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Inflammation-associated genes and genetic variations in colorectal cancer

Nils Elander

Linköping University
FACULTY OF HEALTH SCIENCES

Department of Clinical and Experimental Medicine, Faculty of Health Sciences,
Linköping University, SE-58185, Linköping, Sweden

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

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II. **Elander N**, Zhou J, Ungerbäck J, Dimberg J, Söderkvist P. 2009. Association between adenomatosis polyposis coli functional status and microsomal prostaglandin E synthase-1 expression in colorectal cancer. *Mol Carcinog* 48 (5):401-407.

III. Fransén K, **Elander N**, Söderkvist P. 2005. Nitric oxide synthase 2 (NOS2) promoter polymorphisms in colorectal cancer. *Cancer Lett* 225 (1):99-103.

IV. **Elander N**, Söderkvist P, Fransén K. 2006. Matrix metalloproteinase (MMP) -1, -2, -3 and -9 promoter polymorphisms in colorectal cancer. *Anticancer Res* 26 (1B):791-795.

V. Ungerbäck J, **Elander N**, Dimberg J, Söderkvist P. 2009. Analysis of VEGF polymorphisms, tumour expression of VEGF mRNA and colorectal cancer susceptibility in a Swedish population. *Molecular Medicine Reports* 2:435-439.

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ABSTRACT

Colorectal cancer is a major cause of morbidity and mortality around the world, each year affecting about one million individuals worldwide. The disease is characterized by an accumulation of genetic alterations, and a sequence of events leading to the development of an invasive and metastasising tumour. Chronic or dysregulated inflammation may contribute to tumour initiation and progression via the release and activity of various mediators – *e.g.* cytokines, prostaglandins, inducible nitric oxide synthase (NOS2), matrix metalloproteinases (MMPs), and vascular endothelial growth factors (VEGF). In the present thesis, genes and genetic alterations controlling these events were analysed and discussed within the context of colorectal cancer.

Prostaglandins, being generated from arachidonic acid in reactions dependent on cyclooxygenases (COX-1, COX-2), have been implicated in carcinogenesis of many organs. Since the quite recent characterization of the terminal and specific prostaglandin synthases, which act downstream of COX enzymes, the search for molecular targets which selectively suppress individual prostanoids has been intensified. In *papers I-II*, the role and regulation of inducible prostaglandin E_2 (PGE_2) synthase - mPGES-1 - were explored within the context of intestinal cancer. mPGES-1 was genetically deleted in the $Apc^{Min/+}$ mouse - yielding marked suppression of PGE_2 generation in intestinal and tumour tissue. However, a shift towards enhanced generation of non-PGE_2 prostanoids was observed in mPGES-1 knock out mice, and these mice developed more and larger instestinal tumours. These results therefore indicate that targeting mPGES-1 may paradoxically promote tumourigenesis, most likely by secondary effects on other potentially pro-tumoural mediators. We also explored the relation of the commonly mutated APC gene and mPGES-1 in colon tumour cells, and found that high expression of mPGES-1 was associated with the presence of wild type APC. Rather than by regulating putative β-catenin/Tcf binding sites of the mPGES-1 promoter, APC seems to influence the stabilisation of mPGES-1 mRNA.

In *papers III-V*, the possible contribution of variations in regulatory regions of genes encoding NOS2, MMPs, and VEGF, was assessed in populations of colorectal cancer patients and healthy control individuals. A single nucleotide insertion (1G/2G) at -1607 upstream the transcription start site of the MMP-1 gene was identified to be a susceptibility factor for colorectal cancer development, although no relation with disease characteristics was observed. Except for a rather uncommon combination of two individual polymorphisms of the VEGF gene, investigated genetic variations of VEGF, other MMPs, and NOS2, were not associated with colorectal cancer susceptibility or clinicopathological characteristics. We therefore suggest that other molecular events play more significant roles for the dysregulation of these genes in colorectal tumours.

In summary, accumulating evidence, including the results here presented, suggest significant albeit complex roles of inflammation-induced genes and mediators in colorectal tumourigenesis. The present results may aid in identifying or excluding potential biomarkers and drug targets within cancer-related inflammation.

ABBREVIATIONS

ACF	aberrant crypt focus
APC	adenomatosis polyposis coli
CI	confidence interval
COX	cyclooxygenase
cPGES	cytosolic prostaglandin E synthase
DCC	deleted in colorectal cancer
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
ENU	ethylnitrosurea
FAP	familial adenomatsis polyposis
GSK3β	glycogen synthase kinase 3β
HNPCC	hereditary non-polyposis colorectal cancer
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
mPGES	microsomal prostaglandin E synthase
MSI	microsatellite instability
NF-IL6	nuclear factor–interleukin 6
NFκB	nuclear factor κB
NO	nitric oxide
NOS2	nitric oxide synthase 2
NSAID	non-steoridal anti-inflammatory drug
OR	odds ratio
PG	prostaglandin
PI3K	phosphoinositide-3-kinase
PIGF	placental growth factor
PLA2	phospholipase A2
PPAR	peroxisome proliferator activator receptor
RFLP	restriction fragment length polymorphism
RISC	RNA induced silencing complex
RNAi	RNA interference
SD	standard deviation
SEM	standard error of the mean
siRNA	small inhibitory RNA
SNP	single nucleotide polymorphism
SSCA	single strand conformation analysis
TBE	Tcf binding element
Tcf	T-cell factor
TIMP	tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	tumour necrosis factor
TSS	transcription start site
Tx	thromboxane
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

INTRODUCTION

Colorectal cancer – a brief overview

Colorectal cancer is a common and severe disease, each year affecting nearly one million new individuals and being responsible for about half a million deaths worldwide(Parkin 2004; Potter 1999). In general, colon cancers appear with approximately the same frequency among men and women, while rectal cancers are about twice as common in men(Potter 1999). Incidence rates vary around the world, with the highest rates seen in the Western world and the lowest in Asia, Africa and Southern parts of South America(Parkin and Muir 1992; Parkin 2004). Studies on incidence rates among migrants show that immigrants from low-incidence areas, and their descendants, rapidly reach the incidence rate of the host country, which supports the existence of significant environmental risk factors(Haenszel 1961; McMichael and Giles 1988).

Until today, the intake of red meat, fat, alcohol, and smoking have been identified to enhance the risk of developing colorectal cancer, whereas physical activity, the intake of vegetables, and long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) seem to prevent from tumour development (Potter 1999). Most colorectal cancers appear sporadically in the middle to older ages. By contrast, about 5-10% of all cases arise in families which carry highly penetrant mutations in single genes, giving rise to hereditary syndromes that are mostly transmitted as autosomal dominant traits (Mecklin 2008). In addition, numerous of low-penetrance genes and modifier genes contribute to disease development and progression in both sporadic and familial cases (de la Chapelle 2004).

The adenoma – carcinoma sequence

Nearly two decades ago, Fearon and Vogelstein presented the adenoma – carcinoma step-wise model of colon cancer development (Fearon and Vogelstein 1990). In this model, the transition of normal colonic mucosa into an aberrant crypt focus (ACF) occurs due to the loss of the adenomatosis polyposis coli (APC) gene, which is a tumour suppressor gene located at chromosome 5q21 whose germ-line mutations are responsible for the familial adenomatosis polyposis (FAP) syndrome (Kinzler et al. 1991; Nishisho et al. 1991). Studies of recent decade have revealed that the APC protein is a key controller of the canonical Wnt/β-catenin signalling pathway, by being involved in the intracellular process by which β-catenin is phosphorylated and targeted for ubiquitination and proteasomal degradation (reviewed in

[Schneikert and Behrens 2007]) (figure 1). The large majority of APC mutations result in a premature stop codon and thus a truncated protein(Miyaki et al. 1994; Miyoshi et al. 1992). In the absence of functional APC, β-catenin may translocate to the nucleus, here interacting with members of the T-cell factor (Tcf) family of transcription factors and activating a program of pro-tumoural target genes (Schneikert and Behrens 2007).

Alterations of oncogenes and tumour suppressors such as K-RAS, p53 and SMAD/DCC on chromosome 18q promote further development towards adenoma, carcinoma, and metastasising disease, respectively (reviewed in [Takayama et al. 2006]). K-Ras mutations are found in 40-65% of colorectal cancers, and are thought to activate downstream signals involving Raf/MAPK and PI3K pathways, leading to constitutive growth promotion (Takayama et al. 2006). Further, the presence or absence of K-Ras mutations were found to be prognostic markers for the clinical response to antibodies against the epidermal growth factor receptor (EGFR), which is in line with the current view of Ras/Raf/MAPK as being an important EGFR downstream pathway (reviewed in [Laurent-Puig et al. 2009]).

