, interpain A; PAR-2

Abstract

Background. Oral squamous cell carcinoma (OSCC) is a prevalent cancer worldwide. Oral microbiota dysbiosis causes periodontal diseases and may lead to OSCC. The oral microbiome analysis has indicated that the oral pathogen Prevotella intermedia was more abundant in OSCC patients than in healthy people. Therefore, we investigated the role *P. intermedia* played during OSCC carcinogenesis.

Methods. Human oral epithelial cell line SG as well as OSCC cell lines SCC-15 and SAS were treated with *P. intermedia* conditioned medium (CM). Effect of CM on migration of SCC-15 and SAS cells were examined by wound healing assay and transwell assay. We tested *P. intermedia* in the colorectal cancer mouse model.

Results. CM of P. intermedia ATCC 25611 CM increased cell proliferation of SG, but the CM from clinical strains didn't affect cell proliferation. Comparing the CM proteins composition of cell proliferation-promoting strain ATCC 25611 with other strains, interpain A (InpA) appeared to be the potential key factor promoting cell proliferation. InpA is a cysteine protease secreted by *P. intermedia* and might activate proteinase-activated receptor-2 (PAR-2) signaling pathway. SG cell proliferation was inhibited by adding protease inhibitor E64 or PAR-2 antagonist (FSLLRY-NH2). This phenomenon was also observed in SCC-15 and SAS cells. Additionally, InpA promoted migration of SCC-15 and SAS cells, and enhancement was inhibited by E64 or FSLLRY-NH2. *P. intermedia* increased colorectal tumors development in murine model.

Conclusion. Our results identified InpA as a potential key virulence factor secreted highly by some oral microbiota, such as ATCC 25611. These strains may, therefore, play a role in OSCC development via regulation of PAR-2 signaling pathway.

Introduction

Oral cancer is a prevalent worldwide disease and is the fourth most common

64 cancer among men in Taiwan [1]. It is the most frequent head and neck cancer. Oral
65 cancer could appear in every part of the oral cavity, including lips, tongue, sinuses,
66 cheeks, soft and hard palates, and throat. Most oral cancer types are squamous cell
67 carcinoma (OSCC). It is known for its poor prognosis and high mortality, and the 5-
68 year survival rate is around 50%. Many factors might promote oral cancer, including
69 gender, human papillomavirus (HPV), drinking alcohol, smoking, chewing betel nuts,
70 personal hygiene, and oral microbiota [2, 3]. Chronic periodontitis is highly associated
71 with oral cancer [4-6]. Chronic periodontitis is a gum disease that results in gingiva
72 and teeth loosening or loss [7, 8]. When people have poor oral hygiene, oral bacteria
73 form biofilms, which are called dental plaques, after eating or drinking. Dental plaques
74 causes oral microbiota dysbiosis. Some anaerobic bacteria residing in dental plaques
75 produce acids after people eat or drink [9]. Anaerobic bacteria were considered an
76 important cause of chronic periodontitis [10].

77 Populations of several bacteria, such as *Porphyromonas* spp., *Fusobacterium* spp.,
78 and *Prevotella* spp, are elevated in patients with periodontal diseases as compared to
79 healthy people. [11, 12]. *P. gingivalis* is a Gram-negative anaerobic bacterium and is
80 related to periodontal disease and other systemic disease, such as Alzheimer's disease
81 [13]. Some studies indicated that it is strongly associated with cancers, such as lung
82 cancer, pancreatic cancer, prostate cancer, colorectal cancer (CRC) and OSCC [14-17].
83 *P. gingivalis* secreted gingipain as an important virulence factor promoting cancer
84 invasion and migration [18]. *Fusobacterium nucleatum*, a Gram-negative, anaerobic,
85 rod-shaped bacterium, is reported to be associated with CRC, breast cancer, pancreatic
86 cancer, and OSCC [19-22]. Most research focused on *P. gingivalis* and *F. nucleatum*,
87 however, little is known about *Prevotella intermedia*. *P. intermedia* is a Gram-negative,
88 obligate anaerobic rod-shaped bacterium. It needs vitamin K and hemin for growth and

89 is related to periodontal infections and acute necrotizing ulcerative gingivitis. Previous
90 studies revealed that oral cancer patients had a higher abundance of *P. intermedia* as
91 compared to healthy population. Although the findings showed that the abundance of
92 *P. intermedia* was correlated with the incidence of oral cancer, the role of *P. intermedia*
93 in oral cancer was unclear. We therefore hypothesize that *P. intermedia* is an important
94 bacterium in oral cancer development.

95 In this study, we found that *P. intermedia* and its conditioned medium (CM) could
96 promote cell proliferation in oral normal epithelial cell line SG cells as well as oral
97 cancer cell lines SCC-15 and SAS cells. We discovered that protease interpain A (InpA)
98 in the CM enhanced the cancer cell proliferation and migration. Our findings pointed
99 out the essential role of *P. intermedia* during the OSCC development.

100

101 ## Materials and methods

102 **Bacterial strains and cultures**

103 *P. intermedia* strain ATCC 25611 was purchased from Bioresource Collection and
104 Research Center (BCRC, Hsinchu, Taiwan). Clinical strains were isolated from
105 National Cheng Kung University Hospital (NCKUH) patients' saliva (IRB number: B-
106 ER-106-234). Saliva was diluted with Phosphate Buffer Saline (PBS) and spread on a
107 Kanamycin-Vancomycin Laked Blood (KVLB) plate (TPM, Yilan, Taiwan). After
108 being cultured under the anaerobic condition (5% CO_2, 5% H_2, 90% N_2), black colonies
109 were selected and confirmed by Gram staining and PCR with *P. intermedia* specific
110 primers Pi-192 and Pi-486 (Supplementary Table1). The PCR program was set as
111 follows: 95 ℃ for 5 min, 35 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30
112 s, and a final extension at 72 ℃ for 7 min. Supplemented Tryptic Soy Broth (ATCC
113 2722), which tryptic soy broth (#211825, BD) supplemented with 0.5% yeast extract

114 (#212750, BD), 0.05% L-cysteine (#A3694, Applichem Panreac), 0.1% hemin
115 (#H9039, Sigma) and 0.02% menadione (#M5625, Sigma) was used for *P. intermedia*
116 culturing at 37 ℃ anaerobically.
117
118 **Cell lines and cultures**
119 Immortalized human gingival keratinocyte, Smulow–Glickman (SG) cell [23], was
120 gifted from Dr. Chuan-Fa Chang (NCKU) and was cultured with Dulbecco's Modified
121 Eagle Medium (DMEM) (#11965-092, Gibco). Human tongue carcinoma cells, SAS
122 cells [24] and SCC-15 cells [25] were gifted from Dr. Jhen-Wei Ruan (NCKU) and
123 were cultured with DMEM/F12 (D/F12) medium (#11330-032, Gibco/Life
124 Technology, Carisbad, CA, USA) Both cell culture medium were supplemented with
125 10% fetal bovine serum (FBS) (#10437-028, Gibco) and 1% 100x Penicillin-
126 Streptomycin-Amphotericin B solution (#CC501-0100, Simply Biotechnology, San
127 Diego, CA, USA). Cells were all incubated at 37 °C under 5% CO_2 condition.
128
129 **Conditioned medium (CM) preparation**
130 *P. intermedia* strains were anaerobically cultured in ATCC 2722 broth at 37 °C and
131 were harvested when the OD600 value reached 1.0. The bacterial culture was
132 centrifuged at 3220 xg for 3 min and discarded supernatant. The pellet was resuspended
133 with a double volume of cell culture medium and anaerobically incubated at 37 °C for
134 8 hr. The supernatant was collected after being centrifuged at 3220 xg for 10 min,
135 filtered with 0.22 μm filter (Merck Millipore, Burlington, MA, USA), and stored at 4
136 °C before use.
137
138 **Cell proliferation assay**

139 The cell proliferation assay was followed by previous research [26]. SG, SAS, or SCC-
140 15 cells were dissociated by 0.05% trypsin-EDTA solution (#25300-054, Gibco) and
141 resuspended in DMEM or D/F12 medium. $1x10^4$ cells were seeded per well of 24-well
142 tissue culture plates (Corning, NY, USA) with bacteria or CM treatment. CM was
143 separated into three fractions by three centrifugal filter devices (3 kDa, 10kDa and 30
144 kDa) (Merck Millipore). 10% CM, 20 μM PAR-2 antagonist FSLLRY-NH2 (#4751,
145 TOCRIS/Bio-Techne, Minneapolis, MN, USA) or 0.5 μM cysteine protease inhibitor
146 E64 (#324890, Merck Millipore) and DMEM or D/F12 containing 10% FBS were
147 added to final volume of 1 ml. After 24 hr, 48 hr, or 72 hr of incubation at 37°C, cells
148 were collected by 0.05% trypsin-EDTA solution. The cell suspension was mixed with
149 the same volume of 0.05% trypan blue (#15250-061, Gibco), and the cell number was
150 counted by the Luna cell counter (Logos Biosystems, Gyeonggi-do, South Korea).
151
152 **Capillary electrophoresis/Liquid Chromatography-Mass spectrometry (CE/LC**
153 **Mass) analysis**
154 The mixture of 100 μg proteins in 60 μl rehydration buffer (7 M Urea, 2 M Thiourea
155 and 2% CHAPS), 9.3 μl 7.5% Sodium dodecyl sulfate (SDS) (#75746, Sigma,
156 Burlington, MA, USA) and 0.7 μl 1M Dithiothreitol (#UR-DTT, UniRegion Bio-Tech,
157 Taichung, Taiwan) solution supplemented with 7 M Urea and 0.01 M sodium acetate
158 was heated at 95 °C for 5 minutes and then put on ice for 3 minutes. The mixture was
159 added with 4 μl 500 mM IAM solution (500 mM iodoacetamide dissolved in 50 mM
160 ammonium bicarbonate solution) and incubated at room temperature. After 30 min, 52
161 μl 50% Trichloroacetic acid (TCA) (#100807, Merck Millipore) was added and placed
162 on ice for 15 min. The pellet was resuspended with 150 μl 10% TCA after centrifuging
163 at 15871 xg for 15 min at 4 °C and then spun at 15871 xg for 5 min at 4 °C. This wash

