Published OnlineFirst February 2, 2010; DOI:10.1158/0008-5472.CAN-09-2755

after infection) and in gastric cancer cell lines. All the three genes showed low expression levels in the GECs of noninfected and infected gerbils (Supplementary Fig. S8). *Rnf152* expression was significantly decreased in *HP*-infected gerbils compared with noninfected gerbils (44% and 25% at 10 and 50 weeks, respectively, after infection; $P < 0.001$). None of the three genes were expressed in cancer cell lines with complete methylation of these CGIs (Fig. 1B; Supplementary Fig. S8, top).

The absence of DNA methyltransferase upregulation. DNA methyltransferases (Dnmt) are final effectors of maintenance and induction of DNA methylation, and their overexpression is frequently observed in various types of human cancers (33). To analyze possible upregulation of Dnmts by *HP* infection, expression levels of *Dnmt1, Dnmt3a*, and *Dnmt3b* mRNAs were quantified in GECs of gerbils with and without *HP* infection. Contrary to our initial expectation, the expression levels of the three Dnmts were significantly lower in GECs with *HP* infection (1/2 to 1/3) than those without (Supplementary Fig. S8, bottom).

Discussion

Our study using a gerbil model showed that *HP* infection is causally involved in induction of aberrant DNA methylation in GECs. Thus far, a strong association has been shown between the presence of *HP* infection and high methylation levels or high incidence of methylation in human gastric mucosae (5, 10–12). Taking advantage of an animal model, we were able to conduct an experiment by infecting gerbils with *HP* and showed that *HP* infection was the cause of methylation induction.

The critical role of inflammation in methylation induction was shown. Temporal analysis showed that methylation levels were closely associated with infiltration of inflammatory cells, and suppression of inflammation by CsA markedly repressed methylation induction even in the presence of *HP*. These results indicated that *HP* itself was not necessary for methylation induction once inflammation was induced by it. This finding is important because a direct role of *HP* is suggested by the facts that the SHP2 oncoprotein is deregulated by injection of virulent factors such as CagA into GECs (34) and *HP* possesses multiple DNA (cytosine-5) methyltransferases (35).

Among the inflammation-related genes analyzed, the expression levels of *Cxcl2, Il1b, Nos2*, and *Tnf* were upregulated in the stomach with *HP* infection and decreased after eradication, almost paralleling those of methylation levels. In the CsA treatment, in which methylation induction was markedly suppressed, upregulation of *Cxcl2, Il1b*, and *Nos2* by *HP* infection was significantly suppressed and that of *Tnf* also had a tendency to be suppressed. These results suggest that some specific inflammation-related genes are cooperatively involved in methylation induction by *HP* infection. In human ulcerative colitis and hepatitis (cirrhosis), where aberrant methylation is believed to be induced, increased expression of *IL8* (human functional homolog of *Cxcl2*), *IL1B, NOS2*, and *TNF* was also observed (36–39), suggesting that upregulation of these genes is a common feature of methylation-associated inflammation. Especially for human *IL1B*, its allele with a specific single nucleotide polymorphism is known to be associated with increased gastric cancer risk and increased incidence of *CDH1* promoter methylation in gastric cancers (40, 41). Also, increased production of nitric oxide, due to upregulation of a nitric oxide synthase (*NOS2*) by IL1B or administration of nitric oxide donors, induced methylation of *FMR1* and *HPRT* genes *in vitro* (42).

This study also clearly shows that methylation in gastric mucosae with *HP* infection consists of temporary and permanent components, which has been suggested by studies in humans (5, 10). Methylation that disappeared after eradication corresponds to the temporary component, and methylation that did not disappear corresponds to the permanent component. A pyloric gland (mucosal epithelia) is known to be composed of one or a few stem cells, multiple progenitor cells, and a large number of differentiated cells, and it is renewed within 3 to 14 days (43, 44). Temporary methylation is likely to have been induced in progenitor or differentiated cells, which will finally drop off from the gastric epithelium. Permanent methylation is likely to be induced in stem cells, which will remain for life. In humans, methylation levels in gastric mucosae without *HP* infection correlate with gastric cancer risk (5, 10), and this fact is also in line with the hypothesis that permanent methylation in gastric mucosae without *HP* infection reflects methylation in stem cells.

HG2, SC3, and SD2 were methylated in GECs, although they were located in promoter CGIs, which are generally resistant to DNA methylation (29). Among promoter CGIs, those of genes with low transcription are known to be susceptible to methylation (30, 31, 45), and as expected, all the three genes had low transcription levels in GECs. Transcription levels at 10^{-4} to 10^{-3}/*Gapdh* (*GAPDH*) correspond to 1 to 10 copies of mRNA per cell and are less than 35% of the average expression level of all the genes analyzed by expression microarray (46). Because their methylation levels in GECs of gerbils infected with *HP* for 10 and 50 weeks were less than a few percent, their methylation was unlikely to have affected the overall expression levels in gastric mucosae. As a response to *HP* infection, *Rnf152* was downregulated whereas *Gpr37* and *Nptx2* were not.

Promoter CGIs of *GPR37* and *NPTX2* were highly methylated in human gastric mucosae with *HP* infection and were frequently methylated in human gastric cancers. Because their tumor-suppressive functions have not been reported and they are not expressed in normal gastric mucosae (RefExA database[4]), their silencing is unlikely to be causally involved in gastric carcinogenesis, and they are considered to be passengers. Likewise, methylated CGIs that were not associated with genes were likely to be passengers. However, it is now known that a lot of passengers and limited number of drivers are methylated to high and small degrees, respectively, in human gastric mucosae with *HP* infection (5, 45). Therefore, although most methylation identified here was

[4] http://157.82.78.238/refexa/main_search.jsp

Published OnlineFirst February 2, 2010; DOI:10.1158/0008-5472.CAN-09-2755

considered to be passenger, it is likely that tumor-suppressor genes are also methylated in association with their methylation. Gastric mucosa with accumulation of silencing of various genes, including both drivers and passengers, is considered to form a field where cancers will develop (epigenetic field for cancerization; refs. 7, 10, 47).

As a final effector of methylation induction, we examined overexpression of *Dnmts*, which are implicated in methylation induction in various human cancers (33). Unexpectedly, all the three *Dnmts* were downregulated by *HP* infection. Our recent data in humans also showed that mRNA levels of Dnmts had decreasing tendencies in *HP*-infected gastric mucosae (45). These results indicate that overexpression of *Dnmts* is not involved in *HP*-induced methylation induction, and suggest that local distribution of Dnmts and/or protective factors, such as the presence of RNA polymerase II (48), might be disturbed by inflammation.

Genome-wide screening to isolate DNA fragments methylated by *HP* infection was done by MS-RDA, which is applicable to any species without genome information. We used cell lines as the driver so that we could avoid heterogeneity of primary samples and aberrant methylation will be present in all the DNA molecules in the driver. This was considered to be essential for a genome-wide screening because most methods cannot detect small differences. Although cell lines might have artificial methylation, we confirmed the presence of specific methylation in GECs, and a high-sensitivity method, qMSP, was used for this. As expected, methylation levels of CGIs identified here were small (i.e., a few percent) in GECs with *HP* infection, showing that the strategy was correct.

In summary, *HP* infection was causally involved in induction of aberrant DNA methylation, and a critical role of inflammation in the induction was indicated. This model is expected to be useful in analyzing detailed molecular mechanisms for induction of aberrant DNA methylation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to Dr. Takashi Sugimura for his critical discussion and sustained encouragement.

Grant Support

Grants-in-Aid for Cancer Research and for the Third-Term Comprehensive Cancer Control from the Ministry of Health, Labour, and Welfare, Japan.

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Received 7/24/09; revised 11/10/09; accepted 11/27/09; published OnlineFirst 2/2/10.

References

1. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.
2. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. Mol Cancer Res 2006;4:221–33.
3. Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatology 2000;32:970–9.
4. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res 2001;61:3573–7.
5. Maekita T, Nakazawa K, Mihara M, et al. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 2006;12:989–95.
6. Toyota M, Itoh F, Kikuchi T, et al. DNA methylation changes in gastrointestinal disease. J Gastroenterol 2002;37 Suppl 14:97–101.
7. Ushijima T. Epigenetic field for cancerization. J Biochem Mol Biol 2007;40:142–50.
8. Moss SF, Blaser MJ. Mechanisms of disease: inflammation and the origins of cancer. Nat Clin Pract Oncol 2005;2:90–7.
9. Uemura N, Okamoto S, Yamamoto S, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
10. Nakajima T, Maekita T, Oda I, et al. Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. Cancer Epidemiol Biomarkers Prev 2006;15:2317–21.
11. Perri F, Cotugno R, Piepoli A, et al. Aberrant DNA methylation in non-neoplastic gastric mucosa of *H. pylori* infected patients and effect of eradication. Am J Gastroenterol 2007;102:1361–71.
12. Kaise M, Yamasaki T, Yonezawa J, Miwa J, Ohta Y, Tajiri H. CpG island hypermethylation of tumor-suppressor genes in *H. pylori*-infected non-neoplastic gastric mucosa is linked with gastric cancer risk. Helicobacter 2008;13:35–41.
13. Chan AO, Peng JZ, Lam SK, et al. Eradication of *Helicobacter pylori* infection reverses E-cadherin promoter hypermethylation. Gut 2006;55:463–8.
14. Leung WK, Man EP, Yu J, et al. Effects of *Helicobacter pylori* eradication on methylation status of E-cadherin gene in noncancerous stomach. Clin Cancer Res 2006;12:3216–21.
15. Tatematsu M, Tsukamoto T, Mizoshita T. Role of *Helicobacter pylori* in gastric carcinogenesis: the origin of gastric cancers and heterotopic proliferative glands in Mongolian gerbils. Helicobacter 2005;10:97–106.
16. Nozaki K, Shimizu N, Ikehara Y, et al. Effect of early eradication on *Helicobacter pylori*-related gastric carcinogenesis in Mongolian gerbils. Cancer Sci 2003;94:235–9.
17. Fukase K, Kato M, Kikuchi S, et al. Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. Lancet 2008;372:392–7.
18. Wong BC, Lam SK, Wong WM, et al. *Helicobacter pylori* eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. JAMA 2004;291:187–94.
19. Nozaki K, Tanaka H, Ikehara Y, et al. *Helicobacter pylori*-dependent NF-κB activation in newly established Mongolian gerbil gastric cancer cell lines. Cancer Sci 2005;96:170–5.
20. Shimizu N, Ikehara Y, Inada K, et al. Eradication diminishes enhancing effects of *Helicobacter pylori* infection on glandular stomach carcinogenesis in Mongolian gerbils. Cancer Res 2000;60:1512–4.
21. Cheng H, Bjerknes M, Amar J. Methods for the determination of epithelial cell kinetic parameters of human colonic epithelium isolated from surgical and biopsy specimens. Gastroenterology 1984;86:78–85.

Published OnlineFirst February 2, 2010; DOI:10.1158/0008-5472.CAN-09-2755

22. Yamashita S, Takahashi S, McDonell N, et al. Methylation silencing of transforming growth factor-β receptor type II in rat prostate cancers. Cancer Res 2008;68:2112–21.
23. Ushijima T, Morimura K, Hosoya Y, et al. Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. Proc Natl Acad Sci U S A 1997;94:2284–9.
24. Kaneda A, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T. Identification of silencing of nine genes in human gastric cancers. Cancer Res 2002;62:6645–50.
25. Niwa T, Yamashita S, Tsukamoto T, et al. Whole-genome analyses of loss of heterozygosity and methylation analysis of four tumor-suppressor genes in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced rat stomach carcinomas. Cancer Sci 2005;96:409–13.
26. Kass DH, Kim J, Rao A, Deininger PL. Evolution of B2 repeats: the muroid explosion. Genetica 1997;99:1–13.
27. Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005; 33:6823–36.
28. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 1992;357:695–7.
29. Ushijima T, Watanabe N, Okochi E, Kaneda A, Sugimura T, Miyamoto K. Fidelity of the methylation pattern and its variation in the genome. Genome Res 2003;13:868–74.
30. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 2004;24:4781–90.
31. Song JZ, Stirzaker C, Harrison J, Melki JR, Clark SJ. Hypermethylation trigger of the glutathione-*S*-transferase gene (GSTP1) in prostate cancer cells. Oncogene 2002;21:1048–61.
32. Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: where do they come from? Cancer Sci 2005;96:206–11.
33. Kanai Y, Hirohashi S. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. Carcinogenesis 2007;28:2434–42.
34. Hatakeyama M. Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. Nat Rev Cancer 2004;4:688–94.
35. Vitkute J, Stankevicius K, Tamulaitiene G, et al. Specificities of eleven different DNA methyltransferases of *Helicobacter pylori* strain 26695. J Bacteriol 2001;183:443–50.
36. Cappello M, Keshav S, Prince C, Jewell DP, Gordon S. Detection of mRNAs for macrophage products in inflammatory bowel disease by *in situ* hybridisation. Gut 1992;33:1214–9.
37. Llorente L, Richaud-Patin Y, Alcocer-Castillejos N, et al. Cytokine gene expression in cirrhotic and non-cirrhotic human liver. J Hepatol 1996;24:555–63.
38. McLaughlan JM, Seth R, Vautier G, et al. Interleukin-8 and inducible nitric oxide synthase mRNA levels in inflammatory bowel disease at first presentation. J Pathol 1997;181:87–92.
39. Mihm S, Fayyazi A, Ramadori G. Hepatic expression of inducible nitric oxide synthase transcripts in chronic hepatitis C virus infection: relation to hepatic viral load and liver injury. Hepatology 1997;26: 451–8.
40. El-Omar EM, Carrington M, Chow WH, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398–402.
41. Chan AO, Chu KM, Huang C, et al. Association between *Helicobacter pylori* infection and interleukin 1β polymorphism predispose to CpG island methylation in gastric cancer. Gut 2007;56:595–7.
42. Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. Methylation-dependent gene silencing induced by interleukin 1β via nitric oxide production. J Exp Med 1999;190:1595–604.
43. Hattori T, Fujita S. Tritiated thymidine autoradiographic study of cell migration and renewal in the pyloric mucosa of golden hamsters. Cell Tissue Res 1976;175:49–57.
44. Lee ER. Dynamic histology of the antral epithelium in the mouse stomach: III. Ultrastructure and renewal of pit cells. Am J Anat 1985;172:225–40.
45. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T. The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae. Int J Cancer 2009;124:905–10.
46. Moriguchi K, Yamashita S, Tsujino Y, Tatematsu M, Ushijima T. Larger numbers of silenced genes in cancer cell lines with increased *de novo* methylation of scattered CpG sites. Cancer Lett 2007;249:178–87.
47. Nakajima T, Oda I, Gotoda T, et al. Metachronous gastric cancers after endoscopic resection: how effective is annual endoscopic surveillance? Gastric Cancer 2006;9:93–8.
48. Takeshima H, Yamashita S, Shimazu T, Niwa T, Ushijima T. The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. Genome Res 2009;19:1974–82.

