

Figure 3: A) Intraoperative photography using a left transsylvian approach demonstrating the cystic mass with grayish capsule (T) continuously attached to the oculomotor nerve (cranial nerve III) in the carotid cistern. Histopathological examination of the surgical specimen shows: B) tumor comprising short spindle cells in a palisade-like pattern, corresponding to schwannoma (Hematoxylin and Eosin (HE), $\times 40$); C) hemosiderin deposition (blue signal), suggesting microhemorrhage in the tumor (Berlin-blue stain, $\times 40$). III; oculomotor nerve, IC; internal carotid artery, T; tumor.

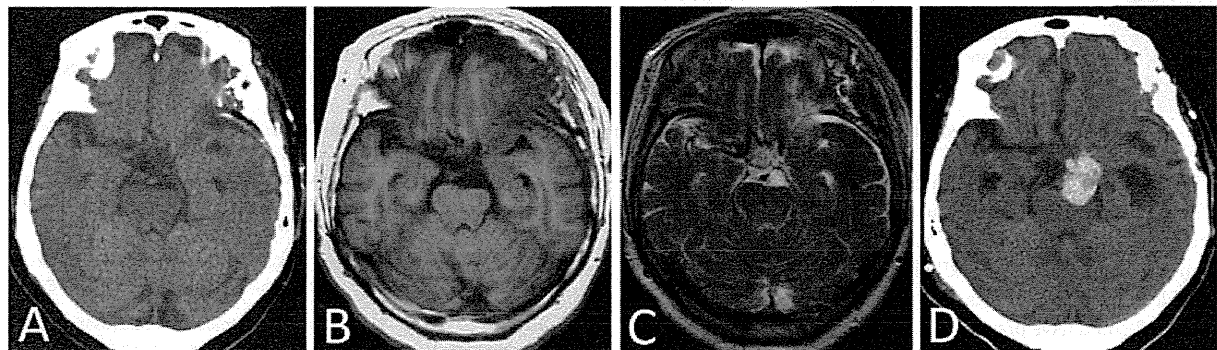


Figure 4: A-C) Postoperative unenhanced axial CT (A), T_1 -weighted MRI (B) and T_2 -weighted imaging (C) showing successful evacuation of cyst fluid. D) One month after first operation, axial unenhanced CT shows hematoma from the residual tumor compressing the brainstem and also causing non-communicating hydrocephalus.

cystic lesions. Neurenteric cyst is one such lesion, although extremely rare [17]. This lesion is more commonly located within the spinal cord, but may develop intracranially; only 140 such intracranial cases have been reported. Although the posterior fossa is a representative location (70–90% of cases) for intracranial lesions, oculomotor nerve neurenteric cyst has been reported [7,18]. These tumors typically do not exhibit enhancement, but two reported oculomotor nerve neurenteric cysts displayed partial or complete rim enhancement [13,15]. While signal intensities are inconsistent on MRI, depending on the protein content, these tumors are usually slightly hyperintense relative to Cerebrospinal Fluid (CSF) on T_1 -weighted imaging. This difference in signal intensity from CSF allows distinction from arachnoid cyst. Neither hemorrhage nor nodules are present in neurenteric cyst, providing one clue for differentiation from oculomotor schwannoma.

Arachnoid cyst should also be considered, and usually exhibits isointensity with CSF on MRI. However, we found one report that documented hemorrhagic arachnoid cyst associated with third nerve palsy [19]. Although positive staining for Epithelial Membrane Antigen (EMA) was shown in that case, staining for S-100 was not described, leaving open the possibility of cystic oculomotor schwannoma. Since other cystic lesions should be differentiated from

entirely cystic schwannoma in the oculomotor cistern or cavernous sinus, the imaging features are summarized in Table 1. Enhancement of the cyst wall [20], fluid intensity of the contents on MRI [21,22], presence of intracystic hemorrhage [23], and lesion location can all help in the differential diagnosis. In particular, we would like to emphasize the importance of intracystic hemorrhage as a clue in favor of schwannoma.

Another issue to consider in a case like the present one is the surgical strategy. When the oculomotor function is intact preoperatively, primary goal of surgery would be preservation of nerve function because schwannoma is benign tumor. When sharp dissection seems difficult, subcapsular or subtotal resection is also recommended to preserve oculomotor function [8]. In previous surgical series of oculomotor schwannoma, oculomotor function became worse after surgery in 50% of case. However, recovery of its function is reported in 11% of case when conservative subtotal or partial resection was tried [2]. Given the age of the patient and partial impairment of the nerve function, we performed palliative partial cyst wall resection to relieve the mass effect to preserve the nerve function in the initial stage of surgery. However, the cyst rapidly expanded again due to repeated hemorrhage from the remaining cyst leading to total loss of the nerve function, which finally necessitated a second

Table 1: Differential diagnosis of whole entire cyst in oculomotor cistern or cavernous sinus.

| | Neurenteric cyst | Arachnoid cyst | Cystic craniopharyngioma | Cystic pituitary adenoma | Rathke cleft cyst | Epidermoid cyst | Dermoid cyst | Cystic oculomotor schwannoma |
|--|--|--|---|--------------------------|---|---|-----------------------------------|------------------------------------|
| Enhancement of the cyst wall [20] | usually none [17] | none | usually none | yes | none or thin cyst wall enhancement | usually none, minimal rim enhancement occurs in 25% | none | none |
| T ₁ /T ₂ -weighted signal intensity of the contents compared to CSF [22] | iso~slightly hyper/hyper | hypo/hyper (similar to CSF) | hyper~hypo on both T ₁ /T ₂ vary as its contents [21] | hyper/extremely hyper | hyper: hypo=1:1 / hyper: iso~hypo=7:3 vary as its contents [21] | iso~hyper on both T ₁ /T ₂ | hyper/hypo~hyper (similar to fat) | hyper/extremely hyper |
| Frequency | extremely rare | common | rare | rare | occasional | rare | extremely rare | extremely rare |
| Intracystic hemorrhage | none | usually none | rare | occasional | usually none [23] | none | none | yes |
| Calcification | usually none | none | yes | rare | none | yes, in 10-25% | yes | none |
| Remarks | FLAIR image show hyperintensity and restricted slightly on DWI | FLAIR image show hypointensity and not restricted on DWI | solid enhancing nodule presents usually in the cyst | | enhancing rim presents in 50% | restricted on DWI located off the midline | located suprasellar at midline | located along the oculomotor nerve |

Metastatic tumor or parasitic lesion is excluded. FLAIR; fluid-attenuated inversion-recovery

operation to resect the tumor with the oculomotor nerve. Gamma Knife Radiosurgery (GKR) after partial debulking might have been one treatment option, but there is only one oculomotor nerve schwannoma case subjected to GKR after surgery [24]. In our case, the tumor rebled as early as 5 weeks after surgery, so it remained unclear whether GKR could have prevented subsequent hemorrhage. Further investigation or experience of stereotactic radiosurgery for entirely cystic masses is needed. It is worth to notify that no other cases have been reported to harbor such oculomotor nerve schwannoma which rebled from the residual tumor necessitating a second operation. Based on our experience, we recommend at least subtotal removal of the cyst wall, preferably preserving the nerve function, to prevent rebleeding in entirely cystic schwannoma with signs of hemorrhage, even in elderly patients.

