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Canonical NF-κB signaling in fibroblasts and cancer-associated fibroblasts in colitis and colorectal cancer.

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1. Introduction

1.1 Colorectal cancer

Cancer represents an immense burden in our society today, with an estimated 14.1million new cases and 8.2 million deaths worldwide in 2012. With an estimated 1.4million cases worldwide, colorectal cancer (CRC) remains as the third commonest diagnosed malignancy globally. CRC is also the third leading cause of cancer related deaths accounting for about 700,000 deaths a year (Torre et al., 2015) and despite significant advances in the detection and treatment in the developed world, it is still associated with relatively poor prognosis and increasing mortality rates since the 1980s (Siegel et al., 2014). At least 40% of patients who undergo resection of the primary tumor die within 5 years either due to local recurrence or metastatic disease.

The factors that increase the likelihood of developing CRC are heterogeneous and diverse but well recognized. While inherited genetic susceptibility has the most striking increase in risk, the majority of CRC are sporadic and caused by a complex interplay between genetic, host, and environmental factors. The environmental factors include the lifestyle and more importantly, dietary factors. Obesity, smoking, physical inactivity together with a diet rich in fat and red meat and excessive alcohol consumption are associated with an increased risk of developing CRC, conversely, fruits and vegetables, micronutrients and fiber in the diet are protective (Huxley et al., 2009; Ferrari et al., 2007; Botteri et al., 2008).

The genetic alterations in hereditary CRC syndromes involve mutations of specific genes that are usually inherited in an autosomal dominant fashion. Mutations in the adenomatous polyposis coli (APC) gene were the first to be identified in predisposing to familial adenomatous polyposis (Valle, 2014). Subsequently, mutations in the genes *STK11*, *BMPR1A*, *SMAD4*, *PTEN* and MutY homologue (*MUTYH*) have also been identified in the pathogenesis of polyposis syndromes (Peters et al., 2015). Hereditary non-polyposis colorectal cancer (HNPCC) also known as Lynch syndrome involves mutations in genes responsible for DNA mismatch repair (MMR). *MLH1* and *MSH2* are the two most commonly mutated MMR genes but mutations in *MSH6*, *PMS2* and *EPCAM* have also been identified (Peters et al., 2015; Carethers, 2015). The loss of MMR activity results in the inability to detect and repair single base mismatches during DNA replication. The ensuing errors in replication lead to high rates of mutations in genes that have microsatellite repeats nested in their sequences. The

increased genome mutability guarantees subsequent mutations will occur and CRC development and progression is usually rapid (Carethers, 2015). The use of high-throughput sequencing technology has enabled extensive and detailed examination of somatic alterations in the genomes of many cancers including CRC. Whole genome or exome-wide examination of genomic mutations involved in the development of sporadic CRC has identified several recurrent mutations involved in the pathogenesis of sporadic CRC which may be potential driver events and thus can serve as new therapeutic targets (Vogelstein et al., 2013). Exome-sequencing analysis of 276 CRC samples from The Cancer Genome Atlas (TCGA) consortium identified 32 somatic genes that were recurrently mutated in both hypermutated and non-hypermutated cancers. In the non-hypermutated tumors, the most frequently mutated genes with a frequency of at least 10% included *APC*, *TP53*, *KRAS*, *PIK3CA*, *FBXW7* and *SMAD4*. In the hypermutated tumors, *ACVR2A*, *APC*, *TGFBR2*, *MSH3* and *MSH6* were recurrent targets of mutation, with a frequency of at least 40% (Muzny et al., 2012). Integrated analysis of the mutations and mRNA expression identified recurrent alterations in several key-signaling pathways including the WNT, MAPK, PI3K, TGF-β and P53 pathways. Not surprisingly, the WNT signaling pathway was altered in 93% of all cases in both hypermutated and non-hypermutated tumors due to either biallelic inactivation of *APC* or activation of *CTNNB1*. In contrast, mutations of *TP53* leading to the inactivation of the P53 pathway were significantly more observed in non-hypermutated tumors compared to the hypermutated tumors (60% vs. 20%). Mutations leading to deregulated TGF-β signaling had an opposite trend to that of *TP53*, with 87% in hypermutated tumors compared to only 27% in the non-hypermutated tumors. This was mainly due to alterations in genes coding for *TGFBR1*, *TGFBR2*, *ACVR2A*, *ACVR1B*, *SMAD2*, *SMAD3* and *SMAD4* (Muzny et al., 2012).

Despite the diversity of the genes that are recurrently mutated, it is estimated that tumor-driving mutations ultimately converge on 12 signaling pathways which regulate three core cellular processes; cell fate determination, cell survival, and genome maintenance. Understanding the altered pathways involved in both the primary tumor and metastatic sites is critical in cancer research in order to understand the pathogenesis of CRC and to guide the development of new and more effective therapeutic agents (Vogelstein et al., 2013; Haan et al., 2014)

1.2 The tumor microenvironment

Although carcinomas originate from the clonal expansion of epithelial cells that have accumulated mutations that override critical pathways regulating cellular homeostasis (Fearon and Vogelstein, 1990), it is now clear that the mere activation of oncogenes and/or inactivation of tumor suppressor genes alone is inefficient for successful tumorigenesis. Accumulating evidence from various tumor models has convincingly demonstrated that tumors are complex tissues and the intercommunication between transformed cells and non-transformed entities of the tumor microenvironment (TME) play critical roles in tumor initiation, progression, invasion, metastasis and response to treatment (Quail and Joyce, 2013; Barker et al., 2015).

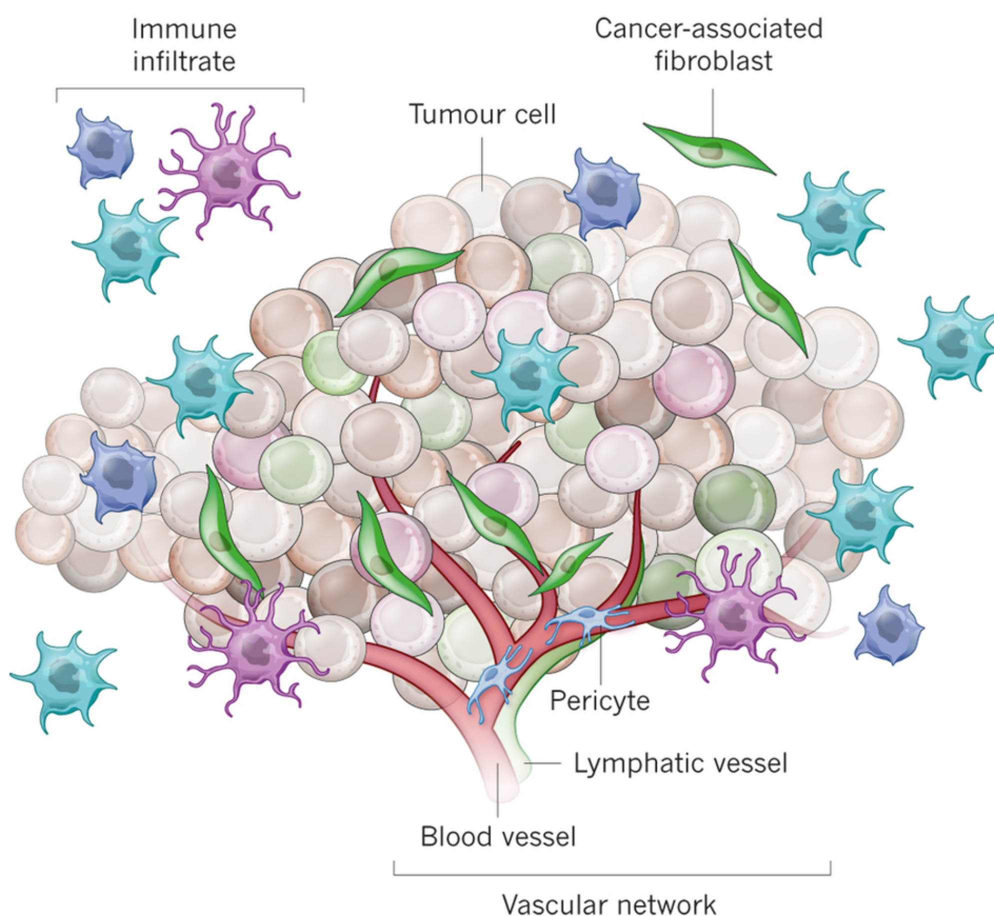


Figure 1: Illustration of the TME.

Components of the tumor microenvironment include the transformed cells, immune cells, vascular cells, and fibroblasts together with components of the ECM. The TME provides critical factors including structural support, access to growth factors, vascular supply and immune cell interactions promoting tumor growth and invasion. Figure from (Junttila and de Sauvage, 2013).

The TME is composed of multiple cellular and non-cellular entities including various types of immune cells, vascular cells, bone marrow derived mesenchymal stem cells, fibroblasts, cancer-associated fibroblasts (CAFs), extracellular matrix (ECM) and their fragments. The TME is initiated early in tumorigenesis, it is dynamic and co-evolves with the transformed cells, providing favorable selection pressures and promoting tumor cell evolution and tumor growth. Most carcinomas depend absolutely on the signals from the TME which influence all the hallmarks of cancer, especially tumor cell survival, proliferation, stemness, migration, invasion, and angiogenesis (Li et al., 2007; Pietras and Ostman, 2010; Hanahan and Coussens, 2012; Barker et al., 2015).

The crosstalk between transformed cells and TME is mediated by direct cell-cell interactions along with paracrine and autocuing stimulation by a variety of soluble factors, including growth factors, chemokines, cytokines and matrix remodeling enzymes (Allen and Louise Jones, 2011; Friedl and Alexander, 2011). This complex heterotypic crosstalk is not only limited to the site of the primary tumor. At the primary site, the TME provides abundant growth factors promoting tumor cell proliferation; reactive oxygen and nitrogen radicals facilitating continued DNA damage; together with matrix remodeling enzymes that fostering invasion (Colotta et al., 2009). At distant sites, stromal cells respond to tumor-derived factors and are involved in establishing a conducive microenvironment (pre-metastatic niche) fostering tumor cell engraftment, survival and proliferation (McAllister and Weinberg, 2014; Quail and Joyce, 2013).

Given the significant contributions of TME, therapeutic approaches targeting only the transformed cells have yielded insufficient results in controlling the growth of both, localized and metastatic cancer. More efficient however are approaches that target the tumor cells and components of TME including stromal fibroblasts, the vascular endothelial network, immune cells and host humoral substances (Brennen et al., 2012; Fang and DeClerck, 2013).

1.3 Inflammation and tumorigenesis

An important component of the TME is the establishment of chronic inflammation. An inflammatory microenvironment has been demonstrated to be a potent initiator and promoter of almost all tumors especially CRC and it is accepted as the seventh “hallmark” of cancer (Shalapour and Karin, 2015). The hallmarks of chronic inflammation include the accumulation of activated immune cells from both the innate and adaptive arm and their inflammatory mediators, along with an accumulation of different types of mesenchymal cells including CAFs (Pietras and Ostman, 2010).

Both sporadic and familial forms of CRC have an element of inflammation which contributes significantly to tumor progression (Grivennikov et al., 2010; Colotta et al., 2009; Ullman and Itzkowitz, 2011).

Among the most important inflammatory mediators are tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which drive cancer-associated inflammation through the activation of important transcription factors, NF-κB and STAT3 respectively, endowing tumor cells with many hallmark capabilities (Bollrath and Greten, 2009; Fan et al., 2013; He and Karin, 2011). Constitutive activation of NF-κB and STAT3 is seen in many tumor and immune cells in CRC and is essential in tumor promotion and progression. However, to date no activating mutations have been found in these signaling pathways in CRC and the aberrant activation is likely due to microenviromental signals acting through paracrine or autocrine stimulation (Grivennikov, 2013).

Consistently, both clinical and experimental studies have shown that prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs) significantly reduce the risk of CRC by up to 50% (DuBois et al., 1996; Hawk and Levin, 2005). The anti-neoplastic effects observed are, in part, due to their inhibitory action on cyclooxygenase-2 (COX-2) which the rate-limiting enzyme for production of prostaglandins resulting into the abrogation of chronic inflammation.

1.4 Cells of the TME

It is now evident that CRC is a heterogeneous, multifactorial disease with different outcomes and response to treatments even in histologically equivalent tumors. It is also clear that the cellular and non-cellular components of the tumor stroma play a significant role in all stages of tumorigenesis, especially invasion and metastasis. The stromal cells that interact with tumor cells can originate from neighboring tissues or can be actively recruited from the bone marrow as endothelial progenitor cells, myeloid and lymphoid inflammatory cells, and mesenchymal cells. Once in the TME, they are "educated" into tumor-associated macrophages or neutrophils (TAMs or TANs), cancer-associated fibroblasts (CAFs), or vascular and perivascular cells. Education of stromal cells has been observed to be a dynamic process where naive cells progressively switch from a neutral or antitumorigenic role toward a protumorigenic role. To fully understand the pathogenesis of CRC, and to be able to develop newer and more potent treatments, it is necessary to understand the contributions of these other components (Wang and DuBois, 2015).

1.4.1 Tumor infiltrating immune cells

Rudolph Virchow and Paul Ehrlich hypothesized the functional relationship between the immune system and cancer over a century ago. Virchow postulated that chronic inflammation is critical to the origin of cancer and Ehrlich hypothesized that the immune system plays a protective role in tumorigenesis. Research conducted since then has elaborated the complex relationships between the immune system and cancer and the ever growing field of tumor immunology has clearly demonstrated that all known innate and adaptive immune cells actively participate in tumor immunosurveillance, but following tumorigenesis they are re-educated and to promote tumor growth. One of the main goals in tumor immunology in the past few decades has been to understand why and how they fail, leading to disease and if immune cells can be educated again to eradicate transformed cells (Shalapour and Karin, 2015; Sidow and Spies, 2015; Ruffell and Coussens, 2015).

Myeloid cells, including macrophages and neutrophils, are the most abundant immune cells in the tumor microenvironment and their accumulation in many tumors is associated with a poor clinical outcome (Ruffell and Coussens, 2015). Macrophages produce a plethora of cytokines, chemokines, growth factors, hormones, MMPs, and metabolites, many of which can significantly alter tumor growth and progression (Noy and Pollard, 2014). The pro-tumorigenic properties of TAMs have been well studied both in primary and metastatic sites, and it is clear that they influence tumor development and progression by stimulating tumor cell proliferation, survival, inducing angiogenesis, facilitating invasion, and suppression anti-tumor immunity (Galdiero et al., 2013; Mantovani and Allavena, 2015). TAMs are recruited early in the TME following a release of a variety of chemokines and cytokines from the tumor cells and resident stromal cells including CCL5, CXCL12, CXC3L1 and colony-stimulating factor-1 (CSF-1) (Zhou et al., 2014). TAMs represents a diverse population of cells which are highly plastic and their gene expression can easily be altered by microenviromental factors during tumor progression. In the early stages of tumorigenesis, TAMs are believed to undergo a classical activation and exhibit an anti-tumor (M1) phenotype. These M1 polarized macrophages are characterized by the efficient production of reactive oxygen and nitrogen intermediates and inflammatory cytokines including IL-1, TNF-α, IL-6, IL-23, iNOS and produce high levels of IL-12 and low levels of IL-10 and thus fostering a cytotoxic microenvironment. Consistent with their anti-tumorigenic functions, M1 macrophages produce CXCL9 and CXCL10/IP-10 thus attracting Th1 lymphocytes (Sica and Mantovani, 2012). Following tumor growth and the acquisition of hypoxic area and accumulation of lactic acid in the TME, macrophage polarization is

skewed towards the alternative (M2) phenotype in the presence of IL-4, IL-13 and TGFβ secreted by the cells in the TME. M2 macrophages are characterized by a high IL-10 and low IL-12 expression together with a poor antigen presenting capacity. M2 macrophages also produce chemokines such as CCL17, CCL22 and CCL24 which are involved in polarizing naïve $CD4^+$ T-cells into Tregs and Th2 phenotype, while inhibiting cytotoxic T-lymphocytes (CTLs) activation along with eosinophil and basophil recruitment (Liu et al., 2011a). Along with their anti-inflammatory properties, M2 macrophages also produce a variety of factors which directly or indirectly stimulate angiogenesis including TGF-β, VEGF, PDGF, MMP-9, thymidine phosporylase (TP) and IL-8 (Galdiero et al., 2013). M2 macrophages expressing TIE2 also promote tumor cell migration and intravasation thus promoting metastasis (Lin and Pollard, 2007). Clinical studies as well as animal models have shown an inverse correlation between M2 macrophage accumulation and tumor progression, indicating their role in tumor promotion. In the setting of CRC however, the role of TAMs is still controversial with several reports indicating a positive prognostic relevance of intratumoral macrophages (Funada et al., 2003; Håkansson et al., 1997; Forssell et al., 2007).

Neutrophils are the predominant circulating leukocytes in humans, accounting for 50–70% of all circulating leukocytes in adults. They have a well-established role in host defense and killing invading micro-organisms, and they have also been observed to interact with tumor cells *in vivo*, however there is surprisingly little data on the significance of their infiltration in tumors (Fridlender and Albelda, 2012). Like TAMs, tumor-associated neutrophils (TANs) are recruited early in the tumors from the blood following the release of pro-inflammatory signals such as chemokines (CXCL1 and CXCL2), cytokines (TNF-a and IFN-γ) as well as cell adhesion molecules like CD11b, selectins, intracellular adhesion molecule 1 and platelet-endothelial cell adhesion molecule (Sanz and Kubes, 2012; Kobayashi, 2008). GM-CSF has been shown to indirectly support the extravasation of neutrophils from the blood by activating the CXCL2–CXCR2 axis in endothelial cells, and it also plays a role in polarizing and preventing neutrophil apoptosis (Wengner et al., 2008). Like most other cells in the TME, TANs exhibit both anti and protumor functions and synonymous to the nomenclature used in TAMs, TANs are classified into antitumor (N1) and protumor (N2) phenotypes (Fridlender and Albelda, 2012). TANs can mediate tumor cell killing indirectly by releasing ROS and neutrophil elastase and directly by mediating Fas-ligand-associated apoptosis (Kobayashi, 2008). N1 neutrophils derived chemokines such as CCL-3, CXCL9 and CXCL10 and pro-inflammatory cytokines including IL-12, TNF-α and GM-CSF have a role in the recruitment and activation of CTLs thus

enhancing anti-tumor immunity. N1 neutrophils also potentiate antitumor T-cell responses by inhibiting TGF-β signaling (Murray et al., 2014) and they also have the ability to cross-present antigens *in vitro*, and antigen-pulsed neutrophils promoted the activation of CTLs (Beauvillain et al., 2007). TAN-derived factors have also been shown to accelerate tumor growth by several mechanisms. Genetic instability is a hallmark of cancer and the reactive oxygen and nitrogen species (RONS) produced by neutrophils are involved in this process (Gregory and Houghton, 2011). TANs also promote angiogenesis directly or indirectly through the secretion of VEGF, MMP-9, and CXCL1(Fridlender and Albelda, 2012; Galdiero et al., 2013). Furthermore TANs can promote tumor invasion and metastasis as they secrete high levels of the basement-degrading enzyme Collagenase IV and heparanase. Neutrophils have also been shown to enhance transendothelial migration, extravasation and formation of new metastases in circulating tumor cells (Cools-Lartigue et al., 2013) It is becoming increasingly clear that TANs are a distinct population of neutrophils polarized by factors in the TME and they have significant supportive roles in tumor biology. High levels of intratumoral neutrophils are significantly associated with unfavorable survival and tumor recurrence in several cancers including; clear cell carcinomas, bronchioloalveolar carcinoma, hepatocellular carcinoma, intrahepatic cholangiocarcinoma, head and neck cancer, non-small-cell lung cancer and renal cell carcinoma and colorectal cancer (Donskov, 2013; Shen et al., 2014). A more complete understanding of the mechanisms underlying the recruitment and polarization of TANs and how they can be educated to fight cancer is important in order to develop strategies to use TANs for cancer therapy (Gregory and Houghton, 2011).

Similarly, the role of tumor-infiltrating T-cells in the TME is complex. Many tumors express antigens that can be recognized by the adaptive immune system, and analysis of the TME usually reveals T-cell infiltrates (Galon et al., 2013). The infiltration of $CD8^+$ CTLs is generally anti-tumorigenic and correlates with a favorable clinical outcome (Fridman et al., 2012). $CD8^+$ T-cells are activated by peptide antigens presented on major histocompatibility class I (MHC-I) molecules which are present on all nucleated cells. Upon recognition of an antigen present on MHC-1 complex of the target cell, the $CD8^+$ T-cell is stimulated to proliferate leading to antigen-specific immune response and killing of the antigen bearing cells (Nolz, 2015). However, the presence of $CD8^+$ T-cells alone is insufficient to characterize the cytotoxic potential of the complex TME, and a more accurate predictor of the net-cytotoxicity potential of the tumor is the ratio of cytotoxic cells vs. immunosuppressive cells.

