学位論文

Doctor's Thesis

Down-regulation of monocyte chemotactic protein-3 (MCP-3) by activated β-catenin

（活性型β-カテニンによるモノサイト ケモタクティック プロテイン-3（MCP-3）の発現抑制）

藤田　学

Manabu Fujita

指導教官

小川道雄　教授

熊本大学大学院医学研究科外科学第二講座

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著　者　名　：　Manabu Fujita

藤田　学

指導教官名　：　外科学第二講座教授

小川　道雄

審査委員名　：　腫瘍医学講座教授　佐谷　秀行

病理学第一講座教授　吉永　秀

胚形成部門初期発生分野教授　永渕　昭良

胚形成部門転写制御分野教授　田賀　哲也

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Contents

Summery

Accumulation of intracellular β-catenin as a result of inactivation of the adenomatous polyposis coli (*APC*) gene or by mutation of the β-catenin gene (*CTNNB1*) itself is involved in a wide range of human cancers. By means of fluorescent differential display using a murine fibroblast cell line (L-MT), which expresses an activated form of β-catenin that accumulates in the cells, we found that expression of murine monocyte chemotactic protein-3 (mMCP-3) was suppressed by activated β-catenin. Inversely, expression of MCP-3 in human colon cancer cells was induced by depletion of β-catenin following adenovirus-mediated transfer of wild-type *APC* genes into the cells. A reporter-gene assay indicated that accumulation of β-catenin in the nucleus suppressed activity of the MCP-3 promoter through a putative Tcf/LEF-binding site, ATCAAAG, but when the promoter sequence contained a two-base substitution in the binding site it failed to suppress reporter-gene (luciferase) activity. An electrophoretic mobility-shift assay using the putative Tcf/LEF-binding sequence revealed interaction of the candidate sequence with the β-catenin complex. Furthermore, induction of MCP-3 cDNA into HT-29 colon cancer cells increased expression of two markers of differentiation, alkaline phosphatase and carcinoembryonic antigen.

Our results implied that activation of β-catenin through the Tcf/LEF signaling pathway may participate in colonic carcinogenesis by inhibiting MCP-3-induced differentiation of colorectal epithelial cells.

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著者名

Manabu Fujita, Yoichi Furukawa, Yutaka Nagasawa, Michio Ogawa and Yusuke Nakamura

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Abbreviations

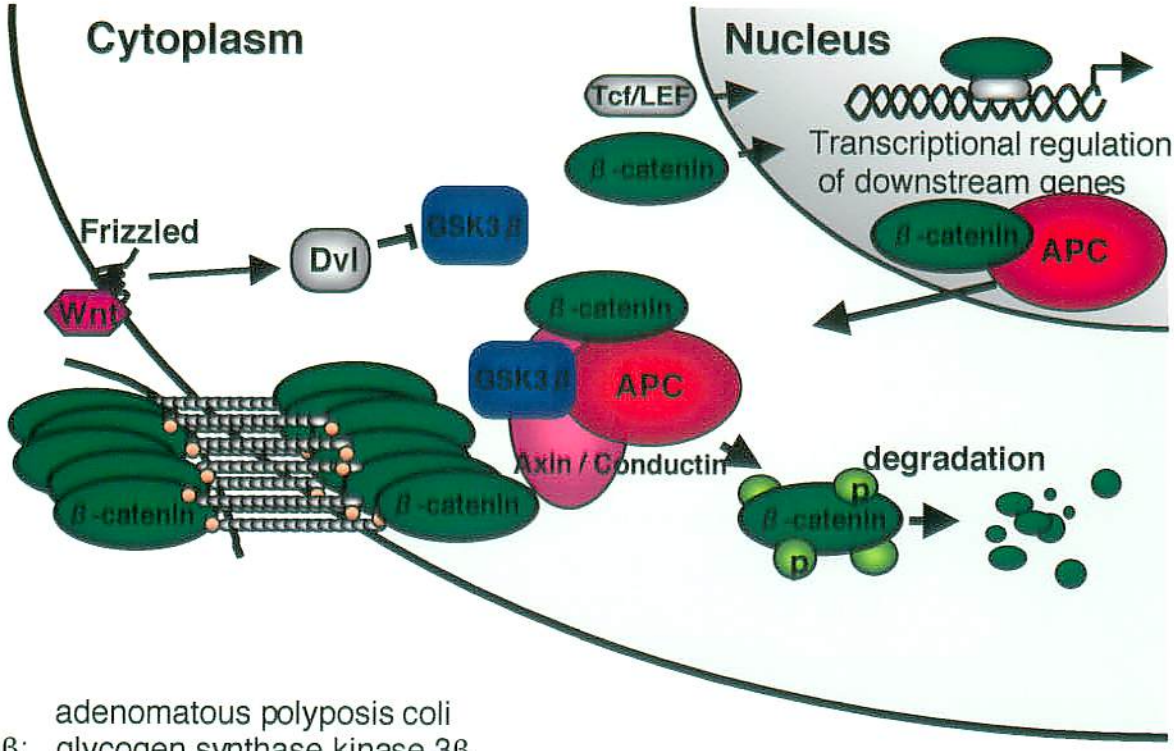
MCP-3:	monocyte chemotactic protein-3
APC:	adenomatous polyposis coli
Tcf:	T-cell transcription factor
LEF:	leukocyte enhancer factor
RT-PCR:	reverse transcriptase polymerase chain reaction
EMSA:	electrophoretic mobility-shift assay
ALP:	alkaline phosphatase
CEA:	carcinoembryonic antigen
GAPDH:	glyceraldehydes-3-phosphate dehydrogenase
NaB:	sodium butyrate

Chapter 1

Background and Purpose

1-1. Wnt/Wingless signaling pathway

The Wnt signaling pathway plays an important role in a number of developmental processes, including body axis formation, development of the central nerve system, axial specification in limb development and mouse mammary gland development (1, 2). The Wingless signaling pathway is the Drosophila homolog of the Wnt signaling pathway and is critical for differentiation and morphogenesis in Drosophila and Xenopus (3), and evidence of aberrant ventralization by ectopic expression of armadillo, the Drosophila homolog of β-catenin, has implicated this protein in the determination of cell polarity (4). The model illustrated in Figure 1 is a proposed mechanism of Wnt signaling which is based on the reported literature.



APC: adenomatous polyposis coli
GSK3β: glycogen synthase kinase 3β
TCF: Tcell specific transcription factor
LEF: lymphoid enhancer binding factor
Dvl: disheveled protein

Figure 1. The model of Wnt signaling pathway

The Wnt family of proteins consists of more than fifteen closely related secreted glycoproteins. The frizzeled receptor, the receptor for Wnt proteins, has a seven-pass transmembrane domain and the Wnt signal is transduced to Dishevelled (Dvl). Dvl suppressed the activity of glycogen synthase kinase-3β (GSK-3β). In the absence of Wnt signals, GSK-3β is phosphorylated, and phosphorylates β-catenin, binding adenomatous polyposis coli (APC) and Axin in a complex. Nuclear β-catenin is transported to cytoplasm by shuttling APC, and is phosphorylated in cytoplasm. Then β-catenin is degradated through ubiquitin-proteasome pathways. However, when Wnt signal stabilizes and causes the accumulation of β-catenin, it translocates into the nucleus, where in association with T-cell factor / lymphocyte enhancer factor (Tcf/LEF), and it modulates transcription of target genes.

1-2. Carcinogenesis and Wnt signaling pathway

Recent progress in cancer research has underscored the importance of β-catenin in the process of tumorigenesis, a molecule that plays pivotal roles in cell-to-cell adhesion and in the Wnt/Wingless signaling pathway (5).

The intracellular amount of β-catenin is negatively regulated by the tumor suppressor APC and Axin. Germline mutations in the *APC* gene

are responsible for the autosomal dominant inherited disease familial adenomatous polyposis (FAP), somatic mutations in APC occur in approximately 80% of sporadic colorectal tumors (6). Consistent with its role as a tumor suppressor, overexpression of APC blocks cell cycle transition from the G1 to S phase (7). Furthermore, introduction of APC induces apoptosis in colon cancer cell line (8). Colorectal tumors with intact *APC* genes were found to contain activating mutations of β-catenin in exon3 that altered functionally significant phosphorylation sites for its degradation (9). Mutations in exon 3 of β-catenin were found approximately 11.5% in colorectal cancers (10) (Table 1). The structures of *APC* and β-catenin genes are described in Figure 2.