The p53 gene is commonly mutated and lost in colorectal cancers(S. J. Baker et al. 1989), and is a key tumour suppressor by regulating cell cycle arrest and apoptosis upon genomic damage or other stresses(Green and Kroemer 2009). Similar to above-mentioned prognostic value of K-Ras, the patient's p53 status has been found to correlate with clinical outcome, response to chemotherapy, and survival time (Russo et al. 2005). Finally, allelic loss of chromosome 18q - carrying candidate genes SMAD4/SMAD2 and DCC - is observed in 70% of colorectal tumours, particularly in advanced cancers with hepatic metastases (Fearon and Vogelstein 1990; Takayama et al. 2006).

In contrast with the Vogelstein sequence, sometimes called the 'chromosomal instability pathway', a subgroup of colorectal tumours arises via the 'microsatellite instability pathway' (MSI), where mutations of mismatch repair (MMR) genes precede microsatellite instability and further genetic alterations. The MSI pathway is related to the hereditary non-polyposis colorectal cancer (HNPCC) syndrome, originally defined by Lynch and co-workers in the 1970-ties(Lynch and Krush 1971), which is characterized by the development of cancers of colon and other organs (de la Chapelle 2004).

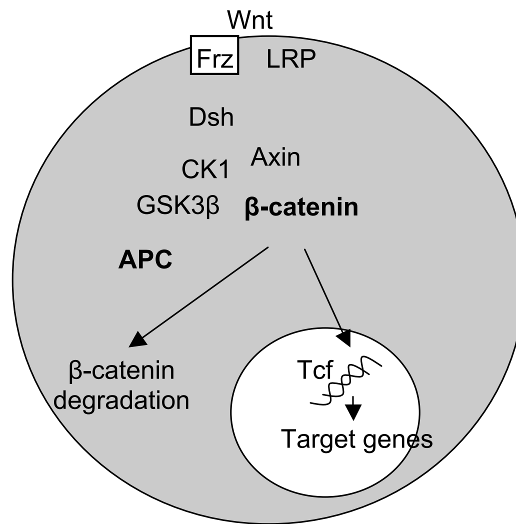

Figure 1. The Wnt/APC/β-catenin pathway. The figure displays a simplified scheme over the Wnt signalling pathway. Wnt ligands interact with membrane bound receptor Frizzled (Frz) and the low-density lipoprotein receptor-related protein (LRP). In the absence of APC, β-catenin is translocated to the nucleus, where it activates the transcription of β-catenin/Tcf target genes in concert with members of the Tcf family of transcription factors. On the contrary, when functional APC is present, a complex of proteins phosphorylates β-catenin, which leads to ubiquitination and degradation via the proteasomal machinery. CK1 = Casein kinase 1. GSK3ß = glycogen synthase kinase 3ß. Tcf = T-cell factor. Dsh = Dishevelled. Modified from (Schneikert and Behrens 2007).

Inflammation-related genes and colorectal cancer

Linking inflammation and cancer

In 2000, Hanahan and Weinberg suggested six 'hallmarks of cancer' which were considered to be fundaments of cancer cell biology, involving self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, the potential of limitless replication, sustained angiogenesis, and the ability to invade tissues and metastasise (Hanahan and Weinberg 2000). Further, since more than a century it has been known that inflammatory cells are commonly present at the site of cancer, although the biological significance of this finding was for long poorly elucidated (reviewed in [Balkwill and Mantovani 2001]). However, incremental clinical and mechanistic evidence during recent decades now support the

existence of a tight link between cancer and inflammation, and, very recently, Colotta *et al* suggested that cancer related inflammation should represent the 'seventh hallmark' in Hanahan and Weinberg's model(Colotta et al. 2009).

Basically, the acute inflammatory response aims at defeating threatening infections or other stressors, and it is characterized by the activation of a broad panel of cytokines, chemokines, interleukins, inducible cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS/NOS2), and matrix metalloproteinases (MMPs), which mediate many features common to both cancer and inflammatory processes, involving tissue degradation, proliferation signals, recruitment of macrophages and other immune cells, and angiogenesis (Aggarwal et al. 2006).

A key factor for orchestrating the innate immune response is the transcriptional factor nuclear factor κB (NFκB). The release of bacterial products or tissue damage leads to NFκB activation via the Toll like receptor (TLR)-MyD88 signalling pathway, or interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) related pathways. Downstream target genes are then induced, with the generation of cytokines, adhesion molecules, COX-2, NOS2, vascular endothelial growth factors (VEGF), and tissue degrading enzymes (Aggarwal et al. 2006; Mantovani et al. 2008).

In contrast with the 'good' inflammatory response, aiming at protecting the host, the sustained inflammatory response may paradoxically result in tissue damage and rather precede cancer development (figure 2). There are many triggers for such inflammation being linked with cancer development, including infectious (*e.g.* helicobacter pylori and gastric cancer, papillomavirus and cervix cancer), chemical (tobacco smoking and lung cancer), and endogenous factors (colitis and colon cancer) (reviewed in [Aggarwal et al. 2006; Mantovani et al. 2008]). Concerning the colon, patients suffering from ulcerative colitis or Crohn´s disease are heavily predisposed to develop colon cancer (Vagefi and Longo 2005), where the stimulation of Toll-like receptors by commensal intestinal flora may contribute to tumourigenesis by activating NFκB and downstream cell survival signals in transformed epithelial cells, and/or by activating submucosal immune cells which release proinflammatory factors (reviewed in [Clevers 2004]). On the contrary, it has for long been known from epidemiological studies that non-steroidal anti-inflammatory drugs (NSAIDs), whose main mechanism of action is to suppress prostaglandin synthesis, may prevent adenomas and cancers in high risk families as well as among the general population(Giardiello et al. 1993; Huls et al. 2003; Kune et al. 1988; Kune 2000; Waddell and Loughry 1983). Taken together,

these findings reveal that inflammatory mediators may significantly contribute to the earliest steps of cancer development.

On the other hand, tumours without any known link with preceding inflammation often contain infiltrating macrophages and other immune cells, indicating that the cancer process itself may initiate the inflammatory process. In fact, some oncogenes and tumour suppressor genes were shown to regulate transcriptional programs which are similar to what occurs during inflammation. Examples of this involves the rearrangement of tyrosin kinase receptor encoding RET/PTC, which commonly occurs in papillary thyroid tumours(Borrello et al. 2005). Mutations affecting Ras-Raf pathway were shown to dysregulate immune responses and angiogenesis in various tumours such as pancreatic cancers and melanomas(Guerra et al. 2007; Sparmann and Bar-Sagi 2004, 2005; Sumimoto et al. 2006), and the inactivation of tumour suppressor von Hippel-Lindau (VHL) may interfere with hypoxia and hypoxia inducible factor-1α (HIF-1α) to induce proinflammatory cytokines, chemokine receptors, and angiogenic factors in concert with NFκB (reviewed in [Mantovani et al. 2008]). Further, and of the highest relevance for colorectal malignancy, accumulating evidence points to the intimate cross-talk between the canonical Wnt signalling pathway, the APC tumour suppressor gene, and genes and mediators of inflammation.

Initially, master regulators of cell cycle and transcription, *e.g.* c-myc (He et al. 1998)and cyclin D1(Tetsu and McCormick 1999), were identified to be targets of β-catenin/Tcf. Further studies however reveal that many genes of inflammation are similarly affected by Wnt/β-catenin signalling, involving COX-2(Araki et al. 2003), NOS2(Du et al. 2006), VEGF(X. Zhang et al. 2001), MMP-2(Wu et al. 2007), MMP-7(Brabletz et al. 1999; Crawford et al. 1999), and MMP-9(Wu et al. 2007).

Taken together, these findings implicate a complex cross-talk between 'classical' cancer genes, and the inflammatory response which takes place in tumour microenvironment. Traditional therapy against cancer focuses on erasing the tumour cells *per se*, while an attractive opportunity is a combination of targeting both malignant cells themselves and inflammatory compartments of the cancer process. Hence, the need for studies defining genes and mediators driving the tumour-promoting inflammation cannot be underestimated.

