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

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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.

64 ## Introduction

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

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

89 however, little is known about *Prevotella intermedia*. *P. intermedia* is a Gram-negative,
90 obligate anaerobic rod-shaped bacterium. It needs vitamin K and hemin for growth and
91 is related to periodontal infections and acute necrotizing ulcerative gingivitis. Previous
92 studies revealed that oral cancer patients had a higher abundance of *P. intermedia* as
93 compared to healthy population. Although the findings showed that the abundance of
94 *P. intermedia* was correlated with the incidence of oral cancer, the role of *P. intermedia*
95 in oral cancer was unclear. We therefore hypothesize that *P. intermedia* is an important
96 bacterium in oral cancer development.

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

102

103 ## Materials and methods

104 ### Bacterial strains and cultures

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

114 s, and a final extension at 72 °C for 7 min. Supplemented Tryptic Soy Broth (ATCC
115 2722), which tryptic soy broth (#211825, BD) supplemented with 0.5% yeast extract
116 (#212750, BD), 0.05% L-cysteine (#A3694, Applichem Panreac), 0.1% hemin
117 (#H9039, Sigma) and 0.02% menadione (#M5625, Sigma) was used for *P. intermedia*
118 culturing at 37 ℃ anaerobically.
119
120 **Cell lines and cultures**
121 Immortalized human gingival keratinocyte, Smulow–Glickman (SG) cell (23), was
122 gifted from Dr. Chuan-Fa Chang (NCKU) and was cultured with Dulbecco's Modified
123 Eagle Medium (DMEM) (#11965-092, Gibco). Human tongue carcinoma cells, SAS
124 cells (24) and SCC-15 cells (25) were gifted from Dr. Jhen-Wei Ruan (NCKU) and
125 were cultured with DMEM/F12 (D/F12) medium (#11330-032, Gibco/Life
126 Technology, Carisbad, CA, USA) Both cell culture medium were supplemented with
127 10% fetal bovine serum (FBS) (#10437-028, Gibco) and 1% 100x Penicillin-
128 Streptomycin-Amphotericin B solution (#CC501-0100, Simply Biotechnology, San
129 Diego, CA, USA). Cells were all incubated at 37 °C under 5% CO_2 condition.
130
131 **Conditioned medium (CM) preparation**
132 *P. intermedia* strains were anaerobically cultured in ATCC 2722 broth at 37 °C and
133 were harvested when the OD600 value reached 1.0. The bacterial culture was
134 centrifuged at 3220 xg for 3 min and discarded supernatant. The pellet was resuspended
135 with a double volume of cell culture medium and anaerobically incubated at 37 °C for
136 8 hr. The supernatant was collected after being centrifuged at 3220 xg for 10 min,
137 filtered with 0.22 μm filter (Merck Millipore, Burlington, MA, USA), and stored at 4
138 °C before use.

139

140 **Cell proliferation assay**

141 The cell proliferation assay was followed by previous research (26). SG, SAS, or SCC-
142 15 cells were dissociated by 0.05% trypsin-EDTA solution (#25300-054, Gibco) and
143 resuspended in DMEM or D/F12 medium. $1x10^4$ cells were seeded per well of 24-well
144 tissue culture plates (Corning, NY, USA) with bacteria or CM treatment. CM was
145 separated into three fractions by three centrifugal filter devices (3 kDa, 10kDa and 30
146 kDa) (Merck Millipore). 10% CM, 20 μM PAR-2 antagonist FSLLRY-NH2 (#4751,
147 TOCRIS/Bio-Techne, Minneapolis, MN, USA) or 0.5 μM cysteine protease inhibitor
148 E64 (#324890, Merck Millipore) and DMEM or D/F12 containing 10% FBS were
149 added to final volume of 1 ml. After 24 hr, 48 hr, or 72 hr of incubation at 37°C, cells
150 were collected by 0.05% trypsin-EDTA solution. The cell suspension was mixed with
151 the same volume of 0.05% trypan blue (#15250-061, Gibco), and the cell number was
152 counted by the Luna cell counter (Logos Biosystems, Gyeonggi-do, South Korea).

153

154 **Capillary electrophoresis/Liquid Chromatography-Mass spectrometry (CE/LC**
155 **Mass) analysis**

156 The mixture of 100 μg proteins in 60 μl rehydration buffer (7 M Urea, 2 M Thiourea
157 and 2% CHAPS), 9.3 μl 7.5% Sodium dodecyl sulfate (SDS) (#75746, Sigma,
158 Burlington, MA, USA) and 0.7 μl 1M Dithiothreitol (#UR-DTT, UniRegion Bio-Tech,
159 Taichung, Taiwan) solution supplemented with 7 M Urea and 0.01 M sodium acetate
160 was heated at 95 °C for 5 minutes and then put on ice for 3 minutes. The mixture was
161 added with 4 μl 500 mM IAM solution (500 mM iodoacetamide dissolved in 50 mM
162 ammonium bicarbonate solution) and incubated at room temperature. After 30 min, 52
163 μl 50% Trichloroacetic acid (TCA) (#100807, Merck Millipore) was added and placed