Table 1. β-catenin mutations in human cancers

Tissue	Frequency	Reference
colorectal	9 / 202	Samowitz 1999
colorectal	2 / 92	Kitaeva 1997
colorectal-w/o APC mutation	7 / 58	Iwao 1998
colorectal-w/o APC mutation	13 / 27	Sparks 1998
colorectal HNPCC	12 / 28	Miyaki 1999
colorectal w/ MSI	13 / 53	Mirabelli-primdahl 1999
colorectal w/o MSI	0 / 27	Mirabelli-primdahl 1999
desmoid, sporadic	1 / 1	Shitoh 1999
desmoid, sporadic	22 / 42	Tejpar 1999
endometrial w/ MSI	3 / 9	Mirabelli-primdahl 1999
endometrial w/o MSI	10 / 20	Mirabelli-primdahl 1999
gastric, intestinal-type	7 / 26	Park 1999
gastric, diffuse-type	0 / 17	Park 1999
hepatocellular w/HCV	9 / 22	Huang 1999
hepatocellular	12 / 35	Van Nhieu 1999
hepatocellular	6 / 26	De la Coste 1998
hepatocellular	14 / 75	Miyoshi 1998
hepatocellular	21 / 119	Legoix 1999
hepatoblastoma, sporadic	8 / 9	Jeng 2000
hepatoblastoma, sporadic	27 / 52	Koch 1999
hepatoblastoma	12 / 18	Wei 2000
kidney, Wilm's tumor	6 / 40	Koesters 1999
medulloblastoma, sporadic	3 / 67	Zurawel 1998
melanoma	1 / 65	Garcia-Rostan 1999
ovarian, endometriod	7 / 13	Gamallo 1999
ovarian, endometriod	3 / 11	Palacios 1998
ovarian, endometriod	10 / 63	Wright 1999
pancreatic tumor	0 / 111	Gerdes 1999
pilomatricoma	12 / 16	Chan 1999
prostate cancer	5 / 104	Voeller 1998
thyroid, anaplastic	19 / 31	Garcia-rostan 1999
uterine endometrium	10 / 76	Fukuchi 1998

(Polakis P. 2000)

The overall frequency (Frequency) of mutations in each tumor type is represented as the number of tumors with mutations/total number of analyzed.

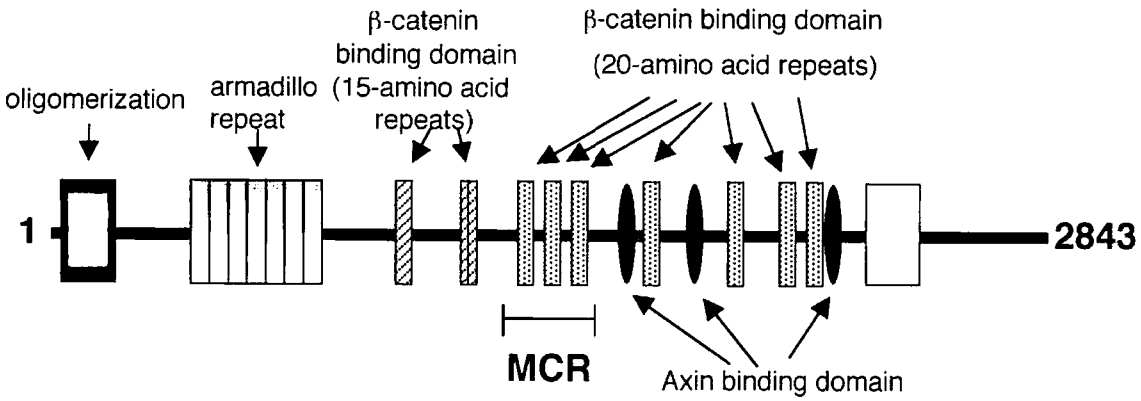


Figure 2A. Structure of APC gene

MCR (mutation cluster region) mutations result in truncated proteins retaining β-catenin binding but not regulatory activity.

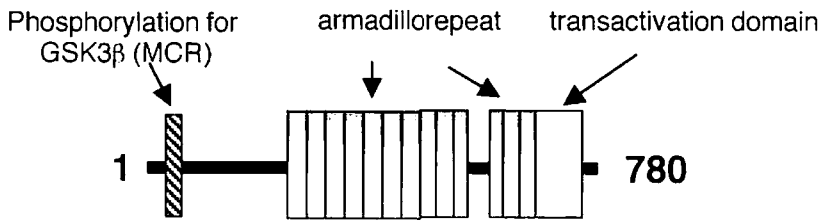


Figure 2B. Structure of β-catenin gene

Mutations in β-catenin (*CTNNB1*) gene affecting the amino-terminal region of the protein, which is essential for the targeted degradation of β-catenin, make it refractory to regulation by APC, Axin and GSK3β (9,11,12). Accumulation of β-catenin, which escaped from degradation pathway, in the cytoplasm or nucleus as a consequence of mutant *APC* or β-catenin (*CTNNB1*) genes is frequently observed in early stages of colorectal tumorigenesis (13, 14). Moreover deletions of, or mutations within, exon 3 of β-catenin (*CTNNB1*) also have been identified in tumors of liver, uterus, prostate, skin, and brain (15-20). Since wild-type APC and Axin1 promotes degradation of intracellular β-catenin through phosphorylation of serine/threonine residues within exon 3, where β-catenin binds with glycogen synthase kinase 3β (GSK-3β) and Axin/Conductin, mutant forms of APC or β-catenin impair the degradation process. Accumulated β-catenin translocates into the nucleus, where in association with T-cell factor/lymphocyte enhancer factor (Tcf/LEF), it modulates transcription of target genes (Figure 3). So far several transcriptional targets of the β-catenin/Tcf/LEF complex have been identified including c-myc, cyclin D1, matrilysin, c-Jun, fra-1, uPAR, ZO-1 (21-25) and NBL-4 (26). However, specific molecular targets that are associated with cell differentiation or cell polarity remain to be defined.

