TTCACGCTAGTGACCG; Phlda3-R, TGGATGGCCTGTT-GATTCTTGA; Gapdh-F, AACTTTGGCATTGTGGAAGG; Gapdh-R, ATGCAGGGATGATGTTCTGG. The amplified products by Ampli*Taq* Gold (Applied Biosystems) were separated on a 2% agarose gel and visualized with ethidium bromide. Otherwise, real-time PCR assay was carried out using Power SYBER green PCR Master kit (ABI).

Chromosome spreads

Mitotic cells were prepared by treatment with 20 ng/ml nocodazole for 6 h and then collected. The collected cells were swollen hypotonically with 75 mM KCl for 15 min, and then fixed with Carnoy's solution (75% methanol/25% acetic acid) for 20 min. After changing the fixative once, the cells were dropped in Carnoy's solution onto glass slides and air-dried. The slides were stained with 4% Giemsa (Merck) solution for 10 min, washed briefly in tap water, and air-dried.

Supporting Information

Figure S1 Representative images of MEFs during the lifespan. MEFs cultivated as in Figure 1A top lead into either immortality development under Std-3T3 or quiescence preservation under tSD-3T3. After serial cultivation, MEFs become morphologically senescent, i.e., flattened and enlarged morphology (P9) under both Std-3T3 and tSD-3T3 conditions. While continuous MEF-culture under tSD-3T3 preserved the quiescent status with continuously senescent morphology, continuous MEF-culture under Std-3T3 lead to the sporadic emergence of immortalized colony from the senescent MEFs. Immortalized MEFs (IP2) are morphologically escaped from senescence and rather similar to that in early passage (P3).
(TIF)

Figure S2 H2AX diminution is also observed in adult mice organs. Samples were prepared from five week (5W), five month (5M) and seven- or nine-month-old mice (7M or 9M). Compared to five months old organs, H2AX protein level is diminished in Testis (9M), Brain (7M), and Colon (7M), in which the diminution levels are lower than those in Liver, Spleen, and Pancreas. In Heart and Thymus, H2AX levels did not altered the alteration in through 5 weeks old to 7 or 9 months old.
(TIF)

Figure S3 H2AX diminution is also shown in damage induced premature senescence. Premature senescence was induced with NCS treatment as shown schematically in the top, in which each red arrowhead represents 100 ng/μl NCS treatment. Premature senescence by damage was induced with H2AX diminution, in which cells showed typical senescent morphology of flattened and enlarged.
(TIF)

Figure S4 H2AX transcript is decreased in quiescent MEFs. Decrease in H2AX mRNA level in senescing MEFs was observed by RT-PCR (right panel) and is compared with protein diminution (left panel).
(TIF)

Figure S5 H2AX over-expression accelerates immortality development in MEFs with tetraploidy. A. Experimental scheme of H2AX over expression. After transfection of H2AX-over expressing (H2AX-OE) or empty control vectors into early passage MEFs (P3), the transformed MEFs were selected, re-plated, and maintained in complete medium until immortalized cells appeared. **B.** Growth curves of MEFs during the experiments in A. MEFs before transfection and re-plating, MEFs transfected with H2AX-over-expressing vector, and MEFs transfected with empty control vector are indicated by black closed squares, red open circles, and black open diamonds, respectively. MEFs over-expressing H2AX showed accelerated development of immortality. **C.** H2AX status was determined as indicated in the figure. Although senescence was induced in the transfected and selected MEFs, H2AX over-expressing MEFs show higher levels of H2AX after the selection resulting in the development of immortality with H2AX recovery. **D.** Representative MEF images during accelerated immortality development with H2AX over-expression and controls. MEFs transfected with the H2AX over-expressing vector showed an efficient escape from senescence, while MEFs carrying the negative control vectors remained senescent with a flattened and enlarged morphology. **E,F.** Genomic instability status in immortalized MEFs (IP3) that were developed with H2AX over-expression was assessed by flow-cytometry (**E**) and Giemsa staining of M-phase chromosome (**F**).
(TIF)

Figure S6 p53 expression in senescing MEFs. To determine p53 expression in the cause of senescence, the expression levels of p53 and the targets (Sidt2 and Phlda3) that are likely associated with tumor suppression were compared between early passage (P2) and senescent MEFs (P7) under tSD-3T3 conditions. Along with H2AX diminution under p53 proficient background after serial cultivation, the expressions of Sidt2 and Phlda3 were observed in senescent MEFs (P7), in which the change in the expressed p53 transcript is limited.
(TIF)

Figure S7 p53 activation shown by miR34a expression in primary wt-MEFs after damage is not directly associated with H2AX expression levels at least for transcript regulation. A. To confirm p53 dependent DNA damage response, wt- and $p53^{-/-}$-MEFs in primary and immortal were treated with 200 ng/ml neocarzinostatin (NCS) for 6 hours and the expression of p53-target miR34a was assessed. As expected, miR34a expression was shown after NCS treatment in primary wt-MEFs (wild type) but neither in immortalized wt-MEFs nor in $p53^{-/-}$-MEFs. **B.** To determine the p53-activation associated change in the expression levels of H2AX transcript, mRNA levels of H2AX in MEFs treated as in **A** were analyzed. Whereas p53 is activated after NCS treatment in primary wt-MEFs, H2AX transcript levels were stable, suggesting no direct regulation by p53 transcription factor for H2AX expression. The PCR primers for miR34a were used from miRNA-specific primers (ABI) with snoRNA202 (ABI) for the control. Real-time PCR assay was carried out TaqMan microRNA assay kit (ABI).
(TIF)

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Author Contributions

Conceived and designed the experiments: KY. Performed the experiments: YA H. Fuji H. Fukuda AI KS YY MS YI JU KY. Analyzed the data: H. Fuji KY. Contributed reagents/materials/analysis tools: SM NT YH HN MM. Wrote the paper: KY H. Fukuda HT.

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Role of the long form leptin receptor and of the STAT3 signaling pathway in colorectal cancer progression

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Abstract. Although a number of recent studies have reported the involvement of leptin in colorectal carcinogenesis, findings are contradictory and difficult to interpret. Our group has previously reported that leptin signaling might have an important role in the development of colorectal adenomas. In this study, we investigated leptin signaling in colorectal carcinogenesis focusing in particular on the differences in leptin signaling between colorectal adenoma and cancer. Whereas no significant differences in the serum leptin levels were observed among normal control subjects and adenoma/cancer patients, increased expression and activation of the long form leptin receptor (ObRL) was observed in colorectal adenoma and cancer tissues compared with the normal colorectal tissues. However, no significant differences were observed between the colorectal adenoma and cancer tissues. Significant increases in the phosphorylation levels of important molecules of the JAK/STAT signaling pathway, located downstream of leptin signaling, and transcriptional regulation of STAT3-downstream target molecules were observed in colorectal adenoma tissue compared with the findings in normal colorectal tissues. Furthermore, these changes were significantly more pronounced in colorectal cancer compared to colorectal adenoma tissues. This is the first analysis of leptin and JAK/STAT signaling in a human colorectal adenoma-carcinoma sequence. These results suggest that the STAT3-mediated leptin signaling through the activation of ObRL may be involved in colorectal carcinogenesis, both in adenoma formation and in the progression to cancer. STAT3 signaling in colorectal cancer may be mediated not only by leptin but by other factors.

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Key words: leptin, colorectal cancer, leptin receptor, STAT3

Introduction

A number of recent studies have reported that leptin is involved in colorectal carcinogenesis (1-3). Although several studies have demonstrated the role of leptin as an important growth factor for colorectal cancer cell lines *in vitro* (1-3), the relationship between leptin signaling and colorectal cancer development *in vivo* still remains controversial. Animal experiments conducted *in vivo* in rodent models of colorectal carcinogenesis have revealed no effects of leptin signaling on colorectal carcinogenesis (2,4,5). In contrast, many previous clinical studies have shown a significant association between serum leptin levels and the presence of colorectal cancer (6-13). Thus, the results of previous clinical studies and animal experiments are contradictory and difficult to interpret.

In a previous study, we demonstrated an important role of leptin signaling in the development of colorectal adenoma (14). Although we found no association between serum leptin levels and the presence of colorectal adenoma, increased expression of the long form leptin receptor (ObRL) and activation of JAK/STAT signaling were observed in colorectal adenoma tissues as compared with the observations in normal tissues. These results clearly suggest that the leptin signaling pathway plays a crucial role in the growth of colorectal adenoma.

Meanwhile, the progression of adenoma to cancer is another important step in colorectal carcinogenesis. However, the role of leptin signaling in the progression of adenoma to cancer is still unclear. Therefore, in this study, we investigated the leptin signaling in colorectal carcinogenesis especially focused on the differences in leptin signaling between colorectal adenoma and cancer.

Materials and methods

Study population. The study protocol was approved by the Yokohama City University Hospital Ethics Committee. Written informed consent was obtained from all the subjects prior to their participation in the study.

Thirty-one patients diagnosed as having colorectal cancer between January 2008 and July 2009 at Yokohama City

Table I. Characteristics of the study patients.

Characteristics	Controls	Adenoma	Cancer	P-value
No. of patients	68	60	31	
Age (year)	62.0±14.0	64.3±11.1	64.3±8.7	0.53
Gender, male/female	37/31	40/20	19/12	0.37
Waist circumference (cm)	84.3±10.1	85.5±8.3	84.0±9.6	0.52
BMI (kg/m^2)	22.6±3.5	23.5±2.7	22.4±3.2	0.10
VFA (cm^2)	74.6±49.7	91.0±39.0	83.7±49.1	0.08
FBS (mg/dl)	111.7±27.2	105.0±25.5	103.9±18.8	0.42
HbA1c (%)	5.7±1.2	5.5±2.9	5.4±0.6	0.99
Leptin (ng/ml)	5.4±4.2	5.4±4.0	4.9±4.3	0.69

Data are shown as the mean ± standard deviation (SD). BMI, body mass index; VFA, visceral fat area; FBS, fasting blood sugar. Statistical analysis was performed by the Kruskal-Wallis test.

