

Fig. 3. Correlation between methylation level and cancer risk. Methylation levels of two marker genes (*FLNc* and *HRASLS*) were quantified in gastric mucosae of healthy individuals (healthy V), non-cancerous gastric mucosae of patients with a single gastric cancer (single GC), and non-cancerous gastric mucosae of patients with multiple gastric cancers (multiple GC) (modified from Nakajima et al.⁽⁴⁷⁾). This showed that accumulation levels of specific genes in non-cancerous gastric mucosae can correlate with gastric cancer risk. Taken together with the findings in other types of cancers, quantification of methylation levels in normal-appearing tissues is a promising cancer risk marker that reflects one's own life history.

tissues might be a weak tumor-suppressor gene that does not induce cellular transformation by itself, such as *SFRP1*,⁽⁵³⁾ or might be a passenger that is methylated in parallel with tumor-suppressor genes.

Inducers of methylation in contrast with those of mutations

Epidemiology indicates that cancer is mainly caused by environmental factors,⁽⁵⁴⁾ and identification of inducers of aberrant DNA methylation, in addition to those of mutations, is critically important. However, only limited information is available for the inducers of aberrant methylation.⁽⁵⁵⁾

Inducers of mutations. Clarification of inducers of mutations, namely mutagens, constitutes a large field of science, and comprehensive description is beyond the scope of this article. Simplistically, mutations are induced by exogenous mutagenic factors, such as chemicals and radiation, and endogenous factors, such as oxygen radicals.⁽⁵⁶⁾ Mutagenic chemicals are contained in diverse sources, including tobacco smoke, overcooked food, and many synthetic chemicals.

Inducers of DNA methylation. To identify inducers of aberrant methylation in humans, analysis of non-cancerous tissues is important because the methylation level in non-cancerous tissues reflects how potentially the methylation was induced by a factor (Fig. 1). Aging was the first factor that was identified to promote accumulation of DNA methylation,⁽⁴⁵⁾ and quantification of methylation in non-cancerous colonic tissues contributed to the identification.

Afterwards, the presence of methylation in colonic mucosae of patients with ulcerative colitis indicated that chronic inflammation is an important inducer of methylation.^(57,58) The importance of chronic inflammation was further supported by the presence of methylation in non-cancerous liver tissue of patients with hepatitis,⁽⁴⁸⁾ in inflammatory reflux esophagitis,⁽⁵⁹⁾ and in non-cancerous gastric tissue of individuals infected by *Helico-*

bacter pylori.⁽⁴⁶⁾ However, the molecular mechanisms of how chronic inflammation induces aberrant methylation are almost unknown.

There can be chemicals that induce aberrant DNA methylation, but few chemicals are known. If we want to identify a chemical whose primary mode of action is induction of gene silencing, methylation induction in NFR of multiple genes should be demonstrated. Methylation of an exon can be induced as a result of gene expression change, and methylation of a NFR of a specific gene can be induced as a result of loss of its expression, as described below. One of the reasons why methylation-inducing chemicals have not been identified might be the lack of suitable assay systems, and efforts to develop such systems are being made.^(55,60)

Gene specificity in methylation induction

Mutations are considered to affect random genes, with some preference for actively transcribed genes.^(18,61) Although there is sequence specificity depending on mutagenic factors,⁽⁶²⁾ there is little gene specificity. Many investigators thought that DNA methylation would have a similar nature in random target genes, but it has now been shown that there is strong target gene specificity in methylation induction.

Presence of target gene specificity in methylation induction. It was initially found that specific CGI are methylated in specific tumor types, and the presence of gene specificity for methylation induction was indicated.^(27,37) However, analysis of a cancer tissue reveals only events in its single precursor cell, and the information obtained is very stochastic. Analysis of a panel of cancers can reflect events in the precursor cells of the cancers, but the number of precursor cells analyzed is still limited to the number of cancers analyzed.

In order to avoid selection bias by gene function, and to analyze as many cells as possible, analysis of a non-cancerous tissue is advantageous. We analyzed methylation of a panel of genes in gastric mucosae with and without *H. pylori* infection, and showed that specific genes are methylated in gastric mucosae with *H. pylori* infection.⁽⁶³⁾ We also analyzed the methylation levels of a panel of genes in esophageal mucosae, and found that specific genes are methylated in correlation with smoking history.⁽⁴⁰⁾ These showed that specific inducers of aberrant DNA methylation induce methylation of specific genes. The presence of a "methylation fingerprint" of individual methylation inducers suggests that the fingerprint can be used as a marker for past exposure to specific carcinogenic factors in our lives.

