Dietary modulation of β-catenin signalling in an experimental model of colon cancer

Marjo Misikangas

ACADEMIC DISSERTATION

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University of Helsinki
Department of Applied Chemistry and Microbiology
Nutrition

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Supervisor

Professor Marja Mutanen

Department of Applied Chemistry and Microbiology (Nutrition)

University of Helsinki, Finland

Reviewers

Professor Sirpa Kärenlampi

Department of Biosciences

University of Kuopio, Finland

Dr. Jan Erik Paulsen

Department of Food Toxicology

Norwegian Institute of Public Health, Norway

Opponent

Professor Hannu Mykkänen

Department of Clinical Nutrition

University of Kuopio, Finland

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To my family

Contents

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Abstract

Colorectal cancer is among the major cancers and one of the leading causes of cancer-related deaths in Western societies. Its occurrence is strongly affected by environmental factors such as diet. Thus, for preventative strategies it is vitally important to understand the mechanisms that stimulate adenoma growth and development towards accelerated malignancy or, in contrast, attenuate them to remain in quiescence for periods as long as decades.

The main objective of this study was to investigate whether diet is able to modulate β-catenin signalling related to the promotion or prevention of intestinal tumourigenesis in an animal model of colon cancer, the Min/+ mouse. A series of dietary experiments with Min/+ mice were performed where fructo-oligosaccharide inulin was used for tumour promotion and four berries, bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), cloudberry (*Rubus chamaemorus*) and white currant (*Ribes x pallidum*), were used for tumour prevention. The adenomas ($Apc^{-/-}$) and surrounding normal-appearing mucosa ($Apc^{+/-}$) were investigated separately due to their mutational and functional differences.

Tumour promotive and preventive diets had opposite effects on β-catenin signalling in the adenomas that was related to the different adenoma growth effects of dietary inulin and berries. The levels of nuclear β-catenin and cyclin D1 combined with size of the adenomas in the treatment groups suggests that diets induced differences in the cancerous process. Adenomas progressing to malignant carcinomas are most likely found in the sub-groups having the highest levels of β-catenin. On the other hand, adenomas staying quiescent for a long period of time are most probably found in the cloudberry or white currant diet groups. The levels of membranous E-cadherin and β-catenin increased as the adenomas in the inulin group grew, which could be a result of the overall increase in the protein levels of the cell. Therefore, the increasing levels of membranous β-catenin in Min/+ mice adenomas would be undesirable, due to the simultaneous increase in oncogenic nuclear β-catenin. We propose that the decreased amount of membranous β-catenin in benign adenomas of berry groups also means a decrease in the nuclear pool of β-catenin.

Tumour promotion, but not the tumour prevention, influenced β-catenin signalling already in the normal appearing mucosa. Inulin-induced tumour promotion was related to β-catenin signalling in Min/+ mice, and in WT mice changes were also visible. The preventative effects of berries in the initiation phase were not mediated by β-catenin signalling. Our results suggest that, in addition to the number, size, and growth rate of adenomatous polyps, the signalling pattern of the adenomas should be considered when evaluating preventative dietary strategies.

List of original publications

This thesis is based on the following original papers, referred to in the text by their Roman numerals, as well as on some unpublished results.

I **Misikangas M**, Tanayama H, Rajakangas J, Lindén J, Pajari A-M, Mutanen M. Inulin results in increased levels of β-catenin and cyclin D1 as the adenomas increase in size from small to large in the Min/+ mouse. Br J Nutr, accepted.

II **Misikangas M**, Pajari A-M, Päivärinta E, Oikarinen SI, Rajakangas J, Marttinen M, Tanayama H, Törrönen R, Mutanen M. Three Nordic berries inhibit intestinal tumorigenesis in multiple intestinal neoplasia/+ mice by modulating β-catenin signaling in the tumor and transcription in the mucosa. J Nutr 2007;137:2285-2290.

III Rajakangas J, **Misikangas M**, Päivärinta E, Mutanen M. Chemoprevention by white currant is mediated by the reduction of nuclear β-catenin and NF-κB levels in Min mice adenomas. Eur J Nutr, submitted, second revision in process.

IV **Misikangas M**, Pajari A-M, Päivärinta E, Mutanen M. Promotion of adenoma growth by dietary inulin is associated with increase in cyclin D1 and decrease in adhesion proteins in Min/+ mice mucosa. J Nutr Biochem 2005;16:402-409.

Contribution of the author to papers I-IV

I The author planned the study together with other authors. The experimental study including most of the empirical work was carried out by the author. The author wrote the manuscript and other authors participated in the writing of manuscript by giving comments and suggestions.

II The author designed the study and wrote the manuscript with other authors. The author was responsible for most of the biochemical and data analyses.

III The author planned the study together with other authors. The experimental study including empirical work and the preparation of the manuscript was carried out by the author and M.Sc. Rajakangas. Prof. Mutanen participated in the writing of manuscript by giving comments and suggestions.

IV The author planned the study together with other authors. The experimental study including empirical work was carried out by the author. The author wrote the manuscript and Prof. Mutanen participated in the writing of manuscript by giving comments and suggestions.

Abbreviations

AC	aberrant crypt
ACF	aberrant crypt foci
AIN	American Institute of Nutrition
AOM	azoxymethane
APC	human adenomatous polyposis coli gene
Apc	murine adenomatous polyposis coli gene
APC	adenomatous polyposis coli protein
BCAC	β-catenin-accumulated-crypts
CDK	cyclin-dependent kinase
CDKI	cyclin dependent kinase inhibitors
CKI	casein kinase I
DMH	dimethylhydrazine
E2F	family of transcription factors
EMT	epithelial-mesenchymal transition
FAP	familial adenomatous polyposis
GSK3β	glycogen synthase kinase 3β
H&E	haematoxylin and eosin stain
IHC	immunohistochemistry
Lef	lymphoid-enhances factor
LOH	loss of heterozygosity
Min/+	multiple intestinal neoplasia
MMP	matrix metalloproteinase
MCR	mutation cluster region
NES	nuclear export signals
NLS	nuclear localization signals
NSAID	non-steroidal anti-inflammatory drugs
Rb	retinoblastoma protein
SCFA	short-chain fatty acids
Tcf	T cell factor

Introduction

Colorectal cancer is the second most common cancer in both incidence and mortality among men and women in more developed countries (Stewart & Kleinhues 2003). It is estimated that 5% of the Western population will develop colorectal malignancy during their lifetime. Nearly 945 000 new colorectal cancer cases are diagnosed worldwide each year and colorectal cancer is responsible for some 492 000 deaths. The highest incidence rates occur in Europe, North America, Australia and Japan. The American Cancer Society estimates that in 2007 around 153 760 people will be diagnosed with colorectal cancer and around 52 180 people will die of the disease (Jemal *et al.* 2007). In Finland, colorectal cancer is among the three most common cancers in both men and women, with incidences in 2005 of 28 and 21 per 100 000, respectively (Finnish Cancer Registry 2007).

Colon cancer most commonly occurs sporadically and is inherited in only 5% of cases (Stewart & Kleinhues 2003). Interactions between genetic and environmental factors play a critical role in its aetiology. Diet is a major environmental factor that affects colon carcinogenesis – it has been estimated that 70% of cases could be prevented by nutritional and life-style interventions (Platz *et al.* 2000). Risk factors and protective factors have been studied extensively (Potter 1999, Donaldson 2004) but the relation between diet and colon cancer as well as the role of specific foods and cellular mechanisms are still not clear.

Interventions that decrease the growth rate of adenomatous polyps have been estimated to be much more effective in reducing the risk of colon cancer than those that decrease the rate of mutations at the APC locus (Adenomatous Polyposis Coli), found in 80% of cases (Luebeck & Moolgavkar 2002). This emphasises the role of diet in lifelong cancer prevention and the importance of understanding how diet modulates adenoma growth, especially the progression of adenomatous polyps to malignancy. It is estimated that one in two people will have a benign colonic tumour during their lifetime and, furthermore, that 10% of those tumours progress to malignancy (Kinzler & Vogelstein 1996). It is therefore important to understand what makes some adenomas grow and develop toward malignancy while the others stay quiescent for decades.

Colon cancer tumourigenesis progresses through epigenetic alterations in colon cancer stem cells that lead to genetic changes (Feinberg *et al.* 2006). One of the earliest mutations is in the *APC* gene, which is also the earliest mutation found in the adenoma-carcinoma sequence of colon cancer (Vogelstein *et al.* 1988). Loss of APC function results in the activation of the Wnt/β-catenin signalling pathway (Morin *et al.* 1997) that is widely studied as a target for cancer drugs (Kundu *et al.* 2006). The aim of this work was to find out whether diet is able to modulate intestinal β-catenin signalling that is related to promotion or prevention of intestinal tumourigenesis in an animal model of colon cancer.

β-catenin signalling and intestinal tumourigenesis

Role of APC in the adenoma-carcinoma sequence

Both the small intestine and colon have specialised epithelial functions. The structure of the epithelium is very similar in the small intestine and colon, even though the overall architecture is different. The small intestine consists of finger-like villi surrounded by the openings of glandular structures, crypts of Liberkühn (Figure 1). This ensures a large absorptive area that is covered by columnar epithelial cells. The colon does not have villi but the invaginations are deeper.

The intestinal mucosa is a place of continuous cell proliferation and migration. Near the base of the crypts are stem cells that give rise to daughter cells in the proliferating zone. As the cells migrate upward the cell cycle is arrested and cells start to express differentiation markers when they reach the top one-third of colonic crypts or the crypt-villus junction in the small intestine. Differentiated enterocytes (or colonocytes in the colon) are the absorptive epithelial cells that constitute the majority of cells and transport nutrients across the epithelial wall. Highly polarized enterocytes have functional cell-cell junctions that enable their migration in coherent bands stretching along the crypt-villus axis. At the top of small intestinal villi or the collar of colonic crypts cells undergo apoptosis and are exfoliated. Other functional cell types in the intestine are mucin-secreting Goblet cells and hormonally active enteroendocrine cells. Paneth cells that secrete antimicrobial molecules are predominantly found at the bottom of the small intestinal crypts. These epithelial cells are known to interact with mesenchymal cells and immunologically active cells and, furthermore, the intestinal microflora affects epithelial cell properties.

The intestinal epithelium constitutes the definitive barrier between the outside world and the body. In this extremely hostile and stressful environment the integrity of the epithelium is ensured by rapid turnover, which usually ensures that oncogenic mutations do not cause much harm. The life cycle of an individual epithelial cell spans less than a week and encompasses the initiation of cell proliferation to sloughing.

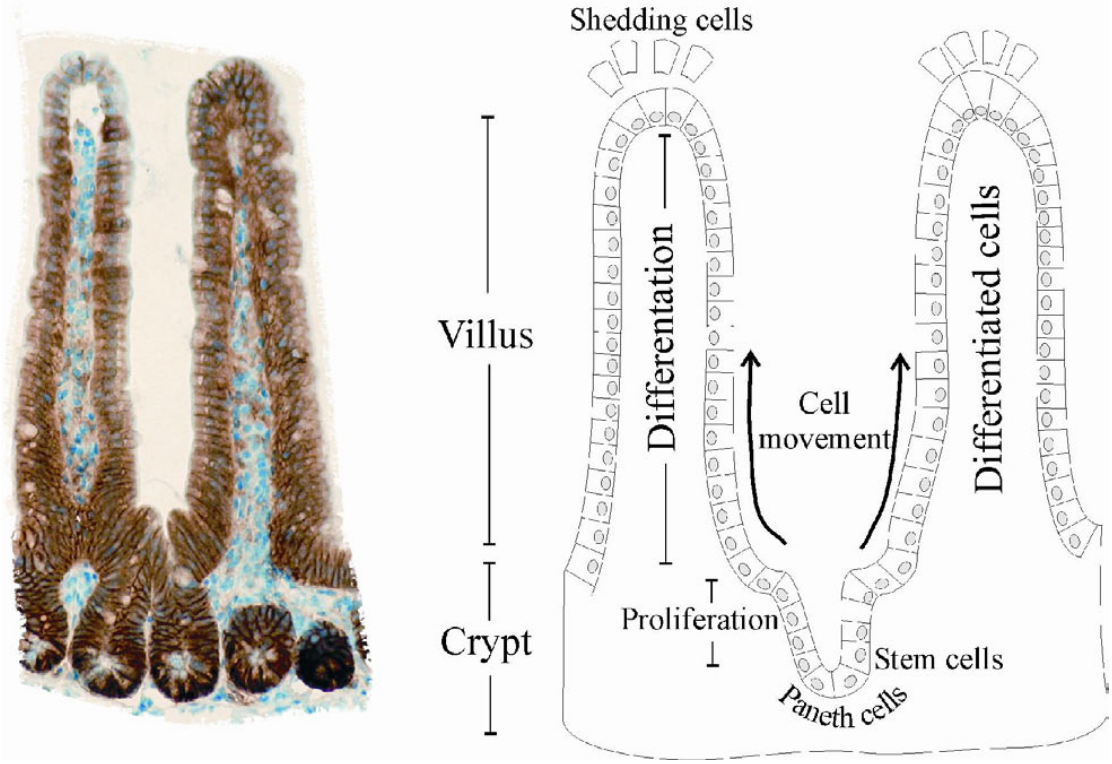

Figure 1. Structure of the small intestine. Stem cells give rise to proliferating progenitor cells that migrate upward. Paneth cells migrate downward and localise at the bottom of the crypt. At the crypt-villus junction progenitors stop proliferating and differentiate to enterocytes, Goplet cells or enteroendocrine cells. At the top of the villus cells undergo apoptosis and are relased to the intestinal lumen (modified from Gregorieff & Clevers 2005).

Vogelstein *et al.* (1988) were the first ones to provide evidence that different pathological stages of colon cancer could be identified by specific successive genetic changes in oncogenes and tumour-suppressor genes. It is now widely accepted that colon cancer proceeds through an adenoma-carcinoma sequence (for example see Fodde *et al.* 2001) (Figure 2).

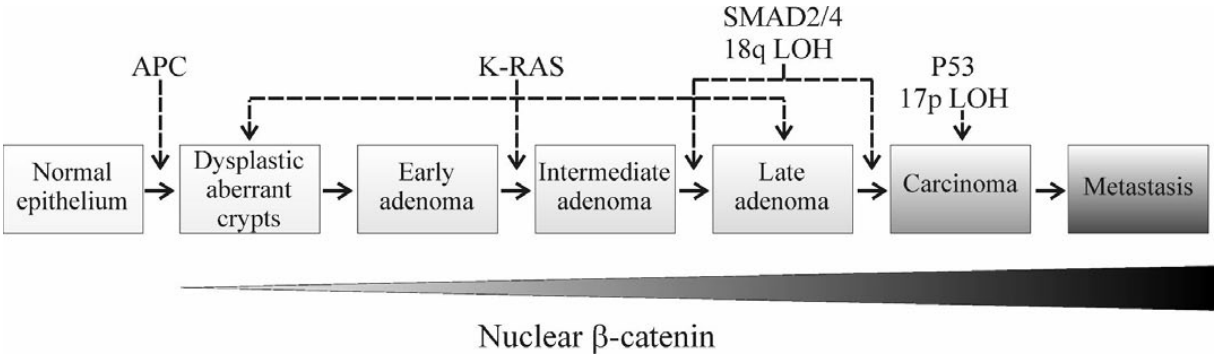

Figure 2. The adenoma-carcinoma sequence for colon cancer. Loss of APC function is one of the earliest events in colon tumourigenesis and it results in the activation of the Wnt/β-catenin signalling pathway. Sequential mutations in K-RAS, SMAD2/4 and p53 lead to progression toward malignancy. Nuclear β-catenin is observed late in de-differentiated tumours, mainly at the invasive front. It increases as tumours progress (modified from Fodde *et al.* 2001, Giles *et al.* 2003, Brembeck *et al.* 2006).

The earliest identified precursors of colon cancer are aberrant crypt foci (ACF) (Takayama *et al.* 1998a). These lesions consist of large, thick crypts that are only visible by methylene blue staining or by microscopy (Bird 1987). A wide range of histologies and biological properties of ACFs causes debate about their role in colon tumourigenesis (Pretlow & Pretlow 2005) and the mechanisms by which polyps or adenomas are formed. Two types of ACFs have been distinguished (Nucci *et al.* 1997). The most common type arising from activating mutations in K-RAS is associated with hypercellular or hyperplastic crypts that seldom develop into malignant carcinomas. The second type, dysplastic or unicryptal adenoma ACFs, bear APC mutations and occur frequently in carcinoma-associated colon mucosa (Nucci et al. 1997). The benign tumour mass that protrudes into the lumen of intestinal epithelium forms polyps that can be of two types: hyperplastic (nondysplastic) polyps that preserve their normal architecture and cellular morphology or adenomatous (dysplastic) polyps that have abnormalities both in inter- and intracellular organization (Fodde *et al.* 2001).

Mutations in the tumour suppressor gene *APC* are one of the earliest genetic alterations as normal intestinal epithelium becomes dysplastic. *APC* is also said to be a gatekeeper gene in the development of colon cancer. Inactivation of both alleles of the *APC* gene triggers the adenomatous process but additional mutations, such as in the oncogene *KRAS* and the tumour suppressor gene *p53*, result in a further growth advantage and lead to the progression to carcinomas. The role of *APC* mutations as initiators of colon tumourigenesis has been, however, challenged as the importance of *KRAS* in ACFs has been revealed (Pretlow & Pretlow 2005). It appears increasingly likely that there are multiple starting points for colon cancer.

Truncation mutations in the gene encoding the APC protein are found in the majority of sporadic colonic tumours. *APC* was originally identified as the gene mutated in Familial Adenomatous Polyposis (FAP) (Grode *et al.* 1991, Kinzler *et al.* 1991) and it is responsible for this inherited form of colon cancer (Su *et al.* 1992, Fodde *et al.* 2002). FAP patients develop large numbers of colonic adenomas early in life, some of which progress to malignancy by about the fourth decade of their life. Loss of APC is an early event in colonic tumourigenesis and precedes the formation of polyps, precursors to adenomas (Näthke 2004). APC controls cellular proliferation, adhesion, migration and differentiation in the self-renewing intestinal crypts and villi. As cancer is often the result of abnormalities in multiple and distinct cellular functions, inactivation of this multi-functional gene may efficiently trigger tumour formation and promote progression towards malignancy (Fodde *et al.* 2003).

In humans, *APC* is located on chromosome five and encodes a large, ubiquitously expressed protein (312 kDa, 2843 amino acids). The structural organisation of the APC protein is well described by Fearnhead *et al.* (2001) and Näthke (2004). The N-terminus contains an oligomerization domain, nuclear localization signals (NLS), and nuclear export signals (NES). The middle of the APC protein

contains the domains important for interactions with proteins in the Wnt signalling pathway, β-catenin, axin, and glycogen synthase kinase 3β (GSK3β). The C-terminal region of APC contains motifs that mediate interactions with a number of structural proteins, microtubules among others.

Both alleles of the tumour suppressor *APC* must become dysfunctional for total loss of growth suppressing activity. Hundreds of different disease-associated mutations of the *APC* gene have been reported in colon cancer (Laurent-Puig *et al.* 1998). The majority of germline and somatic mutations in *APC* occur in the first half of the coding region within the mutation cluster region (MCR) (Nagase & Nakamura 1993). These changes are insertions, deletions, and nonsense mutations that lead to truncation of the central region of the protein containing the β-catenin binding site. The site of the initial truncation mutation in the *APC* gene may predict whether the 'second hit' is another mutation or whether the remaining wild-type *APC* allele is simply lost as in FAP (Lamlum *et al.* 1999, Rowan *et al.* 2000). In sporadic colon cancers, the majority of second mutations result in loss of the wild-type *APC* allele (Miyoshi *et al.* 1992, Fearnhead *et al.* 2001).

