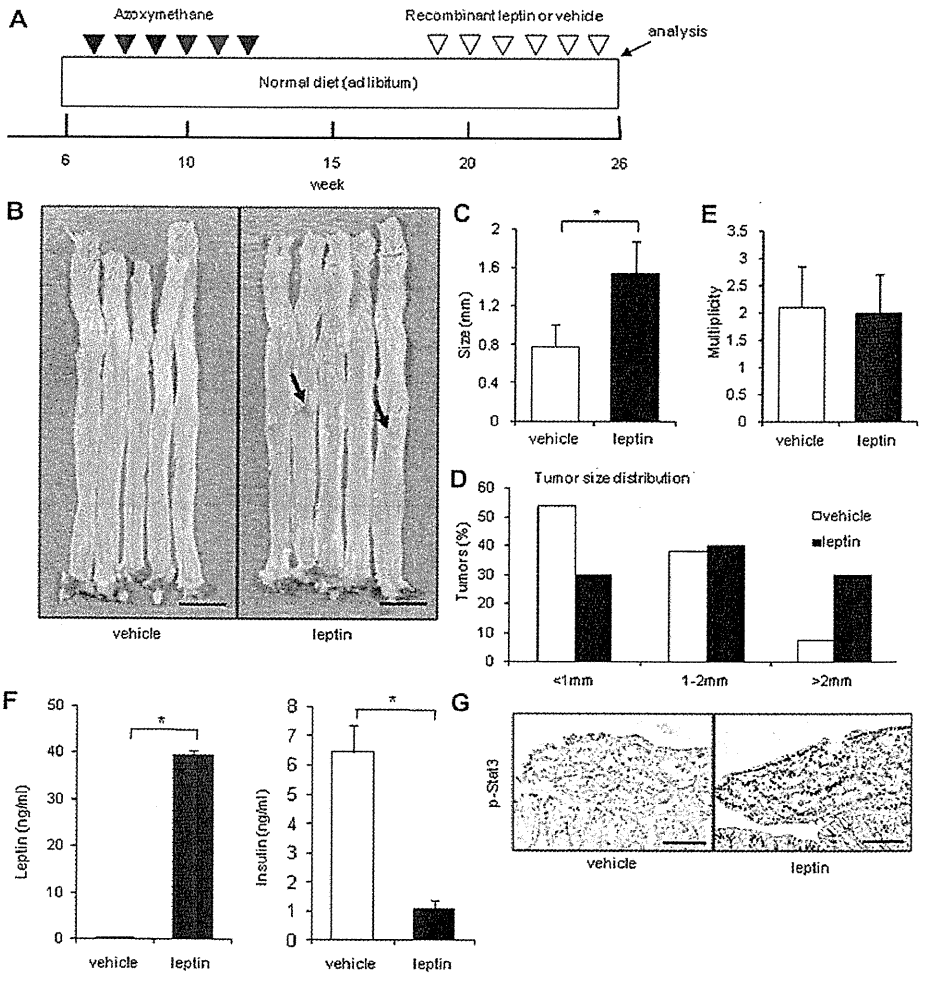

Figure 6 Leptin signalling stimulates tumour growth. (A) Scheme of treatment with recombinant leptin during the late stage of CRC growth. Mice were injected i.p. with 2 μg recombinant leptin, or control saline every day from 15 weeks after initial AOM injection. (B) Macroscopic findings of colon tumours. Arrows indicate large tumours. Scale bars=1 cm. (C) Tumour size. Results are averages ±SEM. (n=6). *p<0.05. (D) Histogram showing size distribution of colon tumours. (E) Tumour multiplicity. Results are averages ±SEM. (n=6). (F) Vehicle- or leptin-treated *ob/ob* mice were bled and the levels of leptin (left panel) and insulin (right panel) in serum were determined by ELISA. Results are averages ±SEM. (n=6). *p<0.001. (G) Paraffin-embedded sections of tumour-containing colons of vehicle- and leptin-treated *ob/ob* mice stained with anti-p-STAT3. Scale bars=100 μm. AOM, azoxymethane; CRC, colorectal cancer.

many factors that influence colon carcinogenesis in an obesity background, and adiponectin may be one of these factors. Further studies in animal CRC models are necessary to address the interaction between adiponectin and leptin.

Using genetic models, we demonstrated that leptin is an important regulator of CRC development. However, leptin signalling did not have a significant effect on promotion of premalignant lesions in the CRC model, because its absence did not alter the number of ACF. These data indicate that leptin does not act as a growth-promoting agent at an early stage of colon carcinogenesis. We observed that the number of ACF was significantly greater in *ob/ob* and *db/db* mice than in WT mice, which suggests that metabolic factors other than leptin act as promoters of early-stage colon carcinogenesis. Furthermore, leptin signalling did not promote normal colonic epithelial cell proliferation either. Why did leptin enhance tumour cell proliferation, but not induce formation of ACF or proliferation of normal colonic mucosa? Here, we noted a difference in ObR between tumours and normal mucosa. A marked increase in ObR expression level was observed in tumours as compared with that in the normal mucosa. Carcinogen-induced tumours frequently show mutation of β-catenin that leads to stabilisation and nuclear translocation of β-catenin, thereby activating the Wnt pathway. On the other hand, mutation and altered cellular localisation of β-catenin are not observed in normal mucosa or ACF.[34] Based on this evidence, we hypothesised that activation of the Wnt pathway not only triggers the formation of colon tumours, but also induces the expression of ObR in colon tumours. To elucidate the roles of Wnt signalling activation on regulation of ObR expression, we examined the effects of β-catenin knockdown on ObR expression in colon cancer cell lines, and confirmed decrease in ObR mRNA and protein levels. Furthermore, we also confirmed increased ObR expression levels in exogenous Wnt-stimulated HEK293 cells. Thus, we propose that Wnt signalling contributes to the upregulation of ObR in colonic epithelium. Based on these results, we conclude that leptin stimulates the proliferation of tumour cells that carry activating alterations in the canonical Wnt pathway (Supplementary figure 11). Furthermore, these data also suggest that leptin is not involved in early-stage colorectal carcinogenesis. Collectively, our observations provided a novel finding that leptin acts as growth factor for CRC only after the tumour initiation stage during the process of colorectal carcinogenesis (Supplementary figure 12).

Our data define a novel role for leptin signalling in the control of tumour growth in addition to its essential role in food intake and energy regulation. The role of leptin signalling is evident from the finding of increased ObR expression in colon tumours, and of such increased expression coinciding with the activation of STAT3. Furthermore, absence of leptin signalling prevented tumour growth, and suppressed STAT3 activation in these tumours. These findings demonstrated that activation of STAT3 in tumours is crucially dependent on leptin signal transduction. Finally, the leptin signalling mechanism of action was revealed

operationally by the finding that treating mice with recombinant leptin increased tumour growth. Taken together, these data provide strong evidence to indicate that leptin signalling controls tumour growth in vivo.

It has been shown previously that recombinant leptin does not stimulate cell proliferation and carcinogenesis in vivo.[16 18 20] While continuous treatment with recombinant leptin enhanced tumour growth in AOM-treated mice, the effect of exogenous leptin was not as strong as we had expected. On the other hand, there is general agreement that leptin acts as a growth factor for colon cancer cells in vitro.[15–17] These discrepancies between in vivo and in vitro studies could be explained by the complicated interaction between various hormones and cytokines. The effects of leptin in vivo are not as simple as those in vitro. Leptin is known to regulate the secretion of several hormones. Importantly, the actions of leptin involve amelioration of hyperinsulinaemia.[35 36] We observed such actions of leptin on insulin levels in mice treated with recombinant leptin. Insulin has the effect of promoting the development of chemically induced tumours in the colon.[37] Therefore, in vivo, the effects of exogenous leptin on promotion of colonic tumourigenesis might be suppressed through a decrease in insulinaemia.

In conclusion, we clearly demonstrated a relationship between leptin signalling and growth of colon tumours, using leptin-deficient or leptin-receptor-deficient mice. The dramatic suppression of colon tumour growth resulting from inhibition of leptin signalling indicates that leptin is an important growth factor for colon cancer progression. We speculate that dietary intake of excessive fat and calories might result in energy storage in the visceral and subcutaneous adipose tissue compartments, and that any surplus energy might be used for growth of CRC through leptin signalling. On the basis of the current results, it is reasonable to conceive that colon tumours might have a tendency to develop in obese individuals who over-eat and who show elevated serum leptin levels. Future study is warranted to address the importance of leptin signalling in the metastatic spread of CRC. Our data provide novel insights into leptin signalling in CRC and suggest novel therapeutic and preventive targets against colon polyps and cancers based on inhibition of leptin-dependent STAT3 signalling.

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Competing interests None.

Ethics approval All animal experiments were conducted with the approval of the institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

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Hiroki Endo, Kunihiro Hosono, Takashi Uchiyama, et al.

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Cancer Epidemiology, Biomarkers & Prevention

Research Article

Investigation of the Prevalence and Number of Aberrant Crypt Foci Associated with Human Colorectal Neoplasm

Eiji Sakai[1], Hirokazu Takahashi[1], Shingo Kato[1], Takashi Uchiyama[1], Kunihiro Hosono[1], Hiroki Endo[1], Shin Maeda[1], Masato Yoneda[1], Masataka Taguri[2], and Atsushi Nakajima[1]

Abstract

Background: Aberrant crypt foci (ACF) are considered to be useful as surrogate biomarker for colorectal cancer (CRC), but the biological significance of ACF remains controversial. We attempted to investigate the relationship between the presence of ACF and human colorectal carcinogenesis using a relatively large sample size.

Methods: We carried out high-magnification chromoscopic colonoscopy to identify ACFs in 861 subjects undergoing a diagnostic endoscopy at the Yokohama City University Hospital. The present study compared the prevalence and number of ACFs in three subject groups (normal subjects, adenoma cases, and CRC cases). The correlations between the demographic and behavioral characteristics of the subjects and the prevalence of ACFs were also assessed.

Results: The prevalence of ACF was 64%, 88%, and 95%, and the mean number of ACF was 3.6, 6.2, and 10.1, in normal subjects, adenoma cases, and CRC cases, respectively. When differences in the prevalence and number of ACFs among age- and sex-stratified subject groups were examined, significant stepwise increments from normal subjects to adenoma cases to CRC cases were apparent ($P < 0.001$). Moreover, an age- and sex-adjusted multiple logistic regression analysis revealed that smoking and alcohol habits had a synergistic effect, increasing the prevalence of ACFs as well as the risk of CRC ($P < 0.001$).