Carcinogenesis vol.31 no.9 pp.1627–1633, 2010
doi:10.1093/carcin/bgq129
Advance Access publication July 9, 2010

Suppressive effect of global DNA hypomethylation on gastric carcinogenesis

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Global DNA hypomethylation and concomitant site-specific gene hypermethylation are among the most common molecular alterations in human neoplasia. Although site-specific DNA hypermethylation has been shown to be associated with the development of various tumors accompanied by transcriptional silencing of target genes, the functional significance of global DNA hypomethylation in tumorigenesis remains unclear. Previous studies have revealed that a genetic reduction of the DNA methylation levels leads to opposing effects on tumor development, depending on the tumor cell type and the stage of tumorigenesis. In the present study, we investigated the effect of DNA hypomethylation on gastric carcinogenesis in mice. The genetic reduction of DNA methylation levels suppressed the incidence, number and size of gastric tumors in two different mouse models for gastric tumorigenesis: the *N*-methyl-*N*-nitrosourea-induced model and the $Apc^{Min/+}$ mouse model that spontaneously develops gastric tumors with aging. Histological analyses revealed DNA hypomethylation to completely inhibit the development of invasive gastric tumors. These findings indicate that the reduction of DNA methylation levels suppresses gastric carcinogenesis and suggest that DNA methylation is closely associated with gastric tumorigenesis.

Introduction

Changes in the DNA methylation status, including global DNA hypomethylation and site-specific gene hypermethylation, are concomitantly found in tumors and are the most common molecular alterations in human neoplasia (1). Site-specific DNA hypermethylation has been extensively analyzed and a number of genes have been shown to be hypermethylated and transcriptionally silenced in various tumors. However, the functional significance of global DNA hypomethylation remains unclear, although this alteration was discovered in a wide variety of human cancers >20 years ago. Global DNA hypomethylation, which is frequently observed at the early stages of tumorigenesis in human cancer (2,3), promotes chromosomal instability *in vitro* and accelerates tumor development in several mouse models (4–6). Although the consequences of global hypomethylation and site-specific hypermethylation have been mechanistically connected to chromosome instability and transcriptional silencing, respectively, the cause of aberrant DNA methylation patterns remains unclear.

Abbreviations: *Cdh1*, *Cadherin 1*; *Cdkn2a*, *Cyclin-dependent kinase inhibitor 2A*; *LINE*, long interspersed nuclear element; *Magea2*, *Melanoma antigen family A, 2*; MNU, *N*-methyl-*N*-nitrosourea; *Sfrp*, *Secreted frizzled-related protein*.

[†]These authors equally contributed to this work.

In human gastric cancers, a variety of aberrant methylations, including global hypomethylation and site-specific DNA hypermethylation, have been identified and the importance of epigenetic alterations in gastric carcinogenesis would be emphasized by the infrequent genetic alterations in gastric cancers (7). Site-specific DNA hypermethylations have been preferentially investigated and it has been revealed that a number of genes are inactivated by promoter hypermethylation in human gastric cancer, including tumor suppressor genes (7–12). On the other hand, global DNA hypomethylation in human gastric cancer has been revealed by quantifications of 5-methylcytosine content (8) and evaluation of methylation status of *long interspersed nuclear elements* (*LINEs*)-*1* repetitive sequences (8,13), but little is known about the role in gastric tumorigenesis.

DNA methylation is catalyzed by a family of three DNA methyltransferases: Dnmt1, Dnmt3a and Dnmt3b (14–16). Although the three Dnmts partially cooperate to establish and maintain genomic methylation patterns, they also have distinctive functions. *Dnmt1* has a preference for hemimethylated DNA and deposits methyl groups on newly synthesized DNA, which results in the faithful replication of methylation patterns. Indeed, a hypomorphic allele of Dnmt1 has been shown to cause global DNA hypomethylation (6). Dnmt1 is therefore considered to be the major maintenance methyltransferase. Using *Dnmt1* hypomorphic alleles as a model for global DNA hypomethylation, previous studies have revealed that global DNA hypomethylation inhibits tumorigenesis in the intestine (17,18), esophagus and tongue (19), whereas it accelerates developments in T-cell lymphomas (6), liver cancers (18) and fibrosarcomas (5), possibly by promoting chromosomal instability. Our previous study also revealed that genetic reduction of DNA methylation eventually suppresses intestinal tumorigenesis but promotes the development of early-stage lesions in the colon of $Apc^{Min/+}$ mice (18). These results indicate that the forced reduction of genomic methylation levels leads to opposing effects on tumorigenesis depending on the cell type and the stage of tumorigenesis. Considering the fact that DNA hypomethylating agents have been used for cancer therapy in a subset of cancer (1), it is important to clarify the effect of global DNA hypomethylation on the risk for tumor development in various organs.

In the present study, we investigated the effect of reduced DNA methylation levels on gastric carcinogenesis in *N*-methyl-*N*-nitrosourea (MNU)-induced mice and $Apc^{Min/+}$ mice (20–22). We herein report that global DNA hypomethylation significantly suppresses the development of gastric tumors.

Materials and methods

Mice

Two mutant alleles of *Dnmt1* were used: the null $Dnmt1^{c}$ allele in the C57BL/6 background (23) and the hypomorphic $Dnmt1^{chip}$ allele in the 129Sv4 background (6). $Dnmt1^{c/+}$ mice (C57BL/6) were crossed with female $Dnmt1^{chip/chip}$ mice (129Sv4) to generate experimental mice in an isogenic F1 hybrid (C57:129) background. A previous study reported that $Dnmt1^{chip/+}$ mice have the same levels of genomic methylation as $Dnmt1^{+/+}$ mice, whereas $Dnmt1^{chip/c}$ mice have reduced DNA methylation contents at pericentromeric satellite repeats (18). We therefore analyzed $Dnmt1^{chip/+}$ mice as a control cohort and $Dnmt1^{chip/c}$ mice as a DNA hypomethylated cohort in this study. All mice were maintained under specific-pathogen-free conditions with isolated ventilation cages in an air-conditioned room with a 12 h light–dark cycle. They were bred and maintained on a basal diet, CE-2 (CLEA Japan, Tokyo, Japan), until the termination of the study.

Methylation analysis of gastric mucosa

The DNA methylation levels in gastric mucosae were examined by methylation-sensitive Southern blot analysis and bisulfite methylation analysis. Genomic DNA were extracted from the homogenized gastric mucosae of $Dnmt1^{chip/+}$ and $Dnmt1^{chip/c}$ mice. Firstly, DNA were digested with the methylation-sensitive enzyme HpaII (New England Biolabs, Ipswich, MA) and the digests were analyzed by Southern blotting using a centromeric

satellite repeat probe as previously reported (6,18,24). Next, the methylation pattern in 5′-noncoding region of *Line-1* was analyzed by bisulfite sequencing in accordance with the previous report (25). Bisulfite treatment of the genomic DNA was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). After polymerase chain reaction amplification using primers for 5′-noncoding region of *Line-1*, the products were cloned into the TOPO vector (Invitrogen, Carlsbad, CA). The inserted polymerase chain reaction fragments of the individual clones obtained from each sample were sequenced with primer for T7 promoter using the ABI Prism Dye Terminator Cycle Sequencing Kit and an ABI Prism 3100 DNA Sequencer. The primers for *Line-1* are shown in supplementary Table 1, available at *Carcinogenesis* Online.

MNU treatment

MNU (Sigma Chemical, St Louis, MO) was dissolved in distilled water at a concentration of 240 p.p.m. and freshly prepared thrice per week for administration in drinking water in light-shielded bottles *ad libitum*. *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ littermates (n = 24 and 18, respectively) from 4 to 6 weeks of age were given drinking water containing 240 p.p.m. MNU on alternate weeks for a total of 10 weeks (total exposure: 5 weeks), according to the protocol described in previous reports (Figure 1A) (20,22). They were thereafter maintained without any further treatment until they were killed at 52 weeks of age.

Apc$^{Min/+}$ *mice model*

Apc$^{Min/+}$ mice in the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). *Apc*$^{Min/+}$ mice were crossed with *Dnmt1*$^{c/+}$ mice (C57BL/6), and male *Apc*$^{Min/+}$; *Dnmt1*$^{c/+}$ mice (C57BL/6) were subsequently crossed with female *Dnmt1*$^{chip/chip}$ mice (129Sv4) to generate experimental mice in an isogenic F1 hybrid (C57:129) background. We analyzed 30 *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice and 21 *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice to quantify gastric lesions at 30–32 weeks of age (Figure 1B). In addition, 9 *Dnmt1*$^{chip/+}$; *Apc*$^{+/+}$ mice and 15 *Dnmt1*$^{chip/c}$; *Apc*$^{+/+}$ mice were analyzed as controls without the *Apc*^{Min} allele. All mice were maintained under the same conditions as those described above.

Preparation of tissue samples for tumor counting and histological analysis

All mice underwent a thorough postmortem examination at the time of killing. The stomach was removed and opened along the greater curvature. The number and the lengths of the major and minor axes of the gastric tumors were determined using a dissecting microscope at ×7 magnification. Tumors >0.5 mm in long axis length were mapped and counted. The sizes of the tumors were determined by multiplying the major axis by the minor axis. To eliminate interobserver error, all counts were performed by a single observer blinded to the genotype of the mice. In addition, all the cases were counted by a second observer to confirm the results of the first observer. After counting the tumors, all the excised stomachs, including the neoplastic nodules, were fixed for 24 h in neutral-buffered 10% formalin and were subsequently cut into eight strips. These strips were processed by standard methods, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The defining characteristics for adenoma and adenocarcinoma were adapted from both the consensus guidelines on murine models of intestinal cancer (26) and previous reports in the literature (20,27).

Immunohistochemistry

The avidin–biotin–peroxidase complex technique was used for immnohistochemical studies. Five micrometers thick sections were cut, deparaffinized, rehydrated in phosphate-buffered saline, placed in 10 mmol/l citrate buffer (pH 6.0) and heated in a 750 W microwave four times for 6 min. The endogenous peroxidase activity was blocked by incubation for 30 min in 0.3% H_2O_2. After washing three times with phosphate-buffered saline, the sections were then pre-incubated with normal blocking serum for 20 min at room temperature and then were incubated with Ki-67 (1:200; DAKO Corporation, Carpinteria, CA) and cleaved caspase-3 (1:400; Cell Signaling Technology, Danvers, MA) antibody overnight at 4°C. Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 30 min, followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min. The sections were developed with 3,3′-diaminobenzidine using DAKO liquid 3,3′-diaminobenzidine Substrate-Chromogen System (DAKO Corporation) and were then counterstained with hematoxylin. No specific staining was observed in the negative control slides prepared without primary antibody. The number of Ki-67-positive cells per gland was calculated as the Ki67-labeling index.

Methylation analysis of MNU-induced gastric tumors

In order to examine the involvement of aberrant site-specific methylations in the development of MNU-induced mouse gastric cancer, the methylation status of the promoter regions of *Cyclin-dependent kinase inhibitor 2A* (*Cdkn2a*), *Cadherin 1* (*Cdh1*), *Secreted frizzled-related protein* (*Sfrp*) *1*, *Sfrp2* and *Melanoma antigen family A, 2* (*Magea2*) were analyzed by bisulfite sequencing. Genomic DNA were extracted from the gastric tumors and the surrounding gastric mucosae of MNU-treated wild-type mouse and bisulfite analysis were performed as described above. The primers for bisulfite sequencing are shown in supplementary Table 1, available at *Carcinogenesis* Online.

Statistical analysis

The incidence of gastric tumors was analyzed using Fisher's exact test. Differences in the number and the size of gastric tumors and Ki-67-positive cell ratio in non-cancerous mucosa were assessed with Mann–Whitney *U*-test.