The best studied function of APC is in relation to Wnt/β-catenin signalling that regulates growth, apoptosis and differentiation. This pathway has a key role during normal development of different tissues but when aberrantly activated is associated with carcinogenesis. To study the role of *APC* in the development of intestinal cancer several *Apc* mutated mouse models have been generated (Fodde *et al.* 2001, Kucherlapati *et al.* 2001, Giles *et al.* 2003, Taketo 2006). The phenotype of the animals depends largely on the precise location of the *Apc* mutation. The best-known and perhaps most widely used model is the Min/+ mouse (Moser *et al.* 1990, Su *et al.* 1992, Clarke 2006) that due to a nonsense mutation in codon 850 produces a truncated Apc polypeptide of approximately 95 kDa. Mice heterozygous for this mutation ($Apc^{+/-}$) develop dozens of intestinal tumours - multiple intestinal neoplasia. Initiation is caused by loss of heterozygosity (LOH) of the remaining wild-type Apc allele and leads to the formation of adenomatous polyps ($Apc^{-/-}$). The precise mechanism is still unknown but it could, for instance, be related to the role of Apc in mitotic events as mitotic defects, like aneuploidy, seem to occur in histologically normal intestinal epithelium before the appearance of dysplacia or adenomas (Caldwell et al. 2007).

Initiation occurs both in the small instestine and colon of Min/+ mouse although most of the adenomas are located in the small intestine with only a few in the colon. In the small intestine the *Apc* mutant cells with uncontrolled levels of β-catenin expand from dysplastic crypts to larger lesions that will eventually give rise to an adenoma (Moser *et al.* 1990, Su *et al.* 1992, Oshima *et al.* 1997 Yamada *et al.* 2002). Small flat dysplastic lesions denoted ACF_{Min} or flat ACF are early lesions in the colon of Min/+ mice that exhibit altered control of β-catenin and proceed from the monocryptal stage to adenoma with fast crypt multiplication (Paulsen *et al.* 1997, Paulsen *et al.* 2000, Paulsen *et al.* 2001).

Due to the molecular biological similarity to human colon cancer Min/+ mice are frequently used when involvement of environmental and genetic factors in tumourigenesis are studied (Shoemaker *et al.* 1997, Corpet & Pierre 2003, http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html).

Regulation of β-catenin signalling

The following presentation of the regulation of β-catenin is mainly based on the extensive reviews of Bienz & Clevers (2000), Bienz (2002), Wong & Pignatelli (2002), Giles *et al.* (2003), Näthke (2004), Hanson & Miller (2005) and Reya & Clevers (2005). The intracellular distribution of β-catenin is of great importance for the functions of β-catenin and the subsequent behaviour of differentiated epithelial cells or tumour cells (Figure 3). β-catenin exists in three subcellular fractions: in the cellular membranes as a part of the adhesion complex, in cytosol where the excessive protein is degraded and in the nucleus where β-catenin can influence transcription.

Most cellular β-catenin interacts with E-cadherin in adherens junctions from where it is continuously released and re-incorporated (Klingelhofer *et al.* 2003). The adherens junctions are essential for the main features of epithelial phenotype: cell-cell adhesion, homophilic cell adhesion and cellular polarity defining basal and apical orientation. It may also modulate the amount of β-catenin available for signalling (Brabletz *et al.* 2005a, Gumbiner 2005).

E-cadherin is a single-span transmembrane-domain glycoprotein that is expressed primarily in epithelial cells. Its extracellular region has a Ca^{2+}-dependent homophilic adhesion function and the cytoplasmic domain interacts with catenins (Cavallaro & Christofori 2004, Gumbiner 2005). The affinity between β-catenin and E-cadherin is high. Binding of β-catenin and E-cadherin takes place immediately after synthesis and guides the complex to the cell surface (Hinck *et al.* 1994a, Cox *et al.* 1996) apparently in an APC-dependent manner (Bienz 1999, Klingelhofer *et al.* 2003). α-catenin binds to cytoplasmic β-catenin and promotes selective binding to cadherin (Gottardi & Gumbiner 2004). Coupled with α-catenin, β-catenin links cadherins at the plasma membrane to the actin cytoskeleton to mediate cellular adhesion (Figure 3). This E-cadherin–β-catenin–α-catenin complex forms a dynamic, rather than a stable, link to the cytoskeleton (Drees *et al.* 2005, Yamada *et al.* 2005).

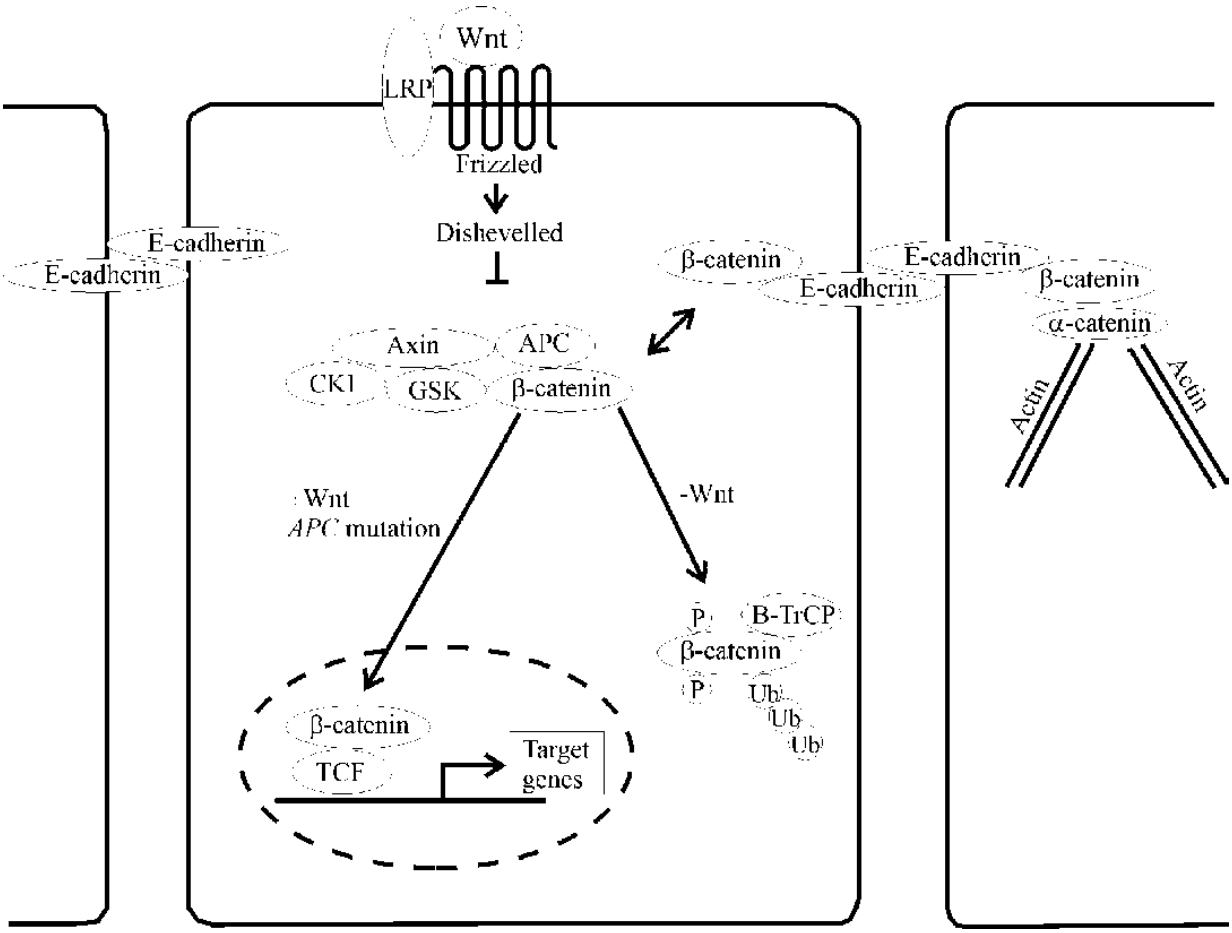

Figure 3. Regulation of β-catenin. In the absence of Wnt signal β-catenin is phosphorylated by an APC-axin-GSK-3β-CKI complex and targeted for degradation by β-TrCP. Wnt signalling proceeding through Frizzed-Dishevelled or *APC* mutation rescues β-catenin from degradation. Translocation of β-catenin to the nucleus enables the transcription of β-catenin/TCF responsive genes. As part of adherens juctions β-catenin with E-cadherin and α-catenin connects actin cytoskeletons of neighbouring cells. β-catenin released from E-cadherin is part of the free intracellular β-catenin pool available for cellular signalling (modified from Bienz & Clevers 2000).

In the absence of activating Wnt signals, free cytoplasmic β-catenin is destabilised by numerous kinases and phosphatases and a multiprotein complex containing APC, axin, GSK3β (Näthke 2004). GSK3β phosphorylates β-catenin and two scaffolding proteins in the complex, which increases their interaction. β-catenin is initially phosphorylated by casein kinase I (CKI) to provide the priming necessary for efficient phosphorylation by GSK3β. Sequential phosphorylation of a set of conserved Ser and Thr residues in the amino terminus of β-catenin recruits a β-TrCP-containing E3 ubiquitin ligase and subsequently leads to degradation of β-catenin by proteasomes.

In the presence of a Wnt signal, the Frizzled-Lrp5/6 receptor complex is activated. This leads to a poorly understood signalling cascade in which the kinase activity of the β-catenin destruction complex is inactivated. The mechanism by which receptor occupancy inhibits the kinase activity of GSK3β seems to involve the phosphorylation of an axin-binding molecule, Dishevelled, that causes

dissociation of the β-catenin destruction complex as it binds axin (Doucas *et al.* 2005). As a consequence, β-catenin cannot be targeted for destruction, but it accumulates and translocates to the nucleus where it acts as a co-activator for T-cell factor (TCF)/lymphoid-enhances factor (LEF)-responsive genes.

Earlier, the presence of APC protein in the nucleus was debated. It is now known to be shuttled into and out of the nucleus by NLS and NES sequences (Bienz 2002). This feature gives APC a dual role in downregulating β-catenin activity. APC promotes the nuclear export of β-catenin to the cytoplasm by direct transport or by indirectly shifting the equilibrium of β-catenin to the cytoplasm. In cytoplasm APC then promotes axin-mediated destabilisation of β-catenin (Rosin-Arbesfeld *et al.* 2000, Henderson *et al.* 2000, Neufeld *et al.* 2000). Other β-catenin interaction partners also retain β-catenin in the compartment in which they are localized and in that way regulate β-catenin subcellular localisation (Krieghoff *et al.* 2006).

The loss of full length APC protein affects β-catenin similarly to Wnt signalling, leading to accumulation and translocation of β-catenin to the nucleus due to non-functional degradation. The mutated APC may also have another effects on β-catenin. The truncated APC typically observed in colon cancer is not exported efficiently from the nucleus due to the lack of necessary central NESs. Mutated APC is also unable to export β-catenin from the nucleus, or may even trap β-catenin in the nuclei of these cells (Bienz & Clevers 2000).

E-cadherin binds to the same region of β-catenin as the destruction complex proteins APC and axin, and TCFs (Harris & Peifer 2005). E-cadherin competes with other binding partners of β-catenin (Orsulic *et al.* 1999) keeping β-catenin away from nuclear signalling (Gottardi *et al.* 2001, Wong & Gumbiner 2003). However, E-cadherin may have a surprisingly small impact on gene expression in the absence of Wnt signalling (Kuphal & Behrens 2006). Any activity capable of dissociating β-catenin from the membranous pool could rapidly increase the level of free β-catenin available for transcription (Harris & Peifer 2005).

Traditionally, the transcriptional activity of β-catenin is considered to hinge on stabilization of cytoplasmic β-catenin and its translocation to the nucleus but Gottardi and Gumbiner (2004), as well as Brembeck and collegues (2004), have challenged this view. According to their discoveries, regulated changes in β-catenin structure alter specific protein interaction affinities to dictate whether β-catenin interacts with adhesion or transcription complexes. Tyrosine phosphorylation of β-catenin may result in dissociation of β-catenin from adherens junctions (Brembeck *et al.* 2006). Of particular importance are two tyrosine residues in β-catenin: tyrosine 654 that is essential for binding to E-cadherin (Roura *et al.* 1999, Piedra *et al.* 2001) and tyrosine 142 that is crucial for binding to α-

catenin (Aberle *et al.* 1996, Piedra *et al.* 2003, Brembeck *et al.* 2004). Tyrosine phosphorylation is also involved in the regulation of the free cytoplasmic pool of β-catenin as tyrosine phosphorylation of β-catenin prevents its association with the destruction complex (Danilkovitch-Miagkova *et al.* 2001).

Formation of β-catenin-TCF complex during Wnt signalling is not only due to elevated β-catenin levels. Conformational changes in cytoplasmic β-catenin due to tyrosine phosphorylation results in selective binding of β-catenin to TCF and lowers the affinity of cadherin interaction (Gottardi & Gumbiner 2004). It is also speculated that APC and axin interactions might be blocked. β-catenin does not bind to DNA directly, but interacts with Tcf/Lef factors, which transiently converts them into transcriptional activators (Giles *et al.* 2003). The vertebrate genome encodes four highly similar Tcf/Lef proteins whose activity is tightly controlled by negative regulators, like Groucho. Tcf/Lefs are normally expressed during embryogenesis, but in most tissues are downregulated once the tissue becomes terminally differentiated. However, in sites of continual cell growth, such as bone marrow, skin, and intestinal mucosa, they are constantly expressed (Giles *et al.* 2003).

β-catenin/Wnt signalling regulates the complex balance of proliferation, migration and differentiation which is essential for normal functioning of the rapidly proliferating intestinal epithelium. In the normal intestinal epithelium β-catenin is located mainly in the proliferative compartment in the crypt and its expression decreases as cells move upward. Conversely, levels of APC increase as differentiating cells move up the crypt-villus axis (Smith *et al.* 1993, Midley *et al.* 1997). Compartmentalisation of β-catenin signalling in the crypt-villus axis has also been proposed (van de Wetering *et al.* 2002, Radtke & Clevers 2005, Clevers 2006). In crypts, Wnt proteins expressed by the crypt epithelial cells (Gregorieff *et al.* 2005) drive the formation of β-catenin/Tcf complexes and thus stimulate the proliferation of crypt progenitors as well as promote the terminal differentiation of Paneth cells, residing at the bottoms of the crypts (van Es *et al.* 2005). This is mediated at least in part through Wnt-controlled expression of the EphB sorting system (Batlle *et al.* 2002, Clevers & Batlle 2006). The absence of Wnt signalling in the villus compartment results in rapid cell cycle arrest and differentiation.

Nuclear β-catenin is a hallmark of an active Wnt pathway. Dozens of Wnt/β-catenin target genes able to regulate different cellular aspects have been identified and an updated list of target genes can be found from http://www.stanford.edu/~rnusse/wntwindow.html. Many of the targets, like c-myc and cyclin D1 (He et al. 1998, Shtutman *et al.* 1999, Tetsu & McCormick 1999), have the potential to change the proliferation, cell-cycle progression and differentiation states of cells. Wnt/β-catenin signalling also influences apoptosis, angiogenesis, extracellular matrix degradation and cell adhesion (Table 1). All these highly regulated processes are needed in the normal physiology of intestinal epithelium.

Table 1. Representative list of β-catenin target genes and their function. Modified from Brablez *et al.* 2005.

Target gene	Function
c-myc	Proliferation
cyclin D1	
Slug	EMT induction
c-jun	Oncogenic transcription factors
ets2	
fra-1	
ITF-2	
MMP-7	Protein degradation
MMP-26	
MT1- MMP	
UPA-R	
VEGF	Angiogenesis
BMP-4	Morphogenesis
Ephrinb2/B3	
Laminin γ2 chain	Migration
Fibronection	
L1	
CD44	Dissemination
Cdx1	Loss of differentiation
Id2	
Enc-1	
Gastrin	Trophic factors
PPARdelta	
MDR	Cell survival
Survivin	Stem cell formation
Conductin/axin-2	Negative feedback and tumour suppression
Tcf-1	

Aberrant β-catenin signalling in intestinal tumourigenesis

Any disturbance in normal intestinal homeostasis may lead to tumour development. Traditionally, the intestinal mucosa of human colon cancer patients has been considered normal. Now there is increasing evidence that mucosa also has genetic and cell signalling alterations early in carcinogenesis (Chen *et al.* 2004, Hao *et al.* 2005a, Hao *et al.* 2005b, Sugiyama *et al.* 2005). The expression of several genes is differently regulated in normal-appearing colonic mucosa from human colon cancer patients when compared with normal colonic biopsies from individuals without cancer (Chen *et al.* 2004). Changes in normal colon may precede or at least accompany the development of cancer as alterations in gene expression patterns in morphologically normal-appearing colonic mucosa are associated with the presence of adenomatous polyps (Hao *et al.* 2005a). It is also known that loss of wild-type Apc protein in the normal-appearing mucosa of Min/+ mice is associated with the earliest stages of dysplasia and moreove,r mitotic defects precede the loss of the second allele of Apc, β-catenin stabilisation, and dysplastic growth (Caldwell *et al.* 2007).

The earliest mutation reported in the adenoma-carcinoma sequence, the *APC* mutation, has deleterious effects on the architecture and function of the intestinal epithelium. The primary consequences of inactivation of *Apc* have been studied with conditional models (Sansom *et al.* 2004, Andreu *et al.* 2005). The inactivation of *Apc* activates Wnt signalling through rapid nuclear relocation of β-catenin which requires Myc as the critical mediator (Sansom *et al.* 2007). This changes both the appearance of enterocytes and the histology of the crypt. Apc-deficient cells maintain a "crypt progenitor-like" phenotype with perturbed differentiation, impaired migration, increased proliferation, and elevated apoptosis. The adenomas are said to result from the unabated expansion of these crypt progenitor-phenotype cells (Gregorieff & Clevers 2005). The role of β-catenin signalling in intestinal tumourigenesis is strengthened by the fact that genetically modified mice lacking β-catenin/Tcf activity lack the proliferative progenitors in the intestine (Korinek *et al.* 1998, Pinto *et al.* 2003, Kuhnert *et al.* 2004).

APC mutation results in the enlargement of the proliferation zone as *APC* is unable to attenuate β-catenin signalling and favour differentiation. Cell migration is slowed down and the initial direction is lost. This might provides an early mechanism for disease progression: an increased number of cells in the crypt-villus compartment allows the opportunity for a 'second hit'. As mutated cells start to accumulate and form a polyp and early adenoma, other mutations may be adopted more easily thereby leading to tumour progression. The enlargement of the proliferation zone has been documented also in the Min/+ mouse. The preneoplastic intestinal epithelium of the Min/+ mouse expresses both the 312 kDa full-length and the 95 kDa truncated Apc proteins ($Apc^{+/-}$). *Apc* mutation results in an extended proliferative compartment, reduced cellular turnover and decreased enterocyte migration in the normal intestinal epithelium of the Min/+ mouse (Mahmoud *et al.* 1997, Mahmoud *et al.* 1999).