Conclusions: These results suggested that ACF may serve as a reliable surrogate biomarker for human colorectal carcinogenesis.

Impact: The use of ACF as an endpoint may enable the size, duration, and cost of CRC chemoprevention studies to be reduced. *Cancer Epidemiol Biomarkers Prev; 20(9); 1918–24. ©2011 AACR.*

Introduction

Despite recent advances in therapeutic modalities, colorectal cancer (CRC) remains one of the most common causes of cancer-related death in developed countries (1). Currently, chemoprevention for CRC has attracted much attention. The purpose of chemoprevention is to reduce the future mortality of CRC using oral agents that can prevent the occurrence of cancer. Although the occurrence of CRC is the most reliable endpoint, such an endpoint is unsuitable for chemoprevention trials because the occurrence of CRC in the general population is relatively infrequent (1) and such trials would require long-term observation periods. Therefore, to evaluate the efficacy of chemopreventive agents in CRC chemoprevention trials, a more common surrogate biomarker that is robustly associated with CRC is required.

Colorectal carcinogenesis is based on the adenoma–carcinoma sequence, wherein adenomas, spurred by acquired genetic mutations, evolve into CRC. Adenomas have been established as premalignant lesions and are characterized by the presence of genetic and histologic changes. Endoscopic screening and the removal of adenomas can reduce the incidence of CRC by as much as 90% (2, 3). Despite retrospective and prospective studies supporting the use of adenomas as a surrogate biomarker of CRC in chemoprevention trials (4), the use of adenomas as a surrogate endpoint biomarker for CRC has some limitations. The most obvious limitation is that using adenoma formation as an efficacy endpoint requires hundreds of subjects and a very long observation period. Furthermore, to assess the effects of chemopreventive agents, the regression or loss of adenomas must be evaluated (5); therefore, a total colonoscopy is necessary. Unfortunately, these limitations result in poor compliance and a high frequency of dropouts over time, preventing a reasonable rate of

Authors' Affiliations: [1]Gastroenterology Division, and [2]Department of Biostatistics and Epidemiology, Yokohama City University School of Medicine, Yokohama, Japan

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Corresponding Author: Atsushi Nakajima, Gastroenterology Division, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama, 236-0004 Japan. Phone: 81-45-787-2640; Fax: 81-45-784-3546; E-mail: nakajima-tky@umin.ac.jp

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progress for clinical research on CRC prevention. Moreover, large adenomas possibly contain cancer cells; therefore, the assessment of chemopreventive efficacy in patients with large adenomas would involve ethical problems. To overcome these problems, a more useful surrogate biomarker that is reliably correlated with the clinical response, that can be modulated by chemopreventive agents or behavioral characteristics (such as diet changes and smoking cessation) within a short period of time, and that is relatively simple to measure is needed.

Aberrant crypt foci (ACF) were discovered as the earliest microscopic lesions to appear in the colonic mucosa of mice treated with azoxymethane (6). Many studies have shown a dose–response relationship between carcinogens, such as azoxymethane and dimethylhydrazine, and the number of ACF induced (7–11). Moreover, in recent studies, numerous chemopreventive agents have been shown to reduce the number of ACFs in animal models of chemical colonic carcinogenesis. Importantly, many agents that block ACF growth were also shown to prevent tumor development in these carcinogen-treated rodent models (11). Thus, in rodent models, ACFs have been established as a precursor of CRC. Shortly after such descriptions in rodent models were made, ACFs were discovered in pathologic specimens of human colonic mucosa (12–14). ACFs were subsequently identified in the colonic mucosa *in vivo* using high-magnification chromoscopic colonoscopy (HMCC) with methylene blue staining (15). Although several previous epidemiologic studies have revealed significant associations between the prevalence and/or number of ACFs and the synchronous presence of advanced neoplasms, including both adenoma and CRC (15–22), most of the sample sizes in these studies were relatively small. Consequently, the findings were somewhat conflicting. In addition, these studies had limited data about other personal characteristics, such as smoking habit, alcohol habit, and obesity—all of which are related to an increased risk of CRC. If ACFs are indeed a surrogate biomarker for CRC, the epidemiology of ACFs would likely be similar to that of CRC. Therefore, we attempted to investigate the relationship between the presence of ACFs and colorectal carcinogenesis using a larger sample size. Here, we compared the prevalence and number of ACFs in 3 subject groups (normal subjects, adenoma cases, and CRC cases). Moreover, we evaluated the association between the presence of ACFs and the adenoma history. The correlations between the demographic and behavioral characteristics in relation to colorectal carcinogenesis and the prevalence and number of ACFs were also assessed. Our results may help to further evaluations of the potential utility of ACF as a surrogate biomarker for CRC.

Materials and Methods

Subjects

The study protocol was approved by the Yokohama City University Hospital Ethics Committee. Between 2004 and 2009, we enrolled 861 subjects who underwent diagnostic endoscopy at the Yokohama City University Hospital, Japan: of the 861 subjects, 383 had no apparent lesions of the colorectum on colonoscopy (normal subjects), 372 had colorectal adenoma(s), and 106 had CRC. Subjects were excluded if they had undergone previous surgical or endoscopic excision of colonic adenomas and/or cancer or if they had familial adenomatous polyposis, inflammatory bowel disease, or radiation colitis. Written informed consent was obtained from all the subjects prior to their participation in the study. Data on the demographic and behavioral characteristics of the subjects pertaining to the risk of the development of CRC, including smoking habit, alcohol habit, and body mass index (BMI), were obtained from the subjects prior to the performance of the colonoscopy.

HMCC

A Fujinon EC-490ZW5/M colonoscope was used for the magnifying colonoscopy (Fujinon Toshiba ES Systems Co., Ltd.). All the subjects were subjected to bowel preparation using a polyethylene glycol–based solution and underwent a total colonoscopy before rectal ACF imaging. Any detected adenomas were biopsied and the histopathologic appearance was analyzed. Advanced adenoma was defined as an adenoma lesion measuring 1 cm or greater in diameter and/or exhibiting a villous histology and/or high-grade dysplasia. Subsequently, 0.25% methylene blue was applied to the mucosa using a spray catheter. On the basis of the results of a previous study, the ACFs were counted in the lower rectal region, from the middle Houston valve to the dentate line (15). To guard against double counting, the ACFs were counted in a sequential fashion during a single withdrawal of the endoscope. We evaluated the presence of ACFs and the category of the subject (normal subjects, adenoma cases, and CRC cases) simultaneously.

Criteria used for the endoscopic diagnosis

ACFs were defined as lesions in which the crypts were larger in diameter and showed a darker staining with methylene blue than normal crypts, often with oval or slit-like lumens and a thicker epithelial lining (ref. 15; Fig. 1).

Statistical analysis

Data were expressed as the mean ± SD for continuous variables and as a proportion (%) for categorical variables. The prevalence of ACFs among the normal subjects, adenoma cases, and CRC cases were compared using age- and sex-adjusted logistic regression analyses. The numbers of ACFs among these 3 groups were also compared using the Kruskal–Wallis test or an age- and sex-adjusted linear regression analysis. The χ^2 test and the Mann–Whitney U test were used to investigate the association between the presence of ACFs and the adenoma status as well as the association between the presence of ACFs and the location of adenoma(s)/CRC. In addition, univariate and multivariate logistic regression

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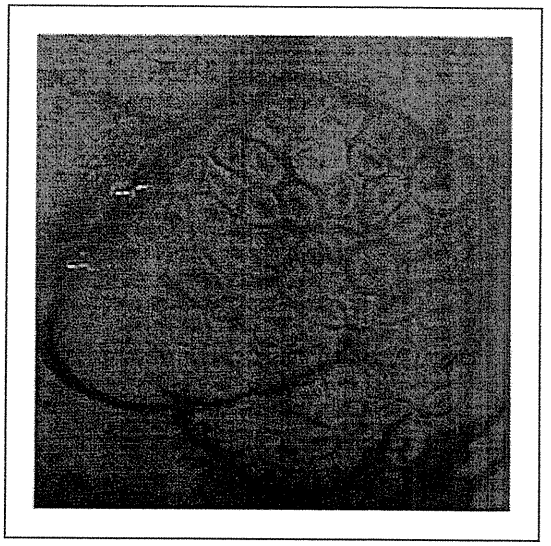

Figure 1. Typical endoscopic appearance of human ACF. This photograph was obtained using a Fujinon EC-490ZW5/M colonoscope after the rectal mucosa had been stained with 0.2% methylene blue.

analyses were used to identify variables with significant independent effects on the prevalence of ACFs among normal subjects. Univariate and multivariate linear regression analyses were also conducted to identify significant variables influencing the number of ACFs. The variables entered in the model included age, sex, smoking habit, alcohol habit, and BMI. Unless otherwise specified, a value of $P < 0.05$ was considered statistically significant. All the analyses were conducted using the SPSS statistical package (version 11.0 for Mac OS X).

Results

Characteristics of the subjects

The characteristics of the subjects according to study group (normal subjects, adenoma cases, and CRC cases) are shown in Table 1. The subjects ranged in age from 19 to 89 years (62.2 ± 12.4): normal subjects, 19 to 85 years (59.3 ± 13.8); adenoma cases, 31 to 88 years (64.2 ± 10.9); and CRC cases, 34 to 89 years (65.6 ± 9.4). A total of 4,742 ACFs were visualized endoscopically in 861 subjects: 1,382 in normal subjects, 2,288 in the adenoma cases, and 1,072 in the CRC cases. The prevalence of ACFs was 64%, 88%, and 95% for the normal subjects, adenoma cases, and CRC cases, respectively. The mean number of ACFs was 3.6 ± 5.2, 6.2 ± 7.0, and 10.1 ± 7.9 in the normal subjects, adenoma cases, and CRC cases, respectively.

