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

Ritesh Kumar[1#], John Taylor[1], Jain Antrix[2], Sung Yun Jung[3], and Yi Xu[1, 4, 5*]

[1]Center for Infectious and Inflammatory Diseases, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, TX

[2]MS Proteomics Core, Baylor College of Medicine, Houston, TX

[3]Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M Health Science Center, College Station, TX

[4]Department of Microbiology and Molecular Genetics, McGovern Medical School, UT Health, Houston, TX

[5]Department of Microbiology and Molecular Genetics, McGovern Medical School, UT Health, Houston, TX

#Current affiliation: IFF Health and Biosciences

*Corresponding author:

Yi Xu

E-mail: yi-xu1@tamu.edu

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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 stimulated the proliferation of CRC cells. However, the molecular mechanism underlying this ability was unknown. The extracellular matrix (ECM) is an important regulator of cell proliferation. 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. Knockdown of ColVI abolished the ability of *Sgg* to induce host cell proliferation. Our data further showed the ECM by itself is a direct mediator of *Sgg*-induced cell proliferation. This is evidenced by the effect of decellularized matrix (dc-matrix), which is free of bacteria or cells. Dc-matrix 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 ColVI knockdown cells incubated in media only, suggesting the importance of matrix composition and property in this process. In addition, we showed that ColVI is important for *Sgg* to promote tumor growth *in vivo*. Taken together, the results reveal a novel mechanism in which *Sgg* stimulates CRC proliferation through modulation of the ECM.

Introduction

Streptococcus gallolyticus subsp. *gallolyticus* (*Sgg*) belongs to the *S. bovis* group of organisms and was previously known as *S. bovis* biotype I [1]. It is an opportunistic pathogen that causes bacteremia and infective endocarditis (IE) [2]. *Sgg* is also known to associate with CRC as documented by numerous case reports and case series over the past several decades [3-6]. A meta-analysis study of case reports and case series published up to 2011 found that among *S. bovis*-infected patients who underwent colonic evaluation, ~60% had concomitant colon adenomas/carcinomas [7]. Furthermore, patients with *Sgg* bacteremia/IE have a higher risk (~ 7 fold) for CRC compared to bacteremia/IE caused by other species in the *S. bovis* group [7], suggesting the existence of a *Sgg*-specific mechanism that promotes the strong association between *Sgg* and CRC. The prevalence of *Sgg* in CRC patients has not been investigated as extensively as the risk for CRC among patients with active *Sgg* infections. However, recent data indicate that *Sgg* was enriched in tumor tissues from CRC patients [8-10], suggesting its potential as a biomarker for CRC.

In addition to the strong clinical association between *Sgg* and CRC, studies have shown that *Sgg* stimulates the proliferation of human CRC cells and promotes the development of tumors in experimental models of CRC [9, 11-14]. *Sgg* treatment of human CRC cells led to larger tumors compared to untreated cells in a xenograft model. β-catenin signaling is required for *Sgg* stimulation of CRC cell proliferation and tumor growth in the xenograft model [9]. In an azoxymethane (AOM)-induced CRC model, mice orally gavaged with *Sgg* had significantly higher tumor burden and dysplasia grade compared to control mice. In a colitis-associated CRC model, oral gavage of *Sgg* augmented tumorigenesis in the colon. Taken together, long-standing clinical observations and recent functional studies indicate that *Sgg* not only has a strong association with

CRC but also actively promotes the development of CRC. The mechanism underlying the cancer-promoting activity of *Sgg*, however, was poorly understood.

The extracellular matrix (ECM) is an important constituent of the tumor microenvironment. It regulates fundamental cell behavior such as cell proliferation, adhesion and migration and plays important roles during normal development as well as in pathological conditions such as cancer [15, 16]. Altered ECM composition, structure and mechanical property are common features in tumor tissues and contribute to tumor progression [17-21]. In the case of CRC, multiple studies have found that various types of collagens are upregulated in tumors compared to matched normal tissues [22-29]. Whether gut microbes can provide exogenous signals to modulate ECM expression and dynamics was unknown.

In this study, we found that *Sgg* upregulates the expression of collagen *in vitro* and *in vivo*. We demonstrated that upregulation of collagen by *Sgg* is important for the promotion of CRC cell proliferation and tumor growth by *Sgg*. Moreover, we demonstrated the importance of the ECM in *Sgg*-mediated cancer cell proliferation by using decellularized matrix from CRC cells cultured under various treatment conditions. Altogether, our results suggest a novel mechanism in which *Sgg* modulation of the ECM plays an important role in regulating CRC cell proliferation. The results highlight a previously under-studied activity of gut bacteria and have important implications in the context of microbial contribution to CRC.

Results

***Sgg* increases collagen expression in human colon cancer cells and in colon tissues *in vivo*.** *Sgg* was previously shown to stimulate the proliferation of certain human colon cancer cells [9, 13]. To understand the changes in host cells induced by *Sgg*, we performed mass spectrometry-

based label-free global proteome profiling of whole cell lysates prepared from HT29 cells cultured alone or in the presence of *Sgg* strain TX20005. Strikingly, the level of several types of collagens was increased in cells cultured in the presence of *Sgg* compared to that in cells cultured alone, with type VI collagen (ColVI) showing the highest relative abundance (Supplemental Table 1). The increased expression of ColVI was confirmed at the transcription and protein level. In RT-qPCR, both COL6A1 and COL6A3 was significantly increased in the presence of *Sgg* compared to cells cultured in media only (Fig. 1A). In western blot, ColVI level was significantly increased in HT29 and HCT116 cells co-cultured with *Sgg*, compared to cells co-cultured with *L. lactis*, a non-pathogenic negative bacterial control, or in media only (Fig. 1B and 1C). Previous studies showed that *Sgg* stimulates the proliferation of HT29, but had no effect on A549 cells, a human lung cancer cell line [9]. No significant changes in ColVI were observed in A549 cells cultured in the presence of *Sgg* when compared to cells cultured in the presence of *L. lactis* or in media only (Fig. 1B and 1C). Using immunofluorescence staining we further observed that *Sgg* induced the expression of ColI (supplemental Fig. 1) and ColVI (Fig. 1D).

We next examined the effect of *Sgg* on collagen expression *in vivo* using colon sections from mice orally gavaged with *Sgg*, *L. lactis* or saline. Sections were stained with Masson's Trichrome stain which stains collagen blue [30]. The results show that colon sections from mice gavaged with *Sgg* had more intense blue staining compared to sections from mice gavaged with *L. lactis* or saline (Fig. 1E), indicating elevated level of collagen in the presence of *Sgg*. Immunofluorescence staining of the colon sections with an anti-ColVI antibody also showed more intense staining of ColVI in colon sections from *Sgg*-gavaged mice compared to control mice (Fig. 1F). Taken together, these results suggest that exposure to *Sgg* results in increased level of collagen *in vitro* and *in vivo*.

Collagen is required for *Sgg* to stimulate human colon cancer cell proliferation. Collagen has been shown to be involved in the proliferation of breast [31, 32] and prostate cancer cells [33, 34] . We investigated the role of collagen in *Sgg*'s stimulation of colon cancer cell proliferation. HT29 COL6A1 and COL6A3 stable knockdown cells were generated. The ability of *Sgg* to stimulate the proliferation of either COL6A1 (Fig. 2A) or COL6A3 (Supplemental Fig. S2A) knockdown cells was significantly reduced compared to that in untransfected cells or cells transfected with control shRNA. *Sgg* was shown to upregulates β-catenin and c-Myc and the pro-proliferative ability is β-catenin-dependent [9]. Knockdown of COL6A1 (Fig. 2B-2D) or COL6A3 (Supplemental Fig. S2B-S2D) abolished the *Sgg*-induced upregulation of β-catenin or c-Myc, suggesting that ColVI acts upstream of β-catenin in the signaling cascade that leads to *Sgg*-induced cell proliferation.