164 on ice for 15 min. The pellet was resuspended with 150 µl 10% TCA after centrifuging
165 at 15871 xg for 15 min at 4 °C and then spun at 15871 xg for 5 min at 4 °C. This wash
166 step was repeated 3 times. The pellet was resuspended in 100 µl 100 mM ammonium
167 bicarbonate and 20 µl trypsin solution (#V5111, Promega, Madison, WI, USA) and
168 then incubated at 37 °C for 16 hr. To inactivate trypsin by storing overnight at -80 °C,
169 Ziptips (Merck Millipore) were used for desalination of samples. Proteins of the
170 samples were identified by CE/LC MASS using Q Exactive Orbitrap Mass
171 Spectrometer (Thermo Fisher Scientific).
172
173 **Interpain A gene expression quantitative analysis (RT-qPCR)**
174 RNA extraction and RT-qPCR were modified from previously described (27). Pellet
175 from 2 ml *P. intermedia* broth of OD600 of 0.8 was mixed with 4 ml RNAprotect
176 Bacteria Reagent (QIAGEN, Venlo, Netherlands). The supernatant was removed after
177 being centrifuged at 2200 xg for 5 min and stored at -80 ºC before RNA extraction.
178 The RNA was purified with RNeasy Mini Kit (QIAGEN), and the residual DNA was
179 eliminated by DNase (Promega). Clean & Concentrator Kit (ZYMO GENETICS,
180 Seatle, WA, USA) was used to concentrate the resulting RNA sample. Then RNA was
181 reverse-transcribed to cDNA with SuperScript III First-Strand System with random
182 hexamers (Invitrogen, Waltham, MA, USA). RT-qPCR was operated with GoTaq
183 qPCR Master Mix (Promega) and MyGO Pro real-time PCR instrument. (IT-IS Life
184 Science Ltd/DKSH, St Leonards, Australia) *inpA* F; *inpA* R; Pi 16s rRNA F and Pi 16s
185 rRNA R (Supplementary Table1). Denaturation at 95 °C for 10 min, amplification for
186 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.
187 All procedures were followed by the manufacturer's instructions.
188

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

190 Genomic DNA of *P. intermedia* ATCC 25611 was extracted with MasterPure^{TM}
191 complete DNA and RNA purification kit (Lucigen Corp, Middleton, WI, USA)
192 following manufacturer's instructions. The gene region of signal peptide, pro-domain
193 and catalytic domain (inpA$_{N381}$) was first amplified by GoTaq G2 Flexi DNA
194 Polymerase (Promega) with *inpA$_{N381}$* F and *inpA$_{N381}$* R (Supplementary Table1). The
195 BamHI & XhoI-digested PCR product was ligated into pET21b (+) expression vector
196 with the same restriction enzymes digestion. The pET21b (+)::*inpA$_{N381}$* plasmid, which
197 the sequence has been confirmed by sequencing, was chemically transformed into
198 *Escherichia coli* C41 (DE3) strain.

199

200 **InpA $_{N381}$ purification**

201 *E. coli* strain C41 (DE3) carrying pET21b (+)::*inpA$_{N381}$* colony was inoculated into 10
202 ml LB broth supplemented with ampicillin (100 μg/ml) and cultured at 37 °C for 14-
203 16 hr with shaking at 170 rpm. Then the bacterial culture was refreshed with 2 liters
204 LB at the ratio of 1:100 and grew at 37 °C with shaking at 170 rpm until OD600 reached
205 0.4. We used a final concentration of 0.2 mM Isopropyl-β-D-thiogalactoside (#101-
206 367-93-1, Cyrusbioscience, Inc., Seatle, WA, USA) for induction, and incubated at 25
207 °C for an additional 16 hr with shaking at 170 rpm (28). After protein induction,
208 bacterial broth was centrifugated at 3300 xg for 40 min at 4 °C. Pellet was resuspended
209 with 5 ml of 1x PBS per gram and ultrasonicated on ice (pulsed on 20 sec and pulsed
210 off 100 sec) until cells lysed properly. Then centrifugated at 16000 xg for 40 min at 4
211 °C after cell lysis. HisTrap HP His-tag protein purification column (Cytiva,
212 Washington D.C., USA) was used for His-tagged protein purification. All procedures
213 were followed by operating recommendations. Purified InpA$_{N381}$ was washed with 100-

214 fold of 1x PBS and stored at -20 °C before usage.

215

216 **Transwell assay**

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

228

229 **Wound healing assay**

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

239

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

241 Mouse experiment was modified from former research (29, 30). Seven-week-old
242 specific-pathogen-free (SPF) male C57BL/B6 mice were purchased from Laboratory
243 Animal Center of NCKU. Mice were once intraperitoneal injected of Azoxymethane
244 (AOM) (Sigma-Aldrich) at a dose of 12.5 mg/kg and combined 2% Dextran Sodium
245 Sulfate (DSS) (MP Biomedicals, Irvine, CA, USA) in drinking water for inducing
246 colorectal cancer. Twenty mice were randomly divided into four groups (MOCK,
247 AOM/DSS, AOM/DSS + *F. nucleatum,* and AOM/DSS + *P. intermedia*). Mice were
248 given three cycles of DSS and bacterial treatment and each cycle lasted for 3 weeks. In
249 the first week, mice drank 2% DSS water for 5 days. And the next 2 weeks, mice were
250 oral gavage for PBS or $1x10^9$ CFU of *F. nucleatum* strain ATCC 23726 or *P.*
251 *intermedia* strain ATCC 25611 once a week. Mice were sacrificed on week 10 and
252 colon tissues were taken out. The colons were cut and opened longitudinally along the
253 main axis, and the tumor numbers were counted.

254

255 **Statistical analysis**

256 Statistical analysis was performed by GraphPad Prism 8 software. Each data was
257 represented in triplicate. T-test was used to compare two groups, and multiple groups
258 were analyzed with one-way ANOVA. Asterisk *, **, ***, **** represented statically
259 significant comparison to control group with $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$,
260 respectively.

