THERAPEUTIC POTENTIAL OF MESENCHYMAL-TARGETED APPROACHES IN INTESTINAL DISEASE

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It was a year full of joy.

Abstract

Intestinal mesenchymal cells (IMCs) comprise a highly heterogeneous population with distinct origin, function and molecular markers that participate actively in intestinal development, as well as in colitis and colitis-associated cancer (CAC), through their involvement in regulation of inflammation and modification of the tumor microenvironment. Recent findings have proposed signaling pathways and specific proteins that could be useful in order to target activated mesenchymal cells, however, the identification of the potential therapeutic effect of diverse IMC subpopulations in intestinal disease remain elusive. In this study we addressed two potential ways to target mesenchymal subpopulations; by their specific deletion based on diphtheria toxin (DT) administration in engineered DT-sensitive transgenic mice, which was applied in homeostasis and CAC, and by transplantation, applied principally in colitis and tissue damage. We analyzed the systemic and topical routes of DT treatment and standardized among different dosing schemes the one that would result in efficient deletion of the ColVI mesenchymal subpopulation and low toxicity to the mice. Hence, we showed that the topical, but not systemic, route of DT administration represents a prosperous strategy of eliminating ColVIcre+ cells of the colon both in homeostasis and colorectal cancer. On the other hand, targeting of mesenchymal cells in colitis by their transplantation, either through implantation to the intestinal mucosa or topical transplantation specifically in the ulcers, would require further standardization to prove a promising therapeutic approach. Collectively, our results reveal the efficiency of the diphtheria toxin system, which in addition to other Cre lines could represent a valuable tool to gain insight in the contribution of mesenchymal subpopulations in intestinal development and tumorigenesis and to design novel therapeutic approaches targeting the tumor microenvironment.

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1. Introduction

1.1 Intestinal Mesenchymal Cells

Intestinal mesenchymal cells (IMCs) are a highly heterogeneous cell type, comprising of subpopulations with different origin and function, which reside in the interstitial space adjacent to the mucosal epithelium of the villi and crypts, known as intestinal lamina propria (Fig.1). These mesenchymal elements of the lamina propria can be divided in myofibroblasts, fibroblasts, mural cells (pericytes), bone marrow-derived mesenchymal stem cells, smooth muscle cells of the muscularis mucosae and smooth muscle cells associated with the lymphatic lacteals.[1]

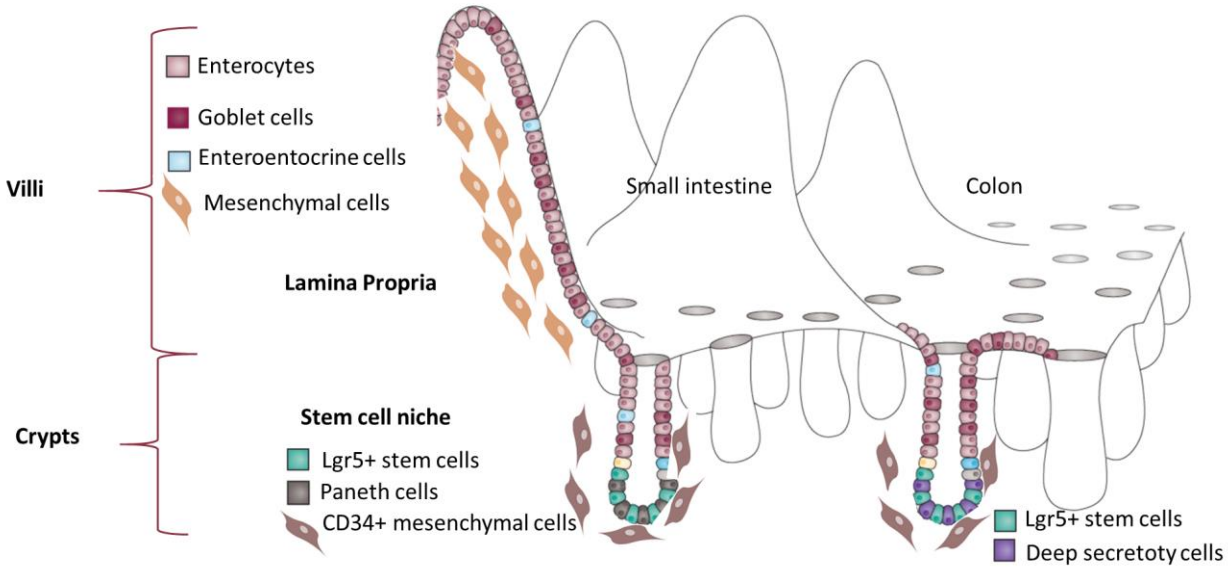


Fig.1 Schematic representation of the cellular hierarchy of the intestine. The major differentiated cell types are enterocytes, goblet cells and enteroendocrine cells and localize in the mucosal epithelium. Paneth cells, Lgr5+ cells, CD34+ mesenchymal cells and deep secretory cells reside in the intestinal stem cell niche. Intestinal mesenchymal cells are found in the lamina propria adjacent to the mucosal epithelium of the villus/crypt axis.[2]

During development, intestinal stromal cells can be found in the lamina propria as early as 21 weeks of gestation in the human embryo and embryonic day (E) 18.5 in the mouse. They originate from the visceral mesoderm, while neural crest cells have also been proposed as a potential source of intestinal stromal cells.[3] In addition, there is strong evidence that fibroblast

and myofibroblasts of the intestinal lamina propria originate from the serosal mesothelium, as lineage tracing of mesothelin positive (Msln+) serosal mesothelial cells demonstrated that at E11.5 these cells undergo epithelial-to-mesenchymal transition (EMT), migrate through the intestine and give rise to mesenchymal cells.[4]

1.2 IMCs heterogeneity

Despite the diverse intestinal mesenchymal cell populations, there is a significant overlap in molecular marker expression, which has hindered so far the delineation of the origin and function of the different subpopulations.[5] IMCs are classically characterized as negative for hematopoietic ($CD45^-$), endothelial ($CD34^-$) and epithelial ($EpCAM^-$) markers and positive for combinations of CD90 (also known as Thy-1), a-smooth muscle actin (a-SMA), S100A4, platelet derived growth factor receptor (PDGFR), vimentin and desmin. Monoclonal antibodies raised against reticular fibroblasts (ER-TR7 and TE-7) also show reactivity against mesenchymal elements of the lamina propria, such as fibroblasts, myofibroblasts, pericytes and lymphatic stromal cells. Moreover, additional candidate markers are fibroblast-specific protein 1 (FSP-1), fibroblast activation protein (FAP), neural glial antigen 2 (NG2, also known as melanoma-associated chondroitin sulfate proteoglycan (MCSP)) and the matrix protein periostin.[6] However, even though the aforementioned molecular markers are useful in the characterization of IMCs, the definitive distinction of mesenchymal subpopulations remains still unclear. Thus, for instance, a-SMA positive expression aids in the separation of myofibroblasts and pericytes from fibroblasts and, accordingly, differential expression of desmin could be effective in the separation of $a\text{-}SMA^+$ myofibroblasts from pericytes, which are weakly positive for desmin, and from the smooth muscle of the muscularis mucosae and lymphatic lacteal, which are strongly $desmin^+$.[1] Other studies have also identified the surface enzyme AOC3 (amine oxidase, copper containing 3) and the transcription factor Nkx2.3 as additional highly distinctive markers for myofibroblasts.[7] (Table 1)

A significant advance in the field of molecular markers that identify subpopulations of mesenchymal cells was accomplished recently by Stzepourginski et al. [8] This research group

revealed that coexpression of gp38 (podoplanin) and CD34 markers can identify a subpopulation of aSMA$^-$ stromal cells localized near the crypts of the colon and small intestine, which are closely associated with Lgr5$^+$ intestinal epithelial stem cells (IESCs) and are distinct from the mesenchymal cells on the top of the villi and crypts. Furthermore, they demonstrated that these gp38+ CD34+ fibroblasts are the principal producers of the intestinal niche factors Wnt2b, Grem1 and Rspo1, supporting the maintenance of the stem cell niche and inhibiting epithelial differentiation at homeostasis. These findings imply that the stromal cells acquire different functions according to their location and the surrounding tissue and microenvironment.

Our lab has also identified another distinct subpopulation of IMCs, which is specifically targeted by the ColVI-cre mouse[9], is characterized as CD201$^+$ and CD34$^-$ and is preferentially located on the top of the villi, rather than the bottom of the crypts. These cells act to support epithelial cell differentiation and proliferation, as well as blood endothelial cell function. Moreover, upon tissue damage, they orchestrate the production of pro-inflammatory cytokines, whereas at the same time maintain their unique expression profile and intrinsic properties (unpublished data).

Table 1 Molecular marker expression and functions of IMC subpopulations in homeostasis and cancer[10]

	Subpopulations	Markers	Functions
IMCs in homeostasis	Fibroblasts	α-SMA$^-$, Vimentin$^+$, CD90$^+$, Desmin$^-$, FSP1$^+$, PDGFRα^+, PDGFRβ^-, VCAM1$^-$, AOC3$^-$, NKX2-3$^-$, SHOX2$^+$, SMM$^-$	Mechanical support, epithelial homeostasis, stem cell niche maintenance, immune regulation, ECM maintenance, regulation of angiogenesis and vascular function
	Myofibroblasts	αSMA$^+$, Vimentin$^+$, CD90$^+$, Desmin$^-$, ER-TR7$^+$, PDGFRβ^+, VCAM1$^-$, MHC class I,II$^+$, CD80/86$^+$, Collagen I$^+$, NG2$^+$, AOC3$^+$, NKX2-3$^-$, SHOX2$^-$, SMM$^-$, FAP$^+$	
	Pericytes	αSMA$^+$, Vimentin$^+$, Desmin$^+$, PDGFRα^+, PDGFRβ^+, VCAM1$^+$, MHC class I,II$^+$, CD80/86$^+$, NG2$^+$, SMM$^-$	Regulation of angiogenesis, cell trafficking, vascular function, stem cell properties
	MSCs	Vimentin$^+$, CD90$^+$, PDGFRα^+, PDGFRβ^+, ICAM1$^+$, VCAM1$^+$, CD73$^+$, CD105$^+$, CD29$^+$, CD44$^+$, SMM$^-$	Stem cell properties
	SMCs	αSMA$^+$, Vimentin$^-$, CD90$^-$, Desmin$^+$, FSP1$^-$, PDGFRα^+, VCAM1$^-$, NG2$^+$, AOC3$^+$, NKX2-3$^+$, SHOX2$^-$, SMM$^+$	Mechanical support, smooth muscle contraction
Nonconventional IMCs	Interstitial cells of Cajal	Vimentin$^+$, c-kit$^+$	Pacemaker of the intestine
	Fibrocytes	CD11α^+, CD11b$^+$, CD13$^+$, CD32$^+$, CD64$^+$, MHC class I/II$^+$, CD40$^+$, CD80$^+$, CD86$^+$, CD34$^+$, CD105$^+$, CD18$^+$, CD29$^+$, VD49$^+$, CD61$^+$, Collagen I$^+$, Fibronectin$^+$, Vimentin$^+$	Mesenchymal progenitor cells
CAFs	αSMA$^+$ myofibroblasts, MSCs	αSMA$^+$, Vimentin$^+$, FAP$^+$	Tumor-promoting role
	Activated tissue fibroblasts	αSMA$^\pm$, Vimentin$^+$, FSP1$^\pm$, PDGFR$\alpha^\pm$	Tumor-promoting role, possible tumor-inhibitory role from subpopulations of non-activated cells
	Originating from EMT/EndoMT	αSMA$^\pm$, Vimentin$^+$, FAP$^\pm$	Tumor-promoting role, metastasis
	Pericytes	αSMA$^+$, Vimentin$^+$, Desmin$^+$, PDGFRα^+, PDGFRβ^+, VCAM1$^+$, MHC type I,II$^+$, CD80/86$^+$, NG2$^+$, SMM$^-$	Tumor-promoting role, regulation of angiogenesis

AOC3, amine oxidase copper containing 3; CD, cluster of differentiation; FAP, fibroblast activating protein; FSP1, fibroblast-specific protein 1; ICAM1, intercellular adhesion molecule 1; NKX2-3, NK2 Homeobox 3; SHOX2, short stature homeobox 2; SMM, smooth muscle myosin; VCAM1, vascular cell adhesion protein 1.

1.3 IMCs in homeostasis

In the normal colon, mesenchymal cells and more specifically fibroblasts are identified as spindle-shaped cells embedded in the ECM and are usually quiescent and inert with negligible metabolic and transcriptomic activity. They create focal adhesions with the ECM and lack smooth muscle myofilaments and external lamina, which are characteristics of smooth muscle cells.[11]

Mesenchymal cells provide structural and mechanical support, as well as growth regulatory elements, which are integral to IMCs' essential role in intestinal development and gut homeostasis. During development, mice deficient for PDGF (platelet derived growth factor), a significant mesenchymal growth factor, which is also important for mesenchymal recruitment, demonstrate depletion of stromal cells in the crypts, resulting in abnormal villus architecture.[12] Intestinal organogenesis is also regulated by mesenchyme-specific transcription factors (Foxl1, Nkx2.3, HOX family) and secreted factors involved in several signaling pathways, such as Notch, Hedgehog, Wnts, Bmps, IGF and FGF.[13] Among these pathways, Hedgehog signaling in IMCs promotes aggregation of PDGFRα^{+}, Hh-responsive mesenchymal cell clusters that form before villus emergence and adhere tightly to the epithelium, regulating thus the initial steps of villus development.[14] Notch signaling is mainly involved in cell fate determination by directing cells towards a specific lineage and in expansion of the crypt progenitor pool, whereas Wnt/β-catenin signaling acts principally to maintain the intestinal stem cell niche and progenitor cell proliferation. In contrast, BMP signaling factors produced towards the tip of the villi, act as antagonists of the Wnt signaling, inhibiting stem cell activation and stimulating differentiation of basal crypt epithelial cells.[15] The major such morphogens expressed in the mesenchymal compartment that regulate the patterning of the crypt-villus axis are Bmp2 and Bmp4, which are identified as downstream targets of Hedgehog secreted factors, as well as of the mesenchymal Foxl1 winged helix and Nkx2.3 transcription factors.[13] On the other hand, Bmp antagonists expressed by pericryptal stromal cells in the bottom of the colon crypts, such as gremlin1, gremlin2 and chordin-like 1, regulate the stem cell niche and inhibit epithelial cell differentiation by activating the Wnt pathway.[16] Moreover, the transcription factor Foxl1 exerts its role in the

Wnt signaling pathway and stem cell maintenance by regulating the expression of extracellular proteoglycans that act as co-receptors for Wnt ligands.[17] Finally, the stromal cell compartment is the predominant site generating R-spondins (Wnt-activating growth factors), non-canonical Wnt ligands and Wnt antagonists, such as Dickkopf proteins (Dkk2 and Dkk3) and secreted-frizzled related proteins (Sfrp1 and Sfrp2), further mediating the crypt-villus patterning during homeostasis (Fig.2).[18,19]

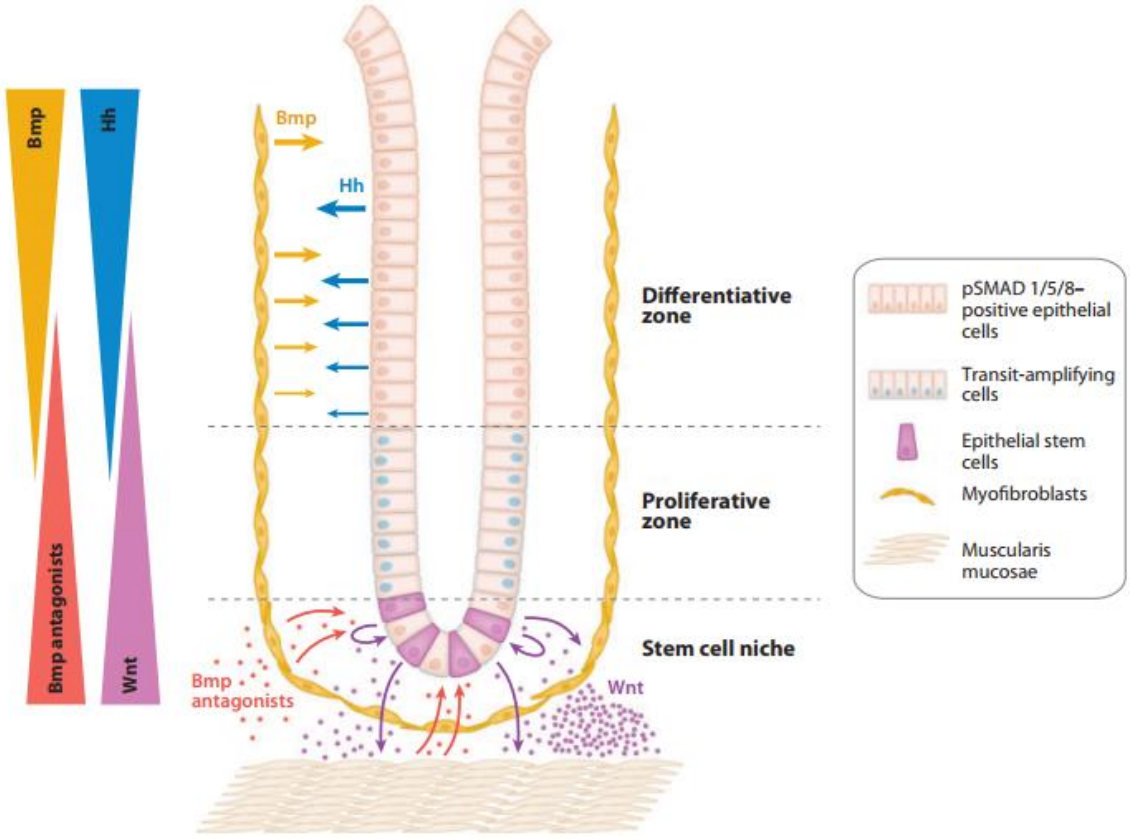


Fig.2 In homeostasis, IMCs generate signals, which are necessary to maintain the different compartments along the crypt villus axis. Expression of BMP antagonists and Wnt molecules in the bottom of the crypts mediates stem cell niche maintenance, whereas activation of the BMP and Hedgehog signaling pathways towards the tip of the villi inhibits proliferation and favors differentiation of epithelial cells.[6]