Decellularized matrix (dc-matrix) derived from *Sgg*-treated cells alone is sufficient to promote cancer cell proliferation. Previous studies indicated that *Sgg* has the ability to attach to several types of collagens [35, 36]. We tested if knockdown of collagen affects Sgg adherence to host cells. The results showed that *Sgg* adherence to COL6A1 knockdown cells is significantly reduced (Fig. 3). The requirement for collagen in *Sgg*-induced cell proliferation could be due to increased collagen deposition in the matrix which results in matrix-induced host cell signaling or/and to its effect on *Sgg* adherence. To investigate if upregulation of ECM molecules by *Sgg* can directly contribute to cell proliferation, dc-matrix was prepared from HT29 cells cultured in media only, and in the presence of *L. lactis* or *Sgg*. We confirmed that no live bacteria were present in the dc-matrix by incubating dc-matrix in antibiotics-free media for 24 hours. No bacterial growth was observed. We also confirmed that no intact cells remained after the decellularization procedure by staining the samples with DAPI (Supplemental Fig. S3). HT29 cells were then seeded onto the

various dc-matrix and incubated in antibiotics-containing media for 24 hours. The dc-matrix prepared from *Sgg*-treated cells stimulated cell proliferation significantly better than dc-matrix from HT29 cells alone or from *L. lactis*-treated cells (Fig. 4), suggesting that the involvement of ECM in *Sgg*-induced cell proliferation includes a direct effect independent of its influence on *Sgg* adherence. Furthermore, the experiment was also performed with COL6A1 knockdown cells. Dc-matrix from *Sgg*-treated COL6A1 cells showed no difference in the ability to stimulate cell proliferation compared to dc-matrix prepared from COL6A1 cells alone (Fig. 4). These results suggest that dc-matrix from *Sgg*-treated cells can stimulate cell proliferation in a manner that does not require live *Sgg* but depends on collagen.

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

Discussion

It is well appreciated that certain gut microbes or microbial communities play important roles in influencing the development of CRC. Long-standing and recent data suggest that *Sgg* is strongly associated with CRC and promotes tumor growth, however the molecular mechanism underlying *Sgg*'s effect was poorly understood. The ECM is a component of the tumor microenvironment and an important contributor to tumor progression. However, insights on the impact of gut microbes on ECM are very limited. Data presented in this study demonstrate that *Sgg* upregulates collagen synthesis. Furthermore, we provide evidence that *Sgg* modulation of the ECM is important for *Sgg*-induced cell proliferation *in vitro* and *in vivo*. To the best of our knowledge, this is the first report that connects a gut microbe with ECM in the context of microbial contribution to cancer.

Our results showed that *Sgg* upregulates the expression of collagen *in vitro* and *in vivo*. We focused on ColVI since it shows the highest relative abundance revealed by MS analysis. ColVI is also implicated in human CRC in previous studies where its level is higher in tumor tissues compared to matched normal tissues [22-24]. We observed that depletion of COL6A1 or COL6A3 rendered the cells insensitive to the proliferation-promoting effect of *Sgg*, while did not affect cell proliferation in the absence of *Sgg* within the time frame of the experiment. Upregulation of β-catenin or c-Myc by *Sgg* is also abolished in these knockdown cells, suggesting that ColVI is required for *Sgg* to stimulate CRC cell proliferation and is upstream of β-catenin. Furthermore, we demonstrated that ColVI is also important for tumor growth *in vivo*.

Our results further suggested that collagen contributes to the ability of *Sgg* to stimulate CRC cell proliferation in multiple ways. Previous studies suggested that close proximity of *Sgg* to host cells is important for the pro-proliferative effect of *Sgg* [9, 11]. Knockdown of COL6A1

impaired the adherence of *Sgg*, suggesting that ColVI can influence the ability of *Sgg* to induce cell proliferation by affecting its adherence. More importantly, our data also showed that collagen can have a direct effect on *Sgg*-induced cell proliferation. This is supported by results using dc-matrix. Our results showed that dc-matrix prepared from HT29 cells that had been co-cultured with *Sgg* was able to significantly increase cell proliferation compared with dc-matrix from cells cultured alone or with *L. lactis*. On the other hand, dc-matrix from *Sgg* treated COL6A1 knockdown cells showed no difference from that prepared from untreated cells. These results suggest that *Sgg* modulation of the ECM directly contributes to the pro-proliferative activity of this organism.

ColVI has a unique supramolecular structure among the members of the collagen family. Its beaded microfilament structure enables it to bind to other components of the ECM such as ColI and ColII and acts as a bridging molecule [37-39]. Thus, depletion of ColVI may affect the overall organization of the ECM [16, 40, 41]. ColVI is special in the sense that a C-terminal cleavage product of COL6A3 chain (endotrophin (ETP)) was found to augment breast tumor growth [42]. ETP is soluble. Our results using dc-matrix speaks against a role of ETP in increased CRC cell proliferation caused by *Sgg*. In addition, we found that knockdown of ColI also abolished the ability of *Sgg* to stimulate CRC cell proliferation. This, combined with the MS result showing increased deposition of several types of collagen in the matrix from cells co-cultured with *Sgg*, suggests that the effect we observed is not ColVI-specific. It is likely that increased deposition of collagen into the matrix changed the composition of the matrix, resulting in enhanced signaling of collagen receptors. Increased deposition of collagen into the matrix may also affect the mechanical property of the matrix. A stiffer matrix can promote cell proliferation by mechanotransduction

[43]. Identifying the downstream signaling pathway involved will shed light on how the ECM contributes to the effect of *Sgg*.

The molecular mechanisms used by other microbes to drive the development of CRC can be loosely grouped into the following categories: 1) by producing genotoxins that directly induce DNA damage in colonic epithelial cells, 2) by modulating host immune responses to generate a microenvironment favorable for CRC, and 3) by shifting host metabolism to support tumor growth [44-52]. Our results suggest a new way by which microbes influence tumor development - by modulating the ECM. In this study, we focused on the effect of *Sgg* on CRC cells. *In vivo*, fibroblasts are major producers of ECM molecules. Tumor-associated macrophages were also shown to regulate the synthesis and assembly of collagenous matrix [53]. Currently the effect of *Sgg* on fibroblasts and macrophages is unknown. It is possible that *Sgg* may also regulate ECM remodeling by engaging fibroblasts and immune cells *in vivo*.

In conclusion, *Sgg* has a long-standing clinical association with CRC and actively promotes tumor development. This study provides the first experimental evidence for *Sgg* modulation of the ECM and a direct role of the ECM in *Sgg*-induced cell proliferation and tumor growth. The results presented here highlight a new way by which microbes contribute to the development of CRC.

Materials and Methods

Bacterial and cell culture conditions. *Sgg* strain TX20005 and *Lactococcus lactis* MG1363 were cultured as described previously [9]. Human colon cancer cell line HT29 and HCT116 were cultured in DMEM/F-12 HEPES (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA). Human lung carcinoma cell line A549 was maintained in

F12-K media supplemented with 10% FBS. All of the cells were cultured in a humidified incubation chamber at 37ºC with 5% CO_2.

Cell proliferation assays. This was performed as described previously [9]. Briefly, cells (~$1x10^4$ cells/well) were cultured in the presence of stationary phase bacteria (*Sgg* or *L. lactis*) at a multiplicity of infection (MOI) of 1 or media only for 24 hours. Trimethoprim was added at 50 µg/ml final concentration after 6 hours of co-culture to prevent media acidification due to bacterial growth. Cells were detached by trypsin treatment and counted in a Cellometer Mini automated cell counter (Nexcelome Biosciences, Lawrence, MA).