The role of infiltrating $CD4^+$ T-cell subpopulations has been shown to either inhibit or promote tumor growth and it is greatly influenced by their signature cytokines that equip the cells with distinct immunological functions. The Th1 polarization is typically driven by IL-12 activation of the signal transducer and activator of transcription 4 (STAT 4) and T-bet transcription factors on naïve T-cells. Th1 polarized T-cells can potentially produce large amounts of IFN-γ and have antitumor properties (Kim and Cantor, 2014). Th2 subsets are characterized by the production of IL-4, IL-5, and IL-13 and are mainly involved in coordinating humoral immunity and allergic responses. IL-4 is primarily accountable for the differentiation of Th2 cells through STAT6 and the transcription factor GATA-3. The role of Th2 effector cells in the TME is still unclear with some studies suggesting they have no effect on the clinical outcome (Tosolini et al., 2011) and others suggesting they accelerate tumorigenesis (Ochi et al., 2012). Regulatory T-cells (Tregs) are characterized by the presence of the activation marker CD25 and expression of the FOXP3 transcription factor. They play a pivotal role in the maintenance of immunologic homeostasis and self-tolerance. In the TME they can turn off immune responses by suppressing the proliferation of effector CTLs in a cell-contact dependent manner or in a cytokine-dependent manner through the secretion of IL-10, TGFβ and IL-35. The accumulation of intratumoral $Foxp3^+$ Tregs suppresses effective anti-tumor immunity and a high Treg–CD8 ratio in tumor infiltrates correlates with poor patient survival (Predina et al., 2013; Sisirak et al., 2012; Preston et al., 2013)

The Th17 subset is characterized primarily by the production of cytokines IL-17A and IL-17F and depends on expression of the transcription factors STAT3 and RORγt. The Th17 lineage is induced by a combination of TGF-β and IL-6 cytokines and represents a third effector arm of CD4-mediated immune response. In addition to IL-17A and IL-17F, human Th17 cells also produce other cytokines including IL-21, IL-22, and IL-26 (Annunziato et al., 2009). IL-17A induces the expression of several chemokines including CCL2, CCL7, CXCL1, and CCL20 as well as MMPs that promote inflammatory responses and provides protection against certain bacteria and fungi, but can also result in the induction of severe inflammation and promote the development of autoimmunity. IL-17 producing Th17 cells have been demonstrated in several human tumors, including ovarian, gastric, prostate, renal, and pancreatic and colorectal cancers, their contribution to tumor progression versus immune protection remains unclear (Bailey et al., 2014). Studies in various murine tumor models have suggested that Th17 cells favor tumor growth by facilitating tumor-related inflammation and promoting angiogenesis (He et al., 2012; Wang et al., 2009), evidence from other studies

demonstrates that adoptively transferred Th17 cells can inhibit tumor growth and mediate effective anti-tumor immunity, in part, by activating endogenous $CD8^+$ T-cells (Martin-Orozco et al., 2009). However, the exact nature of how Th17 cells affect the course of tumor development remains poorly understood, in part because antigen-specificity of tumor-associated Th17 cells has not been defined in most cases. Whether Th17 cells adopt a pro- or anti-tumorigenic role is largely dependent on the stimulation encountered by the cells in the TME and further identification and characterization of the mechanisms involved in the induction of tumor-reactive Th17 effector cells will offer more insight into the development of enhanced vaccine and T cell-based therapies against cancer (Bystrom et al., 2015).

It is clear that the immune system plays many crucial roles in tumor biology and can profoundly influence tumor behavior. Tumor immunity involves complex interactions between tumor and immune cells together with other components in the TME. Given the plasticity of most of the tumor infiltrating immune cells, there is still a lot to learn that can help us develop more robust immunotherapies for the treatment of inflammatory disorders and cancer (Shalapour and Karin, 2015).

1.4.2 Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) also referred to as tumor-associated fibroblasts or reactive myofibroblasts are the most abundant cells in the tumor microenvironment. Accumulating evidence has clearly demonstrated that this cell type confers important structural and functional characteristics to tumor growth especially in CRC, it has been shown that the gene expression profile of CAFs demonstrates an overwhelming dominance in predicting patient prognosis when compared to tumor cells transcription profile (Calon et al., 2015; Isella et al., 2015). While many tumor-promoting stromal signals are attributed to CAFs, their molecular mechanisms are not very well understood, and this has been an area of intense research in the past few decades with many interesting findings (Servais and Erez, 2013).

To be able to fully understand the function of CAFs however, it is important to understand the function of normal fibroblasts. Fibroblasts are non-vascular, non-epithelial and non-inflammatory cells and they are the major cellular constituents of connective tissue in the body. They are of mesodermal origin with a flat and elongated morphology and are usually found embedded within the ECM (Kalluri and Zeisberg, 2006; Tommelein et al., 2015). The main functions of fibroblasts

include maintenance of the ECM through deposition of ECM proteins and ECM-modulating enzymes, regulation of epithelial differentiation through the secretion of Wnts, HGF and EGF and TGF-β (Kalluri and Zeisberg, 2006; Powell et al., 1999; Göke et al., 1998), regulation of inflammation through the production of chemokines and cytokines including IL-1β and IL-6 (Servais and Erez, 2013; Tommelein et al., 2015) and facilitating wound healing (Li and Wang, 2011; Desmoulière et al., 2004; Hinz, 2007).

1.5 Intestinal fibroblasts in wound healing

Due to the close proximity of commensals and other potentially pathogenic microbes in the intestine, the integrity of the gut-barrier is critical for the maintenance of immunological balance. Upon injury of the single-celled intestinal epithelium, abrupt wound healing is critical for preservation of the homeostasis. The wound healing response consists of a series of dynamic but overlapping events that are divided into three phases based on the morphological changes in the tissue, namely, the inflammatory phase, the proliferation phase and the maturation phase (Iizuka and Konno, 2011). Fibroblasts participate in the process of wound healing both functionally and mechanically and their local proliferation and activation follows immediately after epithelial tissue damage in response to growth factors such as PDGF and TGF-β. Activated fibroblasts, also known as myofibroblasts secrete various growth factors such as HGF, FGFs and KGF promoting epithelial cell survival and proliferation, and endothelial cell recruitment and angiogenesis. Through the up-regulation of α-SMA, myofibroblasts are contractile cells and thus reduce the wound size. They also increase the production of ECM, mostly collagen and fibronectin, which constitute the newly formed granulation tissue, providing structural integrity to the wound. Following completion of the wound healing, and resolution of inflammation, these cells are removed by a special type of apoptosis called 'nemosis', the failure to do so results in pathological conditions such as fibrosis (Darby and Hewitson, 2007; Kankuri et al., 2008).

1.6 Fibroblasts and inflammation

Another important function of fibroblasts that has just become apparent in the past decade is their role in regulation of inflammation (De L Karlson et al., 2007; Servais and Erez, 2013; Harper and Sainson, 2014; Shalapour and Karin, 2015). Fibroblasts are non-immune cells and for a long time they were thought to be innocent by-standers during inflammation, accumulating evidence however it is now clear that fibroblasts participate actively in immunological responses and can be

directly stimulated by pro-inflammatory mediators and as a result secrete various chemokine, cytokines, growth factors and proteases (Table 1) and thus significantly affecting leukocyte recruitment, activation and survival in sites of inflammation (Harper and Sainson, 2014; Servais and Erez, 2013). Fibroblasts are implicated in fostering chronic inflammation by prolonging the survival of activated T-cells in rheumatoid arthritis (Harper and Sainson, 2014; Tommelein et al., 2015). Fibroblast-derived factors have been shown to promote T-cell survival by promoting the selective induction of anti-apoptotic genes, *Bcl-2* and *Bcl-xL*, thus allowing T-cells to survive at the site of inflammation, resulting in a prolonged abnormal immunological response leading to a switch from acute resolving to chronic inflammation (Buckley et al., 2001; Gombert et al., 1996). Fibroblasts can respond directly to bacterial products such as LPS and can in turn induce expression of pro-inflammatory cytokines and chemokines such as IL-1α, IL-1β, IL-6, IL-8 and TNF-α (Kalluri and Zeisberg, 2006; Micke and Ostman, 2005).

Quantitative proteomic analysis performed on supernatants of CAFs isolated from AOM/DSS induced CRC showed a clear association between desmoplastic and pro-inflammatory gene signatures (Torres et al., 2013). Evidence was provided for a significant increase in pro-inflammatory cytokines and chemokines including CCL2, CCL11, CCL8, CCL20, CXCL5, and IL-9 in the conditioned media from the CAFs. CCL-2 is involved in attracting macrophages into the TME and to also induce their differentiation into the tumor-promoting M2 phenotype. CCL-8 is also a potent monocytes and lymphocytes chemoattractant. The addition of both CCL2 and CCL8 into human CRC cultures enhanced tumor cell migration, and invasion. CAF-derived CXCL-12 and CXCL-14 contribute to recruiting bone marrow-derived monocytes and endothelial cells thus promoting tumor growth and angiogenesis (Östman and Augsten, 2009). IL-6 and IL-9 are potent immune cell modulators in the TME and affect T-cell survival and $CD4^{+}$ T-cell differentiation in the TME. Additionally, they also promote tumor cell proliferation of by activating STAT3 and other down stream signaling pathways (Nowak et al., 2009). The proinflammatory gene signature of CAFs from skin, breast, and pancreatic and gastrointestinal tumors also includes increased expression levels of Cox-2. COX-2-derived PGE-2 correlates strongly with increased tumor-cell proliferation and invasion in CRC (Quante et al., 2011; Adegboyega et al., 2004).

Table 1: Summary of factors secreted by fibroblasts.

Cytokines	Chemokines	Growth factors	Proteases
IL-1 (Pang et al., 1994)	**CCL2** (Tsuyada et al., 2012)	**PDGF** (Ingram et al., 2004)	**MMP2** (Toullec et al., 2010)
IL-6 (Zhu et al., 2014)	**CCL3** (Sasaki et al., 2014)	**VEGF** (Crawford et al., 2009)	**MMP-9** (Quante et al., 2011)
IL-8 (Terenzi et al., 2013)	**CCL5** (Tang et al., 2010)	**HGF** (Göke et al., 1998)	**MMP-13** (Lederle et al., 2009)
IL-9 (Torres et al., 2013)	**CCL7** (Jung et al., 2010)	**EGF** (Miró et al., 2013)	**MMP-14** (Hawinkels et al., 2014)
IL-10 (Ina et al., 2005)	**CCL8** (Torres et al., 2013)	**TGF-β** (Östman and Augsten, 2009)	**uPA** (Smith and Martínez, 2006)
	CCL11 (Torres et al., 2013)	**FGF2** (Strand et al., 2014)	
	CCL20 (Torres et al., 2013)		
	CXCl1 (Erez et al., 2010)		
	CXCL2(Erez et al., 2010)		
	CXCL5 (Erez et al., 2010)		
	CXCL10 (Laragione et al., 2011)		
	CXCL12 (Sugihara et al., 2015; Orimo et al., 2005)		
	CXCL14 (Augsten et al., 2009)		

1.7 Heterogeneity of intestinal fibroblasts

It has been well demonstrated that fibroblasts are a heterogeneous population of cells with a very high degree of plasticity. In the normal colonic mucosa of humans there are at least two defined subtypes of fibroblasts in the lamina propria. One subtype is the pericryptal sub-epithelial myofibroblasts which are characterized by the expression of α-SMA^{+}, SMM^{+} and desmin^{-}, the other subtype is α-SMA^{-}, SMM^{-} and desmin^{+}, referred to as non-pericryptal fibroblasts (Adegboyega et al.,

2002). Following inflammation and tumorigenesis however, there is a recruitment of cells from various tissue types that are activated to become CAFs (Ohlund et al., 2014; Cirri and Chiarugi, 2011; Hinz et al., 2007). Majority of the CAFs appear to originate from resident fibroblasts and are activated similar to fibroblasts in wound healing, however these cells persist in an activated state and do not undergo nemesis. The CAF phenotype is believed to be due to stable epigenetic mutations and persists even in the absence of the activating signals (Räsänen and Vaheri, 2010). Functionally CAFs are distinctly different from their normal counterparts, they have an increased proliferation rate, enhanced production of collagen and other ECM components, increased production of growth factors and other soluble factors, and they also have unique gene expression programs that influence tumor progression and metastasis. (Tsujino et al., 2007; Berdiel-Acer et al., 2014; Östman and Augsten, 2009; Servais and Erez, 2013).

1.8 CAF markers

Similarly, due to the different origins of CAFs, it is challenging to find a definitive marker for all CAFs. Normal fibroblasts express the mesenchymal markers vimentin, desmin, fibronectin, and type I collagen. Once activated, they up-regulate these mesenchymal markers but they may still express the markers from the cell of origin. The most widely used CAF marker is the de-novo expression of α-SMA. CAFs are also known to specifically up-regulate fibroblast-specific protein 1 (FSP-1), neural/glial antigen-2 (NG2), platelet-derived growth factor receptor-α/β (PDGFRα/β), fibroblast activation protein (FAP), urokinase-plasminogen activator (uPA), CD90, periostin, tenascin-C (TN-C), palladin and podoplanin (Powell et al., 2011). Simultaneously they down-regulate the specific markers of their cell of origin such as the epithelial marker cytokeratin and the endothelial marker CD31. Unfortunately, none of these markers is exclusive to CAFs, or present on all CAFs, for example α-SMA is also highly expressed in vascular smooth muscle cells and pericytes, which also populate the stroma. FSP-1 which is also known as S100A4, is also expressed in metastatic tumor cells and in very early granulocytic lineages from the bone marrow (Bhowmick et al., 2004; Inoue et al., 2005). A combination of multiple biomarkers may help to identify a larger pool of CAFs but would still provide limited information. It is evident that distinct CAF subpopulations with different molecular markers exhibit different biological traits that may promote or suppress tumorigenesis.

1.9 CAFs and tumorigenesis

Similar to immune cells, which can be polarized to either aid or suppress tumorigenesis, fibroblasts have also been shown to exhibit both properties. Fibroblasts have been shown to inhibit tumor growth through the formation of gap junctions between activated fibroblasts (Trosko and Ruch, 1998; Omori et al., 2001). Primary fibroblasts isolated from normal and cancer tissue have also been shown to inhibit the growth of co-cultured cancer cells *in vitro* (Flaberg et al., 2011). In two recent publications, it was demonstrated that ablating CAFs in models of pancreatic cancer results in the growth of undifferentiated, aggressive pancreatic cancer, uncovering a protective role of CAFs in PDAC (Özdemir et al., 2014; Rhim et al., 2014).

As the tumor progresses however, CAFs have been extensively studied in the context of tumor promotion, owing to their many functions on the tumor cells, immune cells and other stromal cells as discussed above. The molecular mechanisms involved in determining the phenotype of CAFs and how they may affect tumor growth are still largely obscure, but the differential activation of different transcription master-regulators such as TGF-β and NF-κB, due to differences in the local TME may play a significant role in this (Augsten, 2014).

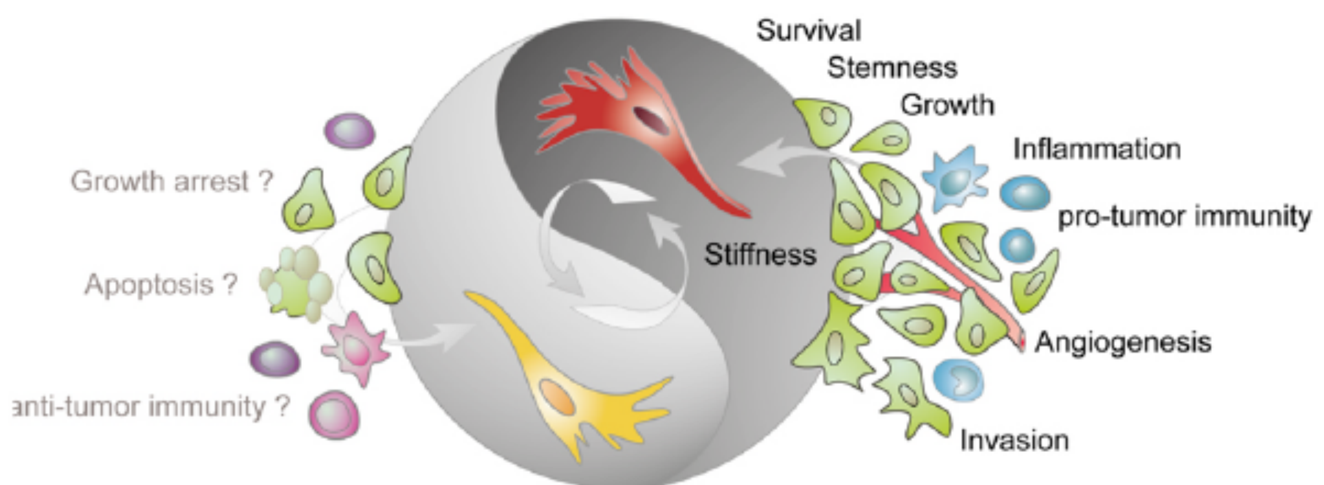


Figure 2: Tumor-stimulatory and tumor-inhibitory effects of CAFs.
CAFs have predominantly been assigned with a tumor-promoting role. CAFs (shown in red) stimulate cancer cell survival, growth, and invasion, enhance the stiffness of the ECM, contribute to angiogenesis by releasing pro-angiogenic factors, contribute to a pro-inflammatory TME, and impact on the activation state of various immune cells. More recent data demonstrate that fibroblasts (depicted in yellow) can also confer tumor-suppressive effects. However, the mechanisms underlying this inhibitory phenotype are not known but may involve direct inhibition of cancer cells and modulation of immune cell behavior. Figure from (Augsten, 2014).

1.10 TGF-β signaling

Transforming growth factor beta (TGF-β) is the prototypic member of a large family of secreted proteins that are involved in a wide range of cellular processes from embryonic development to cell proliferation, apoptosis, differentiation, migration, immune modulation as well as tumor initiation, progression and metastasis (Guo and Wang, 2009; Calon et al., 2014). This signaling pathway is highly conserved in many species and consists of three ligands, TGF-β1, TGF-β2 and TGF-β3; two types of receptors, TGF-β receptor 1 (TβRI) and TGF-β receptor 2 (TβRII) and the signal transducers (Smads).

TGF-β ligands are secreted into the ECM in an inactive form by several cell types, mostly fibroblasts and immune cells, as a heterotrimeric complex together with latency-associated protein (LAP) and latent TGF-β binding protein (LTBP). They remain inactive until they are released from the latent complex by extracellular proteases including thrombospondin1 (TSP1), matrix metalloproteinases (MMPs) and integrins (Stover et al., 2007).

Canonical TGF-β signaling begins with activated TGF-β ligands binding to TβRII, which then recruits and phosphorylates TβRI, present on the target cell surface. The resulting receptor complex phosphorylates the c-terminus of receptor-regulated Smad proteins (R-Smads). Activated R-Smads then heterooligomerize with the common Smad, and translocate to the nucleus where the Smad complex together with co-factors bind to TGF-β elements on the DNA and regulate target gene transcription (Attisano and Wrana, 2002).

1.10.1 Smad Proteins

Smad proteins are the vertebrate orthologs to drosophila protein, “Mothers against decapentaplegic” (MAD), which was the first TGF-β signal mediator identified (Sekelsky et al., 1995). The human genome encodes 8 Smad proteins, which can be grouped into three functional classes; five R-Smads, of which Smad2 and 3 mediate TGF-β/Activin signaling while Smad1, 5 and 8 transduce bone morphogenic protein BMP signaling. The common Smad (Co-Smad) Smad4, and two inhibitory Smads (I-Smads), Smad6 which inhibits the BMP pathway and Smad7 which specifically inhibits TGFβ signaling (Yan et al., 2009a). Individual Smad proteins have relatively low specificity upon DNA binding, thus they co-operate with each other and other transcription factors and co-factors in order to produce specific transcriptional responses. The co-factors are cell-type specific thus enabling different transcriptional responses in different cell types (Shi and Massagué, 2003).

1.10.2 Regulation of TGF-β pathway

Given the potency of TGF-β to act on almost every cell type and its pleiotropic actions, TGF-β signaling is regulated on many levels from ligand activation, receptor complex formation, R-Smad activation as well as nuclear translocation and transcription (Yan et al., 2009a). The negative regulation is mainly through negative feedback by the I-Smads, which are transcriptionally induced by TGF-β itself and NF-κB pathway (Freudlsperger et al., 2013; Bitzer et al., 2000).

When expressed, Smad7 competes with Smad2 and Smad3 for the binding site on TβRI (Nakao et al., 1997; Hayashi et al., 1997), it can also facilitate dephosphorylation of TβRI by protein phosphatase 1 (PP1) through its association with the protein, “growth arrest and DNA damage protein 34” (GADD34) (Shi and Massagué, 2003). Smad7 can also recruits the “Smad-ubiquitin regulatory factor” (SMURF) HECT type E3 ubiquitin ligase proteins, SMURF1 and SMURF2 and promote the ubiquitylation and subsequent proteosomal degradation of the activated TGFβRI complex (Izzi and Attisano, 2004; Chong et al., 2006).

Furthermore, there are several proteins that can affect the stability of Smad7 and thus influence TGF-β signaling either positively or negatively including, “Yes-associated protein” (YAP65) (Ferrigno et al., 2002), Hic-5/ARA55 (Wang et al., 2008), salt-inducible kinase (SIK) (Kowanetz et al., 2008), CRK-associated substrate lymphocyte type (Cas-L) (Inamoto et al., 2007) and serine/threonine kinase receptor associated proteins (STRAP) (Datta and Moses, 2000).