Conclusion

Entirely cystic oculomotor nerve schwannoma is a very rare entity. Intratumoral microhemorrhage is a possible cause of rapid growth within a short span, postoperative hemorrhage from the residual tumor, and cyst formation. In these cystic schwannomas, aggressive tumor resection should be considered as one of the surgical alternatives to avoid rebleeding from the residual tumor.

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Conditional knockout of the leptin receptor in the colonic epithelium revealed the local effects of leptin receptor signaling in the progression of colonic tumors in mice

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Leptin, secreted by the adipose tissue and known to be related to obesity, is considered to be involved in the onset and progression of colorectal cancer. However, the exact role of leptin in colorectal carcinogenesis is still unclear, as several controversial reports have been published on the various systemic effects of leptin. The aim of this study was to clarify the local and precise roles of leptin receptor (LEPR)-mediated signaling in colonic carcinogenesis using intestinal epithelium-specific LEPRb conditional knockout (cKO) mice. We produced and used colonic epithelium-specific LEPRb cKO mice to investigate the carcinogen-induced formation of aberrant crypt foci (ACF) and tumors in the colon, using their littermates as control. There were no differences in the body weight or systemic condition between the control and cKO mice. The tumor sizes and number of large-sized tumors were significantly lower in the cKO mice as compared with those in the control mice. On the other hand, there was no significant difference in the proliferative activity of the normal colonic epithelial cells or ACF formation between the control and cKO mice. In the control mice, marked increase of the LEPRb expression level was observed in the colonic tumors as compared with that in the normal epithelium; furthermore, signal transducer and activator of transcription (STAT3) was activated in the tumor cells. These findings suggest that STAT3 is one of the important molecules downstream of LEPRb, and LEPRb/STAT3 signaling controls tumor cell proliferation. We demonstrated the importance of local/regional LEPR-mediated signaling in colorectal carcinogenesis.

Introduction

Colorectal cancer (CRC) is the most commonly encountered of all malignant neoplasms worldwide, and both the prevalence and mortality of CRC have been increasing (1,2). CRC is known to be associated with lifestyle-related diseases such as hyperlipidemia, diabetes mellitus and obesity (3–6). In particular, obesity has been shown to be associated with the progression of CRC (7). Epidemiological studies have revealed that obesity, characterized by an excess of visceral adipose tissue, is associated with an elevated risk of development of colonic adenomas and CRC (8). In addition to epidemiological reports, several animal studies have also suggested an association between

obesity and CRC (9). Obesity is strongly associated with adipose tissue dysfunction, which may result in increased serum levels of various adipocytokines involved in the onset of the metabolic syndrome.

Among the various adipocytokines, leptin, a 16 kDa product of the *ob* gene, is considered to be closely correlated with the body fat mass, because it is involved in the control of energy balance and regulation of food intake (10–12). In fact, increased serum levels of leptin in obese individuals have been reported (13). Therefore, an association has been suggested to exist between leptin and the risk of CRC. However, there are numerous contradictory reports concerning the involvement of leptin in the onset and progression of CRC (14–23). In humans, while several case-control studies have shown an elevated risk of CRC associated with high serum leptin levels (15,16), others have shown no elevation of the serum leptin levels in patients with CRC (17,18). Although *in vitro* animal experimental studies have indicated that leptin may act as a growth factor for colon cancer cells (18–20), studies have also shown that leptin does not promote the growth of colon cancer, and may even reduce the risk of development of precancerous lesions (19,23). Thus, the exact role of leptin in the development of CRC is still unclear.

To clarify the precise role of leptin in the development of CRC, we recently used mouse models of colon cancer with genetic deficiency of leptin [leptin-deficient; *ob/ob* and leptin receptor (LEPR)-deficient; *db/db*] under the condition of obesity and demonstrated the involvement of leptin in regulating the progression of CRC (24). The *ob/ob* and *db/db* mice are known to show extreme obesity with marked elevation of the serum levels of insulin, glucose and lipids (24). We found in our previous study that *ob/ob* mice, despite showing severe obesity under the high-fat diet condition, showed colonic tumors of much smaller sizes than the corresponding wild-type (WT) mice, suggesting that leptin signaling may play an important role in the regulation of colorectal carcinogenesis. However, the aforementioned mice with genetic leptin deficiency show systemic, rather than local, deficiency of leptin and/or of the LEPR, which may make it difficult to unmask the local and precise roles of leptin signaling in the colonic epithelium. Leptin is an adipocytokine that controls energy balance and regulates food intake, and leptin-deficient (*ob/ob*) and LEPR-deficient (*db/db*) mice develop far greater degrees of obesity than the corresponding WT mice. Furthermore, there are many other differences in the systemic condition between leptin-deficient and WT mice, including in the body weight and blood levels of glucose, insulin, cholesterol and triglycerides. On the other hand, we previously reported that the serum leptin levels in adenoma and CRC patients were not significantly different from those in normal subjects (25,26). In contrast to the absence of any differences in the serum leptin levels between CRC patients and normal subjects, marked increase of the local expression of the LEPR and activation of LEPR-mediated signaling has been observed in the colonic tumors, as compared with that in the normal colonic epithelium in CRC patients (25,26). These observations indicate the importance of local LEPR-mediated signaling in the progression of CRC. Therefore, to definitively resolve the ongoing debate, we were prompted to conduct this investigation on the local and precise roles of LEPR-mediated signaling in the development of CRC. For this purpose, we considered that conditional knockout (cKO) of the LEPR in the colonic epithelium might be useful.