University Hospital, 68 control subjects in whom colonoscopy confirmed the absence of any colorectal polyps, and 60 adenoma patients were recruited for this study. The exclusion criteria were subjects with familial adenomatous polyposis, inflammatory bowel disease, radiation colitis or any malignant disease, and subjects with a previous history of colectomy, gastrectomy or colorectal polypectomy. We also excluded colorectal cancer patients with weight loss of over 5% in the previous 6 months, to avoid the confounding effects of the effects of weight loss on the serum leptin levels.

Collection and analysis of blood samples for determination of the leptin levels. Blood samples were obtained in the morning on the day of colonoscopy, after the subjects had fasted overnight. Serum leptin levels were measured with an enzyme-linked immunosorbent assay of human leptin (SRL Co., Tokyo, Japan).

Immunohistochemical analyses. The expressions of ObR and phospho-STAT3 (p-STAT3) were investigated in normal colorectal, adenoma and cancer tissues. A total 40 samples of normal colorectal and adenoma tissues, and 20 samples of cancer tissues were obtained from the study subjects. The tissue samples were isolated, formalin-fixed and paraffin-embedded; the paraffin-embedded samples were then deparaffinized and rehydrated. Sections prepared from the specimens were incubated with antibodies for ObR (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p-STAT3 (Tyr 705) (1:50, Cell Signaling Technology, Danvers, MA, USA) as the primary antibodies, using an LSAB2 kit (Dako Cytomation, Glostrup, Denmark). They were then incubated with biotinylated immunoglobulin as the secondary antibody and treated with peroxidase-conjugated streptavidin. The antibody complex was visualized with 3,3'-diaminobenzidine, tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan). The expressions of ObR and p-STAT3 were analyzed with light microscopy in 10 different fields of each section, and the mean percentage of the gland cells that showed positive staining was scored by two pathologists. The ObR and p-STAT3 expressions were classified into two categories depending on the percentage of cells showing positive staining: negative, 0-15% of all the gland cells showing positive staining; positive, >15% of all gland cells showing positive staining, as previously described (15).

Western blot analysis. Samples of normal colorectal tissue and colorectal adenoma and cancer tissues were isolated, and the extracted protein was separated with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE); the separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, London, UK) and probed with primary antibodies specific for phospho-ObR (p-ObR) (Tyr 1141), p-ObR (Tyr 985), ObR (Santa Cruz Biotechnology), phospho-JAK2 (p-JAK2), JAK2, p-STAT3 (Tyr 705) STAT3 (Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, MD, USA). Horseradish peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham) were used for the detection of specific proteins.

Real-time RT-PCR. Samples of normal colorectal tissues and colorectal adenoma and cancer tissues were isolated, and total-RNA from the colorectal cancer and normal colorectal tissues was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). For the real-time reverse-transcriptase polymerase chain reaction, total-RNA was reverse-transcribed into cDNA and amplified with the real-time quantitative polymerase chain reaction method using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Probes and primer pairs specific for ObRL, short form leptin receptor (ObRS), BclX, c-Myc, cyclin D1, cdc2, cyclin B1, VEGF and 18S were purchased from Applied Biosystems. The concentrations of the target genes were determined using the competitive computed tomography method and the values were normalized to the internal control.

Statistical analysis. Statistical analyses were performed using the Kruskal-Wallis test and the Fisher's PLSD test. All analyses were performed using the StatView software (SAS Institute, Cary, NC, USA). A P-value <0.05 was regarded as denoting statistical significance.

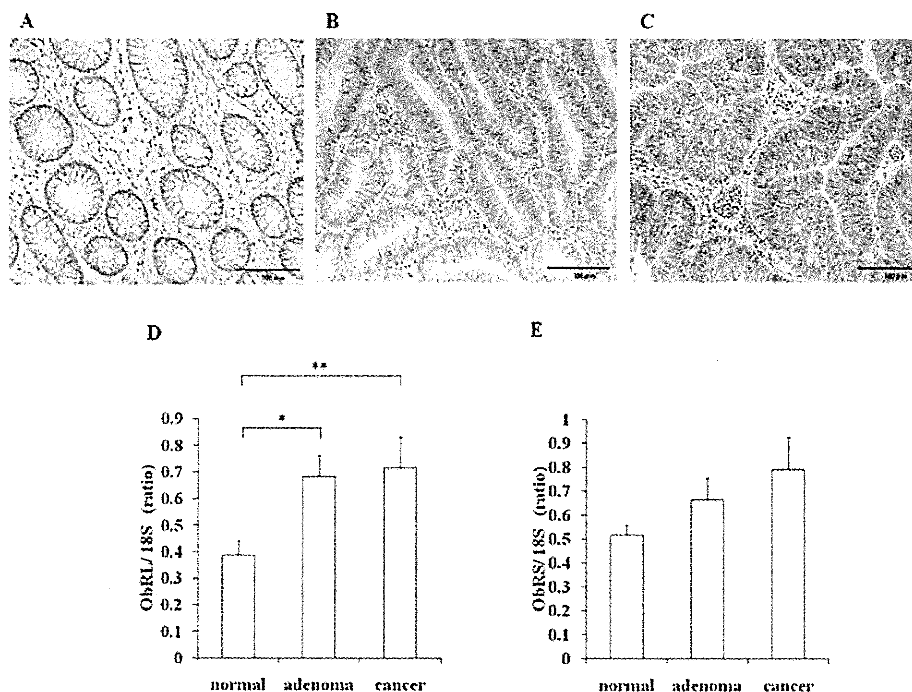

Figure 1. (A-C) Immunohistochemical staining for leptin receptor (ObR) and (D and E) mRNA levels of the long and short forms of ObR, namely, ObRL and ObRS. (A) Normal colorectal tissue, (B) colorectal adenoma tissue and (C) colorectal cancer tissue. Scale bar, 100 μm. The relative mRNA expressions of (D) ObRL and (E) ObRS in normal colorectal, and colorectal adenoma and cancer tissues are expressed as the ratios relative to the expression level of 18S. Each column represents the mean ± SEM. *P<0.05; **P<0.01.

Results

Serum leptin levels and colorectal cancer. The clinical characteristics of the colorectal cancer patients and the control subjects are shown in Table I. No significant differences in the serum leptin levels were observed in these groups. There were also no significant differences in the age, BMI or other obesity-related factors in these groups.

Leptin receptor (ObR) expression in normal colorectal, colorectal cancer and adenoma tissues. To examine the expression levels of the ObR in normal colorectal, colorectal adenoma and cancer tissues, immunohistochemical staining and gene expression analyses were performed. Strong expression of ObR was found in the colorectal adenoma and cancer tissues, but not in the gland cells of normal colorectal tissue (Fig. 1A-C). The detection frequency of ObR in colorectal cancer was 90.0% (18/20) and that in colorectal adenoma was 67.5% (27/40). In addition, the mRNA expression levels of ObRL and the ObRS in the normal colorectal and colorectal cancer and adenoma tissues were investigated. The expression levels of ObRL mRNA were significantly higher in the colorectal adenoma and cancer tissues than in the normal colorectal tissue (Fig. 1D). No significant differences in the expression levels were observed between colorectal cancer and adenoma tissues. The expression levels of ObRS were also higher, but not significantly, in the colorectal adenoma and cancer tissues than in the normal colorectal tissue (Fig. 1E).

Activation of leptin receptor. We also investigated the activation of the leptin receptor with Western blot analysis of the phosphorylation of the cytoplasmic domain of ObRL. A significant increase in ObR expression and phosphorylation of ObRL-Tyr1141 was observed in the samples collected from the colorectal adenoma and cancer tissues as compared with the findings in normal colorectal tissue (Fig. 2A and B). No significant differences were observed between the colorectal cancer and colorectal adenoma tissues. As the phosphorylation of Tyr 1141 in ObRL is required for leptin-induced activation of STAT3 (16), these results indicate that overexpression and activation of ObR are induced in colorectal adenoma and cancer tissues. In contrast, no significant difference was observed in the phosphorylation level of Tyr 985 in ObR, which is required for activation of the ERK (extracellular-signal-regulated kinase) signaling pathway (Fig. 2C) (17).

Phosphorylated STAT3 in colorectal cancer. To confirm the activation of the STAT3 pathway, immunohistochemical staining and Western blot analysis for p-STAT3 were performed. Increased expression of p-STAT3 was observed in the nuclei of gland cells of the colorectal adenoma and cancer tissue specimens, indicating that STAT3 was activated in these tissues. On the other hand, no expression was observed in normal colorectal tissue specimens (Fig. 3). The detection frequency of p-STAT3 in colorectal cancer was 85% (17/20) and that in adenoma was 57.5% (23/40). Western blot analysis demonstrated that the levels of p-JAK2 and p-STAT3 were significantly higher in colorectal cancer tissue than in the normal colorectal and adenoma tissues (Fig. 4). These results suggest that activation of STAT3 was significantly increased in colorectal adenoma and cancer tissues compared with normal colorectal tissues, with significantly increased activation also

