Tumor biology and immunology of colorectal cancer

Studies of prostaglandin E_2 signaling versus clinical outcome in disease models and patients with metastatic disease

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1. Abbreviations

AA – arachidonic acid

Ab – antibody

AC – adenyl cyclase

Ag – antigen

AKAP – A-kinase anchoring protein

APC (*gene*/protein) – adenomatous polyposis coli

AOM – Azoxymethane

ATP – adenosine 5`-triphosphate

b-FGF – basic fibroblast growth factor

cAMP – cyclic adenosine monophosphate

CBP – CREB-binding protein

CD – cluster of differentiation

CEA – carcinoembryonic antigen

CIMP – CpG island methylator phenotype

CIN – chromosomal instability

CK-1 – casein kinase 1

COX – cyclooxygenase

CRC – colorectal cancer

CRLM – colorectal cancer liver metastasis

Csk – C-terminal Src kinase

CTLA-4 – cytotoxic T lymphocyte antigen-4

DNA – deoxyribonucleic acid

DC – dendritic cell

Dsh – Dishevelled

EGFR – epidermal growth factor receptor

EP – E Prostanoid receptor

FAP – familial adenomatous polyposis

FOBT – fecal occult blood test

FOXP3 – forkhead box protein 3

GPCR – G-protein-coupled receptor

GSK-3 – glycogen synthase kinase 3

HLA – human leukocyte antigen

HNPCC – hereditary nonpolyposis colorectal cancer

HSC – hematopoietic stem cell

IBD – inflammatory bowel disease

IFNγ – interferon γ

IGF-1 – insulin-like growth factor 1

IGFBP-3 – insulin-like growth factor binding protein 3

IL – interleukin

IPEX – immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome

Lck – lymphocyte-specific protein tyrosine kinase

LEF – lymphocyte enhancer factor

LPS – lipopolysaccharide

MHC – major histocompatibility complex

MSI – microsatellite instability

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – natural killer

NSAID – nonsteroidal anti-inflammatory drug

p53 – protein 53

PBMC – peripheral blood mononuclear cells

PG – prostaglandin

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

PKB – protein kinase B / AKT

PPARδ – peroxisome proliferator activated receptor

PSC – primary sclerosing cholangitis

PTK – protein tyrosine kinase

SEB – staphylococcal enterotoxin B

TAA – tumor-associated antigen

TAMs – tumor-associated macrophages

TCF – T cell factor

TCR – T cell receptor

TGF – tumor growth factor

Th – T helper

TME – total mesorectal excision

TNFα – tumor necrosis factor α

TNM – tumor, node, metastasis

Tp53 – (*gene*) tumor protein 53

Treg(s) – regulatory T cell(s)

VEGF – vascular endothelial growth factor

WHO – World Health Organization

Wnt – wingless integration 1

2. List of publications included

I   ***Brudvik KW., Paulsen JE., Aandahl EM., Roald B., Taskén K.*** Protein kinase A antagonist inhibits β-catenin nuclear translocation, c-Myc and COX-2 expression and tumor promotion in *Apc*$^{Min/+}$ mice. **Molecular Cancer 2011, 10:149.**

II   ***Brudvik KW., Bains S., Seeberg LT., Labori KJ., Waage A., Taskén K., Aandahl EM., Bjørnbeth BA***. Aggressive Treatment of Patients with Metastatic Colorectal Cancer Increases Survival. **Submitted manuscript.**

III   ***Brudvik KW*., Henjum K*., Aandahl EM., Bjørnbeth BA., Taskén K.*** Regulatory T cell-mediated Inhibition of Anti-tumor Immune Responses is Associated with Clinical Outcome in Patients with Liver Metastasis from Colorectal Cancer. **Cancer Immunology and Immunotherapy 2011 [Epub ahead of print].** *Both authors contributed equally.*

3. Introduction

Current treatment of solid cancers includes a multimodal approach [67,129,159]. New radiological modalities improve disease staging giving the surgeon valuable preoperative data and help the early detection of recurrent disease. Adjuvant chemotherapy and radiotherapy contribute to a prolonged life and increased survival [67,130]. All of the above modalities are relevant in the treatment of cancer in the colon and rectum, colorectal cancer (CRC). The overall treatment of CRC has improved in recent years, and the surgical procedure now has low complication and local recurrence rates [119]. Standardization of the surgical procedure, centralizing the surgery to centers with sufficient patient volume and increased radical resections (e.g. total mesorectal excision in rectal cancer) are some surgical advances in recent years. Laparoscopy has benefits on recovery after surgery [194] leaving the patients available for post-operative adjuvant therapy faster and may indirectly affect the oncologic outcome. In conclusion, treatment of primary CRC is fairly effective with 5-year survival above 90% if the cancer is confined to the submucosal layer of the primary site (Stage I disease, see later regarding CRC staging). However, many patients present with distant metastases, a condition that dramatically reduces the chance of long-term survival, thus leaving a significant challenge for future CRC treatment.

CRC treatment can be directed towards three different stages of the disease. The first is the tumor formation, the second the primary cancer and the third is the systemic disease. The greatest future benefits in CRC treatment may not be at the level of the primary tumor, but in understanding early steps in disease development with respect to prevention, early detection and treatment, as well as in improved treatment of distant disease when it presents.

Colorectal cancer develops from the bottom of normal crypts in the colon or rectum. It is a process thought to progress in multiple steps, both morphologically (adenoma to carcinoma sequence) [158] and at the level of the genome, with genetic and epigenetic alterations different from the normal intestinal epithelial cell [57]. Mutation of the adenomatous polyposis coli (*APC)* gene is considered a gatekeeper of early colorectal tumor development [136], directly affecting cytoplasmic β-catenin levels. β-catenin is the main mediator in the Wnt signaling pathway [63]. Recently, a novel interaction between the Wnt pathway and the PGE_2-cAMP-PKA pathway was described in stem cells [73,83,186]. The PGE_2-cAMP-PKA pathway is previously well characterized and shown to be relevant in a broad variety of cellular mechanisms. We set out to identify the novel interaction between the

two pathways and its relevance in cells from human CRC and in an animal model of CRC (Paper I). Furthermore, we describe the clinical course in patients after liver resection for colorectal liver metastasis (CRLM) and discuss this in relevance to tumor biology supporting the arguments for aggressive surgical and adjuvant treatment (Paper II). Our findings in patients with CRLM suggest that patient selection is important and a challenging task. Based on previous findings, we hypothesized that tumor immunology may play a role in the course of CRC [206]. We therefore investigated the relevance of regulatory T cells (Treg)-mediated anti-tumor immunity acting through the COX-2-PGE$_2$ immunomodulating pathway with respect to outcome in patients with CRLM (Paper III).

3.1 Colorectal cancer (CRC)

Colorectal cancer (CRC) is the fourth leading cause of cancer deaths worldwide in both men and women (WHO, 2008). CRC is the cancer of the colon and the rectum and approximately two thirds are located in the colon. The disease is often considered as one, irrespective of location, but should probably be regarded as two distinct entities as biology, risk factors, treatment and outcome may differ [112,169]. Most CRCs (95%) are epithelial cancers (adenocarcinoma) that originate from glandular tissue. Other, rarer cancers in the colon and rectum are squamous cell carcinoma and lymphoma. For the purpose of this thesis, adenocarcinoma of the colon or rectum will be referred to as CRC.

In Norway, 2405 and 1219 patients were diagnosed with colon and rectum cancer in 2009, respectively (Cancer Registry of Norway). Norway thus has one of the world's highest incidences of CRC (42.2 and 34.8 per 100,000 per year for males and females, respectively). The incidence in the Norwegian population has been increasing more than in neighboring countries, with assumed comparable risk exposure and genetic background [185]. The reason for this remains obscure, but may be explained by alterations in the risk factor exposure panorama at different time periods in the different countries. However, this is not an established fact, and the possibility of previously undetected genetic or environmental risk factors cannot be ruled out. For the last 5-year period this has leveled off and there was no increase in the CRC rates from 2005-2009 compared to the preceding 5-year period (Cancer Registry of Norway, 2009).

Several countries are initiating screening programs to detect and treat early disease stages with the aim of improving long-term survival [85]. In Norway, a CRC screening pilot

study that involves full colonoscopy has been funded and will be initiated in the near future (NORCCAP-2, Cancer Registry of Norway). Fecal Occult Blood Test (FOBT) and flexible sigmoidoscopy have been tested in randomized clinical trials and have shown to reduce mortality from CRC [28]. While the sensitivity and the specificity of the FOBT have improved recent years, it is still not optimal. Sigmoidoscopy, on the other hand, has better sensitivity and specificity, and also has the advantage that adenomas can be removed during the procedure, reducing the incidence of later invasive cancer [27,36]. However, sigmoidoscopy is a procedure that involves cost-benefit discussions and provides discomfort for the patient. In addition, proximal cancers will require colonoscopy to be detected. A need for novel screening methods is emerging, and studies with respect to improving the screening quality are ongoing. Promising studies open for the possibility of using cancer-specific DNA methylation patterns in epithelial colorectal cells in human stools as a non-invasive screening test for CRC and adenomas [96]. Even though screening may detect early cancers, thus leading to increased chance of survival, this does not alter the fundamental problem that CRC incidence is high, and increasing in many countries as diets become more Westernized [35]. Consequently, a better understanding of risk factors and early CRC development must be persued.

The main reason for death in connection with the presentation of primary CRC is complications associated with the onset of bowel obstruction (ileus). Ileus is a symptom seen in circumferentially growing tumors, most often located in the descending or sigmoid colon, and patients may present with a dangerous ileus state as the debut symptom. Tumors located in the cecum and ascending colon tend to grow outwards (exophytic growth) and patients are more likely to present symptoms of anemia and abdominal discomfort before any acute life threatening condition. However, as endoscopy (sigmoid- and colonoscopy) is becoming more available and patients are referred at an earlier stage, most CRCs are discovered before they cause total bowel obstruction and are surgically resectable at presentation. Few patients die from complications related to the primary cancer. Furthermore, the frequency of local recurrence after resection for primary CRC is relatively low (10-20%) [126,149] compared to the frequency of recurrence in distant organs (~55%). Local recurrence is more common after resection for rectum cancer than colon cancer [180], but the frequency of both has declined after the implementation of adjuvant chemotherapy and improved surgical techniques, especially after the introduction of neoadjuvant radiotherapy and total mesorectal excision (TME) of rectum cancer [54].

In colon cancer, the surgical resection technique is under evaluation with respect to whether the resection should include central lymph node stations or not [99,103,114]. Colon is attached to the posterior abdominal wall via mesocolon that contains lymph node stations at different distances from the intestinal segments they drain. A mesocolic resection line close to the colon removes lymph nodes in close proximity to the bowel leaving the possibility of remaining disease in distant lymph nodes. Resections close to the central departure of intestinal vessels includes more draining lymph nodes, increasing the chance of total surgical removal of malignant cells. Central resection however, is technically challenging and may cause intraoperative or post-operative complications as it may threaten the circulation to the anastomosis introducing increased risk of anastomosis leakage, a dangerous complication associated with high mortality and increased risk for local recurrence [131].

Cancer cells metastasize from the primary CRC to distant organs via blood or lymph circulation. The liver is the preferential target organ for malignant cells leaving the primary tumor and entering the circulation. Approximately 20% of the patients have detectable liver metastasis at the time of diagnosis (synchronous metastasis) and ~35% will develop metastasis in the following 5 years after surgery of the primary tumor (metachronous metastasis). Colorectal liver metastasis (CRLM) is the main cause of death in patients with CRC as ~55% of patients with CRC develop liver metastasis. Liver resection remains the only potential for cure but few are available for surgery because of extensive disease. The expected time of survival with synchronous CRLM without any treatment is short [16].

3.1.1 CRC staging

Two different staging systems are used to provide prognostic information and determine treatment strategy in CRC. The Duke classification system from 1932 [52] has been replaced by the more detailed TNM staging system developed and maintained by the American Joint Committee on Cancer (AJCC). Duke staging is no longer recommended for use in clinical practice and is not further discussed here. TNM staging (tumor, node and metastasis) is used on many solid cancers, adapted for CRC and is now in the 7th edition as of 2010. As Table I shows, the tumor is first scored with respect to the TNM variables then assigned to stage I-IV in the CRC specific Table II. However, TNM does not adapt to recent advances in metastatic treatment. For example, the survival of a patient with resectable solitary liver metastasis is better than that of a patient with stage III disease [153]. Current observations regarding the

clinical course of the patients with CRLM (and our findings presented in paper II) support emerging arguments for a new staging system in CRC [152,153,191].

TNM		Disease extension
T1		Tumor invades submucosa
T2		Tumor invades muscularis propria
T3	a	Tumor invades through the muscularis propria
	b	T1 or T2 tumor with satellite deposits in pericolorectal tissues
T4	a	Tumor penetrates the visceral peritoneum
	b	Tumor directly invades or is adherent to other organs or structures
N0		No regional lymph node metastasis
N1	a	Metastasis in 1 regional lymph node
	b	Metastasis in 2 to 3 regional lymph nodes
N2	a	Metastasis in 4 to 6 regional lymph nodes
	b	Metastasis in 7 or more regional lymph nodes
M0		No distant metastasis
M1	a	Metastasis confined to one organ or site (e.g., liver, lung, ovary, non-regional node)
	b	Metastases in more than one organ/site or the peritoneum.

Table 1. The 7th edition of the AJCC-TNM classification system. T; tumor stage, N; lymph node involvement, M; distant metastasis.

Stage		T	N	M
I		T1	N0	M0
		T2	N0	M0
II	a	T3	N0	M0
	b	T4a	N0	M0
	c	T4b	N0	M0
III	a	T1-T2	N1	M0
		T1	N2a	M0
	b	T3-T4a	N1	M0
		T2-T3	N2a	M0
		T1-T2	N2b	M0
	c	T4a	N2a	M0
		T3-T4a	N2b	M0
		T4b	N1-N2	M0
IV		Any T	Any N	M1

Table 2. The 7th edition of the AJCC-TNM classification system. Staging of CRC from I to IV based in the TNM stage.

3.1.2 Risk factors for the development of CRC

Risk factors associated with CRC are either internal (genes/stimuli) or environmental. Some risk factors are known, but as indicated in the previous, unidentified risk factors cannot be

ruled out. Increasing age [9] and a family history of CRC [33] predispose to the development of CRC, and CRC is slightly more common in men. Furthermore, genetic conditions such as familial adenomatous polyposis (FAP) [78], Lynch syndrome (formerly HNPCC; hereditary nonpolyposis colorectal cancer) [65] and Gardner's syndrome (considered a subtype of FAP [74]) are genetic risk factors. The FAP syndrome accounts for approximately 1% of all CRC cases and patients will most likely present with adenocarcinoma before the age of 40. Therefore, prophylactic colectomy is recommended at an early age for FAP patients [193]. Gardner's syndrome is a rare phenotypic variant of FAP, both caused by mutation in the *APC* gene. In addition to colon polyposis, Gardner patients acquire extra-colonic tumors including osteomas, thyroid cancer, epidermoid cysts, fibromas, sebaceous cysts and desmoids tumors [74]. The disease is not curable and life expectancy with the condition is 35-45 years. Lynch syndrome is an autosomal dominant trait (as FAP) predisposing for CRC, the associated mutations impair genes related to DNA mismatch repair. Approximately 1-4% of the cases of colon cancer are related to Lynch syndrome [65]. Interestingly, FAP in contrast to Lynch syndrome seems to be more responsive to chemoprevention by COX inhibition [31], a mechanism that will be discussed in the following sections.

Patients with inflammatory bowel disease (IBD, ulcerative colitis and Crohn's disease) have increased risk of developing CRC and the risk is more likely a result of chronic inflammation rather than genetic predisposition [190]. The progression from adenoma to carcinoma that occurs during development of sporadic colorectal tumors (discussed in the next section) appears to be a sequence of inflammation – dysplasia – carcinoma in IBD-associated CRC [190]. Primary sclerosing cholangitis (PSC) is another chronic inflammatory disease associated with CRC when concomitant with IBD [18]. Pro-inflammatory factors of the innate and adaptive immune system contribute to the development and growth of colon neoplasia.

The geographical difference in CRC incidence is interesting (Central Africa: 2.3 per 100,000 per year, Japan: 49.3 per 100,000 per year) and modifiable lifestyle factors are thought to contribute substantially to these variations [43] (Figure 1). Physical inactivity has been linked to the CRC diagnosis group as a whole, but not convincingly for rectal cancer alone [44,164,198]. Mutations in the genes *K-ras* and *Tp53* are highly relevant for CRC development and the same mutations have been linked to reduced physical activity, smoking and diets high in red meat [26]. Regular exercise may increase the number and activity of macrophages, natural killer cells, and cytokines with the ability to kill cancer cells and thereby improve anti-tumor immune responses [203]. Furthermore, obesity may be a risk

factor for CRC development independently of inactivity. Visceral adiposity is associated with low-grade chronic inflammation leading to up-regulation of the nuclear transcription factor-κB (NF-κB) with transcription of genes that promote tumorigenesis as a consequence [50].

Prostaglandin E_2 (PGE_2) is reduced with high levels of physical activity, possibly through insulin-like growth factor-1 (IGF-1) [124] and our findings show that PGE_2 affects both tumor immunity and tumorigenesis (Paper I and III). The motility of the intestine may affect CRC risk as shortened intestinal transit time reduces the exposure of the mucosa to faecal carcinogens. Exercise is thought to increase the expression of $PGF_{2\alpha}$ and the vagal tone, both stimulating motility. Diets high in fiber, fresh fruits and fresh vegetables may also shorten the intestinal transit time and the formation of fiber may produce mucosa-protecting agents, thereby affecting CRC risk. However, studies regarding diet, transit time and CRC risk are contradictory and firm conclusions cannot be drawn [8,20,25,145].