261

262 ## Results

263 ***P. intermedia* ATCC 25611 provoked human oral cell proliferation**

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

289 kDa, 10~30 kDa, and >30 kDa). To examine which CM fraction has cell proliferation
290 stimulatory effect, we applied these four CM fractions and whole CM to SG cells. It
291 appeared that the major promoting effect came from fraction >30 kDa, which has the
292 same effect as whole CM. Other fractions showed no difference to the un-infected
293 group (Fig. 1C). onto examine the oncogenic effect on OSCC, we treated human oral
294 cancer cell lines, SCC-15 and SAS, with CM of strain ATCC 25611. The results
295 showed that CM significantly promoted the proliferation of cancer cells after 48 hr and
296 72 hr (Fig. 1D & 1E). These results indicated that *P. intermedia* strain ATCC 25611
297 promoted human oral cell proliferation, and this activity may go through protein(s)
298 secreted from strain ATCC 25611.
299
300 **Interpain A played the key role in *P. intermedia* ATCC 25611 promoting oral cell**
301 **proliferation**
302 Following our results, the strain ATCC 25611 and clinical isolated strains exhibited
303 disparate effects on SG cell proliferation, and this difference might be associated with
304 the secreted protein(s). Next, we analyze the CM by CE/LC MASS to compare the CM
305 component of strain ATCC 25611, which had the best-promoting ability, with control
306 strain H31226, which didn't show any promoting capability. Several proteins exhibited
307 higher expression in strain ATCC 25611 as compared to that in strain H31226
308 (Supplementary Table 2). Among these proteins, a protease interpain A (InpA), was
309 secreted from ATCC 25611 thirteen times higher than that in H31226 (Fig. 2A). InpA
310 is a 90 kDa cysteine protease secreted from *P. intermedia*, it was reported to be
311 associated with heme acquisition for bacterial growth and it also could degrade
312 complement factor C3 to escape from the immune system (31-33). In addition, another
313 oral bacterium, *P. gingivalis*, was also strongly related to periodontitis, and it would

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

339 control patients' strains (H31082, H29823 and H31226). From these results, *P.*
340 *intermedia* secreted InpA to accelerate oral cell proliferation by PAR-2 signaling and
341 the proliferative effect is strain dependent.
342
343 **Migration of OSCC cells was affected by *P. intermedia* Interpain A**
344 During cancer metastasis, cancer cells invade into surrounding matrix and metastasize
345 to other organs, causing more severity and higher mortality (36). As a result, we wanted
346 to understand whether *P. intermedia* stimulates oral cancer cells migration. Wound
347 healing assay was used to evaluate the cancer cell migration at different time points.
348 CM of strain ATCC 2561 increased cell migration of both SCC-15 and SAS oral cancer
349 cell lines (Fig. 3A & 3B). Next, we investigated whether cell migration was associated
350 with InpA and PAR-2 signaling. Both E64 and FSLLRY-NH2 inhibited the enhanced-
351 migration of OSCC cell lines. Transwell migration assay also revealed that CM
352 promoted SCC-15 and SAS cell migration (Fig. 3C & 3D). Both E64 and FSLLRY-
353 NH2 suppressed CM-stimulated oral cancer cell migration in transwell migration assay,
354 too. In addition, the recombinant $InpA_{N381}$ from *E. coli* elevated SCC-15 cell migration.
355 However, this induction efefct vanished under the treatment of either E64 or FSLLRY-
356 NH2 (Fig. 3E). In summary, *P. intermedia* InpA also promoted OSCC cell migration.
357
358 ***P. intermedia* accelerated tumor number in colorectal cancer mouse model**
359 Based on the concept of the oral-gut axis, the oral bacteria translocate to the intestine
360 through gastrointestinal diseases and cause gastrointestinal diseases, such as
361 inflammatory bowel disease and colorectal cancer (37, 38). Recent studies revealed
362 that the oral bacterium, *F. nucleatum*, is important during colorectal cancer
363 development (39). We used AOM and 2% DSS to induce colorectal cancer in C57BL/6

364 mice, and oral gavage feeding *F. nucleatum* or *P. intermedia* to examine whether these
365 two bacteria caused severe colorectal cancer. Compared to AOM/DSS-only group,
366 both *P. intermedia* and *F. nucleatum* would increase colon tumor numbers (Fig. 4B-
367 C). According to our results, *P. intermedia* may be an important oral bacterium in
368 colorectal cancer development.
369

370 **Discussion**

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

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

414 diseases.

415 We believed that the effect of *P. intermedia* on regulation of oral cell proliferation was
416 strain dependent. Different *P. intermedia* strains have different amount of inpA
417 secretion. As in our data, clinical strain H31226 showed lower InpA production than
418 strain ATCC 25611 (Fig. 2A) and the clinical isolated strains showed different *inpA*
419 expression levels than ATCC 25611 (Fig. 2B). Also, OSCC-isolated strains displayed
420 higher *inpA* expression levels than control strains. Due to this observation, it might
421 provide an application for periodontal diseases or OSCC diagnosis by detecting the
422 amount of InpA from patients' saliva. Furthermore, we aimed to find the potential
423 mechanism of *P. intermedia* InpA-dependent OSCC cell proliferation. We performed
424 Micro-Western Array, a high-throughput western blot-based platform, on SG cells
425 focusing on signaling pathways associated with cell proliferation (Fig. S4). We
426 observed that the epidermal growth factor receptor (EGFR) signaling pathway was
427 elevated, the RAF expression increased in 8 hr but decreased at 24 hr. EGFR expression
428 and its downstream signaling RAS–RAF–MEK–ERK pathway was reported to evelate
429 in OSCC patients, (56, 57), which is consistent with our Micro-Western Array results.
430 However, the underlying mechanism needs further confirmation. In this study, we
431 discovered that InpA secreted by *P. intermedia* played a crucial role in oral cell
432 proliferation and migration during OSCC development. This might be PAR-2 signaling
433 pathway-dependent. However, the fundamental mechanism needs further investigation.
434

435 **Figure legends**

436 **Figure 1. *P. intermedia* strain ATCC 25611 promoted oral cell proliferation.** (A)
437 Human normal oral epithelial cells, SG, were infected with live *P. intermedia* strain
438 ATCC 25611 or clinical isolated strain H29383 and H31226 at the multiplicity of

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

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

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

498 **Notes**

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512 ***Author Contributions***

513 S.-M. C., Y.-J. C., W.-R. W. and N.-H. W. performed the experiments. S.-M. C. wrote

514 this manuscript. C.-P. C. performed the microwestern array experiments and reviewed
515 this manuscript. J.-R. H., J. S C., and J.-Y. C. provided clinical patients' saliva. I-H. H.
516 contributed to experimental design, co-supervision and review of this manuscript. J.-
517 W. C. contributed to design, provide the concept, and supervise this manuscript. All
518 authors have read and approved the final version of this manuscript.