The *LoxP/Cre* system, used for inactivation of the target gene in a cell- or tissue-specific manner, was developed to resolve the limitation of systemic knockout (27). This system allows inactivation of the target gene in a single cell type, thereby allowing analysis of the physiological and pathophysiological consequences of genetic alteration in a specific cell type. Therefore, we produced mice with 'intestinal epithelial cell (IEC)-specific' cKO of the 'leptin receptor'. In this study, we clarified the 'local' and 'precise' roles of LEPR-mediated

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, 5-bromo-2-deoxyuridine; cKO, conditional knockout; CRC, colorectal cancer; HFD, high-fat diet; IEC, intestinal epithelial cell; LEPR, leptin receptor; ND, normal diet; STAT3, signal transducer and activator of transcription 3; WT, wild type.

signaling in the onset and progression of colonic tumors using mouse models of aberrant crypt foci (ACF) and colonic tumors with IEC-specific cKO of the LEPR.

Materials and methods

Mice

All experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments of the Yokohama City University. These experiments were also approved by the Committee for Animal Research at the Yokohama City University.

We produced IEC-specific LEPR-knockout mice (LEPR^{ΔIEC} mice). Briefly, mice with the leptin receptor b (LEPRb) allele (LEPR^{b^{lox/lox}}), provided by Dr Streamson C. Chua Jr (28), were maintained on a C57BL/6J mixed background (backcrossing C57BL/6J mice over 10 times). Villin-Cre transgenic mice (B6.SJL-Tg(Vil-cre)^{997Gum/J}) were purchased from Jackson Laboratory (Bar Harbor, MA). We crossed the LEPR^{b^{lox/lox}} mice and the Villin-Cre transgenic mice (details are shown in Supplementary Methods, available at *Carcinogenesis* Online) to obtain LEPR^{b^{lox/lox} Villin-cre⁽⁺⁾} mice (control mice) and LEPR^{b^{lox/lox} Villin-cre⁽⁺⁾ ΔIEC} mice (LEPR^{ΔIEC}; cKO) (Supplementary Figure 1A and B, available at *Carcinogenesis* Online). The LEPR^{ΔIEC} mice and LEPR^{b^{lox/lox}} mice were viable and did not show any overt phenotype. There were no differences in the LEPRb expressions in the hypothalamus or other organs between the LEPR^{b^{lox/lox}} and LEPR^{ΔIEC} mice, although the expressions of the receptor in the colon and small intestine were dramatically different between the two models of mice. (Supplementary Figure 1C, available at *Carcinogenesis* Online)

All mice were maintained in filter-topped cages on autoclaved food and water in the animal facility at Yokohama City University.

Tumor induction

Seven-week-old male LEPR^{b^{lox/lox}} (control) and LEPR^{ΔIEC} (cKO) mice were fed either a normal diet (ND) or high-fat diet (HFD) until the end of the study. The compositions of the ND (MF; Oriental Yeast, Tokyo, Japan) and HFD (High Fat Diet 32; CLEA Japan, Tokyo, Japan) have been described previously (29). The protocols for generation of the azoxymethane (AOM)-induced mouse models of ACF, a useful precursor of colorectal carcinogenesis (30,31), or colorectal tumors have been described previously (32). Briefly, mice were given 2 or 6 weekly intraperitoneal injections of AOM at 10 mg/kg (Sigma, St Louis, MO) and killed 6 or 22 weeks later (Figure 1A and B). Eight mice per group were prepared for the ACF model, and 10 mice per group were prepared for the colonic tumor model. A recombinant leptin antagonist (PESLAN-1, PRL, Rehovot, Israel) was administered to the tumor-model LEPR^{b^{lox/lox}} mice ($n = 3$) 12 h before the tissue sample collection. The macroscopic tumors were counted and their sizes were measured with calipers. To facilitate counting of small tumors, the colons were stained with 0.2% methylene blue solution and observed by stereomicroscopy. The number of ACF was counted as described previously (31).

Assay for proliferation and apoptosis

The entire colon was removed, gently flushed with saline to remove any fecal contents, opened longitudinally and fixed in 10% neutralized formalin. Paraffin sections were prepared at 3 μm thickness and stained with hematoxylin and eosin. We determined the 5-bromo-2-deoxyuridine (BrdU) (BD Biosciences, Franklin Lakes, NJ) labeling index as a measure of the proliferative activity of the colonic epithelial cells, as described previously (32). The BrdU uptake analysis was performed in the mouse models of AOM-induced ACF and AOM-induced tumor. The apoptotic tumor cells were stained with a transferase deoxythymine end labeling kit according to the manufacturer's instructions (Wako Pure Chemical, Osaka, Japan).

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and subjected to immunohistochemical staining with primary antibodies using a Histofine kit (Nichirei, Tokyo, Japan) in accordance with the manufacturer's instructions. Nuclear counterstaining was performed with hematoxylin. In the negative controls, the primary antibody was replaced with a non-specific, non-immune immunoglobulin of the same isotype at an equivalent final concentration.

Immunoblot analysis

To obtain appropriate tissue samples, the colon was cut open longitudinally and washed with TBS to remove the fecal contents. Then, it was laid flat on a glass plate and the colon tumors were isolated mechanically. The distal 2 cm of the normal colonic mucosa adjacent to the tumor was scraped with a glass slide (33,34). The sample contained only the mucosal layer, and not the entire colon

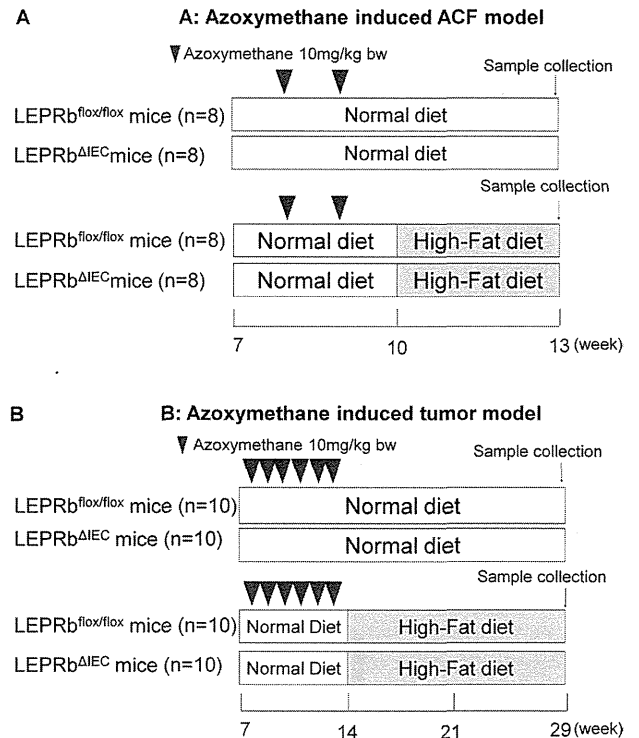


Fig. 1. Study protocol (A) AOM-induced ACF model, (B) AOM-induced tumor model.

thickness was kept at -80°C until further analysis. Protein extracts were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules CA). The membranes were probed with primary antibodies and glyceraldehyde 3-phosphate dehydrogenase (Trevigen, Gaithersburg, MD). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence detection kit (Immobilon, Millipore Corporation, Billerica, MA) were used for the detection of specific proteins. The antibodies used were anti-LEPR (Ob-R B-3:sc-8391) (Santa Cruz Biotechnology, Santa Cruz, CA), and antiphosphorylated-signal transducer and activator of transcription 3 (p-STAT3), or anti-STAT3 (Cell Signaling Technology, Danvers, MA).

Reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from the colonic epithelium using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For the real-time reverse transcription–polymerase chain reaction assay, total RNA was reverse-transcribed into cDNA and amplified using real-time quantitative PCR using the ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Probes and primer pairs specific for LEPRb and β -actin were purchased from Applied Biosystems. The concentrations of the target genes were determined using the Relative Standard curve method and the values were normalized to those of the internal control. The sequences of the primers used are listed in Supplementary Methods, available at *Carcinogenesis* Online.

Cell culture and transfection

Cells of the colon cancer cell line HCT116 were cultured in MaCoy's 5A medium supplemented with 10% fetal bovine serum. Transfection of siRNA was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA interference was confirmed by reverse transcription–polymerase chain reaction. The cells transfected with LEPR siRNA were harvested at 48 h after transfection, and 500 nM recombinant leptin (PeproTech, Rocky Hill, NJ) was added. After 0, 30 and 60 min, immunoblot analyses were performed. The sequences of the primers and LEPR siRNA used are listed in Supplementary Methods, available at *Carcinogenesis* Online.

Serum assay for leptin, insulin, cholesterol and adiponectin

All mice were denied access to food overnight, and blood samples were collected the following morning. The serum leptin concentrations were determined with an enzyme-linked immunosorbent assay kit (Morinaga, Yokohama, Japan),

the serum levels of cholesterol and insulin were measured using a Wako enzyme-linked immunosorbent assay kit (Wako Pure Chemical), and the serum levels of total adiponectin were measured using an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical), according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test and analysis of variance. Values of *P* < 0.05 were regarded as denoting statistical significance. The analysis was performed using SPSS, version 17.0 (SPSS, Chicago, IL).

Results

Comparison of various parameters, including obesity-related factors between the LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice

The experimental protocol based on AOM treatment is shown in Figure 1A and B. The LEPRb^{flx/flx} (control) and LEPRb^{ΔIEC} (cKO) mice were fed a ND or a HFD. The LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice grew to almost the same size, with no significant difference in the mean body weight between the two animal groups (Supplementary Figure 2A, available at *Carcinogenesis* Online). The body weights, serum leptin, serum insulin and serum cholesterol concentrations

were higher in the mice fed the HFD than in those fed ND. Serum adiponectin levels were lower in the mice fed the HFD than in those fed ND (Supplementary Figure 2B, available at *Carcinogenesis* Online). Serum data, including the serum leptin and the serum level of other obesity-related factors were similar in the LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice under both diet protocols (Supplementary Figure 2B, available at *Carcinogenesis* Online).

LEPR deficiency in the colonic epithelium suppressed colorectal tumor growth, but had no effect on the formation of ACF

To investigate the impact of leptin signaling in the colonic epithelium on colorectal carcinogenesis and to determine whether it might act as a tumor promoter, we examined the formation of chemically induced ACF as a marker of experimental colorectal carcinogenesis (30,31), and the formation of tumors in the colon. In both LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice, LEPRb was scarcely expressed in the ACF (data not shown). Although the number of ACF in both the LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice fed the HFD was significantly higher than that in the same mice fed the ND, there was no significant difference in the number of ACF between the LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice under either diet protocol (Figure 2A). Furthermore, there was no significant