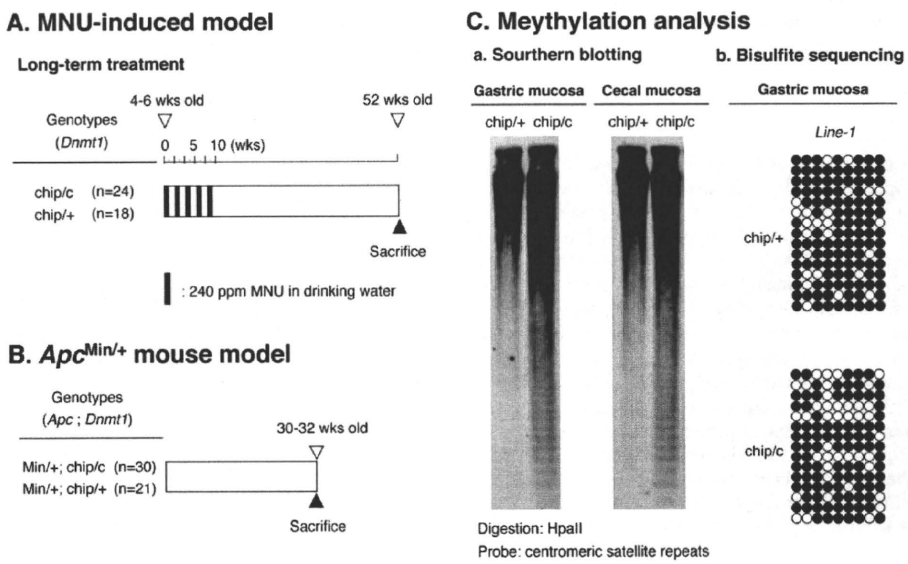

Fig. 1. Experimental design to study the effect of the DNA hypomethylation on gastric carcinogenesis and genomic DNA hypomethylation in *Dnmt1*$^{chip/c}$ mice. (**A**) MNU-induced model. (**B**) *Apc*$^{Min/+}$ mouse model. (**C**) Methylation analysis of gastric mucosa. (a) Methylation-sensitive Southern blot analysis of the pericentromeric regions. Decreased levels of DNA methylation are detectable in both gastric and cecal mucosa of *Dnmt1*$^{chip/c}$ mouse in comparison with those of the *Dnmt1*$^{chip/+}$ mouse. (b) Bisulfite sequencing of the 5′-noncoding region of *Line-1*. A reduction in CpG methylation was observed in the gastric mucosa of *Dnmt1*$^{chip/c}$ mouse as compared with that of the *Dnmt1*$^{chip/+}$ mouse. Filled and open circles represent methylated and unmethylated CpGs, respectively.

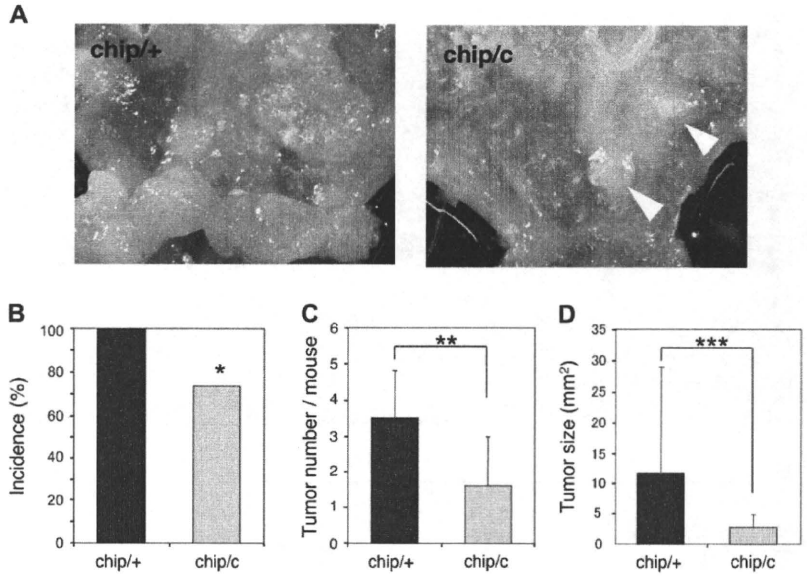

Fig. 2. DNA hypomethylation suppresses gastric tumorigenesis in MNU-treated mice. (**A**) Macroscopic photographs of the glandular stomach in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice at 52 weeks of age. (**B**) Incidence of gastric tumors in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice ($n = 24$ and $n = 18$). The incidence of MNU-treated *Dnmt1*$^{chip/c}$ mice with gastric tumors was significantly lower than that of MNU-treated *Dnmt1*$^{chip/+}$ mice. $^{*}P < 0.05$, by Fisher's exact test. (**C**) Number of gastric tumors per mouse in MNU-treated *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice. MNU-treated *Dnmt1*$^{chip/c}$ mice developed significantly fewer gastric tumors than MNU-treated *Dnmt1*$^{chip/+}$ mice. Columns, mean; bars, SD. $^{**}P = 0.0001$, by Mann–Whitney *U*-test. (**D**) Sizes of gastric tumors in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice. The tumor size of gastric tumors in the MNU-treated *Dnmt1*$^{chip/c}$ mice was significantly smaller than that of the MNU-treated *Dnmt1* $^{chip/+}$mice. Columns, mean; bars, SD. $^{***}P < 0.0001$, by Mann–Whitney *U*-test.

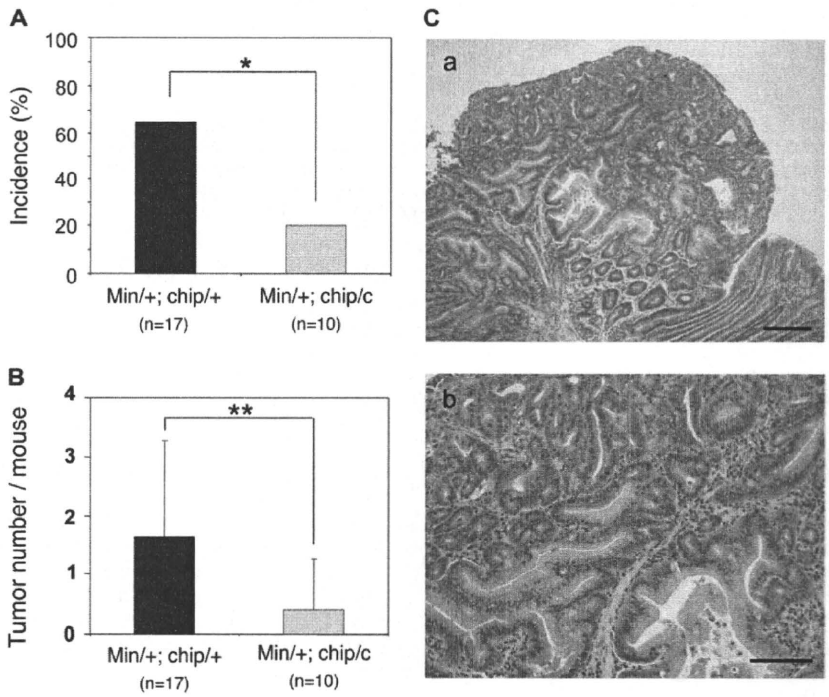

Fig. 3. DNA hypomethylation suppresses the development of gastric tumors in *Apc*$^{Min/+}$ mice model. (**A**) Incidence of gastric tumors in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice ($n = 17$ and $n = 10$). The incidence was significantly smaller in *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice than in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice. $^{*}P < 0.05$, by Fisher's exact test. (**B**) Number of gastric tumors per mouse in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice. *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice developed significantly fewer gastric tumors than *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice. Columns, mean; bars, SD. $^{**}P < 0.05$, by Mann–Whitney *U*-test. (**C**) Histopathology of the gastric tumors in *Apc*$^{Min/+}$ mice. (a) Adenoma in *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice; bar, 200 μm. (b) Higher magnification in (a); bar, 100 μm.

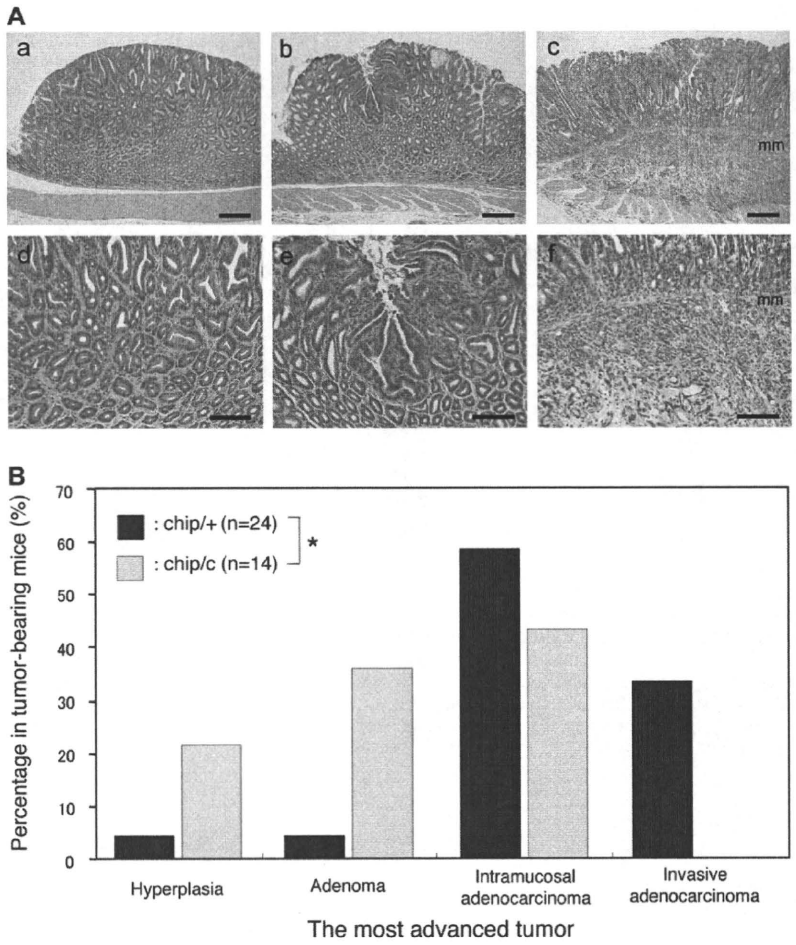

Fig. 4. DNA hypomethylation suppresses the progression of gastric carcinogenesis. (**A**) Histopathologic features of gastric tumors in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice at 52 weeks of age. (a) Adenoma in MNU-treated mice. (b) Intramucosal adenocarcinoma. (c) Invasive adenocarcinoma. (d–f) Higher magnification in (a–c). mm, muscularis mucosae; bars, 200 μm in (a–c) and 100 μm in (d–f). (**B**) Histologic grade of the most advanced tumor in tumor-bearing MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice (n = 24 and n = 14). The development of malignant gastric cancer was significantly decreased in *Dnmt1*$^{chip/c}$ mice as compared with *Dnmt1*$^{chip/+}$ mice. Columns, mean; bars, SD. *P = 0.0005, by Spearman's rank correlation test.

Spearman's rank correlation test was used to determine the relationship between *Dnmt1* genotype and tumor malignancy.

Results

Dnmt1 hypomorphic alleles induce global DNA hypomethylation in gastric mucosa

To determine whether the mutant *Dnmt1* alleles definitely affect DNA methylation in the gastric mucosa, the DNA methylation levels in the gastric mucosae were examined. The methylation-sensitive Southern blot analysis revealed that the gastric mucosa of the *Dnmt1*$^{chip/c}$ mouse was significantly hypomethylated at pericentromeric regions in comparison with that of the *Dnmt1*$^{chip/+}$ mouse (Figure 1C), consistent with both our previous findings and the present observations in the colonic mucosa (18). Additionally, bisulfite analysis showed the reduced CpG methylation of the *Line-1* regulatory region in the *Dnmt1*$^{chip/c}$ mouse gastric mucosa, whereas this region was highly methylated in that of the *Dnmt1*$^{chip/+}$ mouse (Figure 1C). When compared the methylation frequency at each CpG in the *Line-1* regulatory region, the values in the *Dnmt1*$^{chip/c}$ mouse were lower than those in the *Dnmt1*$^{chip/+}$ mouse at almost all CpGs except for the second CpG. These findings indicate that *Dnmt1* hypomorphic alleles lead to the global DNA hypomethylation in the mouse gastric mucosa.

DNA hypomethylation suppresses gastric tumorigenesis in MNU-treated mice

We administered MNU, an alkylating agent that induces the formation of adenomas and adenocarcinomas in murine glandular stomach tissue, to *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice, which express different levels of DNA methyltransferase *Dnmt1*. The MNU-treated *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice (n = 24 and 18, respectively) were examined for gastric lesions at 52 weeks of age. Macroscopically, most tumors developed in the pyloric antrum and showed a sessile and/or polypoid morphology in both *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice (Figure 2A). Both the incidence and the multiplicity of macroscopic gastric tumors in the MNU-treated *Dnmt1*$^{chip/c}$ mice were significantly decreased as compared with those in the MNU-treated *Dnmt1*$^{chip/+}$ mice (Figure 2B and C). The incidence of gastric tumors was 77.8% (14/18) in *Dnmt1*$^{chip/c}$ mice, whereas it was 100% (24/24) in *Dnmt1*$^{chip/+}$ mice ($P < 0.05$). The average numbers of the gastric tumors in *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice were 1.7 ± 1.47 and 3.8 ± 1.33 (±SD) per mouse, respectively (P = 0.0001). Furthermore, the tumor size in the MNU-treated *Dnmt1*$^{chip/c}$ mice was significantly smaller than that of the MNU-treated *Dnmt1* $^{chip/+}$mice ($P < 0.0001$, Figure 2D). Large tumors, namely >5 mm in the longest diameter, were only observed in MNU-treated *Dnmt1*$^{chip/+}$ mice (data not shown). These results clearly indicate that a genetic

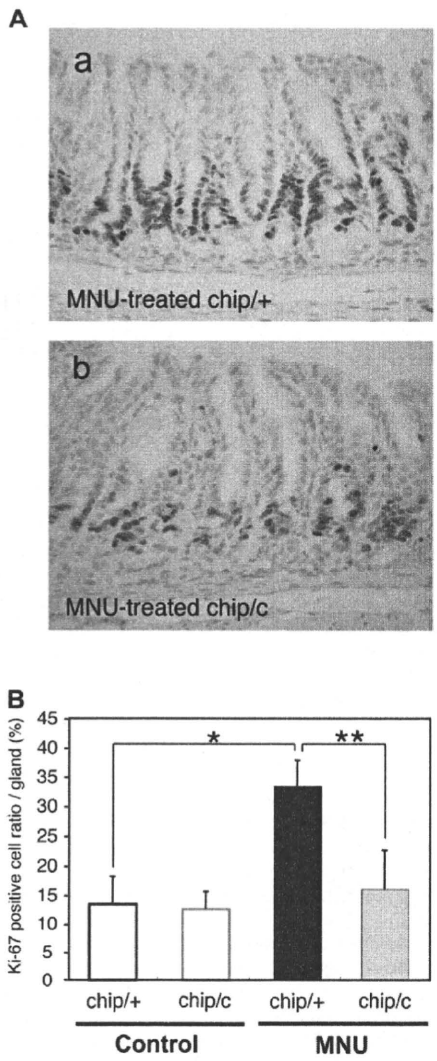

Fig. 5. DNA hypomethylation suppresses abnormal cell proliferation in the non-cancerous gastric mucosa induced by MNU treatment. (**A**) Ki-67 immunostaining of the non-cancerous gastric mucosa in MNU-treated *Dnmt1*$^{chip/+}$ (a) and *Dnmt1*$^{chip/c}$ (b) mice at 52 weeks of age. (**B**) Percentage of Ki-67-positive cells in non-cancerous gastric mucosa of non-treated and MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice ($n = 10$ for each group). The Ki-67-positive cell ratio in MNU-treated *Dnmt1*$^{chip/c}$ mice was equivalent to that in non-treated *Dnmt1*$^{chip/c}$ mice, whereas MNU-treatment significantly elevated the Ki-67-positive cell ratio in *Dnmt1*$^{chip/+}$ mice. Columns, mean; bars, SD. $^{*}P < 0.0005$ and $^{**}P < 0.0005$, by Mann–Whitney *U*-test.

reduction of DNA methylation levels suppresses the initiation and promotion stage of MNU-induced gastric carcinogenesis.