1.10.3 TGF-β signaling in cancer

TGF-β1 was discovered together with TGF-α due to their ability to induce a transformed phenotype in cultured rat kidney fibroblasts (de Larco and Todaro, 1978). Subsequent studies however showed that TGF-β is a potent proliferation inhibitor to most cell types, unlike its mitogenic partner TGF-α. This led to more focus being given to the tumor-suppressive properties of TGF-β and its 'transforming' potential was largely ignored until a decade later when, among other observations, it was shown that *in vitro* stimulation of adenocarcinoma cells with TGF-β1 enhanced their metastatic ability *in vivo* when injected into syngeneic rats (Welch et al., 1990). Several decades of study have shed light on the different roles of TGF-β in cancer and it is has now been clearly demonstrated that TGF-β signaling is a potent regulator of tumorigenesis and can act both as a tumor suppressor or enhancer depending on the cell type and the tumor stage (Akhurst and Derynck, 2001; Calon et al., 2014).

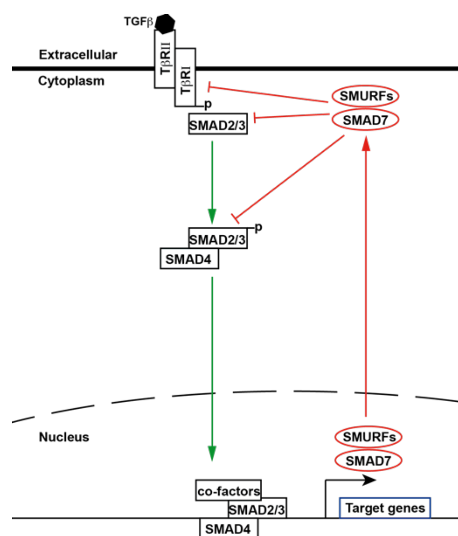


Figure 3: schematic model depicting a simplified overview of TGF- β signaling.

Ligand binding to TβRII results in phosphorylation and activation of TβRI. Consequently, Smads2 and 3 are phosphorylated by TβRI, leading to their heterodimerization and binding to the common Smad4, which then translocates to the nucleus and regulate the transcription of TGF-β responsive genes. Smad7 inhibits the signaling cascade. It can block Smad2/3 binding to TβRI, or their oligomerization with Smad4

1.10.4 Tumor suppressive functions of TGF-β

TGF-β1 signaling is critical for the maintenance of epithelial homeostasis in many tissues. In the normal intestine it is a potent inhibitor of epithelia cell growth and induces apoptosis balancing the rapid epithelia cell proliferation. Indeed mutational inhibition of TGF-β1 signaling through inactivation of *TBRII* or *Smad4* are frequently detected in 40-50% of advanced adenomas in patients with CRC and are commonly seen in patients with HNPCC (Markowitz et al., 1995; Markowitz and Bertagnolli, 2009).

Activation of TGF-β induces cell cycle arrest through the up-regulation of cyclin-dependent protein kinase (CDK) inhibitors $p15^{INK4B}$, $P16^{INK4A}$, $P19^{ARF}$ and $p21^{CIP1}$, as well as the suppression of mitogenic

transcriptional factors such as c-myc, Id1 and Id2 (Massagué, 2008). TGF-β can also activate apoptosis through induction of pro-apoptotic proteins like DAPK and GADD45b, FAS and BIM (Massagué, 2008).

In addition to its direct tumor-suppressive effects on epithelial cells, TGF-β can also restrict epithelia proliferation by inhibiting the secretion of soluble factors from stromal cells. The deletion of Smad4 in T-cells leads to spontaneous development of gastrointestinal tumors in mice (Hahn et al., 2011; Kim et al., 2006). Similarly, the suppression of TGF-β signaling through transgenic expression of a dominant negative TβRII in T-cells accelerates AOM induced colon tumors (Becker et al., 2004). In both cases the loss of TGF-β signaling in T-cells resulted in defective mucosal immunity and excessive expansion of cytotoxic T-cells leading to establishment of a chronic inflammatory microenvironment, promoting tumor growth. (Becker et al., 2004; Kim et al., 2006).

1.10.5 Oncogenic functions of TGF-β

Despite its tumor suppressive properties, the TGF-β pathway is strongly associated with tumor promotion and metastasis (Calon et al., 2012, 2014) and high serum levels of TGFB1 is associated with poor clinical outcome in patient with CRC (Tsushima et al., 2001; Calon et al., 2015). Furthermore HNPCC patients with mutations leading to the loss of function of TGF-β signaling in tumor cells have a better prognosis than patients with sporadic CRC that retain functional TGF-β signaling (Markowitz et al., 1995; Bubb et al., 1996), suggesting that while the loss of TGF-β signaling supports early tumor growth, it paradoxically has a protective effect on tumor progression and metastasis. Supporting this were experiments showing that the reintroduction of TGF-β signaling into colon cancer cells that lacked the receptor expression strongly stimulated their invasive and metastatic capacity (Oft et al., 1998) and recently it has been demonstrated that the inhibition of TGF-β signaling, specifically in the stroma of CRC reduces the metastatic potential *in vivo* (Calon et al., 2015).

It is well established that TGF-β signaling plays a significant role in epithelial-mesenchymal transition (EMT) in embryogenesis (Micalizzi et al., 2010). Activation of EMT in tumor cells is crucial for invasion and metastasis as it confers a junction-free migratory phenotype (Micalizzi et al., 2010; Tommelein et al., 2015). Several signaling pathways have been identified as mediators of TGF-β induced EMT some of which are Smad-dependent and independent. The induction of high-mobility group A2 (HMGA2) which is Smad-mediated, induce the expression of EMT master regulators Snail, Twist and

Slug (Pardali and Moustakas, 2007). Independent of Smad activity, TGF-β induced p38 MAPK and RhoA activation have been shown induce EMT through local actin disassembly in the cytoskeleton leading to tight junction dissolution (Pardali and Moustakas, 2007).

1.10.6 TGF-β signaling in the TME

In addition to its direct effects on the tumor cells, TGF-β also affects tumor growth and invasion by regulating the cells in the tumor microenvironment. While the loss of TGF-β signaling in T-cells leads to spontaneous tumorigenesis due to the persistence of activated pro-inflammatory T-cells, high levels of TGF-β in the TME mediate tumor cell escape from immune surveillance by acting on CTLs and suppressing T-cell cytotoxicity causing local immunosuppression (Thomas and Massagué, 2005). In patients with glioblastoma, TGF-β was shown to suppress NK cell activation by decreasing the expression NKG2D and its ligand MICA which are required for NK activation (Friese et al., 2004).

Another functional consequence of elevated TGF-β activity in the TME is the promotion of tumor angiogenesis. Mice with impaired TGF-β signaling through knockout of TGF-β1, TβRII, TβRI and the endothelial-specific TGF-β co-receptor endoglin have severe defects in angiogenesis, which are usually lethal (Dickson et al., 1995; Oshima et al., 1996; Li et al., 1999). Consistently, mouse models overexpressing TGF-β1 show the opposite phenotype with enhanced angiogenesis (Stearns et al., 1999). These effects are mediated through the induction of angiogenic factors such as connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) (Kang et al., 2003; Sánchez-Elsner et al., 2001) as well as the secretion and activation of matrix metalloproteinase MMP-2 and MMP-9 in endothelial and tumor cells (Derynck et al., 2001).

Another major way in which TGF-β contributes to tumorigenesis is in the recruitment and activation CAFs. As mentioned before CAFs arise from several cellular sources including resident fibroblasts and vascular cells and bone marrow derived mesenchymal cells through MMT and epithelial cells through EMT. This transdifferentiation is dependent on TGF-β signaling (Calon et al., 2014). TGF-β-activated myofibroblasts have a more secretory phenotype, in a model of squamous cell carcinoma (SCC), TGF-β-primed myofibroblasts were shown to secrete high levels of HGF thus enhancing tumor invasion (Lewis et al., 2004). Evidence from two recent studies with cohort of approximately 4000 CRC specimens from 10 datasets demonstrated that stromal gene expression in CRC has an overwhelming dominance over the epithelia gene expression in predicting prognosis and response to treatment (Isella et al., 2015; Calon et al., 2015). Notably, they showed that high stromal TGF-β

levels is a common feature in all CRC subtypes with a poor prognosis (Calon et al., 2015) whereas low stromal TGF-β robustly predicts disease free survival following treatment (Calon et al., 2012).

1.11 HGF-C-Met pathway

Another important signaling pathway necessary for embryonic development, regeneration, and tissue homeostasis that is frequently activated in many human tumors is the HGF/c-Met signaling pathway. This pleiotropic pathway has potent mitogenic and morphogenic functions, which induce cell survival, proliferation, migration and angiogenesis in many different tissue types.

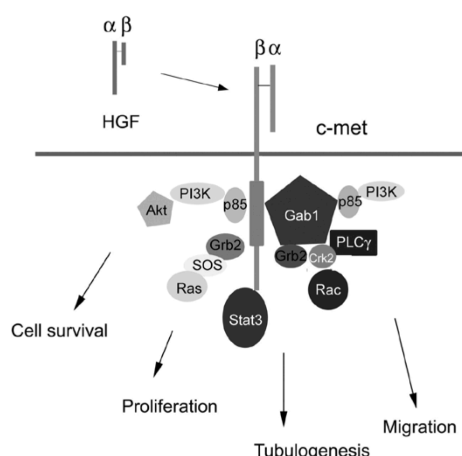


FIGURE 4: Schematic model of the HGF/c-Met signaling pathway
Upon HGF binding, the c-met receptor undergoes autophosphorylation in the tyrosine kinase domain, resulting in the recruitment of various intercellular signal transducers containing the Src homology domain, leading to diverse cellular responses, such as cell survival, proliferation, migration, and tubulogenesis. Figure from (Liu, 2004).

The *MET* proto-oncogene encodes a typeIV receptor tyrosine kinase, which is a single pass transmembrane protein belonging to a subfamily of heterodimeric receptor tyrosine kinases. MET is translated as a single chain precursor with 1,390 amino acids, it then undergoes several posttranslational modifications including glycosylation, it is then cleaved by the cellular protease furin between amino acid residues 307 and 308, into two chains, α and β subunits linked by disulphide bonds (Trusolino et al., 2010; Organ and Tsao, 2011).

The intracellular domain of MET has the tyrosine kinase activity and it is required for signal transduction. The kinase activity is increased by the phosphorylation of Tyr1234 and Tyr1235 and is repressed by that of Ser975. There are also two intracellular multisubstrate docking sites located on Tyr1349 and Tyr1356, which serve as docking sites for multiple transducers and adaptors including GRB2, SHC, CRK, PI3K, PLCγ, SRC, SHIP2 and STAT3 (Fig. 4) (Organ and Tsao, 2011; Weidner et al., 1996).

Hepatocyte growth factor (HGF) is the only known ligand for Met. The molecule was originally named “scatter factor” (SF) due to its ability to promote migration and scattering *in vitro* (Weidner

et al., 1990). HGF/SF is produced by mesenchymal cells and secreted into the ECM as an inactive precursor, similar to TGF-β. Maturation of pro-HGF to its bioactive form occurs in the ECM following cleavage by extracellular proteases, this serves as a critical rate limiting step in HGF signaling (Naldini et al., 1995). Activated HGF is then able to bind to its receptor c-Met which is expressed on adjacent epithelial and endothelial cells and exert a wide-range of effects including cell survival, proliferation, scattering, migration, invasion, branching morphogenesis, angiogenesis, differentiation and EMT (Birchmeier et al., 2003; Zhang and Vande Woude, 2003).

HGF/c-Met signaling is essential for embryonic development and HGF or c-Met knockout is embryonic lethal (Uehara et al., 1995). Wound healing and regeneration is also highly dependent on HGF/c-MET signaling. HGF levels increase dramatically following renal, hepatic or skin damage and induce several cell survival and anti-apoptotic mechanisms (Trusolino et al., 2010; Fan et al., 2005; Chmielowiec et al., 2007). In a murine model of peptic ulcer disease, the loss of HGF/c-Met signaling led to decreased gastric mucosal cell proliferation and delayed healing following mucosal injury emphasizing the role of this signaling pathway in wound healing (Nakahira et al., 2006).

1.11.1 HGF/C-met and cancer

Considering the wide range of effects induced by the activation of HGF/c-Met signaling, it comes as no surprise that many tumors use this signaling axis for survival, invasion and metastasis. Met has been shown to play significant roles in many human tumors including haematpoetic and solid malignancies (Rocci et al., 2014; Gherardi et al., 2012).

HGF/cMet signaling also enhances the malignant capacity of tumors by inducing and promoting angiogenesis through the induction of the VEGF and its receptor VEGFR, and the inhibition of thrombospondin, a negative regulator of angiogenesis (Lin et al., 2012; Garnett et al., 2013). Furthermore, the HGF/c-Met axis has been shown to interact with other membrane kinases, especially members of the epidermal growth factor receptor (EGFR) family, leading to transactivation of MET by stimulation of the EGFRs by their ligands, including EGF and TGF-α, leading to development of resistance in targeted therapies (Bardelli et al., 2014).

MET overexpression is observed in many human tumors and it is associated with metastatic phenotype and poor prognosis (Birchmeier et al., 2003). Amplification of MET has been observed in liver, breast, ovarian, lung, gastro-esophageal and colorectal cancers (Petrini, 2015).

In colorectal cancer, Met amplification correlates with advanced stage, local invasion and the presence of distant metastasis (Takeuchi et al., 2003; Zeng et al., 2008). It has been shown that CRC cells rely on HGF/c-Met signaling for Wnt/β-catenin mediated stemness (Vermeulen et al., 2010).

1.12 NF-κB signaling

The NF-κB family is a ubiquitously expressed family of transcription factors which also plays critical roles in mediating a large number of cellular processes and cell responses to stress, with major roles in cell survival, differentiation, and inflammation (Grivennikov et al., 2010). NF-κB can be activated by a variety of stimuli originating from various sources including cytokines and inflammatory mediators such as TNF-α, IL-1 and ROS, bacterial and viral products and ionizing radiation and it regulates a cohort of over 500 target genes including the production of cytokines, survival proteins and immunoreceptors making it a complex modulator of the immune response (Karin and Greten, 2005).

The mammalian genome contains five NF-κB proteins, which are related through a highly conserved DNA-binding dimerization domain called the Rel homology domain (RHD). These members are RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). NF-κB1 and NF-κB2 are synthesized as precursors with long C-terminal domains containing multiple copies of ankyrin repeats, which act to inhibit these molecules. To become active they undergo posttranslational modification (p105 to p50, p100 to p52) by either limited proteolysis or arrested translation (Ghosh and Karin, 2002).

In unstimulated cells NF-κB complexes are present in the cytoplasm but remain inactive due to their interaction with inhibitor of NF-κB (IκB). Following activating signals, IκB proteins are rapidly phosphorylated by IκB kinases (IKK) followed by polyubiquitination and subsequent proteosomal degradation by the 26S proteasome, liberating NF-κB to enter the nucleus and activate gene transcription (Karin, 2006).

NF-κB can be activated through two distinct pathways, the classical (or canonical) pathway and the alternative (or non-canonical) pathway (Bonizzi and Karin, 2004). Classical activation is triggered by proinflammatory cytokines such as TNF-α and IL-1β, and it is mediated by the IKK complex composed of two catalytic subunits, IKKα and IKKβ and a regulatory subunit IKK-γ. Following IκB degradation, NF-κB dimers, usually composed of p50/p65 subunits, translocate to the nucleus and activate gene transcription.

The alternative activation is solely dependent on IKKα. Activated IKKα homodimers phosphorylate p100 leading to its proteasomal processing to p52. NF-κB p52/RelB complexes then translocate to the nucleus and activate specific gene transcription (Bonizzi and Karin, 2004).

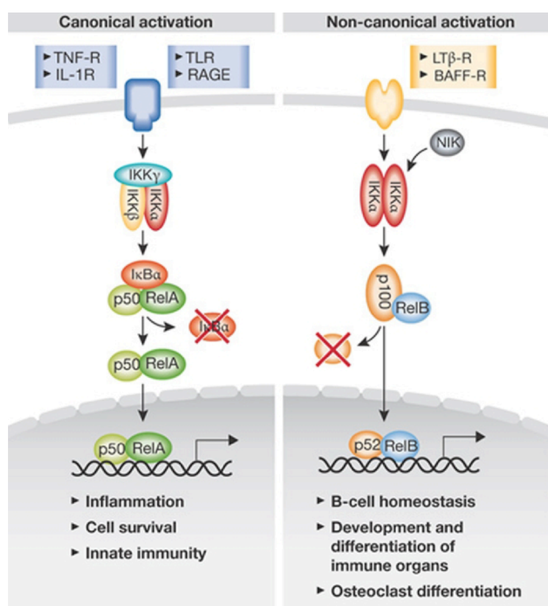


Figure 5: NF-κB activation
The proteins of the NF-κB family of transcription factors are sequestered in the cytoplasm by IκB when inactive. Upon activation through several receptors and afferent signals, IκB is phosphorylated by IKK proteins, thus tagging it for proteolytic degradation. Once liberated from IκB, NF-κB can translocate to the nucleus, where it proceeds to activate a large cohort of genes, including anti-apoptotic and mitogenic genes. Figure from (Bollrath and Greten, 2009).

1.12.1 NF-κB in cancer.

Strict regulation of NF-κB is indispensable for the integrity of cellular functions, which require both its prompt activation and termination. The deregulation/aberrant activation of this well-choreographed pathway is observed in many diseases including inflammatory diseases, metabolic disorders and cancer. Being the central mediator of inflammatory responses, NF-κB serves as the key link between inflammation and cancer but also orchestrates many other tumor-promoting processes beyond inflammation (Ben-Neriah and Karin, 2011). The NF-κB signaling pathway plays critical roles in all the steps of tumor development from tumor initiation, promotion, invasion, metastasis and resistance to therapy in many tumors of hematological and solid tissue origins (Perkins, 2012).

NF-κB signaling is highly multifaceted and depending on the cell type and the context of activation, it can function to either promote or suppress tumorigenesis. Following tissue damage or inflammation, the transient activation of NF-κB is required for the activation of both the innate and adaptive arms of the immune system and also epithelial cell survival and proliferation facilitating tissue repair and resolution of inflammation In the TME however, the sustained activation of NF-κB

functions to enhance tumor cell survival and their exposure to growth promoting pro-inflammatory cytokines leading enhanced primary tumor growth, invasion, metastasis and resistance to therapy (Xia et al., 2014). NF-κB signaling in tumors is a malignant reflection of its normal functioning in protecting cells from danger and facilitating healing and regeneration.

Aberrant activation of NF-κB leads to the induction of crucial genes providing tumor cells with most of the 'hallmarks' of cancer including anti-apoptotic genes (Bcl-2, Bcl-xL, cIAPs and cFLIP) promoting tumor cell survival, cyclins and proto-oncogenes (cyclin-D1, c-myc) promoting tumor cell proliferation, EMT master programs and cell adhesion molecules (Twist1, Snail, ICAM, VCAM) promoting tumor cell migration and metastasis and enzymes that can degrade the basement membrane and remodel the ECM facilitating invasion (MMP-2,3,9) together with the induction of genes responsible for angiogenesis (VEGF, bFGF and IL-8) (Xia et al., 2014). Furthermore, NF-κB can directly remodel tumor metabolism by inducing the expression of glycolytic enzymes (GLUT3, SCO2) and directly suppress mitochondrial function in tumor cells and thus promote a metabolic switch from oxidative phosphorylation to glycolysis (Kawauchi et al., 2008; Johnson et al., 2011). NF-κB can also affect tumor growth indirectly through its reciprocal relationship with various tumor suppressor genes and proto-oncogenes. NF-κB has been shown to inhibit p53 function by interacting with p300 and cAMP response element-binding (CREB)-binding protein (CBP), which are co-activators of p53, and by up-regulating the expression of a potent p53 inhibitor, murine double minute-2 (MDM2) (Perkins, 2012). In experimental mouse models of HCC, growth factor mediated NF-κB activation in hepatocytes involving the PI3K/Akt axis (Cavin et al., 2005), HGF/c-Met signaling (Müller et al., 2002), and TAK1/IKK pathway (Arsura and Cavin, 2005) promotes tumor development and progression. In prostate cancer, constitutive NF-κB activation is also largely attributed to Akt activation, which interacts with and stimulates other signaling pathways including EGFR, HER2, and NIK (Le Page et al., 2012).

A wide variety of mechanisms have been proposed to explain the addiction of cancer cells to the activated NF-κB including viral and fusion proteins, pattern recognition receptors (PRRs), IκB mutations, IKK hyperactivity, overexpression of ligands and receptors of EGF, HER-2/neu, TNF-a, IL-1, integrins, casein kinase 2 (CK2) and mutations in NF-κB (Didonato et al., 2012).

The gastrointestinal tract, and particularly the colon is continuously engaged in a bi-directional crosstalk between the microbiota, most of which are potentially pathogenic, and the immune system. Regulation of inflammatory responses is critical for homeostasis and any deregulation

predisposes the host to IBD, which increases the risk for CRC. Both sporadic and colitis-associated CRC have an element of inflammation, and NF-κB signaling is the key link between inflammatory processes and CRC (Shalapour and Karin, 2015).