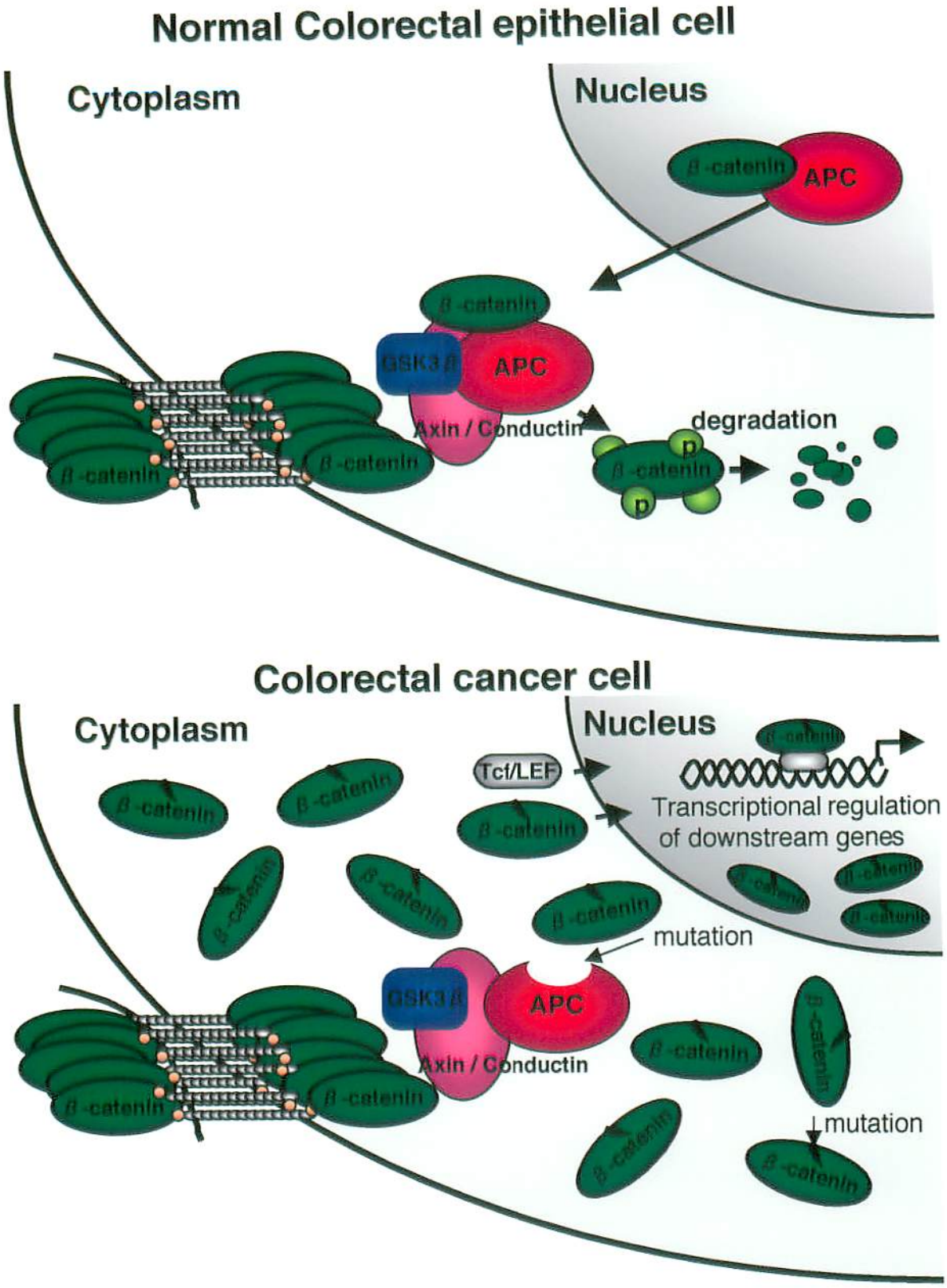


Figure 3. Accumulation of β-catenin in colorectal cancer cell in order to mutations of APC or β-catenin

1-3. Monocyte chemotactic protein 3 (MCP-3)

Cytokines form a family of proteins which are produced in the course of immunitary and inflammatory responses and which serve as signal carriers in a dynamic cellular communication network. One of them, chemokine family can be devided into four subfamilies according to the position of cystein residues. These residues are either separated by one or three amino acid residues CXC, CX_3C, or are contiguous C-C, or possess only the second or the fourth conserved cysteines C chemokines. The subfamilies of chemokines were described in Table 2. Although chemokines display a wide range of biological activities including cell-specific chemotaxis and activation, regulation of cell growth, and differentiation, as well as modulation of immune responses. These functions were initially reported to have strictly cellular specificity only for leukocytes. Recent years, a lot of chemokine receptors were identified and analyzed, then various kinds of cells express chemokine receptors were reported. Furthermore, Muller et al. provided evidence for preferential homing of breast cancer which express chemokine receptors to metastatic sites where their ligands are highly expressed (27). The finding indicates the possibility that tumor cells which express chemokine receptors acquire its ability.

Table 2. Chemokines

C-C chemokine

Monocyte chemotactic protein-1 (MCAF/MCP-1)
Monocyte chemotactic protein-2 (MCP-2)
Monocyte chemotactic protein-3 (MCP-3)
Monocyte chemotactic protein-4 (MCP-4)
Eotaxin
Regulated upon activation, normally T cell expressed and secreted (RANTES)
Macrophage inflammatory protein-1 α (MIP-1α)
Macrophage inflammatory protein-1 β (MIP-1β)
Hemofiltrate C-C chemokine-1 (HCC-1)
Macrophage inflammatory protein-3 α (MIP-3α)
Macrophage inflammatory protein-3 β (MIP-3β)
T cell activation gene-3 (I-398/TCA-3)
Thymus and activation regulated chemokine (TARC)
MIPF-1
MIPF-2

CXC chemokine

Interleukin-8 (IL8)
Growth-related oncogene α (GRO α)
Growth-related oncogene β (GRO β)
Growth-related oncogene γ (GRO γ)
Neutrophil activating protein-2 (NAP-2)
Epithelial neutrophil activating protein-78 (ENA-78)
Granulocyte chemoattractant protein-2 (GCP-2)
Platelet factor-4 (PF-4)
Interferon-γ–inducible protein (IP-10)
Monokine induced by interferon-γ (MIG)
Stromal cell derived factor-1 (SDF-1)
Connective tissue activating protein-III (CTAP-III)
β-Thromboglobulin (βTG)

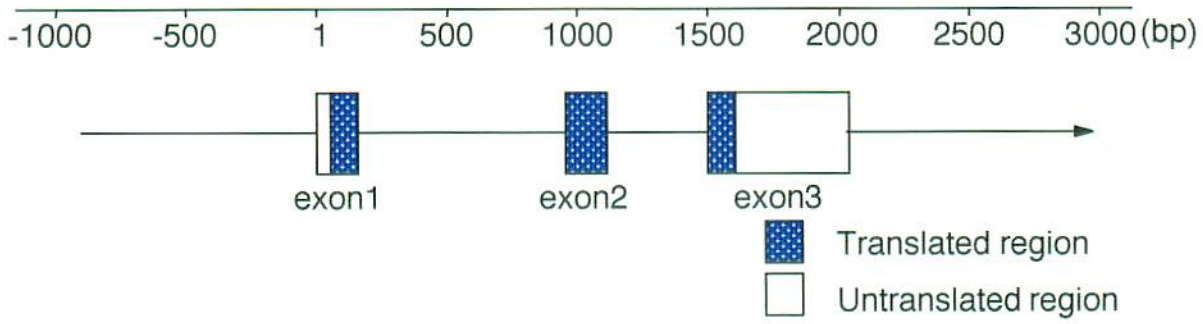
C chemokine

lymphotactin

CX_3C chemokine

fractalkine

The number of chemokines of the C-C subfamily has identified and analyzed considerably during the past several years. Monocyte chemotactic protein-3 belongs to a subgroup of chemotactic factors, the C-C chemokines, and is expressed and secreted by monocyte, fibroblast, platelet, colon epithelial cells and some malignant tumor cells (28-33). MCP-3 attracts and activates various kind of leukocytic cell types through binding to both shared and separated receptors. Monocytes, lymphocytes, dendritic cells, NK cells and granulocytes are attracted and activated by MCP-3 (34), and they infiltrate to sites of inflammation. Furthermore, anti-tumor activities, attracting and activating dendritic cells and type I T cells by MCP-3 were demonstrated (35), but this mechanism is not well understood. The structure and feature are described in Figure 5.



Chromosomal location:	17q11.2-q12
Expression:	Ubiquitous
Amino acids:	premature 99 a.a. mature 76 a.a.
Function:	1. chemotaxis 2. Adhesion 3. Differentiation
Homology:	MCP-1 71% MCP-2 58% NCP-4 78% RANTES 30%
Receptor:	CCR1, CCR2, CCR3

Figure 4. MCP-3 (Monocyte chemotactic protein 3)

1-4. Purpose

To clarify the role(s) of β-catenin in human colorectal carcinogenesis, we aimed to identify and analyze the function of genes regulated by its activated form (36).

Chapter 2

Materials and Methods

2-1. Cell lines

We had previously established a mouse fibroblast cell line, L-MT, by introducing into murine L cells a mutant β-catenin transgene that lacked exon 3 using Tet-off Gene Expression System (37). Briefly, expression of activated β-catenin is induced in the absence of tetracycline or doxycycline, but the transcription is suppressed in cells that are cultured in medium containing either of these drugs. COS-7 cells, human embryonic kidney 293 cell line and human colon-cancer cell lines HT-29, SW480, SW948 and LoVo were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell lines were cultured as monolayers in appropriate media: L-MT, L, and HEK 293 cells in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO), SW480 and SW948 in Leibovitz's L-15 and LoVo in L-12 (GIBCO-BRL Co., Grand Island, NY), and HT-29 in RPMI1640 (Sigma Chemical Co., St. Louis, MO). All media were supplemented with 10% fetal bovine serum (Cansera, Canada) and 1% antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO) and all cells were grown at 37 ^{o}C in an atmosphere of humidified air containing 5% CO_2.