Molecular mechanisms of target gene specificity. As a molecular mechanism for gene specificity, low transcription was suggested in pioneering studies that used an exogenously introduced gene and endogenous genes demethylated by a demethylating agent.^(64,65) Analysis of selected genes in embryonic stem cells, along with normal adult tissue, and cancer cells revealed that genes marked with trimethylation of histone H3 lysine 27 (H3K27me3) in embryonic stem cells are likely to become methylated in cancers.⁽⁶⁶⁻⁶⁸⁾ The finding was further supported by a genome-wide analysis of genes with H3K27me3 in cancer cells and corresponding normal cells.⁽¹⁹⁾

In addition to these factors that confer susceptibility to DNA methylation, the presence of RNA polymerase II (pol II), active or stalled, in NFR was shown to confer resistance to DNA methylation.⁽³⁴⁾ Although the presence of active histone modifications also confers resistance, the effect of active histone modifications was overridden by the presence of pol II in multivariate analysis, suggesting that the presence of pol II is the final effector that protects NFR from DNA methylation. Taken all together, DNA methylation of NFR is protected by the presence of pol II regardless of transcription levels, and promoted by the

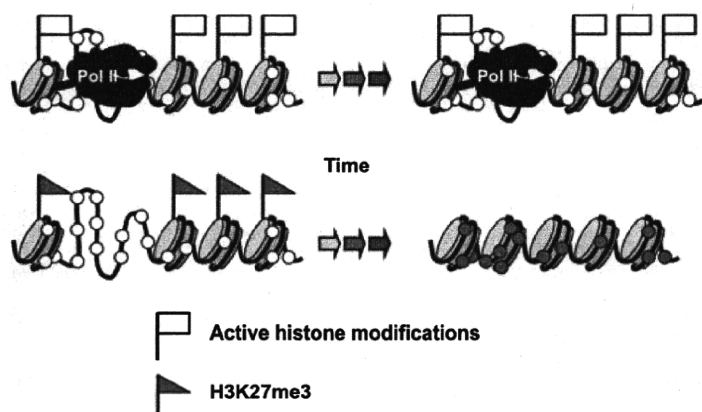


Fig. 4. Determinants of methylation destiny. Genes with RNA polymerase II (pol II), active or stalled, are resistant to DNA methylation, and genes with H3K27me3 are susceptible to DNA methylation. The presence of pol II is associated with the presence of active histone modifications, even if a gene is not actively transcribed. Open and closed circles show unmethylated and methylated CpG sites, respectively.

presence of H3K27me3 (Fig. 4). Once DNA methylation is induced in susceptible NFR, the H3K27me3 mark almost disappears⁽¹⁹⁾ or decreases to a very low level.⁽⁶⁹⁾

Reversibility of alterations

One of the major differences, or most important difference, between mutations and DNA methylation is reversibility. Physiologically, epigenetic modifications undergo dynamic changes during development, differentiation, and reprogramming.^(70,71) In somatic cells the demethylating agents 5-azacytidine and 5-aza-2'-deoxycytidine have long been used in the laboratory.⁽⁷²⁾ Now these agents have come into clinics and are showing very promising effects in hematological malignancies.⁽⁷³⁾ The detailed pharmacological mechanisms and usage are summarized in the reviews cited above.

Future perspectives

Now, unique characteristics of DNA methylation are clear, but many questions still remain. Are there any chemicals that induce aberrant methylation of NFR directly, not as a result of gene expression changes? How does chronic inflammation induce aberrant DNA methylation? Do we know enough about the determinants of gene specificity?

At the same time, the biomedical application of DNA methylation is becoming more promising. The large number of genes

methylated in a cancer increases the chance of successful identification of methylation biomarkers to predict patient prognosis and response to therapeutics. Cancer-specific methylation can be used for detection of cancer cells. The presence of an epigenetic field for cancerization in normal-appearing tissues can be used as a cancer risk marker, which reflects one's own life history. The deep involvement of chronic inflammation in methylation induction indicates that suppression of components involved in the induction can be utilized as a target of cancer prevention. The methylation fingerprint can be used in epigenetic epidemiology.

Mutations have not been considered as a cause of disorders that involve irreversible alteration of cellular functions, such as neurodegenerative disorders, diabetes, immunological disorders, and renal disorders. This was because mutations are rare events and cannot affect as many cells as the function of a tissue is affected as a whole. However, methylation can be induced in many more cells in a tissue, and genes affected are specific. This suggests that a critical gene can be inactivated in a significant fraction of cells, and raises the possibility that aberrant DNA methylation is causally involved in chronic disorders other than cancers.

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Methylation destiny

Moira takes account of histones and RNA polymerase II

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Key words: epigenetics, aberrant DNA methylation, *H. pylori*, tobacco smoking, histone modification, RNA polymerase II

Abbreviations: CGI, CpG island; *H. pylori*, *Helicobacter pylori*; MeDIP, methylated DNA immunoprecipitation; H3K27me3, trimethylation of histone H3 lysine27; PRC, polycomb repressive complex; DNMT, DNA methyltransferase; H3K9me3, trimethylation of histone H3 lysine9; H3Ac, acetylation of histone H3; H3K4me3, trimethylation of histone H3 lysine4; TSS, transcription start site; NFR, nucleosome free region; Pol II, RNA polymerase II

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A aberrant DNA methylation is deeply involved in various human disorders. Contrary to our initial expectation, aberrant methylation is now known to possess several unique characteristics different from mutations, including target gene specificity. Specific cancers have methylation of specific genes and specific inducers of methylation, such as *Helicobacter pylori* infection, induce methylation of specific genes. Mechanistically, it has been known that low levels of transcription of a gene promote its methylation. Multiple studies have shown that high levels of trimethylation of histone H3 lysine27 in normal cells are associated with a risk of becoming methylated during carcinogenesis. We recently demonstrated that genes with high levels of binding of RNA polymerase II, regardless of transcription levels, are resistant to induction of aberrant methylation. Now, epigenetic destiny can be predicted by these factors and interference with these factors might be able to change the destiny.