164 step was repeated 3 times. The pellet was resuspended in 100 µl 100 mM ammonium
165 bicarbonate and 20 µl trypsin solution (#V5111, Promega, Madison, WI, USA) and
166 then incubated at 37 °C for 16 hr. To inactivate trypsin by storing overnight at -80 °C,
167 Ziptips (Merck Millipore) were used for desalination of samples. Proteins of the
168 samples were identified by CE/LC MASS using Q Exactive Orbitrap Mass
169 Spectrometer (Thermo Fisher Scientific).
170

171 **Interpain A gene expression quantitative analysis (RT-qPCR)**

172 RNA extraction and RT-qPCR were modified from previously described [27]. Pellet
173 from 2 ml *P. intermedia* broth of OD600 of 0.8 was mixed with 4 ml RNAprotect
174 Bacteria Reagent (QIAGEN, Venlo, Netherlands). The supernatant was removed after
175 being centrifuged at 2200 xg for 5 min and stored at -80 ºC before RNA extraction.
176 The RNA was purified with RNeasy Mini Kit (QIAGEN), and the residual DNA was
177 eliminated by DNase (Promega). Clean & Concentrator Kit (ZYMO GENETICS,
178 Seatle, WA, USA) was used to concentrate the resulting RNA sample. Then RNA was
179 reverse-transcribed to cDNA with SuperScript III First-Strand System with random
180 hexamers (Invitrogen, Waltham, MA, USA). RT-qPCR was operated with GoTaq
181 qPCR Master Mix (Promega) and MyGO Pro real-time PCR instrument. (IT-IS Life
182 Science Ltd/DKSH, St Leonards, Australia) *inpA* F; *inpA* R; Pi 16s rRNA F and Pi 16s
183 rRNA R (Supplementary Table1). Denaturation at 95 °C for 10 min, amplification for
184 40 cycles at 95 °C for 15 s and 60 °C for 1 m, and melting at 60 to 97 °C for 0.1 °C /s.
185 All procedures were followed by the manufacturer's instructions.
186

187 **InpA$_{N381}$ construction**

188 Genomic DNA of *P. intermedia* ATCC 25611 was extracted with MasterPure^{TM}

189 complete DNA and RNA purification kit (Lucigen Corp, Middleton, WI, USA)
190 following manufacturer's instructions. The gene region of signal peptide, pro-domain
191 and catalytic domain ($inpA_{N381}$) was first amplified by GoTaq G2 Flexi DNA
192 Polymerase (Promega) with $inpA_{N381}$ F and $inpA_{N381}$ R (Supplementary Table1). The
193 BamHI & XhoI-digested PCR product was ligated into pET21b (+) expression vector
194 with the same restriction enzymes digestion. The pET21b (+)::$inpA_{N381}$ plasmid, which
195 the sequence has been confirmed by sequencing, was chemically transformed into
196 *Escherichia coli* C41 (DE3) strain.
197
198 **InpA $_{N381}$ purification**
199 *E. coli* strain C41 (DE3) carrying pET21b (+)::$inpA_{N381}$ colony was inoculated into 10
200 ml LB broth supplemented with ampicillin (100 μg/ml) and cultured at 37 ℃ for 14-
201 16 hr with shaking at 170 rpm. Then the bacterial culture was refreshed with 2 liters
202 LB at the ratio of 1:100 and grew at 37 ℃ with shaking at 170 rpm until OD600 reached
203 0.4. We used a final concentration of 0.2 mM Isopropyl-β-D-thiogalactoside (#101-
204 367-93-1, Cyrusbioscience, Inc., Seatle, WA, USA) for induction, and incubated at 25
205 ℃ for an additional 16 hr with shaking at 170 rpm [28]. After protein induction,
206 bacterial broth was centrifugated at 3300 xg for 40 min at 4 ℃. Pellet was resuspended
207 with 5 ml of 1x PBS per gram and ultrasonicated on ice (pulsed on 20 sec and pulsed
208 off 100 sec) until cells lysed properly. Then centrifugated at 16000 xg for 40 min at 4
209 ℃ after cell lysis. HisTrap HP His-tag protein purification column (Cytiva,
210 Washington D.C., USA) was used for His-tagged protein purification. All procedures
211 were followed by operating recommendations. Purified $InpA_{N381}$ was washed with 100-
212 fold of 1x PBS and stored at -20 ℃ before usage.
213

214 **Transwell assay**

215 Transwell assay was followed by a previous study [29]. SAS or SCC-15 cells were
216 resuspended with D/F12 containing 0.1% Bovine Serum Albumin (BSA) (#A2153,
217 Sigma), and 10% FBS. $1x10^5$ cells in 100 µl were added to the upper chamber of each
218 transwell insert (8 µm pore size, Falcon/Corning, NY, USA). After 10 min incubation
219 at 37 ºC under 5% CO_2, 10% CM, 20 µM FSLLRY-NH2 or 0.5 µM E64 was added in
220 lower chambers of inserts and made up to 600 µl with D/F12 containing 10% FBS.
221 After 24 hr, the medium of upper chambers was discarded and cells above the
222 membrane were wiped away with cotton swabs. Cells below the membrane were fixed
223 with 70% ethanol for 10 minutes and strained with 0.2% crystal violet solution for 5
224 minutes. Five fields on each membrane were randomly selected for counting the visible
225 cells.

226

227 **Wound healing assay**

228 The wound healing assay was followed by a previous study [29]. SAS or SCC-15 cells
229 were seeded on two pores of block insert (SPL Life Sciences Co., Gyeonggi-do, Korea)
230 on each well of 24-well plates and were incubated 24 hr at 37 °C in 5% CO_2 condition.
231 Then inserts were taken out with tweezers and cells were washed with 1X PBS. 10%
232 CM, 20 µM FSLLRY-NH2 or 0.5 µM E64 was added to each well and volume was
233 added up to 1 ml with D/F12 containing 10% FBS. The microscope (OLYMPUS,
234 Tokyo, Japan) was used for taking pictures every 2 hr to measure the gap width changes
235 between cells and analyzed by ImageJ to obtain an average width of the cells. Cell
236 wound healing rate was observed by dividing differences in width by time.

237

238 **Colorectal cancer (CRC) mouse model**

239 Mouse experiment was modified from former research [29, 30]. Seven-week-old
240 specific-pathogen-free (SPF) male C57BL/B6 mice were purchased from Laboratory
241 Animal Center of NCKU. Mice were once intraperitoneal injected of Azoxymethane
242 (AOM) (Sigma-Aldrich) at a dose of 12.5 mg/kg and combined 2% Dextran Sodium
243 Sulfate (DSS) (MP Biomedicals, Irvine, CA, USA) in drinking water for inducing
244 colorectal cancer. Twenty mice were randomly divided into four groups (MOCK,
245 AOM/DSS, AOM/DSS + *F. nucleatum,* and AOM/DSS + *P. intermedia*). Mice were
246 given three cycles of DSS and bacterial treatment and each cycle lasted for 3 weeks. In
247 the first week, mice drank 2% DSS water for 5 days. And the next 2 weeks, mice were
248 oral gavage for PBS or $1x10^9$ CFU of *F. nucleatum* strain ATCC 23726 or *P.*
249 *intermedia* strain ATCC 25611 once a week. Mice were sacrificed on week 10 and
250 colon tissues were taken out. The colons were cut and opened longitudinally along the
251 main axis, and the tumor numbers were counted.
252
253 **Statistical analysis**
254 Statistical analysis was performed by GraphPad Prism 8 software. Each data was
255 represented in triplicate. T-test was used to compare two groups, and multiple groups
256 were analyzed with one-way ANOVA. Asterisk *, **, ***, **** represented statically
257 significant comparison to control group with $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$,
258 respectively.
259
260 **Results**
261 ***P. intermedia* ATCC 25611 provoked human oral cell proliferation**
262 In the previous study from National Cheng Kung University Hospital (NCKUH)
263 [11], the abundance of *P. intermedia* showed a significant increase in oral squamous

264 cell carcinoma (OSCC) patients than control patients. As a result, we determined
265 whether *P. intermedia* plays an important role during oral cancer development. We
266 first examined whether *P. intermedia* could promote oral cell proliferation. We tested
267 different multiplicity of infection (MOI) ratios of *P. intermedia* strain ATCC 25611 to
268 infect human normal oral epithelial cell line, SG cells (Data not shown). After co-
269 cultured for 24 hr, *P. intermedia* appeared with significant cell enhancement at MOI
270 100 than the uninfected group (Fig. 1A). We also co-cultured SG cells with our clinical
271 isolates from NCKUH. Different from strain ATCC 25611, clinical strain H29383
272 reduced number of SG cells after 24 hr (Fig. 1A). Since *P. intermedia* is an obligate
273 anaerobic bacterium and the cell proliferation of SG cells could only last for 24 hours,
274 it was possible that the only short-term effect is because *P. intermedia* can only survive
275 in an aerobic condition for a short time. To clarify the actual effect of *P. intermedia*,
276 we checked whether the conditioned medium (CM) of *P. intermedia* ATCC 25611 may
277 as well promote cell proliferation. As expected, similar to the effect of bacterial co-
278 culture, the CM of strain ATCC 25611 also increased SG cell proliferation after 24 hr
279 (Fig. 1B). To further confirm *P. intermedia*'s cell proliferation induction activity, cell
280 proliferation assay was performed to measure SG cell proliferation (Fig. S1A). Similar
281 to the cell counting method, the CM of the 2 clinical isolates still did not show
282 promotive ability as standard strain ATCC 25611 (Fig. S1B). To recognize which
283 ingredient of CM played an important role in enhancing SG cell proliferation, we used
284 heat-inactivated CM to treat SG cells. The inducible effect vanished with the heat
285 treatment (Fig. 1B), and this result indicated that the active effector may be protein.
286 We used centrifugal filters to separate CM into four different fractions (<3 kDa, 3~10
287 kDa, 10~30 kDa, and >30 kDa). To examine which CM fraction has cell proliferation
288 stimulatory effect, we applied these four CM fractions and whole CM to SG cells. It