Insulin and insulin-like growth factor-1 (IGF-1) are up-regulated in the absence of physical activity and in obesity [44]. Increased levels of insulin and IFG-1 are correlated with risk of CRC [72] possibly through mechanisms that activate the Wnt pathway and increase the expression of pro-angiogenic proteins such as HIF-1α and VEGF [50]. Furthermore, elevated levels of the reciprocally expressed insulin-like growth factor-binding protein-3 (IGFBP-3) have been shown to be associated with reduced risk of CRC [91].
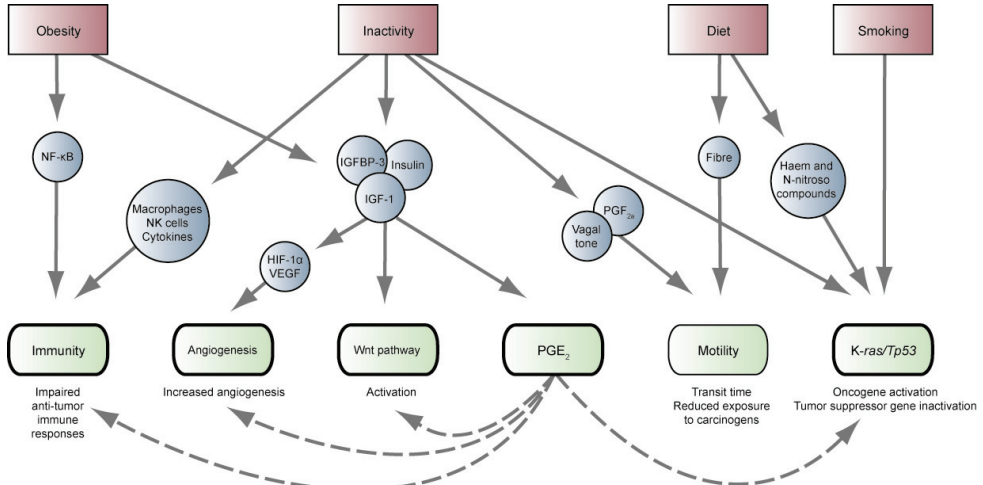

Figure 1. Lifestyle factors associated with risk of developing CRC (red boxes) and selected possible molecular mechanisms (blue circles) that are suggested to connect risk factors and mechanisms towards CRC development (green boxes). Fat lines indicate mechanisms relevant for this thesis.

It is too early to establish a causal relationship between smoking and CRC, however most reports point in the direction that smoking is a risk factor [44,93]. Furthermore, smoking may be associated with different subtypes with different mutational background than non-smokers (e.g. *Tp53*, *APC* and K-*ras*) [48]. Heavy alcohol intake (>45g/day) has been associated with increased risk of CRC, while lower levels were not [41]. The increased CRC risk has been suggested to be associated with lower intake of folate connected with heavy drinking [80].

To conclude, there is evidence that inactivity, red meat, smoking, alcohol, and obesity are connected to increased risk of CRC, but the quality of the observations varies and in some cases, there are contradictory reports. With the geographical differences of CRC in mind, risk/protective factors are an important field and if properly understood, could save many lives.

3.2 Tumor biology in colorectal cancer

The adenoma – carcinoma sequence is a well known and accepted theory describing the development of colorectal cancer. It involves multiple steps of both genetic and morphologic transformation [57,109]. In the morphologic transformation, the first step is recognized as a dysplastic crypt or an aberrant crypt focus and this step may initiate the formation of an adenomatous lesion or an adenoma. Subsequently, some adenomas undergo malignant transformation to carcinomas (Figure 2).
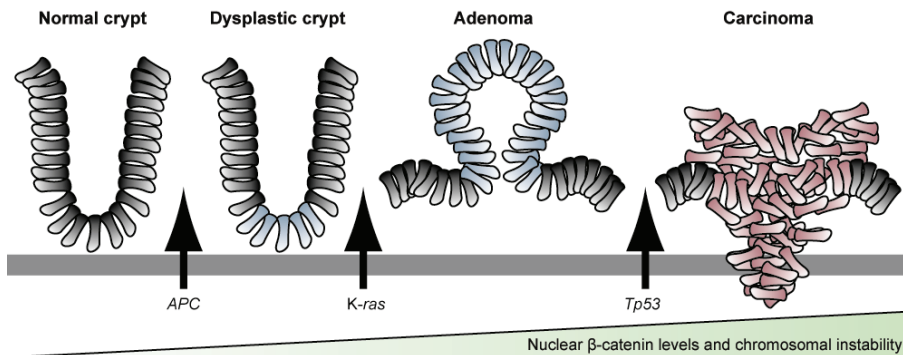

Figure 2. Adenoma – carcinoma sequence of development from normal mucosa to carcinoma. Arrows indicate associated mutations at given step.

Genetic alterations are also thought to progress in a multistep model at different stages in the adenoma – carcinoma sequence. One of the first ("gate-keeper") steps is mutation of the adenomatous polyposis coli (*APC*) gene leading to inactivation of the APC protein [136], which normally serves as a growth control of the colon epithelium, followed by activation of the oncogene K-*ras* [136], and inactivation of the tumor suppressor gene *Tp53* [11]. A mutation in the K-*ras* gene acts as a molecular "on"-switch, leading to recruitment and activation of proteins necessary for the propagation of cell growth. In a clinical setting, mutations in the K-*ras* gene are relevant by predicting the tumor's response to epidermal growth factor receptor (EGFR) inhibiting drugs (chemotherapy), thus K-*ras* positive tumors will respond poorly to these agents [115]. *Tp53* encodes the tumor suppressor protein p53, which is important in cell cycle regulation and therefore relevant in cancer. The *APC*, K-*ras* and *Tp53* mutations are associated with chromosomal instability (CIN) [110], a molecular subtype of CRC [107]. However, accumulating evidence indicates that the adenoma – carcinoma sequence is a too simplified model of a process that in fact is far more complicated and heterogeneous. At least three distinct molecular pathways that promote tumor growth have been described to be activated in CRC, supporting arguments that CRC could constitute several different genetic diseases, all affecting the same organ [140,165]. The CIN pathway, as described, is the main pathway and is observed in up to 70% of CRCs. Microsatellite instability (MSI) occurs in up to 15% of CRCs and is caused by inactivation of DNA mismatch repair genes (MMR) [22]. CpG (-Cytosine-phosphate-Guanine-) island methylator phenotype (CIMP) refers to a widespread hypermethylation (epigenetic modification that represses transcription via the promoter region of tumor suppressor genes) of CpG island loci and is demonstrated in up to 20% of CRCs [104]. All of the above pathways can overlap and the significance of these overlapping features is not fully understood [140].

3.2.1 The Wnt pathway, β-catenin and adenomatous polyposis coli

The *APC* gene is a tumor suppressor gene. In humans, *APC* mutations can be acquired (spontaneous CRC) or inherited, as in the autosomal dominant FAP syndrome, characterized by the formation of multiple colonic adenomatous polyps [78]. Inactivation of both *APC* alleles ($APC^{-/-}$) is considered necessary for tumor formation and patients with FAP carry a heterozygotic mutation, thus leaving the chance of double mutations much higher than in individuals that carry the wild type sequence. The APC protein forms a destruction complex with Axin, glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK-1) [120]. The

destruction complex phosphorylates β-catenin at Ser45, Thr42, Ser37 and Ser33, the first by CK-1, the three latter by GSK3β [186], and the indicated phosphorylation targets β-catenin to degradation by the proteasome system [1] (Figure 3).

In the absence of a normal APC protein, Axin, GSK3β and CK-1 cannot associate and form the destruction complex. The consequence is cytoplasmic accumulation and subsequent translocation of β-catenin to the nucleus [179]. β-catenin, originally discovered as a cadherin-binding protein, is the main mediator in the Wnt (wingless and integration 1) pathway and has been shown to interact with and function as a coactivator of T-cell factor/lymphocyte enhancer factor (TCF/LEF) transcription factors [63]. Human transcription factor 4 (hTCF-4), a TCF family member that is expressed in human colonic epithelium and colon carcinoma cells, transactivates transcription only when associated with β-catenin [29,100]. The result is expression and production of mitogenic and survival genes including c-Myc [81], cyclin D1 [187] and cyclooxygenase-2 (COX-2) [53].
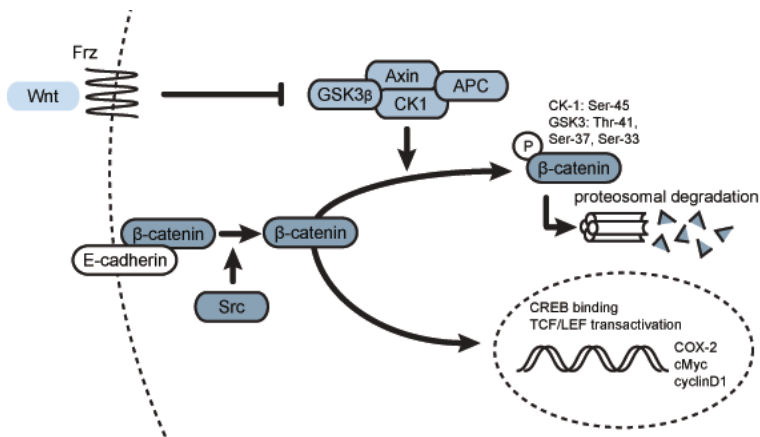
Figure 3. β-catenin is the main mediator in Wnt signaling. The destruction complex composed of GSK3β, Axin, CK1 and APC phosphorylates and thereby targets β-catenin to proteosomal degradation. In the absence of APC/destruction complex β-catenin accumulates in the cytosol and subsequently translocates to the nucleus.

3.2.2 Cyclooxygenase-2 in colorectal cancer

Cyclooxygenase (COX) exists in three isoforms. COX-1 is constitutively expressed in a wide range of cells and COX-2 is expressed in intestinal and other selected tissue in response to the pro-inflammatory cytokines lipopolylsaccharide (LPS), interleukin-1 (IL-1) and tumor necrosis factor- (TNFα) [51,58]. COX-3 is a splice variant of COX-1, thus by some named COX-1b or COX-1*variant* (COX-1v) [39]. COX-2 expression is elevated in inflammation as

well as in several cancers and shown to promote tumor growth via one of its products, PGE_2. COX-2 is expressed in 50% of colorectal adenomas and in 85% of human CRCs [53].
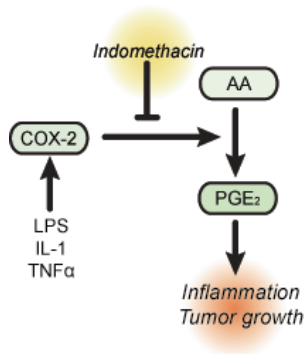
Figure 4. COX-2 cleaves AA to form different prostaglandins (here PGE_2). COX-inhibitors (here indomethacin) block the enzymatic activity of COX-2.

The enzymatic activity of COX cleaves arachidonic acid (AA) to form different prostaglandins (PG), depending on the enzymatic machinery present in the particular cell type (Figure 4). Prostaglandin E_2 (PGE_2) has been shown to be an important mediator of COX-2 associated effects, and PGE_2 levels are elevated in CRC biopsies compared to normal mucosa and even in patient blood samples [206]. PGE_2 binds four cognate prostanoid receptors EP1-4, all part of the G protein-coupled receptor (GPCR) family. EPs activate G proteins and signaling downstream by increasing the intracellular levels of the second messenger cyclic adenosine 3`,5`-monophosphate (cAMP, EP2 and EP4) or phosphoinositide (PI) signal transducers (EP3) inducing a decline in cAMP. EP1 induces calcium mobilization and constitutes a "contractile" receptor group. However, the effects of prostanoids on these G-protein coupled signaling pathways may change as a function of ligand concentration or structure.

PGE_2 stimulated EP receptor signaling events promote tumor growth by a broad range of mechanisms, and COX inhibition is therefore very interesting and relevant in CRC [197]. Peroxisome proliferator activated receptor δ (PPARδ) promotes tumor cell survival through inhibition of apoptosis and has been identified as a direct transcriptional target of the APC-β-catenin-TCF pathway which confirms the relevance in CRC. PGE_2 has been shown to transactivate PPARδ and thereby inhibit apoptosis. Subsequently, pharmalogical inhibition of COX may promote apoptosis [171]. Moreover, PGE_2 induces the expression of NF-κB, a key anti-apoptotic mediator [151].

The Ras-MAP kinase cascade is one of the major intracellular pathways responsible for cell proliferation and has been shown to be activated by PGE_2 [196], thus mediating the stimulatory effects of COX-2 on cell proliferation and transformation [202].

The expression of epidermal growth factor receptor (EGFR) on CRC cells directly correlates with the ability of the CRC cells to metastasize to the liver. Crosstalk between COX-2-PGE_2-PKB and EGFR-PI3K-PKB pathways stimulates cell migration to a further extent than EGFR alone, providing evidence that COX inhibition may block the spread of metastatic disease. COX-2 and PGE_2 over-expression has been shown to correlate with CRC

risk and metastasis of CRC, making this pathway relevant also in follow-up after treatment of the primary cancer [188].

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are angiogenic factors induced by COX-2 overexpression in CRC and important for growth and survival of endothelial cells, as well as stimulation of vascular endothelial cell migration and capillary formation. Reports have indicated that the pro-angiogenic effects of COX-2 are mediated through PGE_2 [82,177] and NSAIDs are shown to have anti-angiogenic effects in CRC through these mechanisms [125,167,174,189].

Local inflammation at the site of solid malignancy leads to accumulation of CD4^{+} T cells and CD8^{+} T cells as well as other immune cells; tumor-associated macrophages (TAMs), monocytes, mast cells, neutrophils and natural killer (NK) cells. Tumor and surrounding stromal cells express chemoattractants and release them locally. The chemoattractants stimulate migration and the infiltration of immune cells and are closely linked to further tumor progression. Furthermore, COX-2 overexpression by CRC cells may also contribute to maintaining a chronic inflammatory state in the tumor and in the tumor vicinity as well as promoting a shift in the tumor microenvironment to an immunosuppressive dominance, preventing an effective anti-tumor immune response.

3.2.3 Animal models studying COX-2-mediated effects in colorectal cancer

The study of cancer in humans is a complicated endeavor, in which ethical aspects and individual considerations often take priority over the research objective. Furthermore, humans are exposed to a variety of known and unknown environmental factors and the genetic heterogeneity can be a challenge when studying biologic mechanisms. Animal models may therefore offer opportunities to study biology *in vivo* where humans are not available or suitable. However, ethical guidelines must be strictly followed also when conducting animal experiments and the animals should not be exposed to distress beyond the purpose of the study. Alternatives to animal experiments should be considered in each case, if the biological effects could be studied in assays not involving animals.

By using a human colon cancer cell line we showed that inhibition of the COX-2–PGE –cAMP–PKA pathway, at the level of PKA, affected the Wnt pathway. We therefore wanted to investigate this in an intestinal tumor model *in vivo*. Three different animal models have been used to test the ability of COX inhibiting agents on CRC in the past. Azoxymethane (AOM) treated rats develop aberrant crypt foci, which are pre-neoplastic lesions that later

progress to carcinomas in the colon [116]. The xenograft mice model, in which cultured colorectal cancer cells are xenografted on the flanks of nude (athymic) mice, has also been used [199]. Tumor volume is measured over time in response to various treatments. The third and most known model is developed from C57BL/6J mice treated with ethylnitrosourea followed by selection for transmission of germ-line mutations. The relevance of this model to human disease was apparent when the phenotype was mapped to a mutation in the *APC* gene and thereby the name *Apc*^{Min/+} mice [182]. The *Apc*^{Min/+} mouse is a well-established model of FAP with a germline mutation in one *APC* allele, thus increasing the probability of a double allele mutation and tumor formation. *Apc*^{Min/+} mice develop multiple adenomas in the intestinal tract, mainly in the small intestine, at an early age which can be blocked effectively by COX inhibition through non-steroid anti-inflammatory drugs (NSAIDs, [15,24,87,88] and our results). In paper I we used the *Apc*^{Min/+} model to study effects of COX and protein kinase-A (PKA) inhibition on tumor formation and interactions with the Wnt pathway.

3.3 Metastatic colorectal cancer

Metastasis from colorectal cancer (CRC) to the liver (colorectal cancer liver metastasis; CRLM) is common [92]. About 20% of the patients present with synchronous metastases at the time of diagnosis and 35% of the patients will later develop metachronous liver metastases [105]. Patients with untreated liver metastases are typically thought to have an expected median survival of less than 6 months [16,111]. Hepatic resection remains the only potentially curable treatment and is now offered to 20-25% (Figure 5) of the patients whereas only 10% were selected for this treatment ten years ago [146]. The main exclusion criteria for liver resection of CRLMs are non-resectable metastasis (tumor growth into both portal branches and/or into both left and right liver vein), inadequately functioning residual liver parenchyma or non-resectable extrahepatic disease. However, these exclusion criteria have all been challenged in recent years. Close follow-up after primary CRC (early detection of metastasis), implementation of new surgical techniques including two-stage hepatectomy with portal vein embolisation [4,89] and transplantation methods, and the introduction of new chemotherapy and biological agents capable of converting inoperable cases to a resectable status by tumor downsizing [3,106,111] have increased the number of patients eligible for resection of liver metastases. Patients with resectable liver metastases have an estimated 5-year survival of 35-50% for selected cases [49]. However, in the recurring population a significant proportion
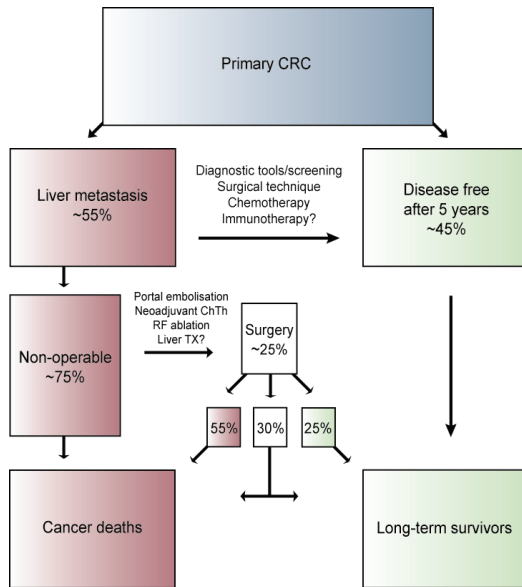
Figure 5. Simplified schematic presentation of the patient population with CRC and CRLMs. Treatment possibilities at different levels and final outcome. ChTh; chemotherapy, TX; transplantation, RF; radiofrequency.

develops rapidly progressing non-re-resectable disease even though receiving chemotherapy, and for those the benefit from surgery is questionable [111].