The genetic evidence linking NF-κB driven inflammation to tumor initiation and promotion in CRC was first shown using mice with a conditional lineage specific knockout of IKKβ, the catalytic subunit required for canonical NF-κB activation, in a model of CAC. The genetic ablation of IKKβ in IECs, not surprisingly, led increased colonic injury and aggravated DSS induced colitis. But despite the increased intestinal inflammation, these mice developed significantly fewer tumors due to the loss of expression of several anti-apoptotic genes, including *Bcl-xL*, *Bcl-2*, and *c-IAP*, thus rendering transformed cells susceptible to apoptosis. The specific deletion of IKKβ in myeloid cells resulted in a reduction of both tumor incidence and size due to reduced secretion of pro-inflammatory cytokines including COX-2, CXCL-1, CXCL-2 and MMP-9 (Greten et al., 2004). This study not only revealed the importance of the IKKβ/NF-κB signaling in the TME, but also highlighted its cell-dependent effects, which in some instances may even have opposing outcomes (Eckmann et al., 2008). Furthermore, constitutive IKKβ/NF-κB activation in IECs was shown to induce mild inflammation in the colon and small intestine, leading to the development of spontaneous adenomas in the duodenum in aged mice. Upon challenge with AOM, intestinal tumor development was markedly elevated. Persistent activation of IKKβ in iecs accelerated APC loss of heterozygosity through iNOS/NO-induced reactive oxygen and nitrogen species leading to enhanced Wnt/β-catenin signaling and CRC (Shaked et al., 2012). In another study, the constitutive IKKβ/NF-κB activation strongly synergized with Wnt signaling to drive intestinal tumorigenesis again by increasing β-catenin activity and promoting proliferation the expression of genes encoding intestinal stem cell–associated factors including *Ascl2*, *Olfm4*, *DLK1*, and *Bmi*-1 (Vlantis et al., 2011). TNFα induced NF-κB activation also promotes β-catenin/TCF signaling activation by recruiting CBP leading to enhanced transcription of Wnt-dependent stem cell genes thus promoting CRC progression and metastasis (Schwitalla et al., 2013a).

However, the relationship between NF-κB signaling and cancer is complex and cannot be generalized into a single unifying theory, several studies have shown NF-κB or its upstream activators can function as tumor suppressor (Hoesel and Schmid, 2013). In a chemical model of HCC using N-nitrosodiethylamine (DEN) the deletion of IKKβ in hepatocytes greatly enhanced tumorigenesis (Karin, 2009). This was due to increased compensatory proliferation of hepatocytes

following increased cell damage after DEN-induced damage in IKKβ-deficient hepatocytes. In another study, p53-mediated apoptosis following cellular stress, DNA damage and oncogene activation was shown to e dependent to NF-κB activation, and this mode of apoptosis is distinct from that induced by TNFα-activation of NF-κB (Ryan et al., 2000). Further evidence of the growth inhibitory functions of NF-κB came from observations that the nuclear translocation and activation of NF-κB contribute to growth inhibition in human basal epithelial layers. Transgenic animals carrying dominant-negative IκBα showed epidermal hyperplasia while those overexpressing nuclear NF-κB showed epidermal hypoplasia thus showing that in the skin, NF-κB activity leads to apoptosis and growth inhibition, whereas loss of NF-κB increases proliferation (Seitz et al., 1998). RelB has also been shown to decrease cell proliferation and tumor growth *in vivo* in a p53-dependent manner. Furthermore, p53-mediated Doxorubicin response was reduced in RelB knockdown MEFs (Jacque et al., 2012). In gastric cancer as well it has been shown that despite the nuclear localization of RelA in facilitating tumor cell proliferation, especially in the early stages of tumorigenesis, NF-κB activation in tumor cells is negatively associated with lymphatic invasion and patients with positive nuclear NF-κB activation have a significantly better prognosis than those negative for NF-κB (Lee et al., 2005). Taken together, these results show that the effect of NF-κB signaling in tumorigenesis is cell dependent and context dependent, it can promote or suppress tumor growth and progression through a variety of mechanisms.

1.12.2 NF-κB and CAFs

CAFs are non-immune stromal cells that readily participate in inflammatory reactions, NF-κB signaling in CAFs has been shown to influence tumorigenesis (Servais and Erez, 2013; Shalapour and Karin, 2015). CAFs from skin, cervical, mammary and pancreatic tumors have express a NF-κB signature genes which promote tumorigenesis through the enhanced the recruitment of innate immune cells and tumor angiogenesis leading to promotion of tumorigenesis (Erez et al., 2010).

CAFs have critical but still underappreciated immune functions as discussed above. They produce numerous cytokines and chemokines, including osteopontin (OPN), CXCL1, CXCL2, IL-6, IL-1β, CCL-5, CXCL12 and CXCL13 (Table 1.1). Early in the tumorigenesis process, fibroblasts respond to the plethora of pro-inflammatory chemokines and cytokines produced by transformed cells and resident/recruited immune cells, along with the changes in tissue architecture caused by increased proliferation of neighboring tumor cells and in turn secrete proinflammatory mediators and growth

factors through the activation of NF-κB (Erez et al., 2010). Stromal myofibroblasts surrounding colon adenocarcinomas also have increased NF-κB activation compared to normal colon, and this up-regulation was also linked to enhanced tumorigenesis (Vandoros et al., 2006). However, due to the heterogeneity and plasticity of CAFs in tumors, and the different microenviromental factors activating different pathways in different subsets of CAFs, and the lack of animal models to directly evaluate the role of NF-κB signaling in fibroblasts, the true impact of NF-κB activation in CAFs remains largely unknown.

Objectives

The presence of large numbers of fibroblasts and myofibroblasts is a hallmark of many solid tumors including colorectal cancer. CAFs provide structural and functional support facilitating tumor growth, invasion, metastasis and resistance to treatment. The activation of NF-κB has been shown to be essential in the pathogenesis of colorectal cancer. Moreover the activation of NF-κB in CAFs has been shown to promote tumor engraftment and growth in other tumor models, but it has not been studied in autochthonous models of colorectal cancer. The aim of this work was to examine the role of IKKβ driven NF-κB activation in mouse model of colitis and colitis-associated colorectal tumorigenesis and to identify the underlying molecular mechanisms involved.

2 Materials and methods

2.1 Mice

Col1a2-creERT2

Transgenic mice with a tamoxifen-inducible Cre-recombinase under the fibroblasts-specific promoter *Collagen1a2* (Zheng et al., 2002).

Ikbkb$^{F/F}$

Generated with a targeting vector inserting 2 loxP sites around exon 3 of *Ikbkb* gene, encoding the IKKβ activation loop. When bred to mice expressing Cre-recombinase they undergo Cre-mediated excision of exon 3 and leading to the translation of non-functional IKKβ protein (Li et al., 2003).

Rosa26R-tdTomato

Rosa-CAG-LSL-tdTomato-WPRE mice contain a loxP-flanked STOP cassette preventing transcription of the downstream red fluorescent protein (tdTomato). When bred to mice that express Cre-recombinase, the resulting offspring have the STOP cassette deleted in Cre-expressing cells resulting in expression of tdTomato (Luche et al., 2007).

2.2 Genotyping

For DNA extraction, mouse tail-biopsies were lysed in a buffer containing 5% Proteinase K (QIAGEN) at 60°C. The enzymatic digestion was stopped by heat inactivation of the samples for 10minutes. The solution was then centrifuged and the DNA in the supernatant used for PCR reactions.

Tail lysis buffer recipe:

- 1,5M Tris/HCl (Roth, #4855.2)
- 200mM NaCl (Fluka, #71376)
- 0,2% SDS (Roth, #2326.2)
- 5mM EDTA (Fluka, #03609)
- 500ml d.H_2O

General PCR reaction mix

10xPCR-Buffer (Invitrogen)	2µl
50mM MgCl2 (Invitrogen)	0,8µl
100mM dNTP mix (Invitrogen)	0,4µl
20pM oligo 144F	0,5µl
20 pM oligo 321R	0,5µl
20 pM oligo 370F	0,5µl
20 pM oligo 370R	0,5µl
Taq polymerase 5U/µL (Invitrogen)	0,15µl
DNA	1,5µl
H_2O	13,15µl
Total	20µl

<u>PCR conditions</u>

Cre recombinase
94°C 30s
58°C 30s
72°C 30s
35 cycles
F 5'-ACC TGA AGA TGT TCG CGA TTA TCT-3'
R 5'-ACC GTC AGT ACG TGA GAT ATC TT-3

Ikbkb
94°C 30s
58°C 30s
72°C 30s
35 cycles
F 5´-CACAGT GCC CAC ATT ATT TAG ATA-3´
R 5´- GTC TTC AAC CTC CCA AGC CTT - 3´

Tdtomato
94°C 60s
61°C 30s
72°C 30s
35 cycles

Wild type
F 5'-AAG GGA GCT GCA GTG GAG TA-3'
R 5'-CCG AAA ATC TGT GGG AAG TC-3'

Mutant
F 5'-GGC ATT AAA GCA GCG TAT CC-3'
R 5'-CTG TTC CTG TAC GGC ATG G-3

2.3 Mouse diets

To ensure Cre-recombination in all recruited fibroblasts throughout the experiments, the mice were kept on a diet containing tamoxifen 400mg/kg (LASvendi) throughout the course of the experiments.

The c-Met inhibitor INCB28060 (Capmatinib) was purchased from Selleck Chemicals LLC. It was incorporated into the rodent diet AIN-76A from Research diets, Inc. with Capmatinib 18mg/kg and tamoxifen 400mg/kg. The mice in the control group received the same rodent diet AIN-76A containing tamoxifen 400mg/kg only.

2.4 Disease models

To induce colorectal tumors in the mice we used a well established model of colitis associated colorectal cancer, using azoxymethane (AOM) and dextran sulfate sodium (DSS)(Neufert et al., 2007)

2.4.1 AOM Administration

Azoxymethane (A5486 Sigma-Aldrich Co. LLC) was injected intraperitoneal at a dose of 10mg/kg at the beginning of the colitis and colorectal cancer treatments.

2.4.2 DSS administration

High molecular weight (36,000-50,000Kda) Dextran Sulfate Sodium (DSS) (0216011090 MP Biomedicals, LLC) was dissolved and administered in the drinking water of the mice for five days, followed by a 14-day recovery period on regular tap water. DSS causes apoptosis of distal colonocytes leading to commensal bacteria translocation into the lamina propria and subsequent inflammation, repeated administration leads to the development of a chronic inflammatory microenvironment that greatly enhances tumorigenesis (Greten et al., 2004).

2.5 Confocal laser endomicroscopy

Probe-based confocal laser endomicroscopy was performed on anaesthetized, tumor bearing mice at the end of the CAC challenge (day 84), in order to evaluate the functional capillary density, which is a surrogate marker for tissue perfusion, in the tumors. The mice were anesthetized with a cocktail containing fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidine (0.5 mg/kg). They were then injected with 10µL of 1% fluorescein and imaging was started immediately and completed within ten minutes using a mini probe (Mauna Kea Technologies, France) for detection of

intravenous fluorescein in the tumors. Minimum of 10images per tumor and 3 tumors per mouse were quantified using the CellVizio software.

2.6 Sacrifice of the mice

90minutes prior to the sacrifice of the mice, they were injected with 75mg/kg of 5-Bromo-2'-deoxyuridine (Sigma). BrdU is a thymidine analogue and its selectively incorporated into newly synthesized DNA at the S phase of cell cycle. Specific antibodies can later be used to identify cells that were actively dividing.

2.7 Histology

2.7.1 Tissue collection and preparation

The organs were removed and fixed overnight in 4% paraformaldehyde/PBS at 4°C. Before fixation, intestines were flushed clean with PBS, opened longitudinally and made into 'Swiss rolls'. The fixed tissues were dehydrated and embedded in paraffin, 4µm sections were prepared on slides. Before staining, the sections were deparaffinized in xylene and rehydrated in alcohols of decreasing strengths and finally into PBS.

2.7.2 Hematoxylin and Eosin staining

The deparaffinized and rehydrated tissue sections were stained for one minute in hematoxylin QS (vector labs) to stain the nuclei of the cells. They were then rinsed in running tap water until the water was colorless. The cytoplasm was then stained with 3% Eosin (Sigma) containing 0,5% acetic acid. The residual eosin was then washed away with running water and the sections dehydrated with alcohols of increasing strengths (50%-100%). The sections were then incubated in xylene for 5minutes and air-dried, covered with mounting medium and a coverslip is placed on top to protect the tissue.

2.7.3 Sirius Red staining

Sirius red is a method performed to histologically visualize collagen I & III fibers. The deparaffinized and rehydrated sections were covered in freshly prepared 0,1% Sirius red solution for two hours and rinsed in distilled water until clear. The slides were then incubated in xylene for 5minutes, air dried and coated with mounting medium (Vector Laboratories, #H5000), covered with a cover slip.

Sirius Red Solution

- 0,1 g Direct Red 80 (Sigma-Aldrich , #365548)
- 0,1 g Fast Green FCF (Sigma-Aldrich, #7252)
- 100 ml Picric acid

2.7.4 Immunohistochemistry/Immunofluorescence

Antigen unmasking was done on the deparaffinized, hydrated sections were brought to a boil in 10mM sodium citrate buffer, pH 6.0 in a microwave and maintained at a sub-boiling temperature for 20 minutes. They were then left on the bench top to cool for another 20minutes. The sections were then washed in dH_2O three times for 5 min each. They were then incubated in 3% hydrogen peroxide for 10 minutes at room temperature to deplete the endogenous peroxidase activity. They were again washed sections in dH_2O two times for 5 min each.

Endogenous avidin and biotin were blocked using Streptavidin-Biotin kit (vector laboratories) following manufacturers recommendations. The sections were then incubated in the primary antibody in working dilutions in the recommended blocking buffer as indicated in Table 2.1 below.

On the following day, the slides were washed in PBS three times for five minutes each and incubated in the appropriate biotinylated secondary antibody for 30minutes, they were again washed in PBS and incubated in ABC solution (vector labs), which was prepared as per manufacturer's recommendation, for 30 minutes. The sections were again washed and DAB solution was added for the color reaction and stopped by rinsing the slides in dH_2O. The slides were counterstained with Hematoxylin for one minute, washed until all residual stain had drained off, dehydrated and mounted with a cover slip.

Table 2.1: Antibodies used for IHC and IF

Antibody	Company	Product #	Dilution
BrdU	Amersham Biosciences	RPN201	1:400
Cl. Caspase 3	Cell Signaling	9661S	1:400
Collagen I	Abcam	ab21286	1:250
Collagen IV	Abcam	ab6586	1:250
E-cadherin	BD	610182	1:500
FSP1	Abcam	ab27957	1:250

p-Akt ser473	Cell Signaling	3787S	1:100
p-Met Y1234/1235	Abcam	ab5662	1:200
p-STAT3 (Y705)	Cell Signaling	9145	1:100
RFP	Abcam	ab34771	1:200
VE-cadherin	Santa Cruz	sc-6458	1:200
Vimentin	Abcam	ab92547	1:250
α-SMA	Dako Cytomation	M0851	1:100
β-catenin	Millipore	06-734	1:200

2.8 Cell isolation procedures

2.8.1 Isolation of primary intestinal fibroblasts

The colon was harvested and thoroughly washed with PBS containing 100units/ml Penicillin/Streptomycin (Invitrogen) and 50µg/ml gentamicin sulfate (Invitrogen). After repeated washing, the tissue was minced and digested in 1mg/ml collagenase I (Sigma CO130), 1mg/ml Dispase II (Roche 04942078001), 50µg/ml DNase (Sigma DN25) in 10ml of RPMI containing 2% FBS for 60min at 37°C, shaking. The supernatant was then filtered through a 40µM cell strainer, and the single cell suspension was washed with sterile PBS containing antibiotics as above. The cells were then plated on T75 flasks. Cells were cultured in complete DMEM containing 10% FCS, 1% glutamax, 1% non-essential amino acids, 1% pen/strep. Cells between 4th and 8th passage were used for experiments. Cell stimulation was done following a 24-hours serum starvation period and using DMEM containing only 1% PenStrep (Gibco), 1% glutamax (Gibco) and 1% non-essential Amino Acids (Gibco). For RNA analysis cells were sorted using flow cytometry and directly collected in RNA lysis buffer.

2.8.2 Intestinal epithelial cell isolation

The colon was harvested and the lumen was thoroughly washed with PBS. The colon was then cut into small pieces and incubated in 1xHBSS (Invitrogen) supplemented with 30mM EDTA (Fluka) for 8minutes at 37°C in a 50mL falcon. The resulting suspension was vortexed for 30s and left on ice until the tissue fragments settled by gravity. The supernatant containing the IECs was carefully transferred into a new 15mL falcon and centrifuged at 500xg for 5mins at 4°C to pellet the cells. The

epithelial cell pellet was collected and washed with sterile PBS and transferred to a new tube. The pellets were snap frozen in liquid nitrogen and stored at -80°C until further use.

2.9 Flow Cytometry

The intestines were harvested from the animals following sacrifice; the lumen was thoroughly flushed with PBS to remove all visible contents and mucus; the tissue was then minced into small pieces using two scalpels and transferred into a 50ml falcon for digestion. The digestion buffer was RPMI containing 2% fetal bovine serum, 1% penicillin/streptomycin, 1mg/ml collagenase I (Sigma), 1mg/ml Dispase II (Roche) and 50µg/ml DNase I (Sigma). The tissue was then digested for 60 minutes and then filtered through a 40µm cell strainer to obtain a single cell suspension. The cells were washed twice with ice cold PBS and centrifuged at 500g.The pellet was then re-suspended in FACS buffer and stained for cell surface antigens. For cytokine stimulation, the cells were plated in a six well dish in RPMI with 10% Fetal bovine serum, 1% Penicillin/streptomycin, 20ng/ml Phorbol 12-Myristate 13-Acetate (PMA), 1µg/mL Ionomycin and 1% Brefeldin A (eBioscience), and kept at 37°C and 5% CO_2 for 5hours. They were then harvested, washed and re-suspended in FACS buffer, then stained for surface antigens and a fixable viability dye Table 2.2. The cells were then fixed in IC fixation buffer (eBioscience) for 30minutes at 4°C, washed and re-suspended in 1x wash-perm buffer (eBioscience) containing the intracellular antibodies. Flow cytometry was performed using BD FACSCanto II Flow cytometer, and analysis was done using FLOW-JO software, v8.8.6 (Tree Star Inc., Ashland, OR).

Table 2.2: Antibodies used for flow cytometry, all antibodies are from e-Bioscience.

Antibody	Product #	Dilution
CD11b-APC-eFluor® 780	47-0112	1:200
CD11c-FITC	11-0114	1:200
CD29-APC-eFluo780	47-0291	1:200
CD3-APC-eFluor® 780	47-0032	1:200
CD31-eFlour450	48-0311	1:200
CD4-eFluor®450	48-0041	1:200
CD44-APC-eFluor780	47-0441	1:200
CD45-APC	17-0451	1:200
CD8a-PerCP-Cyanine5.5	45-0081	1:200

EpCAM-eFlour450	48-5791	1:200
F4/80-APC	17-4801	1:200
Fixable Viability Dye-eFluor® 506	65-0866	1:200
Foxp3-PE	12-4771	1:200
IFN gamma-Alexa Fluor® 488	53-7311	1:200
IL-17A-APC	17-7177	1:200
IL-4-PE-Cyanine7	25-7041	1:200
PDGFRα-APC	17-1401	1:200
PDGFRβ-APC	17-1402	1:200
TER119-FITC	11-5921	1:200

2.10 RNA extraction and RT-PCR analysis

Total RNA was prepared from the tissue samples using RNeasy Mini kit (Qiagen) following manufacturer's recommendations, 1µg of the total RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase First-Strand cDNA Synthesis kit also according to the manufacturer's protocol (Invitrogen).

Real Time PCR was performed on a StepOnePlus Real Time PCR cycler (Applied Biosystems) according to the manufacturer's protocol using primers indicated in table 3 below and 2x SYBR Green MasterMix (Roche). *Cyclophilin* was used as the house-keeping gene and the results were analyzed using the $^{\Delta\Delta}$Ct method (Livak and Schmittgen 2001) and presented as relative gene expression.

The PCR reaction was performed using standard RT-PCR conditions: 50°C for 2min, 10min 95°C, followed by 40 cycles of 95°C for 30s and 60°C for 1min. The results were analyzed using the StepOne Software v2.0.2.