289 appeared that the major promoting effect came from fraction >30 kDa, which has the
290 same effect as whole CM. Other fractions showed no difference to the un-infected
291 group (Fig. 1C). onto examine the oncogenic effect on OSCC, we treated human oral
292 cancer cell lines, SCC-15 and SAS, with CM of strain ATCC 25611. The results
293 showed that CM significantly promoted the proliferation of cancer cells after 48 hr and
294 72 hr (Fig. 1D & 1E). These results indicated that *P. intermedia* strain ATCC 25611
295 promoted human oral cell proliferation, and this activity may go through protein(s)
296 secreted from strain ATCC 25611.
297
298 **Interpain A played the key role in *P. intermedia* ATCC 25611 promoting oral cell**
299 **proliferation**
300 Following our results, the strain ATCC 25611 and clinical isolated strains exhibited
301 disparate effects on SG cell proliferation, and this difference might be associated with
302 the secreted protein(s). Next, we analyze the CM by CE/LC MASS to compare the CM
303 component of strain ATCC 25611, which had the best-promoting ability, with control
304 strain H31226, which didn't show any promoting capability. Several proteins exhibited
305 higher expression in strain ATCC 25611 as compared to that in strain H31226
306 (Supplementary Table 2). Among these proteins, a protease interpain A (InpA), was
307 secreted from ATCC 25611 thirteen times higher than that in H31226 (Fig. 2A). InpA
308 is a 90 kDa cysteine protease secreted from *P. intermedia*, it was reported to be
309 associated with heme acquisition for bacterial growth and it also could degrade
310 complement factor C3 to escape from the immune system [31-33]. In addition, another
311 oral bacterium, *P. gingivalis*, was also strongly related to periodontitis, and it would
312 also secrete cysteine proteases called gingipains. Past studies revealed that Gingipains
313 were associated with invasion of oral cells and it was able to activate the protease-

314 activated receptors (PARs) on oral epithelial cells [18]. *P. gingivalis* was reported to
315 promote cell migration by gingipain to activate PAR-2 signaling [34, 35]. Due to InpA
316 being similar to gingipain, they are both secreted cysteine proteases, we hypothesized
317 that InpA triggers PAR-2 signaling to enhance oral epithelial cell proliferation. We
318 used cysteine protease inhibitor, E64, and PAR-2 antagonist, FSLLRY-NH2, to
319 suppress PAR-2 signaling pathway along with CM of strain ATCC 25611. After 24 hr,
320 proliferation stimulatory effect of CM on SG cell was significantly inhibited by E64 or
321 FSLLRY-NH2 treatment (Fig. 3C). E64 or FSLLRY-NH2 treatment also restricted
322 bacterial culture enhancement of SG cells after 24 hr treatment. Proliferation assay
323 showed similarly results, as CM of ATCC 25611 could promote SG cell proliferation
324 and both E64 and FSLLRY-NH2 appeared to be inhibited (Fig. S3). In SCC-15 and
325 SAS cells, the same inhibition was shown after 48 hr (Fig. 2D & 2E). We used
326 *Escherichia coli* strain C41 to express $InpA_{N381}$ protein and purified with Histag
327 column. Purified $InpA_{N381}$ seemed to increase SCC-15 cell proliferation after 48 hr and
328 it was restrained by E64 or FSLLRY-NH2 (Fig. 2F). Treatment with E64 or FSLLRY-
329 NH2 alone did not affect cell growth in these 3 cell lines (Fig. S2A-C). Due to the
330 difference of InpA production from strain ATCC 25611 and H31226, we suspected
331 that the effect of *P. intermedia* may be strain dependent. We thus examined whether
332 *inpA* expression was different between oral cancer strains and control strains. We
333 compared strain ATCC 25611 and 6 clinical strains of *inpA* expression by RT-qPCR.
334 As predicted, strain ATCC 25611 demonstrated higher *inpA* expression than other
335 clinical isolated strains (Fig. 2B). Furthermore, the isolated strains (H26219-3, H29383
336 and H29703) from oral cancer case patients showed higher expression of *inpA* than
337 control patients' strains (H31082, H29823 and H31226). From these results, *P.*

338 *intermedia* secreted InpA to accelerate oral cell proliferation by PAR-2 signaling and
339 the proliferative effect is strain dependent.
340
341 **Migration of OSCC cells was affected by *P. intermedia* Interpain A**
342 During cancer metastasis, cancer cells invade into surrounding matrix and metastasize
343 to other organs, causing more severity and higher mortality [36]. As a result, we wanted
344 to understand whether *P. intermedia* stimulates oral cancer cells migration. Wound
345 healing assay was used to evaluate the cancer cell migration at different time points.
346 CM of strain ATCC 2561 increased cell migration of both SCC-15 and SAS oral cancer
347 cell lines (Fig. 3A & 3B). Next, we investigated whether cell migration was associated
348 with InpA and PAR-2 signaling. Both E64 and FSLLRY-NH2 inhibited the enhanced-
349 migration of OSCC cell lines. Transwell migration assay also revealed that CM
350 promoted SCC-15 and SAS cell migration (Fig. 3C & 3D). Both E64 and FSLLRY-
351 NH2 suppressed CM-stimulated oral cancer cell migration in transwell migration assay,
352 too. In addition, the recombinant $InpA_{N381}$ from *E. coli* elevated SCC-15 cell migration.
353 However, this induction efefct vanished under the treatment of either E64 or FSLLRY-
354 NH2 (Fig. 3E). In summary, *P. intermedia* InpA also promoted OSCC cell migration.
355
356 ***P. intermedia* accelerated tumor number in colorectal cancer mouse model**
357 Based on the concept of the oral-gut axis, the oral bacteria translocate to the intestine
358 through gastrointestinal diseases and cause gastrointestinal diseases, such as
359 inflammatory bowel disease and colorectal cancer [37, 38]. Recent studies revealed
360 that the oral bacterium, *F. nucleatum*, is important during colorectal cancer
361 development [39]. We used AOM and 2% DSS to induce colorectal cancer in C57BL/6
362 mice, and oral gavage feeding *F. nucleatum* or *P. intermedia* to examine whether these

363 two bacteria caused severe colorectal cancer. Compared to AOM/DSS-only group,
364 both *P. intermedia* and *F. nucleatum* would increase colon tumor numbers (Fig. 4B-
365 C). According to our results, *P. intermedia* may be an important oral bacterium in
366 colorectal cancer development.
367

368 **Discussion**

369 In the present study, we demonstrated that *P. intermedia* play an important role
370 during oral cancer development. InpA, a cysteine protease secreted from *P. intermedia,*
371 appeared as an enhancer for oral cell proliferation (Fig. 2C) and cancer cell migration
372 (Fig. 2D & 2E). The signaling pathways were associated with PAR-2. Several bacterial
373 virulence gene expressions were enhanced in OSCC patients, including *inpA* [40].
374 PAR-2 has been mentioned to be involved in cell proliferation, metastasis and invasion
375 in some cancers, for instance, OSCC, esophageal cancer and pancreatic cancer [41-44].
376 Previous research recognized that gingipains, proteases secreted by *P. gingivalis*,
377 stimulated the proMMP9 expression by PAR-2/NF-kB pathway in OSCC invasion [18].
378 Collectively, these results are consistent with *P. intermedia* InpA acting vitally in
379 OSCC development.
380 *P. intermedia*-CM treatment presented cell proliferation promoting ability in normal
381 oral SG cells (Fig. 1B & 1C) as well as oral cancer SCC-15 and SAS cells (Fig. 1D &
382 1E) Interestingly, the time point of cell proliferation stimulatory effect was different in
383 these two kinds of cell lines, 24 hr for normal cells but 48 hr for cancer cells. The cell
384 number of SG cells decreased after 48 hr while stimulation in SCC-15 and SAS cells
385 lasted until 72 hr. We believed that the signaling of cancer cells has changed. Normal
386 cells still have feedback mechanisms to control cell proliferation properly, but cancer
387 cells lose this control during cancerization. *P. gingivalis* secreted gingipains were

388 implied to cause G1 arrest and cell apoptosis [45, 46]. Gingipains could promote oral
389 epithelial cell apoptosis [47]. InpA may have had the same effect on SG cells after 24
390 hr. Combining with our finding that InpA was an important role, it is necessary to
391 further illustrate whether InpA activated disparate downstream signaling pathways of
392 PAR-2.
393 There is a concept of mouth-gut microbiome axis; oral microbiota dysbiosis not only
394 causes local effects but also systemic effects [48]. A previous study demonstrated that
395 CRC was associated with oral microbiota dysbiosis [49]. Recently, a paper about the
396 relationship between oral microbiome and CRC revealed that *P. intermedia* also had
397 inducible cell proliferation and migration ability in CRC cell lines [50]. The promoting
398 effect was also from *P. intermedia* secreted protein. Those results were similar to our
399 findings (Fig. 2C-E). Also, we discovered *P. intermedia* increased the severity of CRC
400 in our *in vivo* experiments (Fig. 4). Numerous investigations indicated that oral
401 pathogens were considered potential risk factors for other systemic diseases [51]. Oral
402 bacteria might secrete inflammatory factors, causing inflammation of the central
403 nervous system. A study identified serum antibody titers of *P. gingivalis*, *F. nucleatum,*
404 and *P. intermedia* were significantly elevated in Alzheimer's disease patients [52]. In
405 the studies of Alzheimer's disease, *P. gingivalis* was seen as a risk factor, and
406 gingipains were toxic proteases that were found in patients' brains [13]. *P. gingivalis*
407 was proved to be related to another common neuron disease, Parkinson's disease, and
408 gingipains also acted as a key factor [53]. Despite neurologic diseases, cardiovascular
409 diseases were also reported to be associated with periodontal bacteria. Occurrence of
410 *P. intermedia*, *P. gingivalis,* and *F. nucleatum* in atheromatous plaques [54, 55].
411 Together, the oral pathogen, *P. intermedia*, had a potential risk of other systemic
412 diseases.