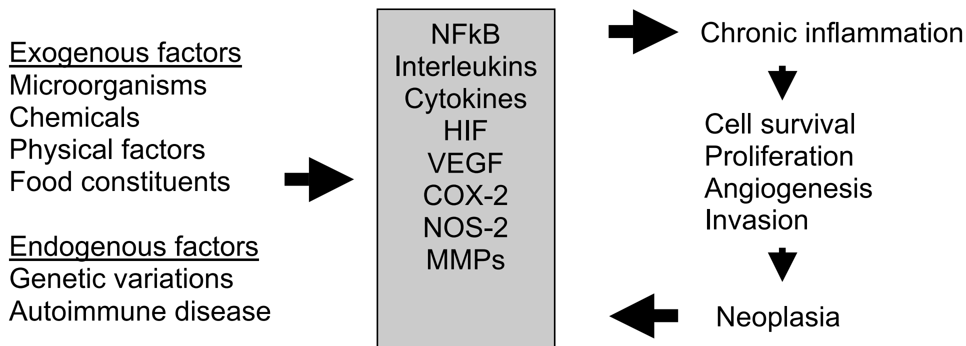

Figure 2. Cancer-related inflammation. The figure displays a schematic view of the link between chronic inflammation and cancer. Exogenous or endogenous stimuli may trigger a chronic inflammatory response, which promotes tumour development via the activation of transcription factors such as NFκB, and downstream effector molecules. Tumours without preceding inflammation may themselves initiate or maintain the release of proinflammatory mediators. Modified from (Aggarwal et al. 2006).

Prostaglandin E_2 and other lipid mediators

In the 1930-ties, the Swedish physician and scientist Ulf von Euler reported that lipid substances from male genital glands induced blood pressure suppression upon injection into experimental animals (reviewed in[Flower 2006]). Initially, the prostate was believed to be the origin of these substances, although further studies defined the seminal vesicles as their true origin in male genital tract, and forthcoming research revealed that the ability to generate prostaglandins was not restricted to the genital tract. In 1971, Sir John Vane reported the mechanism of aspirin's action, *i.e.* through inhibition of prostaglandin synthesis(Vane 1971), and Samuelsson *et al* later characterized the enzymatic way of prostanoid generation (reviewed in [Samuelsson 1983]). In 1982, Vane, Samuelsson, and the biochemist Bergström, jointly received the Nobel Prize in physiology or medicine for their research on prostaglandins(Check 1982).

Prostaglandins are originally derived from membrane phospholipids, which are cleaved by phospholipase A_2 (PLA_2) thereby generating arachidonic acid, the mother substance for both the prostaglandin and leukotrien classes of signalling molecules. Arachidonic acid is converted by the cyclooxygenases into prostaglandin G_2 (PGG_2) and the unstable intermediate prostaglandin H_2 (PGH_2), which is subsequently metabolised by specific enzymes into the main biologically active prostaglandins E_2, D_2, $F_{2\alpha}$, I_2 and

thromboxane A_2 (TxA_2) (fig 3). Prostaglandins and thromboxanes exert a wide plethora of biological actions in the immune system, vascular regulation, central nervous system, reproductive physiology, and cancer biology(Turini and DuBois 2002). In inflammation and tumour biology, most emphasis has been put on the COX-2 isoform, that is induced by mitogenic and proinflammatory stimuli(Turini and DuBois 2002). Rodent models on intestinal cancer reveal that genetic deletion or pharmacological inhibition of this enzyme dramatically suppress tumour development (Barnes and Lee 1998; Jacoby et al. 2000; Oshima et al. 1996; Oshima et al. 2001), and studies on NSAID consumption and colon adenoma and cancer incidences reveal a protective role among humans (Giardiello et al. 1993; Huls et al. 2003; Kune et al. 1988; Kune 2000; Waddell and Loughry 1983).

In colonic cancers, PGE_2 is the most abundant prostaglandin (Pugh and Thomas 1994; Rigas et al. 1993), and promotes cell survival, cell growth, cell migration, invasion and angiogenesis through interference with its G-protein coupled receptors EP1-4 (Chell et al. 2006). The cross-talk between prostaglandins and Wnt/β-catenin pathway was recently dissected by Castellone *et al*, who reported that PGE_2/EP2 signalling interacts with PI3K/Akt and Axin/GSK3β, thereby activating β -catenin activity and stimulating colon cancer cell growth (Castellone et al. 2005). Further, deletion of the EP2 gene suppresses intestinal tumourigenesis in $Apc^{\Delta 714}$ mice(Sonoshita et al. 2001), and other studies reveal additional roles of EP1 and EP4 receptors (Kitamura et al. 2003; Mutoh et al. 2002) in mouse models of intestinal tumourigenesis.

Additional non-PGE_2 prostanoids play major roles as well. TxA_2 synthase and TxA_2 are upregulated in colorectal cancers and TxA_2 was shown to induce tumour cell proliferation *in vitro* and *in vivo* (Pradono et al. 2002; Sakai et al. 2006), and administration of a TxA synthase inhibitor blocked hepatic metastasising upon the injection of colonic tumour cells in portal veins of mice (Yokoyama et al. 1995). Qualtrough *et al* recently reported that $PGF_{2\alpha}$ was highly abundant in colon cell lines and human tumour specimens, and that it was able to strongly induce proliferation and migration of colon cancer cells *in vitro*(Qualtrough et al. 2007). Further, PGI_2 was shown to interact with the nuclear PPARδ receptor and inhibit apoptosis and stimulate cell growth (Cutler et al. 2003; Gupta et al. 2000; D. Wang et al. 2006).

Until today, COX-1/COX-2 inhibition has been the only way of blocking prostaglandins and thromboxanes clinically, which is a powerful but unselective strategy being associated with severe gastrointestinal and cardiovascular side effects, mainly due to the disturbed balance of PGI_2 and TxA_2. Logically, the search for more specific molecular targets

within the prostaglandin pathway may therefore lead to the development of novel anti-inflammatory and anticancer drugs with less severe side effects.

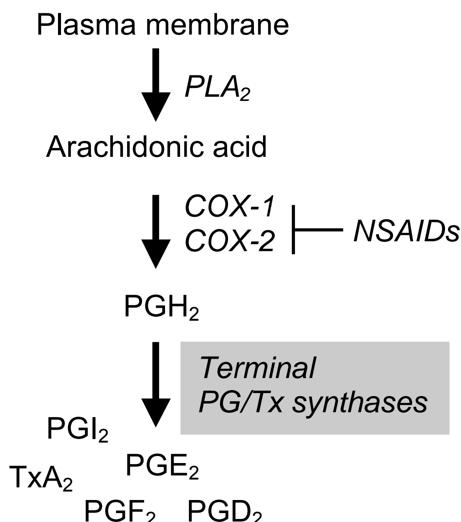

Figure 3. The prostaglandin synthesis cascade. The figure displays how PGE_2 and other prostanoids are generated via membrane phospholipids and arachidonic acid. NSAIDs suppress prostanoid formation by inhibition of COX-1 and COX-2. Terminal PG/Tx synthases, including mPGES-1, selectively catalyse the conversion of PGH_2 to the bioactive prostaglandins and thromboxanes, respectively.

Prostaglandin E synthase

Currently, three isoforms of prostaglandin E synthase are described in the literature; one cytosolic (cPGES) and two microsomal ones (mPGES-1 and mPGES-2). The inducible isoform mPGES-1 is functionally coupled with COX-2 and is commonly upregulated in inflammatory conditions and cancers (Kamei et al. 2003; Murakami and Kudo 2006; Nardone et al. 2004; Sampey et al. 2005; Yoshimatsu et al. 2001b; Yoshimatsu et al. 2001a).

mPGES-1 was originally identified in 1999 by Jakobsson and co-workers(Jakobsson et al. 1999), and the human mPGES-1 gene structure was further characterized by Forsberg *et al* (Forsberg et al. 2000). The gene spans 18.3 kB, contains three exons, and is situated on chromosome 19q34.3 (Forsberg et al. 2000). The gene is induced by bacterial products, inflammation components and mitogenic stimuli, which activate the Toll

like receptor/My88/NF-IL6 (Uematsu et al. 2002) and mitogen-activated protein kinase (MAPK) pathways (R. Han et al. 2002). At the promoter level, interaction between the transcription factor Egr-1 and the proximal GC box in mPGES-1 gene promoter is essential for transcription induction (Naraba et al. 2002).

In colorectal cancers, both mPGES-1 and COX-2 are found upregulated (Eberhart et al. 1994; Kamei et al. 2003; Yoshimatsu et al. 2001b), although marked differences were observed regarding their expression in individual tumours (Yoshimatsu et al. 2001b), implying that COX-2 and mPGES-1 are distinctly regulated.

