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Identification of mesenchymal specific pathways in intestinal carcinogenesis

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ΟΡΚΟΣ ΤΟΥ ΙΠΠΟΚΡΑΤΗ

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε. Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὡρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί. Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν. Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν. Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε. Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἑκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων. Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα. Ὅρκον μὲν οὖν μοι τόνδε ἐπιτελέα ποιέοντι, καὶ μὴ ξυγχέοντι, εἴη ἐπαύρασθαι καὶ βίου καὶ τέχνης δοξαζομένῳ παρὰ πᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τἀναντία τουτέων.

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Table of Contents

Abbreviations

ACF	Aberrant Crypt Foci
ACTA2	Alpha-Actin 2
ACTG2	Actin, Gamma 2
ANGPT2	Angiopoietin 2
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
αSMA	Alpha-Smooth Muscle Actin
ATAC	Assay for Transposase-Accessible Chromatin
BAFF	B-cell Activating Factor
BRAF	v-raf murine sarcoma viral oncogene homolog B
bFGF	Basic Fibroblast Growth Factor
BM-MSCs	Bone Marrow-Derived Mesenchymal Stem Cells
BMP	Bone Morphogenetic Protein
CAC	Colitis-associated cancer
CAFs	Cancer-Associated Fibroblasts
CAR	Chimeric Antigen Receptor
CBF	Crypt Bottom Fibroblast
CCL	Chemokine Ligand
CD	Cluster of Differentiation
CMS	Consensus Molecular Subtype
COL1A2	Collagen 1A2
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CTF	Crypt-Top Fibroblast
CTL	Cytotoxic T Lymphocyte
CXCL	Chemokine (C-X-C motif) Ligand
CXCR	C-X-C Chemokine Receptor
DCN	Decorin
DC	Dendritic Cell

Des	Desmin
DFS	Disease-Free Survival
DSS	Dextran sodium sulfate
ECM	Extracellular Matrix
Ednr	Endothelin Receptor
EGF	Epidermal Growth Factor
EMT	Epithelial-to-Mesenchymal Transition
EndoMT	Endothelial-to-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular Signal-Regulated Kinase
EU	European Union
FACS	Fluorescence-Activated Cell Sorting
FAK	Focal Adhesion Kinase
FAP	Fibroblast Activation Protein
FAP	Familial Adenomatous Polyposis
FGFR2	Fibroblast Growth Factor Receptor 2
Foxl1	Forkhead box protein L1
GAS	Growth Arrest-Specific
GFP	Green fluorescent protein
GIST	Gastrointestinal Stromal Tumor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
Grem1	Gremlin 1
HB-EGF	Heparin-Binding EGF-Like Growth Factor
HER	Human Epidermal Growth Factor Receptor
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-Inducible Factor
IBD	Inflammatory Bowel Diseases
iCAF	Inflammatory Cancer-Associated Fibroblast
IFN-γ	Interferon-gamma
IGF	Insulin-Like Growth Factor
IKK2	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin

IL1-R1	Interleukin-1 receptor type 1
IMCs	Intestinal mesenchymal cells
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
Lin	Lineage
LPS	Lipopolysaccharide
MAM	Methylazoxymethanol
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MCT	Monocarboxylate Transporter
MDSC	Myeloid-Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MMRd	Mismatch Repair Deficiency
MRISC	MAP3K2-Regulated Intestinal Stromal Cell
Myh11	Myosin Heavy Chain 11
NF-KB	Nuclear Factor Kappa B
NG2	Neural/Glial Antigen 2
NK	Natural Killer
NOX4	NADPH Oxidase 4
NPY	Neuropeptide Y
NT5E	Ecto-5'-nucleotidase
OS	Overall Survival
PCA	Principal component analysis
PDAC	Pancreatic Ductal Adenocarcinoma
PDGFR	Platelet-Derived Growth Factor Receptor
PD-L	Programmed Death-Ligand
PDPN	Podoplanin
Ptgs2	Prostaglandin-Endoperoxide Synthase 2
PTX3	Pentraxin 3
RGS5	Regulator of G-protein Signaling 5

ROS	Reactive Oxygen Species
RSPO3	R-Spondin 3
S2	Subset 2
scRNAseq	Single-cell RNA sequencing
SMAD4	Son of mothers against decapentaplegic Family Member 4
SMC	Smooth Muscle Cell
SPP1	Secreted Phosphoprotein 1
STAT3	Signal Transducer and Activator of Transcription 3
TAGLN	Transgelin
TAZ	Transcriptional co-activator with PDZ-binding motif
TCA	Tricarboxylic Acid
TGF-β	Transforming Growth Factor Beta
THBS	Thrombospondin
TIMP	Tissue Inhibitors of Metalloproteinase
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TNF-R1	Tumor necrosis factor receptor type 1
TP53	Tumor Protein P53
Treg	T regulatory cell
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
Wnt	Wingless-related integration site
Yap	Yes-Associated Protein

Abstract

Colorectal cancer (CRC) ranks as the third most prevalent cancer and a leading cause of global cancer-related mortality. Beyond the focus on cancer cells, it is now widely acknowledged that the tumor microenvironment (TME) significantly influences the initiation and progression of CRC. A pivotal component of the TME is the cancer-associated fibroblasts (CAFs), which play indispensable roles in the development and advancement of cancer. Despite recent advances in understanding CAF heterogeneity, a comprehensive understanding of their diverse origins, properties, and functions remains elusive. This thesis focuses on the intricate aspects of CAF origin, elucidating their physiological roles and investigating downstream pathways that orchestrate intestinal tumorigenesis.

By employing the Col6a1Cre mouse, we successfully targeted a distinct CAF population in two mouse models of intestinal carcinogenesis. Col6a1^{+} CAFs maintained key homeostatic characteristics, while they also became activated, supporting CAFs' origin from resident populations. Both bulk and single cell RNA sequencing showed that Col6a1^{+} CAFs were mainly pericytes/vCAFs and to a lesser extent PDGFRahi-like fibroblasts, in accordance with the significant expansion of vCAFs in colon tumors. Both in vitro and in vivo experiments showed that Col6a1+ CAFs had pro-tumorigenic roles, as they could support tumoroid growth in co-cultures and xenograft growth. Mechanistically, Col6a1^{+} IMCs responded to innate immune stimuli in vitro and produced inflammatory mediators, including cytokines, chemokines, and ECM remodeling enzymes. In vivo, TLR4/Myd88 innate sensing by the Col6a1^{+} CAF population was essential for the *Apc* driven tumorigenesis but indispensable for colitis-induced cancer development. The findings contribute to advancing our knowledge of CAF heterogeneity and highlight the role of innate immune sensing as a driver of CAF activation in colorectal cancer.

Περίληψη

Ο καρκίνος του παχέος εντέρου είναι ο τρίτος πιο κοινός τύπος καρκίνου και η δεύτερη πιο συχνή αιτία θανάτου από καρκίνο παγκοσμίως. Είναι πλέον γνωστό πως εκτός από τα καρκινικά κύτταρα, το μικροπεριβάλλον του όγκου συμμετέχει ενεργά σε όλα τα στάδια της καρκινογένεσης. Οι καρκινικοί ινοβλάστες αποτελούν βασικό συστατικό του μικροπεριβάλλοντος του όγκου και διαδραματίζουν σημαντικό ρόλο στην ανάπτυξη και εξέλιξη των όγκων. Παρά τις σημαντικές προόδους των τελευταίων χρόνων σχετικά με τη ετερογένεια που χαρακτηρίζει τους καρκινικούς ινοβλάστες, οι γνώσεις μας όσον αφορά τη προέλευση και την ακριβή τους λειτουργία μέσα στο περιβάλλον του όγκου παραμένουν ελλειπείς. Σκοπός της παρούσας διδακτορικής διατριβής είναι η διερεύνηση της προέλευσης των καρκινικών ινοβλαστών και η ανάδειξη των διαφορικών τους ρόλων μέσω της αναγνώρισης καταβολικών μονοπατιών κατά την εντερική καρκινογένεση.

Χρησιμοποιώντας τον Col6a1Cre ποντικό, επιτύχαμε τη στόχευση ενός διακριτού πληθυσμού καρκινικών ινοβλαστών (KI) σε δύο μοντέλα εντερικής καρκινογένεσης ποντικών. Οι Col6a1+ KI διατηρούσαν κάποια από τα κυριότερα ομοιοστατικά χαρακτηριστικά τους, ενώ ταυτόχρονα ήταν ενεργοποιημένοι, καθιστώντας σαφές ότι οι καρκινικοί ινοβλάστες προέρχονται από ήδη υπάρχοντες πληθυσμούς ινοβλαστών. Ανάλυση του RNA των κυττάρων έδειξε ότι οι Col6a1+ KI ήταν κυρίως περικύτταρα (ή αγγειακοί καρκινικοί ινοβλάστες) και σε μικρότερο βαθμό PDGFRαhi τελοκύτταρα, σε συμφωνία με τη σημαντική αύξηση των περικυττάρων στους όγκους του παχέος εντέρου. In vitro και in vivo πειράματα έδειξαν ότι οι Col6a1+ KI έχουν προ-καρκινικές ιδιότητες, καθώς μπορούσαν να υποστηρίξουν την ανάπτυξη καρκινικών οργανοείδων σε συγκαλλιέργειες, αλλά και την αύξηση ξενομοσχευμάτων. Μηχανιστικά, οι Col6a1^{+} KI ανταποκρίνονταν σε έμφυτα ερεθίσματα in vitro παράγωντας φλεγμονώδεις ουσίες, όπως κυτοκίνες, χημειοκίνες και ένζυμα που διαμορφώνουν την εξωκυττάρια θεμέλια ουσία. In vivo, η απόκριση των Col6a1^{+} KI σε σηματοδοτικά μόρια της έμφυτης ανοσίας μέσω του σηματοδοτικού μονοπατιού TLR4/Myd88 ήταν απαραίτητη για την καρκινογένεση που προκαλείται λόγω της μετάλλαξης στο γονίδιο *Apc* και όχι την καρκινογένεση που σχετίζεται με την κολίτιδα. Τα ευρήματα αυτής της έρευνας συμβάλλουν στην καλύτερη κατανόηση της ετερογένειας των καρκινικών ινοβλαστών και παρέχουν πολύτιμες για την ενεργοποίηση των καρκινικών ινοβλαστών του εντέρου από μηνύματα του έμφυτου ανοσοποιητικού συστήματος.

1. Introduction

1.1. Colorectal Cancer

1.1.1. Cancer

Cancer is a health burden with more than 10 million deaths per year and an ongoing threat for human life globally (Siegel, Miller et al. 2018). Although during the past years, novel therapeutic strategies, including combination of common anti-cancer drugs with immune checkpoint inhibitors have been introduced and have significantly improved the lifespan and quality of life of cancer patients there is still a long way to go before cancer eradication is accomplished.

Cancer development is considered a multistep process with age onset patterns (Nordling 1953) that arises from the progressive accumulation of various mutations, which result in the transformation of normal cells to malignant (Muller 1950, Nowell 1976). For cancer to develop and progress, cancer cells need to acquire key characteristics, which are described by Hanahan and Weinberg in a series of milestone reviews that provide a framework for better understanding carcinogenesis (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Hanahan 2022) (Figure 1). The authors have proposed six hallmarks of cancer that need to be acquired by normal cells in a multistep manner to enable carcinogenesis, while various physiological barriers need to be hijacked by malignant cells to establish tumor growth. One of the core characteristics of carcinogenesis is uncontrolled proliferation, which is further facilitated by the release of growth-promoting molecules. In addition, developing resistance to cell death, which normally serves as a natural barrier for pathogenic conditions, is also required for the establishment of malignant cells and eventually tumor development. Tumor cells are not only proliferating uncontrollably but can also become immortal, in contrast to normal cells that can only undergo a limited number of cell growth and division cycles. Collectively, these three hallmarks of tumors lead to increased metabolic needs in cancer cells, which require high levels of nutrients and oxygen to proliferate. To address these needs, tumors induce neovascularization by the process of angiogenesis. Eventually, once primary tumors have successfully been established, cancer cells may exit and colonize adjacent tissues or travel through the blood stream to distal areas entering the next phase of disease progression: evasion and metastasis (Hanahan and Weinberg 2000).

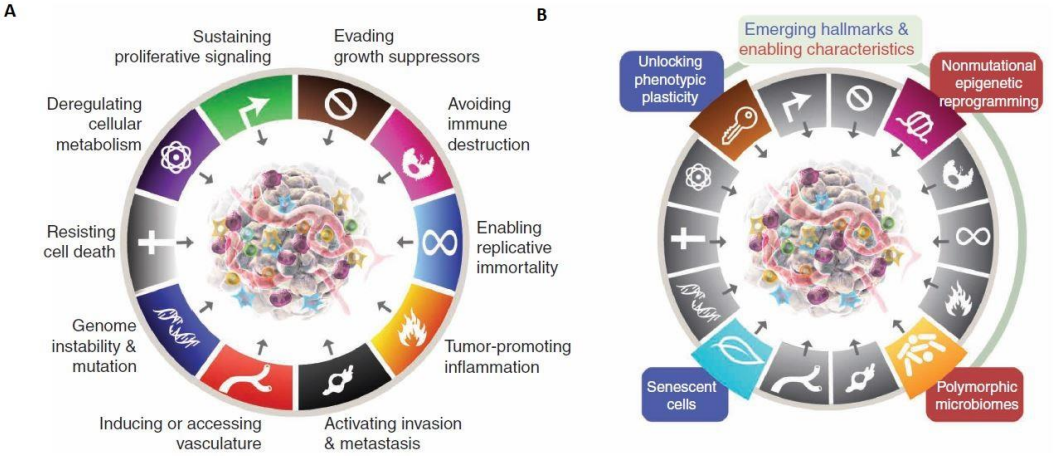

Figure 1. The hallmarks of cancer. *A) The 6 initial hallmarks of cancer as proposed in 2000 by Hanahan and Weinberg along with two 2 new hallmarks ("reprogramming cellular metabolism" and "avoiding immune destruction") introduced in 2011. B) Two emerging hallmarks and enabling characteristics complementing the basic core as proposed in 2022 (Hanahan 2022).*

More recent developments that broaden our knowledge of cancer-related molecular mechanisms have led to the emergence of four new hallmarks: reprograming energy metabolism, evading immune destruction, unlocking phenotypic plasticity, and senescent cells (Hanahan and Weinberg 2011, Hanahan 2022). Briefly, cancer cells reprogram their metabolism through a series of adaptations to cover their high metabolic needs, which even in the presence of sufficient oxygen rely on aerobic glycolysis (Warburg effect) (Hanahan and Weinberg 2011). Moreover, it is now acknowledged that for a tumor to grow, neoplastic cells must escape immune surveillance. Epidemiological data and experiments in genetically engineered mice (Kim, Emi et al. 2007, Teng, Swann et al. 2008, Vajdic and van Leeuwen 2009) provide strong evidence that in immunocompromised hosts tumors develop more frequently and rapidly, suggesting that antitumor immunity is crucial for early cancer eradication. Terminal cell differentiation is a crucial step during organogenesis as it acts as a natural barrier against uncontrolled cell proliferation. Cancer cells are phenotypically plastic and can differentiate into diverse cell states thus bypassing this barrier. Finally, senescent cells of various cellular origins rise as drivers of tumor evolution and a promising therapeutic target.

Additional enabling characteristics may be necessary for cancer cells to acquire the above mentioned hallmarks, including genomic instability in cancer cells favoring the acquisition of additional mutations, the presence of inflammation that supports cancer initiation and progression, epigenetic changes that can shape both cancer cells and the tumor

microenvironment, and finally polymorphic microbiomes, which have emerged as effectors of health and disease (Hanahan and Weinberg 2011, Hanahan 2022).

1.1.2. Colorectal cancer

Colorectal cancer (CRC) is the 3rd most common malignancy worldwide. Epidemiologically, CRC incidence is 5% in the general population and 80% of cases are sporadic in origin (Fearon 2011). As with most cancers, the risk of CRC development increases with age. Environmental factors, such as a western diet, smoking, and lack of exercise are strongly correlated with disease occurrence (Kinzler and Vogelstein 1996). Sporadic CRC is a multifactorial disease that progresses through a well described sequence of events. Fearon and Volgestain first described the consecutive steps of CRC development, which include aberrant crypt foci (ACF), adenoma, and carcinoma development. In this sequence, there is a gradual accumulation of genetic and epigenetic changes that transform normal epithelial cells to neoplastic cells that form small adenomas, large adenomas, and eventually full-grown tumors (Fearon and Vogelstein 1990) (Figure 2). The most common genetic mutations that characterize CRC tumors are in oncogenes and/ or tumor suppressor genes, such as adenomatous polyposis coli *(APC),* kirsten rat sarcoma viral oncogene homolog *(Kras),* tumor protein p53 *(Tp53),* v-raf murine sarcoma viral oncogene homolog B *(Braf)* and son of mothers against decapentaplegic Family Member 4 (*Smad4)* (Terzic, Grivennikov et al. 2010, Armaghany, Wilson et al. 2012). APC plays a detrimental role in colon carcinogenesis. In physiology, the APC protein suppresses the wingless-related integration site (Wnt) signaling pathway by promoting β-catenin disassembly. APC inactivation and aberrant β -catenin activation in pre-neoplastic cells inhibits their shedding into the lumen, allowing more time to acquire mutations that establish early carcinogenesis (Dow, O'Rourke et al. 2015). Kras and Braf are both members of the mitogen-activated protein kinase (MAPK) pathway that is known for its role in the regulation of cell proliferation, differentiation, and apoptosis (Fearon 2011). Mutations in these two genes lead to constitutive activation of the MAPK pathway and hyperplasia. *Tp53* is another tumor suppressing gene that normally controls cell cycle and apoptosis and upon dysregulation can trigger abnormal cell proliferation and adenoma development (Belluco, Guillem et al. 1996). Mutations in the TGF- β/Smad pathway also promote intestinal tumorigenesis (Engle, Hoying et al. 1999).

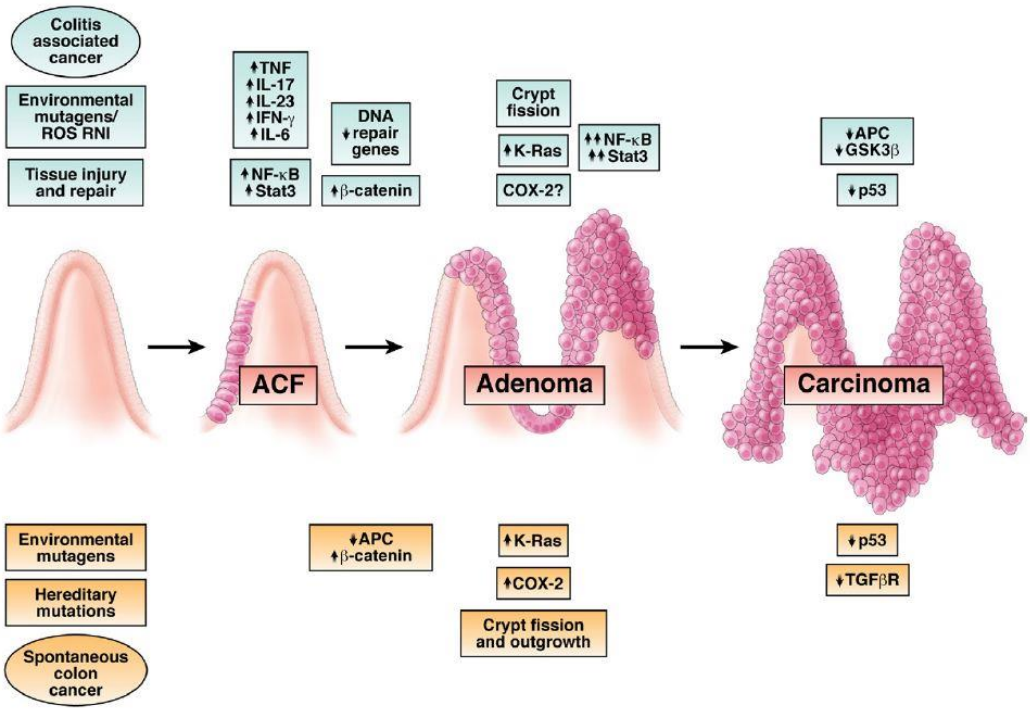

Figure 2. The development of colorectal cancer (CRC) and colitis-associated cancer (CAC) involves specific mechanisms. *Colorectal cancer (CRC) develops through the accumulation of mutations in oncogenes and tumor suppressor genes, particularly in the β-catenin signaling pathway. Mutations in key genes like adenomatous polyposis coli (APC) drive the progression from preneoplastic cells to aberrant crypt foci (ACF), adenomas, and eventually colorectal carcinoma. CAC, associated with chronic inflammation, is characterized by proinflammatory cytokines that induce mutations in oncogenes (Apc, p53, Kras) and promote genomic instability. Persistent inflammation facilitates tumor promotion by activating the proliferation and antiapoptotic properties of premalignant cells, contributing to tumor progression and metastasis. Both CRC and CAC share overlapping mechanisms, involving factors like GSK-3β, reactive nitrogen intermediates (RNI), and transforming growth factor (TGF). (Terzic, Grivennikov et al. 2010)*

CRC development can also be genetic or inflammation related. The Familial Adenomatous Polyposis (FAP) syndrome is a good example, as it genetically predisposes patients to CRC development due to mutation in the *APC* gene (Aaltonen 2000). Genetic analysis has shown that colorectal tumors in FAP patients are closely related to tumors in sporadic CRC, although they are usually benign. *APC* mutations can be detected as early as in the ACF stage of colon carcinogenesis.

Inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn's disease, are characterized by repeated relapsing periods of exacerbated intestinal inflammation, accompanied by barrier disruption and tissue damage. Both UC and CD are highly heterogeneous diseases in their clinical manifestation and multifactorial in their etiology. Epidemiological studies combined with results from animal models have shown that IBD can be attributed to genetic predisposition

combined with compromised immune responses and an altered microbial milieu. It is now well established that chronic intestinal inflammation predisposes IBD patients to cancer development. Colitis-associated cancer (CAC) is a subtype of colorectal cancer with increased mortality rate that is directly linked with IBD. Around 20% of IBD patients will develop CAC depending on the age of occurrence, duration, and severity of the disease, as well as the portion of tissue that is inflamed. Although CAC and CRC share the same adenoma to carcinoma sequence, and their genetic alterations significantly overlap, including mutations in *Apc, p53, b-catenin* and *Kras*, CAC features some major differences mainly due to its inflammatory milieu (Terzic, Grivennikov et al. 2010, Ullman and Itzkowitz 2011) (Figure 2). CAC is believed to arise due to persistent epithelial injury that compromises the intestinal barrier. In IBD, intestinal cells are exposed to inflammatory stimuli, and exhibit constitutive activation of transcription factors, such as NF-Kβ and/ or signal transducer and activator of transcription 3 (STAT3).

In 2015, combination of gene expression profiles of CRC tumors, along with analysis of mutations, microRNAs methylations, copy number variations and proteomics led to a consensus of four molecular subtypes (CMS1-4) in colorectal cancer (Guinney, Dienstmann et al. 2015). These are the following: CMS1 (microsatellite instability immune, 14%), hypermutated, microsatellite unstable and with strong immune activation; CMS2 (canonical, 37%), epithelial with marked WNT and MYC signaling activation; CMS3 (metabolic, 13%), epithelial with evident metabolic dysregulation; and CMS4 (mesenchymal, 23%), with prominent TGF-β pathway activation, stromal invasion, and angiogenesis. Interestingly, the CMS4 mesenchymal subtype strongly correlates with poor patient survival (Ten Hoorn, de Back et al. 2022).

Preventative measures, such as routine colonoscopies, have helped significantly in the early detection of colorectal cancer, leading to better therapeutic outcomes and increased patient survival (Keum and Giovannucci 2019).

1.1.3. Mouse models of colorectal cancer

Mouse models serve for many years as excellent tools for studying human diseases (Phifer-Rixey and Nachman 2015). Especially during the last decades their use has escalated by incorporating new technologies and expanding the number of diseases that can be studied (Ahmad and Amiji 2018). Their use is now evident in almost every biological field, as they provide key information regarding underlying pathogenesis and preclinical testing of new treatments. Only in European Union (EU), mice represent 60% of mammals used for experimentation (Díez-Solinska, Vegas et al. 2022). There are various reasons why *Mus musculus* is the most commonly used species in animal testing. Phylogenetic relation, physiological similarities, ease and cost-effective maintenance and breeding, as well as availability of many different strains are some of them (Phifer-Rixey and Nachman 2015)). The completion of genome mapping for human and mice by the Human Genome project in the 2000s combined with recent advances in sequencing analyses have also highlighted the genetic similarities between the two species.

Two of the most established models of intestinal carcinogenesis in mice are the AOM/DSS model of CAC and the *Apc*$^{min/+}$ model. These are presented in detail below, as they are extensively used in the present thesis.

1.1.3.1. The AOM/DSS model

Administration of Dextran Sulfate polymers (DSS) in the drinking water of mice is one of the most popular methods of modeling intestinal inflammation mainly because it is an easy to use, cost effective, and highly reproducible protocol. DSS is an agent known for its high toxicity for the colonic epithelium, resulting in clinical and histopathological changes that mimic colitis. During DSS administration, mice undergo significant weight loss and display bloody diarrhea, as well as crypt shortening/ loss, ulceration, and immune infiltration (Okayasu, Hatakeyama et al. 1990, Cooper, Murthy et al. 1993). DSS can be administered in the drinking water of mice either for one single cycle resulting in acute colitis or in repeated cycles mimicking chronic colitis (Wirtz, Popp et al. 2017). During the acute phase of DSS induced colitis there is a high production of proinflammatory cytokines (interleukin 1-β (IL1-β), tumor necrosis factor (TNF), interleukin 10 (IL-10) and interferon-gamma (IFN-γ)), as well as increased epithelial apoptosis. Studies in immunocompromised mice have shown that acute intestinal inflammation in this protocol results

from innate immune responses providing key information about the molecular pathways involved (Egger, Bajaj-Elliott et al. 2000). Over the course of 8 weeks some of these mice develop dysplasias and/ or adenocarcinomas in a ratio 15-20%, closely resembling the incidence of CAC in IBD patients (Okayasu, Hatakeyama et al. 1990, Cooper, Murthy et al. 1993).

Pretreatment of mice with a single dose of a genotoxic carcinogen, azoxymethane (AOM), can dramatically accelerate tumorigenesis (De Robertis, Massi et al. 2011), but a single dose of AOM alone fails to induce tumorigenesis. AOM is processed by the cytochrome P450 in the liver, where it is hydroxylated producing methylazoxymethanol (MAM). MAM is then exerted via the bile to the colon where it causes DNA alkylation leading to base mispairings (Papanikolaou, Shank et al. 1998). Following persistent DSS inflammation, AOM results in the production of mutagenic products initiating intestinal tumorigenesis. Inflammatory cytokines, chemokines, growth factors and ROS production caused by DSS inflammation further enhance tumorigenesis (Figure 3).

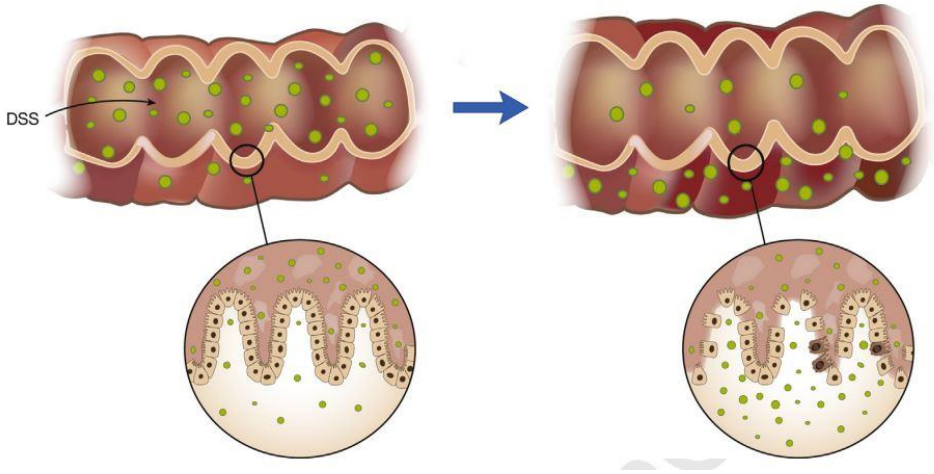

Figure 3. DSS mechanisms of action. *DSS polymers act directly on epithelial cells located in the basal crypts of the gut causing toxicity and disrupting the integrity of the mucosal barrier. (Kiesler, Fuss et al. 2015)*

The AOM/DSS model is an established mouse model for studying CAC sharing many common molecular and pathogenic mechanisms with human pathology (Figure 4) (Meira, Bugni et al. 2008, De Robertis, Massi et al. 2011). AOM/DSS adenomas develop in a multistep manner mimicking CAC pathogenesis, as they follow the aberrant crypt foci- adenoma- carcinoma sequence (De Robertis, Massi et al. 2011) although they rarely progress to carcinomas (Boivin, Washington et al. 2003). Histopathologically, aberrant crypt foci, as well as hyperplastic or dysplastic epithelial lesions can be found as early as 3-4 weeks into the protocol (Roncucci, Stamp et al. 1991, Mori,

Yamada et al. 2004, Mori, Hata et al. 2005). At the end of the protocol, mice usually develop 3- 10 tumors in the distal and middle colon. At the molecular level, most AOM/DSS tumors exhibit aberrant expression of *β-catenin*, as well as activation of the canonical Wnt- signaling pathway, similar to human CAC (Tanaka, Kohno et al. 2003, Bissahoyo, Pearsall et al. 2005). Consistent with studies in humans, *K-ras* mutations have also been identified in mouse colon dysplasias (Jacoby, Llor et al. 1991). However, *Apc* mutations are not as frequent in the AOM/DSS model, whereas no *p53* immunohistochemical signal has been detected in this model (Tanaka, Kohno et al. 2003). Furthermore, inflammatory mediators (e.g. TNF-α, IL-6, IL1-β) and related transcription factors (NF-κB, STAT3) are increased, leading to an inflammatory microenvironment that supports tumor growth (Hanada, Kobayashi et al. 2006, Popivanova, Kitamura et al. 2008, Koliaraki, Pasparakis et al. 2015).

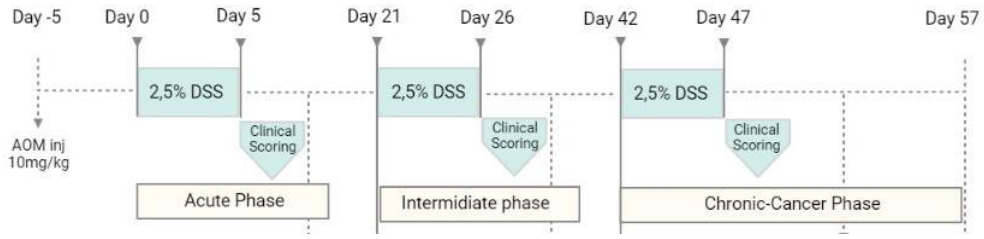

Figure 4. Schematic representation of the AOM/DSS protocol timeline.

To conclude, the DSS model of colitis and the AOM/DSS model of mouse CAC meet all the criteria for in depth exploration of the initiation and progression mechanisms of intestinal inflammation and inflammation-induced colorectal cancer.

1.1.3.2. The Apc$^{min/+}$ model

The *Apc*$^{min/+}$ mouse is another model widely used to study intestinal carcinogenesis (Dove, Clipson et al. 1997). It was developed in 1990 by random mutagenesis experiments using ethylnitosourea and it carries a heterozygous, truncating mutation at the 850 codon of the *Apc* gene. Apc$^{min/+}$ mice develop multiple intestinal neoplasias (around 50) in their small intestine at around 16 weeks of age on a C57BL/6 background, resembling human FAP. However, colonic tumors are significantly less in this model contrary to human FAP, most possibly due to the fact that stem cells in the murine small intestine undergo more cell divisions compared to the large intestine (Tomasetti and Vogelstein 2015, Tomasetti, Li et al. 2017). ACFs are not very common in this model compared to

human pathology and neither is metastasis (Boivin, Washington et al. 2003). Polyps in $Apc^{min/+}$ mice occasionally progress to adenocarcinomas while in human FAP patients, adenocarcinomas are predominant (McCart, Vickaryous et al. 2008). Despite the differences compared to human pathology, $Apc^{min/+}$ mice serve as an excellent tool to study the pathophysiological features and molecular mechanisms of intestinal carcinogenesis, as well as to test new therapeutic approaches.

1.1.4. The tumor microenvironment (TME)

During the past years, the cells surrounding tumor cells were considered bystanders of tumorigenesis; however, in depth exploration of the mechanisms of cancer development has led to the conclusion that the TME can actively affect tumor development by either promoting or inhibiting it. It is now established that in cancer, cellular interactions are disrupted allowing cancer cells to highjack homeostatic barriers and modify surrounding cells (Hanahan and Coussens 2012). Neoplastic cells can also recruit, activate, and reprogram cells, and thus shape the micro-environmental composition of tumors. The TME includes both cell and non-cell components (Hanahan and Coussens 2012, Quante, Varga et al. 2013). The cellular TME involves stromal cells (cancer associated fibroblasts, endothelial cells, and pericytes), immune cells (pro- and anti-tumorigenic lymphoid and myeloid cells), and neural cells. The non-cellular component of the TME includes mainly components of the extracellular matrix (ECM) (Ronnov-Jessen, Petersen et al. 1996) (Figure 5). TME cells, that have acquired new phenotypes, can affect clonal cancer evolution, tumor heterogeneity, invasion, and consequently tumor metastasis, as well as drug resistance (Galon and Bruni 2020). The TME can be highly heterogeneous depending on intrinsic cancer cell characteristics, the type of cancer, the tumor stage, and patient characteristics. It is also dynamic, since it changes as the tumor progresses.

