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## Role of $\beta$ -catenin and Leukotriene in Colon Cancer Progression

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# **Role of $\beta$ -catenin and Leukotriene in Colon Cancer Progression**

## **Tavga Hushiar Salim**

With permission from the Medical Faculty at Lund University for the presentation of this PhD thesis in a public forum in lecture hall Medelhavet, Wallenberg laboratoriet, Skåne University Hospital, Malmö, on Friday June 14<sup>th</sup> 2013 at 1 p.m. for the degree of Doctor of Philosophy, Faculty of Medicine

Faculty opponent: Professor Marene Landström, Department of Pathology,  
Umeå University



**LUND UNIVERSITY**  
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Abstract  Colorectal cancer is the third most common cancer worldwide. Deregulation of Wnt/ $\beta$ -catenin signalling pathway is an early hallmark of colon cancer. Nuclear accumulation of $\beta$ -catenin is a marker of activated canonical Wnt/ $\beta$ -catenin signalling. One of the risk factors for development of colon cancer is inflammatory bowel disease. Inflammatory microenvironment is an essential component of most tumors. Interestingly, high levels of the pro-inflammatory mediator leukotriene D4 (LTD4) in association with its receptor CysLT1R goes with poor prognosis for colon cancer patient. It is therefore of interest to further investigating the effect of LTD4/CysLT1R in tumor progression. The aim of my thesis was to investigate the role of $\beta$ -catenin signalling in colon cancer progression and the effect of LTD4. I found that activation of CysLT1R via LTD4 increased the translocation and accumulation of $\beta$ -catenin to the nucleus, which induce proliferation of HCT116 colon cancer cells through phosphorylation of GSK-3 $\beta$ and activation of TCF/LEF. Furthermore, LTD4 decrease both membranous E-cadherin and $\beta$ -catenin, which lead to an increase migration of HCT116 colon cancer. I also found that membrane expression of $\beta$ -catenin is associated with good prognosis while nuclear GSK-3 $\beta$ is associated with poor prognosis in colon cancer patients. The combination of no membrane $\beta$ -catenin and nuclear GSK-3 $\beta$ is associated with an overall poor survival. In addition I observed that no nuclear GSK-3 $\beta$ in combination with moderate membrane E-cadherin is associated with good prognosis in Duke's B colon cancer patients. It was also found that LTD4 could induce Epithelial-Mesenchymal Transition (EMT) in SW480 colon cancer cells by i) reducing membrane E-cadherin, ii) increase levels of nuclear active $\beta$ -catenin, iii) increase EMT marker such as vimentin and Snail, which lead to increase migration and invasion of SW480 colon cancer cells. In conclusion LTD4 in the tumor microenvironment can induce $\beta$ -catenin signalling leading to increase cell proliferation and migration of colon cancer cells, furthermore, nuclear GSK-3 $\beta$ could be a potential new prognostic marker (target for treatment) for colorectal cancer.		
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# **Role of $\beta$ -catenin and Leukotriene in colon cancer progression**

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For My Father and  
Souls of My Brothers Muhammad and Salar

*The only way to do great work is to love what you do.*

*If you haven't found it yet, keep looking. Don't settle.*

*(Steve Jobs)*



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## **Abbreviations**

AA	Arachidonic acid
APC	Adenomatous Polyposis Coli
AOM	Azoxymethane
CD	Crohn's disease
CDK	Cyclin-dependent Kinase
COX	Cyclooxygenase
CRC	Colorectal cancer
CysLT	Cysteinyl leukotriene
CysLT <sub>1</sub> R	CysLT <sub>1</sub> receptor
CysLT <sub>2</sub> R	CysLT <sub>2</sub> receptor
DAG	Diacylglycerol
DCC	Deleted in colon cancer
Dsh	Dishevelled
E-cadherin	Epithelial cadherin
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FLAP	5-LO-activating protein
FAP	Familial adenomatous polyposis
Fz	Frizzled
GPCR	G-protein-coupled receptor
GRK	G protein coupled receptor kinase
GSK-3 $\beta$	Glycogen synthase kinase 3 $\beta$
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HR	Hazard ratio
HNPCC	Hereditary non-polyposis colon cancer

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IBD	Inflammatory bowel disease
IP3	Inositol triphosphate
LT	Leukotriene
LO	Lipoxygenase
5-LO/5-LOX	5-lipoxygenase
LRP	Low density-lipoprotein-related protein
MET	Mesenchymal-epithelial transition
MMP	Matrix metalloproteinase
NSAID	Non-steroid anti-inflammatory drug
PFA	Paraformaldehyde
PLA <sub>2</sub>	Phospholipase A2
PI3K	Phosphoinositide 3-kinase
PVDF	Polyvinylidene difluoride membrane
Rb	Retinoblastoma
STAT	Signal transducers and activators of transcription
UC	Ulcerative colitis
TCF	T-cell factor
TGF- $\beta$	Transforming growth factor $\beta$
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

## **List of original papers**

The following papers will be included in this thesis and are referred to in the text by their Roman numerals:

- I. **Tavga Salim**, Anita Sjölander, Janna Sand-Dejmek. Nuclear expression of Glycogen synthase kinase-3 $\beta$  and lack of membranous  $\beta$ -catenin is correlated with poor survival in colon cancer. *Int J Cancer*. 2013 Feb 7. doi: 10.1002/ijc.28074. [Epub ahead of print]. \*
- II. **Tavga Salim**, Janna Sand-Dejmek and Anita Sjölander. The Inflammatory Mediator Leukotriene D4 Induces Subcellular  $\beta$ -catenin Translocation and Migration of HCT116 Colon Cancer Cells. *Submitted*
- III. **Tavga Salim**, Katyayni Vinnakota, Janna Sand-Dejmek, and Anita Sjölander. Pro-inflammatory mediator Leukotriene D<sub>4</sub> promotes a more invasive phenotype of SW480 and SW620 cancer cells. *Manuscript*.

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## **Introduction**

Cancer is a major public health problem in the Western part of the world, with a total of 1,660,290 new cancer cases and 580,350 cancer deaths projected to occur only in the United States in 2013<sup>1</sup>. Cancer, however, is not a new disease, the earliest records of cancer are from ancient Egypt, Hippocrate (ca 460 BC- ca 370 BC) also described several kinds of cancer, referring to them with the Greek word for Crab<sup>2</sup>. Cancer is, in essence, a genetic disorder in which mutation in three types of genes are responsible for tumorigenesis: oncogenes, tumor suppressor genes and care-takers or stability genes. However, no single gene defect causes cancer<sup>3</sup>, for example mutations of the KRAS gene in normal intestinal epithelial cells lead to non-malignant hyperplastic lesions that do not progress to malignancy<sup>4, 5</sup>, while the same mutation in an intestinal epithelial cell that already has acquired an adenomatous polyposis coli (APC) mutation results in a clonal expansion that often progresses to cancer<sup>6,7</sup>. There are many factors that increase the incidence of cancer such as chronic inflammation and environmental factors as well as life style factors such as cigarette smoking, alcohol consumption and diets high in saturated fats<sup>8</sup>.

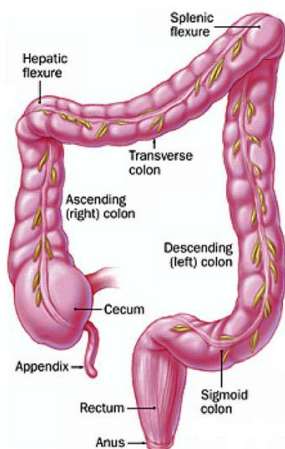
Tumor progression is refers to a tumor becoming clinically and biologically more aggressive over time and includes, in addition to tumor growth, other properties such as invasion and metastasis<sup>9,10</sup>. The role of inflammation in cancer is complex; with level of inflammation ranging from hardly detectable to severe inflammation but an inflammatory tumor microenvironment has been proven to play a crucial role in tumor development and progression<sup>11, 12</sup>. Cysteinyl leukotrienes are powerful proinflammatory mediators that bind to the G-protein coupled receptors CysLT<sub>1</sub>R and CysLT<sub>2</sub>R thereby starting up a cascade of intracellular events leading to uncontrolled cell growth, survival and proliferation<sup>13</sup>. Leukotrienes are associated with the pathogenesis of several inflammatory disorders such as inflammatory bowel disease (IBD), a disease associated with an increased risk of developing colon cancer.

We investigated the effect of cysteinyl leukotrienes on colon cancer cells with various mutations and found that leukotrienes induce tumor progression through the  $\beta$ -catenin signaling pathway. Furthermore, we investigated expression of proteins in the  $\beta$ -catenin signaling pathway in clinical colon cancer samples and correlated them to prognosis.

## **Background**

### ***The Intestine***

The gastrointestinal tract consists of a muscular tube starting from the oral cavity, continuing through the pharynx, esophagus, stomach and intestines to the rectum and anus. The intestine is divided into the small intestine which is approximately 6 meters long and the large intestine (colon) measuring approximately 1.5 meters<sup>14</sup>. The small intestine consists of the duodenum, jejunum and ileum and the large intestine is subdivided into the cecum, ascending, transverse, descending and sigmoid colon (Figure 1). The most distinctive feature of the small intestine is its mucosal lining which consists of finger-like projections covered by epithelial cells called villi. Between the bases of the villi are the pit-like crypts of Lieberkuhn, containing stem cells<sup>15,16</sup>. Villi contain three types of cells; columnar absorptive cells, mucin-secreting goblet cells and endocrine cells. Within the crypt stem cells, goblet cells, more abundant endocrine cells and scattered Paneth cells, which play a role in mucosal innate immunity against bacterial infection, can be found. Unlike the intestinal mucosa, the colonic mucosa has no villi and its morphology is flat. The small intestine accomplishes its absorptive function with a highly liquid luminal stream. The function of the colon is to reclaim luminal water and electrolytes. The regenerative capacity of the intestine is remarkable. Turnover of the surface epithelium in the small intestine takes 4 to 6 days and for colonic surface epithelium 3 to 8 days. The rapid renewal of intestinal epithelium provides a remarkable capacity for repair but also render the intestine very sensitive to agents that interfere with cell replication such as radiation and chemotherapy for cancer.

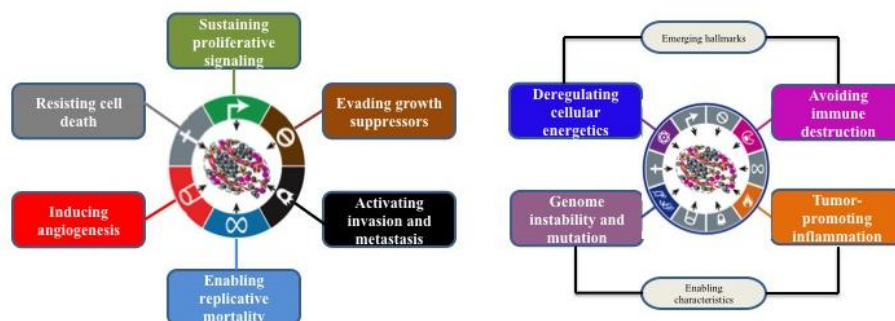


**Figure 1.** Schematic illustration of colorectal anatomy. Adapted from Wolcott, J et al, 2013<sup>180</sup>

### ***Inflammation and cancer***

Cancer is not one disease; it is a group of more than 100 different and distinctive tumor types<sup>17</sup>. The hallmarks of cancer include sustaining proliferative signaling, evading growth suppressor, resisting cell death, enabling explicative immortality, inducing angiogenesis and activating invasion and metastasis. Underlying these hallmarks are genome instability and inflammation, which foster many of the hallmark functions<sup>10</sup> (Figure 2). Inflammation is the response of a tissue to injury, often injury caused by invading pathogens, the signs of inflammation were described in ancient Greece and include swelling, redness, heat, pain and loss of function of the inflamed area<sup>18</sup>. Chronic inflammation seems to be due to persistence of the initiating factors or a failure in mechanisms required to resolve the inflammatory response<sup>11</sup>. The inflammatory response mediated through the innate and acquired immune systems<sup>19</sup>. An association between the development of cancer and inflammation has long-been suspected; in 1863 Virchow hypothesized that cancer originates at sites of chronic inflammation. Tissue injury leads to inflammation causing enhanced cell proliferation although today it is clear that proliferation of cells alone does not cause cancer. However, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma and DNA damage-promoting agents, potentiate or promote cancerous growth<sup>11</sup>.





**Figure 2.** Hallmark capabilities of cancer. Adapted from Hanahan et al, 2011<sup>10</sup>

### ***Inflammatory bowel disease and colitis-associated cancer***

Inflammatory bowel disease (IBD) is an idiopathic chronic intestinal disorder believed to occur due to dysregulated immune response to the intestinal microflora in genetically susceptible hosts. IBD includes Crohn's disease, which can involve any segment of the gastrointestinal tract from mouth to the anus in a discontinuous pattern, and ulcerative colitis (UC), which usually involves the rectum and may affect part of or the entire colon (pancolitis) in a un-interrupted (continuous) pattern<sup>20</sup>. The inflammation in Crohn's disease is mostly transmural whereas in ulcerative colitis it is restricted to the mucosa<sup>21</sup>. Prolonged inflammation in the bowel is a risk factor for developing colon cancer and colitis-associated cancer is defined as colon cancer occurring in a patient with clinically detectable inflammatory bowel disease (IBD)<sup>22,23,24,25</sup>. The increased risk of cancer in IBD patients depends on many factors such as disease severity and duration, efficacy of anti-inflammatory therapies such as TNF $\alpha$  antibodies and IBD management<sup>26,27,28,29</sup>. It has been reported that the carcinogen azoxymethane (AOM) was able to induce multiple colonic tumors after only one injection when coupled with induction of chronic colitis in mice, however, in mice free of chronic colitis, multiple injections of the carcinogen were necessary for tumor induction<sup>30,31</sup>.

### ***Colon cancer***

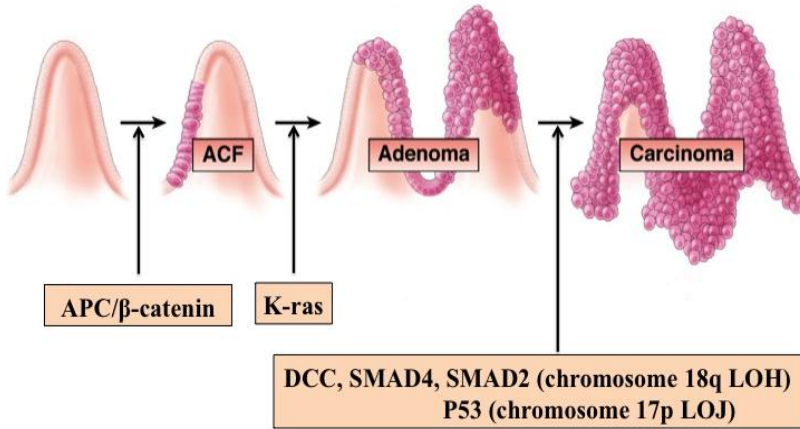
Colorectal cancer (CRC) is the third most common cancer worldwide, surpassed only by lung cancer and breast cancer in women and lung- and prostate cancer in men, and one of the major causes of cancer-related death<sup>32</sup>. The etiology behind CRC is complex; among factors significantly associated with increased risk of developing CRC are dietary and lifestyle factors such as obesity, reduced physical activity, diets rich in unsaturated fats, high intake of red meat and excessive alcohol consumption. Inherited and somatic mutations play an important role in CRC<sup>33-35</sup>. Genetic alterations can lead to constitutive activation of oncogenes such as KRAS, which is mutated in approximately 50% of CRC, or to loss function of tumor suppressor genes such as APC and p53<sup>36</sup>. Fifteen to thirty percent of CRC is hereditary when genetic disorders as well as occurrence of CRC in first or second degree relatives are taken into account<sup>37, 38</sup>. Hereditary non polyposis colorectal cancer (HNPCC) is characterized by a very high risk of adenomas developing into invasive adenocarcinoma and the tumors have a high degree of microsatellite instability (MSI). Familial adenomatous polyposis (FAP) is an autosomal dominant disorder characterized by the presence of hundreds to thousands of adenomatous polyps along the colon and rectum leading to cancer at a young age if left without treatment<sup>39,40</sup>. FAP patients have a germ line mutation in the tumor suppressor gene APC, however, 70-80% of non-hereditary sporadic colorectal adenomas and carcinomas have somatic mutations in APC<sup>41-43</sup>. APC is involved in the regulation of several physiological processes such as cell-cell adhesion, cell migration, apoptosis and regulate  $\beta$ -catenin degradation in the canonical Wnt signaling pathway<sup>44,45</sup>. Up to 20% of colorectal cancers have mutation of  $\beta$ -catenin, which essentially has the same effect as an APC mutation. In normal intestinal epithelium in the absence of Wnt ligand (Wnt off), APC forms a cytosolic complex with Axin and glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) inducing phosphorylation, ubiquitination and subsequently proteasomal degradation of  $\beta$ -catenin<sup>46</sup>. A majority of CRC tumors have a mutation in APC leading to disruption of  $\beta$ -catenin regulation and accumulation of free  $\beta$ -catenin. Cytosolic  $\beta$ -catenin translocates to the nucleus where it functions as a transcriptional co-activator binding to the TCF/LEF family of transcription factors, inducing expression of many proto-oncogenes such as c-myc and cyclin D1 as well as genes encoding membrane factors like matrix metalloproteinase 7 (MMP-7).

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#### The adenoma-carcinoma sequence in colorectal cancer progression

The first histological features of colorectal cancer progression is accumulation of excess cells at the epithelial surface, this results in aberrant crypt foci, cells may still appear normal, leading to hyperplastic tissue or, if cells display abnormal morphological features, give rise to dysplastic tissue. This localized proliferation leads to formation of visible polyps on the inner lining of the intestine<sup>47</sup>. Adenomas are polyps that have the ability to progress to cancer if left untreated<sup>47</sup>. APC mutations occur early during the progression from adenoma to carcinoma<sup>48,49</sup>. Mutations usually occur in the  $\beta$ -catenin binding domain and in most tumors both APC alleles are mutated<sup>50</sup> (Figure 3). Around 20% of tumors have wildtype APC and instead have mutations in  $\beta$ -catenin<sup>51</sup>. In both cases,  $\beta$ -catenin escapes from its repression by APC, ultimately leading to increased transcription of C-MYC and other genes associated with increased cell proliferation. Furthermore, around 50% of adenomas greater than 1 cm and 50% carcinomas have mutations in KRAS. In contrast to APC, mutation of a single allele of KRAS is sufficient to cause progression to CRC<sup>47</sup>. Other genes known to be involved in progression of CRC are DCC, SMAD4 and SMAD2, all located on chromosome 18q21. Deleted in colon cancer (DCC) is a tumor suppressor gene important for maintaining cell adhesion<sup>52</sup>. SMAD2 and SMAD4 mutations lead to loss of response to TGF- $\beta$  and increased proliferation and hence progression of CRC<sup>53</sup>. Seventy-five percent of CRC tumors have mutations in p53<sup>47</sup>, the gard of the genome which controls cell cycle division and induces apoptosis in response to stress<sup>54</sup>.

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**Figure 3.** Progression of colon cancer. Adapted from Terzic J *et al*, 2010<sup>55</sup>

**Tumor classification and staging**

In general, cancer staging offers critical information about the extent of disease, aid in treatment decisions, could predict response to therapy and determines prognosis<sup>56</sup>. In 1926, Lockhart-Mummery offered the first staging system for rectal cancer depending on the extent of disease and lymph node involvement<sup>57</sup>. In 1954, Astler-Coller (MAC)<sup>58</sup> classified CRC. In 1987, the American Joint Committee on Cancer (AJCC) and the International Union against Cancer introduced the cancer staging system based on size and extend of primary tumor (T), number and degree of lymph node involvement (N) and presence or absence of distant metastasis (M)<sup>59</sup>. The TNM staging of CRC was updated in 2002 and is the common staging system used in clinical practice<sup>56, 60</sup> (Table 1).

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*Tumor, Node, Metastasis (TNM) staging of Colorectal Cancer*

**T-Primary tumor**

TX- primary tumor cannot be assessed

T0- No evidence of primary tumor

Tis- Carcinoma in situ: intraepithelial or invasion of lamina propria

T1- Tumor invades sub mucosa

T2- Tumor invades muscularis propria

T3- Tumor invades through muscularis propria in to sub serosa or into non-peritonealized pericolic or perirectal tissues

T4- Tumor directly invades other organs or structures and/or perforates visceral peritoneum

**N-Regional lymph nodes**

NX- Regional lymph nodes cannot be assessed

N0- No regional lymph node metastasis

N1- Metastasis in one to three regional lymph nodes

N2- Metastasis in four or more regional lymph nodes

**M-Distant metastasis**

MX- Distant metastasis cannot be assessed

M0 -No distant metastasis

M1- Distant metastasis

**Table 1.** Comparison of TNM, Dukes and MAC classifications

TNM Classification American Joint Committee on Cancer (AJCC)			Dukes Staging	MAC Staging	
Stages	T	N	M	Stages	Stages
Stage 0	Tis	N0	M0	—	—
Stage I	T1	N0	M0	A	A
	T2	N0	M0	A	B1
Stage II	T3	N0	M0	B	B2
	T4	N0	M0	B	B3
Stage III	T1, T2	N1 or N2	M0	C	C1
	T3, T4	N1 or N2	M0	C	C2/3
Stage IV	Any T	Any N	M1	—	D

### Colorectal cancer screening

Since a premalignant lesion precedes CRC, screening for CRC should be of great value. A benign adenoma grows for a long time (decades) before progressing to carcinoma and the patient can be cured by local excision of the tumor if detected early enough<sup>61</sup>. According to the World Health Organization, screening should be offered to populations with high risk of developing CRC, including family history of CRC or history of polyps or IBD, or hereditary cancer syndromes such as Familial adenomatous polyposis (FAP)<sup>62,63</sup>. Current screening methods are fecal occult blood test, flexible sigmoidoscopy, total colonoscopy and genomic approaches such as detection of mutated DNA/RNA in stool samples including KRAS<sup>64</sup>, APC, or p53<sup>49, 65, 66</sup> and another approach is detection of protein markers in stool or blood<sup>61</sup>.

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#### Treatment of colorectal cancer

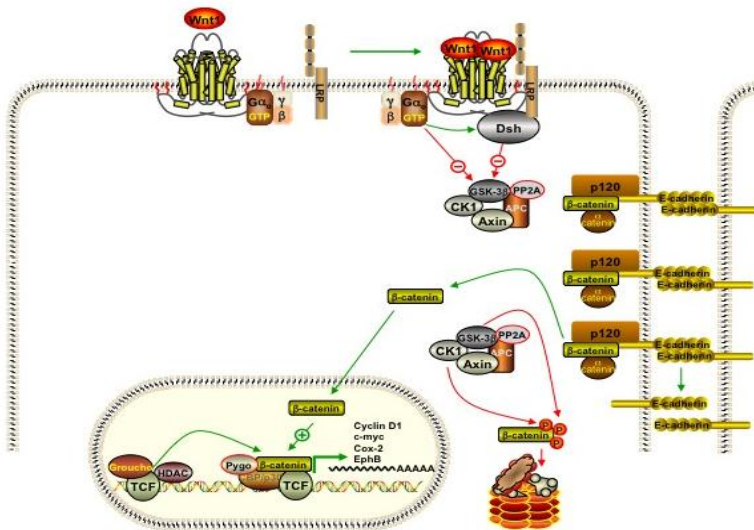
Treatment of CRC depends on many factors such as patient age and disease stage but in general treatment includes surgery (removal of the primary tumor), chemotherapy (if given in the adjuvant setting, to kill remaining cancer cells) and, in case of rectal cancer, radiotherapy<sup>66</sup>. In non-curable, metastatic disease, chemotherapy and targeted therapies are given to decrease tumor load, improve symptoms and increase survival<sup>67</sup>. Chemotherapy combinations with 5-Fluorouracil (5-FU) as a base, and antibodies targeting VEGF (vascular endothelial growth factor) or EGFR (epidermal growth factor receptor) are commonly used<sup>68</sup>.

#### ***Wnt/ $\beta$ -catenin signaling pathway***

The Wnt signaling pathway plays an important role in multiple cellular process such as determination of cell fate, cell polarity, cell migration, neurogenesis and organogenesis in early embryonic development<sup>69</sup>. Deregulation of the pathway occurs in several disorders such as breast cancer, colon cancer, metabolic disorders and congenital anomalies or skeletal defects such as spina bifida<sup>70</sup>. The name Wnt is derived from Wingless, which is the *Drosophila* segment polarity gene and from the vertebrate homolog, Integrated or int-1<sup>71</sup>. Wnt signaling can activate several intracellular signaling pathways, the most important being the canonical or  $\beta$ -catenin dependent pathway and the non-canonical or  $\beta$ -catenin-independent pathway which can be subdivided into the planar cell polarity pathway and the Wnt/ $\text{Ca}^{2+}$  pathway<sup>72</sup>. Wnt are glycoproteins that bind to N-terminal extra-cellular cysteine-rich domains of 7-membrane-spanning transmembrane frizzled (Fz) receptors and, in the canonical  $\beta$ -catenin pathway low density-lipoprotein-related protein LRP5/LRP6 co-receptors<sup>73,74</sup>. The key regulator of the canonical Wnt pathway is  $\beta$ -catenin which is a multifunctional protein with multiple subcellular localizations, at the cell membrane maintaining cell to cell adhesions with E-cadherin, in the cytoplasm where levels of  $\beta$ -catenin are tightly controlled by a complex of proteins, and in the nucleus where  $\beta$ -catenin is free and binds to transcription factors, inducing transcription of target genes<sup>75,76,77</sup>.  $\beta$ -catenin is a member of a family of proteins consisting of  $\alpha$ ,  $\beta$ ,  $\gamma$  and p120 subunits. The structure of  $\beta$ -catenin consist of three domains; the N-terminal domain, the armadillo domain which consist of 12 repeated sequences, and the C-terminal domain<sup>78,79</sup>. The key regulator of canonical Wnt signaling is the  $\beta$ -catenin destruction complex<sup>80</sup>. Axin is a scaffold protein and tumor suppressor playing an important role in the destruction complex<sup>81</sup>. GSK-3 $\beta$  is a serine/threonine protein kinase, under normal conditions active GSK-3 $\beta$  negatively regulates  $\beta$ -catenin levels by phosphorylating  $\beta$ -catenin at its N-terminal binding site<sup>82</sup>. Mutated or inactive GSK-3 $\beta$  leads to

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activation of canonical Wnt signaling<sup>83, 84</sup>. APC is a tumor suppressor and the largest protein of the destructive complex, around 310 kDa, 80% of colorectal cancers have mutated APC and the majority of these mutations are caused by truncation of the  $\beta$ -catenin binding site<sup>85, 86</sup>. APC mutations not affecting  $\beta$ -catenin binding sites may also lead to cancer by affecting the Axin binding region of APC, again leading to destabilization of the  $\beta$ -catenin destruction complex<sup>44</sup>. In the absence of active Wnt signaling cytoplasmic  $\beta$ -catenin is phosphorylated within the destruction complex by GSK-3 $\beta$  and Casein Kinase (CK), leading to ubiquitination and degradation of  $\beta$ -catenin by the proteasome<sup>87</sup>. In the presence of active Wnt signaling with Wnt binding to its trans-membrane receptor, translocation of Axin to the membrane and its binding to the cytoplasmic tails of co-receptor LRP5/6 occurs<sup>88</sup>. Inactivation of Axin is mediated by GSK-3 $\beta$  or CK1<sup>89</sup>, indicating that GSK-3 $\beta$  has two important roles within the canonical Wnt signaling pathway; as a positive mediator for LRP5/6, whereas at the level of  $\beta$ -catenin it has a negative effect<sup>69</sup>. Further, activation of the phosphoprotein Dishevelled (Dsh) induces phosphorylation of GSK-3 $\beta$  and initiates a series of events that prevents degradation of  $\beta$ -catenin and its translocation into the nucleus<sup>90,91</sup>. In the nucleus  $\beta$ -catenin functions as a transcriptional co-activator by binding to TCF/LEF family of transcription factors and inducing transcription of target gene such as cyclin D1, c-myc and COX-2 (Figure 4).