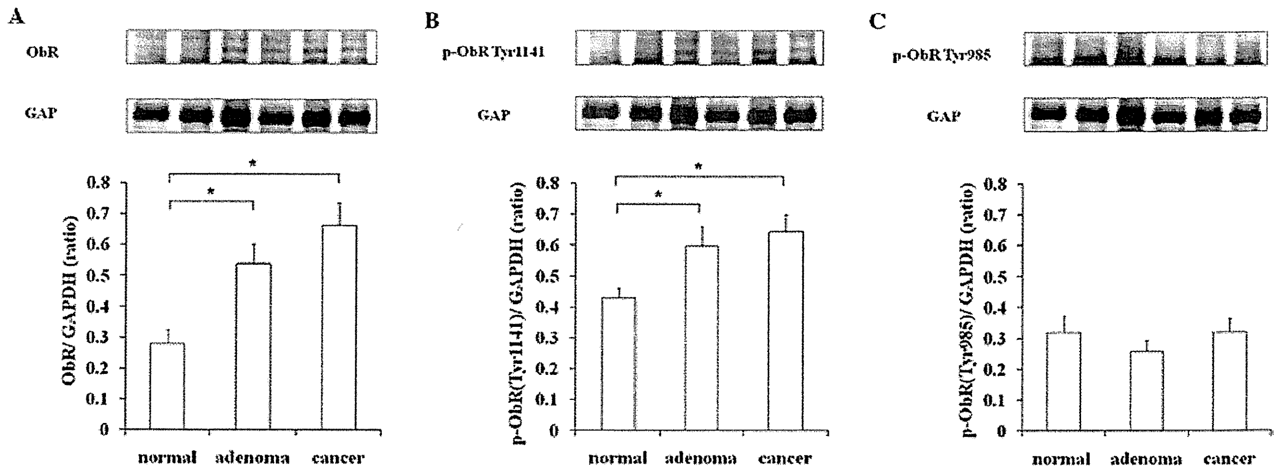

Figure 2. Western blot analysis for ObR and phosphorylated ObR (Tyr 1141 or Tyr 985). (A) ObR, (B) Tyr 1141-phosphorylated and (C) Tyr 985-phosphorylated ObR. Upper panels: representative Western blot analyses for ObR, Tyr 1141-phosphorylated and Tyr 985-phosphorylated ObR. Lanes 1 and 2: normal colorectal tissues; lanes 3 and 4: adenoma tissues; lanes 5 and 6: cancer tissues. Lower panels: ratios of the expression levels of ObR, Tyr 1141-phosphorylated and Tyr 985-phosphorylated ObR to the expression level of GAPDH are shown. Each column represents the mean ± SEM. *P<0.05.

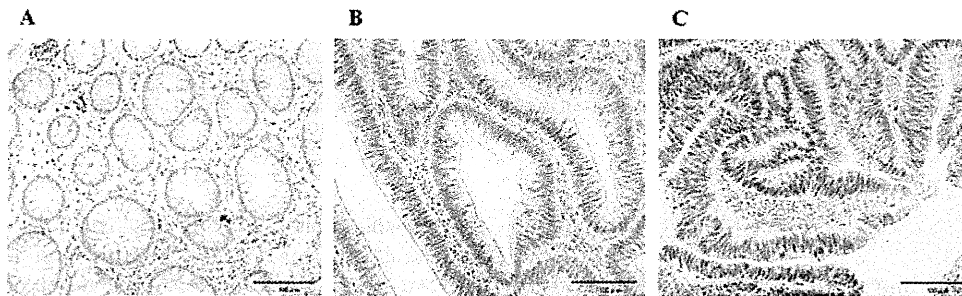

Figure 3. Immunohistochemical staining for phosphorylated STAT3. (A) Normal colorectal tissue, (B) colorectal adenoma tissue and (C) colorectal cancer tissue. Scale bar, 100 μm.

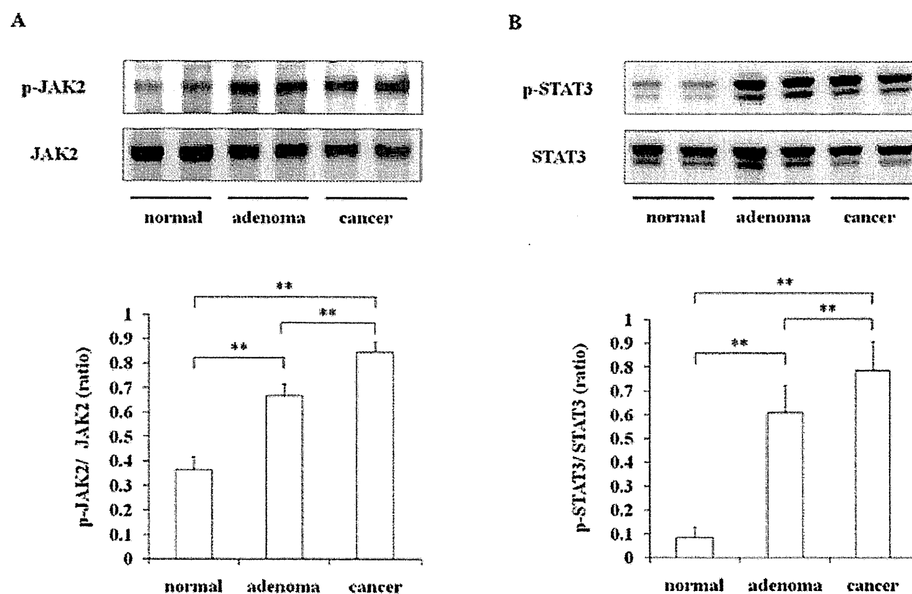

Figure 4. Western blot analysis for phosphorylated JAK2 and STAT3. (A) Phosphorylated JAK2 and (B) phosphorylated STAT3. Upper panels: Representative Western blot analyses for phosphorylated and total levels of JAK2 and STAT3. Lanes 1 and 2: normal colorectal tissue; lanes 3 and 4: adenoma tissue; lanes 5 and 6: cancer tissue. Lower panels: ratios of the levels of the phosphorylated proteins to the total protein level. Each column represents the mean ± SEM. **P<0.01.

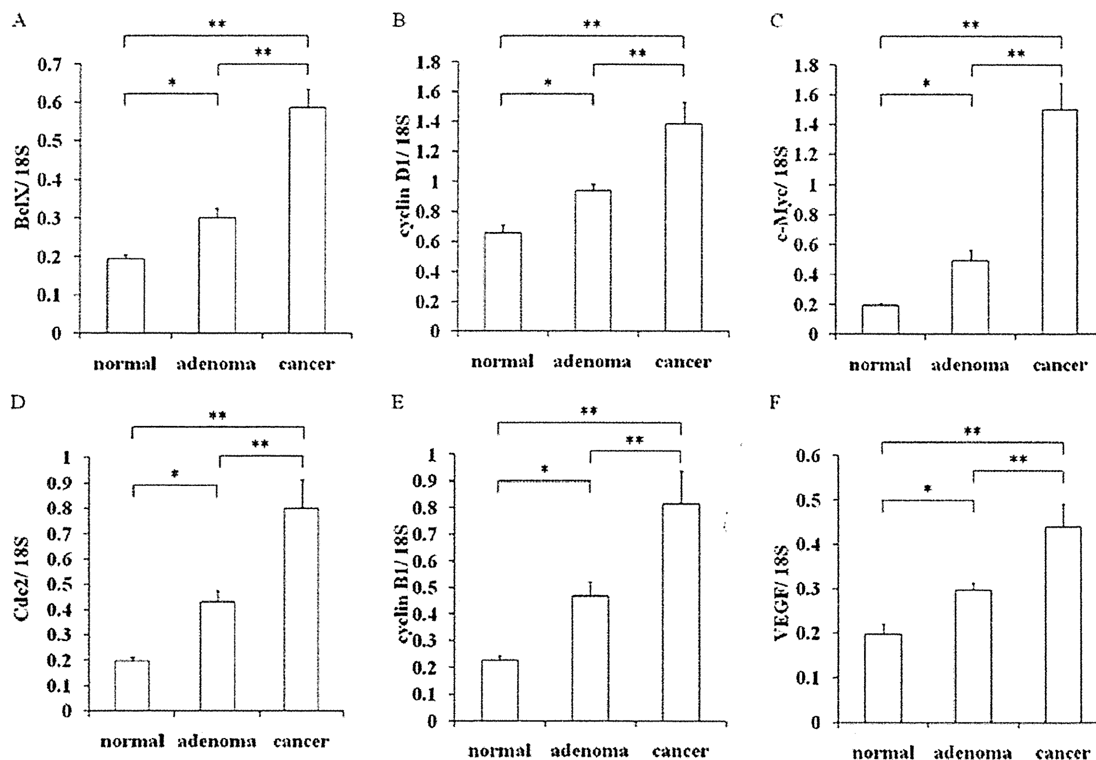

Figure 5. Expressions of downstream genes encoded by STAT3 transcriptional regulation in normal colorectal, colorectal cancer and adenoma tissues. The relative mRNA expressions of (A) BclX, (B) cyclin D1, (C) c-Myc, (D) cdc2, (E) cyclin B1 and (F) VEGF expressions in normal colorectal, adenoma and cancer tissues were expressed as ratios relative to the expression level of 18S. Each column represents the mean ± SEM. $^{*}P<0.05$; $^{**}P<0.01$.

seen in colorectal cancer tissues compared with colorectal adenoma specimens.

STAT3-mediated transcriptional regulation of gene expression. Alterations of the mRNA levels of the genes encoded by STAT3 were investigated with real-time RT-PCR. The expression levels of the apoptosis-suppressing protein BclX, the late G1 to G1/S phase proteins cyclin D1 and c-Myc, the G2/M phase proteins cdc2 and cyclin B1 (18), and of the genes encoding the angiogenesis protein VEGF (19) were significantly higher in colorectal cancer tissue than in the normal colorectal and colorectal adenoma tissues (Fig. 5). These results suggest strong activation of the JAK/STAT signaling pathway in colorectal cancer tissues as compared with normal colorectal and colorectal adenoma tissues.

Discussion

Previous studies have suggested the existence of an association between the serum leptin levels and the development of colorectal cancer (6-13). In this study, however, we observed no statistically significant differences in the serum leptin levels among the normal control and colorectal adenoma/ cancer patients. Stachowicz *et al* reported that the serum leptin levels were not dependent on the clinical or pathological stage of progression of colorectal cancer (20). Combined with this report, the results of our present study suggest that the serum leptin levels are not associated with the presence of tumor in the course of colorectal carcinogenesis.