Adenoma cells of Min/+ mice have homozygous truncating *Apc* mutations ($Apc^{-/-}$) and abnormal migration behaviour due to activated β-catenin/Tcf genes. Activated β-catenin/Wnt signalling leads to the formation of benign intestinal lesions similar to the preneoplastic lesions developed by humans, such as dysplastic crypts and adenomas. In dysplastic crypts the APC mutant cells expand laterally and repopulate the surrounding crypts (Moser *et al.* 1990, Su *et al.* 1992, Yamada *et al.* 2002). Adenomas develop at the crypt-villus junction and form pockets that migrate inside the normal epithelium of the villus (Oshima *et al.* 1997). The cells proliferate inside the mucosa as a disorganized mass that will eventually give rise to a tumour. In the initial process of adenoma formation β-catenin affects the aberrant crypt fission (Wasan *et al.* 1998). Adenomas of Min/+ mice do not go beyond this promotion stage. Progression to carcinoma or invasive activity has not been described in Min/+ mice with a C57BL background as they die early from anaemia due to a large number of tumours. Although in Min/+ mice with an AKR background carcinomas have been observed which is probably due to their higher resistance, lower number of tumours and longer life (Moser *et al.* 1992).

The changes in β-catenin expression and cellular localization are early events in colon cancer development (Valizadeh *et al.* 1997, Sheng *et al.* 1998, Sparks *et al.* 1998, Lifschitz-Mercer *et al.* 1999, Samowitz *et al.* 1999, Lamlum *et al.* 2000). The amount of nuclear β-catenin increases in the course of tumour progression from small to large colorectal adenomas while strongest intensities are found in the dedifferentiated carcinoma cells at the invasive front (Takayama *et al.* 1996, Hao *et al.* 1997b, Brabletz *et al.* 2000, Iwamoto *et al.* 2000). In early colon adenomas nuclear β-catenin is related to the morphogenic changes (Shih *et al.* 2001) and in late dysplastic colon adenomas the increasing accumulation is associated with increasing irregular branching (Kirchner & Brabletz 2000). The increased expression of nuclear β-catenin and the reduced expression of membranous β-catenin in colorectal tumours have been well reported as well as their correlation with metastasis and poor prognosis (Takayama *et al.* 1996, Hao *et al.* 1997a, Valizadeh *et al.* 1997, Takayama *et al.* 1998b, Hugh *et al.* 1999, Wang *et al.* 2002). However, some studies have not seen the connection (Maruyama *et al.* 2000, Chung *et al.* 2001). A reciprocal relationship between reduced membranous and increased nuclear β-catenin expression has also been demonstrated in the development from adenoma to carcinoma (Hao *et al.* 1997b, Hugh *et al.* 1999, Chung *et al.* 2001).

Within the colorectal carcinoma the cells in different areas show different proliferation rates, as tumour cells at the luminal side and central areas proliferate more strongly than at the invasive areas (Palmqvist *et al.* 1999). The staining for β-catenin often shows a heterogeneous pattern with strong nuclear enrichment at the invasion front and mainly cytoplasmic and membrane staining in the central tumour area. Similarly, membranous E-cadherin is found in differentiated central areas of the colorectal carcinoma, whereas in the invasive areas the expression of membranous E-cadherin is decreased (Brabletz *et al.* 2001). Nuclear accumulation of β-catenin appears to predominate in areas of active migration and remodelling rather than sites of proliferation in human tumours (Brabletz *et al.* 1998, Kircher & Brabletz 2000). Even though the target genes of β-catenin known to induce proliferation, cyclin D1 and c-myc, follow the expression of nuclear β-catenin the simultaneous overexpression of the cell cycle inhibitor $p16^{INK4A}$ ceases the proliferation at the invasion site (Brabletz *et al.* 2000, Palmqvist *et al.* 2000, Jung *et al.* 2001). This indicates that high levels of nuclear β-catenin in the tumour margins, as compared with the tumour centre, play a role in the transition to the invasive state of the tumour cells (Brabletz *et al.* 2001) and at the invasive front of well-differentiated colorectal tumours cyclin D1 may have functions other than proliferation (Jung *et al.* 2001).

Capabilities of invasion and metastasis are the hallmarks of malignant transformation. During the carcinoma progression, advanced tumour cells frequently downregulate epithelial markers, like E-cadherin, and loosen the intercellular junctions which result in the loss of epithelial polarity and

reduced intercellular adhesion (Christiansen & Rajasekaran 2006). E-cadherin mutations are very rare in colorectal cancer (Schuhmacher *et al.* 1999) and the loss of E-cadherin observed in colorectal cancers is generally associated with later stages of tumour progression and correlates with increased tumour invasiveness (Birchmeier & Behrens 1994, Mohri 1997, Valizadeh *et al.* 1997, Perl *et al.* 1998, Takayama *et al.* 1998b). Loss of E-cadherin function seems to be a cause of its redisribution from the cell membrane to the cytoplasm by tyrosine phosphorylation rather than due to reduced expression of the protein (Hiscox & Jiang 1997, Wijnhoven *et al.* 2000). Alterations in any of cell adhesion components may lead to disrupted function of the complex. The presence of membranous E-cadherin does not always imply a functional cell adhesion complex as β-catenin may be dysfunctional. Therefore, the combination of E-cadherin and one of the catenins may have a better prognostic value than evaluation of the individual components (Gofuku *et al.* 1999) although some studies have failed to show the relationship (Ilyas *et al.* 1997).

Colorectal carcinomas often retain an epithelial phenotype and grow in tubular structures. A loss of an epithelial and gain of a mesenchyme-like phenotype by re-distribution of β-catenin enables invasion in tumour margins (Brabletz *et al.* 2005a). Oncogenic activation of β-catenin in the tumour invasion front is associated with advanced Dukes' stage, tumour recurrence and the presence of metastasis (Ougolkov *et al.* 2002, Zhang *et al.* 2003). Many β-catenin target genes are involved in epithelial-mesenchymal transition (EMT) by extracellular matrix proteolysis, induced cell migration, loss of E-cadherin function and inhibition of epithelial differentiation (Table 1). Together with the genes involved in formation of stem cells they effectively induce invasion and metastasis (Brabletz *et al.* 2005a). For example, MYC, MMP-7, CD44 and UPA-R, correlate with tumour progression and have been implicated with tumour invasion and metastasis (Fodde *et al.* 2001).

The microenvironment influences the growth and invasion potential of tumour cells by producing various degrading enzymes such as matrix metalloproteinases (MMPs), by storing cytokines and remodelling and supplying new vessels for the tumour (Liotta & Kohn 2001, Geho *et al.* 2005). Environmental factors are the most probable reasons for the heterogeneous intracellular β-catenin distribution and function in colorectal carcinomas (Brabletz *et al.* 2002, Brabletz *et al.* 2005a). Many growth factors are shown to accumulate β-catenin in the nucleus due to release of β-catenin from the cell membranes by tyrosine phosphorylation (Brabletz *et al.* 2002) and, for example, MMPs are able cleave the extracellular domains of E-cadherin that leads to the loss of E-cadherin function.

Role of cyclin D1 in the cell cycle and in intestinal tumourigenesis

Most of the cells in an adult organism are quiescent and only specialized cells in the haematopoietic system or in the gut epithelium maintain active proliferation. β-catenin/TCF4 activity controls proliferation versus differentiation in the intestinal epithelium through its' target genes. At the bottom of the crypt, the progenitor proliferative cells accumulate nuclear β-catenin and express β-catenin/TCF target genes like cyclin D1. As the cells reach the mid-crypt region, β-catenin/TCF activity as well as transcription of cyclin D1 is downregulated which results in cell cycle arrest and differentiation (Giles *et al.* 2003).

The cell cycle consists of four phases (Figure 4). During the two main functional phases, cells generate a single and faithful copy of their genomic DNA (synthesis, S phase) and divide all the cellular components between two identical daughter cells (mitosis, M phase). Between these phases are gap periods, G1 and G2, during which cells prepare themselves for successful DNA replication or mitosis. During development, differentiation, or growth factor withdrawal, cells can enter an inactive period G0. Cell-cycle checkpoints in G1 and G2 ensure proper chromosome replication and separation. One of these in mid G1 is called the restriction point (R) after which cells become independent of growth factors and commit to cell division.

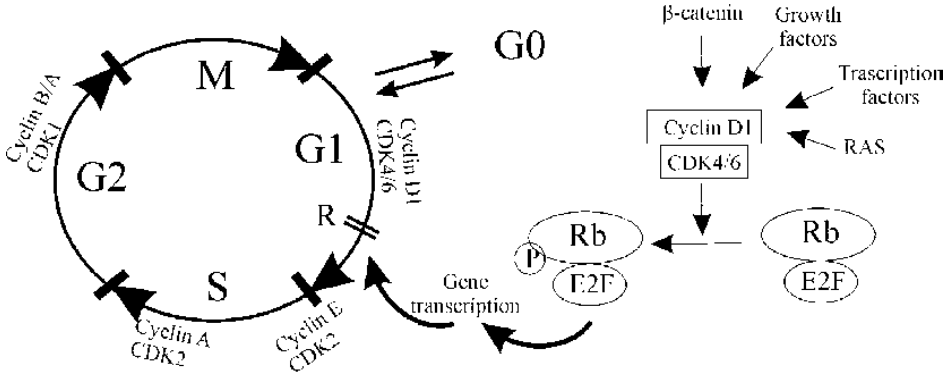

Figure 4. Simplified model of the cell cycle indicating DNA synthesis and mitosis phases as well as gap periods between them. The expression of cyclin D is activated by several growth factors, transcription factors, β-catenin and RAS dependent pathways. Production of D-type cyclins and activation of cdk4/6 in response to mitogens results in phosphorylation and inactivation of Rb with consequent derepression of E2F-dependent transcription (modified from Weinstein 1996, Israels & Israels 2001).

Cell-cycle progression is regulated by two protein classes, the cyclins and their serine/threonine kinase partners, the cyclin-dependent kinases (cdks) (Figure 4). The D-type cyclins bind to and activate cdks 4 and 6, and E-type cyclins interact with and activate cdk2 at restriction point passage. Cyclin-CDK activity is regulated at several levels: through control of cyclin synthesis and degradation, activating and inhibitory phosphorylation of the CDK subunit, subcellular localisation and inhibition by cyclin dependent kinase inhibitors (CKI). Two types of CKI inhibitors are involved: INK4 family proteins

(p15, p16, p18, p19) bind to cdks 4 and 6 preventing their association with D-type cyclins and WAF/KIP family proteins (p21, p27, p57) that have a broader specificity and can bind to all cyclin-CDK complexes (Sherr & Roberts 1999, Besson *et al*. 2004).

The expression of cyclin D is largely dependent on extracellular signals and signalling cascades, and is a fundamental link between mitogens, nutrient stimulation and the cell cycle machinery. Cyclin D1 is a target gene of β-catenin/TCF (Shtutman *et al.* 1999, Tetsu & McCormick 1999) but genes encoding cyclin D1 are also activated by several growth factors, transcription factors and RAS dependent pathways (Coqueret 2002, Diehl 2002, Fu *et al.* 2005, Gladden & Diehl 2005). Active cyclin D1-ckd4/6 complex translocates to the nucleus and partially inactivates the retinoblastoma protein (Rb) by phosphorylation. This allows E2F to transcribe genes required for S phase, such as cyclin E. Binding of WAF/KIP inhibitors to cyclin D1 and ckd4/6 stabilizes the complex without losing the kinase activity and keeps the inhibitors away from the cyclin E-cdk 2 complex. This completes the inactivation of Rb and release of E2F transcription factors (LaBaer *et al.* 1997). Recently, new mechanisms through which cyclin D1-cdk4 drives restriction point passage have been identified: cyclin D1-cdk4 can directly inactivate Smad3, TGF-β signalling protein, by phosphorylation and by that way inhibit its antiproliferative function (Matsuura *et al.* 2004).

In addition to cdk-dependent functions, cyclin D1 also has cdk-independent roles including chromatin remodelling by associating with histone deacetylases and p300 (Fu *et al.* 2005). Furthermore, cyclin D1 can directly associate with and regulate activity of different transcription factors (Fu *et al.* 2004, Coqueret 2002).

Overexpression of cyclin D1 is one of the most commonly observed alterations in human cancers (Diehl 2002). It occurs relatively early during tumourigenesis (Weinstein 1996) and is likely to affect normal intestinal epithelium renewal by increasing the overall proliferation rate. Activated β-catenin signalling favours cellular proliferation as well as exerts anti-apoptotic effects (Peifer 1997) and in human colorectal adenocarcinomas aberrant expression and nuclear accumulation of β-catenin is associated with elevated protein levels of cyclin D1 (Wang *et al.* 2002). Adenomas of FAP patients have significantly increased cyclin D1 levels (D'Orazio *et al.* 2002), similar to those seen in sporadic colorectal tumours (Bartkova *et al.* 1994, Arber *et al.* 1996, Sutter *et al.* 1997, Oda *et al.* 1999, Sugiyama *et al.* 2005). The overexpression of cyclin D1 correlates with advanced cancer stage and poor prognosis (Maeda *et al.* 1997, Oda *et al.* 1999) but the prognostic role of cyclin D1 is not seen in all studies (Palmqvist *et al.* 1998, Cheah *et al.* 2002).

The ability of cyclin D1 to act as an oncogene in the absence of β-catenin demonstrates its functional importance in gastrointestinal tumours (Kazanov *et al.* 2003). Introduction of an antisense cyclin D1

cDNA construct into human colon adenocarcinoma cell lines overexpressing cyclin D1 decreases the levels of cyclin D1 and markedly inhibits growth. These cells also lose the tumourigenity in nude mice (Arber *et al.* 1997). The crossing of Min/+ mice with cyclin $D1^{-/-}$ mice reduces the cyclin D1 abundance as well as intestinal tumour number by approximately 50% upon the loss of a single *cyclin D1* allele (Hulit *et al.* 2004). In the presence of activated β-catenin signalling cyclin D1 inhibits differentiation and promotes proliferation of intestinal epithelium, possibly in a PPARγ dependent manner (Girnun *et al.* 2002, Hulit *et al.* 2004). In the experimental models alterations in subcellular distribution of β-catenin are connected with increased cellular levels of cyclin D1 (Sellin *et al.* 2001) although cyclin D1 might not to be an immediate target of the β-catenin/Wnt pathway *in vivo* as it becomes activated in a delayed manner (Sansom *et al.* 2005).

In addition to the increased expression of cyclin D1, adenomas as well as adenocarcinomas of the colon exhibit a poor staining reactivity of $p21^{waf1}$ (Valassiadou *et al.* 1997, Sinicrope *et al.* 1998, Zirbes *et al.* 2000) as cancer epithelial cells have decreased expression of $p21^{waf1}$ compared with surrounding stromal cells (Sugiyama *et al.* 2005). $p21^{waf1}$ correlates with advanced disease stage (Viale *et al.* 1999) and appears to be an independent prognostic parameter in colorectal cancer that is associated with favourable survival (Zirbes *et al.* 2000). A lack of p27 expression when combined with accumulation of nuclear β-catenin is a marker of poor prognosis (Cheah *et al.* 2002). E-cadherin, generally described as an invasion suppressor, might be a major growth suppressor (Wijnhoven *et al.* 2000) as it has the ability to inhibit proliferation *in vitro* by upregulation of p27 (St Croix *et al.* 1998).

Approaches to target aberrant β-catenin signalling

The implication of deregulated β-catenin signalling in colorectal tumourigenesis has raised the interest for novel cancer drug targets. β-catenin has been seen as both a prognostic marker and a target for drug intervention in colorectal cancer. Many approaches to target the β-catenin pathway at the extracellular/membrane, cytoplasmic, and nuclear levels have been used (Wijnhoven *et al.* 2000, Luu *et al.* 2004, Dihlmann *et al.* 2005, Doucas *et al.* 2005, McMillan & Kahn 2005, van Es & Clevers 2005, Kundu *et al.* 2006). Research has predominately been on nonsteroidal anti-inflammatory drugs (NSAIDs), but interest in natural phytochemicals is increasing.

NSAIDs and a wide variety of naturally occurring anti-inflammatory substances are able to prevent certain forms of cancer (Surh 2002, Chun & Surh 2004, Kundu *et al.* 2006). NSAID treatment significantly lowers nuclear accumulation of β-catenin in adenomas and induces the regression of intestinal tumours in FAP patients (Boon *et al.* 2004) and rodent models of colon cancer (Mahmoud *et al.* 1997, Mahmoud *et al.* 1998, McEntee *et al.* 1999, Brown *et al.* 2001). NSAIDs seem to elicit anti-

proliferative effects in colorectal cancer by inhibiting nuclear accumulation of β-catenin and expression of cyclin D1 (Smith *et al.* 2000, Dihlmann *et al.* 2001, Hawcroft *et al.* 2002, Gardner *et al.* 2004, Dihlmann *et al.* 2005, Kundu *et al.* 2006). Enhanced expression of APC and E-cadherin as well as the relocation of nuclear and cytoplasmic β-catenin to the cell membranes accompany NSAID inhibited growth in colon cancer cells and tumours (Oshima *et al.* 2001, Chang *et al.* 2005, Roy *et al.* 2005, Kapitanovic *et al.* 2006). There is also some evidence that NSAIDs decrease the level of β-catenin and redistribute β-catenin and E-cadherin back to the plasma membrane in normal-appearing mucosa (Mahmoud *et al.* 1998, Roy *et al.* 2005).

Diet and intestinal tumourigenesis

Western-style diets have been hypothesized as contributing to the development of colon cancer (Adlercreutz 1990, Slattery *et al.* 1998, World Cancer Research Fund 2007). Red meat, animal and saturated fat, refined carbohydrates, sugar, alcohol and also total energy intake, appear to relate to risk of colon cancer (Slattery *et al.* 2000, Meyerhardt *et al.* 2007, World Cancer Research Fund 2007). On the other hand, the intake of dietary fibre, whole-grain cereals, vegetables, fruits, antioxidant vitamins, calcium, and folate seem to be negatively associated with the development of colon cancer.

Whether the intake of dietary fibre can protect against colorectal cancer is a long-standing question. Besides intensive research, the relationship between fibre intake and risk of colon cancer is somewhat inconsistent. Many correlational and case-control epidemiologic studies have supported a protective effect of fibres (reviewed in Kim 2000, Young *et al.* 2005); but prospective studies have given confusing results (Young *et al.* 2005, Schatzkin *et al.* 2007). Furthermore, large randomized clinical trials (Alberts *et al.* 2000, Schatzkin *et al.* 2000) and large observational investigations (Peters *et al.* 2003, Bingham *et al.* 2003) have been contradictory. The confounding factors in fibre research are probable the heterogeneous nature of fibre – i.e. fibre from cereals, vegetables, fruits – the mixture of other foods in the diet and different ways in which fibre is measured and recorded.

Diets rich in vegetables and fruit has long been said to protect against cancer. The epidemiological evidence was believed to be strong and firm until recently published studies challenged the view (Kim 2001, Riboli & Norat 2003, Koushik et al. 2007). However, berries and their phenolic compounds have shown promising chemopreventive effects (Duthie 2007, Heinonen 2007). In Finland, the incidence of colorectal cancer differs up to twofold between the North and the South (Finnish Cancer Registry, http://www.cancerregistry.fi/eng/statistics/ updated 12.10.2007). One of the main differences between the diets in the two areas is a significantly higher consumption of wild berries in the North, where colorectal cancer incidence is lower (Similä *et al.* 2005). A lot of effort has been put to finding

the ‘magic bullet’ of cancer prevention from phytochemicals derived from edible plants. Over 5000 individual phytochemicals have been identified from edible plants, including berries (Liu 2004).

Besides synthetic drugs, many extracted naturally occurring phytochemicals are able to target β-catenin signalling related to colorectal cancer (Surh 2003, Clapper *et al.* 2004). The most convinsing evidence is found from curcumin from turmeric (Mahmoud *et al.* 2000, Jaiswal *et al.* 2002, Thangapazham *et al.* 2006) and epigallocatechin gallate (EGCG) from green tea (Orner *et al.* 2003, Ju *et al.* 2005, Dashwood *et al.* 2005) but also resveratrol from grapes, docosahexanoic acid from fish oil, sulforaphane from broccoli, indole-3-carbinol from cabbage, genistein from soybean are widely studied (Oshima *et al.* 1995, Joe *et al.* 2002, Kim *et al.* 2003, Surh 2003, Clapper *et al.* 2004, Kundu *et al.* 2006).