Table 3: Primer sequences used for RT PCR

Gene	**Forward5'→3'**	**Reverse5'→3'**
Ascl2	GCCCGTGAAGGTGCAAAC	ACAGGAAAAGTGCTCGCGAG
bFgf	GTCACGGAAATACTCCAGTTGGT	CCCGTTTTGGATCCGAGTTTA
Cd11c	AACAGAGGTGCTGTCTACATATTTCATG	TGCTGAAATCCTCTGGCTGG
Cd4	GAGGCTCAGATTCCCAACCA	GCAGCAAGCGCCTAAGAGAG
Cd44	CTCCTGGCACTGGCTCTGA	CTGCCCACACCTTCTCCTACTATT

Cd8	CGTGGTGGTGCATGCCT	CCTCGAACTCAGAGATCCGC
Cxcl1	TATCGCCAATGAGCTGCG	GGATGTTCTTGAGGTGAATCCC
Cxcl2	ATCCAGAGCTTGAGTGTGACGC	AAGGCAAACTTTTTGACCGCC
CyclinB	ACTTCAGCCTGGGTCGCC	ACGTCAACCTCTCCGACTTTAGA
CyclinD1	CCCTGACACCAATCTCCTCAAC	GCATGGATGGCACAATCTCCT
CyclinE	ATGTGGCCGTGTTTTGCA	GGTCTGATTTCCGAGGCTGA
Cyclophilin	ATGGTCAACCCCACCGTGT	TTCTGCTGTCTTTGGAACTTTGTC
Epiregulin	GTCTTGACGCTGCTTTGTCTAGGT	TGCATGATGGGATCACGG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Fgf1	AGAAGCATGCGGAGAAGAACTG	CCGAGGACCGCGCTTAC
Foxp3	TAGGAGCCGCAAGCTAAAAGC	TCCTTGTTTTGCGCTGAGAGT
Gr1	GCAATGCAGCAGTTCCCACT	ATTGAATGGATCATCAGAGAAAGGTC
Hgf	AAGCTACAGAGGTCCCATGAATC	CGGTGTGGTGTCTGCTGG
Ifny	TTACTGCCACGGCACAGTCA	AGTTCCTCCAGATATCCAAGAAGAGA
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Il11	CTGCACAGATGAGAGACAAATTCC	GAAGCTGCAAAGATCCCAATG
Il17A	AAGTGAGCTCCAGAAGGCCC	TCATTGCGGTGGAGAGTCC
Il1ß	GTGGCTGTGGAGAAGCTGTG	GAAGGTCCACGGGAAAGACAC
Lgr5	GAGGAAGCGCTACAGAATTTGAGA	GTGGCACGTAGCTGATGTGG
c-Met	GCAGAAACCCCCATCCAGAATGTC	GGCCCCTGGTTTACTGACATACGC
p21	ATTCAGAGCCACAGGCACCAT	TCTCCGTGACGAAGTCAAAGTT
Cxcl12	ACTTTCCCTCTCGGTCCAC	TTGTTTAAAGCTTTCTCCAGGTA
Slug	ATCCTCACCTCGGGAGCAT	GGTAGAGGAGAGTGGAGTGGAGC
Smad7	AGTCAGCTGGTACAGAAAGTGCG	CCATCCACTTCCCGCGT
Smurf1	AGCTCCTGGTTGCCCTGC	AGGAGGGCTGACGATCGAG
Smurf2	TGCCTAGGGATCTTAGCAACATC	TGTTCTGTTGTTATGGTCAACGAA
Sox2	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA
Sox9	GCAGACCAGTACCCGCATCT	TCTCGTTCAGCAGCCTCCA
Tgfß1	GTACAGCAAGGTCCTTGCCCT	TAGTAGACGATGGGCAGTGGC
Tgfß3	TGACCCACGTCCCCTATCA	TCTCCTGAGTGCAGCCTTCC
Tgfß2	GCACAGTTCAGGGTCTTCCG	CAGCGTCTGTCACGTCGAA
Tnfα	AGGCACTCCCCCAAAAGATG	TCACCCCGAAGTTCAGTAGACAGA
Twist1	CTGGCGGCCAGGTACATC	CCATCTTGGAGTCCAGCTCG
Vegf	CATGCGGATCAAACCTCACC	GCATTCACATCTGCTGTGCTGT

2.11 Protein analysis

Tissue samples for protein analysis were snap frozen in liquid nitrogen immediately after harvesting and stored at -80°C. Protein extraction was done by mechanically disrupting the tissue in appropriate amount of protein-lysis buffer on ice. The sample was then centrifuged at 15,000g for

30minutes at 4°C to pellet debris. The supernatant was carefully collected and put into another tube and stored at -80°C until further use.

Protein Lysis buffer recipe

0.5% Nonidet p-40 (Sigma)
1mM Dithiothreitol (Sigma)
1 tablet of complete-protease inhibitor cocktail (Roche)/
50 mL
1% Triton-X 100 (Sigma)
10% Glycerol (Merck)
2mM phenylmethylsulfonyl fluoride(Sigma)
25mM Sodium fluoride (Sigma)
25mM Sodium-pyrophosphate (Sigma)
250mM NaCl (Sigma)
30mM Ethylenediaminetetraacetic acid (Sigma)
30mM Ethyleneglycoltetraacetic acid (Sigma)
5mM Sodium orthovanadate (Sigma)
50mM pH 7.8 Tris (Roth)
50mM β-glycerophosphate (Sigma)

2.12 Protein preparation for western blot

Protein concentration was determined using the Quick Start™ Bradford Protein Assay (Bio Rad) following manufacturer's recommendation. The color intensity, which corresponds to the protein concentration in the samples, was measured at 595 nm using a spectrometer (Bio Rad SmartSpec Plus) and the value of the concentration a standard curve was made using BSA.

Table 2.4: Antibodies used for western blot analysis

Antibody	Company	Product #	Dilution
E-cadherin	BD	610182	1:1000
IKKβ	Millipore	05-535	1:500
Tubulin	Santa Cruz	sc-32293	1:1000
Vimentin	Santa Cruz	sc-7557	1:1000
β-actin	Sigma	A4700	1:5000

2.13 Statistics

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using the standard two-tailed Student's t-test for two data sets and ANOVA followed by Bonferroni post hoc tests for multiple data sets. p-values ≤ 0.05 were considered to be significant. *≤ 0.05; **≤ 0.01; ***≤ 0.001. Error bars indicate standard error of the mean (SEM).

3. Results

3.1 *Col1a2*Cre-ER^{T2} is specific for fibroblasts in the intestine

The rapid progress in cell biology in the last two decades has tremendously been fuelled by the availability of tissue-specific promoters, which allow transgenic expression to be restricted to the cell-type(s) of interest. But for some lineages such as fibroblasts, this has been difficult owing to the lack of naturally occurring lineage-specific markers. To target fibroblasts and cancer-associated fibroblasts (CAFs) in our model of intestinal tumorigenesis, we employed the transgenic mouse model with a tamoxifen inducible Cre-recombinase under the *Proα2(I)collagen* gene (*Col1a2*Cre-ER^{T2}) that directs expression in fibroblasts and cells that are transdifferentiating into myofibroblasts and CAFs but not in other type I collagen-producing cells (Zheng et al., 2002).

To confirm tissue-specificity recombination, *Col1a2*Cre-ER^{T2} mice were crossed to *Rosa26R*$^{CAG\text{-}tdTomato}$ mice to obtain the compound mutant mice *Col1a2*Cre-ER^{T2};*Rosa26R*$^{CAG\text{-}tdTomato}$ that were used for lineage tracing (Fig. 6A). Upon tamoxifen administration, wide spread RFP expression was observed in the stromal cells of untransformed intestine as well as in colon tumors and several other organs including the lungs, liver, kidney, spleen and pancreas (Fig. 6b&C). Further immunofluorescence (IF) characterization showed the co-localization of RFP expressing cells with other stromal markers including collagen type 1 (ColI), collagen type IV (ColIV), fibroblast-specific protein 1 (Fsp1), vimentin and alpha smooth muscle actin (αSMA) positive cells. Co-expression of RFP and the endothelial marker vascular endothelial (VE) cadherin was observed in some colon tumors, which may represent transdifferentiating cells (Fig. 6B). Quantification of the cells undergoing recombination was performed by flow cytometry analysis of digested whole colon mucosa. RFP expression was limited to mesenchymal cells expressing fibroblast markers including CD29, CD44, PDGFRα and β. No RFP expression was observed in CD45^{+} immune cells, EpCAM^{+} epithelial cells, Ter119^{+} erythroid cells or CD31^{+} endothelia cells (Fig. 6D&E). The RTK platelet derived growth factor receptor alpha (PDGFRα) has been described as a robust surface marker for fibroblast, myofibroblasts and CAFs an in many tissues (Erez et al., 2010) and we used this as the marker for IMFs. We found that almost 80% of all PDGFRα^{+} cells were also positive for RFP (Fig. 6E) emphasizing the specificity of the *Col1a2*Cre^{T2} promoter.

Figure 6: Lineage tracing of cells undergoing recombination in *Col1a2*Cre-ER^{T2}

A) Schematic representation of the genetic strategy used to generate mice for lineage tracing. (B) Frozen tissue sections were stained for RFP (red) and the indicated markers (green). The cells co-expressing RFP and mesenchymal markers were marked yellow. Nuclei are stained blue with DAPI. Scale bar = 50µm. (C) RFP expression (red) in other tissues. Nuclei stained blue by DAPI. Scale bar = 100µm (D) Flow cytometry analysis of RFP expressing cells in induced *Col1a2*Cre-ER^{T2};*Rosa26R*$^{CAG\text{-}tdTomato}$ mice using markers for immune cells (CD45), epithelial cells (EpCAM), erythroid cells (Ter119), endothelial cells (CD31) and mesenchymal cells (CD29, CD44, PDGFRα and PDGFRβ). (E) Quantification of the flow-cytometry results. Data are mean± SEM. n≥ 5 animals.

3.2 IKKβ signaling in IMFs is dispensable in steady state

To study the role of IKKβ dependent NF-κB activation in fibroblasts during colitis and colitis-associated cancer (CAC), we crossed the *Col1a2*Cre-ER^{T2} mice with *Ikkβ* floxed mice (*Ikkβ*$^{F/F}$) mice in order to restrict IKKβ deletion in fibroblasts and CAFs. Immunoblot analysis of isolated primary cells from tamoxifen induced *Col1a2*Cre-ER^{T2};*Ikkβ*$^{F/F}$ animals (termed *Ikkβ*$^{\Delta Fib}$) and their Cre-negative littermates (termed *Ikkβ*$^{F/F}$) showed selective IKKβ deletion only in vimentin positive intestinal myofibroblasts from the mutant animals (Fig 7G and H), there was no deletion in the respective splenocytes (Fig. 7I) or sorted lamina propria CD45^{+} cells (Fig. 7J). The loss of *Ikkβ* in fibroblasts did not cause any overt phenotype in unchallenged *Ikkβ*$^{\Delta Fib}$ mice (Fig. 7A&B) and they remained healthy when kept on a tamoxifen containing diet. Collagen fibers visualized by sirius red stain and α-SMA⁺ fibroblasts and were comparable between the two genotypes in unchallenged mice (Fig 7C-F)

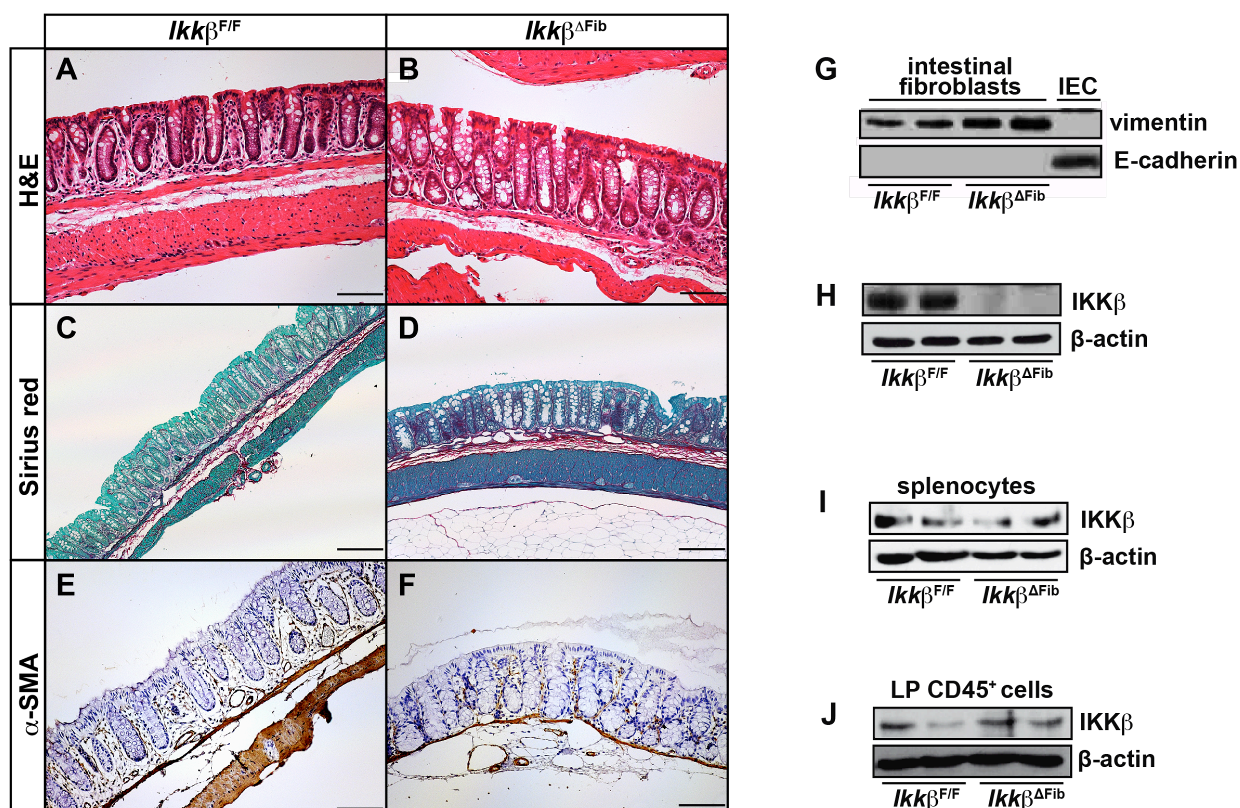


Figure 7: Deletion of IKKβ in myofibroblasts does not affect unchallenged mice

(A-F) Representative images of the colons of tamoxifen-induced unchallenged *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta Fib}$ mice stained with (A) Hematoxylin and eosin stain. (B) Sirius red. (C) αSMA. (G) Immunoblot analysis confirming purity of isolated primary intestinal fibroblasts. (H-J) Immunoblot analysis of IKKβ confirms the deletion is restricted to IMFs (H) but not splenocytes (I) or lamina propria (LP) CD45^{+} cells (J).

3.3 Fibroblast specific IKKβ deletion promotes AOM/DSS induced colon tumorigenesis

To induce colitis-associated colonic tumors, 6-8 week old $Ikk\beta^{F/F}$ and $Ikk\beta^{\Delta Fib}$ mice were challenged with the well established rodent CAC model. At day zero of the challenge the mice receive an intraperitoneal (IP) injection of 10mg/kg azoxymethane, followed by three successive cycles of water-administered 2% high molecular weight DSS. The mice were sacrificed on day 84 of the regimen (Greten et al., 2004). To ensure Cre-recombination in all myofibroblasts that are recruited into the tumor, the mice were kept on a diet containing 400mg/kg tamoxifen during the entire treatment (Fig 8A). The mice showed a consistent pattern of weight loss following each round of DSS treatment, but both groups had recovered completely before the beginning of the following round. There were no significant differences in the relative weight loss between the two groups (Fig. 8A).

To determine if there was any difference in the incidence or size of the tumors at the end of the treatment, the colons were washed and fixed in 4% PFA as 'swiss rolls' (Fig. 8E&F). Tumor analysis was done on 5µm H&E stained colon serial sections. Surprisingly, $Ikk\beta^{\Delta Fib}$ animals were not protected from tumor development and the tumor incidence was similar with the $Ikk\beta^{F/F}$ littermate controls (Fig. 8B). Quantification of the tumor sizes revealed that the $Ikk\beta^{\Delta Fib}$ mice had significantly larger mean tumor size (Fig. 8C) and also had a higher percentage of larger tumors with > 60% of the tumors being larger than 1mm compared to < 20% in the control mice (Fig. 8D). Representative H&E stained swiss rolls are shown in (Fig. 8E). 90 minutes prior to sacrifice, the mice were injected IP with 75mg/kg BrdU to label proliferating cells and unsurprisingly, the increased tumor size of $Ikk\beta^{\Delta Fib}$ mice was accompanied with an increase in the IEC proliferation rate quantified as the percentage of BrdU positive to the total number of epithelia cells in tumors of comparable sizes (Fig 8G-I). $Ikk\beta^{\Delta Fib}$ tumors also had a decreased IEC apoptotic rate quantified with cleaved caspase 3 staining in the tumors. (Fig. 8E-L). These results suggest that IKKβ dependent NF-κB signaling in fibroblasts, while having no effect on tumor initiation, is protective during tumor progression and its loss leads to enhanced tumor growth.

Figure 8: Fibroblast-specific *Ikkβ* deletion promotes AOM/DSS-induced tumorigenesis

(A) Weight curve showing the relative change in body weight of the mice during the CAC treatment. (B) Tumor incidence and (C) Mean tumor size per mouse (D) Histograms showing the tumor size distribution. (E,F) Representative H&E stained sections used for tumor counting; scale bars = 1mm. (G-I) Immunohistochemical analysis of BrdU incorporation Scale bar = 50µm. (I) The proliferation index of tumor cells. (J-K) representative images of cleaved caspase 3 stained sections used for analysis, Scale bar = 50µm. The tumor cell apoptosis index (L). All data are mean from two independent experiments ± SEM; n ≥ 7 mice per group, ; *** $p < 0.0001$, * $p < 0.05$, n.s. not significant by t-test.

3.4 IKKβ-deficient CAFs promote the activation of several key oncogenic pathways in tumor cells

There are several key signaling pathways that are often aberrantly activated in tumor cells and are implicated in driving tumor growth and progression including the PI3K/AKT, STAT3 and the Wnt/β-Catenin pathways (Vogelstein et al., 2013). These pleiotropic pathways are crucial for normal growth and development as they are involved in controlling multiple processes including the cell cycle, metabolism, motility and survival (Fearon, 2011). Immunohistochemical analysis of *Ikkβ*-mutant tumors revealed a significant increase in the activation of these pathways quantified by the phosphorylation of Akt^{S473} and $Stat3^{Y705}$ as well as increased nuclear β-catenin in tumor cells (Fig. 9A-I). This was consistent with our observation before that the loss of IKKβ in fibroblasts promotes CAC.

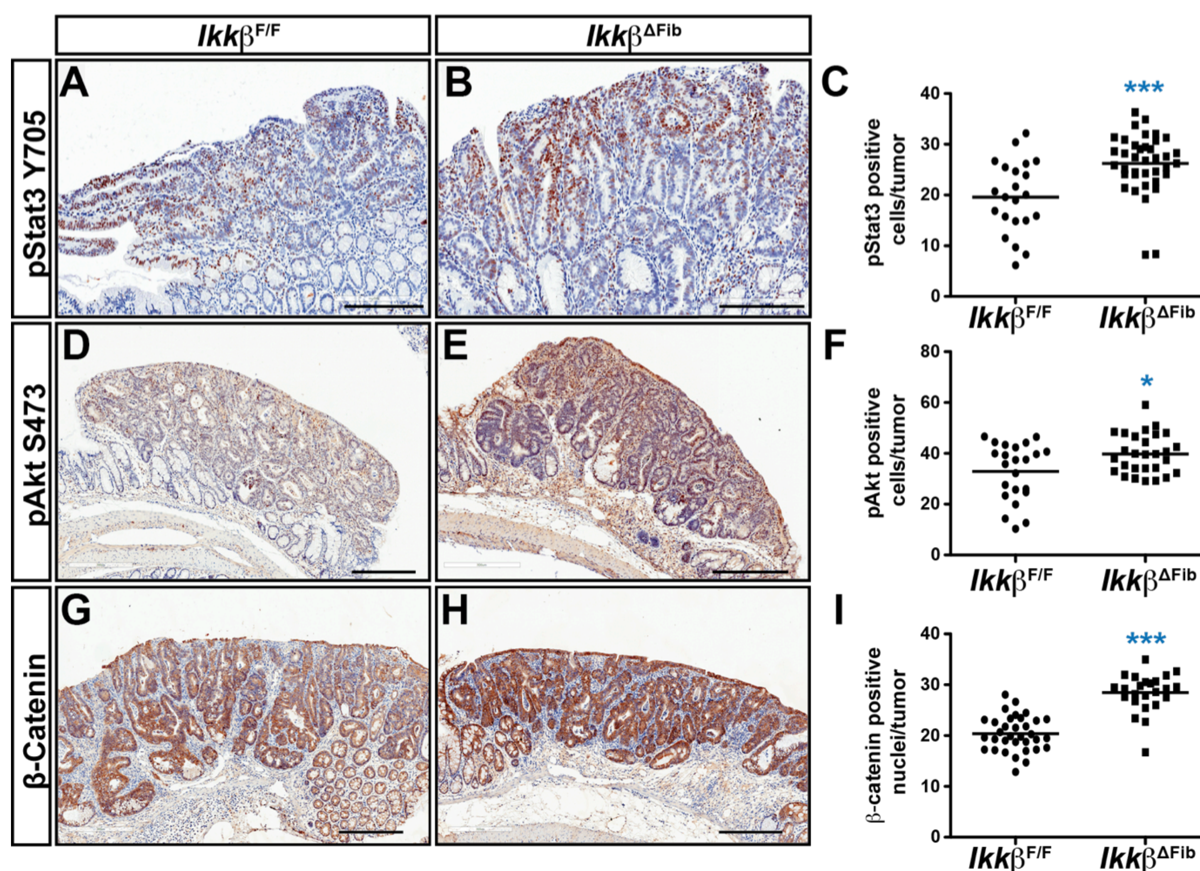


Figure 9: $Ikk\beta^{\Delta Fib}$ tumors show enhanced activation of key oncogenic pathways together with increased angiogenesis

(A,B) IHC analysis of phosphorylated $Stat3^{Y705}$ and quantification (C) in tumor epithelial cells. Scale bar = 200µm. (D,E) IHC analysis of phosphorylated Akt^{S473} and quantification (F). Scale bar = 300µm. (G,H). IHC analysis of nuclear β-catenin and quantification (I). Scale bar = 300µm. Data are mean ± SEM; n ≥ 10 tumors of each genotype; * $p < 0.05$, *** $p < 0.0001$ by t-test.