We observed significantly higher expression and activation levels of ObRL in colorectal adenoma and cancer tissues than in normal colorectal tissue. In addition we focused on the differences in the expression and activation levels between colorectal adenoma and cancer tissues, and observed no significant differences in the expression or activation levels of ObRL between colorectal adenoma and cancer tissues. These results suggest that leptin may play a crucial role not only in the development of colorectal adenoma but equally in that of colorectal cancer.

We also showed activation of the JAK/STAT pathway which is located downstream of the activation of leptin receptor signaling. Several other studies have also reported more pronounced activation of JAK/STAT signaling in colorectal cancer tissues than in normal colorectal tissue (15,21-23). These reports lend support to our present observations. However, our results showed a discrepancy between the expression and activation levels of ObR and the activation of JAK/STAT signaling. Although we observed no significant differences in the expression or activation levels of ObR between colon cancer and adenoma tissues, activation of the JAK/STAT pathway was significantly more pronounced in colorectal cancer tissue than in colorectal adenoma tissue. JAK/STAT signaling has been reported to be mediated not only by leptin receptor signaling, but also by several cytokines and hormones, such as IL-6 (24-26). Therefore, based on our findings we suggested that STAT3 was phosphorylated by leptin signaling in both colorectal cancer and adenoma tissues. In addition, in colorectal cancer, we speculated that STAT3 may also be phosphorylated by other signaling processes.

These results suggest that leptin signaling in the activation of STAT3 was important especially in colorectal adenoma that was in an early phase of colorectal carcinogenesis.

In conclusion, no significant differences in the serum leptin levels were observed among normal control subjects and adenoma/cancer patients. We observed increased expression and activation of the ObRL in colorectal cancer and adenoma tissues compared with normal colorectal tissue. Significantly increased phosphorylation levels of the important molecules of the JAK/STAT signaling pathway and transcriptional regulation of the STAT3-downstream target molecules were observed in colorectal adenoma tissues as compared with the findings in normal colorectal tissue. Furthermore, these changes were more pronounced in colorectal cancer tissue than in colorectal adenoma tissue. Further investigations are required to clarify the exact roles of leptin signaling and JAK/STAT signaling in colorectal carcinogenesis, and the detailed mechanisms underlying colorectal carcinogenesis.

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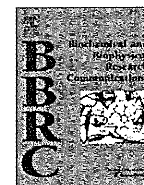

Differential expression of *nanog1* and *nanogp8* in colon cancer cells

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ABSTRACT

Nanog, a homeodomain transcription factor, is an essential regulator for promotion of self-renewal of embryonic stem cells and inhibition of their differentiation. It has been demonstrated that *nanog1* as well as *nanogp8*, a retrogene of *nanog1*, is preferentially expressed in advanced stages of several types of cancer, suggesting their involvement during cancer progression. Here, we investigated the expression of Nanog in well-characterized colon cancer cell lines. Expression of Nanog was detectable in 5 (HCT116, HT29, RKO, SW48, SW620) out of seven cell lines examined. RNA expression analyses of *nanog1* and *nanogp8* indicated that, while *nanog1* was a major form in SW620 as well as in teratoma cells Tera-2, *nanogp8* was preferentially expressed in HT29 and HCT116. In accordance with this, shRNA-mediated knockdown of *nanog1* caused the reduction of Nanog in SW620 but not in HT29. Inhibition of Nanog in SW620 cells negatively affected cell proliferation and tumor formation in mouse xenograft. Biochemical subcellular fractionation and immunostaining analyses revealed predominant localization of Nanog in cytoplasm in SW620 and HT29, while it was mainly localized in nucleus in Tera-2. Our data indicate that *nanog1* and *nanogp8* are differentially expressed in colon cancer cells, and suggest that their expression contributes to proliferation of colon cancer cells.

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1. Introduction

It has been well documented that a set of transcription factors, Oct-3/4, Nanog, and Sox-2, controls self-renewal and pluripotency of embryonic stem (ES) cells [1]. Accumulating reports indicate that these transcription factors, in combination with a set of regulatory microRNAs and transcriptional co-regulators that modify chromatin structure, form a key regulatory network controlling the identity and differentiation of ES cells [2–4].

Recent progress on cancer research revealed that, among the ES-specific transcription factors, Oct-3/4 and Nanog are expressed in a variety of human cancers [5–8]. Especially, expression of Nanog is associated with advanced stage and poor prognosis in some types of cancer [6,9–11].

Nanog is a homeodomain transcription factor that plays a crucial role in maintaining integrity of undifferentiated ES cells, and regulated by a variety of extrinsic and intrinsic signals [12]. Whereas external signal factors including HIF [13], Hedgehog (HH) [14,15], LIF [16], and TGF-β/activin/nodal [17,18] as well as the intrinsic transcription regulators such as Oct-3/4 and Sox-2 [19,20], SMAD [18], Klf4 [21,22], GLI1 [14,15], SATB [23], mSin3A–HDAC [24], β-catenin [25], and Sall4 [26] function to transcriptionally up-regulate Nanog expression, the induction of p53 [27,28] or epigenetic modification of its promoter [29,30] inhibits its expression. Nanog expression is also regulated by ES-specific microRNAs [31,32]. Activated Nanog, in a complex of associated transcription co-factors [33–35], in turn regulates gene transcription of its targets to promote self-renewal of embryonic stem cells and affects differentiation processes [2,34,36]. Presumably through the regulation of the expression of the target genes, Nanog functions to transit to a ground state for pluripotency of ES cells [37], and blocks their differentiation [38].

In addition to *nanog1*, an authentic gene that encodes Nanog, there are 10 pseudogenes for Nanog in the human genome [39,40]. Among them, *nanogp8* is the most recent pseudogene generated during evolution [39], and is regarded as a retrogene because it retains the capacity to code for a 305 amino acid polypeptide that is structurally very similar to the *nanog1* gene product [40], and is expressed in human cells [41]. In fact, ectopic expression of *nanogp8* generates the functional protein [41,42]. Because of high degree of homology between these genes, the protein products of *nanog1* and *nanogp8* are basically indistinguishable on western blot analyses or immunostaining [41,43]. Therefore, protein products of these genes are collectively referred to as Nanog [42,43].

Examination of Nanog expression in clinical studies revealed that Nanog is overexpressed in a variety of cancer [5–11,44,45].

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Indeed, expression of *nanog1* gene was demonstrated at least in some of cancer cell lines [46], and ectopic expression of *nanog1* contributes to stem cell-like properties to cells [5] and promotes cell proliferation [47]. Of note, *nanogp8* gene is rather a major form expressed in many types of cancer [15,41,42], promotes stem cell-like characteristics [15,43], and facilitates progression of cancer [15,41,42]. Thus, it is likely that both *nanog1* and *nanogp8* contribute to cancer development mediated by Nanog.

In human colon cancer, high levels of Nanog expression are associated with advanced stages of cancer and poor prognosis [10]. It is likely that expression of Nanog in colon cancer is functionally important because *nanog1* over-expression in colon cancer cells promotes its proliferation [10]. However, it remains unclear whether *nanog1* or *nanogp8* is a major form that is expressed in colon cancer cells, and the expression and subcellular localization of Nanog in those cells have not been well documented. These issues should be clarified to understand the molecular mechanisms of Nanog in colon cancer development.

In this paper, we investigated expression of Nanog in a set of well-characterized human colon cancer cells. Our data indicate that, while both *nanog1* and *nanogp8* are expressed in most colon cancer cells, they are expressed at varied ratios dependent on cell lines. Examination of subcellular localization indicates Nanog is mainly localized in cytoplasm in at least two colon cancer cells. In addition, Nanog inhibition by shRNAs in colon cancer cells caused growth inhibition in *in vitro* culture and in mouse xenograft, indicating the positive role of Nanog in proliferation of colon cancer cells.

2. Materials and methods

2.1. Cell culture

All colon cancer cells and Tera-2 were cultivated in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). FHC cells were cultivated in a 1:1 mixture of Ham F-12 medium and Dulbecco's modified Eagle medium (Invitrogen) supplemented with 25 mM Hepes (Invitrogen), 10 ng/ml cholera toxin (Calbiochem), 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 100 ng/ml hydrocortisone (Sigma), and 10% fetal bovine serum (Invitrogen).

2.2. Western blot analyses

Cells were lysed in lysis buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with protease inhibitors, and used for Western blot analyses as previously described [48], with anti-Nanog (ReproCELL, Tokyo), anti-Oct-3/4 (C-10, Santa cruz Biotechnology), anti-Sox-2 (H-65, Santa Cruz Biotechnology), anti-Actin (Sigma, A5316), anti-TopoI (C-21, Santa Cruz Biotechnology), or anti-beta-Tubulin (D-10, Santa cruz Biotechnology).

2.3. Lentivirus preparation and Infection

Lentiviral plasmid vectors expressing *Nanog1* shRNAs (#1, #2) or control shRNAs (#1, #2) were purchased from Sigma (the Mission shRNA clones). Note that *nanog1* shRNA (#2) corresponds to the older version of *nanog1* sequence (NM_024865.1) but not to the current version (NM_024865.2). Lentiviruses that express GFP or RFP (pCDH-CMV-MCS-EF1) were purchased from System Biosciences (CA, USA). The lentiviral plasmids were co-transfected with pLP1, pLP2 and pLP/VSVG (Invitrogen) into 293FT cells (Invitrogen), and virus-containing supernatants were prepared according to the manufacturer's instructions. For infection of the lentiviruses, SW620 cells were incubated with virus-containing supernatants in the presence of 6 μg/ml polybrene. Cells infected with the shRNA-expressing viruses or the control viruses were selected in the presence of 1 μg/ml puromycin. For cells infected with GFP or RFP-expressing viruses, flow cytometry analyses (FacsCalibur, Becton Dickinson) were performed to confirm that >90% of cells were infected.