2-2. RNA extraction and fluorescent differential display

Total RNA was extracted from L cells, L-MT cells and four human *colon-cancer* cell lines using TRIZOL Reagent (GIBCO-BRL Co., Grand Island, NY) according to the manufacturer's protocol. The fluorescent differential display (FDD) procedure was performed essentially as described previously (38). PCR products were re-suspended in formamide sequencing dye and electrophoresed for 3 hours at 1800V on sequencing gels containing 4% acrylamide (19:1) with 7 M urea. Gel images were analyzed with an FMBIO II Multi-View fluoroimager (TaKaRa, Tokyo, Japan). Bands that showed differential expression between L cells and L-MT cells were excised from the gels, and DNA was extracted by boiling the gel fragments in Tris-EDTA buffer. Each sample was re-amplified for 30 cycles with the same primer set utilized for the FDD procedure. Re-amplified products were cloned into pBluescript II SK (-) vector (Stratagene, La Jolla, CA) and sequenced using T3, T7 primers and an ABI PRISM Dye Terminator Cycle Sequencing FS Ready Kit (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the protocol provided by the supplier.

2-3. Immunocytochemistry

Cultured cells, re-plated on chamber slides, were fixed with PBS containing 4% paraformaldehyde for 15 min, then rendered permeable by incubation for 3 min at 4 ^{o}C in PBS containing 0.1% Triton X-100. Cells were covered with 2% BSA in PBS for 30 min at room temperature to block non-specific binding of antibody, then incubated with a mouse anti-β-catenin antibody (Transduction Laboratories, Lexington, KY). Antibodies were stained with a goat anti-mouse secondary antibody conjugated to rhodamine (Leinco Technologies, Inc., Ballwin, MO), and viewed with an ECLIPSE E800 microscope (Nikon, Tokyo, Japan).

2-4. Depletion of β-catenin by adenovirus-mediated gene transfer

Expression of the part of APC that corresponds to the 20-amino-acid repeats of its β-catenin-binding domain is able to down-regulate β-catenin (39). Therefore we constructed an adenoviral vector containing this domain (Ad-APC) by inserting a 2.5-kb *Hin*dIII fragment of *APC* cDNA into the HindIII site of the pAd-*Bgl*II vector, which contains the cytomegalovirus promoter/enhancer and a bovine growth hormone polyadenylation signal flanked by Ad5 E1 sequences. The recombinant adenoviruses, constructed as described previously (39), were propagated

in the HEK293 cell line and purified by two rounds of CsCl density centrifugation. Viral titers were measured by a limiting-dilution bioassay using HEK293 cells. Cell monolayers were infected with the viral solutions and incubated at 37 ^{o}C for 1 h, with brief agitation every 15 min. Culture medium was added, and the infected cells were maintained at 37 ^{o}C for 48 h.

2-5. Western Blotting

Western blotting with mouse anti-β-catenin (Transduction Laboratories, Lexington, KY) was performed as described elsewhere (40). Briefly, 20 μg of total protein extracted from each of the two cell lines (L-MT and parental L cells) were electrophoresed, transferred to polyvinylidene difluoride membrane, and visualized with an anti-b-catenin monoclonal antibody (C199220; Transduction Laboratories, Lexington, KY).

2-6. Semi-quantitative RT-PCR analysis

A 3-μg aliquot of total RNA from each cell line was reverse-transcribed for single-stranded cDNAs using oligo$(dT)_{15}$ primer and Superscript II (Life Technologies, Inc., Rockville, MD). Each cDNA

mixture was diluted for subsequent PCR amplification by monitoring *GAPDH* as a quantitative control. The PCR exponential phase was determined on 20-32 cycles to allow comparison among cDNAs developed from identical reactions. As an internal control, the amounts of cDNA were quantified and equalized by amplifying *GAPDH*. The primer sequences used for amplification were 5'-GACAACAGCCTCAAGATCATCA-3' and 5'-GGTCCACCACTGACACTGTG-3' for human *GAPDH*, and 5'-CAACTACATGGTTTACATGTTC-3' and 5'-TGTTCC GAATGTCTGAGGAC-3' for mouse *GAPDH*, and 5'-TCCAATTCTCATG TTGAAGCC-3' and 5'-GAGAAAGGACAGGGTATACAAA-3' for human *MCP-3*, and 5'-CACTCTCTTTCTCCACCATG-3' and 5'-GCTAACACAATGTTAAA GTGAC-3' for mouse *MCP-3*. All reactions involved initial denaturation at 94 ^{o}C for 2 min followed by 20 cycles (for *GAPDH*) or by 32 cycles (for *MCP-3*) at 94 ^{o}C for 30 s, 57 ^{o}C for 30 s, and 72 ^{o}C for 1 min, on a Gene Amp PCR system 9600 (Perkin Elmer). The products were electrophoresed in 3% agarose gels and visualized by fragment Southern-blot analysis followed by the transfer to nylon membranes (Amersham, Cleveland, OH). The membranes were hybridized with ^{32}P-labeled internal oligonucleotide probes. Each

internal oligonucleotide sequence used for Southern-blot analysis as a probe was 5'-CCCAT GGCAAATTCCATGGC-3' for human *GAPDH*, 5'-CTCACGGCAAATTC AACGGC-3' for mouse *GAPDH*, 5'-GCTACAGAAGGACCACCAGTA-3' for human *MCP-3* and 5'-TTCTGTTCAGGCACATTTCTTC-3' for mouse *MCP-3*.

2-7. Deletion mutagenesis and generation of MCP-3 reporter plasmids

To generate a series of 5' deletion mutants of the human MCP-3 gene promoter, two fragments were cloned into appropriate enzyme sites of pGL3-Basic Vector (Promega, Madison, WI). Reporter assay was carried out using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. A plasmid vector, pRL-TK (Promega, Madison, WI), was co-transfected with each reporter construct into SW480, LoVo and SW948 cells using FUGENE6 (Boehringer Mannheim, Mannheim, Germany) according to the supplier's recommendations.

2-8. Electrophoretic mobility-shift assay (EMSA)

Preparation of nuclear extracts and EMSA were performed

essentially as described previously (41). Two pairs of double-stranded, 15-nucleotide DNAs were prepared by annealing two oligonucleotide DNAs;5'-ACCAGAATCAAAGCC-3' and 5'-GGCTTTGATTCTGGT-3' for the wild-type DNA, 5'-ACCAGAGCCAAAGCC-3' and 5'-GGCTTTGGCTCTGGT-3' for the mutant DNA. The wild-type DNA was end-labeled with polynucleotide kinase in the presence of ^{32}P-γATP and served as a probe for EMSA. A typical binding reaction contained 10 μg of nuclear extract in 5 μl of extraction buffer (10 mM HEPES(pH7.9), 400 mM NaCl, 1.5 mM MgCl2, 0.2 mM EGTA, 20 % glycerol), 0.1 ng of the radiolabeled probe and 100 ng of deoxyinosine-deoxycytidine (dI-dC) in 25 μl of binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol). For the competition assays, 50 ng of wild-type or mutant unlabeled DNA was added in the reaction mixture. Samples were incubated for 20 min at room temperature, with or without 0.5 μg of anti-β-catenin antibody and additional incubation for 20 min. Electrophoresis was performed at 4 ^{o}C on 4% non-denaturing polyacrylamide (29:1) gels with 0.25X Tris-boric/EDTA buffer. The gels were dried and autoradiographed.