Prevalence and number of ACF in the three subject groups stratified according to age and sex

The prevalence and number of ACFs in the 3 subject groups according to age and sex are shown in Table 2. The prevalence of ACF was as high as 51% to 74% even in normal subjects. The prevalence of ACFs in the CRC cases was as high as 91% to 100%, whereas that in the adenoma cases was intermediate. An age-adjusted logistic regression analysis for the 3 subject groups stratified according to sex showed that the differences of the prevalence of ACFs among the 3 subject groups (normal subjects, adenoma cases, and CRC cases) were significant ($P < 0.001$ and $P < 0.001$ for men and women, respectively). In addition, an age-adjusted linear regression analysis for the 3 subject groups stratified according to sex showed that the differences of the number of ACF among the 3 subject groups (normal subjects, adenoma cases, and CRC cases) were significant ($P < 0.001$ and $P < 0.001$ for men and women, respectively).

Differences in the presence of ACFs between subjects with nonadvanced and advanced adenomas

The relationship between the presence of ACFs and the adenoma history is shown in Table 3. The prevalence of ACFs in subjects with advanced adenoma(s) did not differ significantly from that of subjects with nonadvanced adenoma(s) (89% and 87%, respectively; $P = 0.41$). However, the number of ACFs in subjects with

Table 1. Characteristics of the subjects

	Normal subjects	Adenoma cases	CRC cases
Number of subjects	383	372	106
Age, y			
Mean ± SD	59.3 ± 13.8	64.2 ± 10.9	65.6 ± 9.4
Median	62	64.5	66
Sex (M/F)	211/172	265/107	73/33
Number of subjects with ACF	246	326	101
ACF prevalence, %	64	88	95
Total number of ACF	1,382	2,288	1,072
ACF number, mean ± SD	3.6 ± 5.2	6.2 ± 7.0	10.1 ± 7.9

NOTE: Normal subjects were defined as subjects with no apparent lesions of the colorectum on total colonoscopy.

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Table 2. Prevalence and number of ACFs among the 3 subject groups stratified according to age and sex

	Male				Female			
	<60 y	60–69 y	≥70 y	Total	<60 y	60–69 y	≥70 y	Total
ACF prevalence[a]								
Normal subjects								
Number of subjects	89	62	60	211	78	52	42	172
Prevalence, %	62	74	67	67	51	71	67	61
Adenoma cases								
Number of subjects	79	92	94	265	29	44	34	107
Prevalence, %	89	88	95	91	76	82	82	80
CRC cases								
Number of subjects	15	34	24	73	9	11	13	33
Prevalence, %	93	94	96	95	100	91	100	97
ACF number,[b] *mean ± SD*								
Normal subjects	2.6 ± 3.2	5.3 ± 7.1	4.6 ± 6.2	3.9 ± 5.6	1.8 ± 2.6	3.8 ± 5.0	5.0 ± 6.5	3.2 ± 4.7
Adenoma cases	5.0 ± 5.5	6.8 ± 7.9	8.1 ± 8.0	6.7 ± 7.4	4.7 ± 5.9	3.8 ± 4.1	6.1 ± 7.8	4.8 ± 6.0
CRC cases	8.7 ± 8.4	10.5 ± 7.4	10.0 ± 7.3	10.0 ± 7.5	10.9 ± 11.6	8.5 ± 9.3	11.8 ± 6.0	10.5 ± 8.7
P	<0.001	<0.001	<0.001	<0.001[‡]	<0.001	0.19	<0.005	<0.001[c]

[a]$P < 0.001$ and $P < 0.001$ (men and women, respectively), calculated using an age-adjusted logistic regression analysis for the 3 subject groups stratified according to sex.
[b]Differences among the age-stratified subject groups (<60, 60–69, and ≥70 years) were analyzed using the Kruskal–Wallis test.
[c]An age-adjusted linear regression analysis was conducted to evaluate the differences among the 3 subject groups stratified according to sex.

advanced adenoma(s) was larger than that in subjects with nonadvanced adenoma(s) (7.8 ± 8.2 and 5.1 ± 6.0, respectively; $P < 0.005$).

Relationship between the presence of ACFs and the location of adenoma/CRC

The relationship between the presence of ACFs and the location of adenoma/CRC is shown in Table 4. Sixty-eight of the 372 adenoma cases (18%) had adenoma(s) only in the proximal colon. No significant differences were observed between the prevalence and number of ACFs and the location of the adenoma(s) ($P = 0.86$ and $P = 0.73$, respectively). Twenty-nine of the 102 CRC cases (28%) had proximal CRC. No significant differences were observed between the prevalence and number of ACFs and the location of the CRC ($P = 0.52$ and $P = 0.26$, respectively).

Correlations between the presence of ACFs and demographic and behavioral characteristics pertaining to the risk of colorectal carcinogenesis

To investigate the risk factors for the prevalence of ACF, univariate and multivariate logistic regression analyses were conducted in normal subjects (Table 5). We defined smoking habit as positive for subjects with more than 10 pack-years who were still smoking or who had quit within the past 10 years; alcohol habit was defined as positive for subjects with alcohol consumption in excess

Table 3. Differences in the presence of ACFs between subjects with nonadvanced and advanced adenoma(s)

	N	ACF prevalence,[a] %	ACF number,[b] mean ± SD
Nonadvanced adenoma	230	87	5.1 ± 6.0
Advanced adenoma	142	89	7.8 ± 8.2
P		0.41	<0.005

NOTE: Advanced adenoma was defined as an adenoma lesion measuring 1 cm or greater in size and/or exhibiting a villous histology and/or high-grade dysplasia.
[a]*P* values were calculated using the χ^2 test.
[b]*P* values were calculated using the Mann–Whitney *U* test.

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Table 4. Relationship between the presence of ACFs and the location of adenoma/CRC

Location	*N*	ACF prevalence,[a] %	ACF number,[b] mean ± SD
Adenoma cases			
Including distal colon	304	88	6.1 ± 6.9
Only in proximal colon	68	88	6.4 ± 7.6
P		0.87	0.73
CRC cases			
Distal colon	77	96	10.4 ± 7.6
Proximal colon	29	93	9.3 ± 8.6
P		0.52	0.26

NOTE: The distal colon was defined as the region of colonic lesion from the splenic flexure to the dentate line. The proximal colon was defined as the region of colonic lesion from the cecum to the splenic flexure.

[a]*P* values were calculated using the χ^2 test.

[b]*P* values were calculated using the Mann–Whitney *U* test.

of 45 g/d. Both of these factors are reported to associate with an increased risk of adenoma and CRC (23–28). Age- and sex-adjusted multivariate analyses revealed that smoking habit [odds ratio (OR) = 1.6; 95% CI = 0.9–3.1] and alcohol habit (OR = 2.0; 95% CI = 0.8–5.0) were not independent risk factor for the prevalence of ACFs ($P = 0.12$ and $P = 0.15$, respectively); however these 2 factors (OR = 5.4; 95% CI = 2.3–13.0) synergistically increased the prevalence of ACFs ($P < 0.001$). Obesity was also reported to associate with an increased risk of adenoma and CRC (28). We defined obesity as positive for subjects with a BMI of 25 or grater. Obesity (OR = 1.5; 95% CI = 0.8–2.6) was also not an independent risk factor for the prevalence of ACFs ($P = 0.17$). We also conducted univariate and age- and sex-adjusted multivariate linear regression analyses to evaluate the correlations between the number of ACFs and these factors. Smoking and alcohol habits also synergistically increased the number of ACFs, but this trend was only borderline significant ($P = 0.06$; Supplementary Table S1).

Discussion

In our study, significant stepwise increments in both the prevalence and the number of ACFs were observed from normal subjects to adenoma cases to CRC cases. In addition, the mean number of ACF was significantly higher in the subject group with advanced adenoma than in the subject group with nonadvanced adenoma. These results indicate that ACF may serve as a reliable surrogate biomarker of human colorectal carcinogenesis.

Most previous studies (15–18, 20–22) have evaluated ACF in the lower rectal region because HMCC is technically easier to conduct at this location, is suitable for use as a follow-up examination, and is well tolerated by patients. Therefore, we evaluated the ACFs in the lower rectal region, similar to previous studies. To evaluate whether the rectal ACF reflects the total colonic adenoma/CRC, we examined associations between the presence of rectal ACFs and the locations of the adenoma/CRC. In our study, no significant differences were observed between the prevalence and the number of ACFs in subjects who had only proximal colonic adenoma/CRC and subjects who had at least 1 distal colonic adenoma/CRC. This result indicates that rectal ACF examinations may be useful as a biomarker not only for distal colonic neoplasia but also for proximal colonic neoplasia.

The development of CRC is influenced by several acquired risk factors including dietary factors and lifestyle factors. If ACFs are indeed a surrogate biomarker of CRC, then their epidemiology is likely to be similar to that of CRC. If risk factors influence colorectal carcinogenesis at an early stage, then they may also be associated with the formation of ACFs. Therefore, we evaluated whether risk factors which associate with the development of CRC were independently associated with the presence of ACFs in normal subjects. In our study, smoking habit

Table 5. Age- and sex-adjusted multiple logistic regression analysis of behavioral characteristics and the prevalence of ACFs in normal subjects

Variable	Proportion, %	ACF prevalence, %	OR (95% CI)			
			Univariate	*P*	Multivariate	*P*
Smoking (–), alcohol (–)	63	58	1 (reference)	–	1 (reference)	–
Smoking (+), alcohol (–)	16	67	1.5 (0.8–2.7)	0.17	1.6 (0.9–3.1)	0.12
Smoking (–), alcohol (+)	7	73	2.0 (0.8–4.9)	0.13	2.0 (0.8–5.0)	0.15
Smoking (+), alcohol (+)	14	87	4.8 (2.1–11.1)	<0.001	5.4 (2.3–13.0)	<0.001

NOTE: Smoking habit was defined as positive if the subject had more than 10 pack-years and was still smoking or had quit within the past 10 years. Alcohol habit was defined as positive if the subject's alcohol consumption exceeded 45 g/d. The multivariate logistic regression analysis was adjusted for age and sex.