2.4. RT-PCR analyses

Complementary DNA (cDNA) was synthesized from total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara). For amplification of *nanog* cDNA, the PCR reaction with the cDNA was performed with ExTaq (Takara) for 35 cycles with the following conditions: 94 °C (1 min), 68 °C (1 min), 72 °C (2 min). For amplification of *gapdh* cDNA, the PCR reaction was performed for 30 cycles with the following conditions: 94 °C (1 min), 55 °C (1 min), 72 °C (2 min). The following primer sets were used for PCR reactions: Nanog forward primer (5′-AACATGAGTGTGGATCCAG-3′), Nanog reverse primer (5′-TCACTCATCTTCACACGTCTTCAGGTTG-3′), GAPDH forward primer (5′-ACCACAGTCCAGTCCATCAC-3′), GAPDH reverse primer (5′-TCCACCACCCTGTTGCTGTA-3′).

2.5. Subcellular fractionation

Cells were rinsed in 1× phosphate-buffered saline (PBS), and cytoplasmic and nuclear fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Thermo Scientific). The prepared lysates were used for western blot analyses.

2.6. Immunostaining

Cells were fixed in 4% paraformaldehyde in PBS for 15 min, washed with 1× PBS, permeabilized in PBS/0.1% Triton X-100 for 5 min at 4 °C, and blocked with PBS containing 3% bovine serum albumin (BSA). The fixed cells were then used for immunostaining with anti-Nanog (ReproCELL) and DAPI.

2.7. GFP/RFP competition assays

The GFP-expressing SW620 cells were infected with the lentiviruses that express Nanog shRNA or the control viruses, selected in the presence of 1 μg/ml puromycin, and mixed with RFP-expressing SW620 at a 1:1 ratio, and used for an *in vitro* culture. For mouse xenograft experiments, the infected cells (1×10^6 cells) were suspended in medium containing 50% Matrigel (Becton Dickinson), and subjected for subcutaneous injection into NOG (NOD/Shi-*scid IL-2rg^null^*) mice. The inhibitory effects of Nanog shRNAs were evaluated by measuring the number of GFP/RFP-positive cells by flow cytometry, and calculating the reduction of GFP/RFP ratios after these experimental procedures.

3. Results and discussion

3.1. Nanog is expressed in a majority of colon cancer cell lines

In order to examine whether ES-specific transcription factors, Nanog, Oct3/4, and Sox2 are expressed in colon cancer cells, we performed western blot analyses of seven colon cancer cells (DLD-1, HCT116, HT29, RKO, SW48, SW480, SW620) as well as normal colon epithelial cells (FHC) and teratoma cells (Tera-2). We observed detectable levels of Nanog and Sox-2 expression in most cell lines, whereas Oct3/4 was not detected in any colon cancer cells

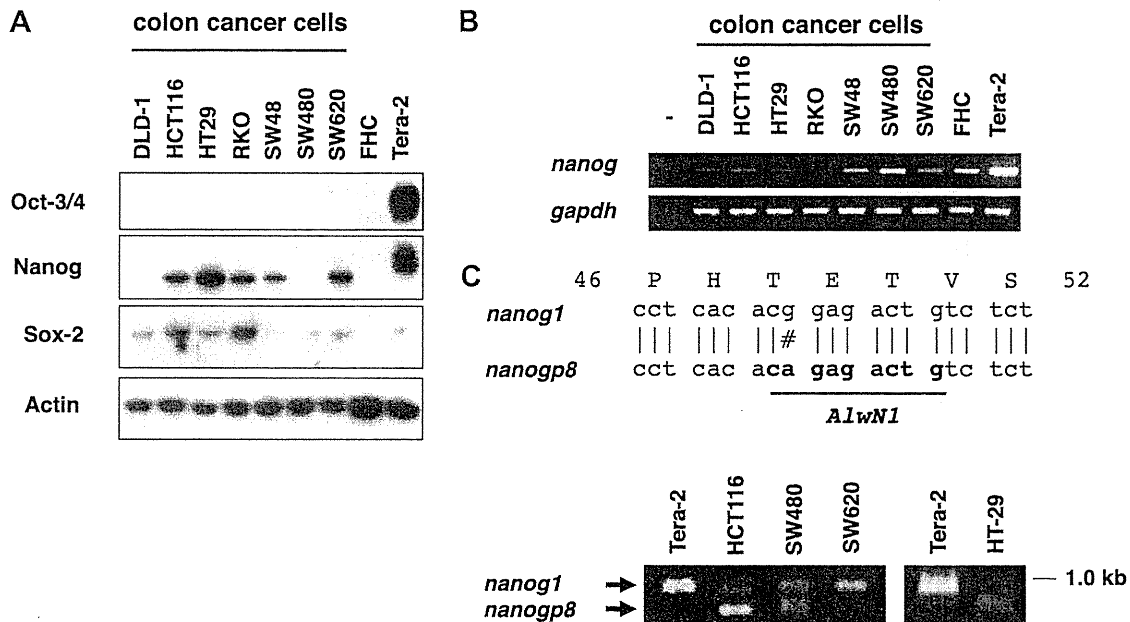

Fig. 1. *nanog1* and *nanogp8* were differentially expressed in colon cancer cells. (A) Western blot analyses of colon cancer cells. Designated colon cancer cells, colon epithelial cells (FHC), and teratoma cells (Tera-2) were used for Western blot analyses with antibodies against Oct-3/4, Nanog, Sox-2, or Actin. (B) RT-PCR analyses of *nanog* and *gapdh* genes for cells shown in A. For PCR amplification of *nanog*, primers that correspond to the N-terminal and the C-terminal sequences of both *nanog1* and *nanogp8* were used. cDNA fragments amplified by PCR were run on a 1% agarose gel and stained with ethidium bromide. (C) Digestion patterns of *nanog* cDNAs with the restriction enzyme *AlwN1* (upper panel). Unique sequence for the restriction enzyme *AlwN1* in *nanogp8* cDNA. Corresponding amino acid sequence is also shown (lower panel). Approximately equal amounts of *nanog* cDNAs from colon cancer cells (HCT116, SW480, SW620, HT-29) and Tera-2, which are shown in B, were digested with *AlwN1* and run on a 2% agarose gel and stained with ethidium bromide. The arrows indicate the position of the predicted size for *nanog1* and *nanogp8* cDNAs after the treatment with *AlwN1*. Note that the digested *nanogp8* cDNA is 148 nt shorter than *nanog1* cDNA.

(Fig. 1A). Nanog in colon cancer cells migrated faster than that in Tera-2 on western blot (Fig. 1A), probably due to its cell type-specific modification [46]. Because of the reported prognostic role of Nanog in progression of colon cancer [10], we focused on investigating the expression of Nanog in the following studies.

3.2. nanog1 and nanogp8 were differentially expressed in colon cancer cells

nanog1 and *nanogp8*, genes responsible for Nanog, encode similar polypeptides that differ from *nanog1* by only six nucleotides and two amino acids [40], and their products are indistinguishable on western blot analyses. Therefore, first we examined the total levels of RNA expression of the *nanog* genes by performing RT-PCR analyses with primers that match with the N-terminal and the C-terminal ends of both genes. The RT-PCR analyses of colon cancer cells indicated that the *nanog* genes were expressed in all colon cancer cells examined, although the expression levels were much lower than that in Tera-2 cells (Fig. 1B).

Next, we performed sequencing of the full-length cDNAs that were amplified by RT-PCR from teratoma cells (Tera-2) and from colon cancer cells (HT-29). 4 out of 4 cDNAs derived from Tera-2 corresponded to *nanog1* (data not shown). In contrast, 6 out of 6 cDNAs derived from HT-29 corresponded to *nanogp8* (data not shown), suggesting that *nanog1* and *nanogp8* are major forms of the *nanog* gene that is expressed in Tera-2 and HT-29, respectively.

In order to determine whether *nanogp8* is a dominant form in other colon cancer cells, we took advantage of the difference in their primary nucleotide sequences of the two genes. One of the nucleotide alterations in *nanogp8* should cause its cDNA to be a substrate for a restriction enzyme *AlwN1* (Fig. 1C, upper panel). Digestion of the full length PCR products with *AlwN1* revealed that each colon cancer cell lines expresses *nanog1* and *nanogp8* at varied ratios; *nanog1* is dominantly expressed in SW620 as well as in Tera-2, whereas *nanogp8* was expressed at a relatively higher level in HT-29 and HCT116 (Fig 1C, lower panel). Thus, two *nanog* genes are expressed at different ratios among colon cancer cell lines.

Of note, RT-PCR analyses in Fig. 1B indicated that *nanog1/nanogp8* was expressed in FHC, SW480, and DLD-1 at a level that was comparable to those in other colon cancer cells, although expression of Nanog protein was very low or undetectable in these cells (Fig. 1A), suggesting that Nanog expression is regulated at post-transcriptional levels.

3.3. nanog1 and nanogp8 are dominant forms of Nanog in SW620 and HT-29 cells, respectively

Next, we attempted to confirm differential expression of *nanog1* and/or *nanogp8* in colon cancer cells by knocking down these genes with shRNAs. Two shRNAs were used; one sh-RNA (sh-Nanog (#1), TRCN0000004887, sigma) is capable of knocking down both *nanog1* and *nanogp8*, whereas the other (sh-Nanog (#2), TRCN0000004884, sigma) can target only *nanog1*. The lentiviruses expressing these shRNAs were infected into SW620 and HT-29 cells to inhibit Nanog. Indeed, as expected from the distinct expression profiles (Fig. 1C), Nanog was inhibited by both shRNAs in SW620 (Fig. 2A), while it was inhibited by sh-Nanog (#1) but not by sh-Nanog (#2) in HT-29 (Fig. 2B). Combined with data shown in Fig. 1C, these data indicate that *nanog1* and *nanogp8* are mainly responsible for Nanog expression in SW620 and HT-29 cells, respectively.