3.5 Accumulation of IKKβ-deficient CAFs in AOM/DSS induced tumors

In the complex TME, these pathways are most commonly activated by the binding of growth factors, cytokines, and other extracellular ligands such as Wnts on their respective receptors and initiate the signaling cascade. Many of these activating ligands are released in a paracrine manner by resident immune and stromal cells including CAFs (Peddareddigari et al., 2010). We therefore examined possible differences in the tumor infiltrating stroma on day 84 of the CAC challenge by flow cytometry using well established lineage specific markers described in Figure 6 above to identify the populations of interest. The gating strategy used is provided in (Fig. 10A) While the relative percentages of $CD45^+$ immune cells and $EpCAM^+$ epithelial cells were comparable between the genotypes (Fig. 10B), there was a significant increase in the percentage of $EpCAM^-CD45^-PDGFR\alpha^+$ fibroblasts (Fig. 10C) and $EpCAM^-CD45^-CD31^+$ endothelial cells in *Ikkβ*$^{\Delta Fib}$ tumors (Fig. 10D).

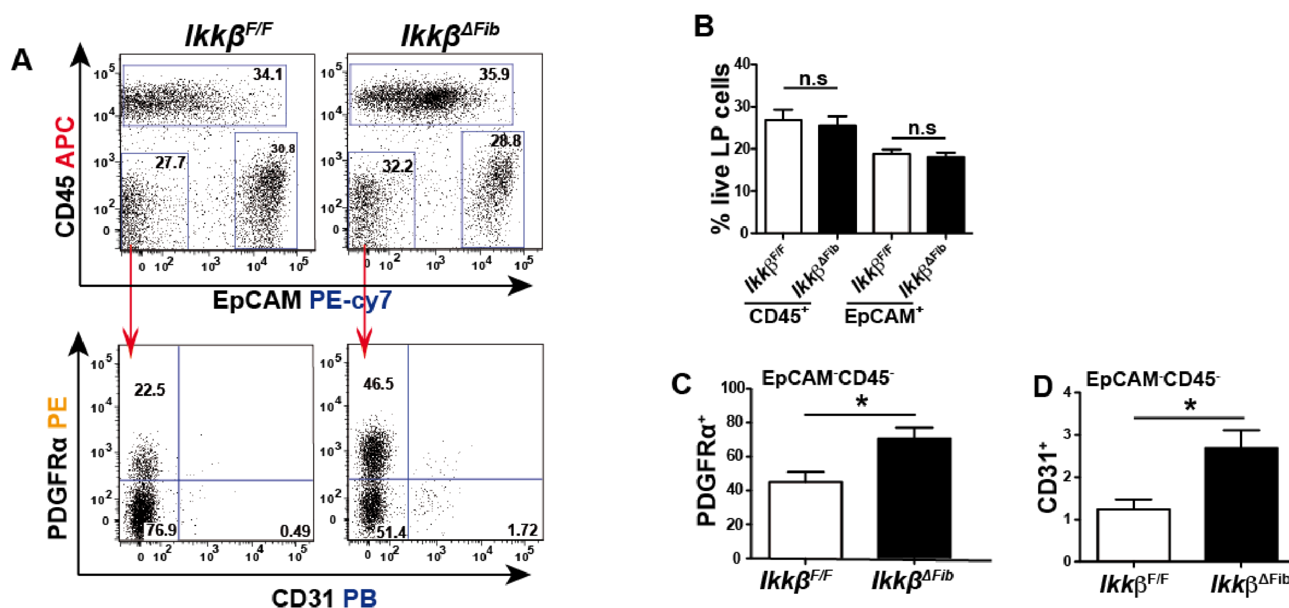


Figure 10: specific Ikkβ deletion in fibroblasts leads to enhanced recruitment of CAFs in the tumors

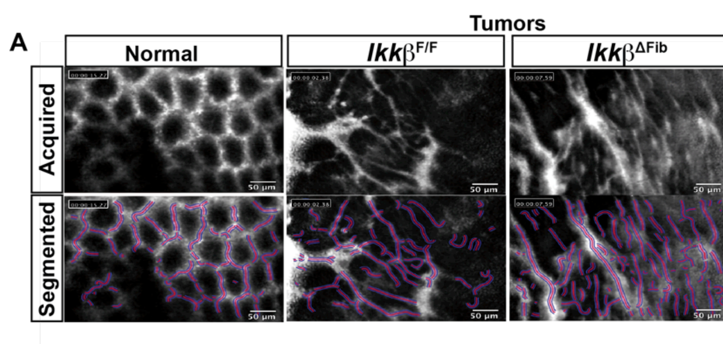
(A) The gating strategy used to identify the different cell lineages (B) Quantification of $CD45^+$ leukocytes and $EpCAM^+$ epithelia cells. (C) Quantification of $PDGFR\alpha^+$ CAFs. (D) Quantification of $CD31^+$ endothelial cells in tumors from *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta Fib}$ mice analyzed by flow cytometry. Data are mean ± SEM; n ≥ 6. * $p < 0.05$ by t-test.

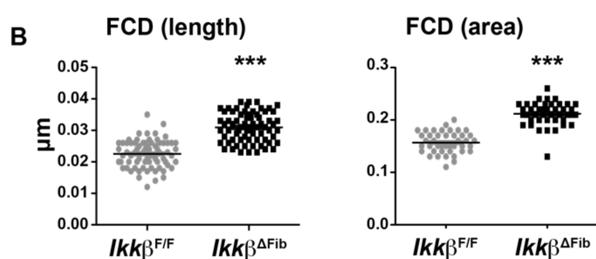
3.6 IKKβ$^{\Delta Fib}$ tumors show enhanced angiogenesis

CAFs mediate many tumor-promoting functions including angiogenesis through the secretion of angiogenic factors including IL-8, VEGF, PDGF, HGF, TGF-β, FGFs, CXCL-12 and MMPs (Tommelein et al., 2015). Given the significantly larger tumor sizes and the increased number of CD31 endothelial cells in IKKβ$^{\Delta Fib}$ tumors, we questioned whether there are any differences in the vascularization of

the tumors. *In vivo* quantification of the tumor microcirculation was done prior to sacrifice of the mice at day 84 of the CAC regimen. The mice were anesthetized and injected intravenously with 10µL of 1% fluorescein, which is rapidly distributed in the vasculature. The capillaries were then visualized by a probe-based confocal laser endomicroscope coupled to a 488nm laser-scanning unit (CLSM). Confocal images were acquired by direct contact of the imaging probe with the colonic mucosa and recorded as video sequences. The videos were then processed using ImageCel software, which performed automated segmentation of the vessels in the images (Fig. 11A). The 'functional capillary density' (FCD) is the ratio of the total vessel area and length to the total area of the image was used as the surrogate marker of microvascular density which represents tissue perfusion (Schmidt et al., 2013). We found a significant increase in the FCD length and area in *Ikkβ*^{ΔFib} tumors suggesting enhanced vascularization and tissue perfusion (Fig. 11B)

Figure 11: *Ikkβ*^{ΔFib} tumors have enhanced vascularization and tissue perfusion





A) Representative acquired and segmented confocal images obtained by CLSM; Scale bar=50µm. (B) Functional capillary density quantification of segmented images obtained from the tumors. Data are mean ± SEM; n ≥ 6 mice per group. *** p < 0.0001 by t-test.

3.7 IKKβ deletion in CAFs leads to reduced transcription of NF-κB-signature genes but enhanced TGFβ transcriptome

In order to identify possible IKKβ-regulated factors that may be responsible for the paracrine stimulation of tumor cell proliferation and recruitment of endothelial cells leading to enhanced angiogenesis, we performed gene expression analysis of fluorescence activated cell sorting (FACS) purified PDGFRα^{+} CAFs from tumors of *Ikkβ*^{ΔFib} and *Ikkβ*$^{F/F}$ mice. To achieve this, single cell

suspensions of digested tumor masses from the colon were stained with lineage specific surface markers as described in (Fig 10A). The $CD45^{+}$ immune cells, $EpCAM^{+}$ epithelia cells and $PDGFR\alpha^{+}$ fibroblasts were sorted and immediately lysed for RNA extraction and cDNA synthesis. The purity of the sort was assessed by qRT-PCR using lineage specific genes (Fig. 12A). The high level of purity of the sorted fractions allowed high quality gene expression analysis of the cells.

In several tumor types, a pro-inflammatory gene signature orchestrated, in part, by NF-κB signaling has been linked to the tumor-promoting behavior of CAFs (Erez et al., 2010). Analysis of NF-κB signature genes in the sorted *Ikkβ*-deficient CAFs showed a down regulation of several pro-inflammatory genes including *Il6*, *Cox2*, *Cxcl1, Cxcl5* and *Cxcl12*, while the expression of *Il1β* and *Cxcl5* remained unchanged (Fig. 12B). Furthermore, we observed a significant increase in the expression of *α-Sma* in *Ikkβ*-deficient CAFs. This finding, together with the increased percentage of CAFs (Fig. 10C) suggested there is an accumulation of activated CAFs in the tumors of $Ikk\beta^{\Delta Fib}$ mice.

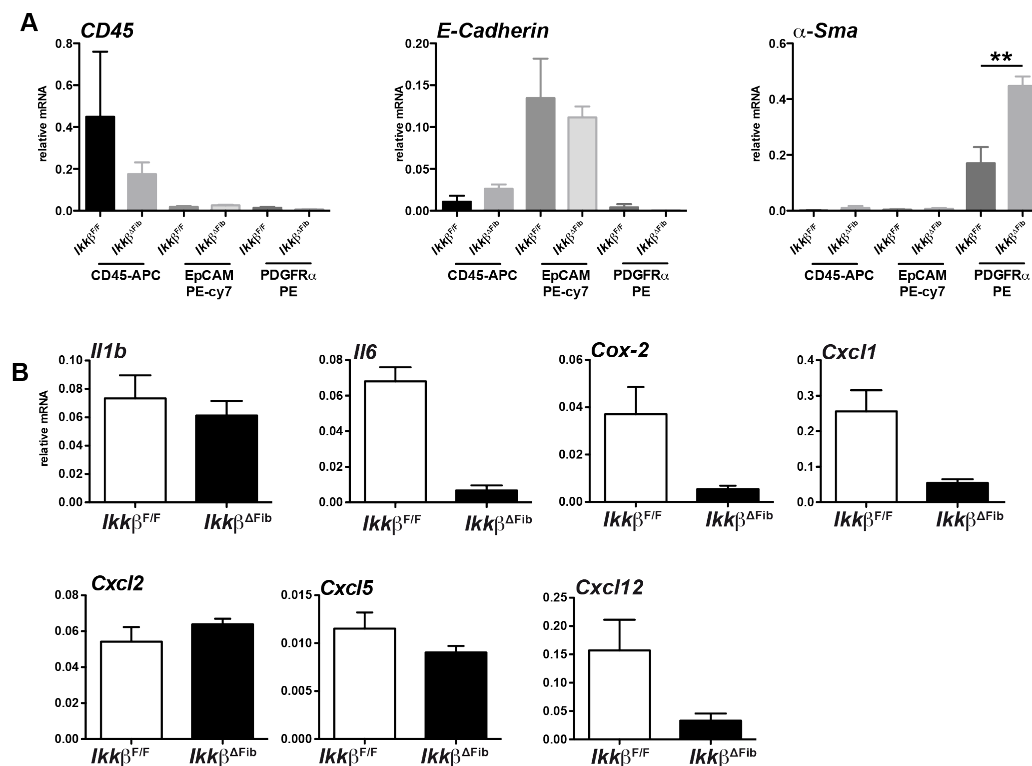


Figure 12: Decreased expression of NF-κB target genes in IKKβ-deficient CAFs

(A) Sort purity assessed by qRT-PCR of cell-specific control genes. (B) Relative mRNA expression of NF-κB target genes from sorted CAFs analyzed by qRT-PCR. Data are mean ± SEM. n≥ 4 mice per group. **p <0.001 by t test

As noted before, CAFs are of very heterogeneous origins; their development however, follows a well-established sequence of events that can be separated into two phases, each of which is characterized by specific cytoskeletal features. In the presence of mechanical stress, the myofibroblast/CAF precursors will first acquire *de novo* contractile bundles and become proto-myofibroblasts. This semi-activation is sufficient to allow cell migration enabling the cells to populate tissue spaces and begin ECM remodeling. In the presence of mechanical stress and TGFβ1, proto-myofibroblasts can further differentiate into myofibroblasts/CAFs, hallmarked by the neo-expression of α-SMA and its incorporation into pre-existing stress fibers. It is this stress fiber incorporation of α-SMA that renders myofibroblasts highly contractile (Hinz et al., 2012). In addition to enhanced ECM remodeling, they also up-regulate their production of chemokines, cytokines, growth factors and can spontaneously generate ROS.

We therefore assessed the expression of genes involved in TGFβ signaling in the purified CAFs. While there were no differences in the genes encoding the TGFβ ligands, *Tgfb1*, *Tgfb2* and *Tgfb3*, there was a down regulation of genes encoding for the negative regulators of TGFβ signaling, notably *Smurf1* and *Smad7* (Fig 13A). The transcription of *Smad7* is known to be controlled by NF-κB (Bitzer et al., 2000; Freudlsperger et al., 2013). In line with increased *α-Sma* expression in *Ikkβ*-deficient CAFs, the mRNA levels of genes coding for other activation markers including *Fap and Collagen1a* were markedly elevated (Fig. 13B). Fibroblasts growth factors 1 (FGF1) and 2 (bFGF) are other stromal derived growth factors that stimulate epithelia survival, proliferation and angiogenesis (Aoyagi et al., 2004), analysis of the mRNA levels of *Fgf1* and *bFgf* were unchanged at this time point (Fig. 13B). TGF-β signaling in CAFs has been shown to promote the growth and metastasis of CRC. A set of TGF-β target genes in CAFs was recently shown to be associated with poor prognosis in CRC patients (Calon et al., 2015), mRNA analysis of the sorted CAFs showed marked up-regulation of these TGF-β target genes including *Decorin, Ctgf, Cald1*, *Postn*, *Il11,* and *Igfbp7*. In addition we found markedly elevated expression of *Hgf* in *Ikkβ*-deficient CAFs (Fig. 13C).

Figure 13: Enhanced TGFβ signaling Ikkβ-deficient CAFs

Relative expression levels of indicated mRNAs from purified PDGFRα$^+$ CAFs analyzed by qRT PCR. (A), genes regulating TGF-β activation (B) CAF activation markers, (C) TGF-β signature genes Data are mean ± SEM±; n ≥ 4 mice per group

3.8 IKKβ-deficient CAFs promote IECs stemness

HGF is a pleiotropic, fibroblast-derived growth factor that acts in a paracrine manner on adjacent cells activating several downstream pathways promoting cell survival, proliferation, scattering, EMT and angiogenesis. The HGF/c-Met pathway is frequently deregulated in many tumors including CRC where it has been shown to be a target of *Wnt* signaling promoting the induction and maintenance of CRC stem cells (Vermeulen et al., 2010). Overexpression of HGF or its receptor c-Met in CRC is associated with metastatic disease and a poor prognosis (Liu et al., 2012; Stein et al., 2009). In light of this, we analyzed sorted EpCAM$^+$ tumor cells and detected consistent up-regulation of several cancer stem cell markers including *Lgr5*, *Ascl2*, *Tnfrsf19, Sox2*, *Sox 9, EphB2, EphB3* and *Dclk1* as well

as EMT master regulators *Snail* and *Slug* in tumor cells derived from *Ikkβ*$^{\Delta Fib}$ tumors indicating increased stemness (Fig. 14A). Furthermore, we also found an up-regulation of genes encoding several cell-cycle regulators including *cyclin A*, *cyclin B*, *Cyclin D1* and *cyclin E* and down regulation of the cell cycle inhibitor *p16*^{INK4A}. This was consistent with the observed increased IEC proliferation and enhanced tumor growth in *Ikkβ*$^{\Delta Fib}$ mice.

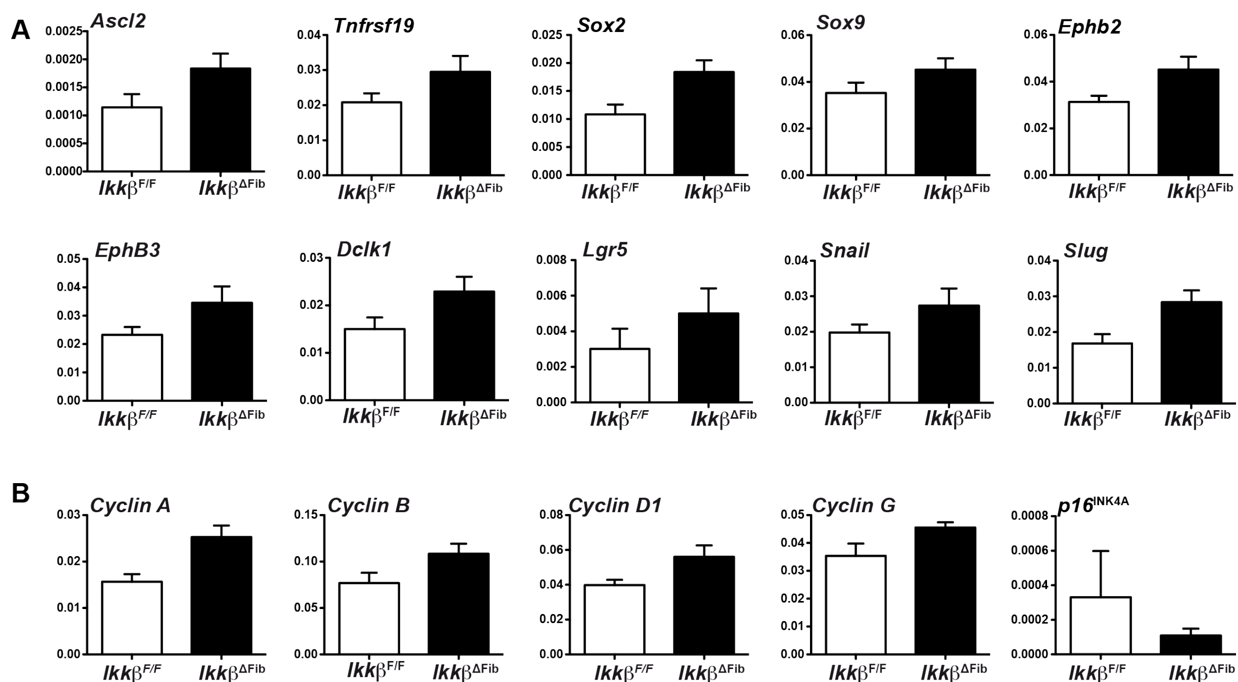


Figure 14: Ikkβ deficient CAFs promote cancer stem cell marker expression on tumor cells

(A) Relative expression levels of stem-cell markers (B) cell cycle markers from purified EpCAM^{+} tumor cells from analyzed by qRT PCR. Data are mean ± SEM; n ≥ 4 mice per group.

3.9 IKKβ$^{\Delta Fib}$ mice show enhanced IEC proliferation following acute colitis

Myofibroblasts have critical functional and structural roles following epithelial damage, orchestrating wound healing and the resolution of inflammation. We therefore assessed if the loss of Ikkβ signaling in fibroblasts and myofibroblasts has any effect on the outcome of DSS-induced colitis. For this we examined animals of both genotypes on day 15 of the AOM/DSS CAC challenge, following the acute phase of inflammation. The loss of *Ikkβ* in fibroblasts had no effect the systemic inflammation, extent of epithelia damage or delay wound healing as reflected by the indifferent relative weight loss, histological score and number of ulcerations (Fig. 15A-C). Whole mucosa mRNA analysis of the distal colon showed no difference in the expression of genes encoding the pro-

inflammatory mediators *Tnfa*, *Il1β* and *Cox-2.* The expression of *Il6* and *Cxcl1 Cxcl2*, *Il11* and *Nos2* were decreased but not statistically significant (Fig. 15D).

Figure 15: Ikkβ-deficiency in fibroblasts does not affect inflammatory response

(A) Weight curves of Ikkβ$^{F/F}$ and Ikkβ$^{\Delta Fib}$ mice in acute colitis. (B) Histological damage score, (C) number of ulcers. (D) Relative expression levels of mRNAs encoding pro-inflammatory genes isolated from whole mucosa on day 15 of the CAC model and analyzed by qRT-PCR. Data are mean ± SEM; n ≥ 7 mice per group. n.s. not significant

The inflamed mucosa of patients with IBD and in mouse models following DSS administration is characterized by elevated levels of TNFα, IL6 and COX-2, which are known to drive CAC by recruiting inflammatory immune cells and activating NF-κB and STAT3 signaling in IECs thus promoting the survival and proliferation of pre-malignant cells (Bollrath and Greten, 2009). Interestingly, despite having no differences in the levels of the classical ligands that activate NF-κB and STAT3 signaling between the genotypes at this early time point, we found increased

proliferation in $Ikk\beta^{\Delta Fib}$ IECs determined by BrdU incorporation (Fig 16A-C). Interestingly, even at this early time point we found over activation of the HGF/c-Met signaling as demonstrated by the enhanced phosphorylation of c-Met in IECs (Fig. 16D&E) which was further confirmed by immunoblot analysis of isolated epithelia cells (Fig 16F&G), mRNA analysis also revealed elevated levels of mRNAs coding for several fibroblast secretory proteins including the c-Met ligand *Hgf* as well as *Fgf1* and *bFgf* in the colonic mucosa of $Ikk\beta^{\Delta Fib}$ mice (Fig. 16H).