Another function of a specific subtype of IMCs, the pericytes of the lamina propria, is their contribution in endothelial cell function. In general, pericytes interact with the endothelial cells of the blood vessels through paracrine and chemo-mechanical signaling so as to regulate vascular

permeability and angiogenesis.[20] Specifically for the intestine, recent data from our lab have shown that a specific mesenchymal subpopulation, ColVI$^+$ fibroblasts, surround blood but not lymphatic vessels, acting thus as pericytes and regulating blood vessel functions, such as vasoconstriction.[21]

In addition, fibroblasts and myofibroblasts play an essential role in the maintenance of extracellular matrix (ECM) and tissue regeneration, since they are the primary ECM-secreting cells, especially during wound healing and fibrosis.[6,22] They express high levels of ECM proteins, such as collagen I, III, V, VI, as well as glycoproteins and proteoglycans (fibronectin, laminin, tenascin). Moreover, due to their contractile properties, they can sense and modulate ECM stiffness through integrin-mediated focal adhesions.[23]

Finally, IMCs play a significant role in the regulation of the local immune landscape by alternating between inflammatory and immunosuppressive (tolerogenic) states, depending on the surrounding dynamic microenvironmental milieu of cytokines, chemokines, growth factors and pathogen-associated patterned molecules (PAMPs). They interact with professional immune cells and shape the innate and adaptive immune responses and, additionally, serve as a conduit for migration of recruited immune cells.[24] Stromal fibroblasts and myofibroblasts are important contributors to the innate immune response by virtue of their location under the epithelial barrier, their number and their capability to express Toll-like receptors (TLRs). Engagement of TLR4, for instance, induces expression of pro-inflammatory soluble mediators and adhesion molecules (ICAMs, VCAMs), which leads to chemoattraction of professional immune cells, such as lymphocytes, macrophages and neutrophils.[25] CD90$^+$ intestinal fibroblasts and myofibroblasts have been shown to sense microbial patterns through TLRs and modulate T cell function. Moreover, the stromal cells of the lamina propria express MHCII molecules, accessory co-stimulatory (CD80, CD86) and inhibitory (PD-L1, PD-L2) molecules, through which they mediate efficient antigen presentation to CD4+ T cells residing in the lamina propria and regulate the activity of B cells, dendritic cells and NK cells.[26] During allogeneic bone marrow transfer, MHC-II$^+$ myofibroblasts also act as antigen-presenting cells to stimulate donor T cell proliferation in the intestine.[27] Finally, mesenchymal cells can act as peripheral educators, instructing the functional

maturation of immunocompetent cells, such as dendritic and mast cells in the peripheral tissues, through the secretion of type I IFN and stem cell factor (SCF).[28]

1.4 IMCs in tissue damage and colitis

Acute damage to the functional parenchyma of the intestine unleashes an inflammatory response that aims to repair the damage and maintain tissue homeostasis, by recruiting immune cells, such as T cells, neutrophils and macrophages. Infiltrating and locally activated immune cells secrete mediators and ECM-modifying and degrading enzymes, paving further the way for the migration of reversibly activated fibroblasts to the inflamed area.[29] These fibroblasts, also known as NAFs (normal activated fibroblasts), show increased expression of a-SMA and vimentin and support the restitution of the tissue by secreting growth factors, such as hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), epidermal growth factor (EGF), transforming growth factor β (TGFβ) and acidic and basic fibroblast growth factors (FGFs). Moreover, fibroblasts acquire a stellate shape, which supports their enhanced contractility, and synthesize and secrete higher levels of ECM proteins. These new secretory and migratory features result in further activation, recruitment and proliferation of fibroblasts and are fundamental events for the production of connective tissue during the initial phase of the wound healing.[30] (Fig.3)

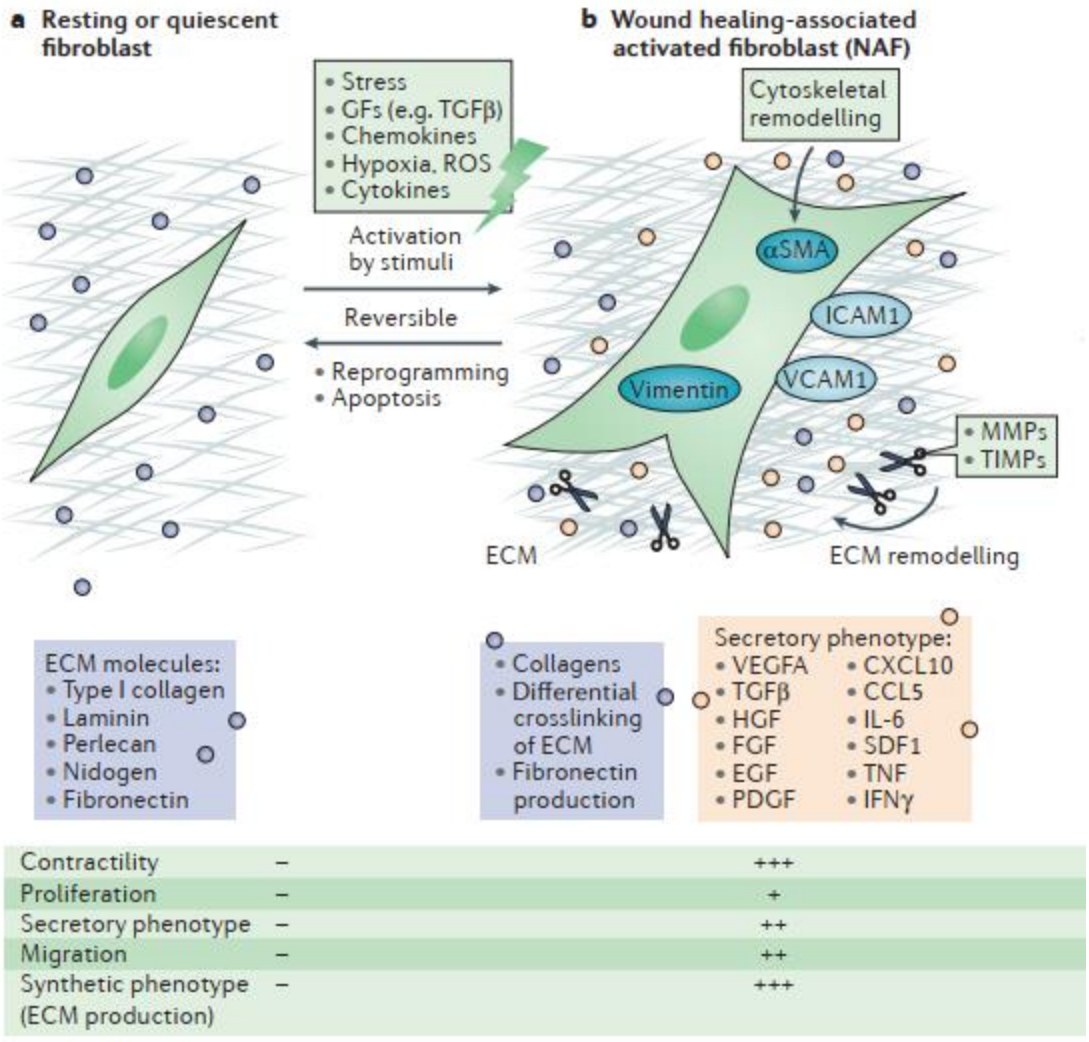


Fig.3 Activation process of fibroblasts. (A) Quiescent fibroblasts are inert and embedded in physiological ECM. (B) Tissue injury and inflammatory stimuli promote their reversible activation to normal activated fibroblasts (NAFs) in order to stimulate tissue repair and regeneration. NAFs gain expression of a-SMA and vimentin and enhance their contractility, as well as their secretory and migratory functions. The resolution of the injury is followed by return of NAFs in the quiescent state by reprogramming or apoptosis.[30]

In normal physiological conditions, once the damage is resolved, local growth factors, such as TGFβ1 and endothelin-1, decrease, which results in apoptosis and reprogramming of the activated fibroblasts, inducing significant reduction in their population (Fig.3)[31].

In pathologic situations, however, such as inflammatory bowel disease (IBD), there is a perpetual wound healing response, accompanied by chronic inflammation and severe mucosal tissue damage.[32] Inflammatory bowel disease (IBD) includes mainly Crohn's disease (CD) and ulcerative colitis (UC). Ulcerative colitis is primarily a mucosal disease of the underlying connective tissue, characterized by persistent ulcerations, whereas the main feature of Crohn's disease, also known as granulomatous colitis, is inflammation that can also extend towards the bowel wall.[33] During such chronic inflammation, activation of anti-apoptotic pathways and hyper-activation of fibroblasts can lead to fibrosis through excessive deposition of collagen-rich ECM. Furthermore, the homeostatic mechanisms of tissue repair of the fibroblasts may be altered, driving further intestinal inflammation and creating a vicious cycle between the inflammatory microenvironment and the activated fibroblasts.[24]

Fibroblast activation in these settings is induced by several signals, including immune mediators from the damaged epithelium, such as TGFβ, Il-1, TNF and transglutaminase-2 (TG-2), but also immune modulators, such as TSLP, Il-25 and Il-33, that activate immune cells subsequently inducing myofibroblasts activation.[34] TGFβ is expressed during inflammation by many cell types and in many organs besides the intestine, and constitutes one of the most potent cytokines that promote fibroblast migration, proliferation and differentiation, as well as recruitment of pericytes, fibrocytes and inflammatory hematopoietic cells.[35] Other inflammatory agents act on diverse resident mesenchymal cell subsets, including fibroblasts and pericytes, and induce their conversion into myofibroblasts that have an altered rate of extracellular matrix production. Such mechanisms are described in models like renal fibrosis, where TGF-β is released by the injured tubular epithelium inducing thus pericyte to myofibroblast transition, or lung fibrosis where lung fibroblasts are differentiated to myofibroblasts with enhanced ECM production and contractility.[36,37] Additionally, epigenetic mechanisms induced by inflammatory mediators can result in the perpetuating and irreversible activation of fibroblasts.[38] Following activation, fibroblasts alter their immunomodulatory functions to produce and secrete cytokines, such as TGFβ1, Il-1β, Il-33, CXC and CC chemokines and prostanoids, which aim to the recruitment of immune cells, while they also regulate the ECM composition facilitating further the migration and retention of inflammatory cells in the affected tissue (Fig. 4)[39].

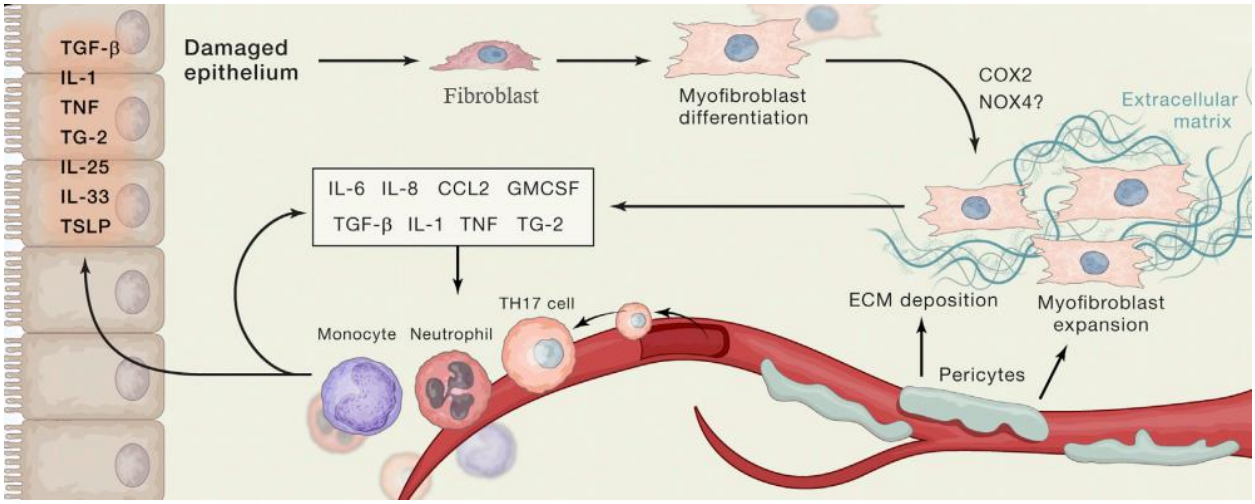


Fig.4 Chronic inflammatory conditions induce differentiation of mesenchymal cells, such as fibroblasts and pericytes into ECM-producing myofibroblasts, thus leading to excessive production of inflammatory cytokines and chemokines, recruitment of immune cells and eventually exacerbation of the tissue damage and perpetuation of the pathological inflammation.[24]

1.5 IMCs in Colorectal Cancer

1.5.1 Colorectal Cancer

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and the 4th cause of cancer death worldwide, representing a major health issue.[40] It is a complex pathology that progresses from aberrant crypt foci to adenoma and carcinoma development and is influenced by multiple factors. Approximately 20% of CRC incidents are linked to genetic predisposition, such as familial adenomatous polyposis (FAP) and hereditary non polyposis colorectal cancer. However, most of the CRC cases are primarily attributed to a combination of genetic and environmental factors. Risk factors involved in disease pathogenesis include diet, exercise, food-borne mutagens, certain commensal bacteria and chronic intestinal inflammation, which precedes tumor development.[41] A subtype of CRC, colitis-associated cancer (CAC), affects patients with long term complications of inflammatory bowel disease (IBD) and is characterized by more rapid progression and increased severity and mortality. Inflammation is present though, in all colorectal cancers, even those without clinically detectable IBD and is associated with

forms of sporadic, as well as heritable colon cancer. In sporadic CRC, inflammation has mostly the role of recruiting immune cells in the tumor microenvironment after tumor formation, rather than contributing initially to tumor development, as it happens with CAC. In CAC, existence of prior chronic inflammation leads to tumor formation by inducing tissue remodeling, promoting angiogenesis and recruiting growth factors.[42] Nevertheless, CAC and sporadic or familial CRC share many common genetic and signaling pathway alterations, such as mutations in adenomatous polyposis coli (APC) suppressor gene, p53, K-Ras, transforming growth factor (TGF)-β and DNA mismatch repair (MMR) proteins. However the timing and the range of mutation acquisition can differ significantly between them. For example, in over 90% of sporadic CRCs mutations in the APC gene happen at the initial stage of tumor formation, whereas CAC is associated with earlier genomic alterations in p53 and K-Ras, resulting in prolonged activation of Nf-κB and further enhancement of inflammation.[43] The inflammatory environment, in turn, stimulates production of reactive oxygen species (ROS), which contribute to DNA damage and accumulation of genetic changes, including mutations in APC gene later during tumor progression (Fig.5).[44]

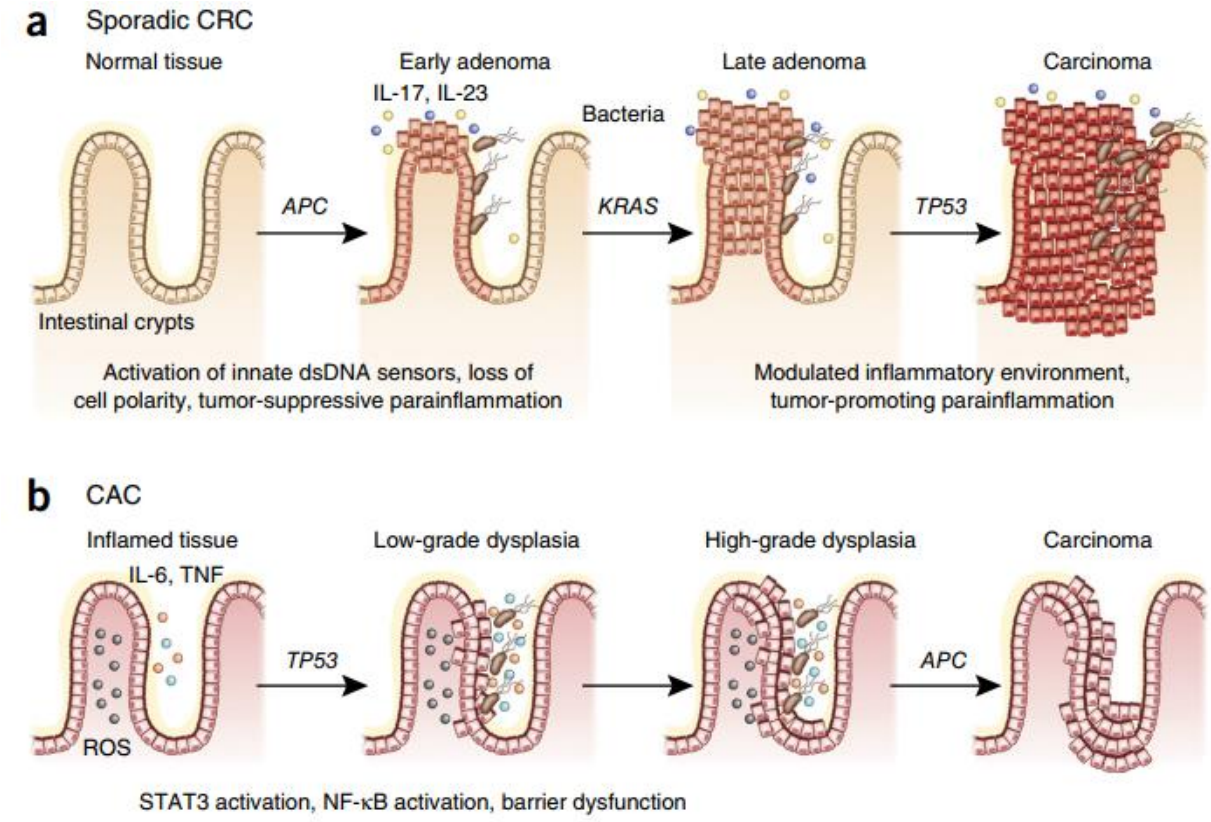


Fig.5 Mechanisms of sporadic CRC and CAC development. (a) Sporadic CRC is associated with early accumulation of mutations in the Wnt pathway, and especially in the gene encoding APC, followed by mutations in Kras and the gene encoding p53. These genetic changes enable the transition of preneoplastic cells to adenoma and then carcinoma. (b) In CAC, chronic inflammation of the intestinal tissue leads to constant activation of NF-κB and mutations in oncogenes and tumor suppressor genes, further facilitating tumor progression.[44]