**Figure 4.** The Wnt/ $\beta$ -catenin signaling pathway



### ***Eicosanoids***

Eicosanoids are biologically active lipids derived from arachidonic acid (AA) which is 20 carbon poly unsaturated fatty acid (the eicosa, derived from the Greek word referring to “twenty”<sup>92</sup>), these AAs are mainly derived from animal fats. Eicosanoids are derived from arachidonic acid metabolism through the cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase (P450) pathways and include prostanoids, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs) and hydroperoxy-eicosatetraenoic acids (HPETEs)<sup>93</sup>. Eicosanoids play important roles in multiple pathological process such as cardiovascular disease, thrombosis, allergic and immunological disease such as IBD, asthma, arthritis and in various malignancies such as colon, breast and pancreatic cancer<sup>944</sup>.

#### Eicosanoids and cancer

Pro-inflammatory mediator including eicosanoids, cytokines and chemokines promote growth and proliferation of tumors through creation of an inflammatory microenvironment<sup>10,94</sup>. Increased expression of inflammatory mediators has been linked to cancer and previous findings showed that levels of LTB<sub>4</sub> were significantly higher in human prostate cancer compared to in precancerous lesions or benign hyperplasia of the prostate<sup>95</sup>. Findings from our lab suggest that CysLT<sub>1</sub>R activation by LTD<sub>4</sub> induces proliferation, survival and migration of intestinal epithelial cells through different pathways including GSK-3 $\beta$ / $\beta$ -catenin, protein kinase C (PKC)-Raf-ERK1 and ERK2, BCL-2 and COX2 pathways, and migration through the PI3K/Rac pathway<sup>96,97,98,99</sup>. Further, high expression levels of CysLT<sub>2</sub>R were associated with better differentiation and prognosis in colon cancer patients<sup>100</sup>. Upregulation of COX-2 in different human cancers including colon cancer has been shown<sup>101,102</sup> and non-selective COX-2 inhibitors such as NSAID including aspirin reduce the risk of colorectal cancer<sup>103</sup> as well as improves survival in patient diagnosed with CRC<sup>104</sup>. However, daily use of aspirin is associated with bleeding in certain patients<sup>105</sup> and more selective COX-2 inhibitors such as celecoxib are only recommended in patients with high risk of CRC<sup>106</sup>.

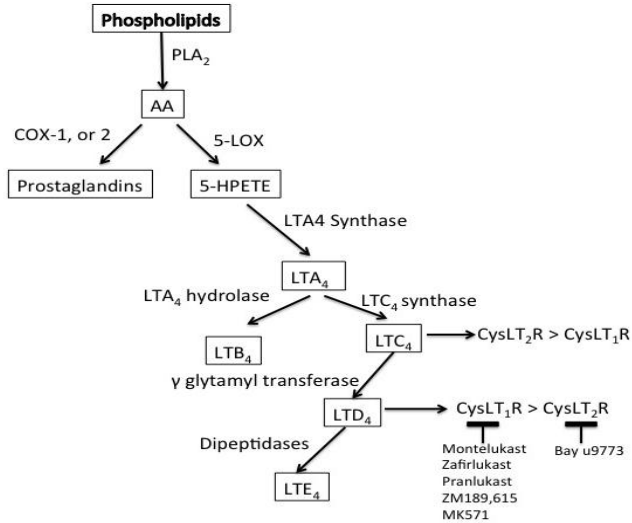
### ***Leukotrienes***

One of biologically active compound of eicosanoids, leukotrienes were first found in leukocytes and they have three conjugated double bonds in the structure<sup>107</sup>. Leukotrienes were discovered by Hamberg and Samuelsson in 1967 when they studied the plant enzyme lipoxygenase<sup>92</sup>. Later they found that platelets

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also have lipoxygenase activity and are able to convert AA into hydroperoxyeicosatetraenoic acid (HPETE)<sup>108</sup>. AA is a polyunsaturated fatty acid stored in the cell membrane and released from the membrane by the effects of cytoplasmic phospholipase A2 (PLA<sub>2</sub>)<sup>93</sup>. Subsequently, released AA is metabolized by COX-1/2 to prostaglandin and thromboxane<sup>109</sup> or/and to unstable 5-HPETE by 5-lipoxygenase which is further metabolized into LTA<sub>4</sub> by LTA synthase to biologically active LTB<sub>4</sub> by LTA<sub>4</sub> hydrolase or to the cysteinyl leukotriene (CysLT) LTC<sub>4</sub> by LTC<sub>4</sub> synthase and glutathione<sup>110</sup>. LTC<sub>4</sub> is further converted into LTD<sub>4</sub> by glytamyl transferase which is further metabolized into LTE<sub>4</sub> by dipeptidase (Figure 5). Those enzymes are present at the surface of cells<sup>111,109</sup>. Leukotrienes are released from different inflammatory cells such as neutrophils, macrophages, basophiles, mast cells<sup>112</sup>, platelets<sup>113</sup>, and endothelial cells as well as epithelial cells in response to receptor stimulation or antigen/antibody reactions<sup>114</sup>. LTB<sub>4</sub> has a biological effect different from that of the CysLTs (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>). In general, these leukotrienes are able to enhance proliferation, increase survival and inhibit apoptosis of human cells. LTB<sub>4</sub> is a very potent chemoattractant for neutrophils and induces neutrophil aggregation. Both LTB<sub>4</sub> and LTD<sub>4</sub> can activate eosinophil, basophils, T cells and macrophages at inflammatory sites and induce leukocyte adhesion to the epithelium and migration of dendritic cells. CysLT is a potent bronchoconstriction agent, LTC<sub>4</sub> and LTD<sub>4</sub> are 1000 time more potent than other bronchoconstrictors such as histamine<sup>115, 116</sup>. CysLTs are also involved in the pathogenesis of allergic rhinitis<sup>115, 116,117</sup>. In addition, CysLTs can induce vascular permeability, edema, and smooth muscle contraction<sup>118</sup> and contribute to the pathology of different disease such as asthma<sup>119</sup>, rheumatoid arthritis<sup>120</sup> and IBD<sup>121</sup>. For example, urinary LTE<sub>4</sub> excretion can be used as a diagnostic biomarker for IBD<sup>122</sup>.

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**Figure 5.** Biosynthesis of leukotrienes and prostaglandins.

***G-protein coupled receptors***

G-protein-coupled receptors (GPCRs) are trans-membrane proteins that span the plasma membrane seven times<sup>123</sup>. GPCR<sub>S</sub> belong to one of the largest families of cell surface receptor<sup>124</sup>. These heterotrimeric G-protein consist of three subunits  $G\alpha$ ,  $G\beta$ ,  $G\gamma$ ; furthermore the  $G\alpha$  subunits are subdivided into  $G\alpha_s$ ,  $G\alpha_i$ ,  $G\alpha_q$  and  $G\alpha_{12}$ <sup>125</sup>. Upon receptor activation by extracellular ligands the  $G\alpha$  protein undergoes conformational changes which increase the affinity of  $G\alpha$  subunit to the GTP to GDP and make it dissociate from the  $G\beta$  and  $G\gamma$  subunits<sup>126</sup>. Receptor activation leads to a subsequent activation of a number of cytoplasmic enzymes such as adenylyl cyclase and phospholipase C which cleave phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3) leading to an intracellular increase of  $Ca^{2+}$ . The activation-inactivation cycle of G proteins is sensitive to bacterial toxins such as pertussis and cholera toxin. Pertussis toxin can inhibit the  $G\alpha_i$ -subunit (except  $G\alpha_{i2}$ )<sup>127</sup> while cholera toxin activate  $G\alpha_s$  by inhibition of GTPase activity<sup>128</sup>. Deregulation of these signaling pathways is associated with cell transformation and tumorigenesis. CysLTRs belongs to the GPCR family and consist of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R. The gene encoding for CysLT<sub>1</sub>R is located on the X chromosome (Xq13-q21) and it encodes about 337 aa with a molecular weight around 38 kDa whereas the CysLT<sub>2</sub>R gene is located on

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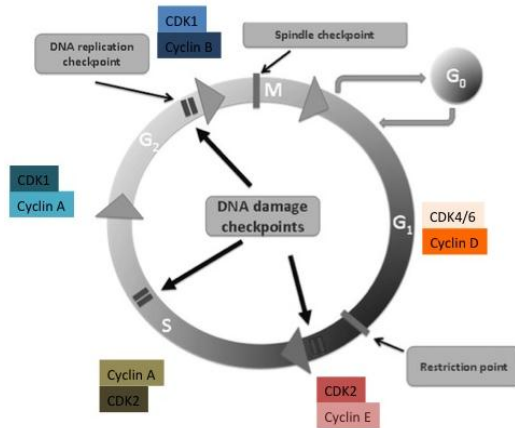
chromosome 13(13q14) which is a region linked to a topic asthma and encodes a 346 aa protein<sup>129,130</sup>. The two receptors share around 28% sequence homology (aa identity)<sup>13,131</sup>. GPCRs are cell surface receptors but can also be located in other intracellular compartment and the signal from the nuclear or cytoplasmic receptors might be different from the receptor signal which is located at the plasma membrane<sup>132</sup>. CysLT<sub>1</sub>R is primarily located in peripheral blood, leukocytes, spleen, intestinal smooth muscle and airway smooth muscles whereas CysLT<sub>2</sub>R is highly expressed in peripheral blood, leukocytes, spleen, adrenal medulla, heart and brain<sup>129, 131, 133</sup>. LTD<sub>4</sub> has higher affinity to CysLT<sub>1</sub>R than LTC<sub>4</sub> and LTE<sub>4</sub> whereas CysLT<sub>2</sub>R binds LTD<sub>4</sub> and LTC<sub>4</sub> with equal affinity; both receptors has less affinity for LTE<sub>4</sub><sup>129, 131</sup>. In addition to CysLT<sub>1</sub>R and CysLT<sub>2</sub>R, there is also another receptor GPR17 (CysLT<sub>3</sub>R) which is expressed mainly in the brain, heart and kidney and it has higher affinity to LTC<sub>4</sub> than LTD<sub>4</sub><sup>134</sup>. The potential response of CysLT<sub>1</sub>R to LTD<sub>4</sub> can be suppressed by selective CysLT<sub>1</sub>R antagonist such as montelukast, pranlukast, ZM189615 and MK571. Montelukast and pranlukast are widely used as for treatment of seasonal allergic rhinitis or as bronchodilators for asthmatic patient<sup>135,136</sup>. The CysLT<sub>2</sub>R antagonist AP-100984 is a selective antagonist where as Bay u9773 is a non selective and the only commersible available pharmacological inhibitor for CysLT<sub>2</sub>R<sup>137</sup>.

### ***Cell proliferation***

The proliferation of cells is a complex series of biochemical changes enabling the cell to double its content and divide into two daughter cells<sup>123</sup>. Cells undergo cycles of growth (increase in cell size) and division (increase in cell number) called the cell cycle. Uncontrolled cell proliferation is one of hallmarks of cancer<sup>10</sup>. In normal cells, transition from G<sub>0</sub>/G<sub>1</sub> arrest phase to active cell cycle leading to cell proliferation is controlled by regulatory cell cycle proteins, but in cancer these mechanisms are disrupted<sup>76, 138</sup>. In eukaryotic cells, the cell cycle consists of four main phases: G<sub>1</sub> (gap 1), S (synthetic), G<sub>2</sub> (gap 2 in which the cell stays for 3 to 5 hrs), and M (mitotic which lasts around 1 hr and includes four sub-phases; prophase, metaphase, anaphase and telophase). Cell in G<sub>0</sub>, which is the resting stage, prepare to enter M phase and cell division, in G<sub>1</sub> phase cells decide either to remain in G<sub>1</sub> or retreat back into G<sub>0</sub> phase or to continue through active cell cycling. And these decisions occur at the restriction point R (Figure 6). Cells respond to extracellular signals and inhibitory factors such as TGF- $\beta$  which has a growth inhibitory effect during early and mid G phase. In S phase, synthesis of new DNA occurs, whereafter cells pass into G<sub>2</sub> during which mistakes in the newly synthesized DNA are corrected or, if the abnormality is major, sends the cell into apoptosis. During M phase the cell divides into two daughter cells<sup>139</sup>. Cell cycle control occurs

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by different serine/threonine kinases named cyclin dependent kinase (CDK)<sup>140</sup>. Further phosphorylation of various proteins such as the retinoblastoma protein (Rb) control different processes during the cell cycle, Rb act as a tumor suppressor by serving as a check point preventing progression of cells through G phase. Mutations of Rb lead to hyper-proliferation and tumor formation and has been shown in malignancies such as retinoblastoma, osteosarcoma and small cell lung carcinoma<sup>141,142,139</sup>. In early and mid G1 phase CDK4 and CDK6 in association with their cyclins (D1, D2, D3) guide cells past the restriction point. In late G1 phase cyclin E1 and E2 associate with CDK2 to enable cell entry into S phase. Progression through S phase is enabled by CDK2 associating with cyclin A1 and A2. In late S phase cyclin A dissociates from the CDK2 and CDK1 and the cell enters G2 phase. Finally, in M phase, cyclin A is replaced by cyclin B<sup>139,143</sup>.



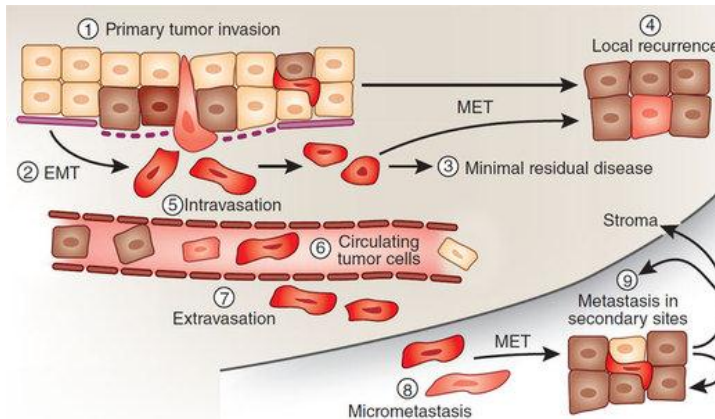
**Figure 6.** The cell cycle. Adapted from Roberti, A et al. 2009<sup>181</sup>

***Cell migration and invasion***

Cell migration describes the movement of cells in a particular direction such as in tissue formation during embryonic development, wound healing and immune responses. Migration requires the cell to undergo conformational changes such as cell polarization and formation of lamellipodia and filopodia, processes regulated by Rho-GTPases, PI3K and integrins<sup>144-146</sup>. Invasion is the process by which cancer cells migrate or move from the primary tumor into adjacent normal tissues. Dissemination of cancer cells from the

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primary tumor to distant organs through the blood or lymphatic systems is known as metastasis<sup>147</sup>. Metastasis is what ultimately causes the majority of cancer patients' death<sup>148,149</sup>. Epithelial mesenchymal transition (EMT) is an early change acquired by epithelial cells located at the edge of the primary tumor acquired in order to gain migratory capacity and induce invasion and metastasis. EMT is a reversible process, for example during embryogenesis such modulation of the epithelial cell phenotype is a normal physiological process. Metastasis from the primary tumor to distant organs<sup>150,151</sup> is a multistep process including detachment of tumor cells from the primary tumor, invasion through the basement membrane and the surrounding tissue by release of proteolytic enzymes such as matrix metalloproteinases (MMPs) and formation of invadopodia which determine the direction of migration and chemotaxis<sup>152, 153</sup>. Subsequently, tumor cells migrate in the extracellular matrix forming pseudopodia which aid the cells to intravasate into the blood or lymphatic systems. Finally, tumor cells extravasate across the endothelium and reach the distant tissue. Here, in the absence of signals induced by the parental cells, tumor cells can revert into an epithelial phenotype by a process called mesenchymal-epithelial transition (MET) and established growth in organs different from where it originated<sup>154</sup>. The most important cellular changes associated with EMT are described in table 2.



**Figure 7.** The EMT-MET process Adapted from E W Thompson et al. 2011<sup>155</sup>

**Table 2.** Cellular changes associated with EMT

Loss of epithelial markers	Acquisition of Mesenchyme markers
<p>Cytokeratin (intermediate filament) expression</p> <ol style="list-style-type: none"> <li>1) Loss of epithelial adherence junction protein (E-cadherin)</li> <li>2) Epithelial cell polarity (<math>\beta</math>-catenin)</li> </ol>	<ol style="list-style-type: none"> <li>1) Fibroblast-like shape</li> <li>2) Motility</li> <li>3) Invasiveness</li> <li>4) Mesenchymal gene expression program (Twist, Snail and Slug)</li> <li>5) Mesenchymal adherence junction protein (N-cadherin)</li> <li>6) Protease secretion; MMP-2, MMP-9</li> <li>7) Vimentin (intermediate filament) expression</li> <li>8) Fibronectin secretion</li> <li>9) PDGF receptor expression</li> <li>10) <math>\alpha\beta_6</math> integrin expression</li> </ol>

## **Aims of the present investigation**

### ***Aims***

- To evaluate GSK-3 $\beta$  and  $\beta$ -catenin as prognostic markers in colon cancer
- To evaluate the role of leukotriene-induced  $\beta$ -catenin signaling in progression of colon cancer
- To investigate the role of leukotrienes in colon cancer migration and invasion



## **Methodology**

### ***Cell lines***

Several different cell lines were used. In paper II I used HCT116 and HT29 colon adenocarcinoma cells, HCT116 cells are poorly differentiated, highly tumorigenic and aggressive colon cancer cells carrying a gain of function mutation in the  $\beta$ -catenin gene (deletion of amino acid serine 45) which prevents  $\beta$ -catenin protein degradation. HT29 cells are highly differentiated colon adenocarcinoma cells with a mutant, carboxy-truncated APC gene<sup>156</sup>. In papers I and III SW480 and SW620 cells were used. SW480 cells were established from the primary tumor in the colon, while the SW620 cell line was isolated a year later, from a metastatic lymph node in the same patient when relapsed with a massive intra-abdominal tumor<sup>157</sup>.

### ***Patients***

All patients that underwent surgery for colon cancer at Malmö University Hospital during the selected time period in 1990 were included in the retrospective study. No stratification or matching was done. All tumors with available slides or paraffin blocks were histopathologically re-evaluated on hematoxylin and eosin (H&E) stained slides. Clinical and histopathological data were retrieved from patient's charts and pathology records. All patients had a pathologically confirmed diagnosis of adenocarcinoma before surgery. Tumors were staged according to the UICC TNM classification of malignant tumors<sup>158</sup>. Tumor grade was determined according to Jass and Sobin as high, medium, or low<sup>159</sup>. Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry with last follow up on December 31<sup>st</sup> 2000. Follow-up started at the time of diagnosis and ended at death, emigration or December 31<sup>st</sup> 2000, Median follow-up time was 4.58 years (range 0.08-10.67) for the full cohort (n=89) and 8.78 years (range 0.33-10.67) for patients alive at the end of follow-up (n=31). The primary endpoint of the study was overall survival. The Ethical Committee at Lund University approved the study.

### ***Tumor tissue microarray (TMA) and immunohistochemistry***

Cases without available paraffin blocks or with an insufficient amount of tumor material were excluded. Out of a total number of 89, 85 patients (95.5%) were suitable for the tissue microarray (TMA)

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construction. To confirm the diagnosis and histological grading, the archival FFPE samples were cut in 1- $\mu$ m sections, dried, deparaffinized, rehydrated and stained with H&E. Two or three 1.5 mm tissue cores from each donor block were placed in a new paraffin block by using an automated Beecher Micro-Arrayer (Beecher Instruments). For samples from distant metastases, whole sections of the tumors were evaluated by H&E to verify the pathological diagnosis. For TMAs as well as larger tumor sections, immunohistochemical staining was performed as previously described<sup>160</sup>. Briefly, the paraffin array blocks were cut in 1- $\mu$ m sections, which were pre-treated as previously described and then placed in citrate buffer and heated  $2 \times 10$  min in a microwave oven. All immunohistochemical procedures were performed using a Dako automatic slide stainer according to the manufacturer's instructions. After immunostaining, all slides were counterstained with H&E. Immunoreactivity was assessed by two investigators (TS and JSD). Disagreement between the observers was less than 10%, and those cases were reviewed until an agreement was reached. Tumors were graded according to staining intensity, percentage of positive cells and subcellular localization. Staining intensity was scored as 0 = no staining; 1 = weak staining, 2 = intermediate staining, and 3 = strong staining. In addition, percentage of positive cells (1 = 0-5%, 2 = 6-25%, 3 = 26-75%, 4 = 76-100%) and subcellular localization (membrane, cytoplasm, or nuclear) was determined. In normal tissue, nuclear GSK-3 $\beta$  was expressed in less than 5% of cells and the staining intensity was weak. For membranous  $\beta$ -catenin, tumors were considered positive if >50% of the cells displayed membranous expression of the protein. Expression of membrane  $\beta$ -catenin was very homogenous, with a majority of tumors being either strongly positive for membrane  $\beta$ -catenin with close to 100% of the cells expressing  $\beta$ -catenin at the membrane, or completely negative, with less than 5% of cells showing immunoreactivity for  $\beta$ -catenin at the cell membrane.

### ***Statistical analysis***

Overall survival (OS) was the primary endpoint. Comparison of clinical data and tumor characteristics according to GSK-3 $\beta$  and  $\beta$ -catenin status was done by Chi-square-test. Kaplan-Meier estimates were used to illustrate survival according to GSK-3 $\beta$  and  $\beta$ -catenin expression and Log rank test used to assess for equality of survival curves. Hazard ratios were estimated using a Cox proportional hazards model for OS in uni- and multivariate analyses. The power to detect a hazard ratio (HR) of 2.0 at the level of significance 0.05 was 77%. Calculations were performed using SPSS version 19.0 (SPSS Inc.). All p-values corresponded to two-sided tests and values equal to or less than 0.05 were considered significant.

### ***Cell fractionation and Western blotting***

Cells were washed in ice cold PBS and lysed by addition of ice-cold buffer A<sup>161</sup> supplemented with 1% Triton X-100, where after cells were kept on ice for 30 min, homogenized by 10 strokes with a Dounce homogenizer and centrifuged for 10 min at 200 x g at 4°C. The supernatant was centrifuged for additional 5 min at 1000 x g at 4°C and the resulting supernatant was considered representing the whole cell lysate. For membrane and cytosol fraction cells were washed with ice cold PBS, covered with buffer A<sup>162</sup>, kept on ice for 30 min, and homogenized with a Dounce homogenizer for 25 strokes and thereafter centrifuged for 10 min at 500 x g. The supernatant was centrifuged for 10 min at 10,000 x g and the resulting supernatant was separated into plasma membrane and cytosol by a 1 h centrifugation at 200,000 x g. For nuclear fractions a Nuclear Extraction Kit from Chemicon was used according to the manufacturer's instructions to isolate nuclear fractions. The Samples were heated to 95°C in sample buffer and DTT for 10 min, loaded onto Mini-Protean TGX Precast gels (Biorad) and subjected to electrophoresis. Separated proteins were transferred to PVDF membranes using Trans-Blot Turbo Transfer Packs (Biorad) blocked with 3% BSA in 1 x PBS for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C. Washing of the membrane was followed by incubation with secondary antibodies at room temperature for 1 h, washed, and, incubated with ECL (enhanced chemiluminescence), and exposed to hyperfilm-ECL to visualize immunoreactive proteins. Densitometric analysis was performed with a fluor-S quantitative imaging system (Biorad).

### ***Immunofluorescence***

Cells were seeded on glass coverslips, grown for 3 days. The medium was removed and after several washes with PBS cells were fixed with 4% paraformaldehyde for 15 min and subsequently permeabilized with 0.1% Triton X-100. Blocking was done with 3% goat serum in PBS for 45 min. Primary antibodies were diluted in PBS with 1% goat serum and cells were incubated at room temperature (RT) for 1 h followed by incubation with secondary antibodies for 1 h at RT. After washing in PBS, coverslips were mounted on glass slides with fluorescent mounting medium. Confocal microscopy images were recorded using Zeiss LSM 700 (Carl Zeiss).

### ***Transfections and luciferase assay***

Luciferase assays were carried out employing the Dual Luciferase Reporter Assay System from Promega. Each plasmid was used at a final concentration of 1  $\mu\text{g/ml}$  except for the control renilla luciferase vector which was present at all time at 0.2  $\mu\text{g/ml}$  to standardize transfection efficiency. Vector DNA was allowed to form complexes with Lipofectamine (Invitrogen; ratio 4:1) and 60% confluent cells in 12-well plates were washed once in medium free from serum and antibiotics, where after the DNA–Lipofectamine mixture was added to the cells<sup>96</sup>.

Cells were kept at 37°C for 24 hrs after which medium was changed to normal growth medium. Cells were allowed to recover for 24 hrs before the addition of stimulation and/or inhibitors. Subsequently, cells were washed in PBS and lysed using 250  $\mu\text{l/well}$  of DRL-passive lysis buffer included in the Dual Luciferase Reporter Assay System (Promega). Lysed samples were collected and briefly centrifuged at 1000  $\times$  g for 5 min to precipitate any debris. A 40  $\mu\text{l}$  portion of each lysate was transferred to a luminometer test tube prefilled with 50  $\mu\text{l}$  of Luciferase Assay buffer II, and the luciferase reaction was immediately read using a MiniLumat LB 9506 (Berthold Technology). The control Renilla luciferase signal was recorded after the subsequent addition of 50  $\mu\text{l}$  of Stop and Glow buffer, and the level of expression is given as a ratio. Triplicate samples were prepared and analyzed for each condition in every set of experiments.