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and alcohol habit synergistically increased the prevalence of ACFs in a significant manner. Interestingly, recent studies have revealed that cigarette smoking and heavy alcohol intake also interact in an additive manner, increasing the risk of CRC, similar to results seen in the aerodigestive tract (29, 30). Tobacco contains a large number of carcinogens that may bind to DNA and form adducts, potentially causing irreversible genetic damage to the normal colonic mucosa (31). On the other hand, alcohol is metabolized to acetaldehyde, which binds to DNA and forms carcinogenic adducts (32). Therefore, these 2 factors may share a common pathway in promoting colorectal carcinogenesis at an early stage and initiating ACF formation. On the other hand, obesity was not strongly associated with the prevalence of ACFs because only a few patients were regarded as obese in our study. In contrast to our hypothesis, no significant associations were observed between the number of ACFs and these factors, although smoking and alcohol habits tended to increase the number of ACFs in a synergistic manner. A not insignificant number of subjects exhibited an extremely high density of ACFs (as high as 30), even in normal subjects; therefore, the wide variance in the number of ACFs might have extinguished the statistical significance (Supplementary Fig. S1).

Although most previous epidemiologic studies of ACFs have shown a significant correlation between the presence of ACFs and synchronous advanced neoplasia (15–22), a recent multicenter study raised serious questions about whether ACFs can be used as a surrogate biomarker for CRC (33). However, their subject groups were determined 8 years, on average, prior to the actual ACF examination. In addition, they determined the subject group on the basis of the results of flexible sigmoidoscopy; thus, proximal adenomas may have been missed. These facts suggest that their control group may have contained a not insignificant number of subjects with adenoma. Therefore, their study may not actually show an association between the presence of ACFs and the adenoma status. However, such considerations are inadequate to explain this discrepancy. Differences in participant characteristics, such as race, age and behavioral factors, may be associated with this discrepancy. Variations in the criteria used to detect ACFs and the method used to visualize ACFs may also affect this discrepancy. A large prospective and cross-sectional study would be useful for resolving this discrepancy.

Recently, several prospective studies have been conducted using the presence of ACF as a surrogate biomarker for CRC in chemoprevention trials in humans (34–36). ACFs are considered to be a heterogeneous group of lesions, some, but not all, of which may be robustly associated with the risk of CRC, as the prevalence of ACF was as high as 70% even in normal subjects. Interestingly, our results suggested that even if a very small subset or none of the ACFs may progress to CRC, ACF may still be useful as a surrogate biomarker for CRC. In humans, Shpitz and colleagues showed that the proliferating cell nuclear antigen (PCNA) labeling indices for ACFs were significantly higher than those for normal mucosa (14). In addition, we previously showed that metformin, which inhibits the mTOR pathway through the activation of AMPK, suppresses cellular proliferation and ACF formation (35). These results suggested that ACF may be a marker for epithelial proliferation. Importantly, previous studies have showed that a high proliferative activity in the colon mucosa is associated with an increased risk of CRC (37).

In conclusion, we confirmed that the prevalence and mean number of ACFs significantly increased with the stage of the adenoma–carcinoma sequence using age- and sex-adjusted analyses of a relatively large sample. We also showed that smoking and alcohol habits synergistically increased the prevalence of ACFs as well as the risk of CRC. These results suggested that ACFs may be useful as a reliable surrogate biomarker for human colorectal carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

Inflammation-induced repression of tumor suppressor miR-7 in gastric tumor cells

D Kong[1,7], Y-S Piao[1,7,8], S Yamashita[2], H Oshima[1], K Oguma[1], S Fushida[3], T Fujimura[3], T Minamoto[4], H Seno[5], Y Yamada[6], K Satou[6], T Ushijima[2], T-O Ishikawa[1] and M Oshima[1]

[1]*Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan;* [2]*Division of Epigenomics, National Cancer Center Research Institute, Tokyo, Japan;* [3]*Department of Gastroenterologic Surgery, Kanazawa University Hospital, Kanazawa, Japan;* [4]*Division of Translational and Clinical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan;* [5]*Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan and* [6]*Faculty of Electrical and Computer Engineering, Institute of Science and Engineering, Kanazawa University, Kanazawa, Japan*

Inflammation has an important role in cancer development through various mechanisms. It has been shown that dysregulation of microRNAs (miRNAs) that function as oncogenes or tumor suppressors contributes to tumorigenesis. However, the relationship between inflammation and cancer-related miRNA expression in tumorigenesis has not yet been fully understood. Using *K19-C2mE* and *Gan* mouse models that develop gastritis and gastritis-associated tumors, respectively, we found that 21 miRNAs were upregulated, and that 29 miRNAs were downregulated in gastric tumors in an inflammation-dependent manner. Among these miRNAs, the expression of miR-7, a possible tumor suppressor, significantly decreased in both gastritis and gastric tumors. Moreover, the expression of miR-7 in human gastric cancer was inversely correlated with the levels of interleukin-1β and tumor necrosis factor-α, suggesting that miR-7 downregulation is related to the severity of inflammatory responses. In the normal mouse stomach, miR-7 expression was at a basal level in undifferentiated gastric epithelial cells, and was induced during differentiation. Moreover, transfection of a miR-7 precursor into gastric cancer cells suppressed cell proliferation and soft agar colony formation. These results suggest that suppression of miR-7 expression is important for maintaining the undifferentiated status of gastric epithelial cells, and thus contributes to gastric tumorigenesis. Although epigenetic changes were not found in the CpG islands around miR-7-1 of gastritis and gastric tumor cells, we found that activated macrophage-derived small molecule(s) (<3 kDa) are responsible for miR-7 repression in gastric cancer cells. Furthermore, the miR-7 expression level significantly decreased in the inflamed gastric mucosa of *Helicobacter*-infected mice, whereas it increased in the stomach of germfree *K19-C2mE* and *Gan* mice wherein inflammatory responses were suppressed. Taken together, these results indicate that downregulation of tumor suppressor miR-7 is a novel mechanism by which the inflammatory response promotes gastric tumorigenesis.

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Keywords: miR-7; gastric cancer; inflammation; macrophages

Introduction

It has been established that inflammatory responses contribute to cancer development through various mechanisms (Coussens and Werb, 2002). The expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for prostaglandin biosynthesis, has an important role in both inflammation and cancer (Wang and DuBois, 2010). Using genetic mouse models, we previously demonstrated that induction of COX-2 and its downstream product, prostaglandin E_2 (PGE_2), is required for gastrointestinal tumorigenesis (Sonoshita *et al.*, 2001; Oshima *et al.*, 2006). The COX-2/PGE_2 pathway, together with a bacterial infection, induces inflammatory responses in the stomach through the recruitment of macrophages, which promotes gastric tumorigenesis (Oshima *et al.*, 2011). However, it remains to be fully elucidated precisely how such inflammatory responses contribute to the promotion of gastric tumors.

MicroRNAs (miRNAs) are a class of single-stranded small noncoding RNAs that regulate gene expression by post-transcriptional interference of specific mRNAs (Ambros, 2004; Bartel, 2004). Through their regulation of cancer-related gene expression, miRNAs can function as either oncogenes or tumor suppressors (Esquela-Kerscher and Slack, 2006; Ventura and Jacks, 2009). Dysregulation of miRNAs in cancer has been shown to be associated with genomic/epigenetic alterations or transcriptional/post-transcriptional mechanisms (Di Leva and Croce, 2010). Moreover, expression of several miRNAs, including oncogenic miRNAs, has been shown to be induced by inflammatory

Correspondence: Professor M Oshima, Division of Genetics, Cancer Research Institute, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192, Japan.
E-mail: oshimam@kenroku.kanazawa-u.ac.jp
[7]These authors contributed equally to this work.
[8]Current address: Department of Pathophysiology, Medical College, Yanbian University, Yanji City 133002, China.
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responses (Sonkoly and Pivarcsi, 2011). For example, miR-155 is induced in macrophages by nuclear factor-κB, interferon-β or Toll-like receptor signaling (O'Connell *et al.*, 2007; Tili *et al.*, 2007), whereas miR-21 is induced by Stat3, a transcription factor activated by interleukin-6 (IL-6) (Iliopoulos *et al.*, 2010). On the other hand, the mechanism responsible for the downregulation of tumor-suppressor miRNA expression in the inflammatory microenvironment has not been fully understood.

Herein, we examined the expression of miRNAs in mouse models of gastritis and gastric tumors, which were developed in *K19-C2mE* and *Gan* mice, respectively (Oshima *et al.*, 2004, 2006). We found the expression level of miR-7 to significantly decrease in gastric tumors in an inflammation-dependent manner. It has been shown that miR-7 has a tumor-suppressor role in several cancers, including glioblastoma, breast cancer and lung cancer (Kefas *et al.*, 2008; Reddy *et al.*, 2008; Webster *et al.*, 2009; Jiang *et al.*, 2010; Saydam *et al.*, 2011). In this manuscript, we demonstrate that miR-7 is induced during the differentiation of normal gastric epithelial cells, and it also has a tumor-suppressor role in the stomach. These results suggest that downregulation of tumor suppressor miR-7 is one of the tumor-promoting mechanisms underlying the role of inflammation in gastric tumorigenesis.

Results

Inflammation-dependent dysregulation of miRNAs in gastric tumors

To examine whether miRNA expression is dysregulated in gastric tumors by inflammatory responses, we examined the miRNA expression profiles in wild-type mouse stomachs, *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors by a microarray analysis. In *Gan* mouse gastric tumors, 50 miRNAs were upregulated (>2.0-fold), whereas 42 miRNAs were downregulated (<0.5-fold) compared with the wild-type mouse stomach level (Figure 1a and Supplementary Table 1). Notably, 21 and 29 miRNAs showed upregulation or downregulation, respectively, in both gastritis and gastric tumors. Therefore, it is possible that dysregulation of these miRNAs is caused by inflammatory responses.

We confirmed the results of the microarray analysis by real-time reverse transcriptase–polymerase chain reaction (RT–PCR). In all, 10 miRNAs randomly selected from the upregulated and downregulated miRNAs (Figure 1a, boxed) showed the same dysregulation pattern in both gastritis and gastric tumors (Figure 1b). Importantly, miR-155 and miR-21, which function as oncogenes (Volinia *et al.*, 2006) were upregulated, whereas miR-145 and miR-7, which function as tumor suppressors (Kefas *et al.*, 2008; Sachdeva *et al.*, 2009), were downregulated in both gastritis and gastric tumors (Figures 1a and b). This suggests that inflammation can induce not only upregulation of oncogenic miRNAs but also downregulation of tumor-suppressor miRNAs.

