Modulation of the extracellular matrix by *Streptococcus gallolyticus* subsp. *gallolyticus* and importance in cell proliferation

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Abstract

Streptococcus gallolyticus subspecies *gallolyticus* (*Sgg*) has a strong clinical association with colorectal cancer (CRC) and actively promotes the development of colon tumors. Previous work showed that this organism stimulates CRC cells proliferation and tumor growth. However, the molecular mechanisms underlying these activities are not well understood. Here, we found that *Sgg* upregulates the expression of several types of collagens in HT29 and HCT116 cells, with type VI collagen (ColVI) being the highest upregulated collagen type. Knockdown of ColVI abolished the ability of *Sgg* to induce cell proliferation and reduced the adherence of *Sgg* to CRC cells. The extracellular matrix (ECM) is an important regulator of cell proliferation. Therefore, we further examined the role of decellularized matrix (dc-matrix), which is free of live bacteria or cells, in *Sgg*-induced cell proliferation. Dc-matrix prepared from *Sgg*-treated cells showed a significantly higher pro-proliferative activity than that from untreated cells or cells treated with the control bacteria. On the other hand, dc-matrix from *Sgg*-treated ColVI knockdown cells showed no difference in the capacity to support cell proliferation compared to that from untreated ColVI knockdown cells, suggesting that the ECM by itself is a mediator of *Sgg*-induced cell proliferation. Furthermore, *Sgg*-treated CRC cells formed significantly larger tumors *in vivo*, whereas *Sgg* treatment had no effect on ColVI knockdown cells, suggesting that ColVI is important for *Sgg* to promote tumor growth *in vivo*. These results highlight a dynamic bidirectional interplay between *Sgg* and the ECM, where *Sgg* upregulates collagen expression. The *Sgg*-modified ECM in turn affects the ability of *Sgg* to adhere to host cells and more importantly, acts as a mediator for *Sgg*-induced CRC cell proliferation. Taken together, our results reveal a novel mechanism in which *Sgg* stimulates CRC proliferation through modulation of the ECM.

Author Summary

Colorectal cancer (CRC) is a leading cause of cancer-related death. The development of CRC can be strongly influenced by specific gut microbes. Understanding how gut microbes modulate CRC is critical to developing novel strategies to improve clinical diagnosis and treatment of this disease. *S. gallolyticus* subsp. *gallolyticus* (*Sgg*) has a strong clinical association with CRC and actively promotes the development of colon tumors. However, the mechanisms *Sgg* utilizes to promote tumors are not well understood. Our results showed for the first time a dynamic interplay between *Sgg* and the extracellular matrix. We found that *Sgg* upregulates the expression of collagens which in turn affects the interaction between *Sgg* and CRC cells and mediates CRC cell proliferation. These findings draw attention to a previously unrecognized dynamic bidirectional interplay between a CRC-associated microbe and the extracellular matrix (ECM). Given the importance of the ECM in normal homeostasis and in tumor microenvironment, these findings have important implications in the context of microbial contribution to cancer.

61 **Introduction**

62 *Streptococcus gallolyticus* subsp. *gallolyticus* (*Sgg*) belongs to the *S. bovis* group of
63 organisms and was previously known as *S. bovis* biotype I [1]. It is an opportunistic pathogen that
64 causes bacteremia and infective endocarditis (IE) [2]. *Sgg* is also known to associate with CRC as
65 documented by numerous case reports and case series over the past several decades [3-7]. A meta-
66 analysis study of case reports and case series published up to 2011 found that among *S. bovis*-
67 infected patients who underwent colonic evaluation, ~60% had concomitant colon
68 adenomas/carcinomas [8]. Furthermore, patients with *Sgg* bacteremia/IE have a higher risk (~ 7
69 fold) for CRC compared to bacteremia/IE caused by other species in the *S. bovis* group [8],
70 suggesting the existence of a *Sgg*-specific mechanism that promotes the strong association between
71 *Sgg* and CRC. The prevalence of *Sgg* in CRC patients has not been investigated as extensively as
72 the risk for CRC among patients with active *Sgg* infections. However, recent data indicate that *Sgg*
73 was enriched in tumor tissues from CRC patients [9-11], suggesting its potential as a biomarker
74 for CRC.

75 In addition to the strong clinical association between *Sgg* and CRC, studies have shown
76 that *Sgg* stimulates the proliferation of CRC cells and promotes the development of tumors in
77 experimental models of CRC [10, 12-15]. *Sgg* treatment of human CRC cells led to larger tumors
78 compared to untreated cells in a xenograft model. In an azoxymethane (AOM)-induced CRC
79 model, mice orally gavaged with *Sgg* had significantly higher tumor burden and dysplasia grade
80 compared to control mice. In a colitis-associated CRC model, oral gavage of *Sgg* augmented
81 tumorigenesis in the colon. Taken together, long-standing clinical observations and recent
82 functional studies indicate that *Sgg* not only has a strong association with CRC but also actively
83 promotes the development of CRC. The mechanism underlying the tumor-promoting activity of

84 *Sgg*, however, is poorly understood. The ability of *Sgg* to stimulate CRC cell proliferation is an
85 important aspect of the tumor-promoting effect of *Sgg*. The Wnt/β-catenin signaling pathway
86 regulates cell fate and proliferation and is a critical pathway in colon tumorigenesis. Previous
87 results indicated that *Sgg* induced upregulation of β-catenin and increased nuclear translocation of
88 β-catenin, and that β-catenin signaling was required for *Sgg* to stimulate CRC cell proliferation
89 and tumor growth [10]. The signaling events that lead to *Sgg*-induced activation of β-catenin
90 signaling and cell proliferation were unknown.

91 The extracellular matrix (ECM) regulates fundamental cell behavior such as cell
92 proliferation, adhesion and migration and plays important roles during normal development as well
93 as in pathological conditions such as cancer [16, 17]. The ECM is an important constituent of the
94 tumor microenvironment. Altered ECM composition, structure and mechanical property are
95 common features in tumor tissues and contribute to tumor progression [18-23]. In the case of CRC,
96 multiple studies have found that various types of collagens are upregulated in tumors compared to
97 matched normal tissues [24-31]. Whether gut microbes can provide exogenous signals to modulate
98 ECM expression and dynamics was unknown.

99 In this study, we found that *Sgg* upregulates the expression of collagen *in vitro* and *in vivo*.
100 We demonstrated that upregulation of collagen by *Sgg* is important for the promotion of CRC cell
101 proliferation, upregulation of β-catenin, and tumor growth by *Sgg*. Moreover, we demonstrated
102 the importance of the ECM in *Sgg*-mediated cancer cell proliferation by using decellularized
103 matrix from CRC cells cultured under various treatment conditions. Altogether, our results suggest
104 a novel mechanism in which *Sgg* actively regulates the expression of ECM molecules which in
105 turn affects the ability of *Sgg* to stimulate CRC cell proliferation in a direct and indirect manner.

106 The results highlight a previously under-studied activity of gut bacteria and have important
107 implications in the context of microbial contribution to CRC.
108

109 **Results**

110 ***Sgg* increases collagen expression in human CRC cells and in colonic tissues *in vivo*.**
111 *Sgg* was previously shown to stimulate the proliferation of certain human CRC cells including [10,
112 14]. To investigate the changes in CRC cells induced by *Sgg*, we performed mass spectrometry-
113 based label-free global proteome profiling of whole cell lysates prepared from HT29 cells cultured
114 alone or in the presence of *Sgg* strain TX20005. Strikingly, the level of several types of collagens
115 was increased in cells co-cultured with *Sgg* compared to that in cells cultured alone, with type VI
116 collagen (ColVI) showing the highest relative abundance (supplemental Table S1). The increased
117 expression of ColVI was further confirmed at the transcription and protein level. In RT-qPCR,
118 both ColVI α1 chain (COL6A1) and α3 chain (COL6A3) was significantly increased in the
119 presence of *Sgg* compared to cells cultured in media only (Fig. 1A). In western blot, ColVI level
120 was significantly increased in HT29 and HCT116 cells co-cultured with *Sgg*, compared to cells
121 co-cultured with *L. lactis*, a non-pathogenic negative bacterial control, or in media only (Fig. 1B
122 and 1C). Previous studies showed that *Sgg* stimulates the proliferation of HT29 and HCT116 cells,
123 but had no effect on A549 cells, a human lung cancer cell line [10]. No significant changes in
124 ColVI were observed in A549 cells cultured in the presence of *Sgg* when compared to cells cultured
125 in the presence of *L. lactis* or in media only (Fig. 1B and 1C). Using immunofluorescence (IF)
126 microscopy, we further validated that *Sgg* induced the expression of ColVI (Fig. 1D). Upregulation
127 of type I collagen (ColI) by *Sgg* was also confirmed by using IF (supplemental Fig. S1).