2-9. ELISA of carcinoembryonic antigen (CEA) and measurement of alkaline phosphatase (ALP) activity

The entire coding region of human MCP-3 was cloned into an expression vector, pcDNA 3.1(+) (Invitrogen, Carlsbad, CA), under control of the cytomegalovirus promoter/enhancer. HT-29 colon cancer cells expressing a high amount of MCP-3 transcript was selected in medium containing 1000 μg/ml geneticin, and subcloned (HT-29-MCP). As a control, HT-29 cells transfected with the empty vector pcDNA 3.1(+) were subcloned as well (HT-29-con). All cells were grown to 30-50% confluence, either with or without 2mM sodium butyrate (NaB). After 72 h of incubation, cells were harvested and lysed with lysis buffer. The amount of CEA was analyzed by an enzyme-linked immunosorbance assay (ELISA) using a commercially available kit (Enzymun-test CEA) (Boehringer Mannheim, Mannheim, Germany). Measurement of alkaline phosphatase activity was performed as described elsewhere (42).

2-10. Statistics

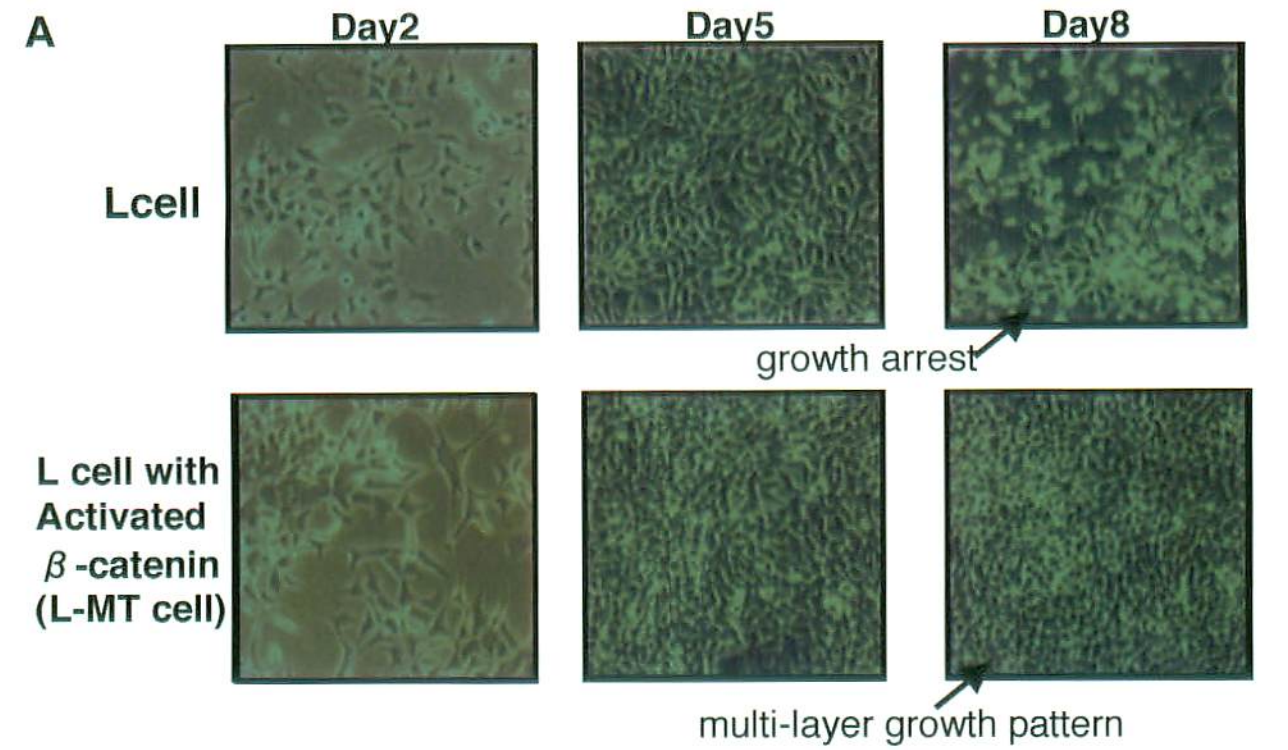
The data were analyzed using an analysis of variance (ANOVA) and the Scheffé's F test.

Chapter 3

Results

3-1. Identification of MCP-3 as a gene down-regulated by activated β-catenin

We previously established a mouse fibroblast cell line, L-MT, in which a mutant form of β-catenin observed in human cancer cells was introduced (36). L-MT cells showed multilayer growth pattern and displayed growth advantage in 10% serum culture media compared with their parent cells, L cells (Figure 5).



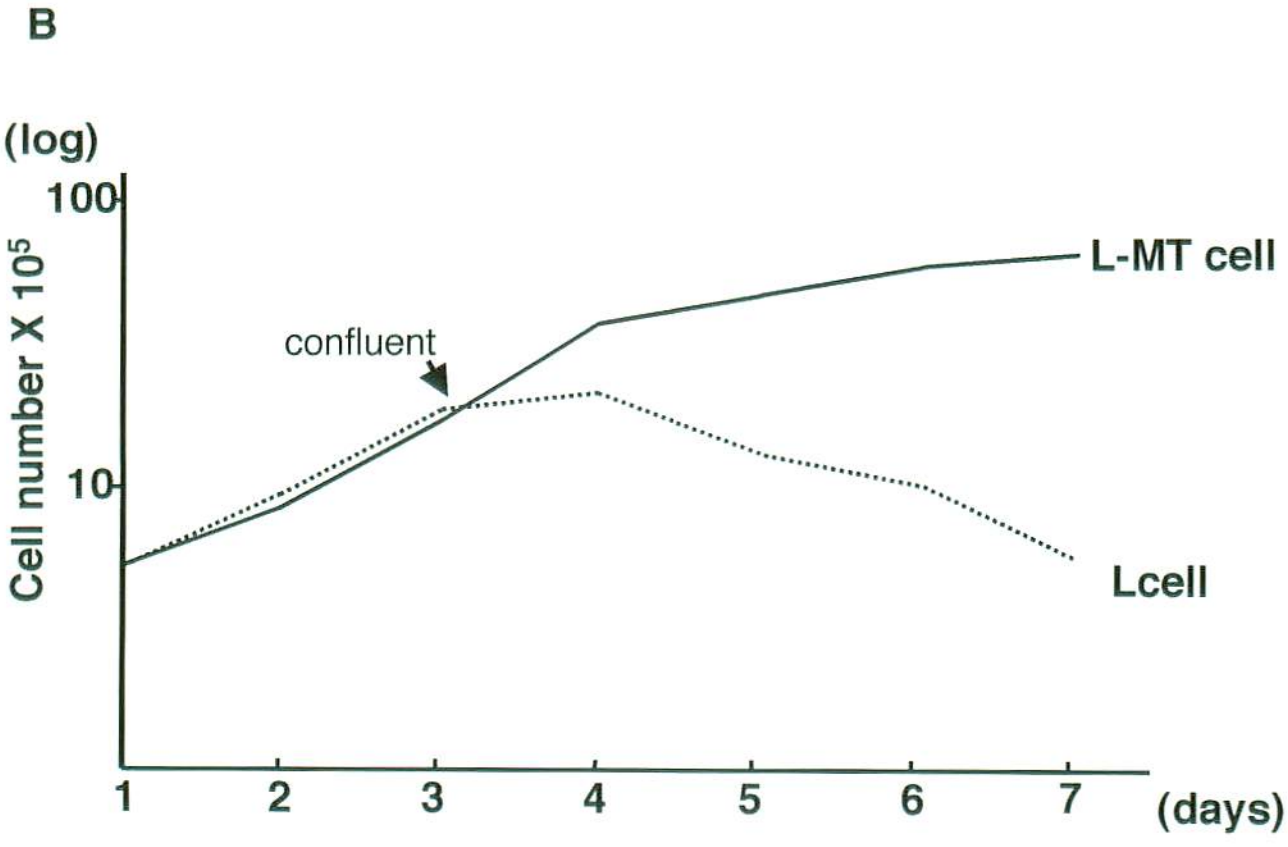


Figure 5. Growth advantage and multi-layer growth by activated β -catenin in L-MT cells

Expression of β-catenin was up-regulated by withdrawal of doxycycline in the L-MT cells but not in L cells. Using these cell lines, we aimed to isolate genes regulated by β-catenin. Fluorescent differential display analysis using L-MT cells revealed a fragment (D15) whose intensity was decreased in response to the accumulation of β-catenin. DNA sequencing and a subsequent search of the databases for homologies revealed identity of the DNA sequence of D15 to sequences of murine MCP-3.