Notably, some TME signatures correlate strongly with patient prognosis. Specifically, in colorectal cancer, the CMS4 subtype, which is characterized by the strong presence of cancer associated fibroblasts (CAFs) is associated with decreased survival (Li, Courtois et al. 2017). Accordingly, a myofibroblastic CAF gene signature correlates with worse prognosis contrary to an inflammatory CAF signature, which is associated with better patient survival, in various cancers (Li, Pei et al. 2021). The immune tumor microenvironment can also drastically affect patient prognosis. For

example, increased tumor associated macrophages are associated with unfavorable outcomes in breast, urogenital and gastric cancer (Fridman, Zitvogel et al. 2017, Pittet, Michielin et al. 2022).

Since stromal cells of the TME are genetically stable, they are considered as promising targets for improving patient survival (Laplane, Duluc et al. 2018, Laplane, Duluc et al. 2019). However, targeting the TME is challenging due to its dual role (Baghban, Roshangar et al. 2020). Targeting the TME includes interfering with the dynamic crosstalk between cancer and stromal cells, and the surrounding ECM. A prime example is immunotherapies, which have revolutionized cancer treatment by training the body's immune system to combat cancer cells. These therapies work by stimulating or enhancing anti-tumor immune responses. Checkpoint inhibitors, for example, block proteins that prevent immune cells from attacking cancer (Topalian, Drake et al. 2015). Chimeric Antigen Receptor- T (CAR-T) cell therapy involves modifying a patient's T cells to recognize and destroy cancer cells (Finck, Blanchard et al. 2022, Irvine, Maus et al. 2022). Anti-angiogenic treatments are also a crucial strategy in cancer therapy, aiming to inhibit the formation of new blood vessels that support tumor growth. Drugs such as bevacizumab target vascular endothelial growth factor (VEGF), a key protein involved in angiogenesis. By blocking VEGF, these treatments disrupt the blood supply to tumors, limiting their ability to thrive and spread. Anti-angiogenic therapies are employed in various cancers, including colorectal, lung, and kidney cancers (Ebos and Kerbel 2011). They are often used in combination with other treatments, such as chemotherapy, to enhance overall efficacy. An in-depth analysis of the TME components is presented in the next sections.

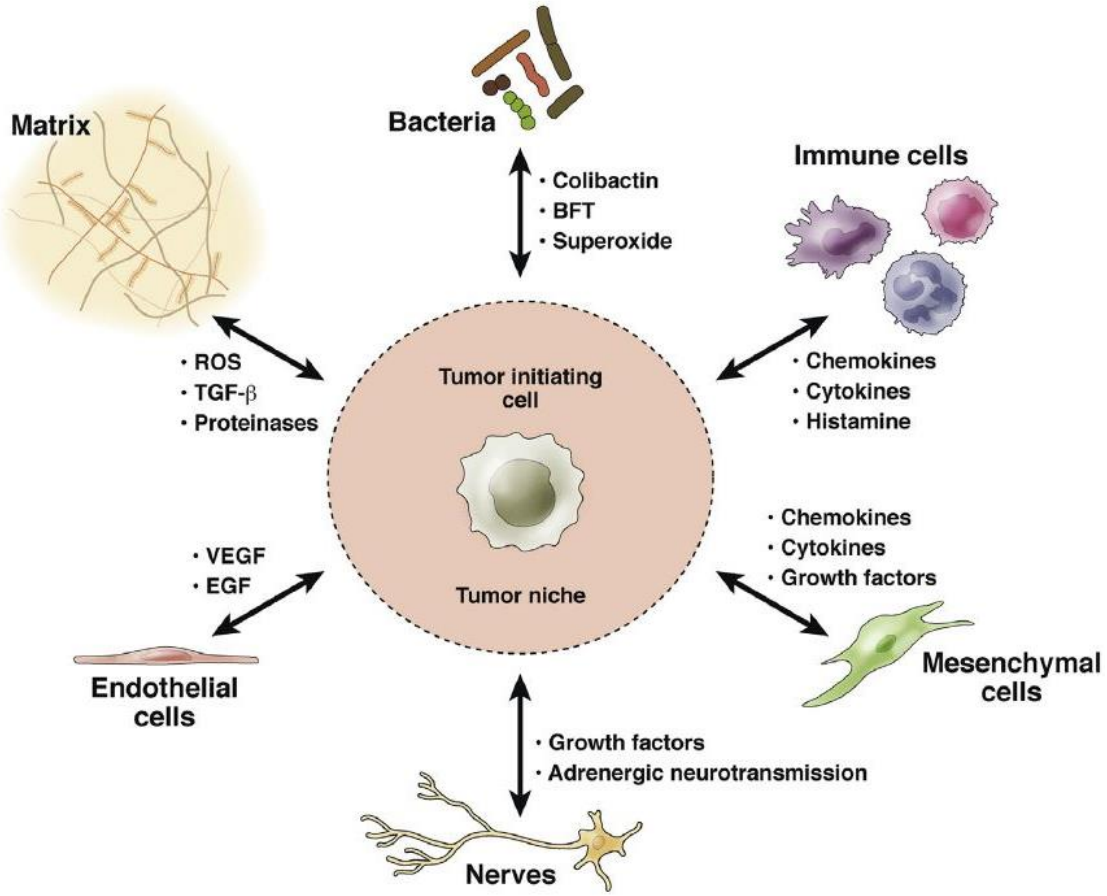

Figure 5. The tumor microenvironment. *The cellular and non-cellular components surrounding tumors can affect tumorigenesis (Quante, Varga et al. 2013).*

1.1.4.1 Tumor Endothelial cells (TECs)

Endothelial cells and their critical role in maintaining homeostasis, including the supply of oxygen and nutrients, angiogenesis, and immune surveillance are well described (Ley, Laudanna et al. 2007, Potente, Gerhardt et al. 2011, Potente and Mäkinen 2017). In cancer, tumor endothelial cells participate in all the steps of tumor development from initiation to progression and metastasis.

During the first stages of tumor development, cancer cells rely on passive transmission for the supply of oxygen and nutrients. Once the tumors grow bigger, the TME becomes more hypoxic and acidic due to the high metabolic rate of proliferating cancer cells. Under these hypoxic conditions, cancer cells express a variety of hypoxia- inducible factors, such as vascular endothelial

growth factor α (VEGFα), platelet derived growth factor (PDGF), hypoxia inducible factor (HIF), or angiopoietin 2 (ANGPT2), which act on endothelial cells to induce neo-angiogenesis (Carmeliet and Jain 2000, Potente, Gerhardt et al. 2011). This process is tightly regulated by proliferating endothelial cells, which form new vessels based on pre-existing ones. Indeed, *in vitro* experiments have shown that tumor endothelial cells (TECs) proliferate more compared to normal endothelial cells (NECs) (Hida, Hida et al. 2004). In addition, upon inflammatory or hypoxic environmental ques, endothelial cells switch to an activated state and produce a variety of molecules (Aird 2012, Klein 2018). Recent sequencing analyses from both humans and mice, comparing the profiles of normal versus tumor endothelial cells, have shown that TECs show increased gene expression of pro-angiogenic factors, stemness genes, and ECM-related genes, as well as chromosomal instability (Akino, Hida et al. 2009, Schaaf, Garg et al. 2018). Endothelial cells also play a crucial role in the metastatic process by providing a physical barrier for cancer cells to cross during extravasation and by secreting pro-angiogenic factors that promote the formation of new blood vessels to support tumor growth at distant sites. TECs have been shown to promote tumor metastasis by chaperoning circulating tumor cells and protecting them from apoptosis by producing pro-survival factors (such as IL-6) or the activation of STAT3 (Whiteside 2018). The VEGF/ VEGFR axis has been proven to be of great importance when it comes to tumor progression and metastasis (Yang and Cao 2022). For example, blocking the VEGF axis, which is a key regulator of angiogenesis, has been shown to reduce tumor growth rate and contribute to blood flow normalization to the tumor, thereby potentially reducing the risk of metastasis (Huang, Goel et al. 2013, Yang and Cao 2022).

As in physiology, inside the tumors, endothelial cells have multifaceted roles. Besides driving angiogenesis, TECs are key regulators of immune responses. Due to their location in the vessel lumen, they serve as the first line encounter for circulating immune cells, thus regulating peripheral immune cell trafficking and migration (Lambrechts, Wauters et al. 2018). TECs can modulate TME immunogenicity by acting as antigen presenting cells, by regulating T cell priming or by modifying effector immune cells (Buckanovich, Facciabene et al. 2008, Kambayashi and Laufer 2014, Goveia, Rohlenova et al. 2020). TECs, while not considered professional antigen presenting cells (APCs), may express MHC class I and II molecules, enabling them to present processed antigens to T cells. Additionally, TECs can actively impact T lymphocyte priming and migration, since they express molecules involved in T cell activation and inhibition, such as immune checkpoint molecules and Fas ligand, which can directly impact T cell activation at the

vessel site. Moreover, the interplay between endothelial and immune cells plays a critical role in promoting metastasis in different types of cancer. In breast cancer, myeloid WNT7b mediates the angiogenic switch and overall metastasis (Yeo, Cassetta et al. 2014). *In vitro*, melanoma cells show metastatic potential upon NF-κB activation in co-cultures with TIM3-expressing endothelial cells (Wu, Yuan et al. 2010) and ovarian cancer cells interact with monocytes/ macrophages to promote angiogenesis, which is considered a key point for metastasis (Wang, Zhao et al. 2013) (Hsu, Pan et al. 2019).

Basic and preclinical research has focused on potential therapies that block factors involved in endothelial cell proliferation, survival, and migration. For example, VEGFR-targeting drugs showed effective results in colorectal cancer patient survival (Ferrara, Hillan et al. 2005), and have since been tested for the treatment of various cancers (Hegde, Wallin et al. 2018). However, repeated use of these drugs often leads to resistance and poor overall patient survival (Heng, Mackenzie et al. 2012). Combination of immune checkpoint inhibitors along with antiangiogenic drugs has shown promising results in restraining immune complications related to therapy and could represent a significant advance in the treatment of metastatic cancer (Fukumura, Kloepper et al. 2018, Zhu, Abbas et al. 2022).

Alternative strategies have also been developed to target TECs rather than the factors related to them. For example, a recent study used dendritic cells to develop a cancer vaccine for directly targeting TECs (Nomura, Yamakawa et al. 2019). Finally, a subpopulation of circulating TECs was found in the periphery blood of colorectal cancer patients, indicating that they could also serve as a potential early diagnostic biomarker (Cima, Kong et al. 2016).

1.1.4.2 Immune cells

During the process of tumor development, host immune suppression and evasion are of great importance for the successful establishment of malignant cells and a compromised immune response is strongly associated with a higher risk of cancer occurrence. Persisting inflammation is also linked with tumor formation in many types of cancer, such as colorectal cancer, hepatocellular carcinoma, and pancreatic ductal adenocarcinoma (Mantovani, Allavena et al. 2008, Terzic, Grivennikov et al. 2010). Unresolved inflammation supports the onset of tumorigenesis by the accumulation of activated immune cells, which help establish a pro-tumorigenic niche through

their secretory phenotype. Therefore, depending on the type of cancer and the type of elicited immune response, the immune microenvironment can have a dual role in carcinogenesis. Recent single cell RNA sequencing analyses have helped to better describe the heterogeneous nature of the tumor immune microenvironment (TIME), and the properties of tumor-antagonizing or tumor-promoting immune cells (Spitzer and Nolan 2016, Zheng, Zheng et al. 2017, Papalexi and Satija 2018).

1.1.4.2.1 Tumor antagonizing immune cells

Tumor antagonizing immune cells include effector T cells ($CD8^+$ cytotoxic T cells and effector $CD4^+$ T cells), natural killer (NK) cells, dendritic cells (DCs), M1-polarized macrophages and N1-polarized neutrophils. $CD8^+$ cytotoxic T cells (CTLs) are well known for their contribution in killing cancer cells that present antigens through major histocompatibility complex class I molecules (MHC-I) (Tanaka, Yoshizawa et al. 1999). They also synergize with DCs and upon antigen presentation they become effector $CD8^+$ T cells and migrate to the tumor area through the expression of C-X-C Chemokine Receptor 3 (CXCR3), where they exhibit cytotoxic capacity, through the production of perforin and granzymes that target the cell membrane of abnormal cells (Farhood, Najafi et al. 2019). Similarly, NK cells mediate tumor immunosurveillance by their recruitment to the tumor site following chemokine secretion from DCs. NK cells kill tumor cells and produce a plethora of factors that promote antitumor immunity, such as IFN-γ and TNF (Voskoboinik, Smyth et al. 2006, Guillerey, Huntington et al. 2016). The main role of DCs inside the tumors is acting as professional antigen-presenting cells (APCs): they recognize, capture and present antigens to T cells. As mentioned before, they interact with T cells, NK cells and B cells (Batista and Harwood 2009, Guillerey, Huntington et al. 2016). Mature, active DCs infiltrate the tumors, thus increasing immune activation and recruitment. As cancer cells establish the cancer TME, DCs are usually suppressed and their tumor antagonizing capability is reduced (Krempski, Karyampudi et al. 2011, Michielsen, Hogan et al. 2011). Macrophages are also an important immune cell type inside the TME. They can be divided in two categories, M1 proinflammatory and M2 anti-inflammatory macrophages. Classically activated M1 macrophages contribute to anti-tumor immunity by promoting cancer cell killing through the production of pro-inflammatory cytokines and ROS (Aras and Zaidi 2017). On the contrary, M2-polarized macrophages have tumor-promoting properties and will be described in the following section. Neutrophils are also part of the TIME with a

potential tumor restricting role through their cytotoxic effect against tumor cells (Di Carlo, Forni et al. 2001, Uribe-Querol and Rosales 2015). Similarly to macrophages, neutrophils can also be divided in N1-polarized and N2-polarized and N2 are considered tumor promoting (Coffelt, Wellenstein et al. 2016).

1.1.4.2.2 Tumor promoting immune cells

In the TIME, there are also tumor promoting immune cells, including T regulatory cells (Tregs), M2 macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs). Tregs are a specialized population of $CD4^+$ T cells that expresses Foxp3 and functions to suppress adaptive immune responses. In cancer, their presence is associated with worse patient prognosis, as they act as immunosuppressors to inhibit $CD8^+$ T cells effector functions and promote thus tumor progression (Wolf, Sopper et al. 2015). MDSCs are a highly heterogeneous population of immature myeloid cells that interact with cancer cells and mediate cancer progression. In response to inflammatory stimuli, MDCs are induced and proliferate to promote angiogenesis through the production of VEGF and MMP9, and migration of cancer cells (Talmadge and Gabrilovich 2013, Zhou, Nefedova et al. 2018). Besides their interaction with cancer cells, MDSCs can affect the tumorigenic process by exerting immunosuppressive functions. More specifically, MDSCs can inhibit T-cell activity by suppressing the activation and proliferation of T cells thus hindering their ability to mount an effective immune response against tumors (Hanson, Clements et al. 2009). Also, they can promote the expansion and activity of regulatory T cells, which further suppress immune responses and contribute to immune tolerance (He, Zhang et al. 2023). Moreover, MDSCs release factors such as arginase-1 and inducible nitric oxide synthase (iNOS), which deplete essential amino acids and generate immunosuppressive molecules, respectively, dampening the immune response (Rodríguez and Ochoa 2008, Groth, Hu et al. 2019). Finally, they can interfere with APCs, specifically dendritic cells and affect their ability to activate T-cells and initiate effective immune responses. M2-polarized macrophages also suppress immunity against tumor cells via the production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor- beta (TGF-β). Moreover, they contribute to tumor growth by promoting neo-angiogenesis, matrix remodeling and tumor progression and metastasis (Aras and Zaidi 2017). N2 polarized

neutrophils can also contribute to tumor promotion through similar mechanisms (Veglia, Perego et al. 2018).

B cells are a controversial cell type regarding their role in the TME. Studies from different cancers have correlated them with either favorable or poor patient prognosis (Schmidt, Böhm et al. 2008, Tsou, Katayama et al. 2016, Garaud, Buisseret et al. 2019). In depth exploration of their role in different context might prove fruitful and dissect their complex role in carcinogenesis.

1.2. Cancer-associated fibroblasts

1.2.1 Fibroblasts in intestinal homeostasis

The mammalian intestine is a self-renewing tissue that ensures nutrient absorption while acting as a barrier against environmental insults. This is achieved by mature intestinal epithelial cells, the renewing capacity of intestinal stem cells at the base of the crypts, the development of immune tolerance, and the regulatory functions of stromal cells. During the last decade, the role of mesenchymal cells in the maintenance of intestinal homeostasis has gained momentum (McCarthy, Kraiczy et al. 2020). Smooth muscle cells constitute the muscularis, and lamina propria fibroblasts produce and remodel the ECM to support intestinal structure and integrity. Fibroblasts also play significant roles in epithelial stem cell maintenance and differentiation, immune homeostasis, and endothelial cell functions (Powell, Pinchuk et al. 2011, Koliaraki, Prados et al. 2020).

Recent data have revealed novel fibroblast specific mechanisms and an unanticipated heterogeneity, which is dependent on the distinct expression profile and location of each subset (McCarthy, Kraiczy et al. 2020). Comparative analysis of single cell RNA sequencing studies of the mouse intestine based on marker gene expression points to the presence of three main functionally distinct fibroblast subsets, similar to the ones described by McCarthy et al., which re-analyzed results from four such studies on a common computational platform (Figure 6). These subsets include:

1) CD81+ fibroblasts (Pærregaard, Schussek et al. 2021), also called trophocytes (McCarthy, Kraiczy et al. 2020), crypt bottom fibroblasts (CBFs) (Brugger, Valenta et al. 2020), MAP3K2-regulated intestinal stromal cells (MRISCs) (Wu, Sun et al. 2021) or $Pi16^{+}$ fibroblasts (Buechler,

Pradhan et al. 2021). They are located within the submucosa, near vascular structures and below crypts, and are the primary cellular source of WNTs (e.g. Wnt2 and Wnt2b), the BMP antagonist Gremlin 1, and R-spondins (Hong, Yang et al. 2020, McCarthy, Manieri et al. 2020, Buechler, Pradhan et al. 2021, Pærregaard, Schussek et al. 2021, Wu, Sun et al. 2021). They mainly function to maintain intestinal stem cell identity and proliferation. *In vitro*, $CD81^+$ trophocytes provide support for intestinal organoid expansion and *in vivo* ablation of Gremlin 1^+ ($Grem1^+$) cells results in extensive intestinal stem cell loss (McCarthy, Kraiczy et al. 2020). In addition, they express the atypical chemokine receptor Ackr4, which marks a distinct fibroblast population that regulates endothelial cells functions (Thomson, van de Pavert et al. 2018).

2) PDGFRαhi fibroblasts (Hong, Yang et al. 2020, Buechler, Pradhan et al. 2021), also called telocytes (McCarthy, Manieri et al. 2020, Wu, Sun et al. 2021), crypt-top fibroblasts (CTFs) (Brugger, Valenta et al. 2020, Fazilaty, Brügger et al. 2021) (Buechler, Pradhan et al. 2021, Melissari, Henriques et al. 2021, Jasso, Jaiswal et al. 2022) and Endothelin Receptor high (Ednrbhi) fibroblasts (Kim, Fei et al. 2020). They are characterized by expression of high levels of platelet-derived growth factor receptor α (PDGFRα), BMPs, among which Bmp3 and Bmp7 are uniquely expressed, *Wnt5a, F3, Sox6, Foxl1*, and low levels of *Alpha-Actin 2 (Acta2*) (Kinchen, Chen et al. 2018, Hong, Yang et al. 2020, Kim, Fei et al. 2020, McCarthy, Manieri et al. 2020, Roulis, Kaklamanos et al. 2020, Buechler, Pradhan et al. 2021, Fazilaty, Brügger et al. 2021, Pærregaard, Schussek et al. 2021, Wu, Sun et al. 2021). They are localized directly under the epithelial layer and are concentrated at the top of crypts and villi (Brugger, Valenta et al. 2020, McCarthy, Manieri et al. 2020, Pærregaard, Schussek et al. 2021). They may also include subepithelial myofibroblasts, as they express alpha smooth muscle actin (αSMA) (McCarthy, Kraiczy et al. 2020, Jasso, Jaiswal et al. 2022). The expression of BMPs and their location suggests an important function in epithelial cell differentiation (Kosinski, Li et al. 2007, Qi, Li et al. 2017, Beumer, Puschhof et al. 2022). Therefore, the relative location of $CD81^+$ and PDGFRαhi fibroblast subsets contributes to the generation of a signaling gradient along the small intestinal villous-crypt and colonic crypt top-bottom axis that facilitates ISC maintenance and differentiation (McCarthy, Kraiczy et al. 2020). Studies using constitutive and conditional Foxl1-Cre strains and immunoelectron microscopy have shown that $Foxl1^+$ cells form a subepithelial plexus along the entire villous/crypt axis and exhibit unique structural characteristics, including long processes called ‘telopodes’, thus leading to the term ‘telocytes’. However, $Foxl1^+$ cells also express stem cell trophic factors, such as Wnt2b and R-Spondin 3 (Rspo3), as well as Sfrp1 and Grem1, which are markers of $CD81^+$ fibroblasts (Aoki,

Shoshkes-Carmel et al. 2016, Shoshkes-Carmel, Wang et al. 2018). Foxl1$^+$ cell depletion or cell-specific deletion of WNT secretion leads to marked changes in the epithelial architecture, including reduced villi length and crypt depth, and a reduction in stem and progenitor cell proliferation (Aoki, Shoshkes-Carmel et al. 2016). These results indicate that telocytes targeted by the Foxl1-Cre mice could include both PDGFRαhi and pericryptal fibroblasts to some extent. Indeed, a recent study differentiated between crypt and Lgr5$^+$ villous tip telocytes (VTTs), and ablation of the latter led to changes in epithelial gene expression at the villus tip, but did not have the detrimental effects of Foxl1$^+$ cell depletion (Bahar Halpern, Massalha et al. 2020). We also recently showed that Col6a1-Cre mice target the entirety of PDGFRαhi fibroblasts, along with pericytes and a small number of PDGFRαlo cells. Depletion of this population in the middle/distal colon did not disrupt intestinal morphology, but led to altered distribution of proliferating epithelial cell and reduced enteroendocrine numbers (Melissari, Henriques et al. 2021). The differences between these experiments most probably reflect the exact specificities of each strain and should be carefully considered.

3) PDGFRαlo CD81- fibroblasts, which reside in the lamina propria, around crypts and inside the villous core (Hong, Yang et al. 2020, Pærregaard, Schussek et al. 2021). They can be further divided into at least two subsets that express Col15a1, and Igfbp5/CD90 or fibroblast growth factor receptor 2 (Fgfr2), Fbln, in the small intestine and colon respectively (Kinchen, Chen et al. 2018, Hong, Yang et al. 2020, Pærregaard, Schussek et al. 2021). They secrete basement membrane proteins and contribute to ECM production and remodeling (Kinchen, Chen et al. 2018). They also maintain lacteal integrity and function through Yes-Associated Protein/ Transcriptional co-activator with PDZ-binding motif (YAP/TAZ)-mediated VEGF-C secretion (Hong, Yang et al. 2020). Notably, CD90$^+$cells have been shown to support epithelial cell growth through the production of class 3 semaphorins (Karpus, Westendorp et al. 2019).

Additional mesenchymal subsets include PDGFRα-NG2$^+$Rgs5$^+$pericytes surrounding blood vessels and capillaries (Muhl, Genové et al. 2020), smooth muscle cells (SMCs) around blood vessels and lymphatic lacteals and in the muscle layer, and myofibroblasts. Varying levels of *Acta2*, myosin heavy chain 11 (*Myh11)* and desmin (*Des)* can help with the distinction between SMCs and myofibroblasts, but the two terms are sometimes used interchangeably in single cell RNA sequencing analyses (Kinchen, Chen et al. 2018, Brugger, Valenta et al. 2020, Hong, Yang et al. 2020, Kim, Fei et al. 2020, Roulis, Kaklamanos et al. 2020, Fazilaty, Brügger et al. 2021, Pærregaard,

Schussek et al. 2021). Notably, the small intestine and colon display similar mesenchymal subsets with location-specific differences in their transcriptional profiles (McCarthy, Kraiczy et al. 2020, Pærregaard, Schussek et al. 2021).

Besides the regulation of epithelial homeostasis and tissue integrity, pseudotime analysis and lineage inference have indicated that $CD81^+/pi16^+$ fibroblasts could also act as mesenchymal stem cells and thus as sources of adult fibroblasts, which pass through intermediate PDGFRαloCD81-$Col15a1^+/CD90^+$ cells towards differentiated subsets (Kinchen, Chen et al. 2018, Buechler, Pradhan et al. 2021, Pærregaard, Schussek et al. 2021). This is in accordance with lineage tracing data of $Grem1^+$ cells, which can renew the entire mesenchymal sheath over a year (Worthley, Churchill et al. 2015). We also recently showed that following depletion of Col6a1-Cre^+ colonic fibroblasts, $CD34^+$ cells could proliferate, occupy subepithelial locations and alter their gene expression profile to support epithelial cell differentiation and regeneration, highlighting the potential plasticity of resident fibroblasts (Melissari, Henriques et al. 2021).

There is fewer insight into the significance of distinct fibroblast subsets in the regulation of intestinal immune homeostasis. Of note, there are also specialized stromal populations that regulate immunity within the topologically restricted structures of the gut-associated lymphoid tissue, including Peyer's patches and isolated lymphoid follicles (Koliaraki, Prados et al. 2020). Still, intestinal fibroblasts, express various chemo-attractants, cytokines, and cytokine receptors and could thus regulate immune cell turnover and function (Barnhoorn, Hakuno et al. 2020, Thomson, Nibbs et al. 2020, Pærregaard, Schussek et al. 2021). Fibroblasts also produce retinoic acid, which synergistically with granulocyte-macrophage colony-stimulating factor (GM-CSF) drives the functional education of migratory dendritic cells (Vicente-Suarez, Larange et al. 2015). B-cell Activating Factor (BAFF) production by lamina propria fibroblasts induces B cell proliferation and differentiation to IgA^+ plasma cells (Cen, Moreau et al. 2019). Human colonic fibroblasts express programmed death-ligand PD-L1 and PD-L2, which suppress $CD4^+$ T-helper cell activation and proliferation through inhibition of IL-2 production (Pinchuk, Saada et al. 2008). Conversely, they also express major histocompatibility complex II (MHC-II) molecules and CD80/86 co-stimulators, suggesting a potential role as non-professional antigen presenting cells, which can stimulate allogeneic $CD4^+$ T-cell proliferation (Saada, Pinchuk et al. 2006) and induce activation of Tregs at least in vitro (Pinchuk, Beswick et al. 2011).

Distinct fibroblast subsets have also been found in the human intestine, and share many similarities with their mouse counterparts, as shown both by direct comparison of single cell transcriptomic data (Kinchen, Chen et al. 2018, Brugger, Valenta et al. 2020, Fazilaty, Brügger et al. 2021) and assessment of marker gene expression. Among them PDGFRα^{+}, WNT5B^{+}, S2, or epithelia proxima fibroblasts express fork-head box protein L1 (*FOXL1)*, *WNT5A*, and *BMPs*, and display a subepithelial localization, correlating with mouse PDGFRαhi fibroblasts (Kinchen, Chen et al. 2018, Huang, Chen et al. 2019, Smillie, Biton et al. 2019, Lee, Hong et al. 2020, Friedrich, Pohin et al. 2021, Qi, Sun et al. 2022). Interestingly, in humans, two clusters have been identified, one expressing *ACTA2* and transgelin (*TAGLN)* and the other Pentraxin 3 (*PTX3)*, and Neuropeptide Y *(NPY)*, but their potential distinct functions are yet unknown (Kinchen, Chen et al. 2018, Smillie, Biton et al. 2019). Additionally, WNT2B^{+} cells that express *RSPO3* are most likely equivalent to mouse CD81^{+} fibroblasts, while WNT2B^{+}FOS^{+} lamina propria fibroblasts correlate with PDGFRαlo CD81- fibroblasts (Kinchen, Chen et al. 2018, Huang, Chen et al. 2019, Smillie, Biton et al. 2019). Additional subsets include myofibroblasts/smooth muscle cells and pericytes (Kinchen, Chen et al. 2018, Huang, Chen et al. 2019, Smillie, Biton et al. 2019, Lee, Hong et al. 2020, Friedrich, Pohin et al. 2021, Qi, Sun et al. 2022). These results further support the value and translatability of mouse studies in modeling human health and disease in the gut.[1]

[1] This section (1.2.1) has been recently published slightly adjusted as a mini-review in the Journal *Frontiers in Immunology* Chalkidi, N., C. Paraskeva and V. Koliaraki (2022). "Fibroblasts in intestinal homeostasis, damage, and repair." Front Immunol **13**: 924866..

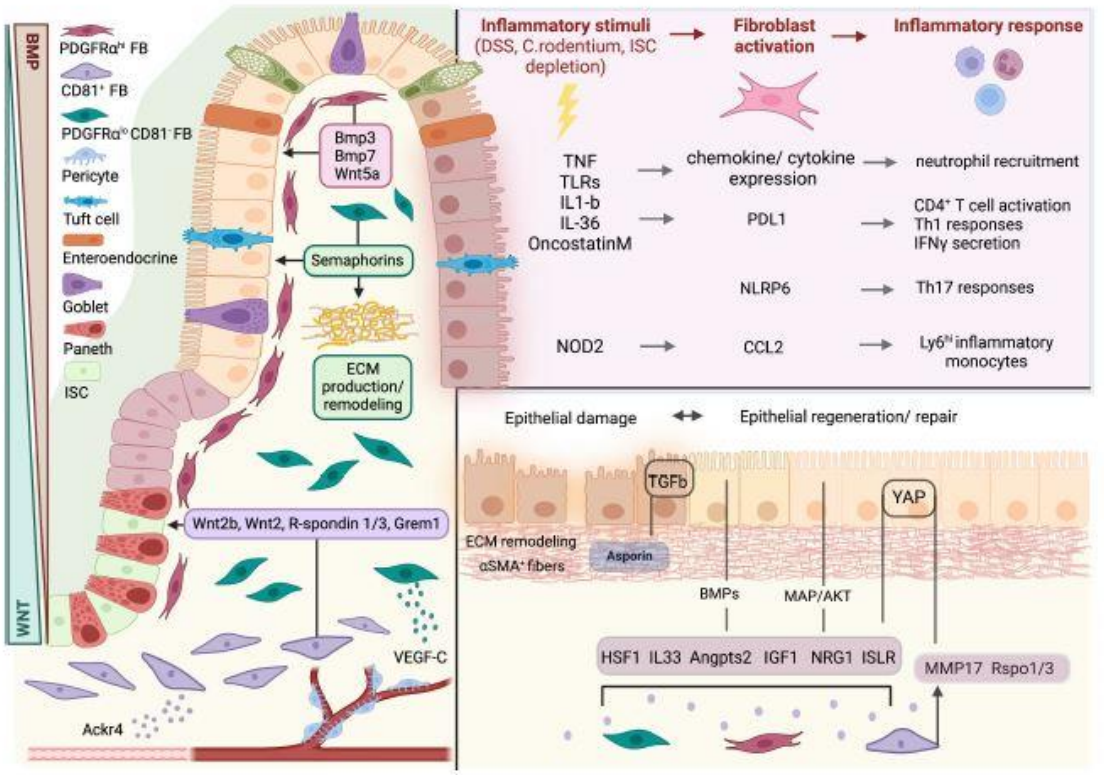

Figure 6. Fibroblasts in intestinal homeostasis, damage, and repair. *Intestinal homeostasis is regulated by 3 distinct fibroblast subsets through the production of effector molecules. WNT ligands, R-spondins and Gremlin 1 are produced by CD81⁺ fibroblasts and maintain intestinal stem cell (ISC) identity. PDGFRα^{hi} fibroblasts orchestrate epithelial differentiation through the production of BMPs and WNT5A. In the lamina propria, PDGFRα^{lo}CD81^{-} fibroblasts contribute to extracellular matrix (ECM) production and remodeling. Upon inflammatory stimuli, fibroblasts are activated and secrete a variety of pro-inflammatory factors to drive immune cell recruitment and function. During damage, intestinal fibroblasts provide paracrine signals to promote epithelial regeneration and ECM remodeling. FB, fibroblast; ISC, intestinal stem cell. (Chalkidi, Paraskeva et al. 2022)*

1.2.2 CAFs

In solid tumors, CAFs constitute a major cell component of the tumor microenvironment. In fact, in some cancers, such as breast and pancreatic, CAFs represent the most prominent cell type of the TME and their abundance is often related with poor patient prognosis (Tsujino, Seshimo et al. 2007, Calon, Lonardo et al. 2015, Isella, Terrasi et al. 2015). CAFs are non-epithelial, non-cancerous, non-endothelial, non-immune cells that can be found within or adjacent to tumors. They were initially thought to be a matrix-producing population with a tumor supportive role. Recent studies show that CAFs are a highly heterogeneous cell type with a complex role that affects all steps of tumor progression and facilitates tumor proliferation, invasion, and metastasis

(Helms, Berry et al. 2022). Besides matrix remodeling, CAFs can actively modulate tumor formation by affecting cancer cell proliferation, tumor immunity, angiogenesis, and metastasis through a plethora of signaling pathways. Numerous studies underscore the fact that CAFs have context dependent functions that can be either tumor promoting or tumor restraining (Dumont, Liu et al. 2013, De Wever, Van Bockstal et al. 2014, Öhlund, Elyada et al. 2014). Interestingly, studies aiming at CAF depletion resulted in accelerated tumor progression due to Treg activation (Özdemir, Pentcheva-Hoang et al. 2014). This bimodal role of CAFs is of crucial importance and supports the need for further investigation. Furthermore, CAFs are characterized by high heterogeneity in their origins, phenotypes and functions indicating that CAFs are composed of multiple subpopulations that might have complementary or opposing effects in tumor growth depending on the conditions (Li, Courtois et al. 2017, Puram, Tirosh et al. 2017, Bartoschek, Oskolkov et al. 2018, Costa, Kieffer et al. 2018, Lambrechts, Wauters et al. 2018, Bernard, Semaan et al. 2019, Elyada, Bolisetty et al. 2019, Hosein, Huang et al. 2019, Peng, Sun et al. 2019, Davidson, Efremova et al. 2020, Dominguez, Müller et al. 2020, Friedman, Levi-Galibov et al. 2020, Kieffer, Hocine et al. 2020, Zhang, Yang et al. 2020).

