

The Texas Medical Center Library

DigitalCommons@TMC

The University of Texas MD Anderson Cancer
Center UTHealth Graduate School of
Biomedical Sciences Dissertations and Theses
(Open Access)

The University of Texas MD Anderson Cancer
Center UTHealth Graduate School of
Biomedical Sciences

5-2017

Stromal Fibroblasts Restrain the Rate of Colon Cancer Progression and Metastasis by Suppressing Regulatory T Cells and Colon Cancer Stem Cells

Changsoo Kwak

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations



Part of the [Cancer Biology Commons](#), and the [Immunity Commons](#)

Recommended Citation

Kwak, Changsoo, "Stromal Fibroblasts Restrain the Rate of Colon Cancer Progression and Metastasis by Suppressing Regulatory T Cells and Colon Cancer Stem Cells" (2017). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 775.

https://digitalcommons.library.tmc.edu/utgsbs_dissertations/775

This Dissertation (PhD) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digitalcommons@library.tmc.edu.

The
TMC LIBRARY
Health Sciences Resource Center

**STROMAL FIBROBLASTS RESTRAIN THE RATE OF COLON CANCER
PROGRESSION AND METASTASIS BY SUPPRESSING REGULATORY T
CELLS AND COLON CANCER STEM CELLS**

A

THESIS

Presented to Faculty of

The University of Texas

MD Anderson Cancer Center UT Health

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Changsoo Kwak, M.S.

Houston, Texas

May 2017

Acknowledgements

It would have been impossible to finish this thesis without the guidance and inspiration of many people. Especially, I would like to thank my advisor Dr. Raghu Kalluri for his tremendous support and excellent mentorship throughout my Ph.D. career. Dr. Kalluri has not only guided me through the challenges of my thesis project but also allowed me to explore my skills to the full extent and provided me the opportunity to develop my ideas freely.

I am also thankful to my Dissertation Advisory Committee members Drs. Jian Hu, Honami Naora, Shao-Cong Sun, and Kwong-Kwok Wong for their suggestions and insightful discussions to improve my dissertation project.

I got so much help from people in Kalluri Lab. They have provided me a very collaborative environment and aided my completion of this study. I am particularly grateful to the assistance of Drs. Valerie LeBleu, Jiha Kim, and Hikaru Sugimoto.

I would like to thank Dr. Adam Boutin for help at the initial stage of this project, providing his valuable genetic mouse model and insightful guidance for this project.

Finally, I thank my parents and wife for their caring support, and the encouragement and motivation they provided. Without them, it would not have been possible to overcome the difficulties of the graduate school experience.

**STROMAL FIBROBLASTS RESTRAIN THE RATE OF COLON CANCER
PROGRESSION AND METASTASIS BY SUPPRESSING REGULATORY
T CELLS AND COLON CANCER STEM CELLS**

Changsoo Kwak, M.S.

Advisory Professor: Raghu Kalluri, M.D., Ph.D.

The initiation, progression, and metastasis of tumors involve not only cancer cells, but also the tumor microenvironment, which consists of immune or inflammatory cells, fibroblasts, endothelial cells, and extracellular matrix components (ECM). Fibroblasts are ubiquitous stromal cells that can influence other neighboring cell types through the secretion of chemokines, cytokines, ECM, ECM remodeling enzymes, and other metabolites. Myofibroblasts are a distinct subtype of fibroblasts characterized by expression α -smooth muscle actin (α SMA). These cells are a dominant component of the microenvironment, and a FSP1 and FAP could be a different clone of fibroblasts. Myofibroblasts also have been known to contribute to cancer progression and metastasis in multiple cancer types including breast and pancreas cancer, both of which are associated with extensive desmoplasia. However, the role of α SMA⁺ myofibroblasts in the context of colorectal cancer has remained largely descriptive and functionally unknown. The studies outlined in this project explored the functional contribution of α SMA⁺ myofibroblasts in the disease progression of colorectal cancer. To address this, we used the spontaneous colorectal cancer model, which consists of 6 alleles: Villin-Cre-

ERT2; APC^{flox/flox}; p53^{flox/flox}; tetO-LSL-Kras^{G12D}; Rosa26-LSL-Luc; Rosa26-LSL-rtTA-eGFP. These mice were bred with α SMA-TK and α SMA-RFP transgenic mice for selective targeting and monitoring of myofibroblasts in the tumor microenvironment in these eight allelic transgenic mice.

Mice with α SMA⁺ myofibroblast-depleted tumors exhibited hind limb paresis, which likely contributed to their decreased overall survival. Intraluminal tumor burden appeared reduced in myofibroblast-depleted tumors; however, the tumors were depressed resulting in more invasion to both the vascular and lymphatic vessels. The depletion of myofibroblast surprising resulted in increased deposition of ECM and generation of an immunosuppressive microenvironment with the accumulation of CD4⁺ Foxp3⁺ Treg. Furthermore, we discovered that the population of cancer stem cells expressing Lgr5 and Dclk1 was increased in α SMA⁺ myofibroblast-depleted tumors. In conclusion, our study reveals the unknown functional role of myofibroblasts in regulating T cell-mediated anti-tumor immunity and stemness features in colon cancer cells.

List of Contents

Approvals	i
Title	ii
Acknowledgments	iii
Abstract	iv
List of Contents	vi
List of Figures	iX
List of Tables	Xi
Chapter 1: Introduction	1
Epidemiology of colorectal cancer	2
Histopathological classification of colorectal cancer	2
Diagnosis and staging of colorectal cancer	2
Standard cares of colorectal cancer	3
Molecular pathogenesis of colorectal cancer	4
Tumor microenvironment	8
Fibroblasts	10
Cancer stem cells	12
Chapter 2: Material and Methods	15
Mice	15
Tumor induction	16
Endoscopic procedures	16

GCV treatment	17
Survival curve	18
Histology scoring for local invasion	18
Immunohistochemistry	19
Flowcytometry	19
Circulating tumor cell (CTC) measurement	20
RNA-Seq	21
Chapter 3: αSMA⁺ myofibroblast-ablated tumors exhibit poor survival in murine CRC model	22
Mouse model for spontaneous colorectal cancer	23
α SMA ⁺ myofibroblasts depletion is associated with decreased survival	25
Chapter 4: αSMA⁺ myofibroblast-depleted tumors present more invasive and metastatic phenotype	32
iKrAP; α SMA-TK ⁺ mice exhibited decreased extraluminal tumor burden	32
α SMA ⁺ myofibroblast-ablated tumors displayed increase frequency of local invasion	34
Depletion of α SMA ⁺ myofibroblast in tumor microenvironment affected to develop depressed tumors	40
Depletion of α SMA ⁺ myofibroblasts increased CTCs	42
Depletion of α SMA ⁺ myofibroblasts increased metastasis to lymphatic system	44
Depletion of α SMA ⁺ myofibroblasts increased metastasis to pericolonic and perirectal fat	44
Depletion of α SMA ⁺ myofibroblasts induced collagen-rich stroma to promote cancer	

cell invasion	48
Chapter 5: Depletion of αSMA⁺ myofibroblasts results in immune- suppressive microenvironment in CRC	52
Chapter 6: Depletion of αSMA⁺ myofibroblasts results in increase of cancer stem cells in CRC	54
Targeting α SMA ⁺ myofibroblasts increased LGR5 ⁺ cancer stem cell population	54
Assessment of Dclk1 ⁺ cells in colon cancer cells	56
Depletion of α SMA ⁺ myofibroblasts caused to up-regulation of cancer stem cell proliferation	57
Chapter 7: Summary and Discussions	59
References	63

List of Figures

Figure 1. Spontaneous mouse colorectal cancer mice	24
Figure 2. iKrAP mice were crossed with α SMA-TK and RFP mice to targeting and monitoring, respectively.	26
Figure 3. Mouse model targeting proliferating α SMA ⁺ myofibroblast by treating GCV	27
Figure 4. Experimental timeline for induction of tumors and ablation of α SMA ⁺ myofibroblasts	28
Figure 5. Decreased α SMA ⁺ myofibroblasts correlated with poor survival	29
Figure 6. Ablation of α SMA ⁺ myofibroblast result in limb paresis whereas non-depleted mice were moribund due to bowel obstruction	30
Figure 7. Decreased α SMA expression in iKrAP; α SMA-TK ⁺ mice	31
Figure 8. Depletion of α SMA ⁺ myofibroblasts results in decreased tumor size and increased inflammation	33
Figure 9. Depletion of α SMA ⁺ myofibroblasts exhibited increased frequency of local invasion	39
Figure 10. α SMA ⁺ myofibroblast depletion affected morphological alteration to depressed CRC	41
Figure 11. iKrAP; α SMA-TK ⁺ showed increased CTC numbers	43
Figure 12. iKrAP; α SMA-TK ⁺ showed increased invasion of cancer cells to lymphatic vessels	45

Figure 13. iKrAP; α SMA-TK ⁺ showed increased metastasis of cancer cells to lymphatic system.....	46
Figure 14. iKrAP; α SMA-TK ⁺ showed increased metastasis of cancer cells to pericolic or perirectal fat	47
Figure 15. α SMA ⁺ myofibroblasts-depleted tumors revealed increased collagen deposition	50
Figure 16. α SMA ⁺ myofibroblasts-depleted tumors displayed increased type I collagen.....	51
Figure 17. Targeting α SMA ⁺ myofibroblasts exhibited immunosuppressive microenvironment.....	53
Figure 18. Targeting α SMA ⁺ myofibroblasts resulted in Lgr5 ⁺ cell among cancer cells.....	55
Figure 19. Targeting α SMA ⁺ myofibroblasts led to increased expression of Dclk15 in cancer cells.....	56
Figure 20. Ablation of α SMA ⁺ myofibroblasts resulted in enrichment of negative regulation of cancer stem cell proliferation in control tumor	58
Figure 21. Summary	62

List of Tables

Table 1. Classification of CRC according to local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage).....	13
Table 2. Table 2. Overall stage classification of CRCs.....	14

Chapter 1: Introduction

Epidemiology of colorectal cancers

Colorectal cancers (CRCs) are a frequently lethal disease with heterogeneous outcomes and drug responses. Incidence is low at ages younger than 50 years but strongly increases with ages. Median age at diagnosis is around 70 years in developed countries [1]. The highest incidence has been reported in countries of Europe, North America, whereas incidence is lowest in some countries of south and central Asia and Africa [2]. In the United States, CRC is the second or third leading cause of cancer and cancer-related deaths, with a 2016 estimate 134,490 new cases diagnosed and 49,190 death. The lifetime risk of developing CRC is as high as 5% in the American population.

The prognosis of patients with CRC has slowly but steadily improved during past decades in many Western countries. Overall survival decreases with age and relative survival at the young age is slightly higher for women than for men. The Stage at diagnosis is the most critical prognostic factor. In the United States in 2001 - 2007, 5-year survival rate for CRC patients with a local stage was 90.1%, 69.2% for patients with regional spread, and 11.7% for patients with distant spread [1].

Epidemiological studies have identified that no single risk factor accounts for most cases of CRC. The following risk factors such as family history of CRC [3], inflammatory bowel disease [4], smoking [5], excessive alcohol consumption [6], high consumption of red and processed meat [7], obesity [8], and diabetes [9] co-occur and interact for development of CRC. Further recent evidence suggested that infection of *Helicobacter pylori* and *Fusobacterium spp.* could be associated with an increased risk of CRC incidence [10, 11]. Established preventive factors contain physical activity [12],

treatment of hormone replacement therapy [13] and aspirin [14, 15], and removal of precancerous lesions via endoscopic procedure showing most promising risk reduction [16, 17].

Histopathological classification of colorectal cancers

CRCs are histopathologically classified according to local invasion depth (T stage), lymph node dissemination (N stage), and the presence of distal metastasis (M stage) (Table 1) [131]. These classifications are combined into overall stage definition to provide the decision for therapeutic treatments (Table 2). Although this classification according to TNM stage provides valuable prognostic information and guide therapeutic decisions, the outcome of CRC patients' treatment is difficult to predict.

Diagnosis and staging of colorectal cancers

A general diagnosis of CRCs depends on biopsy samples taken during endoscopy. Complete endoscopy or computed tomography (CT) endoscopy is necessary to detect synchronous cancers that are present in about 2 - 4 % of CRC patients [18]. For rectal cancer, exact local staging at the time of diagnosis is critical for neoadjuvant treatment. Endoscopic ultrasonography is the accurate method for determination of regional T stage of rectal cancer because of its accuracy to determine between non-invasive and invasive phenotype [19]. Magnetic Resonance Imaging (MRI) is the most accurate method to define advanced T stage of rectal cancer [20].

The most common location of metastasis in CRC patients is the liver, and a meta-analysis with patients without any treatments display that the sensitivity of MRI is slightly higher

than that of CT [21] Especially, MRI had significantly higher sensitivity than CT for small tumors less than 10 mm. Abdominal ultrasonography is the common diagnostic method for detection of many cancers in the abdomen, but its sensitivity to detect liver metastasis was lower than any other staging methods [22]. To identify lung metastasis, the chest radiograph is utilized, and chest CT considers as another option for patients with locally advanced rectal cancers [23].

Standard cares of colorectal cancers

The surgery is the main treatment for early-stage CRCs. The standard surgical procedure for early treatment of rectal cancer patients is excision of total mesorectum [24]. Complete removal of the mesorectum is critical because it contains most of the tumor deposits and involved lymph nodes [25]. For colon cancer patients, the tumors and the surrounding lymphatic vessels are surgically removed. Unlike open surgery, laparoscopic resection provides the patients with reduced blood transfusions, faster return of bowel function, and a shorter duration of hospital stay. However, operating times are longer, and operative costs are higher than open surgery.

The role of neoadjuvant therapies including radiotherapy and chemotherapy is not only to downsizing or staging of tumors but also to reduce local recurrence rates after surgery. Patients with stage I CRCs are not necessary for any treatments before surgery because local recurrence rate is less than 3% [26]. These clinical treatments are known to provide benefits for patients with T4 and advanced T3 tumors infiltrating mesorectal fascia. Patients with stage III CRCs have a high risk of recurrence rating between 15% and 50%. Adjuvant chemotherapy, therefore, is highly recommended for all patients with stage III

CRCs after curative resection. Fluorouracil treatment reduced the recurrence rate by 17% and increased five-year disease free survival (DFS) by 13-15% [27]. Alternatively, Capecitabine, an oral prodrug of fluorouracil has been used for comparable efficacy [28]. Additionally, oxaliplatin increased the 5-year disease-free survival rate by 6.2% to 7.5% and overall survival by 2.7% to 4.2% in patients with stage III CRCs [29, 30].

Molecular pathogenesis of colorectal cancers

The patterns of the molecular pathogenesis of CRCs are highly heterogeneous. The molecular mechanisms underlying development and progression of CRCs are clinically important because they are tightly associated with the prognosis and therapeutic approaches of patients [31, 32]. During past two decades, therefore, the identification of molecular mechanisms including genetic alterations has been focused on improving responses to treatments.

One of the most critical fundamental concepts of colorectal tumorigenesis is adenoma-carcinoma sequence, a term that describes the stepwise progression from normal epithelium to dysplastic lesion called adenoma to carcinoma associated with the accumulation of multiple genetic alterations [33]. The concept was first proposed by Fearon and Vogelstein in 1990 and postulated that mutational activation of oncogenes, coupled with mutational inactivation of tumor suppressor genes leads to the development of colorectal cancer. They tested their hypothesis by measuring the frequency of *ras* gene mutation and loss of p53 in early adenoma, intermediated adenoma, late adenoma, and carcinoma. Approximately 50% of colorectal carcinomas and the similar percentage of intermediated adenoma and late adenoma have been found *ras* mutation. However only

10% of early adenomas have exhibited ras mutation. The loss of a large portion of p53 has been seen in more than 75% of carcinoma, but such loss is infrequent in any stages of adenomas [34].

CRCs often develop over more than 10 years and dysplastic adenomas are the most common form of premalignant precursor lesions [35]. One of the mutations known to occur early in the adenoma-carcinoma progression is the adenomatous polyposis coli (APC) tumor suppressor gene located on chromosome 5q21 in human [132] [36] and, this mutation occurs in more than 70% of adenomas [37]. The protein of *APC* gene is a large 312-kDa protein composed 2843 amino acids. It is known as a multifunctional protein with several domains that interact with several proteins including the β -catenin, glycogen synthase kinase (GSK) 3 β , and end binding protein (EB) 1 [38-40]. One critical function of *APC* to colorectal tumorigenesis is the regulation of intracellular β -catenin levels. Wild-type APC protein forms a complex with β -catenin and GSK-3 β , which leads to an enhanced rate of β -catenin degradation by the ubiquitin-proteasome pathway. C-terminally truncated mutant APC proteins, however, lack of β -catenin binding site, cause to increase intracellular β -catenin level [133] [41]. The importance of β -catenin relates to its capacity to bind T-cell factor (TCF) family of transcriptional factors and activate gene transcriptions [42]. Increased β -catenin-TCF-mediated transcription has been demonstrated in tumors with *APC* mutations and β -catenin mutation and results in increased transcriptional activity of target genes such as oncogenic *c-myc* [134].

An additional genetic alteration presumably occurs early in the adenoma-carcinoma progression is the mutation in the *K-ras* gene. K-ras is one of three members of Ras family located on the short arm of chromosome 12 and associated with somatic mutations

in many human cancers. The type of K-ras somatic mutations found in around 40% of CRCs is point mutations affecting codon 12, 13, or 61. Especially, more than 75% of the K-ras point mutations occur at the codon 12. Only small fraction (< 5%) of CRCs has N-ras mutations at codon 12, 13, or 61 [34]. K-ras encodes 21-kDa protein critically involved in signal transduction of regulatory pathways for proliferation and differentiation [43]. It is guanosine 5'-triphosphate (GTP)-binding protein, located at the cytoplasmic aspect of the cell membrane. When bound to GTP, the ras protein is active but becomes inactive when GTP is hydrolysed to guanosine 5'-diphosphate. Mutations of the *K-ras* oncogene impact the GTP-binding domain, decrease its GTPase activity and result in a constitutively active Ras protein [137].

The p53 gene is the most frequently mutated in human cancers, which located on short arm of chromosome 17 [44]. The functional role of p53 is to block cell proliferation in the presence of DNA damage, to stimulate DNA repair, and promote apoptotic cell death if repair is not sufficient [45]. Loss of p53 function, therefore, results in propagation of damaged DNA to daughter cells. Functional inactivation of p53 most often occurs as a result of missense mutations in the DNA-binding domain, but it can result from oncogenic viral or cellular protein interaction [46, 47]. The alteration of p53 has been reported in 4-26 percent of adenomas, in approximately 50 percent of invasive foci within adenomatous polyps, and in 50-75 percent of adenocarcinomas [48], [135-136]. These results lead to the belief that functional inactivation of p53 protein is associated with the transition from adenoma to carcinoma.

However, more than 15% of sporadic CRCs develop through fundamentally different molecular events instead of generic adenoma-carcinoma sequence. These subtypes of

tumors are originated from serrated precursor lesions and often characterized by the CpG island methylation and activating oncogenic *BRAF* mutations. It is difficult to identify these lesions during colonoscopy because these cancers are relatively flat and inconspicuous characteristics [49]. Most cancers arising from serrated adenomas show the high-level microsatellite instability (MSI-H) phenotype as a result of promoter methylation of *MLH1* gene [50], and appear in the proximal colon of elderly women [51]. Hereditary CRCs contribute to around 3-5% of all CRC cases [52, 53]. Dissecting mechanisms related to hereditary forms is valuable to study molecular pathogenesis of CRCs. Hereditary CRCs display inactivated tumor suppressor genes or DNA repair genes by a germ line mutation and a somatic event abrogating the remaining wild-type allele leading to tumor formation [54]. The two most common forms of hereditary CRCs are hereditary non-polyposis colon cancer (Lynch syndrome) and familial adenomatous polyposis coli (FAP). Both syndromes are autosomal dominant disorders. Whereas Lynch syndrome indicates mismatch repair deficiency and MSI-H [52, 55], FAP syndrome follows the classic adenoma-carcinoma sequence [56].