Multiple lines of evidence suggest that an inappropriate activation of β-catenin signalling contributes to colorectal tumourigenesis. Although extracted phytochemicals have received significant attention for their β-catenin suppressing activity, the potential of foods and dietary constituents to disrupt β-catenin signalling remains less clear. As diet is known to be a major environmental factor that affects colon tumourigenesis it is of great importance to elucidate diet induced cell signalling events that contribute to intestinal tumourigenesis.

Aims of the study

The main objective of this study was to investigate whether diet is able to modulate β-catenin signalling of enterocytes related to the promotion or prevention of intestinal tumourigenesis in an animal model of colon cancer. A series of dietary experiments with Min/+ mouse were performed (Figure 5).

Figure 5. The study outline.

	Adenomas ($Apc^{-/-}$)	**Mucosa ($Apc^{+/-}$)**
Tumour promotive diet inulin	**I**	**IV**
Tumour preventive diet berries	**II, III**	**II, III**

Fructo-oligosaccharide inulin was used in promotion as it has been seen to promote tumourigenesis in Min/+ mice (Mutanen *et al.* 2000, Pajari *et al.* 2003). Berries and their phenolic compounds have shown promising chemopreventative effects (Duthie 2007, Heinonen 2007) and therefore the tumour preventative effects of four berries, bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), cloudberry (*Rubus chamaemorus*), and white currant (*Ribes x pallidum*) were studied.

Dietary experiments were designed to study

- the effects of diet on β-catenin signalling in the adenomas of Min/+ mice
- the effects of diet on β-catenin signalling in the normal-appearing mucosa of Min/+ mice and their wild-type littermates

Study designs and methods

General descriptions of the studies are presented here. More detailed descriptions of the materials and methods used can be found in the original papers I-IV in the appendix. Neither affymetrix microarrays in the original paper II nor NFκβ signalling in the original paper IV were included in this thesis.

Table 2. General overview of materials and methods in the original publications.

Materials or methods	Original publications
Animals	
C57BL/6J WT mice	IV
C57BL/6J-*Apc*$^{Min/+}$ mice	I-IV
Diets	
Control and inulin	I, IV
Control, wild blueberry, lingonberry and cloudberry	II
Control and white currant	III
Tissues	
Adenomas	I-III
Normal appearing mucosa	II-IV
Western blotting	
β-catenin	I-IV
E-cadherin	I-IV
cyclin D1	I-IV
MMP-9	I
p21	II
p27	II
Immunohistochemistry	
β-catenin	I-IV
E-cadherin	I-II, IV
cyclin D1	I-IV

Animals

The Laboratory Animal Ethics Committee of the University of Helsinki, Finland, approved the study protocols of all experiments (I-IV). Male and female C57BL/6J (wt) and C57BL/6J-*Apc*$^{Min/+}$ (Min/+) mice were bred at the Experimental Animal Unit of the University of Helsinki from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). During the suckling time pups had free access to pelleted standard rodent laboratory chow (Altromin, Ringsted, Denmark) and tap water. Mice were genotyped after weaning by PCR assay (Promega Wizard® Genomic DNA Purification Kit) for the *Apc* allele (Dietrich *et al.* 1993). Both Min/+ and wt were used in Study IV and only Min/+ mice in Studies I-III. At five weeks of age, the animals were stratified by litter and sex and assigned randomly to the control or experimental diets, with 8-15 mice per group, depending on the study protocol and genotype. The mice had free access to the semisynthetic diets and tap water for 3 weeks (IV) or 10 weeks (I-IV). Animals were housed in plastic cages, 3-5 mice together in a temperature- and humidity-controlled facility, with a 12-h light-dark cycle. The welfare of the animals was ensured and the development of body weights was recorded weekly. If mice had a rapid decrease in body weight they were killed and excluded from the experiment.

Diets

Mice were fed modified high-fat AIN93-G diets (Reeves *et al.* 1993) from the age of 5 weeks until the age of 8 (III) or 15 weeks (I-IV). The control diet was a high-fat AIN93-G diet with no added fibre. The experimental diets were similar to the control diet but contained 10% (w/w) inulin (polydisperse β(2-1) fructan, RaftilineHP®; Orafti, Tienen, Belgium) (I, III) or freeze-dried wild blueberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), cloudberry (*Rubus chamaemorus*) (II) or white currant (*Ribes* × *pallidum*) (IV). The diets were isocaloric, containing 41% of their energy from fat, 39% from carbohydrate, and 19% from protein. This means that when eating the same amount of energy, the diets provided similar amounts of fat, carbohydrate, protein, as well as other components of the diets, except for those provided by inulin or berries. The fat content of the diets was similar as in an average Western-type diet so that the ratio between saturated, monounsaturated and polyunsaturated fatty acids was close to 3:2:1. The diets were prepared at the beginning of the feeding period, vacuum-packed in weekly portions, and stored at -20 °C.

Tumour scoring and sample collection

The mice were killed at 8 (IV) or 15 weeks (I-IV) of age by CO_2 inhalation. The intestinal tracts were removed, opened along the longitudinal axis, and washed with ice-cold saline. The small intestine was divided into 5 equal sections. The representative tissue samples of variously sized adenomas and normal appearing mucosa were taken from the distal small intestine and fixed in phosphate-buffered 4% paraformaldehyde solution overnight for histology and immunohistochemistry. Two observers blinded to the dietary treatment scored the number, diameter and location of all adenomas in each section using a dissecting microscope under 67 × magnification. Adenomas in each section were categorized as small (diameter < 1.1 mm), medium (1.1-1.5 mm) or large (> 1.5 mm), excised and pooled together according to the size-category (I, II) for Western analysis. In study III adenomas were not categorized to different size-groups but each mouse had one tissue sample containing all adenomas for Western analysis. The normal-appearing mucosa was then gently scraped off with a microscope slide (III, IV). The adenoma burden per mouse was calculated based on the total number and diameter of adenomas (number x πr2). During the procedure samples were kept on ice and only during the adenoma enumeration at room temperature. Because the intestine was divided into several parts enumeration of each section was quick and sample degradation minimal. This was ensured by analysing samples after several standing times (Latvala, 2005). The smallest detectable adenomas had diameters of 0.3 mm. In unclear situations, possible adenomas smaller than 0.3 mm were removed but were not included in the adenoma sample. The samples were snap-frozen in liquid nitrogen and stored at -70°C for further analysis.

Western analysis

Sample preparation and Western analysis are described in detail in original paper IV in the appendix. Briefly, mucosa (III, IV) or adenomas (I, II) in the distal small intestine were fractionated into nuclear, cytosolic, and membranous pools individually for each mouse. For cellular fractionation various centrifugal forces were used: 15 000 × g for the nuclear, 100 000 × g for cytosolic, and Triton X-100 + 100 000 × g for the membranous fraction. All fractions were concentrated using Amicon Ultra-4 Centrifugal Filter Devices (Millipore, Bedford, MA, USA). The purity of the cellular fractions was controlled by determining nuclear lamin B (Sc-6216, Santa Cruz Biotechology, Santa Cruz, CA, USA) levels in the cellular fractions. Both the cytosol and membrane fractions were free of lamin B. We had ensured earlier that our mucosa samples were practically free of COX-2 that is expressed mainly in adenoma tissue. For Western analysis analyses the following primary antibodies were used: anti-β-catenin [Sc-7199, Santa Cruz Biotechology, Santa Cruz, CA, USA (I-IV)], anti-cyclin D1 [Zymed, San Francisco, CA, USA (IV) or RM-9104, NeoMarkers, Fremont, CA, USA (I-IV)], anti-E-cadherin [610182, BD Transduction, San Diego, CA, USA (I-IV)], anti-MMP-9 [M9555, rabbit polyclonal, Sigma-Aldrich Inc, St. Louis, MO, USA (I)], anti-p21 [Sc-397, Santa Cruz Biotechology (II)] and anti-p27 [Sc-528, Santa Cruz Biotechology (II)]. Equal loading of samples was ensured by incubating the blots with β-actin antibody (A5441, Sigma-Aldrich). Blocking peptides, immunoprecipitation, other commercially available antibodies or normal serum were used to ensure detection of the right bands (data not shown). The results are expressed as sample band intensity (optical density of protein band multiplied by band area) divided by intensity of the positive control.

Immunohistochemistry

The fixed tissues were dehydrated, embedded in paraffin, cut in serial 5-µm sections and mounted on slides. Circa 3 sections per tissue sample, enclosing characteristics of adenomatous areas, were selected for immunohistochemisty (IHC) and two for histology. For immunohistochemistry the endogenous peroxidase activity of deparaffinised and rehydrated sections was quenched by H_2O_2. The slides were rinsed in Tris-buffered saline, and an antigen retrieval step was carried out in a microwave oven for 15 min in citrate buffer, pH 6.0. Immunostaining with anti-β-catenin (BD Transduction), anti-E-cadherin (BD Transduction), anti-cyclin D1 (Zymed (III) or NeoMarkers (I, II, IV) was performed using a PowerVision™ Homo-mouse IHC Detection Kit (KDM-7DAB, ImmunoVision Technologies Company, Brisbane, CA, USA) or UltraVision Detection System anti-rabbit, HRP/DAP (Lab Vision Corporation, Fremont, CA, USA). Negative control tissues were prepared in the same manner, except that the primary antibody was replaced with a negative control for the mouse IgG2a Ab-1 (NeoMarkers) or rabbit IgG Ab-1 (NeoMarkers). All immunohistochemical sections were counterstained with Mayer's hemalaum (Merck, Darmstadt, Germany). For histology, the deparaffinized and rehydrated sections were stained with hematoxylin and eosin (H&E).

Immohistochemical and histological evaluation

Two observers blind to the dietary treatment evaluated the IHC stainings. In Studies I and II β-catenin, E-cadherin, and cyclin D1 proteins in adenomas were scored semiquantitatively based on the staining intensity (grade 0 = negative; grade 1 = weak; grade 2 = moderate; grade 3 = strong) and distribution. The percentage of cells with membranous or cytosolic positivity was graded as follows: 0 (<5 %), 1 (5-30%), 2 (30-60%), 3 (> 60%), and nuclear positivity: 0 (< 5%), 1 (5-15%), 2 (15-50%), 3 (> 50%). A total score was obtained for each case by multiplying the 2 respective scores. Staining for β-catenin and E-cadherin proteins in the normal-appearing mucosa of Study II were scored separately in the crypt and villus compartments on the basis of distributions and relative staining intensities. Scales ranged between 0 (no staining) and 5 (very strong staining), at 0.5-units intervals. A scale for staining intensity of cyclin D1 ranged between 0 (no staining) and 3 (very strong staining). In Studies III and IV staining in the normal-appearing mucosa was scored on the basis of distributions and relative staining intensities. A scale for staining intensity ranged between 0 (no staining) and 3 (very strong staining). Histology of the adenomas in Study I was assessed from H&E stained sections by veterinary pathologist Jere Linden, University of Helsinki, blinded to the dietary treatment. Special emphasis was placed on dysplasia (growth pattern and differentiation of the neoplastic epithelium) as well as cellular anaplasia (nuclear and cellular morphology and nucleus to cytoplasm ratio) of the adenomas. These were both graded separately according to the following five-tier system: grade 1 = minimal; grade 2 = mild; grade 3 = moderate; grade 4 = marked; grade 5 = severe. The number of adenomas (adenomatous areas) in the studied tissue samples ranged from one to seven; the most pronounced changes in each sample were used for grading.

Statistics

The results are expressed as the median (min-max). *P* values of 0.05 or below were considered statistically significant. The differences in adenoma number and size between the control and experimental groups were analyzed with the Mann-Whitney U test. Spearman correlation was used for correlation analysis, the Wilcoxon signed rank test for paired comparisons, and the Chi-square test for the IHC data. Statistical analyses were performed with StatView software (StatView, version 5.0.1, SAS Institute Inc., Cary, NC). In Study I data from individual mice were combined to present the average of the treatment group as one curve after a log transformation. The separate curves were statistically tested with linear mixed models for repeated measures data to observe if the effect of diet on the measured parameters (β-catenin, E-cadherin, cyclin D1, MMP-9) was similar in all size-categories (small, medium, and large adenomas). Mixed models takes into account the correlation of the measurements from individual mice. Additionally, the difference between the adenoma size-groups was tested. Explanatory variables included diet, adenoma size and the number of adenomas in different size groups. Statistical analyses were performed with SAS software (SAS, version 8.2).

Results

This section describes how tumour promotive or preventive diets changed the adenoma formation as well as cellular β-catenin signalling in the adenomas ($Apc^{-/-}$) and in the surrounding mucosa ($Apc^{+/-}$). The main focus was in the expression of β-catenin in different cell compartments. Cyclin D1, the target of β-catenin involved in cell cycle progression, and E-cadherin, the membranous protein that in close relationship with β-catenin forms adhesion complexes, as well some other cell signalling parameters relevant for ongoing study were investigated.

Generally, mice grew well in all studies. The body weights were recorded regularly and the final body weights in the treatment groups were similar in each study. Adenoma numbers or sizes did not differ between male and female mice and, therefore, the data from both sexes was pooled in the results. Most (80%) of the adenomas developed into the distal part of the small intestine and this was therefore used for biochemical analyses.

β-catenin signalling in the adenomas of Min/+ mice

The main aim was to see if dietary modifications are able to influence β-catenin signalling in the adenomas of Min/+ mice and if the effects of the tumour promotive (I) and preventive (II, III) diets were opposite.

Tumour promotion by inulin diet (I)

It is earlier shown that dietary inulin promotes adenoma growth in Min/+ mice (Mutanen *et al.* 2000, Pajari *et al.* 2003) and in this study inulin diet was used as a tumour promotive diet. Now we studied whether inulin-induced adenomas were merely larger than the controls or whether the β-catenin signalling changed in the adenomas during their growth. As little as 2.5% inulin in the diet can promote adenoma growth in Min/+ mice (Mutanen *et al.* 2000), but a higher amount (10%) was chosen to enable clearer detection of differences in molecular biological analyses. As expected, inulin feeding increased the size of the small intestinal adenomas of Min/+ mice (Figure 6).

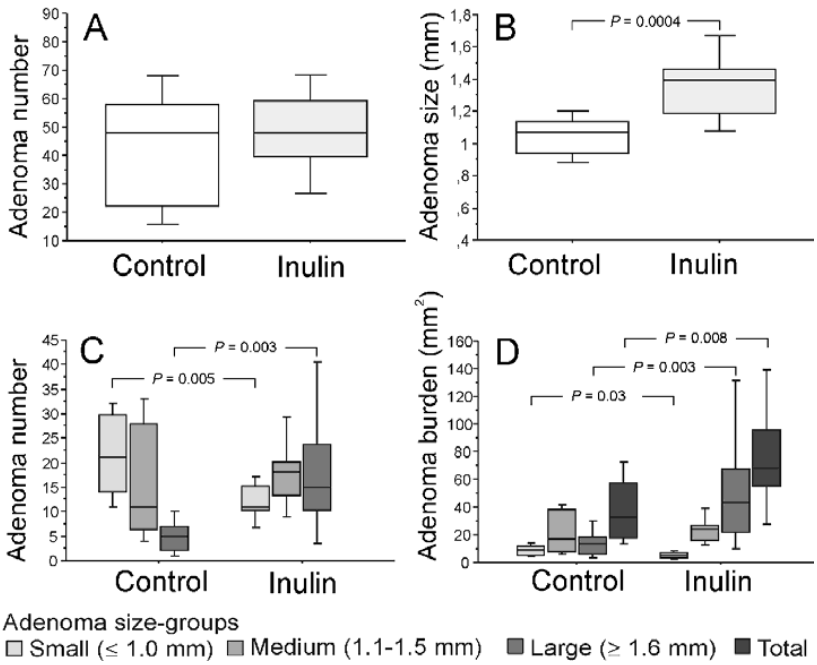

Figure 6. (A) The total number of adenomas in the distal small intestine was similar in both treatment groups, but (B) inulin feeding increased the size (mm) of the adenomas. (C) The majority of adenomas in the inulin group were large and, therefore, (D) the adenoma burden (mm^2) was bigger than in the same category of the control group. *P* is in comparison to the control group by the Mann-Whitney test.

We divided the adenomas of each mouse into 3 size categories: small (diameter < 1.1 mm), medium (1.1-1.5 mm) and large (> 1.5 mm) to be able to follow the cellular signalling as adenomas enlarge. The categories were based on our long-term experience with Min/+ mice but comparable size groups can be found in the literature (Ju *et al.* 2005, Tucker *et al.* 2005, Issa *et al.* 2007). The inulin feeding produced larger adenomas than the control feeding. On the other hand, 50% of the adenomas in the control group remained small, in contrast to 25% in the inulin group. We calculated the adenoma burden as a sum of the areas (πr^2) of all the adenomas for each mouse and found that the adenoma burden was increased by 100% in the inulin group compared with the control group. Histologically adenomas were remarkably similar, regardless of their size or the treatment of the mice (Figure 7, H&E).

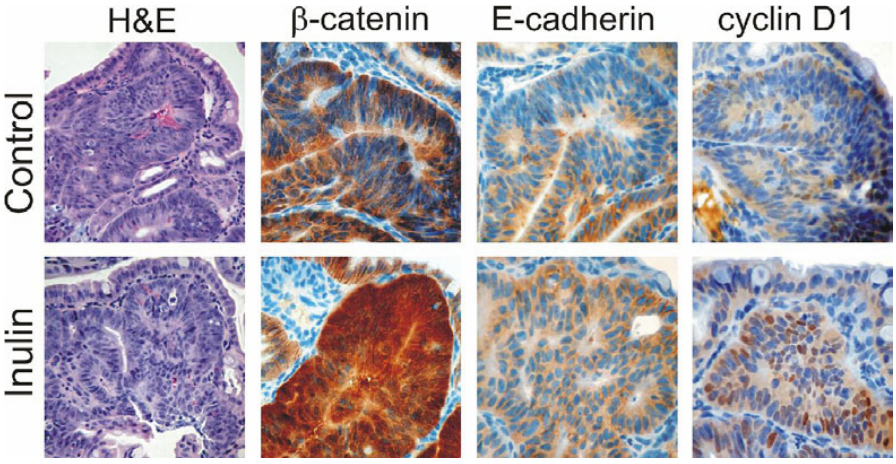

Figure 7. Representative H&E staining and immunohistochemical staining of β-catenin, E-cadherin, and cyclin D1 in the large adenomas of control or inulin-fed mice. Positive cells show dark staining, magnification is 400 ×.

We analysed the level and localisation of cell-signalling proteins in adenomas of three size-categories to determine whether diets regulate the molecular characteristics of adenomas differently. In all size-categories the relevant β-catenin signalling proteins were determined individually for all mice. The diets had different effects on β-catenin signalling as adenomas enlarged, which is presented in Table 3 and in detail in Figures 7, 8 and 9.

Table 3. Effects of diet on β-catenin, E-cadherin, cyclin D1, and MMP-9 during adenoma growth were tested, using linear mixed models for repeated measures data (*P* values are given). Specific diet effects were found for β-catenin, E-cadherin, and cyclin D1 (diet*size interaction $P < 0.05$). The inulin diet increased the amount of nuclear β-catenin ($P = 0.004$) and also membranous β-catenin ($P < 0.001$) and E-cadherin ($P = 0.003$) as the adenomas enlarged. No specific diet effects were found for nuclear cyclin D1, cytosolic β-catenin and membranous MMP-9 and therefore they are not shown in the table.