128 We next examined the effect of *Sgg* on collagen expression *in vivo* using colon sections
129 from mice orally gavaged with *Sgg*, *L. lactis* or saline. Sections were stained with Masson's
130 Trichrome stain which stains collagen blue [32]. The results showed that colon sections from mice
131 gavaged with *Sgg* had more intense blue staining compared to sections from mice gavaged with *L.*
132 *lactis* or saline (Fig. 1E), indicating elevated level of collagen following exposure to *Sgg*. IF
133 staining of the colon sections with an anti-ColVI antibody also showed more intense staining of
134 ColVI in colonic crypts from *Sgg*-gavaged mice compared to control mice (supplemental Fig. S2).
135 Taken together, these results suggest that exposure to *Sgg* results in increased level of collagen in
136 *in vitro* cultured cells and in the intestinal mucosa *in vivo*.

137 **Collagen is required for *Sgg* to stimulate human CRC cell proliferation.** Collagen has
138 been shown to be involved in the proliferation of cancer cells [33-36]. We investigated the role of
139 collagen in *Sgg*'s stimulation of CRC cell proliferation. HT29 COL6A1 and COL6A3 stable
140 knockdown cells were generated. The ability of *Sgg* to stimulate the proliferation of either
141 COL6A1 (Fig. 2A) or COL6A3 (supplemental Fig. S3A) knockdown cells was significantly
142 reduced compared to that in untransfected cells or cells transfected with control shRNA. We
143 confirmed that COL6A1 (Fig. 2B) or COL6A3 (supplemental Fig. S3B) knockdowns reduced the
144 level of ColVI in the cells. *Sgg* was shown to upregulate β-catenin and c-Myc and β-catenin is
145 required for *Sgg* to stimulate cell proliferation [10]. Knockdown of COL6A1 (Fig. 2B-2D) or
146 COL6A3 (supplemental Fig. S3B-S3D) abolished the *Sgg*-induced upregulation of β-catenin or c-
147 Myc, suggesting that ColVI acts upstream of β-catenin in the signaling cascade that leads to *Sgg*-
148 induced cell proliferation. In addition to ColVI, we also carried out knockdown of type I collagen
149 (ColI) using siRNA specific for the α1 chain of ColI (COL1A1). COL1A1 knockdown abolished

150 the ability of *Sgg* to stimulate cell proliferation (supplemental Fig. S4), suggesting that multiple
151 ECM components are involved in *Sgg*-induced cell proliferation.

152 **Collagen is involved in *Sgg* adherence to CRC cells.** *Sgg* TX20005 was previously
153 shown to bind to several type of collagen including type I, IV and V [37, 38]. We found that
154 TX20005 also attached to immobilized ColVI (supplemental Fig. S5). We investigated if
155 knockdown of ColVI affected the adherence of *Sgg* to CRC cells and found that *Sgg* adherence to
156 COL6A1 knockdown cells was reduced by ~40% compared to untransfected cells or HT29 cells
157 transfected with the control shRNA (Fig. 3).

158 **Decellularized matrix (dc-matrix) derived from *Sgg*-treated cells alone is sufficient to**
159 **promote cancer cell proliferation.** The importance of collagen in *Sgg*-induced cell proliferation
160 could be a consequence of its effect on *Sgg* adherence to the ECM molecules around CRC cells,
161 leading to a reduced local concentration of *Sgg*. On the other hand, it is known that increased
162 collagen deposition in the matrix leads to matrix-induced cell proliferation [35, 36]. Therefore, it
163 is also possible that collagen contributes to *Sgg*-stimulated cell proliferation in this fashion. To
164 investigate if upregulation of ECM molecules by *Sgg* can directly contribute to cell proliferation,
165 dc-matrix was prepared from HT29 cells cultured in media only, in the presence of *L. lactis* or *Sgg*.
166 We confirmed that no live bacteria were present in the dc-matrix by incubating dc-matrix in
167 antibiotics-free media for 24 hours. No bacterial growth was observed. We also confirmed that no
168 intact cells remained after the decellularization procedure by staining the samples with DAPI
169 (supplemental Fig. S6). HT29 cells were then seeded onto the various dc-matrices and incubated
170 in antibiotics-containing media for 24 hours. The dc-matrix prepared from *Sgg*-treated cells
171 stimulated cell proliferation significantly better than the dc-matrix from HT29 cells alone or from
172 *L. lactis*-treated cells (Fig. 4). Furthermore, dc-matrices were also prepared from COL6A1

173 knockdown cells that had been incubated with or without *Sgg*. Dc-matrix from *Sgg*-treated
174 COL6A1 cells showed no significant difference in the ability to stimulate cell proliferation
175 compared to dc-matrix prepared from COL6A1 cells alone (Fig. 4). These results indicate that
176 dc-matrix from *Sgg*-treated cells stimulates cell proliferation in a manner that does not require live
177 *Sgg* but depends on collagen. The results also suggest that *Sgg*-induced changes in the ECM play
178 a direct role in stimulating cell proliferation, independent of the effect on *Sgg* adherence.

179 **ColVI is required for *Sgg* to promote tumor growth *in vivo*.** To determine the
180 importance of ColVI in *Sgg*-induced tumor growth *in vivo*, shCOL6A1 knockdown cells and cells
181 transfected with control shRNA were cultured in the absence or presence of *Sgg* and then injected
182 into nude mice. For cells transfected with the control shRNA, *Sgg*-treatment resulted in
183 significantly larger tumors at day 7 and 10 post injection compared to untreated cells (Fig. 5). In
184 shCOL6A1 knockdown cells, *Sgg*-treatment did not cause significant increase in tumor size as
185 compared to untreated knockdown cells. We note that in order to prevent infection caused by *Sgg*,
186 mice were administered with antibiotics following injection of cells to eliminate *Sgg*. Therefore,
187 the effect of *Sgg* on tumor growth was more pronounced at the earlier time point than that at the
188 later one. Altogether these results suggest that ColVI is important for *Sgg* to promote tumor growth
189 *in vivo*.

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193 **Discussion**

194 It is well appreciated that certain gut microbes or microbial communities play important
195 roles in influencing the development of CRC. Long-standing clinical observations and recent

196 functional studies indicate that *Sgg* is strongly associated with CRC and actively promotes tumor
197 growth, however the molecular mechanisms underlying *Sgg*'s effects are not well understood. The
198 ECM is a component of the tumor microenvironment and an important contributor to tumor
199 progression. However, insights on the impact of gut microbes on the ECM are very limited. Data
200 presented in this study demonstrate that *Sgg* upregulates collagen expression. Furthermore, we
201 provide evidence that *Sgg* upregulation of collagen is important for *Sgg*-induced cell proliferation
202 and tumor growth. To the best of our knowledge, this is the first report that connects a gut microbe
203 with the ECM in the context of microbial contribution to cancer.

204 Our results showed that *Sgg* upregulates the expression of collagen *in vitro* and *in vivo*. We
205 focused on ColVI since it shows the highest relative abundance revealed by MS analysis. ColVI
206 is also implicated in human CRC in previous studies where its level is higher in tumor tissues
207 compared to matched normal tissues [24-26]. We observed that depletion of COL6A1 or COL6A3
208 rendered the cells insensitive to the proliferation-promoting effect of *Sgg*. Previous work showed
209 that β-catenin signaling is required for *Sgg*-induced cell proliferation [10]. Results here indicate
210 that upregulation of β-catenin or c-Myc by *Sgg* is abolished in COL6A1 or COL6A3 knockdown
211 cells, suggesting that ColVI is important for *Sgg* to stimulate CRC cell proliferation and acts
212 upstream of β-catenin in the signaling cascade leading to cell proliferation. More importantly, we
213 demonstrated that ColVI is important for *Sgg* to promote tumor growth *in vivo*.

214 Our results further indicate that collagen contributes to the ability of *Sgg* to stimulate CRC
215 cell proliferation in multiple ways. Previous studies suggested that close proximity of *Sgg* to host
216 cells is important for the pro-proliferative effect of *Sgg* [10, 12]. Knockdown of COL6A1 impaired
217 the adherence of *Sgg*, suggesting that ColVI can influence the ability of *Sgg* to induce cell
218 proliferation by reducing the local concentration of *Sgg*. More importantly, our data also showed

219 that collagen can have a direct effect on *Sgg*-induced cell proliferation. This is supported by results
220 using dc-matrices. Our results showed that dc-matrix prepared from HT29 cells that had been co-
221 cultured with *Sgg* was able to significantly increase cell proliferation compared with dc-matrix
222 from cells cultured alone or with *L. lactis*. On the other hand, dc-matrix from *Sgg* treated COL6A1
223 knockdown cells showed no difference from that prepared from untreated cells. These results
224 suggest that *Sgg*-modified ECM is directly involved in mediating cell proliferation.