The expression level of murine *MCP-3* was inversely correlated with the increase of β-catenin in L-MT cells upon the withdrawal of doxycycline (Figure 6).

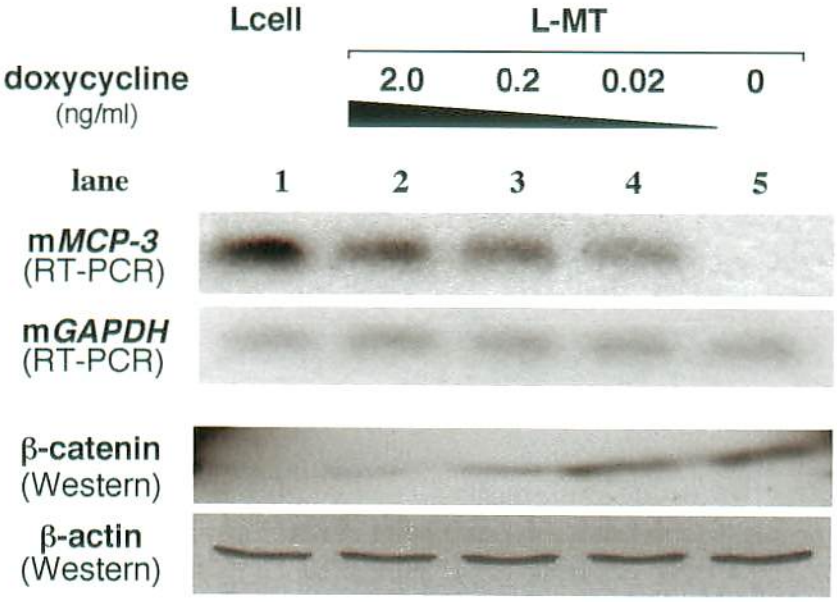


Figure 6. Inverse correlation of expression levels of murine *MCP-3* and β-catenin.

L cells and L-MT cells were grown without or with doxycycline at various concentration for 48 hours. RNAs and protein lysates from these cells were used for either semi-quantitative RT-PCR (for m*MCP-3* and m*GAPDH*) or Western blotting (for β-catenin and β-actin).

That is, when we depleted the culture medium of doxycycline, a significant decrease of *MCP-3* expression in L-MT cells was observed within 4 h and expression became undetectable after 8 h (Figure 7). These results indicated that even a small amount of accumulated β-catenin can reduce the expression of *MCP-3*.

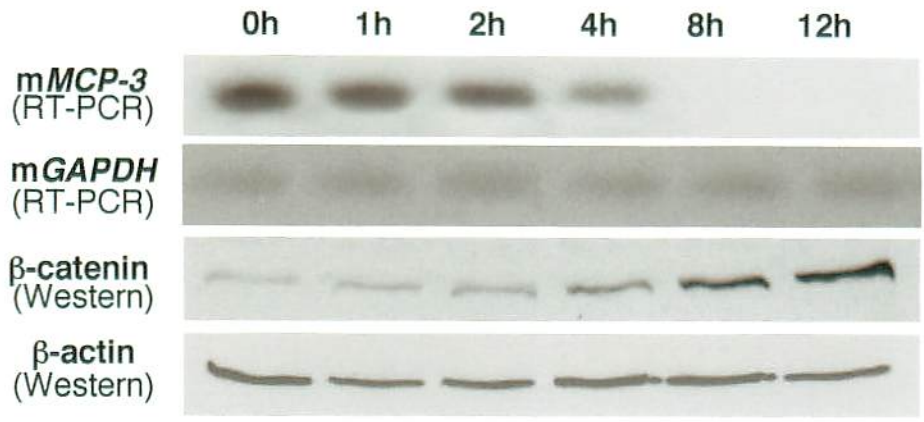
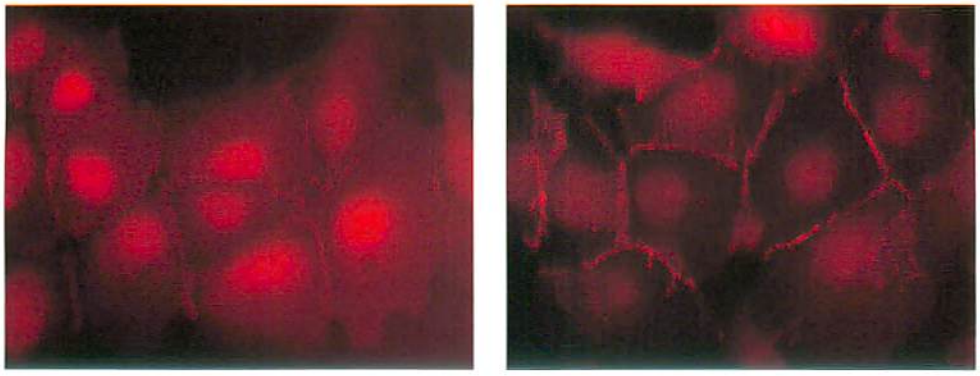


Figure 7. Time-course of reduction of m*MCP-3* in response to the accumulation of β-catenin in L-MT cells.

Prior to the deprivation of doxycycline, L-MT cells were incubated with medium containing 20 ng/ml of doxycycline. RNAs and protein lysates, extracted from these cells at 0, 1, 2, 4, 8 and 12 hours after the deprivation, were used for either semi-quantitative RT-PCR (for m*MCP-3* and m*GAPDH*) or Western blotting (for β-catenin and β-actin).

To confirm the inverse correlation between expression of MCP-3 and accumulation of β-catenin, we used a viral vector designed to express the 20-amino-acid-repeat, β-catenin-binding domain of APC (Ad-APC). Infection of these adenoviruses into colon-carcinoma cell line SW480, in which a large amount of β-catenin accumulates in the nucleus and cytoplasm (43), conferred an evident decrease of β-catenin (Figure 7). However, this change was not observed when SW480 cells were infected with adenovirus containing the LacZ gene (Ad-LacZ) (Figure 8). It was shown in our previous paper that the reduced expression of β-catenin by Ad-APC was correlated with decreased Tcf/LEF specific transactivation activity (44).



Ad-LacZ (100MOI)

Ad-APC (100MOI)

Figure 8. Depletion of nuclear and cytoplasmic β-catenin after adenoviral transfer of APC into SW480 cells.

Cells infected with Ad-APC or Ad-LacZ were fixed and incubated with anti-β-catenin antibody, then stained with a rhodamine conjugated secondary antibody. (Original magnification, x600)

Transfection of the SW480 cells with Ad-APC significantly increased expression of human MCP-3, but no such change occurred in cells transfected with Ad-LacZ (Figure 9).

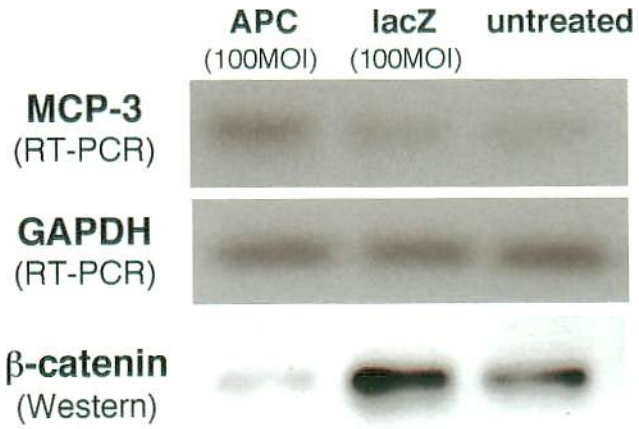


Figure 9. Increased expression of MCP-3 in response to depletion of β-catenin in SW480 cells.

3-2. Putative Tcf/LEF-binding motif in the promoter region of human MCP-3

To identify an element responsible for transcription in the promoter region of h*MCP-3*, we constructed two reporter-plasmid clones containing different lengths of the region upstream of the luciferase gene (Figure 10).