519 ***Potential conflicts of interest.*** All authors: No reported conflict. All authors have
520 submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

521

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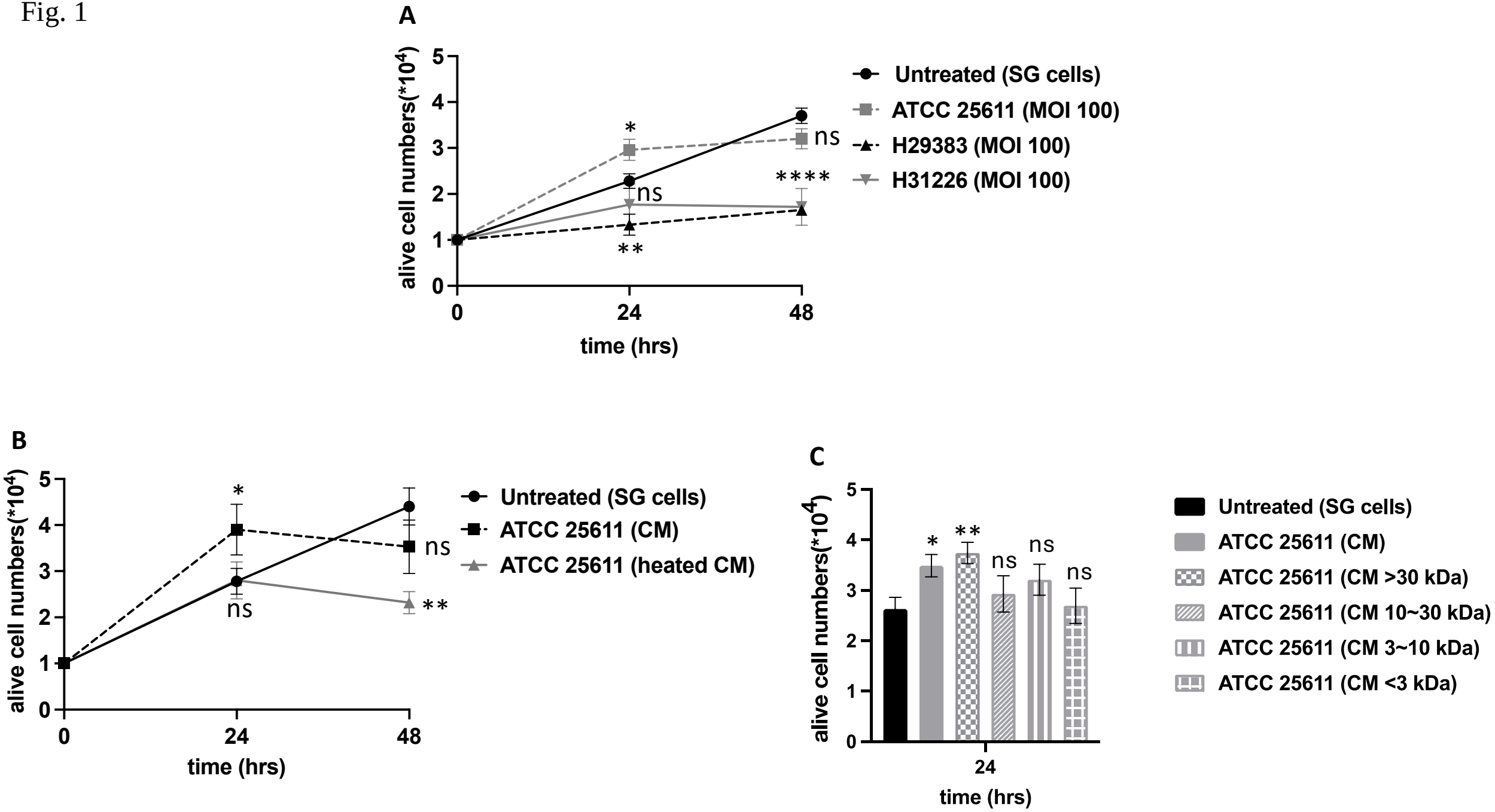
697

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)
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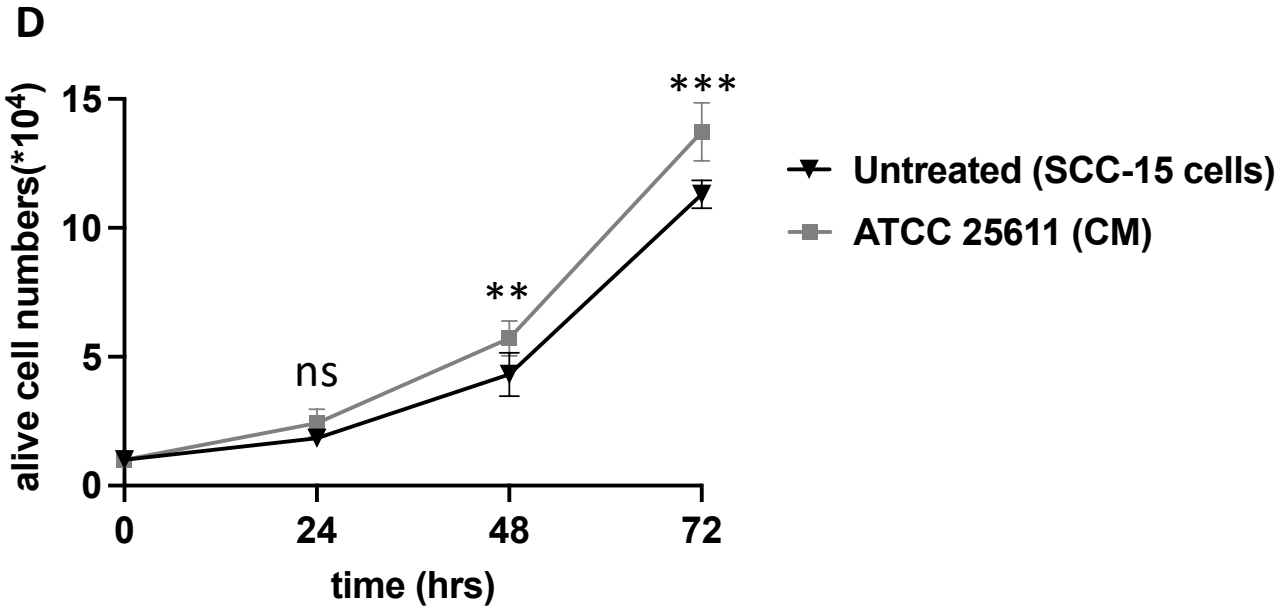
ATCC 25611 (CM 3~10 kDa)
ATCC 25611 (CM <3 kDa)