DNA hypomethylation suppresses the development of gastric tumors in Apc$^{Min/+}$ *mice*

We also investigated the effect of reduced DNA methylation levels on gastric carcinogenesis using a different mouse model. We have previously reported that *Apc*$^{Min/+}$ mice spontaneously develop gastric tumors with aging (21). *Apc*$^{Min/+}$ mice were crossed with *Dnmt1* hypomorphic mice to generate *Apc*$^{Min/+}$ mice that express different levels of *Dnmt1*. Consistent with the results in the MNU-induced gastric tumor model, both the incidence and the number of the gastric tumors were significantly smaller in *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice than in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice (Figure 3A and B, $P < 0.05$ for the incidence and $P < 0.005$ for the number). As previously reported, the gastric tumors were histopathologically classified as adenomas in the *Apc*$^{Min/+}$ mice (Figure 3C) (21). These results provide additional evidence that a genetic reduction of DNA methylation levels suppresses gastric tumorigenesis. Although we assessed the cell proliferative activities in non-cancerous gastric mucosa of *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice, genetic reduction of the DNA methylation level did not affect the cell proliferative activities in the gastric mucosa of *Apc*$^{Min/+}$ mice. The Ki-67-positive cell ratios in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice ($n = 10$ for each animals) were 22.3 ± 5.8 and 19.4 ± 5.4 (average ± SD), respectively.

DNA hypomethylation suppresses the progression stage of gastric carcinogenesis

MNU-induced gastric lesions were evaluated for their histopathologic features and classified as hyperplasia, adenoma, intramucosal adenocarcinoma or invasive adenocarcinoma (Figure 4A). Histopathlogical analyses clearly demonstrated significant decreases in the development of intramucosal and invasive adenocarcinomas in MNU-treated *Dnmt1*$^{chip/c}$ mice as compared with *Dnmt1*$^{chip/+}$ mice (Figure 4B), thus indicating that the reduced DNA methylation levels suppressed the progression into advanced gastric tumors. It is noteworthy that the MNU-treated *Dnmt1*$^{chip/+}$ mice often developed invasive adenocarcinomas, whereas the MNU-treated *Dnmt1*$^{chip/c}$ mice did not (Figure 4B). These findings suggest that DNA methylation may play a role in the progression stage of gastric tumorigenesis.

DNA hypomethylation reduces the persistent increase in the cell proliferative activity in the non-cancerous gastric mucosa exposed to MNU

In order to clarify the inhibitory mechanisms of reduced DNA methylation levels on gastric tumorigenesis, we assessed cell proliferative activity in the non-cancerous gastric mucosa of MNU-treated and non-treated *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice by immunostaining for Ki-67, a marker for proliferating cells (Figure 5A). The Ki-67-positive index of gastric epithelium was significantly higher in MNU-treated *Dnmt1*$^{chip/+}$ mice than in non-treated *Dnmt1*$^{chip/+}$ mice ($P < 0.005$), thus suggesting that MNU induces abnormal cell proliferation even in non-cancerous gastric epithelium to provide favorable conditions for tumor development (Figure 5B). In contrast, the Ki-67-positive cell ratio in MNU-treated *Dnmt1*$^{chip/c}$ mice was equivalent to that in non-treated *Dnmt1*$^{chip/c}$ mice and it was significantly lower than that in the MNU-treated *Dnmt1*$^{chip/+}$ mice ($P < 0.005$), thus indicating that reduced levels of genomic methylation suppress the abnormal cell proliferation induced by MNU (Figure 5B). We also performed cleaved caspase-3 in order to examine the effect of DNA hypomethylation on the induction of apoptosis. However, no significant differences were observed in the induction of apoptosis between *Dnmt1*$^{chip/c}$ mice and *Dnmt1*$^{chip/+}$ mice (data not shown).

The aberrant site-specific methylations are not found in MNU-induced mouse gastric tumors unlike in the case of human gastric cancers

We cannot rule out the possibility that the genetic reduction of DNA methylation suppress gastric tumorigenesis by blocking the aberrant site-specific methylation of specific genes that play a crucial role in gastric tumorigenesis, but there is no available information on hypermethylated or hypomethylated genes in MNU-induced mouse gastric tumors. In order to examine the involvement of aberrant site-specific methylations in the development of MNU-induced mouse gastric tumors, the methylation status of the promoter regions of *Cdkn2a*, *Cdh1*, *Sfrp1*, *Sfrp2* and *Magea2* were analyzed. Though it has been reported that the CpG islands in the promoter regions of *Cdkn2a*, *Cdh1*, *Sfrp1* and *Sfrp2* are highly methylated and those of *Magea2* are frequently demethylated in human gastric tumors (7–12), altered methylation patterns were not found in MNU-induced mouse gastric

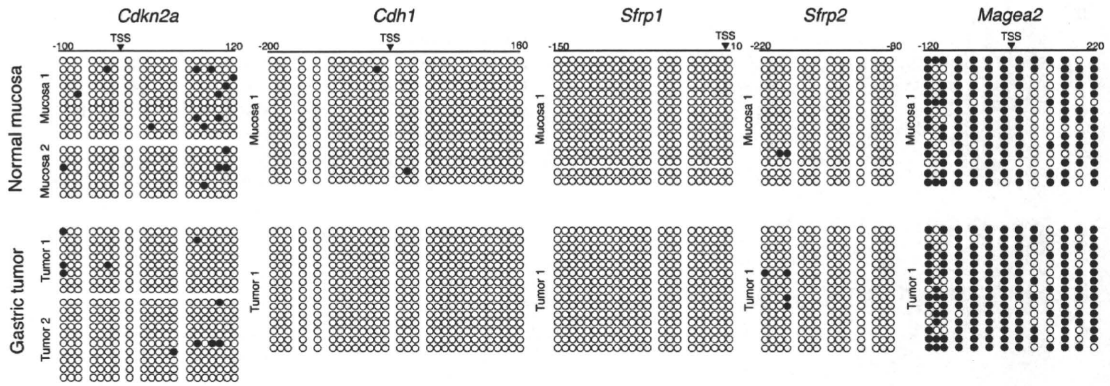

Fig. 6. Results of bisulfite DNA sequencing of the promoter CpG island region of *Cdkn2a*, *Cdh1*, *Sfrp1*, *Sfrp2* and *Magea2*. Numbers represent the positions relative to the transcription start site. TSS, transcription start site. Circles, CpG dinucleotide (potential target of methylation); closed circles, methylated CpG dinucleotide and open circles, unmethylated CpG dinucleotide.

tumors (Figure 6), indicating that the pattern of the site-specific aberrant methylations in mouse gastric tumors is distinctly different from that in human gastric cancers.

Discussion

Previous studies have revealed that a genetic reduction of the DNA methylation levels results in opposing effects on tumor development, depending on the tumor cell type and the stage of tumorigenesis. Consistent with earlier reports concerning tumorigenesis in the intestine (17,18) and the upper digestive tract (19), we herein demonstrated that the genetic reduction of DNA methylation levels suppresses gastric tumorigenesis in two different models of gastric cancer: the MNU-induced model and the $Apc^{Min/+}$ mouse model. Although further long-term observations would be required to determine whether DNA hypomethylation completely suppresses the progression to the invasive tumors or not, it is noteworthy that DNA hypomethylation inhibited the development of invasive adenocarcinoma, suggesting that DNA hypomethylation suppresses the malignant transformation of gastric tumors. This notion is also consistent with previous findings that genetic reduction blocked the development of invasive squamous cell carcinoma in the tongue and esophagus (19). These findings may shed some light on the involvement of epigenetic modification in the acquisition of invasive properties of tumor cells.

The presence of cancer-predisposed mucosa was initially described in the setting of oral carcinogenesis, and the concept of 'field cancerization' (28) has been widely accepted in regard to various organs, including the stomach (29,30). Recent studies suggest the involvement of epigenetic alterations in field cancerization in the human stomach (29–31). In the present study, the administration of MNU induced abnormal cell proliferation in non-cancerous gastric mucosa, which supported the concept of field cancerization. It is important that global DNA hypomethylation significantly suppressed the induction of abnormal proliferation in the MNU-exposed non-cancerous gastric mucosa. Because increased cell proliferation is obviously associated with the risk of cancer development, DNA hypomethylation suppresses the development of gastric tumors by preventing the gastric mucosa from being transformed into a favorable environment for tumor development.

In human gastric cancer, several tumor suppressor genes are inactivated more frequently by epigenetic silencing associated with site-specific DNA hypermethylation than by mutations (7). In addition, higher methylation levels at seven CpG islands in human gastric mucosa have also been reported to significantly correlate with a higher risk of gastric cancer development (29). Therefore, one may hypothesize that the genetic reduction of DNA methylation blocks the epigenetic silencing of the candidate genes that play a crucial role in MNU-induced gastric tumorigenesis. However, it remains controversial whether DNA hypermethylation plays a major role in gene silencing in rodent tumorigenesis. Although previous studies have indicated the presence of genomic hypermethylation in the tumor suppressor genes observed in rodent gastric tumors (32,33), another study revealed no involvement of DNA hypermethylation in the specific genes that are frequently hypermethylated in human gastric cancers (34). In the present study, we also examined the methylation status of the promoter regions of the genes that are frequently hypermethylated or hypormethylated in human gastric tumor (8–12,30) but found no altered DNA methylation patterns. Other epigenetic mechanisms, which are independent of the activation of the silenced genes, might therefore be associated with the suppression of gastric tumorigenesis by the genetic reduction of DNA methylation levels. Further analyses are therefore required to identify the target genes and/or sites of genomic hypomethylation responsible for the tumor suppression in this model.

Our data suggest that a pharmacological modification of the epigenetic status may be a potent strategy for the prevention and treatment of gastric cancers, and this idea is supported by the findings that *Helicobacter pylori*-infected human gastric mucosa harbors genomic hypermethylation (29,31) and several tumor suppressor genes are inactivated by promoter hypermethylation in human gastric cancer (7,9–12). However, possible side effects must be carefully taken into consideration for clinical application of DNA hypomethylating agents because the reduction of genomic methylation levels promotes the development of other type of tumors as previously reported (5,6,18).

In summary, we herein demonstrated that a reduction of the DNA methylation levels consistently suppresses gastric tumorigenesis, thus suggesting that DNA methylation is closely associated with gastric tumorigenesis.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

Funding

Ministry of Health, Labour and Welfare of Japan (22090201); PRESTO (08001433); Ministry of Education, Culture, Sports, Science and Technology of Japan (19689011, 20012021, 20790300).

Acknowledgements

We would like to thank Kyoko Takahashi, Ayako Suga and Yoshitaka Kinjyo for their valuable technical assistance and and help with the animal care.

Conflict of Interest Statement: None declared.