Collagen knockdown. To generate stable knockdown cells, lentiviral plasmids containing COL6A1 or COL6A3 short hairpin RNA (shRNA) (Sigma-Aldrich, TRCN0000116959 and TRCN0000003622), or MISSION pLKO.1-puro Non-Mammalian shRNA Control (Sigma-Aldrich, SHC002) were first transfected into HEK293T cells to produce lentiviral particles. HT29 cells were then infected with the respective lentiviral particles and selected with puromycin (1µg/ml). Gene knockdown was confirmed by western blot assays. Transient knockdown of COL1A1 was carried out using specific siRNA for COLA1 (ThermoFisher).

Decellularization of matrix HT29 cells were decellularized following a protocol described previously [54]. Briefly, cells were washed thrice with ice-cold PBS containing a cocktail of protease inhibitors (GenDEPOT). The cells were incubated in a PBS solution containing 0.25% Triton X and 0.25% sodium-deoxycholate in PBS for 5 minutes. The matrix was gently washed in PBS with 100 mg/mL RNAse A (Roche) and 10 IU/mL DNAse (Sigma) followed by three washes in PBS.

Quantitative reverse transcription PCR (RT-qPCR). HT29 cells were co-cultured with *Sgg* for 6 hrs. Total RNA was extracted from co-cultured cells using the RNeasy Kit (QIAGEN).

cDNA was generated by using the Transcriptor First Strand cDNA Synthesis Kit (Roche). qPCR was performed using FastStart SYBR green master mix (Roche) in a Viia 7 Real Time PCR System (Applied Biosystems). The following cycle conditions were used: 95˚C for 10 minutes followed by 40 cycles at 95˚C for 30 seconds and 60˚C for 1 minute. CT values were first normalized to GAPDH then to cells cultured in media only.

Western blot assays. This was performed as described previously [9]. Briefly, cells were cultured in the appropriate medium in the presence or absence of bacteria for 12 hours. Cells were washed with sterile PBS three times and lysed. Total cell lysates were subjected to SDS-gel electrophoresis and western blot. Rabbit polyclonal antibodies against ColVI (1:500, Abcam), β-catenin (1:4000, Cell Signaling Technology (CST)), c-Myc (1:3000, Abcam), and β-actin (1:5000, CST) were used. Horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (CST) was used as the secondary antibody. Signals were detected using HyGLO, chemiluminescent HRP (Denville, Mteuchen, NJ). Band intensity was quantified using Image J.

Adherence Assay. This was performed as described previously [9]. Briefly, HT29 cells were incubated with or without bacteria at an MOI of 10 for 1 hour. The wells were washed three times with sterile PBS to remove unbound bacteria. To determine the number of bound bacteria, cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. Adherence was expressed as a percentage of total bacteria added.

Animal experiment. Animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Texas A&M Health Science Center, Institute of Biosciences and Technology. The xenograft model experiment was performed as described previously [9]. Tumor diameters were measured with a digital caliper, and tumor

volume calculated using the formula: Volume = (d1xd1xd2)/ 2, with d1 being the larger dimension (Sanceau et al,2002).

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

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Figure Legends

Fig. 1. *Sgg* upregulates collagen expression. A. HT29 cells were co-cultured with *Sgg* (strain TX20005) or media only for 6 hours. RNA is extracted and analyzed by RT-qPCR. **B-C**. HT29, HCT116 and A549 cells were co-cultured with *Sgg*, *L. lactis*, or media only for 12 hours. Cell lysates were subject to western blot with anti-ColVI antibody. Band intensity was quantified using Image J and normalized to GAPDH. **D**. HT29 and HCT116 were co-cultured with *Sgg* or media only for 12 hours. Cells were washed, fixed, incubated with anti-ColVI antibody and counterstained with DAPI. **E** and **F.** A/J mice were administered with 4 weekly i.p. injections of AOM, followed by treatment with ampiliclin for 1 week and then weekly oral gavage of bacteria (*Sgg* and *L. lactis*, respectively) or saline for 12 weeks, as previously described [11, 14]. **E**. Mason's Trichrome stains of colon sections. Collagen is stained blue. **F**. Immunofluorescence staining of colon sections with an anti-COLVI antibody. Representative images are shown. Scale bars represent 25μm. Statistical analysis in A and C was done using unpaired, two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$.

Fig. 2. *Sgg* promotes cell proliferation in a collagen VI dependent manner. **A**. Knockdown of COL6A1 abolished the effect of *Sgg* on cell proliferation. Cell proliferation assays were performed as described in the Materials and Methods section. Untransfected HT29 cells, *COL6A1* stable knockdown HT29 cells or HT29 cells transfected with a control shRNA were used. **B-D**. *Sgg* or *L. lactis* were added to cells (~10^5 cfu/well) as described in the Materials and Methods section and incubated for 12 hours. Total cell lysates were subject to western blot assays to compare COLVI, β-catenin, and c-Myc protein levels. Representative images are shown (**B**). Band intensity was quantified using Image J, normalized to β-actin first and then to the cells only control (**C-D**). Data

are presented as the mean ± SEM. Each experiment was repeated at least three times. Unpaired, two-tailed *t* test was used for statistical analysis. *, $p < 0.05$; **, $p < 0.01$.

Fig. 3. Collagen knockdown impaired the adherence of *Sgg*. HT29 cells, HT29 cells transfected with a control shRNA or COL6A1 stable knockdown HT29 cells were incubated with TX20005 (MOI=10) as described in the Materials and Methods section. Adherence was calculated as the percentage of adhered bacteria vs. total bacterial added and combined from three independent experiments. Mean ± SEM is presented. Unpaired, two-tailed *t* test was used for statistical analysis. *, $p < 0.05$.

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

Fig. 5. Collagen knockdown impairs the ability of *Sgg* to promote tumor growth *in vivo*. 1 x 10^6 HT29sh*COL6A1* or HT29shcontrol cells were treated with *Sgg* or no bacteria, mixed with Matrigel and injected into the dorsal flap of nude mice (n = 5/group). Tumor size was measured at the indicated time point with a digital caliper. Data is presented as the mean ± SEM. Statistical analysis was done using unpaired, two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$.

Supplemental Table S1. Relative abundance of various types of collagen in whole cell 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

[a] HT29 cells cultured in the presence or absence of *Sgg* strain TX20005 (3 biological replicates) were lysed and digested in ammonium bicarbonate buffer and trypsin. The resulting peptide mixtures were analyzed by a nanoLC-1200 system coupled to an Orbitrap Fusion Lumos mass spectrometer. Only the collagen types that show increase in all three biological replicates are listed.

[b] Relative protein abundance is shown as the mean iFOT ± SEM. iFOT is the normalization of individual protein intensity to the total protein intensity within one experiment.

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

[d] Only detected in one biological replicate.

[e] Statistical comparison between HT29 + *Sgg* and HT29 was not performed due to the inability to detect collagen peptide chains in HT29.

References

1. Schlegel L, Grimont F, Ageron E, Grimont PA, Bouvet A. Reappraisal of the taxonomy of the Streptococcus bovis/Streptococcus equinus complex and related species: description of Streptococcus gallolyticus subsp. gallolyticus subsp. nov., S. gallolyticus subsp. macedonicus subsp. nov. and S. gallolyticus subsp. pasteurianus subsp. nov. Int J Syst Evol Microbiol. 2003;53(Pt 3):631-45.

2. Jans C, Boleij A. The Road to Infection: Host-Microbe Interactions Defining the Pathogenicity of Streptococcus bovis/Streptococcus equinus Complex Members. Frontiers in microbiology. 2018;9:603.