Mismatch repair deficient CRCs are defined by the accumulation of insertion and deletion mutations at microsatellites spread along the genome [55]. Clinical characteristics of MSI-H cancers exhibit development of tumors in the proximal colon, the symptom in people younger than 50 years (hereditary form) or elderly people (sporadic form), simultaneous occurrence of additional cancers [57], and relatively large primary tumors with rare distal metastasis. Histopathologically, MSI-H cancers display poor or mixed differentiation with dense infiltration with tumor infiltrating lymphocytes

and expansive pattern of invasion [58]. MSI-H cancers also exhibit loss of expression of at least one DNA mismatch repair protein in greater than 90% of lesions [59].

Tumor microenvironment (TME)

In CRCs, the multistep process from normal colonic epithelium to an adenomatous polyp and ultimately to an invasive adenocarcinoma is associated with a robust desmoplastic reaction, which generates complex tumor microenvironment. Desmoplasia is activation of stroma cells by cancer cells not only to eliminate the pre-existing extracellular matrix but also to induce new matrix formation [60]. The tumor microenvironment consists of cells of hematopoietic origin, cells of mesenchymal origin, active angiogenesis, extracellular matrix (ECM), and proteins and remodeling enzymes [61, 62].

Local inflammation at the site of solid tumors causes the accumulation of a variety of cells, and it is now widely accepted that these cells are intimately linked to the control of tumor growth. CRC, similarly to many other solid tumors, are infiltrated by immune cells such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), monocytes, neutrophils, T cells, B cells, NK cells, and dendritic cells (DCs) and stroma cells such as endothelial cells, endothelial progenitor cells (EPCs), pericytes, platelets, mesenchymal stem cells (MSCs), and cancer-associated fibroblasts (CAFs). TAMs are typically derived from blood monocytes that are recruited to the tumors by growth factors and chemokines such as vascular endothelial growth factor (VEGF), colony-stimulating factor-1 (CSF-1), CCL2, CCL3, CCL4, CCL5, and angiopoietin-2 [63]. Recruited monocytes differentiate into TAM upon the presence of low interleukin (IL)-12 and high IL-10 concentration in TME [64]. It has been well established that

macrophage-deriving tumor necrosis factor (TNF) – α , IL-1 β , and IL-6 induce human colon cancer cell proliferation and migration, and also colon cancer cells stimulate macrophages to produce IL-6 via signal transducer and activator of transcription 3 (STAT3) – mediated IL-10 production [65, 66]. (explaining in detail) Clinically, elevated IL-10 and IL-6 are associated with a poor prognosis in CRC patients [67].

MDSCs are immature myeloid cells that have the immunosuppressive affect. The suppressive effects of MDSCs are associated with metabolism of L-arginine that is the substrate for arginase-1 and inducible nitric oxide synthase (iNOS). Arginase-1 degrades arginine in TME, which affect translational blockade of ϵ chain of CD3 leading to prevention of T cell stimulation from tumor antigens. iNOS-mediated nitric oxide (NO) production inhibits T cell function via a variety of different mechanisms including the blockade of phosphorylation and activation of Janus kinase 3 and STAT5, inhibition of MHC-II gene expression, and induction of T cell apoptosis [68]. Also, there is emerging evidence that MDSCs promote the development of Foxp3⁺ Tregs by releasing IL-10 and TGF- β [69]. It is widely known that the number of MDSCs is increased in the blood of patients with solid cancers including CRCs [70].

CD4⁺ CD25⁺ Foxp3⁺ Tregs are also expanded in TME and are capable of suppressing the proliferation of cytotoxic and effector T cells through direct contact or IL-10 and TGF- β production. IL-17-producing CD4⁺ CD25⁺ Foxp3⁺ Tregs expanded in colonic polyps promotes mastocytosis and tumor-promoting inflammation [71]. Also, CD4⁺ CD25⁺ Foxp3⁺ Tregs express cyclooxygenase-2 (COX-2) which are overexpressed in the majority of CRCs [72] and COX-2-derived prostaglandin E₂ (PGE₂) promote tumor growth by inducing cell proliferation, survival, and invasion [73]. Treatment of long-term

nonsteroidal anti-inflammatory drugs (NSAIDs) increased the levels of MHC II proteins such as HLA-DP, HLA-DQ, and HLA-DR, and infiltration of CD4⁺ effector T cells and CD8⁺ cytotoxic T cells in TME along with decreased Treg-associated molecules including IL-10 and Foxp3 [74, 75].

Fibroblasts

Fibroblasts are dominant population of tumor stroma, and many studies suggested that these cells support cancer progression and metastasis [76, 77]. Fibroblasts in TME have been termed cancer-associated fibroblasts (CAFs), tumor-associated fibroblasts (TAFs), activated fibroblasts or myofibroblasts. CAFs are considered as a major contributor of fibrotic stroma in many solid tumors [78, 79].

Fibroblasts are non-epithelial cells and non-immune cells with the mesenchymal origin and a part of connective tissues [138]. Fibroblasts in healthy tissue are quiescent with negligible metabolic activity. The fibroblasts are usually resting state and become activated in a wound healing response [80]. These activated fibroblasts were termed as myofibroblasts. Myofibroblasts are regarded as involving acute and chronic inflammation and tissue fibrosis [81, 82].

Fibroblasts in mammals are highly heterogeneous, and fibroblasts isolated from different organs reflect tremendous topographic diversity. A recent study by using the genome-wide expression patterns of human fibroblast culture isolated from 16 various sites indicated that the gene expression pattern of human fibroblast lineage is comparable with that of human white blood cells [83]. There are several well-established fibroblast markers including vimentin, alpha-smooth muscle actin (α SMA), Desmin, Fibroblast-

specific protein1 (FSP-1), and Fibroblast-activation protein (FAP), but none of them are commonly expressed fibroblast lineage [84].

Myofibroblasts are spindle shape of contractile cells functioning as remodeling of damaged tissues. The transition from fibroblast to myofibroblasts during wound healing is a stepwise process in which fibroblast first differentiate into proto myofibroblasts by expressing β -cytoplasmic actin and γ -cytoplasmic actin and then fully differentiate into myofibroblasts which express α SMA [85].

α SMA is currently considered the most common marker of intestinal myofibroblasts that also expressed on pericytes, bone marrow-derived mesenchymal stromal cells, muscularis mucosae smooth muscle cells, and mural cell associated with lymphatic lacteals. α SMA expressing cells in the core of villi are mostly pericytes where α SMA expressing cells located in under epithelial layer around crypts of small and large intestines are myofibroblasts [86].

Fibroblasts play a critical role in all stages of cancer progression including tumor initiation, growth, invasion, and metastasis. Mostly this involvement of fibroblasts in cancer is typically based on their specific functions in each stage of cancer. In the 1970s, several studies led to the consideration that cancer cells may recruit activated fibroblasts [139] [87, 88]. This recruitment of fibroblasts is regulated by the growth factors released by cancer cells and other immune cells. The recruitment of activated fibroblasts is mainly associated with transforming growth factor- β (TGF- β) and also the proliferation of CAFs is regulated by TGF- β [89, 90]. The role of CAFs in TME may involve the crosstalk with cancer cell to promote tumor growth and stemness, immunosurveillance, activation of

angiogenesis, tumor-promoting inflammation, and ECM remodeling that facilitate metastasis.

Cancer stem cells

The intestine is one of most rapidly renewing tissues in the human body in that the entire epithelial layer is replaced within every 5 days. This rapid process is maintained by intestinal stem cells (ISCs) that localize in the bottom of the crypt and generate precursor cells. These precursor cells differentiate into specialized cells, mainly enterocytes that uptake nutrients as well as transport and remove faeces that is facilitated by mucin-producing goblet cells [91]. Another abundant differentiated cells are hormone-producing enteroendocrine cells and Paneth cells. Paneth cells reside at the bottom of crypt, function in intestinal immunity, and contribute to the formation of the ISCs niche [92].

Due to its high turnover rate, the intestinal epithelium is subject to malignant transformation like CRCs. Interestingly, the biology of ISCs and CRCs is highly interconnected. Signaling pathways that regulate ISC function such as WNT signaling are aberrantly activated in CRC patients [34]. Most of the intestinal cancers originate from initial transforming events in the ISC compartment. Cancer cells that share properties of ISCs, including multilineage potential and self-renewal, appeared in CRCs and are referred to colon cancer stem cells (CSCs) [93]. These CSCs are thought to contribute to tumor growth and progression, but there are still many unknown properties regarding their identification and importance for clinical implication.

Definition	
T stage	
Tx	No information about local tumour infiltration available
Tis	Tumour restricted to mucosa, no infiltration of lamina muscularis mucosae
T1	Infiltration through lamina muscularis mucosae into submucosa, no infiltration of lamina muscularis propria
T2	Infiltration into, but not beyond, lamina muscularis propria
T3	Infiltration into subserosa or non-peritonealised pericolic or perirectal tissue, or both; no infiltration of serosa or neighboring organs
T4a	Infiltration of the serosa
T4b	Infiltration of neighboring tissues or organs
N stage	
Nx	No information about lymph node involvement available
N0	No lymph node involvement
N1a	Cancer cells detectable in 1 regional lymph node
N1b	Cancer cells detectable in 2–3 regional lymph nodes
N2a	Tumour satellites in subserosa or pericolic or perirectal fat tissue, regional lymph nodes not involved
N2b	Cancer cells detectable in 4–6 regional lymph nodes
N2c	Cancer cells detectable in 7 or greater regional lymph nodes
M Stage	
Mx	No information about distant metastases available
M0	No distant metastases detectable
M1a	Metastasis to 1 distant organ or distant lymph nodes
M1b	Metastasis to more than 1 distant organ or set of distant lymph nodes or peritoneal metastasis

Table 1. Classification of CRC according to local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage)

Adapted by FI. Obrocea et al, (2011) [131]

	T stage	N stage	M stage
Stage 0	Tis	N0	M0
Stage I	T1/T2	N0	M0
Stage II	T3/T4	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
Stage III	Any	N+	M0
IIIA	T1-T2	N1	M0
	T1	N2a	M0
IIB	T3-T4a	N1	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
Stage IV	Any	Any	M+
IVA	Any	Any	M1a
IVB	Any	Any	M1b

Table 2. Overall stage classification of CRCs

Adapted by FI. Obrocea et al, (2011) [131]

Chapter 2: Material and methods

Mice

Mouse colorectal cancer model containing six genetic alleles; *Villin*^{CreERT2}; *tetO-Lox-Stop-Lox-Kras*^{G12D}; *ROSA26-Lox-Stop-Lox-rtTA-IRES-EGFP*; *APC*^{fl/fl}; *P53*^{fl/fl}; *ROSA26-Lox-Stop-Lox-Luciferase* (herein referred to as iKrAP) mice were kindly provided by Dr. Adam T. Boutin in the laboratory of Dr. Ronald A. DePinho at MD Anderson Cancer Center (MDACC). Tamoxifen-dependent Cre recombinase (*Villin*^{CreERT2}) was expressed under the control of a 9 kb regulatory region of *villin* gene and led to spatiotemporally controlled somatic recombination for targeted genes after tamoxifen administration. *tetO-Lox-Stop-Lox-Kras*^{G12D} transgene was controlled by tetracycline-response operon promoter element (tetO) with a lox-stop-lox cassette inserted between the promoter and the start codon of the *Kras*^{G12D} open reading frame. The Cre recombinase-mediated excision of a STOP codon in *ROSA26-Lox-Stop-Lox-rtTA-IRES-EGFP* transgene-induced functional reverse tetracycline transactivator (rtTA) and EGFP activity, which were inserted between exon 1 and exon 2 in the ROSA26 locus. In the presence of doxycycline, rtTA bound to *tetO* and initiated transcription of *Kras*^{G12D}. Conditional APC knockout was regulated by Cre-loxP strategy leading to deletion of exon 14 and generation of a frameshift mutation at AA580 that disrupted that *APC* gene. iKrAP mice were first bred with α SMA-TK mice and then bred with α SMA-RFP mice. All mice were housed under standard housing conditions at MDACC animal facilities, and all animal procedures were reviewed and approved by the MDACC Institutional Animal Care and Use Committee.

Tumor induction

4-OH tamoxifen (Sigma-Aldrich, H7904) was solubilized in 100% ethanol (50 mg/ml) and diluted to 1 mg/ml in 100% ethanol. iKrAP; α SMA-TK; α SMA-RFP mouse (8-10 weeks of ages) of known weight was lightly anesthetized by placing in a plastic chamber containing isoflurane. A 20 gauge and 30 mm plastic feeding tube (Instech, FTP203050) attached to 1 ml disposal syringe was filled with 1mg/ml of 4-OH tamoxifen mixture, and the tube was lubricated by dipping the soft elastomer tip in surgical lubricant. The anesthetized mouse was held by the tail. A plastic feeding tube was inserted into the anus around 2 to 2.5 cm and 50 μ l of 1 mg/ml of 4-OH tamoxifen mixture was injected into its rectum. The mouse was held by the tail in a vertical position, head down, for 30 sec to allow uniform distribution of the 4-OH tamoxifen mixture before putting the mouse back in its cage. The mouse was fed with Doxycycline pellets (200 mg/kg, BioServ, S3888) instead of normal chow after intrarectal administration of 4-OH tamoxifen mixture.

Endoscopic procedures

For monitoring the tumorigenesis, Storz Veterinary video endoscopic system was utilized (Karl Storz, Germany). The endoscopic system consisted of a miniature endoscope (scope 1.9 mm outer membrane) including a telescope and a telescope sheath, a light source, a camera console, USB recorder, a monitor, and an endoflator to regulate inflated mouse colon. The endoscopic procedure was viewed on a color monitor and digitally recorded on USB flash drive. Before anesthetizing the mouse, an endoscope and a camera console were assembled and were sterilized by dipping the tip in 10% bleach and 70% ethanol to prevent disease transmission. The mouse was anesthetized by placing in a

plastic chamber containing isoflurane until completely anesthetized. A 20 ga x 30 mm plastic feeding tube attached to 10 ml disposal syringe was filled with a warm PBS. The tube was coated with the sterile surgical lubricant and inserted into the anus to flush the colon with a warm PBS enema (10ml). The anesthetized mouse was placed on the operation table applied with the nose cone that provided the inhalation anesthesia to the mouse. The lubricated endoscope was inserted into the anus and advanced through the rectum. The air from an endoflator was slowly applied to set apart the intestinal walls. The rectum and colon were carefully assessed and monitored, and the video was recorded if tumors and colonic inflammation were identified. Upon the completion of examination, the endoscope was slowly withdrawn, and the mouse was provided with a warm environment in which its cage was placed on a warm pad. The mouse was monitored closely until full recovery.

GCV treatment

Ganciclovir (GCV) was utilized to obtain a suicide effect in α SMA-TK⁺ cells. GCV is an analog of deoxyguanosine used as a substrate, which is converted into GCV-triphosphate (GCV-TP) by cellular thymidine kinases. GCV-TP incorporated in DNA that led to premature termination of DNA and subsequently cell death. GCV (InvivoGen) was reconstituted with 20 ml of sterile water, and its pH was adjusted to 12 with NaOH 1M because GCV is only soluble at $\text{pH} \geq 12$. GCV solution's pH was lowered to 11, and 5 ml of sterile water was added to obtain a solution at 10 mg/ml. A 0.22 μm sterile filter was used to sterilize GCV solution and its aliquots were stored at -20°C . For in vivo treatment, the concentration of the stock solution (10 mg/ml) was diluted to 5 mg/ml by

adding the same volume of sterile PBS. The mouse was received daily intraperitoneal (i.p.) injections of 2.5 mg of GCV.

Survival study

For survival study, the time of the death is most critical endpoint. In this study, the euthanizing mice with more than 5mm or actively bleeding prolapsed rectum were considered as false results and removed from survival study. Daily GCV treatment via i.p. injection was initiated at 37 days post tumor induction (n = 24 animals for iKrAP; α SMA-TK- group and n = 32 animals for iKrAP; α SMA-TK+ group), and animals continued to receive treatment until they became moribund, which was assessed on the signs of distress and endpoints including weight loss (> 20%), hunching, prostration, impaired motility, and ruffled haircoat. Mice exhibiting limb paresis were evaluated again 6 hours later. Then if mice worsened, they were euthanized and considered as non-survivors.

Histology scoring for local invasion

The H&E slides from each sample were examined blindly by an investigator and were evaluated in the light microscope. The invasion scoring is based upon the measurement of the deepest point of primary tumors. The measurement of invasion depth was categorized into four scales: 0 (no invasion), 1 (invasion of muscularis mucosa), 2 (invasion of submucosa), 3 (invasion of muscularis propria), and 4 (invasion of serosa).

Immunohistochemistry

Harvested tumors and lungs were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. For α SMA immunohistochemistry, the deparaffinized tumor sections were incubated in 10mM citrate buffer (pH 6.0) for 1 hour at boiling temperature before blocking with M.O.M. Mouse IgG Blocking Reagent (Vector Laboratories, West Grove, PA) for 1 hour. Sections were incubated with α SMA antibody (1:200, Daco.) overnight at 4°C, followed by incubation with biotin-conjugated anti-rabbit/rat/mouse IgG and ABC reagent (Vector Laboratories, West Grove, PA) for 30 min each at room temperature. The sections were then developed by DAB staining according to the manufacturer's instructions. For collagen 1(1:200, MP bioscience), β -catenin (1:200, Abcam), LYVE-1 (1:200, Abcam), we used 4% of fish gel for blocking and followed same procedures described above. In the cause of picrosirius red staining, we followed manufacturer's protocol (sigma-Aldrich).

For Dcl1 and CD20 staining, immunostainings were performed on frozen sections. Frozen sections were fixed in acetone for 10 minutes at 4°C and blocked one hour with 5% donkey or goat serum in PBST at room temperature. Following blocking, sections were incubated in 1:200 rat anti- Dcl1 (Abcam) and CK20 (Abcam) at 4°C overnight, followed by fluorescent secondary antibodies. Slides were mounted after DAPI staining, labeling nuclei blue.

Flowcytometry

Tumors were excised and cut into 1-2mm² pieces. Digestion was performed by incubating the small pieces at 37°C for one hour in RPMI 1640 medium supplemented

with 0.15µg/ml of DNase I(Invitrogen), 40U of Dispase I(Sigma), and 100U/ml of collagenase VIII (Sigma). Following initial one-hour incubation, the pieces were vortexed intensively and then incubated at 37°C for 5 min, repeating this step until complete digestion. The digestion solution was passed through a 100µm cell strainer and washed with RPMI 1640 medium. The cells were resuspended with anti-mouse CD16/32 and then staining with anti-CD4, CD8, F4/80, CD3e, Gr1, Ly-6C, CD11b, CD19, NK1.1, and CD45 conjugated with fluorochromes.

For Foxp3 staining, Cells were incubated at 37°C for 3 hours with 50ng/ml of PMA (Sigma), 500ng/ml of Ionomycin (Sigma) to stimulate cytokine production, and Monensin to block protein transport from endoplasmic reticulum to golgi apparatus. Fixation and permeabilization working solution (eBioscience) were added and cells were incubated at 4°C for 30 min in the dark. Following the incubation, the cells were stained with conjugated anti-mouse Foxp3 antibody and fluorescently conjugated antibodies at 4°C for 15 min in the dark and resuspended in appropriate volume for flow cytometric analysis using LSR II.