225 ColVI is special in the sense that a C-terminal cleavage product of COL6A3 chain
226 (endotrophin (ETP)), which is soluble, was found to augment breast tumor growth [39]. Our results
227 using dc-matrix speaks against a role of ETP in increased CRC cell proliferation caused by *Sgg*.
228 ColVI has a unique supramolecular structure among the members of the collagen family. Its
229 beaded microfilament structure enables it to bind to other components of the ECM such as ColI
230 and ColII and acts as a bridging molecule [40-42]. Thus, depletion of ColVI may affect the overall
231 organization and composition of the ECM [17, 43, 44]. In addition, knockdown of ColI also
232 abolished the ability of *Sgg* to stimulate CRC cell proliferation. This, combined with the MS result
233 showing increased expression of several types of collagen in cells co-cultured with *Sgg*, suggests
234 that the effect we observed is not ColVI-specific. It is likely that increased deposition of collagen
235 into the matrix changed the composition of the matrix, resulting in enhanced signaling of collagen
236 receptors. Increased deposition of collagen into the matrix may also affect the mechanical property
237 of the matrix. A stiffer matrix can promote cell proliferation by mechanotransduction [45].
238 Identifying the signaling pathway(s) downstream of the ECM will shed light on how *Sgg*
239 modification of the ECM contributes to the pro-proliferative effect of *Sgg*.

240 The molecular mechanisms used by other microbes to drive the development of CRC can
241 be loosely grouped into the following categories: 1) by producing genotoxins that directly induce

242 DNA damage in colonic epithelial cells, 2) by modulating host immune responses to generate a
243 microenvironment favorable for CRC, and 3) by shifting host metabolism to support tumor growth
244 [46-54]. Our results suggest a new way by which microbes influence tumor development - by
245 modulating the ECM. In this study, we focused on the effect of *Sgg* on CRC cells. *In vivo*,
246 fibroblasts are major producers of ECM molecules. Tumor-associated macrophages were also
247 shown to regulate the synthesis and assembly of collagenous matrix [55]. Currently the effect of
248 *Sgg* on fibroblasts and macrophages is unknown. It is possible that *Sgg* may also regulate ECM
249 remodeling by engaging fibroblasts and immune cells *in vivo*.

250 Components of the ECM such as collagen and fibronectin are commonly targeted by
251 bacterial pathogens to facilitate adherence to and invasion of host cells and colonization of host
252 tissues [56-58]. There is a large body of work investigating the binding interactions between
253 microbial factors and ECM molecules and the biological importance of these interactions. Our
254 results here show that *Sgg* not only targets these ECM molecules for adherence but also actively
255 regulate their expression to provide more attachment sites. More importantly, the *Sgg*-modified
256 ECM is a direct contributor to the pro-proliferative effect of *Sgg*. Thus, our work reveals a novel
257 bidirectional interplay between the pathogen and the ECM. Future work to understand how *Sgg*
258 regulates ECM expression will be important. Given the wide distribution and functional
259 importance of the ECM, modulation of the ECM by *Sgg* is likely to be relevant to *Sgg*
260 pathogenicity in IE. It would also be interesting to see if other bacterial pathogens actively regulate
261 ECM expression to their benefit.

262 In conclusion, the mechanisms underlying the pro-proliferative and pro-tumor activities of
263 *Sgg* are poorly understood. This study provides the first experimental evidence for *Sgg* modulation
264 of the ECM and a direct role of the ECM in *Sgg*-induced cell proliferation and tumor growth. The

265 results presented here highlight a dynamic two-way interplay between *Sgg* and the ECM and call
266 attention to a novel strategy by which microbes contribute to the development of CRC.
267
268
269 **Materials and Methods**

270 **Bacterial and cell culture conditions.** *Sgg* strain TX20005 and *Lactococcus lactis*
271 MG1363 were cultured as described previously [10]. Human colon cancer cell line HT29 and
272 HCT116 were cultured in DMEM/F-12 HEPES (GIBCO, USA) supplemented with 10% fetal
273 bovine serum (FBS) (GIBCO, USA). Human lung carcinoma cell line A549 was maintained in
274 F12-K media supplemented with 10% FBS. All the cells were cultured in a humidified incubation
275 chamber at 37ºC with 5% CO_2.

276 **Cell proliferation assays.** This was performed as described previously [10]. Briefly, cells
277 (~$1x10^4$ cells/well) were cultured in the presence of stationary phase bacteria (*Sgg* or *L. lactis*) at
278 a multiplicity of infection (MOI) of 1 or media only for 24 hours. Trimethoprim was added at 50
279 µg/ml final concentration after 6 hours of co-culture to prevent media acidification due to bacterial
280 growth. Cells were detached by trypsin treatment and counted in a Cellometer Mini automated cell
281 counter (Nexcelome Biosciences, Lawrence, MA).

282 **Collagen knockdown.** To generate stable knockdown cells, lentiviral plasmids containing
283 COL6A1 or COL6A3 short hairpin RNA (shRNA) (Sigma-Aldrich, TRCN0000116959 and
284 TRCN0000003622), or MISSION pLKO.1-puro Non-Mammalian shRNA Control (Sigma-
285 Aldrich, SHC002) were first transfected into HEK293T cells to produce lentiviral particles. HT29
286 cells were then infected with the respective lentiviral particles and selected with puromycin

287 (1μg/ml). Gene knockdown was confirmed by western blot assays. Transient knockdown of
288 COL1A1 was carried out using specific siRNA for COLA1 (ThermoFisher).

289 **Preparation of decellularized matrix (dc-matrix).** HT29 cells were decellularized
290 following a protocol described previously [59]. Briefly, cells were washed thrice with ice-cold
291 PBS containing a cocktail of protease inhibitors (GenDEPOT). The cells were incubated in a PBS
292 solution containing 0.25% Triton X and 0.25% sodium-deoxycholate in PBS for 5 minutes. The
293 matrix was gently washed in PBS thrice and incubated with 100 mg/mL RNAse A (Roche) and 10
294 IU/mL DNAse (Sigma) for 30 minutes followed by three washes in PBS.

295 **Quantitative reverse transcription PCR (RT-qPCR)**. HT29 cells were co-cultured with
296 *Sgg* for 6 hrs. Total RNA was extracted from co-cultured cells using the RNeasy Kit (QIAGEN).
297 cDNA was generated by using the Transcriptor First Strand cDNA Synthesis Kit (Roche). qPCR
298 was performed using FastStart SYBR green master mix (Roche) in a Viia 7 Real Time PCR System
299 (Applied Biosystems). The following cycle conditions were used: 95˚C for 10 minutes followed
300 by 40 cycles at 95˚C for 30 seconds and 60˚C for 1 minute. CT values were first normalized to
301 GAPDH then to cells cultured in media only.

302 **Western blot assays.** This was performed as described previously [10]. Briefly, cells were
303 cultured in the appropriate medium in the presence or absence of bacteria for 12 hours. Cells were
304 washed with sterile PBS three times and lysed. Total cell lysates were subjected to SDS-gel
305 electrophoresis and western blot. Rabbit polyclonal antibodies against ColVI (1:500, Abcam), β-
306 catenin (1:4000, Cell Signaling Technology (CST)), c-Myc (1:3000, Abcam), and β-actin (1:5000,
307 CST) were used. Horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (CST) was used as
308 the secondary antibody. Signals were detected using HyGLO, chemiluminescent HRP (Denville,
309 Mteuchen, NJ). Band intensity was quantified using Image J.

310 **Adherence Assay.** This was performed as described previously [10]. Briefly, HT29 cells
311 were incubated with or without bacteria at an MOI of 10 for 1 hour. The wells were washed three
312 times with sterile PBS to remove unbound bacteria. To determine the number of bound bacteria,
313 cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. Adherence
314 was expressed as a percentage of total bacteria added.

315 **Animal experiment.** Animal studies were performed in accordance with protocols
316 approved by the Institutional Animal Care and Use Committee at the Texas A&M Health Science
317 Center, Institute of Biosciences and Technology. The xenograft model experiment was performed
318 as described previously [10]. Tumor diameters were measured with a digital caliper, and tumor
319 volume calculated using the formula: Volume = (d1xd1xd2)/ 2, with d1 being the larger dimension.