On this background, patient selection is important and predictive factors for the oncologic outcome after resection of CRLM should be pursued. Different predicting tools for survival in patients with resectable CRLM have been proposed. Eight different scoring systems with data from more than 300 patients with resectable CRLMs in each are presented in Table 3 [60,86,94,123,128,137,157,208]. All registered data retrospectively and used Cox proportional hazards regression to identify risk factors associated with survival, presented in Table 3. Five of the studies (Nordlinger *et al*., Fong *et al*., Iwatsuki *et al*., Rees *et al*. (referred to as the Basingstoke Predictive Index, BPI) and Kattan *et al*.) present sets of clinical parameters and merge them to produce a predictive score for the disease-specific survival after resections for CRLM [60,86,94,137,157]. Zakaria *et al*. found only preoperative blood transfusion and positive lymph nodes to be of significant importance and question the value of risk scoring systems [208], while Malik *et al*. found an inflammatory response parameter based on plasma concentrations of C-reactive protein (CRP) or neutrophil/lymphocyte ratio of more than 5:1 [123] to be significant markers. Furthermore, Minagawa *et al*. propose a simplified algorithm to determine the disease stage (defined stage 1 to 4) in CRLMs based on 369 patients and interestingly they use 229 unrelated patients to validate their simplified scoring system [128].

In paper II we examined clinical outcome after surgery for CRLM and how this may support aggressive treatment strategies. Our findings challenge the use of traditional risk factors to decide on treatment strategy. Thus, we suggest that the search for novel markers must continue. Furthermore, clinical parameters may only represent a mirror of the tumor microenvironment where the tumor biology and the extracellular context are primary and secondary determinants for progression, respectively. Clinical parameters may therefore not

be the best predictors. In paper III, we examined whether tumor immunology could affect the clinical outcome. Our results indicate that future prognostication may involve novel biological and immunological markers.

Predictive factors after resection for CRLM	Nordlinger (n=1513)	Fong (n=1001)	Iwatsuki (n=305*/243)	Minagawa (n=369/229**)	Zakaria (n=663)	Malik (n=687)	Rees (n=929)	Kattan (n=1477)
General parameters								
Gender								x
Age	x							x
DF interval from PT to LM	x	x	x					x
The primary tumor								
Site colon/rectum								x
LN status		x		x			x	x
Differentiation grade						x		
Stage	x							
The liver metastasis								
Number of metastases		x	x	x		x	x	x
Size largest tumor	x	x	x				x	x
Bilateral disease			x					x
Number of lobes								x
Hepatic resection margin	x	x	x*			x		
Regional lymph nodes	x		x*	x	x			
Extrahepatic disease		x				x		
Blood transfusion					x			
Biochemical								
CEA at hepatectomy	x	x		x			x	x
Inflammatory response						x		

Table 3. Clinical and biochemical factors reported in indicated studies to be predictive factors with respect to disease-specific survival after resection for CRLMs. *Denotes factors reported in 305 patients including patients with evidence of extrahepatic disease. **Unrelated validation cohort. DF; disease-free, PT; primary tumor, LM; liver metastasis.

3.4 Cancer immunity

From an evolutionary point of view the immune system is designed to protect the organism from foreign microbes such as bacteria, virus and fungi. As modern medicine has evolved, treatment of infections has improved and cancer is now a leading cause of death, accounting for 13.3% of all deaths worldwide (WHO, 2008). Increasing age is associated with many cancer forms. From a theoretical perspective, cancer incidence increases as more people survive other potential causes of death. Efforts should be made to improve knowledge of CRC biology and risk factors with the aim to prevent and treat the disease. In recent years, the

recognition that solid tumors contain infiltrates of immune cells and that cancer patients have higher prevalence of circulating regulatory T cells (Tregs) that potentially prevent a beneficial anti-tumor immune response, has lead to the hypothesis that the immune system may play a central role in the formation and progression of tumor development. Understanding mechanisms of anti-tumor immunity and tumor immune evasion could in the near future lead to development of strategies for prevention, discovery and treatment of cancer.

3.4.1 The adaptive immune response and T cell-mediated immunity

The task of the immune system is simple; to recognize and destroy intruding pathogens, however, the mechanisms by which the immune system performs its task are highly complex. Most microorganisms that meet the host are rejected by internal and external surface barriers. About half of the microorganisms that break the barriers are eliminated by the innate immune system characterized by a direct and universal response. The main players of the innate immune system are phagocytic cells (neutrophile granulocytes, monocytes/macrophages and dendritic cells), cells that release inflammatory mediators (basophile and eosinophile granulocytes and mast cells), antimicrobial peptides and the complement system amplifying the innate immune response. Another important task of the innate immune system is to activate the adaptive immune system by presenting peptides from foreign antigens to the players of the adaptive immune system, B cells and T cells.

The term immunologic memory is based on the ability of the immune system to remember a certain pathogen and thereby increase the response upon reinfection, which upon subsequent encounters leads to more efficient elimination of the pathogen. B cells represent the cellular basis of immunological memory and isotype-switched memory B cells, developing from naïve B cells, may differentiate to immunoglobulin producing plasma cells that migrate to the bone marrow and produce high affinity antibodies for several years. The T cell memory is based on the fact that there are more antigen specific cells than before the primary response, and that surface molecules with homing properties are present, thus the response is faster.

Adaptive immunity is initiated by the presentation of antigen by the innate immune system to B and T cells, the players of the adaptive immune system. Macrophages and dendritic cells, commonly referred to as antigen presenting cells, are equipped with receptors that recognize molecular patterns that are characteristic for different microorganisms. Binding results in uptake and processing of the microorganism by the antigen presenting cells before

presenting it as foreign peptide (antigen) on MHC-class II molecules to the T cell receptor (TCR) in germinal centers of lymph nodes. A large number of naïve T cells circulate through the lymph nodes, and those with a TCR specific for a given antigen induce proliferation and clonal expansion of antigen-specific effector T cells (Figure 6). Depending on the cytokine milieu in the lymph node and the specific antigen presented, the T cells develop to either cytotoxic T cells (CD8$^+$ T cells) or T helper cells (CD4$^+$ T cells), the latter further developing into three different T helper subtypes; T_h1, T_h2 and T_h17. T_h1 with respect to cell-mediated immunity helping macrophages, the T_h2 subtype affecting hormonal immunity helping B cells and T_h17 cells involved in recruitment, activation and migration of neutrophils.

	Immediate 0-4 hours	Early 4-96 hours	Late >96 hours
Transport	No	No	Antigens transported to lymphoid organs
Recognition	Preformed nonspecific effector cells	Microbial-associated molecular patterns Inflammation recruitment and activation of effector cells	Naive B and T cells
Clonal expansion	No	No	Clonal expansion and differentiation to effector cells

Figure 6. Early innate immunity (white boxes) versus late adaptive immunity (green box). Antigens are transported from site of infection by antigen presenting cells to lymphoid organs and presented to naïve B and T cells initiating clonal expansion of antigen specific cells.

Major Histocompatibility Complex (MHC) is found in all vertebrates, originally identified as an antigen system of the leukocytes, therefore called Human Leukocyte Antigen (HLA) in humans. The purpose of the MHC antigens is to serve as identity markers on the surface of different cells and present foreign antigen peptides (MHC-antigen complex) to the TCR on the surface of T cells and thereby cooperate with the T cell to exercise their adaptive

immune functions. MHC-class I molecules are expressed on the surface of all nucleated cells and present antigens to CD8$^+$ cytotoxic T cells while MHC-class II molecules are expressed on the surface of antigen presenting cells and present antigens to CD4$^+$ T helper cells. The CD4 molecule stabilizes the binding between the TCR and the MHC-class II and the CD8 molecule is a co-receptor for MHC-class I.

The T cell receptor undergoes random gene rearrangement in individual developing T cells, thus providing the huge diversity in the T cell repertoire. Each individual possesses millions of different TCRs with individual antigen specificity. The TCR consists of four units, the TCRα and TCRβ unit forming a ligand-binding part and the two CD3ζ units forming a signal-transducing part. The binding of the MHC/peptide complex to the TCR sends the first activation signal to the T cell by triggering phosphorylation cascades downstream and finally resulting in transcription of genes involved in T cell activation and proliferation (TNFα, IFNγ, IL-2 and CD69 among others) [121,156]. However, for full activation a second (co-stimulatory) signal is required. The antigen presenting cells are equipped with CD80 and CD86 molecules interacting with the CD28 or the CTLA-4 co-receptor on the T cell, providing either activation and cytokine production or inhibition of T cell activation, respectively. Thereby, the T cell can fine-tune its immunologic machinery by receiving different co-stimulatory signals. T cells encountering antigen without co-stimulatory signaling undergo apoptosis or become anergic.

3.4.2 T cell activation and regulatory effect of the cAMP - Protein kinase A pathway

Downstream signaling from the TCR and co-receptors defines the fate of the T cell and is a highly complex pathway involving multiple phosphorylation steps and different mediators. A sufficient T cell defense is dependent on initial activation to clear the pathogen followed by inactivation, to switch the immunologic machinery off when the pathogen is cleared.

Cyclic AMP is the first intracellular second messenger described [184] and important in many biological processes. It plays a crucial role in the intracellular signaling of many neurotransmitters, hormones, and other humoral factors, and regulates a wide range of cellular events such as cell division, gene expression, metabolism and regulation of immune responses [133]. In T cells cAMP is important to regulate the cell's response to defined stimuli. Cyclic AMP is synthesized from adenosine 5`-triphosphate (ATP) by adenylyl cyclase which is located in the inner side of the plasma membrane. The synthesizing activity of adenylyl cyclase is activated upon binding of extracellular ligand to the G-protein coupled receptors

(GPCR) and subsequently dissociation of Gα and Gβγ subunits. The Gα subunit activates adenylyl cyclase [79].

Protein kinase A (PKA) is a tetramer consisting of four subunits, two catalytic and two regulatory. Interaction between the regulatory units of PKA and an A-kinase anchoring protein (AKAP) positions the kinase in the vicinity of localized cAMP pools [134]. Each regulatory unit is also equipped with two binding sites for cAMP and cAMP binding dissociates two catalytic subunits from the PKA complex (Figure 7). The free catalytic subunits phosphorylate local target proteins, regulating their activity [173]. In T cells, one major target is C-terminal Src kinase (Csk) and activation and recruitment of the kinase is dependent on phosphorylation at Ser364 by PKA [192,205]. Activated Csk then phosphorylates and turns off Lck, a protein tyrosine kinase (PTK) closely linked to T cell activation allowing signaling through the TCR.

Figure 7. PGE_2 – cAMP - PKA pathway negatively regulates signaling through the T cell receptor via activation of Csk and inactivation of Lck (reviewed by Mosenden and Taskén 2011).

3.4.3 Regulatory T cells

T cells with suppressing properties were discovered in 1970 [71]. Later, they were named regulatory T cells (Tregs) after the finding that a suppressive subset of $CD4^+$ T cells expressed CD25, the IL-2 receptor α chain [162]. However, identifying the regulatory T cell subset by $CD4^+CD25^+$ is not optimal as activated T cells also express CD25. The immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome is a rare disease in humans (spontaneous murine mutant; scurfy mice) and leads to extensive autoimmunity. Investigations to identify underlying causes of IPEX led to the discovery of the transcription factor forkhead box P3 gene (*FOXP3*) [17]. The suppressive function of Tregs is dependent on expression of FOXP3 and stable expression of the FOXP3 protein is restricted to Tregs.

FOXP3 developed as a marker for the suppressive T cell subset and remains the best marker for Tregs today [204]. However, several issues complicate the use of FOXP3 as a unique marker for Tregs. First of all, FOXP3 is expressed intracellularly and for the purpose of isolating a Treg population, surface markers like CD4, CD25, and CD127 would have to be used in order to yield live cells. Furthermore, T cells with a somewhat suppressive phenotype not expressing FOXP3 have been identified [142]. Finally, not all FOXP3^{+} T cells are functionally suppressive [209]. Several new candidate markers have been suggested and validated, including CD147 as a marker for activated Tregs [176], but so far none are unique and this remains a challenge for future Treg studies.

The transmembrane tyrosine phosphatase receptor CD45 antagonizes Csk by dephosphorylating Lck, thereby allowing activation of Lck followed by signaling through TCR [141]. CD45 exists in many isoforms. Positively selected naïve T cells express CD45RO in the thymus, convert to CD45RA expression at the time of peripheral migration, and switch back to CD45RO after antigen recognition (effector/memory cells). A small percentage of antigen-experienced T cells revert from CD45RO^{+} to CD45RA^{+} as they undergo differentiation into terminal effector cells [14]. Recently, CD45RA proved useful as a marker and differentiator for different Treg subsets. Human FOXP3^{+} T cells were shown to comprise three distinct pheno- and functional types based on CD25 and CD45RA expression. Two of the subtypes, CD25^{++}CD45RA^{+} and CD25^{+++}CD45RA^{-} were functionally suppressive resting and activated Tregs, respectively. The third Treg subtype based on the activation marker CD45, the CD25^{++}CD45RA^{+} cells, were shown to be cytokine producing cells with lack of suppressive activity [132]. The latter finding adds to the discussion of using FOXP3 as a marker for suppressive cells.

Tregs require antigen-specific activation or polyclonal TCR stimulation to exert their suppressive function but can suppress effector cells (CD4/8^{+} T cells and NK cells) in an antigen-nonspecific manner [21]. Thymus-derived naturally occurring Tregs (nTregs) are thought to suppress effector T cells in a cell-cell contact-dependent manner although receptors for contact are yet unknown [172]. However, Tregs with a naïve TCR repertoire can also leave the thymus and become induced in lymph nodes, spleen, gut-associated lymphoid tissue or inflamed tissue, and are thereafter called adaptive Tregs (aTregs). In addition to contact-dependent suppression mechanisms (as for nTregs), the suppressive properties of aTregs can also be mediated by soluble factors.

Adaptive Tregs possess a repertoire of different suppressive mechanisms. They can target effector T cells (CD8^{+} cytokine producing T cells) directly. Moreover, they can target

accessory cells (dendritic cells), possibly though CTLA-4 related mechanisms, and thereby indirectly influence effector T cell activation status. Contact-dependent inhibition may be mediated through granzyme expression and surface-bound inhibitory molecules causing cytolysis and inhibition of the effector T cell, respectively. Contact-independent inhibition is mediated through expression of soluble suppressor molecules and cytokines such as IL-10 and TGF-β as well as adenosine or/and through IL-2 consumption, thus depriving surrounding cells of important survival cues and imposing cell-cycle arrest in the effector T cell [161].

In addition, we previously showed that aTregs express PGE_2 [122], thus providing another contact independent regulatory mechanism in which Tregs suppress bystander effector cells (Figure 7). PGE_2 causes immunosuppression by suppression of T, B and NK cell immune responses through stimulation of EP-receptor signaling [134].

3.4.4 Regulatory T cells in colorectal cancer

Tumor infiltrating lymphocytes (TILs) are associated with improved prognosis in many cancers and the type, density and location of immune cells in CRC may have higher predictive power than the prognosis estimated by the conventional AJCC-TNM histological classification [68,143]. In addition, intrinsic immunosuppressive mechanisms in the adaptive immune system such as down-regulation of co-stimulatory receptors and up-regulation of inhibitory co-receptors, secretion of immunosuppressive cytokines and recruitment and induction of Tregs, contribute to the immunosuppressive microenvironment of solid malignant tumors [97].

Tregs are a T cell subset with dominant immunosuppressive properties and in cancer-bearing animals and patients, Tregs expand, migrate to the tumor site and suppress anti-tumor immune responses mediated by NK cells, CD4^{+} and CD8^{+} T cells through molecular mechanisms described in the previous [161]. While high numbers of other TILs such as CD3^{+}, CD8^{+} T cells have shown to be associated with improved prognosis in CRC as well as other cancers, the infiltration and a high number of FOXP3^{+} Tregs are thought to play a significant role by suppressing the anti-tumor immunity, suggesting that this negatively affects the prognosis for the patient [42]. Suppression of adaptive anti-tumor immune responses in the tumor margin shields the tumor from the immune system and contributes to tumor immune tolerance [207]. Treg-mediated suppression of anti-tumor immune responses has been reported in a broad range of human carcinomas, including breast [13], ovarian [201],

lung [148], hepatocellular [69], renal cell [118], pancreatic [84], gastric [147] and cervical carcinomas [90].

