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Epithelial-stromal interaction in the pathogenesis of colorectal cancer

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ABSTACT

Colorectal cancer (CRC) is not a homogenous disease. Recent molecular classification of established tumours based on gene expression and (epi)genetic mutation burden, has revealed considerable disease heterogeneity. The relative importance of the epithelial and stromal tissue compartments varies between different tumour subtypes and this contributes to the observed clinical and molecular heterogeneity of CRC.

The AIM of this study was to explore the role of the stroma in different precancerous pathologies (polyps) and in CRC.

Stromal gene expression varies considerably between the different polyp subtypes (SSA and TVA) with a comparatively greater number of differentially expressed genes in serrated lesion stroma, suggesting the hypothesis that SSA lesions, usually initiated by BRAF mutations and methylation, require the recruitment of pro-tumorigenic stroma to enable lesion progression. In contrast, TVA are initiated by epithelial mutations that disrupt Wnt signaling (such as APC) and this is sufficient to drive tumourigenesis, irrespective of stromal influences.

Moreover, transwell tissue culture 3D techniques and animal models revealed that fibroblasts support the cross species growth of mouse epithelial organoids and abrogate the normal media requirement for Noggin and EGF. Interestingly, mouse epithelium grown in this co culture system develop as spheroids rather than the branching organoids seen with media morphogen supplementation, indicating a phenotype modulating effect of the fibroblasts.

Preliminary results revealed that fibroblasts have effects on cell proliferation and cell cycle regulation by upregulation of genes involved with cell cycle progression, DNA synthesis/repair, protein translation, vesicles mediated transport and lipid metabolism.

Primary stromal cell cultures isolated from adenoma and colon cancer (CMS2 and CMS4) might in part represent the corresponding cancer microenvironment, thus providing a useful complement to the current cellular biochemistry and therapeutic research in CRC.

SOMMARIO

Il cancro del colon-retto (CRC) non è una malattia omogenea. Una recente classificazione molecolare sul CRC basata sull' espressione genica e mutazioni (epi)genetiche, ha rivelato una notevole eterogeneità nella malattia. L' importanza dei compartimenti tissutali, epitelialiali e stromali, varia tra i diversi sottotipi di CRC e questo contribuisce all' eterogeneità clinica e molecolare osservata nel CRC.

Lo scopo di questo studio è stato quello di esplorare il ruolo dello stroma in diverse forme pretumorali (polipi) e nel CRC.

Dalle analisi eseguite, e' emerso che il profilo genetico dello stroma nelle due condizioni pre cancerogene di CRC oggetto di studio (TVA e SSA), differisce in maniera significativa, suggerendo l'ipotesi che i polipi di tipo SSA, in genere promossi da mutazioni nel gene BRAF, richiedono l'assunzione di stroma pro-cancerogeno per consentire la progressione della lesione. Al contrario, in lesioni di tipo TVA, le mutazioni epiteliali che interrompono la via di segnalazione Wnt (es. APC), siano sufficienti per promuovere la tumorigenesi a prescindere dall' influenza dello stroma.

Inoltre, con l'ausilio di tecniche di coltura 3D, inserti e modelli animali, e' emerso che i fibroblasti sostengono la crescita degli organoidi murine senza l'impiego dei fattori di crescita Noggin e EGF. In particolare, i fibroblasti causano lo sviluppo di sferoidi piuttosto che di organoidi, indicando un effetto modulante sul fenotipo.

I risultati preliminary, hanno rivelato che i fibroblasti hanno effetti sulla proliferazione cellulare e la regolazione del ciclo cellulare mediante la regolazione in maniera positiva dei geni coinvolti nella progressione del ciclo cellulare, nella sintesi e/o riparazione del DNA, nella traduzione di proteine, nel trasporto mediato da vescicole e nel metabolismo dei lipidi.

I fibroblasti isolati da adenoma e CRC (CMS2 e CMS4) potrebbero in parte rappresentare il microambiente tumorale, fornendo così un utile complemento biochimico e cellulare sulla ricerca terapeutica nel tumore del colon.

ABBREVIATIONS

AACR	American Association for Cancer Research
ACF	aberrant crypt foci
ACF-D	dysplastic aberrant crypt foci
ACF-H	heteroplastic/hyperplastic type aberrant crypt foci
ACF-S	serrated type aberrant crypt foci
AFAP	attenuated familial adenomatous polyposis
AGA	The American Gastrointestinal Association
AKT	v-akt murine thymoma viral oncogene homolog 1
ANXA10	annexin A10
APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
BM-MSCs	bone marrow derived mesenchymal stem cells
BMI-1	polycomb complex protein
BMP	bone morphogenic protein
BRAF	B-raf proto-oncogene, serine/threonine kinase
CA	conventional adenoma
CAFs	cancer-associated fibroblasts
CAM-DR	cell adhesion-mediated drug resistance
CBCs	crypt base columnar cells
CC	conventional colorectal carcinoma
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CICs	cancer-initiating cells
CIMP	cytosine-phosphoguanine island methylator phenotype
CIMP-H	high-level CIMP
CIMP-L	low-level CIMP
CIN	chromosomal instability
CRC	colorectal cancer
CRM	circumferential resection margin
CRT	chemoradiotherapy
CSCs	cancer stem cells
CSS	cancer-specific survival

CTNNB1	catenin (cadherin-associated protein), beta1
CU	ulcerative colitis
DAB	3,3'-diaminobenzidine
DC	dendritic cell
DCC	deleted in colorectal carcinoma
DCLK1	doublecortin-like kinase 1
DFS	disease-free survival
DNA	deoxyribonucleic acid
ECF	ectopic crypt foci
ECF-d	the density of ectopic crypt foci
ECM	extracellular matrix
e.g.	*exempli gratia*
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial- mesenchymal transition
EndMT	endothelial-mesenchymal transition
Ephrins	Eph receptor-interacting proteins
EphB	Ephrin type-B receptors
ERBB	erythroblastic leukemia viral oncogene homolog
ESGE	The European Society of Gastrointestinal Endoscopy
etc.	*et cetera*
FA	flat adenoma
FAP	fibroblast activation protein
5-FU	5-fluorouracil
FGF	fibroblast growth factor
FSP-1	fibroblast specific protein-1
GTPases	guanosine triphosphatases
HGF	hepatocyte growth factor
HH	hedgehog
HIF	hypoxia-inducible factor
HMPS	hereditary mixed polyposis syndrome
HNPCC	hereditary non-polyposis colon cancer
HP	hyperplastic polyp
HR	hazard ratio

HRWLE	high-resolution white-light endoscopy
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
ICC	immunocitochemistry
i.e.	*id est*
IFN	interferon
IFP	interstitial fluid pressure
IGF	insulin-like growth factor
IGF2	insulin-like growth factor 2
IHC	immunohistochemistry
IL	interleukin
i.e.	*id est*
JPS	juvenile polyposis syndrome
KRAS	Kirsten rat sarcoma viral oncogene homolog
LGR5	leucine-rich repeat containing G protein-coupled receptor
LE	leading edge tumor
LOH	loss of heterozygosity
LRP	low-density lipoprotein receptor-related protein
LS	lynch syndrome
MAP MUTYH	(mutY homolog) gene associated polyposis
MAPK-ERK	mitogen-activated protein kinase extracellular signal-regulated kinase
MCA	methyl cyanoacrylate
MGMT	O-6 methylguanine-DNA methyltransferase
MLH	mutL homolog
MLH1	mutL homolog 1
MMP	matrix metalloproteinase
MMR	mismatch repair
MoAbs	monoclonal antibodies
mo	month
MPO	myeloperoxidase
MSC	mesenchymal stem cell
MSH	mutS homolog
MSH2	mutS homolog 2

MSH6	mutS homolog 6
MSI	microsatellite instability
MSI-H	high-grade microsatellite instability
MSI-L	low-grade microsatellite instability
MSS	microsatellite stable
MUTYH	mutY homolog
MVD	microvascular density
mTOR	mammalian target of rapamycin
MYH	see MUTYH
MYD88	myeloid differentiation primary response gene 88
NF-κ B	nuclear factor-κB
NSAID	non-steroidal anti-inflammatory drug
O.N.	over night
OR	odds ratio
OS	overall survival
PCR	polymerase chain reaction
PCFs	pericryptal fibroblasts
PFS	progression-free survival
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha
PIP3	phosphatidylinositol-3,4,5-triphosphate
PMS2	postmeiotic segregation increased 2
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ROC	receiver operating characteristics
ROS	reactive oxygen species
RT	radiotherapy
RTK	receptor tyrosine kinase
SAC	serrated adenocarcinoma
SSA	sessile serrated adenoma
SSA-D	sessile serrated adenoma with cytological dysplasia
SMAD	small mother against decapentaplegic
α-SMA	α-smooth muscle actin

SP	serrated polyp
SPS	serrated polyposis syndrome
STAT3	signal transducer and activator of transcription 3
TA	tubular adenoma
TAA	tumor-associated antigen
TAM	tumor-associated macrophage
TCF	T cell factor
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor beta
TGFβ-1	transforming growth factor-β1
TGFβR2	transforming growth factor beta receptor 2
Th cell	T helper cell
TIMP	tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	tumor necrosis factor
TMA	tissue microarray
TNM	tumor, node, metastasis
TP53	tumor protein p53
TReg cell	regulatory T cell
TSA	traditional serrated adenoma
TVA	tubulovillous adenoma
sTVA	TVA with serration
RAG-2	recombination-activating gene 2
RNA	ribonucleic acid
RT	radiotherapy
TERT	telomerase reverse transcriptase
VA	villous adenoma
VEFG	vascular endothelial growth factor
WHO	World Health Organization
WNT	Wingless

1. INTRODUCTION

1.1 Structure of the colon and rectum

1.1.1 Anatomy and physiology

The colon is an abdominal organ, covered by the peritoneum except for the most distal part, the rectum; its length is in the order of 100 to 150 cm beginning in the ileocecal valve and ending in the anorectal junction (Ponz de Leon & Di Gregorio 2001).

The large bowel can also be categorized by the regions of vascular supply. “The right colon” (from caecum to splenic flexure) is supplied by the branches of the superior mesenteric artery, while the inferior mesenteric artery feeds “the left colon” (descending and sigmoid colon). The blood supply to the lower rectum is delivered by the branches of the internal iliac artery, the middle and inferior rectal arteries, and this third anatomical region is called “the rectum”.

The colorectal mucosa is covered by two layers of circular and longitudinal smooth muscle cells, which are adjacent to serosa and subserosal tissues; contraction of the external longitudinal muscle layer accounts for the appearance of characteristic haustrations along the colon (Ponz de Leon & Di Gregorio 2001) (Figure 1).

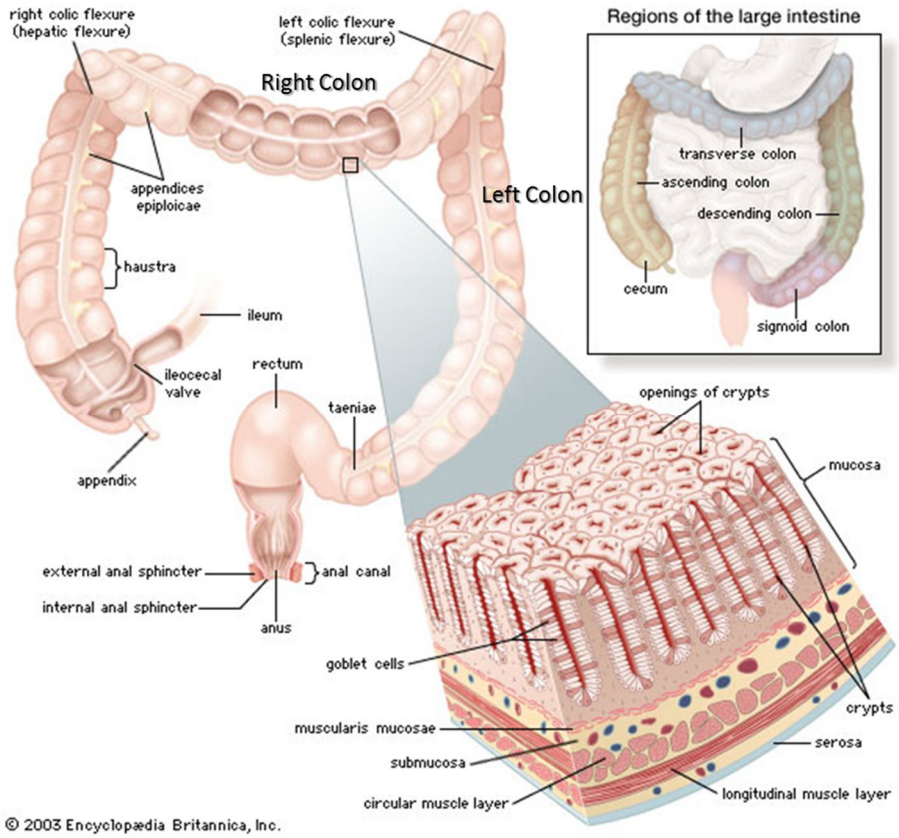


Figure 1. The anatomical subdivision of the large bowel. Adapted from Encyclopaedia Britannica 2003.

1.1.2 Histological structure

The normal colorectal mucosa is constituted by 3 main elements: epithelium (of the surface and crypts), lamina propria and muscularis mucosae; the latter separates the mucosa from the deeper submucosa (Figure 1).

The colonic surface epithelium is composed of a single layer of columnar cells which function as a protective barrier between host and luminal environment. The two main cellular elements of the epithelium are absorptive cells (responsible for ion and water absorption) and goblet cells (which synthesize, store and secrete

mucin); a thin basement membrane (composed of collagen and other proteins) anchors and supports the surface epithelium.

A similar single-layer columnar cell epitheliurn forms the colorectal crypts; besides absorptive and goblet cells, crypt epithelium also contains undifferentiated precursor cells, specialised endocrine cells (containing secretory granules) and rare Paneth cells (pyramid-shaped cells containing eosinophilic secretory granules, the function of which remains unclear), especially in the proximal colon.

The lamina propria extends between crypts and reaches the muscularis mucosae and it contains a wide variety of cells, including fibroblasts, lymphocytes, plasma cells, eosinophils, macrophages and mast cells, which are arranged among strands of collagen tissue. Colonic mucosa also contains well formed gut-associated lymphoid tissue which is responsible for local defence against harmful agents originating in the gut lumen (Intestinal immunity and inflammation: recent progress 1986).

The muscularis mucosae is a thin layer of smooth muscle which separates the epithelium and lamina propria from the submucosa. Contraction of the muscularis may alter the shape of the mucosa, thus affecting normal physiologic processes (absorption of water and electrolytes, mucin secretion, cell replication).

The submucosa is constituted by the same elements that feature the lamina propria (such as lymphocytes that can form lymphatic follicles, fibroblasts, macrophages, mast cells and fibrous tissues). Two neural plexuses are located in the submucosa; one immediately beneath the muscularis mucosae (Meissner plexus), the other (Auerbach plexus) lies in the deeper part of the submucosa, close to the lamina propria. Vascular elements of the submucosa include arterioles, venules and lymphatic vessels; these submucosal structures may, sometimes, appear larger or tortuous even in the absence of any colonic abnormality (Ponz de Leon & Di Gregorio 2001).

1.1.3 Intestinal architecture

1.1.3.1 Intestinal stem cells

The normal colon has two distinct pools of stem cells, which together make up the total population of 16 stem cells (Medema & Vermeulen 2011, Vaiopoulos et al. 2012, Stange & Clevers 2013). The contribution of each pool to the total is not known. The first stem cell pool is localized in the crypt base (crypt base columnar cells (CBCs) and can be characterized by high LGR5 (Leucine-rich repeat containing G protein-coupled receptor) expression and is largely comprised of a proliferating population (Barker et al. 2007, Kemper et al. 2010). The next pool is nearby in the +4 position of the colon base (four cells away from the base of the crypt) and consists of relatively quiescent or dormant cells. This second pool exhibits high expression of BMI-1 (Polycomb complex protein) and telomerase reverse transcriptase (TERT) (Medema & Vermeulen 2011, Bertrand et al. 2012).

Regulation of normal intestinal stem cells occurs at the crypt base, in particular, in the stem cell niche. The stem cell niche consists of epithelial, mesenchymal cells and extracellular substrates which favour the existence of a stem cell in its undifferentiated state. It provides an optimal microenvironment for the production of differentiated progeny by the paracrine secretion of growth factors, cytokines, and morphogens. The phenotypic response of a cell is determined by its position within these concentration gradients. Key constituents of the niche include Paneth cells and pericryptal fibroblasts.

Stem cells at the crypt base produce rigorously dividing transit-amplifying cells. While proliferating, transit-amplifying cells move upward and reach the crypt-villus junction after four to five cell divisions, after which they fulfill their specific function as specialized cells (Heath 1996). To compensate the continuous, conveyor-belt-like flow of cells along the crypt-villus axis, cells at the villus tip undergo apoptosis and are shed into the gut lumen.

This continuous renewal of the epithelium along the vertical (crypt-to-luminal surface) axis of the intestine happen within the 5–7 days that it takes for a cell to migrate from the crypt base to the luminal surface (Biswas et al. 2015). Only one cell type, the Paneth cell, escapes this upward movement. Pushed downward by the repulsive forces of Ephrins (Eph receptor-interacting proteins) and their Ephrin type-B (EphB) receptors, Paneth cells settle at the very base of crypts (Batlle et al. 2002) (Figure 2).

Adult stem cell and daughter cell fate determination is controlled by the same signalling pathways that regulate embryonic stem cell function during development. In the adult, these pathways are stringently controlled with complex interactions used to restrict pathway activity and response to the appropriate cell compartment. Mesenchymal and epithelial-derived pathways result in polarized gradients that regulate stemness, cell proliferation, differentiation, and apoptosis as cells progress along the intestinal vertical axis. Important pathways include Wnt, BMP, Hedgehog (HH), and Notch signaling (Biswas et al. 2015).

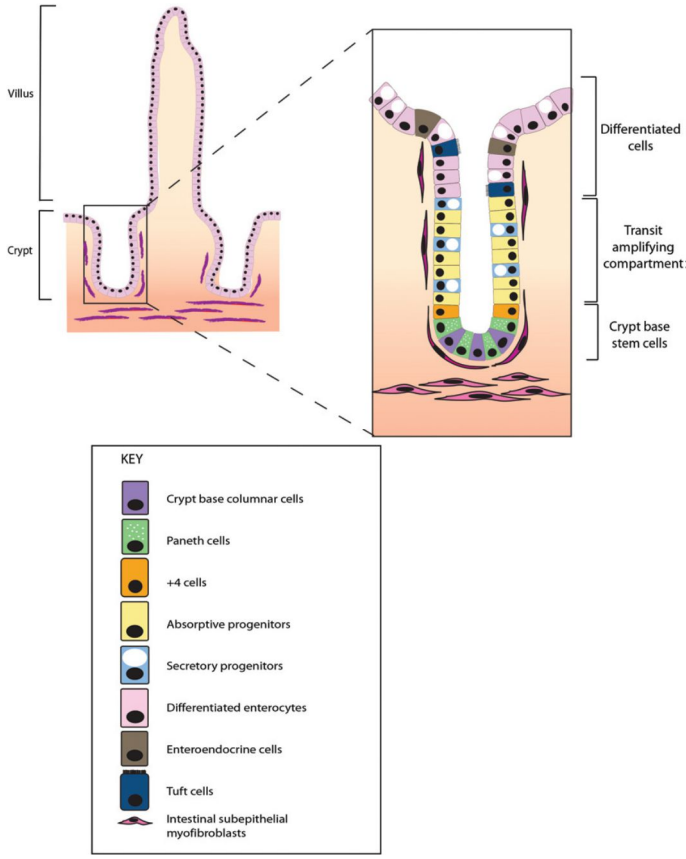


Figure 2. Intestinal crypt architecture and cell types. The intestinal crypt is the basic functional unit of the gut. In the small intestine, several crypts contribute to finger-like projections called villi. In homeostasis, the stem cells (crypt base columnar and +4 cells) are restricted to the crypt base stem cell niche. Immediate stem cell progeny divide rapidly in the bottom half of the crypt, called the transit amplifying zone. Terminal differentiation occurs in the upper part of the crypt, with fully differentiated cells eventually being shed into the intestinal lumen. Under homeostatic conditions in the mammalian gut, transit along the crypt–luminal axis takes 5–7 days . From Biswas *et al*. 2015.

The gut is subdivided anatomically into two parts, the small intestine and the colon.

1.1.3.2 Small Intestine

The small intestine's surface is maximized by millions of protrusions of the epithelium, called villi and by invaginations into the submucosa, called crypt of Lieberkuhn. They are composed of colon stem cells, transit amplifying cells and terminally differentiated goblet cells, enterocytes and endocrine cells (Medema & Vermeulen 2011). Each normal crypt is comprised of about 2,000 cells (Vaiopoulos et al. 2012).

1.1.3.3 Colon

While having an overall similar setup, the colon differs from the small intestine by the absence of villi, creating a flat surface epithelium. While typical Paneth cells are missing in the colon, deep-crypt-secretory cells have been proposed to represent their colonic counterpart. Colonic transamplifying cells differentiate toward the goblet and absorptive cell lineages (Altmann 1983).

Similar to the crypt of the small intestine, less differentiated cells reside in the bottom and terminally differentiated cells reside near the top (Bertrand et al. 2012).

1.2 Colorectal premalignant lesions

Colorectal polyps encompass a range of lesions, widely defined as mucosal protrusions and tumor-like lesions in the large intestine. In addition to premalignant epithelial lesions (CAs and serrated adenomas) and hyperplastic polyp (HPs), the definition also includes inflammatory and reactive polyps (e.g. mucosal prolapse-associated

polyp, inflammatory pseudo-polyp and infection-associated polyp), hamartomatous polyps (e.g. Peutz-Jeghers polyp and Juvenile polyp), stromal polyps (e.g.inflammatory fibroid polyp, Schwann cell hamartoma, and lipoma), lymphoid polyps (e.g. prominent lymphoid follicles and lymphomatous polyposis), endocrine polyps (e.g. well differentiated endocrine tumor) and other lesions (e.g. prominent mucosal fold, elastotic polyp and endometriosis) (Hamilton et al. 2010, Snover et al. 2010). The epithelial polyps are discussed subsequently in more detail because of the biological continuum between these lesions and colorectal adenocarcinoma (CRC).

1.2.1 Aberrant crypt foci

Aberrant crypt foci (ACF), comprising only a few colonic crypts, are the earliest microscopically distinguishable lesions preceding the development of CAs and SPs. ACF were first described in the colorectum of experimental rodent models exposed to carcinogens, and soon after this, in human colonic epithelium (Pretlow et al.1991). Different types of ACF can be histologically characterized. Heteroplastic/hyperplastic type (ACF-H) and serrated type crypt foci (ACF-S) share morphological and molecular similarities with SPs whereas dysplastic aberrant crypt foci (ACF-D) have a resemblance with miniature CAs. The term “microadenoma” includes ACF with dysplasia. Macroscopically, ACF can be detected in colonoscopy only with magnifying chromoendoscopy or other similar techniques because of the minute non-polypoid structure (Inoue et al. 2014, Mäkinen 2007, Rosenberg et al. 2007).

1.2.2 Conventional colorectal polyps (CAs)

In a recently published prospective population-based colonoscopy study on 745 individuals, 10% had adenomas, and 95% of them were CAs (Forsberg et al. 2012). It has been reported that the five-year

incidence rate of adenomas after a negative screening colonoscopy is approximately 15% (Imperiale et al. 2009).

CAs can be classified into tubular (TA), tubulovillous (TVA) and villous adenomas (VA) and represent the most often encountered precursor lesions of the CRC, defined by epithelial dysplasia varying from low to high grade (Buda et al. 2012, Carr et al. 2009). They are more common in males and in patients aged 50 years or more (Hetzel et al. 2010, Neugut et al. 1993, Pendergrass et al. 2008). Most of them are <1 cm in size and macroscopically polypoid or sessile less frequently flat or depressed (Hamilton et al. 2010, Rembacken et al. 2000). Increasing adenoma size has been reported to associate with synchronous and metachronous adenomas (Mattar & Rex 2008), as well as with malignant change (O'Brien et al. 1990, Shinya & Wolff 1979). In addition to large size of the lesions, extensive villous architecture, high-grade epithelial dysplasia (named "advanced adenoma") and flat structure have been shown to increase the tendency toward malignant disease (Hamilton et al. 2010).

The histological appearance of TA is tubular crypts that usually stand closer to each other than in normal mucosa. Villous architecture, defined as leaf- or finger-like projections of the colorectal epithelium, does not represent more than 20 to 25% of the polyp size.

TVAs are formed of a mixture of tubular and villous architectures containing at least 25% villous structures, while VAs are mainly (>75%) comprised of them. All the CAs contain intraepithelial dysplasia ("conventional adenomatous dysplasia") characterized by increasing cellularity, the loss of polarity and the stratification of enlarged, pleomorphic (possibly oval or vesicular) nuclei with increased mitotic activity and reduced mucin (Hamilton et al. 2010, Konishi & Morson 1982).

Flat adenoma (FA) is a rare variant of CA initially thought to be unique to Japanese population but currently known to occur also in

Western population (Rembacken et al. 2000). Histologically, FAs are usually slightly elevated (<2 mm in height), less frequently completely flat or even depressed, making their detection extremely difficult in colonoscopy without special dyeing (methylane blue, cresyl or crystal violet and indigo carmine have all been described). Despite the small size of these lesions, they often show high-grade dysplasia or even harbor invasive cancer, giving ground for the hypothesis of these lesions being behind the "*de novo*" CRCs (Kudo et al. 2008, Rembacken et al. 2000).

1.2.3 Serrated colorectal polyps

Serrated polyp of the large intestine, until recently, was recognized as a common benign lesion, with the small innocuous hyperplastic polyp (HP) as the prototype.

The morphologic complexity of the serrated adenoma varies from being clearly adenomatous to being difficult to distinguish from hyperplastic polyp, which creates a need for more detailed morphologic analysis of all serrated polyps. The suggestion has been made to eliminate the term "hyperplastic polyp" altogether and simply designating these lesions as "serrated polyps" (Iino et al. 1999). SPs represent 35–40% of the colorectal polyps and are classified into Hyperplastic polyp (HP), sessile serrated adenoma (SSA) and traditional serrated adenoma (TSA).

Hyperplastic polyp

Hyperplastic polyp (HP) is the most common SP, accounting for approximately a quarter to a third of all resected colorectal polyps (Carr et al. 2009, Higuchi et al. 2005, Spring et al. 2006).

Macroscopically, HPs are typically small, flat epithelial lesions that rarely reach >1 cm in size. Proximal HPs, which are usually larger

than distal ones, can be covered by a yellow mucus cap that can impede their detection in colonoscopy (Snover et al. 2010). It lacks dysplastic architectural distortion and mutagenic potential for transformation to cancer, and does not represent risk for developing neoplasia (Laiyemo et al. 2009). Management of HP involves at most confirmation by biopsy on colonoscopy.

The histologic appearance of HP is symmetrical, vertically oriented, slender crypts with saw-tooth epithelial serrations generally limited to the superficial half of the crypt. The subepithelial basement membrane and muscularis mucosae are thickened compared with adjacent normal mucosa or the collagen layers of other SPs and the amount of endocrine cells at crypt bases may be slightly increased (Mäkinen 2014). The expanded proliferative zone typically locates symmetrically in the crypts' bases, while the maturation zone is evenly and symmetrically distributed in the luminal compartment (Torlakovic et al. 2008). There are subtle variations in the polyp location in the colorectum, histology and mutation rates between the different subtypes of HPs.

Microvesicular HPs are the most common subtype representing approximately 70% of HPs, encountered mainly in the distal colon and rectum, and largely carrying $BRAF^{V600E}$ mutation (Burnett-Hartman et al. 2013, Spring et al. 2006, Yang et al. 2004). The most designated histological feature among microvesicular HPs are small mucin droplets in the cytoplasm ("microvesicular") of epithelial cells with varying amounts of goblet cells in between (Mäkinen 2014). Based on the similar histology and the frequencies of $BRAF^{V600E}$ mutation, microvesicular HPs and SSAs are hypothesized to form a biological continuum (Bettington et al. 2013).

Goblet-cell rich HPs are predominantly located in the distal colon and rectum and frequently (in approximately half of the cases) harbor the KRAS (Kirsten rat sarcoma viral oncogene homolog) mutation (Spring et al. 2006, Yang et al. 2004). Serration is often less conspicuous compared with microvesicular HPs and more

strictly limited to the upper parts of the crypts abundant in goblet cells. There is lack of evidence of goblet-cell rich HPs being able to progress to CRC (Bettington et al. 2013, Mäkinen 2014).

Mucin-poor HPs are the most infrequently encountered subtype of HPs. It has been suggested that they more likely represent microvesicular HPs with degenerative features than a distinct entity. Fine saw-tooth epithelium is present in microvesicular HPs as in other HPs, but the loss of goblet cells and microvesicular mucin, as well as the degenerative changes in remaining cells, separate the polyp from other subtypes (Mäkinen 2014).

Sessile serrated adenoma

In 1996, Torlakovic & Snover identified a subset of serrated lesions within "hyperplastic polyposis", displaying an abnormal architecture without cytological dysplasia and defined them as "sessile serrated adenomas" (SSA) (Torlakovic & Snover 1996), which are now considered as precursors of microsatellite unstable colorectal carcinomas (Leedham et al. 2005).

SSAs are sessile or flat, rarely pedunculated, lesions with unobtrusive borders and smooth surface, often covered with mucus (Kim et al. 2013, Mäkinen 2014). Their average size is larger than HPs but the sessile structure, as well as the often pale-appearing or slightly reddish surface epithelium, makes their detection difficult during colonoscopy (Snover et al. 2010). A similar surface, with stellate, wide crypt openings ('stellate pit pattern'), is often detected in SSAs and microvescicular HPs (Hasegawa et al. 2011, Kimura et al. 2012).

The estimated proportion of SSAs among colon polyps has varied greatly in previous colonoscopic series and the inter-observer variability of the diagnosis of SSA has been considerable in past studies (Farris et al. 2008, Hetzel et al. 2010, Rau et al. 2014). However, in recently published demographic studies, SSAs were

reported to represent 1.7–11.7% of all colon polyps (approximately a quarter of SPs) and typically, to have a predilection for the proximal colon (Carr et al. 2009, Higuchi et al. 2005, Lash et al. 2010, Spring et al. 2006) and female gender (Carr et al. 2009, Lash et al. 2010, Spring et al. 2006).

Crypt compartmentalization aberration, the dispersed, asymmetrical proliferative zone situated on one or the other side of the crypts and extending to the bottom of the crypts, characterizes the architectural disorganization in SSAs. Prominent epithelial serration lining the crypts' sides extends deep into the bottom of the basally dilated, J-, L- or inverted T-shaped crypts. Irregular crypt branching and pseudoinvasion of the crypts beneath the muscularis mucosae, as well as subtle nuclear atypia (vesicular, oval-shaped nuclei with prominent nucleoli), possibly mitoses dispersed anywhere in the crypt epithelium, dystrophic, irregularly distributed goblet cells and excessive mucin production are also often encountered in SSAs (Hamilton et al. 2010, Mäkinen 2014, O'Brien et al. 2008).

SSAs often harbor $BRAF^{V600E}$ mutation (Jass et al. 2006, Kambara et al.2004, Spring et al. 2006). It is important to stress that SSAs share this molecular feature with microvesicular-hyperplastic polyps which have led Yang et al. to suggest that SSAs may have evolved from this type of hyperplastic polyps (Yang et al. 2004, Bauer & Papaconstantinou 2008). Moreover, BRAF mutation is strongly linked with CIMP-high SSAs or sporadic MSI-high colorectal cancers, as shown by Kambara et al.2004 (Kambara et al.2004). As BRAF mutation and CIMP have been identified in the earliest serrated lesions (microvesicular-hyperplastic polyps, SSAs without dysplasia) they are presumed by experts to be the initial stages of the serrated pathway (Mercer & Pritchard CA 2003).

Silencing of the DNA (deoxyribonucleic acid) mismatch repair (MMR) gene mutL homolog 1 (MLH1) as a consequence of methylation of the CpG (cytosine-phospho-guanine) islands in the promoter area of the gene is another well characterized and

documented feature that may eventually lead to progression into a lesion with cytological dysplasia (Bettington et al. 2013,Mäkinen 2014).The loss of function of hMLH-1 through hyper-methylation of its promoter region is thought to be a later event along this sequence with a higher risk of progression to cancer as suggested by the strong prevalence of hMLH-1 methylation in SSAs with cytological dysplasia (Calon et al 2015, Isella et al. 2015, De Sousa et al. 2013).

Sessile serrated adenoma with dysplasia

Two types of cytological dysplasia have been reported to occur in approximately 15% of SSAs (Lash et al. 2010, Teriaky et al. 2012), giving these polyps the name "SSA with cytological dysplasia" (SSA-D) in the WHO classification (Snover et al. 2010). The conventional adenomatous dysplasia is the same type of epithelial dysplasia as seen in CAs and, in addition to being seen in some SSAs, it is more frequently encountered in TSAs (Bettington et al. 2013, Fujita et al. 2011, Kim et al. 2010). Another type of dysplasia in SSAs is "serrated dysplasia" defined as cells with abundant eosinophilic cytoplasm and basally located vesicular nuclei. Nuclear piling is not as evident as in conventional adenomatous dysplasia and the serrated architecture is typically retained (Mäkinen 2014).

Traditional serrated adenoma

In 1990, Longacre and Fenoglio-Preiser noticed that some "serrated" polyps shared features with both conventional adenomas and hyperplastic polyps and thereby coined these polyps "traditional serrated adenomas" (TSAs) (Longacre & Fenoglio-Preiser 1990). TSAs comprise approximately 1–2% of SPs (Buda et al. 2012, Carr et al. 2009).