1.5.2 The CRC tumor microenvironment

CRCs, like most solid malignancies, are currently recognized as highly heterogeneous tumors. Earlier studies along with recent reports based on single cell sequencing techniques, have analyzed the cellular diversity in human colorectal tumors identifying various distinct cell types. These cellular types comprise the evolving cancer epithelial cells, as well as activated fibroblasts, endothelial cells and immune cell populations, which could be further divided into more subtypes and represent the tumor stroma or tumor microenvironment (TME).[45] The interaction between cancer and stromal cells plays a critical role in tumor growth and involves both

epithelial cell transformation stimulated by stromal changes and paracrine stromal cell activation by transformed epithelial cells.[46] Importantly, the tumor microenvironment also includes extracellular matrix (ECM) components, including proteins, glycoproteins, proteoglycans and polysaccharides, whose deregulated architecture also affects the surrounding stromal cells, further favoring tumor progression.[47] (Fig.6)

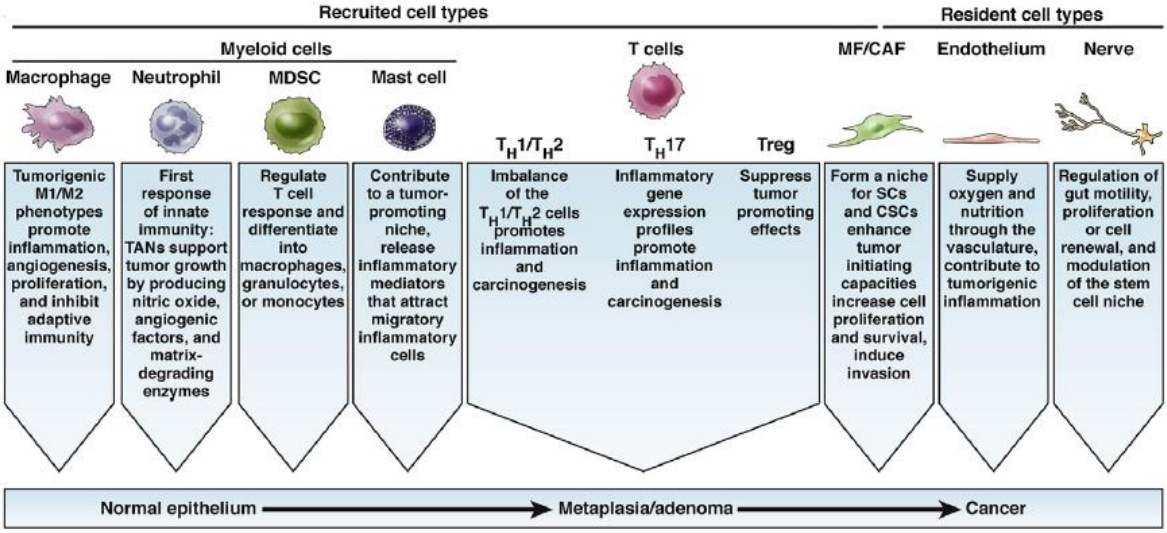


Fig.6 Recruited (myeloid cells, T cells, CAFs) or resident (CAFs, endothelial cells, nerves) cell types in the tumor microenvironment and their functions in tumor development. The complex interaction between these cell types favors the transformation of the normal epithelium to metaplastic and carcinogenic.[48]

The network of infiltrating immune cells interacts with the cancer cells of the microenvironment, through chemokines and cytokines, leading either to tumor suppression or cell survival and tumor progression. Despite the role of both innate and adaptive immune cells to support anti-tumor immunity during the early stages of tumor formation, subsequently, growing premalignant cells evade immune surveillance and become resistant to apoptosis. Additionally, innate and adaptive immune responses are suppressed through activation of several pathways.[49] For instance, cytokines present in the tumor microenvironment, such as Il-10, hinder the differentiation and activation of DCs, which act as key inducers of adaptive immunity, and infiltrating regulatory T cells suppress both innate and adaptive immune cells.[50] Later on,

recruited activated immune cells, contribute to increased intra-tumoral inflammation and promote tumorigenesis through production of cytokines, growth factors, enzymes and angiogenic factors.[48]

Vascular cells, comprising endothelial cells (ECs), pericytes and platelets are also involved in intestinal cancer development, as increased vascular density in the tumor site is closely related to tumor growth, metastasis and poor survival.[49] Highly proliferative ECs are recruited to the TME by angiogenic factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) to establish a vascular niche. From that niche, ECs also secrete angiogenic factors, modulating infiltration of inflammatory cells and remodelling the ECM to create a microenvironment that favors tumor invasiveness and progression. In addition, platelets form aggregates that protect the malignant cells during colonization and metastasis and help them evade immunosurveillance.[51]

Finally, apart from immune and vascular cells, fibroblasts, usually referred to as cancer-associated fibroblasts (CAFs), are a dominant cellular component of the solid intestinal tumors. Over the past decade, many studies have suggested their prominent functional role in tumor initiation, growth and metastasis through interaction with neoplastic cells and other cell types of the microenvironment and regulation of a wide range of fibrotic stromal programs of the tumor.[30] Their implication in cancer is also correlated with their role in tissue repair processes and in inflammation, reminiscently of the consideration of the tumors as wounds that do not heal.[52] Moreover, abundance of CAFs has been associated with poor prognosis of patients, and colon cancer transcriptomic studies have identified their functional importance and their value as prognostic factors in disease progression and recurrence. They will be analyzed in greater detail below.[53–55]

1.5.3 CAFs in Intestinal Cancer

CAFs are a complex and heterogeneous population that comprises several subpopulations of diverse origin and functions, including myofibroblasts, reprogrammed local tissue fibroblasts and

bone marrow derived cells.[56] Emerging data from single cell transcriptomic analysis in human colorectal tumors have further highlighted this diversity by identifying distinct transcriptomic profiles and pathway alterations between CAF subtypes.[45] Several markers have been suggested to identify CAFs, however it is now appreciated that they cannot detect CAFs completely and exclusively. Such general markers of CAFs include a-SMA and FAPα, which are expressed in other cell types as well, such as pericytes and smooth muscle cells for a-SMA and quiescent mesodermal cells of multiple tissues and activated fibroblasts during wound healing for FAP.[57,58] Other markers, such as FSP1 and PDGFRa that characterize unique CAF subpopulations, are usually expressed heterogeneously among CAFs, and they may represent different stages of activation and sources of CAFs.[59]

The recruitment and activation of CAFs in tumors is usually induced by neoplastic cells through secretion of growth factors and cytokines, such as TGF-β, Il-6 and basic fibroblast growth factor (b-FGF), as well as through exosomes transferring protein, RNA and miRNA.[60,61] Among them, TGF-β is the most prominent factor within the tumor microenvironment, since it is known to induce epithelial-to-mesenchymal transition of cancer cells and to promote tumor progression.[62] Moreover, epigenetic changes, such as global DNA hypomethylation, which could be induced through exosome signaling, have been shown to promote reprograming of CAFs and maintenance in an activated state.[63] It should be noted that in contrast to NAFs, CAFs are considered irreversibly activated cells, as they are able to maintain their activated phenotype even when removed from the activated stroma.[64]

CAFs have been shown to have mainly a pro-tumorigenic role and are involved in the regulation of innate and adaptive immunity, the activation of inflammatory pathways, the promotion of cancer cell stemness, the proliferation of nearby epithelial and neoplastic cells, ECM remodeling and the stimulation of angiogenesis. Additional, but not fully characterized, functions of CAFs include the modulation of epithelial cell metabolism and neurotransmission signals (Fig.7).[65,66]

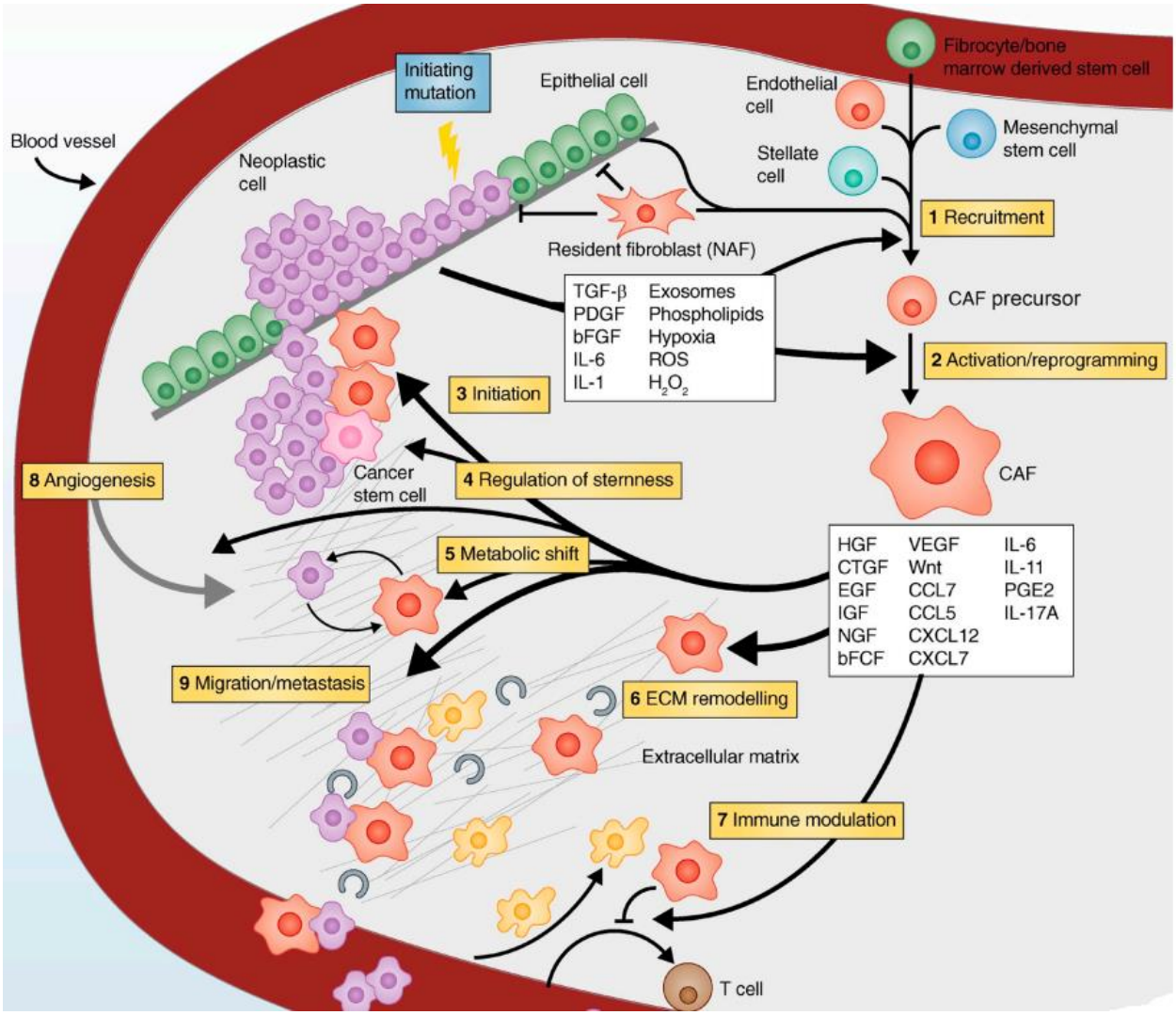


Fig.7 Schematic illustration of the mechanisms, through which CAFs promote tumorigenesis. The transformed epithelium secretes molecules that aim to recruitment (1) and reprogramming (2) of CAFs. Subsequently, activated CAFs promote tumor initiation (3), regulate cancer stemness (4) and alter the tumor metabolism (5). Moreover, they secrete soluble factors that lead to ECM remodeling (6), prevention of immune surveillance of neoplastic cells (7) and enhanced angiogenesis (8). Finally, they facilitate invasion and metastasis of tumor cells (9).[65]

In more detail, CAFs are involved in tumor initiation and progression by cross-talking with the cancer cells through direct cell interaction via cadherin adhesion molecules or paracrine secretion of soluble mediators.[67] CAFs generate and secrete a number of crucial proinflammatory mediators, such as Il1β, Il-6 and Il-11, that potentiate proliferation and inhibit apoptosis of

preneoplastic epithelial cells and cancer cells, as well as matrix metalloproteinases-2,-3 and -9 that promote invasion and cell motility. Moreover, the aberrant paracrine secretion of soluble growth factors and chemokines by CAFs, such as SDF-1, VEGF, HGF, EGF, FGF2, and IGF has been correlated with promotion of tumor growth in the adjacent epithelium and neovascularization within the stroma.[68] Furthermore, CAFs can contribute to tumor resistance to chemotherapy and tyrosine kinase inhibitors through the secretion of Il-6 and HGF.[69,70]

Of particular importance is the mesenchymal secretion of HGF, as it has been shown to induce the tumorigenicity of cancer stem cells by supporting Wnt/β-catenin activation and their clonogenic potential.[71] Secretion of HGF by intestinal myofibroblasts was found to be regulated by the MAP3 kinase Tpl2, which during carcinogenesis, restrains mesenchymal HGF signaling in epithelial cells to hinder tumor formation. Interestingly, pharmacological inhibition of the HGF receptor c-Met in mice bearing ColVI-cre-mediated myofibroblast-specific deletion of Tpl2, was able to prevent the increased tumorigenesis.[72] Contrary to the protective role of Tpl2 in ColVI-cre expressing mesenchymal cells in colitis-associated cancer, Nf-κB is involved in promoting inflammation and cancer initiation in the same model. Specific ablation of IKKβ in ColVI-cre mesenchymal cells was shown to decrease tumor formation in mice subjected to colitis-associated cancer through reduced expression of inflammatory mediators and inflammatory infiltration and by reduction of STAT3 phosphorylation in the epithelium.[73] In contrast to these findings, deletion of IKKβ in Col1a2-cre-expressing cells is associated with enhanced tumor growth in the same model. IKKβ in Col1a2-cre cells increases the expression of TGF-β negative regulators, regulating thus HGF secretion by fibroblasts.[74] The contradictory results between the above studies possibly reflect the heterogeneity of CAFs and the different roles of the subpopulations targeted by the two cre-lines, since Col1a2-cre targets ¬80% of PDGFRα+ intestinal fibroblasts, whereas ColVI-cre a subpopulation of them. In conclusion, all these studies are indicative of the central role that CAFs exert in the tumor microenvironment, however the molecular mechanisms through which these cells, and particularly their subpopulations, act to promote their protective or protumorigenic effect, need to be further elucidated.

1.6 Therapeutic Approaches using IMCs in intestinal disease

The importance of IMCs in intestinal pathologies, such as colitis and colitis-associated cancer (CAC), is currently well established, through their involvement in regulation of inflammation and modification of the tumor microenvironment. Potential ways to target mesenchymal cells therapeutically could be through their transplantation, especially in inflammation and tissue damage, whereas in cancer, CAFs could be targeted through their elimination, reversion to a more mesenchymal state or cell specific drug delivery.