		β-catenin nucleus	**β-catenin** membrane	**E-cadherin** membrane	**cyclin D1** cytosol
Diet-specific interactions					
Diet*size interaction		0.036	0.012	0.009	0.019
Difference between the diets					
	S	0.031	0.047	0.072	ns.
	M	ns.[b]	ns.	ns.	ns.
	L	ns.	0.013	ns.	ns.
Difference between the adenoma size-groups					
Control diet:	S-M-L[a]	ns.	0.026	ns.	ns.
	S-M		0.019		
	S-L		ns.		
	M-L		ns.		
Inulin diet:	S-M-L	0.004	< 0.001	0.003	< 0.001
	S-M	0.064	< 0.001	0.004	< 0.001
	S-L	0.001	< 0.001	0.012	0.010
	M-L	ns.	ns.	ns.	ns.

[a] Adenoma size-groups are as follows: S = small, M = medium, L = large adenomas.
[b] ns. = not significant

During the adenoma growth, inulin diet, but not the control diet, increased the amount of nuclear β-catenin and the amount of its target protein cyclin D1 was also increased (Figure 8). Nuclear β-catenin and cyclin D1 were also strongly correlated ($r = 0.818$, $P < 0.0001$). Based on the statistical analysis (Table 3, linear mixed models for repeated measures data), the inulin diet had a specific effect on β-catenin. This suggests that the increased β-catenin seen in the inulin group was not only due to bigger adenomas in the group, but the inulin diet specifically influenced the level of β-catenin.

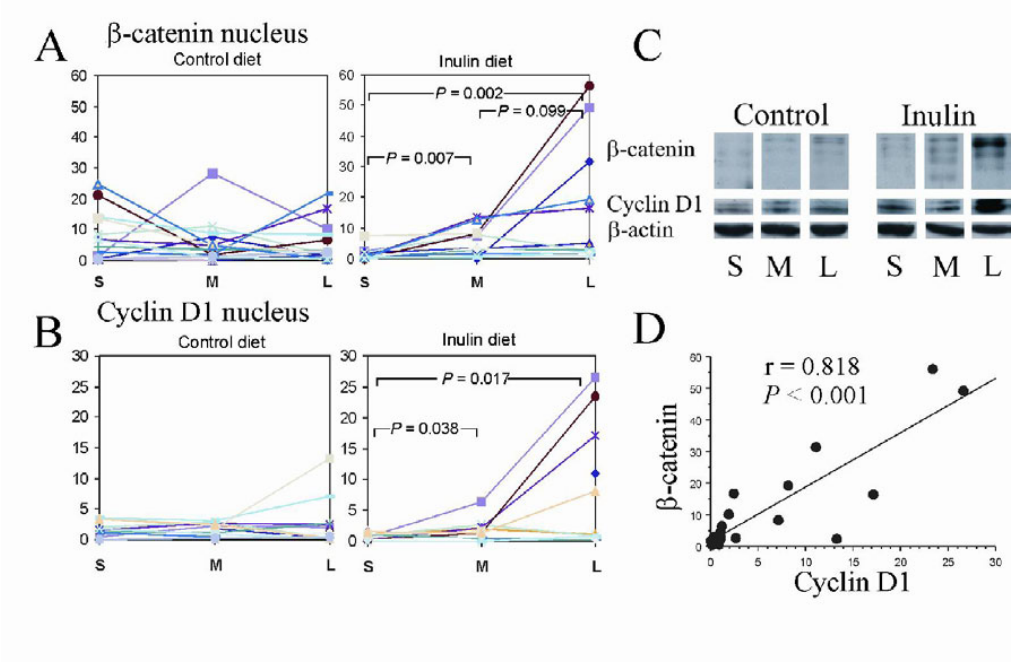

Figure 8. The inulin diet increased the level of β-catenin and cyclin D1 as the adenomas enlarged. (A) Amount of β-catenin and (B) cyclin D1 in adenoma size-groups analysed by Western analysis. For the control (n=15) and inulin (n=13) groups, the results are presented separately for each mouse and the lines show the change during adenoma growth. Each datapoint is a pooled sample that consists of all adenomas the mouse had in the size-group (approx. 10-30 adenomas). The difference between the adenoma size-groups was tested with the Wilcoxon signed rank test (*P* values). The adenoma size-groups are as follows: S = small, M = medium, L = large. (C) In the representative immunoblot of β-catenin and cyclin D1 groups the median intensities are presented. (D) Nuclear β-catenin and cyclin D1 were strongly correlated; presented here is the correlation seen in the large adenomas.

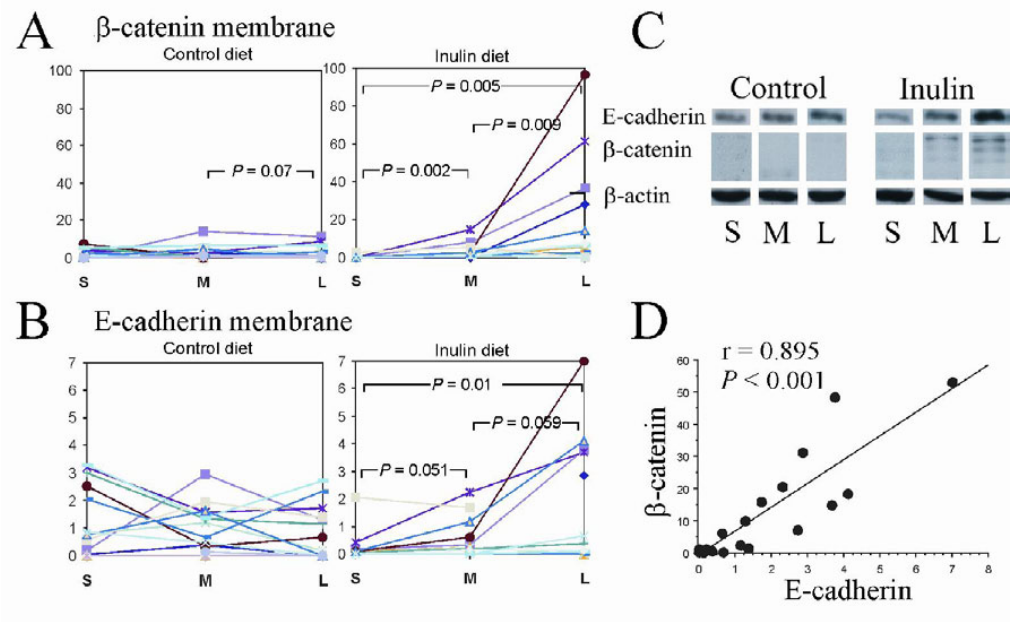

Figure 9. Increased levels of membranous β-catenin and E-cadherin were seen during inulin induced adenoma growth. (A) The amount β-catenin and (B) E-cadherin is presented separately for each mouse and *P* values are the differences between the adenoma size-groups tested by the Wilcoxon signed rank test. (C) The median intensities are presented in representative immunoblots. (D) Membranous β-catenin and E-cadherin had strong correlation in the large adenomas.

We wanted to see if inulin induced adenomas had adopted some malignant properties. Matrix metalloproteinases are related to progression toward malignant metastatic phenotypes (Zucker & Vacirca 2004, Mook *et al.* 2004), and in Affymetrix microarray analysis we have seen that the small intestinal adenomas of Min/+ mice express 20 times more MMP-9 gene than the normal appearing mucosa (Mutanen *et al.*, unpublished observation). MMP-9 could be involved in malignancy also in Min/+ mice and therefore we analysed the level of MMP-9 in different size-categories of adenomas. Neither of the diets changed the amount of MMP-9 as adenomas enlarged. This observation together with the histological similarity seen in H&E staining suggests that adenomas in both diet groups were benign.

The inulin diet, but not the control diet, increased the levels of membranous β-catenin and E-cadherin during adenoma growth (Figure 9) and these proteins had also a strong correlation with each other ($r = 0.895$, $P < 0.001$).

Tumour prevention by bilberry, lingonberry and cloudberry diets (II)

The chemopreventive effects of berries and whether they effected β-catenin signalling during adenoma growth were studied from bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), and cloudberry (*Rubus chamaemorus*). These berries were chosen as they are among the main berries consumed in Finland and they also possess different compositions of phenolic compounds. The major phenolics in bilberry, lingonberry, and cloudberry are anthocyanins, proanthocyanidins and ellagic acid, respectively (Määttä-Riihinen *et al*. 2004a, Määttä-Riihinen *et al*. 2004b).

All three berries provided potent protection against adenoma formation in *Min/+* mice as they decreased the number of adenomas in the small intestine (Figure 10). No differences were found between the groups in the number of colon adenomas. Cloudberry and lingonberry also reduced the size of adenomas and the adenoma burden in the distal small intestine by 60%. Lingonberry and cloudberry diets restricted the growth of the adenomas so that most of the adenomas stayed small ($\leq$ 1.0 mm). They had also less medium size adenomas (1.1-1.5 mm) than the control group and only a few adenomas proceeded to large ($\geq$ 1.6 mm). In the bilberry group the bigger proportion of adenomas grew large and despite the decreased number of adenomas the tumour burden did not differ from the control group. When the analysis for cellular signalling was done, medium size and large adenomas had to be combined (small adenomas = diameter $\leq$ 1.0 mm, large adenomas = diameter $\geq$ 1.1 mm). The reason for this was that berry diets produced few large adenomas and did not provide enough material for Western blotting.

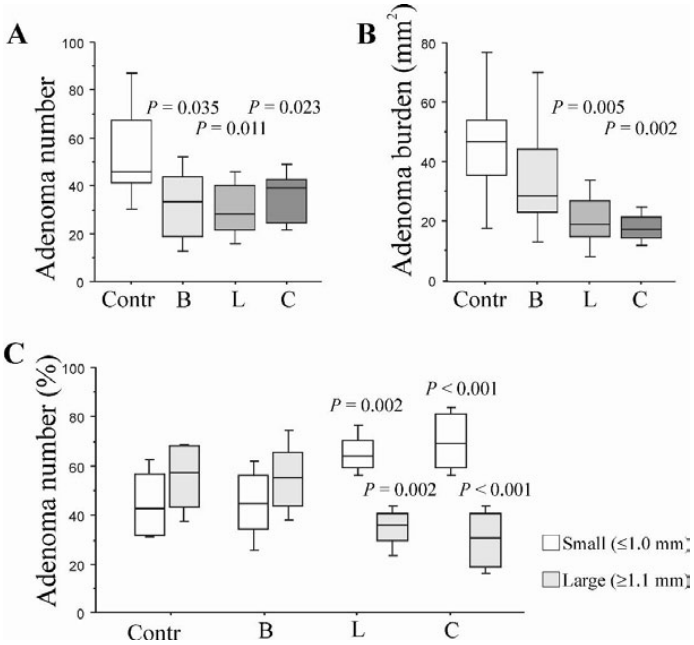

Figure 10. (A) Total number, (B) size (mm) and (C) burden (mm^2) of adenomas in the distal small intestine of 15-week-old Min mice fed either a control diet (n = 10) or diets containing 10% (w/w) freeze-dried bilberry (n = 12), lingonberry (n = 11), or cloudberry (n = 12). *P* is in comparison to the control group by the Mann-Whitney test. Treatment groups as follows: Contr = control, B = bilberry, L = lingonberry, C = cloudberry.

In the inulin study (I) we found that the tumour promotive diet increased the levels of nuclear β-catenin and cyclin D1 during the adenoma growth. Here, with the tumour preventive diets, we wanted to see whether the opposite effects could be found. The cloudberry diet had the strongest inhibitory effect on the adenoma growth which could be explained by the diminished β-catenin signalling. Compared with the control the cloudberry group had less nuclear β–catenin and nuclear cyclin D1 in the large adenomas (Figures 11 and 12).

The results suggest that decreased activity of β-catenin suppresses the expression of cyclin D1 as the levels were also strongly correlated ($r = 0.831$, $P < 0.001$). The levels of β-catenin and cyclin D1 correlated significantly with the tumour burden (Figure 11), strongly suggesting that in the tumour tissue these proteins are associated with the growth of the tumours and are one reason for the decreased adenoma burden seen with the cloudberry diet. The lingonberry diet also decreased the adenoma burden but it had an effect only on nuclear cyclin D1, not on β-catenin. The bilberry and control diets produced comparable adenoma burdens and levels of nuclear β-catenin and cyclin D1 in large adenomas (Figure 12).

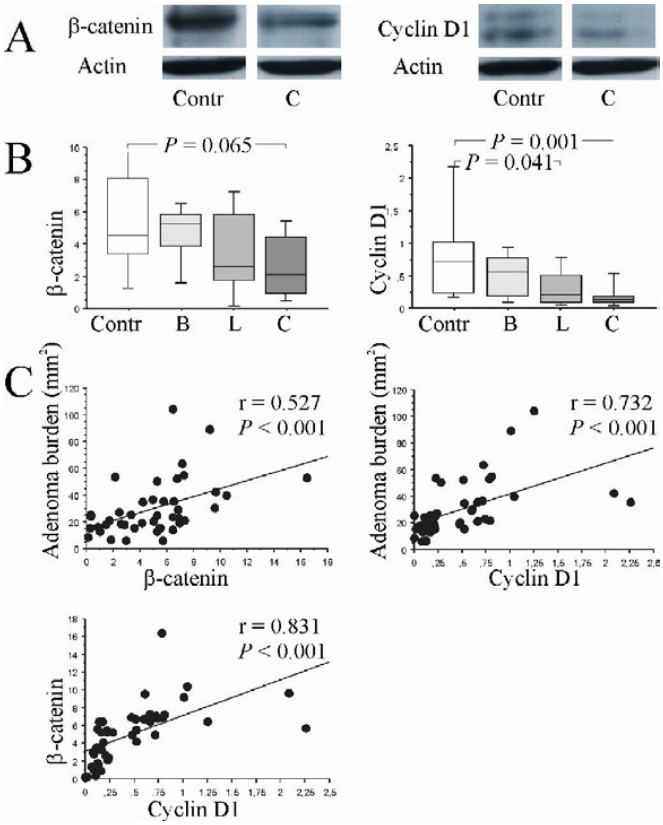

Figure 11. The cloudberry diet decreased the levels of nuclear β-catenin and cyclin D1 in the large adenomas. (A) In a representative immunoblot of β-catenin and cyclin D1 the median intensities are presented. (B) In addition to the cloudberry diet, the lingonberry diet also decreased the level of nuclear cyclin D1. (C) β-catenin and cyclin D1 correlated with the adenoma burden and with each other.

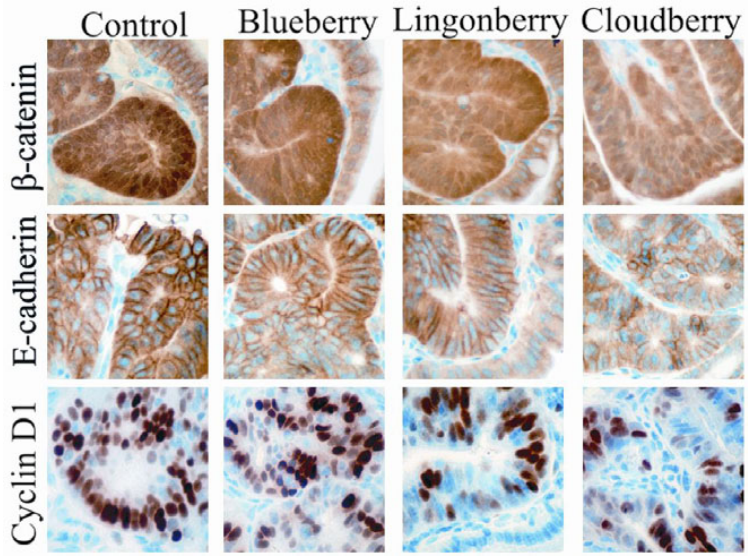

Figure 12. Representative immunohistochemical staining of β-catenin, E-cadherin, and cyclin D1 in the large adenomas of control or berry-fed mice. Positive cells show dark staining, magnification is 400 ×.

To further elucidate the role of β-catenin and cyclin D1 in adenoma growth, we analysed small and large adenomas separately. The results showed that the levels of nuclear β-catenin and cyclin D1 increased several fold from the small to large adenomas in the control, bilberry and lingonberry groups (Figure 13). However, cloudberry prevented the increase in nuclear β-catenin in the large adenomas and actually maintained it at the level found in the small ones [2.47 (0.00-5.85) vs. 2.10 (0.25-6.57) in the large adenomas, $P = 0.999$]. Cloudberry also inhibited the increase in nuclear cyclin D1 although it did not abolish it altogether (Figure 13). It is noteworthy that the variation in cyclin D1 values in large adenomas in the cloudberry group was considerably less than in the other groups.

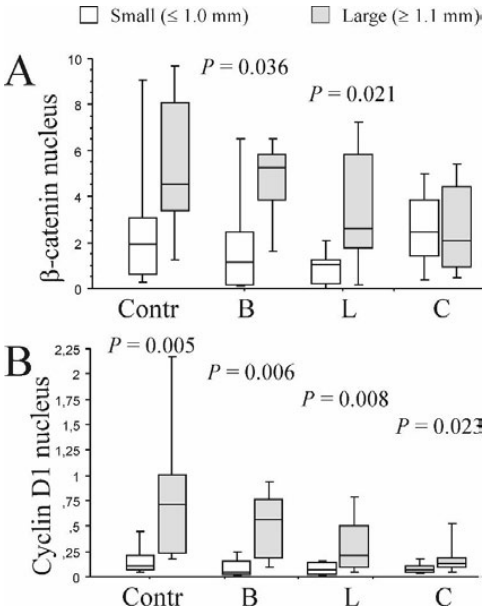

Figure 13. (A) Cloudberry diet inhibited the increase in nuclear β-catenin and (B) only a minor increase was found in nuclear cyclin D1 during the adenoma growth. Difference between the small and large adenomas was tested by Wilcoxon signed rank test (*P* values).

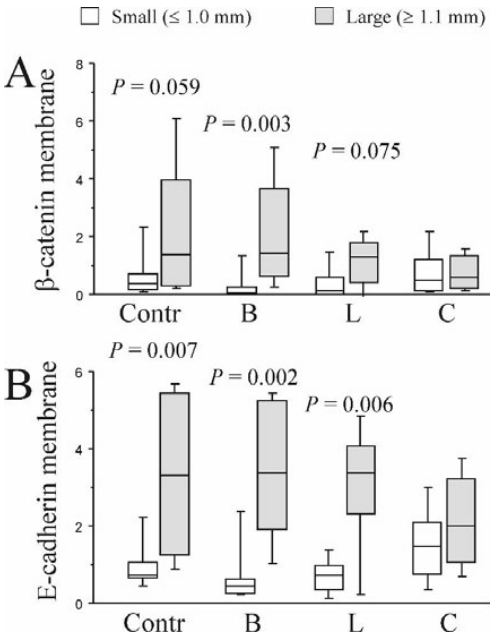

Figure 14. (A) Levels of membranous β-catenin and (C) E-cadherin increased as adenomas grew from small to large. (B) Cloudberry diet inhibited the increase in membranous β-catenin and (D) E-cadherin during the adenoma growth. Difference between the small and large adenomas was tested by Wilcoxon signed rank test (*P* values).

With inulin we found that tumour promotion increased the levels of membranous β-catenin and E-cadherin as adenomas enlarged. Compared with the control group the cloudberry group had less membranous β-catenin [0.56 (0.03-1.36) vs. 1.76 (0.13-4.96) in the controls, $P = 0.056$] in the large adenomas. As we analysed small and large adenomas separately, we found that the levels of membranous β-catenin and E-cadherin increased significantly as adenomas enlarged in the control, bilberry and lingonberry groups (Figure 14). Differing from the other treatments, the cloudberry diet maintained subcellular levels of β-catenin and E-cadherin that were unaltered between the small and large adenomas (Figure 14). A similar effect was not seen with other diets.