We next picked up 74 miRNAs that were not dysregulated in *K19-C2mE* gastritis compared with the wild-type normal stomach (Supplementary Table 2). Among them, three miRNAs were upregulated in *Gan* mouse tumors compared with *K19-C2mE* gastritis tissue samples (>2.0-fold), whereas three miRNA were downregulated (<0.5-fold) (Figure 1c). It is possible that expression of these miRNAs is dysregulated by carcinogenesis-specific mechanisms.

Induction of miR-7 during differentiation of gastric epithelial cells

We further examined the expression of miR-7, because its role(s) in the normal stomach and gastric cancer have never been examined. Gastric glands were isolated from the stomachs of the respective mouse models, and the miR-7 expression was examined by real-time RT–PCR. Notably, miR-7 levels were significantly lower in epithelial cells of *K19-C2mE* gastritis tissues and *Gan* mouse tumors compared with the wild-type mouse stomach (Figure 2a), indicating that miR-7 is predominantly expressed in epithelial cells in an inflammation-dependent manner.

When primary cultured gastric epithelial cells were passaged and maintained for 6 days, the cell morphology appeared to be differentiated, with enlarged and mucin-containing cytoplasm (Figure 2b). Consistently, the expression of differentiation markers, *Muc6* and *Muc5AC*, was elevated on day 6, whereas the expression of the Wnt target gene, *Sox9*, decreased (Figure 2c). These results indicate that cultured gastric epithelial cells underwent differentiation through passage and 6-day culture. Importantly, the miR-7 expression level increased significantly on day 6 to 6.5-fold, compared with the level observed on day 2.

We next examined the miR-7 level in the stomach during development. The expression level of miR-7 in the stomach increased significantly in 14-day-old and adult mice, to >6-fold of that in E15 embryos (Figure 2d). Conversely, expression of *CD44*, one of the Wnt target genes, decreased significantly during development. We confirmed by an immunohistochemistry that most epithelial cells in the gastric mucosa were Ki-67 positive on days 0 and 7, whereas proliferating cells were limited to the gland neck on day 14 and in adult mice (Figure 2e). Accordingly, the ratio of undifferentiated epithelial cells decreased during development. Taken together, these results indicate that miR-7 expression is induced in gastric epithelial cells during differentiation.

Tumor-suppressor role of miR-7 in gastric cancer development

We next examined miR-7 levels in human gastric cancers by real-time RT–PCR. The expression of miR-7 was downregulated in 18 out of 28 human gastric cancer tissue samples (64%) compared with paired non-tumor stomach tissue samples (Figure 3a), suggesting that miR-7 has a tumor-suppressor role in a subpopulation of gastric cancers. We next examined the expression

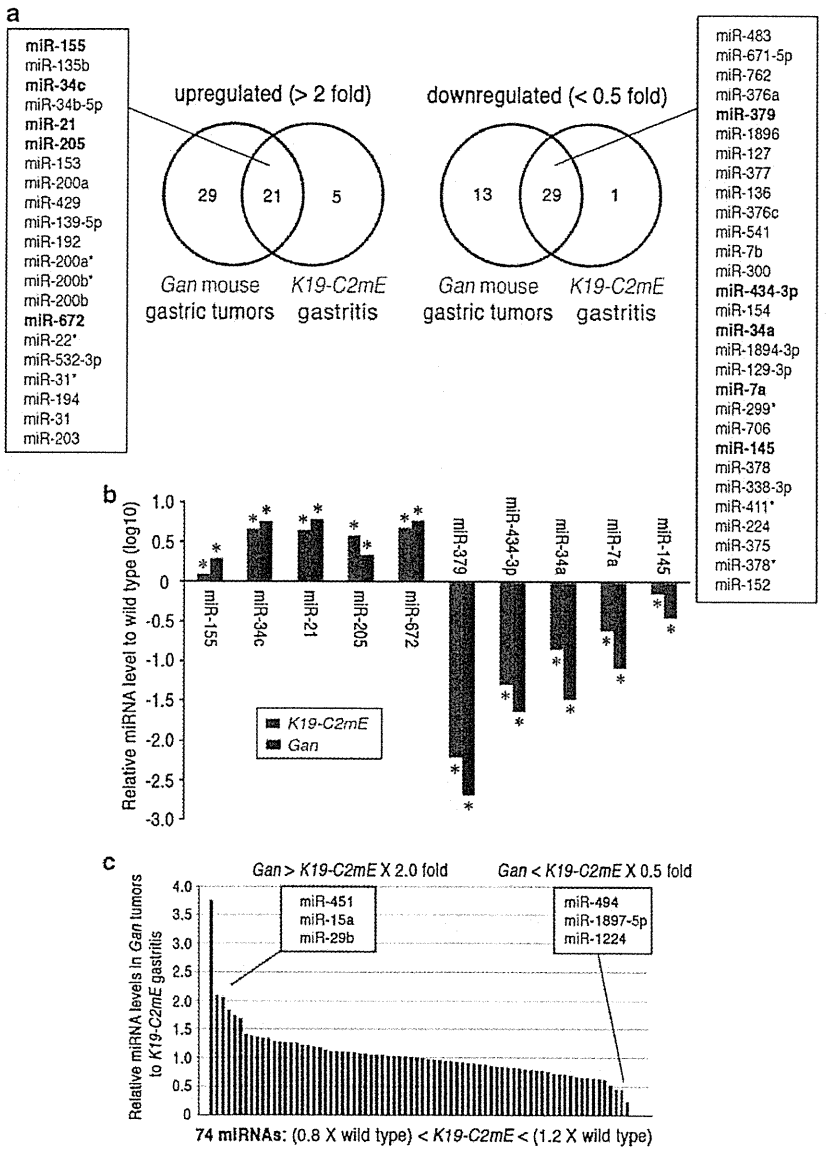

Figure 1 Inflammation-dependent dysregulation of miRNA expression in mouse gastric tumors. (**a**) Venn diagrams of the miRNAs that were upregulated (>2.0) and downregulated (<0.5) in *Gan* mouse gastric tumors and/or *K19-C2mE* mouse gastritis samples as determined by the microarray analysis are shown. The miRNAs listed in boxes were upregulated (left) or downregulated (right) in both gastric tumor and gastritis tissue samples. (**b**) The relative expression levels of selected miRNAs (indicated in bold in the list) for *K19-C2mE* mouse gastritis (blue bars) and *Gan* mouse gastric tumors (red bars) compared with the wild-type levels examined by real-time RT–PCR are shown as the log10 ratios. $^*P<0.05$ versus the wild-type level. The expression levels of miRNAs were normalized to the Sno202 level. (**c**) The miRNA levels in *Gan* mouse tumors relative to those in *K19-C2mE* gastritis tissues examined by the microarray analysis are shown. Red and blue bars indicate upregulated (>2.0) and downregulated (<0.5) miRNAs, respectively, in *Gan* mouse tumors.

level of IL-1β and tumor necrosis factor (TNF)-α, major proinflammatory cytokines, and compared them with the miR-7 level. Importantly, expression levels of miR-7 were inversely correlated with those of IL-1β or TNF-α, suggesting that the downregulation of miR-7 is related to the severity of inflammatory responses (Figure 3b). We also found that miR-7 was markedly downregulated in four out of nine gastric cancer cells lines (Figure 3c).

To examine the tumor-suppressor role of miR-7 in gastric tumorigenesis, we transfected the precursor of miR-7, pre-miR-7, into AZ-521 and Kato-III gastric cancer cells and examined their proliferation and soft agar colony formation. We confirmed that pre-miR-7 transfection into reporter vector-transfected cells resulted in a significant decrease in luciferase activity, indicating an increase of mature miR-7 level (Supplementary Figure 1). Transfection of pre-miR-7

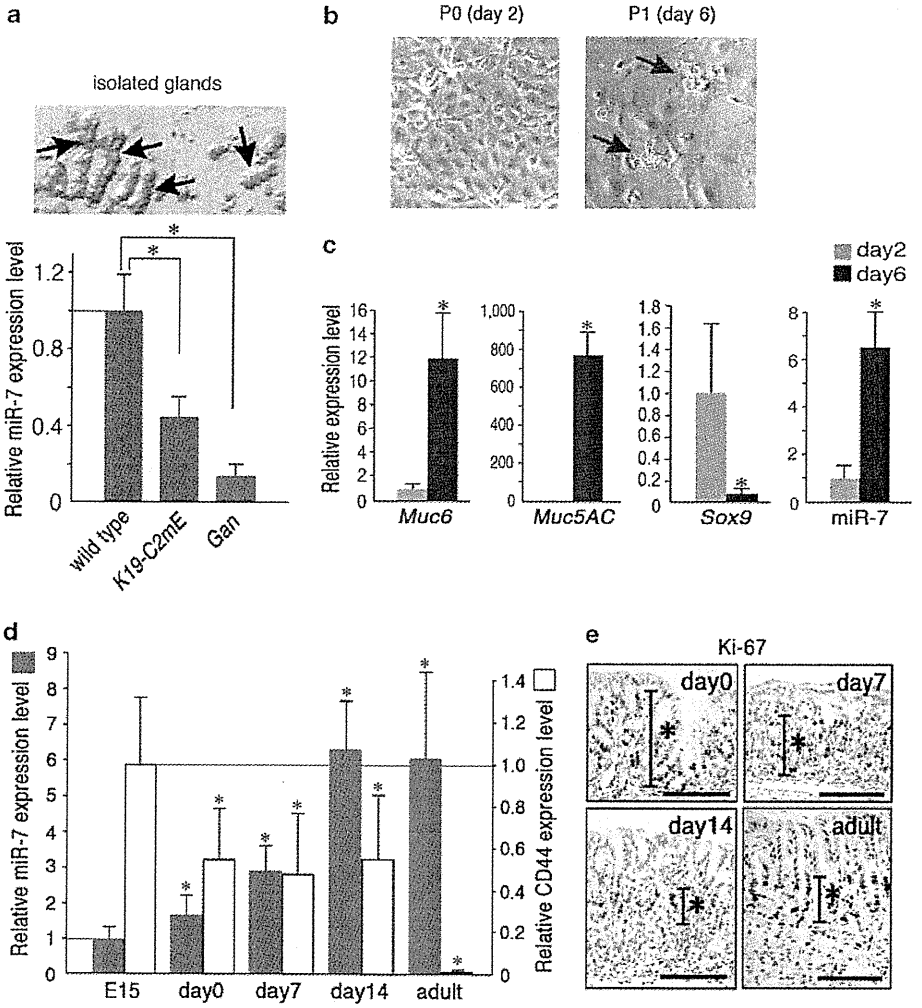

Figure 2 The induction of miR-7 expression in differentiated gastric epithelial cells. (**a**) A representative photograph of isolated gastric glands from wild-type mice (top, arrows). The expression levels of miR-7 in the isolated gastric glands of *K19-C2mE* and *Gan* mice relative to the wild-type level are shown (mean ± s.d.) (bottom). $^*P<0.05$. (**b**) Representative photographs of primary cultured gastric epithelial cells on day 2 (passage 0: P0) and on day 6 (passage 1: P1) (original magnification, × 100). Arrows in P1 indicate mucin-containing enlarged cells on day 6. (**c**) The levels of *Muc6*, *Muc5AC* and *Sox9* mRNA and miR-7 in the primary cultured gastric epithelial cells on day 6 (closed bars) relative to the levels on day 2 (gray bars) are shown (mean ± s.d.). $^*P<0.05$ versus the day 2 level. (**d**) The expression levels of miR-7 (gray bars) and *CD44* (open bars) in the stomach at the indicated ages relative to the levels in E15 embryos are shown (mean ± s.d.). $^*P<0.05$ versus the E15 level. The expression levels of miR-7 were normalized to the Sno202 level. (**e**) Representative photographs of Ki-67 immunostaining in the glandular stomach of mice at the indicated ages. Asterisks indicate proliferative zones. Scale bars indicate 50 μm.