Patients with CRC are reported to have increased frequencies of CD25^{+}FOXP3^{+} Tregs in peripheral blood and in tumor tissue (tumor infiltrating Tregs) [42,117,200]. Tregs in peripheral blood suppress anti-tumor immune responses, as observed by the ability to inhibit proliferation and cytokine release by effector T cells [23,42,117,200,206]. Furthermore, depletion of Tregs from peripheral blood mononuclear cells (PBMC) unmasks immune responses to tumor-associated antigens, e.g. 5T4 and CEA peptides, not seen in healthy controls [42,206]. The findings indicate that Treg-mediated inhibition of anti-tumor immune responses may also be specific to the tumor antigen (or tumor-associated antigen). This opens for the possibility to design specific cancer vaccines and testifies to the importance of controlling Tregs to the benefit of the anti-tumor immunity. Inhibition of Treg activity may represent a future therapeutic avenue to improve anti-tumor immunity. To examine the role of Treg activity versus clinical outcome in CRC, we followed patients undergoing liver resection for CRLM and their levels of Treg suppression of anti-tumor immunity (Paper III).

4. Aims of the study

The aims of this study were to characterize the role of the PGE_2-cAMP-PKA pathway dependent regulatory mechanisms in primary and systemic CRC with respect to biological and immunological mechanisms. When trying to improve the treatment of CRC, three stages of the disease can be targeted: i) the tumor formation, ii) the primary cancer and iii) the systemic disease with distant metastases. While the treatment of primary CRC (stage ii) is associated with low mortality and low frequency of local recurrence, primary prevention targeting tumor formation (stage i) may have better potential for patient benefit. Furthermore, many patients present with distant metastases (stage iii) after treatment for CRC, and this must be considered the main obstacle for long-term cancer-free survival in these patients. The work in this thesis was focused on stage i and iii.

Previous work in our group made it possible to synthesize gram quantities of the cAMP analogue Rp-8-Br-cAMPS which offered the possibility to block the PGE_2-EP2-cAMP-PKA pathway in animal experiments. Based on recent reports of early steps in colorectal cancer tumor biology we found it interesting to study effects of perturbation of this pathway in a CRC animal model. Current treatment controls the primary tumor, but a large proportion of patients present with distant metastasis. We next therefore decided to study the course and outcome for patients selected for resection of CRLMs, with special focus on recently implemented treatment strategies. In previous work we had learned that regulatory T cells suppress anti-tumor immunity in primary CRC patients through a PGE_2-PKA immunosuppressive mechanism [206]. Studying patients undergoing liver resection for CRLM we set out to discover whether tumor immunology could affect the course of the disease or not, and if this could provide valuable prognostic information in these patients. Specific aims of this study included:

1. Does perturbation of the EP2/4 but not the EP3 signaling pathway by inhibition at the level of PKA affect β-catenin levels and tumor formation in $Apc^{Min/+}$ mice?
2. Do new treatment strategies applied to CRLM patients improve survival?
3. Does the level of Treg-mediated suppression of specific anti-tumor immunity affect the outcome in patients with metastatic colorectal cancer?

5. Synopsis of publications included

Paper I

Protein kinase A antagonist inhibits β-catenin nuclear translocation, c-Myc and COX-2 expression and tumor promotion in *Apc*[Min/+] mice. Cyclooxygenase (COX)-2 levels are elevated in most colorectal cancers (CRC) leading to local production of prostaglandin E_2 (PGE_2) that has tumor-promoting properties. The benefits of using COX inhibitors in prevention of primary as well as recurring cancer are therefore currently discussed. While one effect of COX inhibitors is considered to be at the level of β-catenin degradation in epithelial cells, other studies have also shown effects on angiogenesis and on anti-tumor immunity. With respect to control of β-catenin degradation, the adenomatous polyposis coli (*APC)* gene which is the most commonly mutated gene in CRC, plays an important gate-keeping role in tumor formation and progression. APC is part of a destruction complex that phosphorylates and targets β-catenin to proteosomal degradation. The destruction complex receives inhibitory signals both through the Wnt-Frizzled pathway and from PGE_2 via the EP3 prostanoid receptor acting through PI3 kinase and protein kinase B. If not degraded, β-catenin accumulates and translocates to the nucleus leading to expression of mitogenic and survival genes. However, in addition to the PGE_2 mechanism of action through control of the destruction complex, it has recently been shown that protein kinase A (PKA) may directly phosphorylate β-catenin at distinct sites which stabilizes and increases the nuclear activity of the protein [83]; and that PGE_2 through cAMP and PKA may influence the stability of β-catenin via nuclear translocation in zebrafish stem cells [73]. This raises the question of whether COX inhibitors could have a second site of action in β-catenin homeostasis in CRC. Here we used *Apc*[Min/+] mice, a model where mice develop intestinal tumors due to defects in β-catenin degradation and where the effect of COX-inhibitors on tumorigenesis is well-established. We specifically inhibited the PGE_2-cAMP pathway only at the level of PKA, and not through the destruction complex, by treating mice with Rp-8-Br-cAMPS, a PKA antagonist, for 6 weeks. This strategy was only possible as a different project with a spin-out company from the group could deliver gram quantities of this model compound which had also been subject to preclinical testing in animal experiments without any signs of toxicity. We show that treatment of mice with Rp-8-Br-cAMPS not only reduced tumor load but also specifically inhibited β-catenin nuclear translocation and the activation of β-catenin target

genes such as c-Myc and COX-2. This indicates that the direct regulatory effect of PKA on β-catenin nuclear translocation is operative in intestinal cancer, introduces a concept for β-catenin regulation in cancer and also goes directly into the discussion of use of COX inhibitors or aspirin in prophylaxis/secondary prophylaxis in colorectal cancer.

Paper II

Aggressive Treatment of Patients with Metastatic Colorectal Cancer Increases Survival.
In this report, overall and disease-free survival was examined in a cohort of 239 patients with CRC and synchronous or metachronous liver metastases eligible for intentionally curative liver resection. The data have been stratified according to whether the patients received neoadjuvant chemotherapy, the number of resections and the surgical technique applied. Several centers have previously reported successful R0 resections after neoadjuvant chemotherapy to convert patients from inoperable to resectable status; however, little or no evidence is currently available on survival and oncologic outcome. Here, we report that patients with high tumor load that received neoadjuvant chemotherapy achieve oncologic results and life expectancy comparable to those that are primary resectable. The majority of patients with metastatic CRC to the liver experience disease recurrence and about 50% of these patients are available for re-resections. We report that the recurrences present with the same time interval after the primary tumor and subsequent metastases (11±1 months), and that the overall and disease-free survival after each resection is the same, the latter also reported by others. We interpret these findings in light of recent advances in tumor and metastasis biology and suggest that disease recurrence may represent parallel development of metastases that reach level of detection at different time points, rather than a progressing disease that rapidly acquires increased malignant and metastatic potential. In line with these results, we also observed a shift in target organs in later recurrences compared to early ones without affecting the clinical outcome, and that the presence of positive resection margins or presence of resectable extrahepatic disease at the time of the first liver resection did not have a negative impact on overall survival. These observations support emerging arguments for a new classification system in metastatic CRC. Although metastatic CRC has a poor prognosis, surgical treatment has clear patient benefit and strategies to make patients resectable and available for surgery should be pursued.

Paper III

Regulatory T cell-mediated Inhibition of Anti-tumor Immune Responses is Associated with Clinical Outcome in Patients with Liver Metastasis from Colorectal Cancer. Surgical resection of colorectal metastases to the liver (CRLMs) is now offered to an increasing number of patients and neoadjuvant chemotherapy and new advances in surgical techniques further increases the number of patients with resectable disease. However, the rate of recurrence and mortality in patients after resection for CRLMs is still high. For the patients that die from rapid progressive disease within the first year after surgery, the procedure offers little benefit. On this background, tumor and immunological factors that predict or are associated with disease recurrence are important for patient selection and treatment strategy. Tumor-specific regulatory T cells play a significant role in suppressing anti-tumor immunity and negatively affect the prognosis. Based on findings in colorectal cancer, patients have a higher proportion of circulating regulatory T cells that suppress tumor immune responses, and inhibition of their activity may represent a future therapeutic avenue to improve anti-tumor immunity. Here, we examined whether tumor-immune responses are associated with the clinical outcome in patients undergoing liver resection for metastatic colorectal cancer. We found that the levels of regulatory T cell-mediated immune suppression of anti-tumor immune responses *ex vivo* at the time of surgery were more pronounced in patients with later recurring disease. Furthermore, regulatory T cell-mediated immune suppression could be perturbed by blocking the COX-2-PGE_2-cAMP pathway and post-surgery PGE_2 levels were also related to clinical outcome. Our findings could provide valuable prognostic information in these patients.

6. Discussion

The incidence of colorectal cancer (CRC) is high and increasing worldwide. To excel at prevention, early detection and treatment of CRC, it is important to understand early steps of the tumor formation and be able to manage systemic aspects of the disease. An introduction to the field has been given in the previous. In the following, our results will be discussed with focus on how patients in the future may benefit from findings presented in the included publications.

6.1 Connecting COX-2 and Wnt in colorectal cancer

COX-2 is over-expressed in most CRCs compared to normal mucosa, and COX-2 cleaves AA to form PGE_2. PGE_2 is reported to have several local effects promoting the tumor growth in CRC; pro-angiogenic, anti-apoptotic and proliferative [64,170,171,188,189]. Furthermore, COX-2 expression contributes to maintaining a chronic inflammatory state in and around the tumor, thus leading to release of pro-inflammatory cytokines also with proliferative effects and shifting the tumor microenvironment towards an immunosuppressive dominance, which prevents an effective anti-tumor immunity.

Wnt signaling is important in embryogenesis and cancer, and much insight to cancer biology has come from advances in the stem cell field. When a Wnt protein ligand binds the Frizzled receptor (member of the G protein-coupled receptors) the canonical Wnt pathway is switched to an "on" mode resulting in β-catenin translocation to the nucleus where it interacts with the TCF/LEF transcription factor and thereby drives the transcription of genes important for cell proliferation and survival [120]. The main regulatory mechanism in the Wnt pathway is through limiting the availability of β-catenin in the cytosol. The APC protein forms a complex with GSK3β, CK-1 and Axin, the function of which is to phosphorylate β-catenin at defined sites and thereby marking it for proteosomal degradation [160]. Wnt/Frizzled binding results in phosphorylation and activation of Dishevelled (Dsh/Dvl), a cytosolic protein, and activated Dsh/Dvl inhibits the ability of GSK3β to phosphorylate β-catenin by poorly understood mechanisms [34].

CRC has a natural history of transition from precursor to carcinoma that spans 15-20 years, providing a window of opportunity for effective interventions and prevention [6]. Non

steroid anti-inflammatory drugs (NSAIDs) and aspirin are COX inhibiting agents, shown to protect against CRC development in several epidemiological studies [7] and may be beneficial in large population groups at risk [101]. Chemoprevention is a new emerging science which refers to the use of agents to inhibit, delay or reverse the carcinogenesis. In patients with the FAP syndrome, COX inhibition by NSAIDs can cause regression of existing colorectal adenomas and prevent formation of new polyps [195]. The protective effect of COX inhibition on colorectal tumor formation has also been reported in several animal studies. The discussion of whether these mechanisms could be used for clinical purposes in CRC patients is ongoing. One study reported 33-45% reduction in polyp recurrence rate in humans after intake of the selective COX-2 inhibitor celecoxib; however serious cardiovascular events were significantly more frequent in the treated group [19,154], and even more so with the COX-2 specific rofecoxib that was withdrawn from the market because of safety concerns [108]. Nonetheless, the US Food and Drug Administration have approved celecoxib for reducing adenoma frequencies in patients with FAP, a group at extremely high risk of developing carcinomas. As cardiovascular side effects may be a serious issue, these drugs are not recommended for primary protection of CRC. Aspirin and celecoxib have not been compared in randomized clinical trials [12,166]. Aspirin may, however, have an even larger protective effect and at the same time act cardioprotectively [168]. In addition, 600 mg aspirin given daily for more than 25 months to carriers of Lynch syndrome reduced the cancer incidence compared to placebo treatment in the first randomized controlled study to investigate primary chemoprevention with cancer as endpoint [32]. The chemopreventive effects of aspirin or NSAIDs in sporadic CRC need evaluation and further studies are required to establish the optimum dose and duration of treatment. Taken together, it may be that the ideal CRC chemopreventive agent remains to be discovered [7], although both aspirin and NSAIDs may be interesting; especially in secondary prevention, e.g. after surgery.

While the correlation between COX and CRC has been known for some time, the molecular mechanisms responsible for the observed effects are only starting to be mapped out, (some of them introduced in the previous sections of this Thesis). In addition, COX-independent mechanisms of NSAIDs and aspirin may also contribute to the protective effects on CRC including modifications of transcription factors such as NF-κB and DNA stabilization [168]. Knowledge of COX-dependent as well as COX-independent mechanisms is important, as future chemoprevention relies on what molecular mechanisms that are dominant, and if drugs can be designed to avoid serious side effects. However, the COX-independent mechanisms will not be discussed further here.

The COX-dependent molecular mechanisms involved have shown to be highly complex and several pathways are suggested as candidates. Recently PKA was suggested to be a possible link between the Wnt and COX-2-PGE$_2$-cAMP pathway and based on previous experience in our lab with PKA signaling we found this interesting to pursue. Furthermore, we had gram quantities of the PKA inhibitor Rp-8-Br-cAMPS available due to a spin-off project from our lab.
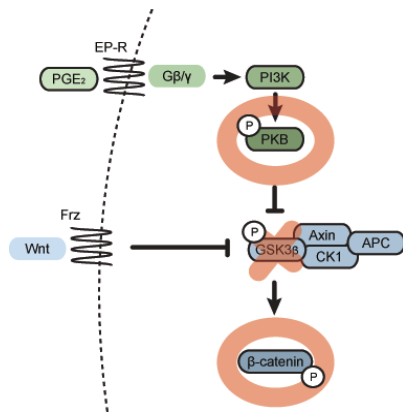
Figure 8. PKA may interact with the Wnt signaling pathway at multiple levels red circles and cross. PKA can activate (indirectly phosphorylate) PKB, inactivate (phosphorylate) GSK3β or stabilize (phosphorylate) β-catenin.

To date PKA has been suggested to interact at the level of GSK3β, PKB and β-catenin; all with the same net effect as activation of the Wnt signaling pathway (Figure 8). Phosphorylation of GSK3β at Ser9 was found to be a target for PKB located downstream of PI3K, however Ser9 at GSK3β is also shown to be substrate for PKA [56,113]. Furthermore, PKA can indirectly phosphorylate and activate PKB, which could provide an indirect mechanism for inhibition of GSK3β by PKA [59]. The result is inactivation of the kinase activity of GSK3β, similarly to the effect of activation of the Wnt signaling pathway. In addition, PKA was recently shown to phosphorylate β-catenin directly at two novel sites, Ser552 and Ser675 [83,186]. While there is agreement that phosphorylation at these sites does not affect regulation of the destruction complex and therefore is via non-canonical mechanisms, there are contradicting reports regarding the effects on β-catenin homeostasis. Two reports find that the PKA phosphorylation of β-catenin affects stability and intracellular distribution [73,83] and one report states the opposite and offers a different mechanism of action via interaction with CREB-binding protein (CBP) [186]. Different experimental conditions may cause these discrepancies.

In addition, non-PKA dependent mechanisms have also been suggested as interaction points between COX-2-PGE$_2$ and Wnt signaling, which emphasize the complexity of the field. Loss of β-catenin phosphorylation, and therefore loss of targeting the protein to proteosomal degradation, arises from the following: Inactivation of GSK3β or CK-1, mutations in the phosphorylation sites of β-catenin or the inability of APC to enhance association with Axin [30]. In 2005, Castellone *et al*. used colon cancer cell lines and presented data suggesting that β-catenin and the Gα subunit of the EP receptor bind the same

Figure 9. Non-PKA specific PGE$_2$/Wnt interactions mediated by Axin displacement and PKB specific phosphorylation of β-catenin.
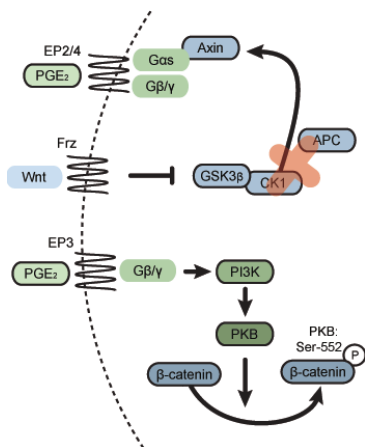
domain of Axin. Consequently, Axin-Gα binding results in displacement of APC and loss of phosphorylation of β-catenin [38] (Figure 9). However, the authors used APC mutant cells in which the destruction complex is already compromised and all studies were done *in vitro*. In addition, PKB may be able to phosphorylate β-catenin directly, representing yet another non-canonical mechanism [183]. In a more recent study, the authors used zebrafish to study PGE$_2$/Wnt interactions in hematopoietic stem cells (HSC) *in vivo* and proposed activation of PKA as a functionally significant effector downstream of PGE$_2$ [73]. The differences could be caused by different experimental conditions, *in vivo* versus *in vitro* and from studying HSC with a functional APC protein versus CRC cancer cells with an *APC* mutation.

In Paper I, we show that PKA specific phosphorylation of β-catenin at Ser552 and Ser675 was reduced after PKA inhibition in HCT-116 cells, a human colon cancer cell line. In *Apc*$^{Min/+}$ mice, animals predisposed to develop intestinal tumors, treatment with the same antagonist reduced the size of the tumors by 36% compared to vehicle-control treated animals. This could be explained by the finding that PKA specific phosphorylation stabilizes β-catenin in the cytosol and facilitates nuclear translocation (Figure 10). Nuclear translocation of β-catenin is a prerequisite for activation of TCF/LEF-mediated transcriptional activation, which is also evident from the finding that PKA antagonist-treated animals expressed less COX-2 and c-Myc which both are expressed upon TCF/LEF transactivation (Paper I).