Circulating tumor cell (CTC) measurement

The blood (100ul) was collected retro-orbitally from iKrAP; αSMA-RFP; αSMA-TK⁻ and iKrAP; αSMA-RFP; αSMA-TK⁺ mice and incubated twice with 1ml of ACK lysing buffer (A1049201, ThermoFish Scientific) at room temperature to lyse red blood cells. ACK-treated cells were washed twice with cold PBS and resuspended in 2% FBS containing PBS. The number of GFP⁺ cells was analyzed by flow cytometry (BD

LSRFortessa X-20 Cell Analyzer, BD Biosciences). The data was indicated by the percentage of GFP⁺ cells among 1×10^5 cells.

RNA-Seq

Tumors were harvested from iKrAP; α SMA-RFP; α SMA-TK⁻ and iKrAP; α SMA-RFP; α SMA-TK⁺ mice. 1ml of TRIzol Reagent (Life Technologies) was added into 50 – 100mg of samples. Tissues were homogenized by a tissue homogenizer, and homogenized sample were incubated for 5 minutes at room temperature. 200ul of chloroform per 1ml of TRIzol Reagent were added, and the mixtures were vigorously shaken by hand for 15 seconds. The mixtures were centrifuged at 12,000 x g for 15 minutes at 4 °C. The aqueous phase of the mixtures was collected by pipetting. 0.5ml of 100% isopropanol was added into the aqueous phase per 1ml of TRIzol Reagent, and incubate at room temperature for 10 minutes. The samples were centrifuged at 12,000 x g for 10 minutes at 4 °C. To wash pellets, 1ml of 75% ethanol per 1ml of TRIzol Reagent was used, and vortex the samples briefly. The samples were centrifuged at 7,500 x g for 5 minutes at 4 °C. We did air dry RNA pellet for 10 minutes, and added DEPC water. The RNA-seq was carried out with three biological replicates. Illumina HiSeq 2000 was utilized with 76nt PE sequencing format. A threshold of q value < 0.05 was used. Gene set enrichment analyses (GSEA) were performed with GSEA platform of the Broad Institute.

Chapter 3: α SMA⁺ myofibroblast-ablated tumors exhibit poor survival in murine CRC model.

CRC irresponsive to therapy is a lethal disease. Therefore, innovative treatments that eradicate primary tumors and prevent metastatic dissemination to distal organs are necessary to improve our capability to cure CRC patients. Tumor microenvironment (TME) had long been regarded as a key cellular regulator of the multi-step tumorigenesis in CRC. The TME in CRC is quite distinct from the standard tissue microenvironment, and it is composed of intra-tumoral bacteria and a particular phenotype of immune cells and stromal cells including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and cancer-associated fibroblasts (CAFs). CAFs have been mainly known as a potential therapeutic target to tumor progression and metastasis in CRC due to their pro-angiogenic and immunosuppressive characteristics in vitro settings. A Recent study has revealed that depletion of CAFs constitutes a protective response from the host rather than the pro-tumorigenic role in pancreatic ductal adenocarcinoma [94]. Another study demonstrated that fibroblast-restricted deletion of *IKK β* upregulated intestinal epithelial proliferation of intestinal epithelial cells, suppressed apoptosis of cancer cells, and promoted the recruitment of CD4⁺Foxp3⁺ Tregs [95].

Based on the previous finding, CAFs have a much wider range of functions. It is not clear that functions of different subtypes and signaling pathway involved between CAFs and other cells in tumor microenvironment.

Mouse model for spontaneous colorectal cancer

In this study, we utilized a spontaneous mouse model for colorectal cancer (iKrAP) containing six alleles: *Villin-Cre-ERT2*; *APC*^{fl_{ox}/fl_{ox}}; *p53*^{fl_{ox}/fl_{ox}}; *tetO-LSL-Kras*^{G12D}; *Rosa26-LSL-Luc*; *Rosa26-LSL-rtTA-GFP* provided by Dr. Adam Boutin in Ronald DePinho lab (Figure 1). Unlike other murine CRC models, iKrAP mice reliably recapitulate the genetic and histopathological features of human diseases including adenoma-carcinoma progression, abundant stroma, and distal metastasis.

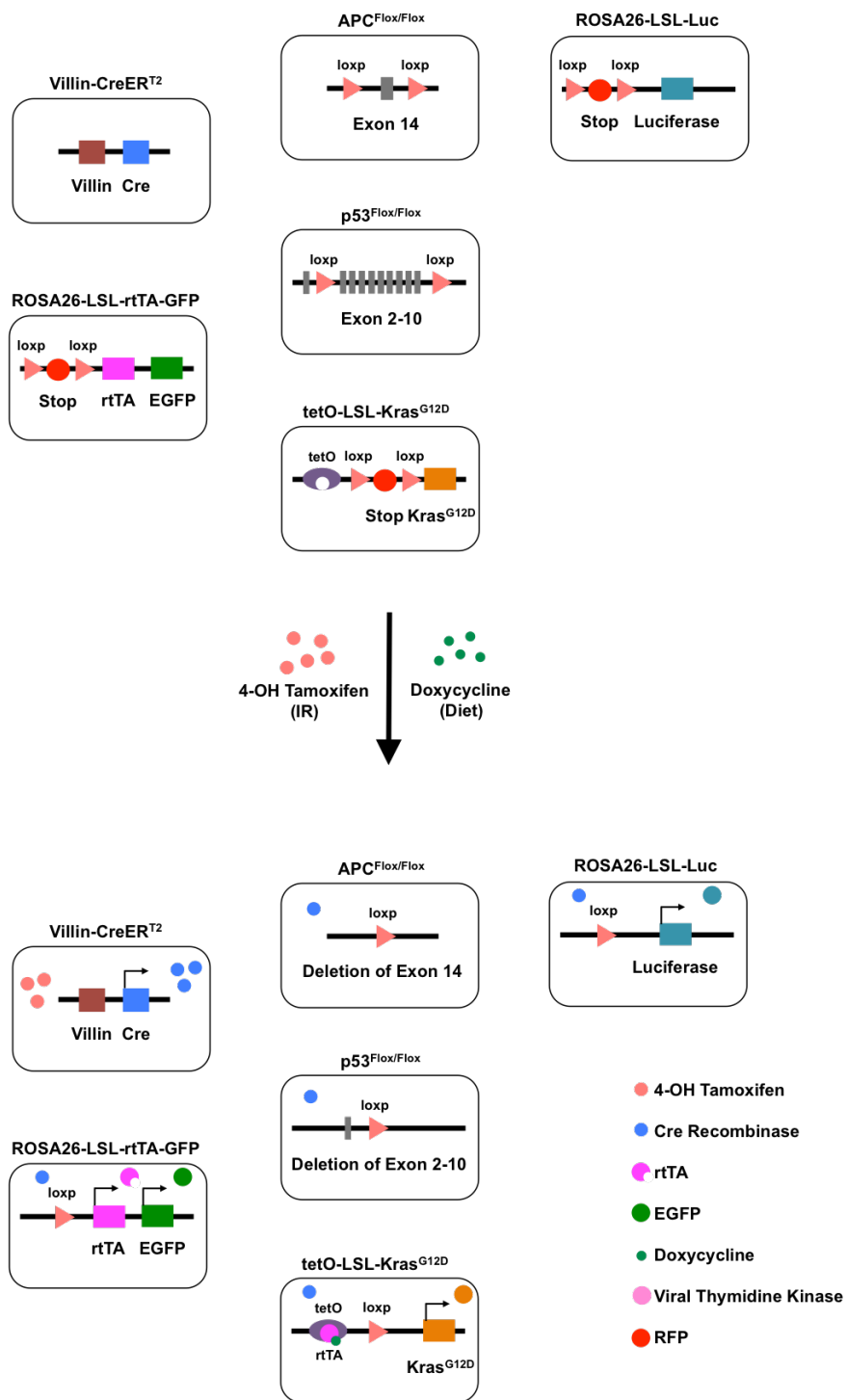


Figure 1. Spontaneous mouse colorectal cancer mice. Mouse colorectal cancer model containing six genetic alleles; *Villin*^{CreERT2}; *tetO-Lox-Stop-Lox-Kras*^{G12D}; *ROSA26-Lox-Stop-Lox-rtTA-IRES-EGFP*; *APC*^{fl/fl}; *P53*^{fl/fl}; *ROSA26-Lox-Stop-Lox-Luciferase*. Tamoxifen-dependent Cre recombinase excision of STOP codon induce deletion of exon 14 in *APC* gene, deletion of exon 2-10 in *p53* gene, and activation of rtTA. In the presence of doxycycline, rtTA binds tetO and initiate transcription of *Kras*^{G12D}.

α SMA⁺ myofibroblasts depletion is associated with decreased survival

To understand the functional significance of α SMA⁺ myofibroblasts in disease progression of colorectal cancer, we crossed iKrAP mice with α SMA-TK and α SMA-RFP mice that express viral thymidine kinase under control of α SMA promoter (Figure 2). Viral thymidine kinase phosphorylates Ganciclovir (GCV) to GCV-monophosphate, which is further converted to GCV-diphosphate and GCV-triphosphate by host kinases. Therefore, the treatment of GCV in α SMA-TK⁺ mice results in the selective elimination of proliferating α SMA⁺ cells due to incorporation of GCV-triphosphate in DNA as an analog of deoxyguanosine by polymerase during replication, leading to termination of DNA synthesis and subsequently cell death while α SMA⁺ cells in α SMA-TK⁻ mice have no effect with GCV treatment [96, 97] (Figure 3).

Tumors were introduced at 8 to 10 weeks ages of iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice by intrarectal injection of 4-hydroxy tamoxifen and doxycycline diet. Villin has expressed microvilli of the brush border of epithelium in both small and large intestine²³. Injection of tamoxifen though IP route causes severe damages to both small and large intestinal epithelium and all of the mice were moribund within two weeks (Data not shown). Therefore, we introduce tamoxifen only via intrarectal anemia. One month later,

endoscopy was performed for tumor incidence. Seven days post endoscopy, we daily treated GCV via intraperitoneal injection (IP) (Figure 4).

α SMA⁺ myfibroblast depletion is associated with significant reduction of survival (Figure 5). Around 60% of iKrAP; α SMA-TK⁺ mice developed hind limb paresis whereas all of the iKrAP; α SMA-TK⁻ mice were moribund because of bowel obstruction (Figure 6).

Immunohistochemical analysis of α SMA revealed around 70% reduction of α SMA⁺ myfibroblasts in α SMA-TK⁺ mice (Figure 7).

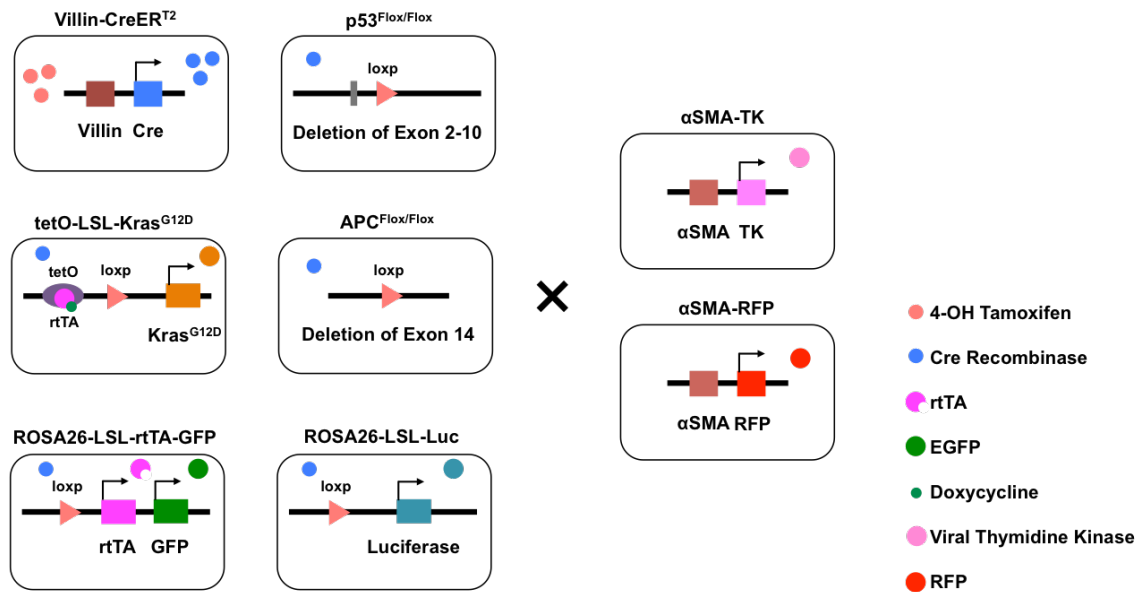


Figure 2. Spontaneous mouse colorectal cancer mice were crossed with α SMA-TK and RFP mice to targeting and monitoring, respectively. Mouse colorectal cancer model containing six genetic alleles; *Villin*^{CreERT2}; *tetO-Lox-Stop-Lox-Kras*^{G12D}; *ROSA26-Lox-Stop-Lox-rtTA-IRES-EGFP*; *APC*^{fl/fl}; *P53*^{fl/fl}; *ROSA26-Lox-Stop-Lox-Luciferase*. These mice were bred with α SMA-TK and α SMA-RFP mice.

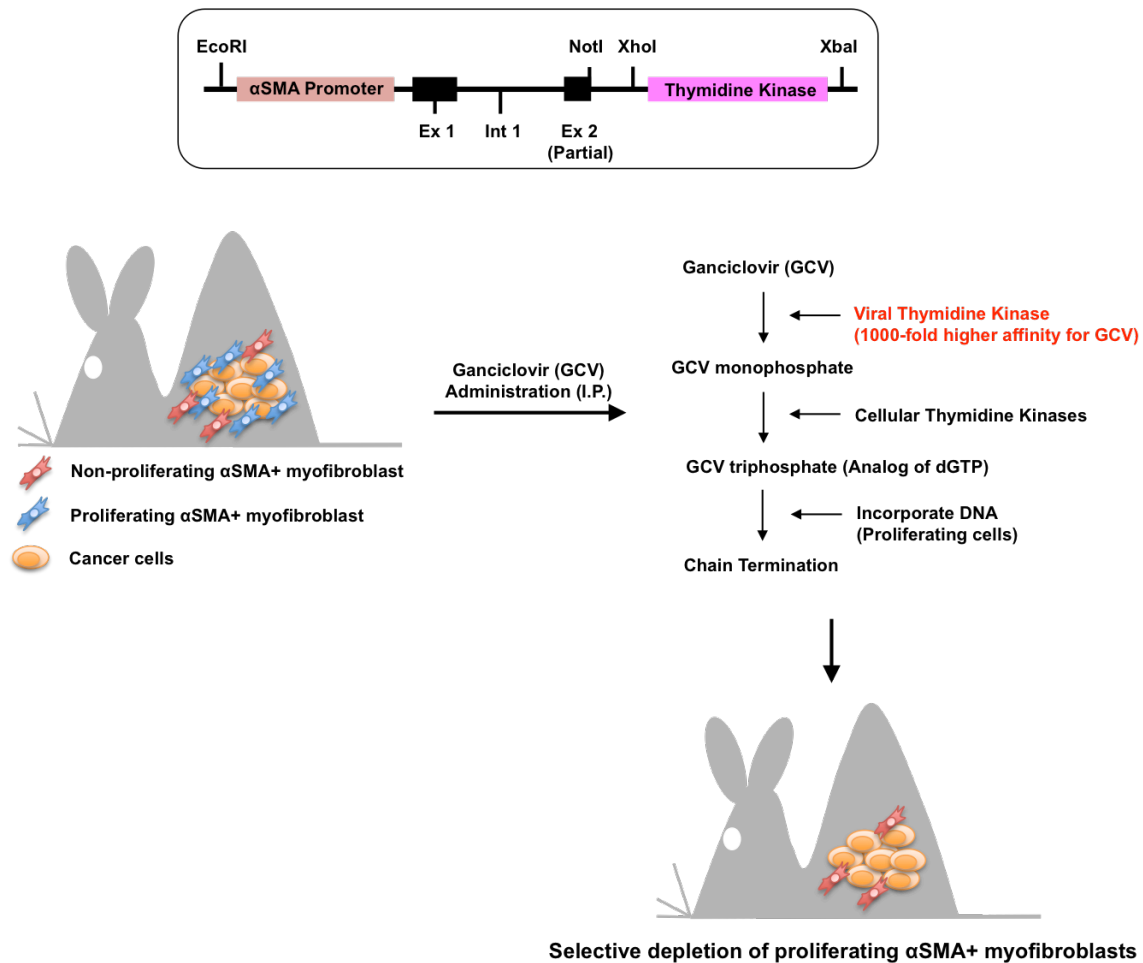


Figure 3. Mouse model targeting proliferating α SMA⁺ myofibroblast by treating GCV. GCV is an analog of deoxyguanosine used as a substrate, which is converted into GCV-triphosphate (GCV-TP) by cellular thymidine kinases. GCV-TP incorporated in DNA that led to premature termination of DNA and subsequently cell death.

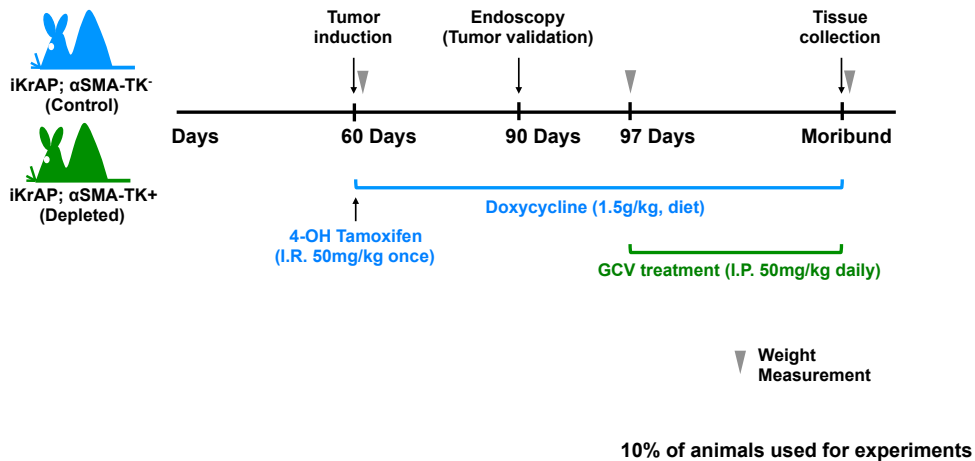


Figure 4. Experimental timeline for induction of tumors and ablation of α SMA⁺

myofibroblasts. Mice were intrarectally treated with 4-OH tamoxifen (50 mg/kg) and were fed with doxycycline pellets (200 mg/kg) at 60 days of age for induction of tumors. 30 days post tumor induction, endoscopy was performed to validate tumors in rectal and colon. At 97 days of age, GCV were intravenously administrated in tumor-bearing mice. The mice continued to receive GCV treatment until they became moribund.