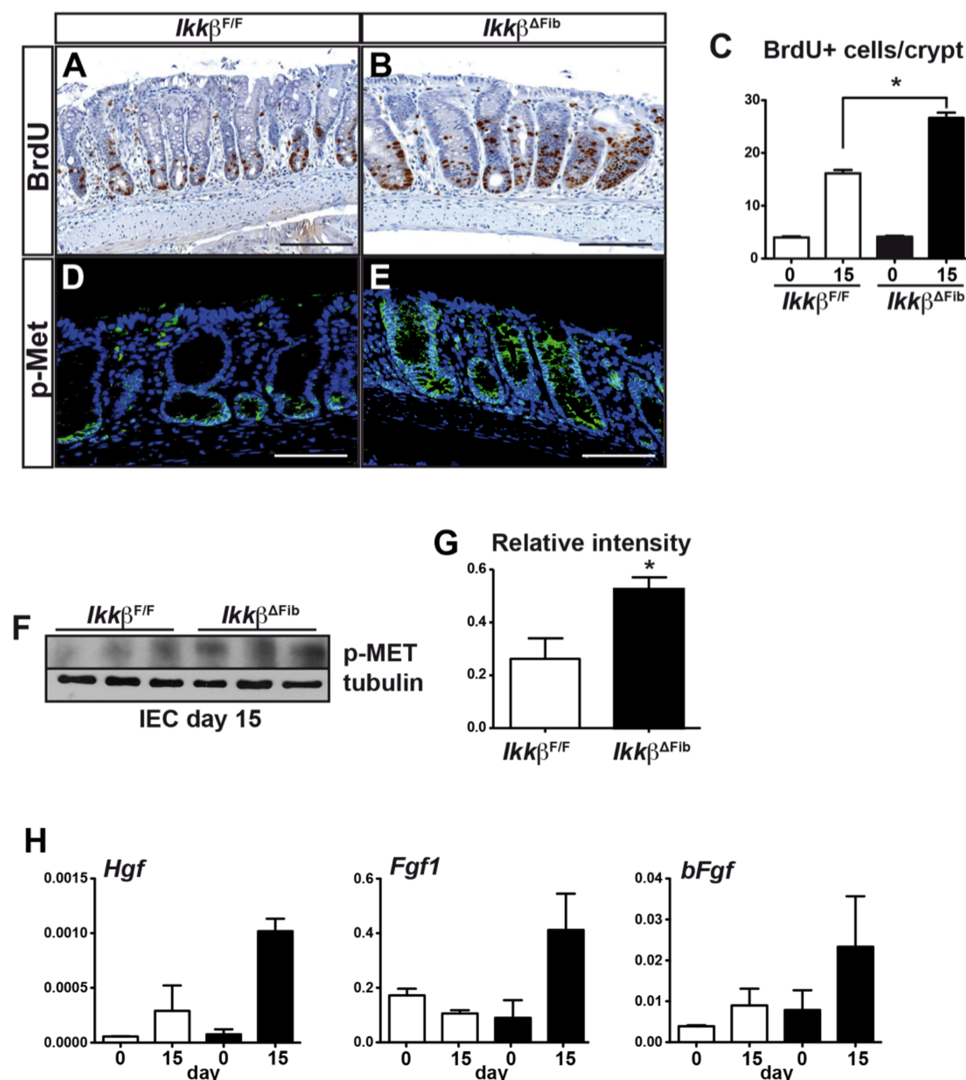


Figure 16 *Ikkβ*-deficiency in fibroblasts stimulates IEC proliferation after acute DSS-induced colitis (A&B) IHC analysis of BrdU stained sections, (C) proliferation index quantification. (D&E) IF staining of p-Met; Scale bar = 20µm. (F) immunoblot analysis of p-Met in IECs (G) Quantification of the immunoblot analysis using Image J. (H) Relative expression levels of mRNAs isolated from whole mucosa on day 15 of the CAC model and analyzed by qRT-PCR. Data are mean ± SEM; n ≥ 7 mice per group. *p < 0.05 by t test

Myofibroblasts actively regulate the recruitment, survival and activation status of immune cells through the production of a plethora of soluble factors and ECM fragments and the deregulation of

this process can lead to inappropriate immune responses leading to chronic inflammation (Östman and Augsten, 2009; Servais and Erez, 2013). In a mouse model of PDAC, depletion of αSMA^{+} myofibroblast was associated with decreased recruitment of CD4^{+} effector T-cells and increased CD4^{+}Foxp3^{+} Tregs, leading to a decreased CD8/Treg ratio. This immunosuppressive TME markedly accelerated disease (Özdemir et al., 2014). We immunophenotyped lamina propria infiltrating immune cells in the two groups following the acute colitis challenge. Flow cytometry analysis using lineage specific markers revealed no differences in the relative percentages of recruited CD45^{+}, EpCAM^{+}, CD31^{+} or PDGFRα^{+} cells at this time point (Fig. 17A). Likewise, there were no differences in the relative percentages of recruited F4/80^{+} macrophages, Gr1^{+} granulocytes or CD11^{+} dendritic cells. We did however find a significant increase in the relative number of infiltrating CD4^{+} and CD4^{+}Foxp3^{+} Tregs. The numbers of IFNγ^{+} Th1 and IL17^{+} Th17 T-cells remained unchanged (Fig. 17A-C).

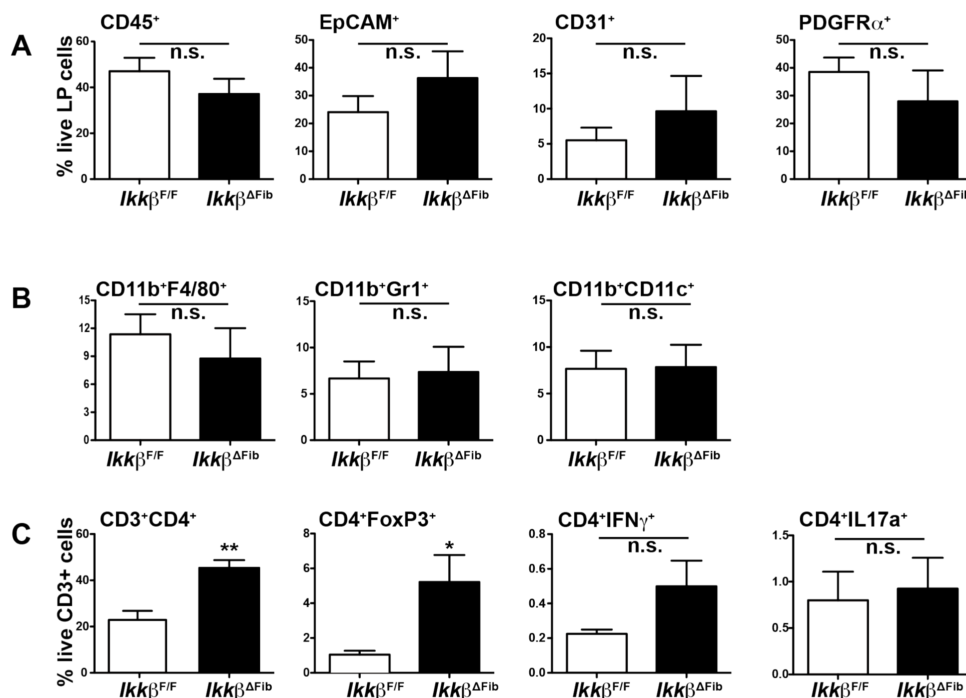


Figure 17: Accumulation of CD4^{+}Foxp3^{+} T cells in lamina propria of *Ikkβ*$^{\Delta Fib}$ mice on day 15 of the AOM/DSS model

(A) Quantification of major cell population in the lamina propria. (B) Quantification of myeloid populations. (C) Quantification of T-cell subsets. Data are mean ± SEM. n≥7 mice per group. *p < 0.05, **p <0.0001 by t test

3.10 Enhanced HGF secretion in the absence of *Ikkβ* is regulated by SMAD7

The immunosuppressive TME coupled with the activation of the c-Met pathway IECs could explain the enhanced proliferation and tumor promotion observed in $Ikk\beta^{\Delta Fib}$ mice following CAC challenge. However, it was still unclear what caused of the elevated *Hgf* transcription in the CAFs. The regulation of *Hgf* transcription, while very interesting, is not clearly understood. TGF-β can suppress the production of HGF and other tumor promoting paracrine signals including TGF-α, and WNTs, and when TGF-β signaling is lost in fibroblasts, which can occur during carcinoma progression, these paracrine ligands may be increased and thus contribute to adjacent carcinoma progression (Cheng et al., 2007). Conversely, TGF-β causes a fibroblast to myofibroblast transition that is also associated with adjacent carcinoma progression, and the TGF-β transcriptome in fibroblasts is associated with CRC progression and metastasis (Calon et al., 2015). At present it is thought that in normal fibroblasts, TGF-β can suppress *Hgf* expression however once a fibroblast is converted to a myofibroblast/CAF, TGF-β signaling up-regulates the expression of *Hgf* by virtue of being a new cell type. It is worth noting that some, but not all fibroblasts are able to become myofibroblasts/CAFs and therefore the mesenchymal lineage must be considered when interpreting the observed TGF-β response. To assess if the cause of the increased *Hgf* transcription we observed was due to a direct cell autonomous effect following the loss of canonical NF-κB signaling or an indirect consequence due the unopposed TGF-β signaling. We examined the HGF production in purified *ex vivo* cultured intestinal fibroblasts and CAFs isolated from $Ikk\beta^{\Delta Fib}$ and $Ikk\beta^{F/F}$ mice following AOM/DSS CAC challenge. Myofibroblasts isolated at day 15 and CAFs isolated at day 84 of the CAC challenge were seeded in 24 well plates and cultured to 80% confluence, after 24 hours of serum starvation they were either left untreated or stimulated with TGF-β1 (10ng/ml) or EGF (10ng/ml) separately or simultaneously over a period of up to 72 hours. Independently of the stimulus, *Ikkβ*-deficient fibroblasts and CAFs secreted significantly higher levels of HGF compared the control (Fig. 18A&B). Since NF-κB has not been shown to directly bind to the *Hgf* promoter, it was becoming increasingly clear that that the enhanced HGF secretion in *Ikkβ*-deficient myofibroblasts and CAFs was due to enhanced TGF-β signaling because of decreased *Smad7* expression. Thus, using a retroviral vector we overexpressed Smad7 or a control green fluorescent protein (GFP) in *Ikkβ*-deficient fibroblasts. Smad7 overexpression completely prevented the TGFβ and EGF dependent HGF secretion in comparison to the control cells (Fig. 18C and D).

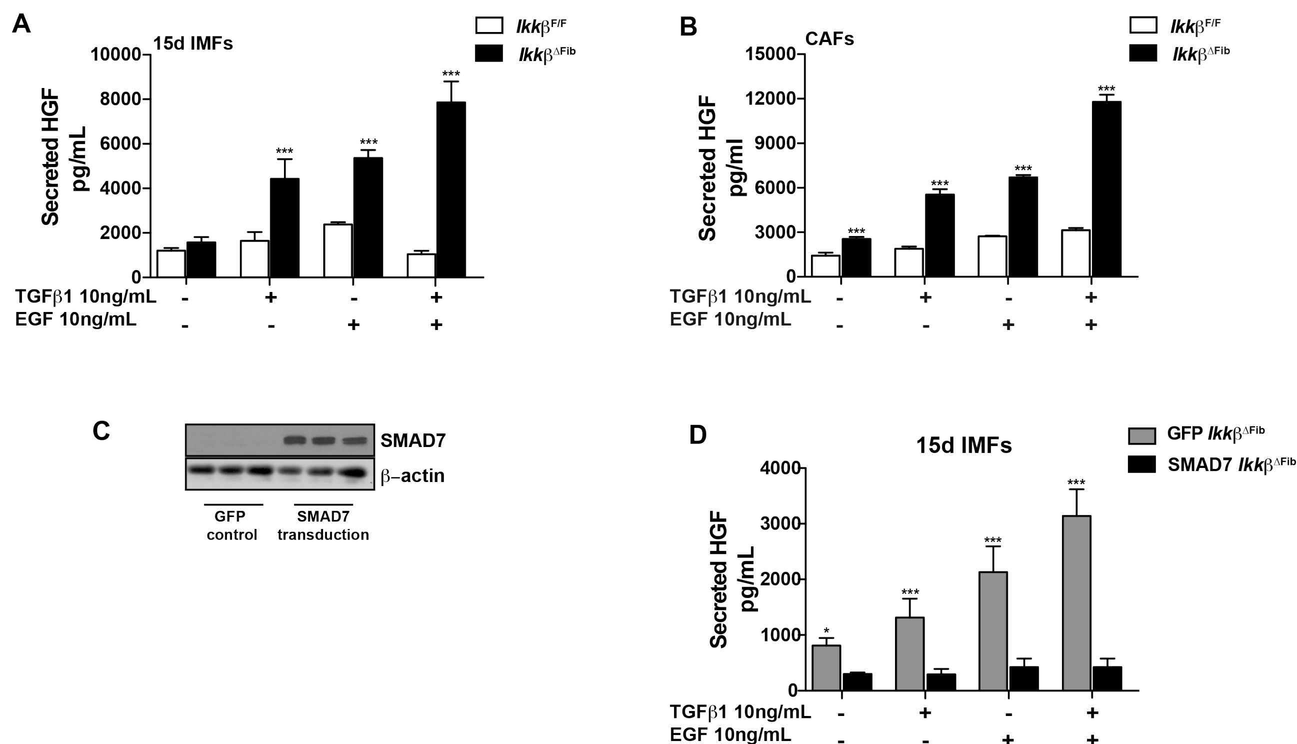


Figure 18: Elevated HGF secretion in *Ikkβ*-deficient fibroblasts depends on SMAD7

(A) ELISA for HGF secreted by $Ikk\beta^{F/F}$ and $Ikk\beta^{\Delta Fib}$ myofibroblasts. Primary myofibroblasts were isolated from the colon on day 15 of the CAC model. Cells were seeded in 48 well plates and stimulated with TGFβ1 10ng/mL or EGF 10ng/mL for 72 hours. (B) ELISA for HGF secreted by $Ikk\beta^{F/F}$ and $Ikk\beta^{\Delta Fib}$ CAFs. Primary CAFs were isolated from the colon tumors on day 84 of the CAC model. Cells were seeded in 48 well plates and stimulated with TGFβ1 10ng/mL or EGF 10ng/mL for 72 hours. (C) Immunoblot analysis of Smad7 in *Ikkβ*-deficient fibroblasts that had been retrovirally transduced with a *pBabe Flag Smad7* plasmid or a GFP control plasmid. (C) *Smad7* overexpression in *Ikkβ*-deficient fibroblasts prevents enhanced HGF secretion determined by ELISA. Data are mean ± SEM of 2 experiments performed in triplicate. $^*P < 0.05$, $^{***}p < 0.0001$ by t-test.

3.11 Blocking c-Met signaling prevents tumor promoting effects of *Ikkβ*-deficient fibroblasts

To examine whether the enhanced HGF secretion by *Ikkβ*-deficient fibroblasts was functionally responsible for the enhanced tumor promotion, we inhibited c-Met activation by feeding mice of both genotypes a diet containing a selective c-Met inhibitor INCB28060 (capmatinib) (Liu et al., 2011b) during the CAC regimen (Fig. 19A). Capmatinib did not affect tumor incidence but blocked tumor growth in control animals (Fig. 19B). However, in $Ikk\beta^{\Delta Fib}$ mice c-Met inhibition was even more apparent and capmatinib suppressed both tumor incidence as well as tumor size. 2 out of the 8 treated $Ikk\beta^{\Delta Fib}$ mice (25%) did not develop any adenomas and capmatinib prevented the enhanced tumor growth in untreated $Ikk\beta^{\Delta Fib}$ mice and consequently tumor size was now comparable to those in capmatinib treated control animals. Moreover, capmatinib blocked the

enhanced angiogenesis observed in $Ikk\beta^{\Delta Fib}$ mice to levels observed in $Ikk\beta^{F/F}$ tumors (Fig. 19D). In line with decreased tumor growth, the activation of $STAT3^{Y705}$, AKT^{S473} and nuclear β-catenin were significantly decreased following c-Met inhibition in $Ikk\beta^{\Delta Fib}$ mice (Fig. 19E-G). $CD4^{+}$ $Foxp3^{+}$ Treg numbers were also normalized following the acute inflammatory response Collectively, these data confirmed that indeed enhanced HGF secretion by $Ikk\beta$-deficient fibroblasts was the main driver of the enhanced tumorigenesis observed in $Ikk\beta^{\Delta Fib}$ mice by activating different tumor-promoting signaling pathways in tumor cells and inducing angiogenesis.

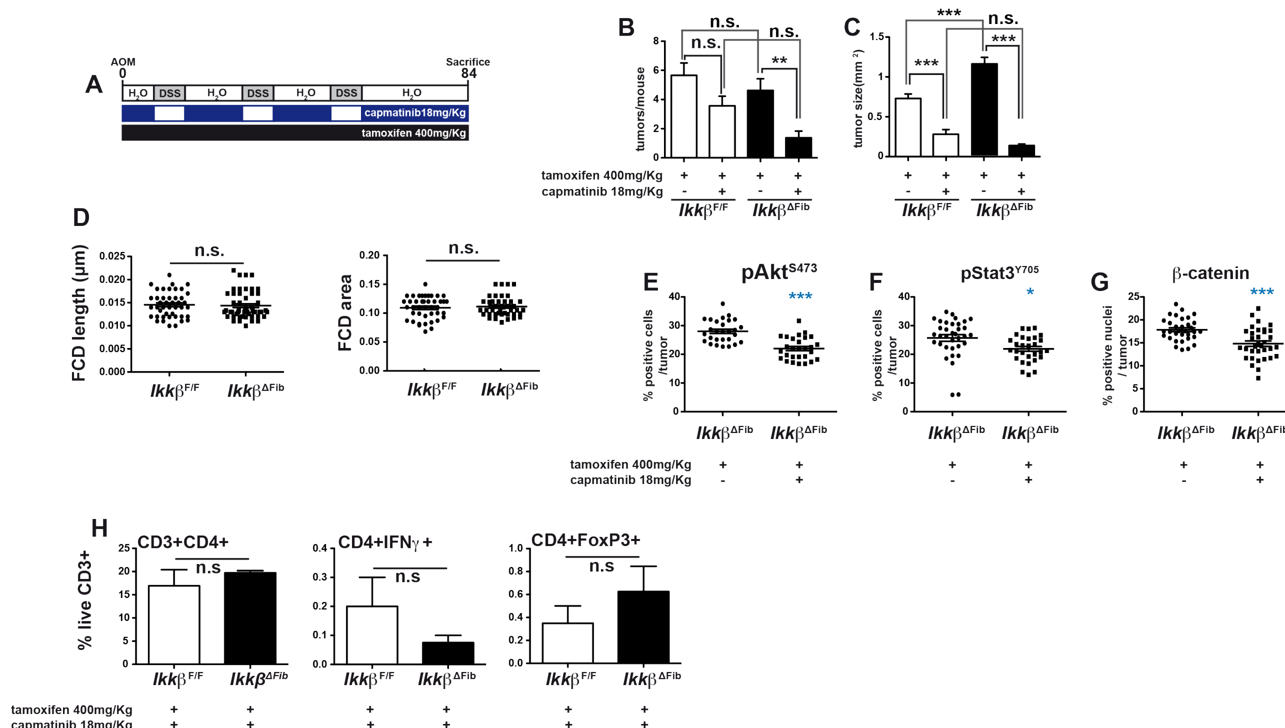


Figure 19: Met inhibition prevents tumor promotion in $Ikk\beta^{\Delta Fib}$ mice upon CAC challenge.

(A) Schematic overview of the CAC model and the mode of capmatinib application. (B) The tumor incidence and average tumor size (C) Data are mean ± SEM; n≥7; *** p < 0.0001 by ANOVA followed by Bonferroni post hoc test for multiple data sets. (D) Quantification of blood vessel FCD length and area in by CLSM. Data are mean ± SEM; n ≥ 6; n.s. not significant. Quantification of IHC analysis of, (E) p-AktS473, (F) p-Stat3Y705 and (G) nuclear β-catenin in tumor epithelial cells from untreated and capmatinib treated mice. Data in E, F and G are mean ± SEM; n ≥ 10 tumors of each genotype; * p < 0.05, *** p < 0.0001 by t-test. (H) Flow cytometry quantification of lamina propria CD4 T-cell subgroups from capmatinib treated mice. Data are mean ± SEM; n≥7; n.s. not significant.

3.12 IKKβ-deficient CAFs promote CRC progression independent of inflammation

Azoxymethane (AOM) is a procarcinogen which requires metabolic activation to induce DNA-reactive adducts in IECs. The repetitive administration of AOM to rodents leads to the development of colonic tumors with pathological features similar to those seen in sporadic CRC. While there is still some element of inflammation in this tumor model, it is not primarily driven by a pro-inflammatory microenvironment (Karin, 2009). To see if canonical NF-κB signaling in CAFs was only protective in the context of inflammation driven tumorigenesis, we induced colorectal tumors in *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta Fib}$ by repeated administration of 10mg/kg AOM once weekly for six straight weeks. The mice were then kept on tamoxifen food for 16 more weeks and sacrificed on week 22 (Fig. 20A). As in the CAC regimen, there was no difference in the tumor incidence (Fig. 20B). The analysis of the tumor area and tumor size distribution also showed the same trend as in the CAC model albeit less pronounced (Fig. 20C&D). Furthermore, these tumors also had a higher proliferating index. Taken together, this data confirms that IKKβ signaling in myofibroblasts and CAFs restrain tumor growth in both colitis-dependent and sporadic models of CRC.