413 We believed that the effect of *P. intermedia* on regulation of oral cell proliferation was
414 strain dependent. Different *P. intermedia* strains have different amount of inpA
415 secretion. As in our data, clinical strain H31226 showed lower InpA production than
416 strain ATCC 25611 (Fig. 2A) and the clinical isolated strains showed different *inpA*
417 expression levels than ATCC 25611 (Fig. 2B). Also, OSCC-isolated strains displayed
418 higher *inpA* expression levels than control strains. Due to this observation, it might
419 provide an application for periodontal diseases or OSCC diagnosis by detecting the
420 amount of InpA from patients' saliva. Furthermore, we aimed to find the potential
421 mechanism of *P. intermedia* InpA-dependent OSCC cell proliferation. We performed
422 Micro-Western Array, a high-throughput western blot-based platform, on SG cells
423 focusing on signaling pathways associated with cell proliferation (Fig. S4). We
424 observed that the epidermal growth factor receptor (EGFR) signaling pathway was
425 elevated, the RAF expression increased in 8 hr but decreased at 24 hr. EGFR expression
426 and its downstream signaling RAS–RAF–MEK–ERK pathway was reported to evelate
427 in OSCC patients, [56, 57], which is consistent with our Micro-Western Array results.
428 However, the underlying mechanism needs further confirmation. In this study, we
429 discovered that InpA secreted by *P. intermedia* played a crucial role in oral cell
430 proliferation and migration during OSCC development. This might be PAR-2 signaling
431 pathway-dependent. However, the fundamental mechanism needs further investigation.
432

433 **Figure legends**

434 **Figure 1. *P. intermedia* strain ATCC 25611 promoted oral cell proliferation.** (A)
435 Human normal oral epithelial cells, SG, were infected with live *P. intermedia* strain
436 ATCC 25611 or clinical isolated strain H29383 and H31226 at the multiplicity of
437 infection (MOI) of 100 for 24 hr and 48 hr. (B) Comparison of conditioned medium

438 (CM) of strain ATCC 25611 and heated CM which were treated to SG cells for 24 hr
439 and 48 hr. (C) Strain ATCC 25611 CM was separated with centrifugal filters into
440 different fractions. Fraction above 30 kDa, 10~30kDa, 3~10 kDa and below 3 kDa. (D-
441 E) CM of strain ATCC 25611 was treated to oral squamous cancer cell lines, SCC-15
442 (D) and SAS (E) for 24 hr, 48 hr and 72 hr. All results are representative of triplicate
443 experiments. Data are presented as mean ±SD. Data were analyzed by one-way
444 ANOVA (A-C) or t-test (D-E). ns: not significant; *p<0.05; **p<0.01; ***p<0.001
445 and ****p<0.0001.
446
447 **Figure 2. Figure 2. Interpain A (InpA) played the key role in *P. intermedia***
448 **promoting oral cell proliferation.** (A) Identification of InpA in strain ATCC 25611
449 and clinical control strain H31226, which did not promote SG cell proliferation, by
450 CE/LC MASS. (B) Bacterial RNA was extraction from broth cultures of *P. intermedia*
451 strains reached OD_{600}=0.8 and performed Real-Time qPCR for *inpA* gene expression.
452 RNA expression ratios of *inpA* between standard strain ATCC 25611 and 6 clinical
453 strains. Three strains, H26219-3, H29383and H29703, from oral cancer patients (CA)
454 and other three strains, H31082, H29823 and H31226, from healthy control patients
455 (CO). Data was performed by $2^{-\Delta\Delta CT}$ method. (C) SG cell proliferation results of *P.*
456 *intermedia* strain ATCC 25611 CM or at MOI 100 treatment with or without 5 μM
457 protease inhibitor (E64) or 40 μM PAR-2 receptor antagonist (FSLLRY-NH2) (D-E)
458 Cell proliferation results of SCC-15 cells or SAS cells. SCC-15 cells (D) or SAS cells
459 (E) treated with CM of strain ATCC 25611 with or without 0.5 μM inhibitor E64 or 20
460 μM antagonist FSLLRY-NH2. (F)Recombinant interpain A (rInpA) was constructed
461 to pET-21b (+), expressed with *E. coli* strain C41 and purified by HisTrap column.
462 Five micrograms of rInpA were treated to SCC-15 cells with or without 0.5 μM

463 inhibitor E64 or 20 μM antagonist FSLLRY-NH2. PBS as the vehicle control and CM
464 of strain ATCC 25611 as the positive control. All results are representative of triplicate
465 experiments. Data are presented as mean ±SD. Data were analyzed by one-way
466 ANOVA. ns: not significant; *p<0.05 and ****p<0.0001.
467
468 **Figure 3. Conditioned medium (CM) of *P. intermedia* also promoted oral cancer**
469 **cell migration in SCC-15 and SAS cells**. (A, C) In the wound healing assay, SCC-15
470 (A) and SAS (C) cells were seeded inside the inserts in a 24-well plate. After 24 hr, the
471 inserts were removed and added D/F12 cell medium or CM of *P. intermedia* strain
472 ATCC 25611 with or without 0.5 μM protease inhibitor (E64) or 20 μM PAR-2
473 receptor antagonist (FSLLRY-NH2). Cell migration was observed at 2 hr and 4 hr with
474 a microscope. Data were calculated by the migration rate (μM/hr). (B, D) In transwell
475 assay, human tongue carcinoma cells, SCC-15 (B) or SAS (D), were seeded on 8 μM
476 transwell inserts in 24-well plates. D/F12 cell medium or CM of *P. intermedia* strain
477 ATCC 25611 with or without 0.5 μM E64 or 20 μM FSLLRY-NH2 were added to the
478 basal chamber. After 24 hr, cells were stained by crystal violet and counted under a
479 phase-contrast microscope. (E) Effect of recombinant InpA (rInpA) on SCC-15 cell
480 migration in the transwell assay. 5 μg/ml rInpA were added to the basal chamber with
481 or without 0.5 μM E64 or 20 μM FSLLRY-NH2. All results are representative of
482 triplicate experiments. Data are presented as mean ±SD. Data were analyzed by one-
483 way ANOVA. ns: not significant; **p<0.01, ***p<0.001 and ****p<0.0001.
484
485 **Figure 4. *P. intermedia* strain ATCC 25611 enhanced tumor formation of**
486 **colorectal cancer (CRC) in mice.** (A)Schematic diagram of CRC mice model.
487 (Created with BioRender.com). Azoxynethane (AOM) was intraperitoneal injection at

488 the dose of 12.5 mg/kg first One week later, mice were given three cycles of Dextran
489 Sodium Sulfate (DSS) treatment. Each cycle was 2% DSS in drinking water for 5 days
490 and 16 days of regular drinking water, and $1x10^9$ CFU of *P. intermedia* ATCC 25611
491 or *F. nucleatum* ATCC 23726 was infected by oral gavage at week 2, 3, 5, 6, 8, 9. Then,
492 mice were scarified at week 10. (B)Morphological images of distal colon with or
493 without *F. nucleatum* or *P. intermedia* infection in AOM/DSS mice model. (C)
494 Number of tumors in distal colon of every group. Each group was quintuplicate. ns:
495 not significant; *$p<0.05$.

496 **Notes**

497 ***Acknowledgements.*** We thank the Laboratory Animal Center, College of Medicine at
498 National Cheng Kung University (NCKU), Taiwan, accredited by AAALAC
499 International and Taiwan Animal Consortium, for their support in taking care of mice.
500 We appreciate the participation of all patients in this study. We are grateful to all
501 the unmentioned clinicians and recruiters. We also thank Dr. Chuan-Fa Chang and Dr.
502 Jhen-Wei Ruan provided the cell lines.

503 ***Authors contributions.*** S.-M. C., Y.-J. C., W.-R. W. and N.-H. W. performed the
504 experiments. S.-M. C. wrote this manuscript. I-H. H. assisted with the experimental
505 design and reviewed this manuscript. C.-P. C. performed the microwestern array
506 experiments and reviewed this manuscript. J.-R. H., J. S C., and J.-Y. C. provided
507 clinical patients' saliva. J.-W. C. contributed to design, provide the concept, and
508 supervise this manuscript. All authors have read and approved the final version of this
509 manuscript.

510 ***Financial support.*** This work was supported by the National Science and Technology
511 Council (Grants: 113-2314-B-006-043-MY3; awarded to J. W. C.). The funding
512 agency played no role in study design, data collection or analysis, manuscript

513 preparation, or decision to publish.

514 ***Potential conflicts of interest.*** All authors: No reported conflict. All authors have

515 submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

516

517 **References:**

518 1. Jhuang, J.R., et al., *Forecast of peak attainment and imminent decline after*
519 *2017 of oral cancer incidence in men in Taiwan.* Sci Rep, 2022. **12**(1): p. 5726.
520 2. Tuominen, H. and J. Rautava, *Oral Microbiota and Cancer Development.*
521 Pathobiology, 2021. **88**(2): p. 116-126.
522 3. Zhang, L., et al., *The Oral Microbiota May Have Influence on Oral Cancer.*
523 Front Cell Infect Microbiol, 2019. **9**: p. 476.
524 4. Krüger, M., et al., *The Correlation between Chronic Periodontitis and Oral*
525 *Cancer.* Case Rep Dent, 2013. **2013**: p. 262410.
526 5. Karmakar, S., et al., *Periodontitis and oral Cancer-A striking link.* Oral
527 Oncology, 2020. **106**: p. 104630.
528 6. Elebyary, O., et al., *The Crossroads of Periodontitis and Oral Squamous Cell*
529 *Carcinoma: Immune Implications and Tumor Promoting Capacities.* Frontiers
530 in Oral Health, 2021. **1**(8).
531 7. Tsai, C.-Y., et al., *Subgingival microbiota in individuals with severe chronic*
532 *periodontitis.* Journal of Microbiology, Immunology and Infection, 2018.
533 **51**(2): p. 226-234.
534 8. Ma, J., et al., *Clinical utility of subgingival plaque-specific bacteria in salivary*
535 *microbiota for detecting periodontitis.* PLoS One, 2021. **16**(6): p. e0253502.
536 9. Kirst, M.E., et al., *Dysbiosis and alterations in predicted functions of the*
537 *subgingival microbiome in chronic periodontitis.* Applied and environmental
538 microbiology, 2015. **81**(2): p. 783-793.
539 10. Benachinmardi, K.K., et al., *Microbial flora in chronic periodontitis: study at a*
540 *tertiary health care center from north karnataka.* J Lab Physicians, 2015. **7**(1):
541 p. 49-54.
542 11. Hsiao, J.R., et al., *The interplay between oral microbiome, lifestyle factors and*
543 *genetic polymorphisms in the risk of oral squamous cell carcinoma.*
544 Carcinogenesis, 2018. **39**(6): p. 778-787.
545 12. Chattopadhyay, I., M. Verma, and M. Panda, *Role of Oral Microbiome*
546 *Signatures in Diagnosis and Prognosis of Oral Cancer.* Technol Cancer Res
547 Treat, 2019. **18**: p. 1533033819867354.
548 13. Dominy, S.S., et al., *Porphyromonas gingivalis in Alzheimer's disease brains:*
549 *Evidence for disease causation and treatment with small-molecule inhibitors.*
550 Sci Adv, 2019. **5**(1): p. eaau3333.
551 14. Wen, L., et al., *Porphyromonas gingivalis Promotes Oral Squamous Cell*
552 *Carcinoma Progression in an Immune Microenvironment.* Journal of Dental
553 Research, 2020. **99**(6): p. 666-675.
554 15. Liu, Y., et al., *Clinical significance and prognostic value of Porphyromonas*
555 *gingivalis infection in lung cancer.* Transl Oncol, 2021. **14**(1): p. 100972.