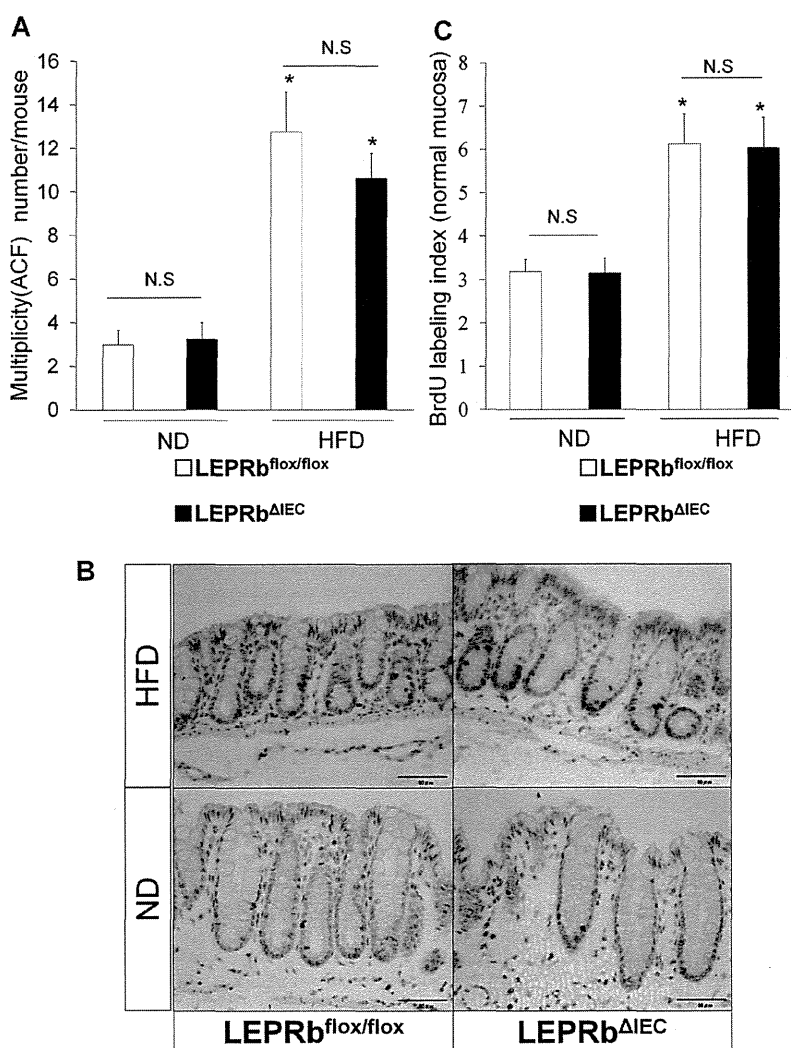


Fig. 2. Leptin is not involved in the early stage of colorectal carcinogenesis. (A) ACF multiplicity. Results represent averages ± SEM (*n* = 8). (B) BrdU incorporation in the normal colonic epithelial crypts of the LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice under both diet protocols (*n* = 8). Scale bars 100 μm. (C) BrdU labeling index in the normal colonic mucosa of the LEPRb^{flx/flx} and LEPRb^{ΔIEC} mice under both diet protocols. Results represent averages ± SEM (*n* = 8). * denotes significant difference between the mice fed HFD and the mice fed ND. ND, normal diet; HFD, high-fat diet. Open squares, LEPRb^{flx/flx} mice; closed squares, LEPRb^{ΔIEC} mice.

difference in the BrdU labeling index of the normal mucosa between the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice, although the BrdU labeling index was higher in the AOM-induced ACF mouse model fed HFD than in those fed ND (Figure 2B and C). These results suggest that HFD-induced obesity enhanced early-stage colorectal carcinogenesis, irrespective of the presence/absence of leptin signaling.

We next focused on the later stages of cancer progression. In the $LEPRb^{flox/flox}$ mice, significantly higher expression levels of the $LEPRb$ mRNA were found in the colonic tumors than in the normal colonic mucosa (Figure 3A and B). In contrast, the expression levels of $LEPRb$ in both the normal colonic mucosa and colonic tumors of the $LEPRb^{\Delta IEC}$ mice were much lower than those in the $LEPRb^{flox/flox}$ mice even under the HFD condition (Figure 4A and B). We observed that the tumor sizes in the $LEPRb^{\Delta IEC}$ mice were significantly smaller than those in the $LEPRb^{flox/flox}$ mice under both the ND and HFD

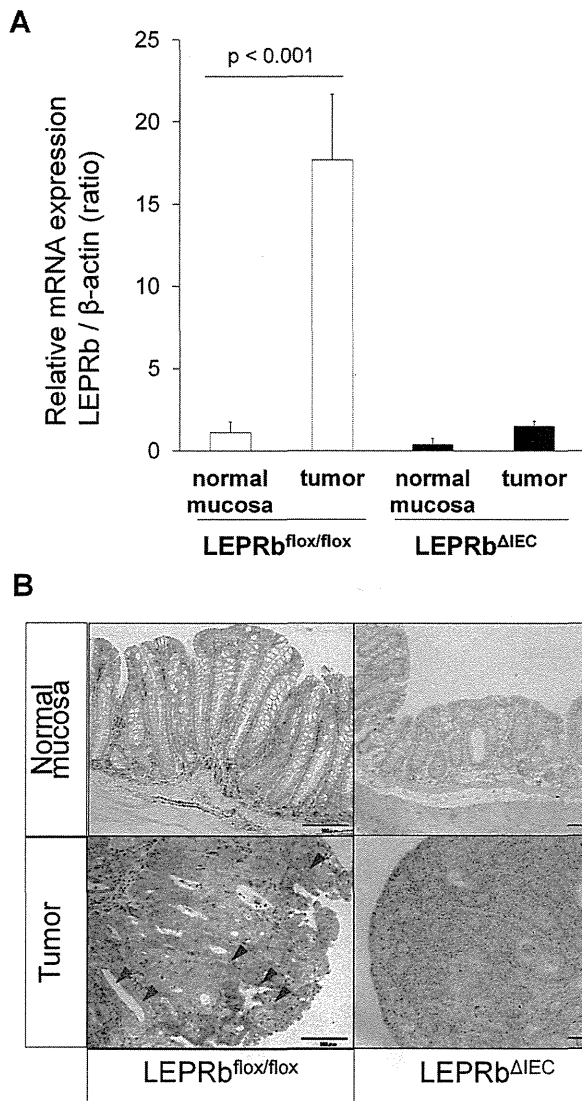


Fig. 3. $LEPRb$ expression in the tumors of $LEPRb^{flox/flox}$ mice, in the normal mucosa and tumors of the $LEPRb^{\Delta IEC}$ mice. (A) Relative expression levels of $LEPRb$ mRNA in the normal colonic mucosa and colonic tumors of the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice, analyzed by real-time PCR. Results represent averages \pm SEM ($n = 10$). Open squares, $LEPRb^{flox/flox}$ mice; closed squares, $LEPRb^{\Delta IEC}$ mice. (B) Microscopic findings in the normal colonic epithelial cells and colonic tumors of the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice stained with antibodies to $LEPR$. Scale bars 100 μ m. Arrows indicate $LEPR$ -positive cells.

conditions (Figure 4A and B). On the other hand, there was no significant difference in the tumor multiplicity between the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice, although there was a tendency towards a decrease in the tumor multiplicity in the $LEPRb^{\Delta IEC}$ mice (Figure 4A and B). Therefore, we analyzed the distribution of the tumor size, and found that the number of comparatively large-sized tumors was much lower in the $LEPRb^{\Delta IEC}$ mice than that in the $LEPRb^{flox/flox}$ mice (Figure 4C). These results indicate that local deficiency of the $LEPR$ resulted in a decrease in the number of large-sized tumors in the colon.

Next, we analyzed the cell-proliferative activity of the tumor cells in the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice to clarify the reason for the differences in the tumor growth rate between the two mouse models observed in this study. We found that BrdU incorporation in the tumors was significantly lower in the $LEPRb^{\Delta IEC}$ mice than that in the $LEPRb^{flox/flox}$ mice (Figure 4D and E), which was consistent with the slower growth of the tumors in the absence of the $LEPR$ and its downstream signaling in this animal model. Therefore, the differences in the cell-proliferative activity between the tumors and normal mucosa might be explained by the differences in the tumor $LEPR$ expression between the two mouse models. On the other hand, transferase deoxytidyl uridine end labeling revealed a reciprocal increase in the apoptotic response of the colon tumors between the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice (Supplementary Figure 2, available at *Carcinogenesis* Online), which suggests that tumor cell survival also relies on leptin signaling. Taken together, these data indicate that leptin signaling in the colonic epithelium enhances the tumor cell-proliferative activity, while not exerting the same effects in the normal mucosa or in premalignant lesions.