320 **Immunofluorescence. 1) Colon sections.** Methcarn-fixed paraffin embedded colon
321 sections were deparaffinized with xylene and rehydrated in an ethanol gradient. The slides were
322 incubated in a citrate buffer at 95°C for 15 minutes, cooled to room temperature (RT), rinsed with
323 PBS and incubated in blocking buffer (PBS containing 1% Saponin and 20% BSA) for 30 minutes.
324 The slides were then incubated with rabbit anti-ColVI (1:200, Abcam) at 4°C overnight, washed
325 with PBS, and incubated with donkey-anti-rabbit Alexa 594 for 1 hour at RT. The slides were
326 washed again, stained with DAPI, mounted and examined in a DeltaVision Elite microscope (GE
327 Healthcare). **2) Cultured cells.** Cells were seeded onto an 8-chambered slide and cultured under
328 various conditions. Cells were washed 3 times in PBS to remove unbound bacteria, fixed with 4%
329 formaldehyde, and permeabilized with 0.1% Triton-X-100 for 30 minutes. Cells were then
330 incubated in a blocking solution (PBS containing 5% donkey serum and 0.3% Triton X-100) for 1
331 hour. The slides were then incubated with anti-Collagen VI or anti-Collagen I antibody (1:100) at
332 4°C overnight, washed with PBS, and incubated with donkey-anti-rabbit Alexa 594 (1:500 dilution

333 in PBS) for 1 hour at RT. The slides were washed again, stained with DAPI, mounted and
334 examined in a DeltaVision Elite microscope (GE Healthcare).

335 **Trichrome staining of colon sections.** Colon sections were deparaffinized, rehydrated and
336 stained using a Trichrome Stain Kit (Abcam, ab150686) following the protocol provided by the
337 manufacturer.

338 **Ethics statement.** Animal studies were performed in accordance with protocols
339 (IACUC#2017-0420-IBT) approved by the Institutional Animal Care and Use Committee at the
340 Texas A&M Health Science Center, Institute of Biosciences and Technology. The Texas A&M
341 University Health Science Center—Institute of Biosciences and Technology is registered with the
342 Office of Laboratory Animal Welfare per Assurance A4012-01. It is guided by the PHS Policy on
343 Human Care and Use of Laboratory Animals (Policy), as well as all applicable provisions of the
344 Animal Welfare Act. Mice were euthanized by CO_2 inhalation followed by cervical dislocation.

345

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351

352 **Figure Legends**

353 **Fig. 1. *Sgg* upregulates collagen expression in cultured cells. A**. HT29 cells were co-cultured
354 with *Sgg* (strain TX20005) or media only for 6 hours. RNA was extracted and analyzed by RT-
355 qPCR. CT values were first normalized to GAPDH then to cells cultured in media only and then

356 converted to fold changes. **B** and **C**. HT29, HCT116 and A549 cells were co-cultured with *Sgg*, *L.*
357 *lactis*, or media only for 12 hours. Cell lysates were subject to western blot with anti-ColVI
358 antibody. Band intensity was quantified using Image J and normalized to GAPDH (**C**). **D**. HT29
359 and HCT116 were co-cultured with *Sgg* or media only for 12 hours. Cells were washed, fixed,
360 incubated with anti-ColVI antibody and counterstained with DAPI. Representative images are
361 shown. Scale bars represent 25μm. **E**. A/J mice were administered with 4 weekly i.p. injections of
362 AOM, followed by treatment with ampicillin for 1 week and then weekly oral gavage of bacteria
363 (*Sgg* and *L. lactis*, respectively) or saline for 12 weeks, as previously described [12, 15]. Colons
364 were harvested one week after the last bacterial gavage, swiss-rolled, fixed with meth-carn,
365 embedded and sectioned. Sections were stained with Trichrome stains. Collagen is stained blue.
366 Statistical analysis in **A** and **C** was done using unpaired, two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$.

367 **Fig. 2. *Sgg* promotes cell proliferation in a ColVI dependent manner**. **A**. Knockdown of
368 COL6A1 abolished the effect of *Sgg* on cell proliferation. Untransfected HT29 cells, COL6A1
369 stable knockdown HT29 cells or HT29 cells transfected with a control shRNA were incubated
370 with media only, *L. lactis* or *Sgg* TX20005 for 24 hours. Cell proliferation assays were performed
371 by counting viable cells as described in the Materials and Methods section. **B-D**. Cells were
372 incubated in media only, *Sgg* or *L. lactis* for 12 hours as described in the Materials and Methods
373 section. Total cell lysates were subject to western blot assays to compare COLVI, β-catenin, and
374 c-Myc protein levels. Representative images are shown (**B**). Band intensity was quantified using
375 Image J, normalized to β-actin first and then to the cells only control (**C-D**). Data are presented as
376 the mean ± SEM. Each experiment was repeated at least three times. Unpaired, two-tailed *t* test
377 was used for statistical analysis. *, $p < 0.05$; **, $p < 0.01$.

378 **Fig. 3. Collagen knockdown impaired the adherence of *Sgg*.** HT29 cells, HT29 cells transfected

379 with a control shRNA or COL6A1 stable knockdown HT29 cells were incubated with TX20005
380 (MOI=10) as described in the Materials and Methods section. Adherence was calculated as the
381 percentage of adhered bacteria vs. total bacterial added and combined from at least three
382 independent experiments. Mean ± SEM is presented. Unpaired, two-tailed *t* test was used for
383 statistical analysis. *, $p < 0.05$.

384 **Fig. 4. Decellularized matrix (dc-matrix) derived from *Sgg*-treated cells is sufficient to**
385 **stimulate cell proliferation.** HT29 cells were co-cultured with *Sgg* strain TX20005, *L. lactis* or
386 media only for 12 hours. The wells were incubated with antibiotics to eliminate bacteria followed
387 by washing. Cells were then stripped away from the underlying matrix as described in the Materials
388 and Methods section. HT29 cells (~ 1 x 10^4) that had not been previously exposed to *Sgg* were
389 seeded on the indicated dc-matrices and incubated for 24 hours. Viable cells were counted. Each
390 experiment was repeated at least three times. Data is presented as the mean ± SEM. Statistical
391 analysis was done using unpaired, two-tailed *t* test. **, $p < 0.01$.

392 **Fig. 5. Collagen knockdown impairs the ability of *Sgg* to promote tumor growth *in vivo*.** 1 x
393 10^6 HT29shCOL6A1 or HT29shcontrol cells were treated with *Sgg* TX20005 or no bacteria, mixed
394 with Matrigel and injected into the dorsal flap of nude mice (n = 5/group). Tumor size was
395 measured at the indicated time point with a digital caliper. Data is presented as the mean ± SEM.
396 Statistical analysis was done using unpaired, two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$.

397

398 **Supplemental Table S1. Relative abundance of several types of collagens in whole cell**

399 **lysates analyzed by Mass Spectrometry [a].**

	HT29[b]	**HT29 + *Sgg*[b]**
COL6A3	ND[c]	11.5 ± 3.1[e]
COL6A1	ND[c]	4.6 ± 1.3
COL3A1	ND[c]	3.9 ± 1.5
COL4A2	ND[c]	2.9 ± 0.9
COL1A1	0.02[d]	0.8 ± 0.5

400

401 [a] HT29 cells were cultured in the presence or absence of *Sgg* strain TX20005 for 24 hours (3

402 biological replicates). The cells were washed, lysed and digested in ammonium bicarbonate

403 buffer and trypsin. The resulting peptide mixtures were analyzed by a nanoLC-1200 system

404 coupled to an Orbitrap Fusion Lumos mass spectrometer. Only the collagen types that show

405 increase in all three biological replicates are listed.

406 [b] Relative protein abundance is shown as the mean iFOT ± SEM. iFOT is the normalization of

407 individual protein intensity to the total protein intensity within one experiment.

408 [c] Not detected. The limit of detection is equivalent to iFOT = 0.0005.

409 [d] Only detected in one biological replicate.

410 [e] Statistical comparison between HT29 + *Sgg* and HT29 was not performed due to the low

411 abundance of collagen peptide chains in untreated HT29.