Introduction

DNA methylation of a promoter CpG island (CGI) causes silencing of its downstream gene by multiple mechanisms.¹ When aberrant methylation occurs in promoter CGIs of genes involved in human disorders, such as tumor-suppressor genes, it inactivates these genes and is causally involved in human disorders.²⁻⁴ To inactivate tumor-suppressor genes, aberrant methylation is an alternative mechanism to point mutations and chromosomal losses.^{5,6} Historically, inactivation of

tumor-suppressor genes by mutations was discovered more than a decade earlier than inactivation by aberrant methylation, and characteristics of aberrant methylation were assumed to be similar to those of mutations.

However, recent studies have revealed that aberrant DNA methylation possesses unique characteristics different from mutations,⁷ such as deep involvement of chronic inflammation in its induction,⁸ target gene specificity in its induction,^{9,10} the presence at high levels in non-cancerous tissues¹¹⁻¹³ and a large number of affected genes in a single cancer cell.¹⁴⁻¹⁶ In a generally accepted multistep carcinogenesis model, a mutation is induced in random genes, with some preference of expressed genes, in a population of cells, and a cell that accidentally harbored mutation of a specific gene, such as tumor-suppressor gene, is selected.¹⁷ In contrast, methylation is now recognized to be induced in specific genes in specific types of cancers^{14,18,19} and by specific inducers, such as *Helicobacter pylori* (*H. pylori*) infection¹¹ and tobacco smoking.¹⁰ Here, we will focus on the presence of target gene specificity in methylation induction and the mechanisms involved in it.

DNA Methylation of Specific Genes in Cancers

The presence of target gene specificity in DNA methylation induction was initially indicated by the presence of methylation of specific genes in cancer cells.^{14,18,19} A pioneering study of 1,184 non-biased CGIs using restriction landmark genomic scanning revealed that some specific CGIs