### ***Wound healing assay***

Cells were grown to confluence in 12-well plates. A wound was inflicted in the monolayer using a pipette tip. Cells were serum-starved for 2 hrs before treatment allowed to migrate for 18-24 hrs at 37°C. Images of wound closure were captured at 0, 18 and 24 hrs with a Nikon phase contrast (DS-Fi1) microscope (Nikon) using a 10x objective and NIS-Elements Basic Research software. The area of the wound was measured at baseline after 18, 24 hrs using Adobe Photoshop CS4 software<sup>163</sup>.

### ***Proliferation assay***

Cells were grown in 96-well plate for 24 hrs. A WST-1 cell proliferation assay (Boehringer Ingelheim) was used following the manufactures protocol.

### **3D Invasion Assay**

To assess if LTD4 induced invasion in SW480 and SW620, an in vitro Boyden chamber assay using Matrigel™ was performed. This assay facilitates easier and controlled quantitation of invasion in 3D in order to mimic the in vivo tumor microenvironment. Invasion in 3D was carried out using an 8  $\mu$ -pore size polycarbonate membrane (Neuro Probe Inc., USA) in a Boyden chamber. The wells in the lower Boyden chamber were filled with either 400  $\mu$ l serum-free medium (control) or serum-free medium containing 100 nM LTD4 as appropriate and covered with the membrane very carefully to avoid air bubbles. The Boyden chamber was then completely assembled, and 100  $\mu$ l of a 1:2 serum-free medium diluted Matrigel™ basement membrane matrix (BD Biosciences, USA) was coated into each well of the upper chamber. The Boyden chamber was incubated for 1 hr at 37°C to allow the matrix to polymerize. The SW480 and SW620 cells were scraped and resuspended in serum-free media and  $2.5 \times 10^5$  cells were added into the matrigel-coated wells of the upper chamber of the Boyden chamber. 5% FCS was used as a positive control in the lower wells of the set-up. The set-up was placed in an incubator at 37°C and cells were then allowed to migrate for 12 hr. After the incubation time, non-migrated cells were carefully removed with a cotton swab, and the migrated cells situated on the lower side of the membranes were rinsed briefly in PBS followed by fixation with 4% paraformaldehyde. The membranes was then mounted on microscopic slides and were stained by DAPI for 10 min. Migrated cells were then counted using a fluorescent microscope (4 x10 magnification fields of view or FOV) and the mean number of migrated cells of each cell-line were then calculated. Data shown here is representative of 1 assay for both cell-lines.

### **Real Time PCR analysis**

The SW480 and SW620 cells were incubated either with 100 nM LTD4 or left unstimulated (control) for 6 hr and then washed in PBS and immediately frozen at -80°C. Thereafter they were scraped in lysis buffer provided in the RNA isolation kit (RNeasy Plus mini kit, Qiagen Nordic, Sweden) and homogenized 10 times with a QIAshredder™. RNA was purified on RNeasy MinElute Spin Columns according to the manufacturer's instructions and eluted with RNase free H<sub>2</sub>O. cDNA synthesis was performed using RevertAid H Minus M-MuLV reverse transcriptase (Fermentas Life Sciences, USA). The mRNA changes in MMP-2, MMP-9, MMP-7 and the endogenous housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT-1) were quantified using real-time PCR analysis using the Maxima

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probe/ROX qPCR master mix (Thermo Scientific, USA) and TaqMan gene expression primers (Applied Biosystems, USA). The cDNA was mixed with 0.9  $\mu$ M TaqMan primers and master mix and amplified at 60°C in an Mx3005P thermocycler (Stratagene, USA). The following TaqMan primers were used: MMP-7 (Hs01042795\_m1), HPRT-1 (Hs99999909\_m1), MMP-2 (Hs 00234422-ml) and MMP-9 (Hs 00957562). The samples were analyzed and normalized against HPRT-1 using the Comparative 2-( $\Delta\Delta$ Ct) method of quantitation on the MxPro software (Stratagene, USA).

## **Results and discussion**

### ***Paper I***

GSK-3 $\beta$  can act both as a tumor suppressor and tumor promotor. These opposing roles of the protein are through repression of Wnt/ $\beta$ -catenin signaling through phosphorylation of  $\beta$ -catenin, and through maintenance of cell survival and proliferation through the NF- $\kappa$ B pathway, respectively<sup>164</sup>. The activity of GSK-3 $\beta$  is regulated by site-specific phosphorylation and the main substrate of GSK-3 $\beta$  is  $\beta$ -catenin. In addition to having its effect in the cytosol, there is evidence that nuclear GSK-3 $\beta$  can form a complex with  $\beta$ -catenin, thereby lowering the levels of  $\beta$ -catenin/TCF- dependent transcription through a mechanism that involves GSK-3 $\beta$ -Axin binding<sup>165</sup>. Furthermore it has been shown that inactivation of GSK-3 $\beta$  can increase transcriptional activity of  $\beta$ -catenin and Snail expression during Fas-induced EMT in gastrointestinal cancer<sup>166</sup>. Some studies suggest that GSK-3 $\beta$  can promote tumorigenesis and cancer development. In pancreatic cancer cells, nuclear localization of GSK-3 $\beta$  was associated with cell dedifferentiation and inhibition of GSK-3 $\beta$ , as well as impaired NF- $\kappa$ B-mediated pancreatic cancer cell survival and proliferation of tumor xenografts<sup>167</sup>. Nuclear accumulation of GSK-3 $\beta$  was found in bladder cancer samples and associated with impaired survival<sup>168</sup>. In colon cancer, the total amount of GSK-3 $\beta$  was higher in tumors than in samples from normal colon and associated with accumulation of  $\beta$ -catenin<sup>169</sup>. Depletion of nuclear GSK-3 $\beta$  or pharmacological inhibition of its kinase activity impaired survival and proliferation of cultured colon cancer cells<sup>164</sup>. In prostate cancer, cytoplasmic accumulation of GSK-3 $\beta$  was associated with more advanced tumors and a two-fold increase in risk of tumor recurrence<sup>170</sup>. More than half of patients with stage III colorectal cancer will experience tumor recurrence. Currently, no biomarker can distinguish patients with good and bad prognosis. Deregulation of Wnt/ $\beta$ -catenin signaling is a hallmark of colon cancer but the role of the GSK-3 $\beta$  has not been evaluated in a clinical setting. In our study, tumor samples from 85 patients were evaluated for expression of GSK-3 $\beta$  expression and  $\beta$ -catenin. We found that nuclear expression of GSK-3 $\beta$  was significantly associated with lack of membrane  $\beta$ -catenin ( $p=0.007$ ), larger size tumor, and distant metastasis. Given the strong correlation of absence membrane  $\beta$ -catenin and presence of nuclear GSK-3 $\beta$  we investigated the prognostic value of this particular expression pattern. We found that these patients were significantly younger, had larger tumors, more lymph node involvement and distant metastasis. When evaluating the effect on overall survival by log rank test we found that nuclear expression of GSK-3 $\beta$  was associated with shorter overall survival Hazard ratio (HR) 2.108, 95% confidence interval (CI) 1.192-3.727). Membrane expression of  $\beta$ -catenin

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was associated with better overall survival hazard ratio (HR) 0.483; 95% CI 0.263-0.889). Traditional prognostic markers such as larger tumor size, distant metastasis, and lymph node involvement were, as expected, significantly associated with poorer survival. The biomarker combination of nuclear GSK-3 $\beta$  expression and lack of membrane  $\beta$ -catenin was significantly associated with poorer prognosis in a univariate analysis (HR 3.290; 95% CI 1.759-6.154). A multivariate analysis confirmed that this biomarker combination in the primary tumor is an independent factor associated with poor prognosis in colon cancer (HR 1.989; CI 1.016-3.894). We further investigated whether expression levels of  $\beta$ -catenin and GSK-3 $\beta$  varied between the primary tumor and distant metastases. For 7 patients, FFPE samples from distant lung- or liver metastases were available. Tumor samples were stained for GSK-3 $\beta$  and  $\beta$ -catenin and the subcellular protein expressions were evaluated and compared to those of the corresponding primary tumor. Regarding both expression and localization of GSK-3 $\beta$  and  $\beta$ -catenin there was tendency towards re-expression of  $\beta$ -catenin at the membrane and a decrease in nuclear GSK-3 $\beta$ . The different expression patterns in primary tumor and corresponding metastases suggests that metastatic tumor cell at secondary sites, having undergone mesenchymal epithelial transition (EMT) during the course of spreading, have reverted back to an epithelial phenotype. In a wound healing assay, we evaluated the migratory capacity of SW480 and SW620 cells and found that cells with low expression of inactive (pGSK-3 $\beta$ ) pGSK-3 $\beta$  in the nucleus in combination with low membrane  $\beta$ -catenin migrated faster compared to cells with low levels nuclear active GSK-3 $\beta$  and high membrane  $\beta$ -catenin<sup>171,171</sup>.

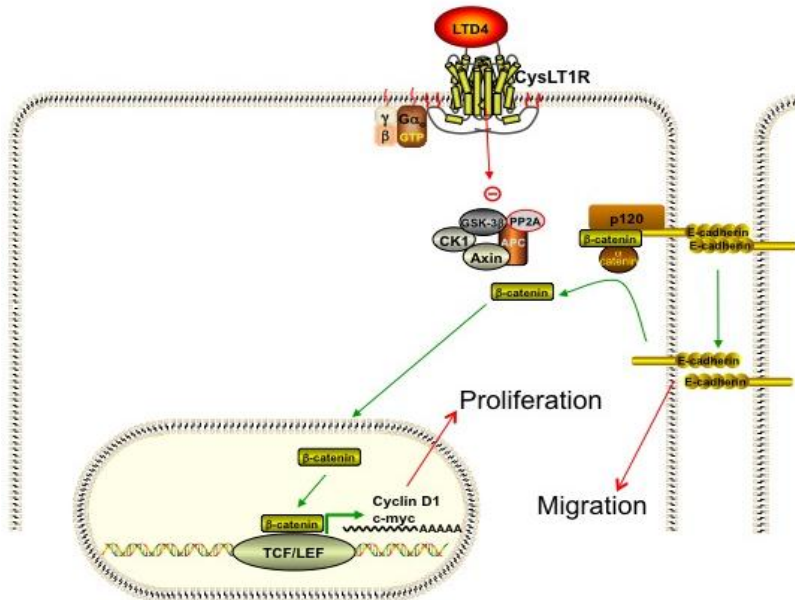
### ***Paper II***

Stabilization and accumulation of  $\beta$ -catenin result from increased canonical Wnt signaling which in turn can be due to mutations of  $\beta$ -catenin itself or in any other regulatory gene product involved in the Wnt pathway, such as APC. More than 80% of colon cancers have APC mutations and a majority of the remaining 20% have  $\beta$ -catenin mutations, the effects of which can be identical to that of an APC mutation. Chronic inflammation plays an important role in tumorigenesis, and an inflammatory microenvironment is an essential component of many tumors<sup>10, 11</sup>. Previous findings from our lab indicate that LTD<sub>4</sub> can increase survival of mammalian intestinal epithelial cells by activation of its receptor CysLT<sub>1</sub>R<sup>98, 160</sup>. Our results show that LTD<sub>4</sub>-induced activation of CysLT<sub>1</sub>R signaling increased  $\beta$ -catenin accumulation. Further, translocation of  $\beta$ -catenin from the membrane to the nucleus through phosphorylation of GSK-3 $\beta$  at Ser-9 in HCT116 cells induced increased transcriptional activity of TCF/LEF target genes c-myc and



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cyclin D1. In functional assays, increased cell proliferation was seen. Inhibition of CysLT<sub>1</sub>R led to complete abolishment of the LTD<sub>4</sub> effect. In HT29 cells, no significant effects of LTD<sub>4</sub> were seen. The reason for this could be that HT29 cells have lower expression levels of CysLT<sub>1</sub>R than HCT116. Furthermore, HT29 have mutated APC, causing the Wnt/ $\beta$ -catenin signaling pathway being constitutively activated, which might make the addition of LTD<sub>4</sub>-induced  $\beta$ -catenin difficult to detect. Subcellular translocation of  $\beta$ -catenin to the nucleus in HCT116 was confirmed by immunofluorescence after stimulation of cells with LTD<sub>4</sub> every 6 hrs for 24 hrs. Further, the active form of  $\beta$ -catenin (pS552- $\beta$ -catenin) we found to significant increase in the nucleus after LTD<sub>4</sub> stimulation. Previous reports have shown that signaling through the PI3K/Akt pathway can synergize with Wnt signaling to increase levels of active  $\beta$ -catenin signaling in the inflammatory microenvironment in colitis<sup>172</sup>. We have shown that LTD<sub>4</sub> can induce  $\beta$ -catenin signaling in intestinal epithelial cells and an association with the anti-apoptotic protein Bcl-2<sup>173</sup>. Further, PI3K signaling activity significantly increased in CRC<sup>174</sup> and it was recently reported that PI3K/AKT is essential for the development of cancer in patient with colitis<sup>175</sup>. We, however, did not find any crosstalk with the PI3K pathway in colon cancer cells, as shown by the inability of wortmannin to inhibit the effects of LTD<sub>4</sub> in HCT116 cells, which is opposed to previous results in cells<sup>173</sup>. In addition to its role in the Wnt signaling pathway,  $\beta$ -catenin, together with E-cadherin, is a key mediator of adherence junctions in epithelial cells. Disruption of these adherence junctions lead to cell depolarization and migration<sup>176</sup>. Previous results from our lab suggested that LTD<sub>4</sub> could induce migration of intestinal epithelial cells<sup>99</sup>. We found a significant reduction of E-cadherin levels at the membrane of HCT116 colon cancer cells after LTD<sub>4</sub> stimulation as well as after inhibition of GSK-3 $\beta$  but no significant changes in HT29 colon cancer cells. The reduction of E-cadherin and  $\beta$ -catenin at the membrane of HCT116 cells induced by LTD<sub>4</sub> was associated with a significant increase in cell migration as seen in Figure 8. Furthermore, inhibition of GSK-3 $\beta$  led to downregulation of E-cadherin at the membrane via up-regulation of Snail transcriptional activity<sup>177</sup>.



**Figure 8.** Leukotriene-dependent  $\beta$ -catenin signaling in colon cancer cells

**Paper III**

The E-cadherin-catenin complex plays an important role in cell to cell adhesions and in maintaining normal tissue architecture<sup>178</sup>. Loss of cell-cell adhesion and polarity can trigger EMT, which is an important step in the invasion and progression of colon cancer. One of the hallmarks of EMT is loss of epithelial marker such as E-cadherin and increased expression of mesenchymal markers such as vimentin. Inflammation and genomic instability foster multiple hallmark functions of cancer. Inflammatory cells which infiltrate the tumor microenvironment such as macrophages, T- and B-lymphocytes release signaling molecules leading to sustained tumor angiogenesis, proliferation, and facilitation of tumor invasion and metastasis<sup>10</sup>. Downregulation of E-cadherin expression was associated with more invasive properties of tumors and poorer prognosis<sup>179</sup>.

We showed that lack of membrane  $\beta$ -catenin in combination with nuclear expression of GSK-3 $\beta$  was associated with poor overall survival in colon cancer<sup>171</sup>. In the same patient material, we found that

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moderate expression of E-cadherin at the cellular membrane, in combination with absence of nuclear GSK-3 $\beta$ , was associated with good prognosis in stage II colon cancer (P= 0.0037). Previous findings from our lab showed that high expression of pro-inflammatory receptor CysLT<sub>1</sub>R was associated with poor prognosis in colon cancer<sup>160, 162</sup>. Here, we further investigated the effects of the combination of moderate membrane E-cadherin and no nuclear GSK-3 $\beta$  in tumors with moderate to high CysLT<sub>1</sub>R expression. We found that this biomarker combination was significantly associated with better survival (P=0.009). There was no significant correlation between E-cadherin intensity and prognosis in the patient cohort investigated. We investigated differences in E-cadherin,  $\beta$ -catenin and pGSK3 $\beta$  protein expression levels and localization in two colon cancer cell lines derived from the primary tumor and from metastatic lymph nodes and found that E-cadherin expression was higher in SW480 than in SW620 cells. For  $\beta$ -catenin, higher expression levels were found both at the membrane, in the cytosol and in the nucleus in the primary tumor compared to the metastatic cells. These differences in protein expression suggest a predominantly mesenchymal phenotype of the metastatic SW620 cancer cells. Furthermore, expression of inactive pGSK-3 $\beta$  was significantly higher in both cytosolic and nuclear fractions of primary SW480 colon cancer cells<sup>171</sup>.

Previous findings from our lab showed that LTD<sub>4</sub> increased proliferation, migration, and survival of intestinal epithelial cells via CysLT<sub>1</sub>R<sup>99</sup>. Given the strong connection between inflammation and colon cancer development and progression we further investigated if the proinflammatory mediator LTD<sub>4</sub> could affect invasiveness of SW480 and SW620 cells. SW480 and SW620 cells were stimulated with 100 nM LTD<sub>4</sub> for 24 hr, where after membrane fractions were isolated. The results showed a significant decrease in levels of membrane E-cadherin and a concomitant increase in vimentin and inactive p-Snail in SW480, but not in SW620 cells. Furthermore, we also found an increase in mRNA expression levels of MMP-2, MMP-7 and MMP-9 in SW480 cells. These data indicate that LTD<sub>4</sub> can induce changes important for EMT, which in turn could explain the effect of LTD<sub>4</sub> on cell motility and invasion.

Furthermore immunofluorescence results showed a significant increase in nuclear active pS552- $\beta$ -catenin and an increase in expression as well as intensity of inactive pGSK-3 $\beta$  in the pre-nuclear area in both primary and metastatic cancer cells after LTD<sub>4</sub> stimulation. Taken together, these findings suggest that an alteration in the E-cadherin/ $\beta$ -catenin/pGSK-3 $\beta$  complex leading to nuclear translocation of  $\beta$ -catenin is involved in LTD<sub>4</sub> induced tumor progression and invasion. We next investigated the effect of an inflammatory milieu on cell migration. SW480 and SW620 cells were stimulated with LTD<sub>4</sub> for 18 hrs and migratory capacity was evaluated in a wound healing assay. Our results show a significant increase in the

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migratory capacity of SW480 cells after stimulation while no significantly increased was seen in SW620 cells. We next investigate effect of LTD<sub>4</sub> on cell invasion we found an increase invasion capacity in SW480 while less effect on cell invasion capacity was seen in SW620 cells. Our results indicate that stimulation colon cancer cells with pro-inflammatory mediator LTD<sub>4</sub> can promote a more migratory and invasive phenotype through induction of EMT with increased expression of mesenchymal markers including vimentin, nuclear  $\beta$ -catenin and inactive pGSK-3 $\beta$ .

## **Conclusions**

### ***Paper I***

- Nuclear GSK-3 $\beta$  is a marker of poor survival in colon cancer (two times increased risk of death compared to patients with no nuclear GSK-3 $\beta$ )
- Membrane expression of  $\beta$ -catenin is associated with a favorable prognosis in colon cancer
- Patients with nuclear GSK-3 $\beta$  in combination with lack of membrane  $\beta$ -catenin have a poor overall survival (independent prognostic marker)
- Tumor cells with high expression of active GSK-3 $\beta$  and low expression of inactive (pGSK-3 $\beta$ ) in the nucleus, in combination with low membrane  $\beta$ -catenin, migrated faster compared to tumor cells with low nuclear active GSK-3 $\beta$ /high membrane  $\beta$ -catenin

### ***Paper II***

- LTD<sub>4</sub> induces stabilization and translocation of  $\beta$ -catenin to the nucleus through inactivation of GSK-3 $\beta$  at serine 9
- LTD<sub>4</sub> induces transcriptional activity of TCF/LEF family of transcriptional factors leading to increased transcription of  $\beta$ -catenin target genes such as c-myc and cyclin D1 and increase proliferation of HCT116 colon cancer cells
- LTD<sub>4</sub> reduces cell to cell adhesion through its effect on the E-cadherin/ $\beta$ -catenin complex at the membrane and increases migration of HCT116 colon cancer cells

### ***Paper III***

- Stage II colon cancer patients with moderate membrane E-cadherin in combination with no nuclear GSK-3 $\beta$  have a good overall survival
- The combination of moderate E-cadherin and no nuclear GSK-3 $\beta$  is associated with better prognosis in colon cancer patients with high expression of CysLT<sub>1</sub>R in the primary tumor
- Pro-inflammatory LTD<sub>4</sub> promotes a more invasive and migratory phenotype of colon cancer cells, possibly through induction of epithelial mesenchymal transition

## **Sammanfattning på svenska**

Kolorektal cancer är den tredje vanligaste cancerformen i världen hos både män och kvinnor. Sjukdomen kan vara genetiskt orsakad och är då oftast av typen FAP (familjär adenomatös polypos) eller HNPCC. Vanligast är dock sporadisk kolorektal cancer. Den absolut största riskfaktorn för att utveckla sjukdomen är, som för de flesta andra cancerformer, ålder; medelåldern för insjuknande i kolorektal cancer är 70 år. Övriga riskfaktorer är bland annat inflammatorisk tarmsjukdom, låg fysisk aktivitet, fettrik diet och alkohol. Inflammation, och då framförallt inflammatoriska mediatorer i tumörens omgivning, har visats ha stor betydelse både för utveckling och tillväxt av kolorektal cancer. Ett tydligt bevis för detta samband är att risken att utveckla cancer vid inflammatorisk tarmsjukdom har minskat i takt med att bättre behandlingar av tarminflammationen börjat användas. Aktivering av Wnt/ $\beta$ -katenin signalvägen har identifierats som en viktig faktor för utvecklingen av kolorektal cancer. Wnt är ett signalprotein med stor betydelse under den embryonala utvecklingen men även vid tillväxt av nya vävnader hos vuxna individer. Wnt-signaleringen är normalt aktiverad längst ned i de så kallade kryptorna inuti tarmväggen där nya epitelceller bildas. Dysreglering av denna signalväg, oftast orsakad av mutationer i något av nyckelproteinerna, antingen APC eller  $\beta$ -katenin, sker i princip 100 % av alla kolorektala tumörer. Wnt-signalering leder till ökade mängder  $\beta$ -katenin i cellen eftersom nedbrytningen som normalt ska ske hindras.  $\beta$ -katenin transporteras därefter in i cellens kärna och påverkar där transkriptionen av gener vilka dels kan leda till utveckling av cancer, så kallade onkgener, men även påverkar tillväxten, proliferationen, av en redan existerande tumör. Inflammatoriska mediatorer tillskrivs ökande betydelse för cancerutveckling och tillväxt av cancertumörer. Leukotriener frisätts från inflammatoriska celler såsom makrofager, mastceller och neutrofiler men även från trombocyter och endotelceller och har visats kunna öka celltillväxt och motverka apoptos (programmerad celledöd).

Kolorektal cancer behandlas i första hand med operation varvid tumören tillsammans med omgivande lymfkörtlar tas bort. De återstående tarmändarna kopplas ihop igen och patienten kan oftast återgå till ett normalt liv. Vid rektal cancer förbehandlas patienten innan operation med strålning och ibland även cellgifter. Efter operationen behöver vissa patienter en uppföljande cellgiftsbehandling, detta avgörs av tumörens storlek, spridning till lymfkörtlar och en rad prognostiska faktorer. Många patienter behandlas dock i onödan, eftersom många botas av endast operation. Det är således angeläget att hitta nya markörer för att korrekt identifiera vilka patienter som är i behov av denna extra behandling för att förebygga återfall. Vid återfall i kolorektal cancer med spridning till lever, lungor och andra organ är sjukdomen oftast inte botbar utan behandlas med cellgifter (5-FU, oxaliplatin, irinotekan och kapecitabin) och sk

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målriktade behandlingar (bevicizumab, aflibercept och EGFR-hämmarna cetuximab och panitimumab) för att krympa tumörerna, minska patientens symtom och förlänga livet.

Jag har i mitt avhandlingsarbete studerat uttrycket av potentiella prognostiska markörer i tumörprover från patienter som behandlats för koloncancer. Vi fann att förlust av  $\beta$ -katenin-uttryck i cellmembranet, tillsammans med överuttryck av GSK-3 $\beta$  i cellkärnan, var starkt associerat med ökad risk att dö i koloncancer. För att försöka förstå mekanismen för detta studerade vi effekten av den inflammatoriska mediatoren LTD<sub>4</sub> (leukotriene D4) på odlade koloncancerceller och fann att LTD<sub>4</sub> kunde öka uttrycket av  $\beta$ -katenin i cellkärnan samtidigt som uttrycket i cellmembranet minskade. I funktionella studier av cellernas förmåga att migrera (förflytta sig) och invadera (sprida sig till omgivande vävnader) kunde vi visa en signifikant effekt av LTD<sub>4</sub> även på dessa mekanismer. Genom att studera effekten av LTD<sub>4</sub> även på andra gener med betydelse för tumörcellers förmåga att sprida sig och i slutändan ge upphov till metastaser (dottertumörer) kan vi konstatera att inflammatoriska mediatorer utsöndrade från celler i tumören omgivning sannolikt bidrar till tumörtillväxt och spridning genom effekter på Wnt/ $\beta$ -katenin signaleringen.

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# Nuclear expression of Glycogen synthase kinase-3 $\beta$ and lack of membranous $\beta$ -catenin is correlated with poor survival in colon cancer

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Dysregulation of Wnt/ $\beta$ -catenin signaling is a hallmark of colon cancer. Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) can be a positive regulator of survival and proliferation of cultured colon cancer cell but its role in clinical colon cancer is unknown. Our objectives were to evaluate the role of GSK-3 $\beta$  in colon cancer. A tumor tissue microarray of primary colon cancers and metastases was used to evaluate expression and subcellular localization of GSK-3 $\beta$  and  $\beta$ -catenin. In total, 85 primary colon cancer samples were evaluated by immunohistochemistry. Immunoreactivity was correlated to known markers of adverse prognosis. Overall survival was the primary end-point. We found nuclear accumulation of GSK-3 $\beta$  in 39% (33/85) of evaluated tumors. Nuclear GSK-3 $\beta$  was significantly associated with shorter overall survival ( $p = 0.008$ ), larger tumor size ( $p = 0.015$ ), distant metastasis ( $p = 0.029$ ) and loss of membranous  $\beta$ -catenin ( $p = 0.007$ ). Loss of membranous  $\beta$ -catenin occurred in 37% (30/82) of the tumors and was associated with poor survival ( $p = 0.016$ ). The combination of nuclear GSK-3 $\beta$  and lack of membrane  $\beta$ -catenin occurred in a total of 26% of the studied tumors (21/61) and was significantly and independently associated with poor prognosis. Our results suggest that nuclear expression of GSK-3 $\beta$  and loss of membrane  $\beta$ -catenin identify a subset of colon carcinomas with worse prognosis.