With regard to COX-2 expression in cancer, several studies imply that loss of APC and subsequent activation of β-catenin/Tcf target genes might contribute to COX-2 induction directly or indirectly (Araki et al. 2003; Dimberg et al. 2001; Eisinger et al. 2006; Hsi et al. 1999; Mei et al. 1999; Prescott and White 1996). Whether mPGES-1 is similarly affected by the APC mutational status has not been investigated until today.

Nitric oxide and nitric oxide synthase

Nitric oxide (NO) was for long only known in non-biomedical situations, *e.g.* as an air pollutant. Surprisingly, biological roles of NO were later described, and in 1998, Furchgott, Murad, and Ignarro received the Nobel Prize in medicine or physiology for their discoveries on NO in vascular tonus regulation, and the finding that NO was identical to the endothelial derived relaxing factor (O. Smith 1998). In addition, NO is essentially involved in immunity/inflammation and neurotransmission (reviewed in [Hofseth et al. 2003]).

In the cells, NO is formed of L-arginine in a NADPH and molecular oxygen dependent reaction that depends on nitric oxide synthases (NOSs). Until today, three different isoforms of NOS are known, *i.e.* endothelial NOS (eNOS/NOS1), inducible NOS (iNOS/NOS2), and neuronal NOS (nNOS/NOS3). NOS1 and NOS3 are constitutively expressed and are activated by calcium and calmodulin, while NOS2 is not expressed in resting cells but can be induced in macrophages and other immune competent cells upon the release of immunostimulatory cytokines or bacterial products. NOS2 generates NO independently of intracellular calcium concentrations, and generally generates higher NO concentrations compared with NOS1 and NOS3 (concentrations measured in micromolars rather than pico- or nanomolars) (Hofseth et al. 2003).

The role of NO in cancer biology appears to be complex due to divergent functional activities under normal and patophysiological conditions. Studies have shown that NO, directly or indirectly via its reactive species, might damage DNA and induce mutations (Lala and Chakraborty 2001; Tamir et al. 1996; Tamir and Tannenbaum 1996; Zhuang et al. 2000). Numerous of reports support its role in tumour vascularisation via induction of HIF1α and VEGF(Jenkins et al. 1995; Kimura et al. 2000; Kimura et al. 2001; Sandau et al. 2000; Sandau et al. 2001a; Sandau et al. 2001b). On the other hand, high concentrations of NO may result in p53 accumulation and induce apoptosis and downregulation of NOS2 in a negative feed-loop manner(Ambs et al. 1998a; Brune et al. 2001; Forrester et al. 1996; Messmer and Brune 1996). With regard to NOS2, which is frequently induced in cancer tissue (Ambs et al. 1998b; Cobbs et al. 1995; Fransén et al. 2002; Thomsen et al. 1994), experiments with targeted deletion or pharmacological inhibition of NOS2 have been performed in murine models to reveal its relevance in intestinal tumourigenesis. Studies demonstrate either suppressed tumour development in the NOS2 deficient state (Ahn and Ohshima 2001; Kawamori et al. 2000; Rao et al. 1999; Rao et al. 2002), or do rather report accelerated tumourigenesis upon NOS2 gene deletion (Fransén 2005; Scott et al. 2001), supporting that the influence of NOS2/NO on tumour development is complex and probably depends on several factors including the tissue context, the supply of substrates and co-factors, the presence of other reactive molecules, host and/or tumour genetic characteristics, and the concentration and temporospatial localisation of generated NO (Hofseth et al. 2003).

The NOS2 gene

The 37 kB, 26 exons spanning NOS2 encoding gene is located on chromosome 17q11.2 (Chartrain et al. 1994) and is under the regulation of pathways related to inflammation. NFκB induces the gene via binding to responsive elements in the NOS2 promoter region (Saura et al. 1999), and interferon-γ (IFNγ) may activate NOS2 via the transcription factor IRF-1(Kamijo et al. 1994; Martin et al. 1994). In hypoxic regions of tumours, HIF-1α is thought to interact with IFNγ to induce NOS2 expression (Melillo et al. 1995; Tendler et al. 2001). The 8.3 kB NOS2 promoter region contains responsive elements for AP-1, various STATs, NFκB, c/EBP and CREB, and IRF-1 (reviewed in [Kleinert et al. 2003]), and it is highly polymorphic with many variants influencing NOS2/NO expression and signalling. The polymorphisms indentified may disturb the interaction between transcription factors and responsive elements, and are thought to influence the outcome of infectious and inflammatory conditions where

NOS2/NO is involved, such as diabetes mellitus, rheumatoid arthritis and malaria (Hobbs et al. 2002; Kun et al. 1998; Morris et al. 2002; Ohashi et al. 2002).

Matrix metalloproteinases and matrix degradation

The first member of the MMP family was discovered in 1962, when Gross *et al* characterized the collagenase that was involved in the metamorphosis of the tadpole (Gross and Lapiere 1962). Since then, a broad family of human MMPs has emerged, being implicated in embryogenesis, wound healing, bone remodelling, inflammatory conditions, cardiovascular disease, and cancer (reviewed in [Zucker and Vacirca 2004]).

Originally, research was mainly focused on the ability of MMPs to degrade extracellular structures such as basement membranes, and these properties of MMPs have for long been associated with cancer cell invasion (Duffy 1987; Liotta et al. 1982; Mignatti et al. 1986; Pauli et al. 1983; Woolley 1984). However, recent findings reveal that the roles of MMPs in tumourigenesis are much more complex and include the mediation of growth factor activation, regulation of cell adhesion and motility, regulation of apoptosis and cell survival, angiogenesis, and immune surveillance (reviewed in [Deryugina and Quigley 2006]). Not surprising, targeting MMP activity was early seen as a promising approach to limit tumour progression clinically. Though, despite the broad implications of MMPs in cancer progression, clinical trials with MMP inhibitors (MMPIs) have been disappointing so far. The reasons for this remain to be fully elucidated, but the broad spectrum MMPIs available until now have been proposed to not only inhibit tumour promoting activities of MMPs, but also target anti-tumourigenic properties of some MMPs (Overall and Kleifeld 2006). First, MMPs are not acting solely, but do rather build a web of proteolytic enzymes in the tissue with abilities to activate or inhibit other members of the enzyme family. Second, some MMP activity does rather limit tumour progression, either by contributing to the immune response to tumour cells, or by inhibiting angiogenesis by cleaving plasminogen and generate angiostatin (Overall and Kleifeld 2006; Zucker and Vacirca 2004). Hence, further characterization of the role and regulation of individual MMPs, and particulary of those carrying the potential of being drug targets, are requested.

The MMP genes

The individual MMPs are encoded by separate genes located on several chromosomes, although a MMP gene cluster is found at chromosome 11q22 (Clark et al. 2008). Numerous of regulatory mechanisms may influence the ultimate activity of an MMP, including regulation of transcription, DNA methylation/acetylation, mRNA stabilisation, activation of latent MMPs, and inhibition of MMPs by naturally existing tissue inhibitors of metalloproteinases (TIMPs)(Clark et al. 2008; Ye 2000). The latter is present with MMP-2, which is widely expressed in 'resting' conditions, but under tight control of activating and inhibiting enzymes in the MMP and TIMP families of enzymes. However, for many MMPs, a key step is transcriptional regulation, because these genes are normally silenced in the absence of inducing signals(Ye 2000). The promoter regions of many MMPs contain AP-1 and PEA-3 consensus elements interacting with members of the Fos/Jun or Ets families of transcription factors, and these elements and transcription factors respond to various MMP inducing stimuli including phorbol ester, cytokines, and growth factors (Angel et al. 1987; Gaire et al. 1994; Ye 2000). Further, MMP-9 is a direct target of NFκB signalling in inflammation and tumourigenesis (St-Pierre et al. 2004), and genes encoding MMPs -2, -7, -9, and -26 contain functional Tcf/Lef responding elements in their 5' regulatory regions(Brabletz et al. 1999; Crawford et al. 1999; Marchenko et al. 2002; Wu et al. 2007), linking the roles of MMPs in inflammation and cancer at the level of transcriptional regulation.