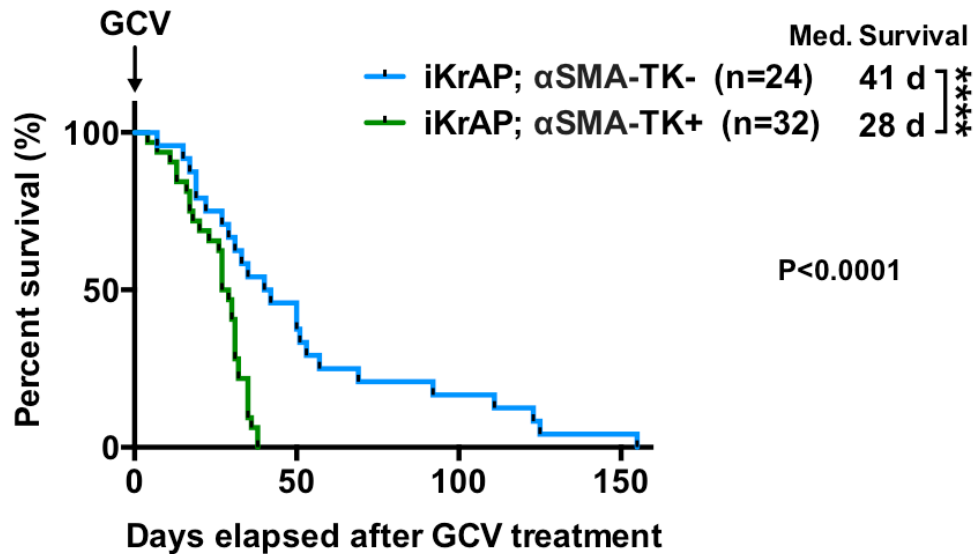
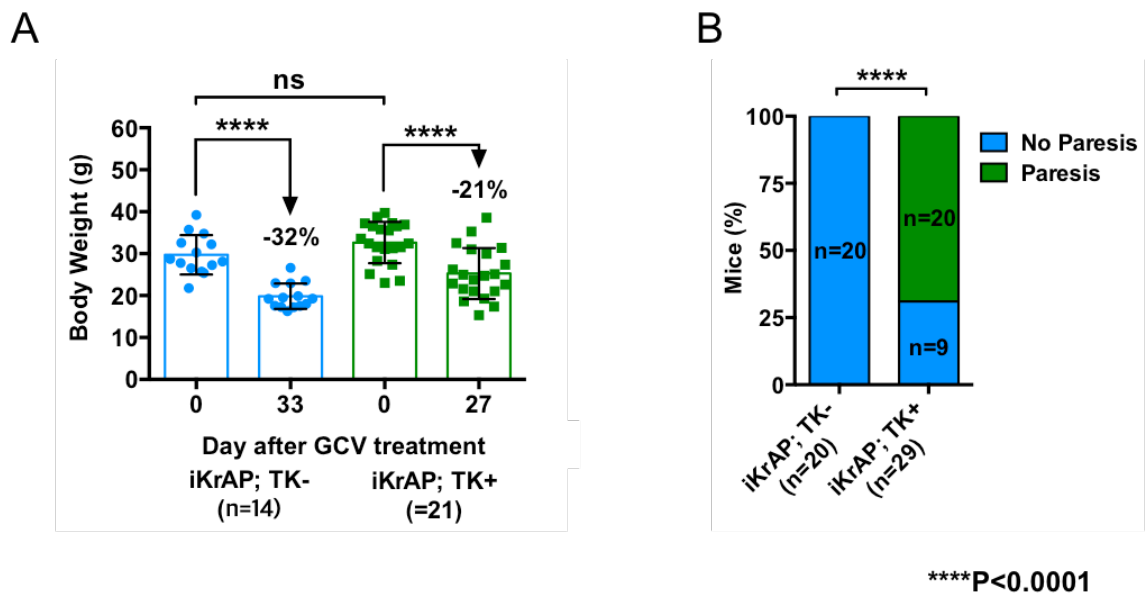


Figure 5. Decreased α SMA⁺ myofibroblasts correlated with poor survival. Daily GCV treatment via i.p. injection was initiated at 37 days post tumor induction. The mice were assessed on the sign of distress and endpoints everyday. The median survival of iKrAP; α SMA-TK+ was 28 days after treatment of GCV whereas that of iKrAP; α SMA-TK- was 41 days. (n=32; iKrAP; α SMA-TK+, n=24; iKrAP; α SMA-, ****p<0.001, Mentel-Cox test)



****P<0.0001

Figure 6. Ablation of α SMA⁺ myofibroblast results in limb paresis whereas non-depleted mice were moribund due to bowel obstruction. (A) (****p<0.0001, T-Test). (B) Comparison of the frequency of paresis (****p<0.0001, Contingency).

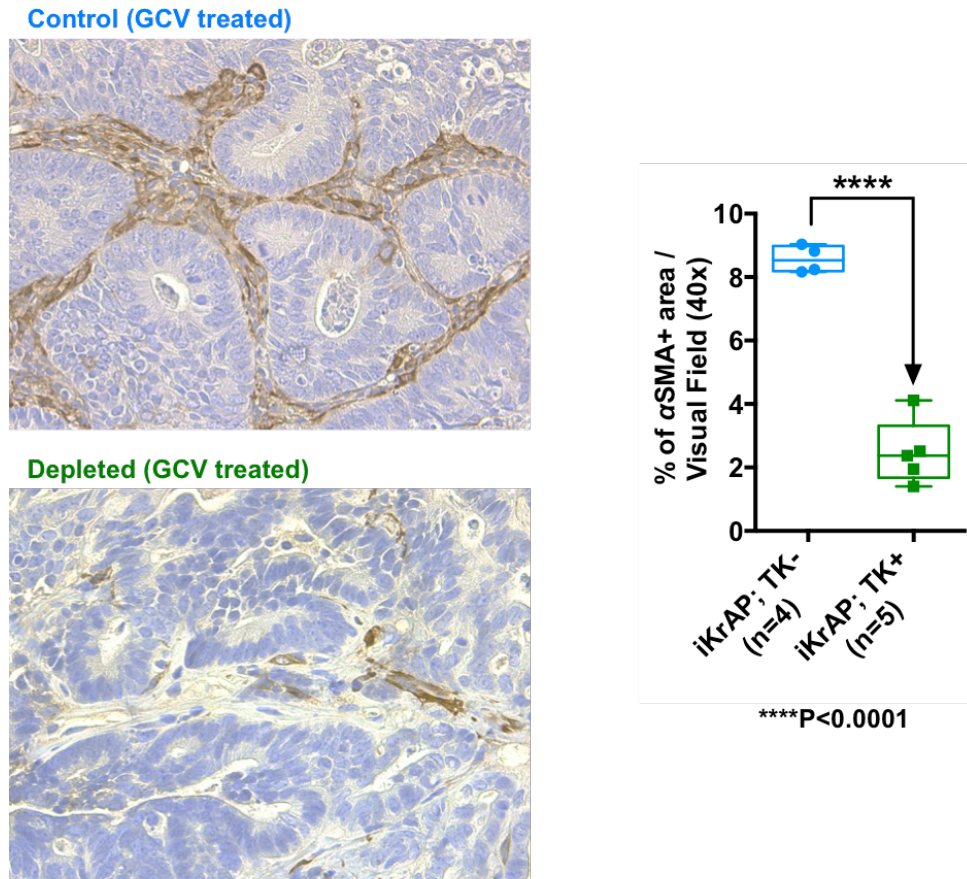


Figure 7. Decreased αSMA expression in iKrAP; αSMA-TK⁺ mice. Tumor sections from iKrAP; αSMA-TK⁻ and iKrAP; αSMA-TK⁺ were immunohistochemically stained with αSMA. Representative microscopic images and corresponding quantification (n=4; iKrAP; αSMA-TK⁻, n=5; iKrAP; αSMA-TK⁺, Student T-Test, ****p<0.0001)

Chapter 4: α SMA⁺ myofibroblast-depleted tumors present more invasive and metastatic phenotype

The major cause of death of CRC is the metastasis in liver and lung. The majority of patients with metastatic CRC remain incurable, and their median survival is around two years. Treatment and investigation of molecular characteristics in CRC only have focused on metastatic cancer cells. However, recent studies revealed that development and progression of CRC linked to many other aspects consisting ECM, vasculatures, CAFs, immune cells. Mainly, CAFs are known as having tumor-promoting characteristics by secreting growth factors, cytokines, and chemokines necessary for promoting tumor growth and metastasis in vitro setting.

Our previous finding demonstrated that decreased α SMA⁺ myofibroblasts correlated with poor survival. Therefore, we hypothesized that α SMA⁺ myofibroblasts-depleted tumors display increased metastasis to blood and lymphatic vessels, lung, and liver.

iKrAP; α SMA-TK⁺ mice exhibited decreased extraluminal tumor burden

Vertical tumor growth is an independent prognostic parameter for patients with CRC. Tumor size [140]. The size was significantly associated tumor progression and cancer-specific survival in most solid tumors. To investigate comparison of tumor size between α SMA⁺ myofibroblasts-depleted tumors and non-depleted tumors, we harvested tissue 30 days post GCV treatment and measure tumor size, tumor number, and colon length. Interestingly, α SMA⁺ myofibroblasts-depleted tumors showed that decreased extraluminal tumor size whereas tumor numbers was no different between two groups (Figure 8A, B). Colon length is one of indicator for colonic inflammation [98]. α SMA⁺

myofibroblasts-depleted colons were shorter than non-depleted colons, suggesting that α SMA⁺ myofibroblasts-depleted colons sustained more colonic inflammation (Figure 8C).

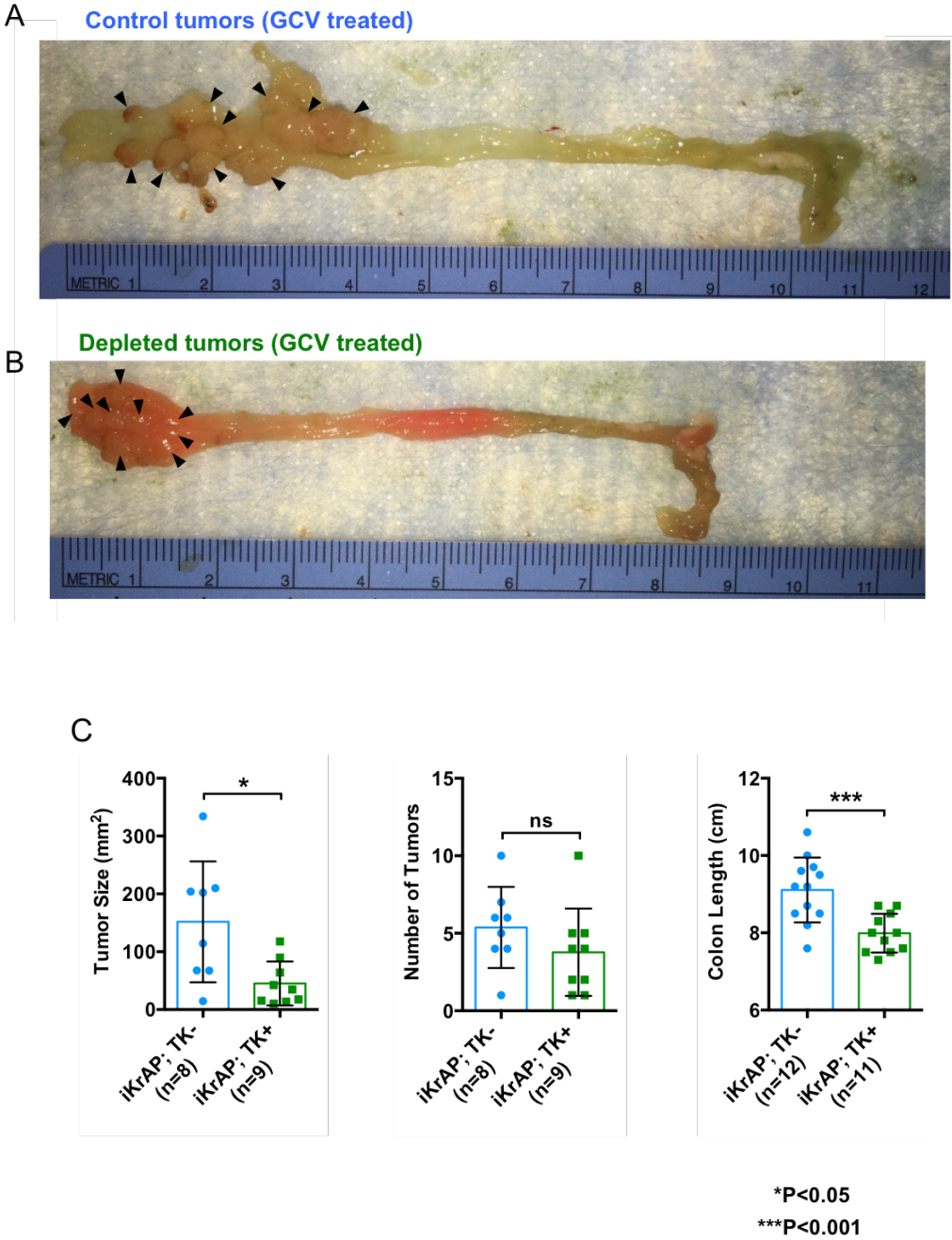


Figure 8. Depletion of α SMA⁺ myofibroblasts results in decreased extraluminal tumor burden and increased inflammation. (A) (B) Representative colon images from iKrAP; α SMA-TK- and iKrAP; α SMA-TK+ mice (C) Comparison of tumor size and number, and colon length (n=8; iKrAP; α SMA-TK-, n=9; iKrAP; α SMA-TK+, Student T-Test, ns; no significant difference, *p<0.05, ****p<0.0001)

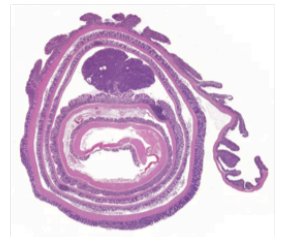
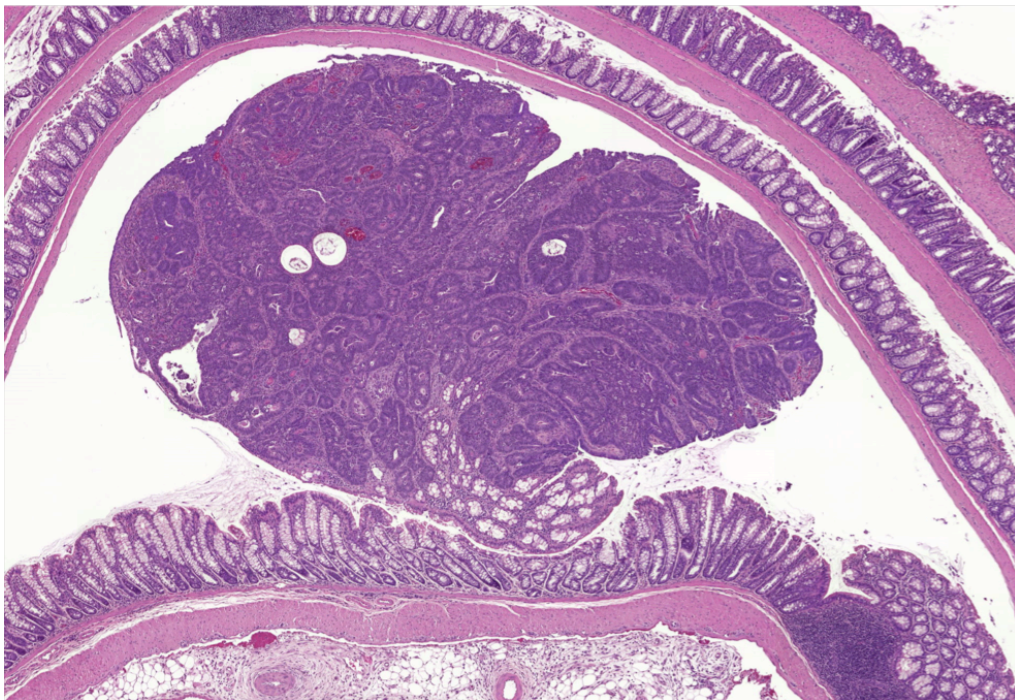
α SMA⁺ myofibroblast-ablated tumors displayed increased frequency of local invasion.

Next, we measured local invasion score to compare aggressiveness between α SMA⁺ myofibroblast-depleted tumors and non-depleted tumors. CRC arose from epithelium and grow vertically, protruding gut lumen. However, aggressive and advanced stages of CRC invade muscularis mucosa, submucosa, muscularis propria, and serosa. We measured this parameter based on local invasion score (Figure 9E). Non-depleted tumors typically exhibited well-differentiated with mostly no local invasion (Figure 9A, B, F). But α SMA⁺ myofibroblasts-depleted tumors displayed poorly differentiated, tumor budding, immune cell infiltration in the submucosa, and increased frequency of local invasion (Figure 9C, D, F). In conclusion, α SMA⁺ myofibroblasts-depleted tumors exhibited increased overall intraluminal tumor burden.

A **Control tumors (GCV treated)**



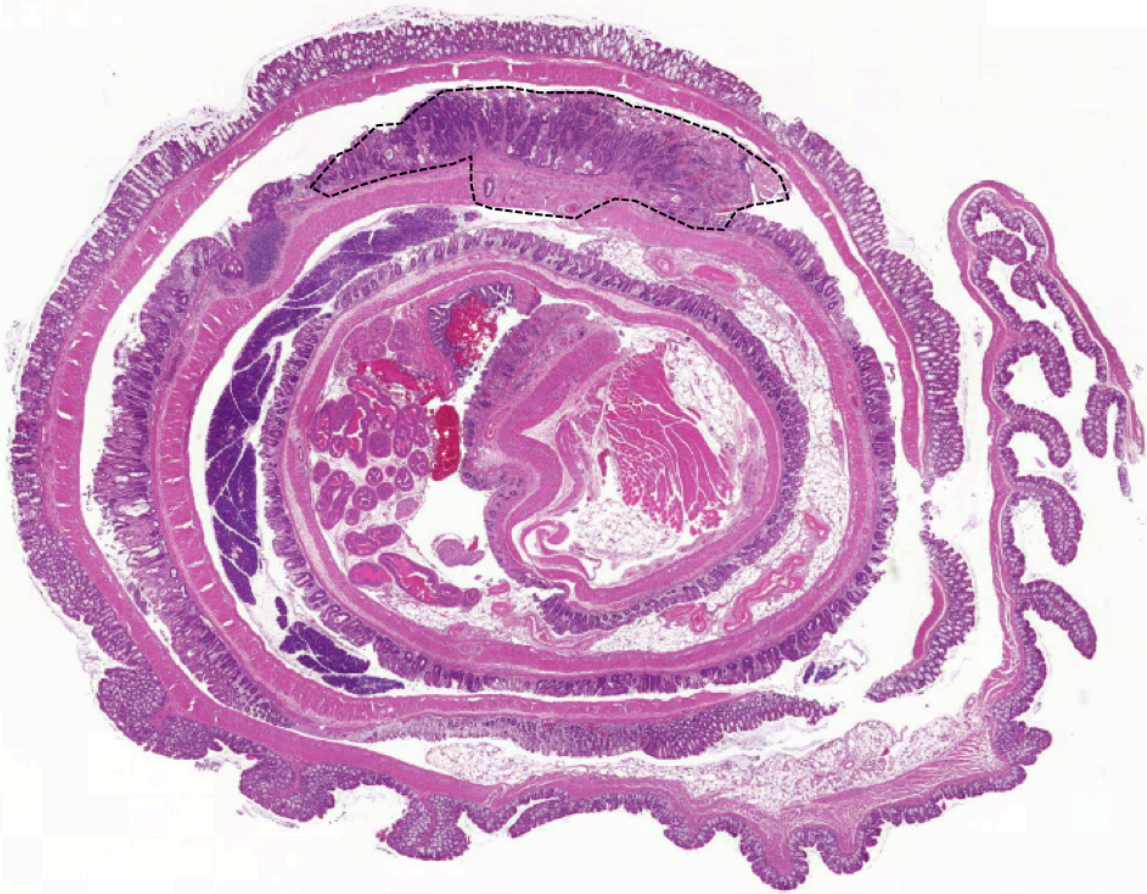
B **Control tumors (GCV treated)**



1. well-differentiated
2. Low frequency of local invasion

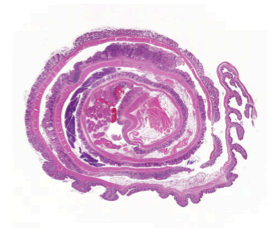
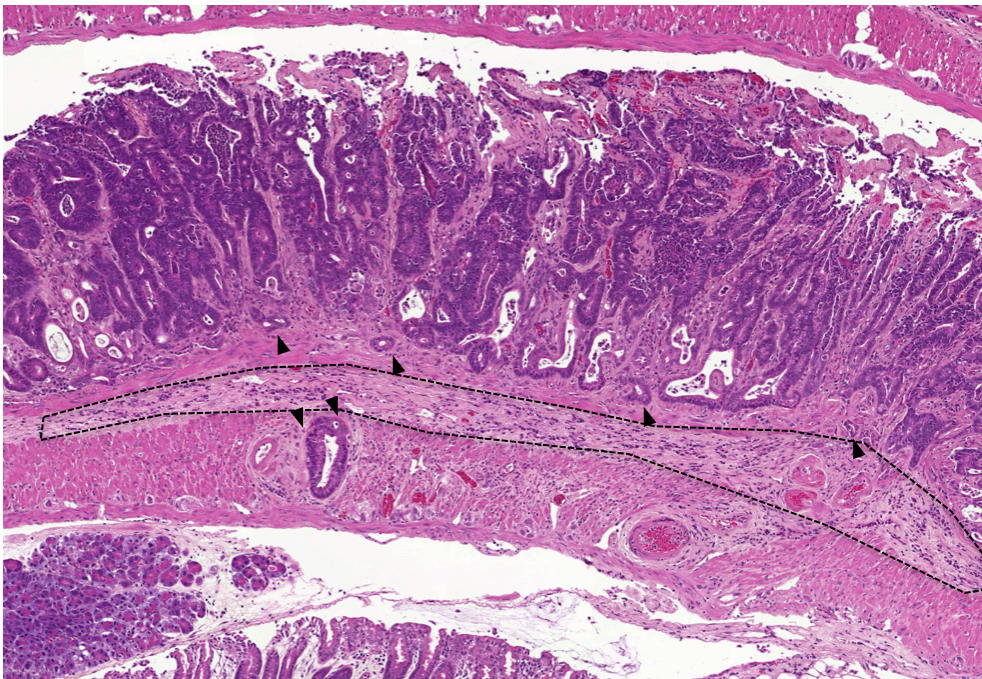
C