significantly decreased cell proliferation in both cell lines compared with control vector-transfected cells (Figure 3d). Moreover, pre-miR-7 transfection significantly suppressed soft agar colony formation in both cell lines (Figures 3e and f). These results strongly suggest that miR-7 has a tumor-suppressor role in gastric cancer development.

Repression of miR-7 in gastric cancer cells by macrophage-derived factor(s)

We detected primary (pri)-miR-7-1, pri-miR-7-2 and pri-miR-7b in the mouse normal stomach by real-time RT–PCR (Supplementary Figure 2), suggesting that mature miR-7 is processed from all these primary miR-7 in the normal gastric mucosa. MiR-7-1 is located in the intron of the *Hnrnpk* gene, and a CpG island is found in the promoter region of *Hnrnpk* (Supplementary Figure 3a). On the other hand, we could not determine CpG islands that regulate the transcription of miR-7-2 and miR-7b. We thus examined DNA methylation in the CpG islands in the *Hnrnpk* promoter region. Notably, DNA methylation levels in *K19-C2mE* gastritis and *Gan* mouse tumor tissues were not increased compared with the wild-type mouse stomach (Supplementary Figure 3a). Consistently, DNA methylation was not detected in the promoter region of the *HNRNPK* gene in human gastric cancer tissues (Figure 4a). We also examined the trimethylation of histone H3 at lysine 27 (H3K27me3) in

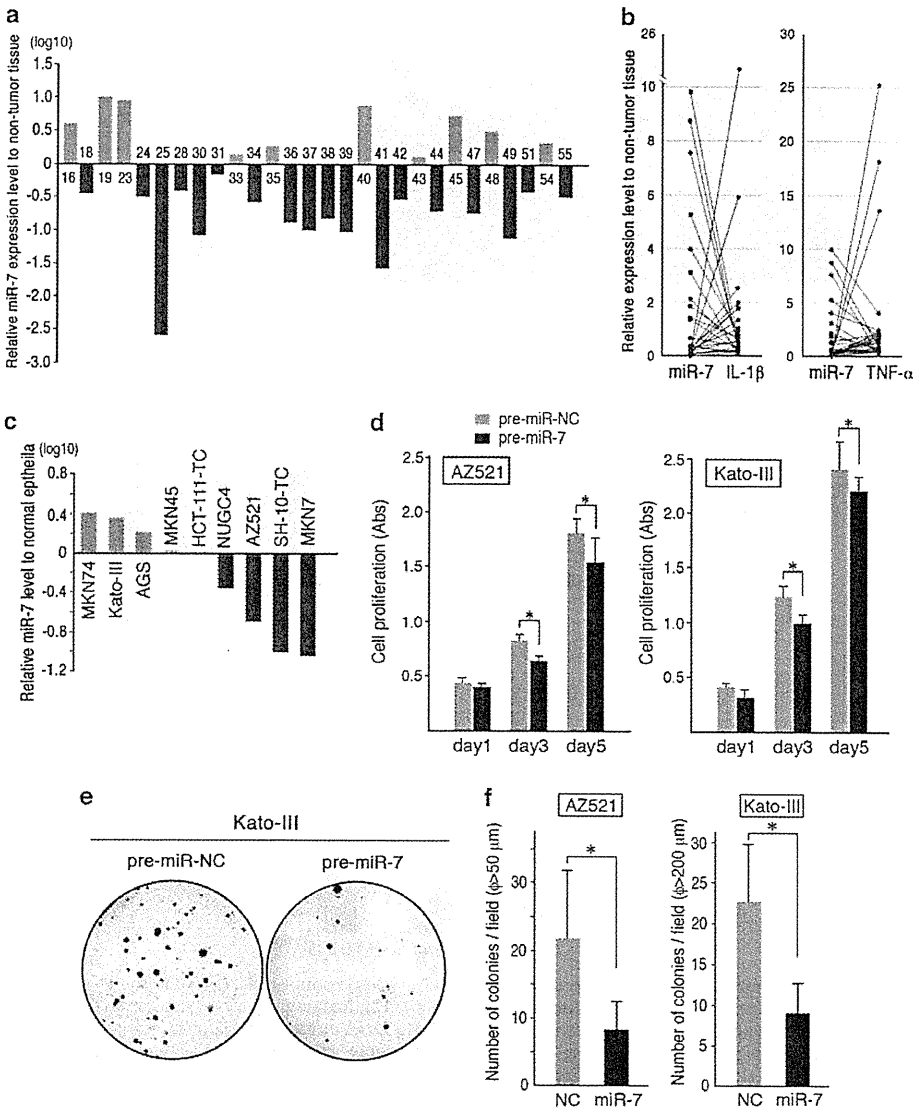

Figure 3 The tumor-suppressor roles of miR-7 in gastric cancer cells. (**a**) The relative miR-7 expression levels in the human gastric cancer tissue samples to the level of non-tumor stomach tissue samples are shown as the log10 ratios. The indicated numbers correspond to the patient ID in the clinicopathological data (Supplementary Table 3). (**b**) Comparison of the relative expression levels of miR-7 and IL-1β (left) or TNF-α (right) in gastric cancer tissues to the non-tumor stomach tissue levels in each patient is shown. Red and blue lines indicate that the expression of miR-7 was increased (>1.0) and decreased (<1.0) in the gastric cancers, respectively. (**c**) The relative expression levels of miR-7 in gastric cancer cell lines compared with the mean level in the human normal gastric epithelial cells are shown as the log10 ratios. The expression levels of miR-7 were normalized to those of U44. (**d**) The proliferation of control (gray bars) and pre-miR-7-transfected (closed bars) AZ521 cells and Kato-III cells at the indicated culture days are shown (mean ± s.d.). $*P<0.05$. (**e**) Representative photographs of soft agar colonies in 6-well plates showing the pre-miR-NC- (left) and pre-miR-7-transfected (right) Kato-III cells. (**f**) The mean numbers of soft agar colonies larger than the indicated diameters in each well of 6-well plate of control (gray bars) and pre-miR-7-transfected (closed bars) AZ521 cells and Kato-III cells are shown (mean ± s.d.). $*P<0.05$.

the upstream CpG islands of *Hnrnpk*. However, the H3K27me3 level was not increased in mouse gastritis and gastric tumors compared with the wild-type stomach (Figure 4b). These results indicate that DNA methylation and trimethylation of H3K27 are not involved in the downregulation of miR-7-1. Moreover, the genomic region including miR-7-1 was not deleted in human gastric cancer cells (Supplementary Figure 3b), suggesting that miR-7 downregulation in gastric cancer is not caused by genomic deletion.

We next examined whether activated macrophages have a role in the downregulation of miR-7, because the major source of proinflammatory cytokines in gastric tumors are macrophages (Oshima *et al.*, 2004, 2011). To monitor miR-7 activity, reporter vector-transfected cells were used. We confirmed that luciferase activity