3.4. Nanog expression mediates cell proliferation and tumor formation of colon cancer cells

In order to examine whether Nanog expression in colon cancer cells promotes their proliferation, we determined the growth rate

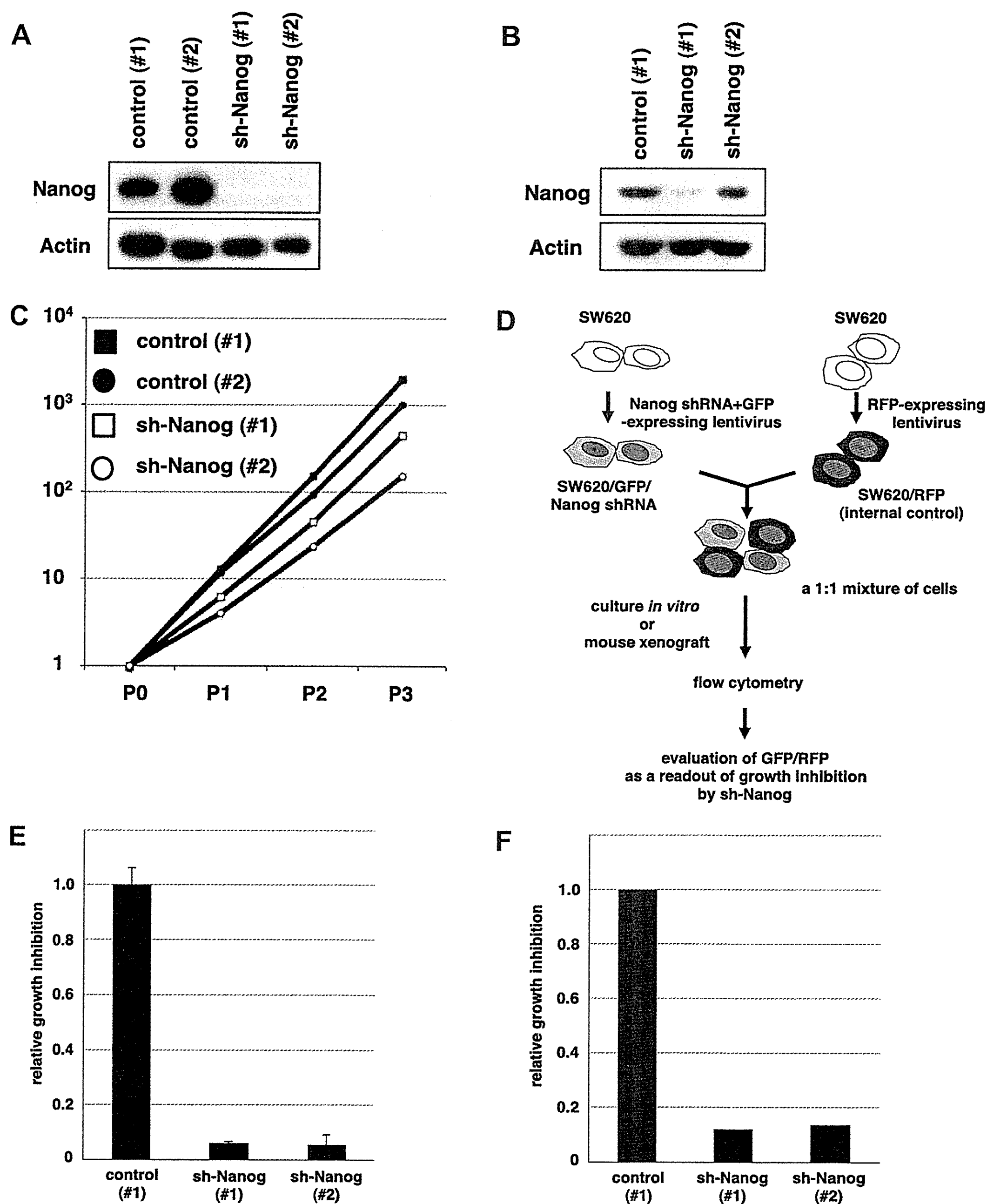

Fig. 2. Nanog expression mediates cell proliferation and tumor formation of colon cancer cells. (A) Western blot analyses of SW620 cells transfected with *nanog* shRNA-expressing lentiviruses or the control viruses. (B) Western blot analyses of HT-29 cells transfected with nanog shRNA-expressing lentiviruses or the control viruses. (C) Growth curves of SW620 cells introduced with lentivirus shown in A *in vitro*. (D) An experimental scheme for the GFP/RFP competition assays (see Section 2). (E) The GFP/RFP competition assays of SW620 cells infected with *nanog* shRNAs under normal culture condition. The shRNA-infected and the control cells were grown *in vitro* under normal culture conditions for seven passages (21 days), and the GFP/RFP ratios before and after the passage was calculated to determine the effects of each shRNA on cell growth. (F) The GFP/RFP competition assays of *nanog* shRNA-infected SW620 cells with in mouse xenograft. The effect of each shRNA on tumor formation was evaluated as described in E.

of SW620 cells after infection of the lentiviruses that express the Nanog shRNA or the control viruses. The inhibition of Nanog negatively affected the proliferation of SW620 (Fig. 2C).

We next questioned whether Nanog inhibition suppresses cell proliferation in a cell autonomous or non-autonomous manner by performing GFP/RFP competition assays under normal cell culture conditions (Fig. 2D). The inhibition of Nanog by shRNAs inhibited cell proliferation of SW620 cells in this assay (Fig. 2E), suggesting that the suppression of cell growth by Nanog inhibition is cell autonomous. Combined with data presented in Fig. 2D, these data indicate that Nanog expression promotes cell proliferation of SW620 cells.

We also performed the GFP/RFP competition assays after the subcutaneous injection of the mixture of GFP/RFP cells in immunocompromised NOG mice. Again, the suppression of Nanog inhibited cell proliferation of the transplanted cells. Thus, these data indicate that the suppression of Nanog in SW620 inhibit cell proliferation in mouse xenograft as well as under normal culture conditions.

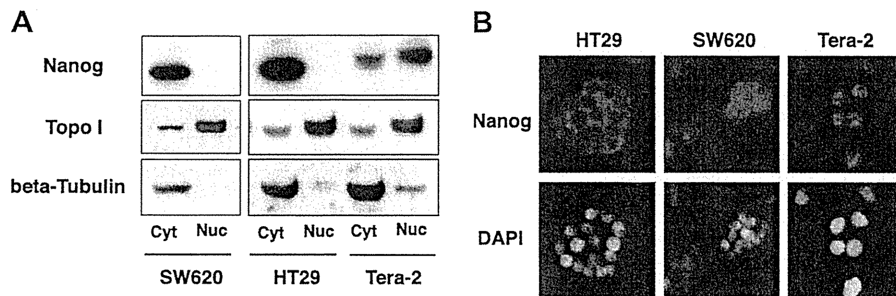

Fig. 3. Subcellular distribution of Nanog in colon cancer cells and teratoma cells. (A) SW620, HT-29, and Tera-2 cells were used for subcellular fractionation, and subcellular localization of Nanog was determined by performing Western blot analyses of the fractionated lysates with anti-Nanog antibody. Anti-TopoI antibody and anti-beta-Tubulin antibodies were also used to evaluate the fractionation. (B) Immunostaining of SW620, HT-29, and Tera-2 cells with anti-Nanog antibody.

3.5. Distinct pattern of subcellular distribution of Nanog in colon cancer cells and teratoma cells

Finally, we determined the subcellular localization of Nanog in colon cancer cells. In agreement with a previous report on immunohistochemical analyses of colorectal cancer [10], both western blot analyses after subcellular fractionation and immunostaining with anti-Nanog antibody indicated that Nanog was mainly localized in cytoplasm in SW620 and HT-29, which was in contrast to its nuclear localization in Tera-2 (Fig. 3A and B). Thus, Nanog is localized mainly in cytoplasm in at least two colon cancer cells.

Taken together, our data indicate that, while Nanog is expressed in a majority of colon cancer cells, *nanog1* and *nanogp8* are expressed with varied ratios among them, and it is likely that the differential expression of both genes contributes to Nanog expression, which promotes proliferation and tumor formation of colon cancer cells. Our data also suggest the importance of post-transcriptional regulation of Nanog expression in colon cancer cells. It will be interesting to determine its molecular mechanism in the future.

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Review Article

Lipoprotein Lipase as a Candidate Target for Cancer Prevention/Therapy

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Epidemiological studies have shown that serum triglyceride (TG) levels are linked with risk of development of cancer, including colorectal and pancreatic cancers, and their precancerous lesions. Thus, it is assumed that serum TG plays an important role in carcinogenesis, and the key enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of plasma TG, may therefore be involved. Dysregulation of LPL has been reported to contribute to many human diseases, such as atherosclerosis, chylomicronaemia, obesity, and type 2 diabetes. Recently, it has been reported that *LPL* gene deficiency, such as due to chromosome 8p22 loss, *LPL* gene polymorphism, and epigenetic changes in its promoter region gene, increases cancer risk, especially in the prostate. In animal experiments, high serum TG levels seem to promote sporadic/carcinogen-induced genesis of colorectal and pancreatic cancers. Interestingly, tumor suppressive effects of LPL inducers, such as PPAR ligands, NO-1886, and indomethacin, have been demonstrated in animal models. Moreover, recent evidence that LPL plays important roles in inflammation and obesity implies that it is an appropriate general target for chemopreventive and chemotherapeutic agents.

1. Introduction

A high-calorie diet and low physical activity, part of the so-called "Westernization" of lifestyle, are associated with elevated incidences of the breast, colon, liver, pancreas, and prostate cancers. Moreover, they are also linked with the risk of obesity, type 2 diabetes, and dyslipidemia. The World Cancer Research Fund and American Institute for Cancer Research have evaluated causal relationships between body fat and cancer and provided strong evidence for roles in such as colorectum and pancreas cancers [1]. In Japan, overweight and obesity (body mass index ≥25) are reported to be associated with cancers of specific organs, such as the colorectum (male), postmenopausal breast (female), and the liver in individuals positive for hepatitis C virus infection [2–4].