Macroscopically, they have been reported to mostly have a protuberant reddish appearance resembling pine cone or coral reef or

a two-tiered appearance and thus, to be structurally closer to CAs than other SPs (Mäkinen 2014, Snover et al. 2010). Sessile appearance, especially in proximal TSAs, is also encountered (Hasegawa et al. 2011, Rex et al. 2012) and in some studies, even more frequently than polypoid appearance (Kim et al. 2013, Wiland et al. 2014). They can be located in any part of the large bowel with a slight predilection for the distal colon and rectum (Bettington et al. 2014, Fu et al. 2012, Wiland et al. 2014).

Histologically, TSAs are typically characterized by tubulovillous structures, eosinophilic, tall columnar cells with penicillate nuclei forming the epithelium with prominent serration and a mixture of variable amounts of goblet cells. Other cytological features include central, elongated nuclei, mild pseudostratification, and eosinophilic cytoplasm (Harvey & Ruszkiewicz 2007). Abnormally developed, small nest-like structures, ectopic crypt foci (ECF), are often dispersed along the epithelial cells (Mäkinen 2014). They lose the orientation toward muscularis mucosae but maintain the orientation toward the mucosal surface of the bowel lumen, thus leading to speculations about ECF's possibly reflecting the disturbances of epithelial-mesenchymal interactions (Bettington et al. 2013, Haramis et al. 2004). Furthermore, ECF are hypothesized to explain the biological basis of protuberant growth in TSAs and they are regarded as the most characteristic histological feature for them, rarely present in any other colon polyps (Haramis et al. 2004, Rex et al. 2012, Snover et al. 2010).

The overall proliferative activity in TSAs is significantly lower compared with CAs and has been reported to have two types of manifestations with either a preferential location in ECF or irregular distribution throughout the intervening surface epithelium (Fu et al. 2012, Kim et al. 2013, Torlakovic et al. 2008).

As SSAs, TSAs show two types of epithelial dysplasia, serrated and conventional, of which conventional adenomatous dysplasia is also frequently (in up to 49% of TSAs) encountered (Bettington et al.

2014, Fu et al. 2012, Kim et al. 2010). Activating mutations in either BRAF or KRAS oncogenes frequently occur in TSAs (Bettington et al. 2014, Fu et al. 2012, Kim et al. 2010, Wiland et al. 2014).The molecular features of the TSA are less well established but include KRAS mutations and aberrant methylation with hypermethylation of the promoter of MGMT (methylguanine DNA methyltransferase) (Landis et al. 1998).

Overlapping features of serrated polyps

Despite the carefully defined histological characteristics, the classification of different colorectal polyps can be challenging due to the overlapping features between the different polyps. The distinction between morphologically reminiscent microvesicular HP and SSA is mainly based on the disordered growth in the lower crypts with a consequence of irregular branching and J-, L- or inverted T-shaped crypts lined by epithelial serration. Whether just one irregular crypt is sufficient for the change of microvesicular HP diagnosis to SSA is undefined (Bateman 2014, Bettington et al. 2014). Furthermore, high-grade dysplastic or carcinomatous areas in SSAs do not always show serrated structure but principally CA-like tubular or tubulovillous structures, despite harboring the same molecular changes that are typical of early SSAs (Fujita et al. 2011, Goldstein 2006).

ECF were regarded as a specific histological feature of TSAs. However, ECF have recently been reported to be encountered also in TVAs, indicating a histological overlap between serrated and conventional colon polyps (Hafezi- Bakhtiari et al. 2015). Moreover, the oncocytic-like cells, formerly associated with the presence of ECF and characterized by the deeply eosinophilic, abundant cytoplasm and elongated nuclei, were also reported to occur in other types of colon polyps, both serrated and conventional ones, highlighting the problems of too straightforward categorizations (Snover 2011).

Although epithelial serration is thought to be the most characteristic feature of SPs, also TVAs have been reported to contain focal areas of epithelial serration. These polyps have been suggested to be named as TVAs with serration (sTVAs) (Bettington et al. 2013, Tsai et al. 2014). Moreover, a recently published study indicated sTVAs being larger, more often proximal, more histologically advanced, and showing more frequent CpG island methylation and higher numbers of KRAS mutation compared with conventional TVAs. Compared to TSAs, they were more often proximal, showed less CpG island methylations, more frequent MGMT (O-6 methylguanine-DNA methyltransferase) methylation and more frequent nuclear translocation of β-catenin (Bettington et al. 2016).

What the histological similarities tell about the background of different colorectal polyps is somewhat undetermined. Some recently published studies have shown that TSAs fairly often contain areas resembling HPs and SSAs within one polyp, a phenomenon that is possibly thought to reflect the biological continuum within the serrated group of colorectal polyps (Bettington et al. 2014, Kim et al.2010, Kim et al. 2013, Wiland et al. 2014).

Risk of malignancy in serrated polyps

In general, HPs, especially when present in multiple numbers in the rectum, are considered to be innocent lesions without the ability to malignant progression and are thus infrequently sampled (Bettington et al. 2013, Mäkinen 2014). However, the estimated share of 30–35% of CRCs originating via the serrated pathway forms a sharp contrast to the reported low prevalence numbers of SSAs, SSA-Ds and TSAs (Mäkinen 2014). Whether this inconsistency tells about the higher risk of malignancy in serrated precursor lesions, their poor recognition in endoscopy or light microscopy, or the shortened time frame for detection due to the SPs' faster growth rate to CRC, is ambiguous (Mäkinen 2014). Multiple, proximally located SPs, as well as large sized (>1 cm) polyps have been reported to associate

with synchronous and subsequent SPs and CRCs, potentially reflecting the fast growth rate and high risk of malignant progression of these lesions (Álvarez et al. 2013, Hiraoka et al. 2010, Lazarus et al. 2005, Schreiner et al. 2010).

Recently, SSAs and HPs, regardless of their location, were reported to often be present in the periphery or stalk of TSAs or even as intimately admixed forms with a typical TSA component suggesting the possibility that also HPs, generally considered as innocent lesions, may progress to TSA and further to CRC (Kim et al. 2013).

1.2.4 Mixed Polyp

The mixed polyp variant displays features of hyperplastic polyp and SSA, and a dysplastic component resembling conventional adenoma. These polyps tend to occur in the right side of the colon, are smaller in size, and show a predominance of BRAF mutation with MSI-H and CIMP-H profile. They may represent a SSA evolving to cytological dysplasia and carcinoma because a mixed serrated and adenomatous transition zone is commonly noted when SSA is found in conjunction with carcinoma (Harvey & Ruszkiewicz 2007).

Table 1 indicates benign and premalignant epithelial tumors of the colon and rectum.

Classification	Designating features
Traditional adenomas	Presence of dysplastic epithelium
Tubular adenoma	Tubular glands
Villous adenoma	Leaf- or fingerlike projections of the epithelium overlying lamina propria
Tubulovillous adenoma (TVA)	Mixture of tubular and villous components; villous component 25–75%
Serrated polyps	Saw tooth-like infolding of the surface and crypt epithelium
Hyperplastic polyp (HP)	Serrations confined to the upper parts of the crypts, no cytological atypia
Sessile serrated adenoma (SSA)	Distortion of the normal crypt architecture: dilated and T- or L-shaped crypts, alterations in the position of proliferative zone; vesicular nuclei
Traditional serrated adenoma (TSA)	Ectopic crypt formation (ECF); cytological atypia
Mixed polyps	Display features of hyperplastic polyp and SSA, and a dysplastic component resembling conventional adenoma

Table 1. Benign and premalignant epithelial tumors of the colon and rectum. Classification and designating features adapted from Hamilton *et al.* 2010, Mäkinen 2007, Torlakovic *et al.* 2008, Snover *et al.* 2010.

1.3 Colorectal cancer epidemiology and classification

1.3.1 Incidence

The gastrointestinal tract is one of the most common sites of carcinogenesis as a consequence of its high number of mitotic events and exposure to carcinogens (Leedham et al. 2005).

Colorectal cancer (CRC) is the third most common cause of cancer death in the world (Parkin 2001, Center et al. 2009, Hutfless & Kalloo 2013) with approximately 5% lifetime prevalence in the Western world (Siegel et al. 2014). The incidence rate (ASRs) in North America and Europe is approximately 30–50/100.000 (Schottenfeld & Winawer 1996).

In Europe, CRC is one of the most commonly diagnosed cancers, with more than 450,000 new cases reported annually. Unfortunately, it remains the second leading cause of cancer deaths in the WHO

European region (12.0% of all cancer-related deaths) (Curado 2011). Almost half of the population will develop at least one benign intestinal tumor during their lifetime and there is a sharp increase in CRC incidence in people over 70 years of age (Siegel et al. 2014). The highest CRC incidence rates have been reported in the developed countries with a westernized lifestyle, while in developing countries, the incidence rates are lower (Center et al. 2009).

1.3.2 Genes and signaling pathways altered in CRC

Several genetic changes are required for the initiation and progression of CRC and they involve several critical genes and important signaling pathways (Fearon 2011, The Cancer Genome Atlas Network 2012).

1.3.2.1 Oncogenes and tumor suppressor genes

Studies in the 1980s and 1990s revealed that important steps in the carcinogenesis include the activation of pro-tumorigenic oncogenes and inactivation of anti-tumorigenic tumor suppressor genes (Vogelstein et al. 1988, Kinzler & Vogelstein 1997, Herman et al. 1999).

Proto-oncogenes distributed throughout the human genome control cell proliferation, differentiation, apoptosis and growth. They can transform into oncogenes, with an ability to promote cancer growth, by point mutations, chromosomal translocations, or gene amplifications and all of these mechanisms result in either a change in the structure of their protein product or an increase in their expression. The products of oncogenes include transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers, and apoptosis regulators (Croce 2008, Markowitz 2009).

In contrast to oncogenes, the tumor suppressor genes inhibit cell growth and differentiation and thus suppress the neoplastic progression (Fearon & Vogelstein 1990). Traditionally, the tumor suppressor genes have been suggested to act "recessively" at the cellular level, denoting that both alleles must be inactivated before the elimination of growth-suppressive function is lost (Knudson 1971). However, subsequent studies have revealed in a proportion of tumor suppressor genes in CRC (e.g. tumor protein p53 (TP53) and deleted in colorectal carcinoma (DCC) that inactivation of one gene copy may be sufficient in a dominant negative fashion (Fearon & Vogelstein 1990). However, most tumor suppressor genes are still considered to follow Knudson's two-hit hypothesis of cancer development, according to which both gene copies need to be inactivated for a phenotype (Knudson 1971).

In cancers with hereditary background, the first hit is an inherited mutation of the tumor suppressor gene in a germline cell, whereas the second hit is restricted to the somatic cancer progenitor cell in target tissue. Conversely, in sporadic cancers, two inactivating hits (one in each allele) occur somatically before the tumor initiation, and these hits can be either genetic (e.g. mutations) or epigenetic (e.g. promoter methylation) (Peltomäki 2014).

The tumor suppressor genes can be classified into three different categories based on the function of their gene products (Michor et al. 2004). In CRC the "gatekeepers" (genes with the ability to directly regulate tumor growth) are adenomatosis polyposis coli (APC) and TP53. The "caretakers" (genes that maintain genomic instability increasing mutations in other genes) are rappresented by MLH1 in CRC (Kinzler & Vogelstein 1997, Michor et al. 2004).

The third class "landscapers" encode gene products that affect the cellular microenvironment: phosphatase and tensin homolog (PTEN) in CRC that, cause the distruption of the normal interactions between cell and stromal environment (Michor et al. 2004). Table 2

shows a group of oncogenes and tumor suppressor genes commonly associated with CRC pathogenesis.

Gene	Significance of the gene product
Oncogenes	
KRAS	Activation of MAPK-ERK signal, transduction, inhibition of apoptosis, promotion of cell survival (Bos *et al.* 1987).
BRAF	Activation of MAPK-ERK signal, transduction, inhibition of apoptosis, promotion of cell survival (Davies *et al*. 2002).
β-catenin	Activation of Wnt signaling that regulates cell proliferation and invasion (Morin et al. 1997).
Tumor suppressor genes	
APC	Inhibition of Wnt signaling via degrading β- catenin (Morin *et al*. 1997).
TP53	Cell cycle regulation (Baker *et al*. 1990).
TGFβR2	Receptor that is responsible for TGFβ pathway signaling mediating growth arrest and apoptosis (Markowitz *et al.* 1995).
SMAD2 and -4	Important component of TGFβ pathway signaling mediating growth arrest and apoptosis (Thiagalingam *et al.* 1996).
MLH1, MSH2, and MLH6	Enzymes contributing to DNA mismatch repair and maintaining the stability of DNA microsatellites (Fishel *et al.* 1993, Herman *et al*. 1998, Miyaki *et al.* 1997, Papadopoulos *et al*. 1994, Strand *et al.* 1993).

Table 2. Oncogenes and tumor suppressor genes commonly associated with CRC pathogenesis. Modified from Markowitz & Bertagnolli 2009.

1.3.2.2 Signaling pathways altered in CRC

The key signaling pathways, according to current understanding, including WNT (wingless), MAPK-ERK (mitogen-activated protein kinase extracellular signal-regulated kinase), PI3K (phosphatidylinositol 3-kinase), TGF-β (transforming growth factor-β), P53 and DNA MMR (Markowitz 2009, The Cancer Genome Atlas Network 2012). Noteworthy, most CRCs show alterations in multiple pathways (The Cancer Genome Atlas Network 2012).

Wnt signaling

Wnt pathway is the most altered in CRC (>90%), most frequently by the biallelic inactivation of APC (Markowitz 2009, The Cancer Genome Atlas Network 2012). APC is a tumor suppressor gene that regulates the phosphorylation of oncoprotein β-catenin/T-cell factor (TCF) dependent transcription and the degradation of β-catenin in proteasomes by ubiquitin ligases as a part of a protein complex modulated by the Wnt signaling pathway (Aoki & Taketo 2007). In CRC the inactivation of APC causes the accumulation of β-catenin in the cytoplasm and nucleus, followed by constitutive, inappropriate activation of the Wnt signaling pathway and the altered expression of multiple genes participating in cell proliferation, differentiation, apoptosis, and migration (Aoki & Taketo 2007).

The mitogen-activated protein kinase extracellular signal-regulated kinase pathway

The MAPK cascades are important pathways mediating the cellular response to extracellular signals that regulate normal cell growth, differentiation and survival. The ERK pathway is the best known of these pathways (Dhillon et al. 2007) and the protein products of the proto-oncogenes KRAS and BRAF are the subsequent mediators in the MAPK-ERK pathway (Fearon 2011, The Cancer Genome Atlas Network 2012).

The proto-oncogene KRAS is a member of the Ras family encoding a group of enzymes named GTPases (guanosine triphosphatases), which function downstream of several different receptor tyrosine kinase (RTK) growth factor receptors, e.g. the EGFR family (Fearon 2011, Wennerberg et al. 2005, Worthley & Leggett 2010). The protein product of KRAS is an important mediator in signal transduction pathways mediating the extracellular signals into intracellular signal cascades including the MAPK-ERK and the PI3K

pathways (Fearon 2011, Malumbres & Barbacid 2003, The Cancer Genome Atlas Network 2012). In approximately 40% of CRCs, KRAS is somatically mutated and the activating mutations of RAS genes result in a constitutive downstream signaling through the MAPK-ERK pathway, and further continuous cell growth (Fearon 2011, Worthley & Leggett 2010). KRAS mutations are often already detected in early adenomas and even in ACFs (Fearon 2011, Rosenberg et al. 2007), as well as other cancers such as pancreatic, lung and thyroid cancers (Dhillon et al. 2007). KRAS mutation is found in 30% of serrated adenomas and is more commonly associated with the traditional serrated adenoma subtype. The serrated pathway exhibits low levels of methylation and microsatellite instability (MSI-L).

The BRAF protein, encoded by the proto-oncogene BRAF, belongs to a family of serine/threonine kinases (known as RAF kinases family) that were originally identified as retroviral oncogenes at the beginning of the 1980s (Rahman et al. 2013). BRAF is one of the direct downstream effectors of KRAS in the MAPK-ERK pathway (Dhillon et al. 2007). Phosphorylated by RAS, BRAF activates its downstream effectors MEK1 and MEK2, which subsequently phosphorylate ERK1 and ERK2. Activated ERK1/2 further transmits the signals to its downstream cytosolic and nuclear effectors regulating normal cell growth, differentiation and survival (Dhillon et al. 2007, Rahman et al. 2013). Over 65 BRAF mutations have been discovered and most of these are found in exon 11 and 15 (Rahman et al. 2013). The most frequent mutation type (>90% of oncogenic BRAF mutations) is a missense mutation in exon 15 (the valine to glutamic acid substitution at the residue position 600) known as $BRAF^{V600E}$ (formerly 599E) (Davies et al. 2002, Wan et al. 2004).

Furthermore, although these genes locate one after another in the MAPK-ERK pathway, the BRAF and KRAS mutated cancers possess distinct clinicopathological characteristics reflecting the numerous downstream effector pathways of KRAS (Dhillon et al. 2007).

Stefanius et al demonstrated a high frequency of KRAS mutations (45.2%) in serrated adenocarcinoma, suggesting that a significant proportion of KRAS mutated CRC originates from serrated polyps (Stefanius et al. 2011). Like KRAS mutations, also BRAF mutations are thought to occur in the early steps of the CRC tumorigenesis, based on the finding that mutations are already detected in small polyps and ACFs (Markowitz 2009). Furthermore, the mutation of BRAF is strongly associated with CIMP (cytosine-phosphoguanine island methylator phenotype), MSI (microsatellite instability) and the serrated pathway of CRC (Fearon 2011, Markowitz 2009, Nagasaka et al. 2004, Stefanius et al. 2011, Weisenberger et al. 2006).

NOTCH

The Notch pathway is highly conserved, with homologs in species ranging from worms through Man (Andersson et al. 2011). The Notch signaling pathway in humans consists of four receptors, Notch-1, -2, -3, -4 and at least five ligands, Jagged-1, Jagged-2, Delta-1, Delta-3 and Delta-4 (Mumm & Kopan 2000). In the canonical Notch pathway, ligand interaction with receptor results in a cascade of proteolytic cleavages mediated first by a metalloprotease and second by a γ-secretase activity. These cleavage steps result in release of a constitutively active intracytoplasmic Notch (ICN) fragment that is then translocated to the nucleus, where it associates with CBF-1 and MAML-1 as part of a larger transcription complex (Kovall 2008). The net effect of ICN is to switch transcriptional complexes of CBF-1 from repression to activation (Lai 2002). Notch signaling is terminated by CDK8-mediated phosphorylation of a PEST domain on the ICN. This then targets ICN for proteosomal degredation and allows the cells to be responsive to new Notch signals (Mumm & Kopan 2011, Andersson et al. 2011, Kovall 2008).

The role of Notch signaling in normal intestinal development has been well-documented and is the subject of several excellent reviews (Geissler & Zach 2012, Medema & Vermeulen 2011, Vaiopoulos et al. 2012, Fre et al. 2011, Vooijs et al. 2011, Miyamoto & Rosenberg 2011). In the colon, Notch signalling is involved in the control of stem cells and transit amplifying cell division (Fre et al. 2005, Van Es et al. 2005). Lateral inhibition in this cell-to-cell signaling pathway means that adjacent cells are driven towards different fates, resulting in a caotic distribution of progenitor cells committed to enterocyte and secretory lineages (Noah & Shroyer 2013).

Transforming growth factor-β signaling

The later genetic step thought to be involved in a fraction of CRCs is the inactivation of the TGF-β signaling pathway (Lampropoulos et al. 2012, Markowitz 2009). TGF-β signaling cascades are normally involved in many cellular processes such as cell growth, differentiation, apoptosis, and migration, triggered by the binding of the TGF-β superfamily ligands (in carcinogenesis TGF-β1) to the type II receptor (TGFBR2; transforming growth factor, beta receptor II) in the cell membrane. The binding to TGFBR2 recruits and phosphorylates a type I receptor (TGFBR1) which further phosphorylates receptor-regulated SMADs (mothers against decapentaplegic homolog), SMAD2 and SMAD3, and triggers a complex formation with SMAD4, which then accumulates in the cell nucleus (where SMAD2 and SMAD3 can also enter in a SMAD4-independent fashion). In the cell nucleus, SMADs act as transcription factors participating in the regulation of target gene expression, engaged in an ambiguous role as both tumor suppressor and cancer promoter, inducing among others, p21, a cyclin-dependent kinase inhibitor, leading to growth arrest (Lampropoulos et al. 2012).

In CRC tumorigenesis, TGF-β and its signaling effectors have been reported to influence cancer biological behavior (disease progression, the degree of differentiation of primary tumor, metastasis and recurrence), as well as to affect multiple components of the human immune system, thus playing a role in carcinogenesis through immune suppression (Lampropoulos et al. 2012).

The most common mechanism, resulting in the alteration of the TGF-β signaling pathway, is the mutation of TGFBR2, detected in about one third of all CRCs (Lampropoulos et al. 2012, Markowitz 2009). In sporadic colon cancer, loss of phosphorylation of SMAD1, SMAD5 and SMAD8 has been observed in 70% of cancers (Kodach et al. 2008). Contrary to SMAD4, SMAD2 mutation occurs more often in the early stages of tumor development and is specifically associated with sporadic cancers (Lampropoulos et al. 2012).

Bone morphogenetic protein

Bone morphogenetic proteinS (BMPs), first identified for their role in controlling bone formation, are members of the TGF β superfamily (Massagué 1998). BMPs bind to the BMP receptors I (BMPRI) or II (BMPRII). BMP binding to BMPRII results in phosphorylation of BMPRI, which subsequently phosphorylates SMAD1, SMAD5 and SMAD8. These then associate with SMAD4, resulting in activation and nuclear localization (Schmierer & Hill 2007).

BMP signalling has a pivotal role in intestinal development and is required for the control of intestinal stem cell replication. It is also needed for terminal differentiation of mature intestinal cells (Auclair et al. 2007). BMP ligands are secreted from both epithelial and mesenchymal cells but act mainly on the epithelial compartment through epithelial cell expression of BMP receptors (Hardwick et al. 2004). BMP signaling is active in the differentiated compartment, and despite the presence of BMP protein, it is relatively inactive in

early compartments in the base of the crypt due to the presence of the BMP inhibitor Noggin (Hardwick et al. 2008, Kosinski et al. 2007).

In colon cancer, mutations in SMAD4 or BMPRI have been shown to be responsible for juvenile polyposis (Hardwick et al. 2008). Loss of SMAD4 or loss or BMPRII is the likely mechanistic basis for loss of BMP signaling in sporadic colon cancers. However, because studies have indicated that loss of BMP signaling in sporadic colon cancers correlates with tumor grade, it is likely that this is not an initiating event (as it is in juvenile polyposis), but rather contributes to tumor progression (Hardwick et al. 2008). There is increasing evidence in sporadic colon cancers (as compared with JP) that mutations affecting BMP signaling corroborate with activated WNT to drive colon cancers, particularly in later stages (Hardwick et al. 2008).

Hedgehog

The Hedgehog (HH) pathway derives its unusual name from the phenotype of hedgehog loss in Drosophila; larvae take on a curled, bristly appearance that may remind some of a hedgehog (Geissler & Zach 2012). In humans, there are three HH proteins, Sonic HH, Indian HH and Desert HH.

HH is synthesized as a 45 kDa precursor that is self-cleaved into C- and N-terminal peptides. The role of the C-terminal peptide is unknown, but the N terminal forms the active HH ligand (Taipale & Beachy 2001). HH can bind to its receptor, Patched, which then de-represses the membrane-bound protein Smoothened (Smo). This cause the activation and release of Gli transcription factors that can translocate to the nucleus. Vertebrates have three Gli proteins. Gli1 will result in activation of HH target genes, while Gli3 is a repressor of signaling. Gli2 serves a dual role, with both repressive and activator functions (Geissler & Zach 2012).

Genes regulated by HH signaling include Myc, Bcl-2 and the Notch ligand, Jagged2. Also induced by HH signaling are the stem cell-associated proteins LGR5, CD133 and CD44, as well as transcription factors that regulate epithelial to mesenchymal transition (EMT) such as Snail, Slug and Twist (Katoh & Katoh 2006).

Indian hedgehog is the main HH protein expressed in the intestine and is secreted in a paracrine manner by differentiated epithelial cells to act on mesenchymal cells. It maintains homeostasis of mesenchymal cells and regulates epithelial cell proliferation through negative feedback to proliferating crypt base columnar cells by increasing BMP signaling (Buller et al. 2012).

Mutations that result in activation of HH signaling are the driver mutations in basal cell carcinomas, for which there are now targeted therapies (Weiss & Korn 2012). Evidence from mouse models indicates that HH may cooperate with activated WNT to drive lethality in colon cells (Varnat et al. 2010). This suggests that HH inhibitors may be an interesting target to consider in colon cancer. HH has moved more to the forefront of a potential targeted therapy in cancer (Tang et al. 2012).

P53 signaling

Another key genetic step in CRC tumorigenesis is the inactivation of the P53 pathway as a result of the mutation of tumor suppressor gene TP53 (Fearon 2011, Markowitz 2009). This gene was already discovered in the late 1970s and has been reported to be mutated in about half of almost all cancers (Johnson et al. 1993).

P53 protein is encoded by the TP53 gene. It is a key transcriptional regulator of genes responsible for the encoding of proteins that function in the cell-cycle checkpoints, restrict angiogenesis and promote apoptosis.

In normal situation, wild-type P53 arrests or slows down the cell cycle in G1/S phase and addresses the DNA damage requiring repair to caretaker genes when mutations or replication errors occur. Furthermore, when the damage is too extensive, P53 may induce apoptosis (Johnson et al. 1993).

In most of the somatic CRCs, both alleles of TP53 are inactivated by a combination of a missense mutation inactivating the transcriptional activity and a chromosomal deletion of a 17p eliminating the second TP53 allele; this occurs typically in the later phase of the tumorigenesis (often with the transition of large adenoma into carcinoma). However, in CRCs with MMR defects, TP53 often remains wild-type (Markowitz 2009). ATM (ataxia telangiectasia mutated), a kinase that phosphorylates and activates P53 after DNA damage, is another altered gene in the P53 pathway, which is found mutated in a trend toward mutual exclusivity with TP53 in CRC (The Cancer Genome Atlas Network 2012).

1.3.2.3 Communication between pathways

There is an increasing body of evidence from a variety of tissues that these developmental pathways exhibit cross-talk or share molecular points (nodes) of intersection (Geissler & Zach 2012, Lin &Hankenson 2011). In addition to cross-talk, these various developmental pathways can also have an impact on cell signaling pathways such as PI3K/Akt and Ras/Raf/Mek/Erk (Ruizi 2011, Beck & Carethers 2007, Beck et al. 2007, Chappell et al. 2011).

WNT signals can control Gli3 from the HH pathway (Alvarez-Medina et al. 2008). HH can antagonize WNT signaling in the colon (Van den Brink et al. 2004, Watt 2004). Likewise, HH has been reported to control the expression of the Notch ligand Jagged2, whereas WNT/β-catenin can control Jagged1 (Estrach et al. 2006, Chen et al. 2010). Hes-1 can be activated by both Notch and HH signaling (Wall & Wallace 2009, Wall et al. 2009, Sang et al. 2010).

BMP and WNT appear to be interconnected via the PI3k/Akt pathway (Tian et al. 2005). TGF β/Smad signaling promotes EMT through WNT, Ras, HH and Notch (Fuxe et al. 2010). Thus, there is interplay between these pathways, and alterations in one could have potential effects on others. Other work has implicated interactions between PTEN/PI3K/Akt signaling and BMP in colon cancers (Beck & Carethers 2007, Chen et al. 2011).

Hedgehog and Ras have been reported to be interconnected in colon cancer (Mazumdar et al. 2011). Connections between HH and p53 have also been proposed, further illustrating the complex interconnectivity between signaling pathways (Ho & Alman 2010, Efstratiadis et al. 2007).

1.3.2.4 Genomic instability

The spontaneous mutation rate in somatic cells is not sufficient to account for the mutational load observed in many human tumors. The loss of genomic stability could explain this discrepancy and the rapid acquisition of new tumor-associated mutations needed for cancer development during a patient's lifetime (Loeb 1991). Subsequent studies confirmed the crucial role of genomic instability for carcinogenesis, which may be acquired by three pathways: chromosomal instability (CIN), MSI, and CIMP (Markowitz 2009, Worthley & Leggett 2010).

Chromosomal instability

In CRC, chromosomal instability (CIN) is the most common type of genomic instability (~70–85% of sporadic CRCs), which causes changes, either gains or losses, of whole or large portions of chromosomes (Bogaert & Prenen 2014, Markowitz 2009). As a result of unequal distribution of DNA, the daughter cells fail to gain the same number or similarly structured chromosomes in the cell

division, leading to karyotypic variability from cell to cell. As a consequence of CIN, an imbalance in chromosome number (aneuploidy), chromosomal rearrangements, and frequent loss of heterozygosity (LOH), inactivating the functioning allele of a tumor suppressor gene, are seen (Bogaert & Prenen 2014, Michor et al. 2004).

Whole-genome sequencing of CRC samples has revealed that the chromosome regions affected by arm-level changes include gains of 1q, 7p and q, 8p and q, 12q, 13q, 19q, and 20p and q, and losses of 18p and q (including SMAD4 in 66% of the tumors), 17p and q (including TP53 in 56% of tumors), 1p, 4q, 5q, 8p, 14q, 15q, 20p, and 22q (The Cancer Genome Atlas Network 2012). The karyotypic abnormalities observed in CIN coupled with the specific mutations of oncogenes and tumor suppressor genes (e.g. APC, CTNNB1, KRAS, BRAF, PIK3CA, SMAD4, TP53) lead to the activation of the previously presented signaling pathways critical for CRC initiation and progression (Bogaert & Prenen 2014). The factors underlying CIN in CRC are poorly defined, but recently published studies have suggested that defects in genes regulating formation of mitotic spindle and proper alignment and segregation of chromosomes at mitosis may be involved. The inactivation of APC may also have some effect in CIN (Fearon 2011, Pino & Chung 2010). CRCs characterized by CIN have been reported to have poor prognosis regardless of tumor stage or therapy, and more commonly favor the distal colon (Kim & Kim 2014).

Microsatellite instability

Another important pathway for genomic instability is caused by the dysfunction of DNA MMR genes and is known as the MSI pathway (Mäkinen 2007). It was first described in Lynch syndrome (LS) patients (formerly known as hereditary non-polyposis colon cancer; HNPCC), with a germ-line mutation in MMR genes, at the beginning of the 1990s (Peltomäki 2005).

Microsatellites are repetitive nucleotide sequence motifs 1–6 base pairs in length within the genome that are prone to mismatch errors during the DNA replication as a result of impaired MMR. If the errors are not corrected, the daughter DNA may gain a different number of sequence repeats within a microsatellite in each replication cycle, resulting in MSI (Bogaert & Prenen 2014, Markowitz 2009).

In sporadic CRCs, the inactivation of MMR gene, typically MLH1, mostly occurs due to the biallelic silencing of the gene expression, as a result of promoter hypermethylation. This phenomenon represents an epigenetic change in tumor development. De novo germline mutations or somatic mutations in MMR genes are infrequent in sporadic MSI-H cancers (Fearon 2011). Overall, MSI-H can be detected in about 15% of all CRCs, while the germ-line mutation, representing the inherited background of CRC in the known MMR genes, is encountered in only ~3% of CRC patients (Fearon 2011, Markowitz 2009).

Several clinicopathological features separate sporadic MSI-H CRCs from non-MSI tumors. In sporadic setting, MSI is often associated with BRAF mutation and high-level CIMP (CIMP-H) and accompanied by histology of serrated pathway neoplasms (Mäkinen 2014, Stefanius et al. 2011). These tumors also typically locate in the proximal colon and they tend to occur in elderly women (Mäkinen 2007, Poynter et al. 2008).

In both inherited and sporadic background of MSI-H CRCs, typical histological features include poor differentiation with mucinous or signet ring cell appearance, tumor-infiltrating lymphocytes and peritumoral Crohn-like infiltrate (Boland & Goel 2010).

While the MSI-H tumors seem to form a distinct clinicopathological phenotype, the concept of MSI-L in CRC is controversial (Pawlik et al. 2004). The biological basis of the MSI-L phenotype is uncertain, as no alterations in MMR genes MLH1, MSH2, MSH6, or PMS2 have been reliably associated with this phenotype. Conversely, the

methylation of the DNA repair gene MGMT has been found to be the most frequent in a fraction of sporadic CRCs with MSI-L and to be associated with the serrated pathway adenomas and SACs. Thus, it has been suggested that an increased production of DNA mismatches due to the loss of expression of MGMT could stress the DNA MMR system and thus lead to the MSI-L phenotype (Jass 2007, Mäkinen 2007).

Epigenetic alterations

Epigenetic alterations modify the transcriptional potential of a gene without changes in the DNA sequence (Bonasio et al. 2010). DNA methylation is the best-known epigenetic phenomenon and is currently considered the most important in CRC pathogenesis (Esteller 2008).

In sporadic CRC, both loss of global DNA methylation (i.e., hypomethylation) and an increase of methylation in the promoter areas of selected CpG islands (i.e., hypermethylation) are present (Fearon 2011, Issa 2004). CIMP was introduced as a pathway of CRC tumorigenesis in 1999 (Toyota et al. 1999). During evolution, most of the CpG dinucleotides (short areas of cytosine nucleotide followed by a guanine nucleotide) were lost in the genome. However, approximately 50% of all genes still contain these dinucleotides (i.e., CpG islands) as dense clusters in their promoter areas (Fearon 2011). The aberrant addition of methyl groups (CH_3) to CpG sites in the promoter region has been associated with inappropriate transcriptional silencing of the genes regulated by these promoters. Deregulation of gene expression of certain key tumor suppressor genes has been reported to enhance tumorigenesis in several other tumor types besides CRC, such as gastric, liver, pancreatic, endometrial, ovarian, breast and lung cancers (Esteller 2008, Issa 2004).