MMPs are generally upregulated and involved in the development, invasion, and metastasis of colonic tumours (*e.g.* [E. A. Baker et al. 2000; Murray et al. 1996; Parsons et al. 1998]). Polymorphic sites in regulatory regions of MMP genes may influence gene and protein expressions and contribute to susceptibility and outcome of autoimmune disorders, cardiovascular disease, and many cancers including those of the lung, the gastrointestinal tract, the mammary, and the gynaecological sphere(Dunleavey et al. 2000; Ghilardi et al. 2001; Grieu et al. 2004; Hinoda et al. 2002; Jones et al. 2003; Matsumura et al. 2005; Miao et al. 2003; Price et al. 2001; Rutter et al. 1998; Y. Wang et al. 2005; E. Xu et al. 2004; Ye et al. 1995; Ye et al. 1996; Yu et al. 2002; B. Zhang et al. 1999; Zinzindohoue et al. 2004; Zinzindohoue et al. 2005).

VEGF and tumour angiogenesis

The ability to form new blood vessels is vital for normal embryogenesis, pregnancy, wound healing and restoration of injured tissues (reviewed in [Birk et al. 2008]), but, if occuring

uncontrolled, it is a hallmark of cancer progression (Hanahan and Weinberg 2000). The first VEGF was originally characterized in 1983 and was initially called vascular permeability factor (Senger et al. 1983). Since then, other VEGFs have been identified, and hence the 'original' VEGF is also called VEGF-A, in contrast with the other family members VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PIGF (placental growth factor)(Birk et al. 2008).

Early studies, together with more recent reports, support a key role for VEGF in initiating tumour angiogenesis in various malignancies. Interestingly, recent clinical studies reveal that VEGF-targeting monoclonal antibodies, in combination with standard chemotherapeutics, are able to retard the progression of metastasised colorectal cancer and improve median survival, progression-free survival, and time to progression (Giantonio et al. 2007; Hurwitz et al. 2004).

The biological effects of VEGFs are exerted via their interaction with the membrane bound tyrosine kinase family receptors VEGFR-1, -2, and -3. Upon ligand-receptor interaction, active receptors are created when two receptors dimerize and autophosphorylate via tyrosine kinase domains. VEGF-A acts as ligand for VEGFR-1 and -2, whereas VEGFR-3 rather interacts with VEGFs -C and –D. Activation of intracellular signalling - including MAPK, PKC, and Akt pathways - results in increased vascular permeability, promotion of cell survival, cell proliferation and cell migration through activation of nitric oxide, eicosanoids, metalloproteinases, and other downstream effectors (reviewed in [Zachary and Gliki 2001]).

The VEGF gene

The VEGF gene is located on chromosome 6p12 and includes a 14 kB coding region consisting of eight exons, and exhibits alternate splicing to form a family of protein isoforms with some variability in function and binding specificity(Tischer et al. 1991). Many stimuli including growth factors, female hormones, inflammatory cytokines, and cellular hypoxia regulate the VEGF expression. The promoter region of VEGF contains response elements for Sp1, STAT3, AP-1, Egr-1, AP-2, oestrogens, progesterone, and HIF-1α, the latter being overexpressed/activated in growing tumours where hypoxia drives angiogenesis(Birk et al. 2008; Kuwai et al. 2003; Pages and Pouyssegur 2005; Zhong et al. 1999). Recently, results of our laboratory suggested that variations of the HIF1α gene may control the growth pattern of colon cancers and precede the development of ulcerative tumours(Fransén et al. 2006),

although it is unclear whether this correlation is directly coupled with VEGF activity, or some other target gene(s) of HIF1α.

In addition, the VEGF gene expression is enhanced upon loss of tumour suppressors VHL, p53 and p73(Igarashi et al. 2002; Salimath et al. 2000; L. Zhang et al. 2000), and activation of oncogenes Ras, Src, HER2/neu, and Bcr/Abl (Mukhopadhyay et al. 1995; Rak et al. 1995b; Rak et al. 1995a).

The promoter is highly polymorphic, and several SNPs which significantly affect VEGF expression have been identified(Renner et al. 2000; Stevens et al. 2003; Watson et al. 2000). These exert possible biomarkers for VEGF activity and disease progression in varying conditions where inflammation and angiogenesis are thought to play roles including Behcet's disease(Nam et al. 2005), adult respiratory distress syndrome(Medford et al. 2005), arthritis(Butt et al. 2007; S. W. Han et al. 2004), and cancer(Howell et al. 2002; Jin et al. 2005; Koukourakis et al. 2004; Sfar et al. 2006).

AIMS

In the present thesis, genes and genetic variations controlling cancer-related inflammation were analysed within the context of colorectal cancer. The specific aims were:

I. To study the role of mPGES-1 in intestinal carcinogenesis, and to define molecular alterations of the APC/β-catenin pathway which are potentially associated with mPGES-1 overexpression in colon tumour cells (*papers I-II*).

II. To study variations of genes encoding NOS2, MMPs, and VEGF, and their relation with colorectal cancer susceptibility and clinicopathological characteristics (*papers III-V*).

MATERIALS AND METHODS

Materials

Animals

In 1990, Moser *et al*(Moser et al. 1990) identified a mouse carrying multiple intestinal neoplasias in a colony of animals treated with the mutagen ethylnitrosurea (ENU). The responsible gentic event was later identified as a germline truncating mutation at codon 850 in one of the APC alleles, hence the mutant was named $Apc^{Min/+}$(Su et al. 1992). Since then, the $Apc^{Min/+}$ mouse has become one of the most widely used animal models for studies on intestinal cancer. In the current thesis, $Apc^{Min/+}$ mice on the C57BL/6 background were intercrossed with mice harboring a targeted deletion of the mPGES-1 encoding gene, previously generated by Uematsu *et al* (Uematsu et al. 2002). Mice of $Apc^{Min/+mPGES-1+/+}$, $Apc^{Min/+mPGES-1+/-}$ and $Apc^{Min/+mPGES-1-/-}$ were generated through subsequent intercrossings. Intestines and tumours were examined and isolated for further analyses. All animal expriments were approved by the Animal Care and Use Committee at the Linköping University.

Human material

Colorectal tumour biopsies, paired intestinal mucosal biopsies approximately 10 cm from the tumour, and blood samples were collected from more than 300 patients at the County Hospital Ryhov, Jönköping and University Hospital, Linköping, Sweden. Blood samples were also obtained from randomly selected healthy individuals from the same geographical area. Clinical information regarding age and gender of the patient, tumour localization, disease staging, and pathological growth pattern were obtained from the clinical records. Tissue and blood samples were immediately snap frozen and stored at -80ºC until further handling, including paraffin embedding and isolation of protein, RNA and DNA. All studies involving human material were approved by the Research Ethics Committee at Linköping University.

Cell lines

Human colon cancer cell lines HT29 and HCT116 were used for studying the regulation of mPGES-1 *in vitro*. HT29 cells lack the expression of full length APC, in contrast with HCT116 cells(K. J. Smith et al. 1993) which express full length APC naturally. In *paper II*, HT29 cells harboring a vector, with a metallothionin driven promoter coupled to the wild type APC gene, were kindly given by Dr. Bert Vogelstein(Morin et al. 1996). By stimulating these cells with zinc, full length APC was expressed. On the other hand, APC was silenced in HCT116 cells with *siRNA*, and naïve HT29 cells were similarly treated with *siRNA* targeting β-catenin.

Analyses of DNA and RNA

Real-time PCR

Real-time PCR provides an ability to monitor the progress of the PCR as it occurs, rather than at the end of the reaction that is done with traditional PCR. The target of interest is amplified in a cyclic manner, and the quantification is based on the time point when the amount of target reaches the point of detection, *i.e.* the C_t value (www.appliedbiosystems.com). Since the method was first developed in the beginning of the 1990-ties(Higuchi et al. 1992), it has revolutionised the way of rapidly and accurately quantifying DNA and RNA. In papers *I, II and V*, RNA was isolated from either tissue specimens or cell lines with commercially available reagents. Equal amounts of RNA were subsequently reversely transcribed to cDNA, and real-time PCR was performed with predesigned primers and probes targeting the human or murine genes of interest. C_t values were related to endogenous control genes (ΔCt), and relative expression ($2^{-\Delta Ct}$) was normalised to the average expression in non-tumour control tissue ($2^{-\Delta\Delta Ct}$) (*paper I*). Alternatively, relative concentrations were translated into a relative concentration unit presented by a standard curve that was co-amplified in each experiment (*papers II and V*).