Greater body fatness is a major risk factor for the metabolic syndrome, which presents as a combination of symptoms, such as dyslipidemia (elevated triglyceride (TG) levels or low high-density lipoprotein (HDL) cholesterol), elevated blood pressure, and elevated fasting glucose levels. Hypertriglyceridemia is associated with the risk of colon cancer in Japanese men (HR = 1.71) and being overweight with the risk of breast cancer (HR = 1.75) [5]. In addition, most epidemiological studies, including our own, have consistently showed that serum TG levels are associated with the risk of colorectal adenoma, a precursor lesion of colorectal cancer [6–11]. Thus, it is assumed that serum TG could play an important role in carcinogenesis and that the key enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of plasma TG, may also be involved. In this paper, we focus on the roles of LPL in cancer development and further discussed possible approaches to cancer prevention/therapy.

2. Function, Structure, and Gene Regulation of LPL

2.1. Functions and Structure of LPL. LPL plays an important role in lipid metabolism as an enzyme responsible for hydrolysis of the TG component in circulating chylomicrons and very-low-density lipoprotein (VLDL) via binding with apolipoprotein C2 [12, 13]. Thus, lowering or deficiency of LPL expression is associated with hyperlipidemia [14, 15]. The LPL enzyme itself is composed of two structurally

distinct regions. The amino-terminal domain is responsible for catalysis with a catalytic center formed by three amino acids (Ser^{132}, Asp^{156}, and His^{241}). The carboxy-terminal domain of LPL is required for its binding to the lipoprotein substrate [3, 16–18].

2.2. LPL Gene Expression and Its Regulation. The human *LPL* gene is located on chromosome 8p22 and composed of 10 exons [19]. LPL is ubiquitously expressed in the whole body, but especially in the adipose tissue and the skeletal muscle [20, 21] and is regulated by hormonal and inflammatory stimuli, such as insulin [22, 23], glucocorticoid [24, 25], adrenaline [26], tumor necrosis factor (TNF)-α [27, 28], transforming growth factor (TGF)-β [29], and interleukin (IL)-1β [27].

The expression of LPL is controlled transcriptionally and posttranscriptionally. Basal promoter activity has been shown to be regulated by Oct-1 and the NF-Y binding motifs [30, 31], and the 5′-CCTCCCCC-3′ motif, which interacts with Sp1 and Sp3 [32]. Induction of *LPL* gene transcription is mediated by the peroxisome proliferator response element (PPRE) and the responsible element which binds to sterol regulatory element-binding protein (SREBP) [33, 34]. The effect of insulin on *LPL* expression is an example of posttranscriptional control, the hormone being suggested to increase *LPL* mRNA levels via mRNA stabilization [23, 35].

3. Relationship between LPL and Cancer: Human Studies

3.1. Loss of LPL and Resultant Common Disease. LPL has been reported to play key roles in many human diseases, such as atherosclerosis, obesity, type 2 diabetes, chylomicronaemia, Alzheimer's disease, and cachexia [15]. Especially, *LPL* gene deficiency is the cause of type I hyperlipoproteinemia (familial hyperchylomicronemia) [36]. Homozygous deficiency of *LPL* in humans is rare, but heterozygous deficiency is observed in around 3% of people with various ethnic backgrounds [37, 38]. Although these individuals have elevated serum levels of TG and decreased HDL cholesterol [39], it is not clear whether they are at increased risk of atherosclerosis, ischemic heart disease, type 2 diabetes, and cancer. There is a report that the *LPL* S447X mutation is associated with a higher risk of pancreatic calcification and steatorrhea in hyperlipidemic pancreatitis [40]. Since LPL provides fatty acids to the tissues and fatty acids evoke insulin resistance, *LPL* gene deficiency could affect glucose metabolism. However, whether heterozygous *LPL* deficiency reduces plasma glucose levels or not is still controversial. One paper described reduction of plasma glucose levels, but two others observed no effects as compared with LPL intact humans [41–43]. On the other hand, it has been reported that patients with poorly controlled diabetes frequently have dyslipidemia due to defects in LPL enzyme activity [44].

3.2. Effects of Chromosome 8p22 Loss and LPL Gene Polymorphisms on Cancer Risk. Alteration in genomic DNA, such as point mutations and deletions/amplifications or epigenetic changes such as CpG island hypermethylation and histone modification, can induce abnormal gene expression, which in the case of tumor suppressor genes or oncogenes could eventually lead to carcinogenesis. The human *LPL* gene has been mapped to chromosome 8p22 and previous studies on loss of heterozygosity (LOH) in colorectal tumors suggested that a putative tumor suppressor gene may lie within the short arm of chromosome 8, that is, 8p22-p21.3. Loss of 8p23.1-22 is also reported to be an important stage in initiation or promotion of hepatocellular carcinoma development and may also be the most frequent chromosomal alteration in prostate cancer [45]. It has been found that deletion of *LPL* is observed in 68% (52/76) of localized prostate cancers by FISH analysis [46]. It has further been reported that chromosomal region 8p23.1-8p21.1 may harbor one or more important prostate-cancer-susceptible loci based on linkage analyses in 159 hereditary prostate cancer families [47, 48]. To date, several new candidate cancer-susceptible genes have been cloned to 8p22, such as *deleted in breast cancer 2* (*DBC2*), *leucine zipper tumor suppressor 1* (*LZTS1*), *deleted in liver cancer 1* (*DLC1*), and *mitochondrial tumor suppressor 1* (*MTUS1*) [49–52]. Thus, cancer-susceptible genes mapped close to the *LPL* gene could be affected by *LPL* gene deletion, and exert combined effects in promoting carcinogenesis.

Moreover, an *LPL* Ser447stop polymorphism has been shown to be associated with prostate cancer risk [53] and the *LPL* gene is commonly methylated in prostate tumors [54]. *LPL* promoter CpG island methylation has been revealed in 45% of LPL-deleted tumors and in 22% of LPL-retaining tumors [54]. Biallelic inactivation of *LPL* by chromosomal deletion and promoter methylation may thus contribute to prostate tumorigenesis, but information is lacking regarding pancreatic cancer.

4. Relationship between LPL and Cancer: Animal Studies

4.1. Dyslipidemia Observed in Cancer-High-Susceptibility Animal Models. Elevated serum TG has been shown to promote carcinogen-induced colon carcinogenesis, and rats with hypertriglyceridemia such as the Zucker obese and Nagase analbuminemic strains and F344 rats fed a high-fat diet are all known to be more sensitive to carcinogen treatments than rats with normal serum lipid levels [55–57].

In the case of mice, the Apc^{1309} (C57BL/6$J^{Apc/Apc\Delta1309}$) [58] and Min (C57BL/6-$Apc^{Min/+}$) animal models of human familial adenomatous polyposis (FAP) feature development of large numbers of intestinal polyps and hypertriglyceridemia [59, 60]. Although no significant differences between Apc^{1309} mice and wild-type mice were observed at 6 weeks of age, the average serum TG value in the former at 12 weeks was obviously increased almost 10-fold (~600 mg/dL) over that at 6 weeks. Similar increase of TG levels (~400 mg/dL) was observed in Min mice at 15 weeks compared to 8 weeks of age (Table 1). Along with TG elevation, mRNA levels of *LPL* in the liver and small intestine of Apc^{1309} and Min mice were suppressed. Of note, other lipogenic genes, such as *FAS* and *stearoyl-CoA*

Table 1: Summary of animal models with dyslipidemia and cancer high susceptibility.

Animal	Strain	Age (week-old)	Serum TG (mg/dL)	Treatment	Tumor	Reference
Mouse	Apc^{1309} (C57BL/6J$^{Apc/Apc\Delta1309}$)	12	~600	—	Intestinal adenoma	[59]
	Min (C57BL/6-$Apc^{Min/+}$)	15	~400	—	Intestinal adenoma	[59, 60]
	KK-A^y	19	481	AOM	Colon cancer	[61]
	ICR	20	159	AOM + DSS	Colon cancer	[62]
Syrian golden hamster	—	6	300	BOP	Pancreatic cancer	[63]

Table 2: Summary of tumor suppressive effects of LPL inducers in animal models.

Agent	Dose	Animal model	Value to the untreated control group	Reference
Pioglitazone	200 ppm	Apc^{1309}	67%	[59]
	1600 ppm	Min	9%	[60]
	800 ppm	BOP-treated hamster	40%	[63]
NO-1886	800 ppm	Min	42%	[65]
Indomethacin	10 ppm	Min	25%	[66]

desaturase-1, β-oxidation genes like *acyl-CoA oxidase* and *carnitine palmitoyl transferase 1*, and gluconeogenesis genes, exemplified by *phosphoenolpyruvate carboxykinase*, demonstrated no variation from wild-type mouse expression.

Obese KK-A^y mice were found to be highly susceptible to azoxymethane- (AOM-) induced colorectal aberrant crypt foci (ACF) and colorectal carcinoma development compared to lean C57BL/6J mice [61]. Surprisingly, colorectal carcinomas developed within a very short-term period, 19 weeks, after AOM injection. The number of total ACF in KK-A^y mice was around 70/mouse and almost 8 times higher than that in lean C57BL/6J mice. The incidences of adenomas and adenocarcinoma were 84% and 88%, respectively, in KK-A^y mice, far higher than the 8% and 4% in C57BL/6J values. KK-A^y mice exhibit abdominal obesity, hypertriglyceridemia, and hyperinsulinemia at the time of ACF and tumor development. At 13 weeks of age, the average serum levels of TG, total cholesterol, and free fatty acids of KK-A^y mice undergoing AOM treatment were 484.1 mg/dL, 101.6 mg/dL, and 1,796 mEq/L, respectively (Table 1). It is interesting that hepatic *LPL* mRNA levels were also suppressed in KK-A^y mice compared with C57BL/6J mice. Moreover, serum proinflammatory adipocytokines, such as IL-6, leptin, and plasminogen activator inhibitor-1 (Pai-1), were elevated. Importantly, expression of proinflammatory adipocytokine mRNAs such as for IL-6, leptin, monocyte chemotactic protein (MCP)-1, Pai-1 and TNF-α was significantly increased in the visceral fat tissue; in contrast, that for adiponectin was decreased.