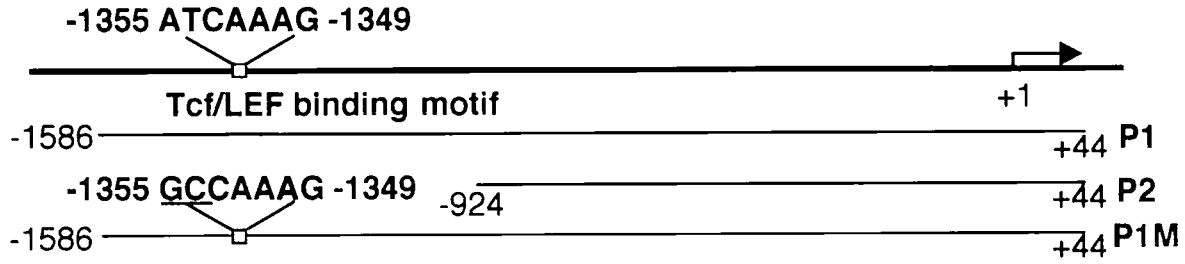


Figure 10. Construction of reporter-gene plasmids representing the MCP-3 was subcloned into a pGL3-Basic Vector.

Plasmid P1M, containing a two-base substitution in the putative Tcf/LEF-binding site.

The luciferase activity of reporter-plasmid P1 was significantly lower than that of P2 in the two colon cancer cell lines examined (Figure 11), indicating the presence of a transcription-suppressing element between -1586 and -924. Since this candidate region contained ATCAAAG, a possible Tcf/LEF-binding motif, we hypothesized that this motif might be responsible for the transcriptional repression. To investigate that hypothesis, we constructed reporter plasmid P1M, in which the candidate Tcf/LEF-binding motif was changed to GCCAAAG (Figure 10). The luciferase assay using these three plasmids revealed that the 1.6-kb fragment containing the mutated motif had lost the ability to suppress transcription of *MCP-3*; its luciferase activity was equivalent to that of the P2 fragment (Figure 11). These results implied that the putative Tcf/LEF-binding motif is involved in repression of MCP-3 transcription.

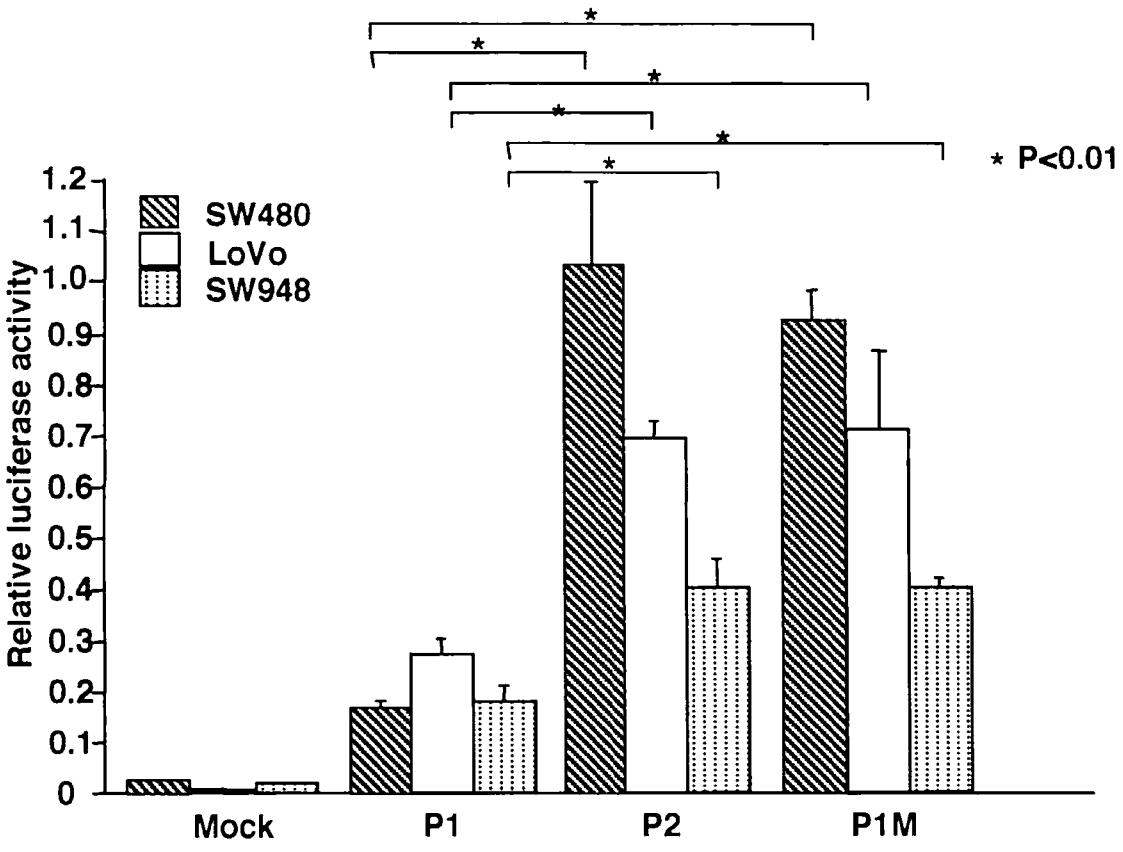


Figure 11. Reporter assay of *MCP-3* promoter using P1, P2 and P1M in two colon cancer cell lines was performed in triplicates.

Error bar, SD. Significantly different from P1 at $p<0.05$ (Scheffé's F test).

To confirm a direct mode of interaction between the β-catenin complex and this promoter element, we prepared a wild-type double-stranded DNA encompassing this sequence and a mutant DNA that involved replacing two base pairs within the motif. Using nuclear extracts from SW480 cells, we performed an EMSA experiment and found that the β-catenin complex bound specifically to this putative element. The specific band was supershifted by the addition of anti-β-catenin antibody (Figure 12).

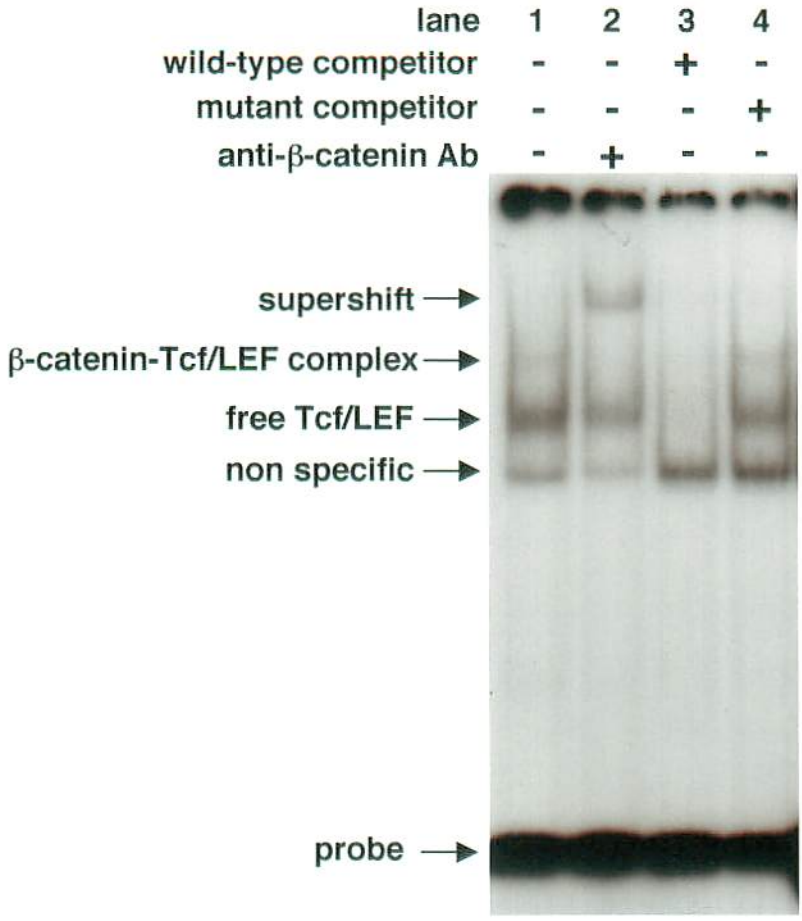


Figure 12. EMSA, using a ^{32}P-labeled DNA probe containing the wild-type putative Tcf/LEF-binding element in the MCP-3 promoter

The specific band corresponding to the DNA-β-catenin complex was supershifted by the addition of anti β-catenin antibody (lane 2). An excess of unlabeled wild-type DNA or mutant DNA was used for competition experiments (lanes 3 and 4).