In sporadic CRCs, the hypermethylation of tumor suppressor gene MLH1 is the major cause of MSI and an important pathway to genomic instability (Leggett & Whitehall 2010). Approximately a third of all CRCs and nearly all MSI-H tumors harbor CIMP. However, conversely, CIMP is not limited to this tumor type, whereas approximately half of all CIMP tumors do not carry MLH1 methylation or MSI (Leggett & Whitehall 2010). CIMP can already be detected in SPs, especially in proximal SSAs (Leggett & Whitehall 2010).

CRCs characterized by CIMP have been reported to differ by clinicopathological characteristics (i.e., by age, sex and location predilection, histology and prognosis) from non-CIMP tumors (e.g. CRCs deriving by traditional adenoma-carcinoma pathway). Typically, they tend to be proximal tumors in older individuals that show mucinous histology, thus sharing many features with MSI tumors (Leggett & Whitehall 2010).

Furthermore, CIMP tumors have been associated with poor prognosis compared with non-CIMP tumors, especially when MSS (Barault et al. 2008, Dahlin et al. 2010, Issa 2004) and they have been reported to have an independent predictive effect on response to chemotherapy treatment (i.e., 5-fluorouracil (5-FU)) (Iacopetta et al. 2008, Jover et al. 2011, Rijnsoever et al. 2003).

1.3.3 Pathways to colorectal cancer

CRC is defined by the invasion of tumor cells through muscularis mucosae to submucosa (Hamilton et al. 2010). The majority of CRC is sporadic. The differences in the incidence between countries around the world (Siegel et al. 2013) as well as immigrant studies (Dunn 1975, Kune et al. 1986, Shimizu et al. 1987) suggest that environmental factors contribute to the development of CRC (Bradbury et al. 2014, Koushik et al. 2007, Larsson & Wolk 2006, Schwingshackl & Hoffmann 2014).

Molecular and morphological developmental pathways have been described to understand the heterogeneity and complexity of CRC development from normal colonic epithelium to adenoma, and further, to adenocarcinoma. Three main pathways based on the developmental events during their progression can be recognized: inherited, sporadic and IBD-associated pathways (Beaugerie & Itzkowitz 2015, Fearon 2011).

1.3.3.1 Inherited pathways

10%–50% of all CRC are hereditary or familial clustering in etiology and they consist of rare conditions known to predispose to development of cancer (Tops et al. 2009). Early-onset diagnosis and multiple affected relatives (either with CRC or with adenomas particularly under 50 years of age) are closely related to the increased risk of CRC. Furthermore, it seems that the familial risk of CRC is greater if relatives have colon rather than rectal cancers, supporting the suggestion of a slightly different etiological basis behind these two diseases (Johns & Houlston 2001).

The most common CRC syndrome, Lynch syndrome (LS) is responsible for 1–3% of all CRC cases (Lynch & de la Chapelle 2003). Another well-described inherited syndrome, familial adenomatous polyposis (FAP), accounts approximately 1% of all CRCs, followed by recently defined MUTYH or MYH (mutY homolog) gene associated polyposis (MAP) (~1%), serrated polyposis syndrome (SPS), hereditary mixed polyposis syndrome (HMPS), and rare hamartomatous polyposis syndrome (Tops et al. 2009, van Herwaarden et al. 2015).

1.3.3.2 Sporadic pathways

The vast majority of CRCs (50%–90% of all CRCs) arise in patients without a family history of CRC and these cancers are termed

"sporadic". As a difference from inherited cancers, a germline mutation as an initiating event is absent and the development of CRC is presumed to occur as a result of somatic changes, which are *per se* more susceptible for environmental factors (Markowitz 2009).

Earlier, the vast majority of CRCs were presumed to develop through a relatively linear sequence of steps known as the Vogelstein adenoma-carcinoma sequence: the suppressor pathway characterized by CIN and initiated with a mutation of the APC tumor suppressor gene (Fearon & Vogelstein 1990, Muto et al. 1975). Later, the discovery of the SPs and the subsequent studies confirming their status as the precursor lesions of a subset of CRCs led to the introduction of a distinct, alternative developmental pathway currently known as the serrated pathway (Jass et al. 2002).

Based on molecular studies, it is estimated that approximately 80% of the CRCs develop along the conventional pathway, which still serves a relevant model for the most common form of sporadic CRC (Jass 2007), whereas up to 20% of all CRCs arise along the serrated pathway (Mäkinen 2014, Snover 2011).

The classical adenoma-carcinoma sequence

Approximately 70-80% of sporadic CRC arise from the 'conventional' adenoma-carcinoma pathway. In the classical genetic model for colorectal tumorigenesis described by Fearon and Vogelstein the evolution of colorectal cancer follows the adenoma-adenocarcinoma sequence which is driven by the progressive accumulation of a number of critical mutations (Fearon & Vogelstein 1990).

The pathogenesis of this pathway has been well studied and is centered around the accumulation of genetic (Parkin 2001, Grady et al. 2005, Lewis et al. 1999) events in the luminal epithelial cells and is classically associated with the gradual development of CIN.

In this pathway, CRC starts by hyperproliferation in the normal epithelium and aberrant cryptic foci forming small adenoma. Thus the genetic inactivation of the adenomatous polyposis coli (APC) gene causes the development of adenomatous polyps, the principal precursor of colorectal cancer (Fearon & Vogelstein 1990, Pino & Chung 2010). The inactivation of the APC occurs in up to 85% of sporadic CRCs and represents an early and critical, possibly rate-limiting, event in the tumorigenesis of most MSS CRCs, found already in microscopic adenomas (Fearon 2011, Markowitz 2009, Powell et al. 1992). This is followed by the stepwise accumulation of other genetic mutations, such as the KRAS that will increase the size into large polyp and with the combination of TP53 genes mutation and the loss of heterozygosity (LOH) at chromosome 18q, malignant cells will appear forming the colon carcinoma (Fearon & Vogelstein 1990) (Figure 3).

While the inactivating mutation of APC is presumed to be the initiating step of the tumorigenesis, mutations of KRAS and TP53 and LOH at chromosome 18q are required for the progression to larger adenomas and early carcinomas (Fearon & Vogelstein 1990, Pino & Chung 2010). The oncogenic mutation of KRAS mostly occurs in early adenomas (Fearon & Vogelstein 1990) and contributes to the transduction of signaling pathways such as MAPK-ERK and PI3K, as previously presented.

The loss of chromosome 18q has been detected in up to 70% of sporadic CRCs (Pino & Chung 2010). Tumor suppressor genes SMAD2 and SMAD4 locate in this chromosome region and are thus mutated in a proportion of CRCs with 18qLOH, contributing to the activation of the TGF-β signaling pathway (Fearon & Vogelstein 1990).

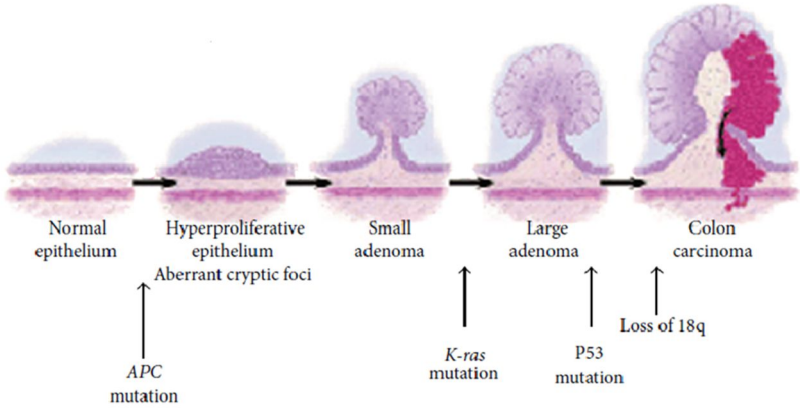


Figure 3. CRC development. From Sandouk *et al.* 2013.

After forming carcinoma, there are five stages for the disease (Figure 4): stage 0 where the tumor locates in the mucosal layer of colon, stage I when it reaches the muscularis layer, stage II when it just perforates the serosa, stage III when the surrounding lymph nodes are involved, and lastly stage IV with distal metastasis (Sandouk et al. 2013).

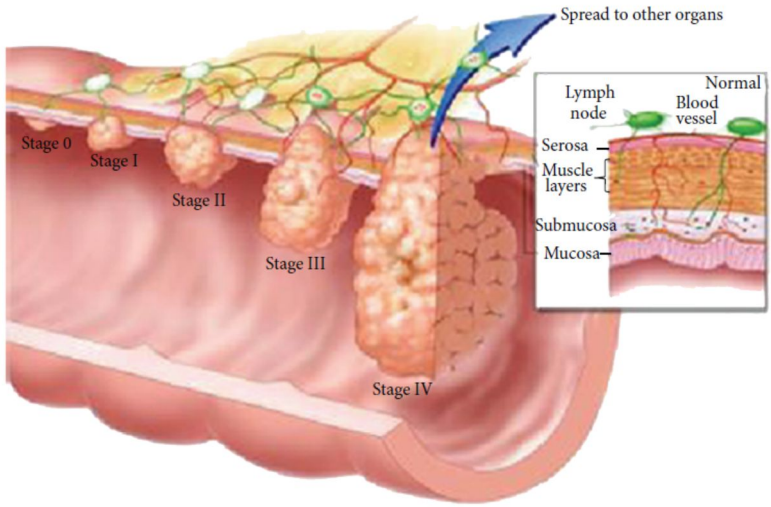


Figure 4. **Stages of CRC** . From Sandouk *et al.* 2013.

The serrated pathway

Approximately 20% of the sporadic CRCs arise from the SPs via the serrated neoplasia pathway.

Until the past two decades, almost all colorectal polyps were divided into hyperplastic (serrated polyps), adenomas (TA, TVA, VA) and mixed polyps. Evidence that adenomas might not represent the only colorectal cancer precursor began to emerge around 1990 when a new category of serrated polyps, biologically different from hyperplastic polyps, were recognized: traditional serrated adenoma (TSA), sessile serrated adenoma (SSA) and mixed polyp, all of which have malignant potential without the villous architecture of classic adenoma. The three variants of serrated adenomas have subtle architectural differences, but all have carcinogenic potential (Longacre & Fenoglio-Preiser 1990).

In contrast with the adenocarcinoma sequence occurring through chromosomal instability, responsible for progressive accumulation of mutations in oncogenes and tumour-suppressor genes, the serrated neoplastic pathway is characterized by aberrant methylation in promoter regions of specific genes based on hyper-methylation of CpGislands resulting in the "CpG islands methylator phenotype" (CIMP) at either low or high degree which reduces gene expression without altering the DNA sequence. Hyper-methylation may occur in DNA mismatch repair gene (MMR) hMLH-1 associated with the development of microsatellite unstable (MSI) cancer. MSI cancers occurring in the course of the serrated pathway are due to a loss of function within DNA MMR system by promoter hyper-methylation of hMLH-1 only.

The mutations of BRAF or KRAS, considered mutually exclusive, are the earliest events of the serrated route (Bettington et al. 2013, Mäkinen 2014). The mutual exclusivity supports the idea of the two individually branched serrated pathways of CRC. While the pathway involving BRAF is well characterized, the role of KRAS in serrated neoplasia is more controversial (Bettington et al. 2013, Mäkinen

2014). Also DNA hypermethylation occurs early (i.e., in ACF and even in the normal proximal colon mucosa in patients with SPS) but is generally more frequent in proximal polyps than in distal ones. The consequences of the hypermethylation are conformable with the target genes involved (e.g. the promoter methylation of MLH1 and MGMT leading to MSI-H and MSI-L, respectively) (Mäkinen 2007, 2014).

Several other genetic changes, such as loss of p16 due to the promoter methylation of CDKN2A (Dong et al. 2005, Kriegl et al. 2011), TP53 mutation (Bond et al. 2012, Gaiser et al. 2013), APC mutation (Jass et al. 2006), somatic mutations of mitochondrial DNA in TSAs (Shimomura et al. 2011) as well as PTPRK-RSPO3 fusions and RNF43 mutations (Sekine et al. 2016) and allelic imbalance of 18q (Yashiro et al. 2005) have been observed in the serrated pathway lesions, although as yet without adequately characterized significance. Furthermore, gene expression profile studies have revealed several differently expressed genes between SACs and CCs, but many of these have not yet been studied on protein level (Conesa-Zamora et al. 2013, Laiho et al. 2007).

1.3.4. Serrated colorectal cancer

Serrated adenocarcinoma (SAC) is a distinct variant of CRC, arising from the serrated precursor lesions via the previously described serrated pathway and representing approximately 20% of CRCs (Bettington et al. 2013, Mäkinen 2014). The clinicopathological features commonly associated with SAC include female gender (Lash et al. 2010) and proximal tumor location (García-Solano et al. 2010, Tuppurainen et al. 2005).

The median age at the time of diagnosis of SAC does not differ notably from that of CCs (García-Solano et al. 2010, Mäkinen et al. 2001), but a rapid tumor progression along the serrated pathway is supported by several case reports of serrated precursor lesions

developing into a carcinoma (Mäkinen et al. 2002, Oono et al. 2009, Takeyoshi et al. 2002), as well as the studies reporting early SACs arising from small SSAs (Fujita et al. 2011, Goldstein 2006, Sheridan et al. 2006).

1.3.4.1 The molecular classification of serrated colorectal cancer

In 2007, Jass proposed a 5-tiered classification of CRC to clarify the origin and clinicopathological features of CRCs with different molecular backgrounds (Jass 2007). The classification is still widely used, providing a helpful tool for researchers to compare the different subtypes of CRC.

The serrated pathway carcinomas belong to three broad molecular profiles: 1. BRAF mutant/CIMP-H/MSI-H, 2. BRAF mutant/CIMP-H/MSI-L or MSS, and 3. KRAS mutant/CIMP-L/MSI-L or MSS. The first two groups are the most strongly associated with the serrated pathway and thus represent cancers most likely arising from SSAs. They also generally show CIMP-H regardless of the CIMP panel used. The last group of the serrated pathway CRCs are thought to develop from TSAs or even CAs and thus conversely to be less strongly associated with the serrated pathway (Bettington et al. 2013, Jass 2007). Furthermore, two more subgroups: 4. CIN/CIMP-neg./MSS (or MSI-L) and 5. LS/CIMP-neg./MSI-H rapresent CRCs arising from CAs, and complete the classification (Jass 2007).

Although the preceding subtypes roughly divide CRCs morphologically into SACs and CCs, the recognition of each subtype is often impossible based on the morphological features alone and thus, the molecular basis is principal for the classification. The advantage of the classification is the understanding of the heterogeneity of the molecular background of CRCs and the possibility to consider each molecular type of cancer as an individual disease, harboring its own clinical, histological, and prognostic features (Jass 2007).

1.3.5 Malignant neoplasms in colorectum

Adenocarcinomas, with CRCs accounting for more than 90%, are the most common cancers in the colorectum (Hamilton et al. 2010, Kang et al. 2007). The great majority of adenocarcinomas are moderately differentiated, showing a less resemblance to well-differentiated adenoma-like epithelium with tubular structures (Treanor & Quirke 2007).

In addition to two most commonly encountered adenocarcinomas, conventional colorectal carcinomas (CCs) and serrated adenocarcinomas (SACs), several somewhat overlapping histopathological variants can be microscopically distinguished (Hamilton et al. 2010) (Table 3).

Classification	Designating features
Adenocarcinoma, not otherwise specified	Glandular differentiation
Mucinous adenocarcinoma	> 50% of the lesion is composed of extracellular mucin
Signet-ring cell carcinoma	Presence of > 50% of tumor cells with prominent intracytoplasmic mucin
Serrated adenocarcinoma	Epithelial serrations, low nucleus-to-cytoplasm ratio, clear or eosinophilic cytoplasm
Micropapillary adenocarcinoma	Tumor cells growing in papillary structures, which lack fibrovascular cores
Medullary carcinoma	Sheets of malignant cells with vesicular nuclei, prominent nucleoli,and abundant eosinophilic cytoplasm; prominent infiltration by intraepithelial lymphocytes
Adenosquamous carcinoma	Areas of glandular and squamous differentiation
Undifferentiated carcinoma	Lack of morphological, immunohistochemical, and molecular biology evidence of differentiation beyond that of an epithelial tumor

Table 3. Histopathological subtypes of colorectal carcinoma. Classification and designating features adapted from Hamilton *et al.* 2010.

1.3.6 Molecular basis and sub-classification of CRC

Recent molecular classification of established tumors based on gene expression and (epi)genetic mutation burden, has revealed considerable disease heterogeneity. Four distinct CRC molecular subtypes (CMS) have been identified, each with a unique pathogenic molecular pathway, response to treatment and prognosis (Dienstmann et. al. 2014).

Established tumor molecular classification subtypes can be partially reconciled with known precursor lesion subtypes. Canonical (CMS2) tumors are driven by disruption of epithelial Wnt signaling through accumulation of genetic mutations and chromosomal instability and arise from tubular and tubulovillous adenomas (conventional adenoma-carcinoma sequence). Poor prognosis mesenchymal (CMS4) molecular signatures are defined predominantly by stromal invasion and angiogenesis and prominent TGFβ activation (Calon et al. 2015, Isella et al. 2015). This subtype has the worst prognosis. These tumors are thought to arise through the serrated neoplasia pathway (De Sousa et al. 2013).

1.3.7 Invasion and metastasis

CRC is defined by the invasion of tumor cells through muscularis mucosae to submucosa although this has more to do with practical issues rather than distinguishing between different biological entities (Hamilton et al. 2010).

The patterns of tumor cell invasion can be classified into individual-cell migration, multicellular migration and expansive growth without migration, which can be further divided into subcategories (Lauffenburger & Horwitz 1996, Ridley et al. 2003). The migration mechanisms of an individual cell are similar to those occurring in normal non-neoplastic cells in physiological conditions, including cell polarization and protrusion, adhesion formation, actinand myosin-based contraction and rear detachment (Lauffenburger &

Horwitz 1996, Ridley et al. 2003). Different patterns of invasion are guided by the expression of cell-matrix adhesion molecules (e.g., integrins), cell-cell adhesion molecules (e.g., cadherins), matrix-degrading enzymes (e.g., MMPs) and cell-cell communication molecules (e.g., chemokines) (Friedl et al. 2012).

Each tumor frequently presents with multiple patterns of invasion (Friedl et al. 2012). About one in four CRCs shows infiltrative tumor border configuration, characterized by finger-like protrusions of the invasive front and representing collective cell migration as strands, while the rest show a rather expansive tumor border configuration (Jass et al. 1996). At high magnification, tumor buds, defined as isolated tumor cells or clusters of two to four cells at the invasive margin of the tumor, can be observed in the majority of CRCs (Hase et al.1993, Ueno et al. 2002) and cytoplasmic pseudofragments, i.e., dendritic processes of the budding cells, are present in half of the patients with highgrade budding, (Shinto et al. 2005). Tumor budding is considered to represent weakening of cell-cell adhesions and it often includes individual cell migration (Natalwala et al. 2008). Accordingly, it has been associated with decreased expression of the cell adhesion molecule E-cadherin (Zlobec et al. 2007).

CRC commonly uses lymphatic vessels (Minsky et al. 1989) and blood vessels (Krasna et al. 1988) as routes of metastasis. The epithelial to mesenchymal transition (EMT) and single cell migration may enhance the efficacy of metastasis (Christiansen & Rajasekaran 2006). However, clusters of circulating tumor cells can be observed in CRC (Molnar et al. 2001) and other carcinomas including lung cancer (Hou et al. 2011), suggesting that collective vascular invasion may also take place. The phenotype of circulating tumor cells may influence the site of metastasis, as proposed by a human colon cancer xenograft mouse model that reported CD110+ cells being more likely to form liver metastases and CUB domain-containing protein 1 expressing cells being more likely to form lung metastases (Gao et al. 2013).

1.3.8 Intratumoral hetereogeneity

Intratumor heterogeneity is a phenomenon characterized by regions and cells with diverse genetic and epigenetic changes, morphology, and behavior within a single tumor and its metastases (Almendro et al. 2013). CRC has been shown to present with heterogeneity within the primary tumors and between primary tumors and metastases in, e.g., activating mutations of KRAS (Baldus et al. 2010). Intratumor heterogeneity may represent a challenge for personalized medicine and biomarker development.Accumulating evidence suggests that not all tumor cells possess equal ability to proliferate.

1.3.9 Colorectal cancer screening and treatment

Colorectal cancer is an ideal disease for population screening as it is common, there is an effective surveillance tool (endoscopy), a well recognised premalignant precursor lesion (the colorectal polyp) and treatment of the premalignant condition reduces the risk of cancer (Leslie et al. 2002).

The aim of the CRC screening is to identify the early stages of cancers which might still be treated with a curative intent and thus have a favorable prognosis (Jellema et al. 2010). The colonoscopy has been validated as the gold standard procedure for both early detection and prevention of CRC as it allows the inspection of the entire colonic mucosa. It also allows resecting pre-neoplastic lesions, which reduces the incidence of colorectal cancer and thus the rate of deaths resulting from it (Zauber et al. 2012).

However, at the time of diagnosis, approximately 15–25% of the CRC patients have metastases outside the bowel, most typically in the liver and another 35–45% of patients will later develop metastases (Poston et al. 2005). Surgery is the primary modality of treatment for CRC, and resection is the only therapy required for early-stage CRC (Nelson et al. 2001). While most of the patients (approximately 80%) with metastatic disease are unresectable,

approximately 2–5% of all CRC patients have one or a few coincident metastases in one organ, staged as having oligometastatic disease potentially treatable in a curative fashion (Van De Velde et al. 2014).

The development of more effective chemotherapeutic agents capable of inducing substantial tumor shrinkage have enabled a significant proportion of patients who were initially thought to be unresectable for cure to undergo metastectomy (Kanas et al. 2012).

1.4 The tumor microenvironment

In cancer research the cancer cell itself is most frequently the object of interest. The majority of human cancers are carcinomas that, by definition, arise from epithelial cells that line glands, ducts, and surfaces of organs (Landis et al. 1998). Consequently, the focus of research to date has been on epithelial cells, or more specifically genetic changes that occur in epithelial cells as they progress from normal to malignant. Multiple genetic alterations are necessary for this transformation to occur (Foulds 1969). It has become clear that a cancer not only consists of neoplastic cells but also contains a stromal infrastructure, including tumor vasculature, which is provided by the host. In fact, a neoplasm constitutes a unique microenvironment in which various subpopulations of tumor cells and tumor stroma interact and together determine the behavior of the neoplasm. A fascinating aspect of the stromal compartment of a tumor is that it appears to be not only a passive scaffold or an inert supply system for cellular nutrients but an active regulatory element. It is in a way responsible for the existence of the tumor: without host stroma there would be no cancer (Bosma et al. 1993).

Over 100 years ago, Paget et al. already proposed the importance of the tumor microenvironment with the theory of “seed & soil” (Paget 1989). It has become increasingly apparent that the stroma plays an

important role in promoting tumour progression (Coussens & Werb 2002, Liotta & Kohn 2001, De Wever et al. 2008).

1.4.1 Characteristics of tumor microenvironment

The tumor microenvironment is composed by both cellular and non-cellular components. The major cellular components include fibroblasts, endothelial and immune cells that, collectively, produce the variety of molecules that represent the non-cellular components of the tumor stroma: i.e. the extracellular matrix (ECM) proteins, proteases, cytokines and growth factors (Hanahan & Weinberg 2000, Matrisian et al. 2001). All these components are functionally organized to promote survival of cancer cells in the host and generate a favorable microenvironment for cancer cells in both primary and metastatic sites (Liotta & Kohn 2001).

Moreover, tumor stroma formation shares many important properties with wound healing, but wound healing is usually self-limited while the growth of tumors and tumor stroma is not. In fact, tumor stroma has been compared to a 'wound that does not heal' (Dvorak 1986, Eyden 2008).

The molecular features of cancer stroma are less well understood than those of cancer cells, thus, in order to control and eradicate cancer, it is very important to take in consideration not only malignant cancer cells, but also the benign stromal cells.

1.4.2 Fibroblasts

Fibroblasts were first described in the late 19th century, based on their location and their microscopic appearance (Virchow 1858, Duvall 1879). They play a critical role in maintaining homeostasis in the microenvironment and in coordinating the complex physiological response to wounds (Martin 1997, Iyer et al. 1999).

Fibroblats are embedded within the fibrillar matrix of the connective tissue and are, to a large extent, responsible for its synthesis. The important functions of fibroblasts include the deposition of extracellular matrix (ECM), regulation of epithelial differentiation, regulation of inflammation and involvement in wound healing (Tomasek et al. 2002, Parsonage et al. 2005). They also contribute to the formation of basement membranes by secreting type IV collagen and laminin (Chang et al. 2002). Fibroblasts are also an important source of ECM-degrading proteases such as matrix metalloproteinases (MMPs), which highlights their crucial role in maintaining an ECM homeostasis by regulating ECM turnover (Chang et al. 2002, Simian et al. 2001).

In addition, fibroblasts are important in maintaining the homeostasis of adjacent epithelia through the secretion of growth factors and direct mesenchymal–epithelial cell interactions (Wiseman & Werb 2002). Activated fibroblasts also have an important role as modulators of the immune response following tissue injury, through the secretion of cytokines such as interleukin-1 and chemokines such as monocyte chemotactic protein 1 (MCP1) (Strieter et al. 1989, Rollins et al. 1989).

Heterogeneity and origins of CAFs

Fibroblasts of the tumor stroma are called activated fibroblasts, peri-tumoral fibroblasts, myofibroblasts, tumor-associated fibroblasts or CAFs. They are the main cellular constituents of stroma associated with primary and metastatic CRC (Herrera et al. 2013, Mueller et al. 2007). Fibroblasts in cancer tissues are similar in morphology to myofibroblasts, which are large spindle-shaped cells that are activated during the wound healing process (De Wever et al. 2008). During the wound healing process, fibroblasts became “activated fibroblasts”, express α-smooth-muscle actin, leading to the term ‘myofibroblasts’(Kalluri & Zeisberg 2006) and actively close the wound by contraction (Garana et al. 1992). During wound

healing, when the process is completed, activated fibroblasts decrease (Tomasek et al. 2002). In contrast, CAFs are perpetually activated and neither revert to a normal phenotype nor undergoes apoptosis and elimination like normal fibroblasts (Li et al. 2007). The continued presence of myofibroblasts within a wound may be associated with fibrous neoplasms called fibromatoses (Fletcher 2000), fibrotic disease (Desmouliere et al. 2005) and a predisposition to cancer (Chang et al. 2004). In addition, epithelial tumors of a number of organs, including breast, are often surrounded by an activated stroma characterized by myofibroblasts that can promote tumorigenesis (Van den Hooff 1988, Olumi et al. 1999, Tlsty 2001, Tlsty & Hein 2001, Bissell et al. 2002, Coussens & Werb 2002, Beacham & Cukierman 2005, Orimo et al. 2005).

It is becoming evident that CAFs origin can vary both between different tumor hystotypes and within different areas of individual tumors. It is possible that CAFs are derived from several cell types and are therefore heterogenous (Sugimoto et al. 2006). There are several theories regarding the origins of CAFs, and this topic is still under debate. They can roughly classify the line of evidence about CAFs origin in: i) resident; ii) mesenchymal stem cell (MSC)-derived; iii) mutational (Cirri & Chiarugi 2011). For example, resident tissue fibroblasts, bone marrow-derived mesenchymal stem cells, hematopoietic stem cells, epithelial cells (epithelial-mesenchymal transition; EMT) and endothelial cells (endothelial-mesenchymal transition; EndMT) are all considered possible predecessors of CAFs. The transdifferentiation of CAFs, a process commonly called mesenchymal-mesenchymal transition (MMT) (Kalluri & Zeisberg 2006), is currently poorly understood. TGF-β1 has been largely acknowledged to be one of the major tumor-cell derived factors affecting CAF activation (Lohr et al. 2001).

Tumor-Promoting Characteristics of CAFs

Fibroblats contribute to tumor proliferation, invasion, and metastasis via secretion of various growth factors, cytokines, chemokines, and degradation of extracellular matrix (ECM) proteins (Yamamura et al. 2015). Colon CAFs secrete epidermal growth factor (EGF), hepatocyte growth factor (HGF), IGF1/2, PGE-2, PDGF, fibroblast growth factor (FGF)-1 and vascular endothelial growth factor (VEGF) (Nakagawa et al. 2004, Peddareddigari et al. 2010, De Boeck et al. 2013, Torres et al. 2013). These growth factors act through activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways, which mediate cell proliferation and cell survival (anti-apoptotic signaling), protein synthesis, cytoskeletal rearrangements, and invasion (Valenciano et al. 2012).

Colon CAFs produce significant amounts of IL-6 and CRC cells further enhance IL-6 production by CAFs. IL-6 is a multifunctional cytokine that plays a central role in the regulation of inflammatory and immune responses, but it is also characterized as an angiogenic cytokine. CAFs play a crucial role for angiogenesis through secretion of various cytokines (Goh et al. 2007). VEGF that is induced by IL-6 and several other factors (FGF, PDGF, and SDF-1) promotes angiogenesis. IL-6 was suggested to stimulate VEGF secretion by the mediation of PGE-2 from CAFs. Secreted VEGF from fibroblasts targets endothelial cells and is known as one of the most important angiogenic factors (Nagasaki et al. 2014). CAF-derived TGF-β and connective tissue growth factor (CTGF) leads to proliferation through the SMAD2/SMAD4 pathway (Nakagawa et al. 2004, Peddareddigari et al. 2010, De Boeck et al. 2013).

CAFs are also able to secrete plasminogen activators as well as several members of the MMP family. These enzymes may be exploited essentially for two purposes: 1) direct degradation of ECM, obviously associated with tumor expansion, invasion and angiogenesis, 2) cleavage of growth factors, pro-inflammatory

cytokines and their receptors, commonly associated with their activation, or cleavage of cell adhesion molecules, leading to increase motility and epithelial –mesenchymal transition (EMT) (Hynes 2009, Roy et al. 2009).

The role of CAFs in tumor progression is multifaceted (Mueller & Fusenig 2004). Similarly to immune cells, which initially repress malignant growth, CAFs inhibit early stages of tumor progression, mainly through the formation of gap junctions between activated fibroblasts. Conversely, later on CAFs become activated by several tumor secreted factors and promote both tumor growth and progression (Nakagawa et al. 2004) (Figure 5).

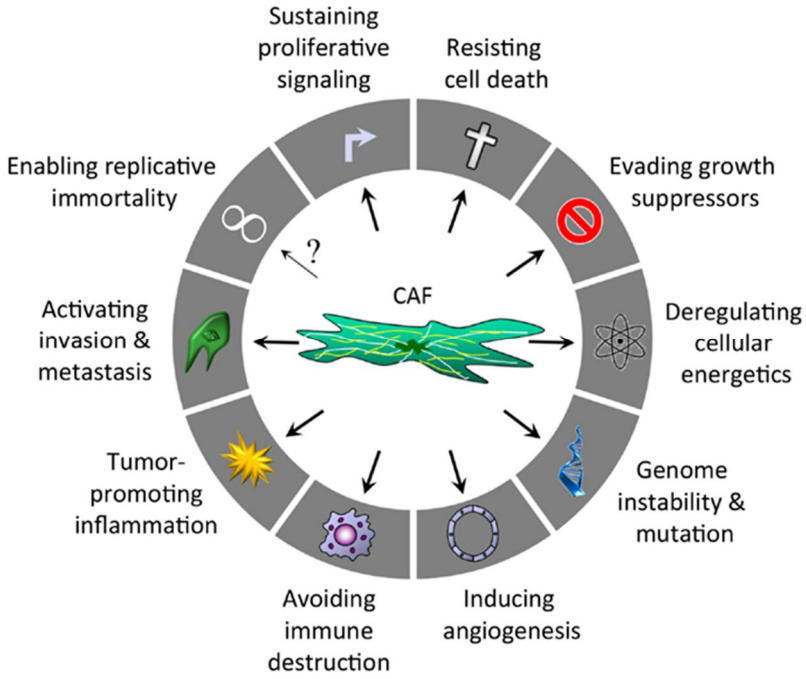


Figure 5. Hallmarks of cancer regulated by CAFs. From Hanahan & Weinberg 2011.

Two closely interactive pathways are established in the crosstalk between cancer and stromal cells: a) in the "efferent" pathway, cancer cells trigger a reactive response in the stroma, and b) in the "afferent" pathway, the modified stromal cells in the surrounding microenvironment affect cancer cell responses (De WO & Mareel 2003, Giannoni et al. 2010).

CAFs support cancer stem cells (CSCs) that are maintained in a quiescent state and are resistant to chemotherapy and radiation (Li & Bhatia 2011). In a recent study, Vermeulen and colleagues described a novel link between CRC stem cells and myofibroblasts, showing that myofibroblast-derived HGF activated CRC Wnt signalling and restored the stem cell phenotype in more differentiated cells (Vermeulen et al. 2010). These data suggest that the microenvironment is a critical regulator of the stem cell niche.

Many clinical and experimental data also support the notion that fibroblasts play crucial roles in immune responses through production of cytokines and chemokines (Bucala et al. 1991). Furthermore, fibroblasts not only mediate the quality but also the quantity of the immune response (Parsonage et al. 2003). In normal physiology, fibroblasts can terminate immune responses by withdrawing survival signals and normalize the chemokine gradients which accelerate the apoptosis or withdraw the tissue through the lymphatic vessels (Buckley et al. 2001). Pro-inflammatory cytokines are secreted by cancer cells and CAFs attract excessive immune cells to the cancer region. Macrophages, neutrophils and lymphocytes could be recruited to the tumor stroma by secreting factors from the CAFs. Macrophages are actively attracted into tumor regions along defined chemotactic gradients and release a number of factors that influence endothelial cell behavior including VEGF, HGF, MMP2 and IL-8. Once macrophages reach the tumor, they start to differentiate into tumor-associated macrophages (TAMs) which further enhance the growth and metastasis of cancer cells (Leek & Harris 2002).