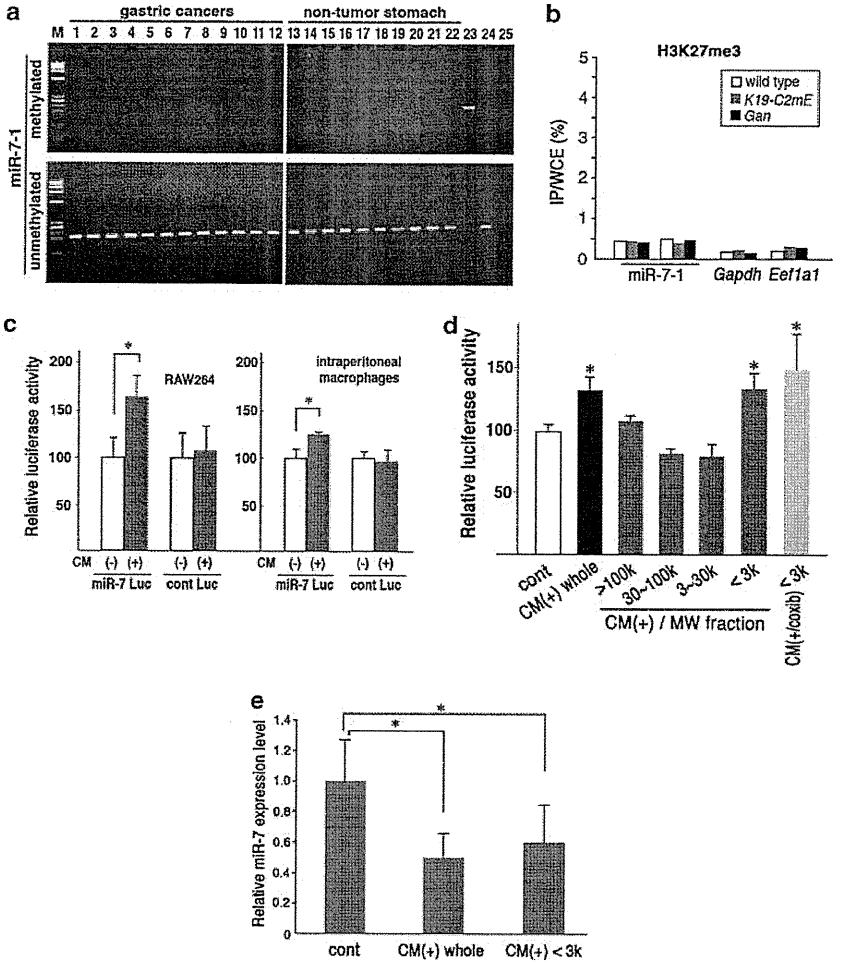

Figure 4 The mechanism responsible for the downregulation of miR-7 in gastric tumor cells. (**a**) Representative results of methylated (top) or unmethylated (bottom) status-specific PCR for miR-7-1. Lanes 1–12, human gastric cancer samples; lanes 13–22, non-tumor stomach samples; lane 23, methylated DNA control; lane 24, unmethylated DNA control; and lane 25, water control. (**b**) The results of ChIP-PCR analyses of miR-7-1 and the housekeeping genes, *Gapdh* and *Eef1a1*, for H3K27me3 in the gastric mucosa of respective genotype mice. The percentages of immunoprecipitated (*IP*)/whole-cell extracts (*WCE*) are shown for each primer set. (**c**) The luciferase activities of miR-7 Luc- or control Luc-transfected AZ521 reporter cells stimulated with CM(+) (gray bars) relative to those with CM(−) (open bars) are shown (mean ± s.d.). $^*P<0.05$. The conditioned medium was prepared from RAW264 cells (left) or intraperitoneal macrophages (right). (**d**) The luciferase activities of miR-7 Luc-transfected AZ521 reporter cells stimulated with whole CM(+) (closed bars), CM(+) fractionated to the indicated molecular sizes (gray bars), or fractionated CM(+) to <3 kDa collected from celecoxib-treated and LPS-stimulated macrophages (light gray bar) relative to the control level (open bar) are shown (mean ± s.d.). $^*P<0.05$ versus the control level. (**e**) The relative miR-7 expression levels examined by real-time RT–PCR in AZ521 cells stimulated with whole CM(+) or fractioned CM(+) <3 kDa relative to the control level are shown (mean ± s.d.). $^*P<0.05$ versus the control level. The expression levels of miR-7 were normalized to the U44 level.

was increased significantly when the miR-7 inhibitor was transfected into reporter vector-transfected Kato-III cells (Supplementary Figure 1c), indicating that the luciferase reporter system was working. Reporter cells were then treated with the conditioned medium of lipopolysaccharide-stimulated RAW264 cells (CM(+)) or unstimulated RAW264 cells (CM(−)). Importantly, the luciferase activity increased significantly when cells were stimulated with CM(+), whereas the luciferase activity was not changed in control vector-transfected cells (Figure 4c). Similar results were obtained when CM(+) and CM(−) were prepared using mouse intraperitoneal macrophages. These results indicate that activated macrophage-derived factor(s) caused miR-7 downregulation in gastric cancer cells.

To identify macrophage-derived factor(s) that suppress miR-7 expression, reporter cells were stimulated with TNF-α, IL-1β, IL-6 or PGE_2. However, none of these factors caused an increase in luciferase activity (Supplementary Figure 4). We thus fractionated CM(+) by ultrafiltration, and separated by molecular weight. Interestingly, a CM(+) fraction of <3 kDa significantly increased the luciferase activity to a similar level as that induced by whole CM(+), whereas the other CM(+) fractions did not (Figure 4d). Moreover, the luciferase activity was still increased when CM(+)

was prepared under co-treatment of RAW264 cells with lipopolysaccharide and a COX-2 inhibitor, celecoxib. We confirmed the decreased level of miR-7 by real-time RT–PCR in CM(+)- or CM(+) fraction <3 kDa-treated AZ521 cells (Figure 4e). These results indicate that small molecule(s) (<3 kDa) derived from activated macrophages are responsible for miR-7 repression in gastric cancer cells, and that such small molecule(s) are expressed in activated macrophages in a COX-2/PGE_2-independent manner.

Downregulation of miR-7 in the stomach by inflammatory responses

We next examined whether inflammatory responses are responsible for miR-7 downregulation in the stomach using different mouse models. The stomachs of wild-type mice were infected with *Helicobacter felis*, and submucosal inflammatory infiltration and mucosal macrophage accumulation were confirmed at 20 weeks after the infection (Figures 5a and b). Notably, the miR-7 expression level was significantly decreased in the *H. felis*-infected inflamed gastric mucosa (Figure 5c).

We recently showed that inflammatory responses and macrophage infiltration were suppressed in *K19-C2mE* mouse gastritis and *Gan* mouse tumors when mice were maintained under germfree conditions (Figure 5d and Oshima *et al.*, 2011). Notably, miR-7 expression levels were increased significantly in germfree *K19-C2mE* and *Gan* mice compared with the levels of mice maintained in a specific pathogen free (SPF) facility (Figure 5e). These *in vivo* experiments suggest that inflammatory responses are responsible for the miR-7 downregulation in the stomach, although further genetic studies are required to examine the role of macrophages in miR-7 downregulation.

Inflammation-dependent upregulation of miR-7 target genes in gastric tumors

The epidermal growth factor receptor (*EGFR*) mRNA is one of the miR-7 target genes (Kefas *et al.*, 2008;

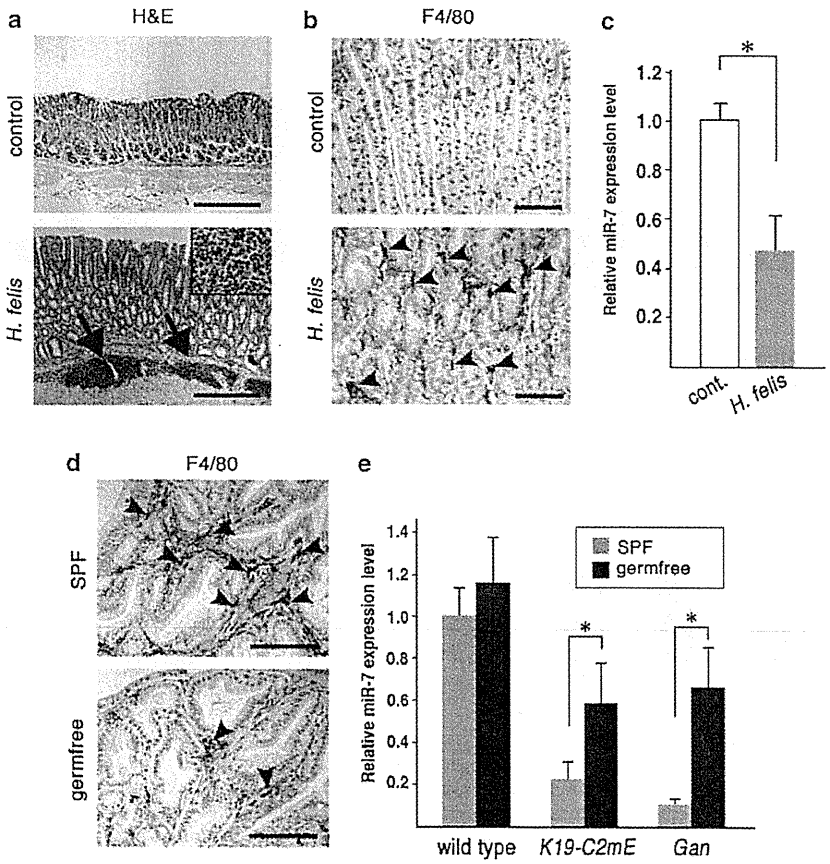

Figure 5 Inflammation-induced miR-7 repression in the mouse stomachs. (**a**) Histology of the wild-type mouse normal glandular stomach (top) and *H. felis*-infected inflamed glandular stomach (bottom). Arrows and inset indicate submucosal inflammatory cell infiltration. Scale bars indicate 0.5 mm. (**b**) Immunostaining of F4/80 in the normal glandular stomach (top) and *H. felis*-infected glandular stomach (bottom). Arrowheads indicate macrophages. Scale bars indicate 100 μm. (**c**) The miR-7 expression level of *H. felis*-infected gastric mucosa (gray bar) relative to that of the control stomach (open bar) is shown (mean ± s.d.). $*P<0.05$. (**d**) Immunostaining of F4/80 in a SPF control *Gan* mouse tumor (top) and a germfree *Gan* mouse tumor (bottom). Arrowheads indicate macrophages. Scale bars indicate 100 μm. (**e**) The expression levels of miR-7 in SPF (gray bars) and germfree (closed bars) K19-*C2mE* mouse gastritis and *Gan* mouse gastric tumors relative to the SPF wild-type stomach levels are shown (mean ± s.d.). $*P<0.05$. The expression levels of miR-7 were normalized to the Sno202 level.

Webster *et al.*, 2009). We thus examined *EGFR* expression levels in pre-miR-7-transfected Kato-III and AZ521 gastric cancer cells. As expected, the *EGFR* expression level was decreased significantly by pre-miR-7 transfection in both cell lines (Figure 6a), suggesting that suppression of *EGFR* expression is one of the tumor-suppressor mechanisms of miR-7 against gastric cancer development.