1.2.2.1 CAF origin

CAFs originate from at least four non-mutually exclusive mechanisms which include: A) proliferation and B) activation of resident fibroblast populations, C) transdifferentiation of other cell types, and D) recruitment of non-tissue resident mesenchymal cells (Hosaka, Yang et al. 2016, Kalluri 2016, Koliaraki, Pallangyo et al. 2017) (Figure 7).

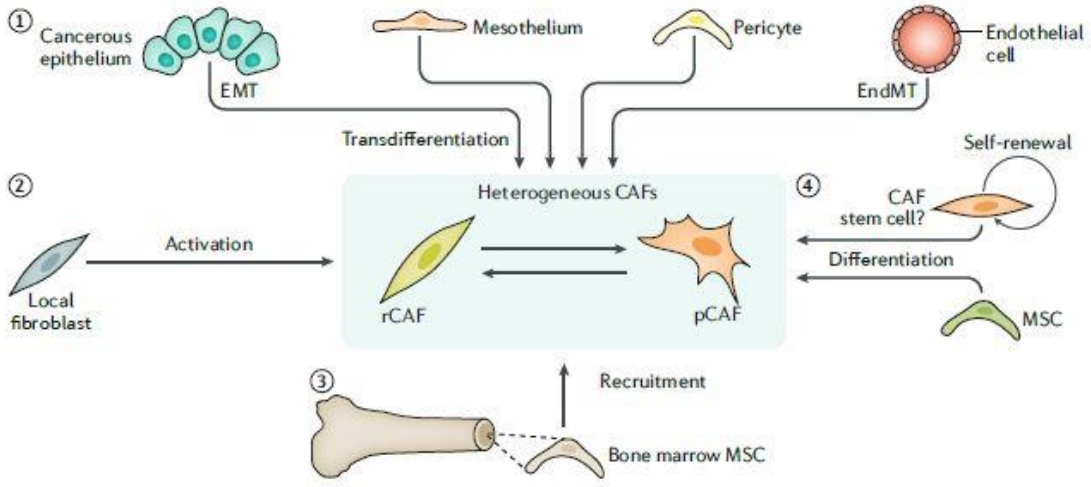

Figure 7. CAF origins. *CAFs are considered to originate from diverse cell types, with the primary source being tissue-resident fibroblasts or fibroblast-like cells that become activated. Bone marrow mesenchymal cells are recruited to tumors and upon stimuli give rise to CAFs. Finally other cell lineages can become CAFs through transdifferentiation, for example epithelial or endothelial cells that have undergone epithelial-to-mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndoMT) (Kobayashi, Enomoto et al. 2019)*

1.2.2.1.1 Proliferation

Studies in autochthonous mouse models of carcinogenesis and human tumor samples show that CAFs can proliferate inside tumors (Özdemir, Pentcheva-Hoang et al. 2014, Bartoschek, Oskolkov et al. 2018). Kobayashi et al showed that there is a large fraction of mouse intestinal CAFs that is characterized by the expression of *MCAM* and *ACTA2* and undergoes cell division during carcinogenesis (Kobayashi, Gieniec et al. 2022). In the same study, single cell re-analysis of human CRC and control samples revealed the presence of a similar population that expressed *ACTA2* along with proliferative markers, suggesting that CAFs in intestinal tumors undergo mitosis. In another study, Bartoschek et al analyzed breast cancer CAFs from mice through single cell RNA sequencing and found a subpopulation of proliferating vascular CAFs that may have implications for breast cancer patient prognosis, particularly in relation to metastatic disease (Bartoschek, Oskolkov et al. 2018). Interestingly, Ozdemir et al identified a proliferative αSMA^{+} fibroblast population in pancreatic tumors from mice, whose deletion led to cancer progression and decreased survival (Özdemir, Pentcheva-Hoang et al. 2014).

1.2.2.1.2 Resident Fibroblast activation

CAFs predominantly originate from tissue resident fibroblasts that get reprogrammed and activated by cancer cell-derived factors or cues from the microenvironment. TGF-β is a dominant effector in many cancers and mediates the conversion of fibroblasts to CAFs (Calon, Lonardo et al. 2015). In pancreatic ductal adenocarcinoma (PDAC), TGF-β downregulates IL-1R1 and blocks the JAK/STAT pathway, favoring thus the generation of CAFs with a myofibroblastic phenotype (myCAFs) (Biffi, Oni et al. 2019). Moreover, TGF-β can synergize with other growth factors, such as PDGF, and drive recruitment and activation of fibroblasts (Ostman and Augsten 2004). It is well established that inflammation is a significant predisposition factor for the initiation and progression of numerous cancers. The abundance of inflammatory signals mediates reciprocal interactions between cancer cells, stromal and immune cells to accelerate the development of an inflammatory TME and the phenotypic switch of fibroblasts to immunoregulatory CAFs (Greten and Grivennikov 2019). Recently, the Tuveson lab (Biffi, Oni et al. 2019) highlighted the importance of IL-1 signaling in shaping CAF functions in PDAC, by showing that tumor-derived IL-1α antagonizes TGF-β signaling and stimulates the production of a cytokine cascade, including LIF, IL-6, and CXCL8. This acts in an autocrine manner to activate the JAK/STAT3 pathway in CAFs, resulting in a positive feedback loop that leads to high IL-1R1 expression and inflammatory CAF formation. Nicolas et al also underscore the significant involvement of inflammatory CAFs in determining the therapeutic response of patients with rectal cancer implicating IL-1 signaling (Nicolas, Pesic et al. 2022). More specifically, they show that oxidative DNA damage is increased in inflammatory cancer-associated fibroblasts (iCAFs) in an IL-1 dependent manner leading to senescence upon irradiation and ultimately resulting in tissue remodeling and resistance to therapy. TNF and members of the IL-6 family are also important inflammatory inducers of fibroblast activation. TNF has been shown to act synergistically with IL-1α to promote proinflammatory gene expression in pancreatic stellate cells (PSCs) through NF-κB activation (Biffi, Oni et al. 2019). Accordingly, *in vivo* inhibition of TNF was able to reduce desmoplasia in mice, which was associated with decreased PSC viability (Zhao, Fan et al. 2016). IL-6 and IL-11 were recently also shown to play a role in fibroblast activation in CRC through STAT3 activation and subsequent expansion of activated fibroblasts and the induction of a proangiogenic profile, which drove colorectal carcinogenesis *in vivo* (Heichler, Scheibe et al. 2019). Similarly, IL-6 was sufficient to induce the trans-differentiation of normal fibroblasts to CAFs via STAT3 phosphorylation and

downstream activation of a Twist1/CXCL12 axis (Lee, Yeo et al. 2015). Besides these major cytokine signals, other cytokines, and chemokines, such as IL-33 and CXCL12 also contribute to fibroblast activation. In humans and mouse models of intestinal cancer, cancer cell-derived IL-33 activates fibroblasts and promotes the expression of ECM components and growth factors associated with intestinal tumor progression (Maywald, Doerner et al. 2015). Other immune cell-derived inflammatory cytokines, such as IL-17, IL-22, IL-31, IL-4 and IL-13, have also been shown to activate quiescent tissue-resident fibroblasts during inflammation or fibrosis, although their role in CAF reprogramming is not yet clear (Andoh, Bamba et al. 2007, Tsuchida and Friedman 2017). In addition to cytokines and chemokines, innate immune signals play a crucial role in CAF activation (Koliaraki, Henriques et al. 2020). The Hedgehog (Hh)/Smoothened (Smo) signaling pathway has also been shown to regulate CAF activation in gastrointestinal cancers, although it seems to exert opposite functions depending on the organ affected. We will further analyze this versatile role of Hedgehog signaling pathway in the chapter of CAF functions. Finally, beyond soluble mediators, the biophysical properties of the TME have been also implicated in CAF activation and the induction of a synthetic phenotype. Mechanical stress induces collagen overexpression and crosslinking, fiber rearrangement, ECM deposition and degradation by fibroblasts, which can thus mediate the remodeling of the ECM and increase matrix stiffness (Mohammadi and Sahai 2018). Matrix stiffness and the resulting mechanical stress further activates fibroblasts in a continuous self-promoting loop resulting in cancer cell proliferation and migration. Several studies in gastrointestinal inflammation and cancer propose that these stimuli activate fibroblasts through FAK, MRTF-SRF, and YAP-TEAD signaling pathways, leading to increased αSMA expression and the regulation of cytoskeletal dynamics (Johnson, Rodansky et al. 2014, Foster, Gualdrini et al. 2017). Many of these effects are also dependent on TGF-β, Rho and ROCK signaling (Zhao, Laschinger et al. 2007, Johnson, Rodansky et al. 2014). Recently, a pan-cancer analysis of tumor stroma identified three common CAF subpopulations in skin, breast, and pancreatic tumors from humans. Interestingly, mechanistic studies in mice showed that genetic ablation of FAK function in the stromal cell compartment leads to a loss of both mechanoresponsive (MR) and immunomodulatory (IM) CAF subpopulations. This suggests that FAK signaling may play a role in the activation and maintenance of specific CAF subtypes within the tumor microenvironment. Also, they observed that tumors with FAK deletion in the stromal compartment grew significantly larger compared to wildtype tumors, suggesting that FAK signaling in CAFs may impact the tumor microenvironment and influence tumor growth and progression (Foster, Januszyk et al. 2022).

HSCs sense mechanical stress through integrins, GPCRs and DDRs, activating Rho, YAP, PAK1, and JAK2/PI3K/AKT-MYOCD, respectively (Martin, Pritchett et al. 2016, Kang 2020). Interestingly, another mechano-sensor, the G protein-coupled estrogen receptor (GPER) shows tumor restricting capacity, as it acts through Rho/myosin axis and YAP deactivation to inhibit the ability of PSCs and HSCs to remodel the ECM (Cortes, Lachowski et al. 2019). [2]

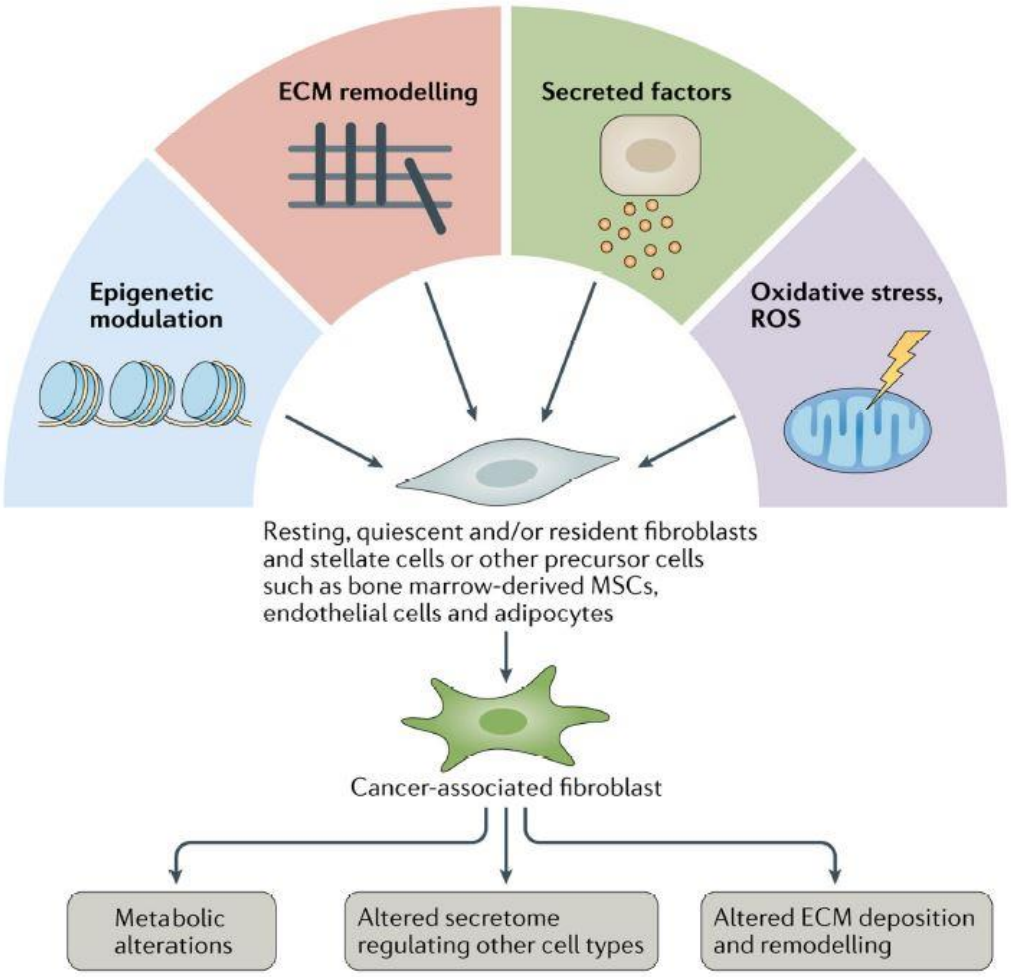

Figure 8. Schematic representation of various CAF activation mechanisms *(Chen, McAndrews et al. 2021).*

[2]Parts of this section (1.2.2.1.2) are published in a review article in the journal *Frontiers in Cell Developmental Immunology* Melissari, M. T., N. Chalkidi, M. E. Sarris and V. Koliaraki (2020). "Fibroblast Reprogramming in Gastrointestinal Cancer." Front Cell Dev Biol **8**: 630.

1.2.2.1.3 Transdifferentiation

Epithelial and endothelial cells can also give rise to CAFs through a process called epithelial or endothelial to mesenchymal transition (EMT and EndoMT), respectively. Through EMT, cancer cells acquire properties linked with a more aggressive phenotype enabling them to invade surrounding tissues and metastasize to distant organs (Nieto, Huang et al. 2016, Brabletz, Kalluri et al. 2018). Stimuli from the microenvironment, such as TGF-β, are capable of converting non-mesenchymal lineage cells to cells that express CAF markers (Polyak and Weinberg 2009, Flier, Tanjore et al. 2010). CXCL12 can also promote epithelial-to-mesenchymal transition, recruit Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs) and drive the expansion of αSMA myofibroblasts and Gremlin 1-expressing mesenchymal stem cells in gastric cancer (Quante, Tu et al. 2011, Shibata, Ariyama et al. 2013).

Furthermore, fate mapping studies in mouse melanomas and pancreatic neuroendocrine tumors indicate that TECs can also disseminate from blood vessels to generate multipotent mesenchymal cells (Zeisberg, Potenta et al. 2007). In esophageal adenocarcinoma, *in vitro* studies have shown that IL-1β and TGF-β2 derived from cancer cells can induce primary human esophageal microvascular cells to undergo EndoMT displaying fibroblast like characteristics and producing VEGF (Nie, Lyros et al. 2014). Experiments using CRC cell xenografts also show that osteopontin interacts with integrin αVβ3 to suppress the transcription of VE-cadherin and facilitate EndoMT. These EndoMT-derived cells can promote tumor growth and metastasis through their secretome (Fan, Chen et al. 2018). *In vitro* experiments also show that colon cancer cells upregulate tubulin-β3 and its phosphorylation to induce EndoMT (Wawro, Chojnacka et al. 2018). Finally, in PDAC, EndoMT-derived cells can drive monocyte M2 polarization and promote tumor growth (Fan, Chen et al. 2019). However, the exact contribution of these cells in the pool of CAFs still remains elusive.

1.2.2.2 CAF heterogeneity

Despite their abundance in solid tumors, CAFs are not targeted in mainstream cancer therapy, in part due to the incomplete understanding of their heterogeneity and diverse functions. CAFs in solid tumors are usually described as negative for epithelial, immune, and endothelial markers. In the past, CAFs were considered a uniform population that expressed αSMA and contributed to tumorigenesis by producing collagen and shaping the ECM. Since then, other CAF markers have

been described, such as fibroblast activation protein (FAP), collagen I or Podoplanin (Tomasek, Gabbiani et al. 2002, Roberts, Deonarine et al. 2013, Nurmik, Ullmann et al. 2020). However, these markers are neither exclusive nor able to identify all CAFs. Recent advancements in single-cell RNA sequencing have shed light on CAF heterogeneity by identifying subsets with gene expression signatures predictive of specific function.

In 2019, Elyada et al., used human PDAC patient samples and the KPC mouse model trying to describe CAF heterogeneity and to assign functional roles to different CAF subclusters (Elyada, Bolisetty et al. 2019). They first introduced the terms inflammatory CAFs (iCAFs), myofibroblastic CAFs (my CAFs) and antigen-presenting CAFs (apCAFs) (Ohlund, Handly-Santana et al. 2017, Elyada, Bolisetty et al. 2019). Since then, many other studies have supported the presence of these populations in different types of cancers. Vascular CAFs have also emerged as another prominent CAF subtype found across cancers (Figure 9).

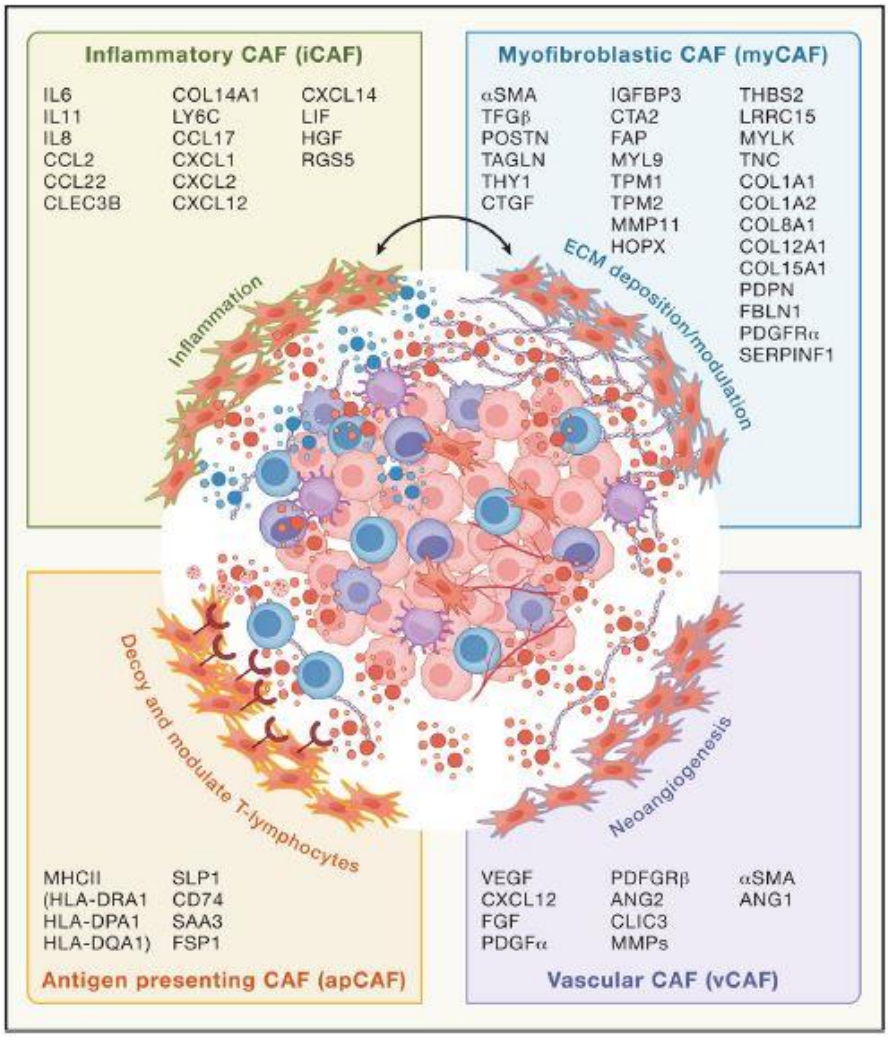

Figure 9. CAF heterogeneity. *The application of single-cell RNA sequencing and multiplex imaging has unveiled distinct subtypes of cancer-associated fibroblasts (CAFs) across various cancer types. These CAFs can be categorized into four main groups: iCAFs (inflammatory CAFs), myCAFs (myofibroblastic CAFs), apCAFs (antigen-presenting CAFs), and vCAFs (vascular CAFs). (Chhabra and Weeraratna 2023)*

myCAFs are present in several solid cancers and are characterized by high αSMA expression (Ohlund, Handly-Santana et al. 2017, Bartoschek, Oskolkov et al. 2018, Lambrechts, Wauters et al. 2018, Davidson, Efremova et al. 2020). In the desmoplastic TME of PDAC (Vera, Garcia-Olloqui et al. 2021), single-cell analysis of human and mouse tumors (KPC model) revealed that myCAFs express genes linked to smooth muscle contraction, focal adhesion, ECM organization, and collagen formation. Importantly, they are situated in close proximity to neoplastic cells, indicating their potential role in the tumor-stromal interaction (Ohlund, Handly-Santana et al. 2017).

Accordingly, several single-cell RNA sequencing (scRNAseq) studies in breast cancer consistently identified myofibroblastic subsets of CAFs (Bartoschek, Oskolkov et al. 2018, Friedman, Levi-Galibov et al. 2020, Sebastian, Hum et al. 2020). TGF-β and other growth factors are considered responsible for their activation. Spatially, myCAFs were more abundant in the invasive regions of tumors, especially within collagen-rich streaks (Bartoschek, Oskolkov et al. 2018). While variations may arise from diverse model systems, these studies emphasize the broad presence of ECM-remodeling/contractile myCAF subpopulations that are associated with a desmoplastic matrix and upregulation of ECM components, contributing to the creation of a fibrotic environment. MyCAFs also exhibit dynamic characteristics in response to microenvironmental stimuli in PDAC and lung cancer (Ohlund, Handly-Santana et al. 2017, Bartoschek, Oskolkov et al. 2018, Lambrechts, Wauters et al. 2018, Davidson, Efremova et al. 2020).

iCAFs is the second major CAF subpopulation usually identified in solid tumors, including PDAC, melanoma, and breast cancer (Biffi, Oni et al. 2019, Elyada, Bolisetty et al. 2019, Davidson, Efremova et al. 2020, Friedman, Levi-Galibov et al. 2020). They are characterized by the secretion of cytokines (IL-6, IL-8, IL-11, etc), chemokines (CXCL1, CXCL2, CXCL12, etc), ECM remodeling enzymes (matrix metalloproteinases like MMP3, MMP10, MMP13, etc), and growth factors (insulin like growth factor 1 (IGF1)) that modulate innate and adaptive immune responses in the tumors. iCAFs are also characterized by low αSMA expression and a more distant location to cancer cells than myCAFs. IL-1 signaling, along with other inflammatory mediators (e.g. TNF), and downstream JAK/STAT and NF-κB signaling, drives this phenotype, as shown first in human PDAC organoids and murine models (Biffi, Oni et al. 2019, Elyada, Bolisetty et al. 2019, Friedman, Levi-Galibov et al. 2020).

apCAFs have been identified in PDAC, lung and breast cancer where they express Major Histocompatibility Complex (MHC) class II and CD74 (Elyada, Bolisetty et al. 2019, Friedman, Levi-Galibov et al. 2020, Kerdidani, Aerakis et al. 2022). In PDAC, they have tumor-restraining functions due to their ability to induce T-cell receptor ligation in CD4+ T cells (Elyada, Bolisetty et al. 2019), while in lung cancer they exert a tumor-suppressive function due to their capability to activate effector T cells (Kerdidani, Aerakis et al. 2022).

vCAFs have been found in multiple tumors, including breast and lung tumors, and melanomas, where they exhibit angiogenic functions and are usually co-clustered with pericytes (Bartoschek, Oskolkov et al. 2018, Lambrechts, Wauters et al. 2018, Davidson, Efremova et al. 2020). They are

characterized by expression of αSMA, regulator of G-protein signaling 5 (RGS5), and MCAM and they may also contribute to the development of a desmoplastic matrix.

Single cell analysis across different solid tumors has further supported the above distinctions and their general applicability. Luo et al., integrated publicly available and in-house scRNAseq data to characterize the TME across ten solid cancer types. The study identified normal like fibroblasts and clusters with enrichment in tumors, characterizing them as CAFs. Comparative analysis of their transcriptional profiles revealed the presence of myCAFs characterized by *ACTA2* expression and iCAFs that expressed FAP and TGFB1 and exhibited an Inflammatory gene signature. Other subtypes were also identified, such as apCAFs, adipogenic CAFs, endothelial-to-mesenchymal transition CAFs and peripheral nerve-like CAFs, each marked by specific gene expression signatures (Luo, Xia et al. 2022). Foster and colleagues also analyzed CAF heterogeneity across species and in different cancers using a multiomics approach. They identified three superclusters: steady state-like (SSL), mechanoresponsive (MR), and immunomodulatory (IM) CAFs (Foster, Januszyk et al. 2022). They discuss the temporal and spatial dynamics of CAF biology, regulatory pathways in CAF differentiation, and the functional relevance of CAF subpopulations. Focusing on the regulatory pathways that determine CAF differentiation, they identify mechanotransduction-related pathways, such as growth arrest specific (GAS), PERIOSTIN, Thrombospondin (THBS), and secreted phosphoprotein 1 (SPP1), which drive MR-CAFs, and cytokine signaling pathways (Chemokine Ligand (CCL), Chemokine (C-X-C motif) Ligand (CXCL), and IL-1) that drive IM-CAFs. Previously functional results have been inconsistent regarding CAF-specific suppression (Demircioglu, Wang et al. 2020, Wu, Hao et al. 2020). Interestingly, they show that genetic ablation of FAK in the stromal cell compartment led to accelerated tumor growth highlighting the potential translational implications of targeting CAF superclusters in cancer therapy.

Another distinction of CAFs is based on their tumor-promoting or restraining roles. Studies suggest that most CAF subpopulation act as cancer-promoting CAFs (pCAFs), which are often linked with the expression of αSMA or FAP and the suppression of antitumor immunity through intricate pathways. The secretion of modulators like TGF-β, IL-6, and CXCL12 by pCAFs creates a tumor-promoting microenvironment, fostering cancer cell proliferation and invasion (Kojima, Acar et al. 2010, Feig, Jones et al. 2013, Costa, Kieffer et al. 2018, Kato, Noma et al. 2018, Lakins, Ghorani et al. 2018). However, tumor-restraining CAFs (rCAFs) have also been reported (Miyai, Esaki et al. 2020). In PDAC mouse models, the depletion or suppression of specific CAFs has been shown to

have tumor-promoting effects (Ozdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014). Moreover, a recent study revealed the presence of unique subset of rCAFs, expressing meflin, with antitumor effects both in mouse models of PDAC and human pancreatic cells (Mizutani, Kobayashi et al. 2019). The presence of rCAFs is not confined to PDAC, as evidenced by studies reporting myofibroblastic CAFs in oral carcinoma inhibiting cancer cell stemness through the secretion of bone morphogenetic protein 4 (BMP-4) (Patel, Vipparthi et al. 2018). Tumor-suppressive CAFs have also been reported in colon and bladder cancers indicating the wide distribution of rCAFs (Shin, Lim et al. 2014, Pallangyo, Ziegler et al. 2015, Gerling, Buller et al. 2016).

1.2.2.2.1. CAF heterogeneity in colorectal cancer

The introduction of the consensus molecular subtype (CMS) classification system for colorectal cancer, which is based on the transcriptomic profiles of tumors, has proven instrumental in predicting disease-free (DFS) and overall survival (OS). Notably, the mesenchymal signature identified as CMS4 has been linked to poorer survival rates compared to other subtypes (Guinney, Dienstmann et al. 2015, Eide, Bruun et al. 2017), underscoring the pivotal role of the TME and more specifically, CAFs in determining disease outcomes in CRC patients. Accordingly, TGF-β signaling in CAFs has been linked to worst prognosis (Li, Courtois et al. 2017, Tauriello, Palomo-Ponce et al. 2018). During the last five years many single-cell transcriptomic studies explored the heterogeneity and role of CAFs in CRC (Table 1). Although the analysis and terminology differ significantly in each of these studies, a consensus on the presence myCAFs, iCAFs and vCAFs emerges also in CRC. In more detail:

myCAFs. Similar to other cancers, many groups have identified one or more CAF subsets in human colorectal tumors with genes related to contractile and/or ECM remodeling functions. Li et al were the first in 2017 to perform scRNAseq in human CRC tumors and identify a myofibroblastic population, which expressed αSMA, transgelin and PDGFRα (Li, Courtois et al. 2017). Pelka et al analyzed mismatch repair deficient and proficient colorectal tumors and also found a myofibroblastic population expressing αSMA (Pelka, Hofree et al. 2021). Qi and colleagues analyzed colonic and rectal adenocarcinomas and identified three myofibroblast-like populations (Qi, Sun et al. 2022). The DES+ myofibroblasts that expressed αSMA and MYH11 and the FAP+ fibroblasts that expressed MMPs were attributed with ECM remodeling related functions.

Interestingly, pathway analysis and imaging techniques showed that FAP+ positive CAFs in CRC tissue are in close proximity to SPP1+ macrophages, suggesting there is a potential crosstalk between these two cell types. High expression of both markers in CRC patients correlated with reduced PD-L1 treatment efficacy, suggesting that FAP+ CAFs contribute to chemoresistance. Khaliq et al used scRNAseq in treatment naïve CRC patients and identified several myCAF subpopulations expressing collagens and FAP and genes related with adhesion and wound healing (Khaliq, Erdogan et al. 2022). Finally, Becker et al., analyzed different stages of colorectal cancer including polyps and fully developed tumors and found myofibroblasts expressing high levels of *ACTA2* and *TAGLN* (Becker, Nevins et al. 2022).

iCAFs. Pelka et al identified, in mismatch repair deficient and proficient colorectal tumors, inflammatory CAF subsets, which were characterized by the expression of tissue remodeling factors (MMP1 and MMP3) and chemokines (CXCL8 and CXCL1) associated with neutrophil attraction, and displayed heightened activity in tumors with mismatch repair deficiency (MMRd) (Pelka, Hofree et al. 2021). Qi et al identified an NT5E+ fibroblast population which expressed CD73 with a role in adenosine production and immunosuppression. Several iCAF populations were also identified by Khaliq and colleagues expressing αSMA, chemokines, SCARA5, DLK1, ADH1B and GPC3 (Khaliq, Erdogan et al. 2022). Nicolas et al., recently described iCAFs in rectal cancer patients which were associated with conferring resistance to neoadjuvant therapy. Experiments in mice showed that upon irradiation, IL1-α induces senescence in iCAFs, subsequently causing extracellular matrix accumulation and resistance to chemoradiotherapy (Nicolas, Pesic et al. 2022). Heiser et al., combined spatial data with scRNAseq and identified two CAF subpopulations: FIB2 and FIB3. FIB2 subpopulation are CXCL14+ CAFs that are suspected to counteract the immune-silencing effect of CXCL12+/FAP+ fibroblasts and are more prominent in MSI-H/iCMS3/HM (Microsatellite Instability-High/ Immune Consensus Molecular Subtype 3/ hypermutated) tumors. FAP and CXCL12 foster an immunosuppressive niche also in other solid tumors, such as pancreatic cancer (Heiser, Simmons et al. 2023). Finally, Koncina et al identified a specific CAF subtype, IL1R1+ iCAFs, characterized by elevated IL-1β signaling and increased expression of IL1R1. This subtype demonstrates a pro-tumorigenic effect both in vitro and in vivo (Koncina, Nurmik et al. 2023).

vCAFs. Tumor pericytes or vCAFs are found significantly enriched in human CRC tumors in several studies, although the terminology differs (Pelka, Hofree et al. 2021, Qi, Sun et al. 2022). For

example, Khaliq and colleagues identified the CAF-S4 population, which was spatially perivascular, expressed pericyte markers (RGS5, CSPG4, and PDGFRβ), CD248 (endosialin) and HIF2, suggesting that this particular subtype is associated with blood vessels, and the likelihood of hypoxia playing a role in invasion and metastasis. Li and colleagues found two RGS5+ pericyte populations (RGS5+αSMAhi, RGS5+CD36+) enriched in colorectal tumors (Li, Lu et al. 2022). The gene expression profiles of CD36hi cells were related to various stimulus-responsive pathways, encompassing cytokine-responsive pathways, signaling pathways regulating immune responses, and fatty acid transport, suggesting that CD36hi myofibroblasts may be responsive to stimuli within tissue microenvironments.