Stromal cells are the main source of thrombospondin-1(TSP-1) which has both positive and negative effects on angiogenesis and interaction with immune cells (Li et al. 2007). As mentioned previously, CAFs excessively secrete MMPs which degrade basement membrane and cleaved products of MMPs such as fibronectin and collagen (Brundula et al. 2002).

In colorectal cancer, CAFs-derived conditioned medium and exosomes promoted clonogenicity and tumor growth of cancer stem cells (CSCs) upon treatment with 5-fluorouracil or oxaliplatin (Hu et al. 2015). Multiple CAF-derived factors sustain proliferative signaling in CRC cells and support the cancer cells to resist cell death (Kalluri & Zeisberg 2006, Tlsty 2001, Liotta & Kohn 2001) and evade growth suppressors. Consequently, it is suggested that CSCs are correlated with recurrence and metastasis of cancer. Chemotherapy-treated CAFs maintain cancer-initiating cells (CICs) and their drug resistance through secretion of IL-17A (Lotti et al. 2013).

Nonetheless, the mechanisms underlying the effects of CAFs on cancer progression are still unclear. Thus, elucidation of these mechanisms is likely to lead to new anticancer treatments targeting CAFs and the cancer-stroma interaction (Shiga et al. 2015).

1.4.3 CAFs markers

To date, fibroblasts have been difficult to positively identify. In some cases, fibroblasts are identified based on their spindle shape combined with positive staining for the mesenchymal marker vimentin and the absence of staining for epithelial or other mesenchymal cell types, such as muscle cells, astrocytes, or hematopoietic cells (Chang et al. 2002). However, this approach is hardly definitive. Fibroblasts can take on a wide array of shapes in different tissues, whereas vimentin-positive cells that are not fibroblasts, including macrophages can also have a spindle-shaped

appearance. Furthermore, vimentin stains a large number of cell types, making it difficult to identify fibroblasts by elimination (Goodpaster et al. 2008).

The most widely used marker for CAFs is α-smooth muscle actin (α-SMA). Upon tissue damage, fibroblasts proliferate and differentiate into myofibroblasts. These myofibroblasts acquire de novo expressed α-SMA, contractile stress fibers, and the ED-A splice variant of fibronectin (Tomasek et al. 2002, Serini et al. 1998). As there are more myofibroblasts in the tumor stroma, α-SMA is widely used as a CAF marker (Orimo et al. 2007, Sappino et al. 1988). α-SMA has been demonstrated not to label CAFs exclusively, but also smooth- muscle cells in the muscularis mucosae and muscularis propria (Herrera et al. 2013, Harper & Sainson 2014).

Another useful marker for CAFs is fibroblast activation protein (FAP) (Park et al. 1999, Kraman et al. 2010). FAP appears to be expressed on pericytes and CAFs (Harper & Sainson 2014). High intratumoral expression of FAP is associated with poor prognosis in colorectal cancer (Wikberg et al. 2013). Nonetheless, the tissue distribution and function of FAP-α are not restricted to stromal fibroblasts: its expression is detectable in epithelial malignant cells (Mentlein et al. 2011, Dohi et al. 2009).

Several other markers have also been reported in previous studies, such as tenascin-C (Yoshida et al. 2015), periostin (Kikuchi et al. 2008), neuron glial antigen-2 (NG2) (Sugimoto et al. 2006), vimentin, desmin, platelet derived growth factor receptor-α and β (PDGFR α and β) and fibroblast specific protein-1 (FSP-1) (Sugimoto et al. 2006). Quiescent resident fibroblasts express vimentin, instead of a-SMA, as intermediate filament proteins (Tsujino et al. 2007). These markers are not necessarily specific for myofibroblasts. Individually, these markers could identify specific subpopulations of fibroblasts, thus it would be more correct to use a combination of markers to select the largest possible population of CAFs (Herrera et al. 2013).

On the other hand, cytokeratin and CD31 are considered negative markers, as CAFs do not have epithelial and endothelial characteristics (Xing et al. 2010, Sukowati et al. 2015).

An exclusive marker for CAFs that can clearly distinguish them from normal fibroblasts from adjacent mucosa or other closely related cell types is yet to be identified.

2. AIMS OF THE STUDY

It has been shown that in cancer, stromal gene dysregulation contributes more to poor prognostic molecular signatures than the epithelium itself (Calon et al. 2015, Isella et al. 2015), supporting the fact that the tumor stroma influence cancer epithelial cell behavior.

Thus, the focus of this thesis has been to try to understand the role of the stroma in CRC initiation and progression. In order to do that:

- DEG in the epithelial and stromal compartment of two CRC human precancerous lesions, have been studied;

- DEG in the epithelial and stromal compartment of two CRC mouse models of precancerous lesions have been analyzed;

- primary human fibroblast cells from normal colonic tissue, adenomas and CRC patients have been isolated and characterized;

- transwells and 3D co culture technics have been used to assess the influence of normal and neoplasia associated fibroblasts on epithelial cell behaviour/expression and *vice versa*.

3. MATERIALS AND METHODS

In order to characterize the transcriptome of the epithelial and stromal compartment of two CRC human precancerous lesions, SSA and TVA, a cohort of these polyps has been collected.

3.1 Human Normal and Polyps (TVAs, SSAs) sample collection for EDTA separation

Human polyp tissues samples (TVAs, SSAs) were obtained from patients undergoing colonoscopy at John Radcliffe Hospital, Oxford with local REC approval (REC 10/H0604/72) (Figure 6). Written informed consent was obtained from all donors.

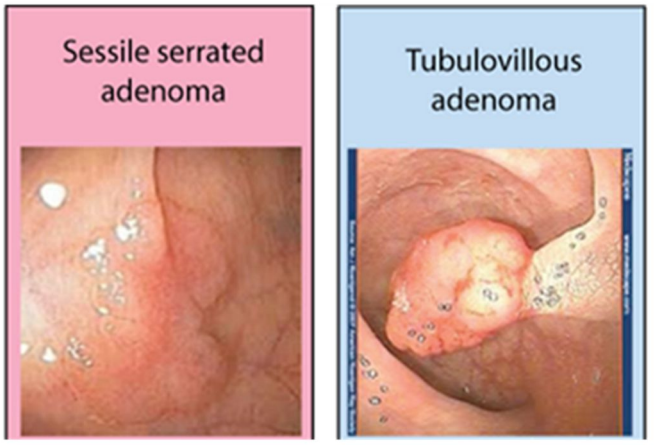


Figure. 6. Representative TVA and SSA before endoscopic resection.

A cohort of SSAs (n=24) and TVAs (n=24) with neighboring normal tissue (n=24) was collected.

1mm^3 biopsies were collected to be analyzed and the rest of the lesion has been processed for histological assessment thus clinical pathological assessment of the lesions will be unaffected.

Table 4 shows the characteristics of the samples collected for this study.

Patient	Organ	Region	Tissue Status	
1	Colon	Rectum	Normal	Polyp (SSA)
2	Colon	Transverse	Normal	Polyp (SSA)
3	Colon	Ascending	Normal	Polyp (SSA)
4	Colon	Ascending	Normal	Polyp (SSA)
5	Colon	Rectum	Normal	Polyp (SSA)
6	Colon	Ascending	Normal	Polyp (SSA)
7	Colon	Caecum	Normal	Polyp (SSA)
8	Colon	Ascending	Normal	Polyp (SSA)
9	Colon	Ascending	Normal	Polyp (SSA)
10	Colon	Ascending	Normal	Polyp (SSA)
11	Colon	Caecum	Normal	Polyp (SSA)

12	Colon	SplenicFlexure Tranverse	Normal	Polyp (SSA)
13	Colon	Ascending	Normal	Polyp (SSA)
14	Colon	Caecum	Normal	Polyp (SSA)
15	Colon	Caecum	Normal	Polyp (SSA)
16	Colon	Transverse	Normal	Polyp (SSA)
17	Colon	Rectum	Normal	Polyp (SSA)
18	Colon	Ascending	Normal	Polyp (SSA)
19	Colon	Rectum	Normal	Polyp (SSA)
20	Colon	Ascending	Normal	Polyp (SSA)
21	Colon	Caecum	Normal	Polyp (SSA)
22	Colon	Ascending	Normal	Polyp (SSA)
23	Colon	Caecum	Normal	Polyp (SSA)
24	Colon	Caecum	Normal	Polyp (SSA)
25	Colon	Caecum	Normal	TubVilAdenoma
26	Colon	Transverse	Normal	TubVilAdenoma
27	Colon	Rectum	Normal	TubVilAdenoma
28	Colon	Hepatic Flexure	Normal	TubVilAdenoma

29	Colon	Rectum	Normal	TubVilAdenoma
30	Colon	Hepatic Flexure	Normal	TubVilAdenoma
31	Colon	Caecum	Normal	TubVilAdenoma
32	Colon	Caecum	Normal	TubVilAdenoma
33	Colon	Transverse	Normal	TubVilAdenoma
34	Colon	Caecum	Normal	TubVilAdenoma
35	Colon	Rectum	Normal	TubVilAdenoma
36	Colon	Hepatic Flexure	Normal	TubVilAdenoma
37	Colon	Rectum	Normal	TubVilAdenoma
38	Colon	Hepatic Flexure	Normal	TubVilAdenoma
39	Colon	Caecum	Normal	TubVilAdenoma
40	Colon	Hepatic Flexure	Normal	TubVilAdenoma
41	Colon	Caecum	Normal	TubVilAdenoma
42	Colon	Caecum	Normal	TubVilAdenoma
43	Colon	Transverse	Normal	TubVilAdenoma
44	Colon	Caecum	Normal	TubVilAdenoma
45	Colon	Rectum	Normal	TubVilAdenoma

46	Colon	Hepatic Flexure	Normal	TubVilAdenoma
47	Colon	Rectum	Normal	TubVilAdenoma
48	Colon	Hepatic Flexure	Normal	TubVilAdenoma

Table 4. Cohort of collected SSAs and TVAs with neighboring normal tissue.

3.2 Mouse sample collection for EDTA separation

The same analysis was performed in mice models of adenomas to see if the results obtained in human adenomas were reproducible in mice models.

3.2.1 Mouse procedures

All procedures were carried out in accordance to Home Office UK regulations and the Animals (Scientific Procedures) Act 1986. All mice were housed at the animal unit at Functional Genomics Facility, Wellcome Trust Centre for Human Genetics, Oxford University. All strains used in this study were maintained on C57Bl/6J background for ≥ 6 generations.

3.2.2 Inducible transgene model Cre

The most popular method for inducible transgene expression is the Cre-ER^{T2} system that was established in the 1990s (Nagy 2000). In this technic, mice carrying a Cre transgene (under the control of an inducible tissue specific promoter) are crossed to mice bearing an inducible allele where the region that is to be deleted is flanked by LoxP recombination sites (Jackstadt & Sansom 2016). This can be either an essential exon(s) of a gene, to produce a conditional

knockout, or a Stop motif that activate an oncogene within adult tissue (Sansom et al. 2006, Jackson et al. 2001).

In this study, the Cre-ER^{T2} fusion protein has been expressed under the control of the Villin promoter (Beuling et al. 2011, El Marjou et al. 2004) and the Lgr5 promoter (Barker et al. 2007). While Cre-ER^{T2} has proven very effective at inducing precisely timed deletion of floxed alleles in the intestine when linked to these promoters, the liability of this approach is that it requires injection of a potent estrogenic compound (tamoxifen) (Hayashi & McMahon 2002).

For this study, inducible Cre C57bl/6j mice were treated with 1 mg tamoxifen by intra peritoneal injection for five days to introduce Apc$^{fl/fl}$ and Braf^{V600E} mutations specifically into adult mouse epithelium.

Thus, VillinCreER^{T2}; Apc$^{fl/fl}$ and VillinCreER^{T2}; Braf^{V600E} mice models were used to reproduce the molecular etiology, pathology and clinical progression of the human TVAs and SSAs precursors lesions respectively. As described above the epithelium and the stroma were separated, isolated and the RNA was extracted for the genes expression analysis.

3.3 Gene expression analysis

3.3.1 Individual crypt and villus isolation, RNA extraction

Human and mouse individual crypts and villus were isolated following Leedham et al. protocol (Leedham et al. 2013). Briefly, biopsies were washed with PBS and incubated in 5 ml dissociation media (30 mM EDTA in DMEM without Ca2+ and Mg2+, 0.5 mM DTT, 2% RNA later (Life Technologies) for 10 min at room temperature.

Immersion in culture medium containing EDTA allows separation of epithelium from the underlying stroma.

The digested tissue was then transferred to PBS and shaken vigorously for 30 s to release individual crypts and villi. Individual structures were selected using a drawn out glass pipette under a dissection microscope and transferred to RLT buffer ready for subsequent RNA extraction with the RNeasy microkit (Qiagen) according to manufacturer's instructions. RNAs were treated with DNase I (Sigma) to degrade residual DNA (Figure 7). The purity and concentration of the RNA samples were determined using NanoDrop ND-1000.

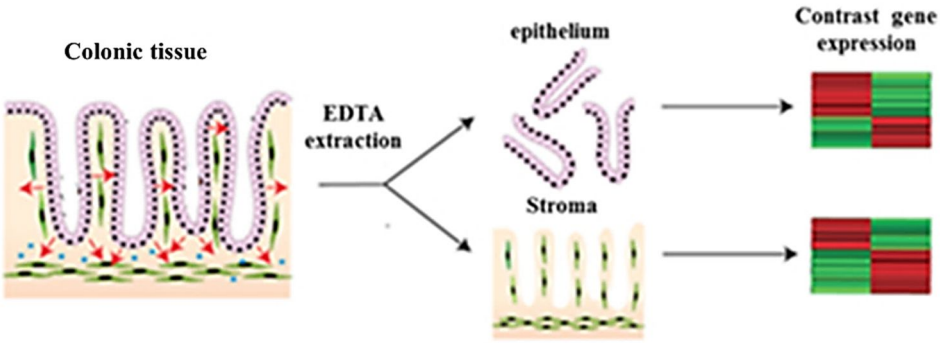


Figure 7. Epithelial and stromal separation from colonic tissue for gene expression profiling.

3.3.2 Gene expression arrays

Gene expression RNA from each sample was checked for quality on the Agilent 2100 Bioanalyzer. Linear RNA amplification was carried out using TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre, Illumina). 20-100ng of total RNA from each sample was passed onto WTCHG Oxford Genomics Centre where hybridization on human Illumina Whole-Genome Gene Expression Beadchips. These platforms allow to capture a broad range of gene expression changes and detect changes in novel transcripts between the experimental samples and the controls. Raw data from Illumina gene expression arrays were processed after removing outlier samples

from initial quality control using the VSN (variance-stabilisation and normalisation) algorithm. A filter was applied by taking a detection score of > 0.95 of the background intensity distribution for all samples to consider a probe detectable.

3.3.3 Bioinformatic analyses

Following, the bioinformatic analyses data normalization were performed and were carried out using the Linear Models for Microarray Data (Limma) by Bioconductor (available at: www.bioconductor.org/packages/2.3/bioc/html/limma.html).
Differentially expressed genes were identified using Student's t-test by running "ttest2" command in MATLAB®. Four lists of differentially expressed gene (DEG) were generated for the comparsonsof the RNA expressed in the experimental groups with the controls groups.

Gene Set Enrichment Analysis (GSEA) was performed using Kolmogorov-Smirnov statistics and gene shuffling permutations. Pathway enrichment analyses using DAVID Bioinformatics Resource 6.7 (NIH) (Huang et al. 2009) and Ingenuity Pathway Analysis (www.ingenuity.com) were used to analyze the gene expression data. Genes were ranked by computing their differential expression in the experimental versus normal samples by the Student's t-test method. If multiple probes were present for a gene, probe with the highest absolute differential expression between experimental and normal was selected. Gene shuffling with 1,000 permutations to compute the P-value for the enrichment score was used.

3.4 Human Normal, Polyps and CRC sample collection for fibroblasts isolation

The results got from the characterization of the epithelial and stromal compartment in human and mouse adenomas led to the study of the stroma major cell component, the fibroblasts.

In order to do that, fresh surgical human tissue samples were obtained with informed consent from different adults who underwent surgical resection for colon cancer (CMS 2 and CMS 4) at the Churchill Hospital. The normal colonic mucosa, the central part and the leading edge (LE) of the tumor where collected from all the patients. Biopsies selected from the same specimens were distal from the outer margin of the cancers masses.

Polyp tissues samples (TVAs, SSAs) were obtained from patients during routine endoscopy by at the John Radcliffe Hospital. The samples are collected from either male or female patients and used without regard to sex. Written informed consent was obtained from all donors. The tumor characteristics of all the patients are listed in Table 5.

Patient	Sex	Age (years)	Sample Type	Polyp/ Tumor site
1	Male	71	Normal	Ascending
2	Male	65	Normal	Transverse
3	Male	75	Normal	Descending
4	Female	65	Normal, Central, Leading Edge	Recto-sigmoid junction
5	Male	79	Normal, Central, Leading Edge	Cecum Right hemicolectomy
6	Male	77	Normal	Ascending
7	Male	74	Normal, Central, Leading Edge	Right hemicolectomy Hepatic flexure
8	Female	65	Normal	Descending
9	Female	34	Normal, SSA	Sigmoid

10	Male	67	Normal, TVA	Ascending
11	Female	36	Normal, SSA 1, 2	Ascending
12	Male	66	Normal, TVA	Sigmoid
13	Female	80	Normal, Central, Leading Edge	Left hemicolectomy Sigmoid
14	Female	67	Normal, SSA	Splenic
15	Female	80	Normal, TVA	Transverse
16	Male	84	Normal, Central, Leading Edge	Cecum Pole- Right hemicolectomy
17	Male	61	Normal, TVA (40 mm)	Recto-sigmoid junction
18	Female	79	Normal, Central, Leading Edge	Right hemicolectomy Hepatic flexure
19	Male	61	Normal, Central, Leading Edge	Left hemicolectomy Sigmoid
20	Female	83	Normal, TVA	Ascending
21	Female	56	Normal, SSA	Ascending
22	Male	79	Normal, Central, Leading Edge	Sigmoid
23	Male	71	Normal, TVA (90 mm)	Descending
24	Female	64	Normal, SSA (10 mm)	Descending
25	Male	73	Normal, Central, Leading Edge	Cecum Right hemicolectomy
26	Male	36	Normal, SSA 1, 2, 3	Transverse
27	Male	72	Normal, TVA (4 cm)	Ascending
28	Male	56	Normal, TVA	Ascending
29	Male	76	Normal, TVA	Sigmoid
30	Female	67	Normal, SSA	Hepatic Flexure
31	Female	69	Normal, SSA (30 mm)	Sigmoid
32	Female	38	Normal, SSA (25 mm)	Transverse
33	Male	70	Normal, TVA (10 mm)	Transverse
34	Male	68	Normal, TVA (4 cm)	Sigmoid
35[1]	Female	65	Normal, TVA (2 cm)	Rectum
36	Female	87	Normal, SSA (31 mm)	Sigmoid

37[2]	Male	24	Normal, TVA (10mm)	Sigmoid
38	Male	90	Normal, TVA (40mm)	Rectum
39	Female	51	Normal, TVA (12mm)	Sigmoid
40	Female	58	Normal, SSA 1 (25 mm), SSA 2 (18 mm)	Transverse
41	Female	71	Normal, TVA (30 mm)	Ascending
42	Male	85	Normal, SSA (25 mm)	Sigmoid
43	Female	65	Normal, TVA (20 mm)	Sigmoid
44	Male	72	Normal, TVA (35 mm)	Recto-Sigmoid Junction
45	Female	51	Normal, SSA (20 mm)	Sigmoid
46[3]	Male	74	Normal, SSA (12 mm)	Hepatic Flexure
47	Male	36	Normal, SSA (13 mm)	Ascending
48	Female	87	Normal, TVA (35mm)	Rectum
49	Male	78	Normal, SSA (15 mm)	Transverse
50	Female	68	Normal, TVA (23 mm)	Sigmoid

Table 5. Characteristics of patients and patient tumours. [1]Adenocarcinoma of the sigmoid and liver methastasis; [2]More than 300 polyps; [3]Resection of lung methastasis and CRC in 2012.

3.4.1 Isolation and culture of primary fibroblastic population

During colonoscopy and surgery, tissue specimens were taken and rapidly dipped into sterile tubes containing 5 mL of medium composed by DMEM supplemented with 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Sigma), Neomycin solution 0.05 mg/ml (Sigma), 1% Fungizone: gibco® amphotericin B 2.5 ug/ml (Gibco), Gentamycin 0.04 mg/ml (Sigma), Ciprofloxacin 10 ug/ml (Sigma) during the transport from the endoscopy room to the cell culture laboratory (approximately 25 minutes).

At the laboratory, biopsy samples were gently washed in phosphate buffered saline (PBS) for several times to remove blood and moved

into a tissue culture dish (60 × 15 mm) and finely chopped into small pieces (1-2 mm) with a disposable surgery knife for approximately 5 minutes; samples were incubated in DMEM (serum free, 1% penicillin/streptomycin (Sigma) containing collagenase type IV (225 units/ml; Sigma) at 37˚C for 3 hours in a shaker.

Then, the digested tissue was centrifugated (1000 × g for 5 minutes) and resuspended in DMEM supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (Sigma), Neomycin solution 0.05 mg/ml (Sigma), 1% Fungizone: gibco® amphotericin B 2.5 ug/ml (Gibco), Gentamycin 0.04 mg/ml (Sigma), Ciprofloxacin 10 ug/ml (Sigma) and 10% fetal bovine serum (Sigma) that stopped the enzymatic activity of the collagenase.

The obtained tissue pieces and floating cells were seeded onto the cell culture Petri dishes (35 × 10 mm) in 2 mL of medium composed by DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Sigma), MEM Non-essential Amino Acid Solution (Sigma), 1% penicillin/streptomycin (Sigma), Neomycin solution 0.05 mg/ml (Sigma), 1% Fungizone: gibco® amphotericin B 2.5 ug/ml (Gibco), Gentamycin 0.04 mg/ml (Sigma), Ciprofloxacin 10 ug/ml (Sigma). The fibroblast cultures were established and maintained at 37°C in primaria plates (Corning).

As previously described for other types of fibroblast (Cristofalo & Pignolo 1993, Cristofalo et al. 1998, Montalto et al. 1999, Pourreyron et al. 2003), colon fibroblasts undergo a phase of senescence after an initial phase of growth. Normal human mitotic cells do not proliferate indefinitely in culture but undergo a limited number of divisions and progressively reach a state of irreversible growth arrest, a process termed replicative senescence caused by reactive oxygen species which are known to apply a genotoxic stress and induce senescence (Nair et al. 2015).

For these reasons, I cultured them in a humidified atmosphere containing 5% CO_2 and 3% O_2. In this way senescence was delayed and they could be kept in culture until cell passage number 18 (P18).

The medium was replaced after 6 days and the Ciprofloxacin was removed from the media after 21 days when the cells were tested for mycoplasma contamination. All the cells treated as described above were mycoplasma free.

When confluent, stromal cells were harvested using 0.1% Trypsin-EDTA (Sigma Aldrich) and split at ratios of 1:3–1:5. Stromal cultures were expanded using primaria T75 flasks and all the different isolated fibroblasts were frozen down and stored in liquid nitrogen for future experiments.

3.4.2 Preparation of fibroblasts conditioned media

Normal, adenomas (SSA, TVA) and CAFs were grown in fibroblasts culture media until they reached a confluency state of approximately 85%. Then, the conditioned media were collected, centrifuged at 400×g for 10 min to remove floating cells and cellular debris, subjected to sterile filtration (pore size: 0.22 mm, Millipore) and protease treated and stored at -80°C until use.

3.4.3 Fibroblasts characterization

3.4.3.1 qRT-PCR

Total RNA from the cultured fibroblasts was extracted to verify the real nature of the fibroblast by performing qRT-PCR for the fibroblasts marker vimentin.

Briefly, the RNA was extracted and treated with DNase I as previously described. Complementary DNA was reverse transcribed in vitro using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). When necessary, pre-amplification of cDNAs was performed prior to qRT-PCR final step. The TaqMan PreAmp (Applied Biosystems) kit was used following manufacturer's instructions. Absolute quantification qRT-PCR was performed in

triplicate using the ABI 7900HT cycler (Applied Biosystems) with GAPDPH/Gapdh serving as an endogenous control.

The primary assumption in analyzing Real time PCR results is that the effect of a gene can be adjusted by subtracting Ct number of target gene from that of the reference gene (ΔCt). The deltaCt for experimental and control can therefore be subject to t-test, which will yield the estimation of ΔΔCt. In all cases the data met the normal distribution assumption of the t-test.

3.4.3.2 Immunocytochemistry

In cell passage 5, the purities of the various fibroblast populations were verified through immunostaining.

For the immunostaining experiments, the cells were cultured overnight on chamber slides, fixed with 4% paraformaldehyde, washed twice in PBS and fixed with 4% formaldehyde in PBS for 20 minutes at room temperature (RT). Fixed cells were blocked with 10% serum for 30 minutes. Slides were incubated with primary antibody for 2 hours: anti-vimentin (1:50, CST), anti-α-smooth muscle actin (α-SMA) (1:1000, Sigma), anti-desmin (1:500, Abcam). Epithelial types were carefully excluded performing anti-human cytokeratin 20 immunocytochemistry (1:200; Abcam) (Table 6). Appropriate secondary antibodies were applied for 1 h at room temperature. Sections were then incubated in ABC (Vector labs) for 30 minutes. The chamber was removed using the supplied tool and DAB solution was applied for 2–5 minutes and development of the colour reaction was monitored microscopically. Slides were counterstained with haematoxylin, dehydrated, cleared and then mounted.

To quantify the percentage of positive cells, the positive cell numbers relative to the total cell numbers (>100 counted cells) were evaluated in 10 independent fields from three different wells of each

fibroblast type. Images were taken with a Digital DS-L1 camera (Nikon).

3.4.4 Identifying fibroblasts in formalin-fixed, paraffin-embedded tissue

In order to localize the fibroblast in the colonic tissue, immunohistochemistry analysis was performed in both human and mouse normal, precancerous and tumor paraffin- embedded tissue.

3.4.4.1 Mouse and human tissue preparation and histology

Wild type (n=3), VillinCreER^{T2}Apc$^{fl/fl}$ (n=3), and VillinCreER^{T2}; Braf^{V600E} (n=3), mice were sacrificed at pre-defined time points or when showing symptoms of intestinal polyps (anaemia, hunching) by cervical dislocation. The intestinal tract was removed immediately and divided into small intestine (proximal/SB1, middle/SB2 and distal/SB3) and colon (Figure 8). The intestines were opened longitudinally, using a gut preparation apparatus, washed in PBS, fixed overnight in 10% neutral buffered formalin (NBF). Specimens of 10% formalin-fixed tissue were embedded in paraffin following standard protocols.

Paraffin-embedded tissues were cut at 4 μm with the microtome and placed on positively charged slides and baked over night (O.N.) at 60°C. Then, deparaffinized and rehydrated through xylenes and graded alcohols to water.

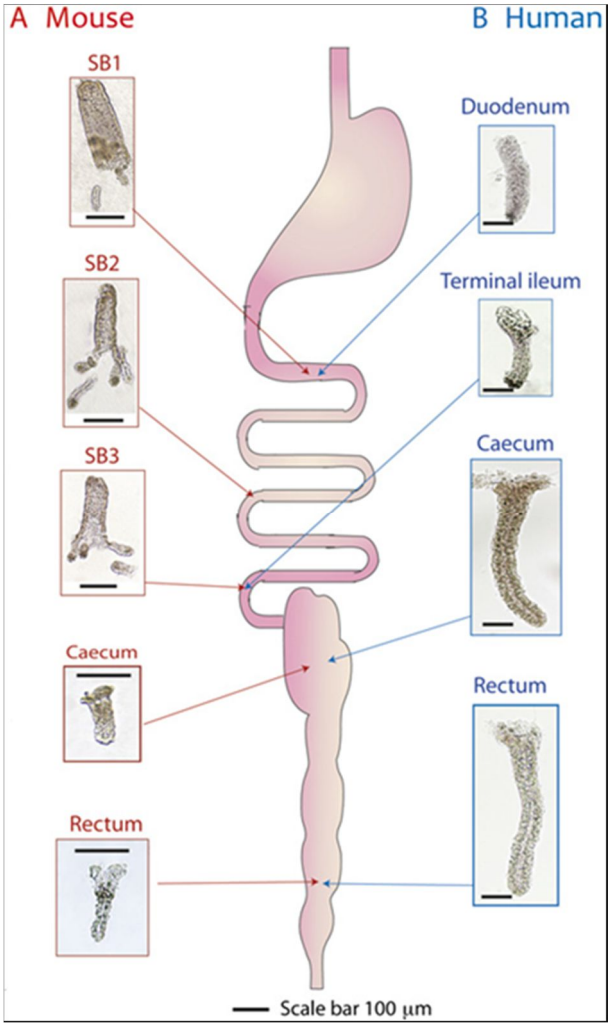


Figure 8. **Correspondences between mouse and human gut**. Adapted from Leedham *et al*. 2013.

Following the same protocol, fresh human colonic normal (n=3), SSA (n=3), TVA (n=3) and CRC (n=3) collected tissue samples were processed for IHC.

3.4.4.2 H&E

On the 4 µm sections hematoxylin-eosin staining was performed using common protocols. Briefly, sections were previously dewaxed

in xylene and rehydrated through graded alcohols to water. Then, each section was treated with the Hematoxylin dye (Merk) that stains of blue-violet nuclei for 30 seconds, washed in running water until clear, followed by a quick dip in acid alcohol and contrasted for 5 minutes in tap water. The sections were treated with eosin (Merck) that stains pink the cytoplasm, for 3 minutes and washed again quickly in tap water, dehydrated, cleared and then mounted.

3.4.4.3 Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (4 μm) were dewaxed in xylene and rehydrated through graded alcohols to water. Endogenous peroxidase was blocked using 1.6% H_2O_2 for 20 minutes. For antigen retrieval, sections were pressure cooked in 10 mmol/L citrate buffer (pH 6.0) for 5 minutes. Sections were blocked with 10% serum for 30 minutes. Slides were incubated with primary antibody for 2 hours.

The following antibodies have been used in this study: anti-vimentin (1:50, CST), anti-α-smooth muscle actin (α-SMA) (1:1000, Sigma), anti-desmin (1:500, Abcam), anti-human cytokeratin 20 (1:200; Abcam), anti-Ki-67 (1 :125, Dako), anti-Alkaline Phosphatase (1:50, Abcam),anti- lysozyme (1:500, Dako) and anti-chromogranin A (1:1.1250, Abcam) (Table 6). Appropriate secondary antibodies were applied for 1 h at room temperature. Sections were then incubated in ABC (Vector labs) for 30 minutes. DAB solution was applied for 2–5 minutes and development of the colour reaction was monitored microscopically. Slides were counterstained with haematoxylin, dehydrated, cleared and then mounted. Images were taken with a Digital DS-L1 camera (Nikon).

Antigen	Antibody type	Clone	Code	Manufacturer	Dilution
Citocheratin 20	Rabbit polyclonal	-	Ab 118574	Abcam	1:200
αSMA	Mouse monoclonal	1A4	A5228	Sigma-Aldrich	1:1000
Desmin	Rabbit polyclonal	-	Ab 15200	Abcam	1:500
Vimentin	Rabbit monoclonal	D21H3	5741	CST	1:50

Table 6. Antibodies used in ICC and IHC.

3.5 *In vitro* organoids co culture experiments

The final aim of the thesis was to try to elucidate the fibroblasts influence on epithelial cells and *vice versa*. For this analysis, different co culture techniques were used.

In particular, co culture is one of culture methodologies that have been performed for the purpose of analyzing epithelial–stromal interactions *in vitro*. Moreover, for this study, I used cell culture inserts with translucent porous membranes that keep the co-cultivated cell populations separated (Figure 9). The membrane material is polyester, which is clear film and can directly examine the cells under the light microscopy. Pore sizes and their density of membrane are 0.4 μm.

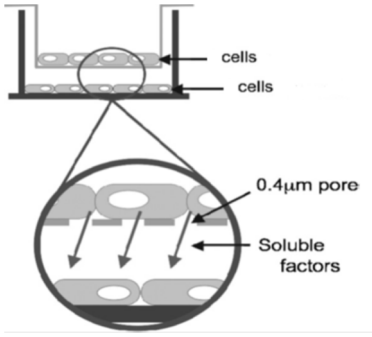


Figure 9. Schema of co-culture system. The membrane separate each cell and allows the cell–cell interactions through the soluble factors cell derived. Adapted from Miki *et al*. 2012.

3.5.1 Small intestinal crypt isolation

The first co culture experiments was performed using small intestinal organoids that display all hallmarks of the small intestinal epithelium in terms of architecture, cell type composition, and self-renewal dynamics.

The protocol used for the isolation of mouse intestinal crypts and organoid culture was adapted from Sato and Clevers (Sato & Clevers 2013) and it is schematically represented in Figure 10.