To identify novel miR-7 target genes that are upregulated in the inflammatory microenvironment,

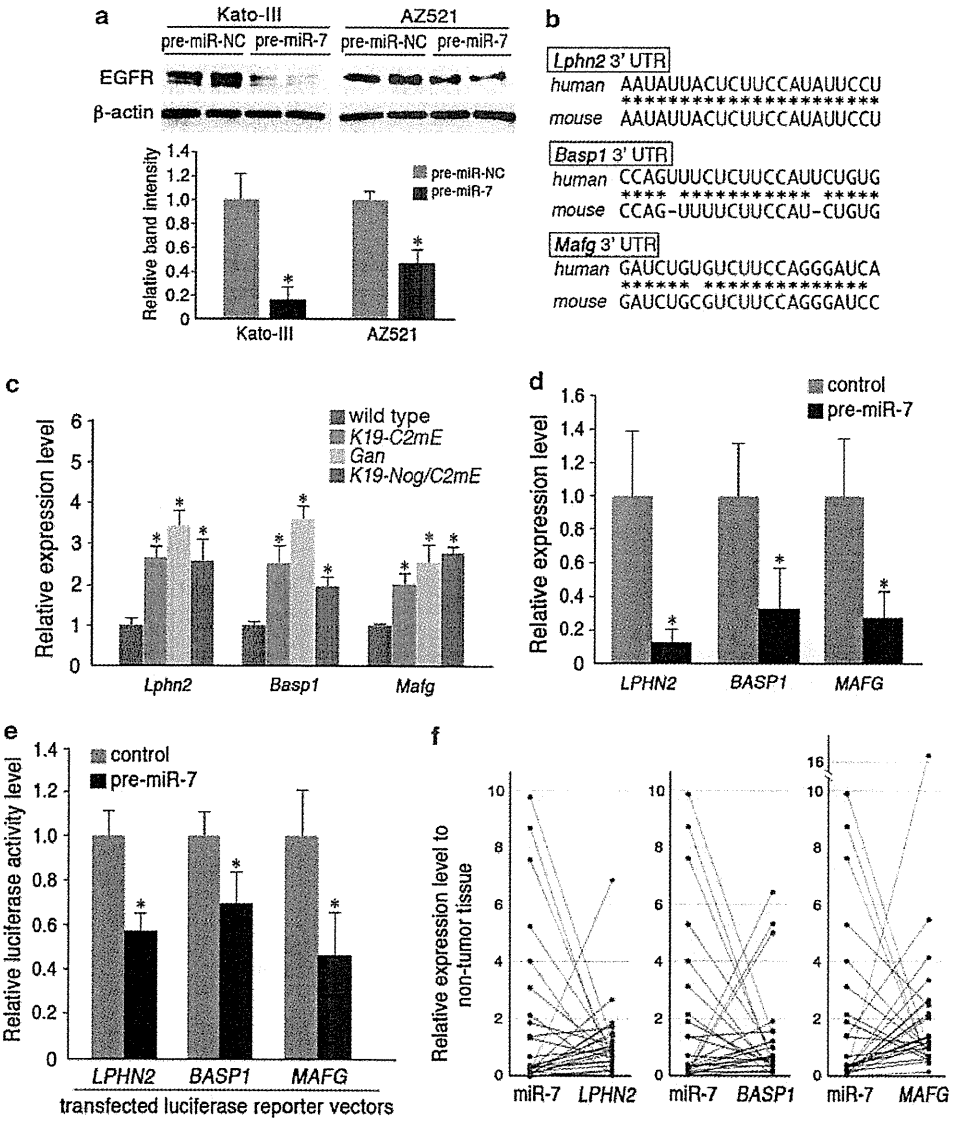

Figure 6 Inflammation-dependent upregulation of miR-7 target genes. (**a**) Representative results of the western blotting analysis of EGFR in Kato-III cells (top left) and AZ521 cells (top right) transfected with pre-miR-NC and pre-miR-7. The results for two independently prepared samples are shown. β-Actin was used as an internal control. The relative band intensities of the western blotting results are shown in the bar graph (mean ± s.d.) (bottom). $*P<0.05$ versus the control level. (**b**) Alignment of the miR-7 target sequences in the 3′-UTR of *Lphn2*, *Basp1* and *Mafg* in human and mouse mRNAs. The seed match sequences for miR-7 are indicated in red. (**c**) The expression levels of the indicated genes in *K19-C2mE* mouse gastritis (green bars), *Gan* mouse gastric tumors (yellow bars) and *K19-Nog/C2mE* mouse gastric hamartomas (blue bars) relative to the wild-type levels (gray bars) are shown (mean ± s.d.). $*P<0.05$ versus the wild-type level. The results were extracted from the microarray data set (Gene Expression Omnibus (GEO), accession GSE16902). (**d**) The expression levels of the indicated genes examined by real-time RT–PCR in pre-miR-7-transfected Kato-III cells (closed bar) relative to those of the control cells (gray bar) are shown (mean ± s.d.). $*P<0.05$ versus control level. (**e**) The relative luciferase activity levels of pre-miR-7-transfected reporter cells (closed bars) to the levels of control pre-miR-NC-transfected reporter cells (gray bars) are shown. The target genes for the respective luciferase reporter vectors are indicated. $*P<0.05$ versus the control level. (**f**) Comparison of the relative expression levels of miR-7 and *LPHN2* (left), *BASP1* (center) or *MAFG* (right) in human gastric cancers with non-tumor stomach levels is shown. Red and blue lines indicate that miR-7 was increased (>1.0) and decreased (<1.0) in gastric cancers, respectively. The expression levels of miR-7 were normalized to the U44 level.

we searched for putative miR-7 target genes from the upregulated gene set in both *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors using the results of the microarray analyses (Itadani *et al.*, 2009). We found that the *Lphn2*, *Basp1* and *Mafg* genes have conserved miR-7 target sequences in their 3′ untranslated region in both mouse and human mRNAs (Figure 6b). Notably, the expression of these genes was significantly increased not only in *K19-C2mE* mouse gastritis and *Gan* mouse tumors but also in *K19-Nog/C2mE* mouse gastric hamartomas (Figure 6c). The gastric mucosa and tumors in these strains are inflamed as a result of induction of the COX-2/PGE_2 pathway (Oshima *et al.*, 2004, 2006, 2009), suggesting that downregulation of miR-7 in inflammatory lesions is involved in upregulation of these genes. Notably, transfection of pre-miR-7 into Kato-III cells resulted in a significant decrease of *LPHN2*, *BASP1* and *MAFG* expressions (Figure 6d). Moreover, we constructed luciferase reporter plasmids that contained the 3′ untranslated region fragment of the putative miR-7 target genes, and transfected these vectors into Kato-III cells. Consistent with the real-time RT–PCR results, luciferase activities of reporter vector-transfected cells decreased significantly when cells were co-transfected with pre-miR-7 (Figure 6e). Taken together, these results indicate that these three genes are miR-7 targets.

Finally, we examined the expressions of *LPHN2*, *BASP1* and *MAFG* in human gastric cancers by real-time RT–PCR and compared their expressions with miR-7 expression levels. We found that expression levels of miR-7 and *LPHN2*, *BASP1* and *MAFG* were inversely correlated (Figure 6f). Accordingly, it is possible that inflammation causes induction of these genes in human gastric cancers through downregulation of miR-7, which may contribute to gastric tumorigenesis, although this will need to be investigated in future studies.

Discussion

It has been shown that miR-155 and miR-21, which function as oncogenes, are induced by inflammatory pathways, providing a link between inflammation and cancer (O'Connell *et al.*, 2007; Tili *et al.*, 2007; Iliopoulos *et al.*, 2010). Consistently, we found inflammation-dependent induction of miR-155 and miR-21 in mouse gastric tumors. On the other hand, miR-145 and miR-7, which function as tumor suppressors, are downregulated in both mouse gastritis and gastric tumor tissues. Moreover, we found that miR-7 levels are inversely correlated with the levels of proinflammatory cytokines, suggesting that the severity of inflammatory response is related to miR-7 downregulation. Therefore, it is possible that inflammation can promote tumorigenesis both by the upregulation of oncogenic miRNAs and by the downregulation of tumor-suppressor miRNAs, possibly through different mechanisms. Such alterations of cancer-related miRNA expression likely link inflammation and cancer.

It has been reported that miR-7 has a tumor-suppressor role in various cancers including brain tumors, breast cancer and lung cancer (Kefas *et al.*, 2008; Reddy *et al.*, 2008; Webster *et al.*, 2009), and we herein showed that miR-7 also functions as a tumor suppressor in gastric cancer. Interestingly, in the normal stomach, miR-7 expression is induced during the differentiation of gastric epithelial cells, suggesting a role of miR-7 in the regulation of epithelial cell differentiation. It has also been reported that miR-7 expression is induced during differentiation of intestinal epithelial cells (Nguyen *et al.*, 2010) and cortical neurons (Chen *et al.*, 2010). These results collectively suggest that miR-7 has a role in regulating cell differentiation in various organs. Therefore, it is conceivable that suppression of miR-7 expression is required for maintenance of the undifferentiated status of stem or progenitor cells in these organs.

These results suggest the possibility that inflammation suppresses epithelial cell differentiation through repression of miR-7. It is not surprising that the inflammatory response suppresses cell differentiation and enhances proliferation when regeneration is required in injured tissues. It has also been shown that a disruption of Toll-like receptor signaling causes an impairment of tissue repair by intestinal epithelial cells (Rakoff-Nahoum *et al.*, 2004). Moreover, activated macrophages are important niche components for intestinal epithelial progenitors in regenerative responses (Pull *et al.*, 2005). Therefore, it is conceivable that downregulation of miR-7 leads to suppression of differentiation, inducing the proliferation of undifferentiated epithelial cells in the inflammatory microenvironment.

Although several target genes of miR-7 have been identified, it is still unclear how miR-7 regulates differentiation. *EGFR* is one of the important miR-7 target genes, and is at least partially responsible for its tumor-suppressor role (Kefas *et al.*, 2008). Moreover, p21-activated kinase 1, Raf1, and the insulin-like growth factor 1 receptor have also been identified as miR-7 target genes that are upregulated in cancer cells (Reddy *et al.*, 2008; Webster *et al.*, 2009; Jiang *et al.*, 2010). Although most of these gene products contribute to cancer cell proliferation, we believe that miR-7 inhibits expression of other factors that have a role in the maintenance of the undifferentiated status of epithelial cells. In this study, we identified three novel miR-7 target genes that are upregulated in gastric cancers in an inflammation-dependent manner. LPHN2 is a G protein-coupled receptor that binds α-latrotoxin (Ichtchenko *et al.*, 1999), whereas BASP1 is implicated in neurite outgrowth (Korshunova *et al.*, 2008). MAFG is one of the small Maf proteins that is important for antioxidant responses (Katsuoka *et al.*, 2005). Although it remains to be investigated, it is of interest to examine whether these molecules have any role in the differentiation or tumorigenesis of gastric epithelial cells.

We examined the mechanisms responsible for miR-7 downregulation in gastric tumor cells. *Helicobacter pylori* infection induces chronic gastritis, resulting in induction of DNA methylation (Niwa *et al.*, 2010). The