Colorectal cancer (CRC) is the third most common cancer worldwide, with over one million cases occurring every year. The mortality rate for CRC is approximately half of its global incidence. Five-year survival estimate for CRC exceeds 50%, but is highly variable depending on the stage of the disease.<sup>1</sup> The etiological factors and pathogenic mechanisms underlying the development of CRC are complex and heterogeneous and include inflammation, with inflammatory bowel disease being associated with an increased risk of CRC; dietary and life style factors, such as diets rich in red meat and unsaturated fat, excessive alcohol consumption and reduced physical

activity.<sup>2</sup> Further, it is estimated that 15–30% of patients have a major hereditary component, only a quarter of which can be attributed to hereditary nonpolyposis colorectal cancer or familial adenomatous polyposis.<sup>3</sup>

Wnt signaling is one of the key signaling pathways controlling proliferation, differentiation and morphogenesis of cells during development. In all, >90% of CRCs have a mutation in a key regulatory factor of the Wnt/ $\beta$ -catenin pathway, most often in APC or  $\beta$ -catenin, resulting in activation of the pathway.<sup>4</sup> Wnt signaling is initiated by the binding of Wnt family members to a receptor complex consisting of the Frizzled family of transmembrane receptors, together with the coreceptors LRP5/6. Wnt signaling inactivates GSK-3 $\beta$  and prevents it from phosphorylating  $\beta$ -catenin, and thus stabilizing  $\beta$ -catenin in the cytoplasm. As  $\beta$ -catenin accumulates, it translocates into the nucleus where it binds to T-cell-specific transcription factor (TCF)/LEF and increases transcription of proto-oncogenes such as *c-myc* and *cyclin-D1*.<sup>5</sup> In addition to its role in embryogenesis and malignant transformation of cells,  $\beta$ -catenin exists at the cell membrane in a complex with E-cadherin and  $\alpha$ -catenin and has a role in cell-cell adhesion and cell polarization, disruption of which results in dissociation of the cells and is an important step in invasion and metastasis.<sup>4</sup> The role of  $\beta$ -catenin in the development of CRC is well documented but as a single biomarker,  $\beta$ -catenin does not appear to have any prognostic value.<sup>6</sup>

GSK-3 is a multifunctional serine/threonine kinase involved in the regulation of cell fate, protein synthesis, glycogen metabolism, cell mobility, proliferation and survival.<sup>7</sup>

**Key words:** GSK-3 $\beta$ ,  $\beta$ -catenin, colon cancer, metastasis, survival  
Additional Supporting Information may be found in the online version of this article.

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**What's new?**

More than half of patients with stage III colorectal cancer will experience tumor recurrence. Currently there is no biomarker capable of distinguishing among patients with good or poor prognosis. In this study, nuclear expression of the Wnt signaling protein glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), combined with lack of membrane  $\beta$ -catenin, was found to be associated with poor prognosis. The findings emphasize the significance of deregulation of the Wnt/ $\beta$ -catenin signaling pathway as a hallmark of colon cancer and reveal a potential role for GSK-3 $\beta$  as a prognostic biomarker for the disease.

There are two mammalian GSK-3 isoforms; GSK-3 $\alpha$  and GSK-3 $\beta$ . Dysregulation of GSK-3 $\beta$  has been implicated in the development of a number of human diseases such as diabetes, cardiovascular disease, some neurodegenerative diseases and bipolar disorder but also in tumorigenesis and cancer progression.<sup>7</sup> Studies indicate that GSK-3 $\beta$  can act both as tumor suppressor and as tumor promoter.<sup>7</sup> The protein is constitutively active in resting cells and undergoes a rapid and transient inhibition in response to a number of external signals. GSK-3 $\beta$  activity is regulated by site-specific phosphorylation with full kinase activity requiring phosphorylation at tyrosine (Tyr216): On the other hand, phosphorylation at serine (Ser9) inhibits GSK-3 $\beta$  activity. One of the most known substrates of GSK-3 $\beta$  is  $\beta$ -catenin. In the absence of Wnt signaling, GSK-3 $\beta$  phosphorylates  $\beta$ -catenin, leading to its ubiquitin-mediated degradation.<sup>7</sup> In addition to having its effect in the cytosol, there is evidence that GSK-3 $\beta$  can enter the nucleus and form a complex with  $\beta$ -catenin, thereby lowering the levels of  $\beta$ -catenin/TCF-dependent transcription.<sup>8</sup>

GSK-3 $\beta$  is also proposed to be involved in cancer cell metastasis with inhibition of GSK-3 $\beta$  promoting epithelial-mesenchymal transition (EMT), a prerequisite for tumor cell invasion and dissemination. Contrastingly though, some studies suggest that GSK-3 $\beta$  can promote tumorigenesis and cancer development. In pancreatic cancer cells, nuclear localization of GSK-3 $\beta$  was associated with cell dedifferentiation and inhibition of GSK-3 $\beta$  impaired NF- $\kappa$ B-mediated pancreatic cancer cell survival and proliferation in tumor xenografts.<sup>9</sup> Nuclear accumulation of GSK-3 $\beta$  was found in bladder cancer samples and associated with impaired survival.<sup>10</sup> In colon cancer, the total amount of GSK-3 $\beta$  was found to be higher in tumors than in samples from normal colon.<sup>11</sup> Depletion of nuclear GSK-3 $\beta$  or pharmacological inhibition of its kinase activity impaired survival and proliferation of cultured colon cancer cells.<sup>12</sup>

The potential dual role for GSK-3 $\beta$  is especially interesting in CRC, given that the Wnt/ $\beta$ -catenin signaling pathway is deregulated in a majority of tumors. Little is known about GSK-3 $\beta$  with respect to the protein's subcellular localization in colon cancer, nor has its role in survival in this cancer type been studied. Therefore, we here investigated the expression and prognostic role of GSK-3 $\beta$  in clinical colon cancer samples. As GSK-3 $\beta$  is a major regulator of  $\beta$ -catenin levels in the cells, we also evaluated  $\beta$ -catenin expression and subcellular localization.

**Material and Methods****Patients**

Patients were selected retrospectively and all patients who underwent surgery for colon cancer at Malmö University Hospital during the selected time period (1990) were included. No stratification or matching was done. All tumors with available slides or paraffin blocks were histopathologically re-evaluated on hematoxylin and eosin (H&E)-stained slides. Clinical, treatment and histopathology data were retrieved from patient charts and pathology records. All patients had a pathologically confirmed diagnosis of adenocarcinoma before surgery. Tumors were staged according to the UICC TNM classification of malignant tumors.<sup>13</sup> Tumor grade was determined according to Jass *et al.*<sup>14</sup> as high, medium or low. Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry until December 31, 2000. Follow-up started at the time of diagnosis and ended at death, emigration or December 31, 2000, whichever came first. Median follow-up time was 4.58 years (range, 0.08–10.67) for the full cohort ( $n = 89$ ) and 8.78 years (range, 0.33–10.67) for patients alive ( $n = 31$ ). The primary endpoint of the study was overall survival (OS). The study was approved by the Ethical Committee at Lund University.

**Tumor tissue microarray and immunohistochemistry**

Cases without available paraffin blocks or with an insufficient amount of tumor material were excluded. Out of a total number of 89, 85 patients (95.5%) were suitable for tumor tissue microarray (TMA) construction. To confirm the diagnosis and histological grading, the archival formalin-fixed, paraffin-embedded (FFPE) samples were cut into 1  $\mu$ m sections, dried, deparaffinized, rehydrated and stained with H&E. Two to three 1.5-mm tissue cores from each donor block were placed in a new paraffin block by using an automated Beecher Micro-Arrayer (Beecher Instruments, Sun Prairie, WI, USA). For the samples from distant metastases, whole sections of the tumors were evaluated by H&E to verify the pathological diagnosis. For TMAs as well as larger tumor sections, immunohistochemical stainings were performed as described previously.<sup>15</sup> Briefly, the paraffin array blocks were cut into 1  $\mu$ m sections, which were pretreated as described previously and then placed in citrate buffer and heated for 2  $\times$  10 min in a microwave oven. All immunohistochemical procedures were performed using a Dako automatic slide stainer (Dako, Glostrup, Denmark)

according to the manufacturer's instructions. After immunostaining, all slides were counterstained with H&E. Immunoreactivity for GSK-3 $\beta$  and  $\beta$ -catenin was assessed by two investigators (Salim T and Sand-Dejmek J). Disagreement between the observers was <10%, and those cases were reviewed until an agreement was reached. Tumors were graded according to intensity and subcellular localization (membrane, cytoplasm and nucleus). Tumors were evaluated for intensity of staining (0 = negative, 1 = weak, 2 = intermediate and 3 = strong), percentage of staining cells (1 = 0–5%, 2 = 6–25%, 3 = 26–75% and 4 = 76–100%) and to subcellular localization (membrane, cytoplasm and nuclear). Tumors were considered positive for nuclear GSK-3 $\beta$  staining if more than 5% of cells exhibited nuclear expression and staining intensity was moderate or high. Normal colon exhibited weak to moderate cytoplasmic GSK-3 $\beta$  expression (Fig. 1a). In normal tissue, nuclear GSK-3 $\beta$  was expressed in <5% of cells and the staining intensity was weak (Fig. 1a). For membranous  $\beta$ -catenin, tumors were considered positive if >50% of the cells exhibited membranous expression of the protein and negative if the expression was below 50%. In reality, however, staining for membranous  $\beta$ -catenin was very homogenous, with a majority of tumors being either strongly positive for membranous  $\beta$ -catenin with close to 100% of the cells expressing  $\beta$ -catenin at the membrane, or completely negative, with <5% of cells exhibiting immunoreactivity for  $\beta$ -catenin at the cell membrane.

A mouse monoclonal antibody against  $\beta$ -catenin (dilution 1:1000, BD Transduction Laboratories) and a rabbit polyclonal antibody against GSK-3 $\beta$  (dilution 1:50, Cell Signaling Technology, Danvers, MA, USA) were used. For Ki67, nuclear immunoreactivity was evaluated by counting Ki67-positive cells in a number of fields of view and estimating the percentage of positive cells in the tumor as a whole. For statistical evaluation, tumors were classified as low proliferation = 0–29% Ki67-positive nuclei, and high proliferation = >29% Ki67-positive nuclei.

#### Statistical analysis

OS was the primary endpoint. Comparisons of clinical data and tumor characteristics according to GSK-3 $\beta$  and  $\beta$ -catenin status were done by *t*-test. Kaplan–Meier estimates were used to illustrate survival according to GSK-3 $\beta$  and  $\beta$ -catenin expression and the log-rank test to assess for equality of survival curves. Hazard ratios (HRs) were estimated using Cox proportional hazards model for OS in uni- and multivariate analyses. The power to detect a HR of 2.0 at the level of significance 0.05 was 77%. Calculations were performed using SPSS version 19.0 (SPSS, IBM, Armonk, NY, USA) All *p*-values corresponded to two-sided tests and values of  $\leq 0.05$  were considered significant.

#### Cell culture

Human colorectal adenocarcinoma cell lines SW480 (DMS ACC 313) and SW620 (ATCC CCL-227) were grown in RPMI

medium (SW480) or Leibovitz's L-15 medium with 2 mM L-glutamine (SW620) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were regularly tested to ensure the absence of mycoplasma. For experiments, cells were used at a confluence of around 70%.

#### Cell fractionation, gel electrophoresis and immunoblotting

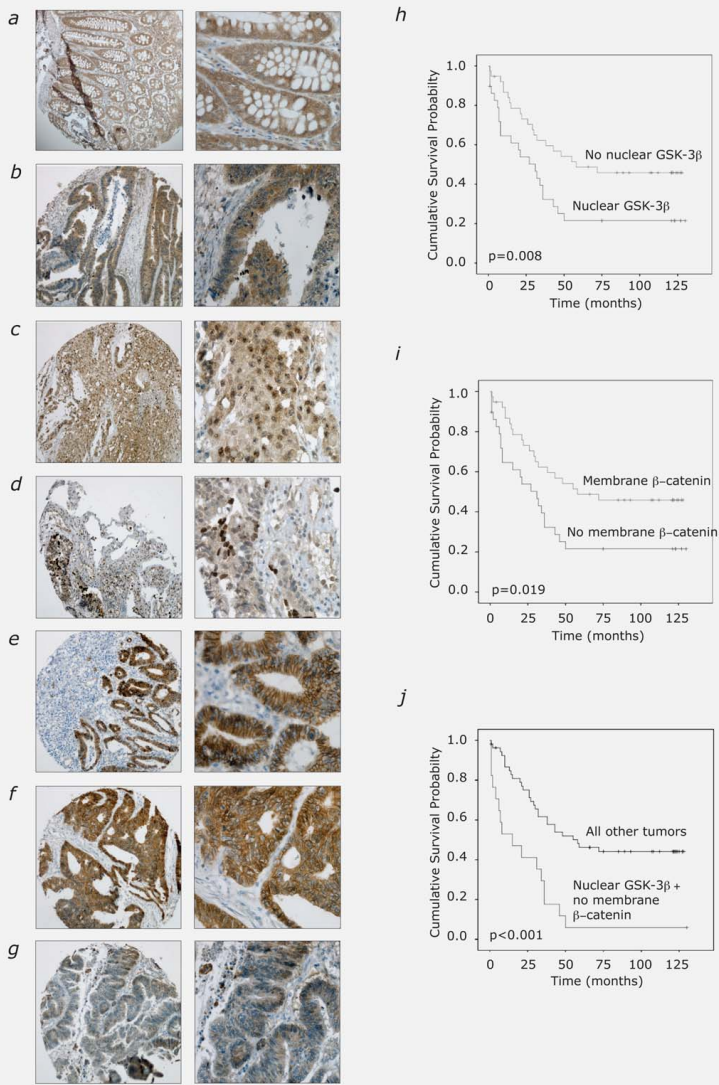
For the preparation of membrane and cytosolic fractions, SW480 and SW620 cells were washed with ice cold PBS (1 $\times$ ), covered with buffer A<sup>16</sup> and kept on ice for 30 min whereafter the cells were homogenized in a Dounce homogenizer and centrifuged for 10 min at 500g. The supernatant was centrifuged for 10 min at 10,000g and the resulting supernatant was separated into plasma membrane and cytosol by centrifugation at 200,000g for 1 hr. For the preparation of nuclear extracts, the Nuclear Extraction Kit from Millipore was used according to the manufacturer's instructions (Millipore, Billerica, MA, USA). Cellular fraction samples were solubilized by boiling in sample buffer and DTT for 10 min. The samples were run on a Mini-PROTEAN TGX Precast gel and were subjected to electrophoresis whereafter the separated proteins were transferred to polyvinylidene difluoride membranes, blocked with 3% BSA in 1 $\times$  PBS for 1 hr at room temperature followed by overnight incubation with primary antibodies at 4°C. GSK-3 $\beta$  expression was detected by probing with the mouse monoclonal antibody at 1:1000 dilution. For  $\beta$ -catenin, the monoclonal antibody was used at a mouse monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA) was used at a 1:1000 dilution. Secondary, horseradish peroxidase (HRP)-coupled, antibodies were from Dako. Immunoreactive proteins were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

#### Immunofluorescence

For immunofluorescence analysis, cells grown on 22-mm glass cover slips were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. Fixed cells were incubated anti-GSK-3 $\beta$  (1:500) or anti- $\beta$ -catenin (1:500) antibodies for 1 hr at 37°C, and subsequently incubated for 45 min at 37°C with goat antirabbit and antimouse secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 fluorochromes (Molecular Probes, Eugene, OR, USA), respectively, at a 1:1000 dilution. DNA was visualized by 4',6-diamidino-2-phenylindole (DAPI) staining (Sigma-Aldrich, Copenhagen, Denmark). Fluorochromes were visualized with a Nikon microscope and imaged with NIS-Elements AR software (Nikon, Tokyo, Japan).

#### Wound-healing assay

SW480 and SW620 cells were grown to confluence in cell-culture dishes. A wound was inflicted on the monolayer using a pipette tip. Cells were serum starved for 2 hr and subsequently incubated in medium containing 20% fetal bovine serum. The cells were allowed to migrate for 24 hr at



**Figure 1.** (a–g) Subcellular localization of GSK-3 $\beta$  and  $\beta$ -catenin determined by immunoreactivity in representative sections of invasive colon carcinomas. (a) Moderate cytoplasmic expression of GSK-3 $\beta$  in normal colon mucosa. (b) Tumor with cytoplasmic expression of GSK-3 $\beta$ , (c) tumor with moderate homogeneous nuclear expression of GSK-3 $\beta$ , (d) tumor with strong heterogeneous nuclear expression of GSK-3 $\beta$ . (e) Tumor with membranous  $\beta$ -catenin expression. (f) Tumor with membranous  $\beta$ -catenin expression and cytoplasmic  $\beta$ -catenin expression, and (g) tumor with weak cytoplasmic expression of  $\beta$ -catenin (microscopy images; left hand panels: 10 $\times$  magnification, right-hand panels: 40 $\times$ ). (h–j) Nuclear GSK-3 $\beta$  and lack of membrane  $\beta$ -catenin is associated with poor survival. Kaplan–Meier survival curves according to (h) nuclear GSK-3 $\beta$  ( $n=82$ ), (i) membranous  $\beta$ -catenin ( $n=82$ ), and (j) nuclear GSK-3 $\beta$  in combination with lack of membrane  $\beta$ -catenin ( $n=82$ ). OS is shown in months and differences between groups were assessed using log-rank testing.

37°C. Pictures were taken at 0, 18 and 24 hr with a Nikon phase contrast (DS-Fi1) microscope using a 10× objective and NIS-Elements Basic Research software. The area of the wound was measured both at the beginning and after 18 and 24 hr with Adobe Photoshop CS4 software.<sup>17</sup>

## Results

### Expression of GSK-3β and β-catenin in colon tumors

In our study, evaluation of GSK-3β and β-catenin expression was possible for 85 and 82 cases, respectively. GSK-3β was expressed in the cytoplasm of all tumors with no tumors exhibiting membranous staining. Thirty-three (39%) tumors showed moderate or strong nuclear staining for GSK-3β. In the remaining 52 (61%) of tumors, GSK-3β was absent from or very weakly expressed in the nucleus. For β-catenin, cytoplasmic, membranous and/or nuclear immunoreactivity was found. Membrane β-catenin was present in 39% of tumors (32/82). Nuclear β-catenin was found in 45% (37/82) of tumors. Figure 1 shows representative images of normal colon mucosa (Fig. 1a) and tumors with different subcellular expressions of GSK-3β (Figs. 1b–1d) and β-catenin (Figs. 1e–1g). For 20 patients, a corresponding sample from normal colon mucosa was included in the TMA. Normal colon mucosa samples expressed various levels of GSK-3β in the cytoplasm but none exhibited any nuclear expression of the protein. Figure 1a shows cytoplasmic expression of GSK-3β in normal colon mucosa (Fig. 1a). Normal colon mucosa did not express nuclear GSK-3β staining.

### Association of nuclear GSK-3β and membranous β-catenin expressions with other clinico-pathological variables

Nuclear expression of GSK-3β was significantly correlated with the absence of membranous β-catenin ( $p = 0.007$ ), larger tumor size ( $p = 0.015$ ) and distant metastasis at the time of primary surgery ( $p = 0.029$ ) (Supporting Information Table 1). Given the strong correlation between nuclear GSK-3β and lack of β-catenin at the cell membrane, we decided to compare patients with tumors with no membranous β-catenin in combination with nuclear GSK-3β with tumors not exhibiting this protein combination. Out of the 82 patients who were evaluated for both GSK-3β and β-catenin, the combination of nuclear GSK-3β and lack of β-catenin was found in 26% (21/82). When examining this subgroup, we found that those patients were significantly younger (average age at diagnosis 68.9 vs. 74.1 years,  $p = 0.031$ ). Moreover, metastatic lymph node involvement was more common in this subset ( $p = 0.009$ ). As for nuclear GSK-3β and membrane β-catenin individually, the presence of the combination was associated with larger tumor size and with distant metastasis at the time of diagnosis. No association was found with proliferation as estimated by Ki67 or with histological tumor grade (Table 1).

### Prognostic role of nuclear GSK-3β and membranous β-catenin expression

For survival analyses, dichotomized variables defined as low or absent staining versus moderate or strong staining were used

Table 1. Clinical and pathological variables in patients with primary tumors expressing the combination of nuclear GSK-3β and no membrane β-catenin versus tumors with no nuclear GSK-3β and/or expression of membranous β-catenin

Characteristic	Nuclear GSK-3β and membrane β-catenin (n = 21)	All other tumors (n = 61)	p-Value
Age at diagnosis (years)	68.9	74.1	0.031
Range	37–84	47–86	
<b>Tumor size</b>			
T1	0 (0%)	3 (5%)	
T2	0 (0%)	8 (13%)	
T3	13 (86%)	48 (79%)	
T4	8 (14%)	2 (3%)	<0.001
Missing	0 (0%)	0 (0%)	
<b>Lymph node metastasis</b>			
N0	6 (28%)	40 (66%)	
N1	10 (48%)	18 (29%)	
N2	3 (14%)	1 (2%)	
N3	2 (10%)	2 (3%)	0.009
Missing	0 (0%)	0 (0%)	
<b>Distant metastases</b>			
M0	12 (57%)	53 (87%)	
M1	9 (43%)	8 (13%)	0.006
Missing	0 (0%)	0 (0%)	
<b>Sex</b>			
Male	10 (53%)	28 (47%)	
Female	11 (47%)	33 (53%)	0.146
Missing	0 (0%)	0 (0%)	
<b>Histological grade</b>			
1	0 (0%)	3 (5%)	
2	15 (71%)	45 (74%)	
3	6 (29%)	13 (21%)	0.497
Missing	0 (0%)	0 (0%)	
<b>Ki67</b>			
Low	7 (50%)	19 (46%)	
High	7 (50%)	22 (54%)	0.528
Missing	7 (33%)	20 (33%)	

for nuclear GSK-3β and for membranous β-catenin. As shown in Figure 1, nuclear expression of GSK-3β protein was associated with shorter OS by log-rank test ( $p = 0.008$ ). For membranous β-catenin, the effect was the opposite, with lack of β-catenin expression at the membrane being associated with improved survival ( $p = 0.016$ ). A univariate analysis according to the Cox proportional hazard regression model confirmed these results. Nuclear expression of GSK-3β in the primary tumor was associated with an increased risk of death (HR: 2.108; 95% confidence interval [CI]: 1.192–3.727).



Table 2. Overall survival according to GSK-3 $\beta$  and  $\beta$ -catenin subcellular localization<sup>1</sup>

Variable	Univariate analysis			Multivariate analysis <sup>2</sup>		
	HR	95% CI	p	HR	95% CI	p-Value
Nuclear GSK-3 $\beta$ + no membrane $\beta$ -catenin	3.29	1.759–6.154	<0.001	1.989	1.016–3.894	0.045
Yes versus no						
Grade	2.374	1.317–4.277	0.004	1.969	0.948–4.091	0.069
High versus low						
Ki67	1.114	0.583–2.127	0.745			
High versus low						
Sex	0.752	0.428–1.321	0.321			
Male versus Female						
Tumor size	4.902	1.188–20.226	0.028	1.773	0.409–7.684	0.444
≤T2 versus >T2						
Lymph node status	3.838	2.095–7.031	<0.001	2.049	0.959–4.376	0.064
≥N1 versus N0						
Distant metastasis	7.741	3.899–15.367	<0.001	4.914	2.239–10.781	<0.001
M1 versus M0						

<sup>1</sup>Cox uni- and multivariate analysis of overall survival in patients with tumors expressing nuclear GSK-3 $\beta$  but no membranous  $\beta$ -catenin compared to the whole cohort.

<sup>2</sup>Multivariate analysis performed only for variables significant in the univariate analysis.

Membranous expression of  $\beta$ -catenin on the other hand was associated with lower risk (HR: 0.483; 95% CI: 0.263–0.889). As expected, larger tumor size, lymph node involvement and distant metastasis at the time of primary surgery were also associated with poorer survival.

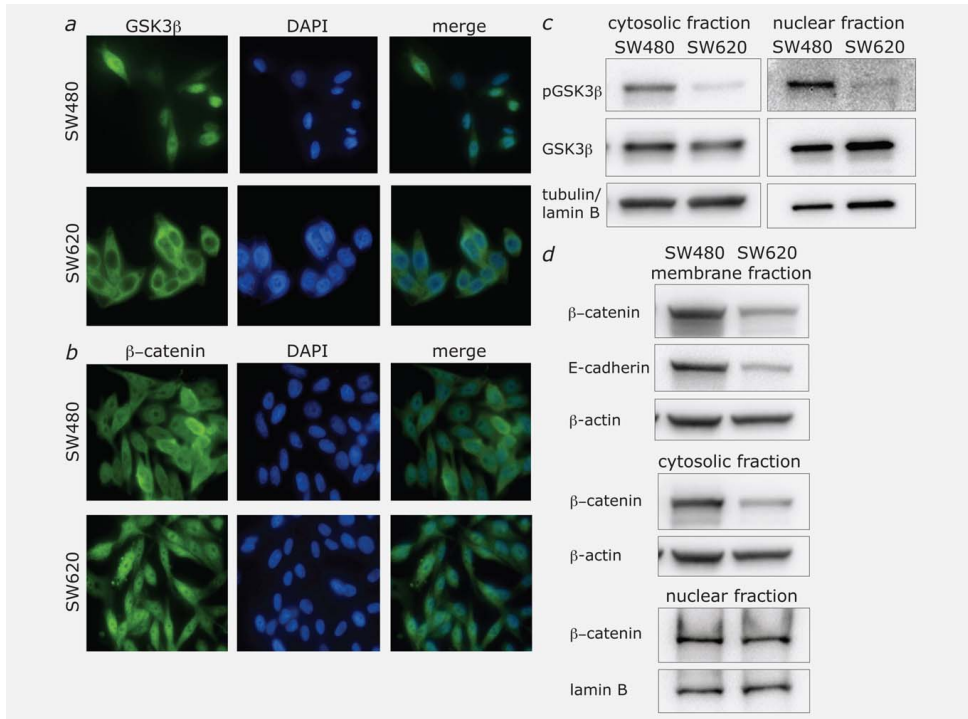
#### Lack of membranous $\beta$ -catenin in combination with nuclear GSK-3 $\beta$ expression is associated with poor prognosis

We found a strong correlation between nuclear expression of GSK-3 $\beta$  and lack of membranous  $\beta$ -catenin ( $p = 0.007$ ). Therefore, we next investigated the effect of this protein combination on survival. In a univariate analysis, the risk of death was significantly increased (HR: 3.290; CI: 1.759–6.154) (Table 2 and Fig. 1). A multivariate analysis confirmed that nuclear GSK-3 $\beta$  in combination with lack of membrane  $\beta$ -catenin in the primary tumor is an independent factor associated with poor prognosis in colon cancer (HR: 1.989; CI: 1.016–3.894) (Table 2).