In most datasets, normal-like fibroblasts have been detected in CRC tumors but in reduced numbers compared to physiology. Notably, the analysis of early-stage polyps and colon carcinomas revealed the presence of normal-like fibroblast populations, exhibiting similar markers as those found in physiological conditions (crypt and villus fibroblasts). This observation suggests that, at least in the initial stages of colon carcinogenesis, fibroblasts retain some of their physiological properties both in terms of markers and functions. (Becker, Nevins et al. 2022). A preCAF population was also found using single cell Assay for Transposase-Accessible Chromatin (sc-ATAC) sequencing providing evidence of intermediate states prior to reprogramming. Finally, proliferating fibroblasts expressing MKI67 have also been described both in human CRC and mouse models of colorectal cancer (Kobayashi, Gieniec et al. 2022, Qi, Sun et al. 2022).

Overall, the comprehensive analysis of CAF heterogeneity in CRC suggests the presence of various subpopulations with distinct marker expressions and functional roles. Myofibroblastic and immunoregulatory CAFs are detected in CRC, similar to other cancers, along with normal-like fibroblasts, albeit in reduced numbers. Notably, tumor pericytes or vCAFs are enriched within colonic tumors.

Table 1 CAF subpopulations in CRC studies

myCAFs				
Publication	**Population**	**Markers**	**Function**	**Stage of cancer**
Li et al., 2017	CAF-A	MMP2, DCN, FAP, PDPN, COL1A2	ECM remodeling	CRC tissue/ cell lines
Pelka et al., 2021	cs26-c28	ACTA2	Myofibroblasts	Colorectal tumors and adjacent normal tissues of MMRp and MMRd patients
Qi et al., 2022	DES+ Myofibroblasts	ACTG2, MYH11, DES	ECM remodeling and angiogenesis	Non-metastatic CRC patients
	MFAP5+ myofibroblasts	ACTG2, MYH11, MFAP	Not reported	
	FAP+ fibroblasts	FAP, MMP1, MMP	Fibroblast activation and extracellular matrix Interaction with SSP1+ macrophages to promote immunosuppression	
Khaliq et al., 2022	myCAFs	COL1A1, COL1A2, FAP, PDPN	Not reported	Treatment naïve CRC patient
	ecm-myCAFs	GJB, ANTXR1, SDC1	adhesion	
	wound-myCAFs	SEMA3C, ANTXR1, CD9	Wound healing	
	TGF-β-myCAFs	CST1, TGFß1, ANTXR1, LAMP5	Not reported	
Becker et al., 2022	Myofibroblasts	ACTA2hi, TANGLN	Not reported	Healthy colon, polyps and CRCs
iCAFs				
Pelka et al., 2021	cs28-c29	MMP1, MMP3, CXCL8, CXCL1	Tissue remodeling and neutrophil attraction	Colorectal tumors and adjacent normal tissues of MMRp and MMRd patients
Qi et al., 2022	NT5E+ fibroblast	CD73	Adenosine production and immune suppression	Non-metastatic CRC patients
Khaliq et al., 2022	iCAFs	chemokines	Not reported	Treatment naïve CRC patients
	IL-iCAF	SCARA5, DLK1	Not reported	

	detox-iCAF	ADH1B, GPC3	Not reported	
Nicolas et al., 2022	iCAFs		ECM remodeling, chemoresistance	Rectal cancer
Heiser et al., 2023	FIB2	CXCL14	Immune-silencing	Regional morphologies representing transitions between tumor-progression stages.
	FIB3	FAP, CXCL12	Immunosuppressive	
Koncina et al., 2023	IL1R1+ iCAF	IL1R1^{hi}	Pro-tumorigenic both in vitro and in vivo	CRC patients integrated with Zang 2020 and Li 2017
vCAFs				
Pelka et al., 2021	tumor pericytes	ACTA2	Not reported	Colorectal tumors and adjacent normal tissues of MMRp and MMRd patients
Qi et al., 2022	Pericytes	Not reported	Not reported	Non-metastatic CRC patients
Khaliq et al., 2022	CAF-S4	RGS5, CSPG4, PDGFRβ, CD248, HIF2	Perivascular/ vessel associated, with hypoxia likely contributing to invasion and metastasis	Treatment naïve CRC patients
Li et al., 2022	N9 RGS5+ACTA2hi	Not reported	Not reported	
	N10 RGS5+CD36+	Not reported	Not reported	
Others				
Qi et al., 2022	Proliferating Fibroblasts	MKI67	Not reported	Non-metastatic CRC patients
Becker et al., 2022	Crypt fibroblasts	WNT2B, RSPO3, semaphorins	Not reported	Healthy colon, polyps and CRCs
	Villus Fibroblasts	WNT5B^{hi}	Not reported	
	preCAFs		May promote cancer progression	
Khaliq et al., 2022	Normal fibroblasts			Treatment naïve CRC patients

1.2.2.3 CAF functions

It is now known that CAFs contribute to all hallmarks of cancer and play an active role in tumor initiation, progression, and metastasis through the promotion of cell proliferation, tumor immunity, angiogenesis, extracellular matrix remodeling, and metastasis (Figure 10).

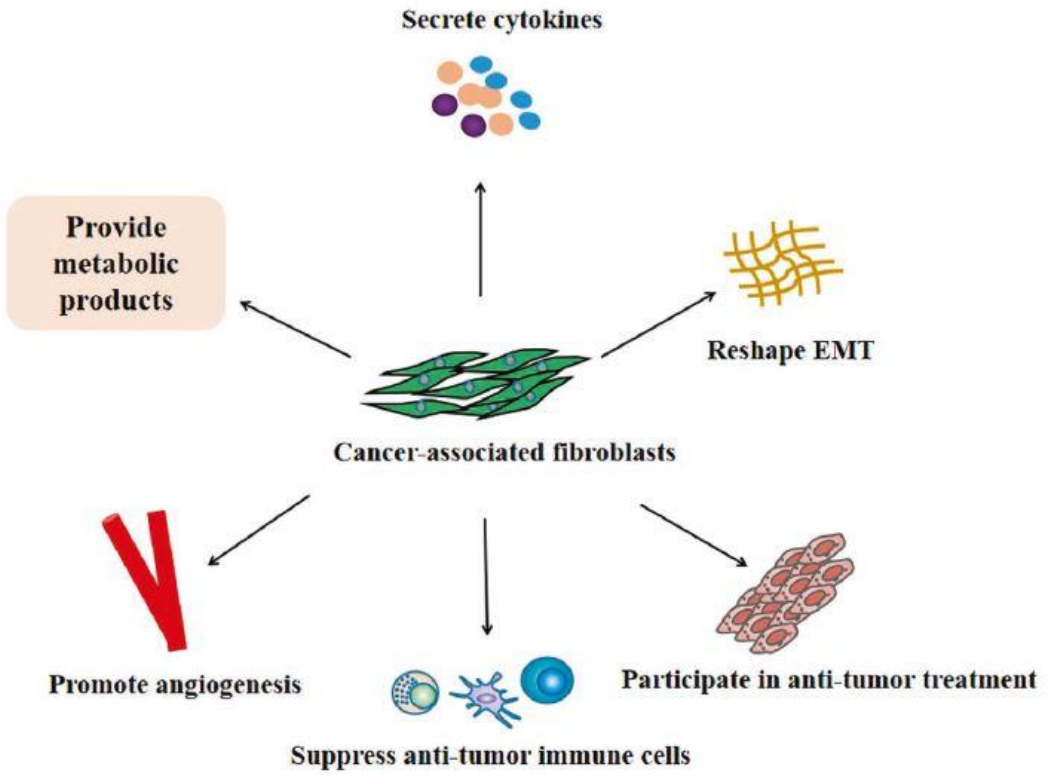

Figure 10 CAF functions. *CAFs affect tumor growth through multiple mechanisms, such as cytokine secretion, ECM remodeling, promotion of angiogenesis, immunomodulation, and regulation of cancer metabolism (Zhao, Shen et al. 2023).*

1.2.2.3.1 CAF interactions with cancer cells

CAFs can actively promote tumor growth by promoting proliferation through paracrine interactions with cancer cells, and specifically through the secretion of various molecules. Chemokines, cytokines, and growth factors, such as CXCL12, hepatocyte growth factor (HGF), epidermal growth factor (EGF), IGF, IL-6, IL-8, and IL-11 play a crucial role in stimulating the growth of epithelial cells and maintaining cancer stem cells (CSCs) (Chen , Orimo, Gupta et al. 2005, Bollrath, Phesse et al. 2009, Grivennikov, Karin et al. 2009, Vermeulen, De Sousa et al. 2010, Calon, Espinet et al. 2012, Putoczki, Thiem et al. 2013, Lau, Lo et al. 2016, Rhee, Kim et al. 2018, Vaquero,

Lobe et al. 2018). For example, CXCL12 produced by activated CAFs can promote breast cancer growth by binding to its receptor CXCR4 on cancer cells (Orimo, Gupta et al. 2005). HGF, when overexpressed, induces cancer stemness and chemoresistance in liver and colon cancer models (Vermeulen, De Sousa et al. 2010). CAFs also secrete EGF family proteins, such as heparin-binding EGF-like growth factor (HB-EGF) and EREG that promote cancer progression through the activation of EGFR receptors (Clapéron, Mergey et al. 2013, Neufert, Becker et al. 2013). IGF2 secreted by CAFs helps maintain the stemness of cancer cells (Chen 2014, Vaquero, Lobe et al. 2018). IL-6 and IL-11 increase cancer cell proliferation and liver metastases in colon cancer models by enhancing STAT3 signaling (Calon, Espinet et al. 2012, Koliaraki, Pasparakis et al. 2015, Koliaraki, Pallangyo et al. 2017). CD10+GPR77 CAFs in breast and lung cancer provide IL-6 and IL-8 to maintain CSCs and promote chemoresistance (Su, Chen et al. 2018). The expression of Gremlin 1, a BMP antagonist, is upregulated in CAFs of gastrointestinal cancers (Worthley, Churchill et al. 2015). Disruption of BMP morphogen gradients by aberrant Gremlin 1 expression can lead to the formation of ectopic crypts and progressive intestinal polyps (Sneddon, Zhen et al. 2006). Accordingly, Gremlin 1 deletion has been shown to ameliorate tumorigenesis in a mouse model of intestinal cancer (Davis, Irshad et al. 2015).

CAFs actively participate also in cancer cell migration, invasion, and metastasis. CAFs can affect the stiffness of the ECM and generate tracks to guide cancer cell invasion at primary sites (Ligorio, Sil et al. 2019). Fibroblasts are recruited to or activated at the metastatic site as a result of cancer cell seeding and inflammatory responses (Quail and Joyce 2013, Altorki, Markowitz et al. 2019, Wang, Liu et al. 2021). They then provide stromal support for disseminated cancer cells and release growth factors and cytokines that stimulate the growth and invasiveness of cancer cells at distant sites (Aggarwal, Montoya et al. 2021) (Soikkeli, Podlasz et al. 2010, Erdogan, Ao et al. 2017, Massagué and Ganesh 2021).

1.2.2.3.2 CAFs and cancer metabolism

Alterations in cancer cell energetics, known as the "Warburg effect," involve a shift towards aerobic glycolysis, providing glycolytic intermediates for macromolecule biosynthesis, which cancer cells utilize to support their rapid growth and proliferation (Vander Heiden, Cantley et al. 2009, Faubert, Solmonson et al. 2020). CAFs play a crucial role in this metabolic adaptation of tumors. They also undergo metabolic reprogramming, including enhanced reliance on anabolic

metabolism and mitochondrial respiration (Zhang 2015). This metabolic shift is influenced by paracrine signaling from cancer cells and direct intercellular contacts between CAFs and cancer cells (Fiaschi, Marini et al. 2012).

The metabolic symbiosis between CAFs and cancer cells can dynamically evolve in response to oxygen levels, extracellular metabolite availability, and chemokine or cytokine signaling. The release of energy-rich fuels (such as lactate, pyruvate, glutamine, and ketone bodies) from CAFs to cancer cells through transporters like monocarboxylate transporter MCT4 and MCT1 facilitates the replenishment of the tricarboxylic acid (TCA) cycle, supporting OXPHOS and biosynthesis pathways that maximize proliferation and reduce cell death (Wilde, Roche et al. 2017, Wu, Zhuo et al. 2017). In colorectal cancer (CRC) and gastrointestinal stromal tumors (GISTs), elevated levels of MCT1 and MCT4 were associated with low patient survival (Lehuede, Dupuy et al. 2016, Martins, Amorim et al. 2016). Pancreatic ductal adenocarcinoma (PDAC) CAFs displayed diverse expression of hypoxic markers and altered metabolic properties, supporting cancer cell invasiveness and correlating with shorter patient survival (Knudsen, Balaji et al. 2016).

Besides glucose metabolism, CAFs also display a lipid metabolic shift. CAFs in CRC accumulate fatty acids and phospholipids, supporting membrane lipid synthesis and inducing cancer cell migration (Auciello, Bulusu et al. 2019, Gong, Lin et al. 2020). The increased autophagy observed in CAFs generates recycled nutrients, such as alanine, supporting the TCA cycle (tricarboxylic acid cycle) and lipid biosynthesis in cancer cells (Sousa, Biancur et al. 2016). Co-culture of fibroblasts and cancer cells in CRC resulted in the upregulation of oxidative stress-related enzymes and autophagy genes, promoting cancer cell proliferation (Zhou, Xu et al. 2017).

The metabolic reprogramming of CAFs has been primarily studied in the context of its influence on cancer cell growth, proliferation, and invasion. However, CAF metabolism can also induce pro-tumorigenic circuits by altering the availability of metabolites such as tryptophan and arginine, which are crucial for T cell activation and lymphocyte function (Chang, Qiu et al. 2015, Turley, Cremasco et al. 2015). This metabolic adaptation of CAFs may contribute to T cell hyporesponsiveness in tumors.

The drivers of metabolic shifts in CAF activation include factors such as TGFβ, PDGF, hypoxia, hypoxia-inducible factor 1α (HIF1α), and reactive oxygen species (ROS)-mediated suppression of CAV1 (Guido 2012, Martinez-Outschoorn, Lisanti et al. 2014, Zhang 2015). These metabolic adaptations may reflect the survival response of CAFs to intratumoral hypoxia and contribute to the repurposing of nutrients for other cells in the tumor microenvironment and cancer cells.

The symbiotic relationship between CAFs and cancer cells creates a tumor metabolic ecosystem that could be a potential target for cancer therapy. Understanding and targeting the metabolic adaptations of CAFs may have implications for inhibiting tumor growth, invasion, and metastasis. However, further research is needed to fully elucidate the specific molecular underpinnings and therapeutic implications of CAF metabolic reprogramming.

1.2.2.3.3 CAFs in ECM remodeling

One of the first steps in tumor initiation is the disruption of the host tissue architecture. CAFs are the main ECM-producing cells in the TME and contribute to desmoplasia and matrix remodeling. They can control ECM remodeling through the production of ECM components (collagens, fibronectin, proteoglycans, periostin, and tenascin C) and remodeling enzymes (MMPs, tissue inhibitors of metalloproteinase (TIMPs) and other proteases) (Bamba, Andoh et al. 2003, McKaig, McWilliams et al. 2003, Levental, Yu et al. 2009, Kessenbrock, Plaks et al. 2010, Kobayashi, Miyoshi et al. 2010). Moreover, ECM remodeling by CAFs allows prolonged secretion of inflammatory cytokines, and ECM sequestered factors, such as TGFβ, sustaining thus signaling pathways that accelerate tumor proliferation. Another result of ECM deposition and remodeling is increased tumor stiffness, which is accompanied by alteration of the physical properties and biomechanical activity of the TME, resulting in tumor growth, invasion, metastasis, and drug resistance (Bonnans, Chou et al. 2014). A dense ECM also shapes the TME by promoting leaky vasculature, T cell migration and recruitment of MSCs which get activated and contribute to the CAF pool (Salmon, Franciszkiewicz et al. 2012, Bordeleau, Mason et al. 2017). High tumor stiffness has been strongly correlated with poor patient prognosis (Henry, Lee et al. 2007, Vellinga, den Uil et al. 2016).

Tumor stiffness relies on the ability of CAFs to transduce mechanical forces and change the orientation of collagen and fibronectin fibers (Ligorio, Sil et al. 2019). The YAP signaling pathway has emerged as a key regulator of ECM stiffness. It has been shown that CAF-specific YAP1 dysregulation leads to transcriptional changes in cytoskelelal regulators such as ANLN, DIAPH3 that promote stiffness and create a self-reinforcing loop that further activates CAFs (Calvo 2013). Overall, understanding the role of ECM and CAFs in tumor progression, as well as the pathways involved in the process, could be important for developing new therapeutic strategies.

1.2.2.3.4 CAFs and tumor immunity

CAFs play a crucial immunomodulatory role inside tumors through both pro-inflammatory and immunosuppressive functions (Monteran and Erez 2019, Gao, Fang et al. 2023, Zhang, Fei et al. 2023).

CAFs can alter the TME through the recruitment and activation of immune cells, especially macrophages and neutrophils via diverse molecular mechanisms (Monteran and Erez 2019, Gao, Fang et al. 2023). Inflammatory cell recruitment is mediated predominantly by the secretion of chemokines, which act through their receptors on neutrophil and macrophages to promote their homing (Ren, Zhao et al. 2012, Yu, Huang et al. 2017, Monteran and Erez 2019). For example, tumor-educated CAFs were found to attract CD11b+Ly6C+ monocytes, F4/80+ macrophages, and CD11b+Ly6G+ neutrophils in a mouse lymphoma model, via the CCL2–CCR2 axis (Ren, Zhao et al. 2012). CAFs preconditioned with TNF were demonstrated to recruit CXCR2+ neutrophils by secreting CXCR2 ligands (CXCL1, CXCL2, and CXCL5), thereby enhancing lung metastasis in a breast cancer model (Yu, Huang et al. 2017). Cytokines and growth factors secreted by CAFs induce the activation of resident and infiltrating innate cells. Specifically, in a breast cancer mouse model, CAF-derived Chi3L1 was identified as a critical factor involved in both recruiting and inducing a functional shift in bone marrow-derived macrophages, leading to an M2-like phenotype (Cohen, Shani et al. 2017). Moreover, prostate CAFs were demonstrated to facilitate both the recruitment and M2-like differentiation of monocytes via the secretion of SDF1 (Comito, Giannoni et al. 2014). Notably, these functions are now attributed to a specific CAF subset (or subsets), the iCAFs, as mentioned above, which induce and maintain an inflammatory milieu through the expression of inflammatory mediators (IL-6, IL-11, CXCL1, CXCL2) (Ohlund, Handly-Santana et al. 2017, Biffi, Oni et al. 2019).

CAFs also exert significant immunosuppressive functions through their crosstalk with both CD4+ and CD8+ cells (Monteran and Erez 2019, Zhang, Fei et al. 2023). They play a pivotal role in orchestrating the recruitment, differentiation, and survival of Treg cells, contributing to the establishment and persistence of an immunosuppressive microenvironment within tumors (Chang, Lin et al. 2012, Anz, Rapp et al. 2015, Costa, Kieffer et al. 2018, Givel, Kieffer et al. 2018, Kuehnemuth, Piseddu et al. 2018, Bourhis, Palle et al. 2021, Kadomoto, Izumi et al. 2021). For example, in breast and high-grade serous ovarian cancers (HGSOC) cancer, the CAF-S1

subpopulation, characterized by the expression of FAP, αSMA, PDGFRβ, and CD29, facilitates the recruitment and retention of Treg cells by secreting CXCL12 and further enhances their differentiation and survival through the expression of OX40L, PD-L2, and Junctional adhesion molecule B (JAM2) (Costa, Kieffer et al. 2018, Givel, Kieffer et al. 2018). Notably, targeting the CXCL12/CXCR4 axis inhibited the recruitment of Treg lymphocytes in ovarian and pancreatic cancer. Other molecules secreted by CAFs, such as CCL5, VEGF-A, CCL1, CCL2, and CCL22, also contribute to Treg recruitment and infiltration, and the development of an immunosuppressive TME (Chang, Lin et al. 2012, Anz, Rapp et al. 2015, Kuehnemuth, Piseddu et al. 2018, Bourhis, Palle et al. 2021, Kadomoto, Izumi et al. 2021). Besides Tregs, CAFs also influence the differentiation of CD4 T cells into various T helper (Th) subpopulations, impacting the balance between Th1 and Th2 responses. In vitro studies using breast cancer models reveal that CAFs regulate the transition from Th1 to Th2 immunity, affecting the expression of cytokines like IL-2 and IL-7 (Liao, Luo et al. 2009). CAFs also show potential interactions with Th17 cells, attracting them through RANTES and monocyte chemoattractant protein-1 (MCP-1) secretion in colorectal tumor areas (Su, Ye et al. 2010).

CAFs also hinder the cytotoxic functions of CD8+ T cells, the key mediators of antitumor immunity (Monteran and Erez 2019, Zhang, Fei et al. 2023). Through the CXCL12-CXCR4 chemokine axis and the secretion of interleukin 6 (IL-6) and transforming growth factor β (TGF-β), CAFs impede the recruitment, homing, and cytotoxic activity of CD8+ T cells in the TME (Wang, Moresco et al. 2022). Inhibition of CAF-derived TGF-β has shown promising results in restoring T cell density, enhancing the effectiveness of immune checkpoint inhibitors, and reducing metastatic burden (Feig, Jones et al. 2013). CAF-derived soluble factors can also downregulate CD69 expression, reduce granzyme B production in activated CD8+ T cells, and dysregulate various immunological checkpoint regulators on CD8+ T cells, increasing the presence of co-suppressive receptors like BTLA and TIGIT. (Érsek, Silló et al. 2021). Besides soluble mediators, CAFs contribute to the dysfunction of antitumor T cells through direct interactions (Lakins, Ghorani et al. 2018, Goehrig, Nigri et al. 2019, Gorchs, Fernández Moro et al. 2019) . In mouse lung adenocarcinoma and melanoma, CAFs can sample, process and cross-present antigens through MHC-I, increasing the expression of PD-L2 and FasL to kill tumor-specific CD8+ T-cells (Lakins, Ghorani et al. 2018). Accordingly, in pancreatic cancer, CAFs exhibit elevated PD-L1 and PD-L2 expression and promote the expression of immune checkpoint receptors proliferating T cells, leading to T cell malfunction and reduced proliferation (Gorchs, Fernández Moro et al. 2019). CAF-mediated inhibition of T lymphocyte function extends

the suppression of the T cell receptor (TCR), leading to a reduction in the proliferation and function of CD8 T cells, thereby disrupting the antigen detection and activation process (Goehrig, Nigri et al. 2019).

Moreover, CAFs can indirectly impede CD8+ T cells' cytotoxic activity by preventing professional antigen-presenting cells (APCs) from carrying out their normal functions or interfering with antigen expression. For instance, CAFs derived from human hepatocellular carcinoma secrete IL-6 that activates the STAT3 signaling pathway in DCs and promotes a regulatory DC (rDC) phenotype that cannot efficiently prime and activate T lymphocytes (Cheng, Deng et al. 2016). Besides direct crosstalk with immune cells, ECM remodeling, a key function of CAFs, also affects immune infiltration, most notably that of CD8+ cells, thus hindering their anti-tumor functions. This ECM remodeling, which involves abnormal deposition of proteins like fibronectin and collagen, functions mainly by spatially segregating CD8+ T cells in regions with sparse ECM networks and inhibiting the contact-dependent death of tumor cells by CD8+ T lymphocytes (Bougherara, Mansuet-Lupo et al. 2015). In addition, CAFs can release angiogenic factors in response to hypoxia, to hinder the expression of adhesion molecules on endothelial cells, and impede the infiltration of CD8+ T cells through the vascular system, limiting their access to tumor sites (Mortezaee and Majidpoor 2021).

1.2.2.3.5 CAFs and angiogenesis

Neovascularization, the process by which new blood vessels form within a growing tumor to supply nutrients and oxygen is regulated both by cancer and stromal cells. CAFs contribute to this process both directly through their secretome or indirectly by orchestrating ECM remodeling. VEGF, which is produced by CAFs, is the most potent pro- angiogenic factor through its binding to its receptor on endothelial cells (Fukumura, Xavier et al. 1998). CAFs also produce several other angiogenic molecules such as PDGF, HGF and CXCL12 that can promote the recruitment and proliferation of endothelial cells and tumor pericytes, promoting thus tumor growth and facilitating metastasis (Jedeszko, Victor et al. 2009, De Francesco, Lappano et al. 2013). Furthermore, CAFs create a hypoxic TME that supports the formation of new vasculature and the angiogenic switch that promotes tumor growth (Ziani, Buart et al. 2021). Indirectly, CAFs can promote the formation of new vasculature through ECM synthesis and remodeling. Stiff matrices

and increased biomechanical forces have been implicated in vascular growth, a process mediated by Rho/ ROCK and Yap pathways (Sewell-Loftin, Bayer et al. 2017). Finally, CAF-derived MMPs degrade the ECM and contributes to angiogenesis, through the release of sequestered growth factors, such as VEGF, basic fibroblast growth factor (bFGF), and TGF-β. (Zigrino, Brinckmann et al. 2016).

1.2.2.4 CAFs and therapy

The diversity of human cancers, arising from different cells of origin in various tissues and organs, along with variable parameters of tumor development and progression, poses challenges in distilling data into effective therapies. Despite advancements in genomic and epigenetic profiling, current therapies for most forms of human cancer remain incomplete and transient. The increased understanding of CAF functions and their association with poor prognosis in cancer patients has highlighted the potential of targeting CAFs for cancer treatment.

1.2.2.4.1 Blocking the interactions with cancer cell

The pivotal role of the TGF-β signaling pathway in CAF activation and tumor promotion has led to the exploration of inhibitors targeting this pathway. LY2109761, a TGF-β receptor inhibitor, has demonstrated its potential to suppress tumor growth and metastasis by inhibiting the release of connective tissue growth factor (CTGF) and disrupting the cross-talk between cancer cells and CAFs (Mazzocca, Fransvea et al. 2010). In pancreatic tumor preclinical models, neuregulin 1 (NRG1), a ligand for human epidermal growth factor receptor HER3 and HER4 receptors, is secreted by both cancer cells and CAFs and has emerged as a target (Ogier, Colombo et al. 2018). The antibody 7E3, designed to inhibit NRG1, was shown to prevent tumor growth and metastasis by disrupting NRG1-mediated HER3 and AKT/MAPK signaling pathways, presenting a novel therapeutic avenue for pancreatic cancer. In gastric cancer, CAF-secreted IL-17a was found to promote the migration and invasion of cancer cells through the activation of the JAK2/STAT3 signaling pathway. Neutralizing antibodies against IL-17a or JAK2 inhibitors, significantly inhibited the impact of CAFs on cancer progression, suggesting potential therapeutic benefits (Zhang, Li et al. 2020). Further investigations in pancreatic cancer unveiled an interaction between CAFs, tumor cells, and hyperactive sonic Hedgehog (SHH) signaling. A SHH inhibitor, was able to reverse fibroblast-induced resistance to doxorubicin in pancreatic cancer cells (Zhou, Zhou et al. 2017). The expression of CXCL12 in fibroblasts was associated with metastases in HER2 breast cancer.

Inhibiting CXCR4, the receptor for CXCL12, through the administration of AMD3100 and TN14003, effectively suppressed tumor growth and metastasis (Lefort, Thuleau et al. 2017). Similar findings were observed in primary esophageal squamous cell carcinoma and colorectal cancer, where interfering with CAF-derived WNT2 restored dendritic cell differentiation and antitumor T-cell responses (Huang, Tan et al. 2022). These studies collectively underscore the potential of interventions targeting specific interactions between CAFs and cancer cells, offering diverse strategies to disrupt critical signaling pathways and enhance therapeutic outcomes.

1.2.2.4.2 CAF specific ablation

An alternative therapeutic strategy for targeting CAFs has focused on the inhibition of FAP, a membrane-bound gelatinase, that is overexpressed in activated fibroblasts. Sibrotuzumab, an antibody targeting FAP, underwent phase I clinical trials in patients with colorectal cancer and non-small cell lung carcinoma (Scott, Wiseman et al. 2003). However, the efficacy in subsequent phase II trials was limited, highlighting the challenges in translating pre-clinical results to clinical success. Another clinical inhibitor targeting FAP, Val-boroPro, was tested in phase II trials for patients with metastatic colorectal cancer, but the results were disappointing, with minimal clinical activity (Narra, Mullins et al. 2007). Innovative strategies involving immunotherapeutic approaches have shown better results. In a mouse model, SynCon, a novel FAP DNA vaccine, demonstrated the ability to break tolerance and induce $CD8^+$ and $CD4^+$ immune responses (Duperret, Trautz et al. 2018). Likewise, the FAP-targeting immunotoxin aFAP-PE38 showed promise in depleting FAP^+ CAFs in a metastatic breast cancer model (Fang, Xiao et al. 2016). This depletion resulted in decreased recruitment of tumor-infiltrating immune cells in the tumor microenvironment, ultimately suppressing tumor growth.

Beyond FAP, efforts have been directed at targeting cells expressing αSMA, a well-established CAF marker. Reduction of αSMA^+ cells in the stroma through Cellax therapy exhibited efficacy in inhibiting tumor progression (Murakami, Ernsting et al. 2013). A neutralizing anti-GPR77 antibody against the $CD10^+GPR77^+$ CAF subsets in breast cancer demonstrated the potential to restore the chemosensitivity of cancer cells, opening avenues for targeted therapeutic interventions (Su, Chen et al. 2018). Despite these advancements, challenges persist in CAF-targeted therapy. CAFs exhibit heterogeneity, lack specific markers, and undergo phenotypic alterations at different stages, making precise and selective targeting challenging. Moreover, non-selective removal of CAFs may

have unintended consequences, such as the observed relationship between the reduction of FAP^+ stromal cells and the loss of muscle mass and anemia. Therefore, a cautious approach is necessary in designing CAF-targeted therapies. The combined application of multiple markers may offer a more accurate localization of specific CAF subtypes, contributing to the development of more effective and tailored therapeutic strategies in cancer treatment.

1.2.2.4.3 CAF reprogramming

The possibility of reversing CAF activation is considered a promising therapeutic strategy, although the potential anti-tumor properties of CAFs should also be taken into account (Hanley, Mellone et al. 2018). Targeting pathways driving CAF activation represents a potential strategy to revert them to a quiescent state, hindering their ability to support tumor growth and even exerting tumor-suppressive effects. Given the crucial role of TGF-β in CAF activation, artesunate and dihydroartemisinin, have shown efficacy in suppressing TGF-β signaling in CAFs, leading to inhibition of tumor growth and metastasis (Yao, Guo et al. 2018). Angiotensin receptor blockers (ARBs), including losartan, have also the potential to convert myofibroblastic CAFs to a quiescent state by decreasing the activation of TGF-β (Chauhan, Chen et al. 2019). This shift in CAF behavior alleviates immunosuppression and enhances T lymphocyte activity. Dasatinib, an inhibitor of PDGF receptor (PDGFR), has also demonstrated the ability to reverse the phenotype of CAFs, transforming them into normal fibroblasts. Incubation of lung cancer cells with conditioned medium from Dasatinib-pre-incubated CAFs resulted in reduced cancer cell proliferation (Haubeiss, Schmid et al. 2010). In addition, combining a Janus kinase (JAK) inhibitor with a DNA methyltransferase inhibitor was found to restore the fibroblast phenotype and reverse the pro-invasive activity of CAFs in lung cancer and head and neck carcinomas (Albrengues, Bertero et al. 2015). The enzyme NADPH Oxidase 4 (NOX4), upregulated by CAFs in various human cancers, plays a role in promoting the fibroblast-to-CAF transition. Inhibition of NOX4, a NADPH oxidase, has shown promise in reversing CAFs to a quiescent state, overcoming cancer immune resistance, and improving the prognosis of multiple cancers in a CAF-rich mouse tumor model (Banerjee, Modi et al. 2016, Hanley, Mellone et al. 2018).