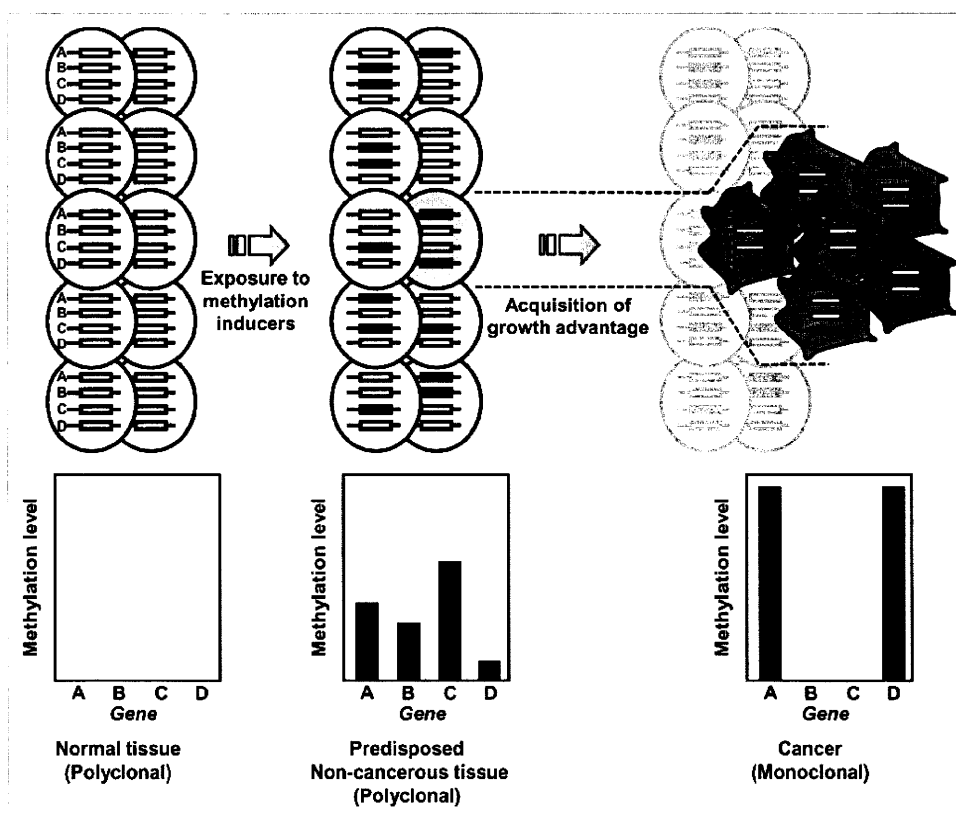


Figure 1. Different meaning of aberrant DNA methylation in non-cancerous and cancer tissues. Cells in an entirely normal tissue contain no aberrant methylation, and, by exposure to methylation inducers, cells come to harbor aberrant methylation of specific genes. A cancer, consisting of many cancer cells, develops from a single precursor cell that contains aberrant methylation of a tumor-suppressor gene (gene D). Since aberrant methylation of a tumor-suppressor gene confers growth advantage, all the cancer cells have its methylation even if it is not a specific target for methylation induction and is rarely induced in non-cancerous tissues. In contrast, methylation of genes whose inactivation does not confer growth advantage (genes A, B and C) is stochastically carried over into a cancer tissue. Therefore, a methylation pattern in a cancer tissue reflects events that incidentally happened in its single precursor cell, and target gene specificity can be assessed only by analyzing a large number of cancers. In contrast, a methylation pattern in a non-cancerous tissue reflects events that happened in any of the many cells in the tissue, and target genes have high levels of methylation.

were methylated at high incidences in specific tumor types among seven tumor types.¹⁴ Analysis of promoter CGIs of mostly tumor-suppressor genes also showed that some CGIs were methylated at high incidences in specific tumor types.¹⁸ A comprehensive analysis of colon cancers using the modern technology of methylated DNA immunoprecipitation (MeDIP)-microarray analysis revealed that most methylated genes were located within defined genomic clusters, were associated with common sequence motifs, belonged to specific functional categories, and had low transcription levels already in normal cells.¹⁹

However, analysis of cancer cells always raises a question about the role of a gene inactivated in a cancer. "Did

the inactivation confer a growth advantage to a cell with it, and thus was the cell selected?" Since inactivation of different sets of genes is expected to confer growth advantage to cells of different tissues, DNA methylation of different sets of genes can be simply explained by functional selection, rather than by methylation induction of specific genes in a specific tissue context (Fig. 1). To avoid this limitation, analysis of non-cancerous tissues where functional selection has not taken place yet is a good solution. Also, it enables us to analyze numerous independent events in different cells while analysis of a cancer, a monoclonal lesion, provides information on the events that took place in a single precursor cell of the cancer.

The Presence of Aberrant DNA Methylation in Non-Cancerous Tissues

Again, going back to the era of mutations, it was a challenging idea to analyze mutations in non-cancerous tissues. Since mutations are present only in a very minor fraction of cells in non-cancerous tissues (1 of 10^3 to 10^5 cells), they cannot be detected by ordinary sequencing techniques and their frequency can be measured only by special methods that introduce positive selection of mutants.²⁰ Instead, if a sequence polymorphism is detected in a cancer tissue, it can be established as a mutation by confirming its absence in the surrounding non-cancerous tissue of the same individual.²¹