3. Abdulamir AS, Hafidh RR, Bakar FA. The association of Streptococcus bovis/gallolyticus with colorectal tumors: The nature and the underlying mechanisms of its etiological role. J Exp Clin Cancer Res. 2011;30:11.

4. Boleij A, Tjalsma H. The itinerary of Streptococcus gallolyticus infection in patients with colonic malignant disease. Lancet Infect Dis. 2013;13(8):719-24.

5. Gupta A, Madani R, Mukhtar H. Streptococcus bovis endocarditis, a silent sign for colonic tumour. Colorectal Dis. 2010;12(3):164-71.

6. Burnett-Hartman AN, Newcomb PA, Potter JD. Infectious agents and colorectal cancer: a review of Helicobacter pylori, Streptococcus bovis, JC virus, and human papillomavirus. Cancer Epidemiol Biomarkers Prev. 2008;17(11):2970-9.

7. Boleij A, van Gelder MM, Swinkels DW, Tjalsma H. Clinical Importance of Streptococcus gallolyticus infection among colorectal cancer patients: systematic review and meta-analysis. Clin Infect Dis. 2011;53(9):870-8.

8. Abdulamir AS, Hafidh RR, Bakar FA. Molecular detection, quantification, and isolation of Streptococcus gallolyticus bacteria colonizing colorectal tumors: inflammation-driven potential of carcinogenesis via IL-1, COX-2, and IL-8. Mol Cancer. 2010;9:249.

9. Kumar R, Herold JL, Schady D, Davis J, Kopetz S, Martinez-Moczygemba M, et al. Streptococcus gallolyticus subsp. gallolyticus promotes colorectal tumor development. PLoS Pathog. 2017;13(7):e1006440.

10. Thomas AM, Manghi P, Asnicar F, Pasolli E, Armanini F, Zolfo M, et al. Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation. Nat Med. 2019;25(4):667-78.

11. Kumar R, Herold JL, Taylor J, Xu J, Xu Y. Variations among Streptococcus gallolyticus subsp. gallolyticus strains in connection with colorectal cancer. Scientific reports. 2018;8(1):1514.

12. Zhang Y, Weng Y, Gan H, Zhao X, Zhi F. Streptococcus gallolyticus conspires myeloid cells to promote tumorigenesis of inflammatory bowel disease. Biochem Biophys Res Commun. 2018;506(4):907-11.

13. Taddese R, Garza DR, Ruiter LN, de Jonge MI, Belzer C, Aalvink S, et al. Growth rate alterations of human colorectal cancer cells by 157 gut bacteria. Gut Microbes. 2020;12(1):1-20.

14. Taylor JC, Gao X, Xu J, Holder M, Petrosino J, Kumar R, et al. A type VII secretion system of Streptococcus gallolyticus subsp. gallolyticus contributes to gut colonization and the development of colon tumors. PLoS Pathog. 2021;17(1):e1009182.

15. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014;15(12):786-801.

16. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139(5):891-906.

17. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. EMBO Rep. 2014;15(12):1243-53.

18. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

19. Crotti S, Piccoli M, Rizzolio F, Giordano A, Nitti D, Agostini M. Extracellular Matrix and Colorectal Cancer: How Surrounding Microenvironment Affects Cancer Cell Behavior? J Cell Physiol. 2017;232(5):967-75.

20. Naba A, Clauser KR, Hoersch S, Liu H, Carr SA, Hynes RO. The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Mol Cell Proteomics. 2012;11(4):M111 014647.

21. Naba A, Clauser KR, Whittaker CA, Carr SA, Tanabe KK, Hynes RO. Extracellular matrix signatures of human primary metastatic colon cancers and their metastases to liver. BMC Cancer. 2014;14:518.

22. Smith MJ, Culhane AC, Donovan M, Coffey JC, Barry BD, Kelly MA, et al. Analysis of differential gene expression in colorectal cancer and stroma using fluorescence-activated cell sorting purification. British journal of cancer. 2009;100(9):1452-64.

23. Qiao J, Fang CY, Chen SX, Wang XQ, Cui SJ, Liu XH, et al. Stroma derived COL6A3 is a potential prognosis marker of colorectal carcinoma revealed by quantitative proteomics. Oncotarget. 2015;6(30):29929-46.

24. Clarke CN, Lee MS, Wei W, Manyam G, Jiang ZQ, Lu Y, et al. Proteomic Features of Colorectal Cancer Identify Tumor Subtypes Independent of Oncogenic Mutations and Independently Predict Relapse-Free Survival. Ann Surg Oncol. 2017;24(13):4051-8.

25. Rolff HC, Christensen IJ, Vainer B, Svendsen LB, Eefsen RL, Wilhelmsen M, et al. The Prognostic and Predictive Value of Soluble Type IV Collagen in Colorectal Cancer: A Retrospective Multicenter Study. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22(10):2427-34.

26. Zhang Z, Fang C, Wang Y, Zhang J, Yu J, Zhang Y, et al. COL1A1: A potential therapeutic target for colorectal cancer expressing wild-type or mutant KRAS. Int J Oncol. 2018;53(5):1869-80.

27. Huang H, Li T, Ye G, Zhao L, Zhang Z, Mo D, et al. High expression of COL10A1 is associated with poor prognosis in colorectal cancer. Onco Targets Ther. 2018;11:1571-81.

28. Jensen C, Nielsen SH, Mortensen JH, Kjeldsen J, Klinge LG, Krag A, et al. Serum type XVI collagen is associated with colorectal cancer and ulcerative colitis indicating a pathological role in gastrointestinal disorders. Cancer medicine. 2018;7(9):4619-26.

29. Shang J, Wang F, Chen P, Wang X, Ding F, Liu S, et al. Co-expression Network Analysis Identified COL8A1 Is Associated with the Progression and Prognosis in Human Colon Adenocarcinoma. Dig Dis Sci. 2018;63(5):1219-28.

30. Gomori G. A rapid one-step trichrome stain. Am J Clin Pathol. 1950;20(7):661-4.

31. JingSong H, Hong G, Yang J, Duo Z, Li F, WeiCai C, et al. siRNA-mediated suppression of collagen type iv alpha 2 (COL4A2) mRNA inhibits triple-negative breast cancer cell proliferation and migration. Oncotarget. 2017;8(2):2585-93.

32. Zhu X, Rao X, Yao W, Zou X. Downregulation of MiR-196b-5p impedes cell proliferation and metastasis in breast cancer through regulating COL1A1. Am J Transl Res. 2018;10(10):3122-32.

33. Koenig A, Mueller C, Hasel C, Adler G, Menke A. Collagen type I induces disruption of E-cadherin-mediated cell-cell contacts and promotes proliferation of pancreatic carcinoma cells. Cancer Res. 2006;66(9):4662-71.

34. Kiefer JA, Farach-Carson MC. Type I collagen-mediated proliferation of PC3 prostate carcinoma cell line: implications for enhanced growth in the bone microenvironment. Matrix Biol. 2001;20(7):429-37.

35. Sillanpaa J, Nallapareddy SR, Singh KV, Ferraro MJ, Murray BE. Adherence characteristics of endocarditis-derived Streptococcus gallolyticus ssp. gallolyticus (Streptococcus bovis biotype I) isolates to host extracellular matrix proteins. FEMS Microbiol Lett. 2008;289(1):104-9.

36. Sillanpaa J, Nallapareddy SR, Qin X, Singh KV, Muzny DM, Kovar CL, et al. A collagen-binding adhesin, Acb, and ten other putative MSCRAMM and pilus family proteins of Streptococcus gallolyticus subsp. gallolyticus (Streptococcus bovis Group, biotype I). J Bacteriol. 2009;191(21):6643-53.