Depleted tumors (GCV treated)



D

Depleted tumors (GCV treated)



1. Poorly differentiated
2. Tumor budding
3. Local invasion
4. Immune cell infiltration

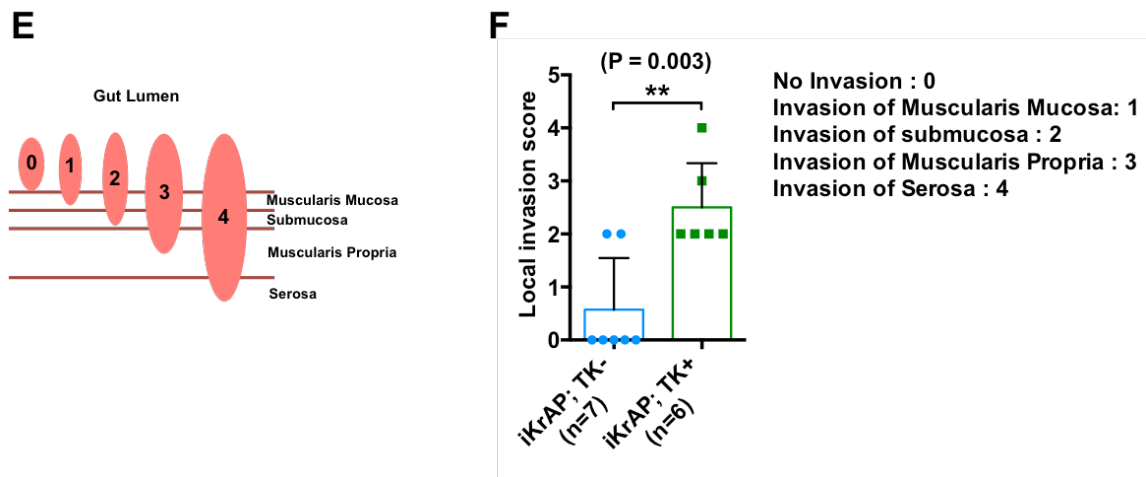


Figure 9. Depletion of αSMA^+ myfibroblasts exhibited increased frequency of local invasion (A) (B) (C) (D) Representative microscopic images tumors from iKrAP; $\alpha\text{SMA-TK}^-$ and iKrAP; $\alpha\text{SMA-TK}^+$ mice and (F) corresponding quantification (Student T-Test, $**p < 0.001$), (E) Schematic diagram of scoring system for local invasion. The invasion scoring is based upon the measurement of the deepest point of primary tumors. The measurement of invasion depth was categorized into four scales: 0 (no invasion), 1 (invasion of muscularis mucosa), 2 (invasion of submucosa), 3 (invasion of muscularis propria), and 4 (invasion of serosa).

Depletion of α SMA⁺ myofibroblast in tumor microenvironment affected to develop depressed tumors

Comparison of histopathological observations from previous data between two groups indicated that α SMA⁺ myofibroblasts-depleted tumors were depressed and nonpolypoid colorectal cancer which are harder to detect by colonoscopy or computed tomography colonography but more aggressive than generic CRC, irrespective of size [99, 100]. To further elucidate this finding, we decided to compare tumor sizes at two-time points: before and after GCV treatment by endoscopy. Strikingly, α SMA⁺ myofibroblasts-depleted tumors mostly exhibited decreased tumor size whereas non-depleted tumors display increased tumor size.

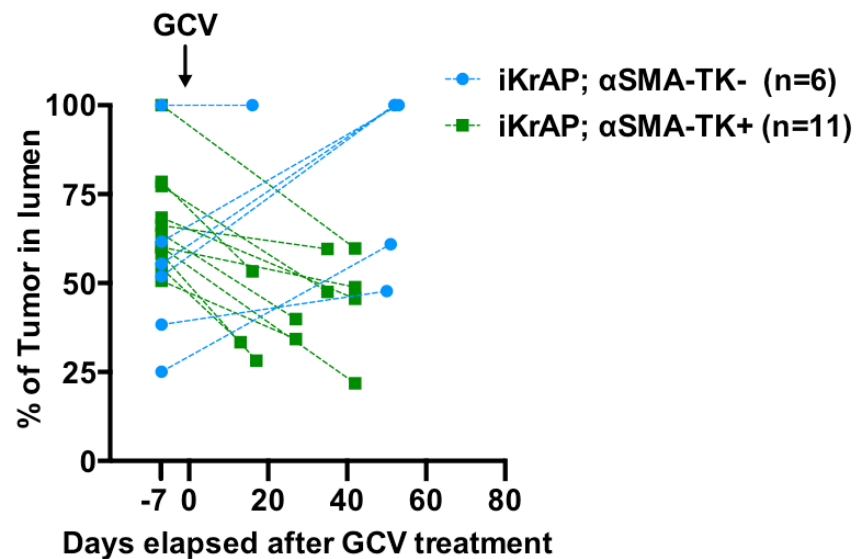
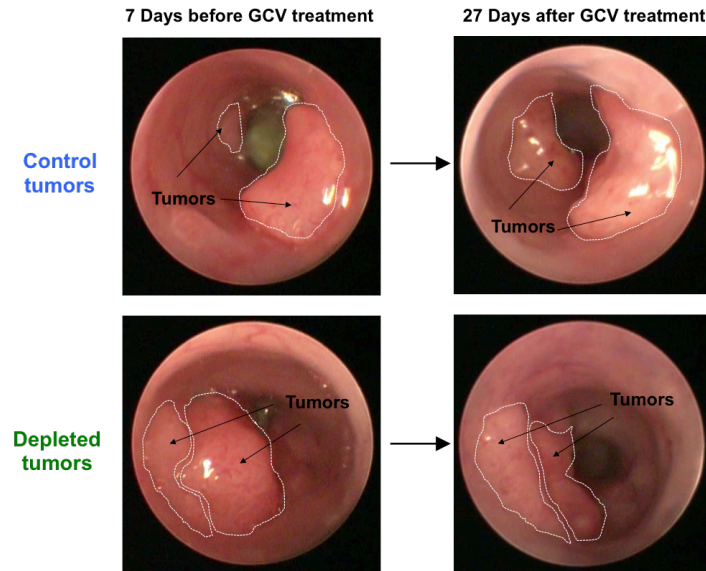


Figure 10. α SMA⁺ myofibroblast depletion affected morphological alteration to depressed CRC. First endoscopy was performed to both iKrAP; α SMA-TK- (n=6) and iKrAP; α SMA-TK+ (n=11)mice before GCV treatment. Once the mice became moribund, the second endoscopy was performed. The occupancy of tumors on the gut lumen was calculated with Photoshop. Representative colonoscopic images and corresponding quantification

Depletion of α SMA⁺ myofibroblasts increased CTCs

The major reason of cancer-related mortality is the metastasis of primary tumors to distal organs. During efficient dissemination, tumor cells invade the nearby tissues and organs of primary tumors, intravasate into blood vessels, migrate distant tissues, extravasate, adapt to new microenvironment, and eventually colonize to form metastasis [101].

The presence of CTCs in cancer patients was first described in 1869 [102] and many studies in past decade have shown that CTCs may be used as a biomarker to predict disease progression and survival in metastatic tumors. High CTCs numbers were relatively correlated with aggressive disease, increased metastasis, and reduced survival.

To investigate whether depletion of α SMA⁺ myofibroblasts impacts metastasis, the number of GFP⁺ CTCs from iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice was counted by FACS. We found that iKrAP; α SMA-TK⁺ showed dramatically increased CTC number (Figure 11).

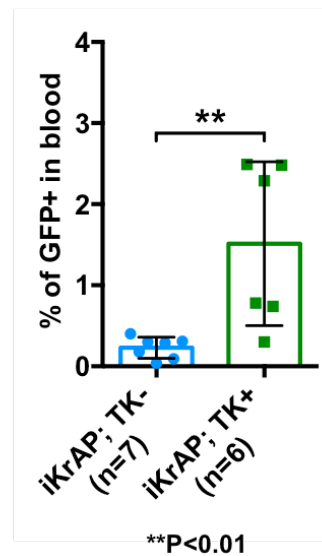
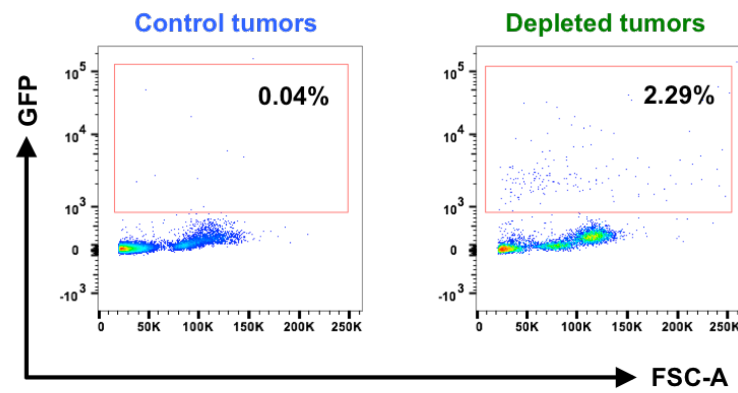


Figure 11. iKrAP; α SMA-TK⁺ showed increased CTC number. The blood was collected from iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice after GCV treatment. GFP⁺ cells were counted by FACS. Representative FACS images and corresponding quantification (Student T-Test, **p<0.01)

Depletion of α SMA⁺ myofibroblasts increased metastasis to lymphatic system

Lymph node metastasis is the key factor for prognosis of CRC. The 5-year survival of patients with lymph node-negative disease is around 70-80% in contrast to 30-60% in those with lymph node-positive disease [103].

To study whether depletion of α SMA⁺ myofibroblasts impacts metastasis of cancer cells to lymph nodes, we first utilized immunohistochemical analysis for lymphovascular invasion with lyve-1. α SMA⁺ myofibroblast depleted tumors exhibited significantly increased invasion of cancer cells to lymphatic vessels (Figure 12).

Next, we further investigated that increased lymphatic vessel invasion correlated with local lymph node metastasis. We harvested mesenteric, lumbar, and inguinal lymph nodes and thymus from iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice to measure GFP⁺ cancer cells by FACS. We found that iKrAP; α SMA-TK⁺ displayed increased GFP⁺ cancer cell number in lymph nodes and thymus (Figure 13).

Depletion of α SMA⁺ myofibroblasts increased metastasis to pericolonic and perirectal fat

Pericolonic tumor deposits (PTDs) are metastatic adenocarcinoma nodules occurring in the pericolonic and perirectal fat. PTDs are an independent prognostic factor for advanced CRCs, and a destructive type of venous invasion different from other types of vessel involvement. Many studies showed that the presence of PTDs correlated with shorter overall survival in patients with stages III and IV CRCs [143]. Therefore, we investigated whether depletion of α SMA⁺ myofibroblast increased dissemination of cancer cells to pericolonic and perirectal fat. Interestingly, depleted animals exhibited the remarkable inflammation of pericolonic fat, and histopathological analysis revealed that

these inflammation lesions contained adipocytes, immune cells, vasculature, and cancer cells (Figure 14A). To confirm histopathological analysis, we performed FACS, and we found increased infiltration of cancer cells and immune cell population in depleted tumors (Figure 14B).

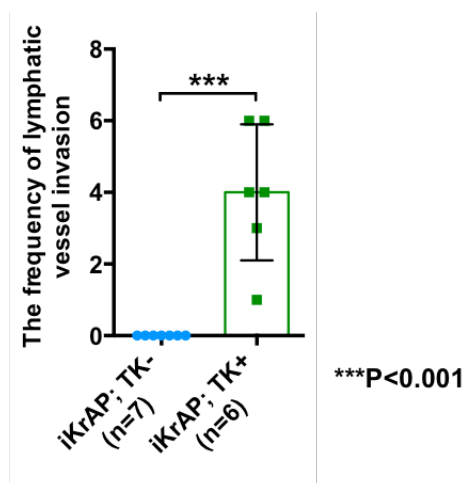
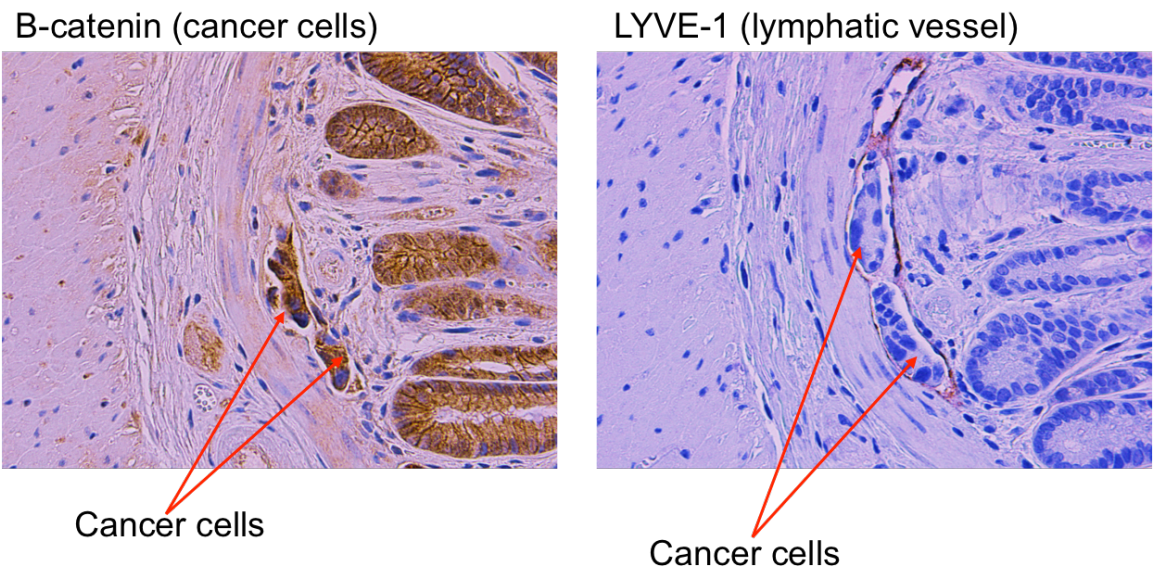
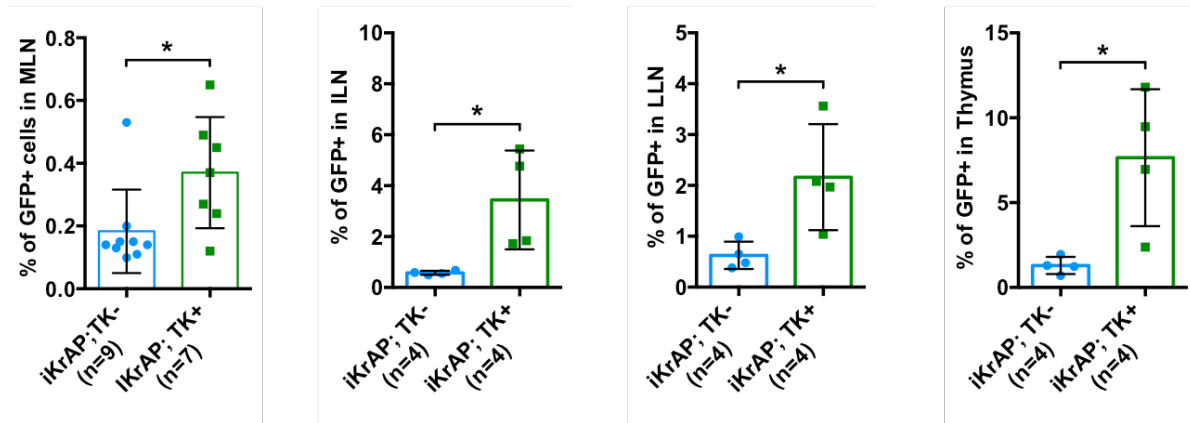


Figure 12. iKrAP; α SMA-TK⁺ showed increased invasion of cancer cells to lymphatic vessels. Tumor sections from iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice were immunohistochemically stained with B-catenin and LYVE-1 to identify cancer cells and lymphatic vessels, respectively. Representative microscopic images and corresponding quantification (n=7; iKrAP; α SMA-TK⁻, n=6; iKrAP; α SMA-TK⁺, Student T-Test, ***p<0.001)



MLN : Mesenteric lymph node (regional)
 LLN : Lumber lymph node (regional)
 ILN : Inguinal lymph node (distant)

*P<0.05

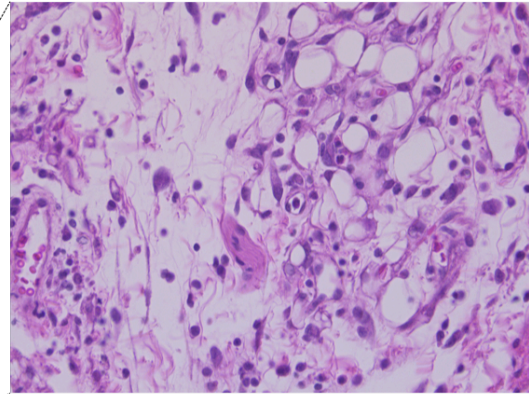
Figure 13. iKrAP; α SMA-TK⁺ showed increased metastasis of cancer cells to the lymphatic system. Mesenteric, lumber, and inguinal lymph nodes and thymus were harvested from iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice after GCV treatment. GFP+ cells were counted by FACS. Representative FACS images and corresponding quantification (Student T-Test, *p<0.05)
 (T-Test, *p<0.05)