Fig. 1


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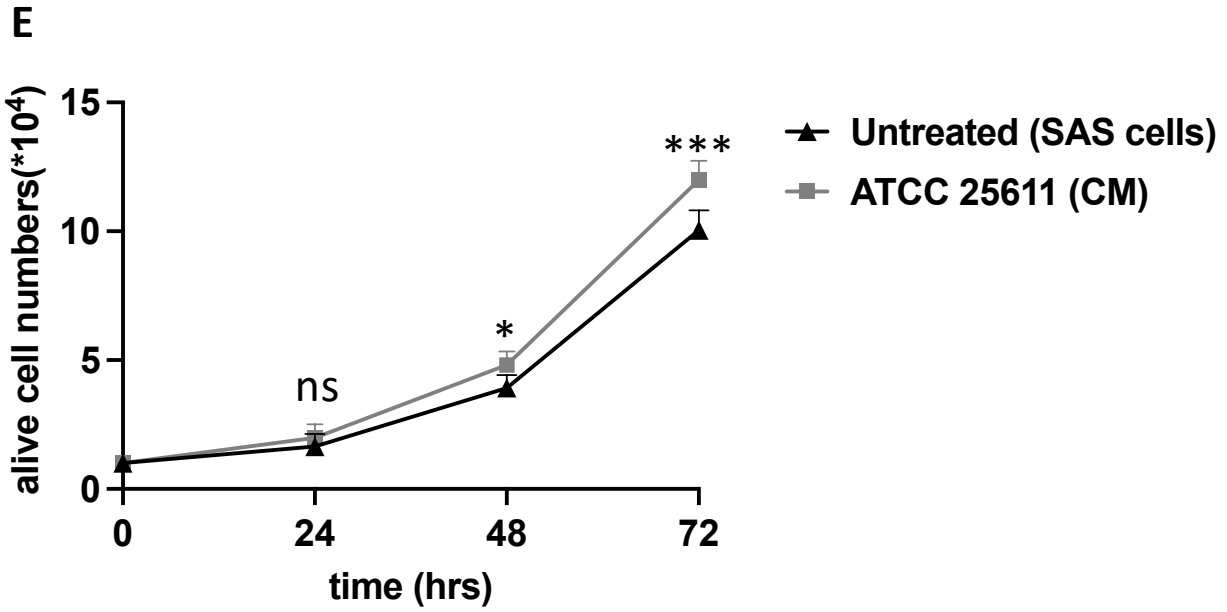
24
48
72
time (hrs)
ns
**
***
Untreated (SCC-15 cells)
ATCC 25611 (CM)