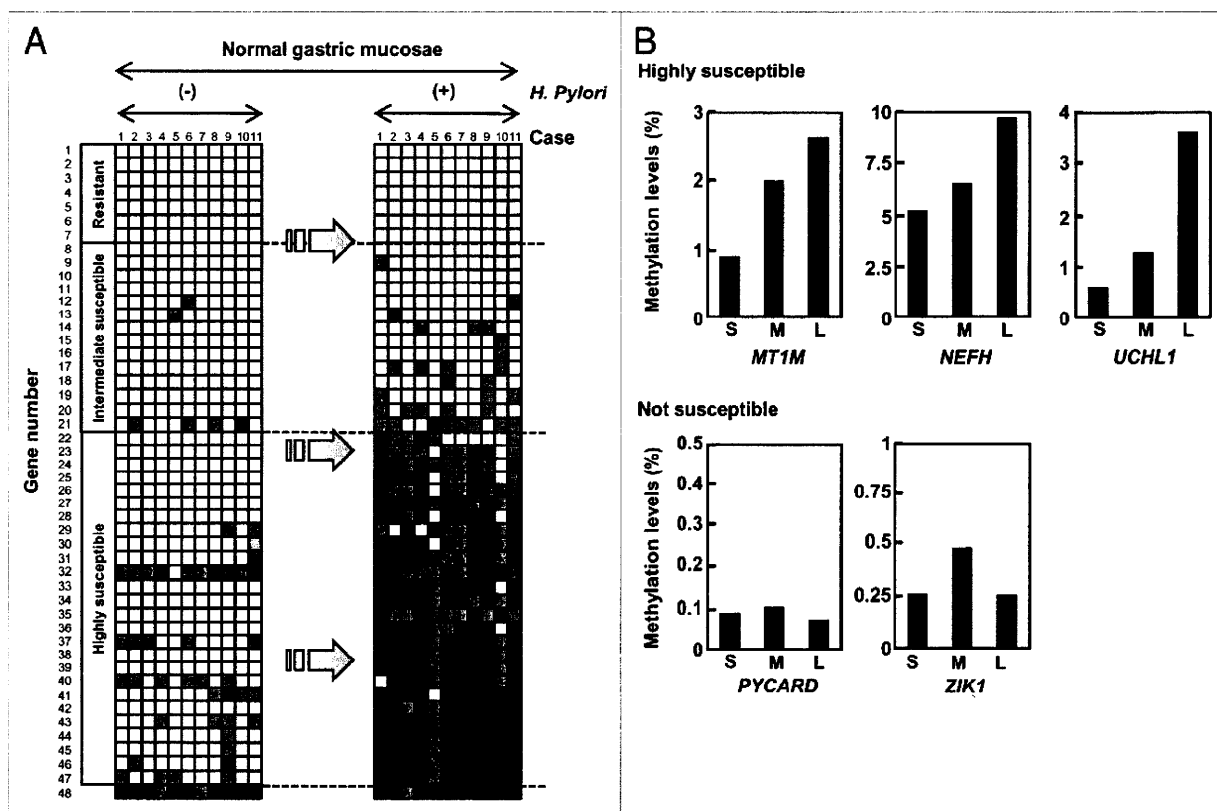


Figure 2. Target gene specificity in DNA methylation induction in non-cancerous tissues. (A) Methylation profile of the 48 genes in normal gastric mucosae with and without *H. pylori* infection (modified from Nakajima et al.⁹). The methylation status of each gene is represented as unmethylated (white), weakly methylated (gray), and highly methylated (black). Seven genes (gene 1 to 7) were completely resistant to aberrant methylation induction. Fourteen genes (gene 8 to 21) were slightly susceptible to methylation induction. Twenty-six genes (gene 22 to 47) were highly susceptible to methylation induction. (B) The correlation between smoking history and aberrant methylation in specific genes (modified from Oka et al.¹⁰). Average methylation levels in non-cancerous esophageal mucosae of individuals with short (S, no or smoking duration <21 years), middle (M, smoking duration; <40 years but more than 21 years), and long (L, smoking duration is more than 40 years) smoking history. *MT1M*, *NEFH* and *UCHL1* were considered to be susceptible to methylation induction by smoking.

In contrast, researchers in cancer epigenetics field became aware that aberrant DNA methylation could be detected in a minor fraction of cells, even in non-cancerous tissues.²²⁻²⁶ Different from mutations, methylation is physiologically present in various regions of the genome and, to demonstrate that methylation of a genomic region is *aberrant*, its absence in the corresponding normal tissue needs to be established. Even adopting this stringent criterion, aberrant methylation was detected in histologically normal non-cancerous liver tissues of patients with a liver cancer²² and in non-cancerous gastric epithelia of patients with a gastric cancer.²³ Possible aberrant methylation was detected in Barrett's esophagus,²⁴ colonic mucosae

of patients with ulcerative colitis²⁵ and gastric tissue of gastric cancer patients.²⁶

To connect the presence of aberrant DNA methylation in non-cancerous tissue to cancer risk, we systematically collected samples from gastric tissues of entirely healthy individuals and non-cancerous gastric tissues of gastric cancer patients, and quantified methylation levels in individual samples.^{11,13} Methylation levels were about 5- to 300-fold higher in the latter samples than in the former samples, among individuals without *H. pylori* infection. At the same time, *H. pylori* infection, a potent risk factor for gastric cancers,²⁷ was associated with temporarily high levels of methylation.^{11,28} Other studies also showed that aberrant methylation is already accumulated in non-cancerous

tissues, and that the accumulation is associated with cancer risk in multiple types of cancers,¹² such as esophageal,²⁹ breast³⁰ and renal cancers.³¹

Target Gene Specificity of Methylation Induction in Non-Cancerous Tissues

It is now clear that aberrant DNA methylation is present in non-cancerous tissues. And, we can analyze methylation induction in a large number of cells, although methylation levels are expected to be low, compared with those in cancers (Fig. 1). However, only limited numbers of specific inducers of aberrant methylation have been established so far,⁸ including *H. pylori* infection,^{9,11} hepatitis virus³²

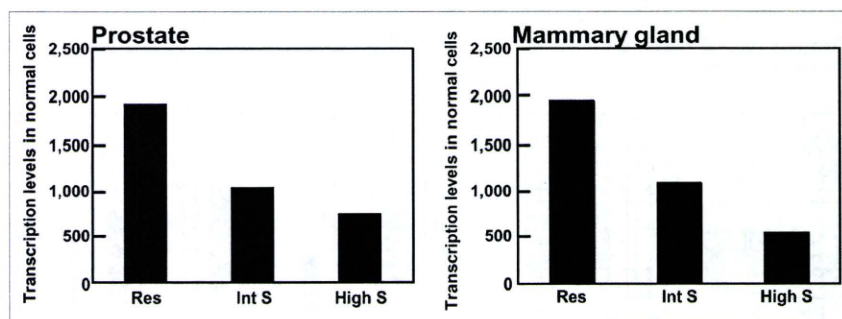


Figure 3. Low transcription levels of genes susceptible to DNA methylation induction (modified from Takeshima et al.³⁹). Genes were classified into those resistant (Res), intermediately susceptible (Int S), and highly susceptible (High S) to methylation induction during carcinogenesis. Their transcription levels in normal prostatic epithelial cells (left) and normal mammary epithelial cells (right) were measured by expression microarray. A gradual decrease of transcription levels in genes with higher susceptibility was observed.