Treatment with indomethacin reduced the number and size of the lesions to a greater extent than the PKA antagonist, and the fact that the indomethacin-treated animals had almost no intestinal tumors precluded us from studying the tumors from these mice by immunohistochemical methods. The difference between COX and PKA inhibition could be explained by the fact that indomethacin is located upstream and thereby could mediate effects also via the PGE$_2$-EP3-PI3K-PKB pathway. However, in the colon cancer cell line, the effect of indomethacin did not differ from the effects of Rp-8-Br-cAMPS with respect to PKA specific phosphorylation, c-Myc expression was, in fact, reduced even more after Rp-8-Br-cAMPS treatment, a finding that indicates that other mechanisms may cooperate to mediate

anti-tumorigenic effects of COX inhibition in the CRC animal model *in vivo*, i.e. angiogenesis, immunology or other important proliferative or apoptotic pathways.
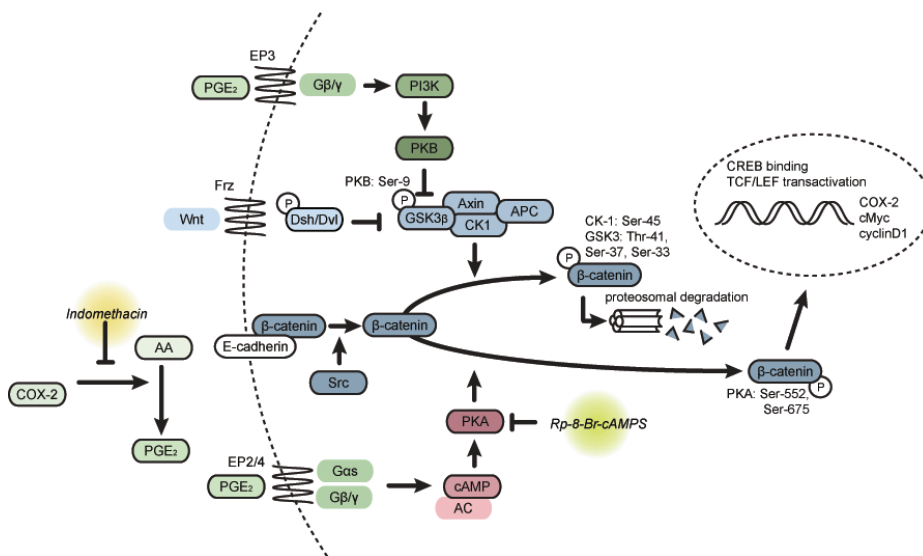
Figure 10. Inhibition of COX-2 upstream by indomethacin and PKA downstream by Rp-8-Br-cAMPS in CRC cells and in a CRC animal model.

In conclusion, treatment of a well-established mouse model of human CRC and FAP with a PKA antagonist not only reduces tumor size but also inhibits β-catenin nuclear translocation and the activation of β-catenin target genes such as c-Myc and COX-2. This indicates that the direct regulatory effect of PKA on β-catenin nuclear translocation described in cell lines and HSC is also operative in intestinal cancer.

6.2 Improving treatment of systemic colorectal cancer

The incidence of CRC is increasing in many countries [35], consequently more patients will present with synchronous or metachronous metastases. The liver (CRLM) is the main organ for metastatic disease from CRC, and may be the main obstacle to cure a large group of patients [45]. For the past 20 years there has been a great development in CRLM treatment. While only 10% were assessed as resectable and consequently curable 10 years ago, 20-25% are today surgically treated with the intent to cure [146]. It is important to constantly

challenge the treatment strategies and disease perception regarding this patient group. Metastatic disease was considered equivalent to incurable status just a few years ago. However, with the treatment options of today, even patients with extensive disease upon presentation may prove to be long-time survivors. Furthermore, even though cure cannot be achieved in many, converting the cancer from rapidly progressing disease and death to a slow progressing or a "chronic state" of disease can improve the quality and time of survival in those that are not curable.

It is a problem to get good data regarding the natural course of patients with untreated liver metastasis as studies would be unethical with respect to currently available treatment options. However, old reports indicate that the survival is short (<6 months) as discussed in the previous [16,111]. Today, patients with non-resectable CRLM have an increased expected survival thanks to modern chemotherapy and other adjuvant treatment options such as RFA [70]. Furthermore, bowel resection followed by chemotherapy appeared to be a better option than chemotherapy alone in patients with CRC and unresectable synchronous CRLM, as shown in an non-randomized trial [66]. The expected 5-year survival after resection for CRLMs is 35-50%. After neoadjuvant downsizing followed by resection of the CRLMs the survival is reported to be comparable 33-50% [70]. This is in line with our results, showing that patients receiving neoadjuvant chemotherapy to downsize the tumor load reached an overall long-term survival comparable to that of primary resectable patients. Furthermore, neoadjuvant chemotherapy serves indirectly as a tool for selection of patients with the best prognosis after surgical resection, as patients that progress during ongoing treatment will probably not benefit from surgical treatment. These results support an aggressive treatment approach to metastatic CRC which also has been suggested by others [2,5,135,150].

Oslo University Hospital has moved from a small to a medium volume center with respect to number of resections performed on CRLM. New treatment strategies applied to this patient group to improve treatment and consequently the survival, includes neoadjuvant and adjuvant chemotherapy, two-stage surgery with portal vein embolisation, laparascopic surgery, surgical techniques adapted from liver transplantation, techniques and surgical tools to limit the blood loss, radio frequency ablation and re-resections for disease recurrence. In Paper II we present data from a time period where most of these strategies were implemented as treatment options for patients with CRLM. We compare outcome with respect to overall and disease-free survival in different subgroups based on established risk factors and the new modalities. The material is based on 239 patients with resectable liver metastasis and even though this is a large single-centre study in Norway, it is a small cohort when doing

comparisons between subgroups. The heterogeneity of the disease and between patients is another problem when performing registrations and doing statistics. Nonetheless, despite shortcomings, we believe our material is interesting with respect to ongoing discussions regarding treatment of CRLM.

We report that a second and third resection of recurring CRLMs should be considered when possible because the survival is the same after each resection and our results point in the direction that resection also should be assessed in patients with extrahepatic recurrences. Extrahepatic metastatic spread has previously been considered an end-stage-disease. In recent years, combined liver and lung resection have produced long-time survivors. Six patients presented with concomitant liver and lung metastases in the material presented in Paper II and only one of them died of progression of the lung manifestation that could not be handled surgically. It is also interesting to observe that patients resected for recurrent disease of the lung after first having been subjected to hepatic resection, had comparable and maybe even better survival compared to those repeatedly resected for recurrent disease of the liver. Consequently, pulmonary metastasis alone should probably no longer be considered an exclusion criterion for surgery [37]. The same could also be the case for non-pulmonary extrahepatic disease, as long as it is resectable or may become resectable after downsizing.

If the resection margin after resection for CRLM contains tumor cells, the disease is by definition not cured and impaired outcome should be expected. Furthermore, the distance of the free margin is reported to be important as a resection margin of 1 cm or more confers a survival advantage over subcentimeter negative margins [47]. However, accumulating data suggesting adjuvant chemotherapy may change the perception of survival after R0 versus R1/R2 resections, and this is important as it allows more patients to undergo resections with curative intent [10,46]. Chemotherapy and repeated resections may help to explain why the discrimination between R0 and R1/R2 resections of CRC metastases is not crucial with respect to overall survival in our material as in other studies [10,46].

Eight different scoring systems for predicting disease-specific survival after resection for CRLMs were presented in the Introduction [60,86,94,123,128,137,157,208]. These systems have proven clinically relevant with respect to survival, but have not been used for risk stratification in areas such as patient selection, administration of chemotherapy or surveillance programs. Furthermore, the data in these reports are from patients who underwent resection from 1968 to 2005. The field has evolved significantly since then and the predictive scores do not account for advances in operative techniques and adjuvant therapies. One problem, for example, is how to score with respect to disease extent as more patients are

receiving neoadjuvant downsizing chemotherapy. Gomez & Cameron recently suggested in their review that there is no ideal prognostic scoring system [75]. In conclusion, one must be careful when using scoring systems for clinical purposes. Patient selection is important and has been a great challenge. Clinical parameters may be nothing more than indirect measurements of tumor biology and tumor microenvironment, and therefore not suitable for patient selection purposes.

A brief introduction to CRC tumor biology was given in the previous sections. Chromosomal instability, mutations in K-*ras* and *Tp53* and tumors expressing high levels of CEA are all associated with poor prognosis [45]. Nuclear β-catenin overexpression may be associated with the ability of the cancer cells to metastasize [40]. In the near future scoring and prognosis estimation of CRC will probably include standardized sets of molecular classifications, tumor immunologic markers and microenvironmental factors in a larger degree than today. A French group reported that type, density and location of immune cells in CRC may have higher predictive power than prognosis estimated by the conventional AJCC-TNM histological classification [68]. In addition, survival following hepatectomy for colorectal metastases should not be considered an isolation of the primary CRC pathology, as tumor cells populating distant organs may continue to evolve and gain different genetic background and the tumor microenvironment may differ from that of the primary tumor [60].

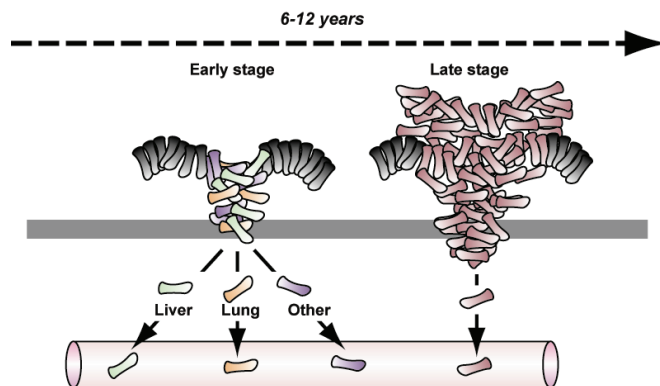**Figure 11.** Parallel development of primary cancer and metastasis in CRC. Cancer cells may metastasize at an early time point and cells metastasizing at late time points may not be as capable as those at early time points to infiltrate and populate distant organs.

Two different models of systemic cancer are accounted for; the linear progression model, in which the primary tumor is the engine of cancer progression and the development

of fully malignant cells occurs locally and they are released at a late time point; and the parallel progression model, where the primary tumor at an early time point releases disseminating tumor cells capable of reaching different target organs and developing into metastases at these sites (Figure 11) [98,181]. The cells may also be released at slightly different, but still early, time points with different mutational backgrounds giving them homing properties specific for different target organs.

Studies designed to describe tumor growth in primary tumor and metastases show that metastases most often grow at the same rate as primary tumors and seldom at more than twice the speed of the primary tumor [62]. Most of these studies are done in breast cancer, showing that a primary cancer uses 12 years to reach a size where it is clinically recognizable and the metastases consequently use 6-12 years to develop to a clinically identifiable size, taking into account the indicated growth rate. If these findings are correct, it would implicate that the disseminating tumor cells would have left the primary cancer at an early time point in patients with synchronous disease having larger metastases than the primary tumor.

A common perception of the linear model is that the disease progresses and acquires more aggressive biological features at later disease stages. However, the parallel model opens for a different interpretation. If the metastasizing process occurs at an early time point in the development of the primary tumor, the disease may not progress and become more aggressive at later stages. The primary tumor may possess more aggressive metastasizing properties early in development rather than late. And the primary tumor and the metastases may develop in parallel, gain different mutations and due to different growth conditions in various tissues be more or less aggressive than the primary tumor [98,181]. Hypothetically, in the parallel model, all the metastases that eventually will present are already present at the time of diagnosis. Metastases in various organs may be manifestations of a process that has taken years to reach the level of detection, and as such, metastases may not be a sign of an explosive metastatic spread [178]. In Paper II we report mean time intervals from primary tumor to liver metastasis and from liver metastasis to presentations of recurrences in patients with resectable CRLM. The disease-free intervals between each event are the same, and even though this is not firm evidence of the disease not becoming more aggressive, it is an interesting finding.

Another interesting issue is the ability of the metastasis to spread beyond its first implantation site. Hypothetically, the metastases that will present are there already at resection of the primary tumor. Thus the patient may actually have a better chance of cancer-specific survival after removal of every "new" disease recurrence as this may not represent new disease, but the last remaining. In line with this thinking, "recurrent disease" may be a

misnomer as the disease may not be recurring, but continues to deliver earlier established metastases growing in parallel and reaching a size that allows diagnosis at different time points following the primary surgery. Thus, resection of metastases may in many cases represent incremental tumor-reductive surgery rather than treatment of recurrent disease.

In reality it is likely that metastases develop on both a linear and a parallel scale and that metastatic disease is systemic or multifocal in its nature and may encompass unrecognized foci at the time of surgery in most patients irrespectively of the presentation at diagnosis. Eradication of all tumor tissue may therefore not be conceivable in the majority of the patients. However, this recognition should not preclude an aggressive treatment approach to induce resectable patients and perform repeated resections that continue to reduce tumor load.

6.3 Treg cancer immunity in liver metastasis from colorectal cancer

To investigate possible prognostic and predictive parameters for use during the assessment and treatment of patients with CRLM, we examined whether immune responses are associated with the clinical outcome. We found that Treg-mediated immune suppression at the time of surgery was more pronounced in patients with later recurring disease.

The frequency of Tregs is increased in blood from CRC patients compared to healthy blood donors [42,117,200]. Depletion of Tregs from the blood of CRC patients unmasks $CD4^+$ and $CD8^+$ T cell responses towards tumor antigens or tumor associated-antigens [42,206]. Tumor infiltrating $CD3^+$, $CD8^+$ and $CD45RO^+$ T cells are associated with improved prognosis in many cancers including CRC. The opposite is observed with tumor infiltrating Tregs, as high density of Tregs associates with poor outcome in many cancer forms. These findings led to the hypothesis that Tregs infiltrating CRC also are associated with poor outcome. However, conflicting and surprising data have accumulated in CRC in which high Treg infiltration in recent studies is reported to be associated with improved survival [61,138,163]. The reports are based on immunohistochemistry on sections of tumors and FOXP3 is used as a Treg marker. However, functional data are missing in most of these reports. Concluding that infiltrating $FOXP3^+$ T cells are protective in CRC before the cells are shown to be functionally suppressive could be a mistake. Other T cells ($CD8^+CD25^+FOXP3^+$ T cells and $CD4^+CD25^+FOXP3^+$ effector T cells) may express some level of FOXP3. However, in one study $CD4^+FOXP3^+$ sorted cells from CRC tumor tissue had the capacity to suppress T cell proliferation and IFNγ production by effector T cells [102]. In another,

CD4$^+$CD25$^+$ Tregs were reported to induce regression of intestinal tumors in $Apc^{Min/+}$ mice [55]. The two latter add functional data supporting the hypothesis that Treg infiltration in CRC may potentially be beneficial.

The immune system is designed to treat infections caused by bacteria, virus and fungi, most of which are infections that last for 3-5 days. After the foreign pathogen is eradicated, the effector T cells may change phenotype and become Tregs expressing FOXP3 to take down excessive immune activity. The high FOXP3$^+$ Treg frequency associated with improved survival in CRC may be a consequence of high frequency of infiltration of previous effector T cells, rather than the suppressive properties of the Tregs at the time of investigation. Furthermore, in a chronic inflammatory state, high activation would need high suppression to balance the response. As high CD8$^+$ T cell density correlates with high infiltration of FOXP3$^+$ T cells in CRC [163], the CD8 response may weigh heavier than the suppressive function of the Tregs. Human Tregs may also express cytotoxic molecules such as perforin and granzyme and could in this way induce death of cancer cells [76], but the direct effect of Tregs on cancer cell still remains to be characterized.

The colon and rectum are highly populated with bacteria which, under normal conditions are kept in the lumen by a monolayer of epithelial cells linked together by tight junctions. This defense is impaired in CRC and bacteria translocate from the lumen to the tissue leading to local inflammation with the release of pro-inflammatory cytokines. The pro-inflammatory cytokines act tumor-enhancing and pro-angiogenic through activation of transcription factors like NF-κB or STAT3 [175]. Thus, by suppressing inflammation and immune responses resulting from bacterial invasion, FOXP3$^+$ Tregs could in fact be anti-tumorigenic in CRC. This could explain a relationship between FOXP3$^+$ T cell abundance inside the tumor and favorable prognosis in CRC (Figure 12).