1.2.2.4.4 CAFs in therapy resistance

Another aspect of CAFs implications in therapy is their contribution to therapy resistance through various mechanisms. These include the modulation of pathways involved in cancer cell-ECM

interactions, CAF-ECM adhesion, and cytokine- or chemokine-mediated signaling pathways. For example, CAFs in BRAF-mutant melanomas generate a fibronectin-rich ECM that enhances β1-integrin-induced focal adhesion kinase (FAK)–SRC-mediated extracellular signal-regulated kinase (ERK) activation, compensating for BRAF inhibition in cancer cells (Hirata, Girotti et al. 2015). These CAF-mediated programs may facilitate new mechanisms of cancer progression, such as promoting the outgrowth of resistant clones. In breast and lung cancer patients, the activation of nuclear factor kappa B (NF-κB) through p65 phosphorylation and acetylation leads to the generation of $CD10^{+}GPR77^{+}$ CAFs. This unique CAF population establishes a supportive environment favoring cancer stem cells survival, facilitating the initiation of tumor formation and promoting resistance to chemotherapy (Su, Chen et al. 2018). Likewise, the secretion of IL-11 by CAFs triggers STAT3 phosphorylation, elevating the expression of anti-apoptotic proteins such as Bcl-2 and Survivin in lung adenocarcinoma (Tao, Huang et al. 2016). These proteins play a crucial role in shielding cancer cells from apoptosis induced by cisplatin, thereby fostering resistance to chemotherapy. Moreover, enhanced adhesion of cancer cells to the ECM offers a signaling platform that enhances pro-survival mechanisms. This adhesion may engage a dormancy phenotype, leading to cell cycle arrest (Hazlehurst, Damiano et al. 2000, White, Rayment et al. 2006). Recently, *in vitro* co-culture experiments using patient-derived organoids showed that CAFs redirect epithelial cells toward a rejuvenated, slow-cycling stem cell destiny by reducing MAPK and PI3K signaling and increasing TGF-β, c-Jun N-terminal kinase (JNK), and NF-kB signaling, providing a protective shield for colorectal cancer cells against the effects of chemotherapy (Ramos Zapatero, Tong et al. 2023). CAFs also contribute to chemoresistance through their secretome, which includes the production of cytokines and the release of miRNAs. Extracellular vesicles originating from $CD63^{+}$ CAFs encapsulated miR-22, fostering resistance to tamoxifen in breast cancer. This resistance mechanism was executed by miR-22 targeting ERα and PTEN (Gao, Li et al. 2020). In gastric cancer, exposure to cisplatin and paclitaxel activated the USP7/hnRNPA1 axis in CAFs, prompting the expression of miR-522. Consequently, ALOX15 inhibition occurred, leading to diminished lipid-ROS accumulation in cancer cells and, ultimately, a reduction in chemosensitivity (Zhang, Deng et al. 2020). In breast, colorectal, and pancreatic tumors, CAFs promote resistance to chemotherapeutic agents like gemcitabine and doxorubicin through the activation of specific pathways in tumor cells (Lotti, Jarrar et al. 2013, Zhang, Yao et al. 2018, Louault, Bonneaud et al. 2019). They also produce factors like TGFβ, IL-6, and HGF, which promote pro-survival signaling cascades in cancer cells (Meads, Gatenby et al. 2009). CAF-derived HGF

promotes resistance to targeted therapies in preclinical cancer models (Wang, Li et al. 2009). Co-targeting CAFs and cancer cells is a promising strategy to overcome resistance, but careful assessment of the CAF secretome and stromal responses is necessary to predict the effectiveness of targeting CAFs in clinical interventions.

Additionally, CAFs participate in immune modulation, promoting cancer cell survival and evasion from therapy. For example, the inhibition of the CXCL12-CXCR4 signaling pathway using AMD3100 (plerixafor), a CXCR4 inhibitor, has resulted in a reduction of CAF-mediated immunosuppression, consequently enhancing the efficacy of anti-PD-1 immunotherapy (Biasci, Smoragiewicz et al. 2020). Moreover, simultaneous administration of TGF-β blocking antibodies and anti-PD-L1 antibodies in mouse tumor models exhibited enhanced treatment responsiveness by to improving T cell infiltration within tumors (Mariathasan, Turley et al. 2018).

Targeting CAFs is a promising approach in cancer therapy. However, due to the dynamic heterogeneity of CAFs and the lack of specific biomarkers, developing effective strategies to target CAFs remains challenging. Current research focuses on understanding the functional roles of different CAF subpopulations and their interactions with tumor cells and the immune system. By identifying specific biomarkers and utilizing novel cell lineage-tracing and genetic manipulation techniques, researchers aim to develop clinically relevant experimental models for real-time tracking and targeting of CAFs. Additionally, the use of high-throughput and non-invasive imaging techniques can provide valuable insights into the evolution of CAFs during tumor progression. Although there are still obstacles to overcome, future research holds promise for the development of precise therapeutic strategies targeting specific CAF subtypes.

2. Aims and scope

CAFs comprise a major component of the intestinal TME and can both structurally and functionally contribute to cancer development through a plethora of mechanisms. Cytokines, chemokines, growth factors, extracellular matrix components, and other signaling molecules produced by CAFs can influence cancer cell proliferation, tumor immunity, angiogenesis, extracellular matrix remodeling and metastasis (Kalluri 2016, Kobayashi, Enomoto et al. 2019, Sahai, Astsaturov et al. 2020). Notably, recent studies suggest that a stromal signature can be also useful for cancer prognosis (Feig, Jones et al. 2013, Tauriello, Palomo-Ponce et al. 2018). Advances in single cell analysis technologies have highlighted the heterogeneity of CAFs in various mouse and human tumors, further classifying them into subpopulations, which can also explain their tumor promoting or tumor retarding functions (Öhlund, Handly-Santana et al. 2017, Raz, Cohen et al. 2018, Elyada, Bolisetty et al. 2019). In colorectal cancer, single cell RNA transcriptomic analyses indicate a similar heterogeneity and potential classification into subpopulations, although the exact pathways involved remain to be identified (Li, Courtois et al. 2017). It is thus important to gain additional mechanistic insights into these processes paving the way for new therapeutic strategies and better prognosis for colon cancer patients.

In the present thesis our aim was to:

- Describe the origin of specific CAF subsets in intestinal carcinogenesis.
- Identify the pathophysiological significance of intestinal CAF subsets.
- Delineate the CAF-specific downstream molecular pathways that orchestrate tumor initiation and progression.

To this end, and based on previous work in our lab, we focused on the origin and properties of Col6a1Cre^{+} intestinal fibroblasts and pathways governing their responses to innate immune signals.

3. Materials and methods

3.1 Mice and Study Approval

Col6a1Cre (Armaka, Apostolaki et al. 2008), Il1r1$^{f/f}$ (Abdulaal, Walker et al. 2016), and p55TNFR1$^{f/f}$ (Van Hauwermeiren, Armaka et al. 2013), MyD88$^{f/f}$ (Vlantis, Polykratis et al. 2016) and Tlr4$^{f/f}$ (Sodhi, Neal et al. 2012) mice have been previously described. Rosa26-mT/mG (Muzumdar, Tasic et al. 2007) and Apc$^{min/+}$ (Moser, Pitot et al. 1990, Su, Kinzler et al. 1992) mice were purchased from the Jackson Laboratory. All mice were maintained on a C57/Bl6 background, and experiments were performed using littermate, co-housed control and experimental mice. Both male and female mice were used at the ages of 2–6 months. Mice were maintained under specific pathogen-free conditions in the Animal House of the Biomedical Sciences Research Center "Alexander Fleming". Animal studies were approved by the Institutional Committee of Protocol Evaluation in conjunction with the Veterinary Service Management of the Hellenic Republic Prefecture of Anika according to all current European and national legislation under the permissions: 1253-10/03/2014, 5759- 26/11/2015, 8443-18/01/2017, 8450-18/01/2017, and 420750-22/06/2020, 1250-10/3/2014, 1252-10/3/2014, 8451-18/01/2017. Experiments were performed in accordance with the guidance of the Institutional Animal Care and Use Committee of BSRC ''Alexander Fleming''.

3.2 Genotyping

Genomic DNA was isolated from tail segments. Tail biopsies were lysed o/n at 56^{o}C with DNA digestion Buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS and Proteinase K (0.5 mg/ml) (Thermo-Fisher). Samples were neutralized with 1:1 volume of phenol/chloroform/iso-amyl alcohol (25:24:1) and centrifuged. The aqueous phase was transferred to a new tube and the DNA was precipitated with 1:1 volume of Isopropanol. Samples were cleaned with 70% Ethanol and the yield was resuspended in concentration of 0.1-0.25 µg/µl DNA in ddH20. PCR master-mix was prepared with the following recipe: Taq Buffer (10x: 100mM Tris HCl pH 8,8, 500mM, KCl, 0,5% NP40, final concentration:1x), 25 mM MgCl2 (final concentration 0.5 mM), DNTPs (final concentration 200 µM), primer forward (final concentration

0.1µM), primer reverse (final concentration 0.1µM), homemade TAQ DNA polymerase (0.3 µL). Genotyping was performed by PCR using the following primers:

Table 2 Primers for genotyping

Mouse line	Primer sequence	Annealing temp/ Time
Col6a1	CAGGTATGCTCAGAAAACGCCT TCAGCTCTGGGCTCTGACT	56^{oC}, 1:00 min
mTmG	CTCTGCTGCCTCCTGGCTTCT CGAGGCGGATCACAAGCAATA TCAATGGGCGGGGGTCGTT	61^{oC}, 1:00 min
Il1R1	CTAGTCTGGTGGAACTTACATGC AACTGAAAGCTCAGTTGTATACAGC	60 °C, 0:30 min
MyD88	GGAGGAAGGCTCAGAGAAGC GTCTGCAGGCAGCTACAGTG	60^{oC}, 1:00 min
TLR4	CAAGGATCCGATGATGAGTACC CTGGGATCAGAGGCTGTCTTATAG	58^{oC}, 1:00 min
p55	CAAGTGCTTGGGGTTCAGGG CGTCCTGGAGAAAGGGAAAG	60 °C, 0:30 min

3.3 Induction of Colitis-Associated Cancer

AOM/DSS colitis-associated cancer (CAC) was induced according to previously published protocols (Neufert, Becker et al. 2007). 8–10-week-old mice received a single intraperitoneal injection of AOM (10 mg/kg) (Sigma) followed by three cycles of 2.5% DSS (MW: 36,000–50,000 Da, MP Biomedicals) in the drinking water. Each DSS cycle was followed by 14 days of regular water. Colitis was monitored by measuring weight loss. At the end of the protocol, mice were sacrificed, the colon was resected, and its length was measured as an indicator of colitis severity. The number of macroscopically visible tumors was counted.

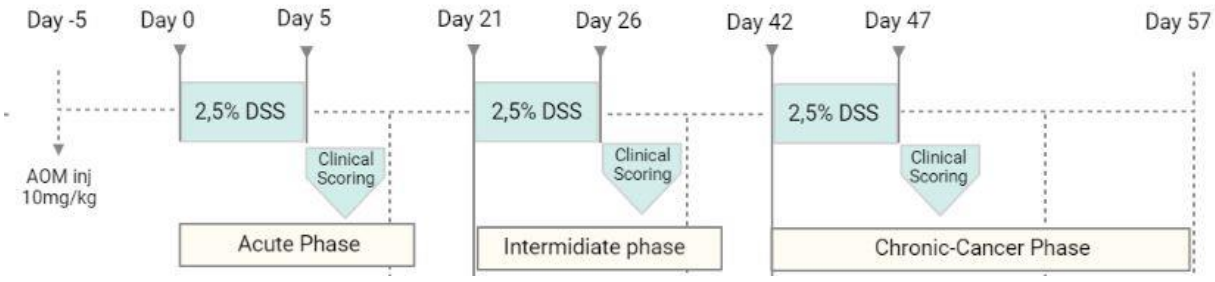

Figure 11 Graphical representation of AOM/ DSS protocol timeline.

3.4 Fluorescence-Activated Cell Sorting (FACS) Analysis and Sorting

Colonic tissues or colonic tumors were dissected, washed with HBSS (Gibco, Waltham, MA, USA), containing antibiotic antimycotic solution (Gibco), and digested. Epithelial cells were removed by incubating tissue with 5 mM EDTA and 1 mM DTT in HBSS for 25 minutes. The colonic tissue was digested in 300 U/mL Collagenase XI (Sigma-Aldrich) and 1 mg/mL Dispase II (Roche) in DMEM for 60 min at 37 °C. The colonic tumors were digested in 1000 U/mL Collagenase IV (Sigma- Aldrich), 1 mg/mL Dispase II (Roche), and 100 U/mL Dnase I (Sigma-Aldrich) in DMEM (BioSera) in three serial 20-min digestions to increase cell viability. The cell suspension was centrifuged, washed three times in FACS buffer (5% FBS (Biowest) in PBS), and cells were counted. For staining, 1–2 million cells/100 µL were incubated with the antibodies shown in **Table 3**. Propidium Iodide (Sigma, St. Louis, MO, USA) was used for live-dead cell discrimination. Sample analysis was performed using the FACSCanto II flow cytometer (BD, San Jose, CA, USA) or the FACSAria III cell sorter (BD) and the FACSDiva (BD) or FlowJo software (v10.2, FlowJo, LLC). Cultured IMCs isolated from Col6a1mTmG mice were used for sorting at passage 2 based on their green fluorescent protein (GFP) or Tomato fluorescent protein expression using a FACSAria III Cell Sorter (BD Biosciences). Cells were grouped as Col6a1+ and Col6a1−IMCs for subsequent experiments.

Table 3 Antibody list

Antigen	Conjugate	Clone/Cat. Number	Company	Use	
CD45	A700	30-F11	BioLegend	FC	Lineage-Antibodies for 3’ mRNA sequencing
CD326 (EpCAM)	APC-efluor780	G8.8	eBioscience	FC	
Ter119	APC-efluor780	TER-119	eBioscience	FC	
CD31	APC/Fire 750	390	BioLegend	FC	
CD45	APC-Cy7	30-F11	BioLegend	FC	Lineage-Antibodies for scRNAseq
CD326 (EpCAM)	APC-efluor780	G8.8	eBioscience	FC	
CD31	PerCP	390	BioLegend	FC	
αSMA	FITC	1A4	Sigma	FC	
PDGFRα	APC	APA5	BioLegend	FC	
CD146 (MCAM)	PeCy7	ME-9F1	BioLegend	FC	
IL-6	PE	MP5-20F3	BioLegend	FC	
CD201	PE/Cy5	eBio1560	Invitrogen	FC	
CD11b	FITC	M1/70	BioLegend	FC	
CD11c	PE/Cy7	N418	BioLegend	FC	

CD19	A700	eBio1D3 (1D3)	eBioscience	FC	
CD4	A700	RM4-5	eBioscience	FC	
CD8a	APC	53-6.7	BioLegend	FC	
Gr-1	A647	RB6-8C5	BioLegend	FC	
F4/80	PE	BM8	eBioscience	FC	
αSMA	unconjugated	1A4	Sigma, St. Louis	IHC	
anti-Phospho-STAT3	unconjugated	Tyr705	Cell Signaling	IHC	
Anti-mouse-IgG	A647	A-21235	Invitrogen	IHC	Secondary
Anti-rabbit-IgG	Biotinylated		Vector Laboratories	IHC	Secondary

3.5 3' RNA Sequencing and Analysis

RNA from FACS-sorted cells was isolated using the Single Cell RNA Purification kit (Norgen), according to the manufacturer's instructions. RNA from tumors and intestinal tissue or was isolated using the RNeasy mini or micro kit (QIAGEN), according to the manufacturer's instructions. 3'RNA sequencing and analysis were performed as previously described (Melissari, Henriques et al. 2021). In more detail, the quantity and quality of RNA samples were analyzed using the Agilent RNA 6000 Nano on an Agilent bioanalyzer. RNA samples with RNA Integrity Number (RIN) > 7 were used for library preparation using the 3' mRNA-Seq Library Prep Kit Protocol for Ion Torrent (QuantSeq− LEXOGEN™), according to the manufacturer's instructions. The quantity and quality of libraries were assessed using the DNA High Sensitivity Kit in the bioanalyzer, according to the manufacturer's instructions (Agilent). Libraries were pooled and templated using the Ion PI IC200 Chef Kit (ThermoFisher Scientific) on an Ion Proton Chef Instrument or Ion One Touch System. Sequencing was performed using the Ion PITM Sequencing 200 V3 Kit and Ion Proton PI™ V2 chips (ThermoFisher Scientific) on an Ion Proton™ System, according to the manufacturer's instructions. The RNA-Seq FASTQ files were mapped using TopHat2 (version 2.1.1) (Kim, Pertea et al. 2013), with default settings and using additional transcript annotation data for the mm10 genome from Illumina iGenomes (hnps://support.illumina.com/sequencing/sequencing_ software/igenome.html, accessed on March 2019). According to the Ion Proton manufacturer's recommendation, the reads, which remained unmapped, were submitted to a second round of mapping using Bowtie2 (version 1.3.1)

(Langmead and Salzberg 2012) against the mm10 genome with the very-sensitive switch turned on and merged with the initial mappings. Through the metaseqr R package (version 4.3.2) (Moulos and Hatzis 2015), GenomicRanges and DESeq were employed to summarize the bam files of the previous step to read counts table and to perform differential expression analysis (after removing genes that had zero counts over all the RNASeq samples).

Downstream bioinformatics analysis and visualization were performed using InteractiveVenn for Venn diagrams (www.interactivenn.net, accessed on October 2023) (Heberle, Meirelles et al. 2015) and Metascape (metascape.org, accessed on October 2023) for network plots (Zhou, Zhou et al. 2019). Heatmaps were generated in R using an in-house developed script utilizing the package pheatmap (version 1.0.12, hnps://cran.rproject.org/web/packages/pheatmap/index.html) (Core Team 2014). We used the Log2-transformed normalized counts of genes for each replicate and performed a z-score transformation for each gene across conditions. Finally, the clustering of gene expression was performed based on pheatmap's default settings. RNA-seq datasets have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev et al. 2002) and are accessible through the GEO Series accession number GSE247089.

3.6 10x library preparation and scRNA sequencing analysis

Sorted stromal cells (Live, CD45-, EpCAM-) were obtained from normal intestinal tissues and mouse intestinal tumors as described in FACs analysis and sorting. To diminish sex bias and biological variation in the analyses, X mice of both sexes were mixed for each sample. Single-cell RNA libraries were prepared according to the 10X Chromium Single Cell 3' protocol. FASTQ files were sent directly from the sequencing company. The final number of reads reached 240,938,543 bp for the AOM/DSS and 237,166,873 bp for the control colon, respectively. Correspondingly total reads reached 544,517,330 bp for the $Apc^{min/+}$and 210,226,057 bp for the control small intestine. Reads were then aligned to the mouse reference genome (mm10, 10X-reference genomes, 2020 version). The steps of read alignment, UMI counting, filtering of empty droplets and creation of sample count matrices were performed using the 10X Genomics Cell Ranger pipeline (cellranger-7.1.0) by utilizing the command cellranger count with default parameters. Since not all samples

were handled at the same day and were not sequenced in the same lane, technical variability (batch effect) could be present in the dataset.

Downstream analysis was performed using the functions of the Seurat package as described below. To compare the experiments, the samples of the two tumor models were merged into a single object based on the intersection of genes expressed in at least 3 cells. Afterwards, cell library normalization was performed using the NormalizeData function with the “Log-Normalise” method and a scaling factor equal to 10,000. To identify the 2000 most variable genes, the FindVariableFeatures function was applied with default parameters. The scaling of gene expression values was achieved by the scaleData function by regressing out the n_UMIs and mt% variables. Finally, principal component analysis (PCA) on scaled values of the most highly variable genes, as identified in the previous step, was performed by the runPCA function. The number of principal components were set to 40, a decision that was based on the corresponding elbow plot showing the variance explained by each principal component.

After exploring the datasets separately, we concluded that batch effect correction was needed to perform an appropriate clustering. To perform batch effect correction, we utilized the command RunHarmony from the Harmony package (Korsunsky, Millard et al. 2019) with the parameters theta equal to 2, sigma equal to 0.1 and the data grouped by the four different samples (colon, AOM/DSS, Small intestine, $APC^{+/min}$).

Cell clustering was approached with a graph-based method based on the construction of a k-nearest neighbor graph of the cells and the utilization of the Louvain community detection algorithm. To achieve this, FindNeighbors and FindClusters functions were used, the first with the parameter dims set to the range 1:40 and the second with the parameter resolution set to 0.3. Finally, UMAP, which is a non-linear dimensionality reduction method, was used for cell visualization and exploration in 2D through the function of seurat runUmap using the optimal number of PCs 1:40.

To identify specific marker genes for each cluster that are conserved across each batch, we utilized the command FindConservedMarkers which is based on a Wilcoxon rank sum test and corrected p-values according to the Bonferroni correction, using all features in the dataset excluding those that exhibit an absolute value of average log fold change less than 0.25. Each such comparison is performed for the cells of each cluster and includes a separate comparison for each batch, against

the rest of the cells of the same batch. The marker genes are filtered based on a batch-combined p-value so that batch-specific markers are excluded. Ucell (Andreatta and Carmona 2021) was used to evaluate, compare, and visualize the expression of gene signatures across cell clusters.

3.7 Isolation and Culture of Primary Mouse Intestinal Mesenchymal Cells

Colons from 6- to 10-week-old mice were isolated, flushed, and washed with ice-cold HBSS (Gibco). The epithelial layer was removed after treatment with pre-warmed 5 mM EDTA (Acros Organics) and 1 mM DTT (Sigma) in HBSS for 20 min at 37 °C. The remaining colonic tissue was then digested using 300 u/mL Collagenase XI (Sigma) and 0.1 mg/mL Dispase II (Roche) for 60 min at 37 °C. Samples were filtered through a 70 µm cell strainer, centrifuged, and the cell pellet was resuspended in complete culture DMEM medium (BioSera) supplemented with 10% FBS (Biowest), 100 U/mL penicillin/100 mg/mL streptomycin (Gibco), 2 mM L-Glutamine (Gibco, Waltham), 1 µg/mL amphotericin B (Sigma), and 1% non-essential amino acids (Gibco, Waltham). Cells were plated in culture flasks and passaged 3–4 times.

3.8. Tumor Organoids and Co-Culture with IMCs

Tumor organoids were isolated from AOM/DSS-induced and $Apc^{min/+}$ tumors and cultured following already published protocols (Xue and Shah 2013). In more detail, colonic tumors were isolated, washed with PBS, treated with chelation buffer (2 mM EDTA, 43.4 mmol/L sucrose, 54.9 mmol/L D-sorbitol, 0.5 mmol/L DL-dithiothreitol in distilled water), and digested using 200 U/mL type IV collagenase, 125 µg/mL type II dispase in DMEM for 2 h at 37 °C. 10.000 tumor fragments were plated at 24-well plates in 30 µL of Matrigel (Cat. No. 356255, Corning). After the Matrigel was polymerized for 15 min at 37°C, 500 µl/well complete basal culture medium was added (1 unit/ml of penicillin, 1 µg/ml of streptomycin, and 2.5 ng/ml of amphotericin B, 10 mmol/L HEPES (Gibco), 2mM Glutamax (Gibco), 1x N2 supplement (Life Technologies 1x), 1x B27 supplement (Life Technologies 1x), 1 mmol/L N-acetylcysteine (Sigma-Aldrich), 50 ng/ml murine EGF (Life technologies) in Advanced Dulbecco's Modified Eagle Medium/F12 (Gibco)). After passaging tumoroids at a 1:4 ratio, they were mixed with 15.000 sorted cultured Col6a1+ or Col6a1− IMCs in 30 µL Matrigel (Corning) in 48 well plates and co-cultured for 72 h. Images were acquired with the

Zeiss Axio Observer Z1 microscope. Organoid measurements were performed using the Fiji/ImageJ software (version 1.53).

3.8 Caco-2 Co-Culture Assay

The Caco-2 co-culture assay was performed following already published protocols (Shaker, Swietlicki et al. 2010). In more detail, cultured IMCs were sorted based on GFP expression and plated in 48 well plates. After forming a monolayer, 37.500 Caco-2 colon cancer cells were added and cultured in 48 well plates for 72 h. Images were acquired with the Zeiss Axio Observer Z1 microscope. Colony size was measured using the ImageJ/Fiji software (version 1.53).

3.9 Allografts

Col6a1-mTmG mice (6 mice/experiment) were sacrificed at a 6–10-week-old age and their colons were dissected and processed, as described in the FACS analysis section. Epithelial, endothelial, immune, and erythroid cells were excluded through negative selection as EpCAM-, CD31-, CD45-, Ter119 cells. Lineage negative cells were sorted based on their GFP/Tomato expression, as Col6a1^{+} and Col6a1^{-} IMCs. Subcutaneous injections were performed in 6-week-old mice with 10.000 MC38 mouse colon cancer cells mixed with 100.000 Col6a1^{+} or Col6a1^{-} IMCs in 100 µL DMEM containing 100 U/mL penicillin/100 mg/mL streptomycin (Gibco), 2 mM L-Glutamine (Gibco), and 1% non-essential amino acids (Gibco, Waltham, MA, USA). 10.000 MC38 cells alone were also injected as controls. Each experimental group included 8 mice. After 15 days, the mice were sacrificed, the allografts were removed, and their width and length were measured using a caliper. Tumor volume was calculated using the ellipsoid volume formula (π/6) x width x length2 (Tomayko and Reynolds 1989).

3.10 Immunohistochemistry

For histopathology, colon and small intestine were fixed overnight in 10% formalin and embedded in paraffin (Medite, TBS88 paraffin embedding system). 4-µm sections were cut (SLEE MEDICAL), mounted on slides and stained with hematoxylin and eosin (Sigma-Aldrich). Colitis and inflammation score was performed on H&E-stained sections according to the parameters described in the Table 5 below. Images were acquired at Nikon E800 upright microscope.

Table 4 Inflammation scoring system

Severity of inflammation	0: rare cells in mucosa, 1: increased cells in lamina propria 2: confluence of cells in the submucosa 3: transmural inflammation
Crypt damage	0: intact crypts, 1: basal one-third damaged, 2: basal two-thirds damaged, 3: only surface epithelium intact
Ulceration	0: absence of ulcers 1: 1 or 2 ulcers 2: 3 or 4 ulcers 3: more than 4 ulcers — extensive ulceration
Percent involvement	1: 1%–25% 2: 26%–50% 3: 51%–75% 4: 76%–100%

The inflammation index was determined by adding the severity of inflammation and the percentage of involvement, ranging from 0 to 7. The tissue damage index was evaluated by combining the degree of crypt damage, ulceration, and percentage of involvement, with a scale ranging from 0 to 10.

For immunohistochemistry, formalin-fixed paraffin-embedded (FFPE) sections underwent de-paraffinization with Xylene and hydration in 100% and 70% ethanol. Antigen retrieval was achieved using heat-mediated Citrate buffer (pH 6.0) for 20 minutes, and the slides were allowed to cool. Subsequently, slides were incubated at in 3% hydrogen peroxide in PBS for 15 minutes to block endogenous peroxidase activity. Tissue sections were washed and blocked with 1% BSA in PBS for 1 hour, followed by overnight incubation with the primary antibody against phospho-STAT3 (1:100, Cell Signaling) at 4°C. A secondary biotinylated antibody targeting rabbit IgG (Vector) was added for 1 hour at room temperature, followed by the Vectastain ABC kit for 30 minutes at room temperature. Peroxidase activity was visualized using ImmPACT DAB Peroxidase (HRP) Substrate

(Vector Laboratories). The sections were counterstained with hematoxylin (Merck), dehydrated through incubation in 70% and 100% ethanol, permeated with xylene, and preserved in mounting medium (Sigma). Images were acquired at Nikon E800 upright microscope.

For immunofluorescence, -intestinal tumors and control samples were isolated, fixed with 4% PFA/PBS overnight, and serially immersed in sucrose solutions (15% and 30% in PBS). Tissues were then embedded in OCT (VWR Chemicals), and cryosections (10µm) were prepared using the LEICA CM1950 cryotome. Stainings were performed using the unconjugated anti-αSMA antibody (1:100, Sigma) and an anti-mouse-A647 secondary antibody (1:500, Invitrogen). A mounting medium containing DAPI (Sigma-Aldrich) was used to stain the nuclei. Images were acquired with a Leica TCS SP8X White Light Laser confocal system.

3.11 Assessment of proliferation

Evaluation of proliferation was conducted by administering mice an intraperitoneal injection (i.p.) of 100 mg/kg BrdU (Roche) 90 minutes prior to sacrifice. Subsequently, formalin-fixed paraffin-embedded (FFPE) tissue sections underwent staining with the BrdU Proliferation detection kit (BD Biosciences), and the tissues were counter-stained with hematoxylin.

3.12 Proteome Profiling

For cytokine determination assays, primary intestinal fibroblasts were plated, serum-starved overnight, and stimulated with 2 mg/mL lipopolysaccharide (LPS) from E. coli (Sigma, St. Louis, MO, USA), 10 ng/mL IL-1β (Peprotech), and 10 ng/mL TNF (Sigma, St. Louis, MO, USA) for 24 h. The expression of a variety of secreted mediators was assessed using the Proteome ProfilerTM Array (R&D Systems Catalog Number: ARY028) according to manufacturer's instructions. Quantification of signal intensity was performed using the ChemiDoc XRS+ instrument and the Image Lab software (version 5.2, Bio-Rad).

3.13 MIP-2 Elisa

Primary intestinal fibroblasts were plated, serum-starved overnight, and stimulated with 10 ng/mL IL-1β (Peprotech), 10 ng/mL TNF (Sigma), and 2 mg/mL LPS from E. coli (Sigma, St. Louis, MO,

USA). Supernatants were collected at 24 h, and MIP-2 quantification was performed using the mouse MIP-2 ELISA kit (Peprotech), according to the manufacturer's instructions.

3.14 IL-6 Elisa

Primary small intestinal fibroblasts were plated, serum-starved overnight, and stimulated with 2 mg/ml LPS from E. coli (Sigma), 10ng/ml IL1b (Peprotech), IL33 (MBL), IL18 (R&D systems) and the mouse TLR1-9 Agonist kit (Invivogen). The quantities used for the TLR agonists were 100ng/ml Pam3CSK4 (TLR1/2), 2x107/ml HKLM cells (TLR2), 10 mg/ml poly(I:C) (TLR3), 100ng/ml FLA-ST (TLR5), 100ng/ml FSL1 (TLR6/2), 1 mg/ml ssRNA/Lyovec (TLR7) and 1mM ODN1826 (TLR9) Supernatants were collected at 24h and IL-6 quantification was performed using the mouse IL-6 Duo-Set ELISA kit, according to the manufacturer's instructions (R&D Systems)

3.15 RNA Isolation and qRT-PCR

RNA was isolated from tumors using the RNeasy mini or micro kit (QIAGEN), according to the manufacturer's instructions. One to three micrograms of total RNA were reverse transcribed using M-MLV reverse transcriptase (Sigma-Aldrich) and oligo-dT primers (Promega) according to the manufacturer's protocol. The cDNA was subsequently used for qRT-PCR using the Platinum SYBR-Green qPCR SuperMix (Invitrogen). Forward and reverse primers were added at a concentration of 0.2 pmol/ml in a final volume of 20 µl. The CFX96 Touch Real-Time PCR Detection System (Biorad). Quantification was performed with the DDCt method. Primer sequences (50-30) are listed below in Table 6.

Table 5 List of qRT-PCR primers.

Gene	Primer (5'-3')	Size (bp)	Anneal. Tem.	Reference
Ptgs2	F: TGAGCACAGGATTTGACCAG R: CCTTGAAGTGGGTCAGGATG	150	58	(Salcedo, Worschech et al. 2010)
Il6	F: GTTCTCTGGGAAATCGTGGA R: TCCAGTTTGGTAGCATCCATC	138	59	(Salcedo, Worschech et al. 2010)
Il11	F: AACTGTGTTTGTCGCCTGGT R: CGTCAGCTGGGAATTTGTCT	199	59	(Salcedo, Worschech et al. 2010)
Cd44	F: TTATCCGGAGCACCTTGGCCAC	143	59	

	R: TGCACTCGTTGTGGGCTCCTGAG			
Tnf	F: CACGCTCTTCTGTCTACTGA R: ATCTGAGTGTGAGGGTCTGG	110	55	
Mmp7	F: GCTGCCACCCATGAATTTGGCC R: GGACCCAGTGAGTGCAGACCG	209	59	
Cxcl1	F: CGCACGTGTTGACGCTTCCC R: TCCCGAGCGAGACGAGACCA	105	59	
Igf1	F: GGGAGATGCAAAGGCCTCCCC R: ACCAGGACTCCCAAATCCCTAGCC	142	56	
Igfbp5	F: ACGGCGAGCAAACCAAGATA R: GAGGGCTTACACTGCTTTCT	382	55	(Ding, Bruick et al. 2016)
Mmp10	F: CACAAGCCCAGCTAACTTCC R: TTTGTCTGGGGTCTCAGGTC	136	59	(Salcedo, Worschech et al. 2010)
Hprt	F: TGCCGAGGATTTGGAAAAAGTG R: CACAGAGGGCCACAATGTGATG	116	55	

3.16 Statistical Analysis

Data were analyzed using the GraphPad Prism v8 software. Statistical significance was calculated by Student's t-test, and p values ≤ 0.05 were considered significant. Data are presented as mean ± SD.

4. Results

We have previously shown that innate immune activation of intestinal mesenchymal cells (IMCs) can drive their pro-tumorigenic functions in the intestine through downstream NF-κB and MAPK activation (Koliaraki, Pasparakis et al. 2015, Henriques, Koliaraki et al. 2018). Notably, using the Col6a1Cre strain, we showed that in vivo deletion of inhibitor of nuclear factor kappa-B kinase subunit b (IKKβ), a key nuclear factor kappa-beta (NF-κB) regulator, led to reduced colon inflammation and associated carcinogenesis in mice (Koliaraki, Pasparakis et al. 2015). However, deletion of IKKβ in a broader population of mesenchymal cells targeted by the Col1a2CreER mouse had the opposite effect, suggesting diverse properties of distinct CAF subsets (Pallangyo, Ziegler et al. 2015, Wagner 2016). Further analysis indeed showed that the Col6a1Cre strain targets a fraction of IMCs in homeostasis and CAFs in intestinal cancer (Koliaraki, Pasparakis et al. 2015, Melissari, Henriques et al. 2021). In more detail, Col6a1Cre mice target the majority of PDGFRαhi fibroblasts and pericytes in the colon, which actively contribute to intestinal morphogenesis during development, as well as epithelial cell proliferation and differentiation in homeostasis (Melissari, Henriques et al. 2021). However, a detailed characterization of Col6a1+ cells and their potential immunoregulatory role in cancer is still missing. Therefore, in this thesis we focused our analysis on 1) the description of the cellular fate and properties of Col6a1+ cells in intestinal tumors and 2) the delineation of their innate-driven functions and impact on intestinal carcinogenesis.