and tobacco smoking.¹⁰ In the case of *H. pylori* infection, we recently demonstrated that inflammation induced by it is critical for methylation induction.³³ To reveal the target gene specificity in aberrant DNA methylation induction by *H. pylori*, we sensitively analyzed methylation of 48 genes, which can be methylated at least in gastric cancer cell lines,³⁴ in human gastric mucosae with and without *H. pylori* infection (Fig. 2A).⁹ It was clearly shown that some genes were susceptible to methylation induction by *H. pylori* infection while others were resistant. The susceptible genes had lower transcription levels in normal gastric mucosae than the resistant genes. Target gene specificity by tobacco smoking was also present in esophageal mucosae. When we quantified methylation levels of 13 genes, which can be methylated in esophageal cancers, methylation levels of only five genes had significant correlations with duration of tobacco smoking (Fig. 2B).¹⁰

Role of Low Transcription in Target Gene Specificity

Regarding the mechanisms underlying the target gene specificity, low transcription in normal cells was proposed in the early 2000s.³⁵⁻³⁸ As mechanistic analyses in vitro, Song et al. demonstrated that disruption of promoter activity (thus low transcription levels) of a transfected gene leads to aberrant DNA methylation of promoter CGIs in a cancer cell line.³⁵ Using an endogenous gene demethylated by a DNA demethylating agent,

5-aza-2'-deoxycytidine, de Smet et al. demonstrated that the demethylated gene becomes remethylated when it is not transcribed.³⁶ As for findings in vivo, we showed that most genes methylated in pancreatic cancers and malignant melanomas had no or low transcription levels in their normal counterpart cells.^{37,38} Genome-wide studies using microarrays in colorectal, prostate, and breast cancers also showed that genes with low transcription in normal cells tend to be methylated in cancers (Fig. 3).³⁹ Even using genes methylated in non-cancerous tissues, genes susceptible to aberrant methylation had lower transcription levels than resistant genes.⁹

Role of Histone Modifications in Target Gene Specificity

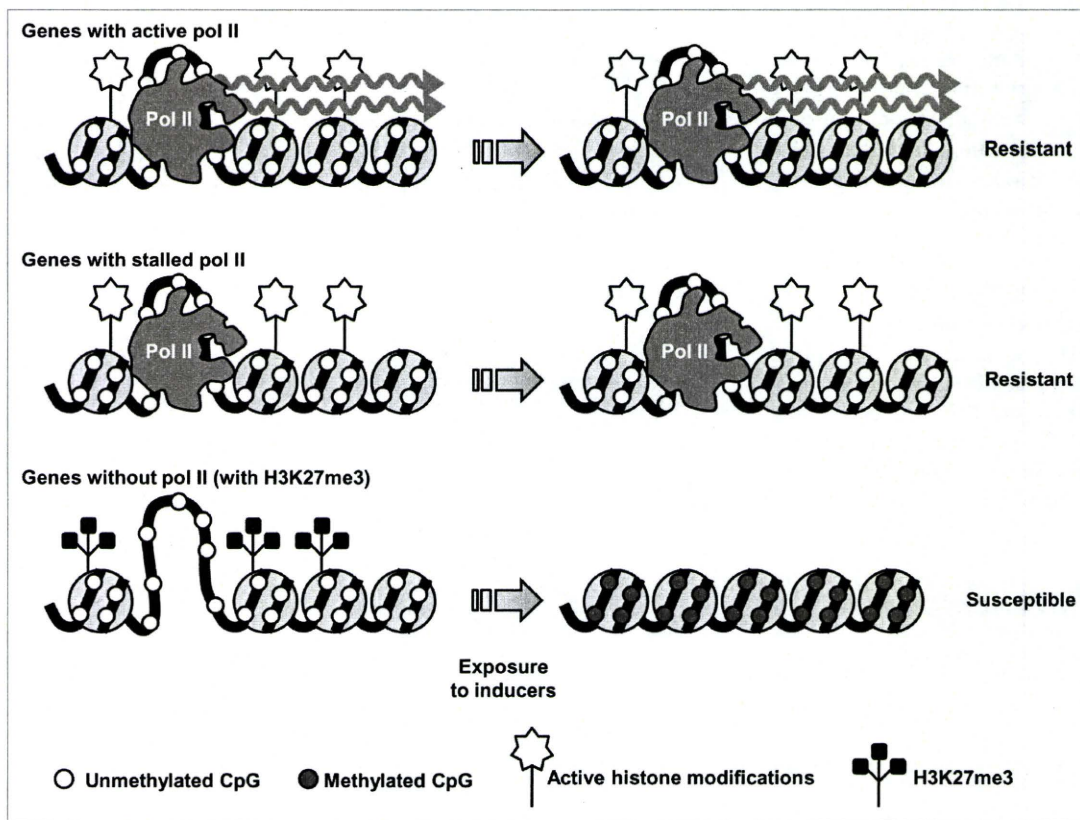
As another mechanism for the target gene specificity, histone modifications have drawn a lot of attention over the last couple of years. Using selected genes, three groups demonstrated that genes methylated in cancers are pre-marked by trimethylation of histone H3 lysine 27 (H3K27me3) in embryonic stem cells⁴⁰⁻⁴² and normal corresponding tissue.⁴² Pre-mark by H3K27me3 of genes that will become methylated in cancers was further confirmed using genes identified by DNA methylation microarray analysis.^{39,43,44} H3K27me3 is known to be recognized by a polycomb repressive complex (PRC).⁴⁵⁻⁴⁷ A component of PRC2, EZH2, and, that of PRC1, CBX7, are known to interact with DNA methyltransferases (DNMTs),^{48,49} and there is a