#### Differences in subcellular localization of GSK-3 $\beta$ and $\beta$ -catenin in primary tumors versus metastases

In clinical samples, expressions of certain proteins have been shown to vary between primary tumors and metastases. An immunohistochemical study of primary colorectal tumors and the corresponding liver metastases demonstrated loss of membranous  $\beta$ -catenin expression in 26% of primary tumors and 60% of liver metastases. No correlation was found between  $\beta$ -catenin expression in primary tumors and metastases from the same patient.<sup>18</sup> Our aim was to investigate the expression of GSK-3 $\beta$  and  $\beta$ -catenin in metastasis from colon cancer. First, we evaluated the expression of GSK-3 $\beta$  and  $\beta$ -catenin in cul-

tured colon cancer cells. To this end, we used the SW480 and SW620 cells, two cell lines derived from a primary Duke B colon cancer and a lymph node metastasis from the same patient. Although the SW480 cell line was established from the primary tumor in the colon, the SW620 cell line was isolated a year later, from a metastatic lymph node in the same patient when he experienced a massive intra-abdominal tumor recurrence.<sup>19</sup> Recently, SW480 and SW620 cells were analyzed using the iTRAQ approach and  $\beta$ -catenin levels were found to be decreased in the metastatic cells; the authors did, however, not further investigate cellular sublocalization of the protein.<sup>20</sup> We investigated untreated cells for their expression and localization of GSK-3 $\beta$  and  $\beta$ -catenin by immunofluorescence and found that SW480 colon cancer cells expressed GSK-3 $\beta$  in the cytosol as well as in the nucleus, whereas the metastasis-derived SW620 cells expressed GSK-3 $\beta$  in the cytosol and at the nuclear membrane (Fig. 2a). For  $\beta$ -catenin, SW480 colon cancer cells express the protein in the nucleus and the cytosol and at the cell membrane. In the metastatic cells, nuclear expression predominated with lower membrane as well as cytosolic expression (Fig. 2b). To confirm our results, we also evaluated the expression of the above-mentioned proteins by Western blot. To this end, nuclear, membrane and cytosolic fractions were isolated from SW480 and SW620 cells. Protein levels of GSK-3 $\beta$  did not differ between primary tumor cells and metastases but the primary tumor cells exhibit significantly higher levels of inactive, phosphorylated GSK-3 $\beta$  in the cytosol as well as in the nucleus (Fig. 2c). For  $\beta$ -catenin, both membranous cytosolic and nuclear protein levels were higher in primary tumor cells compared to the metastatic ones (Fig. 2d). This is in contrast to some previous studies where no differences between subcellular



**Figure 2.** Subcellular expression of GSK-3 $\beta$ , serine-9 phosphorylated GSK-3 $\beta$  and  $\beta$ -catenin in SW480 and SW620 cells assessed by immunofluorescence and Western blotting. Immunofluorescence for (a) GSK-3 $\beta$  and (b)  $\beta$ -catenin, in SW480 primary colon tumor cells and SW620 distant metastasis cells from the same patient. Western blots for (c) GSK-3 $\beta$  and serine-9 phosphorylated GSK-3 $\beta$  in cytoplasmic and nuclear fractions, and for (d) E-cadherin and  $\beta$ -catenin in membrane fractions and  $\beta$ -catenin in cytoplasmic and nuclear fractions of untreated SW480 and SW620 cells.  $\beta$ -actin and lamin B or  $\alpha$ -tubulin was used as loading control.

localization of  $\beta$ -catenin in primary tumors and metastases were found.<sup>21</sup> Expression of E-cadherin at the membrane correlated well with that of  $\beta$ -catenin, with metastatic cells expressing very low levels of E-cadherin, confirming, as expected, the predominantly mesenchymal phenotype of those cells.

To evaluate whether those results could be translated into a clinical setting, we retrieved FFPE samples from distant metastases from patients who already had their primary tumors evaluated for the expression of GSK-3 $\beta$  and  $\beta$ -catenin. A total of 22% (18/85) patients had distant metastases already at the time of primary surgery. For seven patients, FFPE samples from distant lung or liver metastases were available. Tumor samples were stained for GSK-3 $\beta$  and  $\beta$ -catenin and the subcellular protein expressions were evaluated and compared to those of the corresponding primary tumor. As shown in Figure 3, expression and localization of GSK-3 $\beta$  and  $\beta$ -catenin in metastases did not always correspond to that of the primary tumor (Fig. 3a). For GSK-3 $\beta$ , nuclear expression was lost in two

cases, whereas for membrane  $\beta$ -catenin there was a trend toward re-expression of the protein in metastases (Fig. 3b). The apparent discrepancies between primary tumor/matched metastasis in cell lines and clinical samples could be owing to the fact that the cell line metastasis investigated is derived from a lymph node, whereas the clinical metastases samples were distant liver metastases. In contrast to metastases located in the liver, lymph node metastases represent a “in transit” tumor with a retained propensity for migratory behavior. Hence, it is not unlikely that SW620 cells represent tumor cells that have undergone EMT, whereas the clinical liver metastases have reverted back to an epithelial phenotype, and hence their re-expression of epithelial markers.

#### Differences in migration rates between primary tumor cells and metastases

In order for a tumor cell to become metastatic, it needs to acquire the ability to invade. The capacity to migrate is

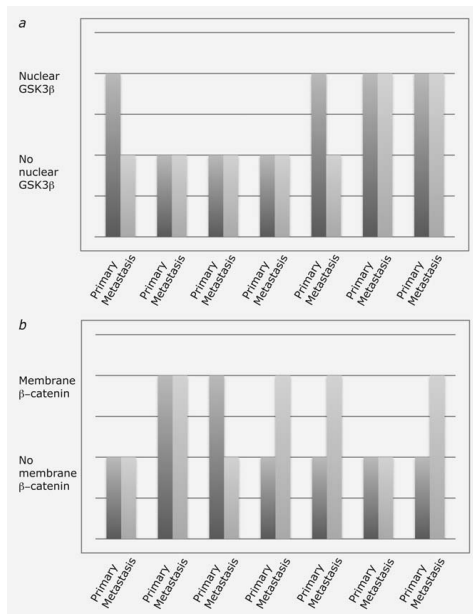


Figure 3. Graphs showing expression of (a) nuclear GSK-3 $\beta$ , and (b) membrane  $\beta$ -catenin, in seven primary tumors and corresponding distant metastases from the same patient. Subcellular expression of the proteins was evaluated by immunohistochemical staining and scored by two independent observers.

required for this initial step toward the establishment of metastatic growth.<sup>22</sup> We evaluated the migratory capacity of SW480 and SW620 cells and found that the metastatic cell line exhibited a significantly faster migratory rate (Fig. 4).

## Discussion

The aim of our study was to examine the role of nuclear expression of GSK-3 $\beta$  in colon cancer. Inactivation of the APC gene or activating mutations of  $\beta$ -catenin is reported in virtually all patients presenting with CRC and is believed to be the critical initiating step in malignant transformation.<sup>4,23</sup> Interestingly, however, although most colon cancers have constitutively activating mutations of the Wnt pathway, such tumors often still reveal a certain degree of regulation of the pathway.<sup>23</sup> In addition to its role in targeting  $\beta$ -catenin for degradation and hence counteracting tumor progression induced by increase transcription of TCF/LEF target genes, GSK-3 $\beta$  has been demonstrated to have an oncogenic role in colon cancer.<sup>9,24</sup> GSK-3 $\beta$  expression levels and kinase activities were markedly and significantly increased in colorectal adenocarcinomas and correlated with increased expression of  $\beta$ -catenin in the nucleus and cytoplasm of colorectal tumors.<sup>11</sup> Downregulation of GSK-3 $\beta$  by siRNA induced tumor cell apoptosis.<sup>12</sup> Moreover, experimental studies

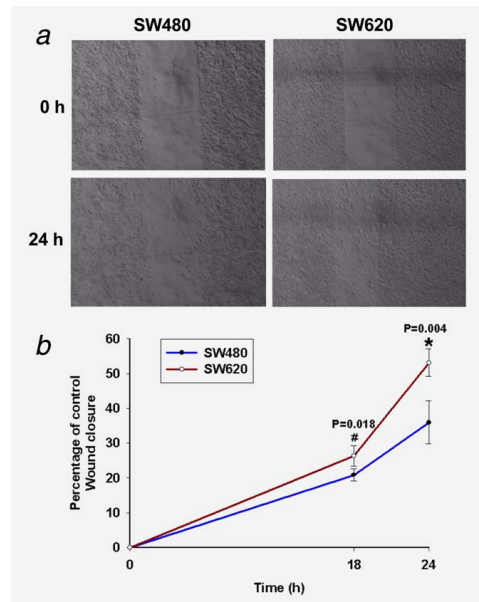


Figure 4. Wound-healing assay comparing migration rates in SW480 and SW620 cells. A scratch was made in a confluent layer of cells. (a) Images of wound closure at 0 and 24 hr in SW480 and SW620 cells. (b) Graph showing the differential migration capacity of the two cell lines. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

suggest that inhibition of GSK-3 $\beta$  activity enhances the  $\beta$ -catenin/E-cadherin-mediated adhesion, presumably through increased expression of  $\beta$ -catenin at the membrane.<sup>25</sup> On the other hand, nuclear GSK-3 $\beta$  has been shown to participate in the degradation of  $\beta$ -catenin and hence, in all logic, rather should have a tumor suppressive role.<sup>8</sup> In cells,  $\beta$ -catenin has both a signaling function, conferred by a soluble cytoplasmic pool that is unstable in the absence of a Wnt signal, and an adhesion function based on a cadherin-bound, stable pool of  $\beta$ -catenin at the membrane. EMT of tumor cells results in the redistribution of  $\beta$ -catenin from the membrane to the cytoplasm.<sup>26</sup> In line with this, loss of membrane  $\beta$ -catenin has been shown to correlate with poor prognosis in CRC.<sup>27</sup> Our results corroborate those results. The strong association with nuclear GSK-3 $\beta$  was, however, somewhat unexpected, given that GSK-3 $\beta$  is known to negatively regulate  $\beta$ -catenin levels in colon cancer and that an experimental study showed that GSK-3 $\beta$  can enter the nucleus and form complexes with  $\beta$ -catenin, leading to decreased transcription of TCF/LEF target genes.<sup>8</sup> A recent publication, however, demonstrated increased levels of functional GSK-3 $\beta$  in CRC, suggesting that GSK-3 $\beta$  can have a tumor-promoting function.<sup>11</sup> In bladder cancer, nuclear accumulation of GSK-3 $\beta$  was associated with metastasis and worse

outcome.<sup>10</sup> It is possible that GSK-3 $\beta$  promotes tumor cell survival as inhibition of GSK-3 $\beta$  activity leads to apoptosis and when GSK-3 $\beta$  was depleted through a siRNA approach, cell viability was reduced<sup>10</sup>. It is not unlikely that nuclear translocation of GSK-3 $\beta$  has a similar function in colon cancer.

Our data indicate that although nuclear GSK-3 $\beta$  is expressed in primary tumors as well as metastases, a larger proportion of the protein is phosphorylated and hence inactive in the primary tumors. Assuming that the function of GSK-3 $\beta$  in the nucleus is equal to that of its cytoplasmic counterpart, this finding offers a clue to the mechanism behind an increased propensity for metastases in nuclear GSK-3 $\beta$  expressing tumors. Moreover, we found that tumor cells with high expression of active GSK-3 $\beta$  in the nucleus in combination with low levels of membranous  $\beta$ -catenin migrated significantly faster compared to their low-active GSK-3 $\beta$ /high-membrane  $\beta$ -catenin counterparts.

Our patient data suggest that analysis of the combination of nuclear GSK-3 $\beta$  expression and lack of membrane  $\beta$ -cate-

nin expression in the primary tumor may be useful to predict prognosis in colon cancer. We performed our study on a small number of patients but nonetheless found that loss of  $\beta$ -catenin expression at the membrane together with nuclear expression of GSK-3 $\beta$  was independently associated with poor prognosis in colon cancer.

Taken together, our results indicate that nuclear GSK-3 $\beta$  could be a potential new target in the development of therapies for CRC. Hence, we believe that our results are very promising and will aim at validating our findings in a larger study.

### Acknowledgements

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# Paper II



# **The Inflammatory Mediator Leukotriene D<sub>4</sub> Induces Subcellular β-catenin Translocation and Cellular Migration in HCT116 Colon Cancer Cells**

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**Running title:** LTD<sub>4</sub> induces β-catenin signaling

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## Abstract

**Background:** Abnormal activation of the Wnt/ $\beta$ -catenin pathway frequently occurs in colorectal cancer. Nuclear translocation of  $\beta$ -catenin leads to activation of TCF/LEF transcription factors and transcription of target genes encoding effectors for cell proliferation, survival, and invasion.  $\beta$ -catenin is also an essential component of the cadherin-based adherent junction complex, changes in which affect cell adhesion and migration. The pro-inflammatory mediator leukotriene D<sub>4</sub> (LTD<sub>4</sub>) exerts its effects on intestinal epithelial cells through the CysLT<sub>1</sub> receptor. We previously showed upregulation of CysLT<sub>1</sub>R in colon tumors, suggesting the importance of leukotrienes in colon cancer. The aim of this study was to investigate the impact of LTD<sub>4</sub> on Wnt/ $\beta$ -catenin signaling and its effects on proliferation and migration of colon cancer cells.

**Methods:** The effects of LTD<sub>4</sub> on  $\beta$ -catenin subcellular localization in human colon cancer cells were evaluated by cell fractionation and Western blot analysis to examine altered protein expression and translocation. Confocal microscopy was used to investigate subcellular localization and protein phosphorylation. Transcriptional effects of LTD<sub>4</sub> and inhibitors were assessed with the TOPflash/FOPflash luciferase reporter assay. The effect of target protein on cell proliferation and migration was determined through proliferation and wound healing assays.

**Results:** Stimulation of HCT116 colon cancer cells with LTD<sub>4</sub> led to an increase in  $\beta$ -catenin expression and nuclear translocation and subsequent transcription of *c-myc* and *cyclin D1*. Furthermore, LTD<sub>4</sub> significantly reduced expression of E-cadherin and  $\beta$ -catenin at the plasma membrane and increased migration and proliferation of HCT116 cells. The effects of LTD<sub>4</sub> were blocked by inhibition of CysLT<sub>1</sub>R and by inhibition of glycogen synthase kinase 3 (GSK)-3 $\beta$  activity, indicating that crosstalk between the G-protein-coupled receptor CysLT<sub>1</sub> and the Wnt/ $\beta$ -catenin pathway occurs upstream of GSK-3 $\beta$ . In contrast, LTD<sub>4</sub>

stimulation of *APC*-mutant HT29 colon cancer cells had a very modest effect on  $\beta$ -catenin levels and did not induce further transcription of  $\beta$ -catenin target genes.

**Conclusion:** LTD<sub>4</sub> induces  $\beta$ -catenin translocation and activation of  $\beta$ -catenin target genes, resulting in increased proliferation and migration of HCT116 colon cancer cells. These results suggest a direct effect of inflammatory mediators on tumor cells via the Wnt/ $\beta$ -catenin pathway.

**Keywords:** LTD<sub>4</sub>, inflammation, colon cancer,  $\beta$ -catenin, cell migration

## Background

Colorectal cancer is one of the most common cancers worldwide and the second leading cause of cancer-related death [1]. Loss of function of the adenomatous polyposis coli (*APC*) gene occurs in 85% of all sporadic and hereditary colorectal tumors [2]. Among the 15% of colorectal cancers that retain wild-type *APC*, point mutations have been identified in one of the four serine/threonine residues in the N terminus of  $\beta$ -catenin, a putative target of glycogen synthase kinase (GSK)-3 $\beta$  [3].

*APC* is a tumor suppressor involved in familial adenomatous polyposis and sporadic colorectal tumors. *APC* mutations occur early during the development of colorectal cancer, suggesting that disruption of *APC* function is a key event in the initiation of colorectal cancer [4].  $\beta$ -catenin is a multi-functional protein that maintains cell to cell adhesions by forming a complex with E-cadherin and mediates Wnt/ $\beta$ -catenin signaling, thus playing an important role in embryogenesis and malignant transformation of cells [5]. E-cadherin interacts with intracellular  $\alpha$ -,  $\beta$ -, and  $\gamma$ -catenin to form a complex that mediates linkage to the actin cytoskeleton [6]. Disruption of this complex and the resulting dissociation of tumor cells is an important step in invasion and metastasis [7]. GSK-3 is a multifunctional serine/threonine kinase involved in the regulation of cell fate, protein synthesis, glycogen metabolism, cell mobility, proliferation, and survival. There are two mammalian GSK-3 isoforms, GSK-3 $\alpha$  and GSK-3 $\beta$ . The Wnt/ $\beta$ -catenin pathway is one of the key signaling pathways controlling cell proliferation, differentiation, and morphogenesis during development and in adulthood. Wnt signaling is transduced through either a  $\beta$ -catenin–dependent pathway (the canonical pathway) or a  $\beta$ -catenin–independent pathway (non-canonical Wnt signaling) [8, 9]. In the canonical Wnt pathway,  $\beta$ -catenin acts as a key transcriptional co-activator and transmits extracellular signals for activation of target genes such as *c-myc* and *cyclin D1*. In the absence of Wnt,  $\beta$ -catenin is phosphorylated by a complex of Axin, *APC*, and GSK-3 $\beta$  and

subsequently targeted for ubiquitin-dependent degradation. Binding of Wnt ligand to the Frizzled receptor and the co-receptor LRP5/6 leads to GSK-3 $\beta$ - or CK1-mediated phosphorylation of the intracellular region of LRP5/6, which prevents Axin from forming a complex and results in dissociation of  $\beta$ -catenin in a Dvl (Dishevelled)-dependent manner [9]. This leads to cytosolic accumulation of  $\beta$ -catenin, which subsequently enters the nucleus and binds to TCF/LEF transcription factors, thereby activating target genes [8, 10]. Abnormal activation of the Wnt/ $\beta$ -catenin pathway is a frequent early event in intestinal epithelial cells during cancer development [11].

The role of inflammation in tumorigenesis has become generally accepted, and it is now evident that an inflammatory microenvironment is an essential component of most tumors [12, 13]. The pro-inflammatory mediators leukotriene (LT) C<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> are termed cysteinyl leukotrienes (CysLTs) due to the presence of the amino acid cysteine and are derived from arachidonic acid through activation of the 5-lipoxygenase (5-LO) pathway [14]. The biological effects of CysLTs are mediated through their binding to two different cell surface receptors, CysLT<sub>1</sub>R and CysLT<sub>2</sub>R [15-17]. Both belong to the family of G-protein-coupled receptors (GPCRs) and their role in inflammatory processes and allergies has been well characterized [18, 19]. Of the two, CysLT<sub>1</sub>R, has been demonstrated to have a higher affinity for LTD<sub>4</sub>. The connection between chronic inflammation and cancer is believed to at least partially occur through infiltration of the tumor microenvironment with inflammatory cells that release different pro-inflammatory mediators such as leukotrienes and prostaglandins [12, 20]. It has been shown that proinflammatory mediators may contribute to the development of esophageal [21] and colorectal cancer, and previous findings in our laboratory indicate that LTD<sub>4</sub> can increase survival of mammalian intestinal epithelial cells by activation of its receptor CysLT<sub>1</sub>R [22, 23]. The mechanism by which inflammation induces neoplastic transformation and progression has not been completely resolved but a strong

association exists between cancer and inflammatory bowel disease [24], which is characterized by tissue injury caused by infiltration of inflammatory leukocytes in the intestinal wall [16]. There is therefore evidence that inflammation has a role in the development of colorectal cancer but the potential role of CysLTs in the progression of already established cancerous growth is less well studied.

To investigate the effect of LTD<sub>4</sub> on  $\beta$ -catenin signaling in colon cancer we used two human colon cancer cell lines with different mutational status. HCT116 is a highly aggressive human colon adenocarcinoma cell line with a mutation of serine at codon 45 of the  $\beta$ -catenin gene. This is one of the four serine/threonine residues that are phosphorylated by GSK-3 $\beta$ , and mutations that prevent their phosphorylation by GSK-3 $\beta$  inhibit subsequent proteosomal degradation of  $\beta$ -catenin. HT29 human colon adenocarcinoma cells carry an *APC* mutation but are more well-differentiated and show a smaller tumorigenic effect in xenograft models than HCT116 [25]. Our findings support the notion that chronic inflammation plays a role in the progression of colorectal cancer by showing that the presence of pro-inflammatory mediators in the tumor microenvironment can induce  $\beta$ -catenin activation and thereby promote tumor cell proliferation and migration.

## Results

### **LTD<sub>4</sub>-induces an increase in $\beta$ -catenin protein levels in colon cancer cells**

In non-cancerous cells, GSK-3 $\beta$  phosphorylates  $\beta$ -catenin and thereby primes it for degradation. We previously showed that LTD<sub>4</sub> stimulation induces an increase in  $\beta$ -catenin protein levels in non-transformed intestinal epithelial cells [26]. Here, we investigated the effect of LTD<sub>4</sub> on  $\beta$ -catenin signaling in colon cancer cells with different mutations in the Wnt signaling pathway. Figure 1 shows the basal levels of  $\beta$ -catenin and CysLT<sub>1</sub>R in HCT116 and HT29 colon cancer cells. Interestingly, the more aggressive cell line HCT116 had higher levels of CysLT<sub>1</sub>R but similar levels of  $\beta$ -catenin to HT29 cells (Figure 1A). Stimulation with 80 nM LTD<sub>4</sub> or 10 mM LiCl (as a positive control) for 24 hrs significantly increased levels of  $\beta$ -catenin in both cell lines (Figure 1B and 1C). Interestingly, there was a significant reduction in  $\beta$ -catenin levels after LTD<sub>4</sub> stimulation in the membrane fraction of HCT116 cells, but not in HT29 colon cancer cells (Figure 1D and 1E).

### **LTD<sub>4</sub> induces subcellular translocation of $\beta$ -catenin in colon cancer cells**

Nuclear accumulation of  $\beta$ -catenin is a recognized characteristic associated with poor prognosis in colon cancer [27]. We therefore examined how the subcellular localization of  $\beta$ -catenin is affected by CysLT stimulation by isolating nuclear and cytoplasmic fractions from untreated or treated colon cancer cells. LTD<sub>4</sub> induced a significant increase in cytoplasmic  $\beta$ -catenin in both cell lines (Figure 2A and 2B). Furthermore, LTD<sub>4</sub> induced a significant nuclear translocation of  $\beta$ -catenin in HCT116 cells (Figure 2C) but not in HT29 cells (Figure 2D). We confirmed the LTD<sub>4</sub>-induced translocation of  $\beta$ -catenin in HCT116 cells by confocal laser scanning microscopy. In untreated control cells  $\beta$ -catenin was localized predominantly to the plasma membrane. After stimulation for 24 hrs, we observed a decrease in  $\beta$ -catenin at the plasma membrane of HCT116 cells (Figure 3A) whereas there was no change in  $\beta$ -catenin

levels in the membranes or nucleus of HT29 cells (data not shown). We next investigated  $\beta$ -catenin localization using an antibody for activated  $\beta$ -catenin (pS552- $\beta$ -catenin) that was previously used to show dual activation of  $\beta$ -catenin by AKT and Wnt signaling during inflammation [28]. We demonstrated a significant increase in pS552- $\beta$ -catenin in the nucleus of HCT116 cells after LTD<sub>4</sub> stimulation for 24 hrs (Figure 3B and 3C), one of the hallmarks of canonical Wnt/ $\beta$ -catenin signaling.

### **LTD<sub>4</sub> induces phosphorylation of GSK-3 $\beta$ in colon cancer cells**

GSK-3 $\beta$  is known to phosphorylate  $\beta$ -catenin, thereby inducing its proteosomal ubiquitination and degradation. The activity of GSK-3 $\beta$  is regulated by site-specific phosphorylation, with full kinase activity requiring phosphorylation at tyrosine (Tyr-216). In contrast, phosphorylation at serine (Ser-9) inhibits GSK-3 $\beta$  activity [29]. Since our data showed that LTD<sub>4</sub> can induce an increase in  $\beta$ -catenin protein levels in colon cancer cells (Figure 1B and 1C), we investigated whether this upregulation involves changes in phosphorylation, and thereby inactivation, of GSK-3 $\beta$ . Following LTD<sub>4</sub> stimulation, we detected a significant increase in cytosolic Ser-9 phosphorylation of GSK-3 $\beta$  in both cell lines, suggesting that LTD<sub>4</sub> mediates  $\beta$ -catenin stabilization and translocation via Ser-9 phosphorylation and inhibition of GSK-3 $\beta$  (Figure 4A and 4B). We also showed nuclear phosphorylation of GSK-3 $\beta$  after LTD<sub>4</sub> stimulation in HCT116 cells (Figure 4C) but not in HT29 cells (Figure 4D). This finding is consistent with the more pronounced effects of LTD<sub>4</sub> on  $\beta$ -catenin levels in HCT116 cells compared with HT29 cells. This increase in inactive GSK-3 $\beta$  after LTD<sub>4</sub> stimulation, especially in the nucleus, might further potentiate the oncogenic signaling by decreasing  $\beta$ -catenin degradation.