1.6.1 Transplantation of IMCs in colitis

Mesenchymal stem cell (MSC)-based therapy carries expectations as a novel treatment strategy to reestablish mucosal barrier function in patients with severe colitis, taking into consideration that the currently available clinical medications have limited success.[75] MSCs possess unique properties of self-renewal and differentiation into diverse mesenchymal lineages, as demonstrated by in vitro studies.[76] More importantly, transplanted mesenchymal cells have the ability to mediate tissue repair and regeneration either from a long distance or by directly migrating to the area of insult and their administration in clinical trials has already demonstrated preliminary feasible and beneficial results.[77,78] Their mechanism of action in inflammation suppression and amelioration of the intestinal pathology involves attenuation of Th1 responses, induction of Il-10 secreting regulatory T cells and polarization of macrophages towards an anti-inflammatory state.[79] Moreover, once located at an inflammatory site, MSCs can exert local functional effects in the resident tissue by secreting an array of trophic molecules that include growth factors (HGF, KGF, VEGF), cytokines (TGF-β, Il-10, Il-6) and soluble extracellular matrix glycoproteins. These trophic molecules play significant role in reducing inflammation, apoptosis and fibrosis, as well as in facilitating tissue regeneration.[80]

The majority of the clinical studies conducted so far use MSCs derived mainly from bone marrow, umbilical cord or adipose tissue and show a reduction in inflammatory activity and stimulation of a reparative process in the intestinal mucosa.[79,81,82] However, MSCs are a

heterogeneous population and distinct subsets can acquire different functions, so there is a substantial interest to determine the cell type or types that demonstrate the therapeutic benefit in colitis.[83] More importantly, the efficient transplantation of MSCs and their homing to the tissue of interest is a crucial consideration. To date, the most common route used in human and animal studies is intravenous injection. Despite the promising results, there are many reports showing that the majority of intravenously injected MSCs become trapped as emboli in the lung, due to their large size, limiting thus their homing and stem cell-related functions.[84] Specifically, a study of intravenous administration of human MSCs in sublethally irradiated mice, demonstrated inefficient homing of the cells in the inflamed intestine, since only 0.13% of MSCs were donor-derived 3 days after the transplantation.[85]

To overcome these difficulties, it has recently been proposed that tissue-resident multipotent stromal cells with overlapping but distinct properties to MSCs, could prove beneficial in cell transplantation therapeutic approaches.[86] In addition, local injection or topical transplantation could prove to be more beneficial in increasing MSCs engraftment and increasing the cell population that participates in healing and repair of the inflamed tissue.[84] Indeed, recent studies introducing to damaged mouse colon either cultured Lgr5+ colon organoids or immature progenitors expanded in vitro as fetal enterospheres showed increased integration to the colonic mucosa. In addition, they contributed to colonic regeneration by creating a single-layered epithelium with functionally and histologically physiological self-renewing crypts that expressed region-specific differentiation markers.[87,88] Furthermore, Manieri et al. demonstrated that mucosally injected colonic MSCs (cMSCs) migrate more efficiently to the damaged colon, preventing thus more effectively the development of penetrating ulcers compared to the intravenously injected MSCs, and promoting mucosal repair by stimulating angiogenesis in a VEGF-dependent manner.[77]

1.6.2 CAF-directed therapy in colorectal cancer

Targeting of mesenchymal stromal cells has emerged as a promising therapeutic approach also in cancer, where CAFs, being genetically more stable than neoplastic cells and retaining distinct

properties, are tempting drug targets.[65] These therapeutic approaches aim to interfere with the protumorigenic properties of CAFs and revert them to a more normal mesenchymal state or to ablate them from the tumor microenvironment and they will be discussed in more detail below. Another, appealing therapeutic intervention could be to deliver drugs specifically to CAFs, based on cell-specific receptors, as this approach could decrease side effects and increase efficiency (Fig. 8).[10] These strategies could be exploited alone or in combination with each other and cancer-targeted therapeutics to advance their therapeutic value in cancer therapy.

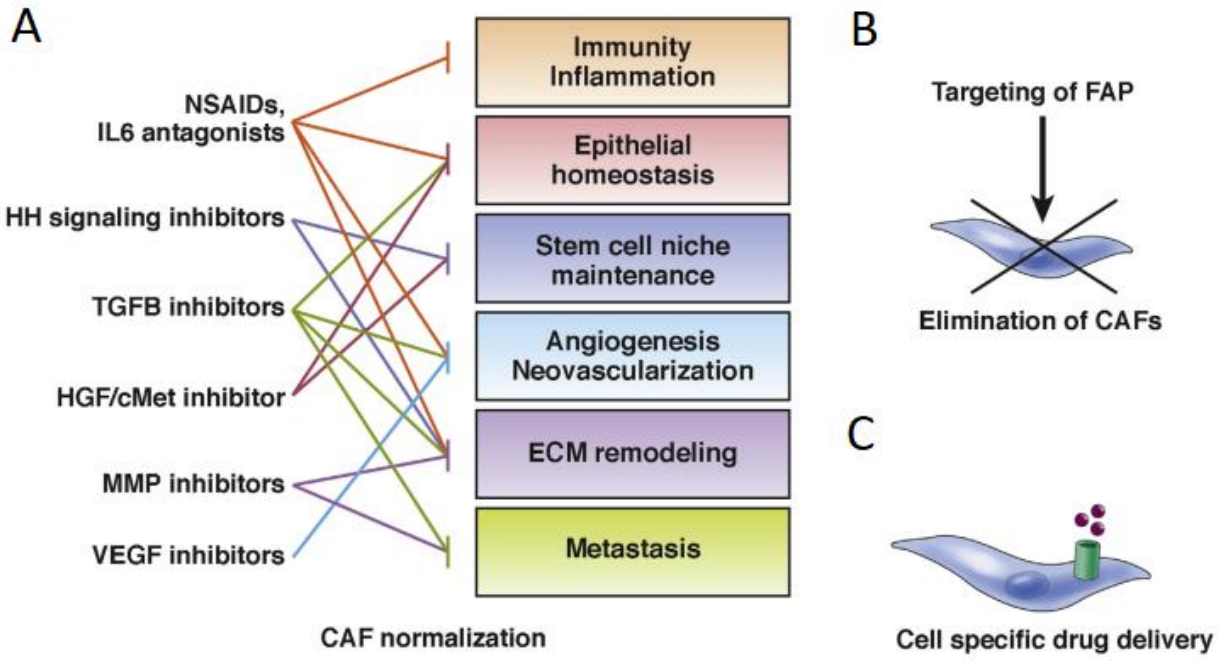


Fig.8 Therapeutic approaches targeting the tumorigenic functions of CAFs in cancer. (A) Normalization of CAFs based on their tumor promoting properties, (B) Elimination of CAFs based on the expression of cell surface markers, such as FAP, (C) CAF-specific drug delivery for reduced side effects and greater efficacy.[10]

1.6.2.1 CAF normalization

As immune modulators, CAFs contribute significantly in the process by which inflammation promotes carcinogenesis. Therefore, targeting the inflammatory properties that they acquire, in order to revert them to a more physiological fibroblastic state, appears as a promising intervention.[10,89] Agents that alter the Hh, Notch, TGF-β and HGF signaling are also evaluated, as

these pathways are a crucial part of the CAF phenotype. Multiple clinical trials that involve TGF-β antagonists or TGF-β inhibitors have been initiated, though the main challenge is that both pro- and antitumorigenic effects have been attributed to TGF-β receptors on fibroblasts.[90,91] Hh inhibitors have not been proven effective in clinical trials either.[92] In contrast, targeting the PDGFR and HGF pathways has shown promising results for the treatment of CAC. Testing of agents that block PDGFR signaling, such as dasatinib, on CAF cell lines demonstrated reduced tumor proliferation, confirming the efficiency of dasatinib in blunting CAF tumor-promoting activity.[93] Moreover, inhibitors of the HGF/c-Met pathway are interesting candidates for tumors, whose growth depends on sustained c-Met activation.[94] Furthermore, intervention in reverting the CAF phenotype is explored with anti-fibrotic agents or agents that block secretion of cytokines by CAFs. For instance, retinoic acid has an indirect effect in inhibition of tumor migration and epithelial to mesenchymal transition (EMT) of tumor cells, by blocking Il-6 secretion of CAFs.[95] Finally, agents that modulate ECM remodeling and angiogenesis, such as MMP and VEGF inhibitors, respectively, could also be used to restrain tumor-stromal alterations. However, the undesirable effect of MMP inhibitors on tumor-suppressive molecules, questions their anti-tumorigenic role and does not show encouraging results in clinical trials.[96]

1.6.2.2 Elimination of CAFs

The targeted elimination of tissue-resident cells using cell depletion strategies opens an alternative new avenue for cancer therapy since elimination of CAFs and their protumorigenic effects could prove highly beneficial in suppressing tumor activity.[97] CAFs express a range of proteins that could serve as targets, although many of them are not CAF-specific. Of these, FAP has emerged over the last decade as the most promising cell surface marker, as it is expressed robustly on CAFs and less in normal fibroblasts. Moreover, experimental tumor models have confirmed that overexpression of FAP promotes tumor growth and progression, whereas clinical studies have shown that colon tumors with high levels of stromal FAP are more likely to have aggressive disease progression.[98,99] Genetic deletion or pharmacological inhibition of FAP activity resulted in significant decrease of tumor burden in mouse models of colon cancer, by increasing

accumulation of collagen, decreasing myofibroblasts content and reducing blood vessel density in the tumors.[100] Furthermore, selective depletion of FAP-expressing cells in a subcutaneous model of pancreatic ductal carcinoma resulted in cytokine-mediated hypoxic necrosis of the tumor and the stroma and permitted immunological control of tumor growth.[101] Current clinical studies assess the effect of different FAP inhibitors, although early-stage results have not indicated their efficacy. An alternative strategy of eliminating CAFs could be through BH3 mimetics which trigger CAFs to apoptosis as a result of heightened PDGF signaling.[102] However, contradictory studies on the effect of CAF ablation, have demonstrated that depletion of a-SMA+ myofibroblasts in either early or late stages of pancreatic cancer, led to invasive, undifferentiated, highly hypoxic tumors, epithelial-to-mesenchymal transition and decreased survival.[103,104] Therefore, given the conflicting results of CAF elimination in tumor promotion or suppression, a crucial question would be which specific CAF subpopulations should be targeted, taking into account the complexity and heterogeneity of solid tumors.

2. Aim

Intestinal mesenchymal cells (IMCs) comprise a highly heterogeneous population with distinct origin, function and molecular markers that participate actively in intestinal development and wound healing. Their importance in colitis and colitis-associated cancer (CAC) is also well established, through their involvement in regulation of inflammation and modification of the tumor microenvironment. Recently, findings have proposed signaling pathways and specific proteins that could be useful in order to target activated mesenchymal cells, however, the identification of the contribution of diverse IMC subpopulations and their potential therapeutic value in intestinal disease remain elusive. In this study we will address two potential ways to target mesenchymal subpopulations; by their specific deletion, principally in CAC and by transplantation, principally in colitis and tissue damage. Ablation of distinct mesenchymal subpopulations will determine the effect of IMCs in intestinal inflammation and tumor initiation and progression and will be accomplished upon administration of diphtheria toxin (DT) in DT-sensitive CollagenVI (ColVI)-cre transgenic mice. Accordingly, transplantation will reveal the contribution of IMCs in repair of mucosal damage and will be achieved through isolation of mesenchymal cells and their subsequent implantation to the intestinal mucosa. Collectively, we aim to increase our knowledge in the function of specific intestinal mesenchymal cell subpopulations and gain novel insight in their potential application in the development of diagnostic biomarkers and therapeutic approaches in intestinal disease.

3. Methods

Mice

The generation of ColVI-Cre mice has been previously described.[9] ROSA^{mTmG},[105] Twist2-Cre,[106] as well as the ROSA26iDTR mice[107] were purchased from the Jackson Laboratory. Briefly, in the iDTR mouse model, the simian diphtheria toxin receptor is inserted into the ROSA26 locus. Widespread expression of DTR is inhibited by an upstream loxP-flanked STOP cassette. The STOP cassette, which prohibits DTR expression, is removed by crossing the iDTR$^{F/+}$ strain to a tissue specific Cre-expressing mouse strain. Consecutive expression of the DTR renders the respective cells sensitive to cell death induced by the administration of diphtheria toxin (Fig.9).

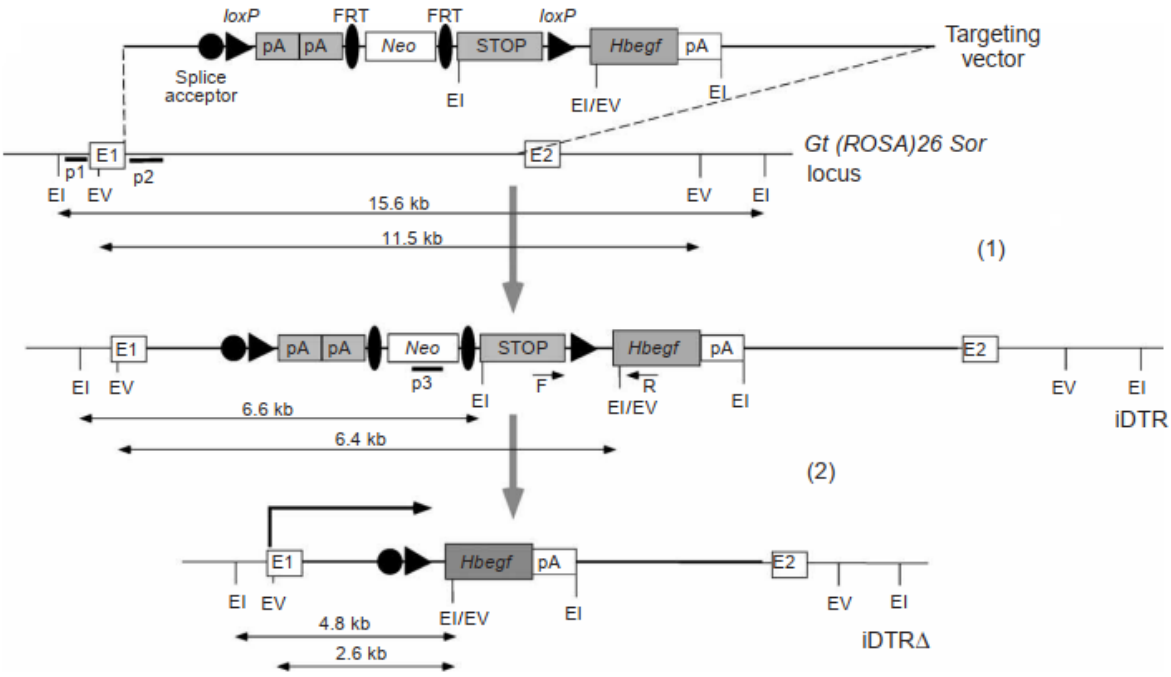


Fig.9 Generation of embryonic stem (ES) cells with Cre-inducible DT sensitivity. A targeting vector was designed with a loxP-flanked STOP cassette upstream of the open reading frame (ORF) of the simian DTR. The loxP-flanked region contains two SV40 polyA signals, an frt-flanked neomycin resistance gene, and a transcriptional STOP cassette. The loxP-flanked STOP cassette and the ORF of the simian DTR were introduced into the ROSA26 locus of the mouse by homologous recombination in ES cells. Correctly targeted ES cells were microinjected in CB20 blastocysts. Chimeric mice were bred to C57BL/6 to generate heterozygous iDTR mice.

In order to study the effect of the deletion of mesenchymal populations in intestinal disease, ColVIcre, ROSA^{mTmG} (referred to as mTmG) mice were crossed with homozygous iDTR mice. Mice were bred and maintained on a C57BL/6J genetic background and experiments were performed in the animal facilities of Biomedical Sciences Research Center (BSRC) "Alexander Fleming" under specific pathogen–free conditions. All experiments were approved by the Institutional Committee of Protocol Evaluation in conjunction with the Veterinary Service Management of the Hellenic Republic Prefecture of Attika according to all current European and national legislation and performed in accordance with the guidance of the Institutional Animal Care and Use Committee of BSRC "Alexander Fleming".

Diphtheria Toxin Administration

Diphtheria toxin (DT) (Sigma-Aldrich) dissolved in 0.9% sodium chloride was administered in the colonic lumen of 6-8 week old mice at 20ng/g body weight. Before DT administration, mice were anesthetized using intraperitoneal injection of ketamine 100mg/ml /xylazine 20mg/ml solution. To setup the system for DT injection into the lumen, a syringe containing the DT solution was attached to an approximately 10 cm long polytetrafluoroethylene tube with outer diameter of 1.5 mm (Braun), which was inserted about 4 cm into the mouse colon. Then, 100μl of the solution was slowly administered in the colon. Mice were administered DT on days 0, 1 and 2 and sacrificed on day 7 or day 14.

DSS and AOM/DSS models

The dextran sodium sulfate (DSS) model was used to induce acute colitis in 6-8 week old mice. DSS is a chemical agent that damages the colonic epithelial cells of the basal crypts exposing the submucosal compartment to luminal antigens and stimulating an inflammatory response similar to human IBD in about 8 days. DSS (MW: 36,000–50,000 Da; MP Biomedicals) solution was used at a concentration of 2.5 % (w/v) in drinking water for 6 days, followed by 1 day of regular water consumption.[108] Colitis induction was monitored by measuring weight loss. Mice were sacrificed

and the colon was removed and subsequently used either for histological analysis or cell isolation.

The azoxymethane (AOM)/DSS model was used to induce inflammation-associated colon cancer. AOM is a mutagenic chemical agent, which alkylates DNA and when combined to DSS it can cause rapid growth of tumors within 8-10 weeks.[109] In the AOM/DSS model, AOM (Sigma-Aldrich) was injected once intraperitoneally at a concentration of 10mg/kg body weight, and then followed by three cycles of 2.5% (w/v) DSS-containing water. Each cycle lasted 5 days and was followed by regular water administration for 16 days. At day 64, macroscopically visible tumors were counted with the endoscope and at day 67 and for three consecutive days DT was administered in the colonic lumen of the mice. Consecutively, the mice were sacrificed at day 75 and their colon was removed and used for cryosections.