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

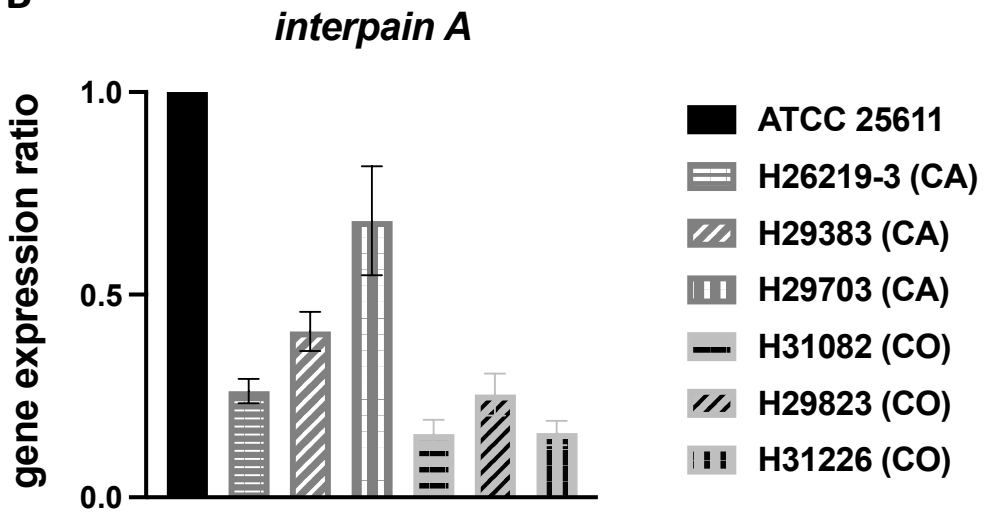
Fig. 2

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



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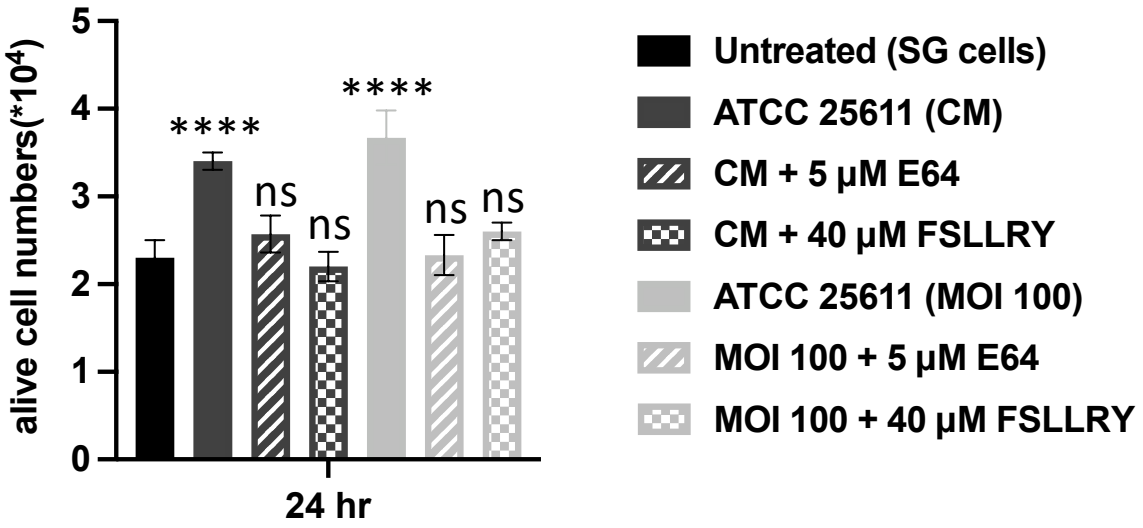
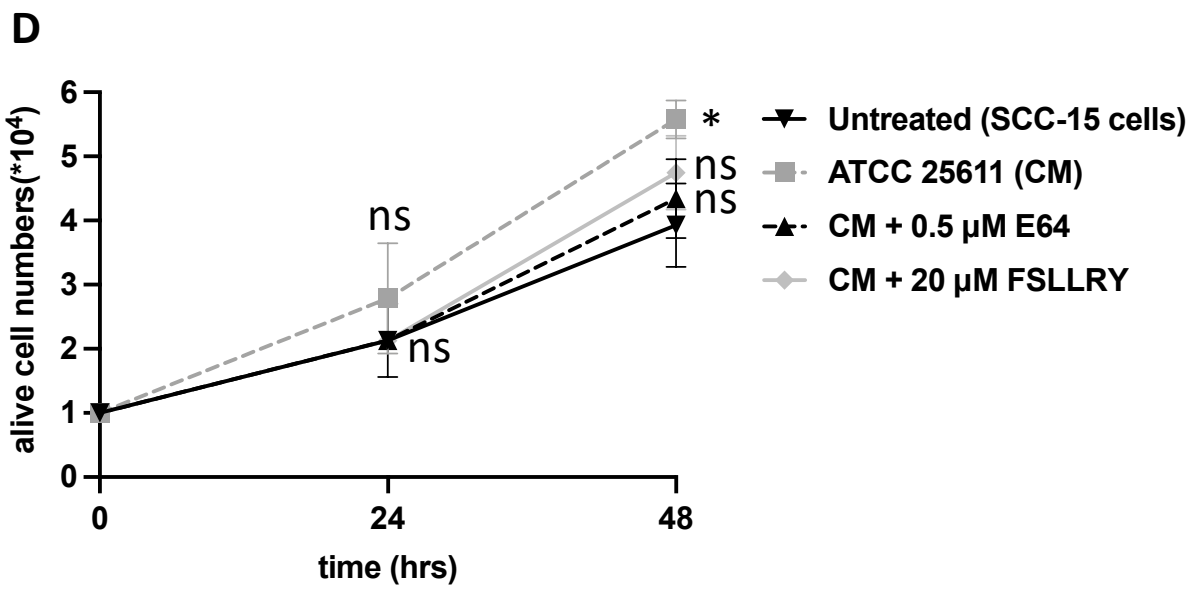


Fig. 2


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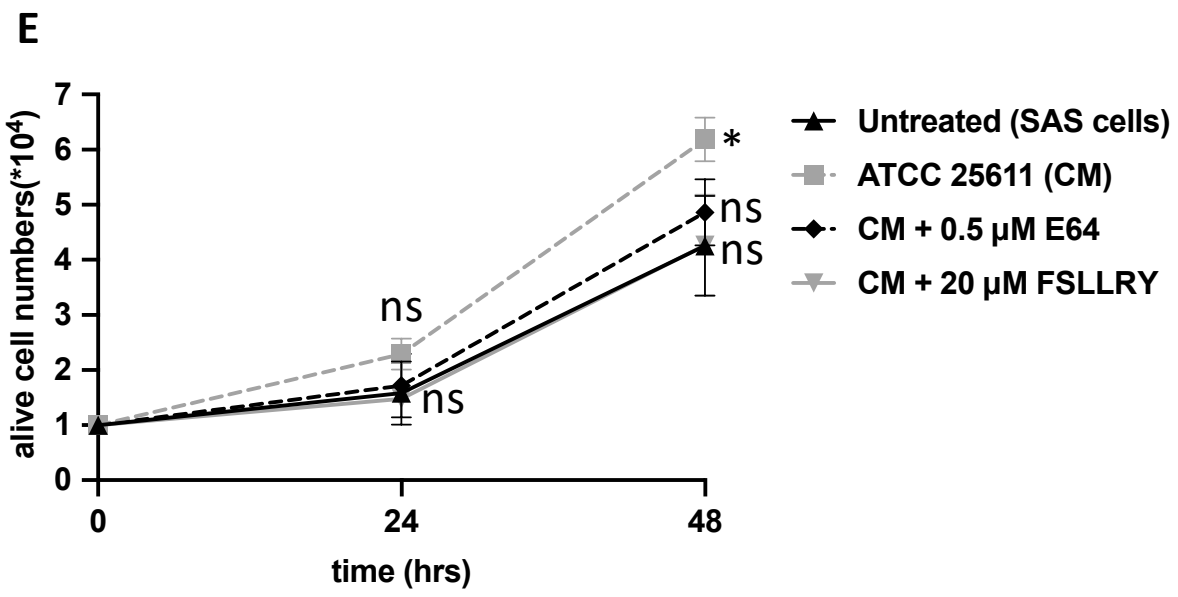
ns
ns
Untreated (SCC-15 cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY


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

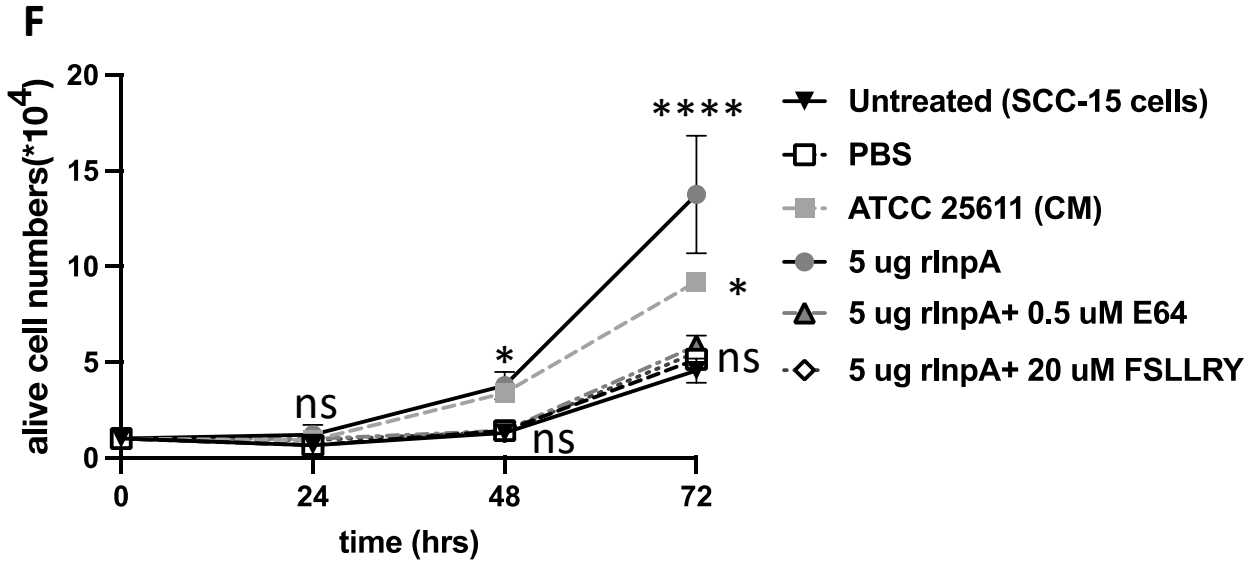

F
alive cell numbers(*10^4)
time (hrs)
0
24
48
72
ns
*
ns
****
*
ns
Untreated (SCC-15 cells)
PBS
ATCC 25611 (CM)
5 ug rInpA
5 ug rInpA+ 0.5 uM E64
5 ug rInpA+ 20 uM FSLLRY