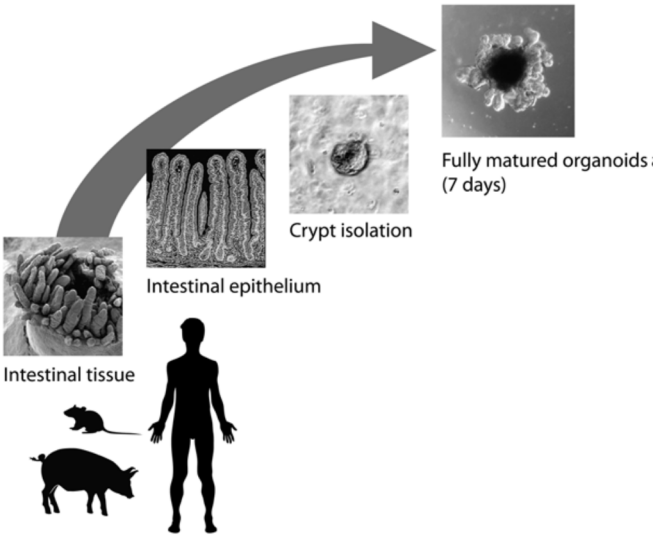


Figure 10. From intestinal epithelial crypt isolation to organoid cultures. From Lukovac *et al.* 2014.

On the day of the experiments, before performing crypt isolation, several aliquots of Matrigel™ Basement Membrane Matrix (BD Biosciences) were thawed on ice and the 24-well plates were pre-incubate in a CO_2 incubator (5 % CO_2, 37°C).

Matrigel™ is a solubilized gelatinous protein mixture and is derived from the Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells. The extract resembles the complex extracellular environment found in many tissues and is commonly used as a substrate for culturing cells. Isolated intestinal cells undergo anoikis outside the normal tissue context (Hofmann et al. 2007) and the matrigel support intestinal epithelial growth (Sato et al. 2011, Sasaki et al. 2002). At room temperature, matrigel polymerizes into a three dimensional structure that is useful for both cell culture and studying cellular processes in three dimensions, including cell migration.

Two C57bl/6j wild type mice were sacrificed and the small intestines were taken (at least 5 cm) and opened longitudinally as previously described. The intestine was washed with ice cold

phosphate-buffered saline (PBS) until most of the luminal contents were cleared and scraped off the villi using a coverslip.

After another washing with ice-cold PBS, the intestine was cut into 2–4 mm pieces with scissors and transferred to a 50-mL tube. 30 mL of ice-cold PBS were added and the fragments were washed by gently pipetting them up and down with a 10 mL pipette. The supernatant was discarded after settling down. This step was repeated for 10 times until the supernatant was almost clear.

Next, 25 mL of ice-cold crypt isolation buffer (2.5 mM EDTA in PBS) were added and the tube was gently rocked at 4 °C for 30 minutes. After settling down the fragments, the supernatant was removed and 10 mL of ice-cold basal culture medium (advanced Dulbecco's modified Eagle medium/F12 (Life Technologies) supplemented with penicillin/streptomycin (Sigma Aldrich), 10 mmol/L HEPES (Sigma Aldrich), Glutamax, 1% N2 (Life Technologies), 10 ml B27 (Life Technologies) and 1 mmol/L N – acetylcysteine (Sigma Aldrich) were added to wash the fragments using a pipette.

When the fragments were settled down, this procedure was repeated in order to release most of the crypts that were also settling down after some time. Villous fractions present in the supernatant were discarded and crypts fractions were passed through a 70-mm cell strainer and collected into 50 mL tube. The crypt fractions was spin down at 800×g for 5 minutes and the pellets were resuspended in 10 mL of ice-cold basal culture medium. The suspension was transferred to a 15 mL Falcon tube and centrifuged at 600×g at 4ºC for 2 minutes to remove single cells (mostly lymphocytes), which end up in the supernatant.

It should be noted that at this point it is difficult to distinguish true crypts from other epithelial debris and the count is only a rough estimate.

3.5.2 *Ex vivo* culture of intestinal crypt organoids

Small intestinal crypts were centrifuged and, as much as possible, supernatant was removed to avoid dilution of Matrigel™ in the next step. The tube kept at 4°C and the crypts pellet was resuspended in Matrigel™ (50 µl basal media/100 µl Matrigel™). 35 µL of the crypt-Matrigel™ suspension were placed into the pre-warmed 24-well plate. The suspension was applied on the center of the transwell so a hemispherical droplet can be formed. The plate was transfer back into the CO_2 incubator (5 % CO_2, 37 °C) as soon as possible after the seeding. After 10 minutes, the Matrigel™ was solidified and 500 µL of complete organoid culture medium was added per well: Epidermal Growth Factor (EGF) (Life Technologies) at 50 ng/ml, R-spondin-1(R&D systems) at 500 ng/ml and Noggin at 100 ng/ml (PeproTech) (Table 7 and Table 8).

Under this culture condition (R-spondin-1, EGF, and Noggin in Matrigel™), small intestinal organoids were established with the purpose to display all hallmarks of the small intestinal epithelium in terms of architecture, cell type composition, and self-renewal dynamics.

	STOCK CONCENTRATION	FINAL CONCENTRATION	FOR 500 ml MEDIA
ADVANCED DMEM/ F12			470~ ml
GLUTAMAX	200 mM	2 mM	5 ml
Pen/Strep	100 X		5 ml
N2	100 X	1X	5 ml
B27	50 X	1X	10 ml
N-ETYLCYSTEINE	500 mM	1 mM	1 ml
HEPES	1M	10 mM	5 ml

Table 7. Basal culture medium for mouse organoid culture.

	STOCK CONCENTRATION	FINAL CONCENTRATION	FOR 500 ml MEDIA
NOGGIN	100 ug/ml	100 ng/ml	1 ul
EGF	50 ug/ml	50 ng/ml	1 ul
R-SPONDIN	100 ug/ ml	500 ng/ml	5 ul

Table 8. Growth factors added to the basal media for mouse organoid culture.

3.5.3 Fibroblasts and small intestine organoids co culture

In order to assess the influence of the fibroblast to the epithelial compartment *in vitro*, the day before the experiment, primary normal (n=3), central (n=3), leading edge (n=3), SSA (n=3) and TVA (n=3) fibroblastic stromal cells (Passage 3-12) were plated at $1x10^5$ cells/well in a 24 well plate (bottom). The cells were allowed to adhere overnight at 37 ◦C and 3% CO_2.

In all the co culture experiments, normal, central and leading edge derived fibroblasts were isolated from the same patients.

The day of the experiment, wild type organoids were isolated as described above and plated on permeable transwells cell culture inserts with 0.4 µm pore size. After few hours, the organoids were moved on the 24 well plate (top) with the fibroblasts. In these conditions, the organoids were grown for 7 days in media supplemented with R-spondin-1 and lacking Noggin and EGF while

the controls were grown with the basal media supplemented with all the growth factors: R-spondin-1, Noggin and EGF (REN) at 37 °C and 5 % CO_2.

The organoid growth and proliferation was monitored on a daily basis and after 7 days of co culture, the organoids were counted and characterized by IHC.

3.5.4 Passaging of organoids

After the intestinal villi had been cultured and had grown into organoids, they were passaged by adding cold media to melt the Matrigel™ and subsequently re-plating in fresh Matrigel™. For passage, the culture medium was replaced with fresh basal culture medium. Organoids and Matrigel™ were mechanically disrupted using a P200 pipette and transferred into a 15 ml falcon tube. Dissociated organoids were washed with 10 ml of basal culture medium and centrifuged at 200 g for 2 minutes. The supernatant was discarded, the pellet resuspended with Matrigel™ and culture medium was added as described above.

3.5.5 Embedding of organoids

To collect material for embedding, the Matrigel™ was melted by adding cold media and then multiple wells were combined. The cells were fixed with 500 µl PFA was 30 min at room temperature, centrifuged at 5000 rpm and resuspended in 150 µl 2% agarose (in PBS). The paraffin embedded cell pellet was then processed and embedded using standard protocols.

3.5.6 Alcian-blue stain for goblet cells

Sections were dewaxed in xylene for 5 minutes, then rehydrated through graded ethanols (100%, 90%, 70%) for 5 minutes each followed by 2 minutes in tap H_20. Slides were then stained in alcian-blue solution (Sigma) for 30 minutes and then washed in running tap H_20 for 2 minutes, before being rinsed in dH_20. Slides were then stained in nuclear fast red solution (Sigma) for 5 minutes and washed in running tap H_20 for 1 minut. Slides were then dehydrated through degraded alcohols for 2–5 minutes each, before mounting a coverslip with DPX. Images were taken with a Digital DS-L1 camera (Nikon).

3.5.7 Organoids immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (4 µm) were dewaxed following the same protocol previously described. Slides were incubated with primary antibody for 2 hours. In particular: anti-Ki-67 (1:125, Dako), anti-Alkaline Phosphatase (1:50, Abcam), anti- lysozyme (1:500, Dako) and anti-chromogranin A (1:1,1250, Abcam) were used for this study (Table 9). Appropriate secondary antibodies and ABC incubation was performed like before.

Antigen	Antibody type	Clone	Code	Manufacturer	Dilution
Ki-67	Rat monoclonal	MIB-5	TEC-3	Dako	1 :125
Alkaline phosphatase	Rabbit polyclonal	-	Ab 65834	Abcam	1:50
Lysozyme	Rabbit monoclonal	A0099	Ec 3.2.1.17	Dako	1:500
Chromogranin A	Rabbit monoclonal	EP1031Y	Ab 15160	Abcam	1:1,1250

Table 9. Antibodies used for organoids IHC.

Moreover, organoids/ spheroids were collected and transferred to RLT buffer ready for subsequent RNA extraction, gene expression arrays and bioinformatics analyses as previously described (Figure 11).

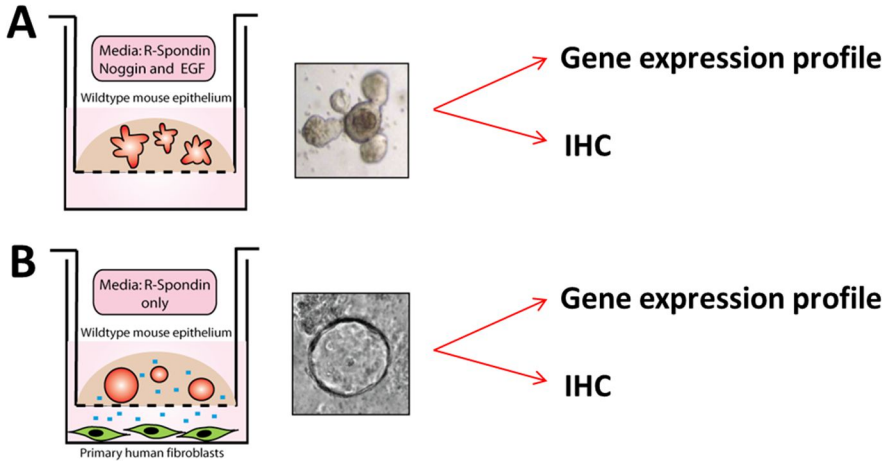


Figure 11. Fibroblasts organoids co-culture. **A** Organoids were seeded on transwells in complete medium (EGF, Noggin, R-Spondin-1) on top of DMEM/10% FBS without fibroblasts. **B**. Organoids were seeded on transwells in medium supplemented with R-Spondin-1, on top of DMEM/10% FBS with $1x10^5$ fibroblasts per well from normal human colon or from adenomas and human CRC (central and leading edge).

3.5.8 Fibroblasts conditioned media and small intestine organoids culture

The influence of the fibroblast on the epithelial compartment was assessed better by culturing organoids with fibroblasts' conditioned media.

Wild type organoids were isolated and plated in 24 well plates for 7 days (5 % CO_2, 37 °C) in normal, central, leading edge, SSA and TVA fibroblasts derivate conditioned medium containing:

1) R-Spondin-1(R);
2) EGF and R-Spondin-1 (RE);
3) EGF, Noggin, R-Spondin-1 (REN).

These 3 different conditions were compared to the controls that were cultured in organoids' medium supplemented with EGF, Noggin, R-Spondin-1. The media were changed every other day and on day 7, the number of organoids and spheroids was counted and pictures collected. Images were taken with a Digital DS-L1 camera (Nikon).

3.6 Human colonic epithelial cell and normal fibroblast co culture

The last co culture experiment was performed in order to look at the influence of normal and mutated epithelial cells to normal stromal cells. In particular, normal and mutated human colonic epithelial cell lines (HCEC) were co cultured with normal fibroblasts as follows.

3.6.1 Human colonic epithelial cell line (HCEC)

Immortalized non-transformed adult human colonic epithelial cell (HCECs) generated by expression of the non oncogenic proteins

cyclin-dependent kinase 4 (Cdk4) and the catalytic component of human telomerase (hTERT) termed 1CT ("C" for CDK4 and "T" for Telomerase) (Roig et al. 2010) were provided by Pr Jerry W Shay.

Moreover, three isogenic cell lines obtained from 1CT cells have been used to model different genetic pathways to CRC (Smith et al. 2002). In particular, 1CT expressing the $KRAS^{V12}$ oncogene (1CT R), a shRNA directed against p53 (1CT P) and $KRAS^{V12}$ oncogene in combination with a shRNA directed against p53 (1CT RP) (Eskiocak et al. 2011) and 1CTA, with a shRNA-mediated downregulation of APC (provided by Dr Laurence Huc) (Graillot et al. 2016).

The human colonic epithelial cell used for this last co cultured experiment are shown in Table 10.

Name	Expressing	Mutations
1CT	CDK_4; hTERT	non-transformed
1CT- R	CDK_4; hTERT	expressing $Kras^{V12}$
1CT- P	CDK_4; hTERT	shRNA directed against TP53
1CT- RP	CDK_4; hTERT	$Kras^{V12}$; TP53
1CTA	CDK_4; hTERT	shRNA-mediated downregulation of APC

Table 10. Nomenclature for the Human colonic epithelial cells (HCECs).

3.6.2 Cell Lines Maintenance

HCECs were maintained on primaria flask in a humidified atmosphere with 3% CO_2 at 37°C, in 4:1 high-glucose Dulbecco modified Eagle medium/ medium 199 supplemented with 2% FBS, epidermal growth factor (EGF 20ng/ml), hydrocortisone (1mg/ml), insulin (10mg/ ml), transferrin(2mg/ml), sodium selenite (5nM) and Gentamycin sulfate (50 µg/ml). In addition, 1CTA cells were selected by puromycin (1 µg/ml).

3.6.3 HCEC co culture

Co-cultures of stromal and epithelial cells were performed using 24 well flat-bottomed plates. Epithelial cell lines were plated at $5x10^4$ cells on the bottom of the wells with their basal media. Normal fibroblasts with their medium were seeded at $5x10^4$ cells on the permeable membrane of the tissue-culture inserts which were introduced into the epithelial cells-containing wells. The medium was changed every other day and the cells were splited when confluent. Co cultures were maintained for 7 days at 37 ◦C and 3% CO_2.

On day 7, cells in the inserts or in the bottom of the wells, were collected and transferred to RLT buffer ready for subsequent RNA extraction, gene expression arrays and bioinformatics analyses as previously described.

4. RESULTS

4.1 DEG analysis

4.1.1 DEG in human TVA and SSA epithelial and stromal compartment

The first goal of this research project was to gain molecular insight into the molecular alterations associated with two key temporal events critical for cancer development and maintenance, specifically that of cancer initiation and cancer progression of the conventional and the serrated pathway.

To address the cancer initiating events, a cohort of SSAs (serrated pathway) and TVAs (conventional pathway) with neighboring normal tissue from patients was collected and the global transcriptomes in the epithelial and stromal compartment were analyzed by microarrays and bioinformatic analyses.

In this study, microarray analysis was employed because allows concurrent measurement of gene expression events on a genome-wide basis.

For the bioinformatic analyses, the data were normalized and four lists of differentially expressed gene (DEG) were generated by comparing SSA epithelial crypt (n=11) RNA expression with adjacent normal crypts (n=16); TVA epithelial GEP (n=6) with corresponding adjacent normal (n=16); SSA stromal GEP (n=7) with normal GEP stroma (n=13); TVA stroma (n=5) with normal counterpart (n=13) (Table 11).

Comparison	Group A	Group B	Sig Genes (FDR<0.1;LOGFC>0.1)
Epi_SSA Versus Normal	Colon_Epithelium_SSA (N=11)	Colon_Epithelium_Normal (N=16)	275
Strom_SSA Versus Normal	Colon_Stroma_SSA (N=7)	Colon_Stroma_Normal (N=13)	104
Epi_TVA Versus Normal	Colon_Epithelium_TVA (N= 6)	Colon_Epithelium_Normal (N=16)	337
Strom_TVA Versus Normal	Colon_Stroma_TVA (N=5)	Colon_Stroma_Normal (N=13)	26

Table 11. Different comparisons and groups used for DEG profile.

Human serrated and tubulovillous polyps show marked variability in the differentially expressed genes in both the epithelial and stromal compartments (Figure 12).

The heatmap showed no overlap between the stromal gene expression profiles of the different polyp types, indicating that different epithelial (epi)mutations provoke variable stromal gene dysregulation in different polyp subtypes (Figure 12A).

Venn diagrams show that stromal gene expression varies considerably between the different polyp subtypes with a comparatively greater number of differentially expressed genes (DEG) in serrated lesion stroma. In fact, DEG in the SSAs were 274 in the epithelium and 103 in the stroma with just 1 gene shared by both tissues (Figure 12B) suggesting adequate separation of epithelial and stromal component. Moreover, the number of genes that were identified as differentially expressed in the epithelial compartment was 284 for the TVA and 222 for the SSA with an overlap of 53 genes (Figure 12C).

In Figure 12D, the DEG in the TVAs were 329 in the epithelium and just 18 in the stroma with 8 overlapping genes. Figure 12E showed stroma gene expression from serrated polyps, has an increased

number of differentially expressed genes (104) than the stroma from TVA (26) with no overlap. Furthermore pathways enriched involved in the immune signaling pathways along with fibroblasts activated pathways were noticed.

My hypothesis is that serrated lesions, usually initiated by BRAF mutations and methylation, require the recruitment of pro-tumorigenic stroma to enable lesion progression. In contrast, tubulovillous adenomas are initiated by epithelial mutations that disrupt Wnt signaling (such as APC) and this is sufficient to drive tumorigenesis, irrespective of stromal influences.

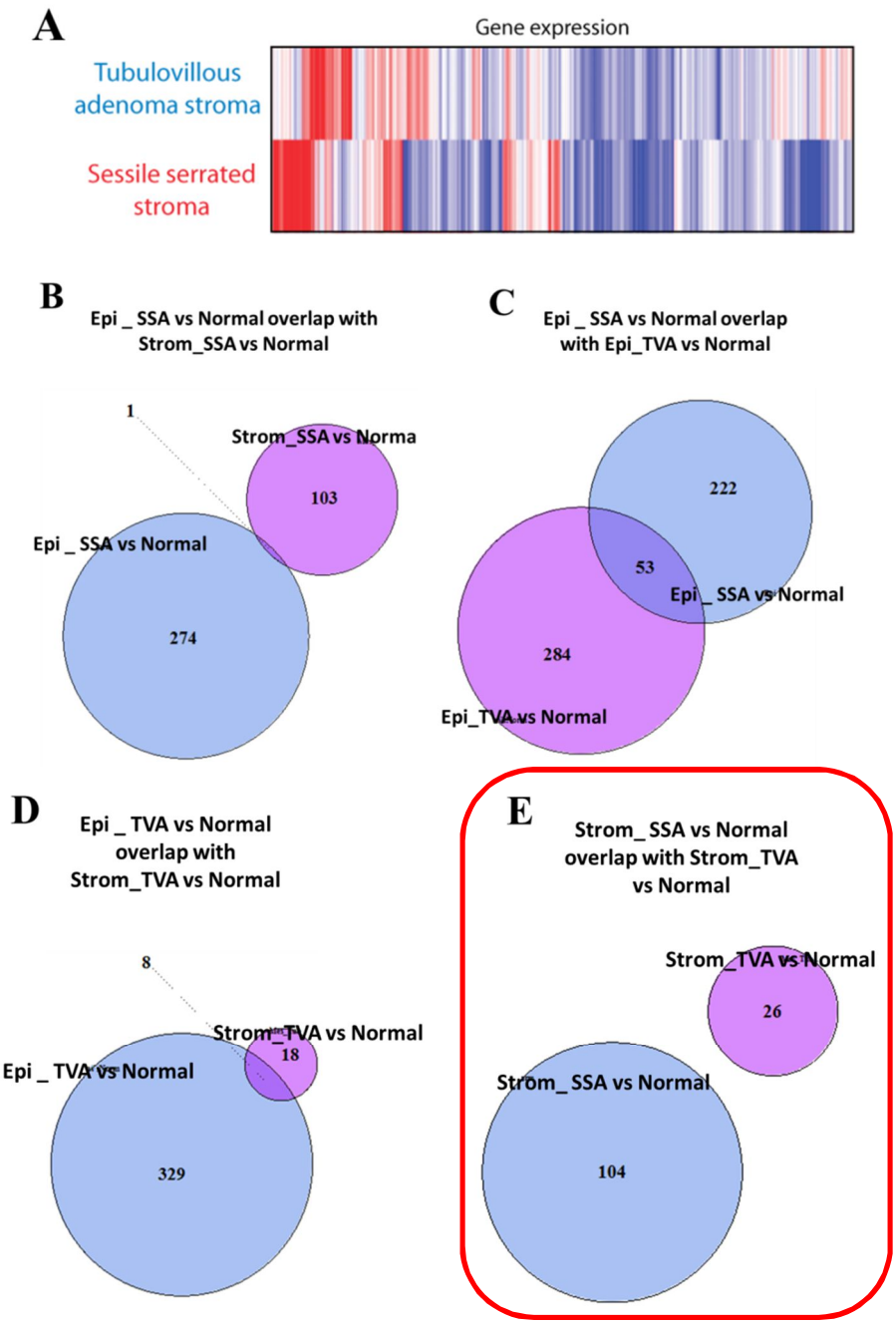


Figure 12. TVAs and SSAs epithelial and stromal gene expression. A) Heatmap showing heterogeneity of differentially expressed genes in the TVAs and SSAs stroma. **B-E Venn diagrams B**) DEG in the SSAs. **C)** DEG in the epithelium. **D)** DEG in the TVAs. **E**) DEG in the stroma. (FDR 0.1; Log FC>0.1).

4.1.2 DEG in mouse epithelial and stromal compartments

DEG analysis was performed in mice models of adenomas with the same purpose to investigate the transcriptome in the epithelial and stromal compartments.

The two mouse models that mimic very closely the mutations that occur in human SSA and TVA adenomas used in this study, were VillinCreER^{T2}; Apc$^{fl/fl}$ (to mimic human TVAs precursors lesions) and VillinCreER^{T2}; Braf^{V600E} (to mimic human SSAs precursors lesions).

The Venn diagrams show the up and downregulated DEG in VillinCreER^{T2}; Apc$^{fl/fl}$ and VillinCreER^{T2}; Braf^{V600E} in the different tissue compartment compared to the wild type control mice. There is a significant difference between the number of genes expressed, positively or negatively, in the epithelium compared to the stroma in both, VillinCreER^{T2}; Braf^{V600E} and VillinCreER^{T2}; Apc$^{fl/fl}$ mice (Figure 13).

This results confirm what seen in the human polyps analysis: the genes up regulated in the stromal compartment are much more in the VillinCreER^{T2}; Braf^{V600E} mouse compared to VillinCreER^{T2}; Apc$^{fl/fl}$ mice.

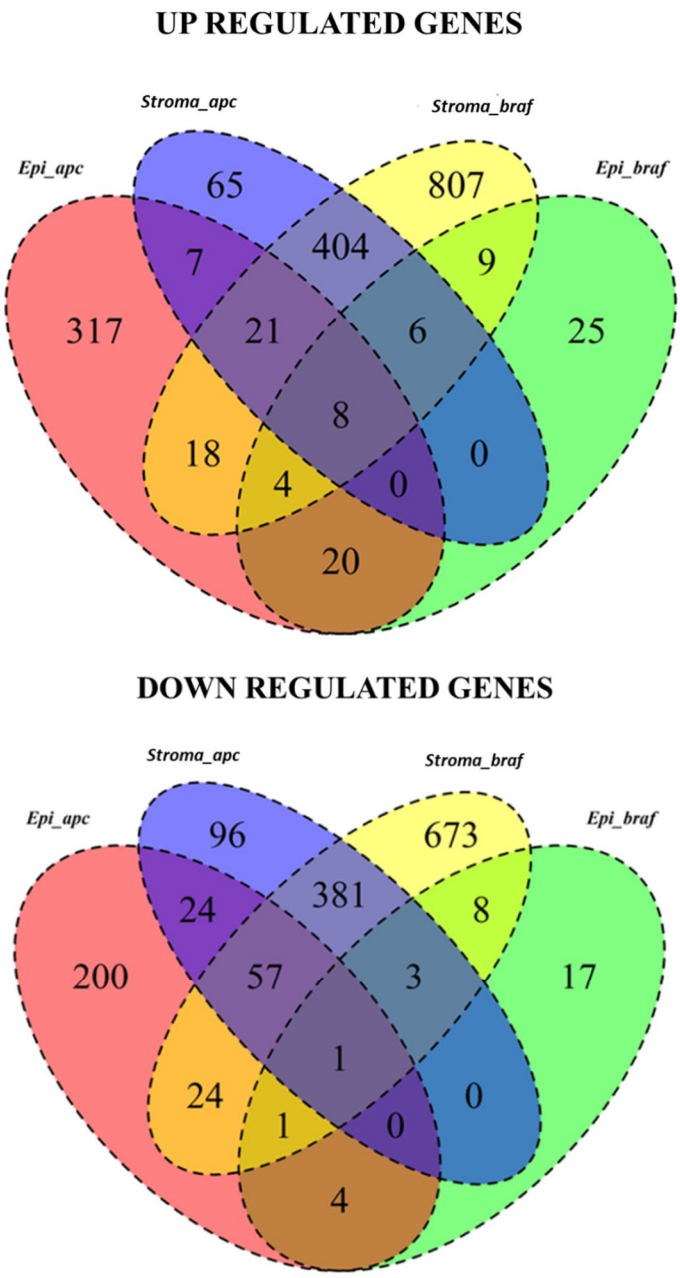


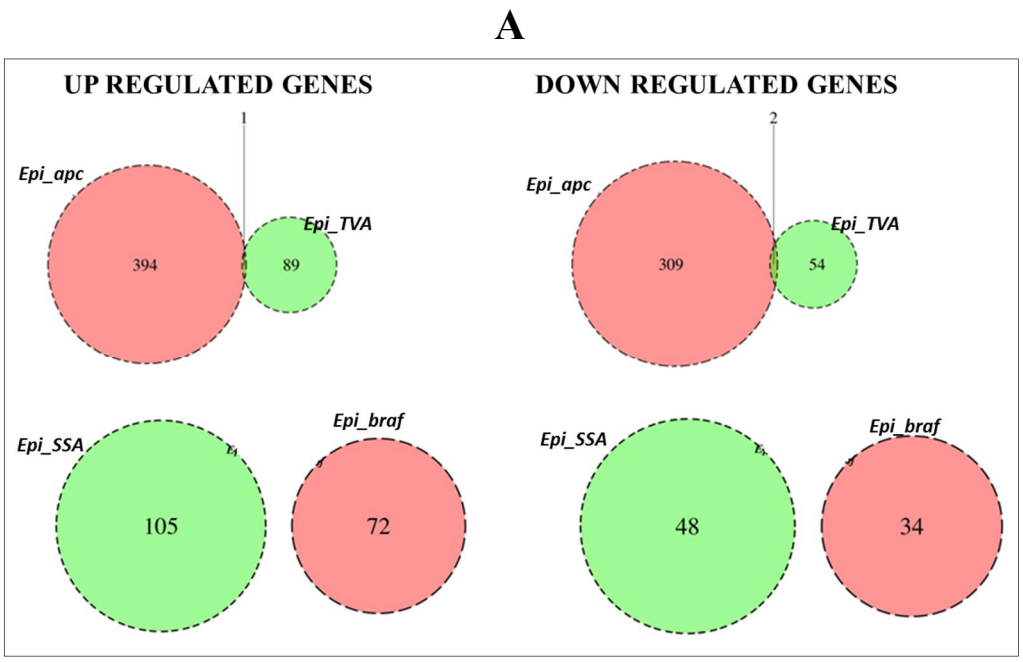
Figure 13. Venn diagrams showing the DEG in VillinCreER^{T2}; Braf^{V600E} (braf) and VillinCreER^{T2}; Apc $^{fl/fl}$ (apc) mice models in the epithelial (Epi) and stromal compartment (Stroma).Overlaps FDR <0.05; Log FC>1.

4.1.3 Mouse and human DEG comparisons

Next, the human and mice results were compared to see if the mice models, employed in this study, were good models.

As shown in Figure 14 by the Venn diagrams, there is no similitude between the DEG found in human and in mice. In fact, the shock effect of pan intestinal changes caused by the induction, determined the loss of the epithelium in favor of the stroma with a consequent increment of the stromal genes expression.

In conclusion, the mouse model used for this study is not a good model of human polyp formation.



B

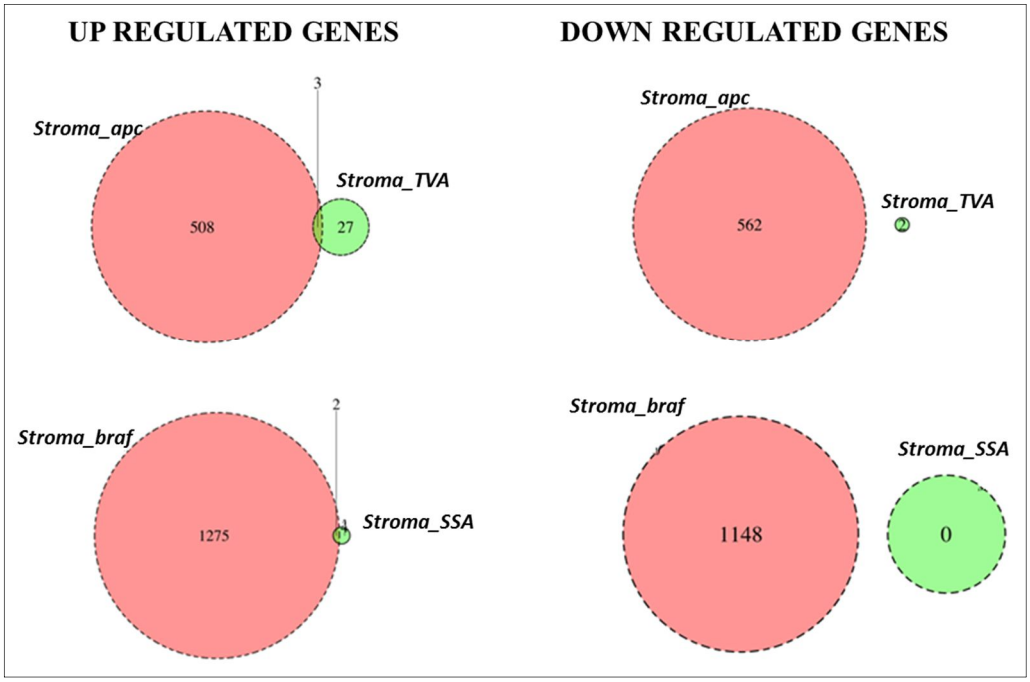


Figure 14. Venn diagrams representing VillinCreER^{T2}; Braf^{V600E} (braf) and VillinCreER^{T2}; Apc $^{fl/fl}$ (apc) mice and human polyps (TVA, SSA) DEG comparisons. A) Up and down regulated genes in the epithelial (Epi) compartment. **B)** Up and down regulated genes in the stromal (Stroma)compartment. Overlaps FDR <0.05; Log FC>1.

4.2 CAFs

The preliminary results show that the genes expressed in polyps stromal compartment are more and different compared to the epithelial compartment. It has been shown that altered stroma can influence cancer development and progression (Bissell et al. 1982, Bissell & Hines 2011). Moreover, epithelial-mesenchymal cross talk plays a vital role in colorectal neoplasia initiation and progression and that variable contribution and importance of the different tissue compartments underpins some of the differences in both polyp and tumor subtype biology.

Thus, in order to elucidate the role of the stroma in CRC initiation and progression, the most abundant cell-type of the stroma, carcinoma-associated fibroblasts (CAFs), were isolated and employed for *in vitro* studies.

4.2.1 Primary culture

Cell or tissue culture techniques have provided numerous pivotal information to understand the basic biology of cancer allowing cancer cells to be maintained outside the body. It was originally developed in the middle of 20th century (Langdon 2003) when "HeLa" was the first established cell line of human cancer originated from surgical pathology specimen of uterine cervical carcinoma from African American patient Henrietta Lacks in 1951 (Hsu et al. 1976). Carcinoma cell lines have been established very important tools to evaluate in depth the biochemistry and molecular biology associated with individual cancer types and have contributed enormously to our understanding of normal as well as malignant cell physiology (Langdon 2003).

Thus, primary culture of the whole cancer tissue derived from surgery or biopsy can indeed provide very important information to our understanding of cancer tissue microenvironment. However, it is

practically impossible to reproduce the cancer microenvironment using the cancer cell line alone in *in vitro* analysis.

4.2.2 Fibroblasts isolation

In order to assess the influence of fibroblast and epithelial cell compartments on each other, I have optimized a protocol for the isolation and culture of fibroblasts from normal and neoplastic (polyp and cancer) biopsy samples.

Fibroblasts were successfully obtained from normal colonic mucosa (n=16), adenocarcinomas (SSAs (n=5), TVAs (n=6) and from 16 CRCs colonic tissue (CMS2 and CMS4) (CENTRALs (n=7) and LEADING EDGEs (n=9) out of a total of 50 samples collected.

The major reasons for unsuccessful culture were bacterial contamination and insufficient material. Intestinal endoscopic biopsies contain a small amount of tissue and can be easily contaminated by common intestinal flora, the manual management of the endoscope and endoscopic forceps, and their passage through the endoscopic channel.

Cultures were monitored for the appearance of highly adherent stromal cells with typical fibroblastic morphology. Fibroblasts start to appear after 4 days (Figure 15) and they could be kept in culture until cell passage number 18 (P18). Stromal cultures were expanded and stored for future experiments.