3-3. Differentiation of colon cancer cells by overexpression of MCP-3

To investigate the biological role of MCP-3 in the colon, we transfected hMCP-3 cDNA into coloncancer cell line HT-29, to establish HT-29-MCP3 in which a high level of MCP-3 was constitutively expressed. The HT-29-MCP3 cells showed no apparent difference from parental HT-29 cells in morphology, growth rate, or ability to form colonies (data not shown). However, we found significant differences in regard to CEA production and ALP activity between HT-29-MCP-3 cells and HT-29-con cells transfected with pcDNA 3.1(+) alone. Sodium butyrate (NaB) is known to promote differentiation of colonic epithelial cells (44-50) and NaB treatment of HT-29 cells increases both the CEA level and ALP activity significantly compared with untreated HT-29 cells. Even in the absence of NaB, HT-29-MCP3 cells revealed 1.7-fold and 1.3-fold excesses of CEA level and ALP activity respectively, compared with HT-29-con cells, while NaB treatment of HT-29-MCP3 cells further enhanced production of CEA and ALP activity (Figure 13).

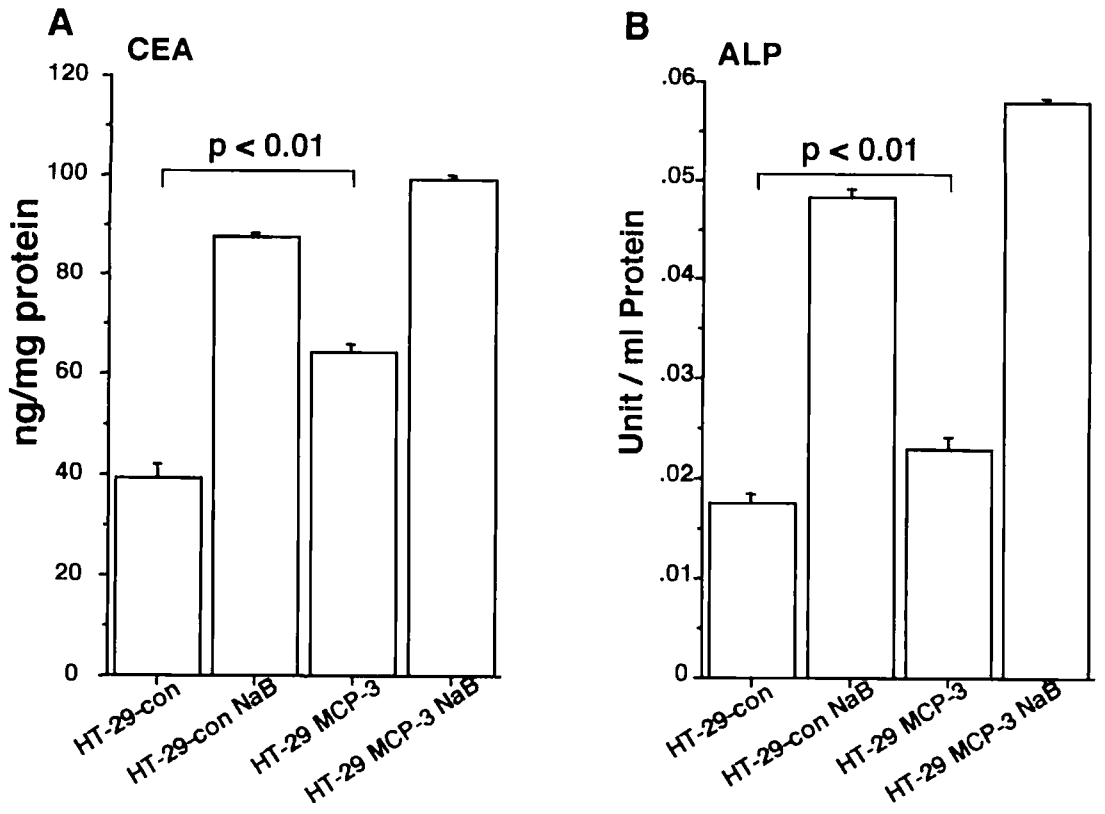


Figure 13. The effect of MCP-3 over-expression on differentiation.

Production of CEA (A) and activity of alkaline phosphatase (B) were measured as markers of differentiation in cells transfected with vector alone (HT-29-con) or with the *MCP-3* gene (HT-29-MCP3) with or without addition of sodium butyrate (NaB), an agent that promotes differentiation. *Error bar,* SD. Significantly different from HT-29 control cells at $p<0.01$ (Scheffé's F test)

Chapter 4

Discussion

The data presented here have demonstrated that MCP-3 is down-regulated by an activated form of β-catenin, and that this decreased expression occurs through direct association of the β-catenin complex with a putative Tcf/LEF-binding motif present in the MCP-3 promoter.

A number of other mammalian genes including c-myc, cyclin D1, matrilysin (matrix metalloproteinase-7), WISP, c-jun, fra-1, uPAR, ZO-1 (21-25,51,52) and NBL4 (26) are known to be regulated by stabilization and activation of β-catenin. Moreover, several target genes for Wnt signaling have been identified in Xenopus and Drosophila, among them the nodal-related 3 gene Xnr3 (a member of the TGF-β superfamily); fibronectin; and homeobox genes engrailed, goosecoid, twin, siamois, and ultrabithorax (53-58). All of them except ZO-1 are transactivated by accumulation of β-catenin. The down-regulation of Tcf-dependent transcription by Groucho, CREB-binding protein (CBP), Sox protein and NEMO-like kinase (NLK) were also reported, although their association with accumulation of β-catenin is unclear at present (59-63). Down-regulation of genes by Tcf/LEF is thought to reflect one of the following possibilities: (1) Tcf/LEF may bind directly to its binding motif and

suppress transcription; (2) since WRM, the homolog of β-catenin in C. elegans, is required to down-regulate the Tcf-like protein POP-1 (64), reduced expression may result from repression of another Tcf-family protein that recognizes a similar binding motif; or (3) the suppression may be a secondary effect of primary targets of Tcf/LEF. These possibilities have not been resolved as yet. However, our data regarding the MCP-3 gene clearly demonstrate that decreased expression by the β-catenin complex is one mechanism by which it regulates downstream genes. Since the Tcf/LEF complex recruits various co-activators or co-repressors to modulate transcription, it is conceivable that these associated molecules in combination may determine its function.

In HT-29 cells, we also found that over-expression of MCP-3 induces CEA and ALP activities, both of which are known to be differentiation markers for the cells (39, 42-47). We did not detect any morphological differences between MCP-3 transfected HT-29 cells and their parent cells, which may suggest that the effect of MCP-3 alone is not enough to induce detectable morphological changes or it is involved in a differentiation process not related to microscopic appearance. Hence, repression of MCP-3 may suppress differentiation of the colonic epithelium; this would represent a heretofore unsuspected mechanism

operating in colorectal tumor cells; i.e., inhibition of differentiation by activated β-catenin. Although the relationship between MCPs and differentiation in colonic cells has not been investigated thoroughly, one group has found that expression of MCP-1 in parenchymal cells was correlated with the histological grade of invasive ductal breast carcinomas (65). In addition, impaired expression of MCP-1 was involved in cervical tumorigenesis (66).

Therefore the novel role of β-catenin revealed in the experiments documented here has brought a more profound understanding of the mechanisms that underlie in the colorectal tumorigenesis. Furthermore, controlling MCP-3 expression may represent a means of therapeutic intervention for treatment of cancer patients in the future.

Chapter 5

Conclusion

Our results implied that activation of β-catenin through the Tcf/LEF signaling pathway may participate in colonic carcinogenesis by inhibiting MCP-3-induced differentiation of colorectal epithelial cells.

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