412

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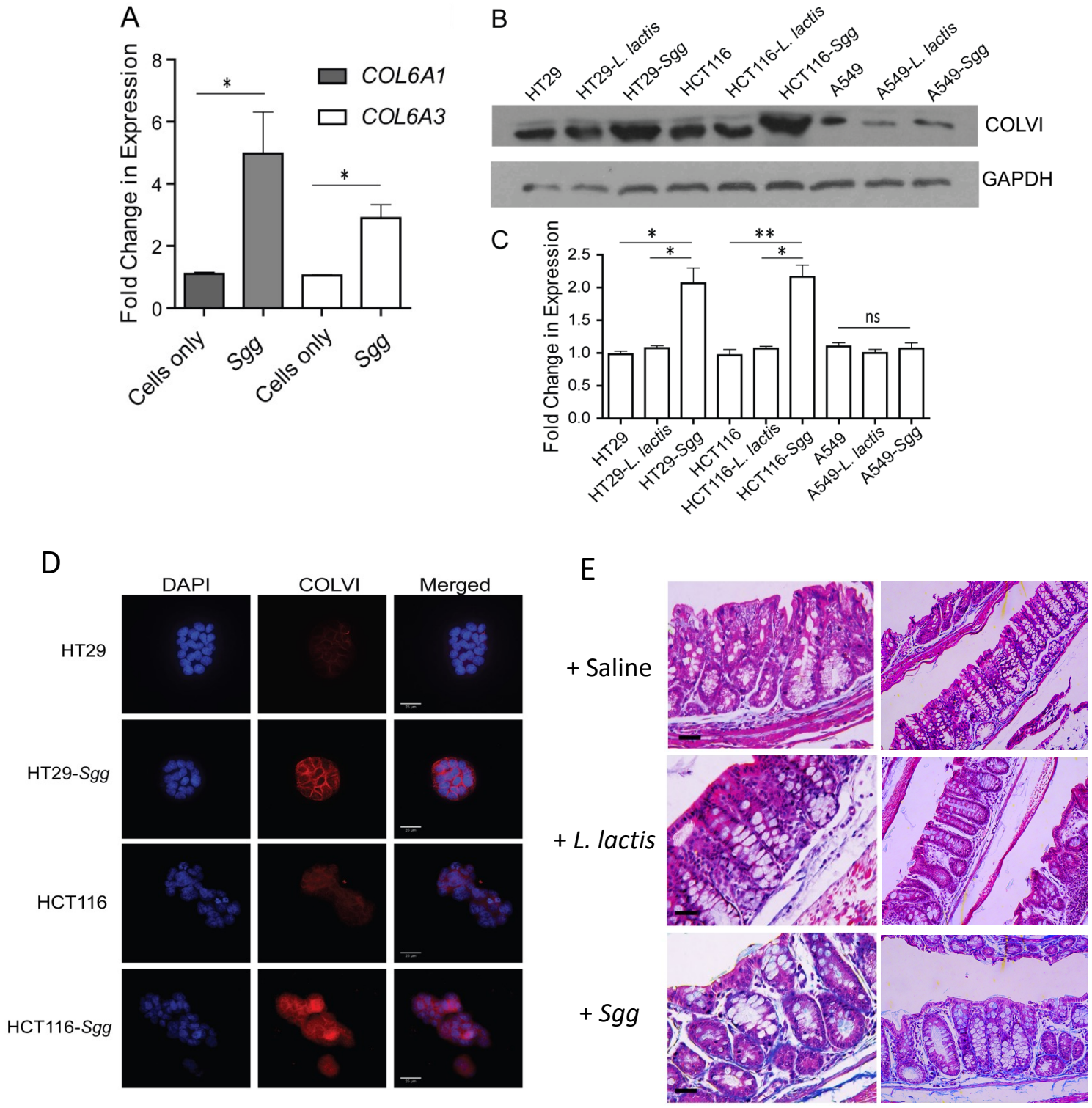

A
Fold Change in Expression
8
6
4
2
0
*
*
COL6A1
COL6A3
Cells only
Sgg
Cells only
Sgg
B
HT29
HT29-L. lactis
HT29-Sgg
HCT116
HCT116-L. lactis
HCT116-Sgg
A549
A549-L. lactis
A549-Sgg
COLVI
GAPDH
C
Fold Change in Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
*
ns
HT29
HT29-L. lactis
HT29-Sgg
HCT116
HCT116-L. lactis
HCT116-Sgg
A549
A549-L. lactis
A549-Sgg
D
DAPI
COLVI
Merged
HT29
HT29-Sgg
HCT116
HCT116-Sgg
E
+ Saline
+ L. lactis
+ Sgg

FIG. 1

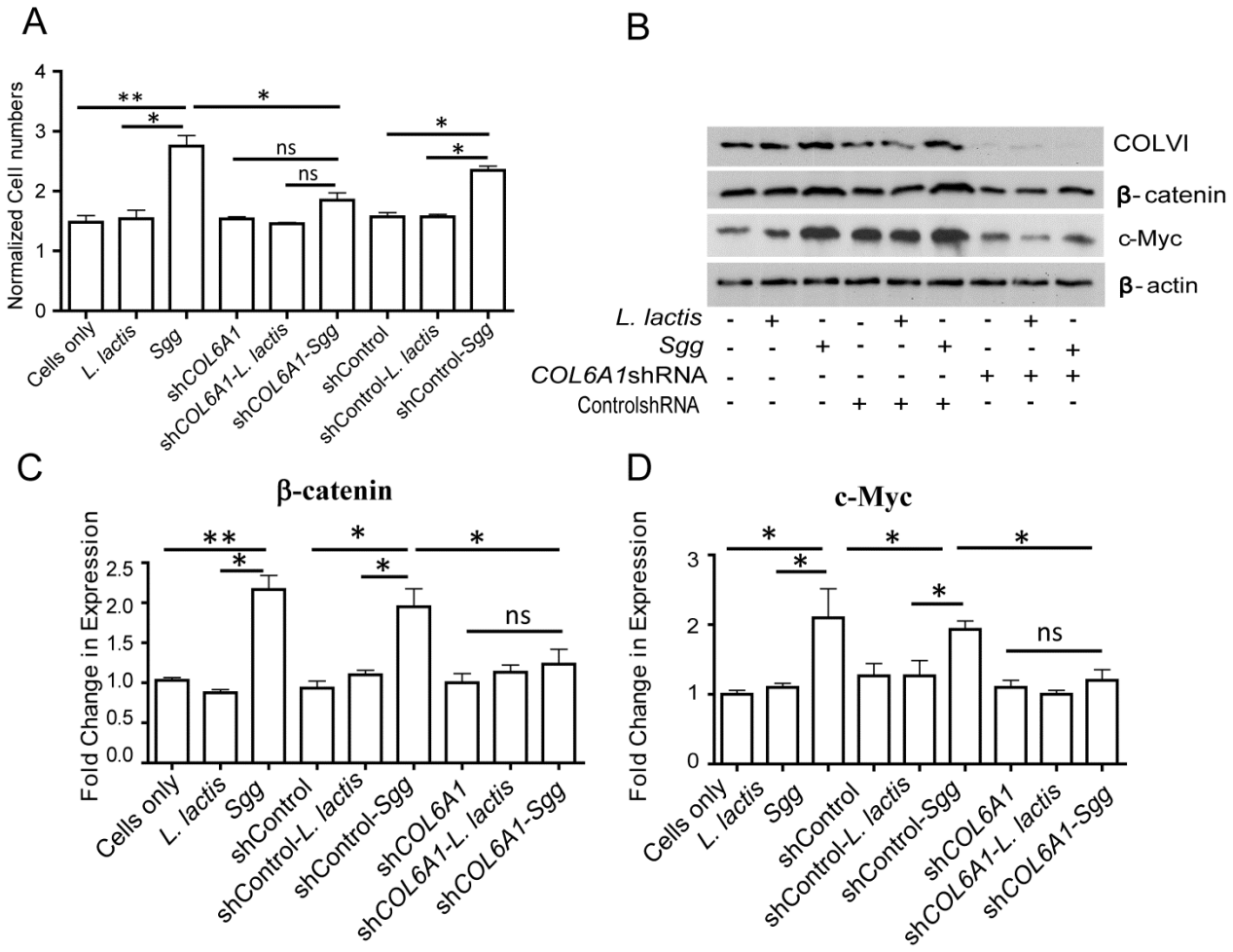

A
Normalized Cell numbers
Cells only
L. lactis
Sgg
shCOL6A1
shCOL6A1-L. lactis
shCOL6A1-Sgg
shControl
shControl-L. lactis
shControl-Sgg
ns
B
COLVI
β-catenin
c-Myc
β-actin
L. lactis
Sgg
COL6A1shRNA
ControlshRNA
C
β-catenin
Fold Change in Expression
D
c-Myc
Fold Change in Expression