4.1. CAFs partly maintain fibroblast identities upon AOM/DSS intestinal carcinogenesis

To define the properties of Col6a1+ cells in intestinal carcinogenesis, we crossed Col6a1Cre mice with the TdTomato-to-GFP replacement (mTmG) reporter strain (hereafter Col6a1-mTmG) and subjected them to the AOM/DSS model of CAC (Neufert, Becker et al. 2007). We isolated colonic adenomas, and after exclusion of immune, endothelial, erythroid, and epithelial cells, using the lineage negative (Lin^-) markers CD45, CD31, Ter119, and EpCAM, respectively, we isolated $Col6a1^+/GFP^+$ and $Col6a1^-/GFP^-$ (or $Tomato^+$) CAFs by FACS sorting and performed 3’ mRNA

sequencing (Figure 12A, B). Results were compared with previously published data of Col6a1^{+} and Col6a1^{-} cells in homeostasis (Melissari, Henriques et al. 2021).

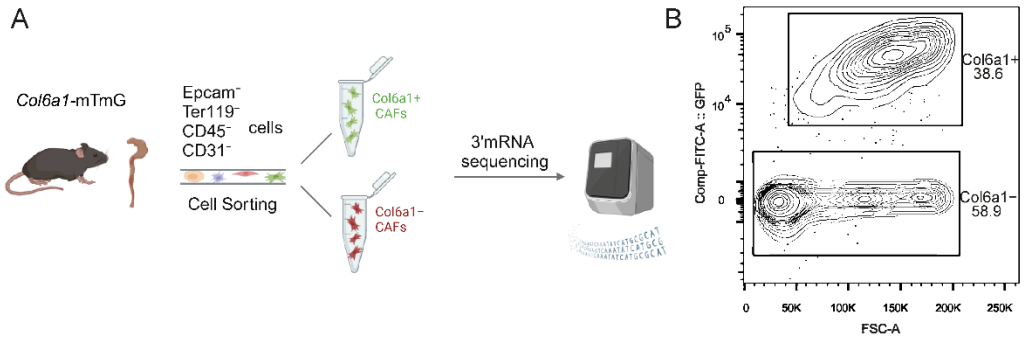

Figure 12 Graphical representaion and gating strategy followed for bulk RNA sequencing. *(A) Schematic showing the procedure for bulk RNA sequencing of fibroblasts isolated from AOM/DSS-induced adenomas (prepared using Biorender.com). A total of three samples were used for bulk RNA sequencing. Each sample originated from a pool of tumors from five to six mice. (B) FACS plot showing the sorting strategy for Col6a1^{+} and Col6a1^{-} CAFs.*

To examine whether the transcriptomic identity of IMCs is maintained in CAC, we assessed the expression of a selected gene signature, previously described for Col6a1^{+} and Col6a1− cells in homeostasis (Melissari, Henriques et al. 2021). This analysis showed that Col6a1^{+} and, to a lesser extent, Col6a1− CAFs maintain some of their physiological characteristics (Figure 13). Col6a1^{+} CAFs, in particular, showed increased expression of telocyte markers (*Foxl1*), BMPs (*Bmp3*, *Bmp5*), and Wnt signaling regulators (*Wnt5a*), which play a significant role in homeostatic epithelial cell differentiation (Helms, Onate et al. 2020, McCarthy, Kraiczy et al. 2020, Melissari, Henriques et al. 2021). Conversely, Col6a1− CAFs sustained their increased expression of stem cell maintenance mediators and trophocyte markers, such as *Wnt2*, *Cd34*, and *Pi16* (Figure 12) (Helms, Onate et al. 2020, McCarthy, Kraiczy et al. 2020, Melissari, Henriques et al. 2021). It should be noted that this subset includes all trophocytes and the majority of PDGFRαlo fibroblasts, which we cannot distinguish in these experiments.

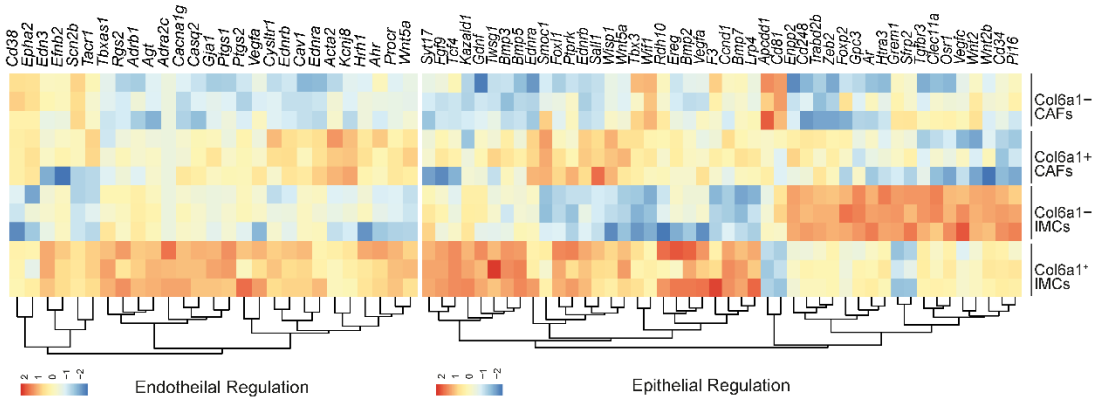

Figure 13 Col6a1+ CAFs and normal fibroblasts display transcriptional similarities. *Heatmap showing gene expression signatures of Col6a1⁺ and Col6a1⁻ fibroblasts in homeostasis (IMCs) and CAC (CAFs). Log2-transformed normalized read counts of genes for each replicate are shown. Red denotes high expression, and blue denotes low expression values. Read counts are scaled per column.*

Notably, Col6a1⁺ CAFs expressed CD201, which we have previously identified as a marker of PDGFRαhi fibroblasts and pericytes (Melissari, Henriques et al. 2021) and localized adjacent to neoplastic cells, similar to their normal counterparts (Figure 14A, B) (Melissari, Henriques et al. 2021).

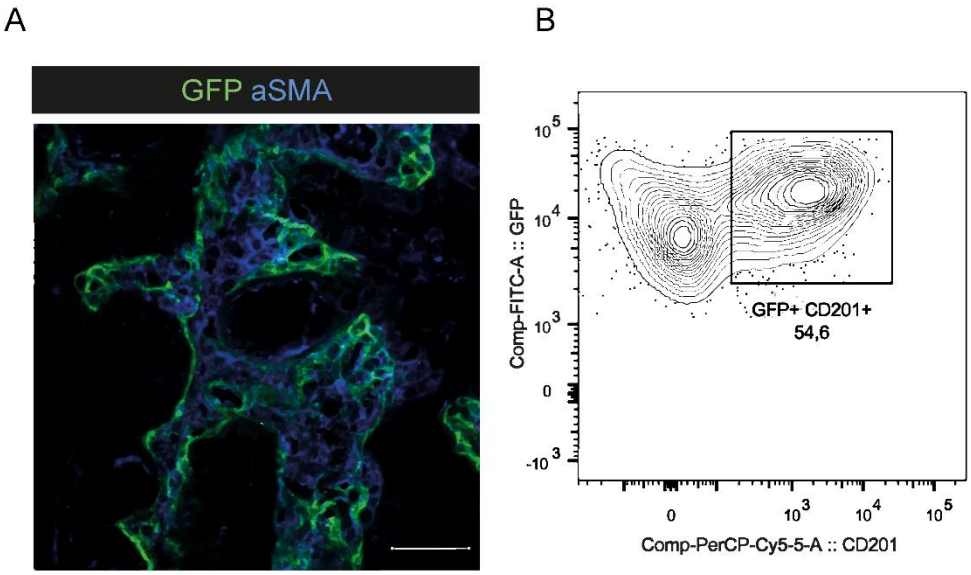

Figure 14 Col6a1⁺ CAFs and normal like fibroblasts show common features. *A) Immunohistochemistry for αSMA in AOM/DSS-induced adenomas of Col6a1-mTmG mice (n = 5 mice, Scale bar: 50 µm). B) Representative FACS analysis of CD201 expression in Lin⁻GFP⁺ cells in AOM/DSS-induced adenomas of Col6a1-mTmG mice (n = 2 mice).*

Overall, these results suggest that CAFs in AOM/DSS-induced adenomas originate from resident mesenchymal cells, including different fibroblast subsets and pericytes, and partly maintain their physiological properties.

4.2. Col6a1^{+} and Col6a1^{-} CAFs are activated in AOM/DSS colon cancer

Despite the maintenance of a minimum homeostatic gene expression signature in Col6a1^{+} and Col6a1− CAFs, deregulated gene expression analysis in comparison to homeostatic Col6a1^{+} and Col6a1− IMCs, respectively, showed significant alterations in the gene expression of both subsets. This analysis showed 1045 upregulated genes in the Col6a1^{+} CAFs and 1906 upregulated genes in the Col6a1− CAFs (Figure 15A, B). Comparison between the deregulated genes in the two subsets revealed both common and distinct transcriptional signatures during cancer (Figures 15C), which was further analyzed through pathway enrichment and network associations using Metascape.org (Zhou, Zhou et al. 2019) (Figures 15D–F).

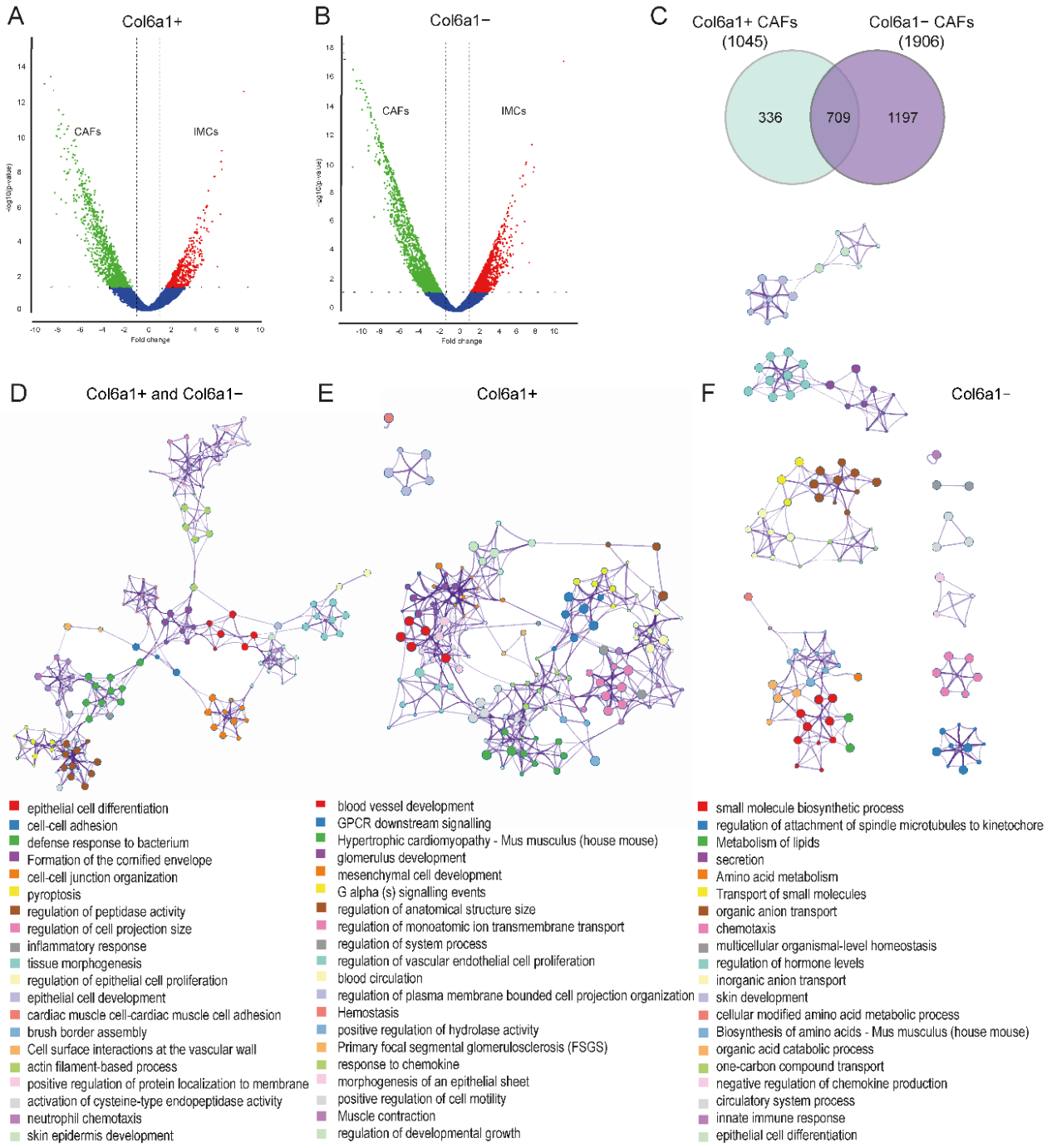

Figure 15 Col6a1⁺ and Col6a1⁻ CAFs are activated upon AOM/DSS colon carcinogenesis. *Volcano plots of deregulated genes in (A) Col6a1⁺ CAFs versus normal Col6a1⁺ IMCs and (B) Col6a1⁻ CAFs versus normal IMCs. (C) Venn diagram showing the differential and common up-regulated genes in Col6a1⁺ and Col6a1⁻ CAFs. (D) Network of enriched terms in Col6a1⁺ CAF and Col6a1⁻ CAF common upregulated gene signature. (E) Network of enriched terms in Col6a1⁺ CAF unique gene signature. (F) Network of enriched terms in Col6a1− CAF unique upregulated gene signature. Networks are colored by cluster ID, where nodes that share the same cluster ID are typically close to each other (generated through metascape.org).*

The upregulated gene signature shared between Col6a1^{+} and Col6a1^{-} CAFs (709 genes) was enriched in biological functions related to epithelial cell differentiation, proliferation, and development, indicating that both Col6a1^{+} and Col6a1^{-} CAFs can directly affect neoplastic cells and drive cancer growth. Accordingly, they can also modulate cell death pathways (formation of the cornified envelope, pyroptosis), whose inhibition is important for cancer progression. Terms associated with cell-cell adhesion and junction organization indicate that CAF activation results in increased cellular interactions, including those between CAFs (muscle-cell adhesion). Notably, several enriched terms (inflammatory response, neutrophil chemotaxis, and response to bacterium) further support the immunoregulatory role of CAFs in CAC and, more specifically, their functions in innate immune responses (Kalluri 2016, Koliaraki, Prados et al. 2020, Kobayashi, Gieniec et al. 2022) (Figure 15D)

Analysis of Col6a1^{+} CAF's uniquely upregulated genes revealed significant enrichment in pathways related to blood vessel development, vascular endothelial proliferation, blood circulation, and hemostasis, indicating that Col6a1^{+} CAFs play a significant role in tumor-associated angiogenesis (Figure 15E). Examples of genes enriched in this process include those encoding for integrins (*Itga7, Itga4, Itgb3*), molecules of the Notch signaling pathway (*Jag1, Hey2*), as well as *Rgs5* and Angpt4 that are typically expressed by pericytes and smooth muscle cells (Dasgupta, Ghosh et al. 2021). Other enriched pathways, specifically in Col6a1^{+} CAFs, include those involved in their activation, both towards a myofibroblastic (mesenchymal cell development, muscle contraction) and inflammatory phenotype (response to chemokine). Notably, genes associated with GPCR signaling are significantly upregulated, suggesting a role of GPCR in Col6a1^{+} fibroblast activation (Figure 15E).

Col6a1− CAFs were highly enriched in metabolic pathways, including the metabolism and biosynthesis of lipids (*Acadl, Alb, Cpt2*, etc.), amino acids (*Arg2, Cs, Gss,* etc.), organic acids (*Cftr, Slc26a3, Acsl1,* etc.), and small molecules (*Alox12, Cbs, Edn2,* etc.). They also expressed genes that are involved in the secretion and transport of small molecules (various solute carrier family members, *Apoc4, Heph*, etc.), suggesting increased secretory functions (Figure 15F). Other enriched terms included unique genes related to immune response and epithelial differentiation, further supporting the pro-tumorigenic role of Col6a1^{-} CAFs in CAC (Figure 15F).

These findings show that both Col6a1^{+} and Col6a1^{-} IMCs cells are activated in AOM/DSS-induced colitis-associated carcinogenesis to exert both similar and unique pro-tumorigenic functions.

Similar transcriptional profiles suggest an important role both in epithelial cell proliferation and in immune regulation. Unique transcriptional profiles highlight a significant role for Col6a1^{+} CAFs in tumor-associated angiogenesis and reveal an increased metabolic and secretory phenotype for Col6a1^{-} CAFs.[3]

4.3. CAFs from the Apc$^{min/+}$ model of carcinogenesis are transcriptionally similar to AOM/DSS CAFs

To further explore CAF heterogeneity across different mouse models, we used a similar strategy by crossing the *Col6a1^{mTmG}* mouse with the *Apc$^{min/+}$* mouse, which spontaneously develops a plethora of tumors in the small intestine after 4-5 months (Dove, Clipson et al. 1997). In both the AOM/DSS and the Apc$^{min/+}$ models, tumors are at the stage of adenomas/adenocarcinomas, but there are also significant differences. These include different driving mutations and downstream mechanisms, the role of inflammation and regeneration caused by dextran sodium sulfate in facilitating AOM/DSS model carcinogenesis, and the location of the tumors. AOM/DSS tumors are located mainly in the middle and distal colon, while Apc$^{min/+}$ tumors are found predominantly in the small intestine.

As we previously showed in our recent publication (Melissari, Henriques et al. 2021), the Col6a1Cre mouse targets a broader mesenchymal cell fraction in the small intestine compared to the colon. More specifically, through FACs analysis we were able to identify a Col6a1+GFP+ hi population and a Col6a1^{+}GFP^{+} low population which represented ~23% and ~29% of the Lin- cells respectively. Through expression analysis we identified that the Col6a1^{+}GFP$^{+\,hi}$ population shares many similarities with the Col6a1^{+} cells in the colon, whereas the Col6a1^{+}GFP^{+} low population represents a cell subset of PDGFRαlo fibroblasts. These cells could potentially be an intermediate

[3] Parts of this section (4.1, 4.2) have been recently published in an article in the international Journal of Molecular Sciences Chalkidi, N., M. T. Melissari, A. Henriques, A. Stavropoulou, G. Kollias and V. Koliaraki (2023). "Activation and Functions of Col6a1+ Fibroblasts in Colitis-Associated Cancer." Int J Mol Sci **25**(1).

cell state between Col6a1^{+} cells and Col6a1- cells or the result of non- specific cell recombination. Accordingly, Col6a1^{-} cells were fewer in the small intestine compared to the colon.

For our analysis, we isolated small intestinal tumors from Apc$^{min/+}$ mice and as previously described in the AOM/ DSS model (Chalkidi, Melissari et al. 2023) we excluded immune, endothelial, erythroid, and epithelial cells, using the lineage negative (Lin^{-}) markers CD45, CD31, Ter119 and EpCAM, respectively. We then isolated Col6a1^{+} and Col6a1^{-} CAFs by FACS sorting and performed 3' mRNA sequencing (Figure 16A). The comparative analysis of small intestinal CAF subsets was performed with the Col6a1^{+}GFP$^{+\,hi}$ and the Col6a1^{-} cells in homeostasis (Melissari, Henriques et al. 2021). (Figure 17A-B).

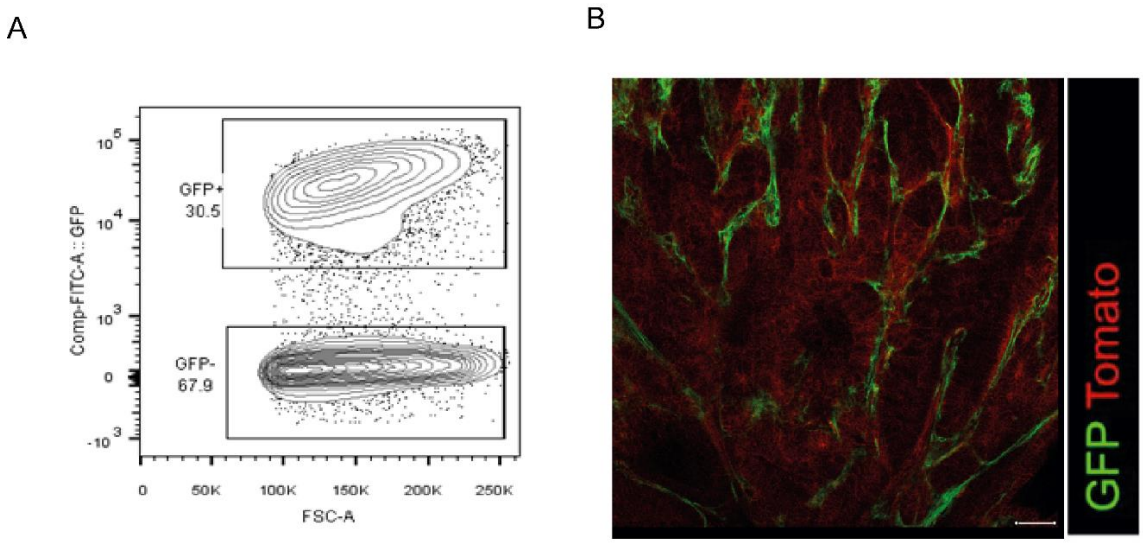

Figure 16 Col6a1^{+} CAF abundance in tumors isolated from the Apc$^{min/+}$ mouse model. *A) FACS plot showing the sorting strategy for Col6a1^{+}GFP^{+} and Col6a^{-}TOM^{+} CAFs. B) Fluorescence microscopy showing the presence of Col6a1^{+}, Col6a1^{-} CAFs inside Ap$^{cmin/+}$ intestinal tumors (Scale bar: 50 μm).*

Significant alterations were observed in the gene expression of both subsets with 1402 upregulated genes in the Apc-Col6a1^{+} CAFs and 1732 upregulated genes in the Apc-Col6a1^{-} CAFs (Figure 17A, B). Comparison between the deregulated genes in the two subsets revealed both common and distinct transcriptional signatures during cancer (Figures 17C), which were further analyzed through pathway enrichment and network associations using Metascape.org (Zhou, Zhou et al. 2019) (Figures 17D–F). Notably, enriched terms and genes significantly resembled the ones found in the AOM/ DSS cancer model.

More specifically, the common upregulated gene signature of Apc-Col6a1^{+} and Apc-Col6a1^{-} CAFs was enriched in pathways related to epithelial cell differentiation and proliferation, as well as

apoptotic signaling, indicative of the pro-tumorigenic role of these cells (Figure 17D). The unique gene signature of Apc-Col6a1^{+} CAFs was enriched in terms related to vascular development, smooth muscle cell development and MAPK signaling, highlighting the significance of these cells in tumor-associated angiogenesis and their potential activation towards both a myofibroblastic and inflammatory phenotype. Analysis of the Apc-Col6a1^{-} CAF unique gene signature revealed terms associated with metabolism pathways (lipid metabolism and monocarboxylic acid metabolic process) and epithelial cell differentiation, supporting a pro-tumorigenic role in mouse intestinal cancer.

Overall, these findings suggest that Col6a1^{+} and Col6a1^{-} CAFs in the Apc$^{min/+}$ model become activated in a similar manner to their counterparts in the AOM/ DSS model, supporting similar functions of the two subsets in driving carcinogenesis across tissues and experimental models.

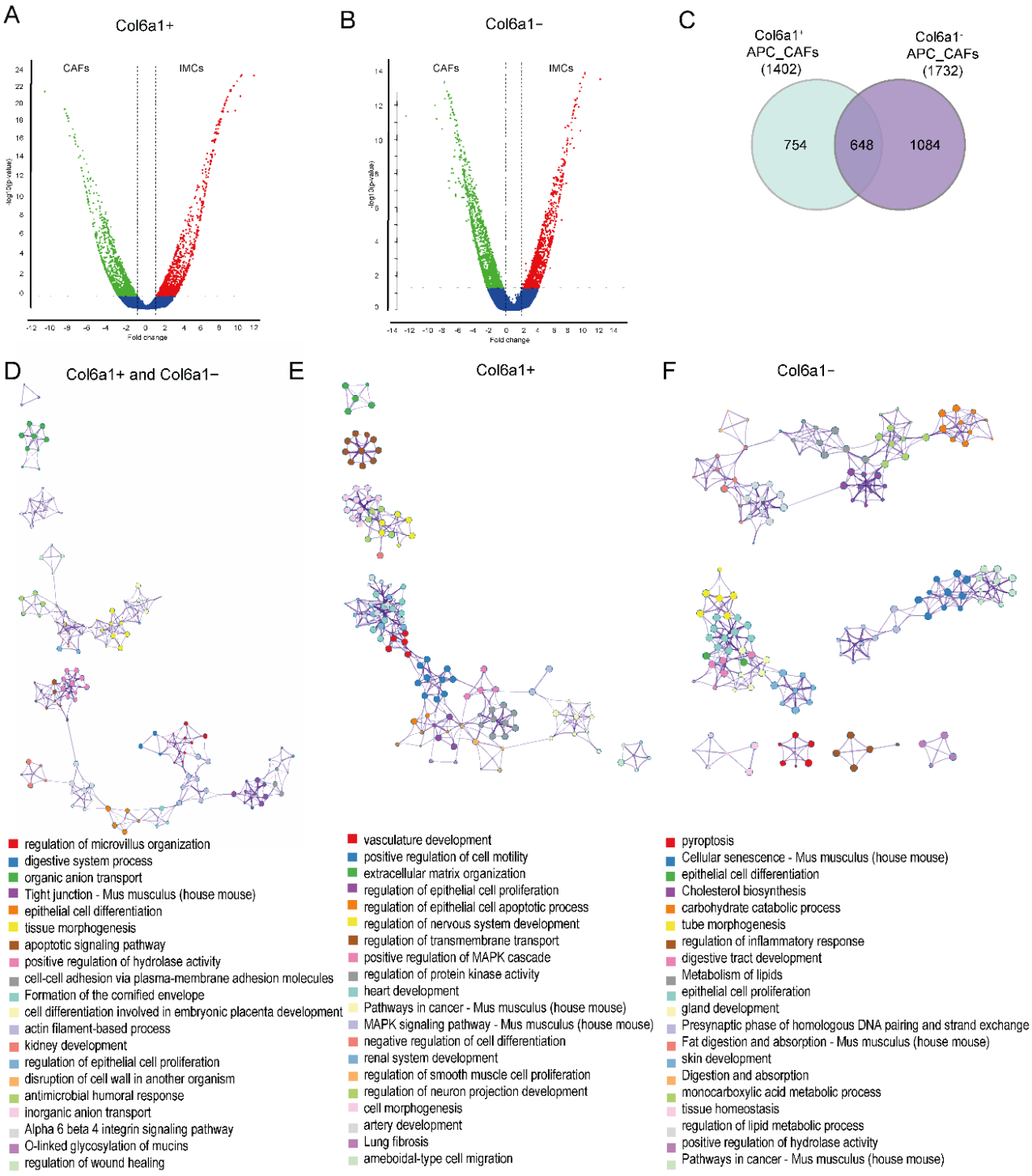

Figure 17 Col6a1+ and Col6a1- CAFs are activated in the Apc$^{min/+}$ model of spontaneous carcinogenesis. *Volcano plots of deregulated genes in A) Col6a1^{+} CAFs versus normal Col6a1^{+} IMCs and B) Col6a1^{-} CAFs versus normal IMCs. C) Venn diagram showing the differential and common upregulated genes in Col6a1^{+} and Col6a1^{-} CAFs. D) Network of enriched terms in Col6a1^{+} CAF and Col6a1^{-} CAF common upregulated gene signature. E) Network of enriched terms in Col6a1^{+} CAF unique gene signature. F) Network of enriched terms in Col6a1^{-} CAF unique upregulated gene signature. Networks are colored by cluster ID, where nodes that share the same cluster ID are typically close to each other (generated through metascape.org).*

Interestingly, the similar gene signatures of Col6a1^{+} CAFs in the two models of intestinal tumorigenesis pointed towards a potential regulation of tumor blood vessel function and angiogenesis. For this reason, we also compared the genes enriched in Col6a1^{+} CAFs with a mural gene signature (Muhl, Genové et al. 2020). We found that 30 of the 45 genes constituting a pan-tissue mural gene signature where specifically enriched in these cells, suggesting a perivascular role of Col6a1^{+} CAFs in intestinal carcinogenesis (Figure 18).

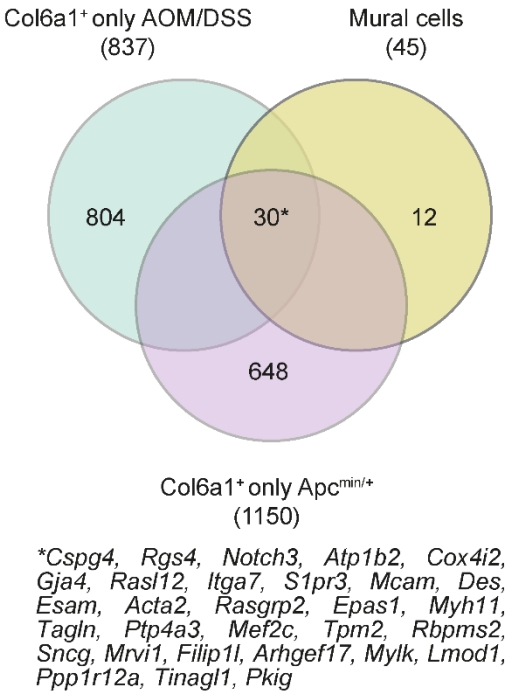

Figure 18 Venn diagram showing common gene expression between Col6a1^{+} CAFs in the AOM/DSS and the Apc$^{min/+}$ models compared to mural cells (Muhl, Genové et al. 2020).

4.4. CAFs are heterogenous in mouse intestinal tumors

To further delineate the stromal heterogeneity in intestinal tumors and define the specificities of Col6a1+ CAFs, we performed single cell transcriptomic analysis of stromal cells from the two mouse models of intestinal carcinogenesis. We used a similar isolation strategy as in the bulk transcriptomic analysis but included all CD45-EpCAM- stromal cells (Figure 19). The isolated fraction comprised ~2% of the single live cells derived from the digestion of the intestinal tumors

of either model. Subsequently, we prepared single-cell RNA libraries using the 10x Genomic platform and performed scRNAseq (Figure 19).

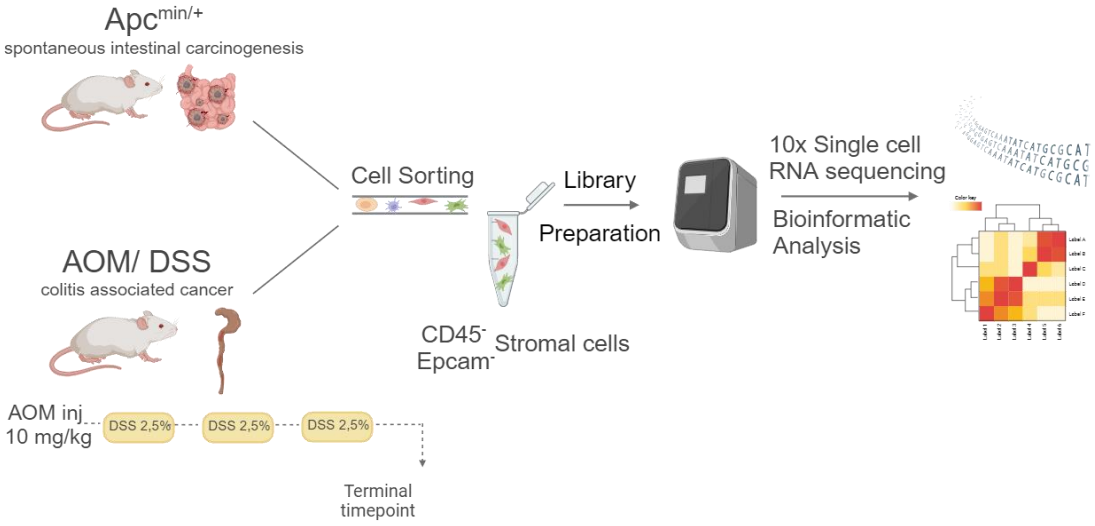

Figure 19 Graphical representation of our scRNA sequncing sample preparation .

Our analysis included the two tumor samples and their control tissue, colon and small intestine, respectively. Samples were integrated using Harmony (Figure 20B) (Korsunsky, Millard et al. 2019). Glial cells (*Gfap*$^{+}$*S100b*$^{+}$), interstitial cells of Cajal (*Kit*$^{+}$ *Ano1*$^{+}$), as well as any remaining immune cells were excluded resulting in 28.236 stromal cells, which were used for subsequent bioinformatic analysis. UMAP visualization revealed clusters of known cell types, including blood and lymphatic endothelial cells, pericytes, smooth muscle cells, and fibroblasts (Figure 20A).