16. Groeger, S., et al., *PD-L1 Up-Regulation in Prostate Cancer Cells by Porphyromonas gingivalis.* Front Cell Infect Microbiol, 2022. **12**: p. 935806.
17. Tan, Q., et al., *Periodontitis pathogen Porphyromonas gingivalis promotes pancreatic tumorigenesis via neutrophil elastase from tumor-associated neutrophils.* Gut Microbes, 2022. **14**(1): p. 2073785.
18. Inaba, H., et al., *Porphyromonas gingivalis promotes invasion of oral squamous cell carcinoma through induction of proMMP9 and its activation.* Cell Microbiol, 2014. **16**(1): p. 131-45.
19. Hong, J., et al., *F. nucleatum targets lncRNA ENO1-IT1 to promote glycolysis and oncogenesis in colorectal cancer.* Gut, 2020.
20. Geng, F., et al., *Fusobacterium nucleatum Caused DNA Damage and Promoted Cell Proliferation by the Ku70/p53 Pathway in Oral Cancer Cells.* DNA Cell Biol, 2020. **39**(1): p. 144-151.
21. Udayasuryan, B., et al., *Fusobacterium nucleatum induces proliferation and migration in pancreatic cancer cells through host autocrine and paracrine signaling.* Sci Signal, 2022. **15**(756): p. eabn4948.
22. Parhi, L., et al., *Breast cancer colonization by Fusobacterium nucleatum accelerates tumor growth and metastatic progression.* Nature Communications, 2020. **11**(1): p. 3259.
23. Babich, H. and D.A. Tipton, *In vitro response of human gingival epithelioid S-G cells to minocycline.* Toxicol In Vitro, 2002. **16**(1): p. 11-21.
24. 高橋, 喜., et al., *ヒト舌癌原発巣より得られた低分化型扁平上皮癌細胞株(SAS)の樹立.* 日本口腔科学会雑誌, 1989. **38**(1): p. 20-28.
25. Rheinwald, J.G. and M.A. Beckett, *Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas.* Cancer research, 1981. **41 5**: p. 1657-63.
26. Yang, Y., et al., *Fusobacterium nucleatum Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor-κB, and Up-regulating Expression of MicroRNA-21.* Gastroenterology, 2017. **152**(4): p. 851-866.e24.
27. Chen, K.Y., et al., *The Transcriptional Regulator Lrp Contributes to Toxin Expression, Sporulation, and Swimming Motility in Clostridium difficile.* Front Cell Infect Microbiol, 2019. **9**: p. 356.
28. Wang, S.T., et al., *OmpR coordinates the expression of virulence factors of Enterohemorrhagic Escherichia coli in the alimentary tract of Caenorhabditis elegans.* Mol Microbiol, 2021. **116**(1): p. 168-183.
29. Pan, Q., et al., *Genomic variants in mouse model induced by azoxymethane and dextran sodium sulfate improperly mimic human colorectal cancer.* Sci Rep, 2017. **7**(1): p. 25.
30. Yu, M.R., H.J. Kim, and H.R. Park, *Fusobacterium nucleatum Accelerates the Progression of Colitis-Associated Colorectal Cancer by Promoting EMT.* Cancers (Basel), 2020. **12**(10).
31. Mallorquí-Fernández, N., et al., *A new autocatalytic activation mechanism for cysteine proteases revealed by Prevotella intermedia interpain A.* J Biol Chem, 2008. **283**(5): p. 2871-82.
32. Potempa, M., et al., *Interpain A, a cysteine proteinase from Prevotella intermedia, inhibits complement by degrading complement factor C3.* PLoS

Pathog, 2009. **5**(2): p. e1000316.
33. Byrne, D.P., et al., *Role of the cysteine protease interpain A of Prevotella intermedia in breakdown and release of haem from haemoglobin.* Biochem J, 2009. **425**(1): p. 257-64.
34. Liu, Y., et al., *Infection of microglia with Porphyromonas gingivalis promotes cell migration and an inflammatory response through the gingipain-mediated activation of protease-activated receptor-2 in mice.* Sci Rep, 2017. **7**(1): p. 11759.
35. Nonaka, S. and H. Nakanishi, *Secreted gingipains from Porphyromonas gingivalis induce microglia migration through endosomal signaling by protease-activated receptor 2.* Neurochem Int, 2020. **140**: p. 104840.
36. Chaffer, C.L. and R.A. Weinberg, *A perspective on cancer cell metastasis.* Science, 2011. **331**(6024): p. 1559-64.
37. Read, E., M.A. Curtis, and J.F. Neves, *The role of oral bacteria in inflammatory bowel disease.* Nature Reviews Gastroenterology & Hepatology, 2021. **18**(10): p. 731-742.
38. Kudra, A., et al., *Insights into oral microbiome and colorectal cancer - on the way of searching new perspectives.* Front Cell Infect Microbiol, 2023. **13**: p. 1159822.
39. Wu, N., et al., *Fusobacterium nucleatum promotes colon cancer progression by changing the mucosal microbiota and colon transcriptome in a mouse model.* World J Gastroenterol, 2022. **28**(18): p. 1981-1995.
40. Moghimi, M., et al., *Interaction of human oral cancer and the expression of virulence genes of dental pathogenic bacteria.* Microb Pathog, 2020. **149**: p. 104464.
41. Tang, K.L., et al., *PAR-2 promotes cell proliferation, migration, and invasion through activating PI3K/AKT signaling pathway in oral squamous cell carcinoma.* Biosci Rep, 2019. **39**(7).
42. Shimamoto, R., et al., *A role for protease-activated receptor-2 in pancreatic cancer cell proliferation.* Int J Oncol, 2004. **24**(6): p. 1401-6.
43. Chen, J., et al., *Effects of silenced PAR-2 on cell proliferation, invasion and metastasis of esophageal cancer.* Oncol Lett, 2017. **14**(4): p. 4115-4121.
44. Sheng, J., et al., *PAR-2 promotes invasion and migration of esophageal cancer cells by activating MEK/ERK and PI3K/Akt signaling pathway.* Int J Clin Exp Pathol, 2019. **12**(3): p. 787-797.
45. Kato, T., et al., *Porphyromonas gingivalis gingipains cause G(1) arrest in osteoblastic/stromal cells.* Oral Microbiol Immunol, 2008. **23**(2): p. 158-64.
46. Inaba, H., et al., *Identification of signaling pathways mediating cell cycle arrest and apoptosis induced by Porphyromonas gingivalis in human trophoblasts.* Infect Immun, 2012. **80**(8): p. 2847-57.
47. Stathopoulou, P.G., et al., *Porphyromonas gingivalis induce apoptosis in human gingival epithelial cells through a gingipain-dependent mechanism.* BMC Microbiol, 2009. **9**: p. 107.
48. Park, S.Y., et al., *Oral-Gut Microbiome Axis in Gastrointestinal Disease and Cancer.* Cancers (Basel), 2021. **13**(9).
49. Zhang, S., et al., *Human oral microbiome dysbiosis as a novel non-invasive biomarker in detection of colorectal cancer.* Theranostics, 2020. **10**(25): p.

11595-11606.
50. Lo, C.H., et al., *Enrichment of Prevotella intermedia in human colorectal cancer and its additive effects with Fusobacterium nucleatum on the malignant transformation of colorectal adenomas.* J Biomed Sci, 2022. **29**(1): p. 88.
51. Bui, F.Q., et al., *Association between periodontal pathogens and systemic disease.* Biomed J, 2019. **42**(1): p. 27-35.
52. Sparks Stein, P., et al., *Serum antibodies to periodontal pathogens are a risk factor for Alzheimer's disease.* Alzheimers Dement, 2012. **8**(3): p. 196-203.
53. Adams, B., et al., *Parkinson's Disease: A Systemic Inflammatory Disease Accompanied by Bacterial Inflammagens.* Front Aging Neurosci, 2019. **11**: p. 210.
54. Romano, F., A. Barbui, and M. Aimetti, *Periodontal pathogens in periodontal pockets and in carotid atheromatous plaques.* Minerva Stomatol, 2007. **56**(4): p. 169-79.
55. Figuero, E., et al., *Detection of periodontal bacteria in atheromatous plaque by nested polymerase chain reaction.* J Periodontol, 2011. **82**(10): p. 1469-77.
56. Xu, M.J., D.E. Johnson, and J.R. Grandis, *EGFR-targeted therapies in the post-genomic era.* Cancer and Metastasis Reviews, 2017. **36**(3): p. 463-473.
57. Cheng, Y., et al., *MAPK Signaling Pathway in Oral Squamous Cell Carcinoma: Biological Function and Targeted Therapy.* Cancers (Basel), 2022. **14**(19).