Endoscopy

For mouse endoscopy we used the Coloview Mini Endoscopic system. This endoscopic system is composed of the miniature endoscope with outer diameter of 1.9 mm, a xenon light source, a digital camera device, as well as an air pump that assures continuous air flow for the inflation of the mouse colon. Instruments such as injection needles, can be introduced through the working channel of the endoscopic sheath. The camera device can be connected to a computer, allowing endoscopic pictures and digital video recording (Fig.10).[110]

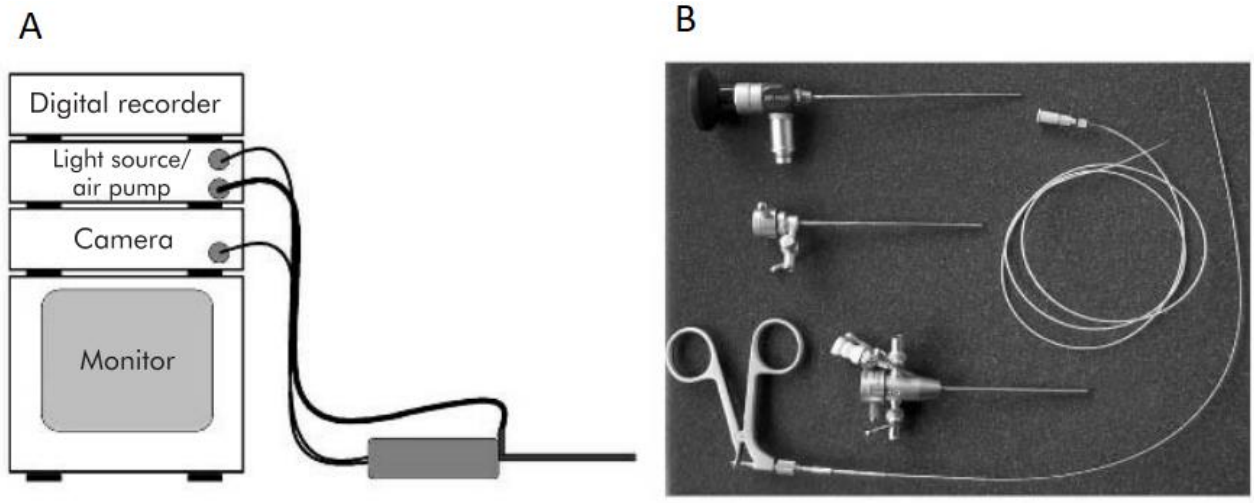


Fig.10 Setup of the endoscopic system. (A) The Coloview Mini Endoscopic system (B)The endoscopic instruments used for mouse examinations: straight forward telescope, examination sheath, manipulation sheath, biopsy forceps, and injection needle.[111]

The endoscopic technique was used to view the colonic tumors of mice subjected to the AOM/DSS model. For the endoscopic procedure, mice were anesthetized by isoflurane and subsequently the mini-endoscope was carefully inserted about 4cm into the mouse colon. During the procedure, continuous inflation of the colon by an air flow, ensured clear analysis and high resolution pictures.

Isolation of IMCs and cell culture

For isolation of IMCs, the colon of 6-8 week old mice was dissected and then washed by flushing with ice cold HBSS (Gibco) containing 2% FBS (Biochrom) and antibiotic-antimycotic (Gibco) (HBSS/Antibiotics). The colon was opened longitudinally and cut into pieces of 0.5-1 cm length, which were washed three times with HBSS/Antibiotics. This was followed by removal of the supernatant and the intestinal pieces were incubated in HBSS/2%FBS solution, containing 5mM EDTA and 1mM DTT for 20 min at 37^{o}C, to remove the epithelial layer. After removal of the supernatant containing epithelial cells, the tissue was incubated with 300 U/ml Collagenase XI (Sigma-Aldrich) and 0.1 mg/ml Dispase (Roche) in DMEM (Biochrom) for 60 min at 37°C. Subsequently, the supernatant was centrifuged at 280g for 10 min and the cell pellet was resuspended in DMEM supplemented with 10% FBS (Biochrom), 1% nonessential aminoacids (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and 1 μg/ml amphotericin B (Sigma-Aldrich) and plated in cell culture flasks.

Cell transplantation

The cells were collected at passage 4, washed in PBS and were assessed for viability using Trypan Blue. Afterwards, they were counted using a Beckman Coulter Z2 cell counter and resuspended in appropriate volume of diluted Matrigel in PBS (1:1) in order to contain 1x10^{6} cells/100μl. In order to administer the cells in the colonic lumen, a syringe containing the cells was attached to a 4cm long 20-gauge x 30mm plastic thin-wall tube (Instech Laboratories) which was introduced into the

colon. Mucosal injection of the cells in specific ulcers, developed following the DSS model, was performed by attaching the syringe to an injection needle inserted in the endoscope through an endoscopic sheath. The 30-gauge needle was positioned at a minimal angle directly into the mucosa with careful steps not to penetrate the muscularis propria. Subsequently, the cells were slowly injected into the mucosa to help them remain at the site of injection.

FACS

The colon of adult mice was removed and then washed by flushing with ice cold HBSS containing 2% FBS and antibiotic-antimycotic (HBSS/Antibiotics). An intestinal piece of 1 cm length from the distal colon adjacent to the rectum was dissected, washed with HBSS/Antibiotics and then incubated with HBSS, containing 0.5mM EDTA, DTT and Penicillin/Streptomycin (Gibco) for 30 min, at 37 C, to remove epithelial cells. After vigorous shaking, the remaining tissue was digested using 300 U/ml Collagenase XI (Sigma-Aldrich) and 0.1 mg/ml Dispase (Roche) for 60 minutes at 37°C. The cell suspension was passed through a 70μm strainer, centrifuged and resuspended in PBS supplemented with 2% FBS at a concentration of $2x10^6$ cells/ 100μl. Samples were analyzed using the FACS Canto II flow cytometer (BD) and the FACSDiva (BD) or FlowJo (LLC) software.

RNA Extraction and qRT-PCR

Total RNA extraction of intestinal tissue 0.5-1cm length was performed with the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. RNA of each sample was quantified using NanoDrop spectrophotometer. 1 microgram of total RNA was reverse transcribed to cDNA using oligo-dT primers (Promega) and M-MLV reverse transcriptase (Sigma-Aldrich) according to the manufacturer's protocol. Quantitative RT-PCR was performed using SYBR Green qPCR Supermix (Invitrogen) and the following primers specific for Bmp5: Forward: 5'-ACCTCTTGCCAGCCTACATG -3' and Reverse: 5'- TGCTGCTGTCACTGCTTCTC -3' and for Gremlin1: Forward: 5'- ACTCGTCCACAGCGAAGAAC -3' and Reverse: 5'-TCATTGTGCTGAGCCTTGTC -3'. Forward and reverse primers were used at a concentration of

5pmol/µl, in a sample volume of 20µl. The expression of candidate genes was normalized to β2-microglobulin (B2m). For B2m the primers used were: Forward: 5'-TTCTGGTGCTTGTCTCACTGA-3' and Reverse: 5'- CAGTATGTTCGGCTTCCCATTC-3'. The analysis was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using Opticon Monitor 3 software (Bio-Rad).

Histology imaging

The colon was dissected and fixed in 10% formalin or 4% PFA for 24 hours, for H&E sections and cryosections, respectively. The colon sections directed for H&E staining were washed in PBS and then paraffin-embedded. Afterwards, histological analysis was performed. The samples directed for cryosections were washed in PBS and then snap-frozen in an OCT-filled scaffold on a liquid nitrogen-cooled metal surface. The tissue samples in OCT were cryotomized at -20 °C into slices 10 µm thick and then washed three times with PBS. Tissue sections of colon were visualized using the Leica DM2000 fluorescence microscope (Leica microsystems) in order to assess endogenous EGFP fluorescence.

Apoptosis assessment

Colon sections from formalin-fixed, paraffin-embedded tissues were assessed for apoptotic cells using the DeadEnd Fluorometric TUNEL System (Promega) and DAPI (Sigma-Aldrich) was used to stain the nuclei. TUNEL-positive cells were quantified in each slide. Quantifications were performed using ImageJ software analysis.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was calculated by Student's t-test or two-way ANOVA for multiple comparisons. The D'Agostino Pearson test was used to test if the dataset followed a normal distribution. P-values ≤ 0.05 were considered significant. Data were analyzed using GraphPad Prism 6.

4. Results

4.1 Effect of the deletion of mesenchymal cells in intestinal disease

The diphtheria toxin system

The diphtheria toxin (DT) system permits specific lineage ablation and has proved very useful in studying the role of distinct cell populations. The DT system is based on the transgenic expression of the diphtheria toxin receptor (DTR) under a specific promoter, which drives its production in restricted cell populations (Fig.1).[107] Mice are normally resistant to diphtheria toxin, but expression of the DTR in cells, renders them sensitive to DT, inducing cell-specific apoptosis upon administration of the toxin.

In the iDTR system, expression of DTR is inhibited by an upstream loxP-flanked STOP cassette. This STOP cassette is removed by crossing the iDTR strain to a tissue specific Cre-expressing mouse strain. Consecutive expression of the DTR renders the respective cells sensitive to cell death induced by the administration of diphtheria toxin.

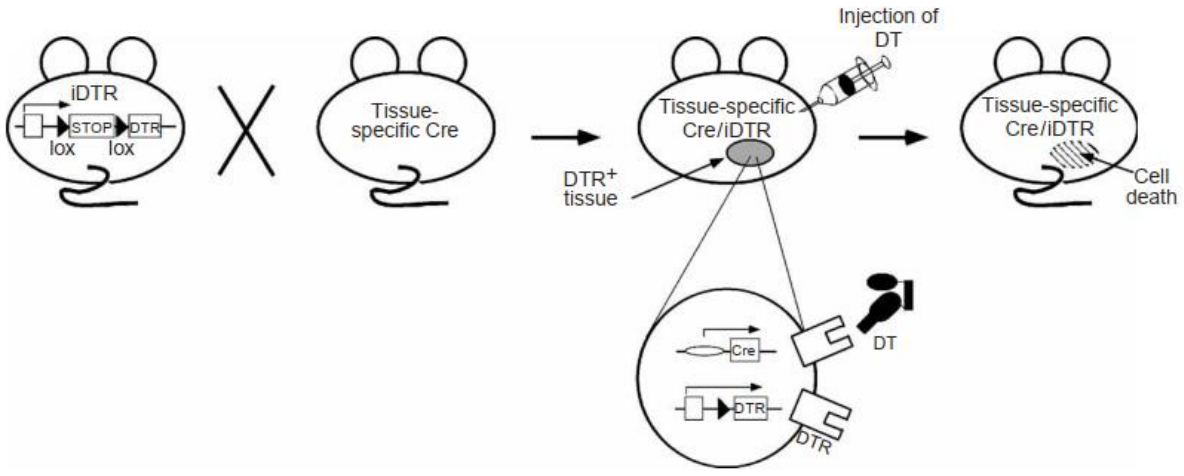


Fig.1 Crossing of the iDTR strain to a tissue specific Cre-expressing mouse strain induces expression of DTR in the respective tissues. Following diphtheria toxin injection these tissues are rendered sensitive to cell death.[107]

There are many studies that have used DT administration to explore the effect of genetic ablation of specific cells, such as hepatocytes, neutrophils and myeloid derived macrophages.[112–114] Recently, specific deletion of a subset of mesenchymal subepithelial cells expressing the

winged-helix transcription factor forkhead box l1 (Foxl1) through the DT system elucidated also the critical role of this mesenchymal subpopulation in maintaining the stem cell niche of the intestine.[115]

As shown in the past, the ColVIcre transgenic mouse model shows specificity for mesenchymal cells in the joints, skin, heart and intestine.[9] In the small intestine and colon, the ColVIcre transgene targets a subpopulation of mesenchymal cells that is approximately 30% of the total mesenchymal cell population.[73] Therefore, to assess the contribution of the ColVI subpopulation in intestinal disease, we aimed to specifically ablate the ColVI cells from the intestine of mice by crossing ColVIcre mice with the iDTR mouse model.

4.1.1 Evaluation of different routes of diphtheria toxin administration for the deletion of ColVIcre+ mesenchymal cells in the intestine

4.1.1.1 Systemic administration

To accomplish efficient deletion of ColVI-expressing cells, we evaluated different routes of diphtheria toxin administration. The first route examined was the systemic route of DT treatment through single or twice-daily injections, different DT concentrations and varying periods of DT exposure. In more detail, we injected diphtheria toxin intraperitoneally (ip) to ColVI-iDTR$^{F/+}$ mice, whereas iDTR$^{F/+}$ mice treated with DT and ColVI-iDTR$^{F/+}$ mice that did not receive DT were used as controls. The dosing schemes initially used included DT administration of 10ng/gr of body weight either twice daily for 7 consecutive days or once daily for 4 consecutive days. However, at day 3 (after 4-5 injections) and day 4 (after 4 injections) of the dosing schemes respectively, the mice became moribund and were sacrificed. Additional injection schemes of various concentrations, namely 20ng/g, 10ng/g, 5ng/g or 2ng/g every 48 hours resulted in the same moribund appearance of the mice at day 2 or 3, whereat they were sacrificed and their colon and small intestine were dissected. H&E sections of the colon and small intestine revealed that there was no difference in the morphology of the villus and crypt architecture between the three groups (Fig.2A). We also performed TUNEL assay in both the

small intestinal and colon, and found that the ColVI-iDTR$^{F/+}$ mice demonstrated significantly increased cell death upon DT administration (Fig.2B).

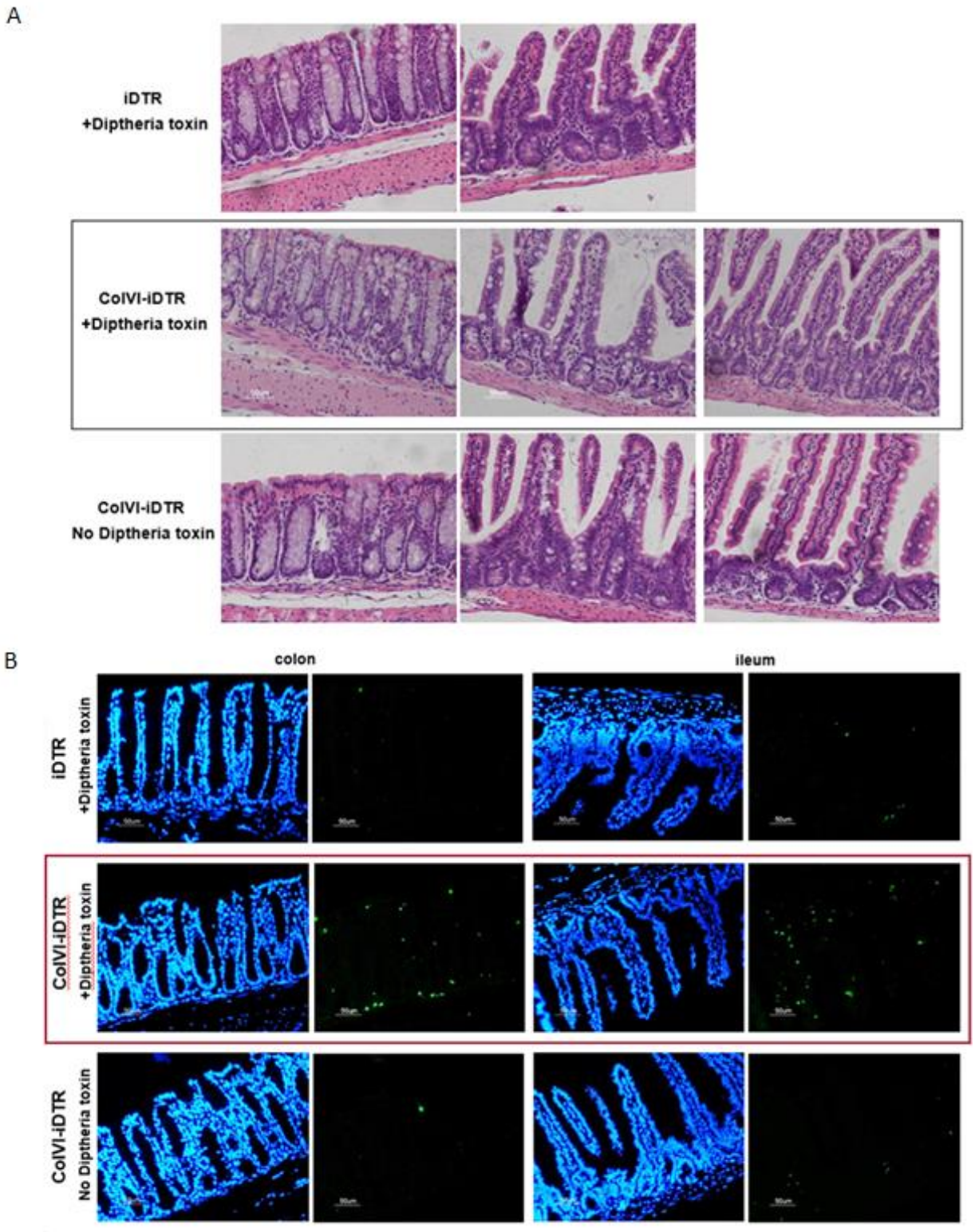


Fig.2 Systemic diphtheria toxin administration of 10ng/gr of body weight twice daily for 3 consecutive days to ColVI-iDTR$^{F/+}$ **mice does not affect the morphology of either the colon or the small intestine, though it increases apoptosis.** (A) Representative images of H&E stained colon and small intestine sections from iDTR$^{F/+}$ mice, ColVI-iDTR$^{F/+}$ mice that received DT and ColVI-iDTR$^{F/+}$ mice that did not receive DT. (B) Representative images of TUNEL staining of colon and ileum sections from the above genotypes. DAPI was used to stain nuclei in the TUNEL assay. The experimental group used included 1 iDTR and 3 ColVI-iDTR$^{F/+}$ mice that were treated with DT and 1 ColVI-iDTR$^{F/+}$ mouse in which DT was not administered.

Besides the colon and the small intestine, we also removed the spleen and the kidney, in order to evaluate the effect of systemic DT administration in various vital organs. We performed H&E staining, as well as immunohistochemical staining for CD3 and B220 of the spleen to determine whether there was an alteration in its morphology. Indeed, the structure of the spleen in ColVI-iDTR$^{F/+}$ mice receiving DT was significantly altered and was representative of their moribund state (Fig.3A). Kidney tissue stained with H&E appeared to have no morphological difference between the mice, in which the ColVI cells were ablated, and their littermate controls (Fig.3B).

Therefore, we concluded that the systemic treatment with diphtheria toxin is not a promising approach for deleting ColVI cells from the intestine, as it causes increased mortality, most possibly due to multi-organ failure attributed to the deletion of other mesenchymal cells, also targeted by the ColVIcre mouse (e.g. the skeletal muscle, cardiac fibroblasts, etc).