References

1. Jones,P.A. *et al.* (2002) The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.*, **3**, 415–428.
2. Feinberg,A.P. *et al.* (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. *Cancer Res.*, **48**, 1159–1161.
3. Feinberg,A.P. *et al.* (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, **301**, 89–92.
4. Chen,R.Z. *et al.* (1998) DNA hypomethylation leads to elevated mutation rates. *Nature*, **395**, 89–93.
5. Eden,A. *et al.* (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. *Science*, **300**, 455.
6. Gaudet,F. *et al.* (2003) Induction of tumors in mice by genomic hypomethylation. *Science*, **300**, 489–92.
7. Ushijima,T. *et al.* (2004) Focus on gastric cancer. *Cancer Cell*, **5**, 121–125.
8. Kaneda,A. *et al.* (2004) Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. *Cancer Sci.*, **95**, 58–64.
9. Kang,G.H. *et al.* (2003) Profile of aberrant CpG island methylation along the multistep pathway of gastric carcinogenesis. *Lab. Invest.*, **83**, 635–641.
10. Nojima,M. *et al.* (2007) Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. *Oncogene*, **26**, 4699–4713.
11. Suzuki,H. *et al.* (1999) Distinct methylation pattern and microsatellite instability in sporadic gastric cancer. *Int. J. Cancer*, **83**, 309–313.
12. Tamura,G. *et al.* (2000) E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. *J. Natl Cancer Inst.*, **92**, 569–573.
13. Chalitchagorn,K. *et al.* (2004) Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. *Oncogene*, **23**, 8841–8846.
14. Li,E. *et al.* (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.
15. Linhart,H.G. *et al.* (2007) Dnmt3b promotes tumorigenesis *in vivo* by gene-specific *de novo* methylation and transcriptional silencing. *Genes Dev.*, **21**, 3110–3122.
16. Okano,M. *et al.* (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell*, **99**, 247–257.
17. Laird,P.W. *et al.* (1995) Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*, **81**, 197–205.
18. Yamada,Y. *et al.* (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc. Natl Acad. Sci. USA*, **102**, 13580–13585.
19. Baba,S. *et al.* (2009) Global DNA hypomethylation suppresses squamous carcinogenesis in the tongue and esophagus. *Cancer Sci.*, **100**, 1186–1191.
20. Lu,J. *et al.* (2005) Chemopreventive effect of peroxisome proliferator-activated receptor gamma on gastric carcinogenesis in mice. *Cancer Res.*, **65**, 4769–4774.
21. Tomita,H. *et al.* (2007) Development of gastric tumors in Apc(Min/+) mice by the activation of the beta-catenin/Tcf signaling pathway. *Cancer Res.*, **67**, 4079–4087.
22. Yamachika,T. *et al.* (1998) N-methyl-N-nitrosourea concentration-dependent, rather than total intake-dependent, induction of adenocarcinomas in the glandular stomach of BALB/c mice. *Jpn. J. Cancer Res.*, **89**, 385–391.
23. Lei,H. *et al.* (1996) *De novo* DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development*, **122**, 3195–3205.
24. Sanford,J. *et al.* (1984) Methylation patterns of repetitive DNA sequences in germ cells of Mus musculus. *Nucleic Acids Res.*, **12**, 2823–2836.
25. Kuramochi-Miyagawa,S. *et al.* (2008) DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev.*, **22**, 908–917.
26. Boivin,G.P. *et al.* (2003) Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology*, **124**, 762–777.
27. Nam,K.T. *et al.* (2004) The selective cyclooxygenase-2 inhibitor nimesulide prevents *Helicobacter pylori*-associated gastric cancer development in a mouse model. *Clin. Cancer Res.*, **10**, 8105–8113.
28. Slaughter,D.P. *et al.* (1953) Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*, **6**, 963–968.
29. Nakajima,T. *et al.* (2006) Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. *Cancer Epidemiol. Biomarkers Prev.*, **15**, 2317–2321.
30. Ushijima,T. (2007) Epigenetic field for cancerization. *J. Biochem. Mol. Biol.*, **40**, 142–150.
31. Maekita,T. *et al.* (2006) High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. *Clin. Cancer Res.*, **12**, 989–995.
32. Bai,H. *et al.* (2003) p16 hypermethylation during gastric carcinogenesis of Wistar rats by N-methyl-N'-nitro-N-nitrosoguanidine. *Mutat. Res.*, **535**, 73–78.
33. Guo,W.H. *et al.* (2002) Inhibition of growth of mouse gastric cancer cells by Runx3, a novel tumor suppressor. *Oncogene*, **21**, 8351–8355.
34. Niwa,T. *et al.* (2005) Whole-genome analyses of loss of heterozygosity and methylation analysis of four tumor-suppressor genes in N-methyl-N'-nitro-N-nitrosoguanidine-induced rat stomach carcinomas. *Cancer Sci.*, **96**, 409–413.

Received January 10, 2010; revised June 11, 2010; accepted June 13, 2010

Fibroblasts associated with cancer cells keep enhanced migration activity after separation from cancer cells: A novel character of tumor educated fibroblasts

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Received February 12, 2010; Accepted April 29, 2010

DOI: 10.3892/ijo_00000680

Abstract. It is now clear that the association between cancer cells and recruited fibroblasts (cancer-associated fibroblasts; CAFs) leads to alteration of the biological properties of both types of cells and creates a specific microenvironment. Here we report a novel biological property of CAFs and its cellular mechanism using *in vivo* and *in vitro* model. Cultured CAFs derived from human lung cancer tissue displayed significantly higher migration activity in response to PDGF-BB than that of fibroblasts from corresponding non-cancerous tissue (NCAFs). Moreover, KM104^{GFP} (GFP-labeled human fibroblast cell line) co-cultured with human cancer cell line Capan-1 showed significantly higher migration activity than KM104^{GFP} alone. No such phenomenon occurred when KM104^{GFP} and Capan-1 were cultured separately. Even after KM104^{GFP} were sorted from co-cultured Capan-1, KM104^{GFP} retained their enhanced migration activity until passage-5 of culture in the absence of cancer cells. Despite a similar level of phosphorylation of ERK1/2 after exposure to PDGF-BB, the inhibitory effect of MEK inhibitor was significantly higher on migration of KM104^{GFP} that had been sorted from co-cultured Capan-1 than of KM104^{GFP} alone. This higher dependence on ERK1/2 signaling for cell migration was also seen in CAFs obtained from cancer tissue. The results of this study indicate that by association with cancer cells, CAFs can acquire enhanced migration activity which could be kept after separation from cancer cells and suggest the possibility that higher dependence on ERK1/2 signaling for enhanced migration activity would be one of the biological properties of CAFs.

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Key words: cancer associated fibroblast, MAP kinase, migration activity

Introduction

During the process of cancer cell invasion, the cancer cells associate with several types of stromal cells that together create the specific microenvironment of the cancer tissue. It is becoming clear that the microenvironment plays an important role in allowing the tumor to express its full neoplastic phenotype (1,2). Fibroblasts, which are the major component of the newly created stroma, are recruited from neighboring tissues and remote organs (3-7). This recruitment step is called the 'desmoplastic reaction' and is a kinetic sequence of events in the invasion process. Within the cancer tissue, the fibroblasts directly communicate with the cancer cells and other types of stromal cells and acquire a specific biological phenotype (8,9). The type of fibroblast in cancer tissue that has acquired a specific biological phenotype is called cancer-associated fibroblast (CAF), and such fibroblasts have been postulated to perform supportive roles that promote tumor progression and metastasis by secreting growth factors (10,11), chemokines (12,13), and matrix metalloproteinases (MMPs) (14,15). Although an increasing number of translational studies have emphasized the prognostic significance of the altered phenotype of CAFs (16-18), the cellular mechanisms of these alterations remain unclear.

It is noteworthy that expression of specific molecules on CAFs is maintained under *in vitro* conditions, which means that alterations of gene expression in CAFs are capable of being stably maintained even in the absence of continued exposure to cancer cells. Actually, in a comparison with cultured non-cancerous fibroblasts Nakagawa *et al* found that approximately 170 of 22,000 genes were up-regulated in cultured CAFs (fold change >2, $P<0.05$), and that the upregulated genes included many genes that encode cell

adhesion molecules and growth factors (19). However, the mechanisms by which specific gene expression is induced in CAF are controversial. In some studies, the frequency of LOH in CAFs has been reported to be similar to the frequency observed in the epithelial components (20-22). Qiu *et al*, on the other hand, reported that LOH and copy number alterations were extremely rare in CAFs from breast and ovarian cancers (23). Very little is known as to how genetic or epigenetic status can modify gene expression and cellular signaling(s) in CAFs.

The desmoplastic reaction during cancer progression centers on various functions of fibroblasts (24). One of the representative phenotypes involved in this reaction is migration activity, which is thought to be related to cancer progression (25). In this study we used *in vivo* and *in vitro* models to investigate the biological characteristics of CAFs, with a special focus on their migration activity and the cellular mechanism.

Materials and methods

Human fibroblast culture. Cancer-associated fibroblasts (CAFs) and non-cancerous tissue-associated fibroblasts (NCAFs) were obtained from the surgically resected lungs of lung cancer patients as previously reported (26). Briefly, approximately 5-mm^3 carcinoma tissue and non-cancerous lung tissue specimens were cut into about ten pieces and each was placed in α-MEM (Gibco, Grand Island, NY) culture medium with 10% heat-inactivated FBS and antibiotics (penicillin and streptomycin) (Sigma, St. Louis, MO). The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. The tissue was then removed and cultured for two more days. When the cells reached 80% confluence they were harvested with 0.25% trypsin and 1 mmol/l EDTA and then replated at a density of $1x10^4$ cells/cm^2. The fibroblasts were separated from contaminating epithelial and macrophages by differential trypsinization and used between passages 3 and 7. All specimens were collected after the subjects had given their written informed consent and it was approved by the Institutional Review Board of the National Cancer Center.

Animals. Six-week-old female severe combined immunodeficient (SCID) mice (C.B-17 background) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained at the National Cancer Center Research Institute East (Chiba, Japan). All animals were maintained under specific-pathogen-free, temperature-controlled environmental conditions throughout this study, in accordance with the Institutional Guidelines. Written approval for all animal experiments (K03-011) was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute.

Cell lines and cell cultures. KM104 cells, SV-40 transformed human fibroblast cell line derived from bone marrow, were originally established from male patient as described previously (27). This cell line was cultured in RPMI-1640 medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Human pancreatic cancer cell line Capan-1 was purchased from the American Type Culture Collection (Rockville, MD). The Capan-1 cells were maintained in DMEM (Sigma) medium with 20% heat-inactivated FBS and antibiotics. All cells were maintained in a 5% CO_2 incubator at 37°C.

Green fluorescent protein (GFP) labeling and cell sorting. KM104 were transfected with pEGFP-C1 vector encoding EGFP (Clonetech, Palo Alto, CA) by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). GFP-positive KM104 ($KM104^{GFP}$) cells were sorted by using a FACSCalibur sorting system (Becton-Dickinson, San Jose, CA). After confirming that over 90% of cell line was GFP positive, they were used in the following experiment.

Intraperitoneal xenotransplantation of Capan-1 and $KM104^{GFP}$. Capan-1 cells ($5x10^6$ cells per animal) were injected into the peritoneal cavity of SCID mice as described previously, and 1 h later, $5x10^6$ $KM104^{GFP}$ were injected into each peritoneal cavity at a different injection site (28). The animals were sacrificed on day 21, and parapancreatic tumors were removed, minced, and cultured in 10% FBS RPMI-1640. Under subconfluent conditions, the adherent cells were harvested, and the GFP-positive cells were sorted with the FACSCalibur sorting system.

Co-culture system. $KM104^{GFP}$ ($1x10^6$) and Capan-1 ($2x10^5$) were co-cultured in RPMI-1640 medium with 10% FBS. After 48 h, cells were collected, and GFP-positive cells were sorted with the cell sorter, and the cultured. $KM104^{GFP}$ used in experiments were passaged by harvesting and distribution into five new plates. To obtain cell-conditioned medium, Capan-1 were cultured until semiconfluent conditions. The plate was then washed with sterile PBS, and the medium was changed to RPMI-1640+10% FBS. After incubation for 24 h, the Capan-1 culture supernatant was collected and added to the pre-seed $KM104^{GFP}$, and their chemotactic activity was assayed 48 h later.

Migration assays. In the preliminary study, we tested the ability of 10 synthetic chemoattractants and growth factors (b-FGF, CCL-21, EGF, IGF-1, IL-8, PDGF-AA, PDGF-BB, SDF-1, TGF-ß, VEGF-A), and found that PDGF-BB was the most powerful chemoattractant at the same protein concentrations (data not shown). PDGF-BB was therefore used as the chemoattractant in the subsequent experiments. The migration assay was performed by using 24-well culture chambers (Becton-Dickinson Labware, Bedford, MA) and a polycarbonate filter with an 8 μm pore size (Becton-Dickinson Labware) as previously described (28). Briefly, the lower chamber contained 0.6 ml of RPMI-1640 + recombinant PDGF-BB (final concentration; 0-100 ng/ml), or 0.6 ml of RPMI-1640 as a control. In the upper compartment, $2x10^4$ cells/well were placed in triplicate wells and incubated for 6 h at 37°C in a humidified incubator under a 5% CO_2 atmosphere. At the end of the incubation period, the cells that had passed through the filter into the lower wells were stained with hematoxylin, and the cells in nine predetermined fields were counted under a microscope.

Western blotting. Western blot analysis was performed as follows. Cells were lysed in whole-cell extraction buffer

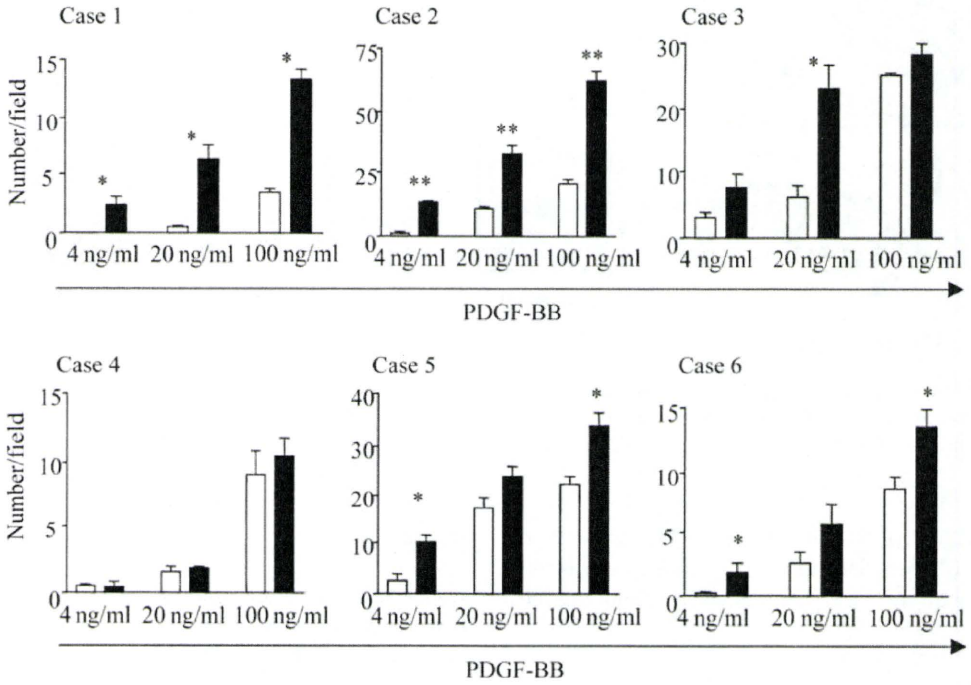

Figure 1. Cancer-associated fibroblasts (CAFs) isolated from human cancer tissue displayed enhanced migration activity in response to PDGF-BB. Fibroblasts isolated from human lung cancer tissue (cancer-associated fibroblasts; CAFs) and fibroblasts from corresponding non-cancerous lung tissues (non-cancerous-tissue-associated fibroblasts; NCAFs) were isolated and cultured, and their migration activity was compared. Except for case 4, migration activity of the CAFs in response to PDGF-BB was significantly higher than that of the NCAFs. ($^{*}P<0.05$, $^{**}P<0.01$).