possibility that H3K27me3 functions as a recruiting signal for DNMTs. Another representative repressive histone modification, trimethylation of histone H3 lysine 9 (H3K9me3), in normal cells was not associated with genes that become methylated during carcinogenesis.³⁹

Regarding histone modifications of active chromatin, we observed that genes resistant to aberrant DNA methylation tend to have acetylation of histone H3 (H3Ac) and trimethylation of histone H3 lysine4 (H3K4me3) in normal cells.³⁹ Active histone modifications are known to be recognized by proteins involved in transcriptional activation, such as ATP-dependent chromatin remodeling complex, SWI/SNF⁵⁰ and the basal transcription factor, TFIID,⁵¹ and are associated with high levels of transcription. The resistance of genes with active histone modifications to methylation induction may be dependent upon high levels of gene transcription.

Role of RNA Polymerase II Binding, Active or Stalled, in Target Gene Specificity

Although genes with low transcription levels are susceptible to DNA methylation induction, many such genes are still resistant. Even if limited to genes that have low transcription and H3K27me3 in normal cells, 16% of them are still resistant to methylation induction during carcinogenesis.³⁹ This indicates that there are additional factors that confer resistance to methylation induction. At individual gene



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Figure 4. A scheme of the instructive mechanism of aberrant DNA methylation induction. Both genes with active Pol II and genes with stalled Pol II are resistant to aberrant methylation induction. In contrast, genes without Pol II but with H3K27me3 are highly susceptible to aberrant methylation induction.

levels, SP1/3 and MLL have been reported to be involved in resistance of the *APRT* and *Hoxa9* genes, respectively, to methylation induction.⁵²⁻⁵⁴

A region just upstream of a transcription start site (TSS), designated as a nucleosome-free region (NFR),⁵⁵ is most resistant to DNA methylation induction,⁵⁶ indicating that something there is associated with resistance to methylation induction. Recent studies showed that RNA polymerase II (Pol II) is stalled at NFRs for some genes with low transcription levels,^{57,58} and we decided to focus on Pol II as a factor that confers resistance to methylation induction. Genome-wide analysis of histone modifications and Pol II binding in normal cells revealed that, even among genes with low transcription, high levels of Pol II binding and active histone modifications were associated with resistance to methylation induction during carcinogenesis.³⁹ By multivariate analysis,

Pol II binding had stronger influence on the resistance than active histone modifications. These results showed that the presence of Pol II, active (high transcription levels) or stalled (low transcription levels), is associated with resistance to methylation induction during carcinogenesis (Fig. 4).

Pol II forms a large complex with several general transcription factors,⁵⁹ and such a large complex around NFRs might inhibit the recruitment of DNMTs. Further analysis is needed to establish cause-consequence relationship between the presence of Pol II and resistance to DNA methylation induction, and to clarify molecular mechanisms of why genes with high Pol II binding are resistant to methylation induction.

Concluding Remarks

The presence of target gene specificity in DNA methylation induction indicates that a methylation profile specific to a

carcinogenic factor can be used as a methylation fingerprint that tells past exposure to the factor. Since target genes are pre-marked by the presence of H3K27me3 and the absence of Pol II binding, methylation fingerprints are likely to be present for various inducers of aberrant methylation. Methylation fingerprints in individual tissues are likely to become available in the coming years and, if such fingerprints are also present in peripheral leukocytes, a new field of epigenetic epidemiology will be opened up.

The fact that genes with active transcription are resistant to DNA methylation induction can be rephrased as “iron (a gene) rusts (is methylated) from disuse (without Pol II binding),” or “use it, or lose it.” An important implication is that we might be able to protect a gene from becoming methylated by bringing Pol II to it. Although the distribution of H3K27me3 is likely to be predetermined

by cell types, the distribution of Pol II is considered to be modifiable, for example by inducing gene transcription. If we can develop a method, including use of chemicals, which can change the thread by Goddess Moira, it is likely to be a novel method for disease prevention by keeping our epigenome fresh.