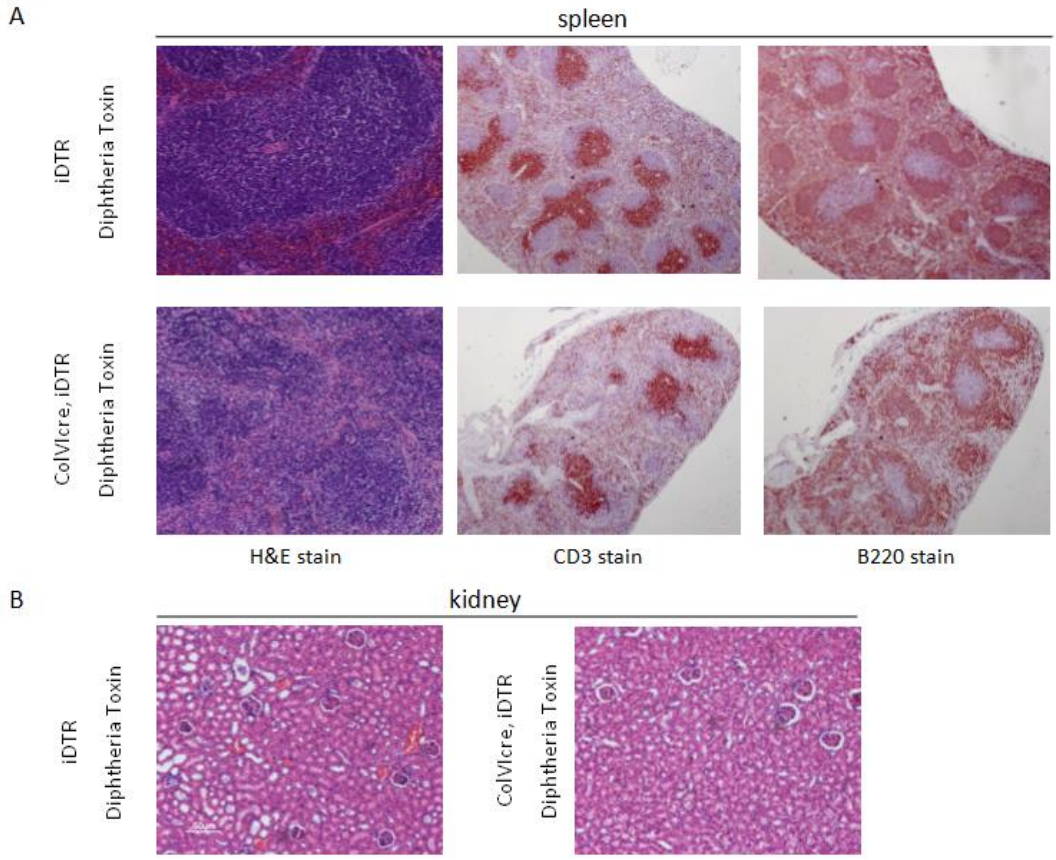


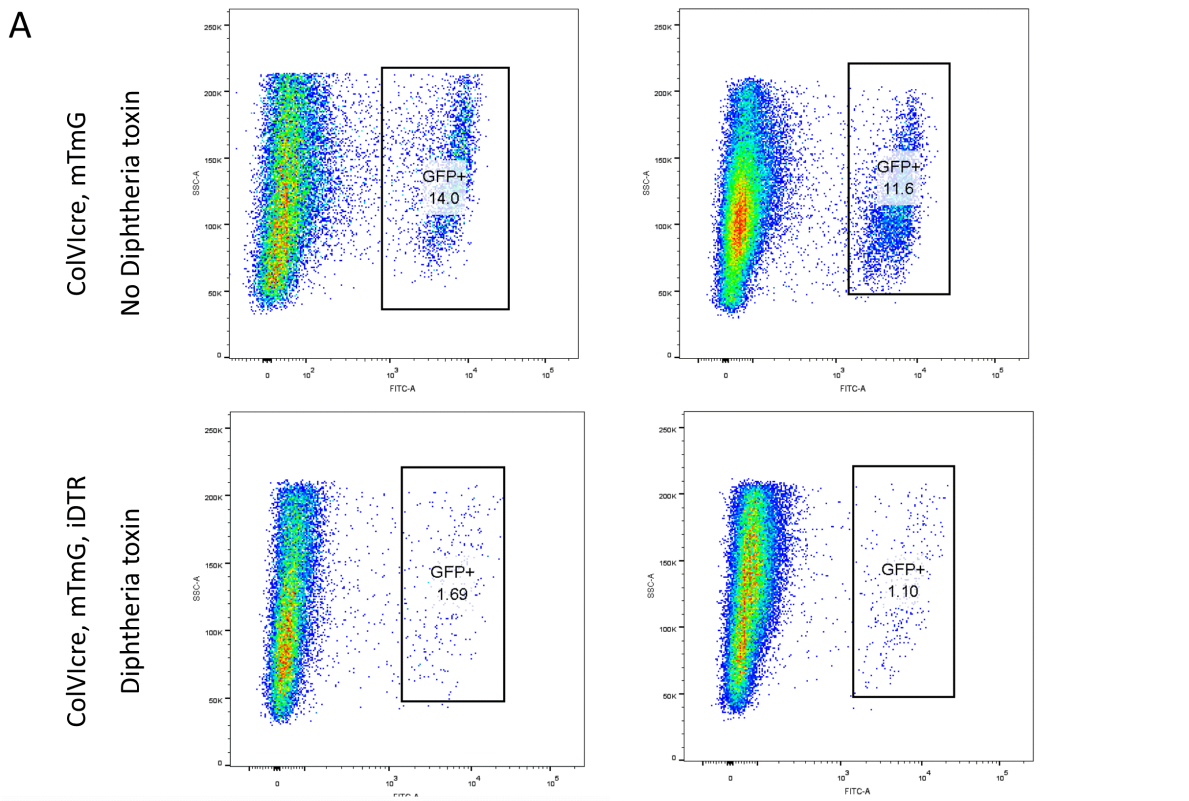
Fig.3 Systemic diphtheria toxin administration of 10ng/gr of body weight twice daily for 3 consecutive days to ColVI-iDTR$^{F/+}$ mice induces changes in the structure of the spleen, whereas it does not affect the structure of the kidney. (A) Representative H&E-staining and immunohistochemical staining for the T cell marker CD3 and the B cell marker B220 of the spleen from iDTR$^{F/+}$ and ColVI-iDTR$^{F/+}$ mice upon systemic administration of DT. (B) Representative images of H&E staining of colon and ileum sections from iDTR$^{F/+}$ and ColVI-iDTR$^{F/+}$ mice upon systemic administration of DT. The experimental group used included 1 iDTR$^{F/+}$ and 3 ColVI-iDTR$^{F/+}$ mice that were treated with DT and 1 ColVI-iDTR$^{F/+}$ mouse in which DT was not administered.

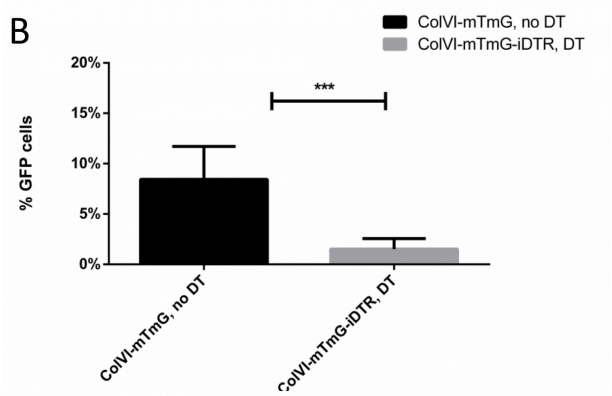
4.1.1.2 Topical administration

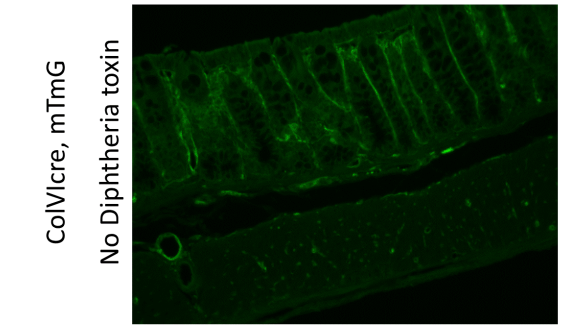
We next hypothesized that topical rectal administration of DT could result in less undesired effects in comparison to systemic administration. To test this hypothesis, we used ColVI-iDTR$^{F/+}$ and iDTR$^{F/+}$ littermates controls, which received 20ng/g diphtheria toxin dissolved in 0.9% sodium chloride intrarectally. The mice received 3 DT doses once daily every 48h. In contrast to the moribund condition observed in mice that received i.p DT injection (systemic effect), the mice that received DT intrarectally (local effect) were healthy and had a normal physical appearance.

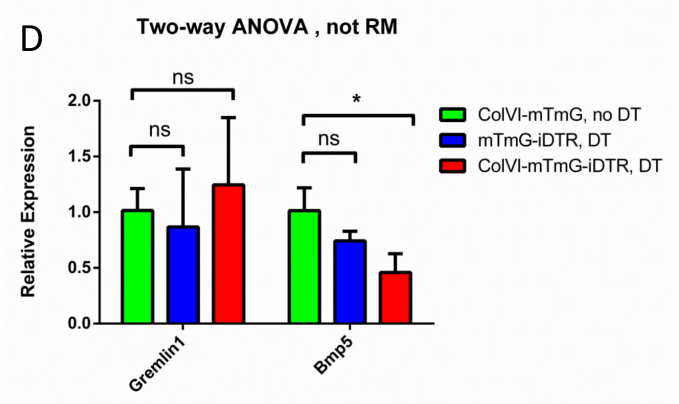
To examine the efficient deletion of the ColVIcre+ cells in the ColVI-iDTR$^{F/+}$ mouse, we crossed them with the reporter mouse line ROSA$^{mT/mG}$ (referred to as mTmG).[105] In this mouse strain, all cells express the membrane-targeted Tomato sequence. Upon Cre-mediated recombination, this sequence is excised, enabling the expression of GFP in the mesenchymal compartment. Thus, when the ColVI-mTmG-iDTR$^{F/+}$ mice are treated with diphtheria toxin, ColVI-GFP expressing cells are rendered sensitive to DT and are deleted. To quantify the deletion of the ColVIcre+ cells, we performed FACS analysis of colon from ColVI-mTmG-iDTR$^{F/+}$ mice and ColVI-mTmG mice treated with 20ng/g diphtheria toxin for 3 consecutive days and sacrificed at day 4. This analysis showed that there was indeed a significant decrease in the population of ColVI-GFP+ cells, upon local DT administration (Fig.4A). While the ColVI-GFP+ cells constituted approximately 10% (8.44%±1.15%) of mesenchymal cells in the colon, their proportion was decreased almost four times (1.51%±0.32%) after DT-induced depletion (Fig.4B). We also verified these results through imaging of colon sections, which also showed reduced GFP expression in the colon of ColVI-mTmG-iDTR$^{F/+}$ mice that received diphtheria toxin in comparison to untreated controls (Fig.4C). It should also be noted that this reduction in GFP expression was principally observed in the distal part of the colon, as expected, since the DT administration, and subsequently the ColVI-GFP expressing cells ablation, affects mainly the first 4 cm of the colon.

Recent evidence from our lab showed that the intestinal ColVIcre+ subpopulation is highly enriched in the expression of Bmps (Bmp3, Bmp7, Bmp5), Wnt inhibitors (Wif1, Wnt5a, Wisp1) and genes related to the differentiation of epithelial cells (Fgf1), while ColVIcre- cells express stem cell niche factors, such as Grem1, Wnt2 and Nog (unpublished data). For this reason, we also performed qRT-PCR of the colon tissue on day 4 to assess expression levels of genes preferentially expressed by the ColVI subpopulation. In agreement with our data, expression of Bmp5 was significantly decreased in the colon of ColVI-mTmG-iDTR$^{F/+}$ mice that were treated with diphtheria toxin, compared both to mTmG-iDTR$^{F/+}$ treated mice and ColVI-mTmG untreated mice. Accordingly, there was no significant alteration in Gremlin1 expression between the three groups (Fig.4D).


A
ColVIcre, mTmG
No Diphtheria toxin
GFP+
14.0
GFP+
11.6
ColVIcre, mTmG, iDTR
Diphtheria toxin
GFP+
1.69
GFP+
1.10
SSC-A
FITC-A


B
ColVI-mTmG, no DT
ColVI-mTmG-iDTR, DT
% GFP cells
20%
15%
10%
5%
0%
***
ColVI-mTmG, no DT
ColVI-mTmG-iDTR, DT


C
ColVIcre, mTmG
No Diphtheria toxin


D
Two-way ANOVA , not RM
Relative Expression
2.0
1.5
1.0
0.5
0.0
ns
ns
*
ns
ColVI-mTmG, no DT
mTmG-iDTR, DT
ColVI-mTmG-iDTR, DT
Gremlin1
Bmp5

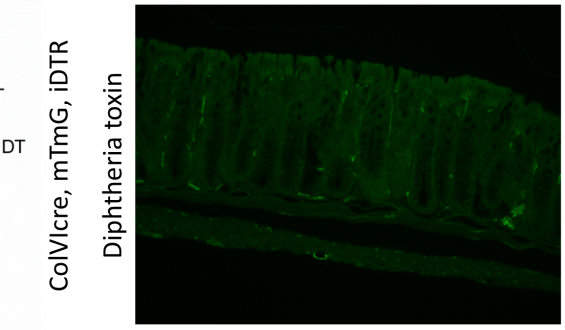

ColVIcre, mTmG, iDTR
Diphtheria toxin

Fig.4 Efficient deletion of the ColVI-GFP expressing cells upon topical administration of diphtheria toxin 20ng/g for 3 consecutive days. (A) FACS analysis of colon samples from ColVI-mTmG-iDTR and mTmG-iDTR mice treated with DT (n=10 mice), as well as ColVI-mTmG untreated controls (n=8 mice). (B) Quantification of ColVI-GFP+ expressing cells in the colon by FACS analysis (n=10 ColVI-mTmG-iDTR mice treated with DT, n=8 ColVI-mTmG mice not treated with DT). (C) Representative images of GFP expression in colon sections from ColVI-mTmG-iDTR where the ColVI-GFP+ experssing cells were ablated compared to untreated controls. (D) Relative expression of Gremlin1 and Bmp5 by ColVI-mTmG-iDTR that received DT treatment compared to littermate controls that either received or did not receive DT (n=5 Cre-mTmG , no DT mice, n=4 mTmG-iDTR mice, n= 6 ColVI-mTmG-iDTR mice). Data are presented as mean ± SEM. (***; P<0.0005, *; P<0.05, ns;nonsignificant, using Student's t test and two-way ANOVA)

Following validation of the efficient deletion of the intestinal ColVI subpopulation upon the diphtheria toxin topical administration, we focused on the investigation of the effect of ablation of this specific subpopulation in intestinal homeostasis, using the same protocol. Therefore, we examined histopathologically colon sections of ColVI-iDTR$^{F/+}$ and iDTR$^{F/+}$ mice that received DT and ColVIcre mice without DT administration. We could not detect any obvious differences in the morphology and structure of the H&E stained colon sections between the three groups (Fig.5A). We also studied apoptosis, by performing TUNEL staining; however, the three groups appeared to have no difference in cell death (Fig.5B). This could be related to the time-point of analysis, as GFP+ cells were already absent at this stage.

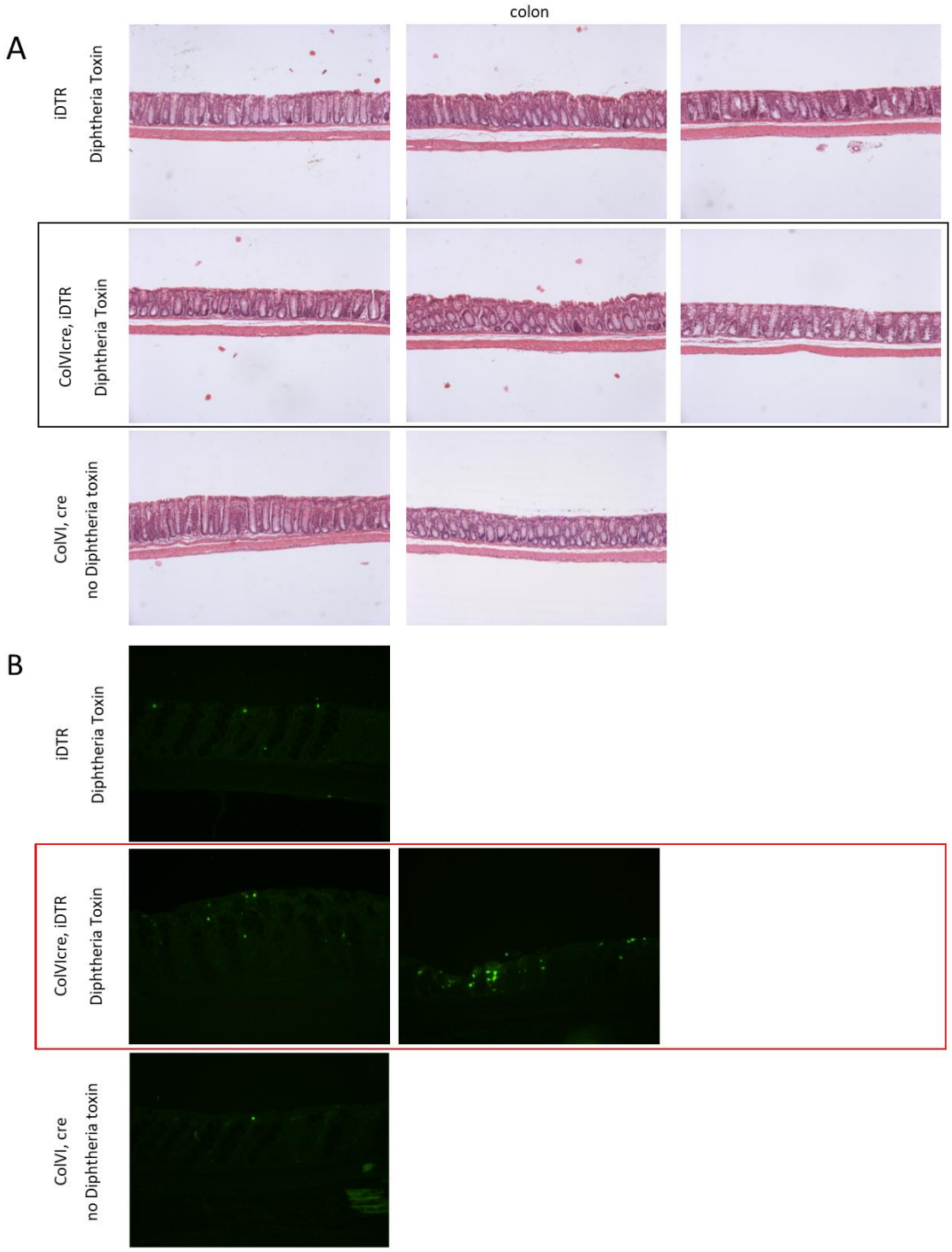


Fig.5 Topical diphtheria toxin administration of 20ng/gr of body weight once daily for 3 consecutive days to ColVI-iDTR$^{F/+}$ mice does not affect the morphology of the colon. (A) Representative images of H&E stained colon sections from iDTR$^{F/+}$ and ColVI-iDTR$^{F/+}$ mice that received DT and untreated ColVIcre controls. (B) Representative images of TUNEL staining of colon sections from the above mice. DAPI was used to stain nuclei in the TUNEL assay. The

experimental group included 3 iDTR$^{F/+}$ and 3 ColVI-iDTR$^{F/+}$ mice that were treated with DT and 3 ColVI-iDTR$^{F/+}$ untreated controls. Images are representative from one of three individual experiments performed.