FIG. 2

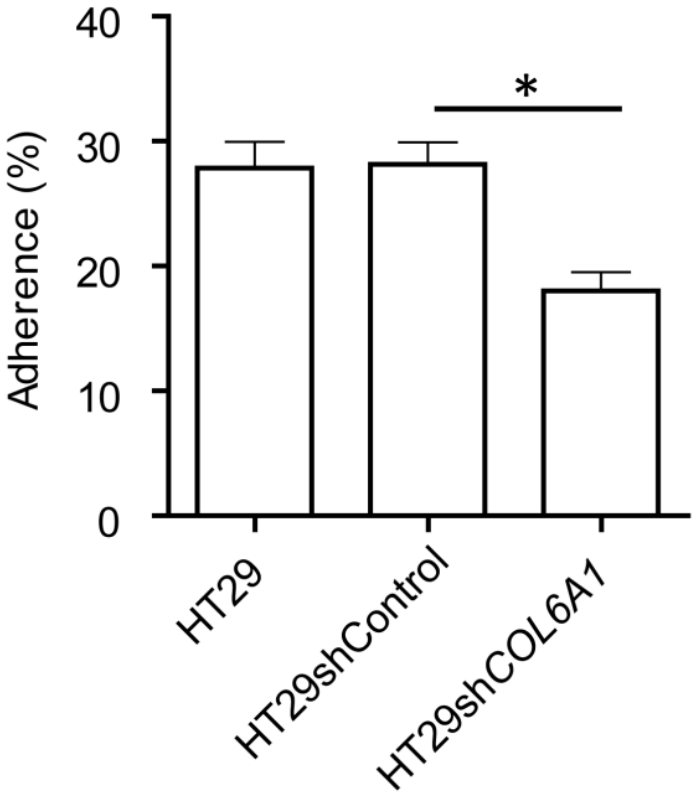


Fig. 3

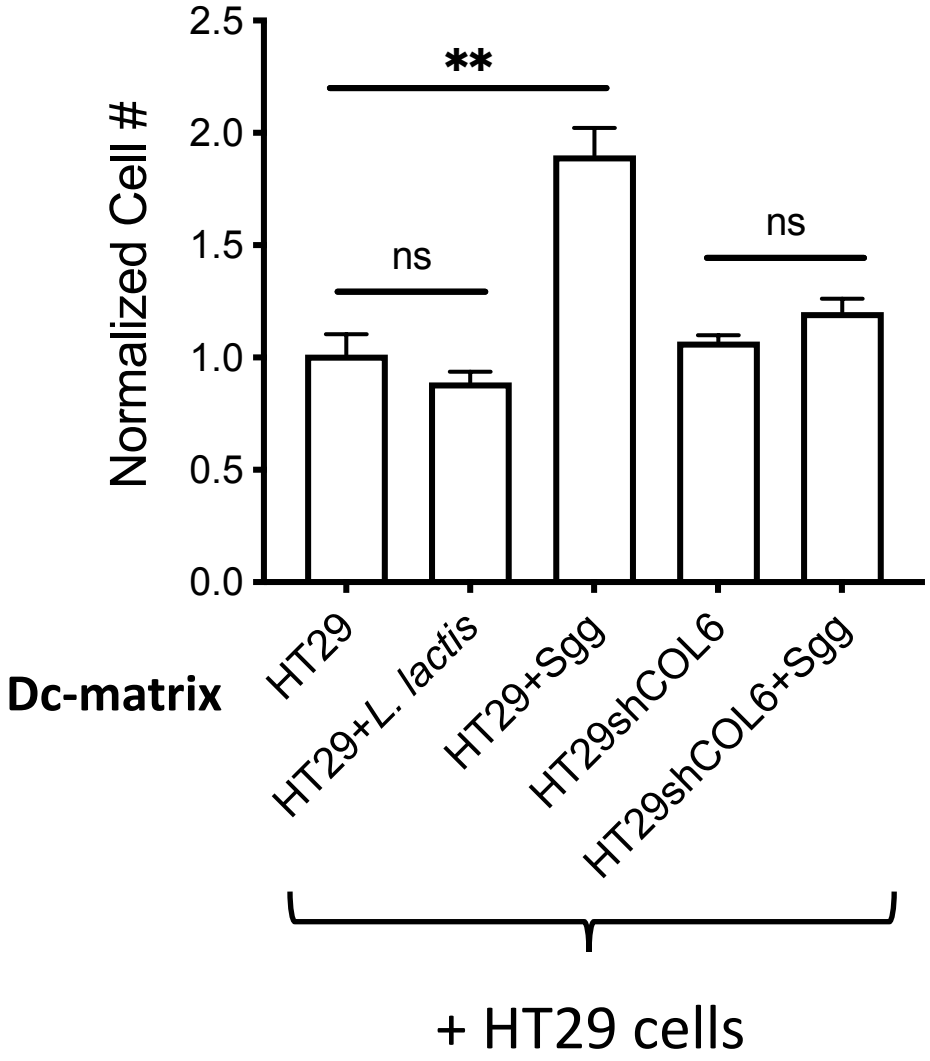

2.5
2.0
1.5
1.0
0.5
0.0
Normalized Cell #
**
ns
ns
Dc-matrix
HT29
HT29+L. lactis
HT29+Sgg
HT29shCOL6
HT29shCOL6+Sgg
+ HT29 cells

Fig. 4

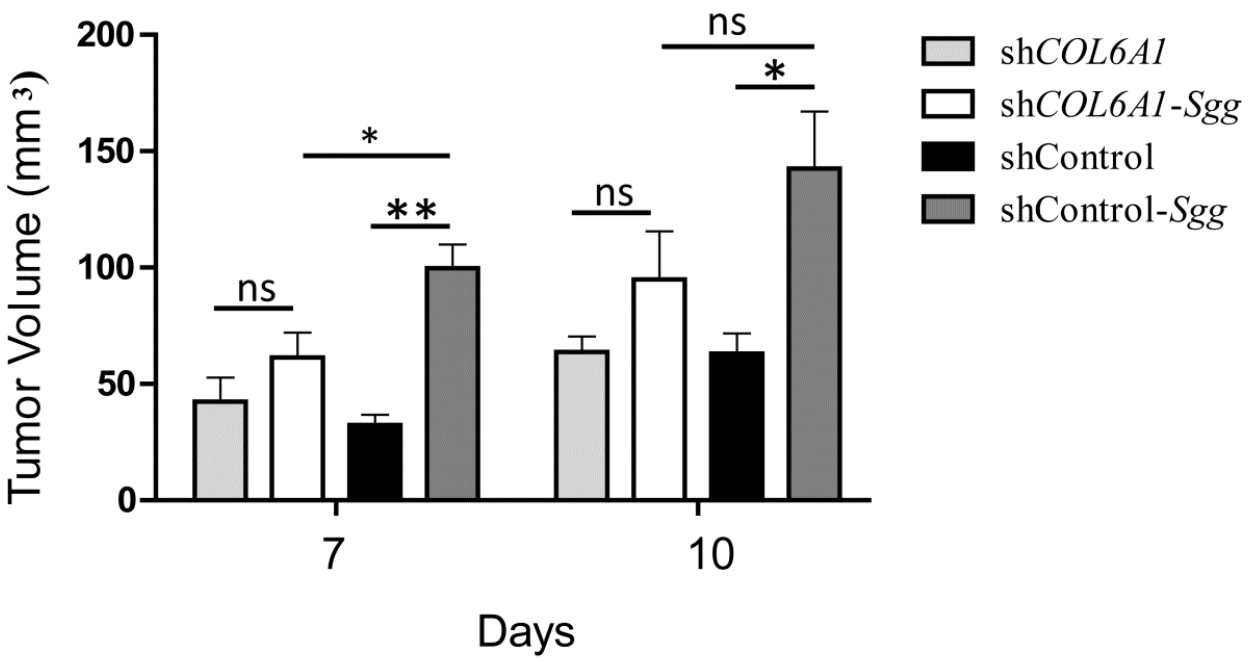
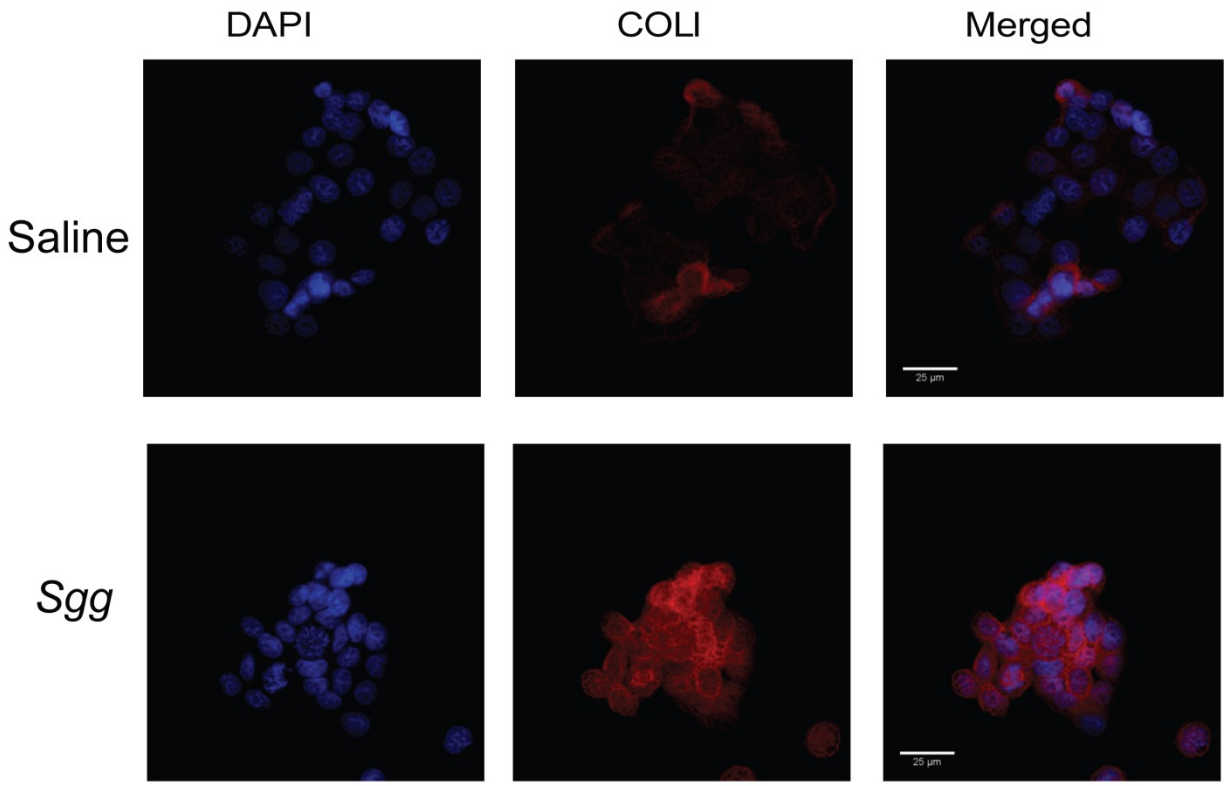
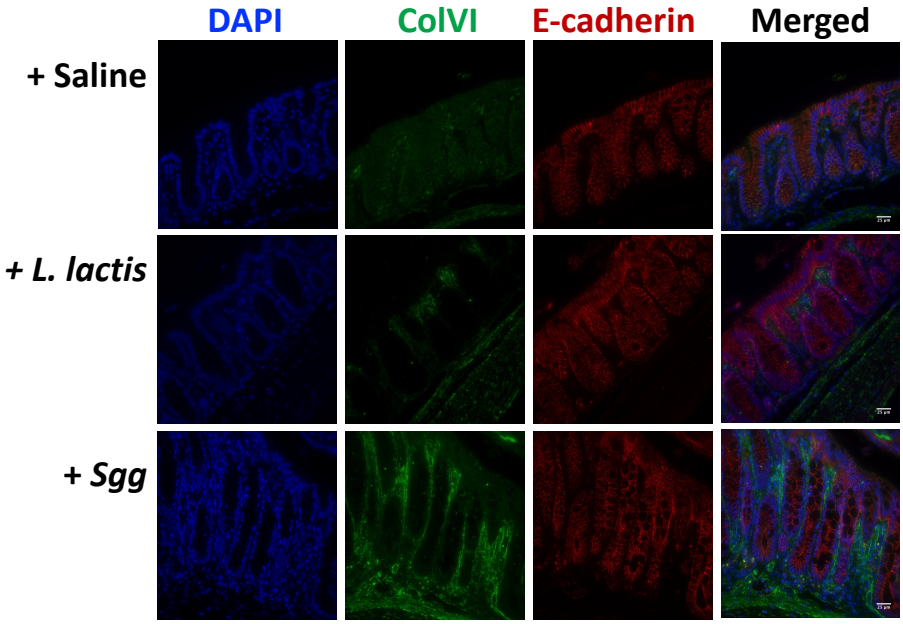