We focused our work on peripheral immunology and studied Treg mediated anti-tumor responses in blood versus outcome in patients with resectable CRLM. Our main finding was that Treg inhibition of anti-tumor activity was more pronounced in patients that later presented with recurrent disease suggesting that Tregs may affect the clinical course of metastatic CRC. Furthermore, our data suggest that Tregs express COX-2 and that one important mechanism by which they suppress surrounding effector cells is by perturbation of the COX-2- PGE$_2$-cAMP-PKA pathway. PGE$_2$ plasma levels were related to disease activity, but did not decline as rapidly as expected after surgery, suggesting that COX-2 may be expressed by other sources than the cancer cells and Tregs may be a substantial contributing factor. The study presented in Paper III involved a small cohort of patients (n=18) and some

were lost from follow-ups as they died or were otherwise prevented from meeting. The group was split 18 months post-operatively into a disease-free and a recurrent disease group. We checked for group-differences regarding tumor size, number of metastasis, chemotherapy, sex and age as it is possible that the anti-tumor immune function is determined by the extent of the disease at the time of surgery, but we were not able to identify such correlation in the cohort.
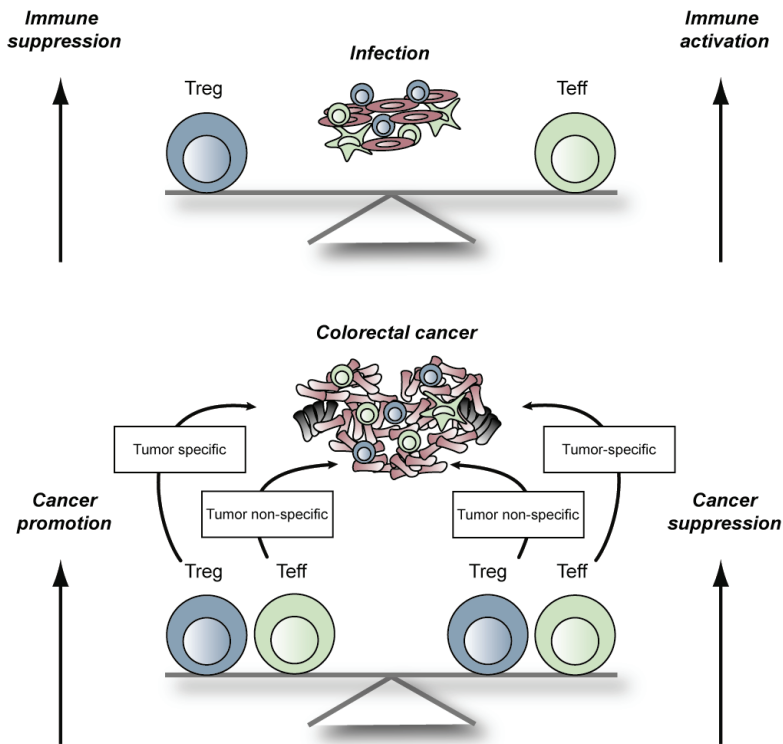
Figure 12. During the course of an infection (top) the immune response balances activation to fight foreign pathogens and suppression to reduce the response when a pathogen has been eradicated preventing autoimmunity. Lymphocyte infiltration in CRC (bottom) could relate to foreign pathogen invasion as well as antigens originating from the tumor cells. An inflammatory response towards microbes with the release of pro-inflammatory cytokines may be unwanted as it may promote tumor growth. However, an inflammatory response targeting the cancer cells may be beneficial. Tregs serve as breaks in both of the described mechanisms and may therefore possess both tumor-suppressive and tumor-promoting effects in primary CRC which could explain conflicting reports.

A quality of our study that distinguishes it from many other Treg-cancer studies is the temporal aspects, as we followed the patient over time post-surgery and have thereby samples from different disease-stages. This allowed us to use each patient as its own control. The most interesting time-related finding was the observation that PGE_2 levels related to disease

activity. Furthermore, that FOXP3$^+$ Tregs in patients with recurrent disease expressed higher levels of COX-2 than in the disease-free patients, which may be a sign of activation. Lastly, the activation marker CD69 was up-regulated in CD4$^+$ T cells in patients with active disease at follow-ups compared to disease-free patients.

Another asset in our study of Treg-mediated effects in CRLMs is that we included and based our findings on functional data and not exclusively on phenotypical data. A challenge when studying cancer specific anti-tumor immunity is that the expected effects on cytokine production or proliferation are very low, as compared to the massive response to pan-clonal stimulation with anti-CD2/CD3/CD28 beads or staphylococcal enterotoxin B (SEB). Furthermore, the HLA-specificity of the tumor-associated antigens (CEA peptides) used to trigger an immune response has been discussed. We found that 2 of 9 patients with recurrent disease and 6 of 7 patients that remained disease-free were HLA-A2 positive. If the peptides were HLA-A2 restricted, this could implicate that patients with recurrent disease do not respond to CEA stimulation and our results would be based on failure of stimulation rather than the suppressive effect of the depleted Tregs. However, the peptides we used have been reported to have preference, but not restriction, to HLA-A3 [95] and HLA-A24 [139] (for CEA$_{61-69}$ and CEA$_{318-327}$, respectively) and are not reported to be HLA-A2 restricted. Indeed,
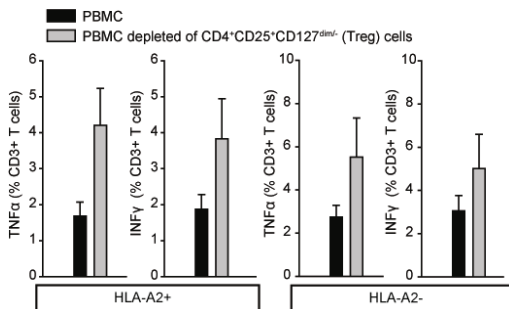
Figure 13. Cytokine responses in HLA-A2 positive and HLA-A2 negative individuals after stimulation with CEA peptide in PBMC (black bars) and Treg depleted PBMC (grey bars).

we observed that these two peptides in our earlier study [206] appeared to give good responses in all patients independently of HLA status. Furthermore, in Figure 13 we showed that TNFα and IFNγ responses to CEA-peptide stimulation in PBMC and Treg-depleted PBMC stratified on HLA-A2 status, revealed no significant differences upon Treg depletion, although there is a tendency for basal levels to be higher in HLA-A2 negative patients.

Cancer vaccines are currently being tested for many forms of cancer. The concept is to trigger a cancer-specific immune response to provide a maximal attack of the tumor cells. One challenge is to identify immunogenic target molecules and several candidates have been suggested and are currently entering controlled clinical trials. So far, results from cancer vaccine trials have reported promising results [127] and synergistic effects can be expected if vaccine is given in combination with chemotherapy [155]. Furthermore, vaccines are safe and

administrable. If we could manipulate the regulatory pathways (e.g. Tregs) of the immune system, this could provide further beneficial effects for patients subjected to vaccination. A problem with CRC when developing immune based therapy is that CRC is not as immunogenic as for instance melanoma, which expresses several tumor-associated antigens (TAA) resulting in a strong immunological response to the tumor. In CRC, p53, CEA, Her2 and heparanase have been proposed as antigen targets [23]. In recent years the surface marker CD55 and the surface glycoprotein oncofetal antigen 5T4 have been found to be expressed by human adenocarcinoma and may therefore constitute good targets for vaccine development [144].

In conclusion, regulatory T cells protect many cancers from anti-tumor immune responses and may help the tumor cells to evade detection by the immune system. This information can be used and may provide valuable prognostic information. A more complete understanding of Treg function and suppressive mechanisms should be persued and will in the future hopefully allow us to modulate the immune response to an optimal defense.

6.4 Perspectives

In Paper I, we discovered that PKA inhibition affects tumor formation in a CRC animal model and this goes directly into the discussion regarding COX inhibition in human CRC and FAP. COX inhibition was far more tumor-reductive than PKA inhibition alone in the $Apc^{Min/+}$ mice. Protein kinase A phosphorylation of β-catenin is therefore probably only one of many mechanisms responsible for the effect on tumor load. Future research should focus on mapping yet other molecular pathways responsible for the COX-associated effects. The next step would be to develop drugs directly targeting these pathways and thereby increase beneficial effects and, if possible, at the same time avoid dangerous side effects. In addition, use of different NSAIDs should be evaluated further. PKA is a common messenger for PGE_2 in all cells, not exclusively in cancer cells, and other effects of perturbation of the PGE_2-cAMP-PKA pathway would have to be addressed if this target is to be pursued. Furthermore, *APC* mutation is only one out of many mechanisms responsible for progression towards CRC development, and PKA inhibition would therefore also have to be tested in cancers where the APC protein is intact.

In Paper II we highlight the effect of implementation of novel treatment strategies with respect to disease-free and overall survival in patients directly subjected to liver surgery or

after downsizing to resectable CRLMs. We address the question of whether neoadjuvant chemotherapy works, whether re-resection is indicated, and how extrahepatic disease should be assessed. Whether the oncologic outcome after laparascopic surgery equals that of open surgery and what benefits we get from clinical scoring systems. Furthermore, we discuss the validity of using predictive variables based on old patient materials on today's patients. Our report adds arguments to ongoing discussions in the field, however, it cannot be used to develop guidelines, as that would have to be based on randomized clinical trials. Furthermore, the search for novel biologics and chemotherapeutics as well as improving the overall treatment from disease detection to surgery must continue. Personalized medicine will continue to evolve, and the discovery of new tumor-specific molecular signatures could open new avenues of drug targeting.

In Paper III we suggest that Tregs, a suppressive T cell subset, may affect the course of the disease in patients with CRLMs. However, the mechanisms of how Tregs suppress bystander T cells, B cells and NK cells are far from fully understood. Ongoing projects in our lab are aiming to discover a main suppressive function; if such a mechanism exists it could be a potentially important drug target. In a recent project, we found that Tregs maintain their suppressive function after fixation, but not after trypsination, indicating that a cell-surface protein is directly involved. Furthermore, conventional T cell inhibitors do not affect Treg activation, indicating pathway redundancy or that Treg activation proceeds by distinct signaling mechanisms. Finally, Tregs suppress effector cells in a time window of immune suppression [77]. The significance of TILs in CRC is controversial and in need of further characterization. One possibility could be to study Treg infiltration in CRC and synchronous CRLM, the latter being an environment shielded from the bowel microflora. Tregs affect other immune cells such as T cells, B cells and NK cells and are thereby thought to affect cancer development and progression. However, it would also be interesting to study potential effects of Tregs on cancer cells directly. Immune cell homing mechanisms could serve as drug targets and if we can identify molecular mechanisms that are unique for Tregs versus effector T cells, this could be used to modulate the tumor microenvironment to help us in the fight against cancer progression.

7. Conclusions

1. a) Treatment with a PKA antagonist decreased the size of intestinal tumors in $Apc^{Min/+}$ mice, an animal model of human FAP and CRC, suggesting that one of the beneficial effects of COX inhibition in CRC is through perturbation of the PGE_2-cAMP-PKA pathway, as PKA is located downstream of EP2/4, but not EP3.

   b) Our data suggest that the mechanism of action is at the level of β-catenin phosphorylation and subsequent cytoplasmic accumulation and nuclear translocation.

2. a) Patients with high tumor load of CRLMs receiving neoadjuvant chemotherapy achieve oncologic results and life expectancy comparable to those that are primary resectable with low tumor load, indicating that pre-operative chemotherapy may be beneficial to these patients.

   b) Patients with resectable recurrent CRLM have the same estimated survival as after the first resection. The recurrences present with the same time interval after the primary tumor and subsequent metastases, indicating that recurrent disease may not be a sign of explosive fast progressing metastatic disease.

3. a) Tumor-associated antigen-specific $CD4^+CD25^+CD127^{low}FOXP3^+$ regulatory T cells with suppressive properties can be identified in blood from patients with CRLM.

   b) Patients with CRLM and high levels of Treg mediated anti-tumor immune suppression in peripheral blood have increased risk of disease recurrence compared to patients that have low levels of Treg suppression.

   c) Tregs appeared to suppress through $COX-2-PGE_2$ dependent mechanisms, PGE_2 plasma levels correlated with disease progression in metastatic CRC and Tregs may be a source of PGE_2 in these patients.

8. References

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I

MOLECULAR CANCER

SHORT COMMUNICATION   **Open Access**

Protein kinase A antagonist inhibits β-catenin nuclear translocation, c-Myc and COX-2 expression and tumor promotion in *Apc*[Min/+] mice

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Abstract

Background: The adenomatous polyposis coli (APC) protein is part of the destruction complex controlling proteosomal degradation of β-catenin and limiting its nuclear translocation, which is thought to play a gate-keeping role in colorectal cancer. The destruction complex is inhibited by Wnt-Frz and prostaglandin E_2 (PGE_2) - PI-3 kinase pathways. Recent reports show that PGE_2-induced phosphorylation of β-catenin by protein kinase A (PKA) increases nuclear translocation indicating two mechanisms of action of PGE_2 on β-catenin homeostasis.

Findings: Treatment of *Apc*[Min/+] mice that spontaneously develop intestinal adenomas with a PKA antagonist (Rp-8-Br-cAMPS) selectively targeting only the latter pathway reduced tumor load, but not the number of adenomas. Immunohistochemical characterization of intestines from treated and control animals revealed that expression of β-catenin, β-catenin nuclear translocation and expression of the β-catenin target genes c-Myc and COX-2 were significantly down-regulated upon Rp-8-Br-cAMPS treatment. Parallel experiments in a human colon cancer cell line (HCT116) revealed that Rp-8-Br-cAMPS blocked PGE_2-induced β-catenin phosphorylation and c-Myc upregulation.

Conclusion: Based on our findings we suggest that PGE_2 act through PKA to promote β-catenin nuclear translocation and tumor development in *Apc*[Min/+] mice *in vivo*, indicating that the direct regulatory effect of PKA on β-catenin nuclear translocation is operative in intestinal cancer.

Keywords: *Apc*[Min/+]/b-catenin, Colorectal cancer, COX-2, protein kinase A

Findings

The adenomatous polyposis coli (*APC)* gene is thought to play a gate-keeping role in the tumor formation and progression and is the most commonly mutated gene in all colorectal cancers. In humans, *APC* mutations can be acquired (spontaneous CRC) or inherited as in the autosomal, familiar adenomatous polyposis (FAP), characterized by the formation of multiple colonic adenomatous polyps [1]. Inactivation of both *APC* alleles ($APC^{-/-}$) is considered necessary for tumor formation. The APC protein forms a destruction complex with Axin, glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) which phosphorylates β-catenin at multiple sites [2], and targets β-catenin for ubiquitination and to degradation

by the proteasome system [3]. A defective APC protein leads to cytoplasmic accumulation and translocation of β-catenin to the nucleus [4]. β-catenin, originally discovered as a cadherin-binding protein, has been shown to interact with and function as a coactivator of T-cell factor/lymphocyte enhancer factor (TCF/LEF) transcription factors. Human transcription factor 4 (hTCF-4), a TCF family member that is expressed in human colonic epithelium and colon carcinoma cells, transactivates transcription only when associated with β-catenin [5]. The result is expression and production of mitogenic and survival genes including c-Myc [6], cyclin D1 [7] and cyclooxygenase-2 (COX-2) [8].

COX-2 levels are elevated in as many as 85% of human CRCs and approximately 50% of colorectal adenomas [8]. Studies have shown that COX inhibition by non-steroidal anti-inflammatory drugs (NSAIDS) or aspirin reduces the risk of CRC and may be beneficial in large population

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groups at risk [9]. Selective COX-2 inhibitors are also associated with a decline in the incidence of CRC and reduced mortality rate, although COX-2 inhibitors have been associated with serious cardiovascular events in this context [10]. Prostaglandin E_2 (PGE_2) has been shown to be an important mediator of COX-2 associated effects, and PGE_2 levels are elevated in CRC biopsies compared with normal mucosa and even in patient blood samples [11]. Beside an anti-angiogenic effect [12], COX inhibition promotes apoptosis and alters tumor growth [13]. PGE_2 and COX-2 over-expression also correlates with CRC risk and metastasis of CRC [14], making this pathway relevant also in follow-up after treatment of the primary cancer. Furthermore, our observations show that the PGE_2 produced also inhibits anti-tumor immunity through the EP2 prostanoid receptor - cAMP - protein kinase A (PKA) - Csk pathway in effector T cells that inhibit T cell activation [11].

Both the Wnt-Frz and the PGE_2-EP3 pathway acting through phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB) negatively regulates the APC destruction complex that controls β-catenin proteosomal degradation. COX inhibitors are thought to reverse the inhibitory effect of PGE_2-EP3 receptor signaling on the APC destruction complex promoting β-catenin degradation and reversing the mitogenic effects. However, homozygous deletion of the gene for the PGE_2 receptor EP2 also reduced the number and size of colorectal polyps in a polyposis mouse model [15]. Furthermore, recent reports have shown that PKA can phosphorylate β-catenin at Ser552 [16] and Ser675 [16,17] and that the effect of β-catenin phosphorylation at the latter site is mediated by non-canonical mechanism(s) that does not involve regulation of the formation of the destruction complex. While Taurin *et al.* show that Ser675 phosphorylation promotes β-catenin interaction with the transcriptional coactivator CREB-binding protein in the nucleus and does not affect β-catenin stability and intracellular location [16], Hino *et al.* report that PKA phosphorylation of the same site stabilizes β-catenin and affects its intracellular localization [17]. These differences highlight the complexity of regulation of Wnt-β-catenin signaling and may relate to the experimental conditions and system examined. Finally, PGE_2 has been shown to control β-catenin homeostasis in zebrafish stem cells by signaling through both the EP3 receptor to the destruction complex and through the EP2 and EP4 receptors via cAMP to PKA affecting β-catenin stability [18]. Given the importance of β-catenin as a trans-activator in CRC and the interest in COX chemoprevention, the question of whether the PGE_2-EP2/4-cAMP-PKA pathway is also active in controlling β-catenin levels in CRC is highly relevant [19].
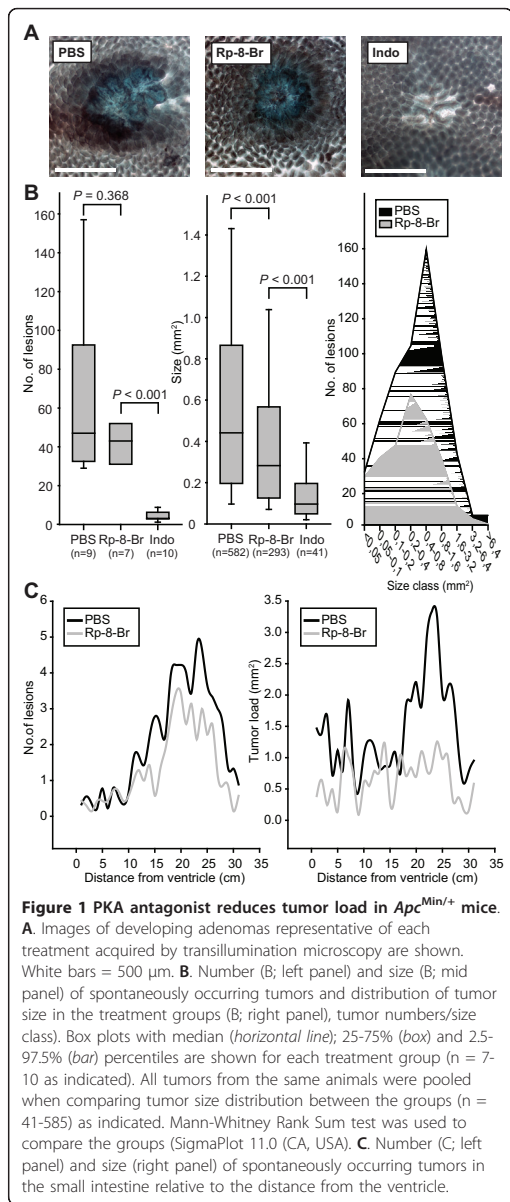
The $Apc^{Min/+}$ mouse is a well-established model of FAP with a germline mutation in one *APC* allele, thus increasing the probability of a double allele mutation and tumor formation. $Apc^{Min/+}$ mice develop multiple adenomas in the intestinal tract, mainly in the small intestine, at an early age which can be blocked effectively by COX inhibition through NSAIDS. Here, we asked whether perturbation of the EP2/4 but not the EP3 pathway by inhibition at the level of PKA, could affect β-catenin levels and tumor formation. We show that treatment of $Apc^{Min/+}$ mice with a PKA antagonist, Rp-8-Br-cAMPS, reduces tumor load, β-catenin levels and nuclear translocation as well as expression of β-catenin target genes in $Apc^{Min/+}$ mice *in vivo*.