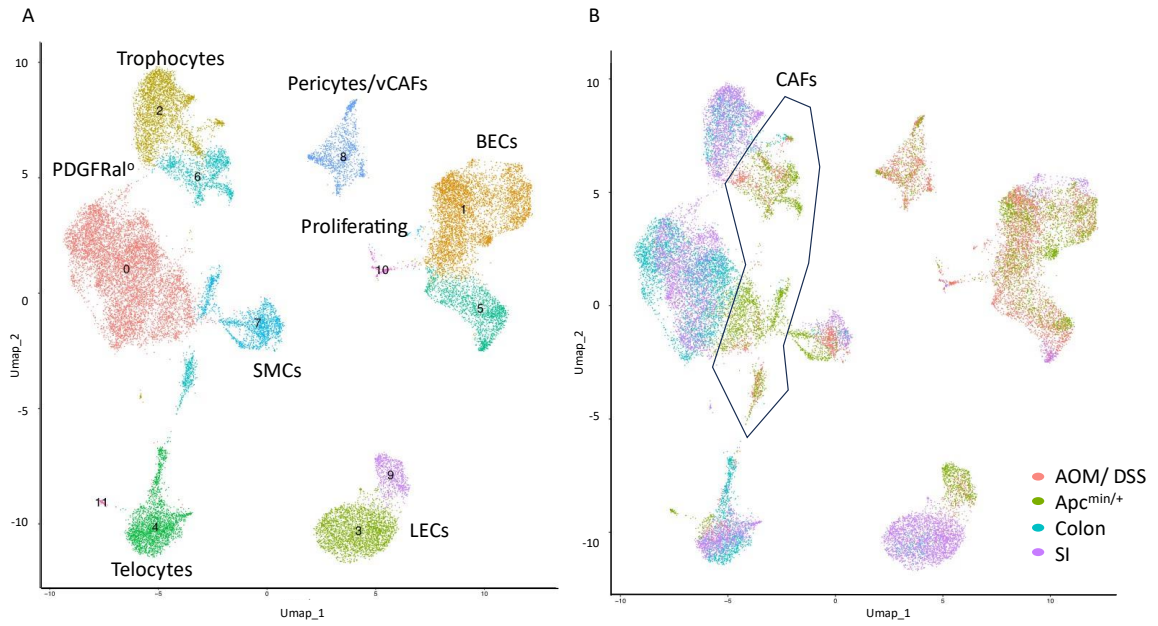

Figure 20 Single cell analysis of tumor stroma in two models of mouse intestinal carcinogenesis, the AOM/DSS and the Apc$^{min/+}$ model. *A) UMAP visualization of stromal cells in intestinal tumors and normal tissue (colon and small intestine). Cell clusters are color coded. SMCs: smooth muscle cells, BECs: blood endothelial cells, LECs: lymphatic endothelial cells. B) UMAP visualization of integrated tumor versus control samples. Bioinformatic analysis was performed by Athanasia Stavropoulou.*

Within fibroblasts, we were able to identify three main subclusters, including PDGFRαhi telocytes, PDGFRαlo fibroblasts, and trophocytes (McCarthy, Kraiczy et al. 2020, Chalkidi, Paraskeva et al. 2022) (Figure 20A-B). CAFs could be identified within each fibroblast subset. Especially for trophocytes and PDGFRαlo fibroblasts, they also formed distinct subclusters. Tumor pericytes or vascular CAFs (vCAFs) were also identified by the expression of classic pericyte markers, such as *Notch3*, *Rgs5*, *Acta2*, along with a small fraction of proliferating stromal cells that was characterized by the expression of *Mki67*, *Birc5*, and *Top2a,* and SMCs that expressed *Actin, Gamma 2 (Actg2)*, *Acta2* and *Myl9*. Notably, tumor samples from both models showed increased numbers of blood endothelial cells, pericytes/vCAFs, and smooth muscle cells, and less fibroblasts (Figure 21). Similar changes in the proportions of stromal cells, as well as the presence of a proliferative stromal subset have been reported in single cell transcriptomic analysis of human CRC (Lee, Hong et al. 2020, Pelka, Hofree et al. 2021, Becker, Nevins et al. 2022, Li, Lu et al. 2022, Qi, Sun et al. 2022).

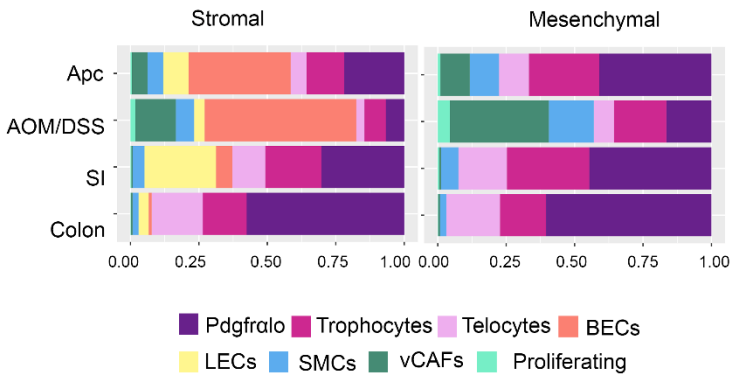

Figure 21 Blood endothelial cells, pericytes/vCAFs, and smooth muscle cells increase in numbers in mouse intestinal tumors. *Proportions of stromal and mesenchymal cells in tumor and control samples. SI: small intestine. Bioinformatic analysis was performed by Athanasia Stavropoulou.*

Differences in mesenchymal cluster abundancies were further verified by FACs analysis, which showed a significant decrease of PDFGRa^{+} fibroblasts inside tumors along with an increase in CD146^{+}αSMA^{+} pericytes/vCAFs and αSMA^{hi} smooth muscle cells (Figure 22). An increase in CD146^{+}αSMA^{+} cells in the AOM/DSS model has also been previously reported (Kobayashi, Gieniec et al. 2022).

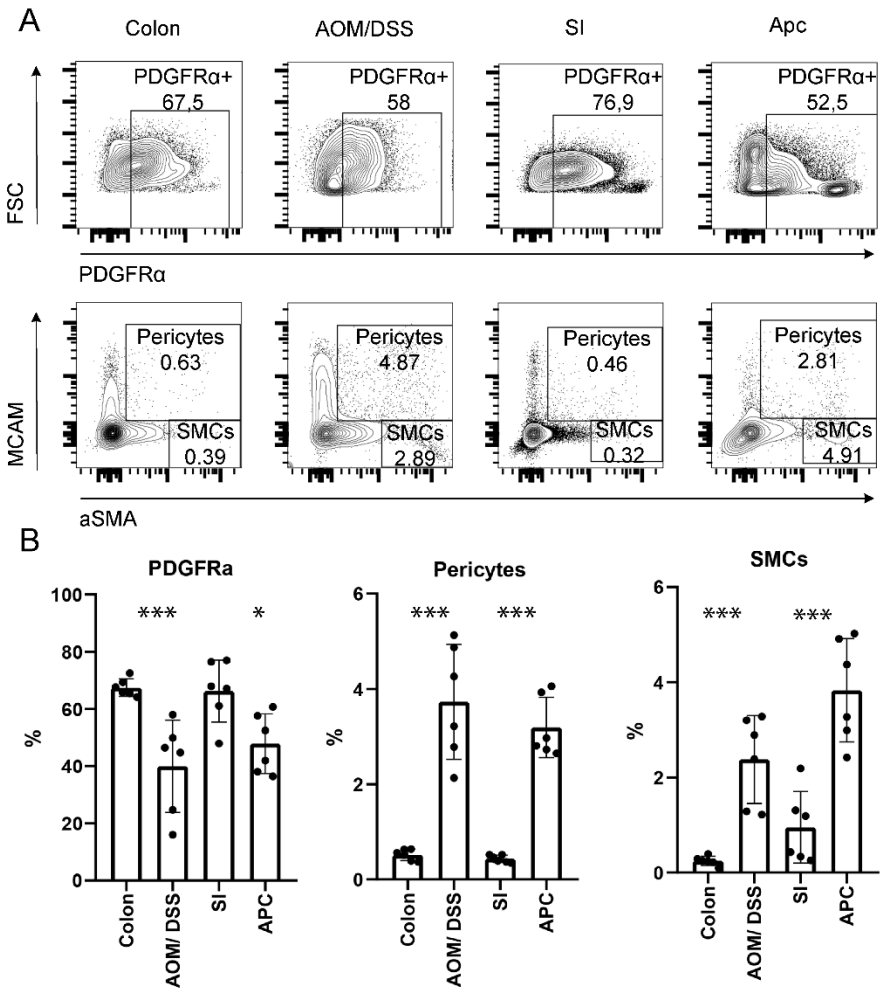

Figure 22. PDGFRα⁺ fibroblast decrease inside tumors whereas pericytes and smooth muscle cells increase. *A) FACs analysis and B) quantifications showing the abundance of PDGFRα⁺ fibroblasts, pericytes/vCAFs and smooth muscle cells in tumor and control samples (n=6) gated in EpCAM⁻CD45⁻ cells. Data represent mean ± SEM. *p < 0.05, ***p < 0.001.*

To verify the specificities of Col6a1⁺ cells in homeostasis and intestinal tumors at the single cell level, we plotted their gene signatures on the respective single-cell analysis UMAPs (Figure 23). This analysis verified that in tumors, Col6a1⁺ cells were mainly pericytes/vCAFs and to a lesser extent PDGFRαhi telocytes, in accordance with the significant expansion of vCAFs in tumors.

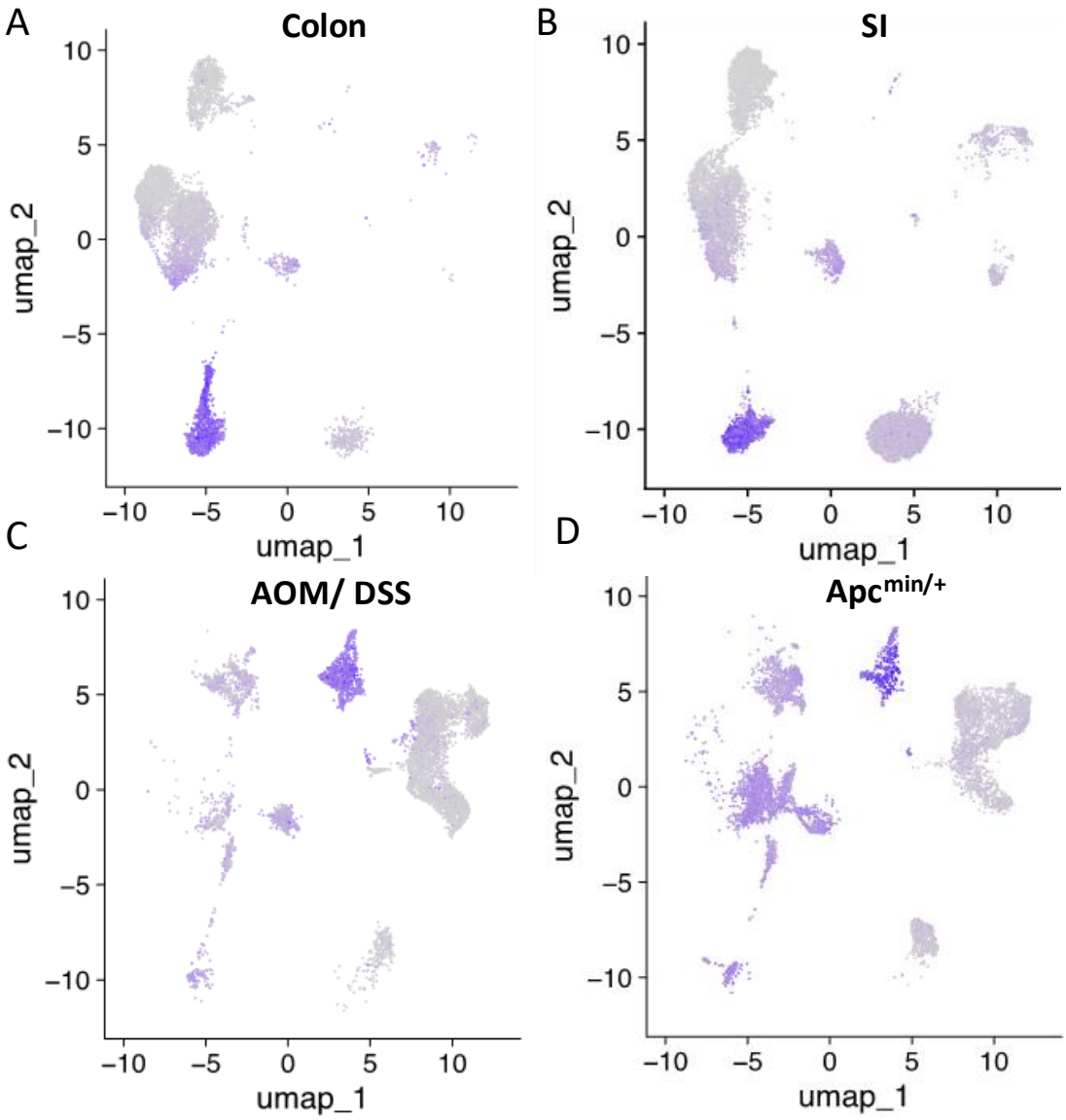

Figure 23 The Col6a1 cell gene signature is specific for PDGFRαhi telocytes and pericytes. *UMAP visualization of the gene signatures of A) Col6a1⁺GFP⁺ colonic fibroblasts, B Col6a1⁺GFPhi small intestinal fibroblasts, C) Col6a1⁺GFP⁺ colonic fibroblasts in AOM/ DSS, and D) Col6a1⁺GFP⁺ colonic fibroblasts in Apc$^{min/+}$ tumors,in the respective datasets. Bioinformatic analysis was performed by Athanasia Stavropoulou.*

4.5. Both Col6a1^{+} and Col6a1^{-} CAFs support cancer cell growth in vitro and in vivo

To define the physiological importance of the two CAF subsets on cancer growth, we next performed a series of *in vitro* and *in vivo* experiments. Initially, we assessed the colony formation potential of Caco-2 colon cancer cells grown on top of either Col6a1^{+} or Col6a1^{-} IMCs. The size of Caco-2 colonies on Col6a1^{+} IMCs after 3 days in culture was statistically significantly larger in comparison to Col6a1^{-} IMCs; however, the difference was small (Figure 24A–C).

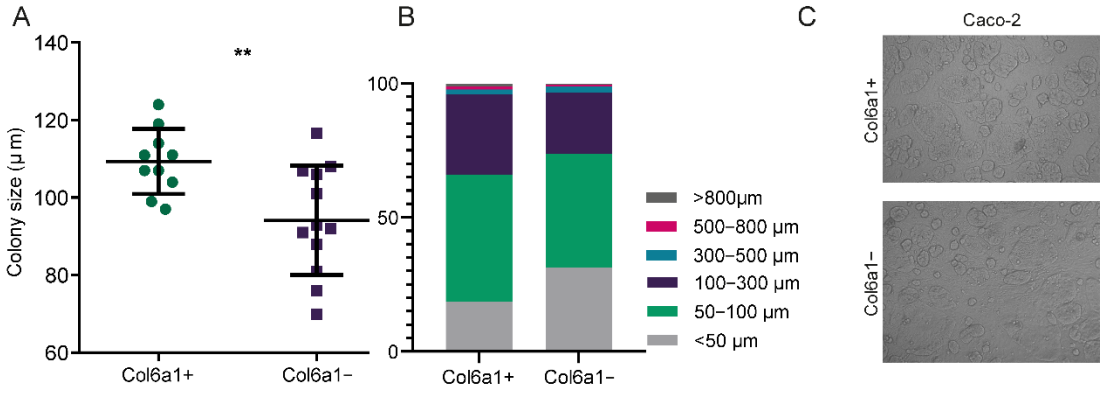

Figure 24 Co-culture experiments supporting roles of Col6a1^{+} and Col6a1^{-}IMCs on colony size formation of Caco-2 cancer cells. *(A) Average colony size per well, (B) size distribution, and (C) representative bright field images of Caco-2 colonies after 3 days of culture on sorted Col6a1^{+} and Col6a1^{-} colonic IMCs. Data represents mean ± SD from one of three experiments performed, n = 10–12 wells, ** p < 0.01, Scale bar = 0.5 mm.*

We then used a more physiologically relevant *in vitro* model by co-culturing Col6a1^{+} or Col6a1^{-} cells with AOM/DSS or Apc$^{min/+}$ tumoroids for 3 days. The size of AOM/DSS tumoroids was similar in both co-culture conditions (Figure 25A–C).

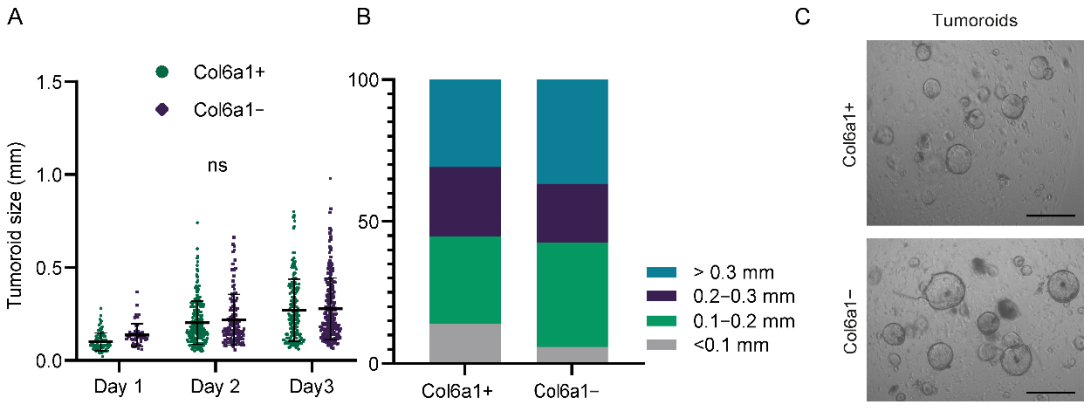

Figure 25 Co-culture experiments supporting roles of Col6a1⁺ and Col6a1⁻IMCs on AOM/ DSS tumoroid growth. *Tumoroid size per condition for each day of the co-culture, (E) size distribution of tumoroids at day 3 of the co-culture, and (F) representative bright-field images of AOM/DSS tumoroids at day three of their co-culture with sorted Col6a1⁺ and Col6a1⁻ colonic IMCs. Data rep-resents mean ± SD of tumoroids from one of three experiments performed. ns = not statistically significant, Scale bar = 1 mm.*

However, the size of Apc$^{min/+}$ tumoroids co-cultured with Col6a1^{+} IMCs was statistically significantly smaller in comparison to Col6a1^{-} IMCs (Figure 26A-B). These results indicate that both fibroblast subsets can support tumor organoid growth, although Col6a1^{-} IMCs may be more potent at least *in vitro*.

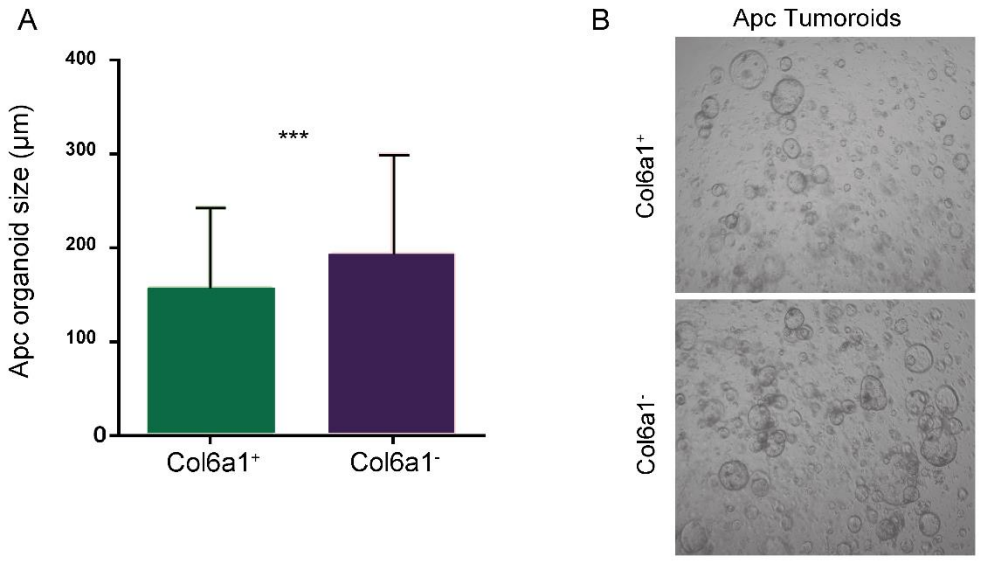

Figure 26 Co-culture experiments supporting roles of Col6a1+ and Col6a1-IMCs on Apc$^{min/+}$ tumoroid growth. *A) Size, B) Representative bright field images of Apc$^{min/+}$ tumoroids after 3 days of co-culture with sorted Col6a1⁺ and Col6a1⁻ colonic IMCs. Data represents mean ± SD from one of three experiments performed in quadruplicates. *p<0.05, **p<0.01, ***p<0.001.*

To further examine the *in vivo* role of the two subsets in cancer growth, we performed allograft experiments. We isolated fresh colonic Col6a1^{+} and Col6a1^{-} IMCs through FACS sorting and co-injected them with MC38 colon cancer cells subcutaneously in the flanks of C57/Bl6 wild-type mice. MC38 cells alone were used as controls. After 15 days, allograft tumors containing either Col6a1^{+} or Col6a1^{-} IMCs showed no difference in tumor size between the two subsets (Figure 27A). Notably, both IMC subsets were equally represented inside the tumor allografts, as shown by fluorescent microscopy (Figure 27B). Taken together, our results show that both Col6a1^{+} and Col6a1^{-} IMCs can support cancer growth *in vitro* and *in vivo*.[4]

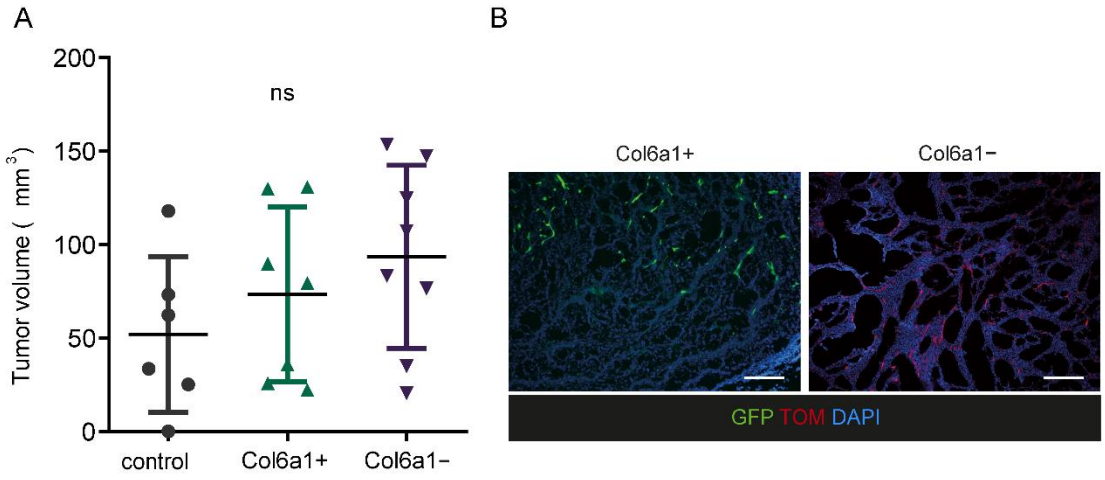

Figure 27 Allograft experiments show similar effects of Col6a1^{+} and Col6a1^{-} CAFs on cancer cell growth. *A) Total volume of allografts after 15 days of growth with Col6a1^{+} and Col6a1^{-} colonic IMCs. Data represents mean ± SD from one of three experiments performed (n = 6–8), ns = not statistically significant. (H) Representative fluorescent images of allografts with sorted Col6a1^{+} and Col6a1^{-} colonic IMCs. Scale bar = 50 µm.*

4.6 Intestinal fibroblasts respond to inflammatory signals

Next, we aimed to better understand the pathways driving diverse and subset-specific fibroblast activation in intestinal carcinogenesis. Previous studies have shown that fibroblasts respond to cytokines (TNF, IL-1β) in the tumor microenvironment to drive their proinflammatory activation

[4]*Parts of this section (4.5) have been recently published in an article in the international Journal of Molecular Sciences Ibid.*

(Koliaraki, Pasparakis et al. 2015, Kalluri 2016, Biffi, Oni et al. 2019, Koliaraki, Prados et al. 2020). To further identify the innate molecular mechanisms in IMCs we incubated colon and small intestinal IMCs with various inducers. First, IMCs were induced with TLR ligands and interleukins IL-1β, IL-18, and IL-33. We used measurement of IL-6 as the readout of this initial analysis, based on our previous studies. Our results showed that IL-6 was increased in response to IL-1β and ligands for TLR1/2, TLR4, and TLR6 and that lipopolysaccharide (LPS) and IL-1β were the stimuli that produced the most abundant effect (Figure 28).

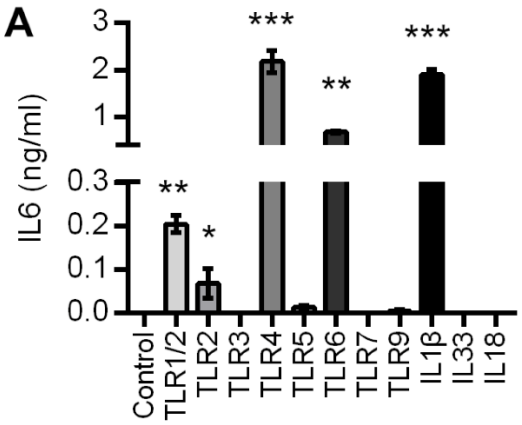

Figure 28. IMCs respond to innate inducers producing IL-6. *IL-6 quantification by ELISA in the supernatants of IMCs stimulated for 24 h with TLRs and interleukins. One representative of three independent experiments performed in triplicates is presented.*

IMCs were then stimulated with IL-1β, LPS, as well as TNF, and their secretome was analyzed using a Proteome Profiler Array (Figure 29A, B). TNF was included in these experiments, since it has been previously shown to induce proinflammatory gene expression in fibroblasts from different tissues, including the intestine (Armaka, Apostolaki et al. 2008). We found that IMCs respond to all three factors, and the most robust response was upon LPS and IL-1β stimulation (Figure 29A, B). Overexpressed secreted mediators included mainly cytokines (IL-6, IL-11, IL-23, and IL-1β), chemokines (e.g., CXCL1, CXCL2, and CXCL10) and matrix metalloproteinase (MMPs) (MMP-3, MMP-9) (Figure 29A, B). We then used the CXCL2 (or MIP-2) chemokine as a readout and measured the response of sorted Col6a1^{+} or Col6a1^{-} IMCs in culture supernatants. Interestingly, both IMC subsets responded similarly to all three inducers in vitro (Figure 29C), suggesting that different intestinal fibroblast subsets can respond to inflammatory stimuli and become activated.[5]

[5]Parts of this section (4.6) have been published in an article in the international Journal of Molecular Sciences Ibid. and the Journal of Experimental Medicine Koliaraki, V., N. Chalkidi, A. Henriques, C. Tzaferis,

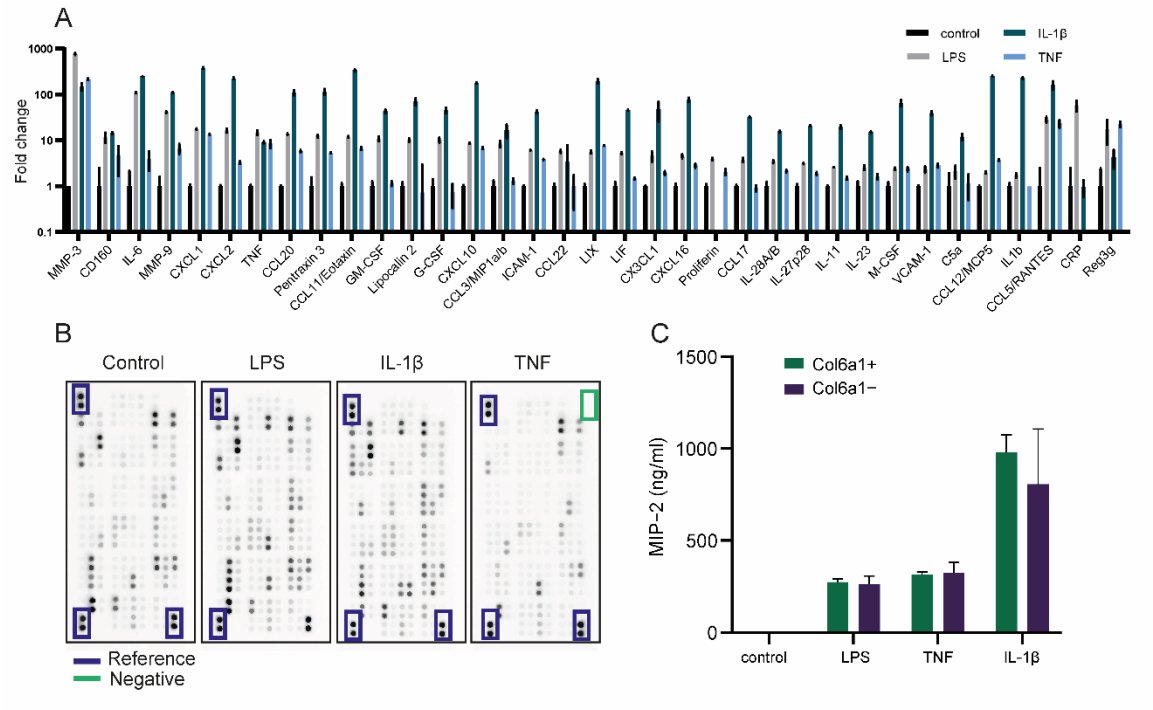

Figure 29 Col6a1+ and Col6a1- fibroblasts respond similarly to innate inducers. *A) Proteome profiling of cultured unsorted IMCs upon LPS, IL-1β, and TNF stimulation. Only factors with differences in fold change > 2 in at least one condition are shown. Data represents mean ± SD from one experiment performed in duplicates. (B) Image showing the signal intensity of the proteome profile assay as obtained from the ChemiDoc XRS+ instrument. (C) MIP-2 quantification in the supernatants of Col6a1+ and Col6a1− IMCs stimulated for 24 h with LPS, TNF, and IL-1β. One representative of two independent experiments performed in triplicates is presented.*

4.7. Deletion of innate immune receptors in Col6a1⁺GFP⁺ IMCs is not sufficient to ameliorate colitis-associated carcinogenesis

Given our previous results on the pro-inflammatory and pro-tumorigenic role of Col6a1⁺ cells in CAC, and the role of NF-κB in driving their activation, we next assessed whether cell-specific *in vivo* deletion of immune-related pathways could lead to reduced carcinogenesis in the AOM/DSS

A. Polykratis, A. Waisman, W. Muller, D. J. Hackam, M. Pasparakis and G. Kollias (2019). "Innate Sensing through Mesenchymal TLR4/MyD88 Signals Promotes Spontaneous Intestinal Tumorigenesis." Cell Rep **26**(3): 536-545.e534.

model. For this reason, we crossed Interleukin-1 receptor type 1 f/f (*Il1r1*$^{f/f}$) (Abdulaal, Walker et al. 2016), tumor necrosis factor receptor 1 f/f (*p55*$^{f/f}$) (Van Hauwermeiren, Armaka et al. 2013), toll like receptor 4 f/f (*Tlr4*$^{f/f}$) (Sodhi, Neal et al. 2012) and *Myd88*$^{f/f}$ (Vlantis, Polykratis et al. 2016) mice with the Col6a1Cre strain to specifically inhibit the IL-1R, TNFR, TLR4 and MyD88 pathways in Col6a1^{+} IMCs and then subjected the mice to the AOM/DSS protocol of colitis-associated carcinogenesis. Surprisingly, all conditional knockout mice developed an equal number of tumors in comparison with their littermate controls (Figure 30A-D).