Leptin signaling activated $STAT3$ in the colorectal tumor cells

In the $LEPRb^{flox/flox}$ mice, a higher number of phosphorylated $STAT3$ (p- $STAT3$)-positive cells were observed in the tumors as compared to that in the normal mucosa (Figure 5A). In contrast, in the $LEPRb^{\Delta IEC}$ mice, p- $STAT3$ -positive cells were scarcely expressed in either the tumors or in the normal mucosa, similar to the case for $LEPR$ expression (Figure 5A). These data suggest that leptin signaling may promote the growth of colonic tumors through activation of the $LEPRb/STAT3$ pathway. Immunohistochemical analysis revealed tumor cell nuclear localization of p- $STAT3$ in the colonic tumor cells in the $LEPRb^{flox/flox}$ mice, while this signal was almost completely absent in the tumors of the $LEPRb^{\Delta IEC}$ mice (Figure 5A). The number of p- $STAT3$ -positive cells was significantly higher in the tumors of the $LEPRb^{flox/flox}$ mice fed the HFD than in the same mice fed ND (data not shown). Meanwhile, p- $STAT3$ -positive cells were almost undetectable in the normal mucosa in both the $LEPRb^{flox/flox}$ and $LEPRb^{\Delta IEC}$ mice (Figure 5A); importantly, this lack of $STAT3$ activation in the colonic mucosa was closely associated with the lack of colonic $LEPR$ expression. Immunoblot analysis for $LEPR$ and p- $STAT3$ in the normal colonic mucosa and tumors revealed increased expression of p- $STAT3$ in the tumors as compared with that in normal mucosa in the $LEPRb^{flox/flox}$ mice, but not in the $LEPRb^{\Delta IEC}$ mice (Figure 5B). Therefore, our data indicate that leptin signaling in the colon tumor is crucial for $STAT3$ activation during tumor growth in colonic tumors.

To further clarify the relation between $LEPRb$ and $STAT3$ activation, we conducted two further experiments. First, a recombinant leptin antagonist was administered to the tumor-model $LEPRb^{flox/flox}$ mice. Immunohistochemical analysis revealed tumor cell nuclear localization of p- $STAT3$ in the colonic tumor cells in the $LEPRb^{flox/flox}$ mice (control), while this signal was almost entirely absent in the tumors of the $LEPRb^{flox/flox}$ mice administered the leptin antagonist (Figure 5C). Immunoblot analysis for p- $STAT3$ in the tumors of the mice administered the recombinant leptin antagonist revealed decreased tumor expression of p- $STAT3$ as compared with that in the control mice (Figure 5D). Second, immunoblot analysis was performed of the cells of the human CRC cell line HCT116 transfected with $LEPR$ siRNA and treated with recombinant leptin (500 ng/ml). Treatment with recombinant leptin did not activate $STAT3$ (increase