RNA interference (RNAi)

In 1998, Andrew Fire and co-workers described the principles of RNA interference (RNAi) - a gene silencing mechanism by which double stranded RNA induces the degradation of corresponding mRNAs (Fire et al. 1998). The discoveries yielded the Nobel Prize in 2006, and RNAi is now not only known as a natural process by which cells regulate gene expression, but also as a powerful method for gene silencing in molecular biology research(Couzin 2006). Upon introduction of short double stranded RNA oligonucleotides, complementary host mRNA is degraded in an intracellular process driven by the RNA induced silencing complex (RISC) (Hutvagner 2005). In *paper II,* RNAi was performed by transfecting colon cancer cells *in vitro* with small inhibitory RNA oligonucleotides (*siRNA*). To ensure successful gene silencing, pools of four different oligonucleotides were used, targeting the genes of interest, *i.e.* APC or β-catenin. The attenuated expression of the targets was confirmed at the protein level with Western blot, and functionally the experiments were evaluated by assessing the expression of previously known target genes of APC and β-catenin.

Luciferase reporter assays

Luciferase is the generic name for naturally occurring enzymes, produced by fireflies and other organisms, which are characterized by their ability to emit light upon the oxidation of their substrates, *i.e.* luciferins. In biomedical studies, luciferase activity is often used as a reporter to assess the transcriptional activity in cells that have been transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest(Fan and Wood 2007). In *paper II*, HT29 colon cancer cells were transfected with a luciferase reporter vector containing the -1455/+70 promoter sequence of the mPGES-1 gene, or a series of deletion construct of this. The luciferase activity was then assessed in cells with and without the induction of full length APC and normalized to the activity of β-galactosidase control vector, to characterize whether any parts of the promoter region were influenced by the current APC status.

Single strand conformation analysis (SSCA)

Since the publications of Orita *et al* 1989(Orita et al. 1989b; Orita et al. 1989a), SSCA has become widely used as a rapid and sensitive method for the detection of DNA point mutations. Most often, PCR is used to generate multiple copies of the DNA fragment of interest. In a secondary reaction, the fragments are labelled with radioactivity and then electrophoretically separated on a gel, usually of poly-acrylamide, under non-denaturing conditions. Depending on whether any mutation is present or not, the single-stranded DNA fragments form different secondary structures with distinct migration patterns on the gel. Final detection and interpretation is performed upon the exposure of the gels to radiosensitive films. The above mentioned PCR-SSCA approach was used in *paper III* to detect polymorphisms in the promoter region of the NOS2 gene.

Restriction fragment length polymorphism (RFLP)

The discovery and characterization of the restriction enzymes rendered the Nobel Prize in 1978 for Werner Arber, Dan Nathans and Hamilton Smith(The Nobel prizewinners 1978: medicine. From modest beginnings 1978). In mutation analysis, restriction enzymes can be used to discriminate between two alleles if the mutation or polymorphism creates or abolishes a restriction site. In *papers III-IV*, the RFLP principle was used for the detection of some of the polymorphisms of interest. Following PCR amplification, the DNA fragments were incubated with the appropriate restriction enzyme. Cleaved or uncleaved fragments were then separated electrophoretically on agarose gels and visualized with ethidium bromide and UV-light.

Primer extension

In *papers IV-V*, a PCR/primer extension approach was used to discriminate between different alleles of the polymorphic sites in the MMP and VEGF promoter regions. DNA fragments were amplified with PCR and the fragments underwent a primer extension reaction with ~20 bp primers matching a region in the close vicinity of the SNP of interest. Under the appropriate conditions, the primer will hybridize to its complementary sequence, and a thermosequenase will introduce single nucleotides complementary to the template at the 3' end of primer. For the MMP promoter fragments, three standard deoxyribonucleotides (dNTPs) were included, but one of the dNTP was replaced with a didexyribonucleotide

(ddNTP), which terminates the reaction upon its introduction in the elongating DNA strand. The size of the fragments generated by primer extension will differ depending on whether the mutation is present or not, and for the MMP polymorphisms, fragments were finally separated by high performance liquid chromatography (HPLC). Similarly, the extension reactions of VEGF gene fragments included dideoxynucleotides, but these were rather labelled with different dyes, enabling subsequent computerised detection and allele-discrimination.

Analyses of proteins and lipids

Enzyme Linked ImmunoSorbent Assay (ELISA)

ELISA as a novel immunoassay was originally described in 1971 by two independent laboratories(Engvall and Perlmann 1971; Van Weemen and Schuurs 1971). Basically, in ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. The antibody is linked to an enzyme, and in the final step a substrate is added which the enzyme can convert to a detectable signal. In *papers I-II*, predesigned ELISA kits were used to determine the amounts of PGE_2, $PGF_{2\alpha}$, PGI_2, PGD_2 and TxA_2, or their stable metabolites, in samples of tumour and normal intestines from $Apc^{Min/+}$ mice or the growth media of cell lines, respectively. Tissue samples were collected and immediately homogenized with a buffer containing indomethacin which blocked any remaining COX activity, before undergoing ELISA analyses. The absorbance was measured and converted to a concentration unit which was given by the standard curve that was co-run in every experiment.

Western blot

Western blot was originally described in the late 1970-ties(Burnette 1981; Renart et al. 1979; Towbin et al. 1979), and has since then become a standard method for the detection and quantitation of specific proteins in a sample homogenate. It combines the ability to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native conditions) in gel electrophoresis with the ability to easily detect them following transfer to a nitrocellulose or PVDF membrane. In *paper II*, denatured protein lysates from colon cancer cell lines or tissue specimens were seperated on poly-

acrylamide gels. Electroblotting to PVDF membranes was then performed, with subsequent incubation in primary and secondary antibody solutions, respectively. Secondary antibodies were linked to horse radish peroxidase (HRP), enabling subsequent detection with enhanced chemiluminiscence and exposure to a digital camera.

Immunohistochemistry (IHC)

The first immunohistochemical staining was performed in the 1940ties, when Coons et al identified pneumococci using a direct immunofluorescent method. Since then, the method has undergone major development and become a routin method for the detection of proteins in tissue specimens *in situ*. Today several IHC variants exist with different types of antibodies, enzymes, and detection methods (reviewed in [Burnett et al. 1997]). In *paper II*, paraffin embedded slides from colorectal tumours were stained for mPGES-1. Before incubation with the primary polyclonal antibody, antigen retrieval was performed through boiling the slides for 30 minutes in a citrate buffer. The secondary antibody used was conjucated with horse-radish peroxidise (HRP), and visual detection followed the oxidation of the substrate (DAB+), which yields a brown colour upon oxidation. Slides were finally counterstained with hematoxylin.

Statistics

Quantitative variables were generally expressed as means ± standard error of the mean (SEM) and compared with Student's t-test. Categorical variables such as geno/allelo-types were analysed with Yates-corrected $\chi 2$-test or Fischer's exact test, and the odds ratios (OR) and 95% confidence intervals (CI) were determined. For genotype distributions in the control populations, Hardy-Weinberg equilibrium was confirmed for all polymorphisms investigated. When appropriate, power analyses were performed with either PS v2.1.31, or the open access calculator found at UCLA:s homepage (http://calculators.stat.ucla.edu/powercalc/). If not stated otherwise, all calculations were performed with the SPSS software (SPSS UK Ltd., Woking, UK). In general, *p*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Papers I-II

In *paper I*, $APC^{Min/+}$ mice with homozygous deletion of the mPGES-1 encoding gene were generated to asses the role of mPGES-1 in intestinal carcinogenesis, and to determine whether blocking of mPGES-1/PGE_2 render any secondary effects on the generation of other prostanoids downstream COX-1/COX-2.

Briefly, we observed a two fold enhanced tumour frequency among the mPGES-1 knock out mice, which was also accompanied by increased mean tumour size. By contrast, previous studies with deletion or inhibition of COX-2 rather suppressed tumour development in animal models of intestinal cancer (Barnes and Lee 1998; Jacoby et al. 2000; Oshima et al. 1996; Oshima et al. 2001). The mPGES-1 lacking animals of the present study however presented with a shifted balance of non-PGE_2 eicosanoids in tumours and corresponding normal mucosa, with enhanced tissue concentrations of $PGF_{2\alpha}$, PGI_2, TxA_2, and PGD_2. Similar findings have been previously described in neuronal tissue, gastric mucosa and macrophages where mPGES-1 was deleted *in vitro* and *in vivo* (Boulet et al. 2004; Brenneis et al. 2008; Trebino et al. 2005), indicating that 'selectively' targeting mPGES-1/PGE_2 might still indirectly influence the general prostaglandin balance. Interestingly, recent reports put forward significant roles for these non-PGE_2 mediators in tumourigenesis, including stimulation of tumour growth and motility, angiogenesis and metastasis (see introduction) (Cutler et al. 2003; Gupta et al. 2000; Mutoh et al. 2002; Park et al. 2007; Pradono et al. 2002; Qualtrough et al. 2007; Sakai et al. 2006; D. Wang et al. 2006; D. Wang and Dubois 2008; Zamuner et al. 2005). Hence, a redirected prostanoid generation, with enhanced tissue concentrations of these mediators, exerts a possible mechanism which accelerates tumourigenesis in mPGES-1 deficient animals.