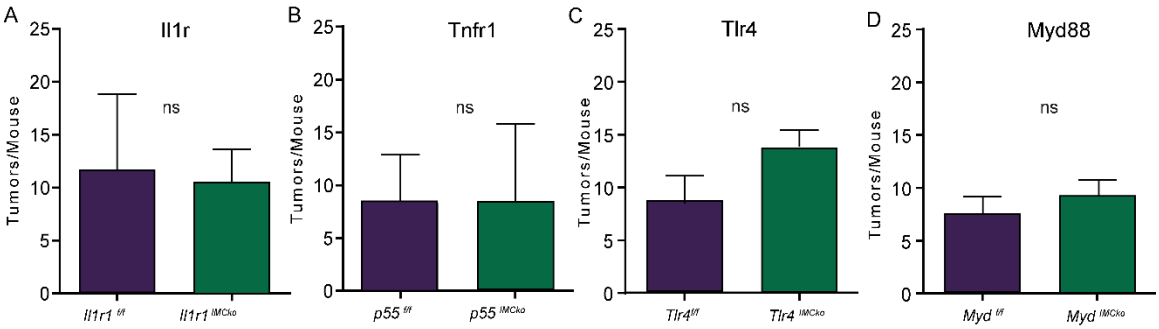

Figure 30 Deletion of IL-1R1, TNFR1, TLR4 and Myd88 in Col6a1+ IMCs is not sufficient to ameliorate CAC. *Number of tumors in A) Il1r1*^{IMCko} *mice (n = 6) and their littermate controls (n = 6) at the end of the AOM/DSS protocol (one representative experiment of four performed) B) p55*^{IMCko} *mice (n = 8) and their littermate controls (n = 9) at the end of the AOM/DSS protocol (one representative experiment of two performed). C) Tlr4*^{IMCko} *mice (n = 8) and their littermate controls (n = 8) at the end of the AOM/DSS protocol (one representative experiment of three performed). D) Myd88*^{IMCko} *mice (n = 8) and their littermate controls (n = 7) at the end of the AOM/DSS protocol (one representative experiment of four performed). n.s= not significant.*

Colon length and colitis scoring were also similar between control and experimental mice, indicating similar levels of inflammation (Figures 31). These results show that the deletion of a single inflammatory inducer in Col6a1^{+} cells is not sufficient to reduce inflammation and tumorigenesis in CAC, indicating that potential synergistic activation of NF-κB could be driving the pro-inflammatory properties of these cells. Nevertheless, we cannot exclude that deletion of a single inducer in a larger fibroblast subset or in cells preferentially activated by it due to their microenvironment milieu could have a significant effect.[6]

[6]Parts of this section (4.7) have been published in an article in the international Journal of Molecular Sciences Chalkidi, N., M. T. Melissari, A. Henriques, A. Stavropoulou, G. Kollias and V. Koliaraki (2023). "Activation and Functions of Col6a1+ Fibroblasts in Colitis-Associated Cancer." Int J Mol Sci **25**(1). and the Journal of Experimental Medicine Koliaraki, V., N. Chalkidi, A. Henriques, C. Tzaferis, A. Polykratis, A. Waisman, W. Muller, D. J. Hackam, M. Pasparakis and G. Kollias (2019). "Innate Sensing through

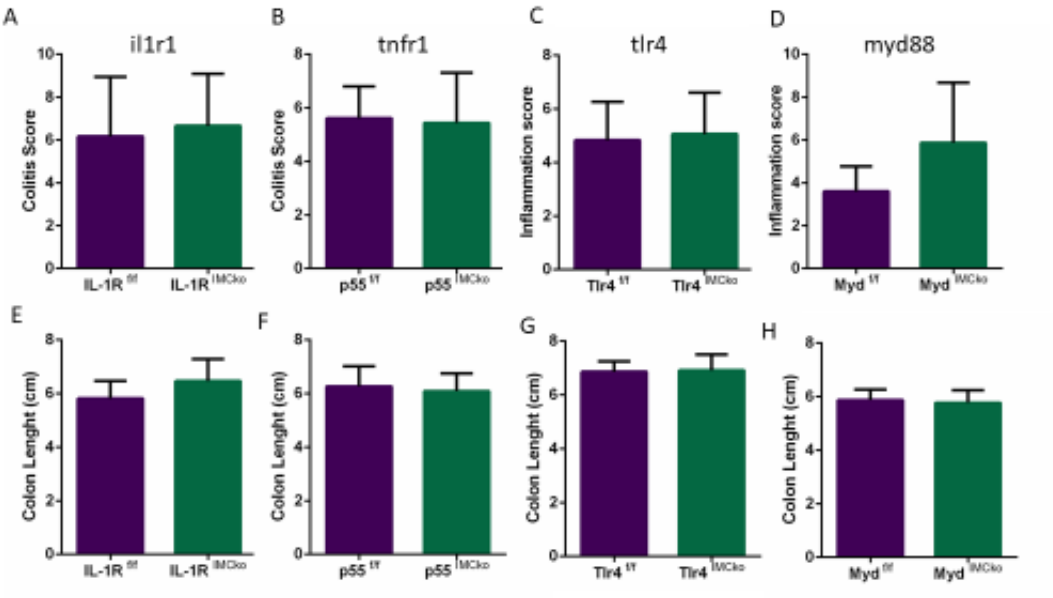

Figure 31 Deletion of IL-1R1 and TNFR1 in Col6a1⁺ IMCs is not sufficient to ameliorate CAC. *Colitis score in A) Il1r1^{IMCko} mice (n = 6) and their littermate controls (n = 6) at the end of the AOM/DSS protocol (one representative experiment of four performed). B) p55^{IMCko} mice (n = 8) and their littermate controls (n = 9) at the end of the AOM/DSS protocol (one representative experiment of two performed).C) Tlr4^{IMCko} mice (n = 8) and their littermate controls (n = 9) at the end of the AOM/DSS protocol (one representative experiment of three performed D) Myd88^{IMCko} mice (n = 8) and their littermate controls (n = 10) at the end of the AOM/DSS protocol (one representative experiment of four) performed. Colon length in E) Il1r1^{IMCko} mice (n = 6) and their littermate controls (n = 6) at the end of the AOM/DSS protocol (one representative experiment of four performed). F) p55^{IMCko} mice (n = 8) and their littermate controls (n = 9) at the end of the AOM/DSS protocol (one representative experiment of two performed). G) Tlr4^{IMCko} mice (n = 8) and their littermate controls (n = 9) at the end of the AOM/DSS protocol (one representative experiment of three performed H) Myd88^{IMCko} mice (n = 8) and their littermate controls (n = 10) at the end of the AOM/DSS protocol (one representative experiment of four) performed. ns: not statistically significant.*

Mesenchymal TLR4/MyD88 Signals Promotes Spontaneous Intestinal Tumorigenesis." Cell Rep **26**(3): 536-545.e534.

4.8. *Innate immune functions of Col6a1⁺ IMCs in Apc driven intestinal tumorigenesis*

The AOM/DSS model requires acute and extensive inflammation followed by regeneration to drive carcinogenesis. As such, the lack of a phenotype in our previous in vivo studies could be due to robust inflammatory activation during the early stages of the protocol. Therefore, we also assessed these immune-related pathways in the $Apc^{min/+}$ mode of spontaneous carcinogenesis.

For this reason, we first targeted Myd88, a central regulator of innate immunity that acts directly downstream of Toll-like receptors (TLRs). Previous studies have shown that complete Myd88 deletion led to a significant reduction in tumor load and size in the $Apc^{min/+}$ mouse model, which was dependent on its role in non-hematopoietic cells (Salcedo, Cataisson et al. 2013). Indeed, Myd88 deletion in Col6a1⁺ IMCs resulted in reduced tumorigenesis both in tumor load and size and a similar phenotype with $Apc^{min/+}$-Myd88 knockout mice (Figure 32A-B), suggesting that MyD88 signaling in Col6a1⁺ IMCs is responsible for the reduction of intestinal tumorigenesis in these mice. To further explore the upstream MyD88 inducers driving fibroblast activation, we then deleted IL-1R1 and TLR4 in Col6a1⁺ cells. Similar to the AOM/DSS model, deletion of IL-1R1 from Col6a1⁺ IMCs did not affect Apc-driven intestinal carcinogenesis (Figure 32E-F). However, TLR4 deletion in Col6a1⁺ IMCs resulted in a statistically significant smaller number of tumors, as well as tumor size compared to littermate controls (Figure 32C-D).

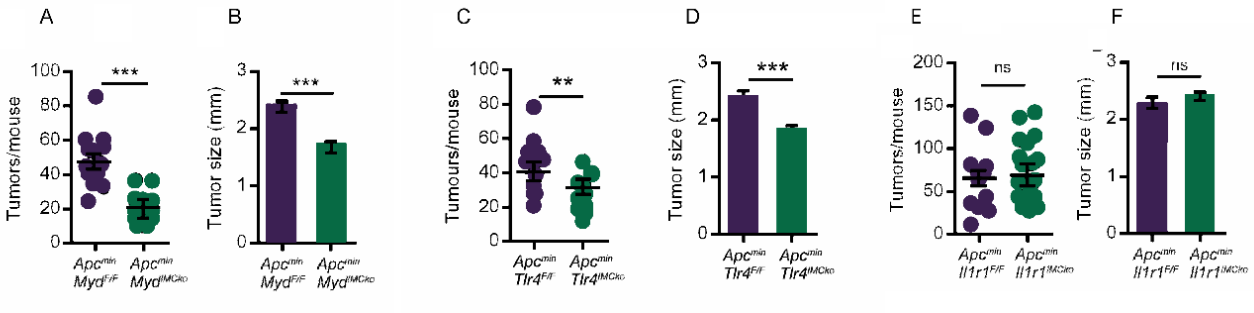

Figure 32. Deletion of MyD88 and TLR4 but not IL1-R1 in Col6a1Cre IMCs reduces tumorigenesis in the $Apc^{min/+}$ model of sporadic intestinal cancer. *A) Total number of tumors per mouse in 4-month-old $Apc^{min/+}$ $Il1r1l^{IMCko}$ mice (n = 14) and their littermate controls (n = 24). B) Size of small intestinal tumors presented as mean tumor size in the two genotypes. C) Total number of tumors per mouse in 4-month-old $Apc^{min/+}$ $Tlr4^{IMCko}$*

*mice (n = 13) and their littermate controls (n = 20). D) Size of small intestinal tumors presented as mean tumor size in the two genotypes. E) Total number of tumors per mouse in 4-month-old Apc$^{min/+}$ Myd88^{IMCko} mice (n = 15) and their littermate controls (n = 18). F) Size of small intestinal tumors presented as mean tumor size in the two genotypes. Data represent mean ± SEM. **p < 0.01, ***p < 0.001,*

Consistent with these data, BrdU staining in size-matched tumors from the Apc$^{min/+}$-Myd88^{IMCko} and Apc$^{min/+}$-TLR4^{IMCko} mice showed decreased proliferation in comparison to their respective littermate controls (Figure 33).

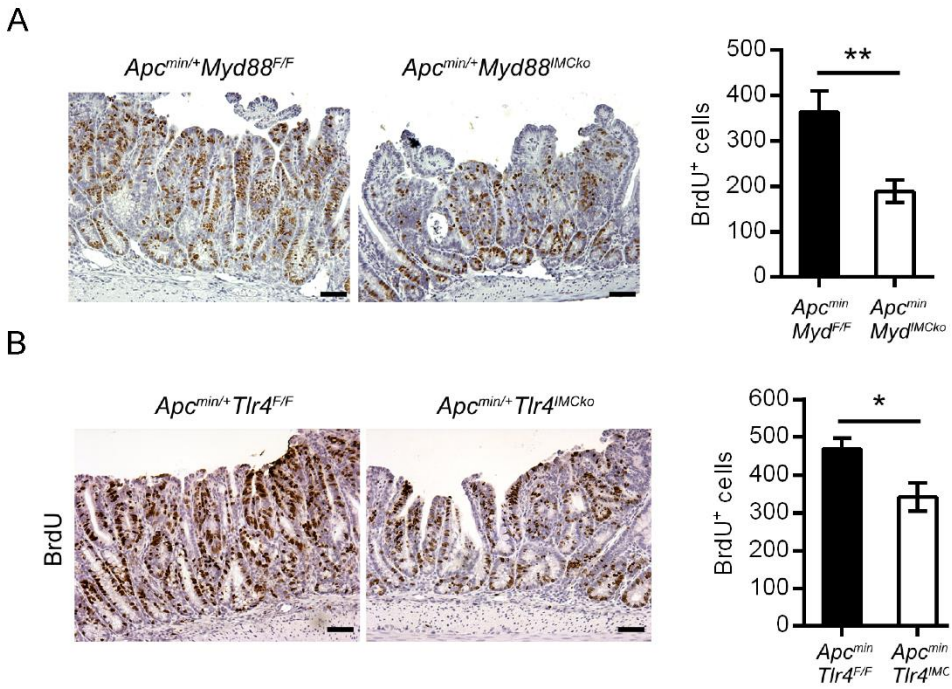

Figure 33 BrdU+ proliferating cells are decreased in Apc$^{min/+}$-Myd88^{IMCko} and Apc$^{min/+}$-TLR4^{IMCko} tumors. *A) Representative BrdU staining and quantification per field in equal-sized small intestinal tumors of Apc$^{min/+}$Myd88^{IMCko} mice and their littermate controls. (n = 12–14 tumors from 5 mice per genotype) B) Representative BrdU staining and quantification per field in equal-sized small intestinal tumors of Apc$^{min/+}$TLR4^{IMCko} mice and their littermate controls. (n = 12–14 tumors from 4 mice per genotype). Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001*

4.9. Deletion of MyD88 in IMCs results in deregulated gene expression, reduced STAT3 phosphorylation, and altered inflammatory cell infiltration

To further analyze the phenotype of $Apc^{min/+}$-$Myd88^{IMCko}$ mice and their similarities with the complete knockout mice, we next analyzed the gene expression profile of $Apc^{min/+}$-$Myd88^{IMCko}$ tumors by measuring the expression level of genes that were differentially regulated in the complete MyD88 knockout mice (Rakoff-Nahoum and Medzhitov 2007). We found significant deregulation in many genes, especially those encoding pro-inflammatory mediators and matrix metalloproteinases (MMPs), while *Igf1* and *Igfbp5* were not upregulated in our tumor samples (Figure 34).

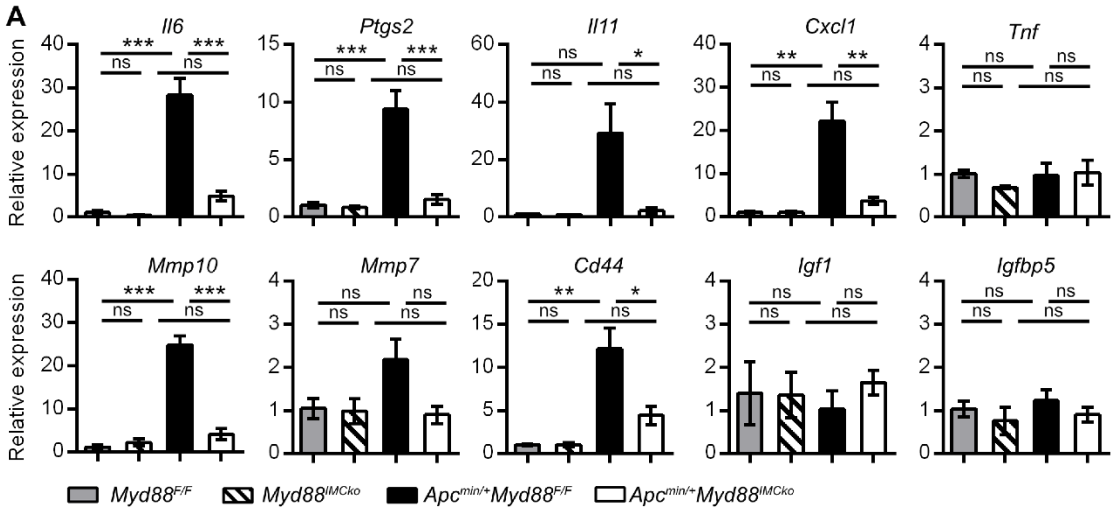

Figure 34 Tumors from Mice with Deletion of MyD88 in IMCs Show Differential Gene Expression. *Gene expression analysis in the small intestine of $Myd88^{F/F}$ and $Myd88^{IMCko}$ mice (n = 3) and in tumors from $Apc^{min/+}Myd88^{F/F}$ and $Apc^{min/+}Myd88^{IMCko}$ mice (n = 6). Hprt was used for normalization.*

Interestingly, among the deregulated genes were *Il6* and *Il11*, two pro-inflammatory cytokines with important functions in enhancing epithelial cell proliferation and tumorigenesis through STAT3 activation. Accordingly, we found that $Apc^{min/+}$-$Myd88^{IMCko}$ and $Apc^{min/+}$-$TLR4^{IMCko}$ mice showed decreased pSTAT3 staining both in tumors and normal villi in comparison to controls (Figures 35).

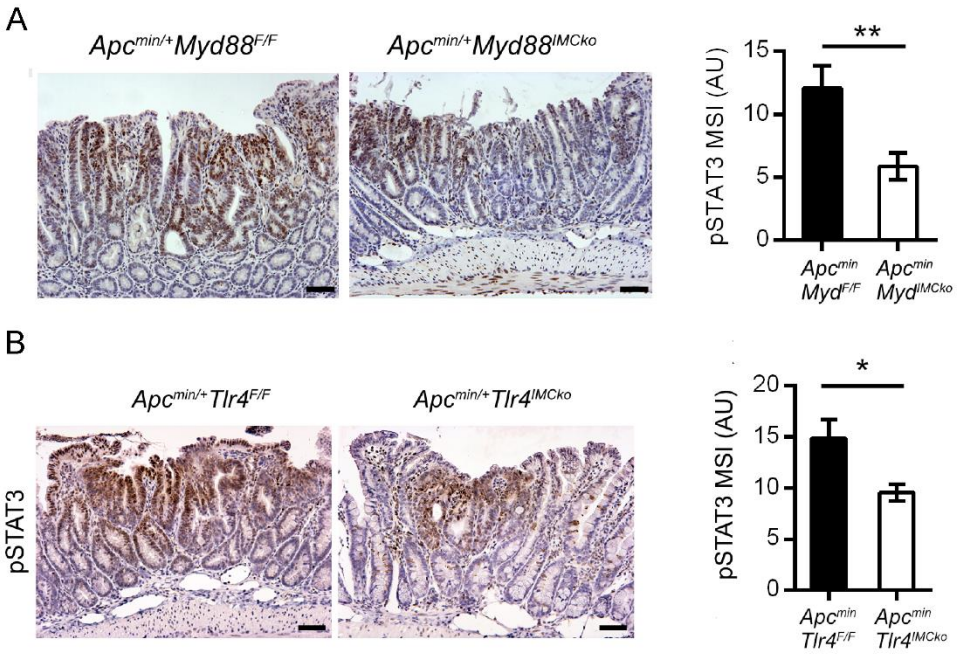

Figure 35 STAT3 phosphorylation is decreased in Apc$^{min/+}$-Myd88^{IMCko} and Apc$^{min/+}$-TLR4^{IMCko} tumors. *A) Representative STAT3 staining and quantification per field in equal-sized small intestinal tumors of Apc$^{min/+}$Myd88^{IMCko} mice and their littermate controls. (n = 12–14 tumors from 5 mice per genotype) B) Representative STAT3 staining and quantification per field in equal-sized small intestinal tumors of Apc$^{min/+}$TLR4^{IMCko} mice and their littermate controls. (n = 12–14 tumors from 4 mice per genotype). Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Mean signal intensity (MSI) of pSTAT3*

Myd88^{IMCko} mice, were also examined for inflammatory cell infiltration in tumors by fluorescence-activated cell sorting (FACS) analysis. We did not find statistically significant differences in the numbers of infiltrating CD45$^+$ hematopoietic cells between the two genotypes (Figure 36A); however, we found a reduction in CD45$^+$CD11b$^+$F4/80$^+$ macrophages, CD45$^+$CD11b$^+$Gr1$^+$ neutrophils, and CD45$^+$CD4$^+$ T cells, while CD45+CD8+ T cells were increased in the Apc$^{min/+}$-Myd88^{IMCko} tumors (Figures 36B and 36C). Therefore, Apc$^{min/+}$-Myd88^{IMCko} mice display altered balances in immune infiltration toward a less pro-inflammatory microenvironment and interestingly enhanced cytotoxic T cell infiltration, both being directly associated with the decreased number and size of tumors. These results indicate that a MyD88-dependent pathway in IMCs and/or CAFs regulates the infiltration of immune populations creating a pro-tumorigenic inflammatory milieu in the Apc$^{min/+}$ model, which potentially acts as an additional mechanism to accelerate tumorigenesis.

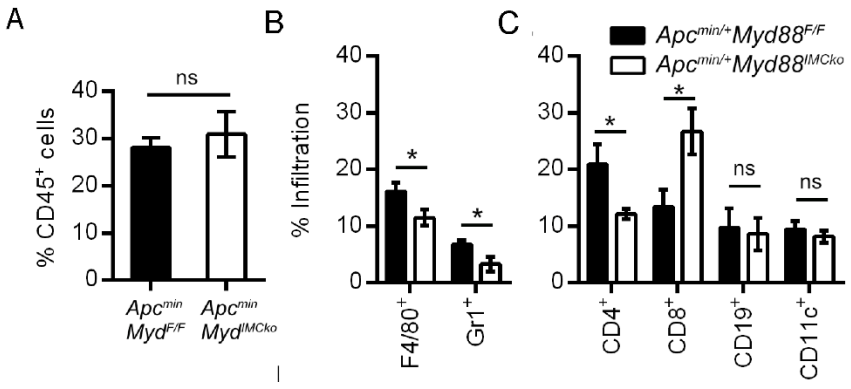

Figure 36 Apc$^{min/+}$-MyD88^{IMCko} tumors display altered immune cell infiltration. *A) Infiltration of CD45$^+$ cells B), CD11b$^+$F4/80$^+$ macrophages and CD11b$^+$Gr1$^+$ neutrophils C), and CD4$^+$ T cells, CD8$^+$ T cells, CD19$^+$ B cells, and CD11c$^+$ dendritic cells in tumors from 4- to 5-month-old Apc$^{min/+}$-Myd88$^{F/F}$ and Apc$^{min/+}$-Myd88^{IMCko} mice (n = 4–7), quantified by FACS analysis (from two independent experiments). Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant.*

Finally, to identify the IMC-specific MyD88-dependent gene expression changes, we next performed RNA sequencing of Col6a1Cre$^+$, wild-type (WT) and MyD88 knockout IMCs before and after treatment with LPS for 6 h. Interestingly, even unstimulated MyD88 knockout cells showed a significantly altered gene expression profile in comparison to the unstimulated control cells. Gene Ontology (GO) analysis of the mostly downregulated genes in non-induced MyD88 knockout cells revealed differences in pathways related to inflammatory and/or immune response and cell proliferation, indicating an intrinsic defect of these cells in acquiring an innate identity under homeostatic conditions (Figures 37A and 37D). Comparisons with the LPS-stimulated control and MyD88 knockout samples further showed a significant number of MyD88-regulated genes, which either remained unchanged or were altered upon LPS stimulation (Figure 37B). GO analysis of these MyD88- regulated genes showed enrichment in inflammatory and/or immune response and regulation of cell proliferation (Figure 37C). Related genes included mainly chemokines (*Cxcl1, Cxcl2, Cxcl5, Ccl2, Ccl7, Ccl8, Ccl11*), cytokines (*Il6, Il34*), growth factors (*Fgf7, Fgf10, Tgfa, Ctgf, Igf1, Igfbp4*), and MMPs (*Mmp3, Mmp8, Mmp9, Mmp10, Mmp13*) (Figure 37D). This MyD88-dependent gene signature is in agreement with the deregulated gene expression of the IMC-specific MyD88 knockout tumors and the accompanied reduced proliferation and altered inflammatory infiltration.[7]

[7] Parts of this section (4.8, 4.9) have been published in an article in the Journal of Experimental Medicine Koliaraki, V., N. Chalkidi, A. Henriques, C. Tzaferis, A. Polykratis, A. Waisman, W. Muller, D. J. Hackam, M.

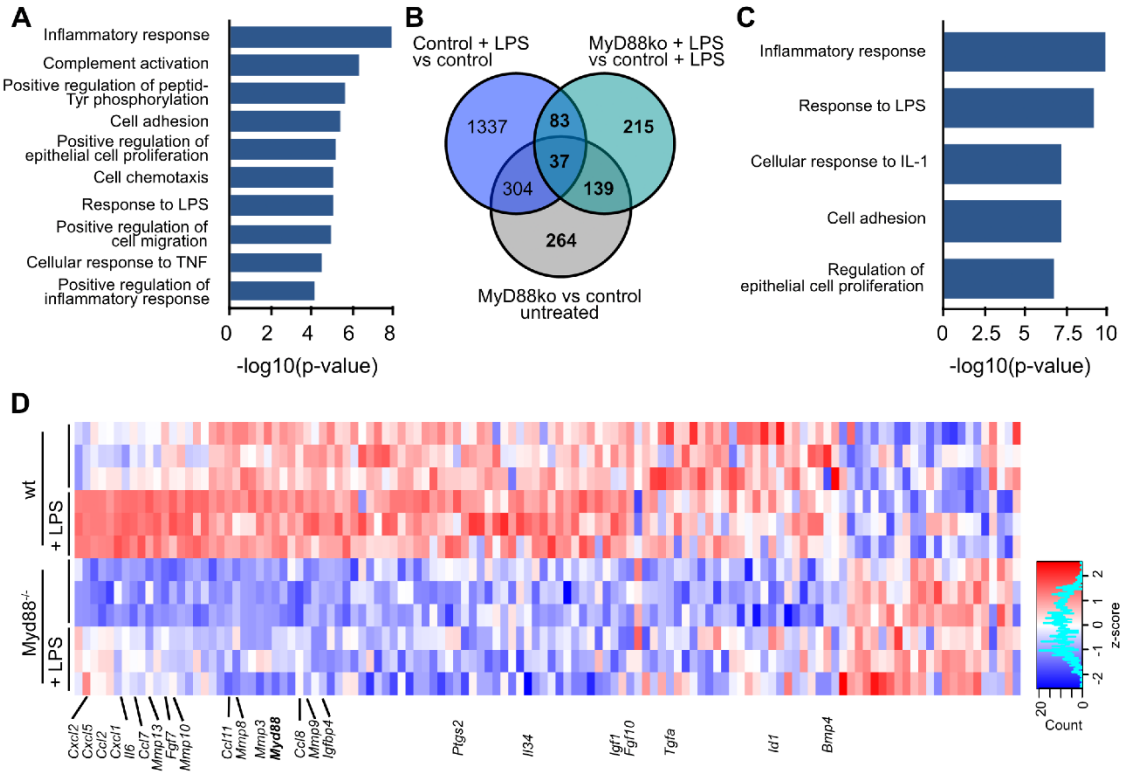

Figure 37 TLR4/MyD88 Signaling Induces a Pro-Tumorigenic Gene Signature in IMCs. *(A) Gene Ontology (GO) terms enriched in differentially expressed genes between untreated control and MyD88 knockout IMCs. (B) Venn diagram showing the number of overlapping differentially regulated genes between untreated and LPS-treated control and MyD88 knockout IMCs. (C) GO terms enriched in MyD88-regulated genes indicated as bold in (B). (D) Heatmap of differentially expressed genes between control and LPS-induced WT and MyD88 knockout IMCs that belong to the GO terms shown in (C). Log2 transformed normalized read counts of genes are shown. Read counts are scaled per column. Red denotes high expression values, and blue denotes low expression values.*

Pasparakis and G. Kollias (2019). "Innate Sensing through Mesenchymal TLR4/MyD88 Signals Promotes Spontaneous Intestinal Tumorigenesis." Cell Rep **26**(3): 536-545.e534.

5. Discussion

During the last decade, many studies have highlighted the multiple functions of CAFs, as well as their heterogeneity, which may be attributed to their different topology, microenvironmental cues or origins at the cell type (fibroblasts, epithelial cells, endothelial cells, etc) or subpopulation level (Kalluri 2016, Koliaraki, Prados et al. 2020, Kobayashi, Gieniec et al. 2022).

In this study, we explored the origin and role of CAFs in two mouse models of intestinal carcinogenesis and we showed both through single cell and bulk RNA sequencing that resident fibroblast populations are activated upon carcinogenesis to give rise to intestinal CAFs. As we have previously shown Col6a1^{+} and Col6a1^{-} cells in the intestine represent two distinct fibroblast subsets (Melissari, Henriques et al. 2021). In more detail, colonic cells targeted by the Col6a1Cre strain include PDGFRαhi telocytes, pericytes, and a small fraction of PDGFRαlo fibroblasts, while Col6a1^{-} fibroblasts include trophocytes and the majority of PDGFRαlo fibroblasts. In the small intestinal, the Col6a1Cre mouse targets similar populations, but a higher number of PDGFRalo cells (Melissari, Henriques et al. 2021). Bulk RNA sequencing analysis of Col6a1^{+} and Col6a1^{-} CAFs in the AOM/DSS and Apc$^{min/+}$ models showed that both CAF subsets displayed a significant transcriptional similarity with their respective normal fibroblast subsets, indicating that CAFs can maintain some of their key physiological identities at least at the early stages of adenoma development, represented in the mouse models analyzed. More specifically, Col6a1^{+} CAFs displayed a dual gene expression signature, reflecting a role both in epithelial cell differentiation and vascular function, similar to their normal counterparts. Accordingly, Col6a1^{-} CAFs maintained some of their epithelial cell differentiation gene signature. This similarity in the gene expression signatures of CAF and normal fibroblast subsets supports the significance of resident fibroblast populations as a source of intestinal CAFs upon mouse intestinal carcinogenesis. It also indicates that activation of CAFs could be a stepwise process towards their complete reprogramming at the late stages of the disease.

Differential gene expression analysis, as well as functional *in vitro* and *in vivo* experiments, showed that both Col6a1^{+} and Col6a1^{-} IMCs could support tumor growth. Focusing on the molecular pathways governing the activation of the two CAF subsets in intestinal tumor, we found that both Col6a1^{+} and Col6a1^{-} CAFs displayed a significant deregulation of genes implicated in carcinogenesis in comparison to normal fibroblasts. These included both common and uniquely deregulated

expression profiles between the two subsets. Col6a1$^-$ CAFs displayed a unique activated gene signature representing an enhanced metabolic and secretory activity, in agreement with the secretory and metabolic reprogramming of CAFs (Kalluri 2016, Gieniec, Butler et al. 2019, Koliaraki, Prados et al. 2020). On the other hand, pathway analysis highlighted the significant and unique enrichment of Col6a1$^+$ cells in functions related with angiogenesis, which agrees with the targeting of both pericytes and fibroblasts near capillaries by the Col6a1Cre mouse in homeostasis (Melissari, Henriques et al. 2021).

Single-cell RNA sequencing further supported these conclusions, as CAF populations in both animal models could be clustered together with their normal counterparts. Nevertheless, cancer-associated PDGFRalo interstitial fibroblasts and trophocytes also clustered as distinct subsets, indicative of their significantly altered gene expression profiles. In addition, fibroblasts were reduced in numbers in intestinal tumors, while pericytes and smooth muscle cells were enriched, which was further verified through FACs analysis. These results were in line with already published data from human single cell transcriptomic studies, which show similar changes in mesenchymal subsets (Berger, Bergers et al. 2005, Li, Lu et al. 2022, Qi, Sun et al. 2022). Notably, the Col6a1$^+$ signature was enriched in tumor pericytes and to a less extent to PDGFRahi CAFs, confirming the bulk RNA sequencing analysis and suggesting that Col6a1+ CAF functions could be related to their pericyte-specific roles. Accordingly, increased expression of pericyte markers, such as PDGFRβ and Mcam, has been reported for human colorectal CAFs and perivascular CAFs have been detected in multiple human CRC single cell analyses and have been shown to exert pro-tumorigenic functions (Berger, Bergers et al. 2005, Foster, Januszyk et al. 2022, Verginadis, Avgousti et al. 2022). (Lee, Hong et al. 2020, Pelka, Hofree et al. 2021, Becker, Nevins et al. 2022, Li, Lu et al. 2022, Qi, Sun et al. 2022).

We next focused our analysis on the potential innate activation of distinct CAF subsets and their role in tumorigenesis. Previous results have shown that deletion of IKK2 in Col6a1$^+$ fibroblasts, and thus inhibition of NFκB signaling, resulted in reduced tumorigenesis in the intestine (Koliaraki, Pasparakis et al. 2015). To identify the upstream signals driving NFκB activation in IMCs, we incubated them with common immune stimuli, such as IL-1β, TNF, and LPS, all of which induced a robust pro-inflammatory response, including cytokines, chemokines, matrix metalloproteinases, and other inflammatory mediators. Interestingly, we showed that both Col6a1$^+$ and Col6a1$^-$

subsets responded similarly to these innate stimuli at least *in vitro* and are thus not characterized by inherent differences in the ability to mount an inflammatory response.

Next, we evaluated the pathophysiological role of these pathways in intestinal carcinogenesis. We showed that deletion of Myd88, as well as upstream TLR4 or IL1-R1 specifically in Col6a1Cre-expressing cells was not sufficient to reduce AOM/DSS induced colon tumorigenesis. Accordingly, deletion of TNF-R1 signaling in these cells had a similar effect. However, deletion of TLR4, but not IL1R1, and its downstream target MyD88 in Col6a1^{+} IMCs significantly reduced the number of tumors in the Apc$^{min/+}$ model, suggesting an important tumor-promoting role of the TLR4/MyD88 signaling pathway in Col6a1^{+} IMCs in spontaneous intestinal tumorigenesis. Consistent with these data, *in vitro* activation of Myd88 knockout IMCs with LPS demonstrated an altered inflammatory gene signature underscoring the pivotal role of this pathway in regulating IMC-specific inflammatory response.

These results may seem to be in contrast with the anti-tumorigenic effect of IKK2 deletion using the same genetic tools and mouse model (Koliaraki, Pasparakis et al. 2015). However, each one of these stimuli can drive activation of NF-κB signaling and the absence of more than one may be necessary to dampen NF-κB in these cells. Furthermore, since Col6a1^{-} cells are also able to respond to inflammatory stimuli, the microenvironmental milieu of individual cells plays a crucial role in their *in vivo* activation. As such, deletion of individual receptors in another or in multiple subsets may be sufficient to inhibit carcinogenesis *in vivo*. Indeed, IL-1 signaling in Grem1^{+} cells is sufficient and necessary for recovery after DSS-induced colitis (Cox, Storm et al. 2021). In addition, AOM/DSS carcinogenesis is driven by robust and recurring inflammation, in contrast to the Apc-driven adenomas, in which inflammation emerges subsequent to their establishment. This disparity in the timing and intensity of inflammatory stimuli could significantly influence the activation signals received and responded to by resident fibroblast populations, thereby contributing to distinct TME characteristics.

In conclusion, this work offers valuable insights into intestinal CAF heterogeneity and the role of subset-specific innate immune sensing in driving intestinal carcinogenesis.

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