In addition to results reported in original paper II, regulators of the cell cycle p21 and p27 were analysed from the cytosolic fractions. The levels of p21 or p27 did not explain the adenoma growth as only in the bilberry group was p21 slightly increased during adenoma growth [0.72 (0.47-2.39) in the small adenomas vs. 1.49 (0.59-4.17) in the large adenomas, $P = 0.012$]. MMP-9 was not measured in this study as the adenomas of berry-fed mice seemed to be more quiescent than the adenomas of inulin fed mice on the earlier study (I) and even there the level of MMP-9 stayed constant.

Tumour prevention by white currant diet (III)

The original aim of the study of preventative effects of white currant was that we chose to use this colourless berry as a negative control that would not affect tumour formation in Min/+ mouse. White currant contains only very low levels of phenolic compounds, at least compared to bilberry, lingonberry and cloudberry (Häkkinen *et al*. 1999, Määttä *et al*. 2001). Nevertheless, the white currant diet decreased the number and size of the adenomas so that there was 65% reduction in the adenoma burden in the distal small intestine (Figure 15).

The lower adenoma burden in the white currant group was associated with decreased levels of nuclear β-catenin in the adenomas (Figure 16). The result was similar to that obtained in the cloudberry group in the previous study (II). We assumed that the level of nuclear cyclin D1 could also be affected by the white currant diet but due to a lack of sample material for Western analysis we could perform only the immunohistochemical staining of adenomas. The staining revealed a decreasing trend for nuclear cyclin D1 in the white currant group.

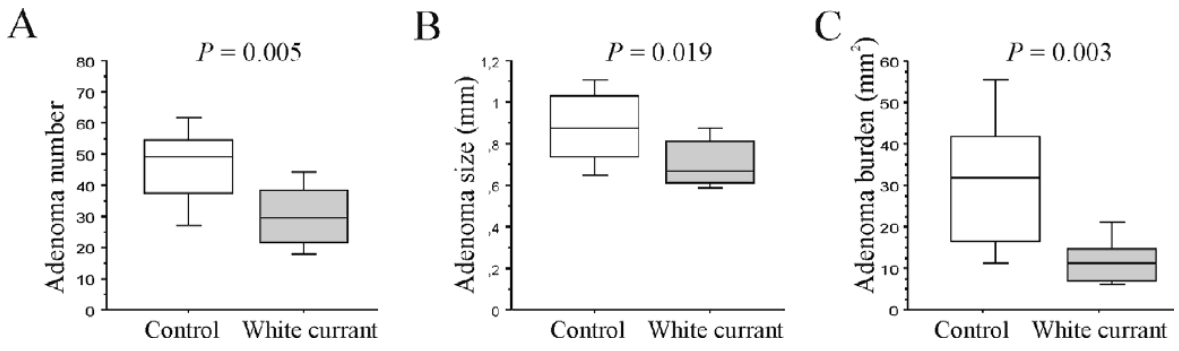

Figure 15. (A) Total number, (B) size (mm) and (C) area (mm^2) of adenomas in the distal small intestine of 15-week-old Min mice fed either a control diet (n = 11) or a diet containing 10% (w/w) freeze-dried white currant (n = 12). *P* is in comparison to the control group by the Mann-Whitney test.

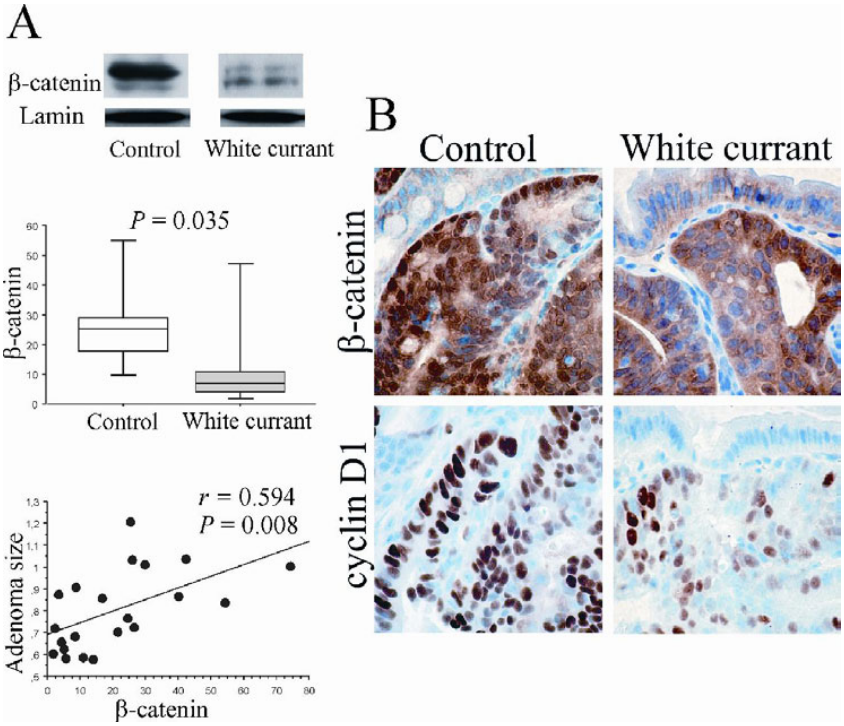

Figure 16. (A) White currant feeding decreased the level of nuclear β-catenin in the adenomas and nuclear β-catenin correlated with the size of the adenomas. (B) Immunohistochemical staining also revealed the decrease in nuclear cyclin D1 in the adenomas of the white currant group. Positive cells show dark staining, magnification is 200 ×.

Once more we wanted to evaluate the role of membranous β-catenin in tumour prevention. This time adenomas were not divided into different size groups but all adenomas were pooled and analysed together. We found the same effect as with the cloudberry: the level of membranous β-catenin was decreased by the tumour preventive white currant diet [1.28 (0.18-22.30) vs. 8.26 (2.40-12.00), *P*=0.012)]. This strengthens our observations of earlier studies.

β-catenin signalling in the normal appearing mucosa of Min/+ and WT mice

The main objective of these studies was to see whether tumour promotive (IV) or preventive (II, III) diets influenced the normal appearing mucosa ($Apc^{+/-}$) surrounding the adenomas. It is of great importance to elucidate cell signalling events in the surrounding mucosa that influence the initiation of the tumourigenic process. We hypothesised that dietary components could change the cellular integrity in the mucosa, the levels of β-catenin in the cell membranes and further the activity in the nucleus.

Effect of the tumour promotive inulin diet (IV)

In Study IV the influence of the tumour promotive inulin diet on the normal appearing mucosa was tested. Min/+ mice were fed for 3 or 10 weeks with the control and inulin diets (Figure 17). The adenoma data at the age of 8 weeks was not presented earlier but is introduced here. The adenomas of Min/+ mice were bigger in the inulin group. At the age of 8 weeks the inulin group had relatively more medium size adenomas and less small adenomas than the control group. The proportion of large adenomas was similar. At the age of 15 weeks the proportion of large adenomas was bigger in the inulin group. This indicates that inulin accelerated the growth of some adenomas while others stayed small.

The promotion of adenoma growth in the inulin group was associated with increased level of cytosolic cyclin D1 in the normal appearing mucosa of Min/+ mice (Figure 18). The most probable reason for that was activation by nuclear β-catenin. Inulin feeding seemed to reduce the levels of membranous β-catenin and E-cadherin (Figure 18) and lead to the accumulation of cytosolic and nuclear β-catenin. The β-catenin signalling in adenomas at 15 weeks is presented in study I (p. 33).

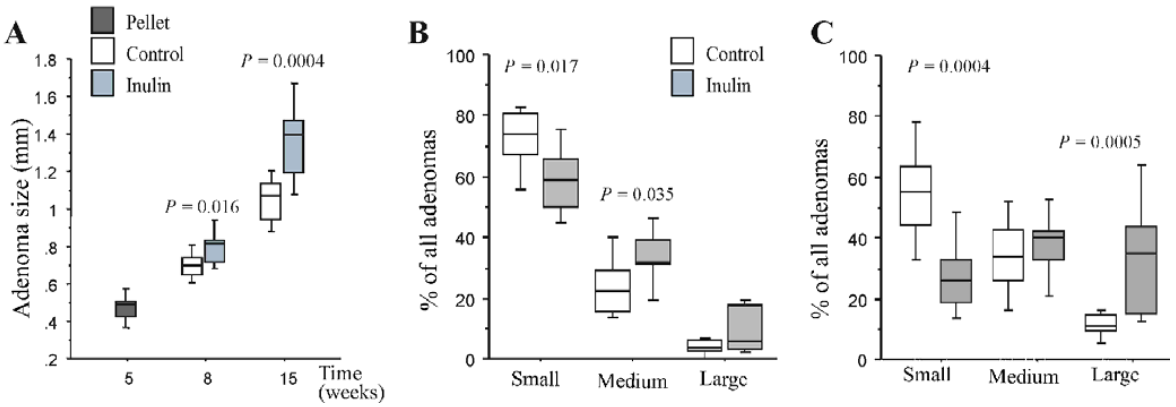

Figure 17. The Min/+ mice (n = 10-15 per group) were fed either a non-fibre control diet or 10 % inulin diet from the age of 5 weeks until the ages of 8 or 15 weeks. (A) Adenomas were larger in the inulin group at both time points. (B) At the age of 8 weeks the proportion of small adenomas in the inulin group was much lower than in the control group and a larger proportion of adenomas had reached the medium size. (C) By the age of 15 weeks inulin had induced the growth of the adenomas so that the proportion of large adenomas was nearly three-fold compared to the control group. The proportion of medium size adenomas remained unchanged but the proportion of small adenomas was decreased to half. *P* is in comparison to control mice of the same age by Mann-Whitney test.

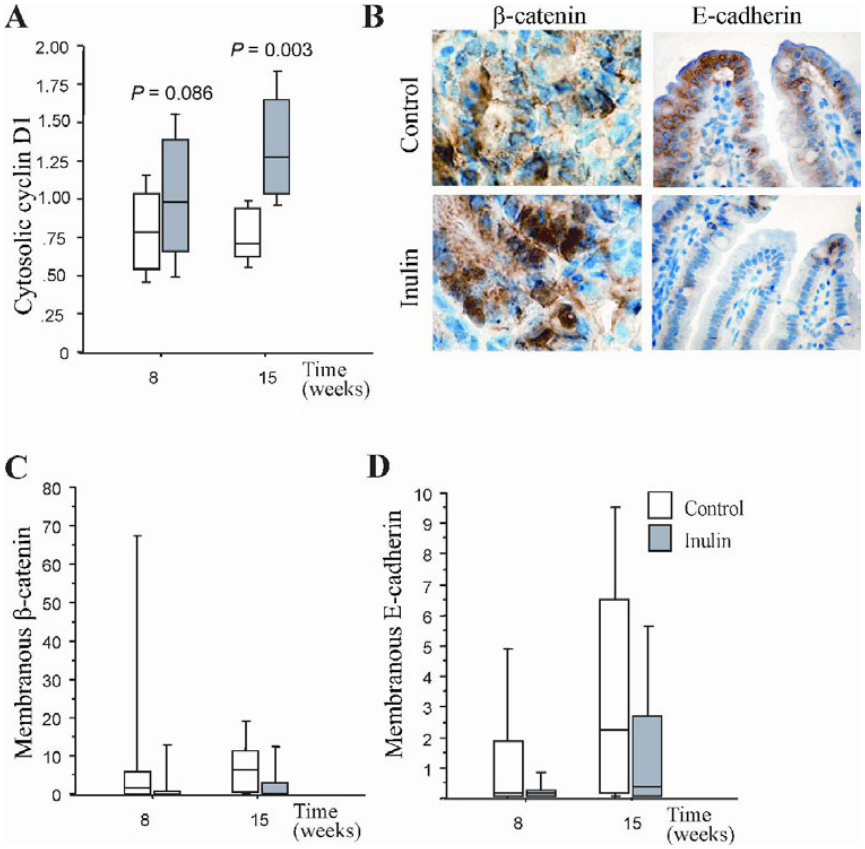

Figure 18. (A) The level of cytosolic cyclin D1 was increased in the normal appearing mucosa of the inulin group. (B) Immunohistochemical staining showed that inulin feeding reduced the levels of membranous β-catenin and E-cadherin and induced nuclear accumulation of β-catenin. (C, D) Similar trends were found by Western analyses. Positive cells show dark staining, magnification for β-catenin 400 × and E-cadherin 100 ×. *P* is in comparison to control mice of the same age by Mann-Whitney test.

The influence of the inulin diet on the normal appearing mucosa was also tested in wild-type (WT) mice. These were fed either with the control or inulin diet from the age of 5 weeks until the age of 8 weeks. WT mice did not develop intestinal adenomas. In the WT mice, that have no *Apc* mutations, the inulin induced drop in membranous β-catenin was clear (Figure 19). In addition, a subset of crypts also had enhanced nuclear β-catenin staining.

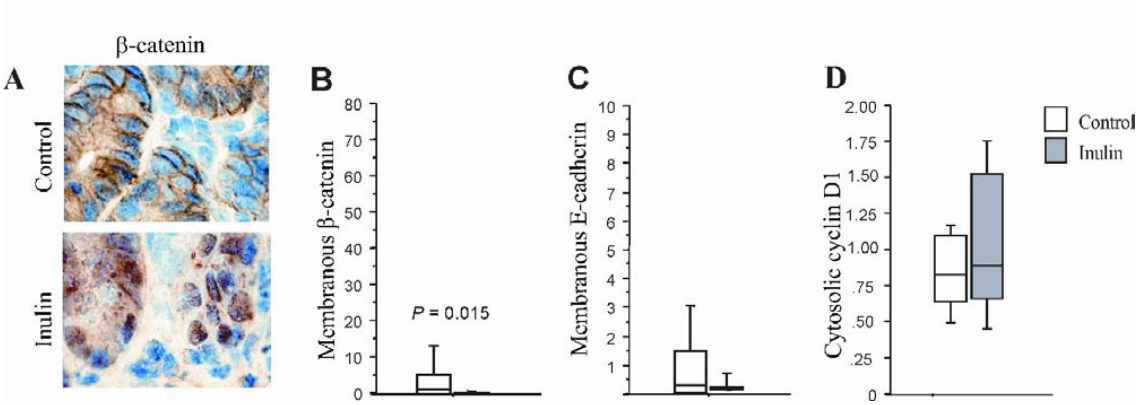

Figure 19. Wild-type mice were fed with the control or inulin diets for 3 weeks (n = 8-9 per group). (A) The control fed mice had clear membranous staining for β-catenin. Positive cells show dark staining, magnification 400×. (A, B) Inulin feeding caused a reduction in membranous β-catenin, while subsets of crypts had nuclear staining of β-catenin. (C) There was also trend for membranous E-cadherin but (D) not for cytosolic cyclin D1.

Effect of the tumour preventive berry diets (II, III)

As tumour promotion by dietary treatment was able to modify β-catenin signalling in normal appearing mucosa, the main objective of these studies was to see if tumour prevention by berries can also influence the same parameters but in reverse direction. The decreased number and size of the adenomas in bilberry, lingonberry, and cloudberry groups is presented in Figure 10. In addition to the analysis of the adenoma tissues (II) also the normal appearing mucosa was used for immunohistochemistry. The expression of β-catenin, E-cadherin, and cyclin D1 were scored separately from the crypts and villus compartments (Figure 20).

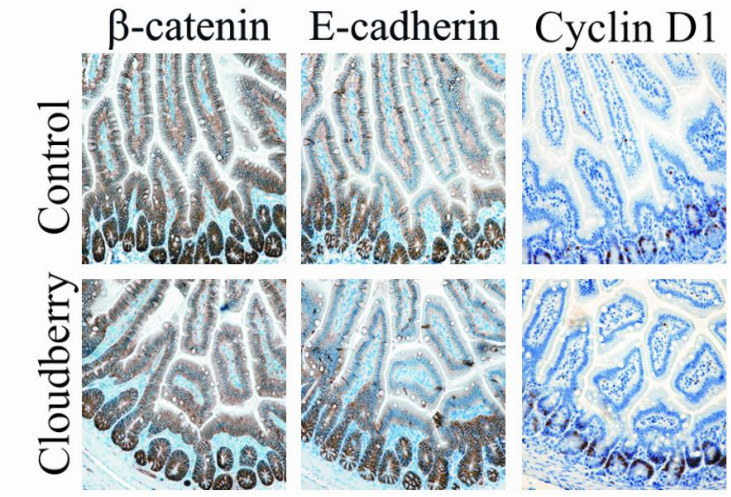

Figure 20. Representative immunohistochemical staining for β-catenin, E-cadherin, and cyclin D1 in the normal appearing mucosa of control or cloudberry-fed mice. Cloudberry was chosen here as tumour prevention was the best among berry groups. However, no difference was found between the treatment groups. Magnification is 100×.

The inhibition in adenoma formation was not related to changes in subcellular levels of β-catenin, E-cadherin, and cyclin D1 in mucosal tissue. No differences between the diets were observed in either the crypts or the villus compartments (Table 4). The intensities of β-catenin and E-cadherin were stronger in the crypt than in the villus in all subcellular fractions. Staining for cyclin D1 was predominantly nuclear in the proliferation zone of the crypt, while the villus had no cyclin D1 staining.

Table 4. Immunohistochemical staining intensities (relative units) for β-catenin, E-cadherin and cyclin D1 in the normal appearing mucosa of Min/+ mice after bilberry, lingonberry and cloudberry diets, median (min-max).

			Villus	
	Control (n=10)	**Blueberry (n=12)**	**Lingonberry (n=11)**	**Cloudberry (n=12)**
β-catenin,				
- cytoplasmic	2.4 (1.5-2.8)	2.1 (1.8-3.0)	2.3 (1.8-2.8)	2.3 (1.5-2.8)
- nuclear	1.6 (0.3-2.3)	1.3 (1.0-1.8)	1.8 (0.0-2.0)	1.5 (0.8-2.3)
- membranous	1.9 (0.8-2.5)	1.8 (0.5-2.8)	1.8 (0.5-2.3)	1.8 (0.8-3.0)
E-cadherin,				
- cytoplasmic	1.3 (0.5-1.8)	1.3 (0.5-2.0)	0.8 (0.5-1.8)	1.3 (0.5-2.0)
- membranous	0.4 (0.0-1.5)	0.6 (0.0-2.3)	0.5 (0.0-1.8)	0.8 (0.0-2.0)
			Crypt	
	Control (n=10)	**Blueberry (n=12)**	**Lingonberry (n=11)**	**Cloudberry (n=12)**
β-catenin,				
- cytoplasmic	2.5 (2.3-2.8)	2.5 (2.3-2.8)	2.5 (2.0-3.0)	2.5 (2.3-2.8)
- nuclear	2.6 (2.0-3.3)	2.8 (2.0-3.0)	2.3 (2.0-3.0)	2.5 (2.0-3.0)
- membranous	3.1 (2.8-3.5)	3.0 (3.0-3.8)	3.3 (2.8-3.5)	3.0 (0.8-2.5)
E-cadherin,				
- cytoplasmic	2.0 (1.8-2.3)	2.0 (1.5-2.3)	2.0 (1.5-2.3)	2.0 (1.5-2.0)
- membranous	2.4 (1.8-3.0)	2.5 (1.5-3.0)	2.3 (1.3-2.8)	2.3 (1.8-2.8)
Cyclin D1,				
- nuclear	2.0 (1.0-3.0)	2.0 (1.0,3.0)	2.0 (1.0,3.0)	2.5 (2.0,3.0)
- thickness of stained cell layer (cells)	9 (6-12)	10 (7-10)	9 (8-11)	11 (8-12)
- % of stained cells/crypt	50% (20-70%)	50% (20-60%)	50% (30-70%)	50% (30-60%)

As the white currant diet had also tumour preventive effects we wanted to study the normal appearing mucosa in more detail in this group. In this experiment, β-catenin signalling could be analysed by Western analysis. The tumour prevention had no effect on β-catenin signalling in the normal appearing mucosa: the levels of β-catenin, E-cadherin and cyclin D1 were similar in both treatment groups. However, cytosolic cyclin D1 in the normal appearing mucosa might influence the initiation phase of intestinal tumourigenesis as it significantly correlated with the number of adenomas ($r = 0.736$, $P < 0.001$).