A

Control



Tumor



Depleted



Tumor

B

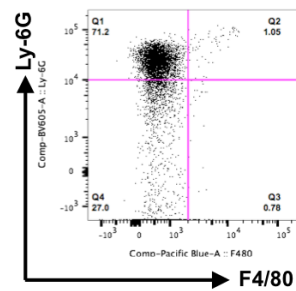
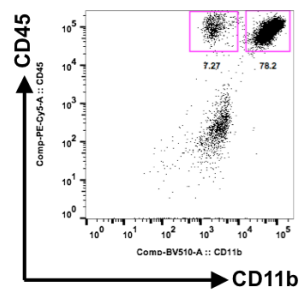
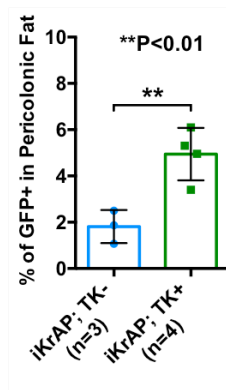
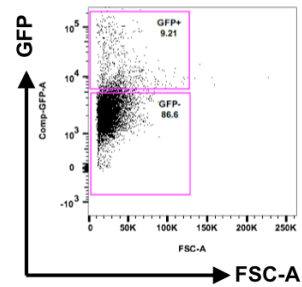


Figure 14. iKrAP; α SMA-TK⁺ showed increased metastasis of cancer cells to pericolonic and perirectal fat. (A) Pericolonic and perirectal fat were harvested from iKrAP; α SMA-TK⁻ and iKrAP; α SMA-TK⁺ mice after GCV treatment. (B) GFP⁺ cells were counted by FACS. Single cells suspension was stained with anti-CD3, anti-CD45, and anti-CD11b. Representative FACS images and corresponding quantification (Student T-Test, *p<0.05)

Depletion of α SMA⁺ myofibroblasts induce collagen-rich stroma to promote cancer cell invasion

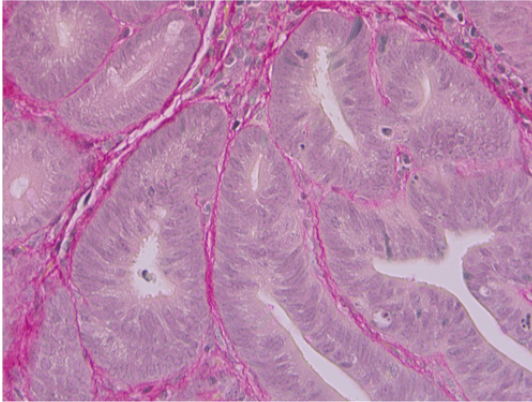
ECM remodeling is tightly regulated during development in normal colon. Abnormal ECM production can upregulate integrin signaling and thus can improve cell survival and proliferation [104]. Increased collagen contents by LOX overexpression promote ERK and PI3 kinase signaling in cancer cells [105]. The properties of ECM also play a significant role in regulating both stem cell and cancer stem cells. Abnormal changes of ECM disrupt cellular differentiation, resulting in loss of differentiation and increased stem cell pool [106].

A Recent study showed that collagen-1 promotes mesenchymal gene expression and stimulate tumor cell invasion and collagen-1 is the most enriched genes in recurrence-prone tumors in CRC [107].

Previous our findings revealed that ablation of α SMA⁺ myofibroblasts induced poorly differentiated, more invasive and metastatic tumors. Therefore, it is impossible that aberrant expression of collagen contents alters the behavior of cancer cells, aggressive phenotype.

To test this possibility, picrosirius red staining was performed and revealed that a significant increase of collagen contents in αSMA^+ myofibroblasts-depleted tumors (Figure 15). Additionally, Tumor collagen I assayed by type 1 collagen immunostaining was significantly increased αSMA^+ myofibroblasts-depleted tumors (Figure 16).

Control tumors



Depleted tumors

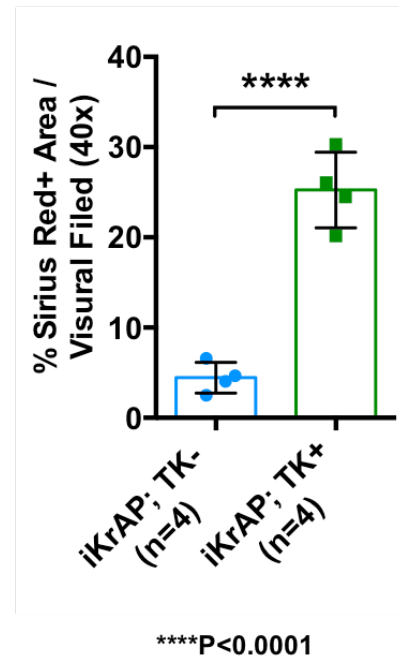
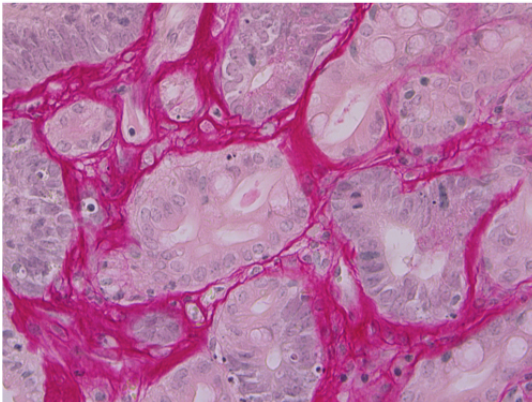
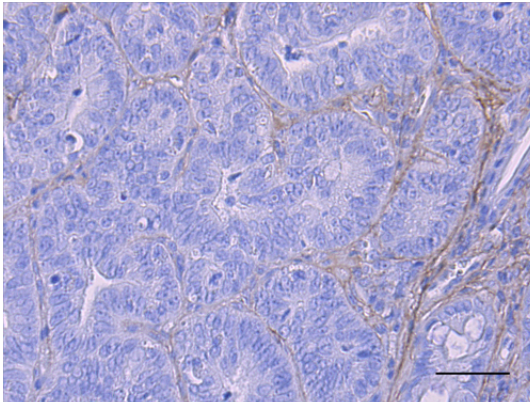


Figure 15. α SMA⁺ myofibroblasts-depleted tumors revealed increased collagen deposition.

Tumor sections from iKrAP; α SMA-TK- and iKrAP; α SMA-TK+ mice were immunohistochemically stained with Sirius Red. Representative microscopic images and corresponding quantification (n=4; iKrAP; α SMA-TK-, n=5; iKrAP; α SMA-TK+, Student T-Test, ****p<0.0001)

Control tumors



Depleted tumors

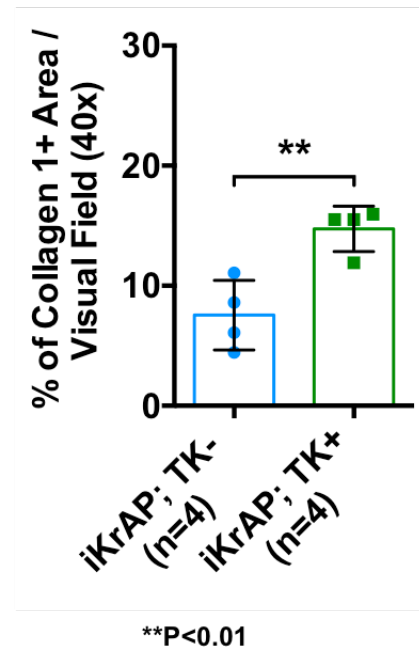
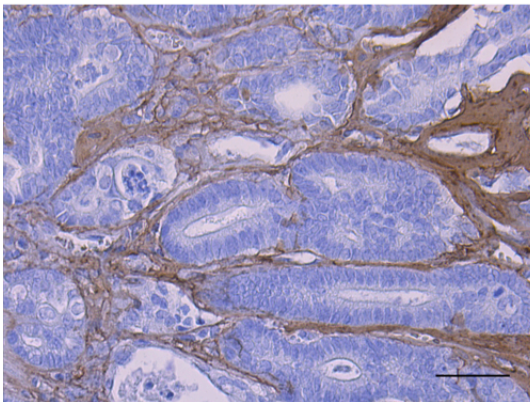


Figure 16. α SMA⁺ myofibroblasts-depleted tumors displayed increased type I collagen.

Tumor sections from iKrAP; α SMA-TK- and iKrAP; α SMA-TK+ mice were immunohistochemically stained with collagen I. Representative microscopic images and corresponding quantification (n=4; iKrAP; α SMA-TK-, n=5; iKrAP; α SMA-TK+, Student T-Test, **p<0.01)

Chapter 5: Depletion of α SMA⁺ myofibroblasts results in immune-suppressive microenvironment in CRC

Cancer cells interact with their surroundings for growth, invasion, and metastasis. The tumor microenvironment is composed of a diverse range of cells including stroma cells and immune cells. The immune system is capable of controlling and shaping tumors through a process known as immunoediting; elimination, equilibrium, and escape [108]. Experimental evidence has shown that inflammation support tumor development and growth [109], but the role of adaptive immune responses is still widely unclear based on murine and human studies. Although the presence of high proportion of cytotoxic CD8 T cells has been most associated with a better prognosis in CRC, the prognostic assessment to infiltration of Foxp3⁺ regulatory T cells (Tregs) has been controversial [110].

About the prognostic value of Foxp3⁺ Tregs, low CD3⁺ T cells and Foxp3⁺ Tregs in stage II and stage III CRC is associated with shorter patient survival [111]. Another study investigated that the proportion of Foxp3⁺ Tregs in healthy colon versus tumor tissue and found that high Foxp3⁺ Tregs proportion in tumor tissue was associated with improved survival, whereas the high density of Foxp3⁺ Tregs in the normal colon was associated worse prognosis [112].

Additionally, suppressive immune profiles with increased CD4⁺Foxp3⁺ Tregs and decreased CD8⁺ cytotoxic T cells were observed in α SMA⁺ myofibroblasts-depleted mouse pancreatic tumors [94].

To interrogate whether depletion of α SMA⁺ myofibroblasts leads to immunosuppressive microenvironment, we performed FACS analysis. We noted that α SMA⁺ myofibroblasts-depleted tumors displayed increased percentage of CD4⁺ Foxp3⁺ Tregs leading to the

overall decrease in Teff/Treg ratio together with reduced percentage of CD8⁺ cytotoxic T cells (Figure 17).

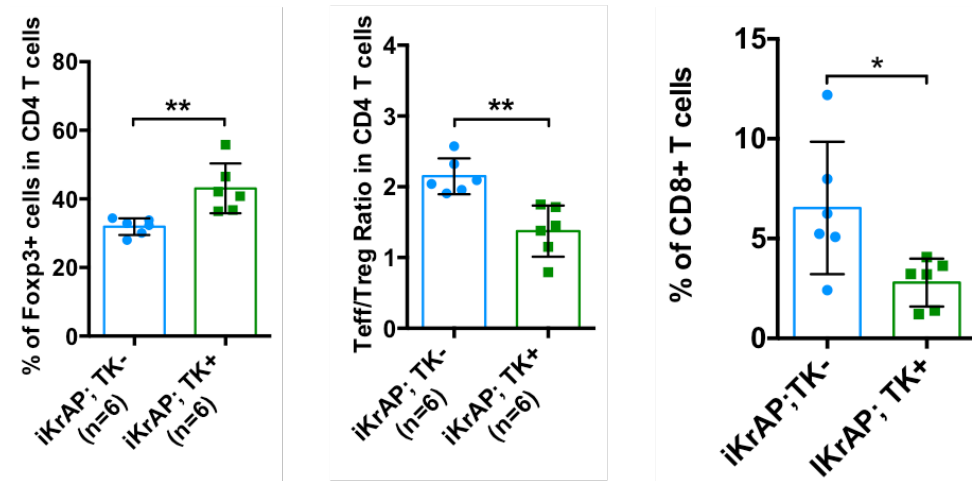


Figure 17. Targeting α SMA⁺ myofibroblasts exhibited immunosuppressive

microenvironment. Tumor tissues were harvested and digested with enzyme mixture including collagen 8, dispase I, and DNase I. For surface staining, single cell suspension was stained with anti CD3e, CD4, CD8, and CD45. For foxp3 staining, cells were fixed with Foxp3 staining kit and staining with anti-Foxp3. (n=6; iKrAP; α SMA-TK-, n=6; iKrAP; α SMA-TK+, Student T-Test, **p<0.01, *p<0.05)

Chapter 5: Depletion of α SMA⁺ myofibroblasts results in increase of cancer stem cells in CRC

Many emerging evidence suggests that a small subpopulation of cancer cells, termed cancer stem cells (CSCs), are responsible for propagation and dissemination in a very efficient manner [113]. Compared normal stem cells, CSCs show slow rate of cell cycling responsible for resistance to treatment such as chemotherapy and radiotherapy and recurrence [114, 115]. Also, CSCs have a critical feature to initiate new tumors for metastatic colonization to the distal organ.

In CRC, gene expression array to human patient samples revealed that aggressive CRC is enriched in intestinal stem cell gene expression including Lgr5 [116]. Also, there was a significant increase in Lgr5 expression in patients at stages III and IV comparing to stages I and II of CRC. The expression of Dclk1 was also elevated in advanced stages [117].

Since pathological morphology of α SMA⁺ myofibroblasts-ablated tumor revealed more undifferentiated tumors with enhanced invasiveness, we expected increased expression of Lgr5 and Dclk1 in cancer cells of the α SMA⁺ myofibroblasts-ablated tumor.

Targeting α SMA⁺ myofibroblasts increased LGR5⁺ cancer stem cell population

To investigate whether α SMA⁺ myofibroblasts depletion is associated with increased Lgr5⁺ cancer stem cells in CRC, we decided to perform FACS analysis. As we expected, tumors with α SMA⁺ myofibroblasts depletion revealed increased Lgr5 expression on cancer cells (Figure 18).

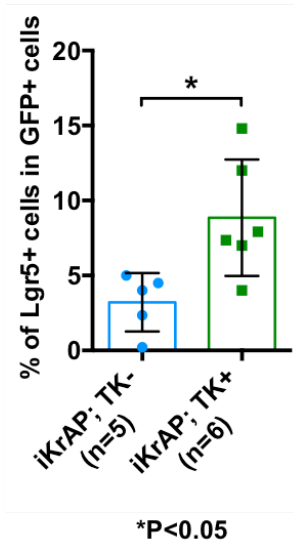
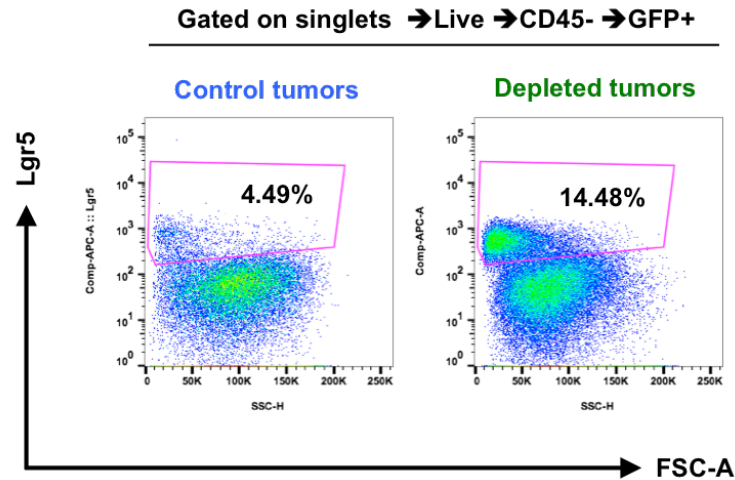


Figure 18. Targeting α SMA⁺ myofibroblasts resulted in the Lgr5⁺ cell among cancer cells.

Tumor tissues were harvested and digested with enzyme mixture including collagen 8, dispase I, and DNase I. Single cell suspension were stained with anti Lgr5 and CD45. Representative FACS images and corresponding quantification (n=5; iKrAP; α SMA-TK-, n=6; iKrAP; α SMA-TK+, Student T-Test, *p<0.05)

Assessment of Dcl⁺ cells in colon cancer cells

Next, we tested whether depletion of α SMA⁺ myofibroblasts increases Dcl⁺ cancer stem cell population. Immunohistochemical analysis revealed that dramatic increased Dcl⁺ cancer stem cell population when we targeted α SMA⁺ myofibroblasts (Figure 19).

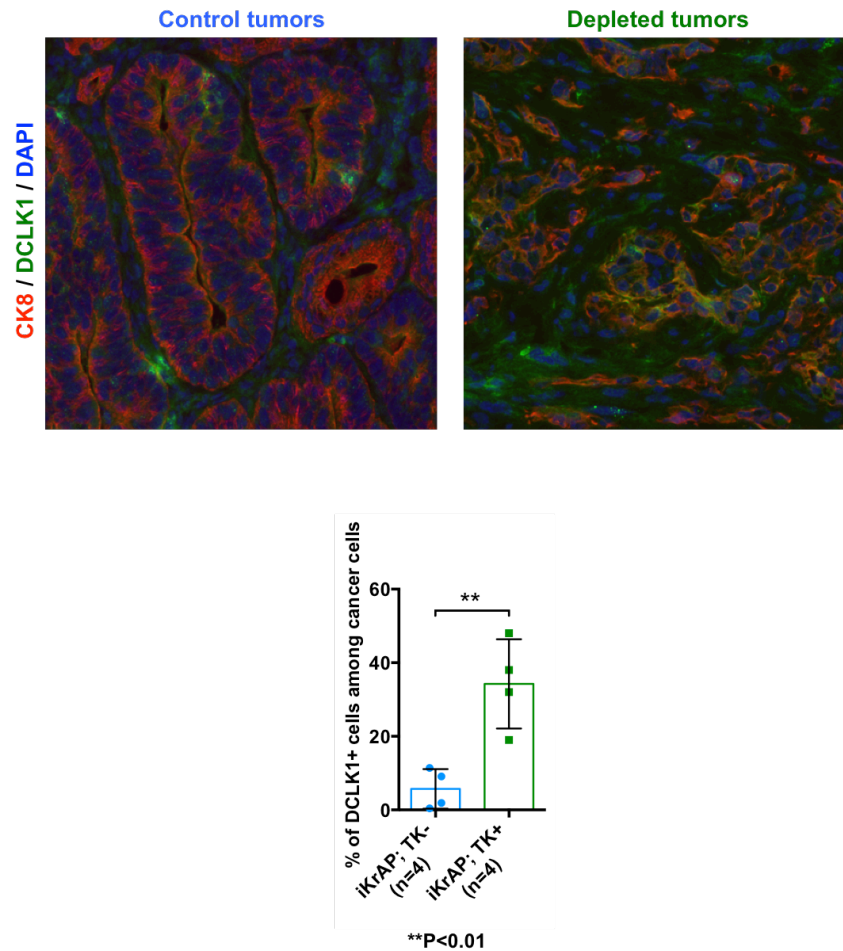
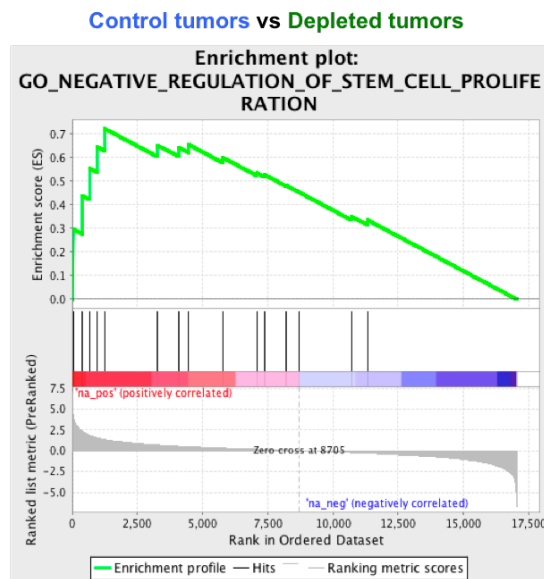


Figure 19. Targeting α SMA⁺ myofibroblasts led to increased expression of DCLK1 in cancer cells. Tumor sections from iKrAP; α SMA-TK- and iKrAP; α SMA-TK+ mice were immunohistochemically stained with CK20 and DCLK1. Representative microscopic images and corresponding quantification (n=4; iKrAP; α SMA-TK-, n=4; iKrAP; α SMA-TK+, Student T-Test, **p<0.01)

Depletion of α SMA⁺ myofibroblasts caused to up-regulation of cancer stem cell proliferation

Previous our observation indicated that ablation of α SMA⁺ myofibroblasts increased the frequency of Lgr5⁺ or Dclk1⁺ cancer stem cell population in TME. To address molecular mechanism regarding this phenotype, we performed RNA sequencing (RNA-seq). We found that a significant enrichment of the signature genes in negative regulation of stem cell proliferation in control tumors, including pleiotrophin (PTN), singles-type MMTV integration site family member 5A (WNT5A), snail homolog 2 (SNAI2), fibulin 1 (FBLN1), and bone morphogenetic protein 4 (BMP4) (Figure 20). This result highlight α SMA⁺ myofibroblasts play an essential role in regulation of cancer stem cell proliferation.