Tumor Volume (mm³)
200
150
100
50
0
ns
*
**
ns
ns
*
7
10
Days
shCOL6A1
shCOL6A1-Sgg
shControl
shControl-Sgg

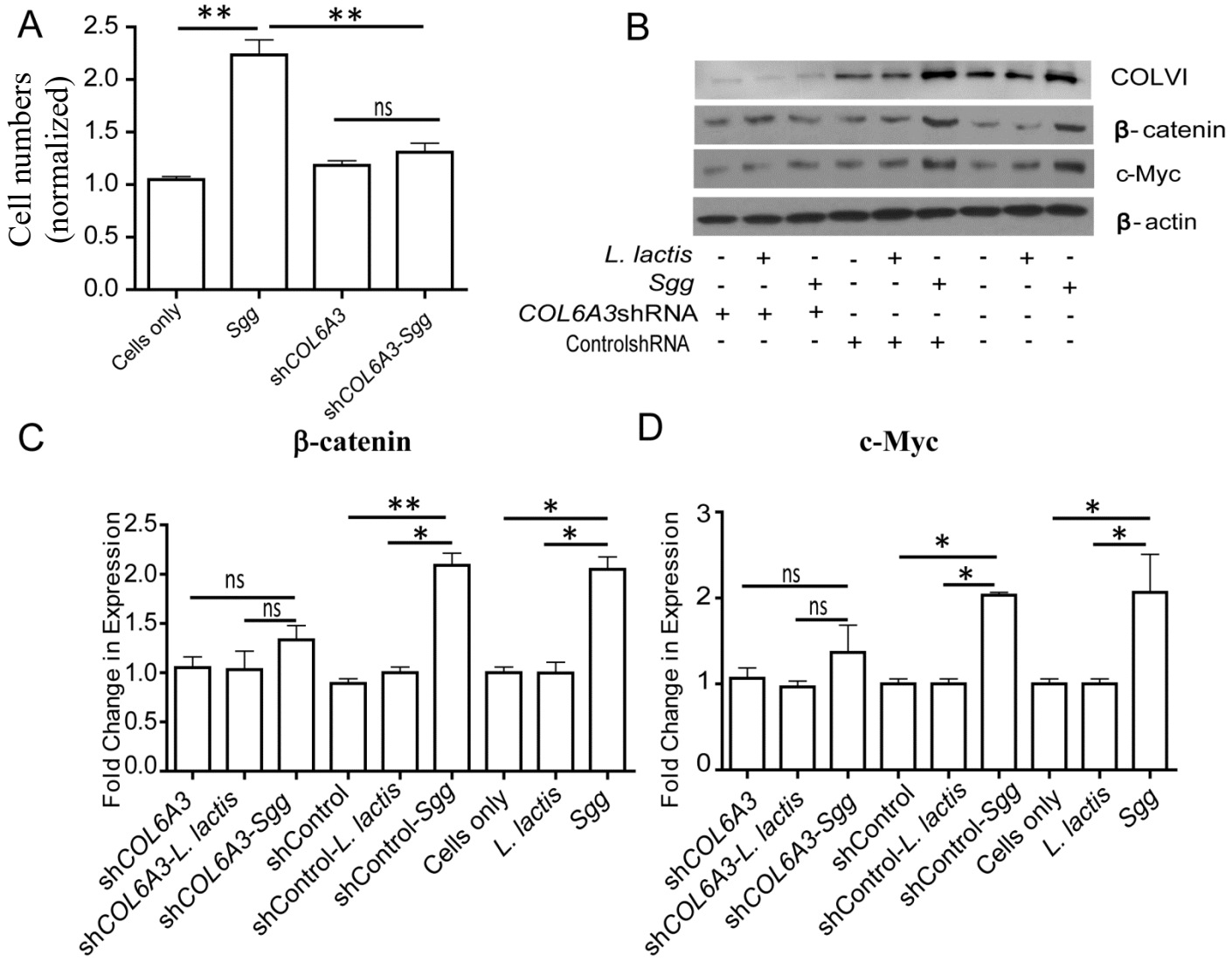
Fig. 5



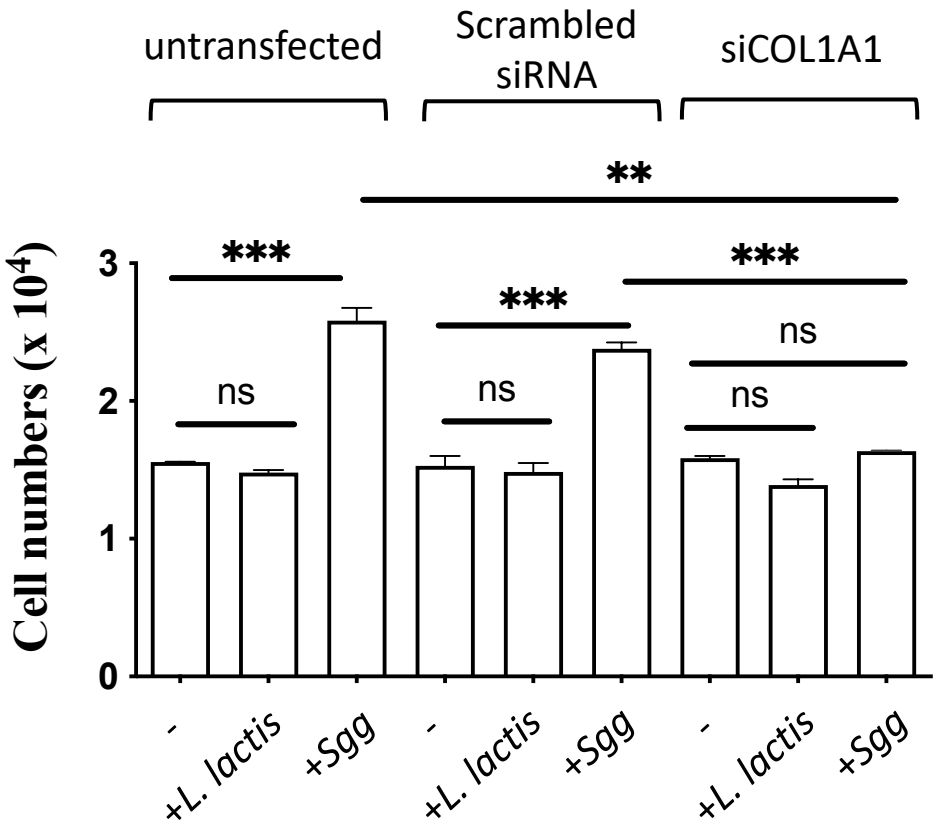
Supplemental Fig. S1. ***Sgg* upregulates type I collagen.** HT29 cells were co-cultured with *Sgg* TX20005 or media only for 12 hours. Cells were washed, fixed, incubated with anti-ColI antibody and counterstained with DAPI.



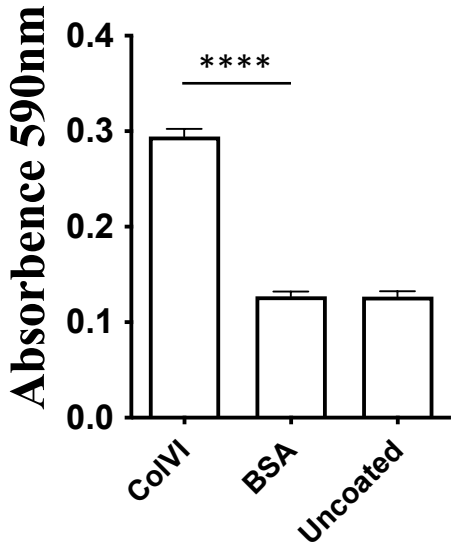
Supplemental Fig. S2. ***Sgg* upregulates type VI collagen *in vivo*.** A/J mice were administered with 4 weekly i.p. injections of AOM, followed by treatment with ampicillin for 1 week and then weekly oral gavage of bacteria (*Sgg* and *L. lactis*, respectively) or saline for 12 weeks. Colons were harvested one week after the last bacterial gavage, swiss-rolled, fixed with meth-carn, embedded and sectioned. Colon sections were incubated with antibodies against COLVI and E-cadherin (to indicate colonic epithelial cells), followed by appropriate secondary antibodies and counterstained with DAPI.



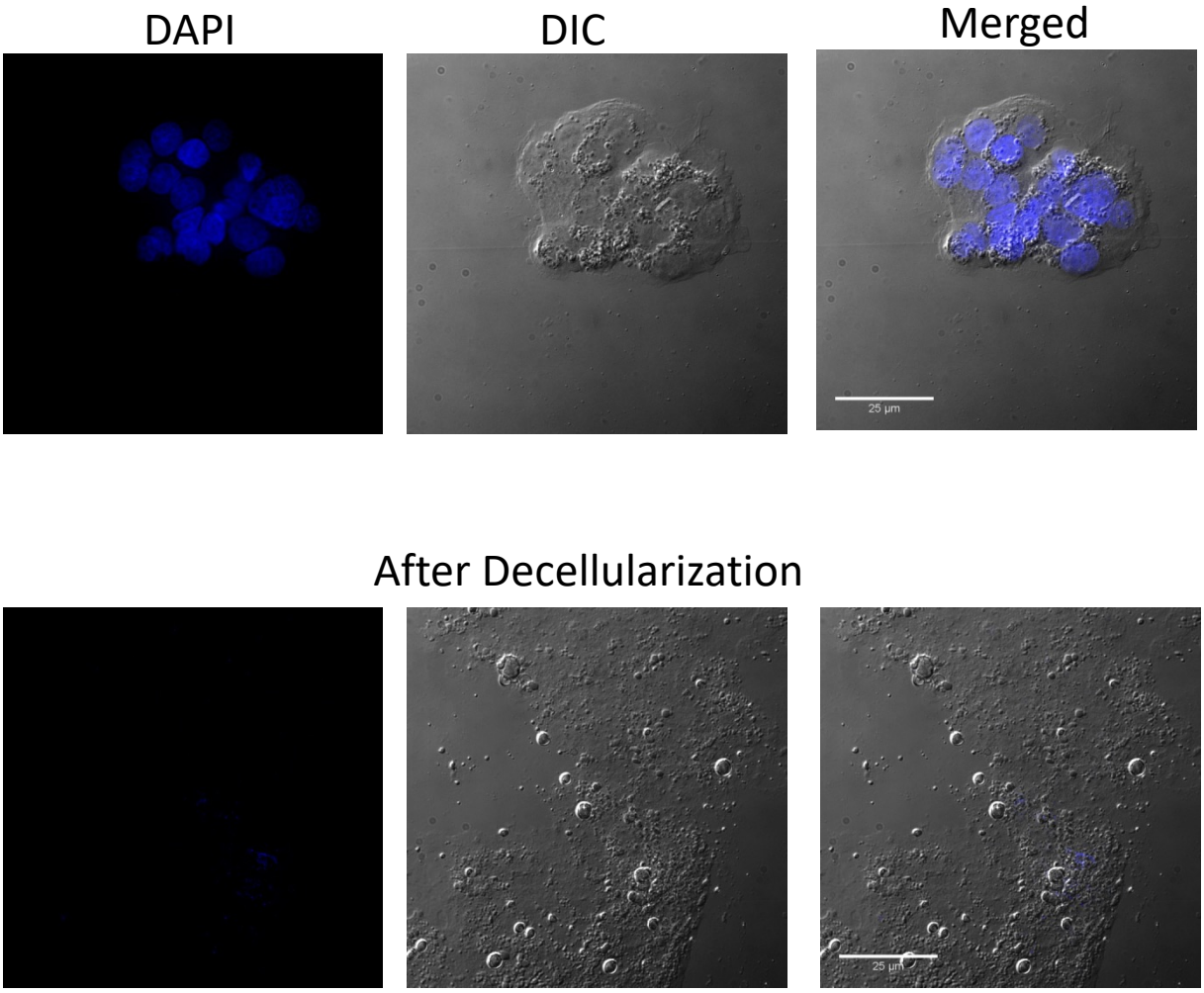
Supplemental Fig. S3. **Knockdown of Col6A3 rendered *Sgg* unable to stimulate cell proliferation.** The experiments were carried out as described in Materials and Methods section. **A. Cell proliferation assay.** HT29 cells and COL6A3 stable knockdown HT29 cells were incubated with *Sgg* TX20005 or media only for 24 hours. Viable cells were counted using an automated cell counter. **B-D. Western blot** of whole cell lysates from HT29 cells, HT29 cells with control