37. Bonaldo P, Russo V, Bucciotti F, Doliana R, Colombatti A. Structural and functional features of the alpha 3 chain indicate a bridging role for chicken collagen VI in connective tissues. Biochemistry. 1990;29(5):1245-54.

38. Bidanset DJ, Guidry C, Rosenberg LC, Choi HU, Timpl R, Hook M. Binding of the proteoglycan decorin to collagen type VI. J Biol Chem. 1992;267(8):5250-6.

39. Cescon M, Gattazzo F, Chen P, Bonaldo P. Collagen VI at a glance. Journal of cell science. 2015;128(19):3525-31.

40. Du J, Zu Y, Li J, Du S, Xu Y, Zhang L, et al. Extracellular matrix stiffness dictates Wnt expression through integrin pathway. Scientific reports. 2016;6:20395.

41. Tan F, Huang Y, Pei Q, Liu H, Pei H, Zhu H. Matrix stiffness mediates stemness characteristics via activating the Yes-associated protein in colorectal cancer cells. J Cell Biochem. 2018.

42. Park J, Scherer PE. Adipocyte-derived endotrophin promotes malignant tumor progression. J Clin Invest. 2012;122(11):4243-56.

43. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. Nat Rev Mol Cell Biol. 2014;15(12):802-12.

44. Goodwin AC, Destefano Shields CE, Wu S, Huso DL, Wu X, Murray-Stewart TR, et al. Polyamine catabolism contributes to enterotoxigenic Bacteroides fragilis-induced colon tumorigenesis. Proc Natl Acad Sci U S A. 2011;108(37):15354-9.

45. Arthur JC, Perez-Chanona E, Muhlbauer M, Tomkovich S, Uronis JM, Fan TJ, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science. 2012;338(6103):120-3.

46. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009;15(9):1016-22.

47. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14(2):207-15.

48. Housseau F, Wu S, Wick EC, Fan H, Wu X, Llosa NJ, et al. Redundant innate and adaptive sources of IL-17 production drive colon tumorigenesis. Cancer Res. 2016.

49. Chung L, Thiele Orberg E, Geis AL, Chan JL, Fu K, DeStefano Shields CE, et al. Bacteroides fragilis Toxin Coordinates a Pro-carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells. Cell Host Microbe. 2018;23(2):203-14 e5.

50. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nature reviews Microbiology. 2014;12(10):661-72.

51. von Frieling J, Fink C, Hamm J, Klischies K, Forster M, Bosch TCG, et al. Grow With the Challenge - Microbial Effects on Epithelial Proliferation, Carcinogenesis, and Cancer Therapy. Frontiers in microbiology. 2018;9:2020.

52. Donohoe DR, Holley D, Collins LB, Montgomery SA, Whitmore AC, Hillhouse A, et al. A gnotobiotic mouse model demonstrates that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. Cancer discovery. 2014;4(12):1387-97.

53. Afik R, Zigmond E, Vugman M, Klepfish M, Shimshoni E, Pasmanik-Chor M, et al. Tumor macrophages are pivotal constructors of tumor collagenous matrix. J Exp Med. 2016;213(11):2315-31.

54. Burns JS, Kristiansen M, Kristensen LP, Larsen KH, Nielsen MO, Christiansen H, et al. Decellularized matrix from tumorigenic human mesenchymal stem cells promotes neovascularization with galectin-1 dependent endothelial interaction. PLoS One. 2011;6(7):e21888.

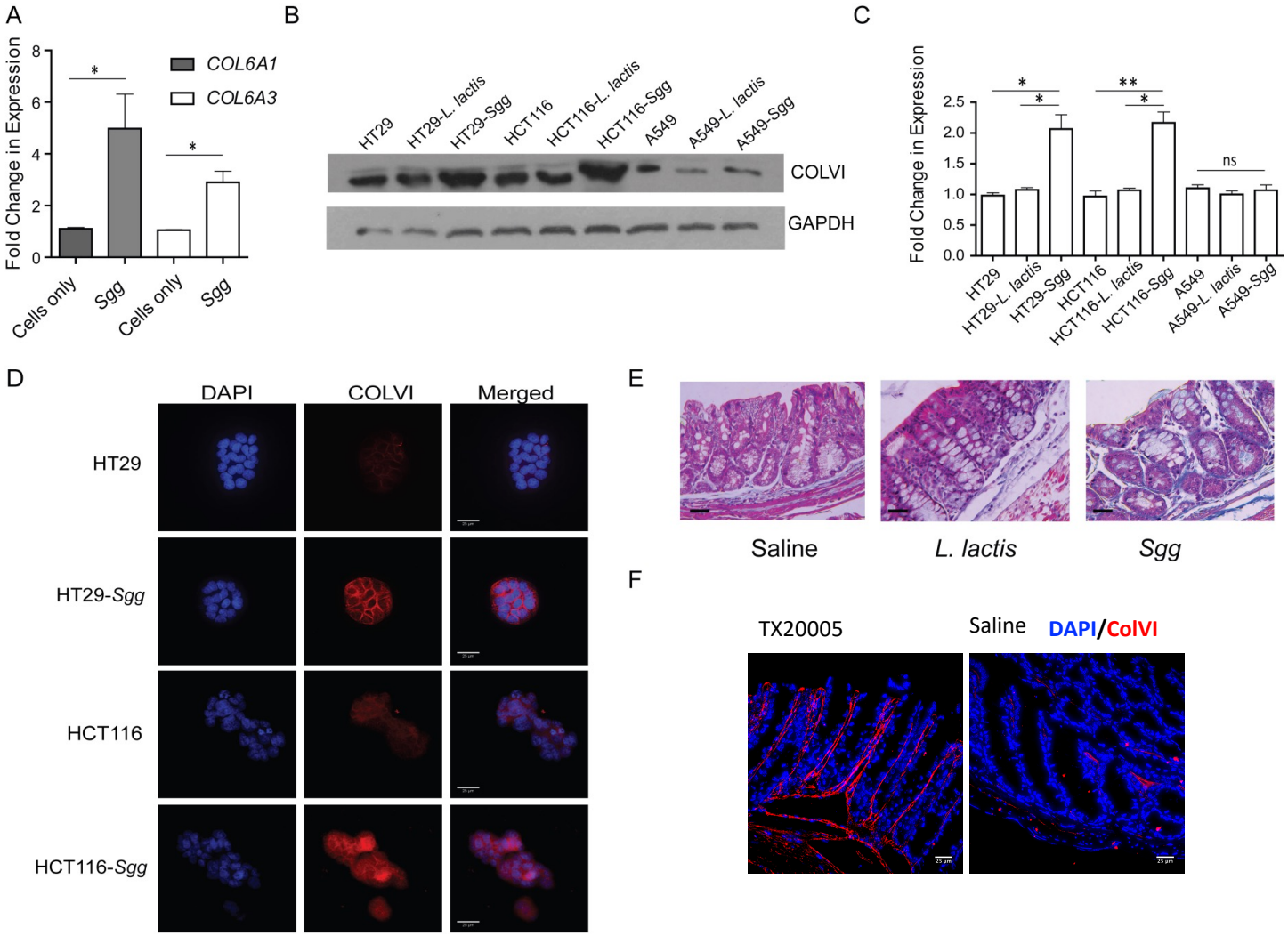

A
Fold Change in Expression
COL6A1
COL6A3
Cells only
Sgg
Cells only
Sgg
B
HT29
HT29-L. lactis
HT29-Sgg
HCT116
HCT116-L. lactis
HCT116-Sgg
A549
A549-L. lactis
A549-Sgg
COLVI
GAPDH
C
Fold Change in Expression
ns
D
DAPI
COLVI
Merged
HT29
HT29-Sgg
HCT116
HCT116-Sgg
E
Saline
L. lactis
Sgg
F
TX20005
Saline
DAPI/ColVI