All experiments were performed using stromal cells from passage 3.

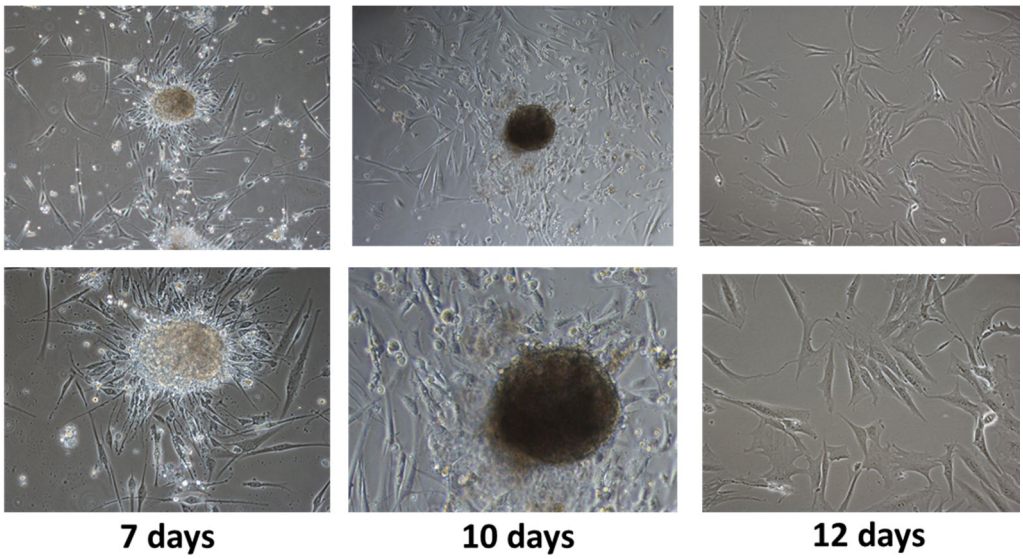


Figure 15. Cellular growth from a chopped and enzymatically digested fragment of endoscopic duodenal biopsy at different times after seeding (7-10 days) and at passage 1 in culture (12 days); (100 × magnification, upper panel and 200 x magnification, bottom panel).

4.2.3 Characterization of human fibroblast primary cultures

4.2.3.1 Morphological features

The fibroblast cell population was first verified by cell morphology under the microscope.

The established primary fibroblast cell cultures grew as a monolayer of cells and showed the typical fibroblast-like features, with spindle-like shapes and elongated projections. The CAFs were slightly more slender than the normal fibroblasts but without significant differences. Human normal, adenoma and tumor derived colon fibroblasts presented as large mesenchymal cells, characterized by an abundant cytoplasm provided with many cellular extensions. Figure 16 shows the morphology of isolated fibroblasts.

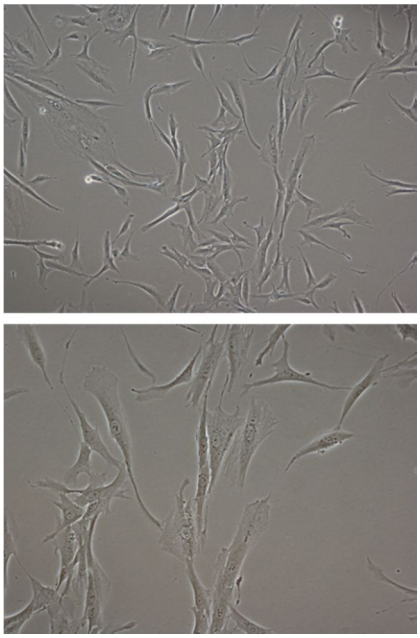


Figure 16. Morphological features. Representative microscopic fields showing cancer-associated fibroblasts. At passage 4 morphologies of CAFs were dendroidal-spindle-shaped. Top: low magnification; bottom: high magnification.

4.2.3.2 Immunophenotyping of stromal cell primary cultures

Immunocytochemistry

At cell passage 5, the purities of the various fibroblast populations were verified through immunostaining by detection of specific fibroblast biomarkers.

For the immunocytochemistry (ICC) experiments, the following primary antibodies have been used: anti-vimentin, anti-α-SMA, anti-desmin and epithelial types were carefully excluded performing anti-human cytokeratin 20 immunocytochemistry.

For fibroblast cultures derived from normal and cancerous colon tissues, immunocytochemical assays revealed that they highly expressed the fibroblastic marker vimentin, a common mesenchymal

cytoskeletal marker and the smooth muscle cell marker desmin. Very few cytokeratin-positive cells were detected which may be due to epithelial-mesenchymal transition.

In the present study, all the tested fibroblasts express the myofibroblast α-SMA marker. During the tumorigenesis process, stromal fibroblasts acquire some of the characteristics of smooth muscle cells that specifically express α-SMA. This result indicates that the isolated CAFs contain a high proportion of myofibroblasts. This high expression of α-SMA in cancer stromal cells is in agreement with a previous study (Orimo et al. 2005).

None of the used antibody showed a specific pattern between the five types of fibroblasts, indicating that although they were good mesenchymal markers, they did not discard between normal fibroblasts and CAFs (Figure 17).

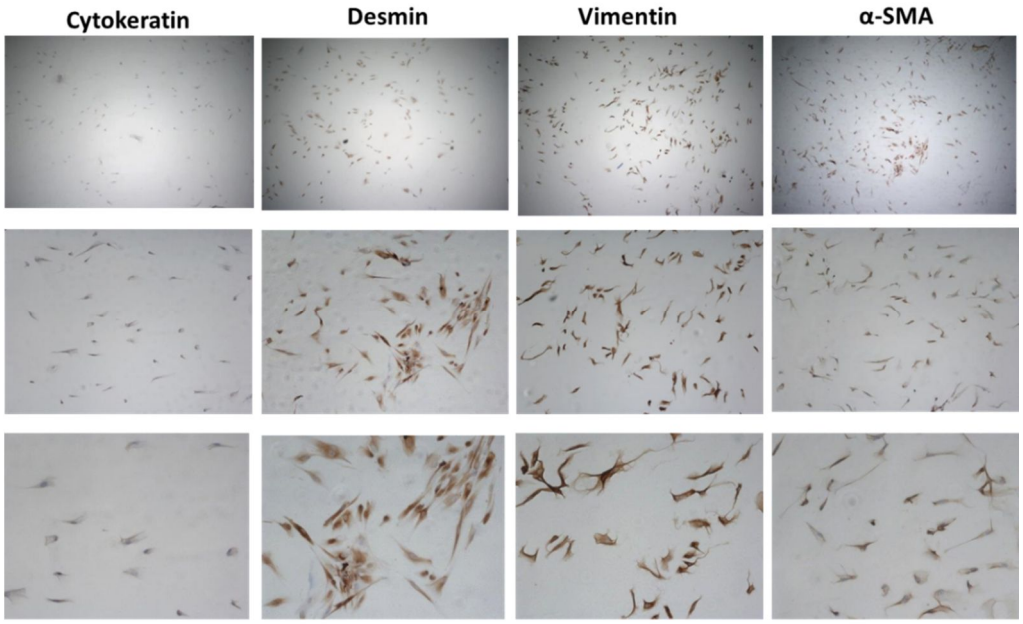


Figure 17. Immunostaining characteristics of primary fibroblastic cells. All the primary fibroblastic cells strongly expressed fibroblastic markers vimentin, desmin and α-SMA but were negative for epithelial cell marker cytokeratin. (40 × magnification, upper panel; 100 × magnification, middle panel and 200 x magnification, bottom panel).

4.2.3.3 Identifying Fibroblasts in paraffin-embedded tissues

In the present study, immunostaining analysis for stromal markers was used to check the localization of the fibroblast in paraffin-embedded tissue and in particular, in the crypt-villi axis. In order to visualize fibroblasts in the colonic tissue specimens by light macroscopy mouse and human paraffin sections were analized.

Mouse tissue sections

In normal wild type C57Bl/6J mouse the sections show the normal cell morphology at the crypt bases and villi tips. Cytokeratin-positive cells show epithelial cells of the crypts and villi. Pericryptal myofibroblasts and nonpericryptal stromal cells (fibroblasts) have similar morphologic appearance (hematoxylin-eosin). Muscularis mucosae cells and pericryptal myofibroblasts are both α-SMA and desmin positive. Diffuse expression of vimentin by both pericryptal and nonpericryptal stromal cells is evident along the SB1, SB2, SB3 and colon sections but it is not present in the muscularis mucosae. Networklike connections are are easily discernible and demonstrated between the αSMA positive pericryptal myofibroblasts, the non pericryptal stromal cells and the muscularis mucosae (Figure 18).

In VillinCreER^{T2}; Apc$^{fl/fl}$ mouse, the histology revealed disturbed crypt-villi architecture and the strong presence of the stroma. Cytokeratin-positive cells show epithelial cells of the crypts and villi. Morphologically, the small bowel sections (SB1, SB2, SB3) show ectopic crypts and large polyps are evident in the colon. Pericryptal myofibroblasts and nonpericryptal fibroblasts have similar morphologic appearance (hematoxylin-eosin). Muscularis mucosae cells and pericryptal myofibroblasts are both α-smooth muscle actin positive. Furthermore, muscularis mucosae, pericryptal and nonpericryptal stromal cells are positive for desmin. Of note the

striking absence of vimentin positivity in the muscularis mucosae (Figure 19).

VillinCreER^{T2}; Braf^{V600E} mouse histology revealed the loss of crypts architecture. Cytokeratin-positive cells show epithelial cells of the crypts and villi. In the colon, pedunculated colonic adenomas have central dysplastic areas with a sharp cut off between enclosing serrated epithelium. Morphologically, all the small bowel sections (SB1, SB2, SB3) show prominent serrations, branching crypt with basilar dilatation and asymmetric proliferative zone. Pericryptal myofibroblasts and nonpericryptal stromal cells (fibroblasts) have similar morphologic appearance (hematoxylin-eosin). Muscularis mucosae cells and pericryptal myofibroblasts are both α-smooth muscle actin positive. Muscularis mucosae, pericryptal and nonpericryptal stromal cells are also positive for desmin whereas only pericryptal and nonpericryptal stromal cells are positive for vimentin which is not expressed in the muscularis mucosae (Figure 20).

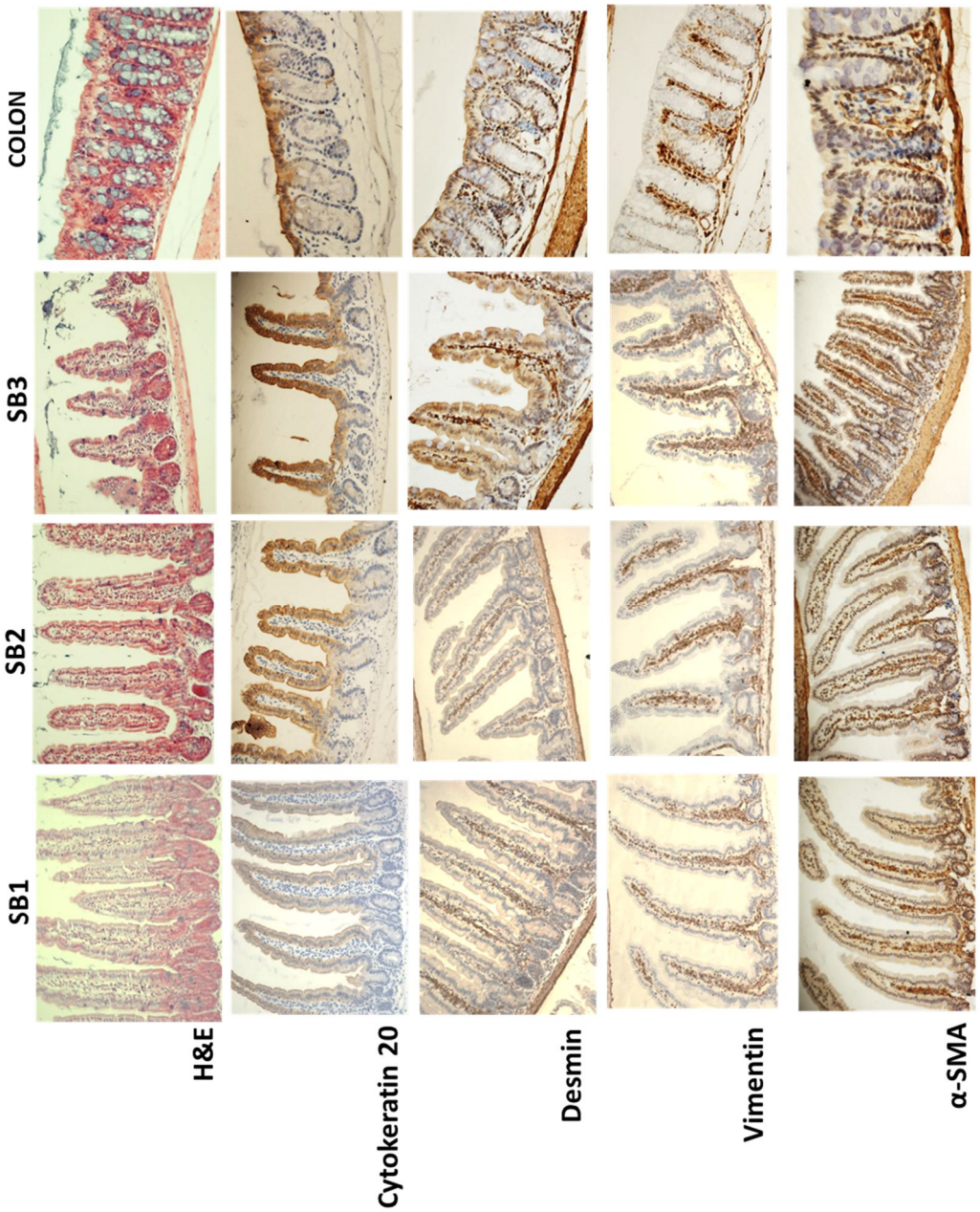


Figure 18. Step sections of normal wild type C57Bl/6J mouse proximal small bowel (SB1), mid-small bowel (SB2), distal small bowel (SB3) and colonic mucosa showing the distinctly different immunophenotypic characteristics of the muscularis mucosae and interstitial stromal cells of the lamina propria. Nuclei are stained blue with hematoxylin. Antibody staining is shown in brown from DAB plus substrate (original magnification x 200).

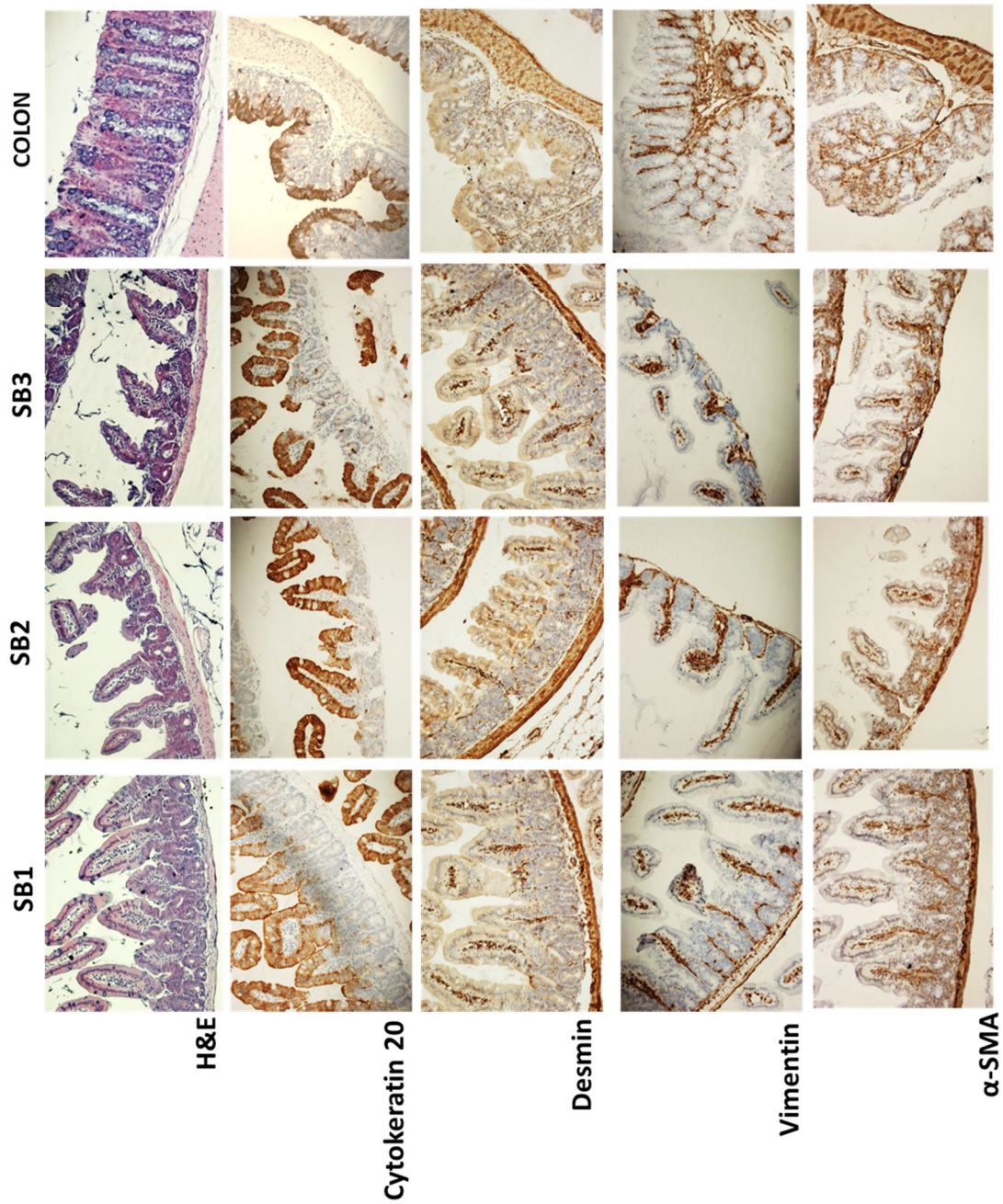


Figure 19. Step sections of *VillinCreER^{T2}; Apc$^{fl/fl}$* mouse proximal small bowel (SB1), mid-small bowel (SB2), distal small bowel (SB3) and colonic mucosa showing the distinctly different immunophenotypic characteristics of the muscularis mucosae and interstitial stromal cells of the lamina propria. Nuclei are stained blue with hematoxylin. Antibody staining is shown in brown from DAB plus substrate (original magnification x 200).

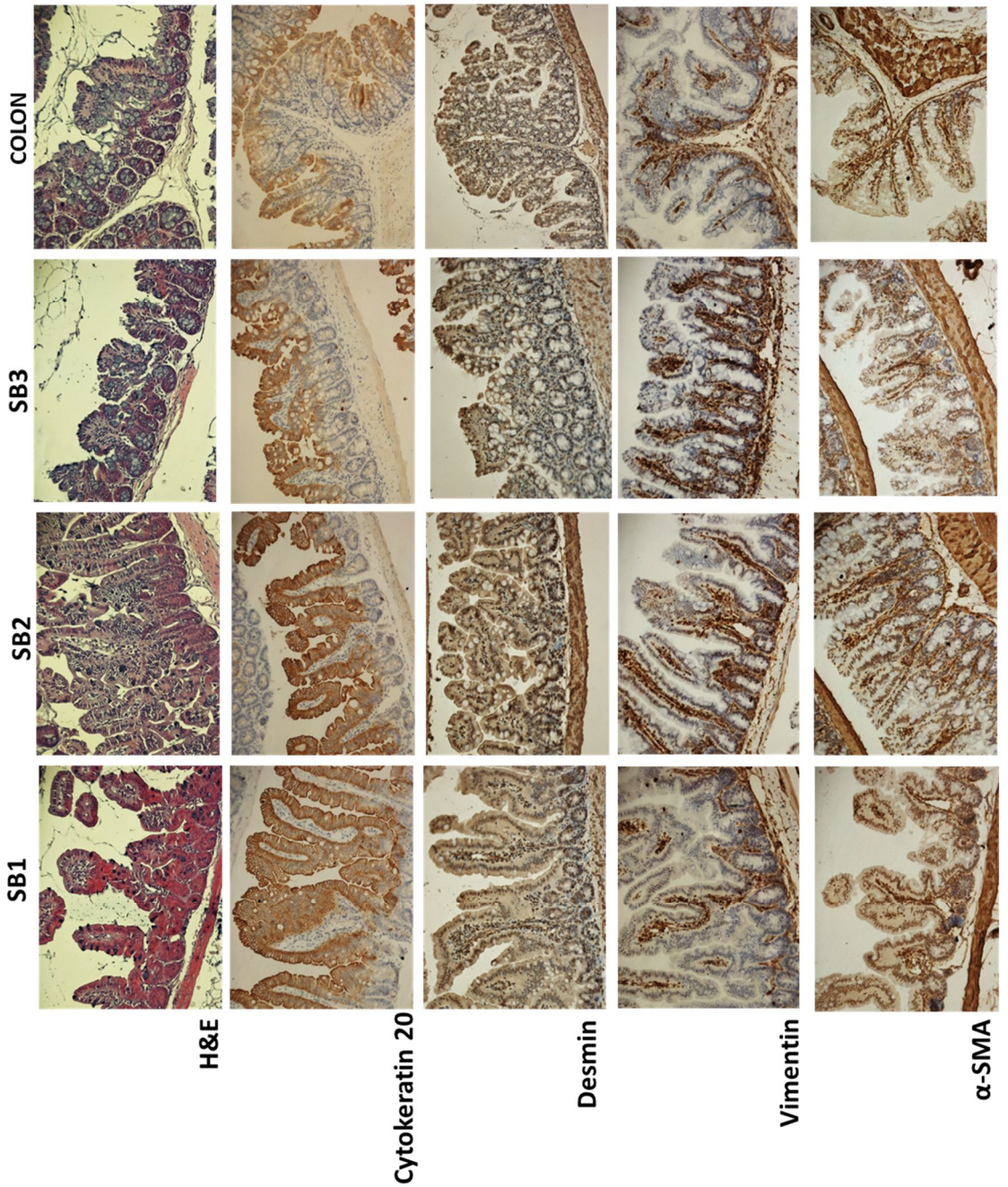


Figure 20. Step sections of *VillinCreER^{T2}; Braf^{V600E}* mouse proximal small bowel (SB1), mid-small bowel (SB2), distal small bowel (SB3) and colonic mucosa showing the distinctly different immunophenotypic characteristics of the muscularis mucosae and interstitial stromal cells of the lamina propria. Nuclei are stained blue with hematoxylin. Antibody staining is shown in brown from DAB plus substrate (original magnification x 200).

Human tissue sections

H&E staining allows seeing the normal colonic tissue, SSA with epithelial infoldings and ectopic crypt formation. TVA staining shows diffuse high grade dysplasia. In CRCs paraffin sections, it is shown disrupted crypts and villi structure compared to the normal crypts and the infiltration of the lamina propria by tumor cells.

Cytokeratin-positive cells show epithelial cells of the crypts and villi, in the disorganized adenomas and in the chaotic CRCs parenchimas.

Diffuse expression of α-smooth muscle actin by muscolaris mucosae, pericryptal myofibroblasts and nonpericryptal stromal cells with direct connections between the pericryptal myofibroblasts and the muscularis mucosae is evident in normal mucosa, adenomas and CRCs. Immunohistochemical staining of desmin expression by both pericryptal and nonpericryptal cells is seen as well as the vimentin antibody in the stromal cells of the lamina propria (Figure 21)

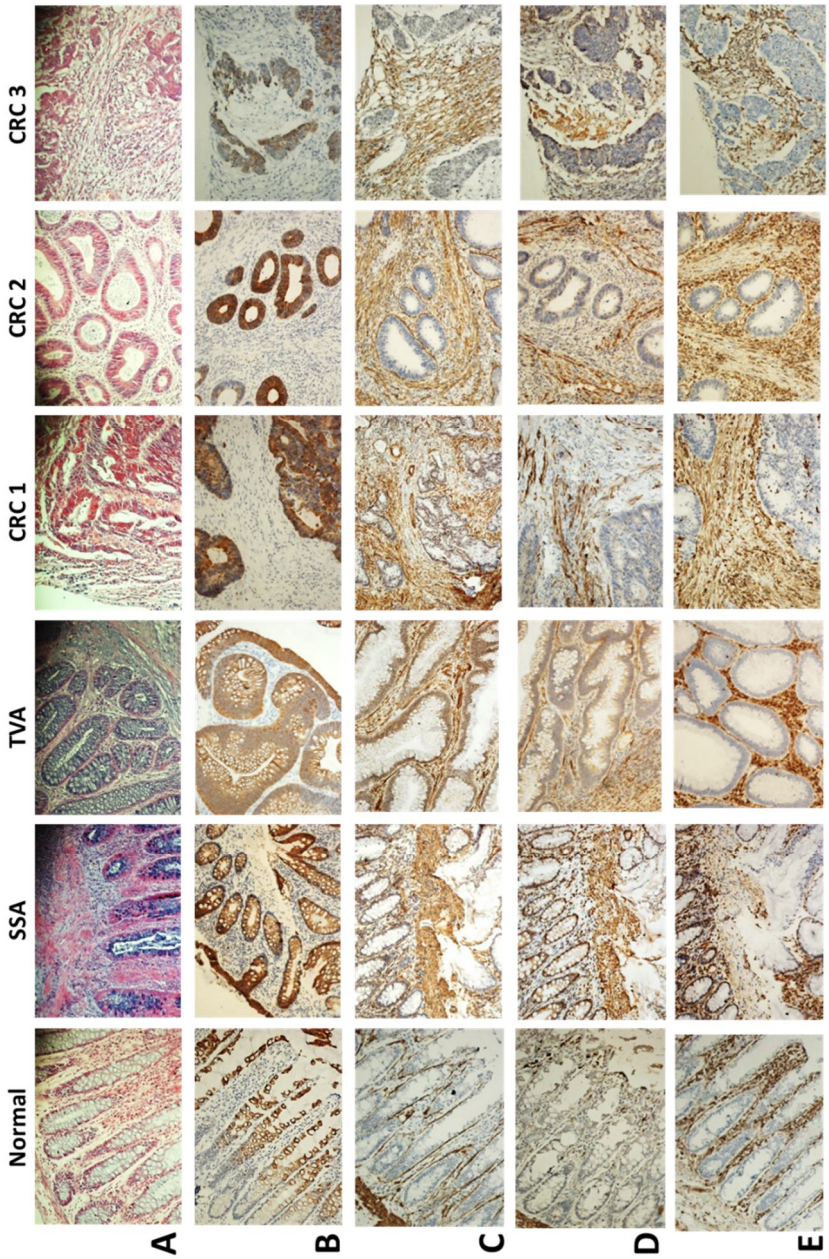


Figure 21. Representative sections from human colon, SSA, TVA, and three different CRCs (CRC1, CRC2, CRC3) showing the distinctly different immunophenotypic characteristics of the muscularis mucosae and interstitial stromal cells of the lamina propria for H&E (**A**), cytokeratin 20 (**B**), α-SMA (**C**), desmin (**D**) and vimentin (**E**). Nuclei are stained blue with hematoxylin. Antibody staining is shown in brown from DAB plus substrate (original magnification x 200).

4.2.3.4 Genetic analisys

qRT-PCR

Next, the real nature of the isolated fibroblasts was further checked by qRT-PCR.

In particular, the expression of the stromal marker vimentin was checked in the different isolated fibroblast from passage 5 to passage 13 (P5-13). All the analyzed fibroblasts strongly expressed the marker vimentin (Figure 22), confirming what seen in the immunocytochemistry analysis.

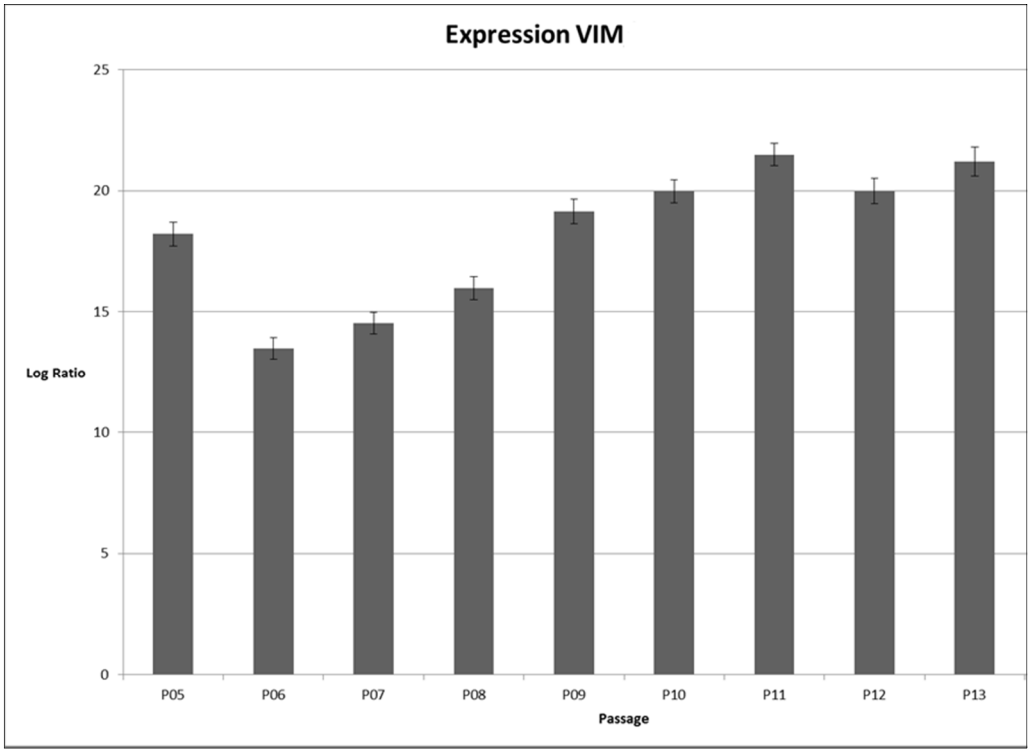


Figure 22. Relative expression of specific mesenchymal marker such as vimentin in fibroblasts from passage 5 to 13.

Passage-dependent variations of human colon fibroblast in culture

The use of cultured fibroblasts made it possible to undertake a more detailed chronologic study of age-dependent variations induced in mesenchymal cells maintained in culture. It has been shown that in MCF-7 cell line there were some karyotype differences, which is generally considered due to the differences in cell passage number and maintenance or culture conditions among different laboratories (Wenger et al. 2004).

In order to see if the culture conditions (early primary cultures and late primary cultures) have caused possible phenotypic and functional alterations on the isolated colon fibroblasts, gene expression profile was performed. Such information is important for devising experimental models of physio pathologic relevance.

Gene expression RNA microarray analysis for normal (n=3), SSAs (n=3), TVAs (n=3) and CAFs (Central n=3 and Leading edge n=3) pairs of early passage (P3-6) and late passage (P16-17) was performed. Raw data from Illumina gene expression arrays was processed after removing 2 outlier samples from initial quality control (detection score of < 0.95 of the background intensity for majority of probes) using the VSN (variance-stabilisation and normalisation) algorithm. A filter was applied by taking a detection score of > 0.95 of the background intensity distribution for all samples to consider a probe detectable, resulting in a total of 24,262 detectable probes. Differentially expressed genes between early (n=13) and late passages (n=15) were identified using Student's t-test by running "ttest2" command in MATLAB®. Unsupervised hierarchical clustering of the 24,262 genes was allowed and it is shown in the heat map (Figure 23).

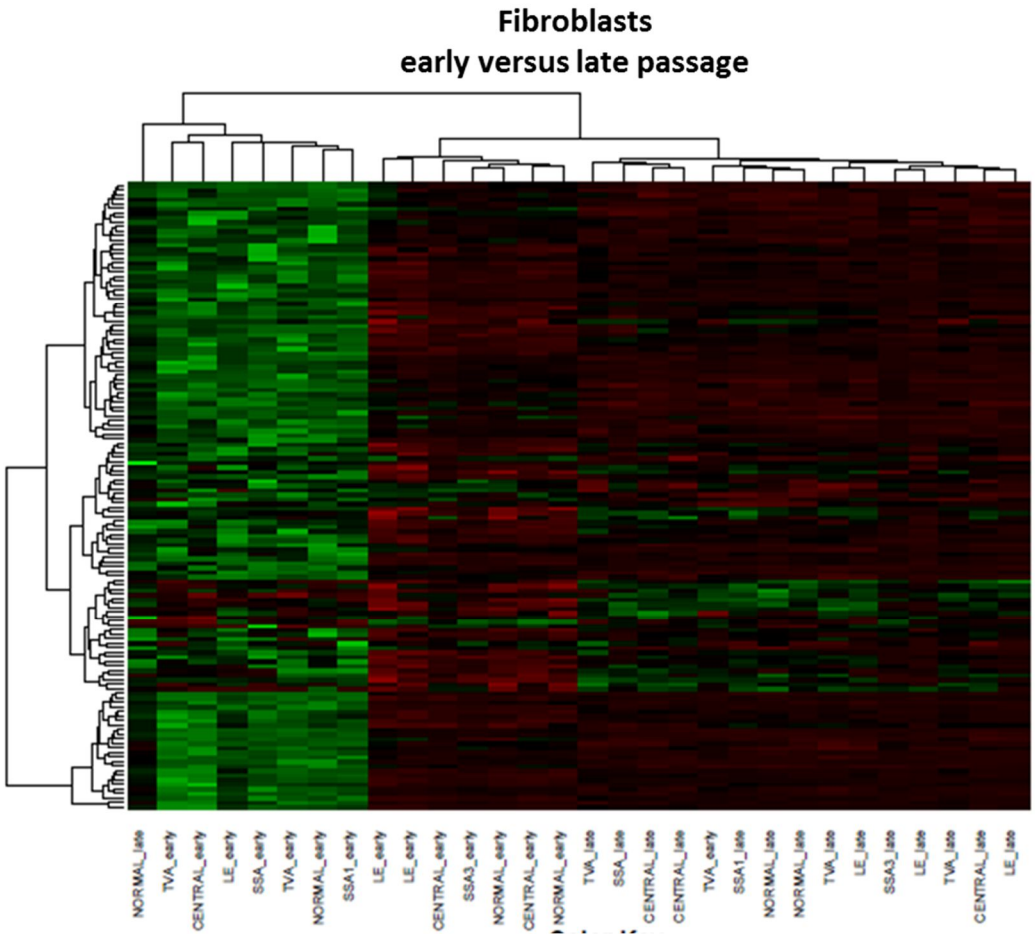


Figure 23. Hierarchical clustering heat map of the genes based on custom gene sets with significant differentially expressed changes with *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR<5%. Each column represents a sample; each row refers to a gene. The dendrograms that determine the ordering of the rows (genes, left side) and columns (samples, upper side). The color bar shows the fold change and corresponding color depth: red, up-regulated (ratio ≥1.5); green, down-regulated (ratio <1.5); and black, unchanged.