Fig. 3

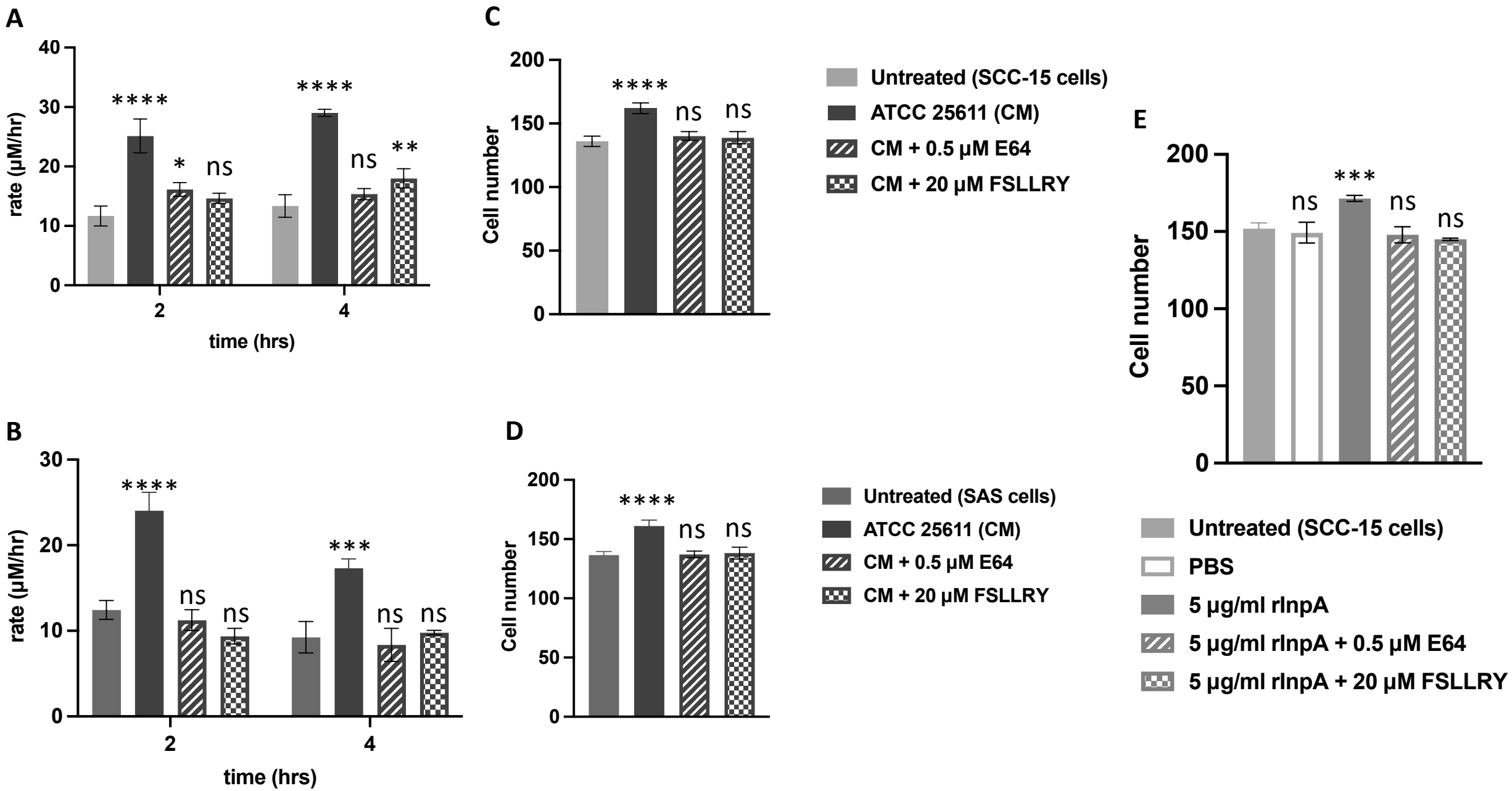
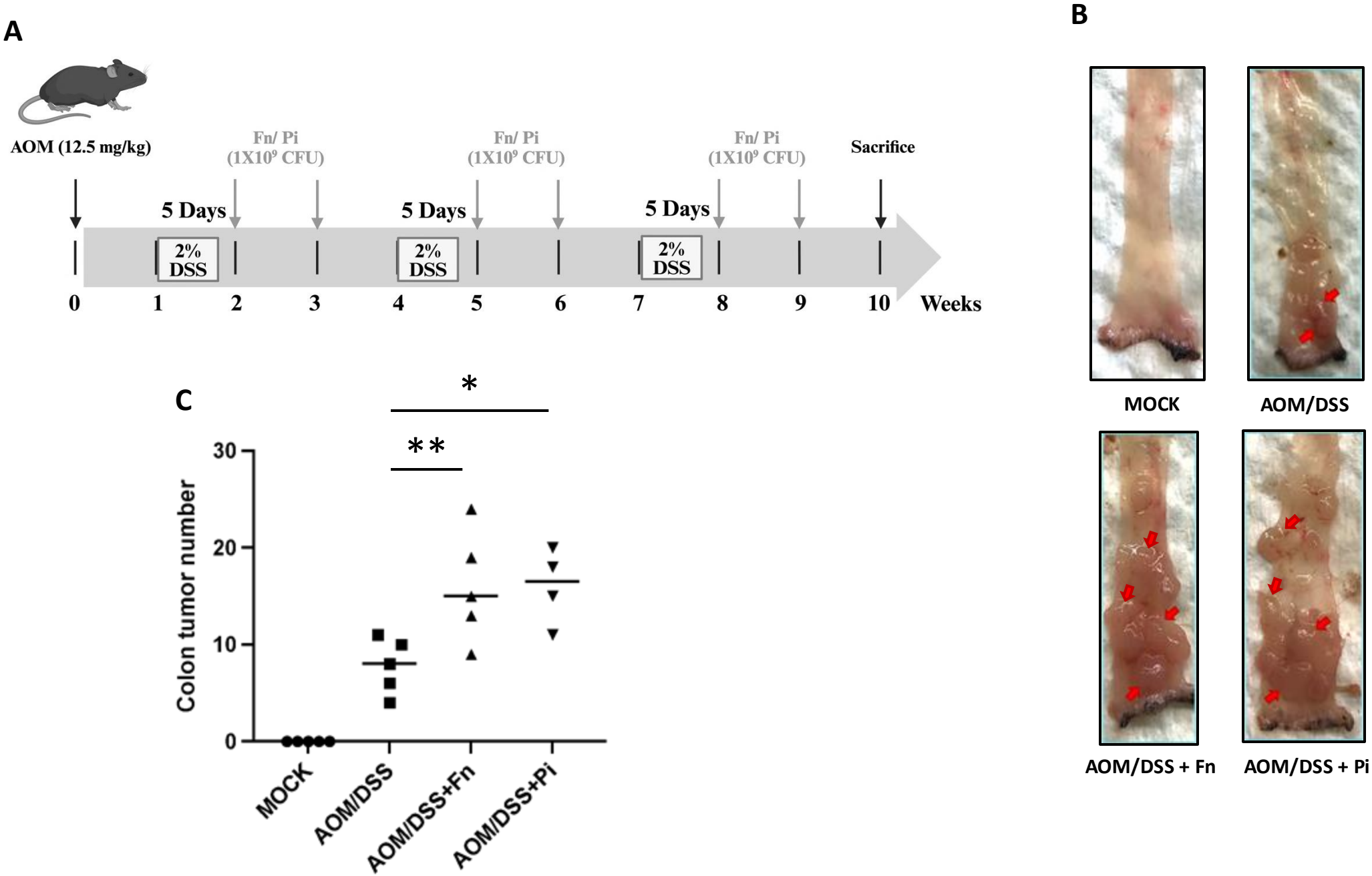

A
rate (μM/hr)
time (hrs)
****
*
ns
****
ns
**
B
rate (μM/hr)
time (hrs)
****
ns
ns
***
ns
ns
C
Cell number
****
ns
ns
D
Cell number
****
ns
ns
Untreated (SCC-15 cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY
Untreated (SAS cells)
ATCC 25611 (CM)
CM + 0.5 μM E64
CM + 20 μM FSLLRY
E
Cell number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