Fig. 1

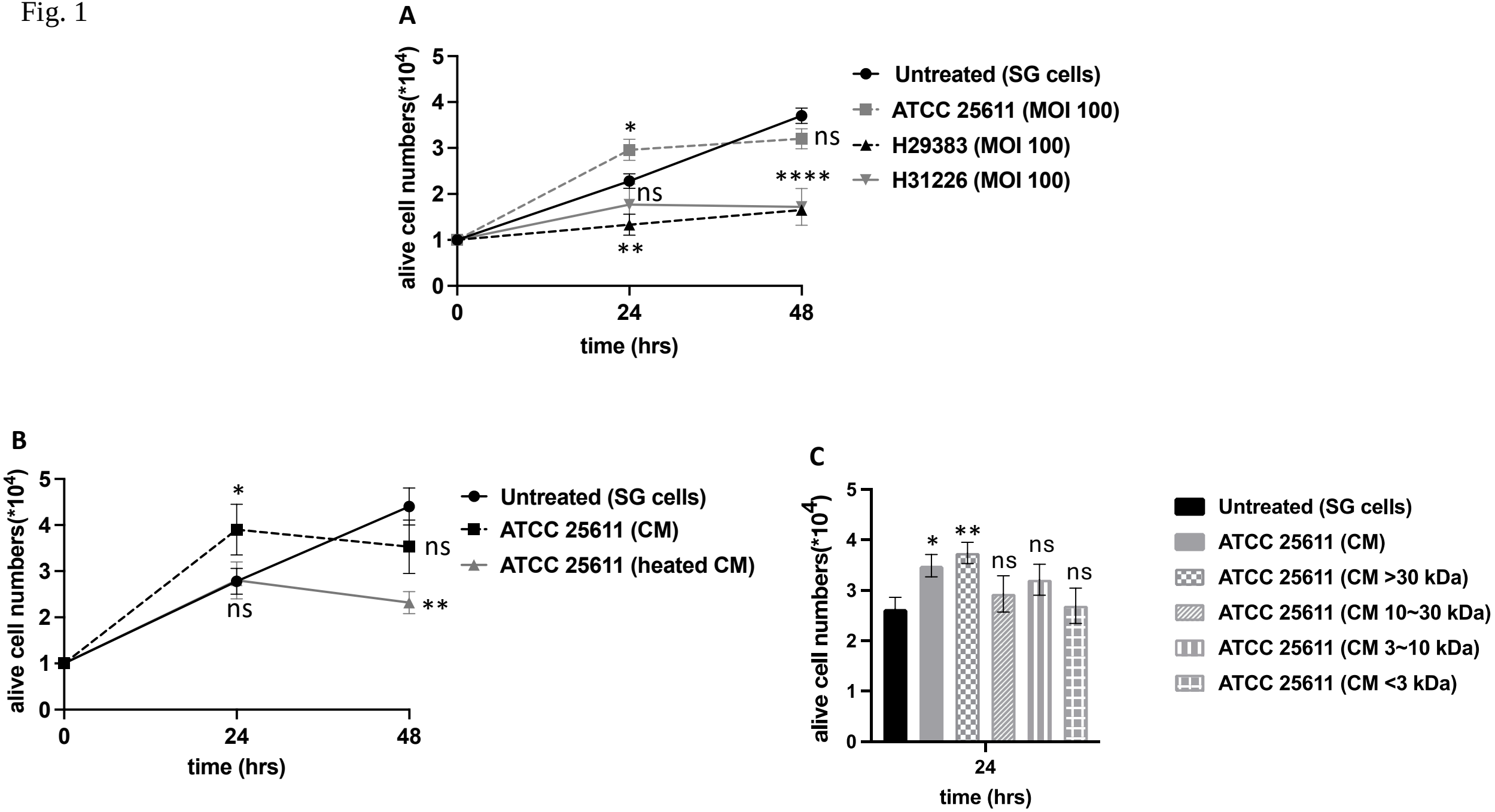
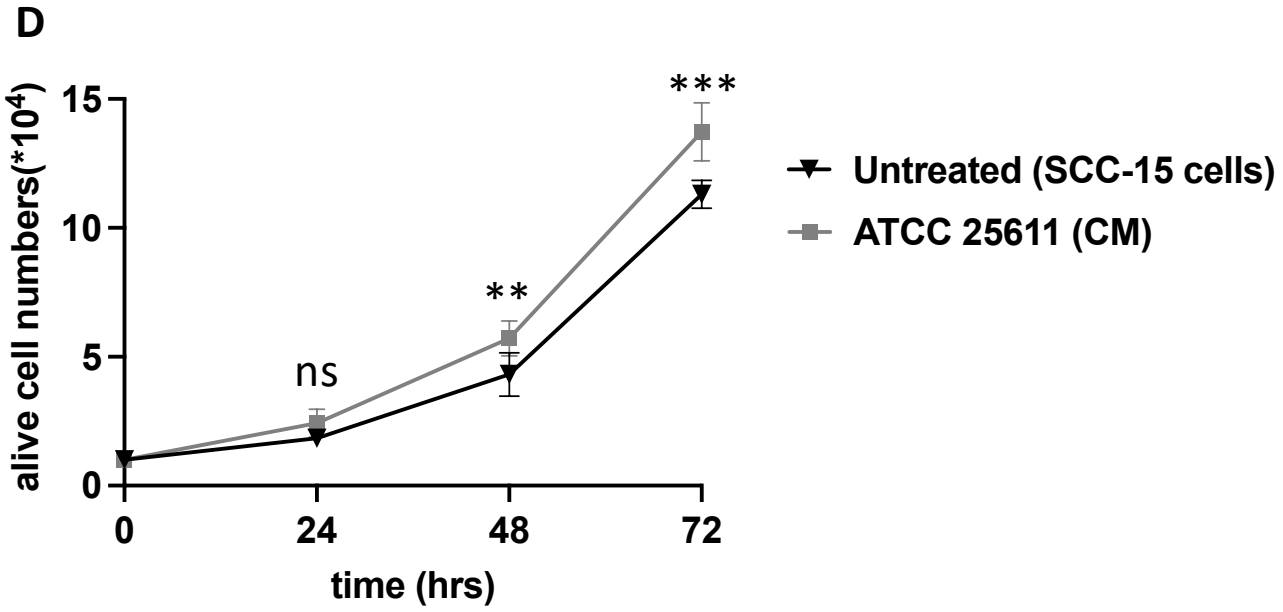

A
alive cell numbers(*10^4)
time (hrs)
Untreated (SG cells)
ATCC 25611 (MOI 100)
H29383 (MOI 100)
H31226 (MOI 100)
ns
****
**
B
alive cell numbers(*10^4)
time (hrs)
Untreated (SG cells)
ATCC 25611 (CM)
ATCC 25611 (heated CM)
C
alive cell numbers(*10^4)
time (hrs)
Untreated (SG cells)
ATCC 25611 (CM)
ATCC 25611 (CM >30 kDa)
ATCC 25611 (CM 10~30 kDa)
ATCC 25611 (CM 3~10 kDa)
ATCC 25611 (CM <3 kDa)

Fig. 1


D
alive cell numbers(*10^4)
15
10
5
0
0
24
48
72
time (hrs)
ns
**
***
Untreated (SCC-15 cells)
ATCC 25611 (CM)

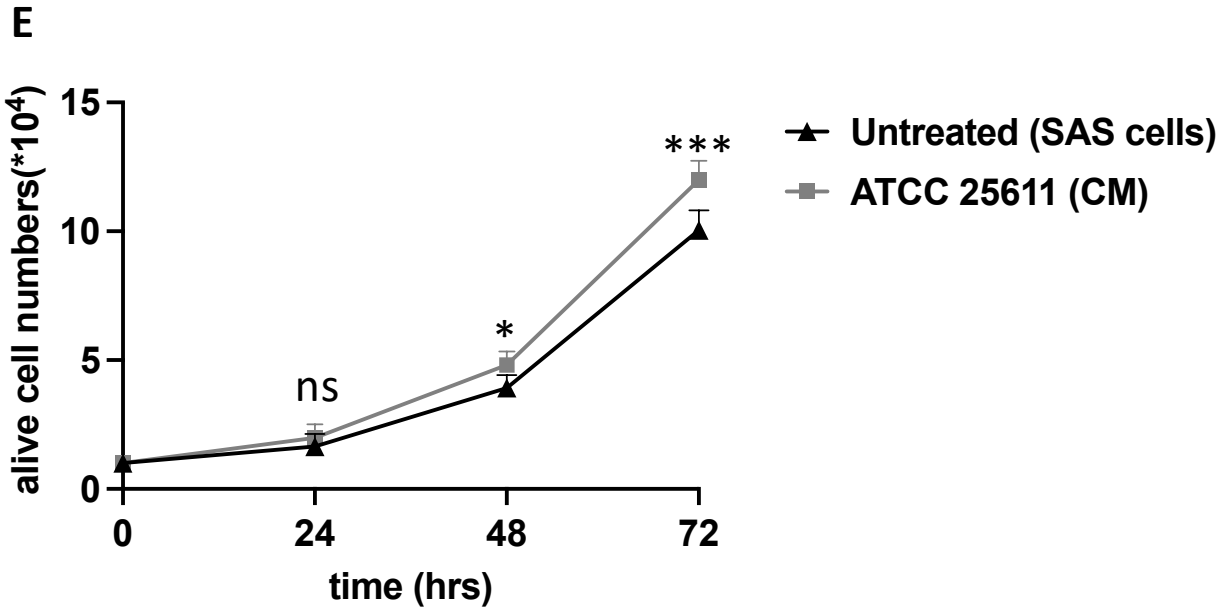

E
alive cell numbers(*10^4)
15
10
5
0
0
24
48
72
time (hrs)
ns
*
***
Untreated (SAS cells)
ATCC 25611 (CM)