FIG. 1

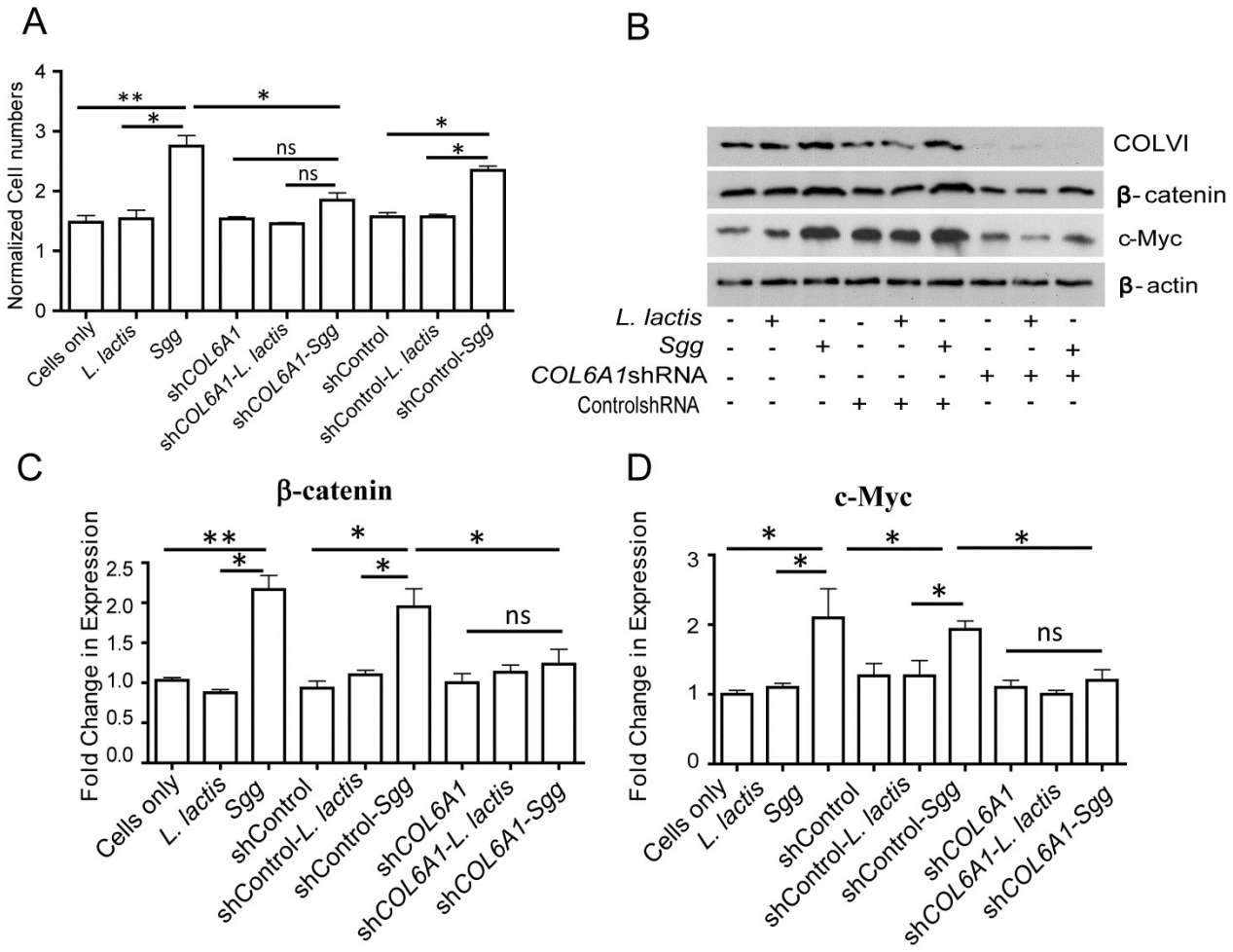

A
Normalized Cell numbers
Cells only
L. lactis
Sgg
shCOL6A1
shCOL6A1-L. lactis
shCOL6A1-Sgg
shControl
shControl-L. lactis
shControl-Sgg
ns
B
COLVI
β-catenin
c-Myc
β-actin
L. lactis
Sgg
COL6A1shRNA
ControlshRNA
C
β-catenin
Fold Change in Expression
D
c-Myc
Fold Change in Expression