Nakanishi *et al.*(Nakanishi et al. 2008) very recently demonstrated conflicting data with regard to ours. They employed two other animal models of intestinal cancer, *i.e.* $Apc^{\Delta 14/+}$ and *AOM*-injected mice, where targeted mPGES-1 gene deletions were introduced. In their models, tumourigenesis was suppressed in the mPGES-1 deficient state, and PGE_2 was lowered without altering the profile of other primary bioactive PGH_2 metabolites (*i.e.* TxA_2, PGD_2, $PGF_{2\alpha}$, and PGI_2). The reasons for the diverging data are obscure, but different animal models have indeed been used, with different features regarding the APC mutational status and the distribution of intestinal tumours. $Apc^{Min/+}$ and $Apc^{\Delta 14/+}$ mice predominantly

present with tumours of small intestine, in contrast with AOM-treated mice which rather develop lesions of colon and rectum. With regard to the prostaglandin concentrations, the metabolites were determined in both papers with slightly different approaches. Nakanishi *et al.* used the entire colon or distal part of small intestine for metabolite measurements with a HPLC based analysis, whereas we used ELISA, which allowed measurements in polyps and non-polyp, healthy biopsies *per se*, rather than pooled tissue.

In *paper II*, we continued with analysing the association between the APC tumour suppressor gene and mPGES-1 in colon tumour cells of cell cultures and tumour specimens. Previously, the loss of wild type APC has been proposed to be an event contributing to COX-2 upregulation in colon cancer cells(Araki et al. 2003; Dimberg et al. 2001; Eisinger et al. 2006; Hsi et al. 1999; Mei et al. 1999; Prescott and White 1996), while a similar possible relation for mPGES-1 has not been studied so far.

The expression of mPGES-1 and COX-2 was determined in relation with APC gene status in HT29 and HCT116 colon cancer cells. Induction of APC in HT29 cells was followed by upregulation of mPGES-1 and PGE_2, whilst COX-2 mRNA was unaffected and COX-2 protein was downregulated, indicating that mPGES-1 rather than COX-2 was the rate-limiting step in PGE_2 generation under the current conditions. Results on COX-2 supports those obtained by Hsi *et al*(Hsi et al. 1999), who suggested that loss of APC influenced COX-2 at the protein rather than gene/mRNA level in HT29 cells. Silencing of APC in HCT-116 cells yielded the opposite outcome, revealing downregulation of mPGES-1 mRNA and protein.

A similar relationship, however of border-line significance (p=0.059), was observed in biopsies of human colorectal tumours, where the absence of mutant APC correlated with enhanced mPGES-1 expression. In line with previous reports (Kamei et al. 2003; Yoshimatsu et al. 2001b), tumours in general displayed induced mPGES-1 expression, and mPGES staining was essentially limited to tumour epithelial cells.

Luciferase assays with the putative mPGES-1 promoter region, and deletion constructs of it, revealed similar activity in both APC- and mock-induced HT29 cells, implying that the association with APC status and mPGES-1 regulation was not occurring at the promoter level. Further, since the mPGES-1 promoter region contains several potential β-catenin/Tcf binding elements (*TBEs*) within the first 1500-bp upstream of TSS, we explored whether the β-catenin status could influence the mPGES-1 expression. However, no altered mPGES-1 expression was observed upon *siRNA* silencing of β-catenin in HT29 cells, indicating no or a limited biological significance for the potential *TBEs* identified in the

mPGES-1 promoter. In contrast, RNA degradation assays revealed retarded degradation of mPGES-1 mRNA in APC-induced HT29 cells, suggesting that APC influences RNA stability, rather than transcriptional activity, of mPGES-1.

Former reports have mainly focused on factors and signals involved in the induction of mPGES-1 transcription, such as NF-IL6(Uematsu et al. 2002) or the MAPK pathway(R. Han et al. 2002), while, to our knowledge, until now little is known about regulation at the mRNA stability level. Although the molecular mechanisms need further dissection, the results of *paper II* imply that mPGES-1 mRNA might be a target for post-transcriptional regulation in tumour cells, and that this process is influenced by the APC mutation status.

Taken together, *papers I-II* confirm that mPGES-1 is crucial for the generation of PGE_2 in colon tumour cells, although deletion of the gene may lead to significant shift towards the generation of other potentially pro-tumoural mediators downstream COX-2/PGH_2. The results therefore question previously made expectations on inhibiting mPGES-1 for anticancer purposes, and support a view where the entire prostanoid balance, rather than one mediator solely, influences tumour biology. Further, a complex interaction between APC gene status and COX-2/mPGES-1/PGE_2 emerges, suggesting that wild type APC influences mPGES-1 mRNA stability rather than mPGES-1 transcriptional regulation.

Paper III

In *paper III*, 131 colorectal cancer patients and 160 healthy controls were genotyped for four different polymorphisms in the 5' regulatory region of the NOS2 gene. The polymorphisms investigated have previously been found to affect the development and outcome of various infectious and inflammatory diseases where NOS2 is involved, including malaria, diabetes, and rheumatoid arthritis (Boutlis et al. 2003; Gonzalez-Gay et al. 2004; Hobbs et al. 2002; Kun et al. 1998; Levesque et al. 1999; Morris et al. 2002; Ohashi et al. 2002; Pascual et al. 2002; W. Xu et al. 1997).

First, two SNPs at -954 (G/C) and -1173 (C/T) upstream of the NOS2 transcription start site (TSS) were analysed among more than 100 patients and control individuals, revealing no polymorphic individuals at all. Other studies on Caucasian populations are in agreement with our findings, and to our knowledge these SNPs have until now only been identified in African populations, where they in some studies have been associated with the outcome and clinical characteristics of malaria(Boutlis et al. 2003; Hobbs et al. 2002; Kun et al. 1998; Levesque et al. 1999; Pascual et al. 2002). While performing the SSCA for the -1173 polymorphism, we however found a novel T/C substitution at -1144 among ~2% of patients as well as control individuals. Whether this SNP rends any influence on NOS2 promoter activity or has any impact for any other clinical conditions remains to be elucidated.

A $(TAAA)_n$ repeatable variant located -756 to -716 upstream TSS was identified by Bellamy and co-workers(Bellamy and Hill 1997), and later found in association with diabetic complications(Morris et al. 2002). In the present material, no association with colorectal cancer susceptibility or clinicopathological features was observed. Another multiallelic $(CCTTT)_n$ polymorphism, located 2.5 kB upstream TSS(W. Xu et al. 1997), was previously shown to influence the severity of malaria(Ohashi et al. 2002) and the outcome of atopic conditions(Konno et al. 2001). When analysing the present population, the alleles were however similarly distributed among both patients and controls, with copy numbers reaching from eight to seventeen in the present material. A stratification system according to Hobbs *et al*(Hobbs et al. 2002) was employed to stratify the fragments in 'long' (12 and more) vs. 'short' (8-11) alleles. The proportions of 'long' and 'short' alleles were similar in both populations, implying a limited role for this polymorphism in colorectal cancer development.

Later studies have however indicated a contribution for this polymorphism in some other cancers including gastric and urothelial carcinomas, where the high activity

‘longer’ alleles (n > 11) generally enhance disease susceptibility or progression(Kaise et al. 2007; Sawa et al. 2008; Shen et al. 2007; Tatemichi et al. 2005).

Collectively, none of the investigated polymorphisms in the NOS2 promoter region seem to contribute to colorectal cancer development or clinicopathological features. Most likely, other events contribute to the high expression of NOS2 that is seen in many colorectal tumours, including the possible modulation of upstream regulatory genes and pathways.

Paper IV

In *paper IV*, a possible association between MMP promoter polymorphisms and the susceptibility for colorectal cancer was investigated. In total, 127 patients and 208 randomly chosen control individuals from the same geographical area were genotyped for four different SNPs in the regulatory regions of the MMP-1, -2, -3, and -9 genes, respectively.