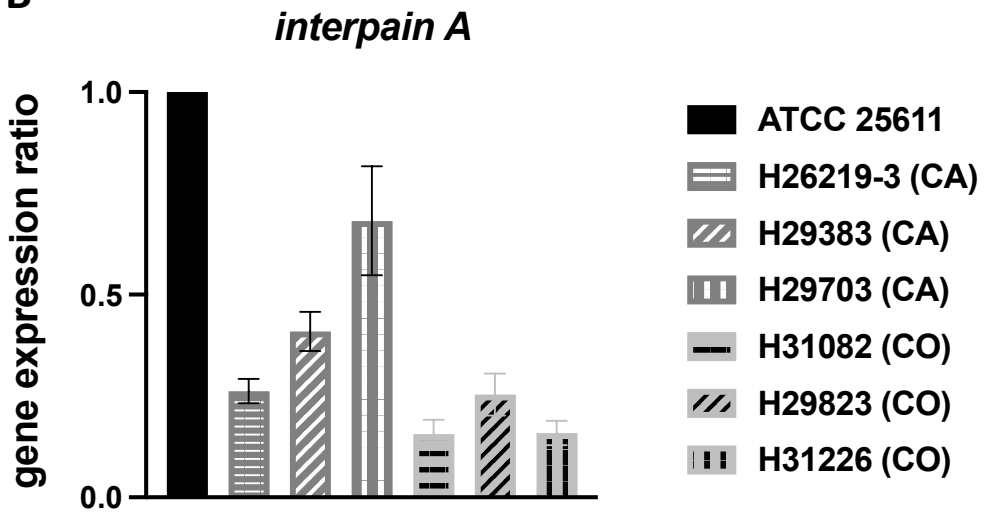
Fig. 2

A

Strain	Protein	Prot_acc	Prot_hit_num
ATCC 25611	Interpain A	A0A0D9NCF8 A9J7N5	273 273
H31226		A0A0D9NCF8 A9J7N5 A0A0S3UMK9	21 21 21

13-fold

B



C

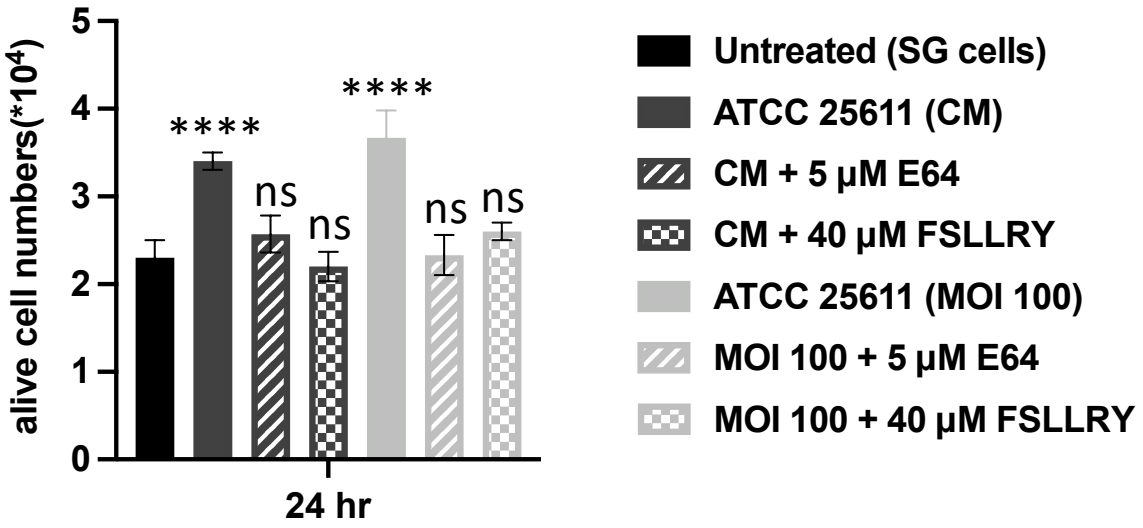
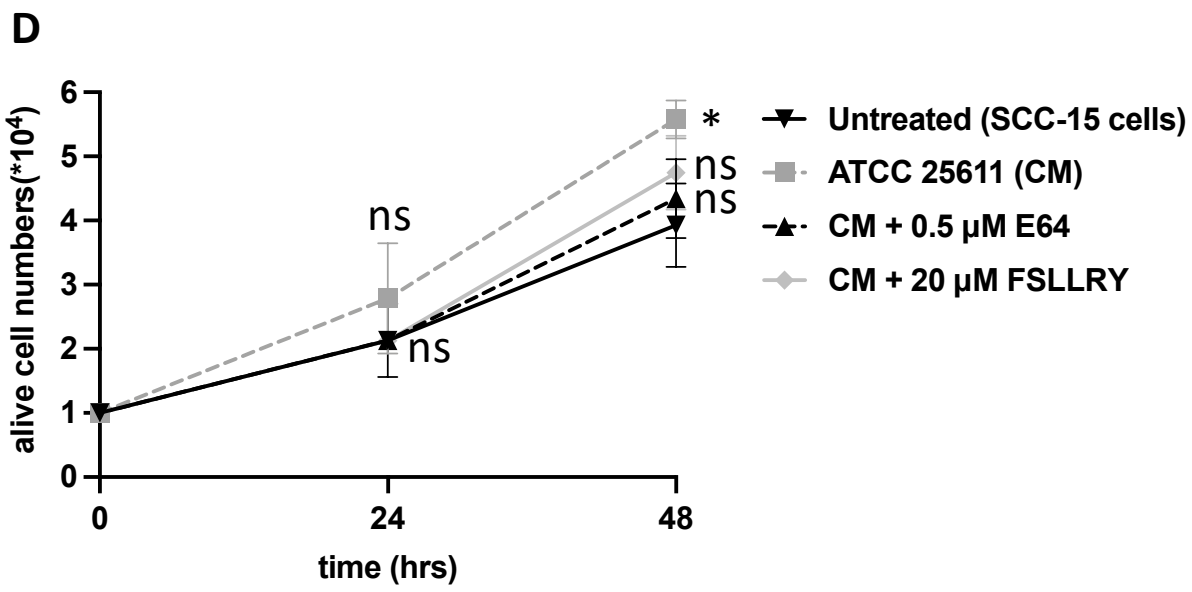
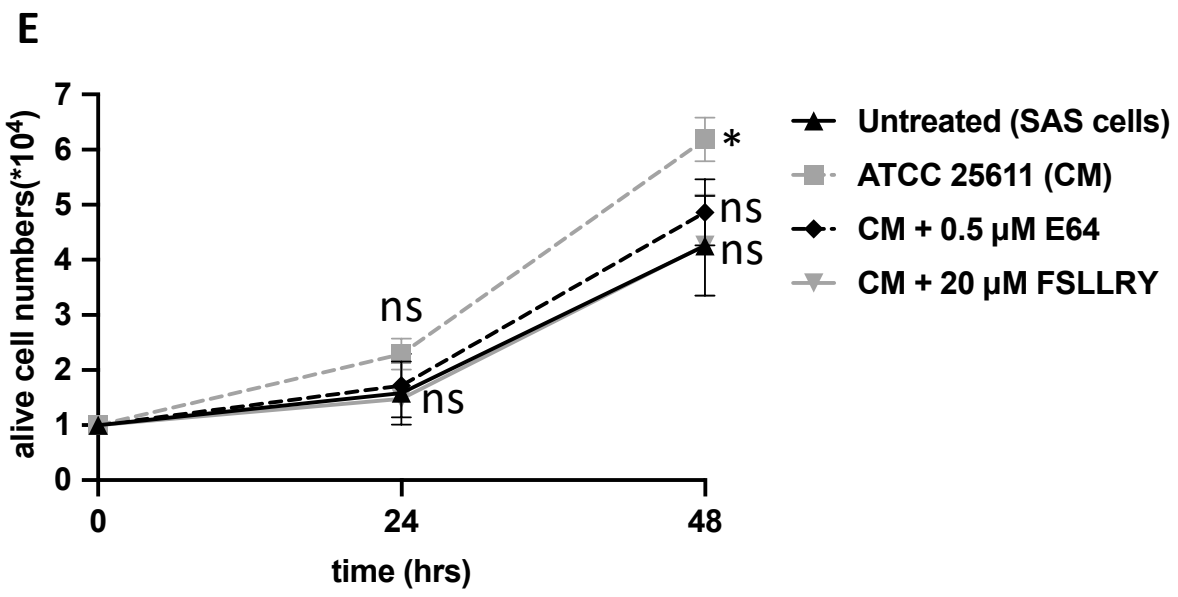


Fig. 2


D
alive cell numbers(*10^4)
time (hrs)
0
24
48
ns
ns
*
ns
ns
Untreated (SCC-15 cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY


E
alive cell numbers(*10^4)
time (hrs)
0
24
48
ns
ns
*
ns
ns
Untreated (SAS cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY

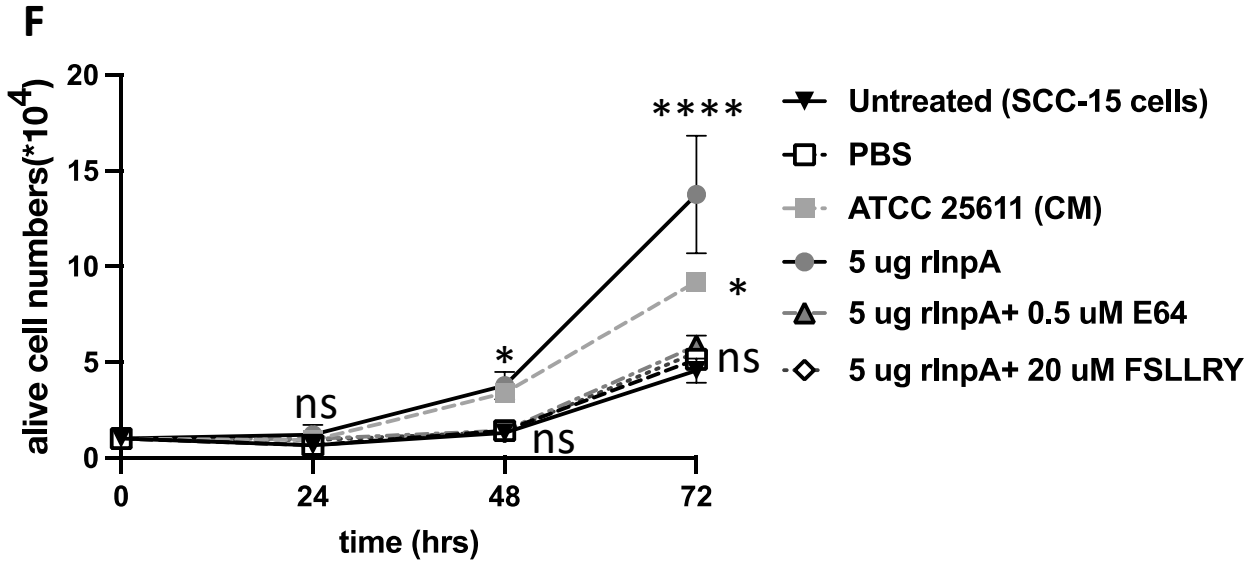

F
alive cell numbers(*10^4)
time (hrs)
0
24
48
72
ns
*
ns
****
*
ns
Untreated (SCC-15 cells)
PBS
ATCC 25611 (CM)
5 ug rInpA
5 ug rInpA+ 0.5 uM E64
5 ug rInpA+ 20 uM FSLLRY