A



Normalized enrichment Score (NES): 1.865

FDR q-value: 0.0166

Control tumors (n=3)

Depleted tumors (n=3)

1. PTN

pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)

2. WNT5A

wingless-type MMTV integration site family, member 5A

3. SNAI2

snail homolog 2

4. FBLN1

fibulin 1

5. BMP4

bone morphogenetic protein 4

B

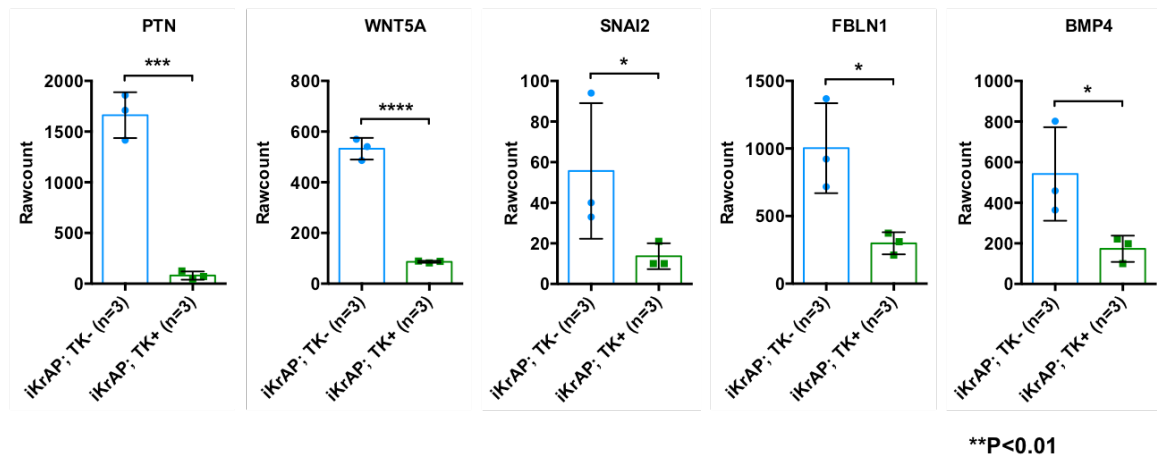


Figure 20. Ablation of α SMA⁺ myofibroblasts resulted in enrichment of negative regulation of cancer stem cell proliferation in control tumor. (A) GSEA revealed significant enrichment of gene signatures with negative regulation of stem cell proliferation. (B) Comparison of RNA rawcount between control tumors and depleted tumors (n=3; iKrAP; α SMA-TK⁻, n=3; iKrAP; α SMA-TK⁺, Student T-Test, *p<0.05)

Chapter 6: Summary and Discussion

It is widely believed that CAFs contribute to tumor growth, invasion, and metastasis by secreting various growth factors, ECM proteins, chemokines, and cytokines. These cellular and extracellular components alter genetic and epigenetic features of cancer cells. In this regard, several studies were reported that CAFs correlated with poor prognosis in cancer patients. LOXL2 is highly expressed in CAFs, which is associated with poor colon cancer survival [118]. Expression of PDGF receptor on CAFs is associated with metastasis and poor survival in CRC [119]. Our data demonstrate that ablation of α SMA⁺ myofibroblasts results in multiple effects likely leading to reduced survival. These results indicated that α SMA⁺ myofibroblasts have a protective role in CRC.

Several studies have been proposed that myofibroblasts can be a putative target for cancer therapy because of their pro-invasive activity in vitro culture and tumor xenograft mouse model. Only when adding conditioned media from myofibroblast culture isolated from surgical colon cancer fragments, colon cancer cells invaded collagen gel [141]. Coimplantation of colon cancer cells and myofibroblasts dramatically increased invasiveness of colon cancer cells [80] [120]. We demonstrated that α SMA⁺ myofibroblasts-ablated tumors exhibited increased local invasion, lymphovascular invasion, CTCs number, and lymph node metastasis. Additionally, the morphology of α SMA⁺ myofibroblasts-ablated tumors was similar with non-polypoid tumors that are relatively depressed and flat but have a high risk of local invasion regardless of size. Therefore, we speculated that α SMA⁺ myofibroblasts served as the physical barrier to prevent migration of cancer cells to blood and lymphatic vasculatures.

One of our interesting findings is that depletion of α SMA⁺ myofibroblasts results in remodeling of the ECM with increased collagen contents in TME. Many studies suggested that α SMA⁺ myofibroblasts and type I collagen are associated with tumor progression [121-123]. It is widely accepted that myofibroblasts mainly contribute to the production of type I collagen in TME but recent studies demonstrated that cancer cells and other stroma cells secrete Type I collagen [124] [142]. Also, fibroblasts such as FAP⁺, FSP1⁺, or vimentin⁺ subpopulation could compensate collagen deposits. Myofibroblasts are also the primary source of ECM-degrading proteins including MMPs. In these respects, we can postulate that cancer cells and other stroma cells may compensate production of type I collagen or lack of MMP2 and MMP7 secreted by α SMA⁺ myofibroblasts. This causes a reduction in the efficacy of degradation of collagen contents in TME.

Another impact of α SMA⁺ myofibroblast depletion was the alteration of immune cell infiltration. Although most of the studies elucidating tumor-promoting functions of CAFs were driven by co-culture experiments, CAFs are considered as a facilitator of immunosuppressive TME. CAFs stimulated IL-6 signaling pathway on dendritic cells to inhibit maturation, resulting in T cell anergy [125]. CAFs isolated human non-small cell lung cancer expressed PD-L1 and PD-L2 [126]. In vivo study, depletion of α SMA⁺ myofibroblasts led to immunosuppressive adaptive immunity with increased Foxp3⁺ Treg infiltration in PDAC [94]. We demonstrated that T cell-mediated immune responses were impaired when α SMA⁺ myofibroblasts were ablated. α SMA⁺ myofibroblast-depleted tumors exhibited increased Foxp3⁺ Treg infiltration along with decreased recruitment of CD8⁺ cytotoxic T cells. Also, tumor-expressing collagens can trigger an inhibitory

signaling via leukocyte-associated Ig-like receptors (LAIRs) for inactivation of cytotoxic NK cells [127]. This study supported that aberrant deposition of collagen contents promoted immunosuppressive TME in α SMA⁺ myofibroblast-depleted tumors. The most striking effect when α SMA⁺ myofibroblasts were depleted is increased stemness in cancer cells. CAFs are considered as a putative regulator of cancer stem cells because of major capability to secrete ECM proteins that interact ECM receptors to acquire or maintain stem cell properties. CAFs may induce epithelial-mesenchymal transition (EMT)-driven cancer stemness in prostate cancer via reciprocal interaction between cancer cells and CAFs [128]. IGF1R signaling in cancer cells was activated in the presence of CAFs that induce Nanog expression and promote stemness in non-small cell lung cancer [129]. Our data showed that the proportion of cancer stem cell population expressing Lgr5⁺ or Dclk1⁺ was increased in α SMA⁺ myofibroblast-depleted tumors. RNA-seq data revealed that gene signatures associated with down-regulation of stem cell proliferation were enriched in non-depleted tumors. Therefore, it is possible that aberrant collagen deposition in α SMA⁺ myofibroblast-depleted tumors led to stem cell overexpression and loss of differentiation.

In summary, our data demonstrated a protective role of α SMA⁺ myofibroblasts in CRC and targeting α SMA⁺ myofibroblasts resulted in diminished survival with the aggressive tumor, aberrant collagen deposit, accumulation of Foxp3⁺ Tregs, and increased cancer stem cell population (Figure 21).

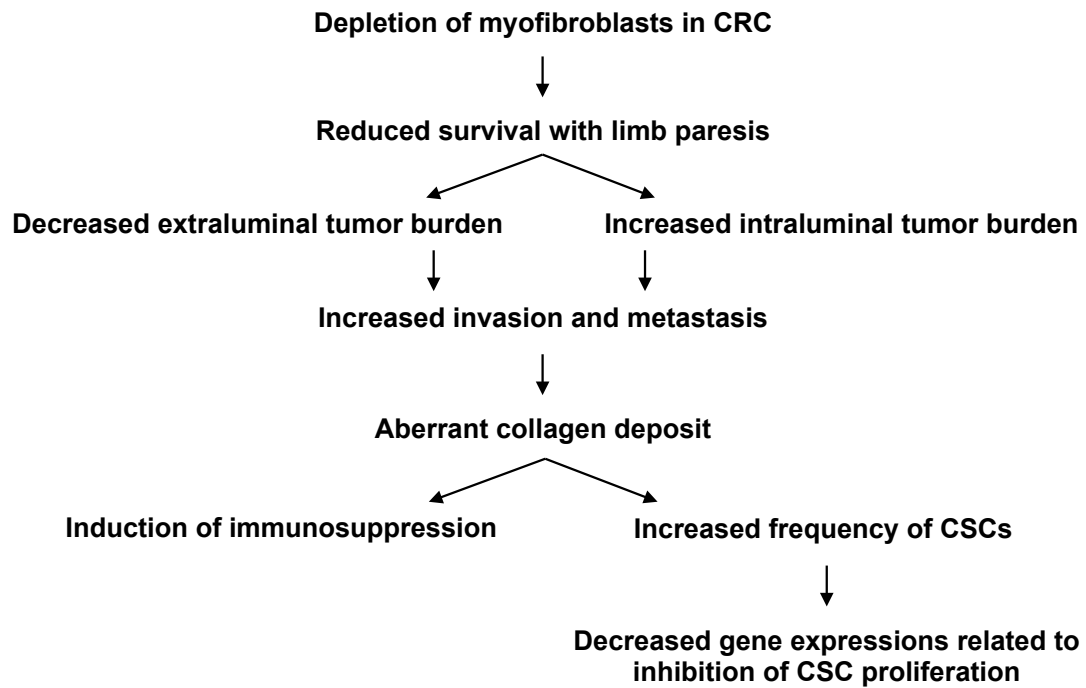


Figure 21. Summary. Our study reveals unknown functional role of myofibroblasts in regulating T cell-mediated anti-tumor immunity and stemness features in colon cancer cells

References

1. Siegel, R., et al., *Cancer treatment and survivorship statistics, 2012*. CA: A Cancer Journal for Clinicians, 2012. **62**(4): p. 220-241.
2. Center, M.M., et al., *Worldwide Variations in Colorectal Cancer*. CA: A Cancer Journal for Clinicians, 2009. **59**(6): p. 366-378.
3. Taylor, D.P., et al., *Population-Based Family History–Specific Risks for Colorectal Cancer: A Constellation Approach*. Gastroenterology, 2010. **138**(3): p. 877-885.
4. Jess, T., C. Rungoe, and L. Peyrin–Biroulet, *Risk of Colorectal Cancer in Patients With Ulcerative Colitis: A Meta-analysis of Population-Based Cohort Studies*. Clinical Gastroenterology and Hepatology, 2012. **10**(6): p. 639-645.
5. Liang, P.S., T.-Y. Chen, and E. Giovannucci, *Cigarette smoking and colorectal cancer incidence and mortality: Systematic review and meta-analysis*. International Journal of Cancer, 2009. **124**(10): p. 2406-2415.
6. Fedirko, V., et al., *Alcohol drinking and colorectal cancer risk: an overall and dose–response meta-analysis of published studies*. Annals of Oncology, 2011. **22**(9): p. 1958-1972.
7. Chan, D.S.M., et al., *Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies*. PLOS ONE, 2011. **6**(6): p. e20456.
8. Ma, Y., et al., *Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies*. PLOS ONE, 2013. **8**(1): p. e53916.

9. Jiang, Y., et al., *Diabetes mellitus and incidence and mortality of colorectal cancer: a systematic review and meta-analysis of cohort studies*. European Journal of Epidemiology, 2011. **26**(11): p. 863-876.
10. Sonnenberg, A. and R.M. Genta, *Helicobacter pylori is a Risk Factor for Colonic Neoplasms*. Am J Gastroenterol, 2013. **108**(2): p. 208-215.
11. Kostic, A.D., et al., *Genomic analysis identifies association of Fusobacterium with colorectal carcinoma*. Genome Research, 2011.
12. Boyle, T., et al., *Physical Activity and Risks of Proximal and Distal Colon Cancers: A Systematic Review and Meta-analysis*. JNCI: Journal of the National Cancer Institute, 2012. **104**(20): p. 1548-1561.
13. Lin, K.J., et al., *The effect of estrogen vs. combined estrogen-progestogen therapy on the risk of colorectal cancer*. International Journal of Cancer, 2012. **130**(2): p. 419-430.
14. Bosetti, C., et al., *Aspirin and cancer risk: a quantitative review to 2011*. Annals of Oncology, 2012. **23**(6): p. 1403-1415.
15. Rothwell, P.M., et al., *Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials*. The Lancet. **377**(9759): p. 31-41.
16. Brenner, H., et al., *Protection from colorectal cancer after colonoscopy: A population-based, case-control study*. Annals of Internal Medicine, 2011. **154**(1): p. 22-30.
17. Elmunzer, B.J., et al., *Effect of Flexible Sigmoidoscopy-Based Screening on Incidence and Mortality of Colorectal Cancer: A Systematic Review and Meta-*

- Analysis of Randomized Controlled Trials*. PLOS Medicine, 2012. **9**(12): p. e1001352.
18. Park, S.H., et al., *CT colonography for detection and characterisation of synchronous proximal colonic lesions in patients with stenosing colorectal cancer*. Gut, 2012. **61**(12): p. 1716-1722.
 19. Puli, S.R., et al., *Can Endoscopic Ultrasound Predict Early Rectal Cancers That Can Be Resected Endoscopically? A Meta-Analysis and Systematic Review*. Digestive Diseases and Sciences, 2010. **55**(5): p. 1221-1229.
 20. Al-Sukhni, E., et al., *Diagnostic Accuracy of MRI for Assessment of T Category, Lymph Node Metastases, and Circumferential Resection Margin Involvement in Patients with Rectal Cancer: A Systematic Review and Meta-analysis*. Annals of Surgical Oncology, 2012. **19**(7): p. 2212-2223.
 21. Niekel, M.C., S. Bipat, and J. Stoker, *Diagnostic Imaging of Colorectal Liver Metastases with CT, MR Imaging, FDG PET, and/or FDG PET/CT: A Meta-Analysis of Prospective Studies Including Patients Who Have Not Previously Undergone Treatment*. Radiology, 2010. **257**(3): p. 674-684.
 22. Floriani, I., et al., *Performance of imaging modalities in diagnosis of liver metastases from colorectal cancer: A systematic review and meta-analysis*. Journal of Magnetic Resonance Imaging, 2010. **31**(1): p. 19-31.
 23. Choi, D.J., et al., *Preoperative chest computerized tomography in patients with locally advanced mid or lower rectal cancer: Its role in staging and impact on treatment strategy*. Journal of Surgical Oncology, 2010. **102**(6): p. 588-592.

24. Heald, R.J. and R.D.H. Ryall, *RECURRENCE AND SURVIVAL AFTER TOTAL MESORECTAL EXCISION FOR RECTAL CANCER*. The Lancet, 1986. **327**(8496): p. 1479-1482.
25. Nagtegaal, I.D. and P. Quirke, *What Is the Role for the Circumferential Margin in the Modern Treatment of Rectal Cancer?* Journal of Clinical Oncology, 2008. **26**(2): p. 303-312.
26. van Gijn, W., et al., *Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial*. The Lancet Oncology, 2011. **12**(6): p. 575-582.
27. Gill, S., et al., *Pooled Analysis of Fluorouracil-Based Adjuvant Therapy for Stage II and III Colon Cancer: Who Benefits and by How Much?* Journal of Clinical Oncology, 2004. **22**(10): p. 1797-1806.
28. Twelves, C., et al., *Capecitabine versus 5-fluorouracil/folinic acid as adjuvant therapy for stage III colon cancer: final results from the X-ACT trial with analysis by age and preliminary evidence of a pharmacodynamic marker of efficacy*. Annals of Oncology, 2012. **23**(5): p. 1190-1197.
29. Schmoll, H.-J., et al., *Capecitabine Plus Oxaliplatin Compared With Fluorouracil/Folinic Acid As Adjuvant Therapy for Stage III Colon Cancer: Final Results of the NO16968 Randomized Controlled Phase III Trial*. Journal of Clinical Oncology, 2015. **33**(32): p. 3733-3740.
30. Yothers, G., et al., *Oxaliplatin As Adjuvant Therapy for Colon Cancer: Updated Results of NSABP C-07 Trial, Including Survival and Subset Analyses*. Journal of Clinical Oncology, 2011. **29**(28): p. 3768-3774.

31. Sadanandam, A., et al., *A colorectal cancer classification system that associates cellular phenotype and responses to therapy*. Nat Med, 2013. **19**(5): p. 619-625.
32. De Sousa E Melo, F., et al., *Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions*. Nat Med, 2013. **19**(5): p. 614-618.
33. Leslie, A., et al., *The colorectal adenoma–carcinoma sequence*. British Journal of Surgery, 2002. **89**(7): p. 845-860.
34. Fearon, E.R., *Molecular Genetics of Colorectal Cancer*. Annual Review of Pathology: Mechanisms of Disease, 2011. **6**(1): p. 479-507.
35. Jass, J.R., *Classification of colorectal cancer based on correlation of clinical, morphological and molecular features*. Histopathology, 2007. **50**(1): p. 113-130.
36. Groden, J., et al., *Identification and characterization of the familial adenomatous polyposis coli gene*. Cell, 1991. **66**(3): p. 589-600.
37. Kinzler, K.W. and B. Vogelstein, *Lessons from Hereditary Colorectal Cancer*. Cell, 1996. **87**(2): p. 159-170.
38. Su, L.-K., et al., *APC Binds to the Novel Protein EBI*. Cancer Research, 1995. **55**(14): p. 2972-2977.
39. Rubinfeld, B., et al., *Binding of GSK3 β to the APC- β -Catenin Complex and Regulation of Complex Assembly*. Science, 1996. **272**(5264): p. 1023-1026.
40. Kaplan, K.B., et al., *A role for the Adenomatous Polyposis Coli protein in chromosome segregation*. Nat Cell Biol, 2001. **3**(4): p. 429-432.
41. Molenaar, M., et al., *XTcf-3 Transcription Factor Mediates β -Catenin-Induced Axis Formation in Xenopus Embryos*. Cell, 1996. **86**(3): p. 391-399.