shRNA, or COL6A3 stable knockdown HT29 cells incubated with *Sgg* TX20005 or *L. lactis* for 12 hours. Band intensity from three independent experiments were quantified and normalized to β–actin. Fold change was against shCOL6A3 cells incubated in media only. Unpaired two tailed t test was used for pairwise comparison. *, $p < 0.05$; **, $p < 0.01$; ***; ns, not significant.



Supplemental Fig. S4. **Knockdown of Col1A1 rendered *Sgg* unable to stimulate HT29 cell proliferation.** Cell proliferation assay was carried out as described in the Materials and Methods section. HT29 cells were transfected with siRNA for COL1A1 or scrambled control siRNA and incubated for 24 hours. The cells were then incubated with *L. lactis* or *Sgg* TX20005 for 24 hours. Viable cells were enumerated using an automated cell counter.



Supplemental Fig. S5. ***Sgg* binds to immobilized ColVI in a dose-dependent and saturable manner.** ColVI or BSA were coated into 96-well plate at 1μg/well and blocked. Uncoated wells were incubated with PBS. Overnight cultures resuspended in PBS were then added to the immobilized proteins and incubated for 1 hour at room temperature. The wells were washed three times with PBS. Bound bacteria were detected by using the crystal violet staining method.



Supplemental Fig. S6. Decellularization of HT29 cells. HT29 cells were treated with 0.25% Triton-X and 0.25% Sodium-Deoxycholate for 5 minutes and then with DNAse and RNAse for 30 minutes, as described in Materials and Methods. The samples were washed thrice with PBS and then fixed and stained with DAPI.