Differential effects of COX and PKA inhibition on tumor formation in $Apc^{Min/+}$ mice

To more closely delineate the effect of PKA in the COX-2 - PGE_2 pathway active in colorectal cancer, we treated $Apc^{Min/+}$ mice with the PKA antagonist Rp-8-Br-cAMPS for 6 weeks using earlier established doses (see Additional file 1, Supplementary information) and compared the result with that of treatment with the COX inhibitor indomethacin, previously shown to inhibit tumor development in the $Apc^{Min/+}$ model [20]. Phosphate buffered saline (PBS) was used as vehicle control for the Rp-8-Br-cAMPS. Examination revealed that indomethacin reduced the number and area of tumors in the small intestine of the $Apc^{Min/+}$ mice compared to PBS (from 47 to 3 tumors per mouse and from 0.44 mm^2 to 0.10 mm^2 tumor area; $P <$ 0.001; Figure 1A, B). The PKA antagonist Rp-8-Br-cAMPS did not significantly reduce the number of adenomas (47 versus 43 tumors; $P = 0.368$, Figure 1A, B), but reduced the tumor area by 36% (from 0.44 mm^2 to 0.28 mm^2; $P <$ 0.001; Figure 1). Specifically, tumor load was reduced in the distal part of the small intestine (Figure 1C). The differential effect of COX inhibitor and PKA antagonist on tumor numbers and tumor size indicated to us that the mechanisms of action could be distinct and were examined in more detail in the following.

Inhibition of PKA does not affect lymphocytic tumor infiltration or HIF-1α expression in $Apc^{Min/+}$ mice tumors

Lymphocytic tumor infiltration affects the course of human CRC where type, density and location of immune cells are shown to have higher prognostic power than the classical UICC-TNM staging [21]. Furthermore, the hypothesis of adaptive regulatory T cells (Treg) inhibiting anti-tumor immune responses has been subject to considerable interest [22]. Previously, we found that upon activation, Tregs express COX-2 and suppress effector T cells by PGE_2 - cAMP dependent mechanisms that may be of clinical relevance in patients with CRC [11]. However,

Figure 1 PKA antagonist reduces tumor load in *Apc*^Min/+ mice. **A**. Images of developing adenomas representative of each treatment acquired by transillumination microscopy are shown. White bars = 500 μm. **B**. Number (B; left panel) and size (B; mid panel) of spontaneously occurring tumors and distribution of tumor size in the treatment groups (B; right panel), tumor numbers/size class. Box plots with median (*horizontal line*); 25-75% (*box*) and 2.5-97.5% (*bar*) percentiles are shown for each treatment group (n = 7-10 as indicated). All tumors from the same animals were pooled when comparing tumor size distribution between the groups (n = 41-585) as indicated. Mann-Whitney Rank Sum test was used to compare the groups (SigmaPlot 11.0 (CA, USA). **C**. Number (C; left panel) and size (right panel) of spontaneously occurring tumors in the small intestine relative to the distance from the ventricle.

immunohistochemical characterization of small intestinal tumors from PKA antagonist Rp-8-Br-cAMPS treated animals did not reveal any significant changes in the number of CD3+ T cells, CD8+ cytotoxic T cells, Foxp3+ Tregs or CD56+ natural killer (NK) cells (Figure 2A, B and Additional file 2, Figure S1). In contrast, levels of granzyme B
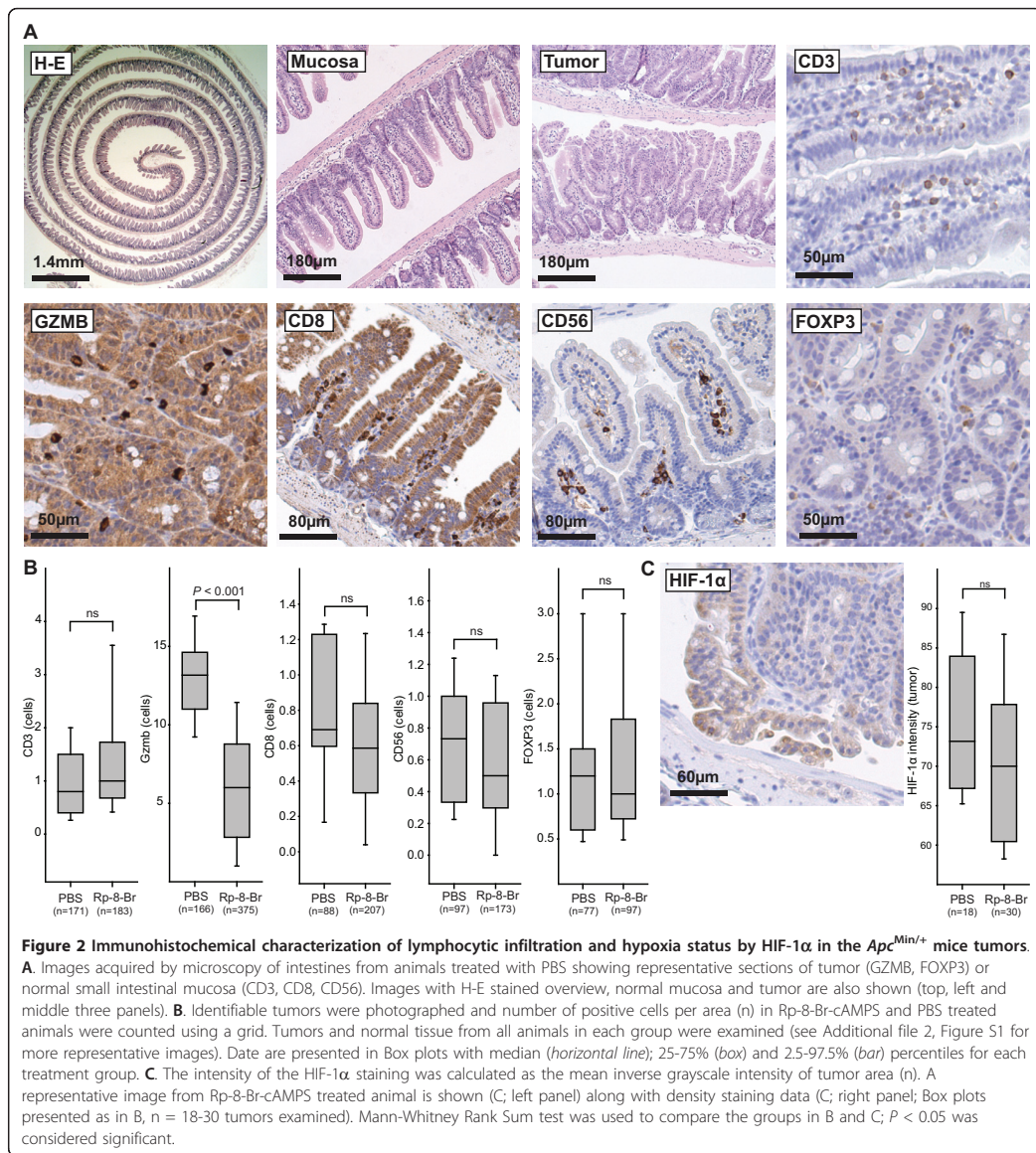
(GZMB), a protein expressed in the cytotoxic T lympho-cytes (CD8+ T cells) and NK cells, were reduced in Rp-8-Br-cAMPS treated animals which may indicate more degranulated cytotoxic cells post activation (Figure 2B). Our observations indicate that intestinal immune responses play a minor role in the development of the *Apc*^Min/+ mice tumor load, consistent with other observa-tions [23].

PGE$_2$ also affects angiogenesis and up-regulates vascular endothelial growth factor receptor-1 (VEGFR-1) in a human colon cancer cell line [12] whereas indomethacin inhibits the expression of VEGF and thereby angiogenesis [24]. To assess treatments effects on angiogenesis, we examined levels of the hypoxia-inducible transcription fac-tor (HIF)-1α which regulates the expression of target genes important in angiogenesis by accumulation and translocation to the nucleus under hypoxic conditions. While apical regions of all tumors showed higher cytoplas-mic intensity and nuclear staining of HIF-1α, no differ-ences between treatment groups were observed (Figure 2C and Additional file 2, Figure S1).

PKA antagonist treatment of *Apc*^Min/+ mice decreases the levels β-catenin signaling to the nucleus and of COX-2 and c-Myc expression in *Apc*^Min/+ mice tumors

We next examined the effect of treatment on the activity of the PGE$_2$- β-catenin pathway in tumor cells. As evident from image analysis of immunohistochemically stained sec-tions, levels of β-catenin were significantly decreased in tumors from the animals treated with the PKA antagonist Rp-8-Br-cAMPS compared to tumors from the control-treated group (138 versus 117 median inverse grayscale intensity units; *P* < 0.001, Figure 3A, B) Furthermore, the median number of β-catenin positive nuclei were reduced from 20% in tumors in the control group to 10% in tumors in the Rp-8-Br-cAMPS treated group (*P* = 0.024, Figure 3A, B and Additional file 3, Figure S2). In addition, expression of the β-catenin/TCF/LEF transcription complex-regulated genes c-Myc and COX-2 were reduced in tumor cells upon treatment with the PKA antagonist Rp-8-Br-cAMPS as evi-dent from the median number of nuclei positive for c-Myc (reduced from 60% in control to 20%; *P* < 0.001, Figure 3A, B and Additional file 3, Figure S2) and from the cytoplas-mic expression levels of the COX-2 enzyme (reduced from 136 in control to 106 median inverse grayscale intensity units in the treated group; *P* < 0.001, Figure 3A, B and Additional file 3, Figure S2).

For further quantification of the observed effects on tumor tissue and validation of the observed effects without dilu-tion into normal mucosa, we next looked at the regulation of β-catenin phosphorylation and c-Myc regulation in a human colonic cancer cell line, HCT 116 (Figure 4A and 4B). While treatment of HCT 116 colon carcinoma cells with PGE$_2$ for 30 min (phosphorylated β-catenin) or 1 h

Figure 2 Immunohistochemical characterization of lymphocytic infiltration and hypoxia status by HIF-1α in the $Apc^{Min/+}$ mice tumors. **A**. Images acquired by microscopy of intestines from animals treated with PBS showing representative sections of tumor (GZMB, FOXP3) or normal small intestinal mucosa (CD3, CD8, CD56). Images with H-E stained overview, normal mucosa and tumor are also shown (top, left and middle three panels). **B**. Identifiable tumors were photographed and number of positive cells per area (n) in Rp-8-Br-cAMPS and PBS treated animals were counted using a grid. Tumors and normal tissue from all animals in each group were examined (see Additional file 2, Figure S1 for more representative images). Date are presented in Box plots with median (*horizontal line*); 25-75% (*box*) and 2.5-97.5% (*bar*) percentiles for each treatment group. **C**. The intensity of the HIF-1α staining was calculated as the mean inverse grayscale intensity of tumor area (n). A representative image from Rp-8-Br-cAMPS treated animal is shown (C; left panel) along with density staining data (C; right panel; Box plots presented as in B, n = 18-30 tumors examined). Mann-Whitney Rank Sum test was used to compare the groups in B and C; $P < 0.05$ was considered significant.

(c-Myc) increased phosphorylation of both Ser552 and Ser675 as well as c-Myc levels, treatment with indomethacin or Rp-8-Br-cAMPS reduced levels compared to untreated sample. The latter indicates some basal prostaglandin production and PKA activation, although COX-2 levels are not sufficiently high to allow detection by Western blot in HCT 116 cells [25] (and our observations).
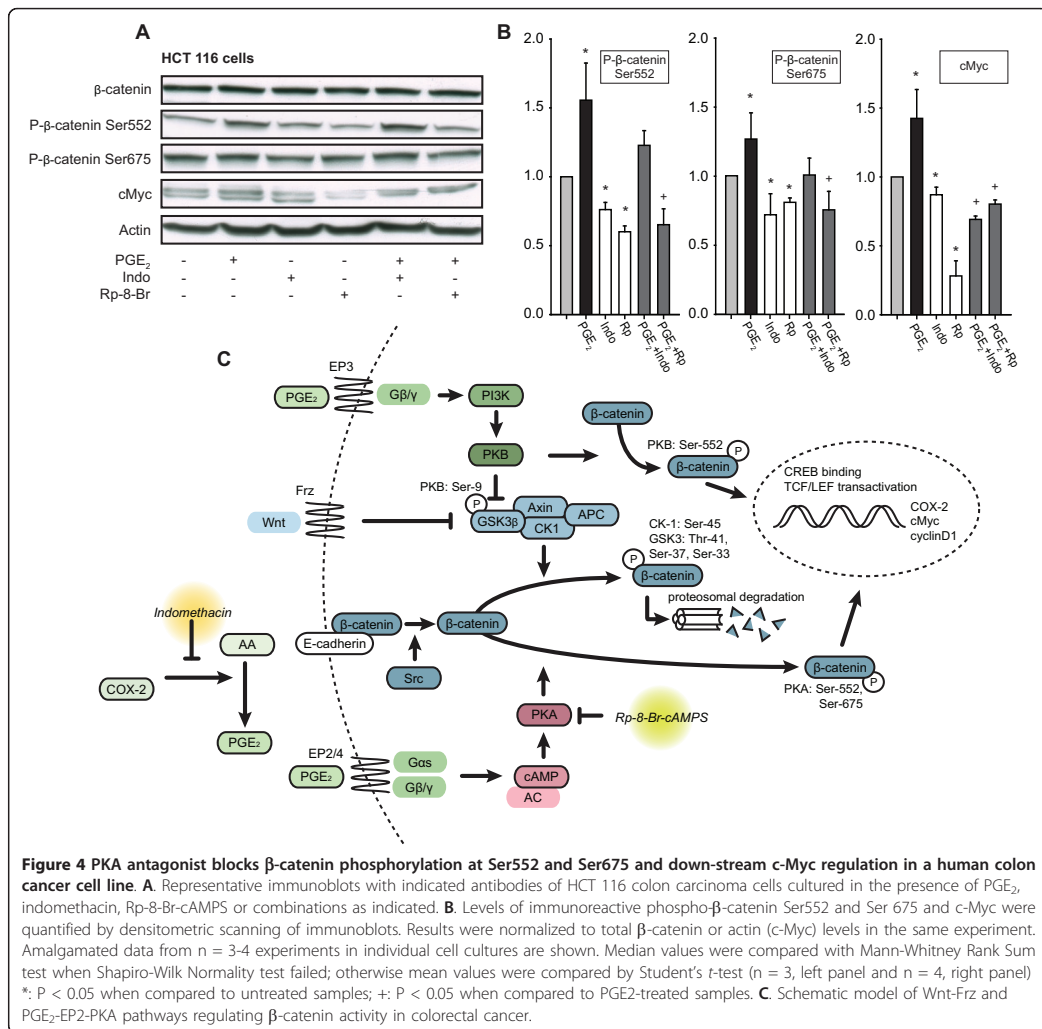
Furthermore, the effect of exogenously added PGE_2 on β-catenin Ser552 and Ser 675 phosphorylation could be blocked by Rp-8-Br-cAMPS but not to the same extent by indomethacin which cannot inhibit the down-stream effect of adding PGE_2 to the cultures. In contrast, PGE_2-mediated upregulation of c-Myc levels could be blocked in the presence of indomethacin, which may indicate that the

Figure 3 PKA antagonist treatment of *Apc*[Min/+] mice decreased COX-2, β-catenin and c-Myc expression in small intestinal tumor tissue.
A. The intensity of the β-catenin and COX-2 staining was calculated as the mean inverse grayscale intensity of tumor area (Image J 1.43u software package). Percent β-catenin and c-Myc positive nuclei were also assessed. Data are presented as Box plots with median (*horizontal line*); 25-75% (*box*) and 2.5-97.5% (*bar*) percentiles shown for each treatment group. Median values compared with Mann-Whitney Rank Sum test when Shapiro-Wilk Normality test failed; otherwise mean values compared by Student's *t*-test. N = no. of tumors examined (tumors from every animal in each treatment group were investigated). **B**. Micrographs of representative tumors from the PBS and Rp-8-Br-cAMPS (Rp-8-Br) treated groups stained with β-catenin, c-Myc and COX-2 antibodies.

regulation at this later time point relies more on endogenously produced PGE$_2$.