The results of this study confirmed the notion that tumour prevention by berries does not influence β-catenin signalling in the normal appearing mucosa.

Discussion

The development of colon cancer is a long process. The progression of the disease from benign adenoma through early carcinoma to malignant metastatic cancer requires years or even decades. Due to a long asymptomatic period, all too often the disease is life-threatening before it is diagnosed. As food derived compounds are constantly present in the intestine diet can both prevent and induce colon carcinogenesis, through for instance epigenetics, cellular signalling and mutations. It is also suggested that from the chemoprevention point of view, inhibiting the growth of existing tumours is even more important than preventing the initiating mutations (Luebeck & Moolgavkar 2002). Thus, it is vitally important to understand the mechanisms that stimulate adenoma growth and development towards accelerated malignancy or, in contrast, attenuates them to remain in quiescence for periods as long as decades.

At the time this thesis work began there was already some evidence that diet could influence the number and size of intestinal adenomas (Corpet & Pierre 2003). Whether diet is able to alter the metabolism of these adenomas, which could result in very early prediction of their eventual outcome was, however, unknown. The main objective of the thesis was to find out whether it is possible to modulate cellular signalling in colon cancer by dietary constituents. β-catenin signalling was chosen as it has a well characterised role in intestinal tumourigenesis. The diet was defined as tumour inducing inulin diet and preventive berry diets. Evaluation of other possible colon cancer related foods and cell signalling pathways would give valuable information about cancer biology but is outside the scope of this thesis.

To address β-catenin signalling in the adenoma promotion phase, we used dietary inulin that based on earlier studies was known to induce growth of intestinal adenomas in the Min/+ mouse. In fact, inulin had the most foreseeable and constant effects when compared to other tumourigenic foods in experimental model of colon cancer (Mutanen *et al.* 2000, Pajari *et al.* 2003). To find the foods or dietary ingredients that could clearly prevent adenoma formation in the Min/+ mouse, the different kind of brans and sources of lignans were earlier studied (Mutanen *et al.* 2000, Oikarinen 2005). Their preventive effects were not very pronounced and, therefore, we decided to evaluate the preventive effects of berries. The idea of studying the effects of berries came from the notion that in Finland the incidence of colorectal cancer differs up to twofold between the North and the South (Finnish Cancer Registry, http://www.cancerregistry.fi/eng/statistics/ updated 12.10.2007). One of the main differences between the diets in the two areas is a significantly higher consumption of wild berries in the North, where colorectal cancer incidence is lower (Similä *et al*. 2005). In fact, the adenoma burden was significantly decreased by four berries studied, therefore, they were chosen for use in the tumour

prevention studies. With inulin and berry diets we could create situations where adenoma burden was doubled or reduced by over 60 % compared to the control diet. Inulin accelerated adenoma growth so that most of the adenomas grew large but cloudberry, lingonberry, and white currant prevented adenoma growth, thus most of the adenomas stayed small and did not progress to large ones.

This was a fascinating starting point for the analyses of β-catenin signalling. By dividing adenomas to different size-categories we were able to track cell signalling changes that were diet-specific and not just due to the differences in adenoma sizes. We studied if adenomas were different from the controls in size or if the cellular signalling changed differently during growth of the adenomas. We consider that it is important to understand the early markers for the growth process of adenomas to be able to evaluate the quality of adenomas in regard to their possible future aggressiveness.

Our results showed that the tumour promoting inulin diet doubled the total area of the adenomas and also reinforced the early expression of nuclear β-catenin and cyclin D1 in the adenomas of Min/+ mice. On the other hand, cloudberry, lingonberry, and white currant diets prevented tumour initiation and growth as well as the accumulation of nuclear β-catenin and cyclin D1 in the adenomas.

Inulin resulted in increased levels of β-catenin and cyclin D1 as adenomas enlarged

β-catenin signalling is involved in the initial expansion of cancer cells as well as in tumour progression. Nuclear β-catenin correlates with tumour size in colon cancer patients (Brabletz *et al.* 2000) and it may be necessary for exceeding a certain tumour size. This has been seen in human adenomas (Brabletz *et al.* 2000) and our studies also showed a similar pattern as the level of nuclear β-catenin increased when adenomas grew from small to large. Additionally, increase in the number of cells with nuclear β-catenin is significantly related to the tubular branching that is the main way how adenomas of FAP patients and Min/+ mice enlarge (Wasan *et al.* 1998, Kirchner & Brablet 2000). Our observations that the expression of β-catenin was related to the increased growth potential of inulin-fed Min/+ mice fit well with these findings. Furthermore, cloudberry and white currant feeding diminished the adenoma growth by attenuating β-catenin signalling.

The predictive value of nuclear β-catenin expression in the progression of colorectal cancer has been proven (Hugh *et al.* 1999, Cheah *et al.* 2002) and the amount of nuclear β-catenin increases as early adenomas progress to adenocarcinomas (Takayama *et al.* 1996, Hao *et al.* 1997b, Brabletz *et al.* 2000). Nuclear β-catenin, which seems to be the key player in the transition to the tumour invasive state, has several target genes that regulate tumour proliferation, malignancy, and invasiveness. One of

the targets and a positive effector of the cell cycle, cyclin D1, is frequently overexpressed in colon adenomas and tumours (Arber *et al.* 1996, Zhang *et al.* 1997, Bartkova *et al.* 2001, Chen *et al.* 2007). Nuclear staining of cyclin D1 increases significantly from low-grade dysplastic adenomas to high-grade dysplasia (Zhang *et al.* 1997) and it also correlates with the early onset of cancer and risk of tumour progression and metastasis (Arber *et al.* 1996, Maeda *et al.* 1997, Oda *et al.* 1999, Kristt *et al.* 2000, Bartkova *et al.* 2001, Utsunomiya *et al.* 2001, Bahnassy *et al.* 2004, Mermelshtein *et al.* 2005). In light of these observations, the inulin induced increase in the levels of nuclear β-catenin and cyclin D1 as adenomas enlarged indicates acceleration in tumour promotion.

Histological evaluation of the adenomas of the inulin and control groups showed similar patterns in their growth as well as the dysplasia and anaplasia stages. All histologically examined tumours were also clearly benign. This is in agreement with the amounts of MMP-9 in the tumours, which did not differ between the diets or the adenoma size-groups. The results support the idea that the adenomas of Min/+ mice are still benign and that the MMP-9 gelatinase, the level of which increases in the transition from colon adenoma to adenocarcinoma (Mook *et al.* 2004, Wagenaar-Miller *et al.* 2004, Zucker & Vacirca 2004), is not yet needed for tumour cell invasion. Despite the similar histology of the adenomas, all molecular biological changes occurred earlier in the inulin group, and the linear mixed model analysis showed the specific diet effect on β-catenin signalling. The increase in nuclear β-catenin during inulin induced adenoma growth was not just due to bigger adenomas but to a specific effect of the inulin diet on β-catenin signalling. The increase in nuclear cyclin D1 seemed to be a secondary event. This indicates that diet may cause qualitative changes in adenomas so that even in the same size-category of histologically uniform adenomas, the diets reinforced β-catenin signalling differently and predisposed to accelerated growth. We propose that some dietary constituents are able to induce cellular signalling in a subset of adenomas so that they are more prone to malignancy than they would have been under another dietary treatment.

Our notion that not all adenomas have equal potential for developing to cancer is supported by studies of cancer stem cells and β-catenin-accumulated-crypts. The cancer stem cell hypothesis is increasingly gaining evidence that colorectal cancer is created and propagated by a small number of undifferentiated tumourigenic cells able to self-renew and differentiate into the bulk tumour population (O'Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007). Cancer stem cells may arise when the function of tumour-progenitor genes are disrupted by epigenetic changes that might be frequent in a subpopulation of stem cells owing to, for example, chronic inflammation, injury and nutrition (Feinberg *et al.* 2006). Good candidates for such tumour-progenitor genes are proposed to be *APC*, *β-catenin* and *E-cadherin*. Targeting this small subset of colon cancer cells able to initiate tumour growth might offer effective therapeutic strategies.

The notion that most colorectal carcinomas in humans and also in Min/+ mice arise from pre-existing adenomas is supported by the similar gene expression profiles of adenomas and carcinomas (Paoni *et al.* 2003, Nosho *et al.* 2005). However, all early intestinal lesions do not act similarly and some have higher tumourigenic potential than others. β-catenin and its target genes have invincible roles in creating stem cell phenotypes for cancer cells progressing toward malignancy (Brabletz *et al.* 2005b). Furthermore, only some of the ACFs are prone to expand to malignant tumours and this process also involves β-catenin (Yamada *et al.* 2003). Small dysplastic crypts with excessive β-catenin (BCAC, β-catenin-accumulated-crypts) and microadenomatous crypts, which are similar lesions found in the colon of Min/+ mice (Yamada *et al.* 2002), have histologal dysplasia with disruption of cellular morphology. Some of them are also recognised as adenomatous crypts with extensive branching (Yamada *et al.* 2000). These β-catenin-accumulated-crypts are thought to be more likely to progress into malignant transformation than classical ACFs (Yamada & Mori 2003). These studies highlight the importance of analysing also the signalling pattern of adenomas, not just the number or size of the adenomas when evaluating the health claims for dietary constituents.

Studies of inulin in various colon cancer models

Inulin is a fructo-oligosaccharide fibre found for example in garlic, onion, artichoke and asparagus. Mammals have no digestive enzymes to break β(2-1) glucoside bonds and, therefore, inulin-type fructans escape digestion in the upper intestinal tract but are fermented by intestinal microbiota. The extensive fermentation of inulin-type fructans results in the formation of short-chain fatty acids (SCFA) that have been associated with a variety of positive health effects (Roberfroid 2005). The food industry is able to enrich inulin from its natural sources and inulin is used in foods to enhance the fibre content and to give special texture usually to low-fat foods.

Pool-Zobel (2005) has written an extensive review of inulins in the reduction of colon cancer risk and, therefore, only a few facts are pointed out here. Most of the studies determining the effect of inulin on colorectal cancer have been conducted in rats injected with dimethylhydrazine (DMH) or its metabolite, azoxymethane (AOM), that specifically targets the colon of rats (Corpet & Tache 2002, Pool-Zobel 2005). When comparing the results between Min/+ mice and the chemical rodent model one should keep in mind that in different colon cancer models the mutational background varies.

The molecular genetics of chemical rodent models are different from that found in human colon tumourigenesis. AOM causes β-catenin and K-ras mutations (Takahashi & Wakabayashi 2004) that are usually found in the later stages of human adenoma-carcinoma sequence. For example mutation in *Apc*, the earliest change found in inherited FAP as well as in most of sporadic colon cancers, is less frequent and mutation in *p53* is totally absent in chemically induced rodent models (Song *et al.* 2000,

O'Shaughnessy *et al.* 2002, Takahashi & Wakabayashi 2004). However, like *APC* mutated human tumours, rat tumours may accumulate β-catenin in the nucleus due to degradation resistant mutations in β-catenin (Sheng *et al.* 1998, Corpet & Pierre 2005). Chemically induced tumours arise from flat foci of dysplasia rather than from adenomatous polyps but similarly to human tumours go through ACF, adenoma and carcinoma. Adenomas are considered one of the most established markers of colon cancer risk and are reported as suitable targets for treatment intervention because of their phenotypic and genotypic similarities and evolutionary proximity to invasive cancer (O'Shaughnessy *et al.* 2002).

The tumour preventive action of inulin is mainly demonstrated by the suppression of ACF formation. In chemically induced rodent models the numbers of ACFs and of aberrant crypts per focus seem to be reduced when inulin-type fructans (2-15%) are added to the diet on their own or as part of synbiotic diets (Koo & Rao 1991, Gallaher *et al.* 1996, Reddy *et al.* 1997, Rao *et al.* 1998, Rowland *et al.* 1998, Bolognani *et al.* 2001, Perrin *et al.* 2001, Verghese *et al.* 2002a, Verghese *et al.* 2002b, Poulsen *et al.* 2002, Femia *et al.* 2002). Only some ACFs develop into tumours and the actual tumours have been investigated in only few experiments (Verghese *et al.* 2002b, Femia *et al.* 2002) which found dietary inulin alone or as a part of synbiotic diet to decrease the number of AOM induced colon tumours.

Different colon cancer models present different stages of colorectal tumourigenesis which can influence the chemoprevention. Dietary compounds may have different effects depending on the stage of preneoplastic lesion and also the life stage timing, dose, and genetic background that determines how cells respond to specific dietary compounds (Fenton & Hord 2006). For example, the chemopreventive effects of β-carotene and isoflavones of soy are dependent on the stage of carcinogenenis in which they are presented. Short-term animal bioassays have shown that some dietary constituents, like cholic acid (Reddy *et al.* 1977, Magnuson & Bird 1993) and genistein (Steele *et al.* 1995, Rao *et al.* 1997), inhibit the development of carcinogen-induced colonic ACF, but long-term studies using tumours as the end points have demonstrated tumourigenic effects. The studies with cholic acid have revealed that the ability of dietary agents to modulate the multiplicity and size of BCACs is more important for its potential action than the modulation of ACF (Hirose *et al.* 2003). The studies with inulin have concentrated on the ACF, and not on BCAC.

The ability of inulin to reduce the risk of colon cancer in humans has been studied only in the SYNCAN project (Van Loo *et al.* 2005, Rafter *et al.* 2007, Roller *et al.* 2007). Two groups of volunteers, polypectomised subjects at high risk for colon cancer and colon cancer subjects who had previously undergone 'curative resection' for colon cancer but were not currently having treatment, received inulin as a part of synbiotic diet for 12 weeks. The health effects were neutral or slightly positive. The synbiotic intervention resulted in probiotic survival during gastrointestinal transit and modification of the intestinal flora. The DNA damage in the colonic mucosa of the polyp patients was

slightly decreased but a similar effect was not seen in the cancer patients. The effects of fecal water samples on tight junction permeability was monitored with cell cultures *in vitro* and some positive effects were seen with the faeces obtained from the polyp patients but not from the cancer patients. The synbiotic treatment had only minor stimulatory effects on the systemic immune system mainly on polyp patients and the reduction of the colorectal proliferation was not statistically significant. The results obtained from the SYNCAN project attest that chemopreventive effects of dietary compounds are dependent on the stage of preneoplastic lesion and genetic background.

The inulin chain length may have different effects on ACF multiplicity. Poulsen *et al.* (2002) found that long-chain inulins significantly inhibited the number of small and total ACFs but short-chain inulins significantly increased the numbers of medium and large ACFs. Although we used long-chain inulin in our studies, our results that inulin accelerated adenoma growth resulting in a higher proportion of large adenomas is in accordance with the study of Poulsen *et al.* (2002). In addition to our experiment, there are only a few other reports using Min/+ mouse. Pierre *et al.* (1997) fed short-chain fructo-oligosaccharides to Min/+ mice which caused a reduction in the incidence of colonic tumours but not of small intestinal tumours: fructo-oligosaccharides decreased the number of small adenomas (diameter, $\leq$ 1 mm) but increased the number of large adenomas (diameter, > 1 mm). A result similar to this statistically nonsignificant trend was found in our study. Another Min/+ mouse study that Pool-Zobel (2005) reports to be under preparation is not yet published but the author claims a positive effect was found.

It can be speculated that the distal small intestine of mice resembles the colon, and that the molecular biological background of the distal small intestine resembles the colon more than the proximal small intestine as seen in the expression of D-type cyclins (Yang *et al.* 2006). In inherited and somatic colorectal cancers tumours develop in distinct regions of the colon. The similar regionality in the small intestine is seen in animal models of these cancers, which reflects the mechanism of loss of Apc function (Haigis *et al.* 2004). For still unexplained reason mouse models of colon cancer develop small intestinal tumours that are absent in human (Taketo 2006).

The promotional activity of inulin in our studies in contrast to others who found protection may be due to the different test systems. However our controversial results against the healthiness of inulin are not unique, since inulin type fructans have also been shown to impair the mucosal barrier by the rapid production of fermentation metabolites (Ten Bruggencate *et al.* 2004, Ten Bruggencate *et al.* 2005). This may indicate that inulin and other more fermentable fibres may cause mucosal irritation in humans (Ten Bruggencate *et al.* 2006) or even be associated with a greater risk of cancer (Jacobs 1987, Wasan & Goodlad 1996). Transgenic and chemically induced rodent models of carcinogenesis provide tools for mechanistic chemoprevention studies in different stages of carcinogenesis, but their

usefulness to predict efficacy in humans has been questioned (Corpet & Pierre 2005). The rodent models roughly predict the effect in humans, but the prediction is not accurate for all agents. Keeping this in mind it must be emphasised that the results obtained in this thesis are from Min/+ mice experiments. The conclusions for human colon carcinogenesis may be suggestive but molecular biological backgrounds in human and Min/+ mouse tumourigenesis are similar and therefore the Min/+ mouse is seen as a relevant model to study diet-induced mechanisms.

The aim of this work was to investigate if a tumour promoting inulin diet is able to modulate β-catenin signalling in Min/+ mice adenomas. Previous studies have not investigated the effects of inulin-type fructans on cellular signalling as they have mainly concentrated on ACF multiplicity, fermentation and possibly on proliferation. Our studies reported in this thesis and in other publications (Mutanen *et al.* 2000, Pajari *et al.* 2003, Rajakangas *et al.* 2006) are the only ones describing the mechanisms of inulin action. It was found in this thesis that adenomas in the inulin group were not merely larger but dietary inulin was able to alter the cellular signalling of the adenomas, i.e. to result in qualitative differences that may predict the future outcome of the adenomas.

Four berries inhibited intestinal tumourigenesis by modulating β-catenin and cyclin D1 in the adenomas

As the tumour promoting inulin diet induced β-catenin signalling in the adenomas of Min/+ mice, the tumour prevention by berry diets was studied to see whether opposite effects on β-catenin could be found. Indeed, cloudberry, white currant, and lingonberry diets significantly decreased the adenoma size as well as accumulation of nuclear β-catenin and cyclin D1 in the adenomas. The levels of β-catenin and cyclin D1 correlated significantly with the tumour burden and with each other. These results suggest that cloudberry, white currant and to a lesser extent lingonberry are able to inhibit the growth of tumours by preventing the nuclear accumulation and thus presumably the transcriptional activity of β-catenin, which leads to the decreased expression of cyclin D1. Our results are in line with observations that declining tumour burden by dietary treatment decrease cyclin D1 in ACF and tumours (Wali *et al.* 2002, Issa *et al.* 2007, Xiao *et al.* 2007) and the loss of cyclin D1 in mice prevents the growth of intestinal lesions (Samson 2005). Decreases in nuclear β-catenin and cyclin D1 caused by the berries could be a significant marker of their chemopreventive activity since both β-catenin and cyclin D1 are recognised as targets for drug development (Kundu 2006), and also NSAIDs mediate their effects through this pathway.