Moreover, we examined whether higher DT concentration or administration of DT for more consecutive days could achieve higher deletion efficiency of the ColVI subpopulation (Table 1).

Concentration of DT administration	Days of administration (consecutive)	Sacrifice of mice
20 ng/g	3 days	day 5
20 ng/g	5 days	day 8
40 ng/g	3 days	day 5
20 ng/g	3 days	day 14

Table 1. Dosing schemes used for the standardization of DT treatment. The dosing schemes included distinct DT concentrations, days of DT administration and time points of sacrifice of the mice.

Therefore, ColVI-mTmG-iDTR$^{F/+}$, mTmG-iDTR$^{F/+}$ and ColVI-mTmG mice were treated either with 40ng/g DT for 3 consecutive days and sacrificed on day 5 or with 20ng/g DT for 5 consecutive days and sacrificed on day 8. FACS analysis of the colon of ColVI-mTmG-iDTR$^{F/+}$ and ColVI-mTmG mice revealed that the deletion of ColVI-GFP+ expressing cells in either experimental dosing schemes was comparable to the ablation accomplished upon 20ng/g DT treatment for 3 consecutive days (Fig.6). Finally, we explored whether colonic ablation of the ColVIcre+ cells could affect the morphology of the colon after a more prolonged period of time. Hence, mice were treated with 20ng/g DT for 3 consecutive days and were sacrificed on day 14. However, we observed again no difference in the morphology of the colonic tissue.

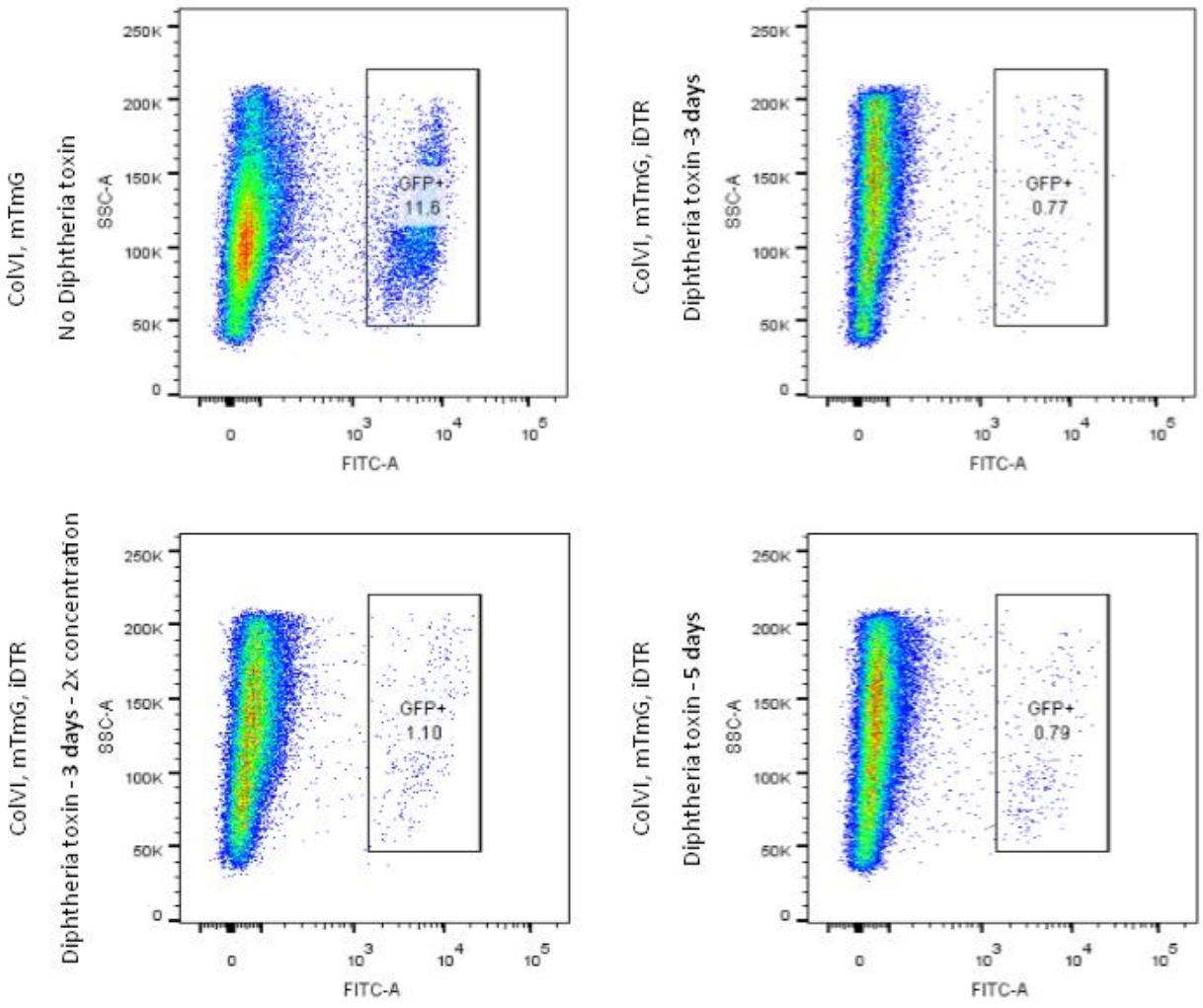


Fig.6 Efficiency of deletion of the ColVI-GFP expressing cells upon topical administration of different dosing schemes of diphtheria toxin treatment. FACS analysis of colon samples from ColVI-mTmG-iDTR mice treated with DT, as well as ColVI-mTmG untreated controls.

4.1.2 Deletion of intestinal mesenchymal populations in colorectal tumors.

To investigate the role of the ColVIcre+ subpopulation in inflammation-induced intestinal carcinogenesis, we used the well-established AOM/DSS model of CAC in ColVI-mTmG-iDTR$^{F/+}$, mTmG-iDTR$^{F/+}$ and ColVI-mTmG mice. In this model, mice receive a single injection of the carcinogen AOM followed by three cycles of DSS administration in the drinking water (Fig.7A). The chronic inflammation induced by the repeated DSS treatments accelerates both tumor

initiation and progression in the colon.[116] During the DSS cycles, there was a constant monitoring of the body weight loss, which displayed no statistically significant difference between the three groups (Fig.7B). On day 70, the mice were subjected to endoscopy to assess tumor formation (Fig.7C). Once confirmed that the colon tumors had developed, we proceeded at day 75 with 20ng/g rectal diphtheria toxin intrarectal administration, once daily, for 3 consecutive days (Fig.7A).

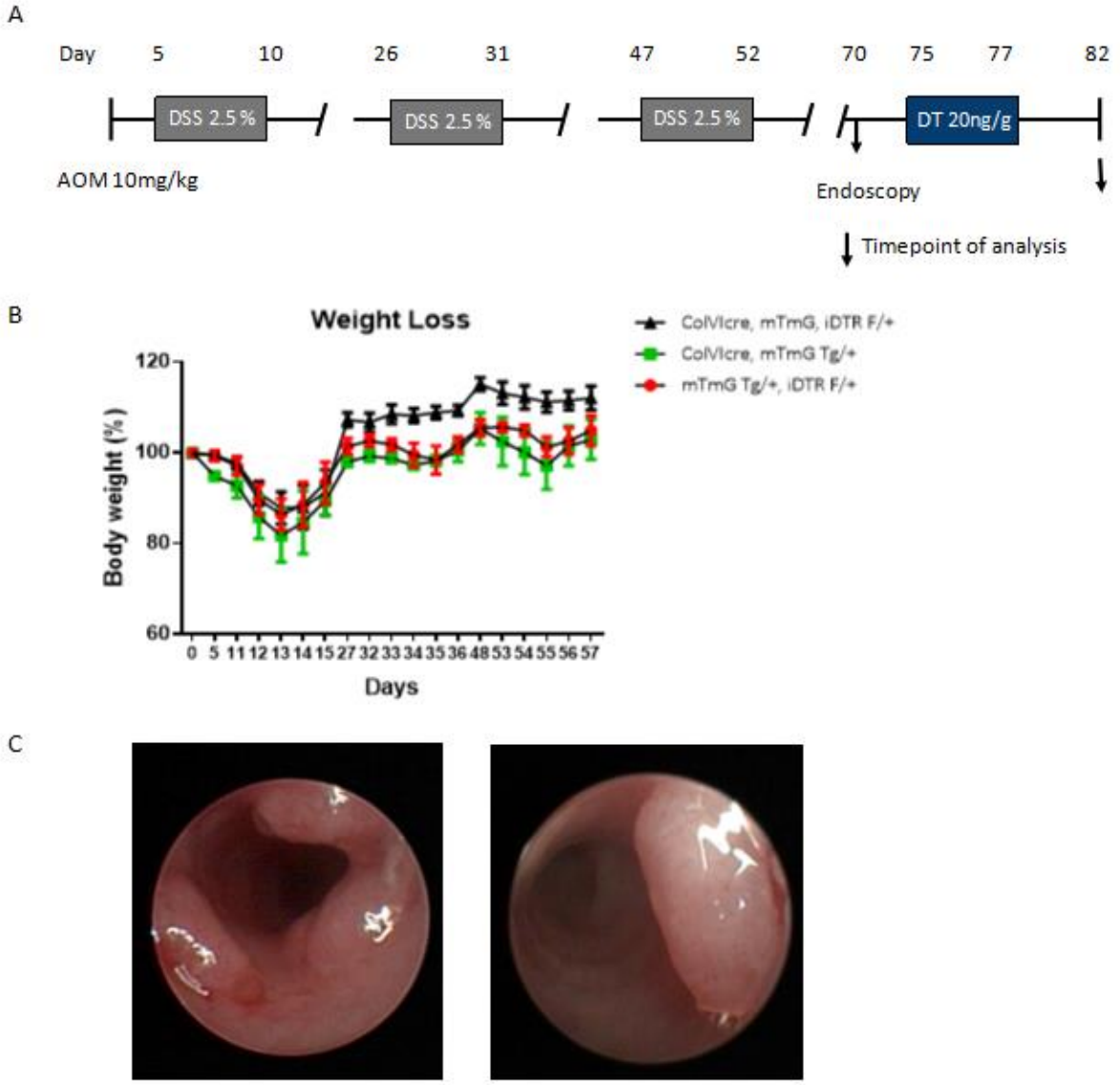


Fig.7 Assessment of the AOM/DSS model of carcinogenesis. (A) Schematic diagram of the AOM/DSS model of CAC. A single AOM injection (10 mg/kg) is followed by three cycles of 2.5% DSS administration in the drinking water. Following endoscopy to assess the tumor development, mice were subjected to 20ng/g DT treatment for 3

consecutive days. (B) Schematic diagram of the weight loss of ColVIcre-mTmG-iDTRF/+, mTmG-iDTRF/+ and ColVIcre-mTmG mice during the AOM/DSS induced carcinogenesis. (C) Representative images of the tumors developed during the AOM/DSS induced carcinogenesis as assessed through endoscopy with the Coloview Mini Endoscopic system at day 70 of the AOM/DSS model. The experimental groups used included 5 ColVI-mTmG-iDTR, 5 mTmG-iDTR and 2 ColVI-mTmG mice.

At day 82, the mice were sacrificed and their colon was removed and macroscopically visible tumors were counted. Both the colon length and the number of tumors per mouse demonstrated no significant difference between the three groups (Fig.8A, B).

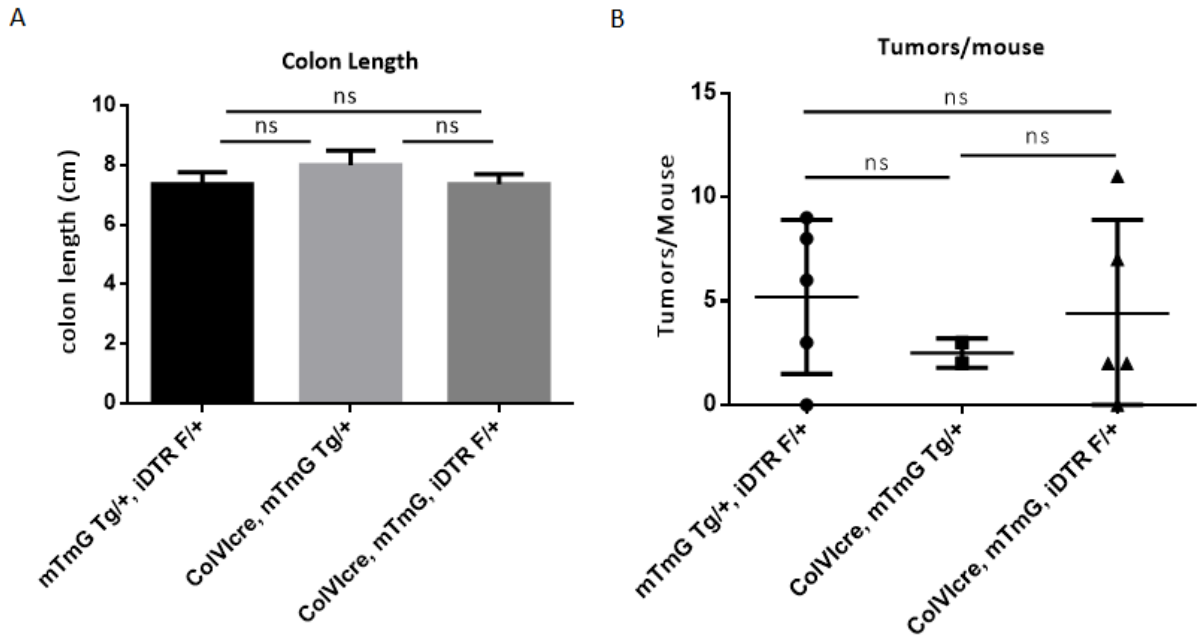


Fig.8 The ablation of ColVI intestinal mesenchymal cells upon DT administration does not affect the colon length and the number of tumors developed in the AOM/DSS model of CAC. (A) The mean colon length was measured and the macroscopically visible tumors were counted in ColVI-mTmG-iDTR$^{F/+}$ mice and the mTmG-iDTR$^{F/+}$ and ColVI-mTmG littermates at day 82 of the CAC model. The experimental groups used included 5 ColVI-mTmG-iDTR$^{F/+}$, 5 mTmG-iDTR$^{F/+}$ and 2 ColVI-mTmG mice. All data are presented as mean ± SEM (ns, nonsignificant, using Student's t test).

We initially assessed the efficiency of ColVIcre+ cell deletion, by examining GFP expression in colon sections from ColVI-mTmG-iDTR$^{F/+}$ and ColVI-mTmG mice subjected to DT administration

following the AOM/DSS model. We found that the ColVI-mTmG-iDTR$^{F/+}$ mice showed indeed deletion of ColVI-GFP expressing cells in colonic tumors located in the distal part of the colon (Fig.9A). These data suggest that the local diphtheria toxin administration can be successfully employed to accomplish efficient deletion of ColVI cells inside the developed tumors.