## **LTD<sub>4</sub> induces transcription of $\beta$ -catenin target genes and proliferation in HCT116 colon cancer cells**

Because the oncogenic effects induced by  $\beta$ -catenin signaling are mediated through its target genes we evaluated whether LTD<sub>4</sub> induces the expression of known  $\beta$ -catenin targets. After synchronization, cells were stimulated with LTD<sub>4</sub> and protein levels of c-Myc and cyclin D1 were analyzed. The basal levels of cyclin D1 and c-Myc were higher in HCT116 cells than in HT29 cells (Figure 5A), consistent with the fact that HCT116 is a more aggressive colon cancer cell line than HT29. LTD<sub>4</sub> stimulation induced a significant increase in c-Myc (Figure 5B) and cyclin D1 (Figure 5D) protein levels in HCT116, but there was no change in either c-Myc (Figure 5C) or cyclin D1 (Figure 5E) expression in HT29 cells. Furthermore, the LTD<sub>4</sub>-induced increase in  $\beta$ -catenin was prevented by pretreatment with the CysLT<sub>1</sub> receptor antagonist ZM198615 (Figure 5F). Stabilized “free”  $\beta$ -catenin translocates to the nucleus where it acts as a regulator of the TCF/LEF family of transcription factors and initiates transcription of Wnt target genes such as *c-myc* and *cyclin-D1* [30]. We have previously shown that leukotrienes can increase TCF/LEF transcriptional activity in intestinal epithelial cells [26]. We therefore investigated whether LTD<sub>4</sub> also induced  $\beta$ -catenin/TCF/LEF activity in HCT116 colon cancer cells. TCF transcription was significantly increased by LTD<sub>4</sub> and slightly decreased in the presence of CysLT<sub>1</sub>R antagonist (ZM198615) (Figure 5G). As a control we also used the selective GSK-3 $\beta$  inhibitor (CHIR-99021), which significantly increased TCF luciferase activity (Figure 5G). Given that induction of c-myc stimulates cell cycle entry of quiescent cells [31, 32], we further investigated whether LTD<sub>4</sub> increased the proliferation of colon cancer cells. Indeed, LTD<sub>4</sub> induced proliferation of HCT116 cells, which could be abolished by pretreatment of cells with the CysLT<sub>1</sub>R antagonist ZM198615 (Figure 5H).



**Effect of LTD<sub>4</sub> on the E-cadherin-catenin complex and migration of colon cancer cells**

An intact E-cadherin-catenin complex is required for maintenance of normal cell-cell adhesions and polarity [33]. E-cadherin and  $\beta$ -catenin levels at the plasma membrane are reduced in many poorly differentiated tumor cells that have lost cell-cell adhesion and show strong invasive behavior. A reduced level of E-cadherin correlates significantly with increased “free” cellular  $\beta$ -catenin, and abrogation of E-cadherin-mediated adhesion correlates with increased transcription of  $\beta$ -catenin target genes, a phenomenon associated with cancer progression and poor prognosis [34, 35]. We found that, in accordance with their levels of differentiation, HT29 cells expressed higher levels of E-cadherin than HCT116 cells (Figure 6A). We next investigated the effect of LTD<sub>4</sub> on the E-cadherin-catenin complex. Analysis of isolated membrane fractions from LTD<sub>4</sub>-stimulated cells revealed a significant reduction in E-cadherin levels in HCT116 cells (Figure 6B) but no significant change in HT29 cells (Figure 6C). This is interesting because cell migration is an essential component of cancer invasion and metastasis [35, 36] and our previous studies showed that LTD<sub>4</sub> induces migration of intestinal epithelial cells through activation of the PI3-Kinase/Rac cascade [37]. Stimulation with 100 nM LTD<sub>4</sub> or 2.5 nM CHIR-99021 for 24 hrs induced a significant increase in migration of HCT116 cells (Figure 6D). However, LTD<sub>4</sub> had no effect on migration of HT29 cells (data not shown). This could potentially be related to the high levels of E-cadherin (Figure 6A) and the weak effects of LTD<sub>4</sub> on  $\beta$ -catenin signaling in HT29 cells, which might reflect the lower expression of the CysLT<sub>1</sub> receptor.

## Discussion

Disruption of the Wnt/ $\beta$ -catenin signaling pathway is one of the hallmarks of colorectal cancer [38, 39]. Increased stabilization and accumulation of  $\beta$ -catenin can result from increased Wnt signaling and/or from mutations in  $\beta$ -catenin itself or in any of the Wnt-regulatory genes, such as *APC* [40]. We have recently shown that a lack of plasma membrane  $\beta$ -catenin and high levels of nuclear GSK-3 $\beta$  is associated with poor prognosis in patients with colorectal cancer [41].

The role of inflammatory mediators in the development and progression of colorectal cancer is well established [42]. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the long-term risk of cancer death [43]. Pro-inflammatory leukotrienes and prostaglandins are important mediators in the crosstalk between tumor epithelial cells and their surrounding stroma cells and play an important role in a tumor microenvironment with chronic inflammation [20]. One possible mechanism for a direct effect of NSAIDs on tumor cells is suppression of nuclear translocation of  $\beta$ -catenin, which can be induced in colon cancer cells by treatment with Ibuprofen [44]. In epithelial cells, activation of LTD<sub>4</sub>-CysLT<sub>1</sub>R signaling was shown to promote cell proliferation and survival through multiple pathways [22, 26, 45]. Furthermore, our previous findings showed that increased expression of CysLT<sub>1</sub>R in patients with colorectal cancer and inhibition of LTD<sub>4</sub> signaling by blocking the CysLT<sub>1</sub>R receptor induced apoptosis in cancer cells [46, 47].

In the present study, we investigated the effect of LTD<sub>4</sub> on the  $\beta$ -catenin signaling pathway and its role in the proliferation and migration of colon cancer cells. We compared HCT116 cells, a poorly differentiated colon adenocarcinoma cell line, and well-differentiated HT29 cells. Our data showed that LTD<sub>4</sub> signaling via CysLT<sub>1</sub>R induced translocation of  $\beta$ -catenin from the plasma membrane to the nucleus, where it can act on Wnt/ $\beta$ -catenin target genes such as *c-Myc* and *cyclin-D1* via binding to the TCF/LEF complex to initiate

transcription. Our results suggest that, in addition to their role in the tumor microenvironment, leukotrienes can directly increase proliferation and migration of HCT116 colon cancer cells through activation of the Wnt/ $\beta$ -catenin signaling pathway. This effect was not seen in HT29 cells, perhaps because HT29 cells harbor an *APC* mutation that results in an activated Wnt/ $\beta$ -catenin signaling pathway and any additional effect of LTD<sub>4</sub> on downstream events such as proliferation and migration cannot be detected. Another explanation could be that HT29 cells have much lower protein expression of the CysLT<sub>1</sub>R than HCT116 cells, which is logical as they are more differentiated. Furthermore, nuclear APC can oppose  $\beta$ -catenin-mediated transcription through nuclear sequestration of  $\beta$ -catenin from the TCF/LEF transcription complex, as well as through interaction of APC with the transcription co-repressor CtBP [48]. In mature non-cancerous cells, GSK-3 $\beta$  phosphorylates  $\beta$ -catenin in the cytoplasm and induces its degradation. GSK-3 $\beta$  can also enter the nucleus and form a complex with  $\beta$ -catenin, decreasing levels of  $\beta$ -catenin/TCF-dependent transcription through a mechanism thought to involve GSK-3 $\beta$ -Axin binding [49]. Our data show accumulation of nuclear  $\beta$ -catenin, together with a significant increase in inactive phospho-GSK-3 $\beta$  in both the cytosol and the nucleus, after stimulation of HCT116 colon cancer cells with LTD<sub>4</sub>. This suggests that leukotriene signaling both reduces GSK-3 $\beta$ -induced degradation of  $\beta$ -catenin in the cytosol and increases the amount of  $\beta$ -catenin in the nucleus that can interact with the TCF/LEF transcription machinery.

Disturbance of the E-cadherin-catenin adhesion complex is one of the main events in the early and late stages of cancer [7]. Inhibition of GSK-3 $\beta$  leads to upregulation of Snail followed by downregulation of E-cadherin, which also could lead to cytoplasmic mobilization of  $\beta$ -catenin [50]. Relatively little is known about the ability of leukotrienes to regulate tumor cell migration and invasion, although LTB<sub>4</sub> was shown to inhibit metastatic spread to the liver and other organs in an *in vivo* study of pancreatic cancer [51] and previous

results from our laboratory suggested that LTD<sub>4</sub> could induce migration of non-transformed intestinal epithelial cells through activation of PI3-Kinase/Akt signaling [37]. In addition, prostaglandins were shown to induce migration and invasion of colon carcinoma cells via epidermal growth factor receptor (EGFR)-PI3-Kinase/Akt [52]. Interestingly, in this study we observed a significant reduction in E-cadherin at the plasma membrane after CysLT<sub>1</sub>R activation and an even more pronounced effect with GSK-3 $\beta$  inhibitors as LiCl and CHIR-99021. A similar effect on wound healing/cell migration was observed, stressing the importance of phospho-GSK3 $\beta$ . Furthermore, we did not observe any crosstalk with the PI3-kinase pathway because inhibition of the PI3-kinase pathway with wortmannin did not inhibit the effects of LTD<sub>4</sub> in HCT116 cells (data not shown). This is in contrast to the results reported in non-transformed intestinal epithelial cells [37], and suggests that the effect of LTD<sub>4</sub> on  $\beta$ -catenin in colon cells occurs at the level of GSK-3 $\beta$ . This fits nicely with our recent finding that a lack of plasma membrane  $\beta$ -catenin and high levels of nuclear GSK-3 $\beta$  is associated with poor prognosis in patients with colorectal cancer [41].

## **Conclusions**

Our data suggest a direct effect of LTD<sub>4</sub>, a component of the tumor microenvironment, in regulating the degradation and relocalization of  $\beta$ -catenin in colon cancer cells via CysLT<sub>1</sub>R signaling. Furthermore, LTD<sub>4</sub> increases both proliferation and migration of tumor cells, probably via GSK-3 $\beta$ / $\beta$ -catenin signaling pathways. Finally, this study provides additional evidence linking inflammation to the progression of colon cancer.

## **Materials and Methods**

### ***Antibodies and reagents***

Antibodies used were as follows: mouse monoclonal antibody against  $\beta$ -catenin (dilution 1:500 for immunofluorescence and 1:1000 for Western blotting; BD Transduction Laboratories, Franklin Lakes, NJ, USA); rabbit phospho-specific pSer552- $\beta$ -catenin antibody [28], a gift from Dr Linheng Li (Kansas University Medical Center, Kansas City, KS); rabbit monoclonal antibody against total GSK-3 $\beta$  and phospho-GSK-3 $\beta$ -3 $\alpha$ / $\beta$  (Ser-21/9), (dilution 1:500, Cell Signaling Technology, Beverly, MA, USA); mouse monoclonal anti  $\alpha$ -tubulin and goat polyclonal anti-Lamin B antibodies (dilution 1:1000; Santa Cruz Biotechnology, Dallas, TX, USA); mouse anti-actin antibody (dilution 1:2000; Abcam Cambridge, MA, USA); rabbit polyclonal antibodies against c-Myc (dilution 1:1000), E-cadherin (dilution 1:500), and cyclin D1 (dilution 1:200) (Calbiochem). Secondary peroxidase-linked goat anti-rabbit, goat anti-mouse, and rabbit anti-goat antibodies (dilution 1:5000) and fluorescent mounting media were from DAKO (Glostrup, Denmark). The conjugated secondary antibody AlexaFluor 488 was from Molecular Probes (Eugene, OR, USA) and the enhanced chemiluminescence (ECL) reagents and Western blot detection reagents were from GE Healthcare Biosciences (Pittsburgh, PA, USA). LTD<sub>4</sub> and CHIR-99021 were from Cayman Chemical Company (Ann Arbor, MI, USA), and ZM198615 was a gift from B. Andersson, AstraZeneca (Lund, Sweden).

### ***Cell culture***

Human colorectal adenocarcinoma cell lines HCT116 (ATCC # CCL-247) and HT29 (ATCC # HTB-38) were grown in McCoy's 5A medium with glutamine and without sodium bicarbonate, supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were regularly

tested for the absence of mycoplasma. Cells were cultured as monolayers for 5 days to approximately 80% confluence before stimulation.

#### ***Preparation of whole cell lysates***

Cells were cultured for 5 days and serum-starved for 2 hrs before stimulation with 80 nM LTD<sub>4</sub> or 10 mM LiCl for 24 hrs in the absence of serum. Treatment was terminated by the addition of ice-cold buffer [53] supplemented with 1% Triton X-100. Cells were incubated on ice for 30 min, homogenized by 10 strokes with a Dounce homogenizer, and centrifuged for 10 min at  $200 \times g$  at 4°C. The supernatant was centrifuged for an additional 5 min at  $1000 \times g$  at 4°C and the resulting supernatant was taken as whole cell lysate.

#### ***Preparation of membrane and cytosol fractions***

Cells were washed with ice-cold phosphate-buffered saline (PBS), covered with buffer A [54], and incubated on ice for 30 min. Cells were homogenized with a Dounce homogenizer for 25 strokes and centrifuged for 10 min at  $500 \times g$ . The supernatant was centrifuged for 10 min at  $10,000 \times g$  and the resulting supernatant was separated into plasma membrane (pellet) and cytosol (supernatant) fractions by further centrifugation at  $200,000 \times g$  for 1 hr.

#### ***Preparation of nuclear fractions***

Nuclear extracts were isolated using a Nuclear Extraction Kit according to the manufacturer's instructions (Chemicon, Temecula, CA, USA).

#### ***Gel Electrophoresis and immunoblotting***

Samples were heated to 95°C in sample buffer and dithiothreitol for 10 min, loaded onto Mini-Protean TGX Precast gels (Bio-Rad, Hercules, CA, USA), and subjected to

electrophoresis. Separated proteins were transferred to polyvinylidene difluoride membranes using Trans-Blot Turbo Transfer Packs (Bio-Rad) and blocked with 3% bovine serum albumin in  $1 \times$  PBS for 1 hr at room temperature (RT) followed by overnight incubation with primary antibody at 4°C. After washing, the membrane was incubated with secondary antibodies at RT for 1 hr. The membrane was washed, processed with enhanced chemiluminescence (ECL), and exposed to hyperfilm-ECL to visualize immunoreactive protein. Densitometric analysis was performed with a Fluor-S quantitative imaging system (Bio-Rad).

### ***Immunofluorescence***

Cells were seeded on glass coverslips, grown for 3 days, and stimulated with or without 2.5  $\mu$ M CHIR-99021 and 100 nM LTD<sub>4</sub> every 6 hrs for 24 hrs in the absence of serum. The cells were washed several time with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100. After blocking with 3% goat serum in PBS for 45 min, cells were incubated with primary antibodies against  $\beta$ -catenin (1:500) and pS552- $\beta$ -catenin (1:200) in PBS with 1% goat serum at RT for 1 hr followed by incubation with secondary antibodies at 1:1000 dilution for 1 hr at RT. After washing with PBS, coverslips were mounted on glass slides with fluorescent mounting medium and confocal microscopy images were recorded using a Zeiss LSM 700 (Carl Zeiss Microscopy GmbH, Jena, Germany).

### ***Transfections and luciferase Assay***

Luciferase assays were performed with the Dual Luciferase Reporter Assay System from Promega (Madison, WI, USA). TOPFlash and FOPFlash vectors were used at a final concentration of 1  $\mu$ g/ml except for the control Renilla luciferase vector, which was used at

0.2 µg/ml to standardize transfection efficiency. Vector DNA was allowed to form complexes with Lipofectamine (Invitrogen, Carlsbad, CA, USA) (ratio 4:1). Cells were grown to 60% confluence in 12-well plates, washed once in medium lacking serum and antibiotics, and incubated with the DNA-Lipofectamine mixture at 37°C for 24 hrs. The medium was replaced with normal growth medium and cells were allowed to recover for 24 hrs before the addition of LTD<sub>4</sub> and/or inhibitors. When applicable, cells were pretreated with 20 µM ZM198,615 and/or 1.25 mM or 2.5 mM CHIR-99021 for 30 min before the addition of 100 nM LTD<sub>4</sub> every 12 hrs for 24 hrs. Cells were washed in PBS and lysed by addition of 250 µl/well DRL-passive lysis buffer included in the Dual Luciferase Reporter Assay System. Lysed samples were collected and briefly centrifuged at 1000 × g for 5 min to precipitate any debris. A 40-µl sample of each lysate was transferred to a luminometer test tube containing 50 µl Luciferase Assay buffer II, and the luciferase reaction was immediately read using a MiniLumat LB 9506 (Berthold Technologies GmbH, Düsseldorf, Germany). The control Renilla luciferase signal was recorded after subsequent addition of 50 µl Stop and Glow buffer, and the level of expression was given as a ratio relative to control. Triplicate samples were prepared and analyzed for each condition in every set of experiments.

### ***Wound healing assay***

HCT116 and HT29 cells were grown to confluence in 12-well plates and a wound was made in the monolayer using a pipette tip. Cells were serum-starved for 2 hrs before treatment with or without 100 nM LTD<sub>4</sub> or 2.5 mM CHIR-99021 for 24 hrs and allowed to migrate for 24 hrs at 37°C. Images of wound closure were captured at 0 and 24 hrs with a Nikon phase contrast (DS-Fi1) microscope (Nikon, Tokyo, Japan) using a 10× objective and NIS-Elements Basic Research software. The area of the wound was measured at baseline and after 24 hrs using Adobe Photoshop CS4 software.



### ***Proliferation assay***

HCT116 colon cancer cells were grown in 96-well plates for 24 hrs and pretreated with or without 20  $\mu$ M ZM198,615 for 30 min before stimulation with 100 nM LTD<sub>4</sub> every 24 hrs for 72 hrs. A WST-1 cell proliferation assay was performed following the manufacturer's protocol (Boehringer Mannheim GmbH, Mannheim, Germany).

### ***Authors Contributions***

Conceived and designed the experiments: TS, JSD, AS. Performed the experiments: TS.

Analyzed the data: TS, JSD, AS. Wrote the paper: TS, JSD, AS.

### ***Competing Interests***

The authors have declared that no competing interests exist.

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## Figure legends

### **Figure 1 The pro-inflammatory mediator LTD<sub>4</sub> increases $\beta$ -catenin levels in colon**

**cancer cells.** (A) Western blot analysis of expression of CysLT<sub>1</sub>R and  $\beta$ -catenin in HCT116 and HT29 colon cancer cells. (B, C) Western blot and densitometric analysis of LTD<sub>4</sub>-induced accumulation of  $\beta$ -catenin in whole cell lysates from HCT116 and HT29 cells. (D, E) Western blot and densitometric analysis of the effect of LTD<sub>4</sub> on  $\beta$ -catenin in membrane fractions from HCT116 and HT29 cells. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti- $\beta$ -catenin antibody and subsequently reprobed with an antibody against  $\beta$ -actin to ensure equal loading. Data are presented as the percent of untreated control cells and represent means  $\pm$  standard error of the mean (SEM) of at least three separate experiments. Statistical analysis was by unpaired Student's t-test (\* P<0.05, \*\* P<0.01).

### **Figure 2 Translocation of $\beta$ -catenin in colon cancer cells after LTD<sub>4</sub> stimulation.**

Western blot and densitometric analysis of LTD<sub>4</sub>-induced accumulation of  $\beta$ -catenin in the cytoplasm (A, B) and nucleus (C, D) of HCT116 and HT29 cells. Proteins were separated by SDS-PAGE and subjected to immunoblotting with anti- $\beta$ -catenin antibody. The membrane was reprobed with antibody against  $\beta$ -actin (cytoplasm) or Lamin B (nuclear fractions) to ensure equal loading. Data are presented as percent of untreated control cells and represent means  $\pm$  SEM of at least three separate experiments. Statistical analysis was by unpaired Student's t-test (\* P<0.05, \*\* P<0.01).

### **Figure 3 LTD<sub>4</sub>-induced translocation and serine-phosphorylation of $\beta$ -catenin in colon**

**cancer cells.** (A, B) Confocal microscopy immunofluorescence showing LTD<sub>4</sub>-induced translocation of (A)  $\beta$ -catenin from the plasma membrane and increased (B) nuclear levels of  $\beta$ -catenin (pS552- $\beta$ -cat) in HCT116 colon cancer cells. After stimulation every 6 hrs for 24

hrs, the cells were fixed, permeabilized, and stained with antibodies against  $\beta$ -catenin or pS552- $\beta$ -catenin. Left panels show primary antibody immunoreactivity, middle panels show nuclear staining with DAPI, and right panels show a merged image. (C) Graph showing the number of cells showing positivity for nuclear pS552- $\beta$ -catenin in the presence or absence of 100 nM LTD<sub>4</sub>, calculated as the number of positive cells per 100 cells. The data are given as means  $\pm$  SEM. Statistical significances were calculated using unpaired Student's t test, \* P<0.05.

**Figure 4 LTD<sub>4</sub> induces phosphorylation of GSK-3 $\beta$  in colon cancer cells.** (A, C)

HCT116 and (B, D) HT29 colon cancer cells were left untreated, or treated with 80 nM LTD<sub>4</sub> for 3 hrs or 10  $\mu$ M LiCl for 5 hrs. Cytoplasmic (A, B) and nuclear (C, D) fractions were assayed for phosphorylated GSK-3 $\beta$  using a phospho-specific antibody against Ser-9 GSK-3 $\beta$ . The membranes were stripped and reprobbed with antibodies against total GSK-3 $\beta$  and  $\alpha$ -tubulin, as a cytoplasmic control, or Lamin B, as a nuclear control, to ensure equal loading. Data are given as percent of untreated control cells and represent means  $\pm$  SEM of at least three separate experiments. Statistical analysis was by unpaired Student's t-test (\* P<0.05, \*\* P<0.01).

**Figure 5 LTD<sub>4</sub> induces transcription of  $\beta$ -catenin target genes and proliferation of**

**HCT116 colon cancer cells.** (A) Western blot analysis of the expression levels of c-Myc and cyclin D1 protein in whole cell lysates of HCT116 and HT29 cells. (B-E) LTD<sub>4</sub> induced increased expression of c-Myc and cyclin D1 in HCT116 (B, D) and HT29 (C, E) cells. Cells were synchronized overnight and subsequently stimulated with 80 nM LTD<sub>4</sub> or 10  $\mu$ M LiCl for 24 hrs in medium containing 10% serum. After probing with primary antibodies against cyclin D1 or c-Myc, the membranes were reprobbed for  $\beta$ -actin to ensure equal loading. (F) HCT116 cells were pretreated with 20  $\mu$ M ZM198,615 for 30 min before stimulation with

100 nM LTD<sub>4</sub> for 24 hrs. Western blot analysis was performed with anti-β-catenin antibody and reprobed with β-actin to ensure equal loading. (G) HCT116 cells were seeded in 12-well plates for 48 hrs and pretreated with or without 20 μM ZM198,615 or CHIR-99021 (1.25 mM or 2.5 mM) for 30 min before stimulation with 100 nM LTD<sub>4</sub>. Luciferase values were measured and standardized to Renilla values. Results are given as ratios between TOP/Flash reporter activity and inactive mutant FOP/Flash reporter activity. (H) LTD<sub>4</sub> induced proliferation in HCT116 cells. Cells were grown in 96-well plates for 24 hrs and pretreated with or without 20 μM ZM198,615 for 30 min before stimulation with 100 nM LTD<sub>4</sub> every 24 hrs for 72 hrs. Proliferation was measured using the WST-1 assay. Data are given as percent of untreated controls and represent means ± SEM of at least three separate experiments. Statistical analysis was by unpaired Student's t-test (\* P<0.05, \*\* P<0.01).

**Figure 6 Effect of LTD<sub>4</sub> on the E-cadherin-catenin complex and migration of colon cancer cells.**

(A) Western blot analysis of whole cell lysates of E-cadherin expression in HCT116 and HT29 cells. Membrane fractions were isolated from HCT116 (B) and HT29 (C) cells following stimulation for 24 hrs with 80 nM LTD<sub>4</sub> or 10 μM LiCl. Proteins were separated on a SDS-PAGE and immunoblotted with anti-E-cadherin antibodies with subsequent reprobing for β-actin as a loading control. (D) Wound healing assay. HCT116 cells were grown to a confluent monolayer in 12-well plates and a wound was inflicted with a pipette tip. Cells were grown for additional 24 hrs in the presence or absence of 100 nM LTD<sub>4</sub> or/and 2.5 μM CHIR-99021, or with 2.5 μM DMSO as vehicle control for CHIR-99021. The width of the wound was measured by phase-contrast microscopy. Data are shown as percent of untreated controls and represent means ± SEM of at least three separate experiments. Statistical analysis was by unpaired Student's t-test (\* P<0.05, \*\* P<0.01).