It seems that the major difference seen in the cultured fibroblasts is due to the different types of fibroblast analized, confirming the heterogeneous origin of the CRC derived fibroblast population. Stable gene expression changes in CAFs may be due to epigenetic changes (Hu et al. 2005) versus somatic mutations (Kurose et al. 2002, Weber et al. 2006). It is now known that somatic mutations in

the DNA sequence of CAFs are rarely, if ever, encountered (Campbell et al. 2009, Jiang et al. 2008) and thus, the acquisition of tumor-promoting activities by CAFs, in part, are due to epigenetic alterations in the DNA (Hu et al. 2005, Mitra et al. 2012).

Comparison of gene expression patterns in Adenomas, Cancer stroma and Normal fibroblasts

Next the global transcriptome of resident fibroblasts in the normal colon mucosa (NF) versus adenomas and CAFs in human colorectal cancer was analyzed. The differentially expressed genes for each experimental group are shown in the Venn diagrams. There is a significant difference in the DEG, between normal fibroblasts, adenomas fibroblasts (SSA and TVA) and CAFs (Central and Leading Edge). The numbers of genes that are shared by two or more groups are indicated in the intersections (Figure 24).

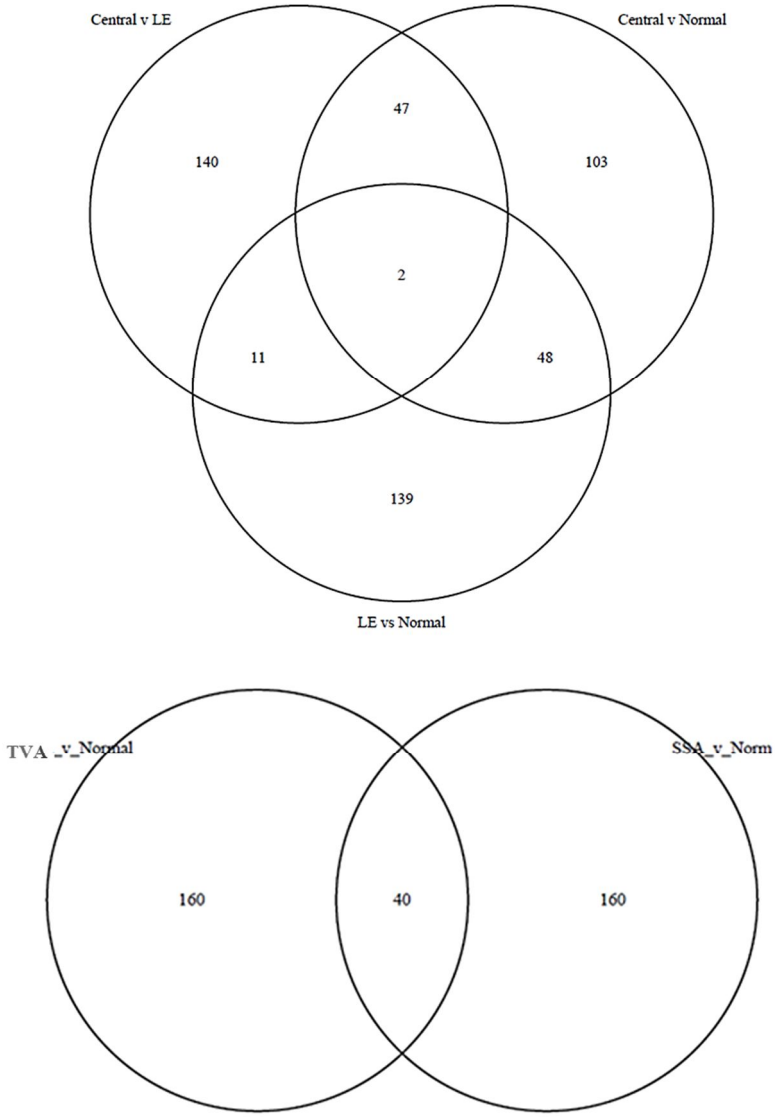


Figure 24. Venn diagram based on the top 200 genes from each list showing the differentially expressed genes overlaps between Normal, Central and Leading edge fibroblasts (LE). *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR <5%.

Gene Set Enrichment Analysis (GSEA) derives its power by focusing on gene sets, that is, groups of genes that share common biological function, chromosomal location or regulation.

Genes were ranked by computing the differential expression in the adenomas and CAFs versus normal fibroblasts by the Student's t-test method. If multiple probes were present for a gene, probe with the highest absolute differential expression between experimental and normal was selected. Gene shuffling with 1,000 permutations to compute the P-value for the enrichment score was used.

The analysis of each comparison revealed expression patterns correlated with cell proliferation and cell cycle regulation marked by upregulation of genes involved with cell cycle progression, DNA synthesis/repair, protein translation/folding, vesicles mediated transport and lipid transport/metabolism. Moreover, pathway enriched with up-regulated genes included CAFs genes list based on Calon et al. 2015 (Figure 25- 28).

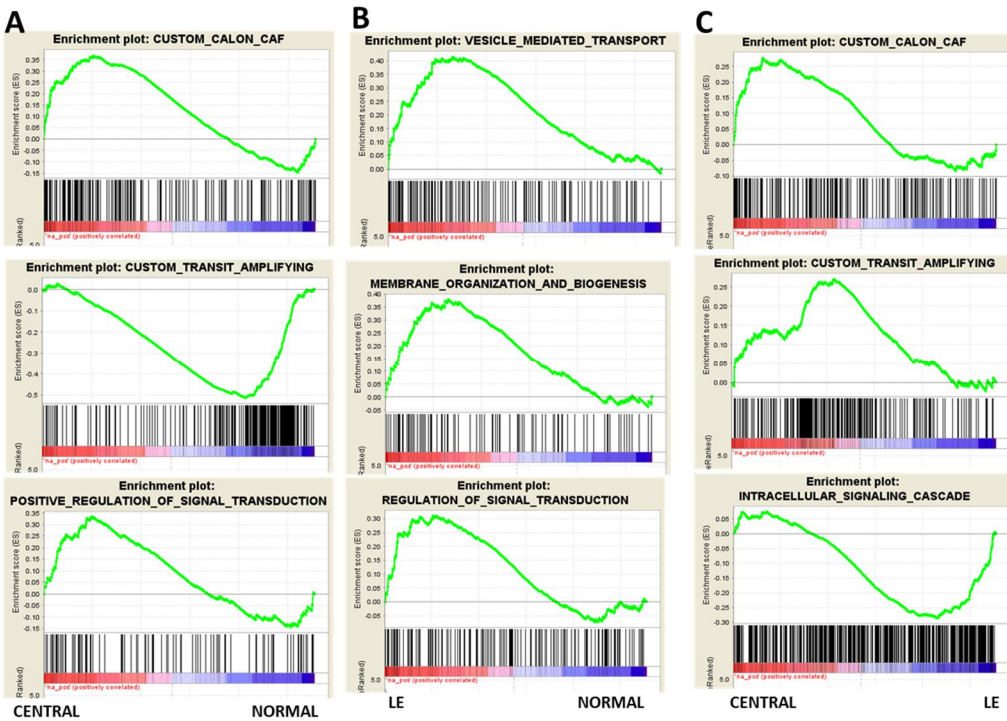


Figure 25. GSEA analysis using established gene program sets of cultured fibroblasts. GSEA plots shown are for **A)** CENTRAL versus NORMAL fibroblasts, **B)** LEADING EDGE (LE) versus NORMAL fibroblasts and **C)** CENTRAL versus LEADING EDGE (LE) fibroblasts. Enrichment score is calculated using Kolomogrov-Smirnov test. Gene shuffling with 1,000 permutations to compute the P-value for the enrichment score was used.

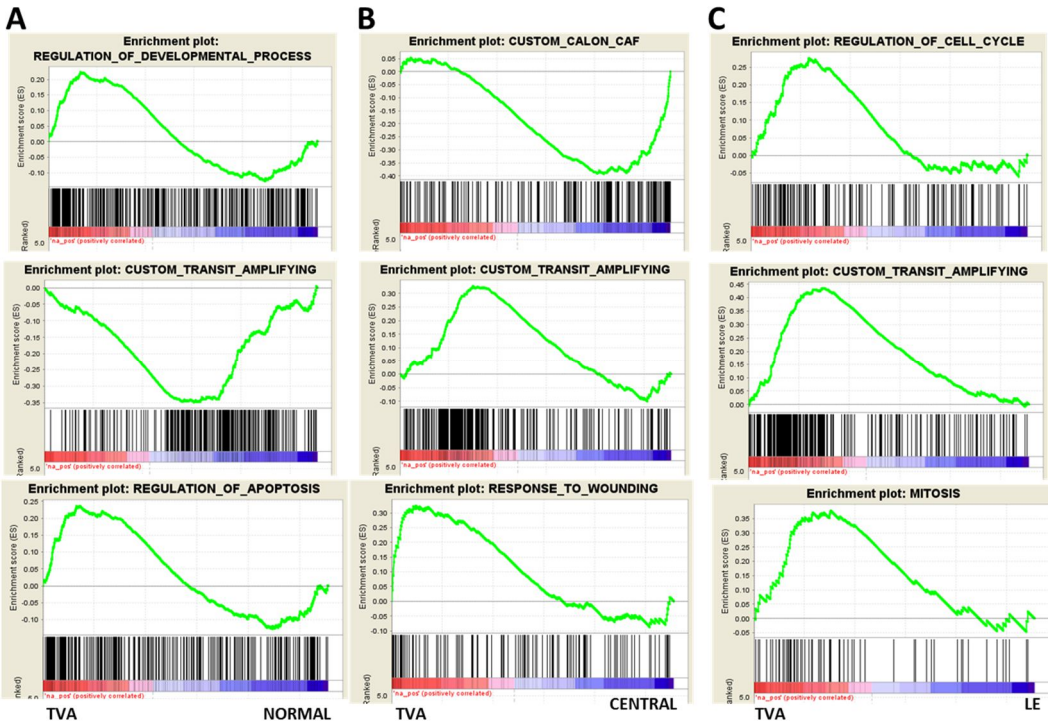


Figure 26. GSEA analysis using established gene program sets of cultured fibroblasts. GSEA plots shown are for **A)** TVA versus NORMAL fibroblasts, **B)** TVA versus CENTRAL fibroblasts and **C)** TVA versus LEADING EDGE (LE) fibroblasts. Enrichment score is calculated using Kolomogrov-Smirnov test. Gene shuffling with 1,000 permutations to compute the P-value for the enrichment score was used.

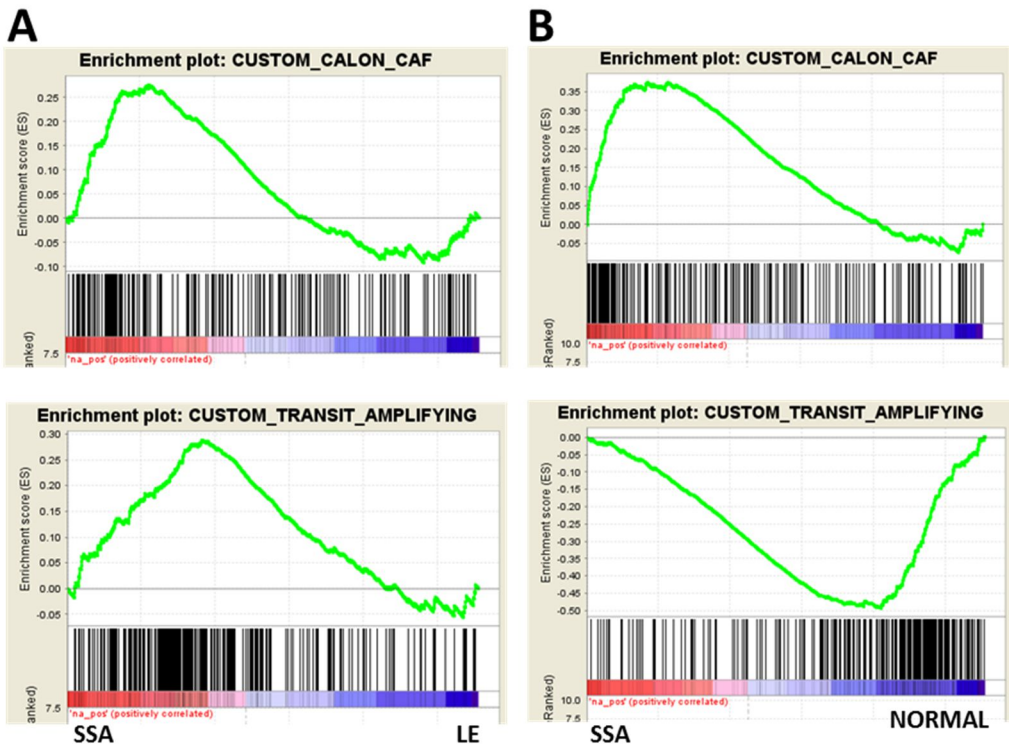


Figure 27. GSEA analysis using established gene program sets of cultured fibroblasts. GSEA plots shown are for **A)** SSA versus LEADING EDGE (LE) and **B)** SSA versus NORMAL fibroblasts. Enrichment score is calculated using Kolomogrov-Smirnov test. Gene shuffling with 1,000 permutations to compute the P-value for the enrichment score was used.

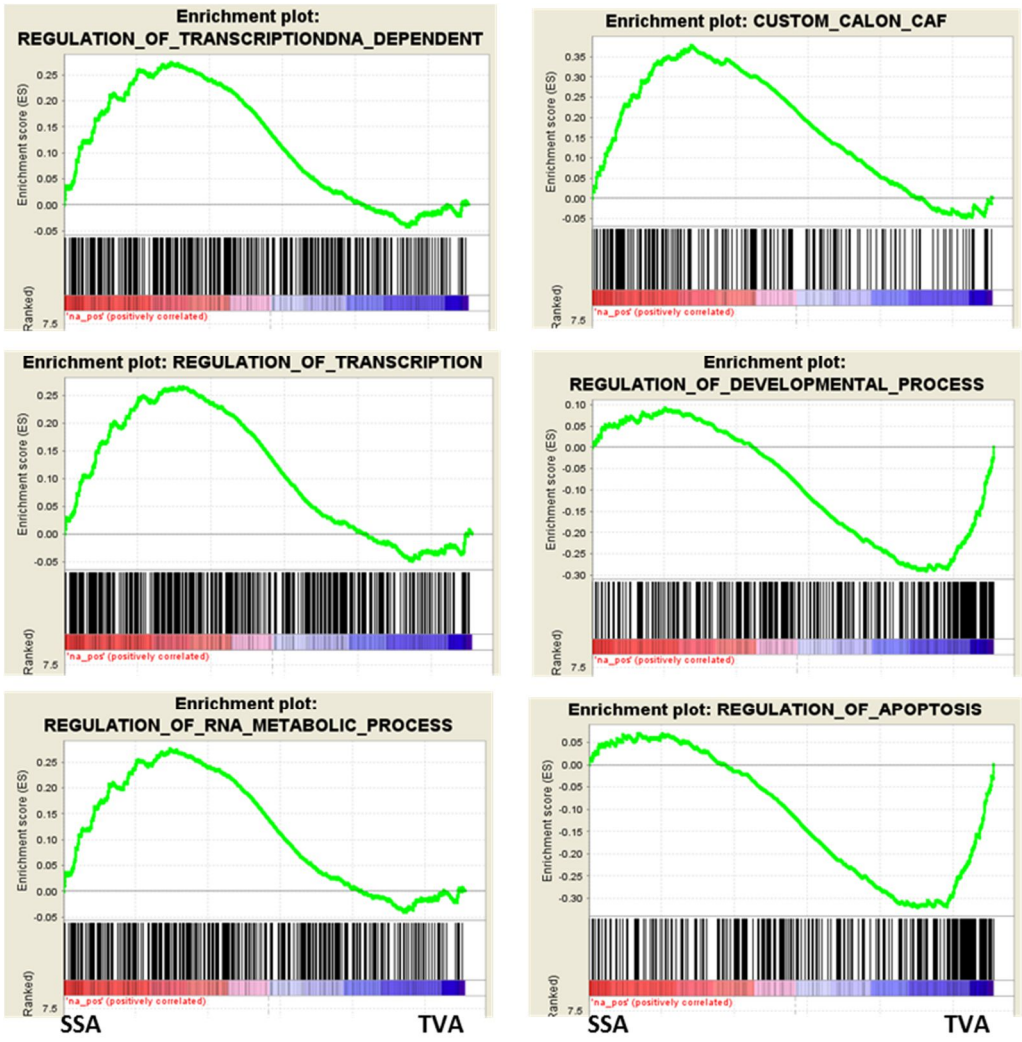


Figure 28. GSEA analysis using established gene program sets of cultured fibroblasts. GSEA plots shown are for SSA versus TVA fibroblasts. Enrichment score is calculated using Kolomogrov-Smirnov test. Gene shuffling with 1,000 permutations to compute the P-value for the enrichment score was used.

4.2.4 Modeling stromal- epithelial interactions

Tumor/cancer stromal cells have been considered not only as a mere physical supporting cell of the parenchymal or carcinoma cells but also functional or regulatory cells in tumor/cancer microenvironment. Therefore, endocrine, autocrine and paracrine interactions between parenchymal and stromal cells are considered pivotal for metastasis, proliferation and angiogenesis in CRC microenvironment.

As a matter of fact, cell lines cannot recapitulate the complex spatial (3D) organization of the intestinal epithelium. Furthermore, cell lines have undergone significant molecular changes to become immortal and do not represent all intestinal subsets, hence do not represent the *in vivo* situation accurately.

In recent years, paradigm shift from two-dimensional (2D) to 3D cell culture techniques have been developed rapidly. 3D culture affects cell functions and behaviors including morphology and gene expression in a similar fashion to the *in vivo* response.

Recently, Sato et al. presented a novel method that allows long-term culture of isolated intestinal crypts or intestinal stem cells (Sato & Clevers, 2013). This method takes advantage from the presence of intestinal stem cells in the crypts and makes use of a mixture of extracellular matrix proteins (Matrigel) that allows three-dimensional growth. Supplemented with the appropriate growth factor cocktail (epidermal growth factor, Noggin, R-spondin-1) and cultured in a three-dimensional extracellular matrix, these intestinal stem cells are capable of developing into organoids, displaying many important functions of the normal intestinal epithelium (mini-guts). Of note, the supplemented growth factors in the culture medium are identical with the signals that regulate intestinal stem cell niches in vivo (Sato et al. 2011a).

In particular, Wnt signaling is a pivotal requirement for crypt proliferation, (Korinek et al. 1998, Kuhnert et al. 2004) and the Wnt

activator R-spondin1 induces dramatic crypt hyperplasia in vivo (Kim et al. 2005). It has been shown that R-spondin-1 is a ligand for Lgr5, a marker for intestinal stem cells (Barker et al. 2007) and an essential factor to activate Wnt signal in intestinal crypts (de Lau et al. 2011, Sato et al. 2011a). Second, epidermal growth factor (EGF) signaling is associated with intestinal proliferation (Dignass & Sturm 2001). Third, transgenic expression of Noggin induces expansion of crypt numbers (Haramis et al. 2004). Fourth, isolated intestinal cells undergo anoikis outside the normal tissue context (Hofmann et al. 2007). Under this culture condition (R-spondin-1, EGF, and Noggin in Matrigel), small intestinal organoids can be ever-expanding and display all hallmarks of the small intestinal epithelium in terms of architecture, cell type composition, and self-renewal dynamics (Sato et al. 2011a).

Similar to the intestinal epithelium, stem cells and highly proliferative transit amplifying cells reside in the crypt-like domain of the organoids (Figure 29). These stem cells are able to differentiate in to all intestinal epithelial cells (enterocytes, Paneth cells, Goblet cells, enteroendocrine cells, but also stem and progenitor cells), as shown *in vivo* and in culture (Sato et al. 2009).

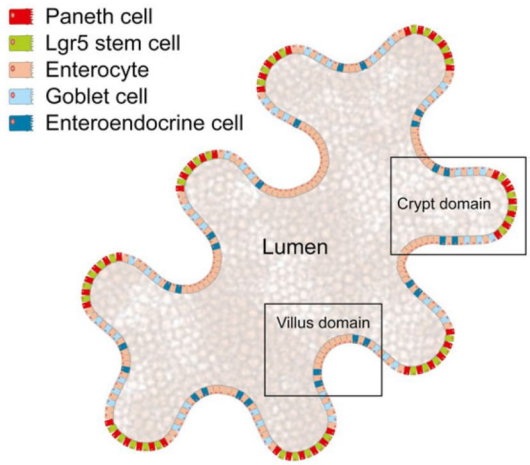


Figure 29. Schematic image of an intestinal organoid. All epithelial cell types normally present *in vivo* are also present in the cultured intestinal organoids. Secretion of mucins into the luminal space and lysozyme production by Paneth cells illustrate the functionality of this epithelium. From McCracken *et al*. 2011.

Intratumoral fibroblastic stromal cells are heterogeneous in individual patients. Therefore, the co culture system makes it possible to study epithelial–stromal interactions and vice versa through maximum simulation to *in vivo* microenvironment of CRC.

Thus, in order to assess the paracrine effect of secreted signaling pathway cross-talk between tissue compartments on each other, small intestinal mouse crypts were co cultured in combinations with human normal, adenoma (SSA and TVA) and neoplasia associated (Central and Leading Edge (LE) fibroblasts.

When isolated crypts were cultured in matrigel and media supplemented with all the growth factors, they closed and formed organoids - transparent sphere-like- structures within the first hours of culture. On day 5, the spheres started to bud, and after 7 days, organoids with numerous crypt-like structures were formed.

Instead, the isolated crypts co cultured on top of normal, adenomas and cancer associated fibroblasts in medium supplemented with R-spondin-1 (which is crucial for organoid culture) and lacking in

Noggin and EGF, in addition to adult-type minigut organoids, a proportion of hollow spheres (hereafter referred to as ‘‘spheroids’’) were generate. This spheroid phenotype was not seen in the controls that received all the growth factors (Figure 30).

It seems that the fibroblast supported the cross-species generation of normal mouse epithelial organoids and developed spheroids organoids, indicating a phenotype modulating effect of the underlying fibroblasts (Figure 31).

Mustata et al. concluded that spheroids are made of poorly differentiated intestinal cells with progenitor/stem cell characteristics different from those of adult crypt base columnar cells (CBCs) and that fetal spheroids have the potential to generate adult-type CBCs. Spheroids cells correspond to incompletely caudalized progenitors (Mustata et al. 2013).

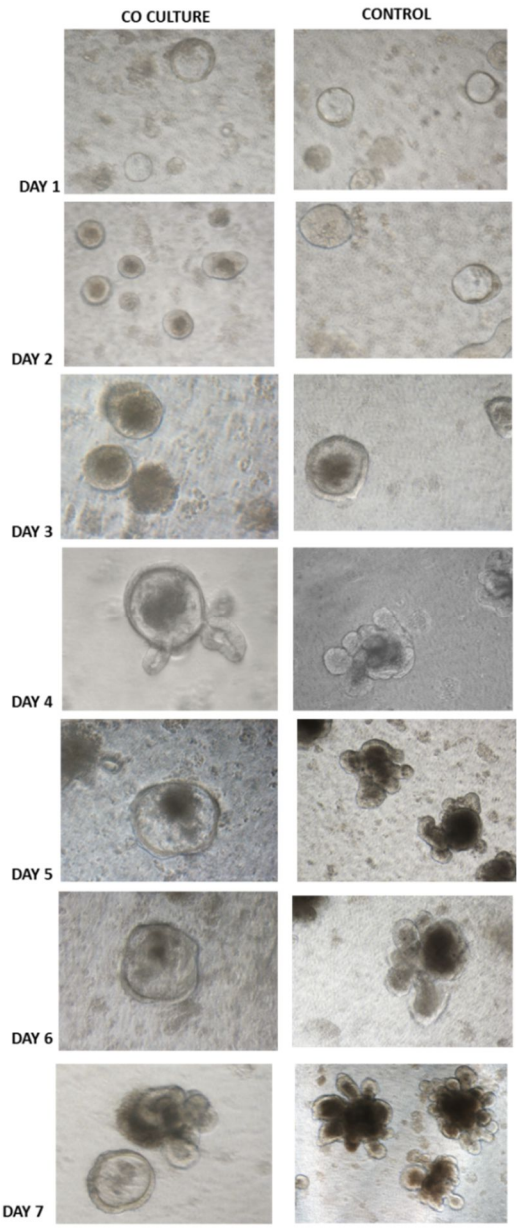


Figure 30. Phase contrast images of developing mouse intestinal crypts into organoids or spheroids in culture from day 1 to 7. **A.** Macroscopic image of the co culture spheroids development after crypt isolation; **B.** Organoid in culture with all the growth factors (EGF, Noggin, R Spondin1) from isolation (DAY 1) to mature organoid 7 days after crypt isolation, ready to be passaged (note the accumulated debris of dead cells in the lumen of the organoid). (original magnification x 200).

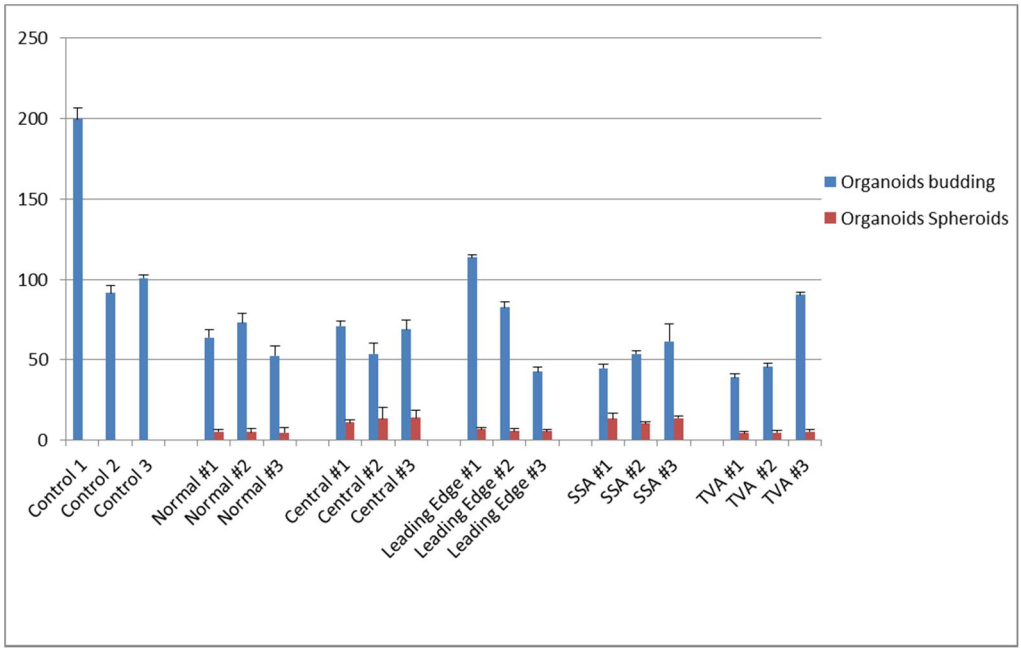


Figure 31. *Ex vivo* co culture of fibroblasts and mouse small intestine mini gut generates mixed populations of spheroids and organoids. Quantification of the percentage of spheroids and organoids obtained at day 7 in 3 different experiments.

4.2.4.1 Characterization of small intestinal organoids

After the intestinal villi had been co cultured for 7 days and had grown into organoids buddy or spheroid structures, they were collected and different immunostainings were performed.

In particular, H&E staining showed that the organoid were composed of a monolayer of polarized columnar epithelial cells. Alcian-blue staining for goblet cells revealed presence of mucus producing cells and the secretion of mucus into the lumen. Besides differentiated zones characterized, for example, by the presence of mucus producing cells, in small intestinal organoids proliferative zones could be distinguished. Moreover, lysozyme staining show the localization of Paneth cells, chromogranin A the endocrine cells and Alkaline P (Phosphatase) revealed the presents of the enterocytes

Interestingly, Ki-67 (a non-histone nuclear protein detected in the G1 through M phase of cell cycle staining) staining shows that cells with proliferative activity seemed to be localized along the crypts in the control whereas in the co cultured spheroids, they are distributed all around the spheroid structure (Figure 32).

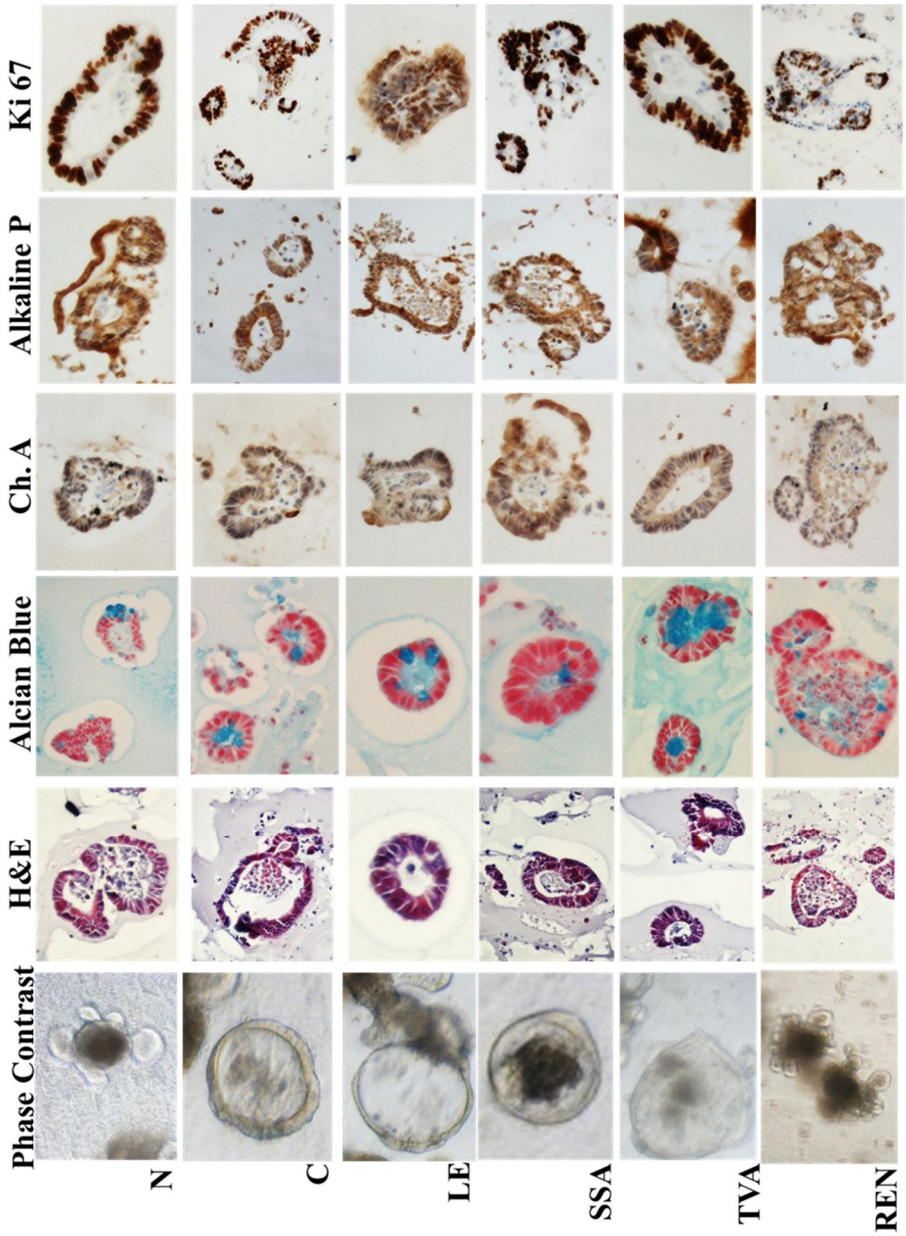


Figure 32. Mouse intestinal organoid cell type composition. Phase contrast images of spheroids and organoids (note the accumulated debris of dead cells in the lumen of the organoids). H&E staining. Markers of the different cell types were used to show differentiation. Alcian Blue staining (*blue*) for goblet cells, Chromogranin A (*brown*) for enteroendocrine cells, Alkaline phosphatase (*brown*) for mature enterocytes, and and Ki-67 (*brown)* for the evaluation of mitotic activity staining of small intestine organoids (original magnification x 200).

Microarray analysis and gene expression arrays were performed to better characterized co cultured organoids /spheroids. In particular, differentially expressed genes in the experimental groups (2x normal, 2x central, 2x leading edge (LE), 2x SSA and 2x TVA) were contrasted with the controls (2x control (REN).