Tanaka et al. have developed a novel colitis-related colorectal carcinogenesis model, using AOM plus dextran sodium sulfate (DSS), a colitis-inducing agent [64]. In this model (AOM + 2% DSS in ICR mice), numerous colorectal adenocarcinomas occur within a short-term period and the serum TG levels demonstrate increase to about 134, 175 and 159 mg/dL at 5, 10, and 20 weeks, respectively [62] (Table 1).

Injection of *N*-nitrosobis(2-oxopropyl)amine (BOP) into Syrian golden hamsters is known to induce pancreatic ductal adenocarcinomas, with a histology very similar to typical human pancreatic ductal adenocarcinomas. Moreover, associated genetic mutations, that is, K-*ras* point mutations and *p16* aberrant methylation/homozygous deletions, are found in common in both hamster and human lesions. Interestingly, Syrian golden hamsters exhibit a hypertriglyceridemic state, almost 300 mg/dL at 6 weeks of age, even when not fed a high-fat diet [63] (Table 1). Also, in the case of this animal model, a low activity of LPL could be one of the causes of hypertriglyceridemia, activity of this enzyme in the liver being only 20% and 30%, respectively, of the values in C57BL mice and F344 rats.

5. Tumor Suppressive Effects of LPL Inducers

Pioglitazone, {(±)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]thiazolidine-2,4-dione monohydrochloride}, is a potent peroxisome proliferator-activated receptor (PPAR)γ ligand with a weak binding affinity for PPARα. In the promoter region of the *LPL* gene, there exists a PPRE, and pioglitazone treatment successfully induced LPL expression in the liver and intestinal epithelial cells in *Apc*-deficient mice. The total numbers of polyps in the groups treated with 100 and 200 ppm pioglitazone in the Apc^{1309} were reduced to 67% of the value in the untreated control group [59] (Table 2). With another *Apc*-deficient model, Min mice given 100–1600 ppm pioglitazone for 14 weeks showed decrease of intestinal polyps to 63–9% of the control number [60] (Table 2 and Figure 1).

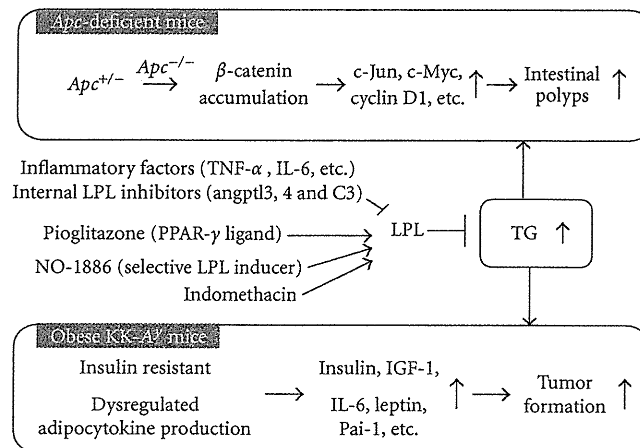

Figure 1: Involvement of triglycerides in animal intestinal carcinogenesis models. Angptl-3,4: angiopoietin-like protein-3,4; IGF-1: insulin like growth factor-1; IL-6: interleukine-6; LPL: lipoprotein lipase; Pai-1: plasminogen activator inhibitor-1; PPAR: peroxisome proliferator-activated receptor; TG: triglyceride; TNF-α: tumor necrosis factor-α.

Pioglitazone possesses other functions rather than just simply inducing LPL, such as causing cell growth arrest and apoptosis. Thus, data regarding LPL selective inducers are necessary for determining the relationship between hypertriglyceridemia and intestinal carcinogenesis. NO-1886, 4-[(4-bromo-2-cyanophenyl)carbamoyl] benzylphosphonate, chemically synthesized at Otsuka Pharmaceutical Factory [67] is one useful tool for clarifying this issue. Using a reporter gene assay, NO-1886 demonstrated no PPAR agonistic activity, unlike bezafibrate and pioglitazone [68].

Administration of 400 and 800 ppm NO-1886 also significantly decreased the total number of intestinal polyps to 48% and 42% of the untreated control value, respectively, in Min mice, along with causing marked increase in *LPL* mRNA levels in the liver and the small intestine. Moreover, treatment with NO-1886 also significantly decreased the numbers of colon polyps [65] (Table 2, Figure 1).

In the case of BOP-treated hamsters, pioglitazone has been demonstrated to improve hyperlipidemia and suppress ductal adenocarcinoma development. The incidences of ductal adenocarcinoma in the BOP plus 800 ppm pioglitazone and BOP alone groups were 38% and 80%, and the multiplicities were 0.55 and 1.37, respectively [63] (Table 2). Expression levels of hepatic LPL mRNA were elevated by treatment with 800 ppm pioglitazone. Moreover, quantitative real-time RT-PCR assays demonstrated almost 1.7-fold higher mRNA levels of LPL than that of pioglitazone-nontreated hamsters.

Indomethacin is a conventional nonsteroidal anti-inflammatory drug which has long been clinically employed to improve inflammation. It has demonstrated potent chemopreventive activity against intestinal tumor development in animal models, and a clinical trial in FAP patients also showed reduction in intestinal polyp development [69, 70]. We earlier reported that indomethacin suppresses intestinal polyp formation in Min mice together with ameliorating the hyperlipidemic state by regulating LPL, other lipid metabolic factors and inflammatory pathways [66]. Reduction of serum TG levels was 90% in Min mice with 10 ppm indomethacin treatment and higher than that with 400 ppm pioglitazone (83%) observed in our other previous study [59, 60]. The PPARγ agonistic activity of indomethacin is reported to be 50 times weaker than that of troglitazone, a well-established PPARγ agonist [71]. These results indicate that functions other than agonistic activity of indomethacin are responsible for its strong lipid-lowering effects (Figure 1).

6. Involvement of LPL in Inflammation, Obesity, and Others

6.1. LPL and Inflammation and Apoptosis. In addition to the lipid modifying function of LPL, two different mechanisms might be involved in *LPL* influence on carcinogenesis. The first involves anti-inflammatory action of LPL. It has been reported that LPL suppresses TNF-α- and interferon (IFN)-γ-evoked inflammation-related gene expression in endothelial cells through inactivation of transcription factor nuclear factor kappa B (NF-κB) [72]. Conversely, TNF-α, IFN-γ, IL-1β, IL-6, and leukemia inhibitory factor (LIF) decrease LPL activity.

It is well known that cyclooxygenase-2 (COX-2) is markedly elevated in human colon cancers, in AOM-treated rats, and in intestinal polyps of *Apc*-deficient mice. COX-2 is in fact thought to play important roles in both cancer cell proliferation and angiogenesis. Experiments conducted to clarify the mechanisms of NO-1886 effects on colon carcinogenesis revealed that the expression levels of mRNA for COX-2, in DLD-1 human colon cancer cells, were reduced under conditions of TGFα stimulation. On the other hand, there was no obvious change in the mRNA levels for COX-1 and inducible nitric oxide synthase (iNOS). The results obtained by RT-PCR analysis were also confirmed by

β-gal reporter gene assay in DLD-1 cells [65]. Consistent with the *in vitro* data, administration of 400 and 800 ppm NO-1886 reduced COX-2 mRNA levels in normal parts of small intestine of Min mice at 20 weeks of age [65]. In addition, NO-1886 ameliorates and induces regression of experimental steatohepatitis through increasing LPL activation and suppression of proinflammatory agents, such as TNF-α, IL-6, and COX-2 [73]. Recently, mice lacking *angiopoietin-like protein family 4* (*Angptl4*), which is the inhibitor of LPL, showed a severe and lethal phenotype characterized by fibrinopurulent peritonitis, ascites, intestinal fibrosis, and cachexia in response to a saturated fat diet [74].

The second mechanism is modification of the apoptosis pathway by LPL activation. Phosphatase type 2Cβ activation by unsaturated fatty acids has been demonstrated to induce apoptosis [75]. Unlike ester bodies of fatty acids, free fatty acids have cytotoxic effects *in vitro* and the products produced by hydrolysis of plasma TG may be implicated in such an apoptotic effect.

6.2. LPL and Obesity. Given the importance of LPL for lipid metabolism, its activity would be expected to be intimately involved in obesity effects and development of the metabolic syndrome. A large number of studies in rodents and humans have revealed that obesity results in increased LPL activity in adipose tissue [15, 35, 76–78]. Interestingly, LPL is regulated in opposite directions in adipose tissue and muscle. Feeding increases adipose LPL activity with a corresponding decrease in muscle LPL activity [35, 79]. Exercise stimulates LPL activity in the muscle and leads to increase fatty acid oxidation [80]. In an animal study, NO-1886 suppressed high-fat diet-induced fat accumulation in rats due to the increase of muscle LPL activity [81].

7. Conclusion

Targeting LPL activity or expression levels for development of reagents against cancer seems particularly challenging, because LPL is expressed ubiquitously and plays essential roles in maintaining homeostasis in the body. Data from LPL homozygous knockout mice, which die within one day of birth, underline its importance. However, appropriate suppression of serum TG levels could be achieved by using drugs, even if the number of selective inducers of LPL is limited. Thus, it might be important to develop selective LPL inducers or search for agents focusing on the aspect of "drug repositioning" to obtain the tools for investigating correlation between LPL and cancer. It should be borne in mind that LPL is inhibited by intrinsic factors, such as angptl3, angptl4, and C3 (Figure 1). These could clearly be candidate target molecules for development of LPL inducers. Considering that LPL activity has impact on obesity and metabolic syndrome, its targeting may also affect the regulation of adipocytokines, which may also be involved in carcinogenesis. Further investigations are warranted to clarify the importance of LPL and to accumulate evidence as to the worthiness as a target for cancer chemopreventive and chemotherapeutic agents.

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