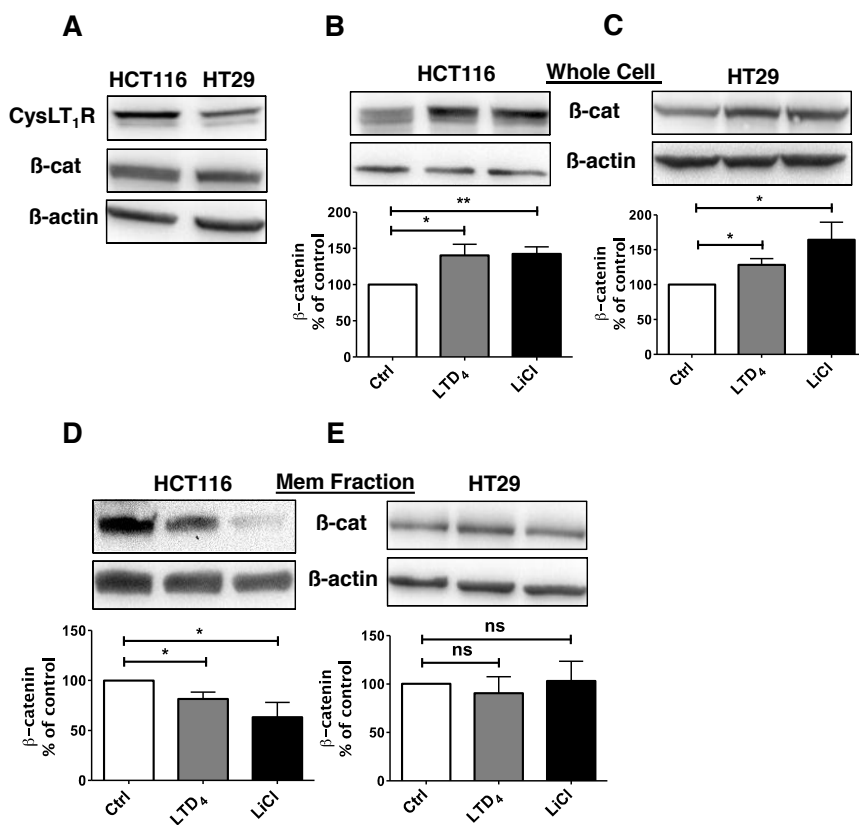


Figure 1

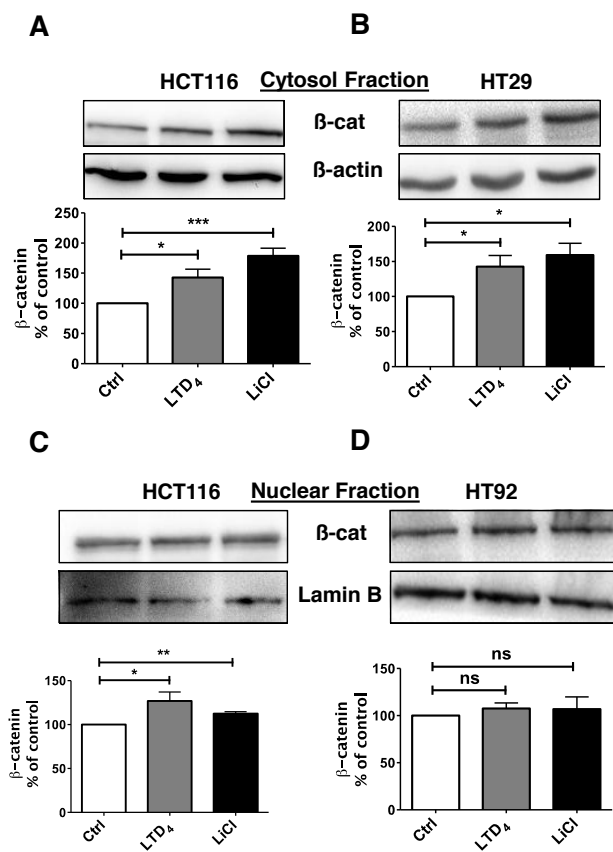


Figure 2

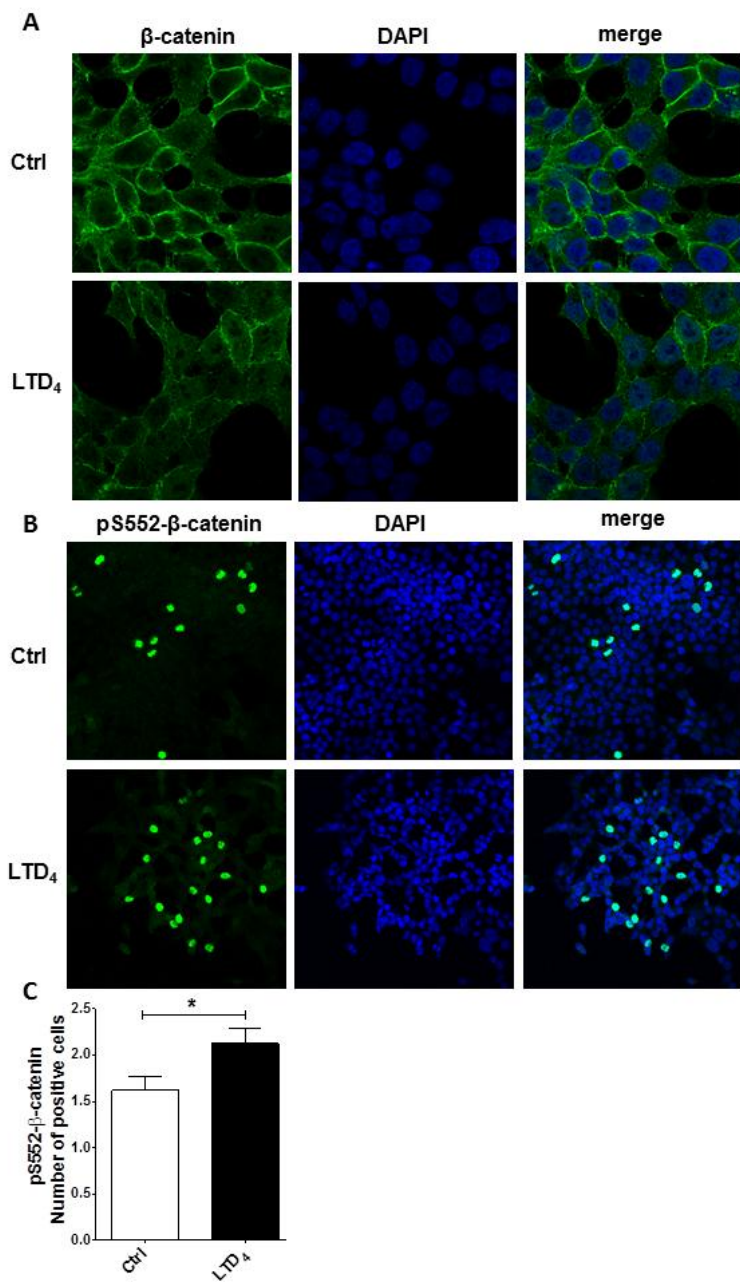


Figure 3

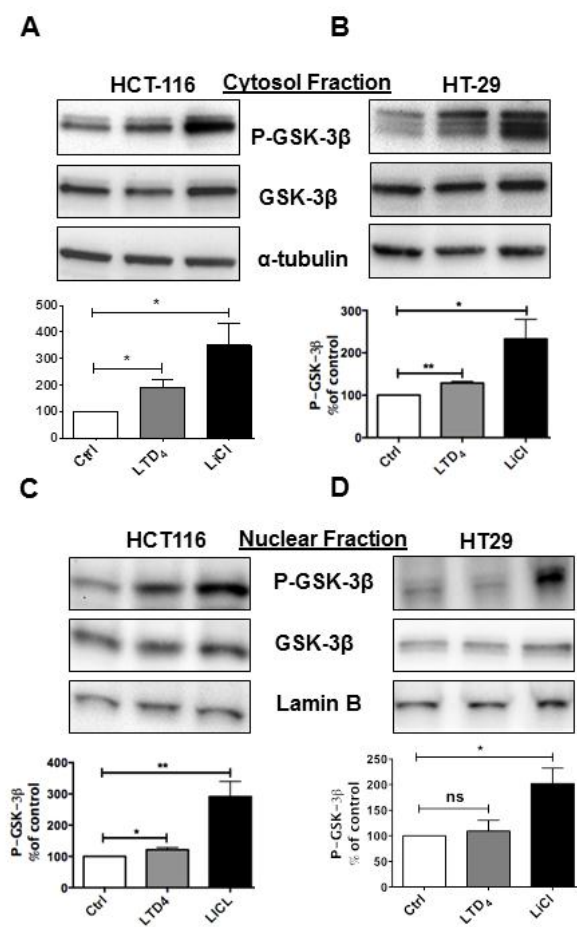


Figure 4

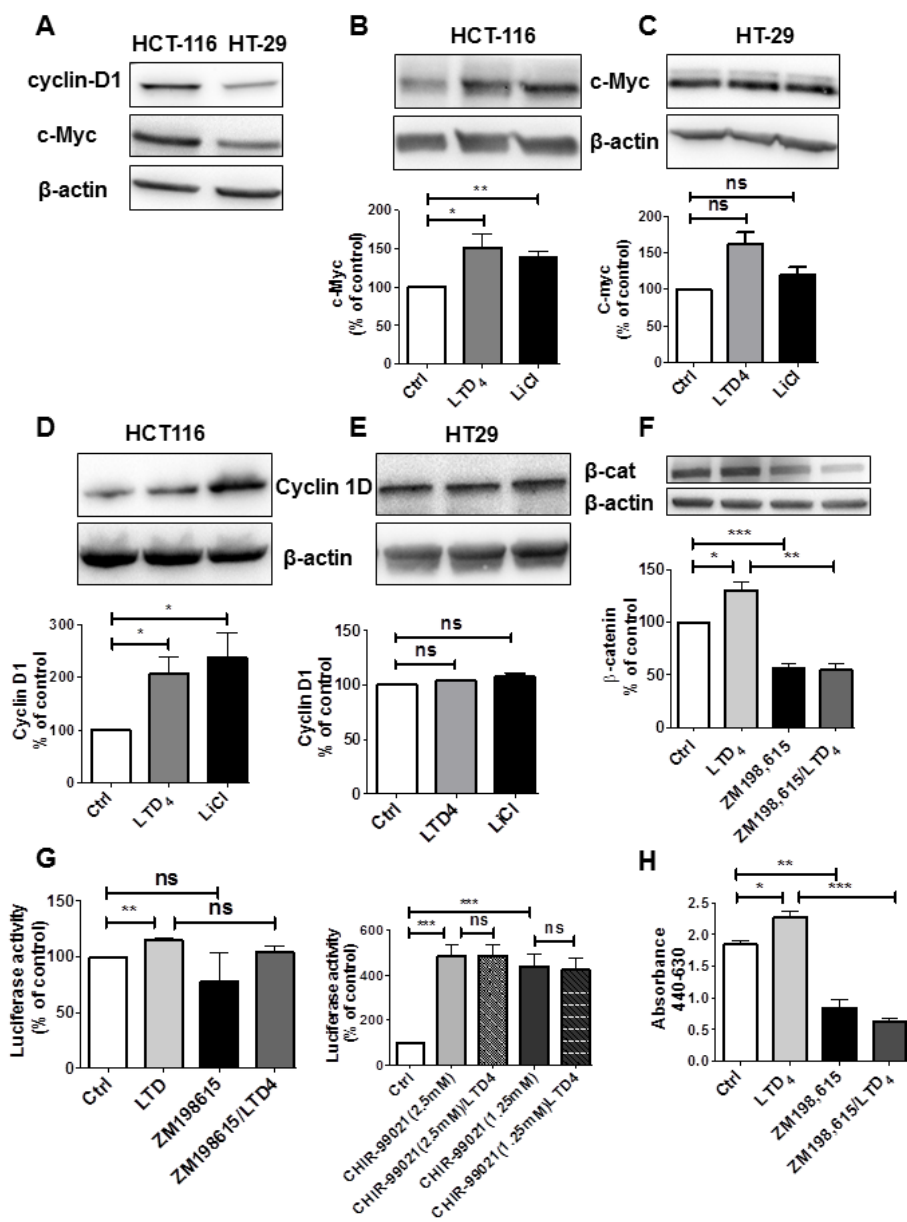


Figure 5

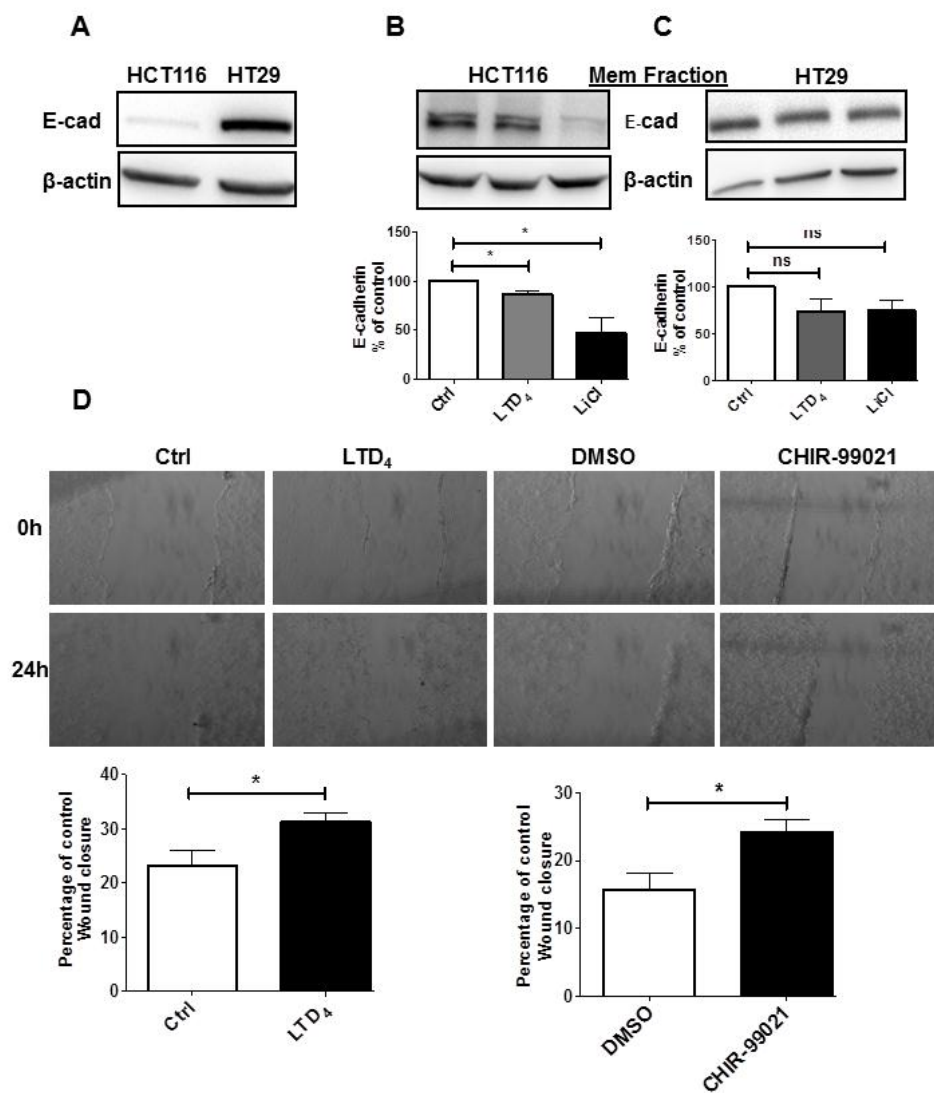


Figure 6



# Paper III





# **Pro-inflammatory mediator Leukotriene D<sub>4</sub> promotes a more invasive phenotype of SW480 and SW620 cancer cells**

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Running title: LTD<sub>4</sub> promotes cell migration

Keywords: E-cadherin,  $\beta$ -catenin, CysLT<sub>1</sub>R, GSK-3 $\beta$ , leukotriene D<sub>4</sub>, cell migration, MMP, invasion, colon cancer, metastasis

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## **Abstract**

We have recently shown that nuclear GSK-3 $\beta$  was significantly associated with poor prognosis in colon cancer. We have also shown that high expression levels of the pro-inflammatory receptor CysLT<sub>1</sub>R is associated with poor prognosis in colon cancer. Here we show those patients with membrane E-cadherin in combination with no nuclear GSK-3 $\beta$  have good prognosis. To study the mechanisms and impact of the inflammatory milieu on tumor invasion and metastasis, we used the ligand of CysLT<sub>1</sub>R called leukotriene D<sub>4</sub> (LTD<sub>4</sub>) on SW480 colon cancer cells and its metastatic counterpart SW620. Interestingly, we found that the pro-inflammatory mediator LTD<sub>4</sub> increased expression levels of vimentin and decreased membrane levels of E-cadherin only in SW480 cells. LTD<sub>4</sub> also induced nuclear translocation of active  $\beta$ -catenin as well as pre-nuclear accumulation of GSK-3 $\beta$  in these cells. Furthermore, LTD<sub>4</sub> induced a robust increase in MMP-2 and MMP-9 gene expressions while it induced a modest increase in MMP-7 gene expression in SW480 cells. In conclusion, our results indicate that LTD<sub>4</sub>, which can be released by macrophages in the tumor microenvironment, has the capacity to change cancer cells into a more invasive phenotype and thereby potentially contribute to increase tumor progression and metastasis.

## **Introduction**

Colorectal cancer is the third most commonly diagnosed cancer in the world, and the second leading cause of cancer related deaths around the world. Patients suffering from chronic inflammatory conditions like inflammatory bowel disease, Crohn's disease and ulcerative colitis, have an elevated risk of developing colon cancer<sup>1</sup>. In recent years, the properties of the tumor microenvironment have been highlighted as an important hallmarks of cancer<sup>2</sup>. In the tumor microenvironment, macrophages constitute the major inflammatory component of the stroma and affect many properties of the neoplastic tissue<sup>3</sup>. Studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) can prevent many different types of cancers, especially colorectal cancer by cyclooxygenase inhibition<sup>4</sup>. Cyclooxygenase (COX) and lipoxygenase (LO) are enzymes metabolised from arachidonic acid by two different pathways<sup>5</sup>. These enzymes catalyse important mediators involved in both acute and chronic inflammation and cancer<sup>5 6</sup>. Leukotrienes are metabolites from the 5-LO pathway and are released predominantly by inflammatory cells such as macrophages, mast cells, eosinophils and polymorphonuclear leukocytes<sup>5</sup>. Since the past few years a great deal of attention has been given to investigate the role of stromal microenvironment on colon cancer progression<sup>7</sup>. We have previously shown that the pro-inflammatory cysteinyl leukotriene D<sub>4</sub> (LTD<sub>4</sub>) has been implicated in colon cancer progression<sup>8</sup>, and that LTD<sub>4</sub> increases proliferation, migration, and survival of intestinal epithelial cells via CysLT<sub>1</sub> receptor (CysLT<sub>1</sub>R) signaling<sup>9, 10</sup>. Equally important are our results which show that colon cancer patients with high CysLT<sub>1</sub>R expression in the primary tumors have poor prognosis<sup>11 12</sup>.

It is well know that mutations in the adenomatous polyposis coli (APC) or other "gatekeeper gene" are frequent in colorectal cancer.

A complex of APC/GSK-3 $\beta$  participates in phosphorylation of  $\beta$ -catenin and ubiquitination by the proteasome<sup>13</sup>. In the absence of an intact APC/GSK-3 $\beta$  complex,  $\beta$ -catenin translocates to the nucleus where it activates the TCF/LEF transcription family, which in turn activates genes such as cyclin D1, c-myc and COX-2.  $\beta$ -catenin also exists in a complex with E-cadherin which is important for cell-cell contact and polarity<sup>14</sup>. Alteration of the E-cadherin/ $\beta$ -catenin complex increases cell motility and metastasis. The  $\beta$ -catenin target gene COX-2 is overexpressed in many colorectal tumours<sup>6</sup>. COX-2 has been shown to be a powerful colon cancer target. However, selective COX-2 inhibitors such as Rofecoxib would be of limited usefulness due to their side effects on cardiovascular functions. Therefore other targets need to be investigated. Our results indicated that pharmacological targeting of CysLT<sub>1</sub>R or its ligand LTD<sub>4</sub> by inhibiting 5-LO in colon cancer might be a good candidate for effective therapy. Our results shows that LTD<sub>4</sub> affects proteins which are important for epithelial to mesenchymal transition (EMT), cell migration and invasion.

## **Material and Methods**

### *Materials*

The rabbit phospho-specific  $\beta$ -cat-pS552 antibody<sup>15</sup> was a kind gift from Professor Linheng Li (Department of Pathology & Laboratory Medicine, University of Kansas School of Medicine, Kansas, USA) diluted 1:400 used for immunofluorescence and, rabbit monoclonal antibody against p-GSK-3 $\alpha/\beta$  (Ser21/9) antibody (1:200 for immunofluorescence) was from Cell Signaling Technology (Beverly, MA, USA). The mouse anti-actin (1:5000 for ?), rabbit anti-E-cadherin polyclonal antibodies (1:500 for Western blot) and mouse anti Vimentin (1:1000 for Western blot) were from Santa

Cruz Biotechnology (Dallas, TX, USA). Secondary peroxidase-linked, goat anti-rabbit, goat anti-mouse and rabbit anti-goat antibodies (1:5000) and fluorescence mounting media were from DAKO (Glostrup, Denmark). The conjugated secondary antibody Alexa-488 was from Molecular Probes (Eugene, OR, USA) and the enhanced chemiluminescence (ECL) reagents and Western blot detection reagents were from GE Healthcare Biosciences (Pittsburgh, PA, USA). LTD<sub>4</sub> was from Cayman Chemical. All other reagents were from Sigma Chemicals Co. (St. Louis, MO).

#### *Cell culture*

Human colorectal adenocarcinoma cell lines SW480 (ATCC CCL-228) and SW620 (ATCC CCL-227) were grown in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were regularly tested to ensure the absence of mycoplasma. For experiments, cells were used at a confluence of around 70%.

#### *Cell lysates and membrane fractions*

Cells were washed with ice cold PBS, covered with buffer A, kept on ice for 30 min, and homogenized with a Dounce homogenizer for 25 strokes and thereafter centrifuged for 10 minutes at 500 x g. The supernatant was centrifuged for 10 min at 10,000-x g and the resulting supernatant was separated into plasma membrane and cytosolic fractions by a 1 h centrifugation at 200,000 x g.

#### *Gel electrophoresis and immunoblotting*

To ensure equal loading, all samples were evaluated and compensated for protein concentration by the Coomassie blue protein assay. Proteins were denatured by boiling in sample buffer for 10 min. The samples were subjected to electrophoresis on 8% or 10% polyacrylamide gels in the presence of 10% SDS. The immunoblotting and developing were performed as described in<sup>16</sup>.

#### *Real-time PCR analysis*

The SW480 and SW620 cells were incubated either with 100 nM LTD<sub>4</sub> or left unstimulated (control) for 6 hr and then washed in PBS and immediately frozen at -80°C. Thereafter they were scraped in lysis buffer provided in the RNA isolation kit (RNeasy Plus mini kit, Qiagen Nordic, Sweden) and homogenized 10 times with a QIAshredder™. RNA was purified on RNeasy MinElute Spin Columns according to the manufacturer's instructions and eluted with RNase free H<sub>2</sub>O. cDNA synthesis was performed using RevertAid H Minus M-MuLV reverse transcriptase (Fermentas Life Sciences, USA). The mRNA changes in MMP-2, MMP-9, MMP-7 and the endogenous housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT-1) were quantified using real-time PCR analysis using the Maxima probe/ROX qPCR master mix (Thermo Scientific, USA) and TaqMan gene expression primers (Applied Biosystems, USA). The cDNA was mixed with 0.9 μM TaqMan primers and master mix and amplified at 60°C in an Mx3005P thermocycler (Stratagene, USA). The following TaqMan primers were used: MMP-2 (Hs00234422\_m1), MMP-7 (Hs01042795\_m1), MMP-9 (Hs00957562\_m1) and HPRT-1 (Hs99999909\_m1). The samples were analyzed and normalized against HPRT-1 using the Comparative 2<sup>- $\Delta\Delta C_t$</sup>  method of quantitation on the MxPro software (Stratagene, USA).

#### *3D Invasion Assay*

To assess if LTD<sub>4</sub> induced invasion in SW480 and SW620, an *in vitro* Boyden chamber assay using Matrigel™ was performed. This assay facilitates easier and controlled quantitation of invasion in 3D in order to mimic the *in vivo* tumor microenvironment. Invasion in 3D was carried out using an 8 μ-pore size polycarbonate membrane (Neuro Probe Inc., USA) in a Boyden chamber. The wells in the lower Boyden chamber were filled with either 400 μl serum-free medium (control) or serum-free medium containing 100 nM LTD<sub>4</sub> as appropriate and covered with the membrane very carefully to avoid air bubbles. The Boyden chamber was then completely assembled, and 100 μl of a 1:2 serum-free medium diluted Matrigel™ basement membrane matrix (BD Biosciences, USA) was coated into each well of the upper chamber. The Boyden chamber was incubated for 30-60 min at 37°C to allow the matrix to polymerize. The SW480 and SW620 cells were scraped and resuspended in serum-free media and 2.5x 10<sup>5</sup> cells were added into the matrigel-coated wells of the upper chamber of the Boyden chamber. 5% FCS was used as a positive control in the lower wells of the set-up. The set-up was placed in an incubator at 37°C and cells were then allowed to migrate for 12 hr. After the incubation time, non-migrated cells were carefully removed with a cotton swab, and the migrated cells situated on the lower side of the membranes were rinsed briefly in PBS followed by fixation with 4% paraformaldehyde. The membranes was then mounted on microscopic slides and were stained by DAPI for 10 min. Migrated cells were then counted using a fluorescent microscope (4 x10 magnification fields of view or FOV) and the mean number of migrated cells of each cell-line were then calculated. Data shown here is representative of 1 assay for both cell-lines.

*Wound healing assay*



SW480 and SW620 cells were grown to confluence and serum starved for 2 hr. A sterile pipette tip was used to inflict a scratch in the cell monolayer. Non-adherent cells were gently washed away with PBS. Cells were allowed to migrate for 18 hr at 37 °C with or without LTD<sub>4</sub> (100 nM) stimulation. Pictures were taken with a Nikon DS-Fi1 microscope using a 10x objective and NIS-Elements Basic Research software. The area of the wound closure was measured by the Adobe Photoshop CS4 software.

#### Patient samples

Archival formalin fixed and paraffin embedded primary colon tumors and control colon specimens from colon cancer patients who underwent surgery in 1990 were obtained from the bio bank of Malmö University Hospital. Tissues from 78 patients with varying grades and stages of the disease were included. Staging of tumors was done according to the Dukes' system of classification. Matched control samples of normal colon tissue were taken from the borders of the surgical specimens.

#### Immunofluorescence

SW480 and SW620 cancer cells were grown on coverslips for approximately three days, washed with PBS and fixed in 4% paraformaldehyde for 15 min, then washed with PBS and permeabilized in 0.1% Triton X-100 / PBS for five minutes. After repeated washing, cells were incubated at room temperature in 3% goat serum in PBS for 45 min, and thereafter for 1 hr with  $\beta$ -catenin-pS552 antibody (1:400) and phospho-GSK-3 $\alpha/\beta$  (Ser21/9) antibody (1:500) in PBS containing 1% goat serum. Coverslips were washed with 0.05% Tween / PBS and incubated at room temperature for 1 hr with goat anti-rabbit Alexa-488 secondary antibody (1:1000). Then after stained with DAPI for 3 min (1:1000) Coverslips were subsequently washed and

mounted in fluorescent mounting medium. Fluorescent images were taken using the Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

#### Statistical analyses

Statistical significance was determined by Student's t-test. Pearson's correlation test was used when comparing different sets of immunohistochemical stainings. SPSS software 16.0 (SPSS, Inc.) was used for patient material analysis. A p value less than 0.05 was considered to be significant.

### **Results and Discussion**

#### *Expression of E-cadherin, GSK-3 $\beta$ and CysLT<sub>1</sub>R in colon tumors*

We have previously reported that high levels of the pro-inflammatory receptor CysLT<sub>1</sub>R are associated with poor prognosis in colorectal cancer patients<sup>11, 17</sup>. In a recent study we showed that nuclear expression of GSK-3 $\beta$  and lack of membranous  $\beta$ -catenin also serve as markers for poor prognosis in colon cancer<sup>16</sup>. Here, we evaluated the role of E-cadherin, GSK-3 $\beta$  and CysLT<sub>1</sub>R expression in a colon cancer tissue microarray. We wanted to clarify if the expression levels and/or localization of these proteins were of clinical relevance. For E-cadherin membrane expression was present in 68 of tumors (Fig. 1A). GSK-3 $\beta$  was expressed in the cytoplasm of all tumors with no tumors exhibiting membrane staining. Over one third of tumors also showed moderate to strong nuclear staining for GSK-3 $\beta$ , and in the other two third tumors, GSK-3 $\beta$  was absent from or very weakly expressed in the nucleus (Fig. 1B). CysLT<sub>1</sub>R expression was shown at moderate to high expression levels in over 50% of

the tumors. Here we observed that patients with more aggressive colon cancers had less expression of E-cadherin and nuclear expression of GSK-3 $\beta$  as well as high expression levels of CysLT<sub>1</sub>R. Figure 1A shows representative images of patient tumors with expressions of E-cadherin (Fig. 1A), tumor with cytoplasm expression of GSK-3 $\beta$  (Fig. 1B) and CysLT<sub>1</sub>R (Fig. 1C). We found a statistically significant correlation ( $p = 0.037$ ) between moderate membrane E-cadherin combine to no nuclear GSK-3 $\beta$  compared to all other tumors (low or no membrane E-cadherin and nuclear GSK-3 $\beta$  expressing tumors) of overall survival in Duke's B colon cancer patients. We also found a statistically significant correlation ( $p = 0.009$ ) between membrane E-cadherin/no nuclear GSK-3 $\beta$  compared to all other tumors in that group of patients with moderate to high expression levels of CysLT<sub>1</sub>R. Our results show that patients with high CysLT<sub>1</sub>R levels, indicative of inflammation, have a much poorer prognosis compared to patients with low expression levels of CysLT<sub>1</sub>R. These results show the importance of the inflammatory milieu around the tumor and highlight the crosstalk between immune cells and cancer cells possibly through inflammatory lipid mediators.