The Venn diagrams based on the top 200 genes, show the differentially expressed genes between the different experimental groups (Figure 33). Of note, the number of differentiated expressed genes in the SSA and TVA co cultured organoids is significant higher compare to the others comparisons (Figure 33 B).

The hierarchical clustering heat maps reveal that by looking to the co cultured organoids with Normal, Central and Leading edge (LE) fibroblasts, there is an upregulation of the genes implicated in stemness and proliferation. In the co cultured organoids with Central and LE fibroblasts, there is a down regulation of the genes involved in differentiation whereas no differences are evident considering the apoptotic genes expression (Figure 34).

Gene Set Enrichment Analysis (GSEA) confirmed what seen in the heat maps. An enrichment was seen in the proliferative or transit amplifying pathways (DNA replication, DNA repair, RNA processing) and in the stem cells gene expression related pathway (Figure 35).

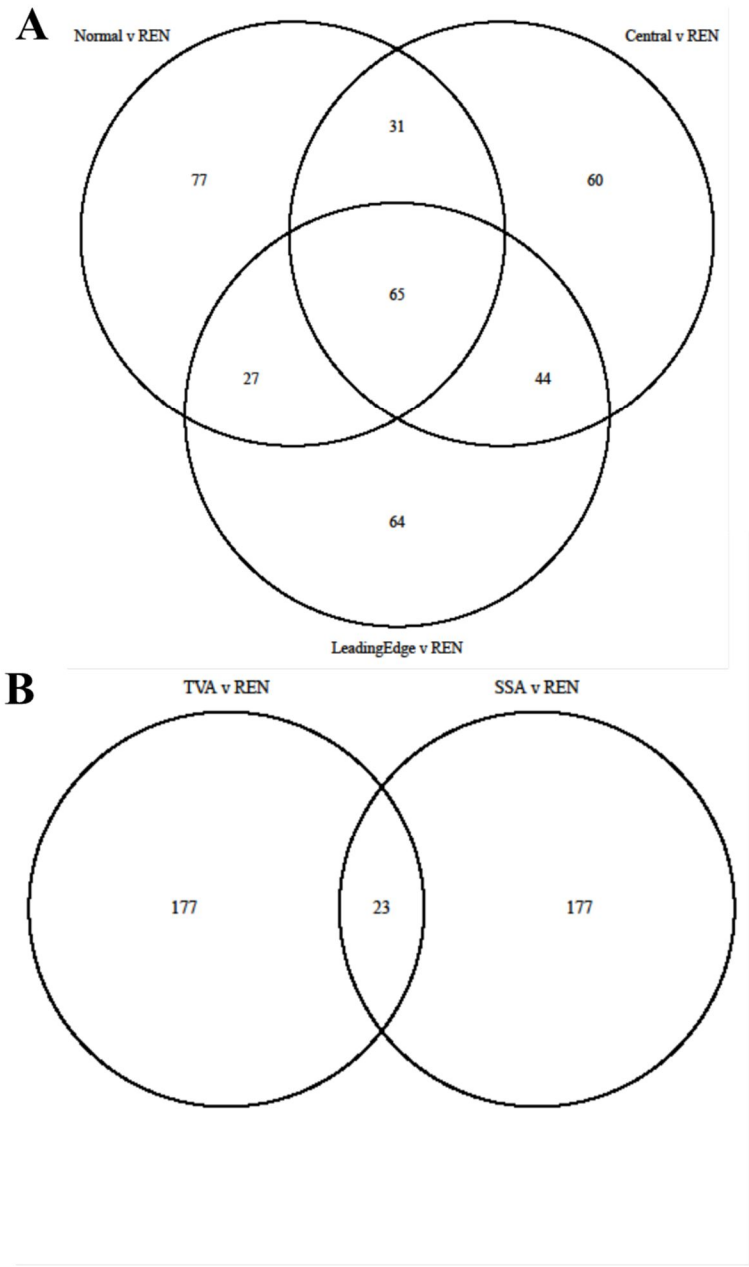


Figure 33. Venn diagrams showing the differentially expressed genes overlaps in **A)** NORMAL, CENTRAL, LEADING EDGE, **B**) SSA and TVA co cultured organoids compared to organoids mono cultured (REN). *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR <5%.

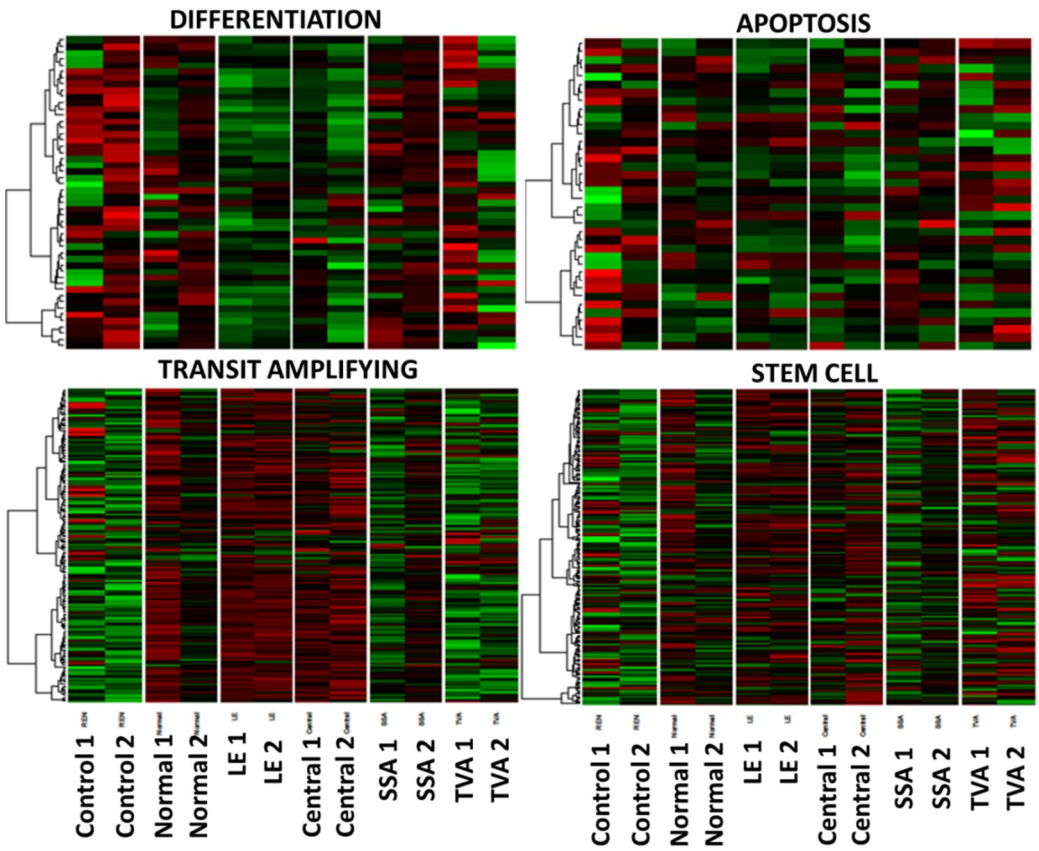


Figure 34. Hierarchical clustering heat map of the genes based on 4 custom gene sets with significant differentially expressed changes with *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR<5%. Each column represents a sample; each row refers to a gene. The color bar shows the fold change and corresponding color depth. Gene expression changes with respect to median changes are denoted by: red, up-regulated (ratio ≥1.5); green, down-regulated (ratio <1/1.5); and black, unchanged.

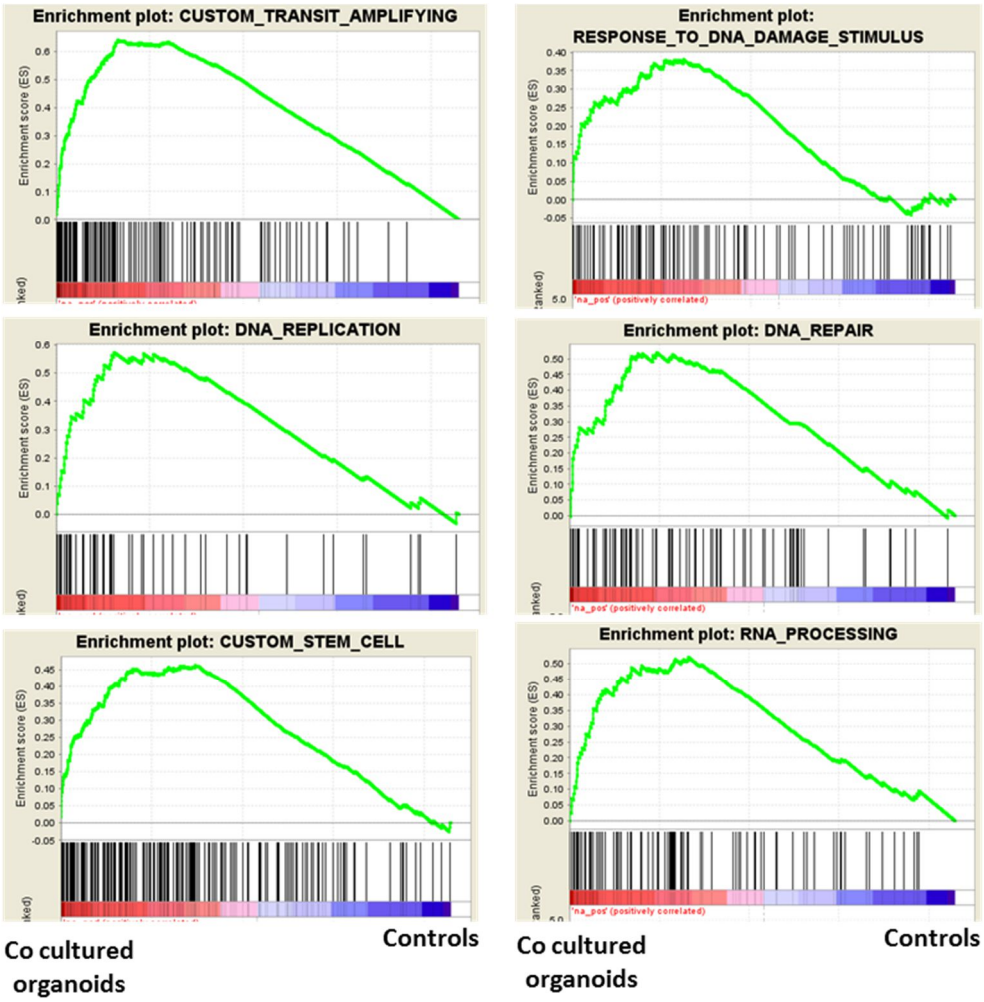


Figure 35. GSEA analysis using established gene program sets of co cultured mouse organoids. GSEA plots shown are for NORMAL, CENTRAL, LEADING EDGE, SSA and TVA co cultured organoids versus organoids mono cultured (REN). Enrichment score is calculated using Kolomogrov-Smirnov test. Gene shuffling with 1,000 permutations to compute the *P*-value for the enrichment score was used.

4.2.4.2 Fibroblasts conditioned media and small intestine organoids culture

Collected fibroblasts conditioned media was used to better understand the influence of the fibroblasts secreted factors to the organoids culture system.

This experiment validate and confirm what see in the in the previuos co culture experiment. In fact, the controls that were cultured in organoids' medium supplemented with EGF, Noggin, R-Spondin-1 (REN) formed organoids (Figure 36 A). When cultured with conditioned media supplemented with EGF and R-Spondin-1 (RE), the spheroids phenotype was decreased (Figure 36 B, E). If the organoids where grown in the presents of all the growth factors, the size of the spheroids was increased as well as the number of organoids (Figure 36 C, E). Lastly, when organoids were grown in media supplemented by the R-Spondin-1(R) an increase in the number but not in the size of the spheroids was seen (Figure 36 D, E).

In a similar manner to the fibroblasts, fibroblasts secreted factors present in the conditioned media supported the organoids/spheroids development.

4.2.4.3 Assessment of organoid tissue reprogramming

To see whether this is a co-culture dependent phenomenon or an imprinted change in the epithelium, the established spheroids were removed from the co culture and after 72 hours, in the absence of fibroblasts, the organoids lose the spheroid morphology and become "budding" organoids.

Thus, this result demonstrates that the induced epithelial tissue reprogramming was lost in the absence of fibroblasts or the fibroblast derived secreted factors.

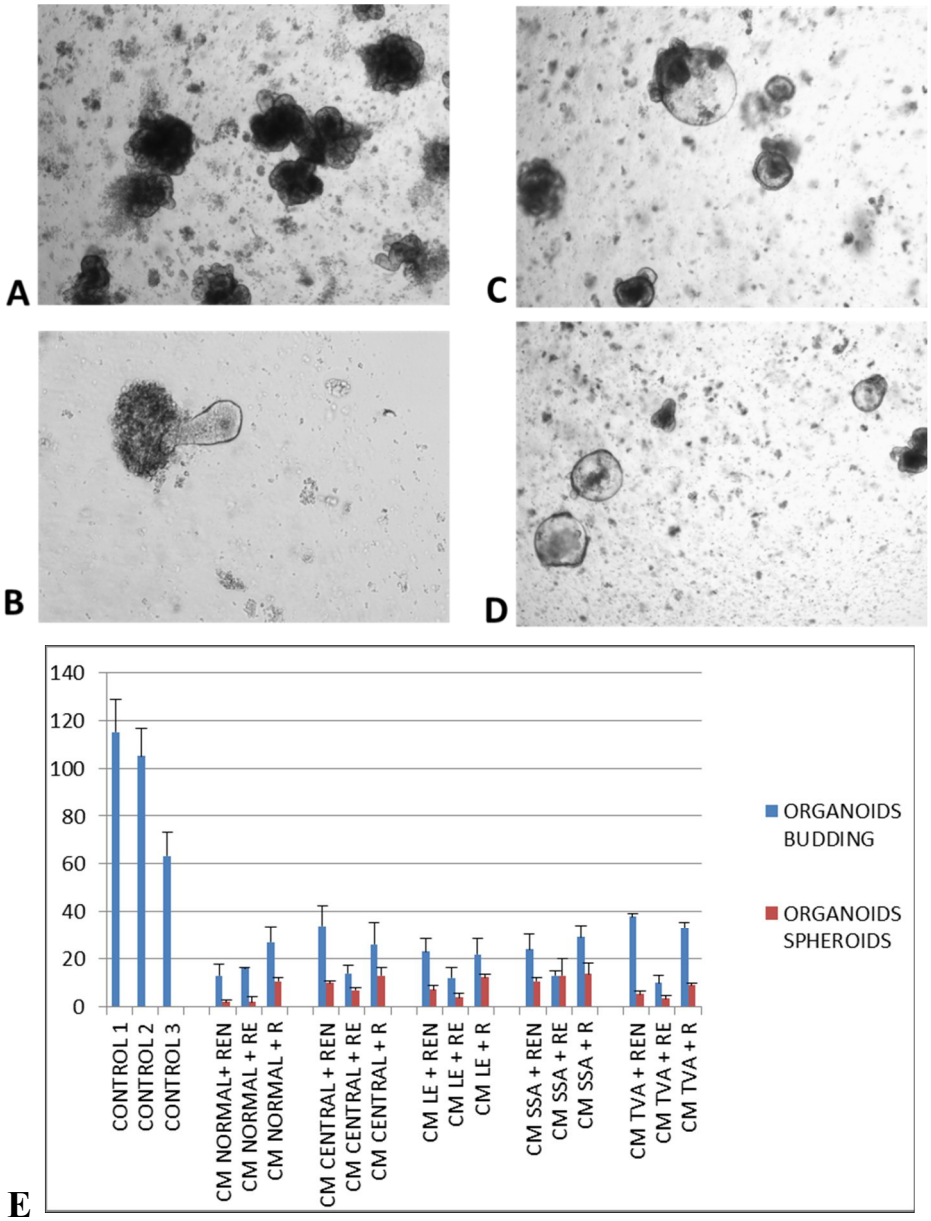


Figure 36. Organoids budding and spheroids after 7 days of culture. A) Wild type organoids cultured in organoids' medium supplemented with EGF, Noggin, R-Spondin-1 (Control) **B**) Wild type organoids cultured in fibroblasts conditioned medium supplemented with EGF and R-Spondin-1 (RE); **C**) Wild type organoids cultured in fibroblasts conditioned medium supplemented with EGF, Noggin, R-Spondin-1 (REN). **D**) Wild type organoids incubated for 7 days in fibroblasts conditioned medium containing R-Spondin-1(R); (original magnification x100). **E**) Histogram showing the numbers of organoids or spheroids in each experimental condition.

4.2.5 Modeling epithelial - stromal interactions

Each tissue compartment profoundly influences the behavior of the other with epithelial and stromal compartments co-evolving as neoplasia progresses to result in an optimal tumour microenvironment.

Co culture of normal fibroblast with non-transformed human colonic epithelial cells and their isogenic derivative was performed in order to see how individual epithelial mutations influence stromal gene expression.

The Venn diagram shows an higher number of differentiated expressed genes in normal fibroblasts co cultured with HCEC APC cells (DEG=67), followed by HCEC R cells (DEG=33), HCEC RP cells (DEG=21) and finally non mutated HCEC OneCT (Figure 37).

The hierarchical proliferation clustering heat map reveals, once again, the variability of the genes expression in the transit amplifying or proliferative gene list. The first difference that it is evident is related to the origin of the fibroblasts (from Patient 1 and 2). Moreover, APC epithelial mutated cells seems to cause a negative regulation of the genes expressed by the fibroblasts whereas KRAS alone (P) or in combination with TP53 (PR), cause a positive regulation of the genes expression in the fibroblasts (Figure 38).

It seems that, initiating APC and KRAS epithelial (epi)mutations, have variable effects on the underlying stroma and that confirm the bi-directional epithelial-stromal interaction seen *in vivo*. However, the variability seen in the experiment did not allow drawing a definitive conclusion.

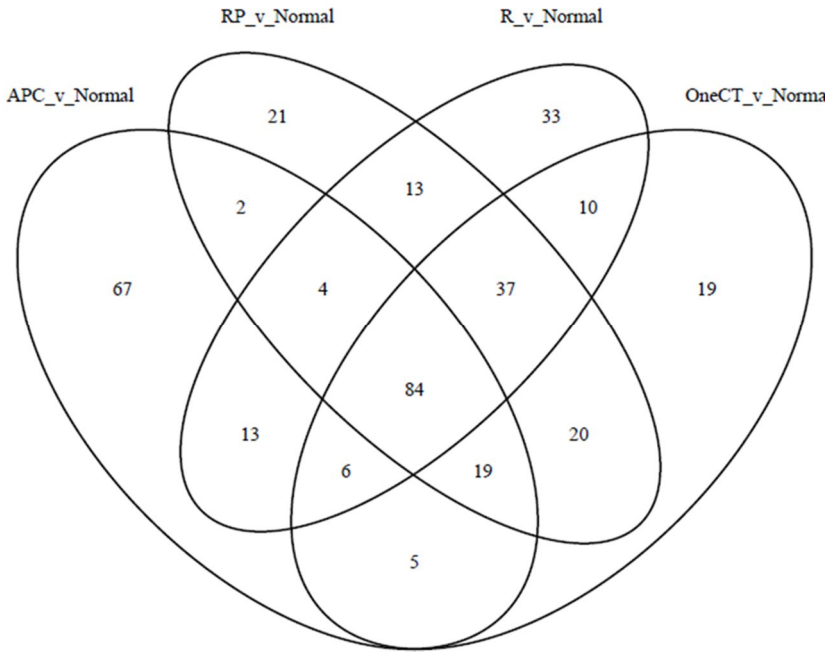


Figure 37. Venn diagram based on the top 200 genes from each list showing the differentially expressed genes overlaps in fibroblasts co cultured with HCEC APC (APC), HCEC R (R), HCEC RP (RP) and non mutated HCEC (OneCT) compared to normal fibroblasts (Normal). *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR <5%.

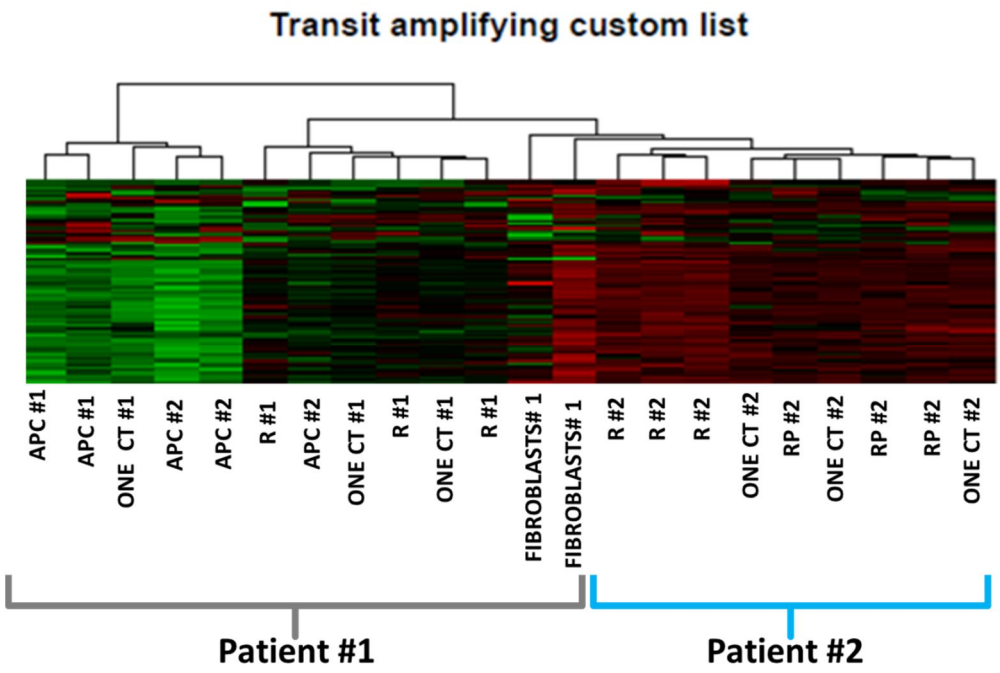


Figure 38. Hierarchical clustering heat map of the genes based on 4 custom gene sets with significant differentially expressed changes with *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR<5%. Each column represents a sample; each row refers to a gene. The color bar shows the fold change and corresponding color depth. Gene expression changes with respect to median changes are denoted by: red, up-regulated (ratio ≥1.5); green, down-regulated (ratio <1/1.5); and black, unchanged.

CONCLUSIONS

The focus of the majority of studies regarding colorectal cancers has been the genetic abnormalities of the cancer cell itself, treating colorectal tumorigenesis as a cell-autonomous process governed exclusively by the abnormal cancer genes (Calvert & Frucht 2002, Chang et al. 2000, Oving & Clevers 2002, Lin et al. 2002, Frederiksen et al. 2003, Jubb et al. 2003). However, it is becoming evident that, if clinically relevant discoveries are to be made in cancer biology and treatment, emphasis needs to be refocused to the "entire" tumor, which includes the epithelial cancer cells and surrounding reactive stromal components.

Recent analysis has demonstrated that in cancer, stromal gene dysregulation contributes more to poor prognostic molecular signatures than the epithelium itself (Calon et al. 2015, Isella et al. 2015), highlighting the important influence of the tumor microenvironment on cancer epithelial cell behavior. What is less clear is whether these dynamic and important mesenchymal changes arise reactively, in response to invasion by malignant epithelium, or occur earlier and have a role in driving lesion initiation and progression.

Moreover, the relative importance of the epithelial and stromal tissue compartments varies between different tumour subtypes and this contributes to the observed clinical and molecular heterogeneity of CRC (Calon et al. 2015, Isella et al. 2015). However, the role of the stroma in different precancerous pathologies and in CRC, is not clear. Furthermore, epithelial and stromal separation is technically difficult in established tumours preventing insight into the tissue compartmental origin of the disrupted gene expression signatures.

Thus, the main goal of this thesis has been to gain molecular insight into the development of CRC. Preliminary results have shown that in SSAs and TVAs, the transcriptome of the stromal compartment is

very different from the epithelium. In particular, in the SSAs, that are the precursors of the serrated adenocarcinomas, the number of differentially expressed genes in the stroma was greater compared to that found in the TVAs, the precursor lesions of the conventional adenocarcinomas. This led to the conclusion that serrated lesions, usually initiated by BRAF mutations and methylation, require the recruitment of pro-tumorigenic stroma to enable lesion progression. In contrast, tubulovillous adenomas are initiated by epithelial mutations that disrupt the Wnt-β-catenin signaling pathway (such as APC) and this is sufficient to drive tumourigenesis, irrespective of stromal influences.

Unfortunately, the differentially expressed genes found in the epithelial and stromal compartment of VillinCreER^{T2}; Apc$^{fl/fl}$ and VillinCreER^{T2}; Braf^{V600E} mouse models used in this study, were not comparable to those found in human adenomas showing the limitations of these two mouse models in mimicking genomic alterations in the development and progression of CRC.

The study of experimental colon carcinogenesis in rodents has a long history, dating back almost 80 years (Krebs 1928). Numerous mouse models of CRC have been developed, providing insights into pathogenesis mechanisms, tools for discovery, validation of novel therapeutic targets and a predictive platform in which to test new chemoprevention strategies (Young et al. 2013, Tong et al. 2011, Kobaek-Larsen et al. 2000, Rosenberg et al. 2009, Karim & Huso 2013, Johnson & Fleet 2013).
The hypothetical ideal animal model should mimic the human disease in terms of morphology, biochemical alterations and biological behaviour (Kobaek-Larsen et al. 2000). The “ideal animal model” that resembles the human situation in all aspects does not exist, but available models approximate many of the characteristics of human colonic carcinogenesis and metastasis. For these reasons, it is important to use a specific model to address a particular scientific question. CRC mouse model can be grouped as genetically-engineered, chemically-induced and inoculated models.

Genetically engineered mice models are useful for studying the importance of specific genomic alterations in the development and progression of CRC and their sensitivity to various therapies (Tong et al. 2011). The versatility of genetically engineered mouse models has not only facilitated identification of a wide range of potential therapeutic targets, but also enabled the study of environmental factors, such as diet, on the occurrence and severity of CRC. Conditional genetic models of colorectal cancer Cre-lox technology is one of the most frequently used methods to study the very early stages of tumourigenesis by conditionally inactivating genes of interest to produce an almost endless possibility of different DNA disruptions allowing having full spatial and temporal control over DNA mutations (El Marjou et al. 2004).

The chemically induced mouse models mimic human sporadic colorectal cancer and are often used to study effect on the treatment or prevention of CRC formation (Tong et al. 2011).

The inoculated colorectal cancer models recapitulate some features of colorectal cancer metastasis and are useful models for anti-metastatic drug evaluation (Tong et al. 2011, Hung et al. 2010). Other models have been used to address specific questions like how aging, or alcohol consumption, or diabetes affects the risk of developing CRC (Karim & Huso 2013).

There are many advantages to studying the pathogenesis of carcinogen-induced CRC in mouse models, including rapid and reproducible tumour induction and the recapitulation of the adenoma– carcinoma sequence that occurs in humans. Mouse models do recapitulate the complex, nuanced and intercompartmental signaling that can't be easily achieved *in vitro*. Moreover, the availability of recombinant inbred mouse panels and the ever-increasing number of transgenic knock-out and knock-in genetic models further increase the value of the studies (Rosenberg et al. 2009).

These models allow testing various therapeutic modalities that would not be possible in humans. However, the sequence of genetic events that are thought to promote tumorigenesis from epithelial

cells to carcinomas in humans has only partially been successful modelled in mice. For example, the role of oncogenes such as KRAS has not yet been recapitulated in mice (Karim & Huso 2013). Also, differences in mouse size and physiology, as well as variations in colon cancer that develops in mice and humans may also lead to translational limitations (Tong et al. 2011).

Despite the progress made in the development of animal models of human CRC, there are several clear weaknesses with these models.

First, all of the global gene deletion and chemically-induced models develop cancer outside the colon; in some models colon cancer is a minor phenotype. As a result, long-term studies of CRC development are limited due to high morbidity and mortality from these other phenotypes. Induced mutations of the Apc gene in mice have provided animal models that are similar to human colon polyposis, although most polyps are formed in the small intestine rather than in the colon (Taketo 2006). It should be noted that whereas FAP patients mainly develop polyps in the colon-rectum, Apc mouse models are characterized by adenomas clustering in the upper gastrointestinal tract, mainly in the duodenum. This anatomical difference between the mouse and human adenomas may exert a confounding effect in the analysis as duodenum and colon-rectum represent distinct organs (Gaspar et al. 2008).

Second, only some of the genes relevant to human colorectal cancer have been modified to make mice with floxed alleles. As a result, we are often left with models where the cancers develop early in life and their development may be confounded by the anabolic environment of growth. In addition, the lack of mice with floxed gene alleles limits the use of tools for both intestine-specific and inducible gene modifications in mice.

Third, only a few of the animal models for colorectal cancer are relevant to natural initiating events that drive human colorectal cancer. This limits our ability to study the initial stages of colorectal carcinogenesis as well as primary cancer prevention in animal models. Finally, only a few mouse models have been developed to study metastatic events (Johnson & Fleet 2013).

In summary, despite the inability of a model of CRC to completely recapitulate all stages of the human disease, the use and impact of the available models has been far reaching (Young et al. 2013). All of the models available have their own strengths and weaknesses, however it is the sheer range of mouse models available that make them the most versatile tool for researchers (Johnson & Fleet 2013). The careful selection of an appropriate model, allow asking questions regarding the initiation, progression and development of CRC. However, as none of these models recapitulate the process of CRC development in its entirety, it is important to use a specific model to address a particular scientific question and bypass the imperfections of each individual model using supporting data gained from a different model. Moreover, mouse models can be combined with the use of computational modelling and bioinformatics in order to increase the impact of the data that they produce (Young et al. 2013). With this in mind, the development of targeted therapeutics for the treatment of CRC can only be possible through the use of a combination of the different model platforms (Young et al. 2013). Therefore developing mouse models and related methods to discover and validate candidate genomic CRC drivers that play an important role in human CRC is urgently needed for translation of CRC sequencing advances into new, safe and effective chemopreventives and treatments.

In this study, in order to better characterize the stroma, I also focused on CAFs, the main cellular components of reactive stroma in primary and metastatic cancer. It has been shown that they play a key role in CRC development (Kalluri & Zeisberg 2006). In particular, preliminary results have shown that human normal, adenomas and CRC primary fibroblasts support the cross-species generation of normal mouse epithelial organoids and abrogate the normal requirement for Noggin and Epidermal growth factor (EGF) supplementation in the media. Moreover, they promote the poorly differentiated intestinal cells with progenitor/stem cell characteristics in the epithelial compartment (spheroid phenotype). Furthermore, I have found that in all the co culture experiments,

stromal fibroblasts isolated from normal colonic tissues, precursor lesions and CRC promote cell proliferation and cell cycle regulation by upregulation of genes involved with cell cycle progression, DNA synthesis/repair, protein translation, vesicles mediated transport and lipid metabolism.

Studying the genetics of normal, adenomas and cancer associated fibroblasts can help to identify those genes and the pathways responsible for the stromal gene dysregulation that contributes to poor prognostic molecular signatures seen in CRC (Calon et al. 2015, Isella et al. 2015).
In this study, the majority of the genes used for the transcriptome characterizations, were based on genes lists created for epithelial intestinal cells. Few studies have, indeed, explored the characteristics of CAFs in primary colon adenomas and cancers. The heterogeneity that exists in CRC patients was found as well in the isolated fibroblasts analyzed by this thesis and that didn't allow, in some cases, to draw valid and reliable conclusions.
As a matter of fact, primary culture has limitations. In fact, it cannot recapitulate the complex endocrine, autocrine and paracrine interactions between parenchymal and stromal cells that are considered pivotal for metastasis, proliferation and angiogenesis in CRC microenvironment. Furthermore, cell lines undergo molecular changes, hence do not represent the *in vivo* situation accurately.

Indeed, RNA microarray studies provide the potential to greatly enhance our knowledge of the genes and pathways involved in the physiological responses to physiological stressors, drugs, environmental stimuli and in pathogenesis of diseases. Managing and mining the huge amount of data generated by microarray experiments remains a major challenge. This is because microarray analysis challenges the traditional hypothesis driven method of investigation and shifts the emphasis towards hypothesis generation. Validation of microarray expression trends using a second readout remains a critical requirement. This is especially important if the sample size is too small to allow rigorous statistical analysis (as

shown in this study). It is also worth remembering that gene expression studies measure mRNA levels thereby provide a snapshot of relative mRNA abundance at the time of measurement and no more. Since most genes are also highly regulated at the post-transcriptional stage, changes in mRNA levels may not necessarily reflect changes at the protein level. Thus, follow-up studies in a larger sample will have more power to find subtle differences.
Furthermore, the integration of this technology with the appropriate post-microarray validation experiments like, for example, the power of mouse models, as testing in humans is limited. Mouse models can be used to explain the underlying biological mechanisms found in this study with the purpose to better understand the role of CAFs in human CRC pathogenesis. Mouse studies are time- and cost effective and they share 99% of their genes with humans. This will allow drawing more confirmative conclusions.

In conclusion, primary fibroblast cell cultures might in part represent the corresponding cancer microenvironment. Preliminary results, confirm that cancer cells and surrounding stroma cooperate in tumor angiogenesis and invasion through an active autocrine and/or paracrine fashion. Importantly, the expression of genes related to angiogenesis, invasion and metastasis, cell adhesion and proliferation were altered, thus further supporting the proposed critical role of cancer stroma in providing a favorable environment for cancer proliferation and invasion. Understanding the cellular and molecular processes governing stromal influence on epithelial cell biology at all stages and in all subtypes of colorectal tumours will be vital to clinically risk stratify patients with colorectal polyps, identify novel therapeutic targets and assist in effectively harnessing the power of the endogenous immune system. Future therapies directed to blocking the cross-talk between stromal elements and epithelial cells may provide a more effective approach to prevention and treatment of colon cancer.

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