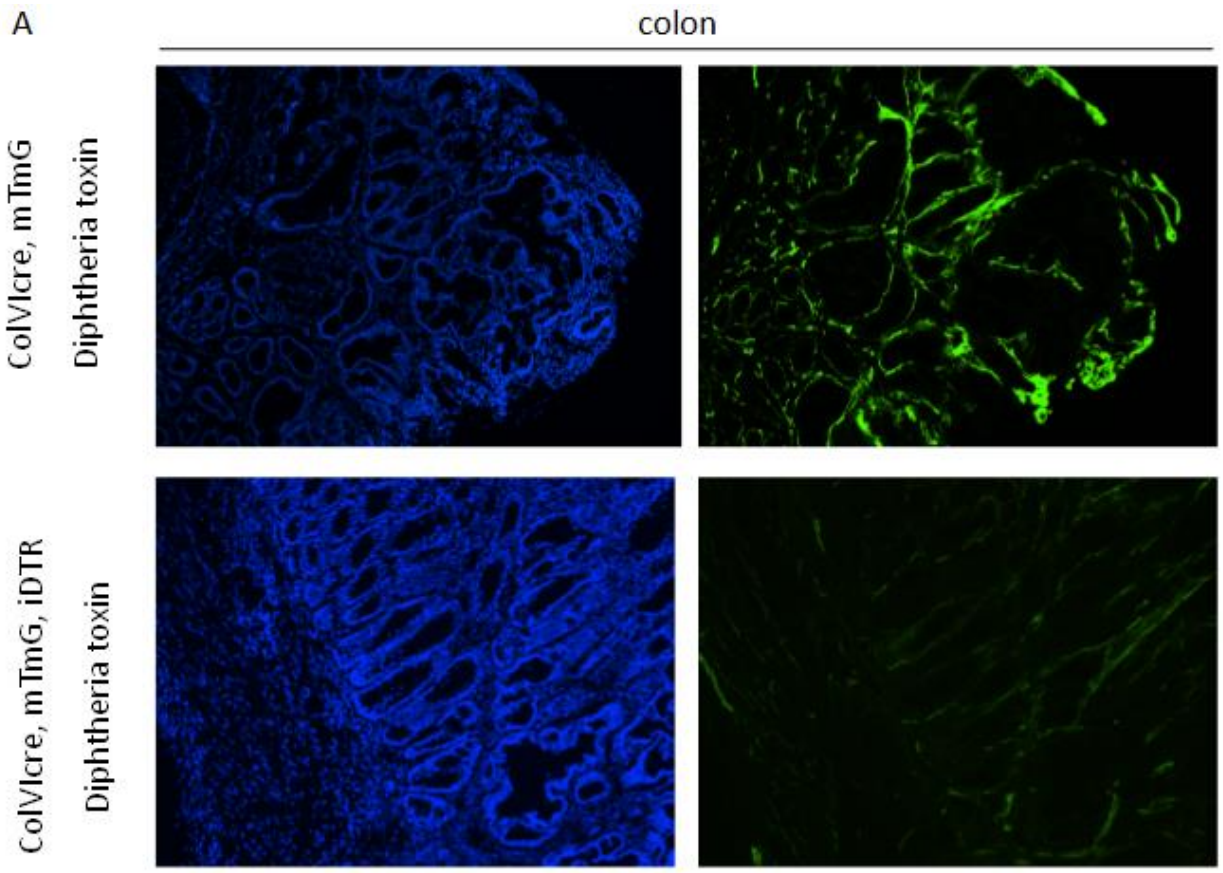


Fig.9 Topic diphtheria toxin administration of 20ng/g of body weight once daily for 3 consecutive days following the AOM/DSS model of carcinogenesis accomplishes efficient ablation of ColVI-GFP expressing cells inside the tumors. (A) Representative images of GFP expression in tumors developed in the colon. The ColVI-mTmG-iDTR$^{F/+}$ mice subjected to DT treatment after the AOM/DSS induced colon cancer demonstrate ablation of ColVI-GFP-expressing cells. DAPI was used to stain the nuclei. The experimental groups used included 5 ColVI-mTmG-iDTR, 5 mTmG-iDTR and 2 ColVI-mTmG mice.

4. Transplantation of colonic mesenchymal cells in acute colitis

To investigate the therapeutic potential of cell transplantation in acute colitis, we aimed to transplant mesenchymal cells in the colonic mucosa of mice subjected to DSS-induced colitis. To accomplish this, we isolated and cultured colonic mesenchymal cells, which were then collected and resuspended in PBS/Matrigel in a ratio of 1:1. To induce colitis, we used the chemical agent DSS which was administered at a concentration of 2.5 % in drinking water of wild type mice for 5 days, followed by regular water consumption (Fig.10A).

Evaluation of the homing potential of mesenchymal cells following different approaches of colon mesenchymal cell transplantation in mice with colitis

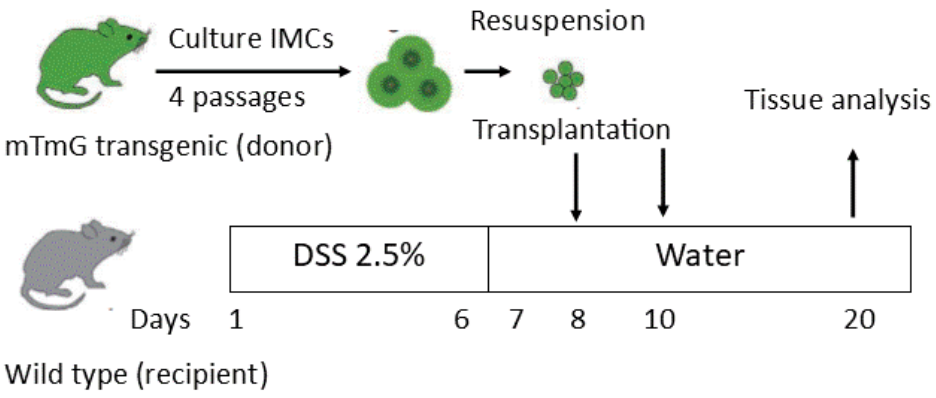
Initially, we aimed to assess whether colonic mesenchymal cells would be able to home in the injured mucosa of wild type mice with colitis. For this reason, we used the reporter mouse line $ROSA^{mT/mG}$, in which all cells express the membrane-targeted Tomato sequence.

Firstly, we tested transplantation of mesenchymal cells in the colonic lumen. The cells were transplanted at days 8 and 10 of the DSS protocol, when the weight loss of the mice was approximately 15% of their body weight (Fig.10B). To examine the colonic lumen transplantation, Tomato-expressing cells were administered intrarectally at a concentration of $1x10^6$ cells/100μl in 1:1 PBS/Matrigel in mice subjected to DSS induced colitis. This was accomplished through a syringe attached to a plastic thin-wall tube, which was introduced 4 cm into the colon. At day 20, the mice were sacrificed and their colon was removed. However, there was no Tomato expression, as assessed both using a fluorescent stereoscope and in colon sections suggesting that the transplanted cells could not home efficiently in the injured mucosa.

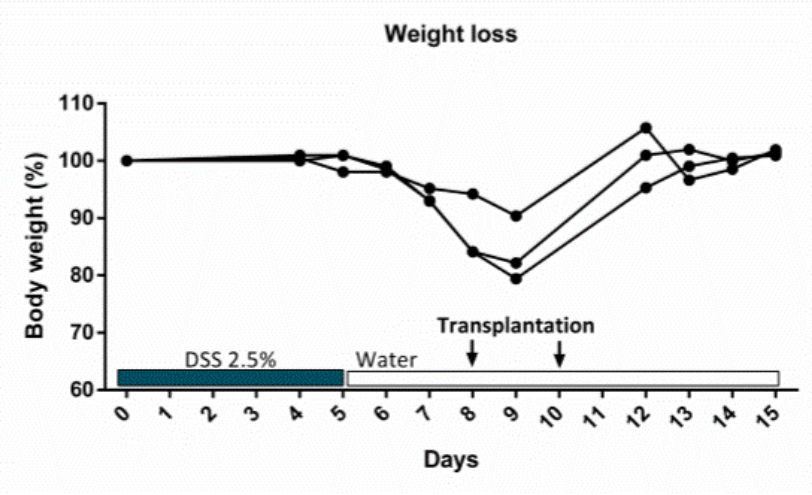
Therefore, we hypothesized that a more topical approach, where cells would be transplanted specifically in the ulcers formed following DSS administration, would have higher probability of homing of the cells. To test this, Tomato-expressing mesenchymal cells at a concentration of $1x10^6$ cells/50μl were introduced to a syringe, which was attached to an injection needle inserted in the endoscope. With the aid of the endoscope and the digital camera, we managed to find ulcers developed in the colonic mucosa and specifically inject the cells there (Fig.10C).

The transplantation was performed slowly and carefully to help the cells remain at the site of injection and to prevent the needle penetrating in the muscularis propria. At day 20 the mice were sacrificed and their colon was removed and fixed in PFA. However, again we could not see Tomato+ cells in the colonic mucosa, indicating that the homing of the transplanted cells in the injured mucosa was not achieved.

A



B



C

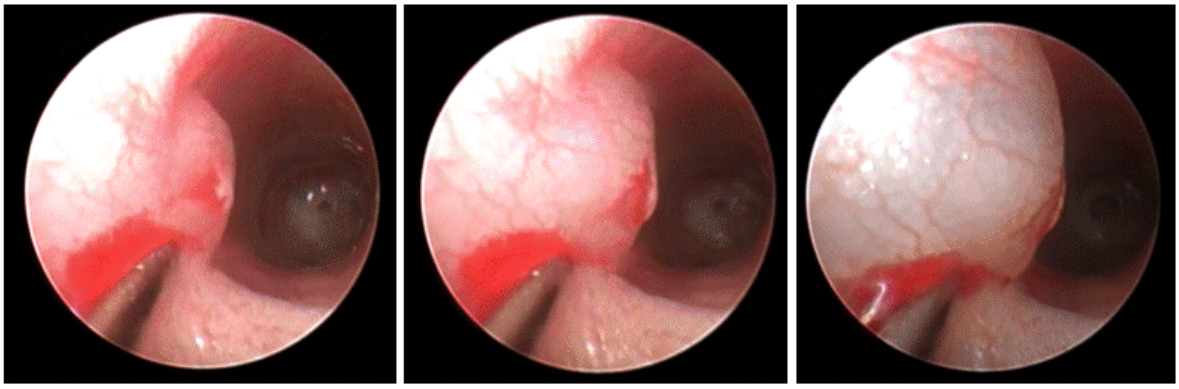


Fig.10 Transplantation of mTmG cultured cells in mice subjected to acute colitis. (A) Schematic diagram of the experimental protocol. The IMCs were isolated from the colon of an mTmG transgenic mouse and cultured for 4 passages. Meanwhile, wild type mice were subjected to acute colitis through 2.5% DSS administration in the drinking water for 6 days, followed by regular water consumption. At day 8, the mTmG cultured IMCs were resuspended in PBS/Matrigel and transplanted in the mucosa of the mice with colitis. At day 20, the mice were sacrificed and the colon tissue was analysed. (B) Schematic diagram of the weight loss of wild type mice during the DSS induced colitis and following colonic lumen cell transplantation. (C) Representative consecutive time frames obtained during the local infusion of IMCs in the mucosa of wild type mice with the aid of the endoscopic system.

These results suggest that IMC transplantation, following acute colitis induced by the chemical agent DSS, needs further standardization in order to increase the homing potential of the transplanted cells.

5. Discussion

In this study, we employed two different strategies to target intestinal mesenchymal cells; through their elimination, specifically in colorectal cancer, and through their transplantation, especially in inflammation and tissue damage. Elimination of IMCs was based on a diphtheria toxin system, characterized by engineered sensitivity to DT, to study the contribution of the ColVI intestinal mesenchymal subpopulation in intestinal homeostasis and disease, and specifically in tumorigenesis. By using the ColVI-cre transgenic mouse we specifically ablated this defined subpopulation of IMCs. However, because the DT sensitivity may vary according to the route of administration and the DT dosing scheme for every Cre line used, the DT system has to be standardized to ensure that the desired degree of ablation can be achieved.[112] The second strategy we used to target IMCs as a therapeutic approach was based on the transplantation of these cells, aiming to evaluate their contribution in intestinal healing following mucosal damage induced by colitis. We investigated this approach since transplanted mesenchymal cells mediate tissue repair and regeneration either from a long distance or by directly migrating to the area of insult and their administration in clinical trials has already demonstrated preliminary feasible and beneficial results.[78,84]

Therefore, initially, we employed the DT system to study the deletion of ColVI-expressing cells. We examined the systemic route of DT administration first, by using single or twice-daily injections for varying periods of time and different DT concentrations. However, all the injection schemes employed, resulted in a moribund appearance of the mice after approximately 3-4 i.p. injections. Analysis of the histopathological phenotype of the colon and small intestine of ColVI-iDTR mice that were treated with DT, showed no difference in the morphology of the crypt, whereas assessment of the apoptosis rate demonstrated increased cell death upon DT treatment. Moreover, evaluation of the morphology of other vital organs, such as the spleen, revealed that there was a significant alteration in its structure in ColVI-iDTR mice receiving DT, which was representative of their moribund state. In contrast to the moribund condition observed in the ColVI-iDTR mice, Aoki et al. showed efficient deletion of the Foxl1-expressing IMCs through i.p. DT injection, suggesting thus more firmly that each Cre-line targeting a distinct

mesenchymal population responds differently, depending on its specificity both in the intestine and in other tissues.[115] Hence, we concluded that the systemic treatment with diphtheria toxin is not a promising approach for ablating ColVI cells from the intestine, as it causes increased mortality, most likely due to multi-organ failure attributed to the deletion of other mesenchymal cells also targeted by the ColVIcre mouse, such as the skeletal muscle, and fibroblasts in the heart valve and the skin.[9,117]

We next hypothesized that topical rectal DT administration could help to avoid the undesired systemic effects of the i.p. injections. Indeed, DT treatment intrarectally did not affect the survival and healthy appearance of the mice and, thus, we proceeded to standardize the DT administration scheme and to evaluate its deletion efficiency. We concluded in administration of 20ng/g DT for 3 consecutive days, which caused a significant reduction in the ColVI-GFP expressing cells in the last 4 cm of the colon, as well as in the expression of genes specifically enriched in the ColVIcre+ subpopulation, such as Bmps. The latter results in gene expression are in high accordance with recent evidence from our lab that demonstrated the enrichment of the intestinal ColVIcre+ subpopulation in the expression of Bmps (Bmp3, Bmp7, Bmp5), Wnt inhibitors (Wif1, Wnt5a, Wisp1) and genes related to the differentiation of epithelial cells (Fgf1) (unpublished data). Further experiments to define the effect of ablation of the ColVI cells during homeostasis showed no difference in the morphology of the distal colon, when the ColVIcre+ subpopulation is deleted. These results suggest either that there could be an alternative intestinal mesenchymal subpopulation that compensates for the loss of colonic ColVI cells upon DT treatment or that the remaining ColVIcre+ cells are adequate to maintain intestinal homeostasis. Additionally, the above results could indicate that the adult intestine is fully developed and subsequent elimination of the ColVI cells has no effect in altering the colon structure. Furthermore, assessment of the apoptosis rate following intrarectal DT administration indicated no significant change in cell death. However, this is probably due to the time point of analysis. As a future goal of the study, it would be interesting to investigate whether the ablated ColVIcre+ cells can be replenished by new ColVI-GFP expressing cells in later time points or other mesenchymal populations compensate for the loss of the eliminated population. Moreover, it would be interesting to device ways to target ColVIcre+ cells in the small intestine, where the

morphology of the villi is more prominent and their deletion could have a more evident effect. Accordingly, deletion of these cells during embryonic development could provide valuable information on their role in the morphogenesis and maintenance of the crypt/villous axis.

Following the effect of elimination of the ColVIcre+ subpopulation in homeostasis, we aimed to elucidate whether this route of DT administration could be also applied to the ablation of the ColVI cells inside colonic tumors during AOM/DSS induced carcinogenesis. By employing the standardized DT protocol used during homeostasis, we demonstrated that topical rectal DT administration can accomplish efficient deletion of the ColVIcre+ subpopulation inside the developed tumors. Therefore, in the future, it would be interesting to examine if topical injection of DT on each individual tumor can lead to higher ablation efficiency and if this has any effect on tumor growth. In addition, it would be also interesting to see if the deletion of the ColVIcre+ subpopulation before inducing the AOM/DSS carcinogenesis can affect the subsequent tumor development. Furthermore, future plans could be directed to investigate if elimination of other mesenchymal populations can have an effect on tumor growth. Thus, we could explore the effect of elimination of the Twist2cre+ and Col1a2cre+ subpopulations compared to the ColVIcre+. The Twist2cre line targets all mesenchymal cells[118] and the Col1a2cre line a larger subpopulation of CAFs in the tumor microenvironment in comparison to the ColVIcre mouse, while previous studies have shown that they can have contradictory roles in the promotion of carcinogenesis. Specifically, our research group and Pallangyo et al. have shown that by employing the same model of colitis-associated cancer, ablation of IKKβ in ColVI-cre mesenchymal cells decreases tumor formation while deletion of IKKβ in Col1a2-cre-expressing cells is associated with enhanced tumor growth.[73,74] Consequently, elimination of specific heterogeneous CAF subpopulations could further elucidate their contribution in tumor promotion or suppression.

Finally, we studied the transplantation of intestinal mesenchymal cells as a therapeutic approach in the DSS model of acute colitis. Since the majority of the intravenously injected mesenchymal cells become entrapped in the lung, limiting thus their ability to exert their beneficial effect in the site of tissue damage, we selected to examine the local IMC transplantation in the colonic

mucosa. However, when the cells were transplanted in the injured mucosa either through transplantation in the colonic lumen or through a more topical approach where cells were injected specifically in the existing ulcers, we could not accomplish efficient homing of the cells. The inability of the mesenchymal cells to home in the injured tissue is controversial to other studies employing mesenchymal cell transplantation. However, it could be explained by the fact that Yui et al. used transplantation of organoids derived from Lgr5+ stem cells to achieve engraftment in the colonic epithelium, whereas Manieri et al. applied mucosal MSC transplantation following a different acute injury model, which focally injures the mucosa through endoscopy-directed biopsy.[87,119] In conclusion, taking into consideration our results and the above studies, topical transplantation of IMCs in the injured mucosa following the DSS induced acute colitis, would require further standardization in order to emerge as a promising therapeutic approach.

In conclusion, both approaches that we have used need further standardization; however, the ablation of mesenchymal cells through the iDTR system seems the most promising and further experiments also with the use of additional Cre lines could help identify the role of specific subpopulations in homeostasis and cancer in the intestine. Such information could prove valuable in the effort to design novel therapeutic approaches targeting the tumor microenvironment either alone or in combination with treatments targeting cancer cells, aiming to increase both therapeutic efficiency and drug delivery.

6. References

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