FIG. 2

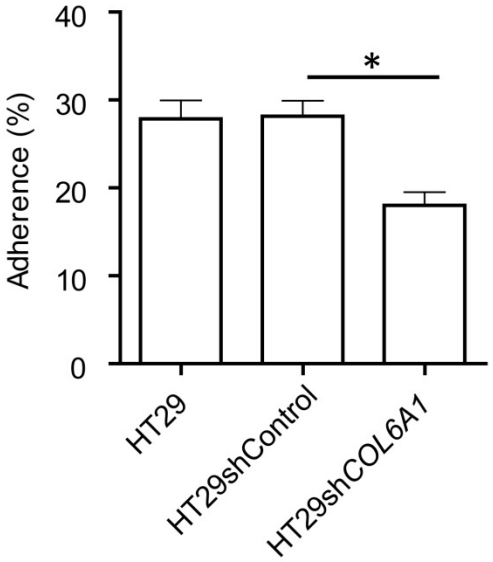


Fig. 3

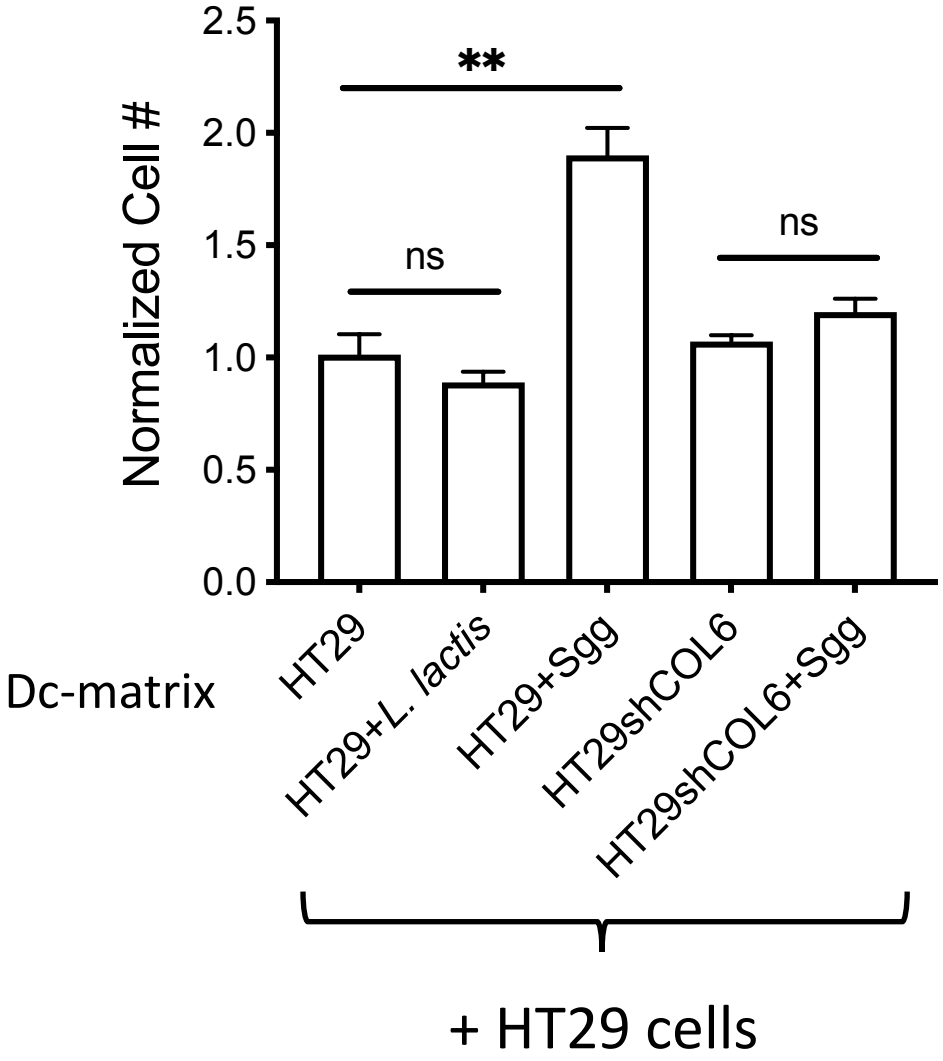

2.5
2.0
1.5
1.0
0.5
0.0
Normalized Cell #
**
ns
ns
Dc-matrix
HT29
HT29+L. lactis
HT29+Sgg
HT29shCOL6
HT29shCOL6+Sgg
+ HT29 cells

Fig. 4

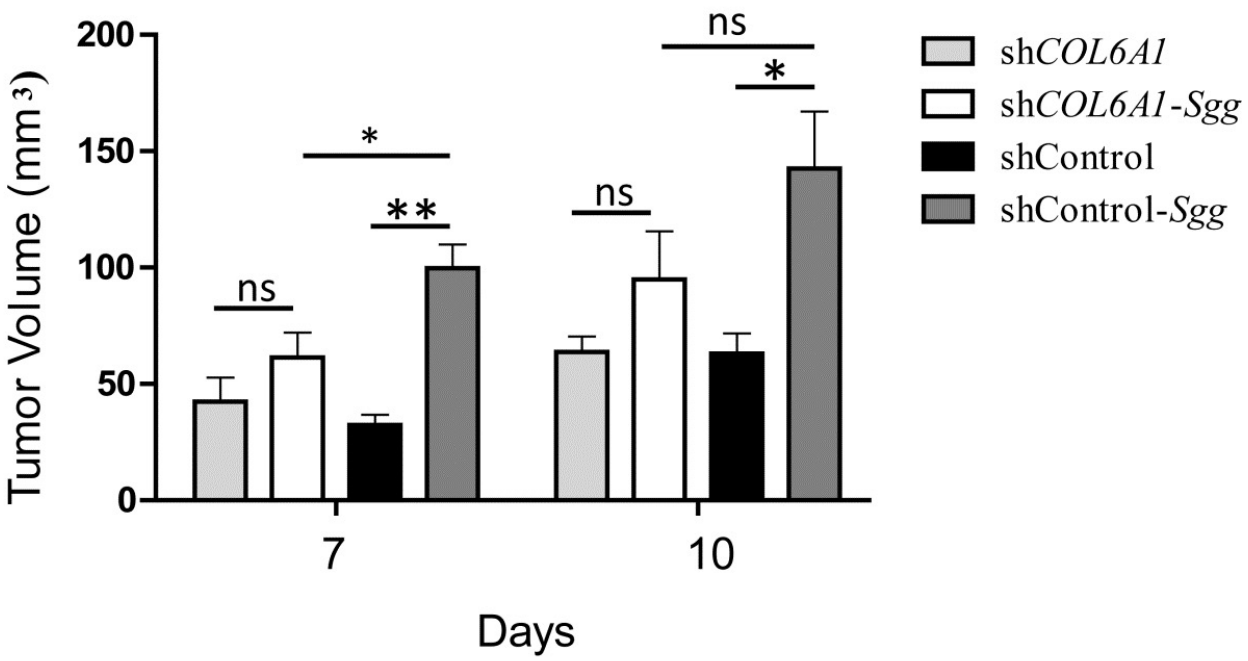
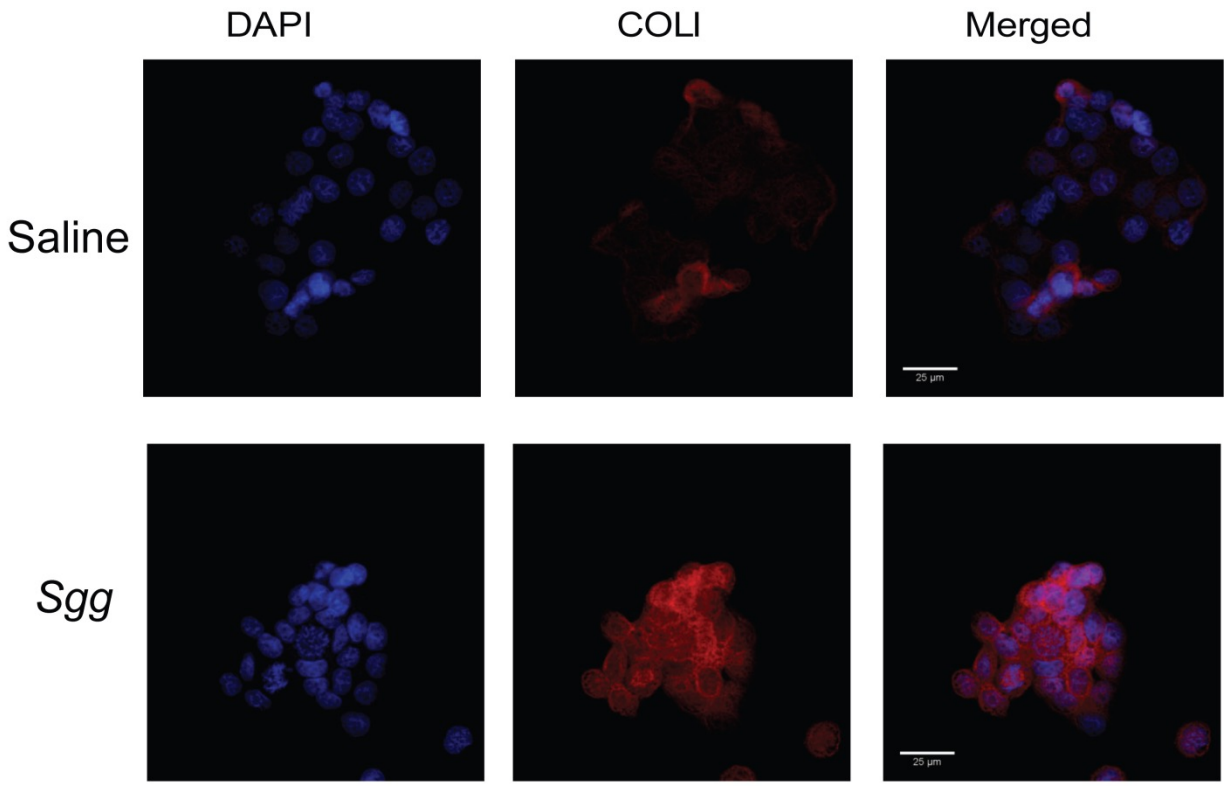
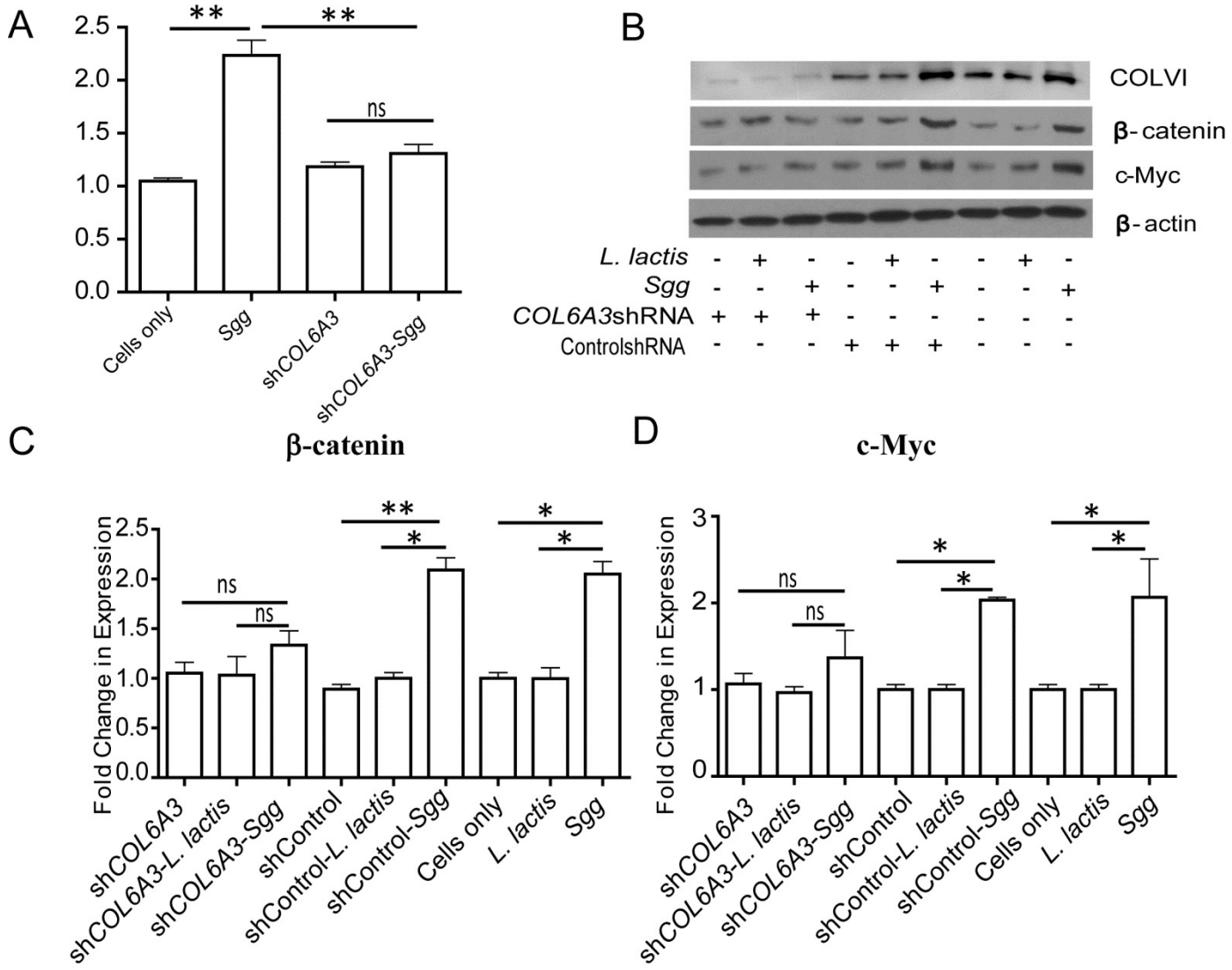