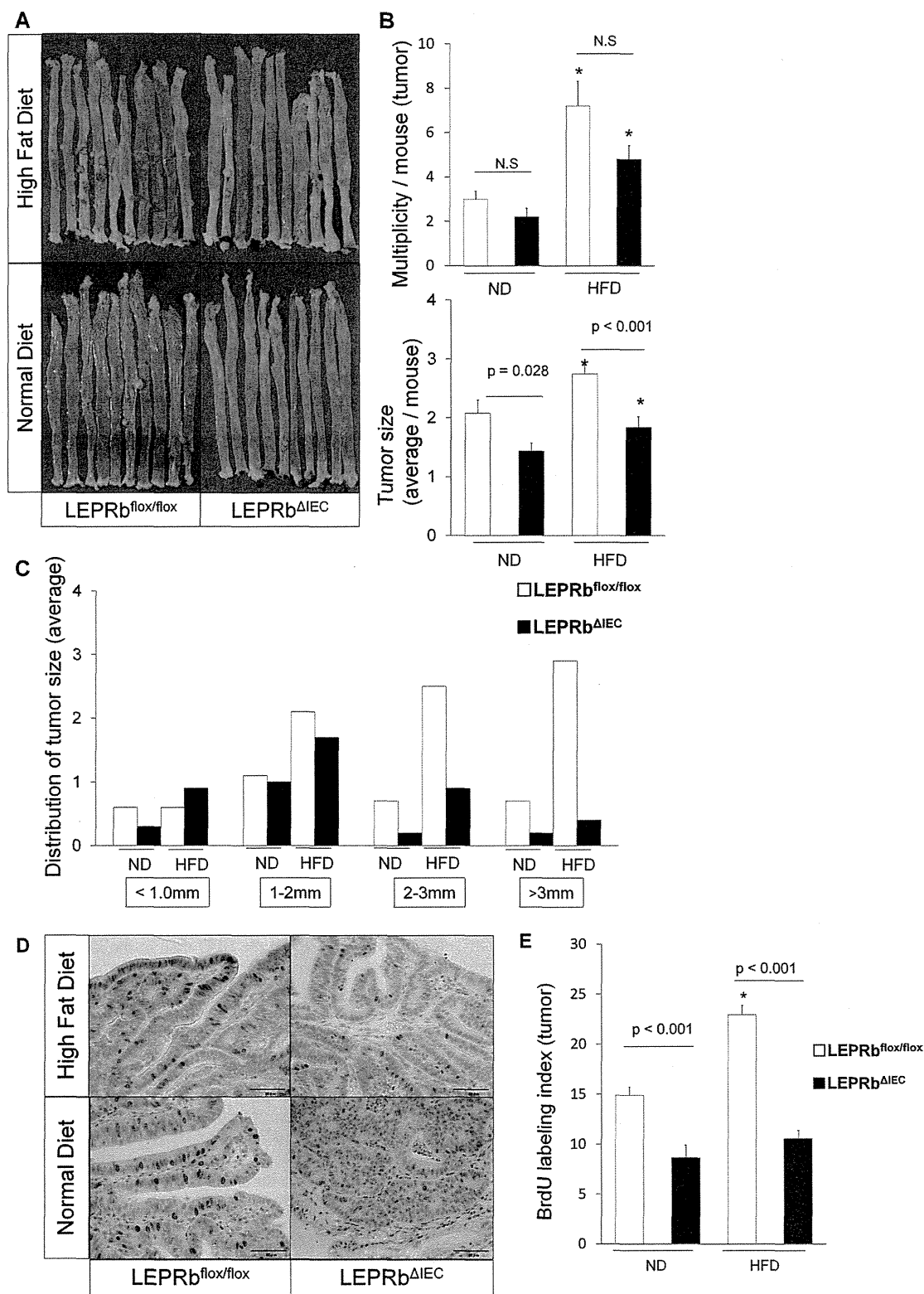


Fig. 4. Leptin/LEPRb signaling regulates AOM-induced colonic tumor growth. **(A)** Macroscopic findings of the colonic tumors. **(B)** Tumor multiplicity and tumor sizes in the LEPRb^{lox/lox} and LEPRb^{ΔIEC} mice under both diet protocols. Results represent averages ± SEM (n = 10). **(C)** Histogram showing the size distribution of the tumors. Results represent averages (n = 10). **(D)** BrdU incorporation in the colonic tumors of the LEPRb^{lox/lox} and LEPRb^{ΔIEC} mice. Scale bars 50 μm. **(E)** BrdU labeling index in the colonic tumors of the LEPRb^{lox/lox} and LEPRb^{ΔIEC} mice under the two diet protocols. Results represent averages ± SEM (n = 10). * denotes significant difference between the mice fed HFD and the mice fed ND. Open squares, LEPRb^{lox/lox} mice; closed squares, LEPRb^{ΔIEC} mice.

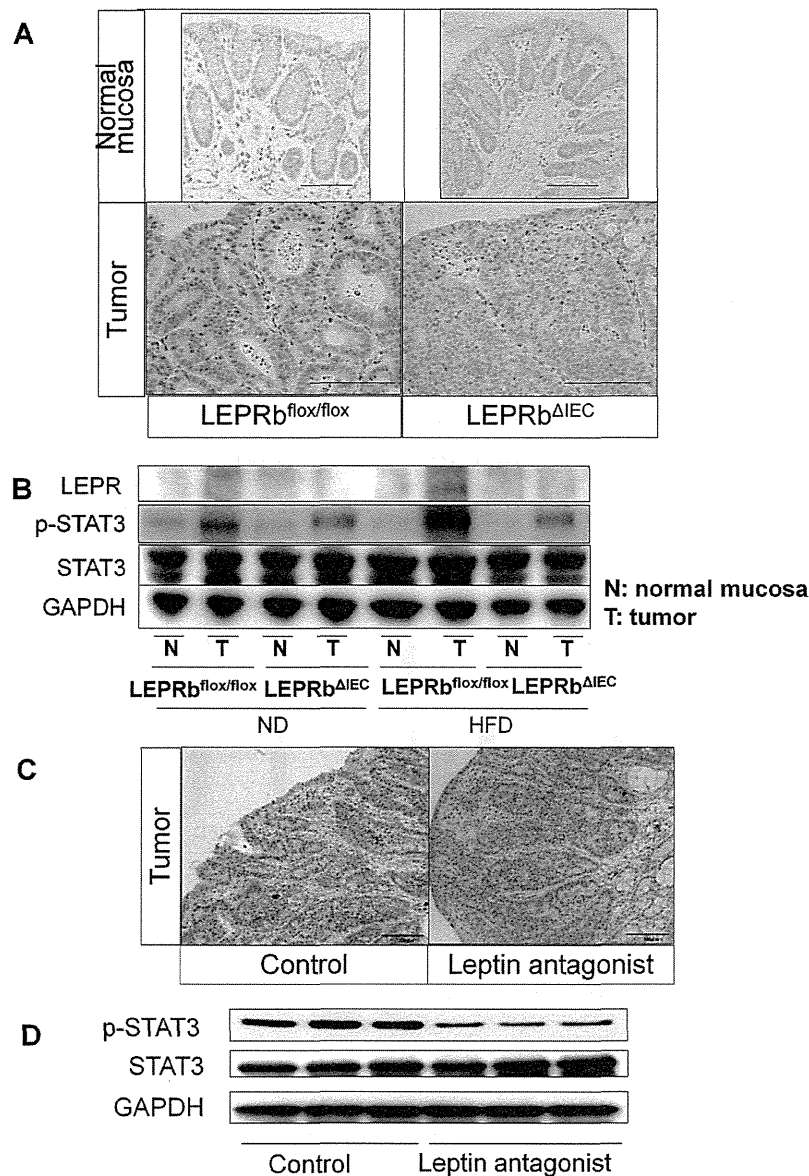


Fig. 5. Increase of STAT3 phosphorylation by leptin/LEPRb signaling in the colonic tumors of the LEPR^{flox/flox} mice. (A) Microscopic findings in the normal colonic mucosal cells and colonic tumors stained with antibodies to phosphorylated STAT3 (p-STAT3). Scale bars 50 μ m. (B) Immunoblot analysis for LEPR and p-STAT3 in the normal colonic mucosa and colonic tumors of the AOM-treated LEPR^{flox/flox} and LEPR^{ΔIEC} mice under the two diet protocols. Inhibition, using a leptin antagonist, of STAT3 phosphorylation in the colonic tumors of the LEPR^{flox/flox} mice. (C) Microscopic findings in the colonic tumors stained with antibodies to p-STAT3 in the AOM-treated LEPR^{flox/flox} mice administered normal saline or the leptin antagonist. Scale bars 50 μ m. (D) Immunoblot analysis for p-STAT3 in the colonic tumors of the AOM-treated LEPR^{flox/flox} mice administered normal saline or the leptin antagonist. N, normal mucosa; T, tumor.

of p-STAT3) in the LEPR-knockdown CRC cells (Supplementary Figure 4, available at *Carcinogenesis* Online).

Taken together, these findings suggest that STAT3 is one of the main molecules downstream of the LEPR involved in regulating colorectal carcinogenesis, and that LEPR/STAT3 signaling controls tumor cell proliferation.

Analysis of molecules involved in tumor cell growth downstream of STAT3 activation

Next, we analyzed the expressions of the cell cycle genes and of the genes encoding the cyclin-dependent kinase inhibitor p21^{cip} in the tumors. We found a greater degree of increase in the mRNA expressions of cyclin D1, c-Myc, cyclin B1, cyclin E and cdc2 in the tumors of the LEPR^{flox/flox} mice than in those of the LEPR^{ΔIEC} mice

(Figure 6). This finding suggests a stimulatory effect of STAT3 on the cell cycle, an observation that was consistent with downregulation of the cyclin-dependent kinase inhibitor p21^{cip} in the tumors of the LEPR^{flox/flox} mice. Furthermore, we observed elevated expression levels of Bcl-X_L and survivin in the tumors of the LEPR^{flox/flox} mice than in those of the LEPR^{ΔIEC} mice. These results suggest that impaired induction of Bcl-X_L and survivin protein expression may account for the increased rate of apoptosis observed in the tumors of the LEPR^{ΔIEC} mice. On the other hand, there were almost no differences in the expressions of the cell-cycle genes in the normal colonic mucosa between the LEPR^{flox/flox} and LEPR^{ΔIEC} mice (data not shown). Collectively, these results strongly support the notion that STAT3-associated cell-proliferative and antiapoptotic effects are important for tumor growth.