In FAP patients and rodent models of colon cancer a wide variety of anti-inflammatory substances are reported to mediate their chemopreventive effects in colorectal cancer by lowering nuclear accumulation of β-catenin and expression of cyclin D1 (Mahmoud *et al.* 1995, Mahmoud *et al.* 1998,

McEntee *et al.* 1999, Brown *et al.* 2001, Boon *et al.* 2004, Chang *et al.* 2005, Kundu *et al.* 2006). Besides synthetic drugs, many extracted naturally occurring phytochemicals are able to target β-catenin signalling related to colorectal cancer (Surh 2003, Clapper *et al.* 2004). The most convincing evidence is found from curcumin from turmeric (Mahmoud *et al.* 2000, Jaiswal *et al.* 2002, Thangapazham *et al.* 2006) and epigallocatechin gallate from green tea (Orner *et al.* 2003, Ju *et al.* 2005, Dashwood *et al.* 2005, Xiao *et al.* 2007). Also resveratrol from grapes, docosahexanoic acid from fish oil, sulforaphane from broccoli, indole-3-carbinol from cabbage and genistein from soybean are widely studied for chemoprevention (Oshima *et al.* 1995, Joe *et al.* 2002, Kim *et al.* 2003, Surh 2003, Clapper *et al.* 2004, Kundu *et al.* 2006).

Studies of berries in various colon cancer models

Studies using extracts of blackberry, black currant, black raspberry, bilberry, blueberry, cloudberry, cowberry, cranberry, gooseberry, lingonberry, red raspberry, sea buckthorn, strawberry, tart cherry, and white currant *in vitro* have found promising chemopreventive antiproliferative and proapoptotic effects against colon cancer (Kang *et al*. 2003, Katsube *et al.* 2003, Olsson *et al.* 2006, Parry *et al.* 2006, Seeram *et al*. 2004, Seeram *et al*. 2006, Boivin *et al.* 2007, Coates *et al.* 2007, Wu *et al*. 2007). The anticarcinogenic effects of the berries could be explained by their high concentration of phenolics, although the studies have not found consis roles for different compounds or determined the concentrations needed. The major phenolic compounds in bilberry, lingonberry, and cloudberry are anthocyanins, proanthocyanidins and ellagic acid, respectively (Häkkinen *et al.* 1999, Määttä *et al.* 2001, Määttä-Riihinen *et al*. 2004a, Määttä-Riihinen *et al*. 2004b, Koponen *et al.* 2007). White currant contains only very low levels of phenolic compounds and it practically lacks antocyanins and other flavonoids (Häkkinen *et al.* 1999, Määttä *et al.* 2001).

Many of the phenolic compounds of the berries have been studied on their own or in mixtures (Wargovich *et al.* 2000, Surh 2003). For example, anthocyanins (Kang *et al.* 2003, Yi *et al.* 2005, Katsube *et al.* 2003, Seeram *et al.* 2004, Zhao *et al.* 2004), proanthocyanidins (Seeram *et al.* 2004, Ferguson *et al*. 2006), phenolic acids (Yi *et al.* 2005) and ellagitannins (Adams *et al.* 2006) were found to have chemopreventive effects in colon cancer cells *in vitro*. However, based on a subsequent experiment (Päivärinta *et al.* 2006), we can exclude the possibility of ellagic acid being the main compound in cloudberry to prevent adenoma formation in Min/+ mice. In this study, the effects of the berries on tumour number were similar despite their different phenolic profiles, suggesting that the anticarcinogenic effects of the berries may result from compounds other than phenolics. This result is in agreement with the study that found 10 different extracts of fruits and berries with great differences in the contents of antioxidants and phenolic compounds inhibited cell proliferation of HT29 colon cancer cells surprisingly similarly (Olsson *et al.* 2004).

None of the four berries studied have previously been examined in animal models of colon cancer or in β-catenin signalling. However, some other berries or berry extracts have demonstrated chemopreventive effects *in vivo* and they seem to have stronger effects than the purified compounds alone (Adams *et al.* 2006). Black raspberry has been shown to inhibit carcinogen-induced tumourigenesis in the rat colon (Harris *et al.* 2001) possibly through AP-1 and NFκB pathways (Lu *et al.* 2006). Anthocyanin-rich tart cherry extract added to drinking water has been associated with fewer and smaller tumours in the caecum of Min/+ mice (Kang *et al*. 2003). In combination with sulindac, tart cherry extract has resulted in fewer tumours and a smaller tumour burden in the small intestine of Min/+ mice when compared to mice fed sulindac alone (Bobe *et al*. 2006) but the studies were not able to explain the possible chemopreventive mechanism. Anthocyanin-rich extracts of bilberry, chokeberry and grape have been shown to have antiproliferative effects and to reduce the number of ACFs compared with the control in AOM treated rats (Lala *et al.* 2006). Also in Min/+ mice an anthocyanin mixture from bilberry has been shown to decrease the number of intestinal adenomas (Cooke *et al.* 2006).

In addition to modulation of the levels of cyclin D1, the bioactive compounds of vegetables, fruits, and berries can cause cell-cycle arrest and/or apoptosis through upregulation of CDK inhibitors p21 and p27 (Manson 2003, Manson 2005). The specific roles for p21 and p27 in colon tumourigenesis have also been pointed out as the effect of a Western type diet have been investigated (Yang *et al.* 2001, Philipp-Staheli *et al.* 2002, Yang *et al.* 2003b, Yang *et al.* 2005). In an Apc deficient background p21 and p27 mutations have additional influence on the incidence, multiplicity, and size of intestinal tumours that can be, however, modulated by the composition of the diet. In our berry experiments the levels of p21 and p27 were analysed from the different-sized adenomas of bilberry, lingonberry, and cloudberry fed mice. p21 and p27 could not explain the adenoma growth and they stayed relatively constant in different size-categories. Our findings are contradictory to those of Wu *et al.* (2007) who found bilberry, black currant, cloudberry, lingonberry, raspberry and strawberry extracts to induce the expression of p21 that was related to inhibition of cancer cell proliferation *in vitro*. It is possible that the berries studied did not have specific effects on the levels of p21 or p27 *in vivo* and tumour preventive functions in Min/+ mice are mediated by other signalling components. Another explanation for the null result is a methodological problem with p21 and p27 analyses. These proteins are supposed to function as cell cycle inhibitors in the nucleus (Coqueret 2003), but in our Western analyses they were detected only in the cytosol. This was probably due to the antibodies used and may have been non-specific cross-reaction.

In this thesis, Western analysis was the main method for analysing the levels of the proteins due to its semiquantitative nature and thus its ability to allow the estimation of the average quantities of the

protein of interest in the whole sample. Immunohistochemistry was used mainly to confirm the results as one can focus on selected areas of the intestine and to visualise the location and to some extent also the levels of analysed proteins. Our Western analysis and immunohistochemical stainings were validated using commercially available antibodies. The antibody specificity i.e. the ability to detect the correct proteins was ensured by blocking peptides, immunoprecipitation, other commercially available antibodies and normal serum. The analysis of p21 and p27 was also proved to work specifically but they detected only in the cytosolic fractions of the tissue samples. Therefore, the p21 and p27 results could be questioned.

Our results indicate that the berries studied are potential sources of chemopreventive compounds against colon cancer. Importantly, no adverse effects on weight gain by any of the berries were seen. Furthermore, contrary to some drugs (Yang *et al.* 2003a), none of the berries increased tumour formation in the colon, suggesting that the chemopreventive components of the berries are likely to have a good safety profile. The inhibition of nuclear β-catenin and cyclin D1 is comparable to substances studied as cancer drugs (Luu *et al.* 2004, Kundy *et al.* 2006) and recently also green tea and its constituents has been shown to decrease nuclear expression levels of β-catenin and cyclin D1 (Issa *et al.* 2007, Xiao *et al.* 2007). The effects of berries most probably are not mediated by one class of phenolics but rather by mixture of different compounds acting in synergistic and additive ways (Harris *et al.* 2001, Adams *et al.* 2006). As demonstrated in this study, chemoprevention using natural, non-toxic foods can achieve the same effect as drugs, both in terms of reduction in tumour burden, and inactivation of cell signalling.

Effects of diets on membranous β-catenin in the adenomas

Staining for β-catenin in colorectal carcinomas often shows a heterogeneous pattern with strong nuclear enrichment at the invasive front and mainly cytoplasmic and membranous staining in the central tumour areas (Behrens 2005). In our studies a heterogeneous staining pattern of β-catenin was found but it seemed to be the same adenomatous areas that were enriched with both nuclear and membranous β-catenin within the adenoma. This controversy could be due to the fact that adenomas of Min/+ mouse are benign and cells accumulating nuclear β-catenin at the invasive front is a phenomenon found in invasive carcinomas.

β-catenin shuttling between the nucleus, cytoplasm and cell membranes is the key for maintaining the balance between the role of β-catenin in cell-cell adhesion at the surface and transcription in the nucleus. Therefore, oncogenic nuclear activation of β-catenin could lead to tumour formation in somatic cells (van Es *et al.* 2003). The loss of E-cadherin may release β-catenin from cell membranes

and increase the cytoplasmic pool of β-catenin that is available for nuclear signalling (Smits *et al.* 2000, Gottardi *et al.* 2001). Our findings that the levels of membranous E-cadherin and β-catenin increased as the adenomas enlarged after the tumour promoting inulin diet contradicts the view that tumour growth induces nuclear β-catenin due to release of β-catenin from the membranous pools (Hao *et al.* 1997). Although this link between membranous and nuclear expression of β-catenin is known (Hao *et al.* 1997), contrasting or neutral findings are also described (Herter *et al.* 1999). Participation of β-catenin in adhesion or Wnt signalling may be dictated by the regulation of distinct molecular forms of β-catenin with different binding properties, rather than simple competition between cadherins and Tcfs for a single constitutive form (Gottardi & Gumbiner 2004).

Our findings that the amounts of membranous E-cadherin and β-catenin increased as the adenomas enlarged could be a result of the overall increases of protein in contrast to increases only in some parts of the cell. Therefore, the increasing levels of membranous β-catenin in Min/+ mice adenomas is not desirable, due to simultaneous increase in oncogenic nuclear β-catenin. Our results are in line with other reports (Carothers *et al.* 2001, Ju *et al.* 2005) showing that the expression and association of E-cadherin, β-catenin, and α-catenin are increased in Apc-/- adenomas compared with normal-appearing Apc+/- mucosa and may result in tighter adhesion of tumour cells. Indeed, in tissue culture cells, increased levels of β-catenin induced by Wnt signalling led to saturation of β-catenin binding to cadherin at the plasma membrane and increase in cell-cell adhesion (Hinck *et al.* 1994b).

Tumour preventive cloudberry and white currant diets decreased the levels of membranous β-catenin and E-cadherin compared to the control diet. In other diet groups the levels of these membranous proteins were increased as adenomas enlarged but the cloudberry maintained the level of membranous β-catenin in the large adenomas at the level found in the small ones. These results are in contrast to the published reports where tumour prevention by NSAIDs redistributed β-catenin and E-cadherin back to the plasma membrane (Mahmoud *et al.* 1998, Roy *et al.* 2005, Kapitanovic *et al.* 2006). Our results suggest that a loss of membranous β-catenin indicates an overall reduction of β-catenin in the cell, as there was also a strong correlation between nuclear and membranous β-catenin and no increase in the cytosol could be seen. Based on the findings from tumour promoting and preventive experiments, we propose that the decreased amount of membranous β-catenin in a benign adenoma also means a decrease in the nuclear pool and may thus be desirable. This would be in line with studies where treating colorectal cancer patients with sulindac resulted in reduced membranous staining of β-catenin (Koornstra *et al.* 2005) and treating human colon cancer cells with the anticarcinogenic NSAID celecoxib induced β-catenin degradation, seen as decreased levels of cytosolic, nuclear, and membranous β-catenin, and E-cadherin (Maier *et al.* 2005).

β-catenin signalling in the normal-appearing mucosa was modulated by inulin diet but not by berry diets

Dietary compounds influence the intestinal microenvironment that directs the cellular functions and influences the clonal expansion of tumour cells. The extracellular signals may activate the normal-appearing mucosa and the adenomas differently and cause diverse cellular responses as seen with metabolic pathways (Koukourakis *et al.* 2006). It would be important to understand how to control the microenvironment and to find biomarkers for the physiological status of the mucosa. As the dietary modifications changed the β-catenin signalling in the adenomas, this approach was also chosen to investigate normal-appearing mucosa.

The inulin-induced growth promotion was accompanied by accumulation of cytosolic and nuclear β-catenin, and an increased amount of cytosolic cyclin D1 in the normal-appearing mucosa of Min/+ mice. Furthermore, inulin feeding reduced the levels of β-catenin and E-cadherin in the mucosal membranes. In the wild-type mice the drop in membranous β-catenin was clear and, in addition, subset of crypts also had enhanced nuclear β-catenin staining. Increasing levels of nuclear β-catenin and the activation of β-catenin/Tcf pathway are the primary transforming events in colon cancer as the target genes of β-catenin control whether cells in the intestinal epithelium proliferate or differentiate (Bienz & Clever 2000, Giles *et al.* 2003). E-cadherin can influence the availability of β-catenin by competing with other binding partners of β-catenin and, therefore, β-catenin is suggested to have nuclear activity only when it is free of E-cadherin (Orsulic *et al.* 1999, Gottardi *et al.* 2001) as was seen also in the normal-appearing mucosa of inulin-fed mice.

Histologically normal-appearing enterocytes in the small intestine of Min/+ mice show 25% reduction in migration rate compared to those in their wild-type littermates (Mahmoud *et al.* 1997). Min/+ mice have impaired enterocyte proliferation and apoptosis, as well as defects in cell-cell adhesion, reflecting the reduced association between E-cadherin and β-catenin (Carothers *et al.* 2001). It has been proposed that *APC* mutations result in increased localisation of β-catenin at the membrane, increasing adhesion and consequently decreasing migration (Gilet *et al.* 2003). Our results are in accordance with these observations since at 5 weeks of age the Min/+ mice had less membranous E-cadherin and β-catenin in the mucosa than their wild-type littermates. Cyclin D1 has also been suggested to be involved in signalling pathways regulating cellular differentiation and migration (Hulit *et al.* 2004, Fu *et al.* 2004) and the Min/+ mice had less cytosolic cyclin D1 than WT mice.

Epithelial cell proliferation rate in the normal-appearing mucosa predicts the risk for colorectal cancer and is associated with diet (Akedo *et al.* 2001). The precise action of different dietary fibres and the fermentation products in colorectal tumourigenesis still remains to be determined but it is already

known that both the amount and the nature of dietary fat influence the function of fibre (Lupton 2000, Lupton 2004). The most fermentable dietary fibres have been shown to increase the cells in the S phase of the cell cycle and to stimulate intestinal cell proliferation by lowering intestinal pH (Jacobs & Lupton 1984, Lupton *et al.* 1985, Lupton *et al.* 1987). This could be one reason for increased cyclin D1 found in the inulin group. The role of cyclin D1 in colon tumourigenesis is well defined and there are reports indicating that cyclin D1 in the normal-appearing mucosa responds to dietary treatment and may reflect adenoma formation (Wali *et al.* 2002, Shinozaki *et al.* 2003, Orner *et al.* 2003, Yang *et al.* 2003a). It has been suggested that cyclin D1 overexpression is not a mere consequence of cellular proliferative activity but rather represents a true difference between the normal and tumorous states (Hur *et al.* 2000) which could explain the tumour promoting effects of the inulin diet.

Our results showed that cloudberry, white currant, and lingonberry were able to target both the initiation and promotion stages of colon cancer, as they prevented the formation and growth of intestinal adenomas in Min/+ mice. Since some chemopreventive compounds have resulted in changes in the levels of β-catenin (Schmelz *et al.* 2001) and E-cadherin (Carothers et al. 2001, Moran *et al.* 2005) in Min/+ mice mucosa *in vivo* and *ex vivo*, and in colorectal cancer patients (Koornstra *et al.* 2005), we investigated if berries affected the levels of β-catenin, E-cadherin and cyclin D1 in the normal-appearing mucosa. *In vitro* studies have shown that citrus flavonoids are able to promote cell migration in *Apc* deficient colon epithelial cells (Fenton & Hord 2004). The stabilisation of the migration up the crypt by bioactive compounds in the berries could allow cells to migrate normally, undergo apoptosis and in that way prevent adenoma formation. However, the levels of analysed proteins were similar in the control and berry groups. The β-catenin signalling gives a convincing explanation for the induction and inhibition of tumour growth in Min/+ mice. It does not, however, explain the inhibition of tumour initiation by berries as the effects were found in the adenomas but not in the normal-appearing mucosa, suggesting that other pathways are responsible for the reduced number of adenomas. It is also possible that the reduced formation of adenomas was due to slow growth and regression in berry diet group as it was seen in our study IV that at 5 weeks of age Min/+ mice already have visible adenomas. The strong correlation between cytosolic cyclin D1 and the number of adenomas after the white currant diet suggests a possible mechanism for initiation of adenoma formation. However, new studies claim that upregulation of cyclin D1 in intestinal neoplasia may be an important factor in adenoma establishment and growth rather than initiation (Sansom *et al.* 2005). Some authors also propose that cyclin D1 may not be essential for the development of intestinal tumourigenesis but may modify adenoma frequency (Wilding *et al.* 2002, Hulit *et al.* 2004).

As this thesis was unable to find a clear explanation for the prevention of tumour initiation from the β-catenin signalling, some other possibilities were also evaluated. In addition to the proteomic approach, an Affymetrix microarray technique on normal-appearing Min/+ mucosa was used to identify early

changes in intestinal tumourigenesis. When the cut-off point was set at a 2-fold difference between the berry and the control treatments, Affymetrix microarray revealed changes in genes implicated in colon carcinogenesis, including the decreased expression of the adenosine deaminase, ecto-5'-nucleotidase and PGE2 receptor subtype EP4 (results shown in the original paper II). It is noteworthy that the changes in the cancer-related genes took place already in the normal-appearing mucosa and that all the berries resulted in similar changes in the expression of these genes. The results are in line with the inhibitory effects of the berries on tumour formation rather than explaining their effects on tumour growth. The gene expression data give valuable information and a good starting point to further study the chemopreventive effects of the berries.

Conclusions

In conclusion, diets were able to modulate β-catenin signalling in the adenomas of Min/+ mice, tumour promotive and preventive diets having opposite effects on β-catenin signalling in the adenomas. The changes in β-catenin signalling in adenoma tissues seemed to be related to the different adenoma growth capacity in the presence of dietary inulin or berries. The levels of nuclear β-catenin and cyclin D1 combined with the size of the adenomas in the treatment groups suggest that diets induced differences in the cancerous process and adenomas progressing to malignant carcinomas are most likely found in the sub-groups having the highest levels of β-catenin. On the other hand adenomas staying quiescent for long perioid of time are most probably found from cloudberry or white currant diet groups.

That levels of membranous E-cadherin and β-catenin increased as the adenomas in the inulin diet group grew could be a result of the overall increase in protein levels of the cell. The increasing levels of membranous β-catenin in Min/+ mice adenomas would be undesirable, due to simultaneous increase in oncogenic nuclear β-catenin. We propose that the decreased amount of membranous β-catenin in benign adenomas of berry diet groups implies also a decrease in the nuclear pool of β-catenin and may thus be desirable.

Tumour promotion, but not tumour prevention, influenced β-catenin signalling already in the normal-appearing mucosa. Inulin-induced tumour promotion was related to β-catenin signalling in Min/+ mice and in WT mice changes were also visible. The chemopreventative effects of berries in the initiation phase were not mediated by attenuated β-catenin signalling, but cyclin D1 might have its own independent role in this.

One should bear in mind that the results obtained in this thesis are applicable in an Apc deficient background. All other tumour promotive or preventive diets do not necessarily cause similar β-catenin signalling as seen in this study. However, it could be argued that if a similar signalling pattern is found the tumourigenic effect of diet are most probably similar. Our results suggest that in addition to the number, size, and growth rate of adenomatous polyps, the signalling pattern of the adenomas should also be considered when evaluating preventative dietary strategies.

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