Tumor Volume (mm³)
200
150
100
50
0
ns
*
**
ns
ns
*
7
10
Days
shCOL6A1
shCOL6A1-Sgg
shControl
shControl-Sgg

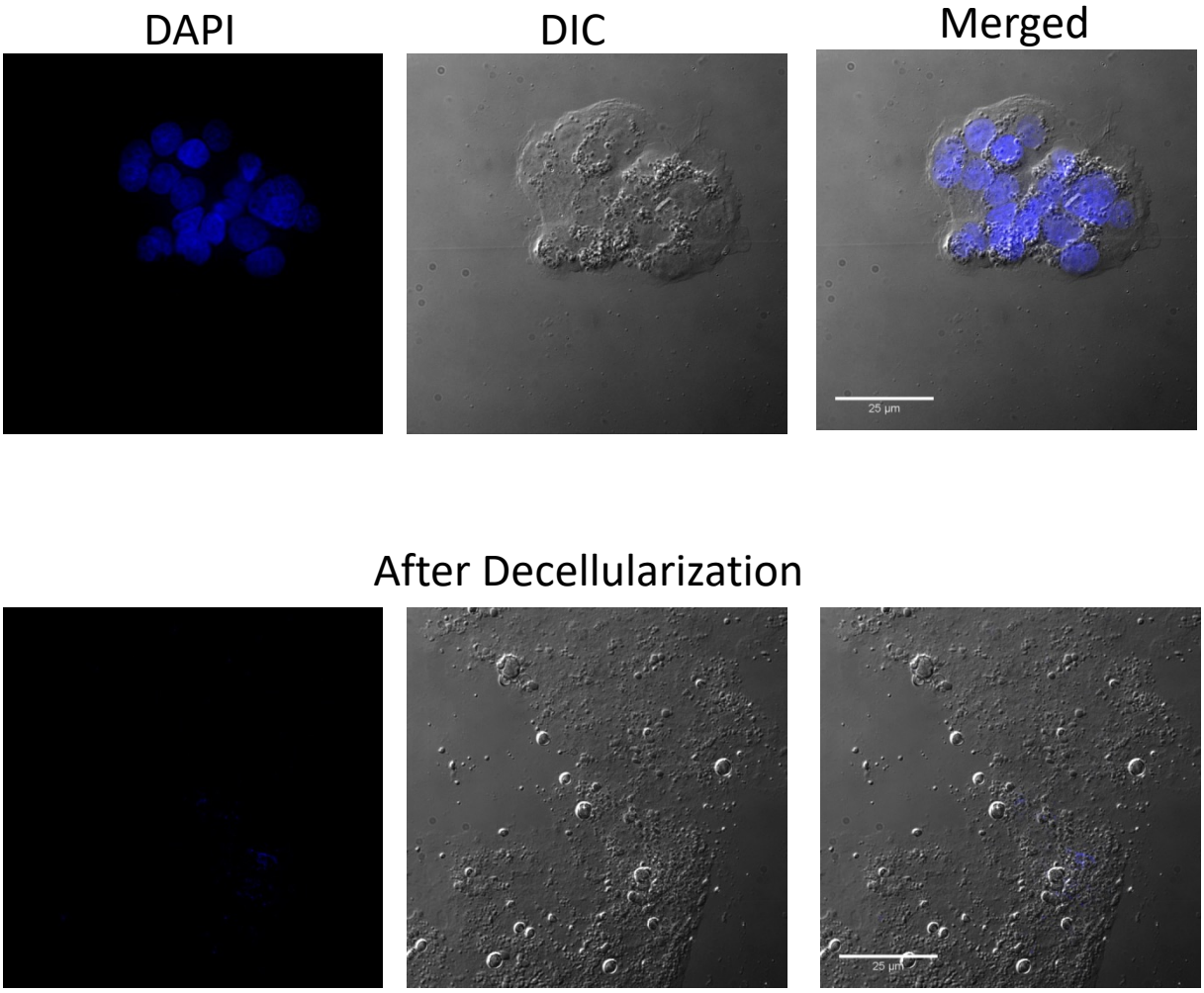
Fig. 5



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. **Knockdown of Col6A3 rendered *Sgg* unable to stimulate cell proliferation.** The experiments were carried out as described in Fig. 2 legend.



Supplemental Fig. S3. Decellularization of cells. 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. Cells were washed thrice with PBS and then fixed and stained with DAPI as explained in Materials and Methods section.