Cytoplasmic β-catenin may be targeted to proteosomal degradation through the destruction complex consisting of GSK3β, Axin, CK1 and APC (Figure 4C). However, in the presence of active Wnt signaling, β-catenin accumulates in the cytosol and translocates to the nucleus to act in a mitogenic fashion by transactivation of TCF/LEF leading to expression of target genes in a cell proliferation and survival program [5]. As is well established, the Wnt-Frz pathway inhibits the destruction complex at the level of GSK3β, leading to less proteosomal degradation and more nuclear translocation and activation of β-catenin [2]. Similarly, the up-regulation of COX-2 in colorectal cancer leads to production of PGE$_2$ which binds to the EP3 receptor leading to PI3K and PKB activation, phosphorylation and dissociation of GSK3β and thereby inhibition of the destruction complex [26] (Figure 4C). In zebrafish stem cells, PGE$_2$ acting through an EP2/4-cAMP-PKA pathway was recently shown to induce direct phosphorylation of β-catenin, thereby stimulating its translocation to the nucleus and mitogenic effect [18]. Here, we tested whether this second pathway was providing a mitogenic drive in

intestinal cancer. Using *Apc*[Min/+] mice with a disturbed β-catenin degradation, we specifically inhibited the PGE$_2$-cAMP pathway at the level of PKA by treating mice with Rp-8-Br-cAMPS for 6 weeks (see, Figure 4C for point of action). We show that this not only reduces tumor load but also specifically inhibits β-catenin nuclear translocation and the activation of β-catenin target genes such as c-Myc and COX-2 which may indicate that the direct regulatory effect of PKA on β-catenin nuclear translocation is also operative in intestinal cancer cells. Furthermore, the fact that COX inhibitors may block the effect of PGE$_2$ both in the β-catenin degradation and β-catenin nuclear translocation pathways while Rp-8-Br-cAMPS only affects the latter may explain why inhibitory effect of the PKA antagonist on tumor promotion is comparably weaker than that of indomethacin. Finally, our observation that COX inhibitor abolishes tumor numbers whereas PKA antagonist reduces tumor load but not tumor numbers may indicate that the anti-tumorigenic and anti-proliferative effects are distinct and relate to different points of action in PGE$_2$ signal pathways. It is interesting to speculate that stem cells in crypt foci that give origin to adenomas may be more sensitive to regulation via the Wnt-Frz and PGE$_2$-EP3

Figure 4 PKA antagonist blocks β-catenin phosphorylation at Ser552 and Ser675 and down-stream c-Myc regulation in a human colon cancer cell line. **A**. Representative immunoblots with indicated antibodies of HCT 116 colon carcinoma cells cultured in the presence of PGE_2, indomethacin, Rp-8-Br-cAMPS or combinations as indicated. **B**. Levels of immunoreactive phospho-β-catenin Ser552 and Ser 675 and c-Myc were quantified by densitometric scanning of immunoblots. Results were normalized to total β-catenin or actin (c-Myc) levels in the same experiment. Amalgamated data from n = 3-4 experiments in individual cell cultures are shown. Median values were compared with Mann-Whitney Rank Sum test when Shapiro-Wilk Normality test failed; otherwise mean values were compared by Student's *t*-test (n = 3, left panel and n = 4, right panel) *: $P < 0.05$ when compared to untreated samples; +: $P < 0.05$ when compared to PGE2-treated samples. **C**. Schematic model of Wnt-Frz and PGE_2-EP2-PKA pathways regulating β-catenin activity in colorectal cancer.

pathways than via the EP2/4-cAMP-PGE_2 pathway whereas this balance may shift during tumor development.

Additional material

Additional file 1: Supplementary information. Materials and Methods. Reference List [27-29].

Additional file 2: Figure S1. Immunohistochemical staining with indicated antibodies of tumor and normal mucosa from small intestines from $Apc^{Min/+}$ mice treated with PBS or Rp-8-Br-cAMPS.

Additional file 3: Figure S2. Immunohistochemical staining with indicated antibodies of tumor and normal mucosa from small intestines from $Apc^{Min/+}$ mice treated with PBS or Rp-8-Br-cAMPS.

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Authors' contributions
KWB, EMA and KT designed the experiments; KWB performed animal and WB experiments; KWB and JEP characterized intestinal lesions; KWB and BR performed and analyzed IHC images; KWB and KT wrote the manuscript with comments from all authors; all authors read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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Brudvik et al. *Protein kinase A antagonist inhibits β-catenin nuclear translocation, c-Myc and COX-2 expression and tumor promotion in Apc$^{Min/+}$ mice*

Materials and Methods

Six-week old, female *Apc*$^{Min/+}$ mice (PCR-genotyped) obtained by breeding C57BL/6J wild-type (*Apc*$^{+/+}$) females with C57BL/6J *Apc*$^{Min/+}$ males (Jackson Laboratory, Bar Harbor, ME) were randomized into three groups of 10 animals and treated with Rp-8-Br-cAMPS (Rp-8-Br; 75 mg/kg, prepared by Lauras AS (Oslo, Norway) [27], previously dose and toxicity tested [28], injected i.p. every second day), indomethacin (Indo; 5 mg/kg in ethanol; Sigma-Aldrich, MO, USA, delivered by gavage every second day [29]) or PBS (vehicle control , injected i.p. every second day) for 6 weeks (standard chow and maintenance, Norwegian Institute of Public Health), sacrificed and intestines subsequently examined. Two animals in the Rp-8-Br-cAMPS group were euthanized during the first 3 weeks due to rectal prolapse; two animals, one each from the Rp-8-Br-cAMPS and control groups, were excluded as outliers due to tumor numbers > mean + 3SD.

Small and large intestines were cut longitudinally, spread on filter paper, fixed (10% formalin, 48h), stained with 0.2% methylene blue in 10% formalin and examined by transillumination in an inverse light microscope (320x; blinded data acquisition), as described in detail elsewhere [30]. The study was approved by the National Animal Research Authority (id: FOTS1597) before conducting the experiments. We fully complied with the guidelines issued and exercised due consideration. The health of the animals was checked daily.

Immunohistochemistry was performed on sections from the distal third part of the small intestine and tumors were analyzed from all animals in each group. Formalin fixed intestines were rolled (Fig. 1), embedded in paraffin, subjected to serial sectioning at 3.5 µm and stained routinely with hematoxylin and eosin (H-E) or subjected to immunohistochemistry (Lab Vision Autostainer 480, AH-Diagnostics, Denmark) using antibodies to CD3 (Abcam, UK; 0.5 µg/ml), Granzyme B (Abcam, UK; 2.5 µg/ml), CD8 (Santa Cruz, CA, USA; 0.025 µg/ml), CD56 (Abcam, UK; 5 µg/ml), FOXP3 (Aviva Systems Biology, CA, USA; 1.67 µg/ml), HIF-1α (Novus Biologicals, CO, USA; 1.67 µg/ml), β-catenin (Thermo Fischer Scientific, CA, USA; 0.8 µg/ml), c-Myc (Santa Cruz, CA, USA; 4 µg/ml) and COX-2 (Thermo Fischer Scientific, CA, USA; 0.25 µg/ml) followed by blocking with endogenous peroxidase and staining with horseradish peroxidase, then examination in a DM 3000 microscope with DFC 420 camera (Leica, Germany).

Images captured with a 10x objective were analyzed using Image J 1.43u software package (National Institutes of Health, USA). On average 10 sections from each animal were created and a total of 1014 images were captured and analyzed (CD3; 138, CD8; 76, CD56; 96, GZMB; 155, FOXP3; 100, b-Catenin; 139, COX-2; 161, cMyc; 69 and HIF-1a; 80). The intensity of the HIF-1α, β-catenin and COX-2 staining were calculated as the mean inverse grayscale intensity of tumor area: The area of the lesions was marked with the Freehand Selection tool and then analyzed by the measure tool to calculate the mean grayscale intensity in the area. The mean grayscale was then inverted on the grayscale intensity scale (1-255). To exclude the possibility of bias because of background staining, the normal mucosa was evaluated using the same method with no differences observed between the treatment groups. Furthermore, the size of the tumor area used for grayscale calculations was registered, but there was no correlation between tumor number or tumor size and the staining intensity

(Spearman Correlations R < 0.16, P > 0.05). Moreover, a direct coupling between observations grayscale intensity (i.e. β-catenin levels) and tumor size was precluded by the experimental setup where immunohistochemistry was done on sections (which may not represent the true size of the tumor) whereas the tumor size observations came from examinations in an inverse light microscope directly on whole mice intestines. SigmaPlot 11.0 (CA, USA) was used to compare groups and create graphs. A *P*-value < 0.05 was regarded significant. Median values were compared with Mann-Whitney Rank Sum test when Shapiro-Wilk Normality test failed; otherwise mean values were compared by Student's *t*-test. Animals with tumor numbers outside three standard deviations from the mean of the group were excluded when comparing the groups.

Western blotting was performed on cell lysates from HCT 116 colorectal carcinoma cell line (ATCC, VA, USA) after stimulation (PGE$_2$) in the absence or presence of inhibitors (indomethacin or Rp-8-Br-cAMPS) for different periods of time (30 min., 1 hour and 2 hours). The following antibodies were used β-catenin, phospho-β-catenin (Ser552), phospho-β-catenin (Ser675), (Cell Signaling Technology, MA, USA), c-Myc and Actin (Santa Cruz, CA, USA). Cell lysates and were analyzed on a 10% SDS/PAGE and blotted onto PVDF membranes. The filters were blocked in 5% non-fat dry milk in TBST or 5% BSA (according to manufacturers protocol) for 30 min at RT, incubated overnight at 4°C with primary antibodies, washed five times 5 min in TBST and incubated with a horseradish-peroxidase-conjugated secondary antibody. Blots were developed by using Supersignal West Pico substrate (Pierce, Rockford, IL, USA) and exposure of film and Image J software was next used for quantification of the immunoreactive signals.
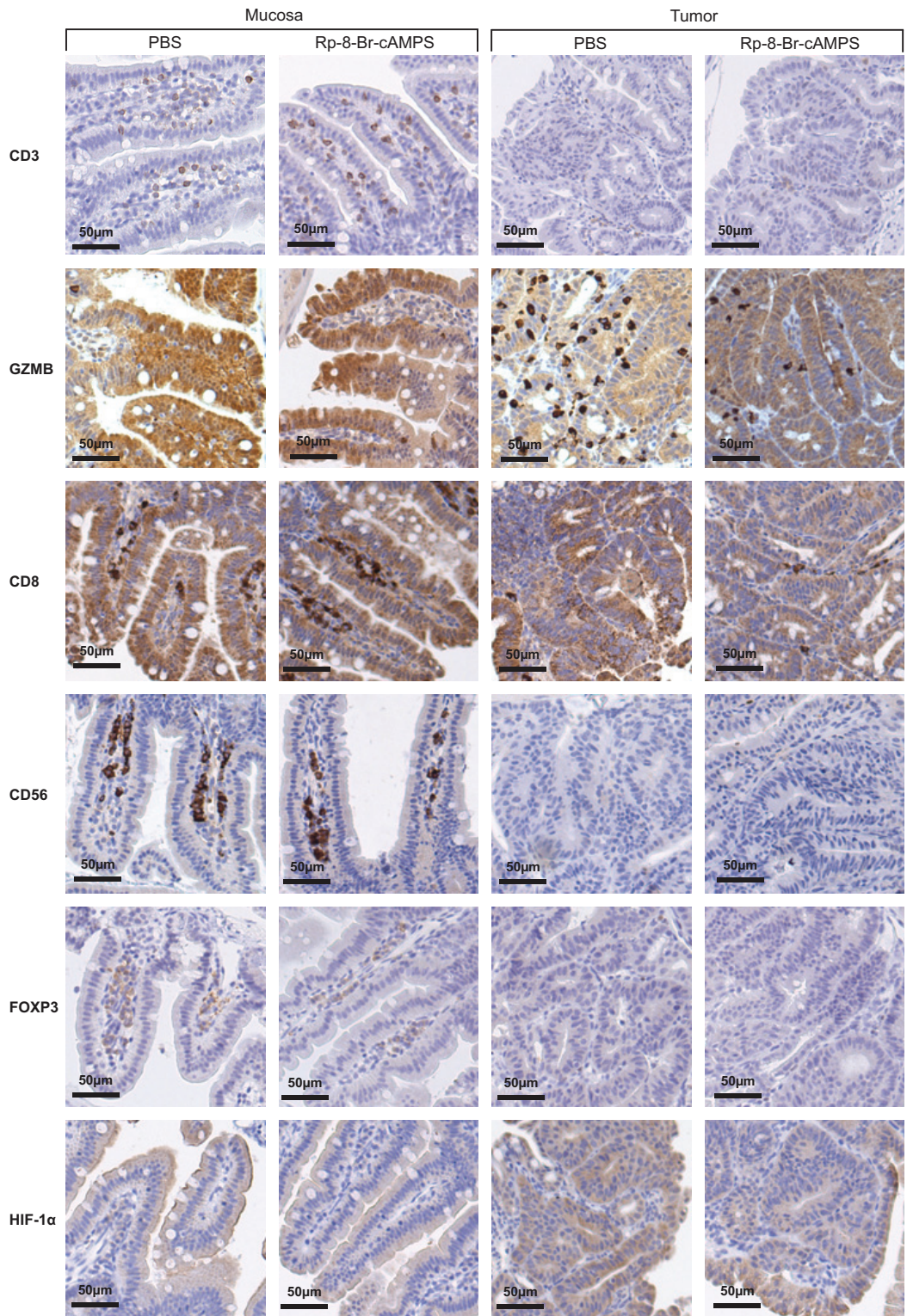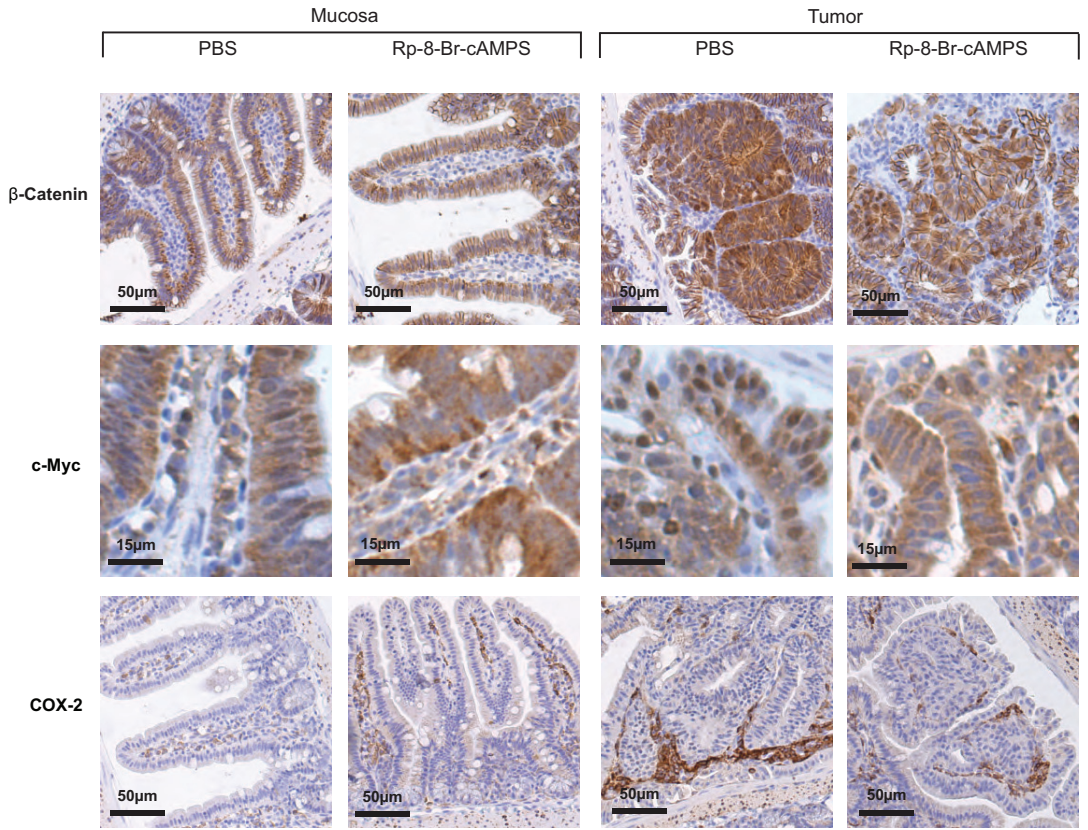
Mucosa — PBS | Rp-8-Br-cAMPS
Tumor — PBS | Rp-8-Br-cAMPS

CD3

GZMB

CD8

CD56

FOXP3

HIF-1α

50µm

Fig. S1 - Brudvik et al.

	Mucosa		Tumor	
	PBS	Rp-8-Br-cAMPS	PBS	Rp-8-Br-cAMPS

β-Catenin

50µm 50µm 50µm 50µm

c-Myc

15µm 15µm 15µm 15µm

COX-2

50µm 50µm 50µm 50µm

Fig. S2 - Brudvik et al.

II

III