#### *Effect of LTD<sub>4</sub> on E-cadherin and Vimentin expression levels in SW480 and SW620 cells*

E-cadherin is important for normal cell-cell adhesion and loss of function in this cell junction complex is one of the hallmarks of epithelial cancer<sup>14, 18</sup>. Loss of cell-cell adhesion and polarity triggers epithelial-mesenchymal transition (EMT), which is an important step in the invasion and progression of colon cancer. One of the features of EMT is loss of membrane E-cadherin and increased expression of vimentin. We therefore investigated the possibility of LTD<sub>4</sub> inducing metastatic

capacity in colon cancer cells by inducing EMT-like properties. Indeed, we found that LTD<sub>4</sub> induced a dramatic reduction in membrane E-cadherin expression levels in SW480 cells after 24 hr stimulation (Fig. 2A) and no significant change in membrane E-cadherin expression in SW620 cells (Fig. 2B). We next evaluated the effect of LTD<sub>4</sub> on vimentin expression in these cells and found a significant increase after 24 hr of LTD<sub>4</sub> stimulation in SW480 cells (Fig. 2A) and no significant change of vimentin expression in SW620 cells (Fig. 2B). We also found an increase in the expression levels of phosphorylated Snail after LTD<sub>4</sub> stimulation in SW480 cells (data not shown). Taken together these data show that LTD<sub>4</sub> alters expression levels of proteins which are important for EMT

*LTD<sub>4</sub> induced nuclear translocalization of  $\beta$ -catenin in SW480 and SW620 cells:*

E-cadherin/ $\beta$ -catenin complex alteration is an important event for the outcome of colon cancer. We therefore next investigated if  $\beta$ -catenin localization was changed after LTD<sub>4</sub> stimulation. Besides the role of  $\beta$ -catenin in cell-cell adhesion in the complex with E-cadherin, it can also act as a transcription factor. Thus the intracellular levels of  $\beta$ -catenin in non-transformed cells are tightly regulated by a degradation system<sup>19</sup>. However, 80-90% of colon cancers have mutations in the APC or  $\beta$ -catenin genes, resulting in inhibition of  $\beta$ -catenin degradation leading to transcriptional activation of a number of genes by active/nuclear  $\beta$ -catenin<sup>19</sup>. LTD<sub>4</sub> induces nuclear translocation of  $\beta$ -catenin in SW480 and SW620 cells as shown by confocal laser scanning microscopy. We have shown a significant increase of activated  $\beta$ -catenin in the nucleus (pS552- $\beta$ -catenin) after LTD<sub>4</sub> stimulation in SW480 (Fig. 3A) and SW620 (Fig. 3B) cells. Furthermore, LTD<sub>4</sub> induced phosphorylation of GSK-3 $\beta$  in both SW480 and SW620 colon cancer cells. Interestingly, this

phosphorylation of GSK-3 $\beta$  at serine 9 (Ser9) inhibits GSK-3 $\beta$  activity<sup>20</sup>. We found that in un-stimulated (control) cells, pGSK-3 $\beta$  was localized predominantly to the cytosol but after stimulation with LTD<sub>4</sub> every 12 hr for 24 hr we observed a tendency of increased pGSK-3 $\beta$  in peri-nuclear regions in both SW480 and SW620 cells. These findings suggest an alteration in the E-cadherin/ $\beta$ -catenin/pGSK-3 $\beta$  complex leading to nuclear translocation of  $\beta$ -catenin that is involved in LTD<sub>4</sub> induced tumor progression and invasion.

*Effect of pro-inflammatory mediator LTD<sub>4</sub> on migration and invasion of SW480 and SW620 colon cancer cells*

In a recent study, we evaluated the migratory capacity of SW480 primary tumor cells and SW620 metastases-derived cells and found that SW620 cells had a significantly faster migratory rate<sup>16</sup>. In our present study, we investigated the effect of the inflammatory milieu on cell migration and invasion *in vitro*. We therefore stimulated SW480 and SW620 cells with LTD<sub>4</sub>. In SW480 cells, LTD<sub>4</sub> significantly increase cell migration via CysLT<sub>1</sub>R signaling (Fig. 5A) while no significant changes in SW620 cell migration capacity after stimulation with LTD<sub>4</sub> were observed (Fig. 6A). Moreover, stimulation with LTD<sub>4</sub> also resulted in increased invasive ability of both SW480 (Fig. 5B) and SW620 cells (Fig. 6B) in an *in vitro* 3D matrigel invasion assay. Since several matrix proteases are linked to cancer invasion and metastasis, we explored if an inflammatory stimulus from LTD<sub>4</sub> could induce changes in expression of key matrix metalloproteases, namely MMP-2, -7 and -9. Indeed, there was a robust induction of MMP-2 and -9 following LTD<sub>4</sub> stimulation in SW480 cells (Fig. 5C and 5D) compared to the un-stimulated control after 6 hr. We also found a modest increase in mRNA levels of MMP-7 after 6 hr of LTD<sub>4</sub>

stimulation in SW480 (Fig. 5E). Taken together these data show that LTD<sub>4</sub> alters gene expression important for EMT, cell motility and invasion.

These results support the notion that an ability to migrate is required as the first step towards establishment and progression towards metastasis and invasion. Colon cancers with high levels of CysLT<sub>1</sub>R have high migratory capacity in an inflammatory milieu in a manner similar to other types of tumors<sup>9</sup>. Several tumors, including breast, liver and colon cancer display increased levels of infiltrating inflammatory cells such as macrophages and tumor associated macrophages (TAM) and thus associated with poor prognosis<sup>3</sup>. In addition, eosinophils, mast cells and lymphocytes in the tumor microenvironment have the capacity to secrete various soluble factors such as leukotrienes and prostaglandins<sup>6</sup>. Taken together, the results from our present study underscore the importance of the inflammatory microenvironment for tumor progression and metastasis.

### **Acknowledgements**

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### **Figure Legends**

#### **Figure 1**

Expression of E-cadherin, GSK-3 $\beta$  and CysLT<sub>1</sub>R determined by immunoreactivity in representative sections of colon carcinomas. (A) Tumor with moderate/high expression of membrane E-cadherin. (B) Tumor with no nuclear expression of GSK-3 $\beta$ , or strong cytoplasmic expression of GSK-3 $\beta$  (C) moderate to high CysLT<sub>1</sub>R expression (microscopy images; left hand panels: 10x magnification, right hand panels: 40x). Kaplan-Meier survival curves according to, (D) moderate membrane E-cadherin and no nuclear GSK-3 $\beta$  (n=28) is associated with better survival in Dukes B colon adenocarcinomas as compared to all other tumors (E) moderate membrane E-cadherin/no nuclear GSK-3 $\beta$  are associated with better survival in moderate/high CysLT<sub>1</sub>R (n=54) expression patient groups (F) no/weak membranous E-cadherin (n=28) is associated with poor survival in moderate/high CysLT<sub>1</sub>R expression patient groups (n=54) but the p-value is not significant. Overall survival is shown in months and differences between groups were assessed using log-rank testing.

### **Figure 2**

Pro-inflammatory mediator LTD<sub>4</sub> increases vimentin and decreases E-cadherin expression levels in SW480 colon cancer cells. Western blot analyses showed expression levels of E-cadherin, Vimentin with or without LTD<sub>4</sub> stimulation for 24 hr in SW480 colon cancer cells (A) and SW620 cells (B). Proteins were separated by SDS-PAGE and immunoblotted with anti-E-Cadherin, and anti-Vimentin antibodies and re-probed with an antibody against  $\beta$ -actin to ensure equal loading. The experiments was repeated at least 3 times.

### **Figure 3**

Pro-inflammatory mediator LTD<sub>4</sub> induces nuclear translocation and serine-phosphorylation of  $\beta$ -catenin in SW480 and SW620 cells. Confocal microscopic

images with or without LTD<sub>4</sub> stimulated cells showed increased nuclear levels of active  $\beta$ -catenin-pS552 in SW480 colon cancer cells (A) and SW620 cells (B). After stimulation every 12 hr for 24 hr, the SW480 and SW620 cells were fixed, permeabilized and stained with antibodies against  $\beta$ -catenin-pS552. Left panels show  $\beta$ -catenin-pS552 immunostaining, middle panels the nuclear staining with DAPI and right panels are a merge of both. Corresponding graphs show the consolidated results of nuclear  $\beta$ -catenin-pS552-positive cells with or without 100 nM LTD<sub>4</sub>, calculated as number of  $\beta$ -catenin-pS552 positive cells per 100 cells. The data are given as mean  $\pm$  SEM. Statistical significance was calculated using unpaired Student's t-test (\*P < 0.05).

**Figure 4**

Pro-inflammatory mediator LTD<sub>4</sub> induces peri-nuclear accumulation of phospho-GSK-3 $\beta$  in SW480 and SW620 cancer cells. Confocal microscopic images of these cells with or without LTD<sub>4</sub> showed peri-nuclear intensity of p-GSK-3 $\beta$  in SW480 (A) and SW620 colon cancer cells (B). After stimulation every 12 hr for 24 hr, cells were fixed, permeabilized and stained with antibodies against phospho-GSK-3 $\beta$ . Left panels show p-GSK-3 $\beta$  immunostaining, middle panels nuclear staining with DAPI and right panels a merge of both.



**Figure 5**

Effect of LTD<sub>4</sub> on cell migration and invasion of SW480 cells. Wound healing assay with or without LTD<sub>4</sub> stimulation of SW480 cells (A), Invasion assay with or without LTD<sub>4</sub> stimulation of SW480 cells (B). Real time PCR quantification of mRNA expression of MMP-2 (C), MMP-9 (D) and MMP7 (E).

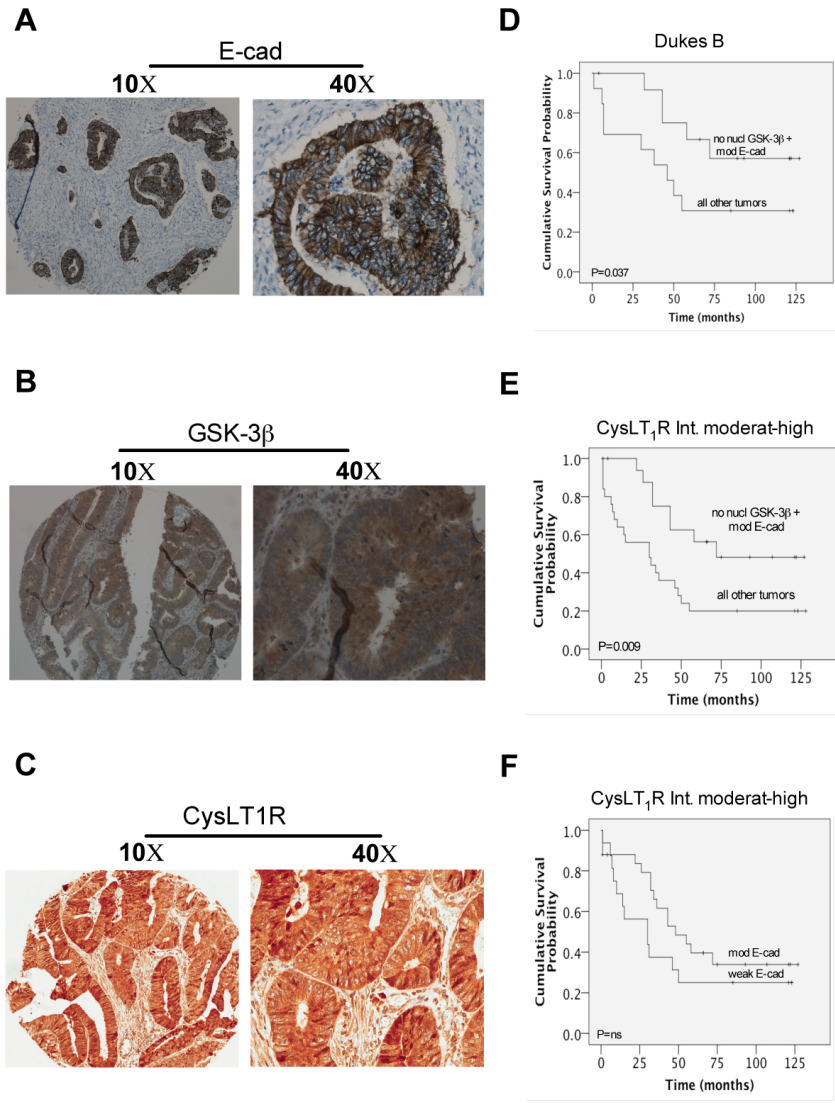
**Figure 6**

Effect of LTD<sub>4</sub> on cell migration of SW620 cells. Wound healing assay with or without LTD<sub>4</sub> stimulation of SW620 cells (A). Invasion assay with or without LTD<sub>4</sub> stimulation of SW620 cells (B).

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**Figure 1**

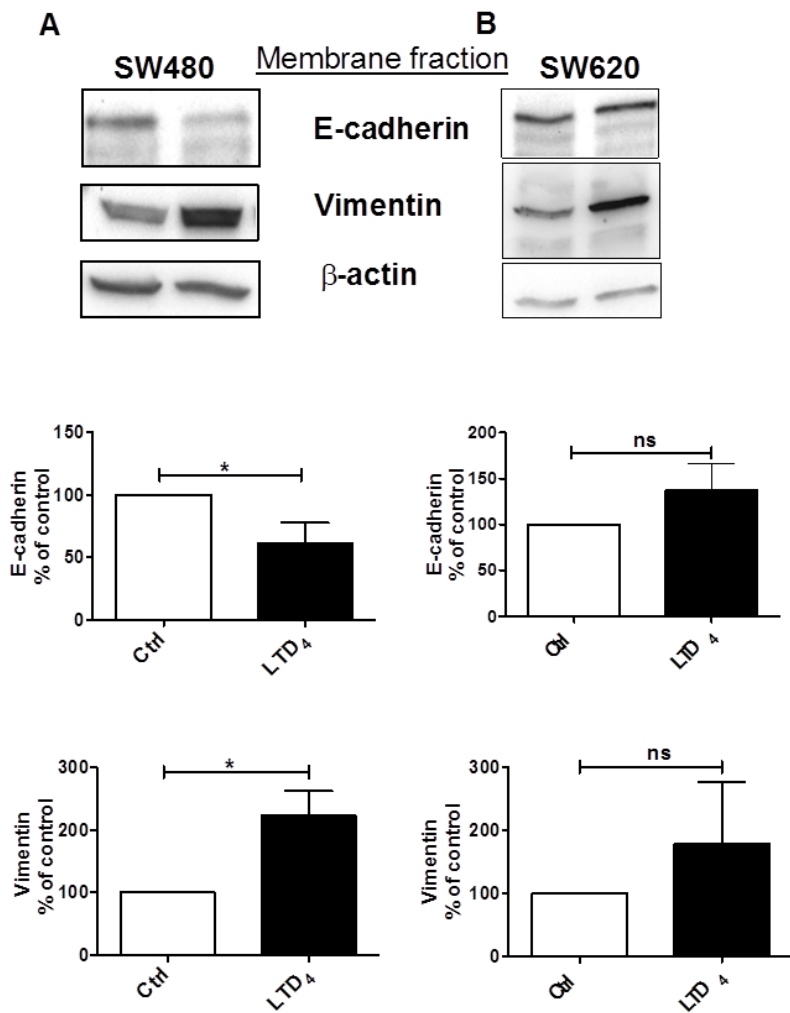


Figure 2

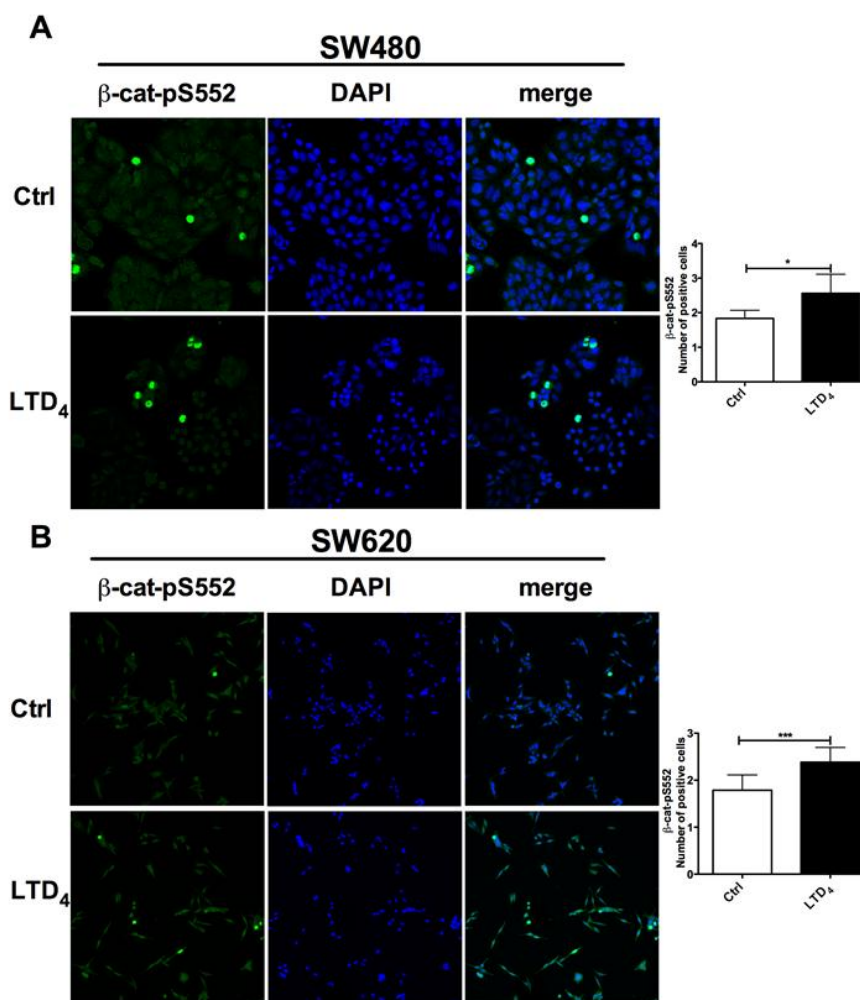


Figure 3

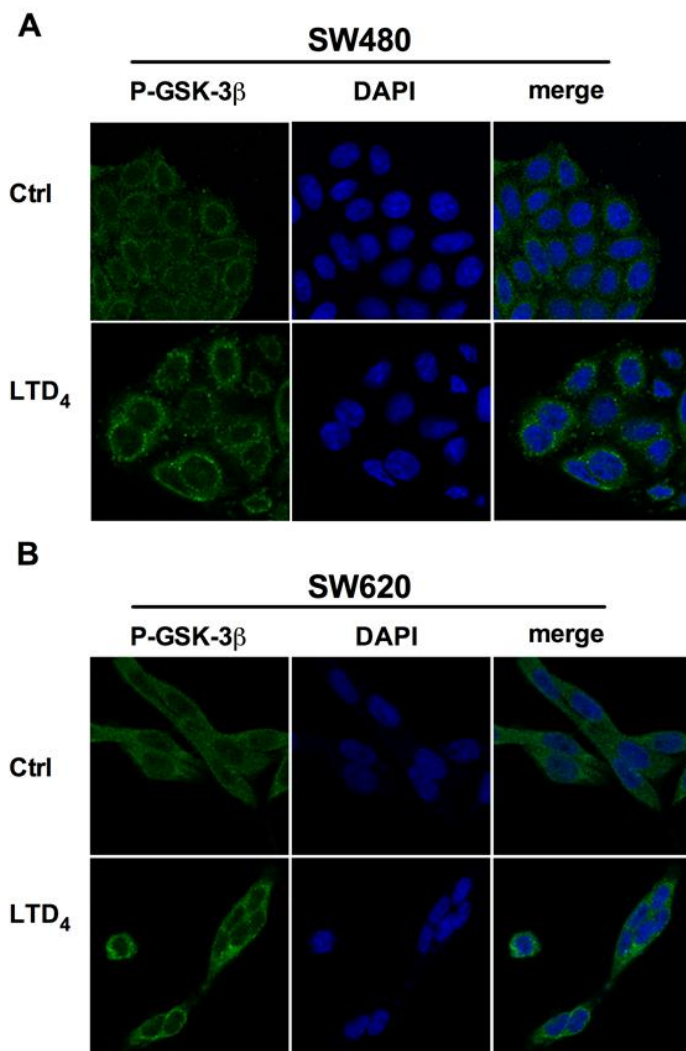


Figure 4

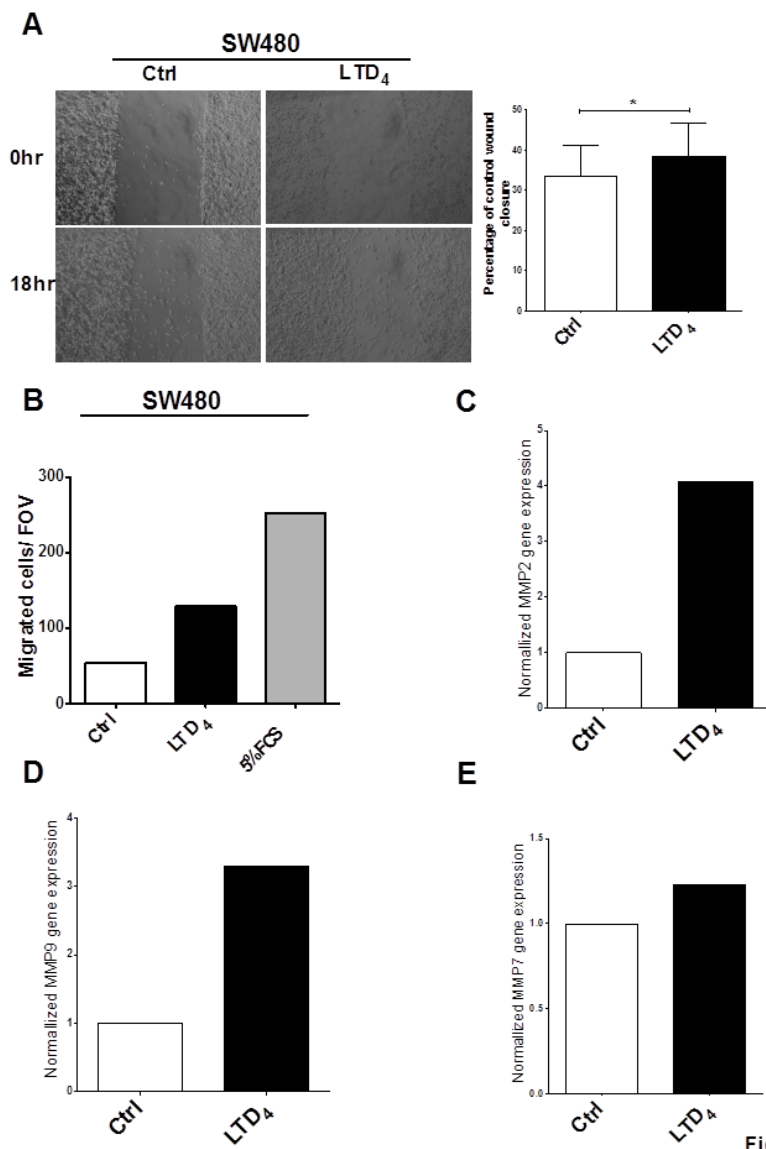


Figure 5



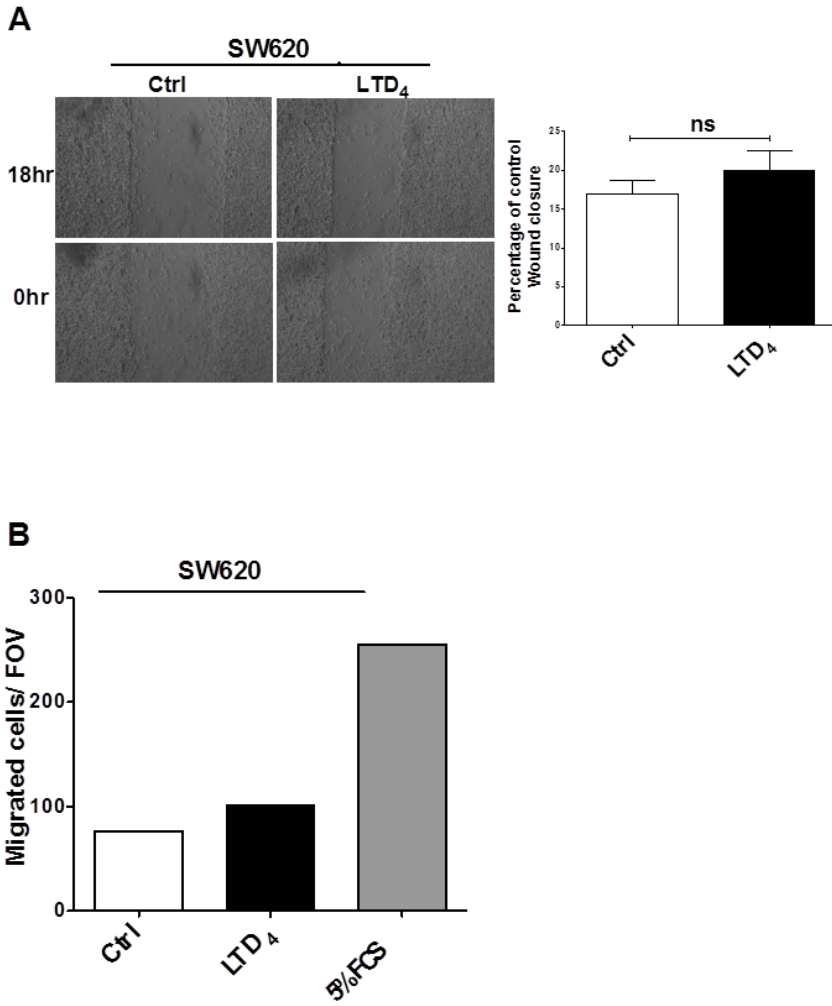


Figure 6