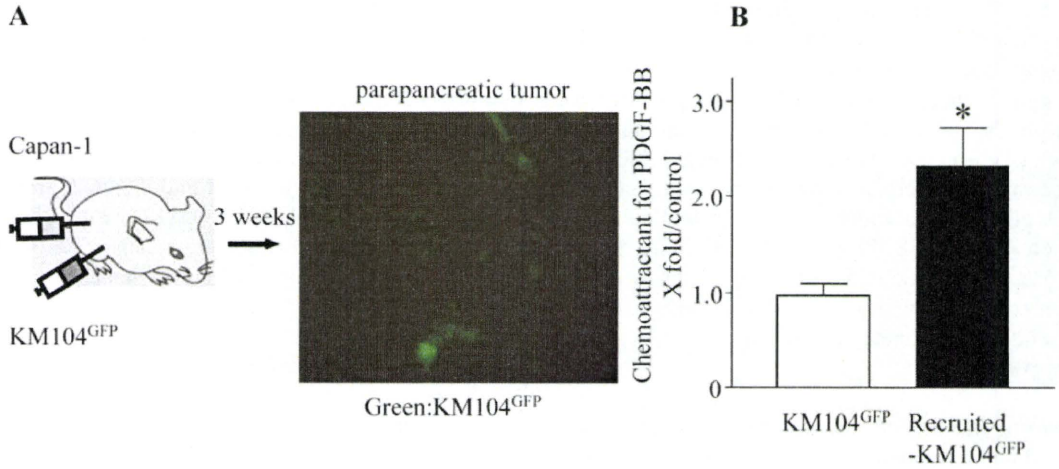

Figure 2. KM104^{GFP} recruited into cancer stroma *in vivo* display enhanced migration activity in response to PDGF-BB. (A) GFP-positive cells (KM104^{GFP}) are scattered within this abdominal tumor. (B) GFP (+) cells were sorted from the minced abdominal tumor and cultured *in vitro* (rec-KM104^{GFP}). Their migration activity in response to PDGF-BB was then compared with that of the parent cells (KM104^{GFP}). Rec-KM104^{GFP} exhibited a 2.4-fold higher migration activity than parent KM104^{GFP} (n=3, $^{*}P=0.03$).

(20 mM Hepes-NaOH, 0.5% NP-40, 15% glycerol) containing a Complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Proteins were separated on 12% SDS-polyacrylamide gels and then transferred to an Immobilon-P PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA). The blots were saturated with blocking buffer (5% skim milk in TBS-T) for 1 h at room temperature and then incubated for 1 h at 4°C with anti-phospho-ERK1/2 antibody (mouse monoclonal IgG, E10, phospho-p44/42 MAPK antibody-Thr 202/Tyr 204) and total ERK1/2 antibody (rabbit polyclonal, p44/42 MAPK antibody) according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). After washing in TBS-T, the membranes were incubated for 1 h at room

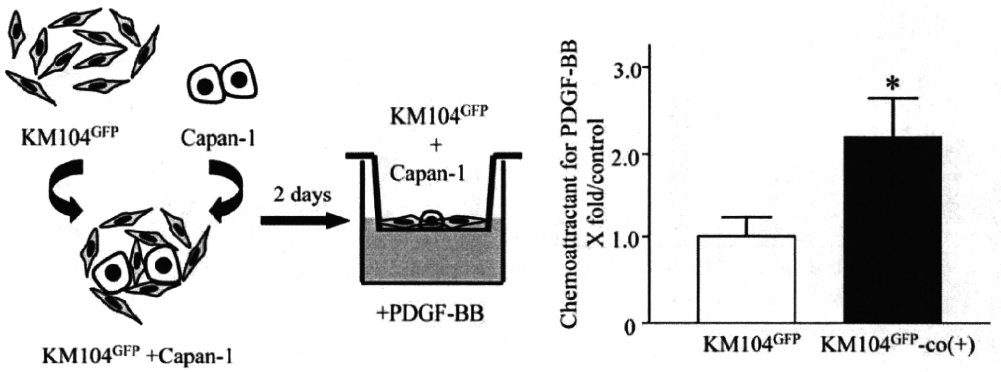

Figure 3. KM104^{GFP} co-cultured with a cancer cell line display enhanced migration activity in response to PDGF-BB. KM104^{GFP} were co-cultured with Capan-1 cells for two days [KM104^{GFP}-co(+)], and their migration activity in response to PDGF-BB was assayed. KM104^{GFP}-co(+) displayed 2.2-fold higher migration activity than KM104^{GFP} cultured in the absence of Capan-1 (n=9, *P=0.04)

temperature with HRP-Rabbit anti-mouse IgG or HRP-goat anti-rabbit IgG (Zymed, San Francisco, CA). Antibody binding was detected with an Amersham enhanced chemiluminescence system.

Methylated DNA immunoprecipitation-chip (MeDIP-chip) assay. A 5 μg sample of genomic DNA was sonicated to fragment lengths between 200 and 800 bp. After heat denaturation, the DNA was incubated with 5 μg of antibody against 5-methyl cytidine (Diagnode, Liège, Belgium) at 4°C overnight. Immuno-complexes were collected with Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), treated with Proteinase K, and purified by phenol and chloroform extraction and isopropanol precipitation. The chip assay was carried out with a human CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA). Immunoprecipitated DNA from 4.5 μg of sonicated DNA and 1 μg of input DNA were labeled with Cy-5 and Cy-3, respectively, using an Agilent labeling kit (Agilent Technologies), and then hybridized with the microarray. The microarray was scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). The methylation statuses of 8,528 promoter CGIs were evaluated by using Me values, which have been shown to have a higher correlation coefficient with the fraction of methylated DNA molecules (29,30), the methylation status of 8,528 promoter CGIs was evaluated.

MEK inhibitor treatment. Cells were pretreated with the MEK1/2 inhibitor compound UO126 (Promega, San Diego, CA) or with DMSO vehicle alone before harvesting. UO126 was used at a concentration of 25 μM, which was shown to maximally inhibit MEK in fibroblasts. In the upper compartment, 2x10^4 cells/well were placed in medium containing 25 μM of U0126 and incubated for 6 h.

Statistical analyses. Three or four independent experiments were performed for each protocol. Results were expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed by using Prism 3.03 software. Differences in measured variables between experimental groups and control groups were assessed by using an unpaired t-test. P<0.05 was considered statistically significant.

Results

Cancer-associated fibroblasts (CAFs) isolated from human cancer tissue displayed enhanced migration activity in response to PDGF-BB. Fibroblasts isolated from human lung cancer tissue (cancer-associated fibroblasts; CAFs) and fibroblasts from corresponding non-cancerous lung tissues (non-cancerous tissue-associated fibroblasts; NCAFs) were isolated and cultured, and their migration activity was compared. Except for Case 4, migration activity of the CAFs in response to PDGF-BB was significantly higher than that of the NCAFs (Fig. 1).

Fibroblast cell line KM104^{GFP} recruited into cancer stroma displays enhanced migration activity in response to PDGF-BB. Capan-1 and KM104^{GFP} were injected intraperitoneally at a different site. Immunofluorescence examination of the parapancreatic tumor revealed the presence of KM104^{GFP} within the cancer tissue (Fig. 2A). GFP (+) cells were sorted from the minced parapancreatic tumor and cultured, and the chemoattractant response of the KM104^{GFP} to PDGF-BB was compared with that of the parent cells. KM104^{GFP} recruited into the tumor exhibited a 2.4-fold higher migration activity than parent KM104^{GFP} (n=3, Fig. 2B, P=0.03).

KM104^{GFP} co-cultured with the cancer cell line displayed enhanced migration activity in response to PDGF-BB. We co-cultured KM104^{GFP} and Capan-1 for two days and assayed the migration activity of KM104^{GFP} in response to PDGF-BB. No Capan-1 cells were able to migrate through the cell culture insert (8.0 μm pore size) under these conditions. KM104^{GFP} co-cultured with Capan-1 [KM104^{GFP}-co(+)] displayed 2.2-fold higher migration activity than KM104^{GFP} cultured in the absence of Capan-1 (n=9, P=0.04, Fig. 3).

Cell-cell contact is necessary for enhanced migration activity. To explore whether cell-cell contact is necessary for KM104^{GFP} to exhibit enhanced migration activity, we cultured KM104^{GFP} with KM104 cell conditioned medium (control) or Capan-1 cell conditioned medium for 2 days, and assayed their migration activity, but no significant difference in migration activity was found between the two groups (Fig. 4A). Next,

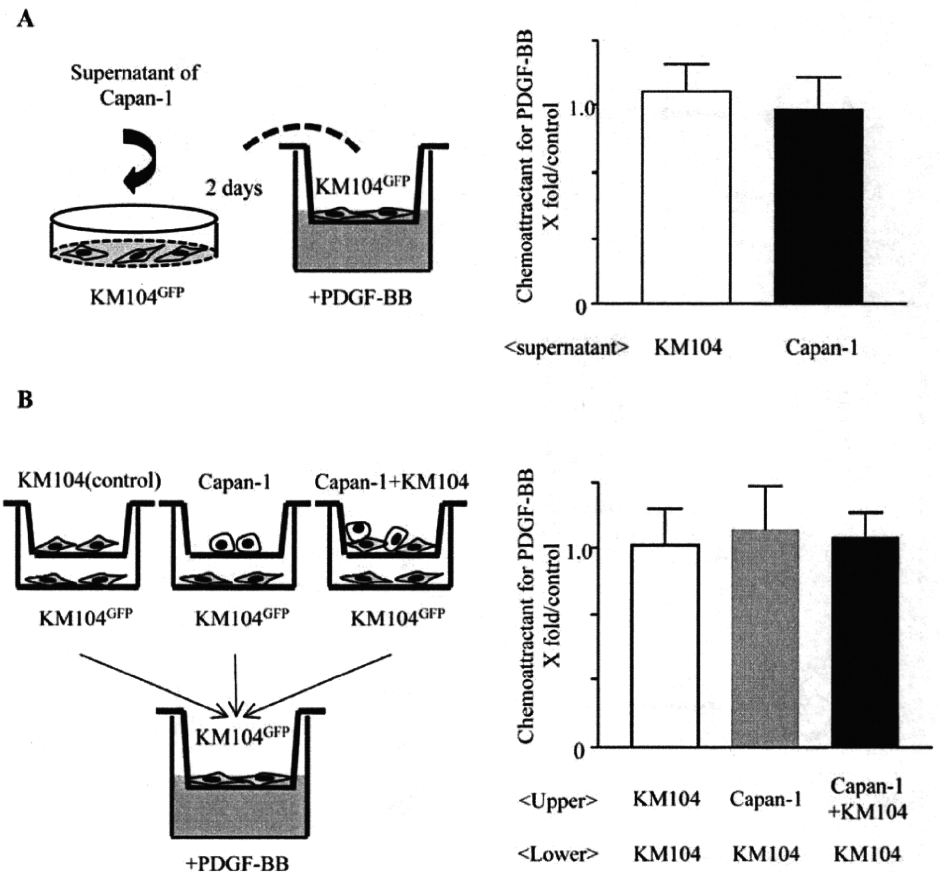

Figure 4. Cell-cell contact is necessary for enhanced migration activity. (A) KM104^{GFP} cells were cultured with conditioned medium from KM104 cell cultures (control) or Capan-1 cell cultures for 2 days, and their migration activity was assayed. (B) KM104^{GFP} were co-cultured in the 2-chamber system (upper chamber; KM104, Capan-1, or Capan-1 +KM104, lower chamber; KM104^{GFP}) for 2 days, and the migration activity of the KM104^{GFP} cultured in the lower chamber was assayed.

KM104^{GFP} were co-cultured in the 2-chamber system (upper chamber: KM104, Capan-1, or Capan-1+KM104; lower chamber: KM104^{GFP}) for 2 days, and the migration activity of the KM104^{GFP} cultured in lower chamber was assayed (Fig. 4B). Migration number of KM104^{GFP} exposed to Capan-1 cell supernatant (middle column) and KM104^{GFP} exposed to Capan-1+KM104 cells supernatant (right column) was almost the same as that of control group (left column). These findings suggested that direct KM104-Capan-1 contact is necessary for KM104^{GFP} to exhibit enhanced migration activity.