42. Felsher, D.W. and J.M. Bishop, *Transient excess of MYC activity can elicit genomic instability and tumorigenesis*. Proceedings of the National Academy of Sciences, 1999. **96**(7): p. 3940-3944.
43. Bos, J.L., *Oncogenes in Human Cancer: A Review*. Cancer Research, 1989. **49**(17): p. 4682-4689.
44. Fromentel, C.C.D. and T. Soussi, *TP53 tumor suppressor gene: A model for investigating human mutagenesis*. Genes, Chromosomes and Cancer, 1992. **4**(1): p. 1-15.
45. Lane, D.P., *p53, guardian of the genome*. Nature, 1992. **358**(6381): p. 15-16.
46. Momand, J., et al., *The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation*. Cell, 1992. **69**(7): p. 1237-1245.
47. Mietz, J.A., et al., *The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein*. The EMBO Journal, 1992. **11**(13): p. 5013-5020.
48. Kaklamanis, L., et al., *p53 expression in colorectal adenomas*. The American Journal of Pathology, 1993. **142**(1): p. 87-93.
49. Bettington, M., et al., *The serrated pathway to colorectal carcinoma: current concepts and challenges*. Histopathology, 2013. **62**(3): p. 367-386.
50. Herman, J.G., et al., *Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma*. PNAS, 1998. **95**(12): p. 6870-6875.
51. Parsons, M.T., et al., *Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a*

- literature review assessing utility of tumour features for MMR variant classification.* Journal of Medical Genetics, 2012. **49**(3): p. 151-157.
52. Jasperson, K.W., et al., *Hereditary and Familial Colon Cancer.* Gastroenterology, 2010. **138**(6): p. 2044-2058.
 53. Lynch , H.T. and A. de la Chapelle *Hereditary Colorectal Cancer.* New England Journal of Medicine, 2003. **348**(10): p. 919-932.
 54. Knudson, A.G., *Antioncogenes and human cancer.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(23): p. 10914-10921.
 55. Boland, C.R. and A. Goel, *Microsatellite Instability in Colorectal Cancer.* Gastroenterology, 2010. **138**(6): p. 2073-2087.e3.
 56. Segditsas, S. and I. Tomlinson, *Colorectal cancer and genetic alterations in the Wnt pathway.* Oncogene, 0000. **25**(57): p. 7531-7537.
 57. Jung, S.-B., et al., *Clinico-pathologic Parameters for Prediction of Microsatellite Instability in Colorectal Cancer.* Cancer Research and Treatment : Official Journal of Korean Cancer Association, 2012. **44**(3): p. 179-186.
 58. Jinru, S., *Value of Histopathology in Predicting Microsatellite Instability in Hereditary Nonpolyposis Colorectal Cancer and Sporadic Colorectal Cancer.* The American Journal of Surgical Pathology, 2003. **27**(11): p. 1407-1417.
 59. Lindor, N.M., et al., *Immunohistochemistry Versus Microsatellite Instability Testing in Phenotyping Colorectal Tumors.* Journal of Clinical Oncology, 2002. **20**(4): p. 1043-1048.

60. Ohtani, H., *Stromal reaction in cancer tissue: Pathophysiologic significance of the expression of matrix-degrading enzymes in relation to matrix turnover and immune/inflammatory reactions*. Pathology International, 1998. **48**(1): p. 1-9.
61. Pattabiraman, D.R. and R.A. Weinberg, *Tackling the cancer stem cells [mdash] what challenges do they pose?* Nat Rev Drug Discov, 2014. **13**(7): p. 497-512.
62. Peddareddigari, V.G., D. Wang, and R.N. DuBois, *The Tumor Microenvironment in Colorectal Carcinogenesis*. Cancer Microenvironment, 2010. **3**(1): p. 149-166.
63. Lewis, C.E. and J.W. Pollard, *Distinct Role of Macrophages in Different Tumor Microenvironments*. Cancer Research, 2006. **66**(2): p. 605-612.
64. Fricke, I. and D.I. Gabrilovich, *Dendritic Cells and Tumor Microenvironment: A Dangerous Liaison*. Immunological Investigations, 2006. **35**(3-4): p. 459-483.
65. Jedinak, A., S. Dudhgaonkar, and D. Sliva, *Activated macrophages induce metastatic behavior of colon cancer cells*. Immunobiology, 2010. **215**(3): p. 242-249.
66. Herbeuval, J.-P., et al., *Recruitment of STAT3 for Production of IL-10 by Colon Carcinoma Cells Induced by Macrophage-Derived IL-6*. The Journal of Immunology, 2004. **172**(7): p. 4630-4636.
67. Galizia, G., et al., *Prognostic Significance of Circulating IL-10 and IL-6 Serum Levels in Colon Cancer Patients Undergoing Surgery*. Clinical Immunology, 2002. **102**(2): p. 169-178.
68. Nagaraj, S. and D.I. Gabrilovich, *Tumor Escape Mechanism Governed by Myeloid-Derived Suppressor Cells*. Cancer Research, 2008. **68**(8): p. 2561-2563.

69. Huang, B., et al., *Gr-1⁺CD115⁺ Immature Myeloid Suppressor Cells Mediate the Development of Tumor-Induced T Regulatory Cells and T-Cell Anergy in Tumor-Bearing Host*. Cancer Research, 2006. **66**(2): p. 1123-1131.
70. Mandruzzato, S., et al., *IL4Rα⁺ Myeloid-Derived Suppressor Cell Expansion in Cancer Patients*. The Journal of Immunology, 2009. **182**(10): p. 6562-6568.
71. Gounaris, E., et al., *T-Regulatory Cells Shift from a Protective Anti-Inflammatory to a Cancer-Promoting Proinflammatory Phenotype in Polyposis*. Cancer Research, 2009. **69**(13): p. 5490-5497.
72. Gupta, R.A. and R.N. DuBois, *Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2*. Nat Rev Cancer, 2001. **1**(1): p. 11-21.
73. Williams, C.S., M. Mann, and R.N. DuBois, *The role of cyclooxygenases in inflammation, cancer, and development*. Oncogene, 1999. **18**(55): p. 7908-7916.
74. Yaqub, S., et al., *Regulatory T cells in colorectal cancer patients suppress anti-tumor immune activity in a COX-2 dependent manner*. Cancer Immunology, Immunotherapy, 2008. **57**(6): p. 813-821.
75. Lönnroth, C., et al., *Preoperative treatment with a non-steroidal anti-inflammatory drug (NSAID) increases tumor tissue infiltration of seemingly activated immune cells in colorectal cancer*. Cancer Immunity : a Journal of the Academy of Cancer Immunology, 2008. **8**: p. 5.
76. Öhlund, D., E. Elyada, and D. Tuveson, *Fibroblast heterogeneity in the cancer wound*. The Journal of Experimental Medicine, 2014. **211**(8): p. 1503-1523.

77. Kalluri, R. and M. Zeisberg, *Fibroblasts in cancer*. Nat Rev Cancer, 2006. **6**(5): p. 392-401.
78. Östman, A. and M. Augsten, *Cancer-associated fibroblasts and tumor growth – bystanders turning into key players*. Current Opinion in Genetics & Development, 2009. **19**(1): p. 67-73.
79. Marsh, T., K. Pietras, and S.S. McAllister, *Fibroblasts as architects of cancer pathogenesis*. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2013. **1832**(7): p. 1070-1078.
80. Gabbiani, G., G.B. Ryan, and G. Majno, *Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction*. Experientia, 1971. **27**(5): p. 549-550.
81. Micallef, L., et al., *The myofibroblast, multiple origins for major roles in normal and pathological tissue repair*. Fibrogenesis & Tissue Repair, 2012. **5**(Suppl 1): p. S5-S5.
82. Desmouliere, A., I.A. Darby, and G. Gabbiani, *Normal and Pathologic Soft Tissue Remodeling: Role of the Myofibroblast, with Special Emphasis on Liver and Kidney Fibrosis*. Lab Invest, 0000. **83**(12): p. 1689-1707.
83. Chang, H.Y., et al., *Diversity, topographic differentiation, and positional memory in human fibroblasts*. Proceedings of the National Academy of Sciences, 2002. **99**(20): p. 12877-12882.
84. Kalluri, R., *The biology and function of fibroblasts in cancer*. Nat Rev Cancer, 2016. **16**(9): p. 582-598.

85. Desmoulière, A., C. Chaponnier, and G. Gabbiani, *Tissue repair, contraction, and the myofibroblast*. Wound Repair and Regeneration, 2005. **13**(1): p. 7-12.
86. Mifflin, R.C., et al., *Intestinal myofibroblasts: targets for stem cell therapy*. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2011. **300**(5): p. G684-G696.
87. Durning, P., S.L. Schor, and R.A. Sellwood, *Fibroblasts from patients with breast cancer show abnormal migratory behaviour in vitro*. Lancet (London, England), 1984. **2**(8408): p. 890-892.
88. Ryan, G.B., et al., *Myofibroblasts in human granulation tissue*. Human Pathology, 1974. **5**(1): p. 55-67.
89. Aoyagi, Y., et al., *Overexpression of TGF- β by infiltrated granulocytes correlates with the expression of collagen mRNA in pancreatic cancer*. British Journal of Cancer, 2004. **91**(7): p. 1316-1326.
90. Löhr, M., et al., *Transforming Growth Factor- β 1 Induces Desmoplasia in an Experimental Model of Human Pancreatic Carcinoma*. Cancer Research, 2001. **61**(2): p. 550-555.
91. Cheng, H. and C.P. Leblond, *Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine V. Unitarian theory of the origin of the four epithelial cell types*. American Journal of Anatomy, 1974. **141**(4): p. 537-561.
92. Sato, T., et al., *Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts*. Nature, 2011. **469**(7330): p. 415-418.

93. Vermeulen, L., et al., *Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity*. Proceedings of the National Academy of Sciences, 2008. **105**(36): p. 13427-13432.
94. Özdemir, Berna C., et al., *Depletion of Carcinoma-Associated Fibroblasts and Fibrosis Induces Immunosuppression and Accelerates Pancreas Cancer with Reduced Survival*. Cancer Cell, 2014. **25**(6): p. 719-734.
95. Pallangyo, C.K., P.K. Ziegler, and F.R. Greten, *IKK β acts as a tumor suppressor in cancer-associated fibroblasts during intestinal tumorigenesis*. The Journal of Experimental Medicine, 2015. **212**(13): p. 2253-2266.
96. Wilhelmus, K.R., *Antiviral treatment and other therapeutic interventions for herpes simplex virus epithelial keratitis*. The Cochrane database of systematic reviews, 2015. **1**: p. CD002898-CD002898.
97. LeBleu, V.S., et al., *Origin and function of myofibroblasts in kidney fibrosis*. Nat Med, 2013. **19**(8): p. 1047-1053.
98. Chae, W.-J. and A.L.M. Bothwell, *Spontaneous Intestinal Tumorigenesis in Mice Requires Altered T Cell Development with IL-17A*. Journal of Immunology Research, 2015. **2015**: p. 11.
99. Facciorusso, A., et al., *Non-polypoid colorectal neoplasms: Classification, therapy and follow-up*. World Journal of Gastroenterology : WJG, 2015. **21**(17): p. 5149-5157.
100. Soetikno, R.M., et al., *Prevalence of nonpolypoid (flat and depressed) colorectal neoplasms in asymptomatic and symptomatic adults*. JAMA, 2008. **299**(9): p. 1027-1035.

101. Plaks, V., C.D. Koopman, and Z. Werb, *Circulating Tumor Cells*. Science, 2013. **341**(6151): p. 1186-1188.
102. Cristofanilli, M., et al., *Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer*. New England Journal of Medicine, 2004. **351**(8): p. 781-791.
103. Ong, M.L.H. and J.B. Schofield, *Assessment of lymph node involvement in colorectal cancer*. World Journal of Gastrointestinal Surgery, 2016. **8**(3): p. 179-192.
104. Paszek, M.J., et al., *Tensional homeostasis and the malignant phenotype*. Cancer Cell, 2005. **8**(3): p. 241-254.
105. Wozniak, M.A., et al., *ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix*. The Journal of Cell Biology, 2003. **163**(3): p. 583-595.
106. Lu, P., V.M. Weaver, and Z. Werb, *The extracellular matrix: A dynamic niche in cancer progression*. The Journal of Cell Biology, 2012. **196**(4): p. 395-406.
107. Vellinga, T.T., et al., *Collagen-rich stroma in aggressive colon tumors induces mesenchymal gene expression and tumor cell invasion*. Oncogene, 2016. **35**(40): p. 5263-5271.
108. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape*. Nat Immunol, 2002. **3**(11): p. 991-998.
109. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, Inflammation, and Cancer*. Cell, 2010. **140**(6): p. 883-899.

110. Ladoire, S., F. Martin, and F. Ghiringhelli, *Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer*. Cancer Immunology, Immunotherapy, 2011. **60**(7): p. 909-918.
111. Hanke, T., et al., *High intratumoral FOXP3(+) T regulatory cell (Tregs) density is an independent good prognosticator in nodal negative colorectal cancer*. International Journal of Clinical and Experimental Pathology, 2015. **8**(7): p. 8227-8235.
112. Shang, B., et al., *Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis*. Scientific Reports, 2015. **5**: p. 15179.
113. Puglisi, M.A., et al., *Colon cancer stem cells: Controversies and perspectives*. World Journal of Gastroenterology : WJG, 2013. **19**(20): p. 2997-3006.
114. Moore, N. and S. Lyle, *Quiescent, Slow-Cycling Stem Cell Populations in Cancer: A Review of the Evidence and Discussion of Significance*. Journal of Oncology, 2011. **2011**.
115. Pannuti, A., et al., *Targeting Notch to Target Cancer Stem Cells*. Clinical Cancer Research, 2010. **16**(12): p. 3141-3152.
116. Merlos-Suárez, A., et al., *The Intestinal Stem Cell Signature Identifies Colorectal Cancer Stem Cells and Predicts Disease Relapse*. Cell Stem Cell, 2011. **8**(5): p. 511-524.
117. Chandrakesan, P., et al., *DCLK1 facilitates intestinal tumor growth via enhancing pluripotency and epithelial mesenchymal transition*. Oncotarget, 2014. **5**(19).

118. Torres, S., et al., *LOXL2 Is Highly Expressed in Cancer-Associated Fibroblasts and Associates to Poor Colon Cancer Survival*. Clinical Cancer Research, 2015. **21**(21): p. 4892-4902.
119. Peña, C., et al., *STC1 Expression By Cancer-Associated Fibroblasts Drives Metastasis of Colorectal Cancer*. Cancer Research, 2013. **73**(4): p. 1287-1297.
120. Orimo, A., et al., *Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis through Elevated SDF-1/CXCL12 Secretion*. Cell, 2005. **121**(3): p. 335-348.
121. Merika, E.E., K.N. Syrigos, and M.W. Saif, *Desmoplasia in Pancreatic Cancer. Can We Fight It?* Gastroenterology Research and Practice, 2012. **2012**: p. 781765.
122. Karnoub, A.E., et al., *Mesenchymal stem cells within tumour stroma promote breast cancer metastasis*. Nature, 2007. **449**(7162): p. 557-563.
123. Vong, S. and R. Kalluri, *The Role of Stromal Myofibroblast and Extracellular Matrix in Tumor Angiogenesis*. Genes & Cancer, 2011. **2**(12): p. 1139-1145.
124. Afik, R., et al., *Tumor macrophages are pivotal constructors of tumor collagenous matrix*. The Journal of Experimental Medicine, 2016.
125. Chomarat, P., et al., *IL-6 switches the differentiation of monocytes from dendritic cells to macrophages*. Nat Immunol, 2000. **1**(6): p. 510-514.
126. Nazareth, M.R., et al., *Characterization of Human Lung Tumor-Associated Fibroblasts and Their Ability to Modulate the Activation of Tumor-Associated T Cells*. The Journal of Immunology, 2007. **178**(9): p. 5552-5562.

127. Rygiel, T.P., et al., *Tumor-expressed collagens can modulate immune cell function through the inhibitory collagen receptor LAIR-1*. Molecular Immunology, 2011. **49**(1–2): p. 402-406.
128. Giannoni, E., et al., *Reciprocal Activation of Prostate Cancer Cells and Cancer-Associated Fibroblasts Stimulates Epithelial-Mesenchymal Transition and Cancer Stemness*. Cancer Research, 2010. **70**(17): p. 6945-6956.
129. Chen, W.-J., et al., *Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via paracrine signalling*. Nature Communications, 2014. **5**: p. 3472.
130. Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. CA: a Cancer Journal for Clinicians, 66(1), 7–30.
131. Fl. Obrocea et al., Colorectal cancer and the 7th revision of the TNM staging system: Review of changes and suggestions for uniform pathologic reporting. (2011). Rom J Morphol Embryol 52(2):537-544
132. Bodmer WF, Bailey CJ, Bodmer J, Bussey HJR, Ellis A, Gorman P et al. Localization of the gene for familial adenomatous polyposis on chromosome 5. Nature 1987; 328:614±16.
133. Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. Regulation of intracellular b -catenin levels by the adenomatous polyposis coli [36] tumor-suppressor protein. Proc Natl Acad Sci U S A 1995; 92: 3046±50.
134. Yin XY, Grove L, Datta NS, Long MW, Prochownik EV. C-myc overexpression and p53 loss cooperate to promote genomic instability. Oncogene 1999; 18: 1177±84.

135. Kaserer K, Schmaus J, Bethge U, Migschitz B, Fasching S, Walch A et al. Staining patterns of p53 immunohistochemistry and their biological significance in colorectal cancer. *J Pathol* 2000; 190: 450±6
136. Boland CR, Sato J, Appelman HD, Bresalier RS, Feinberg AP. Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. *Nat Med* 1995; 1: 902±9.
137. King RJB. *Cancer Biology*. 2nd ed. Harlow: Prentice Hall, 2000
138. Tarin, D. & Croft, C. B. (1969) Ultrastructural features of wound healing in mouse skin. *J. Anat.* 105, 189–190.
139. Ryan, G. B. et al. (1973). Myofibroblasts in an avascular fibrous tissue. *Lab. Invest.* 29, 197–206.
140. Kornprat, P., Pollheimer, M. J., Lindtner, R. A., Schlemmer, A., Rehak, P., & Langner, C. (2011). Value of Tumor Size as a Prognostic Variable in Colorectal Cancer. *American Journal of Clinical Oncology*, 34(1), 43–49.
141. De Wever O, Nguyen Q-D, Van Hoorde L, Bracke M, Bruyneel E, Gespach C, Mareel M. Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *FASEB J* 2
142. Januchowski, R., Świerczewska, M., Sterzyńska, K., Wojtowicz, K., Nowicki, M., & Zabel, M. (n.d.). Increased Expression of Several Collagen Genes is Associated with Drug Resistance in Ovarian Cancer Cell Lines. *Journal of Cancer*, 7(10), 1295–1310.
143. Giacomo Puppa et al., Pathological assessment of pericolonic tumor deposits in advanced colonic carcinoma: relevance to prognosis and tumor staging. *Modern*

Pathology (2007) 20, 843-855