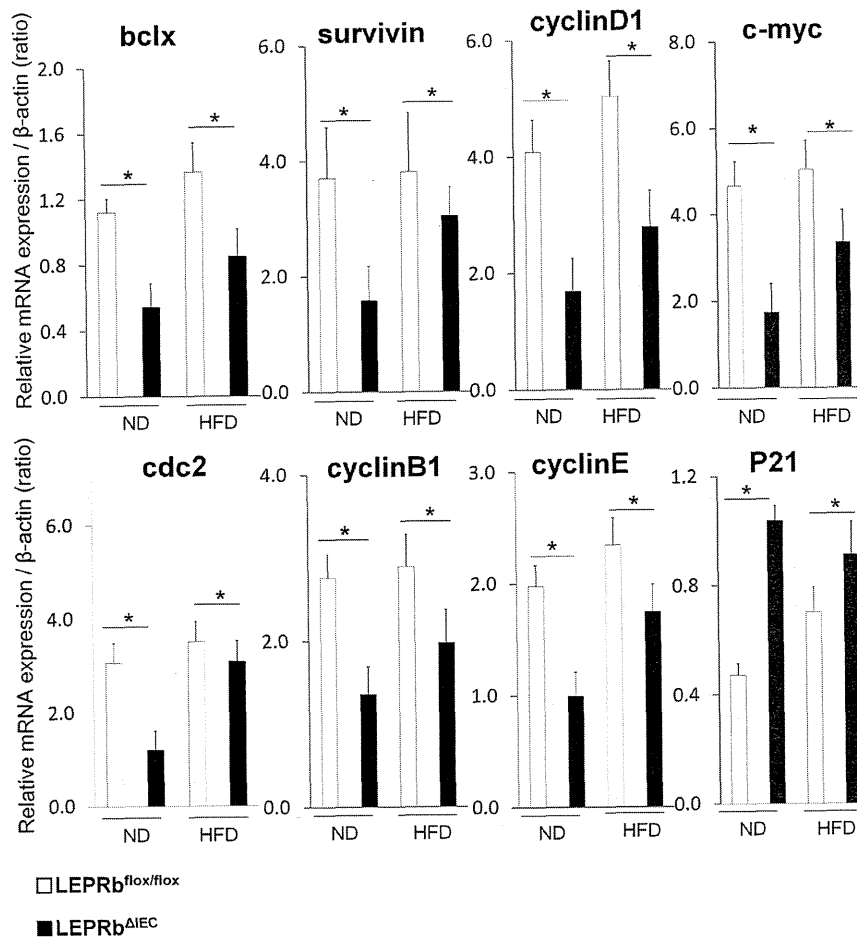


Fig. 6. Expression levels of the cell cycle and apoptosis regulators in isolated colonic tumors from the LEPR^{b^{flox/flox}} and LEPR^{b^{ΔIEC}} mice. Relative mRNA expression levels were determined by real-time PCR. Results represent average ± SEM, (n = 6) *P < 0.05. Open squares, LEPR^{b^{flox/flox}} mice; closed squares, LEPR^{b^{ΔIEC}} mice.

Discussion

Existence of a relationship between obesity-related factors and CRC has been speculated in recent years, although no definitive conclusions have been arrived at as yet. Previous studies have provided evidence of increase in the risk of colorectal carcinogenesis associated with obesity-related factors (9). However, *in vivo*, the various obesity-related factors are intricately related, so that with the knockdown of some signaling, other signalings may exert compensatory effects. Thus, it is difficult to analyze the local effects of hormones that also exert systemic effects. To clarify the local and precise roles of LEPR-mediated signaling in the development of CRC, we considered that cKO of the LEPR in the colon using the LoxP/Cre system would be useful. Therefore, using the LoxP/Cre system, we produced mouse models of ACF and colon tumors with IEC-specific cKO of the LEPR.

There were no significant differences in systemic parameters such as the body weight, serum insulin, serum cholesterol or serum adiponectin concentrations between the LEPR^{b^{flox/flox}} and LEPR^{b^{ΔIEC}} mice. Despite the absence of significant differences in the systemic parameters between the two models of mice, we observed that the LEPR^{b^{ΔIEC}} mice developed colonic tumors of much smaller sizes than the LEPR^{b^{flox/flox}} mice. On the other hand, there was no significant difference in the formation of ACF or proliferative activity of the normal colonic epithelial cells between the LEPR^{b^{flox/flox}} and LEPR^{b^{ΔIEC}} mice. These results indicate that LEPR signaling may have little effect in the very early stage of colorectal carcinogenesis due to the scarce expression of the LEPR in the normal mucosa in both the LEPR^{b^{flox/flox}}

and LEPR^{b^{ΔIEC}} mice. On the other hand, in the LEPR^{b^{flox/flox}} mice, a marked increase in LEPR expression was observed in the tumors as compared with that in the normal mucosa. Such drastically increased expression of the LEPR in the tumors of the LEPR^{b^{flox/flox}} mice may cause activation of the STAT3 pathway, whereas in the cKO (LEPR^{b^{ΔIEC}}) mice, absence of the LEPR suppressed STAT3 activation, resulting in the inhibition of tumor growth; moreover, treatment with the LEPR antagonist also suppressed STAT3 activation. The STAT3 signal may cause activation of the genes involved in the cell cycle and suppress those involved in apoptosis, such as cyclins, cdc, c-myc, survivin and bclx, to stimulate the tumor cell-proliferative activity. These findings also suggest that the activation of STAT3 in the tumors is crucially dependent on LEPR-mediated signaling.

Meanwhile, the role of LEPR-mediated signaling in the formation of ACF may be limited, because no significant difference in the formation of ACF was observed between the control and cKO mice. These results may also be due to the absence of significant increase in the LEPR expression level in either the ACF or the normal epithelium in these mice, as the degree of increase of LEPR expression under the HFD condition was much lower in the ACF than that in the tumors. Therefore, the role of LEPR-mediated signaling in the formation of ACF from normal epithelium might be more limited than that in the transformation of ACF to colonic tumors. We previously reported an increase in local expression of the LEPR and activation of LEPR-mediated signaling in colonic tumors as compared with that in the normal colonic epithelium in human CRC patients (25,26). Although CRC has been said to be related to obesity, it is suggested that CRC