With regard to the MMP-1 1G/2G polymorphism, located at -1607 of TSS, the 2G allele creates an Ets binding site which is associated with enhanced promototer activity(Rutter et al. 1998). The 2G allele was more frequently occurring among the patients than healthy controls in the present study (OR=1.4, 95% CI 1.02-1.96, *p*=0.037), although no associations with disease characteristics, including age of diagnosis, Duke's staging, tumour growth pattern and anatomical localisation, were identified. Other studies on colorectal cancer populations of different nationalities and ethnicities have described similar results(Ghilardi et al. 2001; Hinoda et al. 2002), collectively indicating an association with the 2G allele and the enhanced susceptibility of developing colorectal cancer.

Regarding overall survival and disease progression, previous investigations reveal varying results. Ghilardi *et al*(Ghilardi et al. 2001) studied an Italian population of 60 colon cancer patients, and revealed an association between the 2G allele and metastasising disease. In consistence, results obtained by Zinzindohoue *et al* (Zinzindohoue et al. 2005) pointed to an inverse relationship between the number of 2G alleles and disease survival. On the other hand, Hettiaratchi *et al*(Hettiaratchi et al. 2007) unexpectedly found that the 5-year survival was more favourable among 2G/2G carriers in a population of 503 Australian colorectal cancer patients. The reasons for the inconsistent results obtained are not well known, but speculatively they might reflect the dichotomous functions of MMPs in tumour biology with both pro- and anti-tumourigenic properties under different disease stages. However, by chance associations, varying patient/tumour characteristics, or variations depending on different ethnical populations can not be excluded.

Concerning MMP-3, a -1171 5A/6A SNP was identified by Ye and co-workers and found to be associated with suppressed promoter activity and coronary atherosclerosis(Ye et al. 1996). The 6A allele has been associated with reduced as well as enhanced susceptibility for varying types of cancer(Ghilardi et al. 2001; Hinoda et al. 2002; Zinzindohoue et al. 2004), but in the present study, no relation with colorectal cancer susceptibility or clinicopathological features was observed.

MMP-2 and -9 has for long been known to degrade the basement membrane component collagen IV, and have been implicated in cancer invasion and metastasis due to this ability (reviewed in [Mook et al. 2004]). A -1306 C/T substitutive polymorphism was identified in the regulatory region of the MMP-2 gene, being associated with lower promoter activity(Price et al. 2001). In contrast with our findings, revealing no relation to either susceptibility or disease characteristics, the high activity C allele was associated with colon cancer susceptibility and invasiveness in a Chinese population of similar size as ours (E. Xu et al. 2004).

Finally, a C/T substitution, located at -1562 in the MMP-9 promoter, was previously associated with poor outcome of gastric cancer(Matsumura et al. 2005), but positive prognostic findings of mammary cancer(Grieu et al. 2004). However, no association with colorectal cancer susceptibility or characteristcs was observed in the present material.

Taken together, several studies including ours reveal an association between the MMP-1 -1607 2G allele and the susceptibility for colorectal cancer, although diverse results remain regarding its relation with disease progression and characteristics. With regard to MMP-2, -3 and -9, the investigated SNPs appear less likely to influence colorectal cancer development or progression.

Paper V

VEGF induced angiogenesis is a crucial event during the growth and progression of solid malignancies, including colorectal cancers. Previously, variation of the upstream HIF1α gene was correlated with growth pattern of colorectal tumours(Fransén et al. 2006), although the role of variations of the VEGF gene has been scarcely investigated until now.

In *paper V*, five polymorphisms located in the regulatory regions of the VEGF gene were examined in a colorectal cancer population of 302 individuals, and allelotypes, genotypes, and haplotypes were compared with 336 healthy controls. Further, possible associations with VEGF mRNA expression in tumours and normal colonic mucosa were assessed among a subset of patients.

The genetic variants investigated were situated in the promoter and 5'-untranslated region at -2578 (C/A), -2549 (del/ins 18 bp), -1154 (G/A), and -634 (G/C) compared to TSS. These have previously been found to influence promoter activity and VEGF expression levels(Brogan et al. 1999; Stevens et al. 2003; Watson et al. 2000). In addition, one SNP in the 3'-UTR was examined (+936 C/T), with suppressive influence on VEGF plasma levels(Renner et al. 2000). The SNPs and insertion in the promoter/5'-UTR were found to be in tight LD (|D'| = 0.91-1.00) and hence considered to be a block, while the +936 C/T was only weakly associated with the other polymorphisms (|D'| = 0.05-0.19) and hence not included in haplotype analyses. The -2578 C/A and the -2549 del/ins site were confirmed to be in complete LD and -2578 C/A was therefore used as a marker for these SNPs in further analyses.

Generally, the genotypes and haplotypes investigated were similarly distributed among cases and controls, and no association with clinicopathological parameters (age, gender, tumour localisation, Duke's stage, pathological phenotype) was observed. However, when combining the -2578C and +936T alleles a small yet significant overrepresentation in the colorectal cancer population was revealed (OR=1.6, 95% CI 1.3-1.6).

Not surprisingly, VEGF mRNA was ~2-fold enhanced in tumours compared to corresponding normal mucosa, which is in agreement with previous papers reporting enhanced plasma and tumour VEGF expression among colorectal cancer patients(Garcia et al. 2008; Yamamori et al. 2004), although no genotypes or haplotypes were able to significantly discriminate between high vs. low-expressing samples in the present study.

Other recent studies reveal contradictory data regarding the influence of VEGF gene polymorphisms on colorectal cancer susceptibility and outcome. Very recently, Lurje *et*

al reported that the high expression +936 C/C genotype was associated with shorter time to disease recurrence among stage III colon cancer patients(Lurje et al. 2008). On the contrary, Dassoulas *et al* reported an association between the +936 T/T, as well as the 2578 A/A, and -634 C/C genotypes and lower overall survival among Greek colorectal cancer patients (Dassoulas et al. 2008), and Kim *et al* similarly reported that -634 C and +936 C alleles were independent positive prognostic factors among colorectal cancer patients(Kim et al. 2008). Chae and co-workers presented results in line with those obtained by Dassoulas and Kim, supporting similar influences of +936 C/T and -460 T/C polymorphisms, the latter being in strong LD with -634 G/C, on colorectal cancer progression in a Korean population (Chae et al. 2008).

In the present study, genotype/haplotype frequencies were analysed in strata according to Duke's stage and other clinicopathological parameters without revealing any significant associations. Taken together, results of different studies are still diverse, and the role and contribution of VEGF gene variations in colorectal carcinogenesis can not yet be concluded. A general disadvantage with the above-mentioned studies, including ours, is the relatively small sample sizes included. Although the power for analysing associations in the entire population may be sufficient, analyses of possible associations among smaller subgroups result in weaker power and considerable risks of type II errors. Hence, larger studies are requested before any general conclusions can be drawn regarding VEGF polymorphisms as potential biomarkers. Further, it would be of considerable interest to analyse VEGF polymorphisms in relation with clinical response to VEGF targeting therapy. Very recently, Schneider et al(Schneider et al. 2008) reported an association of -2578 A and -1154 A alleles and improved overall survival among breast cancer patients treated with VEGF inhibitor in combination with chemotherapeutics, and Jin et al earlier reported associations with VEGF genotypes and breast tumour characteristics (Jin et al. 2005). Whether similar relations are evident with regard to colorectal cancer therapy and prognosis need future elucidation.

CONCLUSIONS

Accumulating evidence suggest that inflammation-related genes and mediators play significant roles in the development and progression of neoplasias, and the search for biomarkers and potential drug targets of cancer-related inflammation is an area of major interest. In the current thesis, cell cultures, animal models, and biopsies of human tumours were employed to characterize the role and relevance of certain inflammation-related genes and genetic variations within the colorectal cancer context.

First, we conclude that mPGES-1 is critical for PGE_2 generation in colon tumour cells, although blocking mPGES-1/PGE_2 may paradoxically promote tumourigenesis by shunting the eicosanoid synthesis towards other pro-tumoural mediators. The mutational status of the APC gene is associated with tumour cell expression of mPGES-1 by a mechanism that involves mRNA stabilisation, and points to a complex relationship between the APC and PGE_2 pathways.

Second, analyses of variations of the NOS2, MMP, and VEGF encoding genes reveal that a SNP located at -1607 in the MMP-1 gene promoter constitutes a potential susceptibility factor for developing colorectal cancer. Similarly, a rather uncommon combination of two individual SNPs (-2578 and +936) of the VEGF gene may enhance the risk of developing colorectal cancer, although other investigated variations of VEGF, NOS2, and MMP genes do not contribute to colorectal cancer occurrence or characteristics.

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