Enhanced migration activity of KM104^{GFP}-co(+) is kept after separation from cancer cells. GFP-positive KM104 were sorted ($3.0x10^5$) from cocultured Capan-1 and cultured (Fig. 5A). In a control experiment, KM104^{GFP} alone were cultured, and GFP-positive cells were sorted (sham-sorted) and cultured. Cells from subconfluent cultures ($1.0x10^6$) were then assayed for migration activity in response to PDGF-BB. The results showed that the sorted KM104^{GFP}-co(+) displayed 2.3-fold higher activity than sham-sorted KM104^{GFP} (P=0.02), indicating that the sorted KM104^{GFP}-co(+) displayed enhanced migration activity for almost 2 popu-lation doublings after separation from the cancer cells.

Sorted KM104^{GFP}-co(+) were then serially passaged by harvesting and distribution into five new plates, and migration activity was compared in each passage culture. Three independent experiments were performed, and the results are shown in Fig. 5B. The sorted KM104^{GFP}-co(+) kept their enhanced migration activity even after the passage-5 culture in every experiment. In experiments 2 and 3, the enhanced migration activity of the sorted KM104^{GFP}-co(+) returned to the levels of passaged sham-sorted KM104^{GFP} cells in the passage-6 culture. These results indicate that the sorted KM104^{GFP}-co(+) retained the enhanced migration activity for at least 13 population doublings after separation from the cancer cells *in vitro*.

MeDIP-chip analysis of CAFs. Because of the prolonged enhanced migration activity of CAFs, it was important to analyze the epigenetic changes that accompany this phenomenon. We used a methylation screening approach called MeDIP-chip to compare the DNA methylation profiles of the CAFs and corresponding NCAFs from two cases. The results of the MeDIP-chip analysis showed that 2.2% (188 promoter CGIs) (Case 1) and 1.1% (98 promoter CGIs) (Case 2) of the CGIs were hypermethylated in CAFs compared with NCAFs.

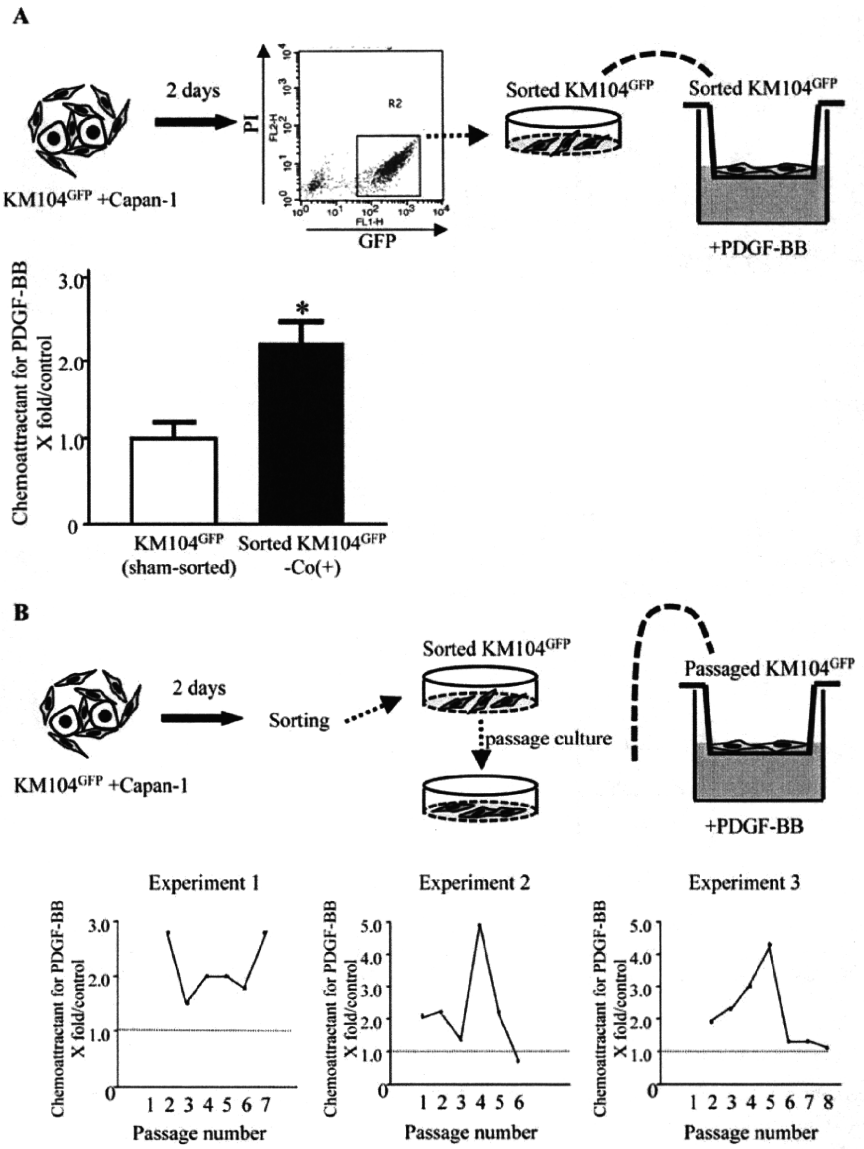

Figure 5. Sustained enhanced migration activity of KM104^{GFP}-co(+) after separation from the cancer cells. (A) After sorting from the co-cultured Capan-1, GFP-positive KM104 were sorted and cultured [Sorted KM104^{GFP}-co(+)]. In a control experiment, KM104^{GFP} alone were cultured, and the GFP-positive cells were sorted (sham-sorted) and cultured. Cells from a subconfluent culture were then examined for migration activity in response to PDGF-BB. Sorted KM104^{GFP}-co(+) displayed 2.3-fold higher activity than sham-sorted KM104^{GFP} (n=3, *P=0.02). (B) Sorted KM104^{GFP}-co(+) were serially passaged by harvesting and distribution into five new plates, and their migration activity was compared at each passage culture. Representative data of three independent experiments are shown.

0.5% (39 promoter CGIs) (Case 1) and 0.6% (50 promoter CGIs) (Case 2) were hypomethylated in CAFs compared with NCAFs (Table I). Ten promoter CGIs (*ALDH1L2*, *AP1G2*, *CENPJ*, *KBTBD6*, *MAB21L1*, *MED4*, *RCBTB2*, *SH2B3*, *TBC1D4* and *UBL3*) were hypermethylated in the CAFs from both cases (Table II), but no promoter CGIs were hypomethylated in the CAFs from either of the cases.

Role of extracellular signal-regulated kinase (ERK) 1/2 in PDGF-BB-induced migration of sorted KM104^{GFP}-co(+) and CAFs. Activation of extracellular signal-regulated kinase (ERK) 1/2 has been reported to play crucial role in PDGF-induced cell migration. We analyzed the effects of PDGF-BB

Table I. Aberrantly methylated promoter CGIs in CAF to be compared with NCAF.

	Pair	No. of genes
Hypermethylated genes		
	Case 1	188
	Case 2	98
Hypomethylated genes		
	Case 1	39
	Case 2	50

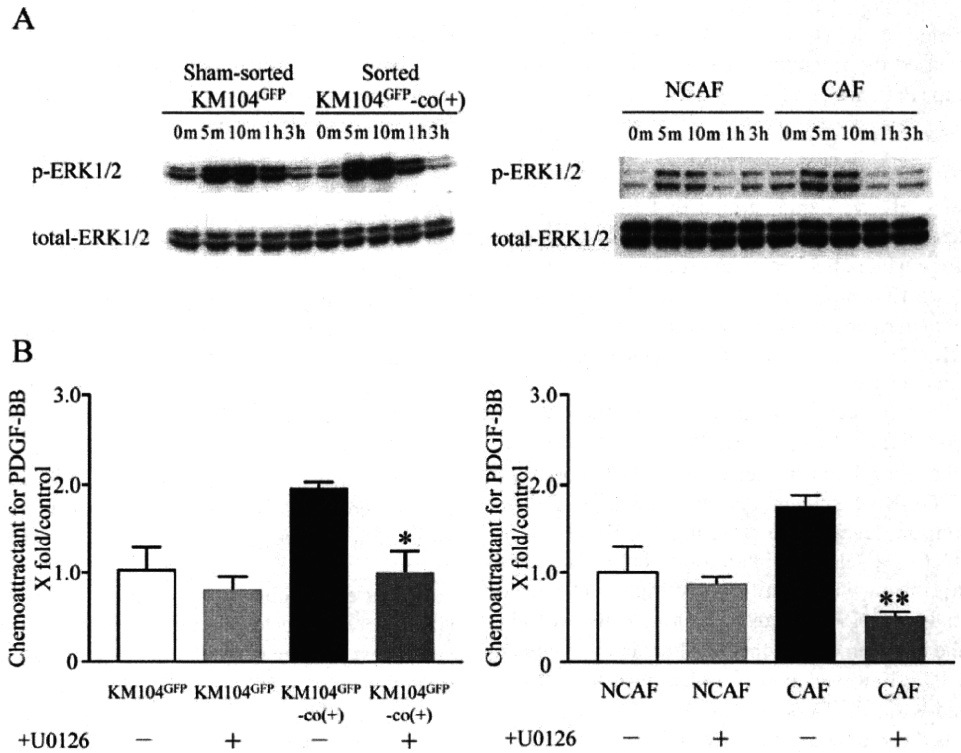

Figure 6. Role of extracellular signal-regulated kinase (ERK) 1/2 in PDGF-BB-induced migration by sorted KM104^{GFP}-co(+) cells and CAFs. (A) Western blotting of phosphorylated ERK1/2. PDGF-BB increased the level of ERK1/2 phosphorylation in sham-sorted KM104^{GFP} cells, sorted KM104^{GFP}-co(+) cells, NCAFs, and CAFs in a time-dependent manner, reaching a maximum at 300-600 sec, and the phosphorylation level decreased slightly thereafter. (B) Inhibitory effect of treatment with UO126 (25 μM) on cell migration. Treatment of the sham-sorted KM104^{GFP} and KM104^{GFP}-co(+) with UO126 decreased migration to 80.0±15.3% and 50.1±10.8% of untreated group, respectively (P=0.01). UO126 decreased the migration of NCAFs and CAFs to 86.7±8.9% and 28.1±1.6% of untreated group, respectively (P=0.001).

Table II. Genes hypermethylated in both CAFs.

Gene ID	Gene name
160428	Aldehyde dehydrogenase 1 family, member L2 (ALDH1L2)
8906	Adaptor-related protein complex 1, γ 2 subunit (AP1G2)
55835	Centromere protein J (CENPJ)
89890	Kelch repeat and BTB (POZ) domain containing 6 (KBTBD6)
4081	Mab-21-like 1 (C. elegans) (MAB21L1)
29079	Mediator complex subunit 4 (MED4)
1102	Regulator of chromosome condensation and BTB domain containing protein 2 (RCBTB2)
10019	SH2B adaptor protein 3 (SH2B3)
9882	TBC1 domain family, member 4 (TBC1D4)
5412	Ubiquitin-like 3 (UBL3)

Hypomethylated genes in both CAFs were not found.

on ERK1/2 activation of sorted KM104^{GFP}-co(+). As shown in Fig. 6A, PDGF-BB increased ERK1/2 phosphorylation in both sham-sorted KM104^{GFP} and sorted KM104^{GFP}-co(+) in a time-dependent manner up to a maximum at 300-600 sec, and the phosphorylation level decreased thereafter. The levels of phosphorylated ERK1/2 in both sham-sorted KM104^{GFP}and sorted KM104^{GFP}-co(+) was similar, and this phenomenon was also observed in CAFs and NCAFs isolated from lung cancer tissue.

Treatment of the sham-sorted KM104^{GFP} with UO126 (25 μM), a highly selective inhibitor of both MEK1 and MEK2, decreased migration to only 80.0±15.3% of untreated group, whereas it decreased migration of the sorted KM104^{GFP}-co(+) to 50.1±10.8% of untreated group (P=0.01) (Fig. 6B).

Furthermore, although U0126 decreased the PDGF-BB-induced migration of NCAFs to only 86.7±8.9% of untreated group, it decreased the migration of CAFs to 28.1±1.6% of untreated group (P=0.001).

Discussion

In the current study we demonstrated that fibroblasts that have associated with cancer cells retain their enhanced migration activity for a while after separation from the cancer cells. Furthermore, we first report the possibility that one of the biological characteristics of CAFs would be higher dependence on ERK1/2 signaling for their enhanced migration activity even in the absence of cancer cells.

It is now clear that activation of ERK1/2 is important for PDGF-induced cell migration (31-33). We examined the levels of ERK1/2 phosphorylation of sorted-KM104^{GFP}-co(+) and CAFs after PDGF-BB treatment, but there was no significant difference from the level in the control cells. However, the inhibitory effect of a selective inhibitor of both MEK1 and MEK2 on migration was significantly higher in sorted-KM104^{GFP}-co(+) and CAFs than in control cells. We also investigated the involvement of other MAPK family, including the JNK and P38, however, phosphorylation of these molecules in sorted-KM104^{GFP}-co(+)and CAFs were almost the similar level as those of control cells with or without PDGF-BB stimulation. Moreover, the inhibitory effect of their specific inhibitor SP600125 or SB203580 on cell migration in sorted-KM104^{GFP}-co(+) and CAFs and in untreated cells was almost equal (data not shown). This finding indicated that the fibroblasts that had associated with cancer cells were more dependent on the ERK1/2 pathway for cell migration. Activated ERKs regulate membrane protrusions and focal adhesion dynamics via myosin light chain kinase (MLCK), calpain, paxillin, and focal adhesion kinase (FAK) activation (32), however, the MeDIP-chip analysis did not reveal any promoter CGIs involved in these molecules. After association with cancer cells, pairs of factors that have a synthetic relationship in CAFs may differ from those in naive fibroblasts, thus increasing the dependence of cells on the downstream ERK1/2 pathway. Alternatively, the downstream ERK1/2 signaling pathway may play a more essential and qualitatively different role in a given pathway in CAFs than in naive fibroblasts, making them much more dependent on the activity of a specific signaling pathway. The direct association with cancer cells may cause 'signaling addiction' to occur in fibroblasts, which would be a similar condition explained by the concept of 'oncogene addiction' that some cancer cells are dependent on or addicted to one or a few genes for both maintenance of their malignant phenotype and cell survival (34,35).