Fig. 3

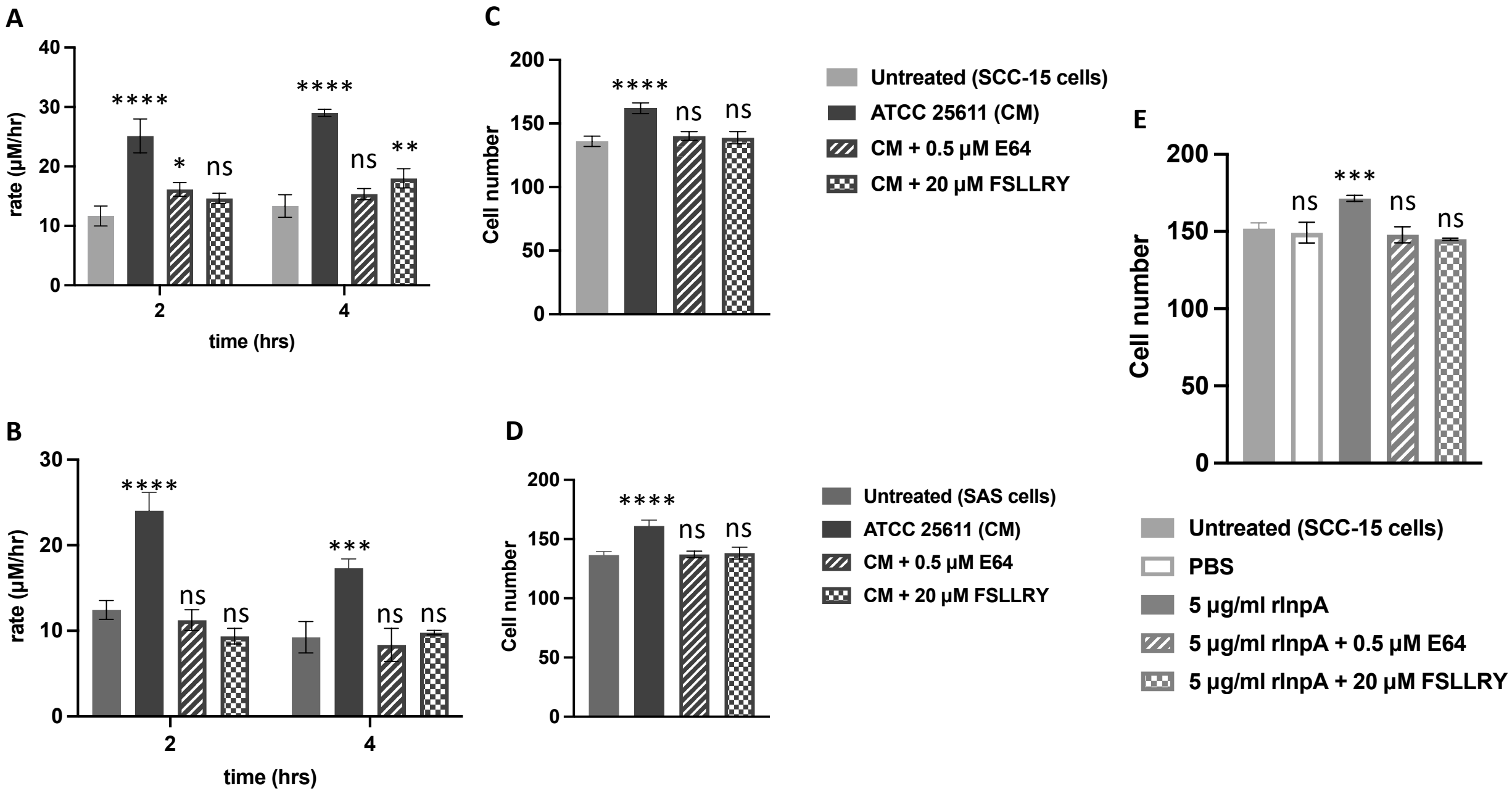
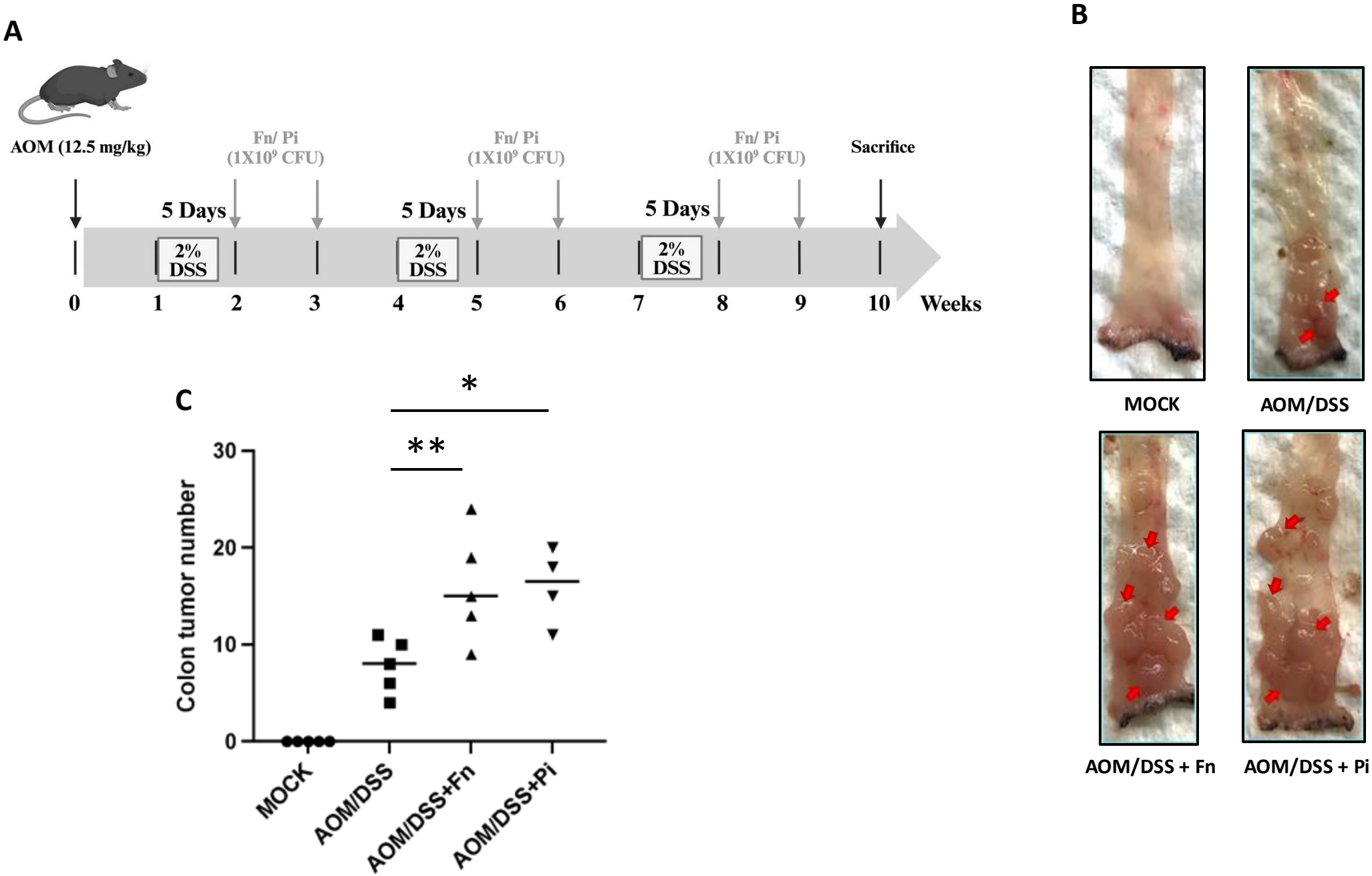

A
rate (μM/hr)
time (hrs)
****
*
ns
****
ns
**
B
rate (μM/hr)
time (hrs)
****
ns
ns
***
ns
ns
C
Cell number
****
ns
ns
D
Cell number
****
ns
ns
Untreated (SCC-15 cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY
Untreated (SAS cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY
E
Cell number
ns
***
ns
ns
Untreated (SCC-15 cells)
PBS
5 μg/ml rInpA
5 μg/ml rInpA + 0.5 μM E64
5 μg/ml rInpA + 20 μM FSLLRY

Fig. 4


A
AOM (12.5 mg/kg)
Fn/ Pi
(1X10⁹ CFU)
Fn/ Pi
(1X10⁹ CFU)
Fn/ Pi
(1X10⁹ CFU)
Sacrifice
5 Days
5 Days
5 Days
2% DSS
2% DSS
2% DSS
0 1 2 3 4 5 6 7 8 9 10 Weeks
B
MOCK
AOM/DSS
AOM/DSS + Fn
AOM/DSS + Pi
C
*
**
Colon tumor number
30
20
10
0
MOCK
AOM/DSS
AOM/DSS+Fn
AOM/DSS+Pi