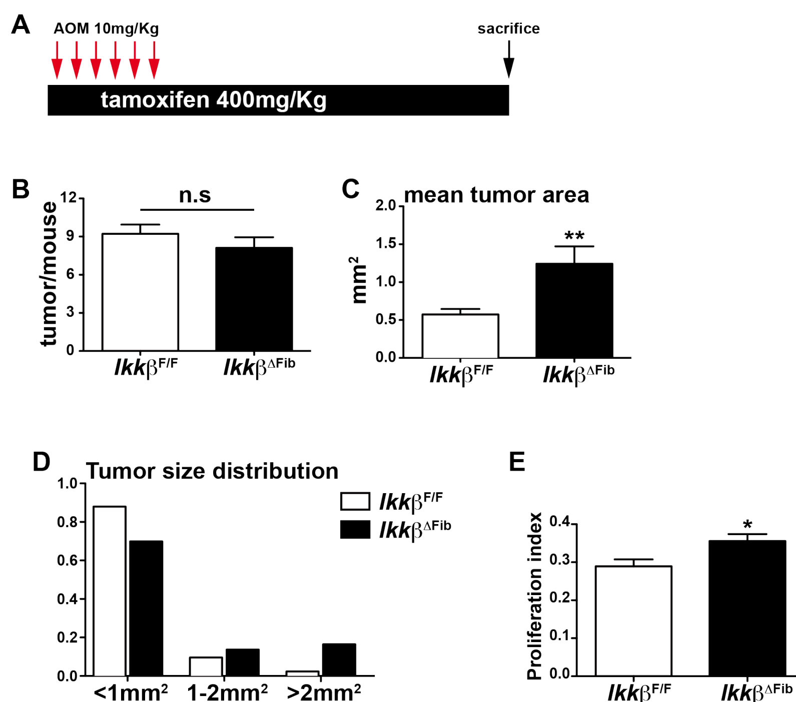


Figure 19: Met inhibition prevents tumor promotion in *Ikkβ*$^{\Delta Fib}$ mice upon CAC challenge.
(A) Schematic overview of the model used to induce sporadic CRC. (B) The tumor incidence and (C) the average tumor size. (D) Histogram showing tumor size distribution. (E) the proliferation index. Data are mean ± SEM; n≥9 animals per group; ** $p \leq 0.01$, * $p \leq 0,05$ by t test.

4. Discussion

The ability to generate transgenic animals and to restrict transgene expression to the tissue of interest has made very significant progress during the last few decades. This has proven extremely valuable in regulating gene expression and elucidating disease mechanisms *in vivo* thus facilitating basic research as well as biotechnological applications. It is now evident that fibroblasts play critical roles in the pathogenesis of many diseases including inflammatory conditions, fibrosis and cancer initiation and progression, but understanding the mechanisms involved has been hampered by the absence of specific markers. Fibroblast-activated protein (FAP), fibroblast-specific protein-1 (FSP1/S100A4), neural-glial antigen-2 (NG2), platelet-derived growth factor receptor beta (PDGFR-β) and CollagenVI (ColVI) have all been used to define fibroblasts and CAFs *in vivo*, but it is clear that these markers are not exclusive to fibroblasts and CAFs and in the heterogeneous CAF population, they only define small, distinct subsets which does not reflect the overall roles of CAFs (Orimo and Weinberg, 2007; Ohlund et al., 2014). Our data suggests that the expression of the regulatory sequence *proα2(I)collagen* is restricted to fibroblast as described before (Zheng et al., 2002), and this promoter is expressed by a large proportion of fibroblasts and CAFs from different subpopulations, and can thus be used to as a robust marker to target fibroblasts, myofibroblasts and CAFs *in vivo.*

4.1 IKKβ dependent NF-κB signaling in CAFs suppresses tumorigenesis through negative regulation of HGF transcription

Canonical NF-κB activation has been demonstrated to be the key signaling pathway linking inflammation and tumorigenesis. In tumor cells as well as inflammatory immune cells, NF-κB controls a variety of functions that ultimately promote all stages of tumorigenesis (Karin, 2009). In line with this notion, CAFs from pancreatic, skin and mammary cancer express a pro-inflammatory gene signature that is partly orchestrated by NF-κB, which is suggested to drive tumorigenesis. In this study by Erez et. al, CAFs were found to be activated from the earliest discernable stages of tumorigenesis and overexpress classical NF-κB signature genes including *Cox-2*, *Il-6*, *Cxcl1*, *Cxcl2*, and *Il-1β.* This NF-κB gene signature was shown to mediate tumor related inflammation and induce angiogenesis by recruiting macrophages. The expression of dominant negative IKKβ in co-

transplanted CAFs inhibited macrophage recruitment and the observed tumorigenesis (Erez et al., 2010).

In our model however, canonical NF-κB signaling in *Col1a2* expressing fibroblasts suppressed tumor growth in mouse model of CRC. We found that in the absence of IKKβ, intestinal myofibroblasts (IMFs) and CAFs secrete elevated HGF levels, which through the activation of several key downstream signaling pathways, stimulated epithelial cell proliferation and recruited endothelia cells promoting angiogenesis.

Since there is no known direct crosstalk between NF-κB and *Hgf* transcription, we examined further on the possible mechanisms by which *Ikkβ* deficiency would lead to the up regulation of CAFs. Gene expression analysis of sorted purified CAFs revealed there was increased TGF-β signaling, upon loss of *Ikkβ*. This also correlated with the upregulation of several key TGF-β target genes, which have been shown to drive human CRC. Furthermore, the loss of *Ikkβ* in CAFs promoted a tumor suppressive microenvironment characterized by increased infiltration of $CD4^{+}FoxP3^{+}$ Tregs following acute colitis, which may assist transformed cells to escape immune destruction. Interestingly, the tumor-promoting roles of IKKβ-deficient CAFs was not limited to inflammation-associated tumorigenesis and similar results were obtained in a model of sporadic colon tumorigenesis.

The MAPK Tpl2 has been suggested to be involved in the upstream activation of NF-κB (Vougioukalaki et al., 2011). Interestingly, mice that lack *Tpl2* in ColVI expressing intestinal myofibroblasts show a very similar HGF dependent phenotype in the CAC model but not in an inflammation-independent model (Koliaraki et al., 2012) suggesting that in contrast to *Col1a2* expressing CAFs, ColVI expressing cells may play a more prominent role during inflammatory processes. This raises the possibility that NF-κB may have distinct function in different fibroblast subpopulations possibly explaining the discrepancy between these results and the previously reported findings when CAFs had been co-injected in xenografts of pancreatic and mammary cancer (Erez et al., 2010). Considering the high degree of plasticity in fibroblasts NF-κB signaling may confer distinct functions depending on the respective activation status of CAFs. In other mesenchymal cells as well including adipocytes, macrophages and muscle-derived stem cells, NF-κB negatively regulates *Hgf* transcription in response to pro-inflammatory stimuli (Yin et al., 2014; Proto et al., 2015).

HGF is a pleiotropic growth factor with important roles in embryogenesis, regeneration and homeostasis in many adult tissues (Trusolino et al., 2010). Secreted by fibroblasts in a proform, HGF

requires cleavage by proteases to produce two chains, which bind to form the biologically active HGF heterodimer, which can then bind to epithelial/endothelia c-Met and activate signaling. The C-terminal motif of c-Met contains docking sites for numerous downstream signaling pathways including PI3K, GAB1, phospho-lipase Cγ1 and STAT3 (Ponzetto et al., 1994; Trusolino et al., 2010) promoting cell survival, proliferation, and enhance angiogenesis. HGF has also been identified as the stromal factor responsible for maintenance of the cancer stem cell niche in CRC (Vermeulen et al., 2010) and aberrant activation of this signaling pathway correlates with a poor prognosis CRC (Arlt and Stein, 2009). Indeed, sorted tumor cells from $Ikk\beta^{\Delta Fib}$ mice expressed higher levels of several stem cell and EMT markers. Interestingly, HGF seemed to be also responsible for Treg polarization during acute colitis considering the normalization upon capmatinib administration. This is consistent with findings in preclinical models of autoimmune diseases such as osteoarthritis, autoimmune myocarditis and in CNS demyelinating autoimmune diseases when HGF promoted Treg accumulation by suppressing dendritic cell function (Okunishi et al., 2007; Benkhoucha et al., 2010).

4.2 TGFβ-HGF double paracrine signaling is important for tumor progression in different tumors

It is now clear that TGF-β can either positively or negatively regulate tumor growth depending on the cell type and the context of activation. At early stages TGF-β inhibits epithelial cell proliferation directly, and indirectly by suppressing the stromal production of HGF, Mst-1, Wnts and TGF-α. And upon loss of TGF-β signaling in fibroblasts, these paracrine factors are able to promote lung, liver and spleen metastasis of adjacent carcinoma cells (Cheng et al., 2007, 2008). In addition, complete ablation of TGF-β signaling in stromal fibroblasts was also shown to initiate and promote adjacent carcinoma progression. The conditional loss of function of TβRII in stromal fibroblasts $T\beta RII^{FSPKO}$ developed intraepithelial neoplasia in the prostate and invasive squamous cell carcinoma of the forestomach with 100% penetrance (Bhowmick et al., 2004). The carcinomas in this mouse model were associated with elevated levels of HGF production and c-Met activation.

At the same time, TGF-β induces a myofibroblast/CAF conversion of adjacent fibroblast cell populations. Resulting myofibroblasts produce more HGF than their fibroblast precursors and the HGF produced by myofibroblasts was shown to drive SCC invasion (Lewis et al., 2004). This study was one of the first to demonstrate this double paracrine interaction directly associating tumor cell derived TGF-β to myofibroblast activation and production of pro-invasive HGF.

High stromal TGF-β expression has also been convincingly demonstrated as a common feature of all

poor-prognosis subtypes of CRC. It is now clear that TGF-β signaling in CAFs boosts the tumor-initiating capacity and the metastatic potential of CRC cells and pharmacological inhibition of TGF-β signaling in the TME blocks metastasis (Calon et al., 2015).

In this study as well we found TGFβ1 induces the expression of a set of genes associated with poor outcome including *Hgf*, following genetic ablation of canonical NF-κB signaling. and we could demonstrate that SMAD7 plays a key role in the crosstalk between NF-κB and TGF-β in murine CAFs as it has been demonstrated before (Yan et al., 2009b; Baugé et al., 2008; Freudlsperger et al., 2013).

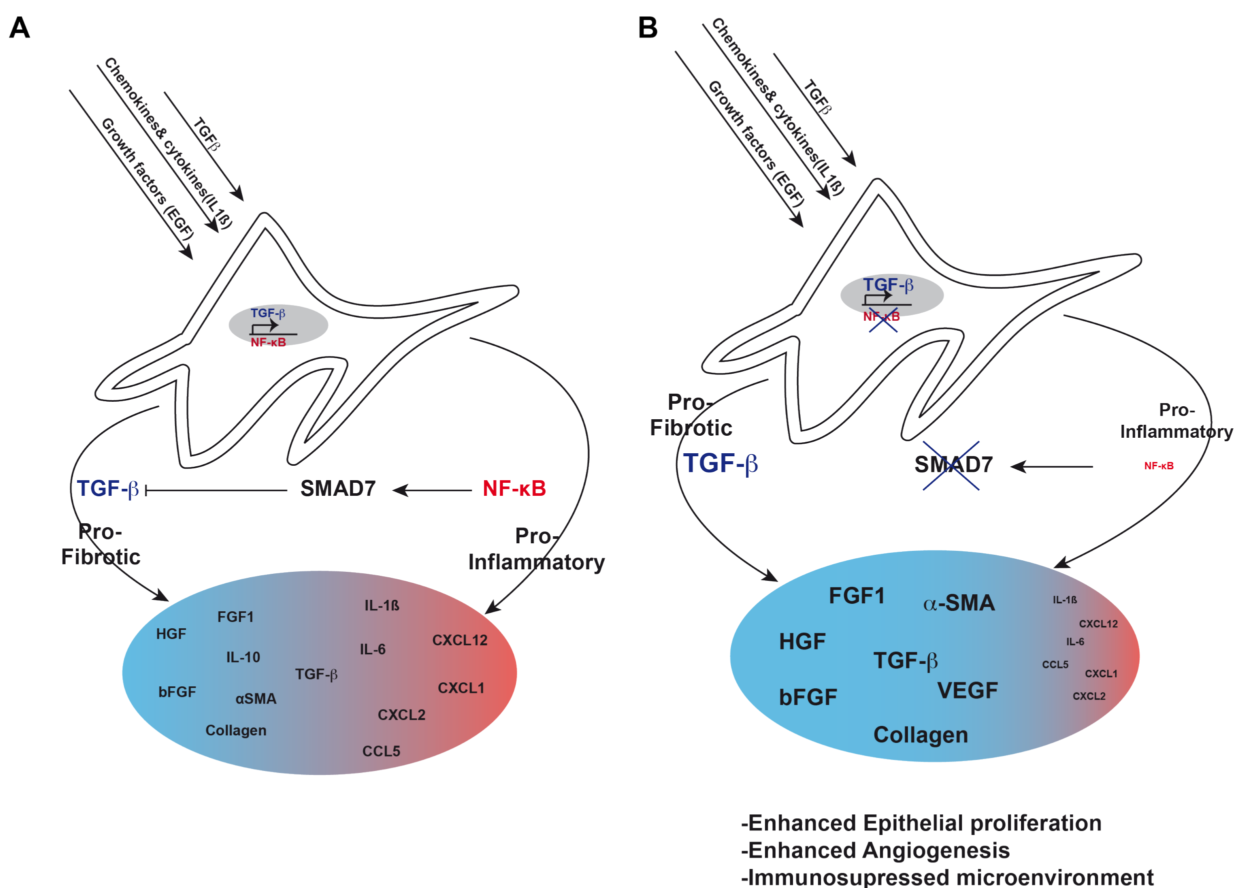


Figure 21: Illustration of crosstalk between NF-κB and TGF-β signaling in CAFs in the TME

Depending on the local microenvironment, CAFs are activated early in the TME. (A) In an inflammatory microenvironment, they up regulate NF-κB signature genes and mediate tumor-related inflammation. (B) In the absence of NF-κB signaling however, they attain a secretory phenotype mediated by TGF-β due to the loss of SMAD7.

4.3 NF-κB inhibition in CRC may have many adverse and yet unidentified side-effects and caution should be exercised using it as therapy

The crosstalk between NF-κB and TGF-β signaling via SMAD7 has already been described in head and neck cancer (Freudlsperger et al., 2013), osteosarcoma (Eliseev et al., 2006) and breast and gastric cancers (Hong et al., 2007). Thus, in addition to the previously identified negative regulation of IL-1β secretion causing increased susceptibility to sepsis when inhibiting IKKβ (Greten et al., 2007) our data here raise additional concern regarding the use of specific IKKβ inhibitors for the treatment of CRC. However, considering that these effects seem to be restricted to CAFs and that in intestinal tumor cells and myeloid cells NF-κB clearly confers tumor promoting functions (Greten et al., 2004; Schwitalla et al., 2013a; b) it remains to be determined what net effect IKKβ inhibition would have.

5. Summary

The microenvironment of solid tumors (TME) has a significant impact on all stages of tumorigenesis including response to treatment. Cancer associated fibroblasts (CAFs) are the most abundant cells in the TME, and through the secretion of multiple factors, they are known to be key effectors for many of the functions attributed to the TME. IKKβ driven NF-κB activation is a central driver for many inflammation-driven tumors. A pro-inflammatory NF-κB gene signature in CAFs has been suggested to promote tumorigenesis in models of pancreatic, mammary skin cancer. Using an autochthonous model of colitis-associated carcinogenesis (CAC) we provide evidence for a tumor suppressive function of IKKβ /NF-κB in CAFs. Fibroblast restricted deletion of $Ikk\beta$ stimulates intestinal epithelial cell proliferation, suppresses tumor cell death, enhances accumulation of $CD4^{+}Foxp3^{+}$ Treg cells and promotes angiogenesis ultimately promoting colonic tumor growth. In *Ikkβ*-deficient fibroblasts transcription of negative regulators of TGFβ signaling including Smad7, Smurf 1 and Smurf2 is impaired causing up-regulation of a TGFβ gene signature and elevated HGF secretion. Overexpression of Smad7 in Ikkβ-deficient fibroblasts prevents HGF secretion and - pharmacological inhibition of Met during the CAC model confirms that enhanced tumor promotion is dependent on HGF/Met signaling in mucosa of Ikkβ-mutant animals. Collectively, these results highlight an unexpected tumor suppressive function of IKKβ/NF-κB in CAFs linked to HGF release and raise potential concerns about the use of IKK inhibitors in CRC patients.

5.1 Zusammenfassung

Die Tumor Mikroumgebung (TMU) spielt in allen Stadien der Tumorentwicklung eine essentiell wichtige Rolle, der Verlauf der Behandlung eingeschlossen. Der Großteil an Zellen in der TMU wird durch krebs-assoziierte Fibroblasten representiert. Durch die Sekretion diverser Faktoren spielen sie eine Schlüsselrolle in der Funktion der TMU. Die Aktivierung von NF-κB durch IKKβ ist ein zentraler Punkt in entzündungs-assoziierten Tumoren. Dadurch unterstützt die entzündungsfördernde NF-κB Signatur in krebs-assoziierten Fibroblasten die Tumorprogression in Modellen von Bauchspeicheldrüßenkrebs und Brustkrebs. Durch die intensive Charakterisierung und Analyse unseres etablierten kolitis-assoziierten Krebs(KAK)-models können wir eine tumorhemmende Funktion der IKKβ/NF-κB Aktivierung in krebs-assoziierten Fibroblasten zeigen. Eine auf Fibroblasten beschränkte IKKβ deletion stimuliert die Proliferation intestinaler Epithelialzellen, die Ansammlung von $CD4^{+}Foxp3^{+}$ Treg Zellen und die Gefäßneubildung und verringert zusätzlich den Zelltod der Tumorzellen, was letztendlich zur Unterstützung des Darmkrebswachstums beiträgt. In den IKKβ-defizienten Firbroblasten ist die Transkription von negativen Regulatoren von TGFβ, wie Smad7, Smurf1 und Smurf2 beeinträchtigt, was eine Hochregulation von TGFβ zur Folge hat und zu einer erhöhten HGF Sekretion führt. Die Überexpression von Smad7 in IKKβ-defizienten Fibroblasten hingegen, verhindert die HGF Sekretion und die pharmakologische Inhibition von Met während des KAK-models bestätigt, dass eine dramatischere Tumorentwicklung von der HGF/Met Produktion in der Mukosa in IKKβ mutierten Tieren abhängt. Zusammengefasst, zeigen diese Ergebnisse eine unerwartete tumorunterdrückende Funktion von IKKβ/NF-κB in krebs-assoziierten Fibroblasten, die auf die HGF Ausschüttung zurückzuführen ist. Diese Ergebnisse stellen die Behandlung von Patienten mit Dickdarmkrebs mit IKK Inhibitoren in Frage.

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Abbreviations

AOM	Azoxymethane
BrdU	Bromodeoxyuridine
CAC	Colitis-associated cancer
CAFs	Cancer-associated fibroblasts
Cas-L	CRK-associated substrate lymphocyte type
CCL	C-C motif ligand
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
Col1	Collagen type 1
ColIV	Collagen type 4
Cox	Cyclooxygenase
CTL	Cytotoxic T-lymphocytes
CXCL	C-X-C motif ligand
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EndMT	Endothelial-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activating protein
FCD	Functional capillary density
FGF	Fibroblast growth factor
FSP	Fibroblast specific protein
GADD	Growth arrest and DNA damage protein
GFP	Green Fluorescent protein
HGF	Hepatocyte growth factor
HNPCC	Hereditary non-polyposis colorectal cancer

IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IF	Immunofluorescence
IHC	Immunohistochemistry
IKK	I kappa B kinase
IL	Interleukin
IκBα	Inhibitor of kappa B
LAP	Latency-associated protein
LPS	Lipopolysaccharide
LTBP	Latency TGF-β binding protein
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
MMT	Mesenchymal-mesenchymal transition
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neuro/glial antigen 2
NK	Natural killer cells
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PSA	Paraformaldehyde
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
SMA	Smooth muscle actin
SMURF	Smad-ubiquitin regulatory factor
STAT	Signal Transducer And Activator Of Transcription
STRAP	Serine/threonine kinase receptor associated proteins
TAM	Tumor-associated macrophage

TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alpha
Treg	Regulatory-T cell
TRβ	Transforming growth factor beta receptor
TSP-1	Thrombospindin-1
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular-endothelial growth factor receptor
YAP	Yes-associated protein

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At the heart of science is an essential balance between two seemingly contradictory attitudes—openness to new ideas, no matter how bizarre or counterintuitive they may be, and the most ruthless skeptical scrutiny of all ideas, old and new.
This is how deep truths are winnowed from deep nonsense.

— Carl Sagan