DNA methylation is one of the molecular mechanisms that have long-term effects on gene expression. Since the enhanced migration activity of fibroblasts continues after being separated from cancer cells, we speculated that the association with cancer cells might change the methylation status of certain DNA(s) involved in cell migration. MeDIP-chip analysis identified that 10 promoter CGIs (*ALDH1L2, AP1G2, CENPJ, KBTBD6, MAB21L1, MED4, RCBTB2, SH2B3, TBC1D4* and *UBL3*) that were hypermethylated, no promoter CGIs were hypomethylated in both CAFs, and we were unable to identify any well-known methylated genes that were directly involved in cell migration. The cause of the persistent enhanced migration activity remains unknown, and additional research on chromatin modifications, such as histone deacetylation, will be necessary to understand the mechanisms underlying this phenomenon in CAFs.

Jiang *et al* detected global hypomethylation of genomic DNA by means of a methylation-sensitive SNP array analysis (MSNP), a restriction enzyme-based analysis, in the stromal myofibroblasts of gastric carcinomas (36). By contrast, when we focused on promoter CGIs in the current study, the results showed a higher number of hypermethylated genes than hypomethylated genes in CAFs (hypermethylated: 188 CGIs in Case 1 and 98 CGIs in Case 2; hypomethylated: 39 CGIs in Case 1 and 50 CGIs in Case 2). This difference might be caused by CGIs to be focused on. Our data showed for the first time that a few hundred genes were aberrantly hypermethylated in CAFs, suggesting that some regulators can be epigenetically targeted by the cancer microenvironment (37,38).

Cultured fibroblasts from idiopathic pulmonary fibrosis (IPF) were found to migrate faster than those from control fibroblasts, indicating that they also retained enhanced migration activity *in vitro* (39). Since there are many parallels between cancer and chronic active inflammation, as described in the saying, 'tumours are wounds that never heal', enhanced migration may be a common characteristic of fibroblasts recruited into fibrotic lesions.

Our current results may imply that by using this special property of CAFs, strategies that are directed to block the recruitment and tumor-associated functions of CAFs could be a possible method against tumors. Although the exact mechanisms of this phenomenon are not fully understood, cellular and molecular studies on the role of 'educated fibroblasts' should provide novel insights into the pathogenesis of a unique microenvironment of cancers.

Acknowledgements

This study was supported in part by the Grant-in-Aid for Cancer Research (19-10) from the Ministry of Health, Labour and Welfare, the Grant for Scientific Research Expenses for Health Labour and Welfare Programs, the Foundation for the Promotion of Cancer Research, 3rd-Term Comprehensive 10-year Strategy for Cancer Control, and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

References

1. Lin EY, Nguyen AV, Russell RG and Pollard JW: Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 193: 727-740, 2001.
2. Hartel M, Di Mola FF, Gardini A, Zimmermann A, Di Sebastiano P, Guweidhi A, Innocenti P, Giese T, Giese N, Büchler MW and Friess H: Desmoplastic reaction influences pancreatic cancer growth behavior. World J Surg 28: 818-825, 2004.
3. Iwano M, Plieth D, Danoff TM, Xue C, Okada H and Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 110: 341-350, 2002.

4. Ishii G, Sangai T, Oda T, Aoyagi Y, Hasebe T, Kanomata N, Endoh Y, Okumura C, Okuhara Y, Magae J, Emura M, Ochiya T and Ochiai A: Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. Biochem Biophys Res Commun 309: 232-240, 2003.
5. Wels J, Kaplan RN, Rafii S and Lyden D: Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev 22: 559-574, 2008.
6. Sangai T, Ishii G, Kodama K, Miyamoto S, Aoyagi Y, Ito T, Magae J, Sasaki H, Nagashima T, Miyazaki M and Ochiai A: Effect of differences in cancer cells and tumor growth sites on recruiting bone marrow-derived endothelial cells and myofibroblasts in cancer-induced stroma. Int J Cancer 115: 885-892, 2005.
7. Chiba H, Ishii G, Ito TK, Aoyagi K, Sasaki H, Nagai K and Ochiai A: CD105-positive cells in pulmonary arterial blood of adult human lung cancer patients include mesenchymal progenitors. Stem Cells 26: 2523-2530, 2008.
8. Bissell MJ and Radisky D: Putting tumours in context. Nat Rev Cancer 1: 46-54, 2001.
9. Hawsawi NM, Ghebeh H, Hendrayani SF, Tulbah A, Al-Eid M, Al-Tweigeri T, Ajarim D, Alaiya A, Dermime S and Aboussekhra A: Breast carcinoma-associated fibroblasts and their counterparts display neoplastic-specific changes. Cancer Res 68: 2717-2725, 2008.
10. Okusa Y, Ichikura T and Mochizuki H: Prognostic impact of stromal cell-derived urokinase-type plasminogen activator in gastric carcinoma. Cancer 85: 1033-1038, 1999.
11. Tokunou M, Niki T, Eguchi K, Iba S, Tsuda H, Yamada T, Matsuno Y, Kondo H, Saitoh Y, Imamura H and Hirohashi S: c-MET expression in myofibroblasts: role in autocrine activation and prognostic significance in lung adenocarcinoma. Am J Pathol 158: 1451-1463, 2001.
12. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121: 335-348, 2005.
13. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R and Weinberg RA: Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449: 557-563, 2007.
14. Coussens LM, Tinkle CL, Hanahan D and Werb Z: MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 103: 481-490, 2000.
15. Ito TK, Ishii G, Chiba H and Ochiai A: The VEGF angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells. Oncogene 26: 7194-7203, 2007.
16. Hasebe T, Sasaki S, Imoto S and Ochiai A: Proliferative activity of intratumoral fibroblasts is closely correlated with lymph node and distant organ metastases of invasive ductal carcinoma of the breast. Am J Pathol 156: 1701-1710, 2000.
17. Kawase A, Ishii G, Nagai K, Ito T, Nagano T, Murata Y, Hishida T, Nishimura M, Yoshida J, Suzuki K and Ochiai A: Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. Int J Cancer 123: 1053-1059, 2008.
18. Nakao M, Ishii G, Nagai K, Kawase A, Kenmotsu H, Kon-No H, Hishida T, Nishimura M, Yoshida J and Ochiai A: Prognostic significance of carbonic anhydrase IX expression by cancer-associated fibroblasts in lung adenocarcinoma. Cancer 115: 2732-2743, 2009.
19. Nakagawa H, Liyanarachchi S, Davuluri RV, Auer H, Martin EW Jr, de la Chapelle A and Frankel WL: Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. Oncogene 23: 7366-7377, 2004.
20. Tuhkanen H, Anttila M, Kosma VM, Yla-Herttuala S, Heinonen S, Kuronen A, Juhola M, Tammi R, Tammi M and Mannermaa A: Genetic alterations in the peritumoral stromal cells of malignant and borderline epithelial ovarian tumors as indicated by allelic imbalance on chromosome 3p. Int J Cancer 109: 247-252, 2004.
21. Fukino K, Shen L, Matsumoto S, Morrison CD, Mutter GL and Eng C: Combined total genome loss of heterozygosity scan of breast cancer stroma and epithelium reveals multiplicity of stromal targets. Cancer Res 64: 7231-7236, 2004.
22. Weber F, Shen L, Fukino K, Patocs A, Mutter GL, Caldes T and Eng C: Total-genome analysis of BRCA1/2-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. Am J Hum Genet 78: 961-972, 2006.
23. Qiu W, Hu M, Sridhar A, Opeskin K, Fox S, Shipitsin M, Trivett M, Thompson ER, Ramakrishna M, Gorringe KL, Polyak K, Haviv I and Campbell IG: No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. Nat Genet 40: 650-655, 2008.
24. Korc M: Pancreatic cancer-associated stroma production. Am J Surg 194 (Suppl. 4): S84-S86, 2007.
25. Grum-Schwensen B, Klingelhofer J, Berg CH, El-Naaman C, Grigorian M, Lukanidin E and Ambartsumian N: Suppression of tumor development and metastasis formation in mice lacking the S100A4(mts1) gene. Cancer Res 65: 3772-3780, 2005.
26. Hoshino A, Chiba H, Nagai K, Ishii G and Ochiai A: Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells. Biochem Biophys Res Commun 368: 305-310, 2008.
27. Harigaya K and Handa H: Generation of functional clonal cell lines from human bone marrow stroma. Proc Natl Acad Sci USA 82: 3477-3480, 1985.
28. Ishii G, Sangai T, Ito T, Hasebe T, Endoh Y, Sasaki H, Harigaya K and Ochiai A: In vivo and in vitro characterization of human fibroblasts recruited selectively into human cancer stroma. Int J Cancer 117: 212-220, 2005.
29. Takeshima H, Yamashita S, Shimazu T, Niwa T and Ushijima T: The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. Genome Res 19: 1974-1982, 2009.
30. Yamashita S, Hosoya K, Gyobu K, Takeshima H and Ushijima T: Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis. DNA Res 16: 275-286, 2009.
31. Lundberg MS, Curto KA, Bilato C, Monticone RE and Crow MT: Regulation of vascular smooth muscle migration by mitogen-activated protein kinase and calcium/calmodulin-dependent protein kinase II signaling pathways. J Mol Cell Cardiol 30: 2377-2389, 1998.
32. Huang C, Jacobson K and Schaller MD: MAP kinases and cell migration. J Cell Sci 117: 4619-4628, 2004.
33. Lee CK, Lee HM, Kim HJ, Park HJ, Won KJ, Roh HY, Choi WS, Jeon BH, Park TK and Kim B: Syk contributes to PDGF-BB-mediated migration of rat aortic smooth muscle cells via MAPK pathways. Cardiovasc Res 74: 159-168, 2007.
34. Weinstein IB and Joe AK: Mechanisms of disease: Oncogene addiction-a rationale for molecular targeting in cancer therapy. Nat Clin Pract Oncol 3: 448-457, 2006.
35. Weinstein IB and Joe A: Oncogene addiction. Cancer Res 68: 3077-3080, 2008.
36. Jiang L, Gonda TA, Gamble MV, Salas M, Seshan V, Tu S, Twaddell WS, Hegyi P, Lazar G, Steele I, Varro A, Wang TC and Tycko B: Global hypomethylation of genomic DNA in cancer-associated myofibroblasts. Cancer Res 68: 9900-9908, 2008.
37. Fiegl H, Millinger S, Goebel G, Muller-Holzner E, Marth C, Laird PW and Widschwendter M: Breast cancer DNA methylation profiles in cancer cells and tumor stroma: association with HER-2/neu status in primary breast cancer. Cancer Res 66: 29-33, 2006.
38. Hanson JA, Gillespie JW, Grover A, Tangrea MA, Chuaqui RF, Emmert-Buck MR, Tangrea JA, Libutti SK, Linehan WM and Woodson KG: Gene promoter methylation in prostate tumor-associated stromal cells. J Natl Cancer Inst 98: 255-261, 2006.
39. Suganuma H, Sato A, Tamura R and Chida K: Enhanced migration of fibroblasts derived from lungs with fibrotic lesions. Thorax 50: 984-989, 1995.

J Gastroenterol (2010) 45:37–44
DOI 10.1007/s00535-009-0142-7

ORIGINAL ARTICLE—ALIMENTARY TRACT

Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication

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Received: 22 June 2009 / Accepted: 14 September 2009 / Published online: 10 October 2009

Abstract

Background *Helicobacter pylori* (*HP*) infection potently induces aberrant DNA methylation in gastric mucosae, and its accumulation is associated with gastric cancer risk. Cross-sectional analysis of methylation levels (fraction of methylated DNA molecules) and temporal analysis of methylation incidence suggested that methylation levels decrease after *HP* infection discontinues. We aimed to demonstrate the decrease in methylation levels.

Methods Thirty-five patients with *HP* infection who had undergone curative endoscopic resection and 11 healthy volunteers were recruited. Methylation levels were quantified by real-time methylation-specific PCR. Histology was evaluated according to the updated Sydney System.

Results In the 20 patients with successful eradication, the *FLNc* methylation level, along with infiltration of inflammatory cells, decreased from 0.6 to 0.4% at 6 weeks ($P = 0.049$) and remained low at 1 year. The *THBD* methylation level (30.1%) remained high at 6 weeks, but decreased to 19.0% at 1 year ($P = 0.0032$). Nine healthy volunteers with successful eradication tended to show a decrease of both *FLNc* and *THBD* at 6 weeks. However, the methylation levels after the decrease were still higher than those of healthy individuals without *HP* infection. In the 15 patients with persistent infection, the methylation levels remained the same. Before eradication, the *THBD* methylation level correlated with the degree of inflammatory cell infiltration ($P < 0.05$).

Conclusions Methylation levels in gastric mucosae decreased to certain levels after *HP* eradication in profiles unique to individual markers. Involvement of chronic inflammation in methylation induction was suggested.

Keywords DNA methylation · Epigenetics · *Helicobacter pylori* · Eradication · Molecular epidemiology

Electronic supplementary material The online version of this article (doi:10.1007/s00535-009-0142-7) contains supplementary material, which is available to authorized users.

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Introduction

Gastric cancer is one of the most common cancers in the world, especially in eastern Asia [1]. To reduce its morbidity and mortality by early detection and early treatment, identification of individuals with high risk is important. Also, endoscopic resection (ER) is becoming a standard treatment for early gastric cancer [2, 3], and this