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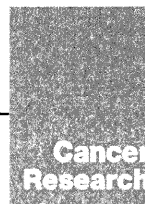
ヒトがんにおけるエピジェネティックな異常の
解明と応用に関する研究

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Inflammatory Processes Triggered by *Helicobacter pylori* Infection Cause Aberrant DNA Methylation in Gastric Epithelial Cells

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Abstract

Altered patterns of DNA methylation associated with *Helicobacter pylori* (*HP*) infection of gastric epithelial cells are thought to contribute to gastric cancer risk. However, it is unclear whether this increased risk reflects an infection-associated inflammatory response or the infection itself. In this study, we sought to clarify mechanisms in a gerbil model of gastric cancer where we showed that *HP* infection is causally involved in induction of aberrant DNA methylation. By genome-wide screening, CpG islands that were aberrantly methylated in gerbil gastric cancer cell lines were isolated, and 10 islands were shown to be specifically methylated only in gastric mucosae infected with *HP*. By temporal analysis, methylation levels in gastric epithelial cells started to increase at 5 to 10 weeks after infection and reached high levels by 50 weeks. When *HP* was eradicated, methylation levels markedly decreased 10 and 20 weeks later, but they remained higher than those in gerbils that were not infected by *HP*. Expression levels of several inflammation-related genes (*CXCL2*, *IL-1 β* , *NOS2*, and *TNF- α*) paralleled the temporal changes of methylation levels. Significantly suppressing inflammation with the immunosuppressive drug cyclosporin A did not affect colonization by *HP* but blocked the induction of altered DNA methylation. Our findings argue that DNA methylation alterations that occur in gastric mucosae after *HP* infection are composed of transient components and permanent components, and that it is the infection-associated inflammatory response, rather than *HP* itself, which is responsible for inducing the altered DNA methylation. *Cancer Res*; 70(4): 1430–40. ©2010 AACR.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGI) is one of the major inactivating mechanisms of tumor-suppressor genes and is deeply involved in human carcinogenesis (1). Nevertheless, there is only limited information on its inducers and induction mechanisms. Chronic inflammation, known to promote certain types of cancers (2), is one of the possible inducers of aberrant methylation. The presence of aberrant methylation is frequently observed in non-cancerous tissues of patients with inflammation-associated cancers, such as liver cancers, ulcerative colitis-associated colon cancers, and gastric cancers (3–7). However, a causal role of chronic inflammation in methylation induction remains to be established.

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In human gastric mucosae, the presence of *Helicobacter pylori* (*HP*) infection, a well-known inducer of chronic inflammation and gastric cancers (8, 9), is associated with high methylation levels or high incidences of methylation (5, 10–12). In addition, among individuals without *HP* infection, noncancerous gastric mucosae of gastric cancer patients have higher methylation levels than gastric mucosae of healthy individuals (5, 10). In addition, eradication of *HP* leads to a decreased incidence of *CDH1* (*E-cadherin*) promoter methylation (11, 13, 14). These findings suggest that *HP* infection induces aberrant methylation in gastric mucosae and indicate that levels of accumulated methylation are associated with gastric cancer risk. However, because infection experiments are impossible in humans, it needs to be clarified in animal models whether or not *HP* infection induces methylation and what mechanisms are involved.

HP infection in humans is best modeled in Mongolian gerbils (*Meriones unguiculatus*). As in man, *HP* infection induces severe inflammation in gerbil gastric mucosae and promotes gastric carcinogenesis induced by administration of *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N*'-nitrosoguanidine (15). The incidence of gastric cancers in gerbils depends on the duration of *HP* infection, and eradication of *HP* significantly reduces the incidence (16), as in man (17, 18). Thus, we can expect that the gerbil model is also useful in analyzing whether *HP* infection induces aberrant methylation and what mechanisms are involved *in vivo*. However, unfortunately,

little information is available for the gerbil genome, and the genetic and molecular analysis of this model has been hampered.

In this study, we aimed to show that *HP* infection is causally involved in induction of aberrant DNA methylation and to clarify a critical factor involved. For this, we first isolated CGIs that could be methylated in gerbil gastric cancers by a genome-wide screening method, methylation-sensitive representational difference analysis (MS-RDA). Using the CGIs isolated, we then showed that methylation was induced specifically in gerbils with *HP* infection and that inflammation induced by *HP* infection, not *HP* itself, was critically involved in methylation induction.

Materials and Methods

Cell lines. Two gerbil gastric cancer cell lines, MGC1 and MGC2, were established from a single gastric cancer induced in a gerbil by MNU and *HP* infection (19). They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum on a type I collagen-coated dish (Asahi Techno Glass). Although we did not check the cross-contamination of cell lines biochemically or genetically just before use, they had the same morphology and growth rates as described previously (19).

Animal experiments and sample preparation. Male Mongolian gerbils (MGS/Sea) were purchased from Kyudo. To induce gastric cancers, male gerbils were administered with 30 ppm of MNU (Sigma-Aldrich) in drinking water for a week at 7, 9, 11, 13, and 15 wk of age, and then inoculated with *HP* (ATCC 43504, American Type Culture Collection) by gavage at 17 wk of age (20). At 57 wk, gerbils were sacrificed and stomachs were resected. Because it was difficult to identify cancers macroscopically in gastric mucosae with severe hyperplasia, we dissected an area of gastric cancer tissue by an apparatus for laser microdissection (ASLMD, Leica Microsystems) after histologic confirmation. For temporal analysis of methylation levels, male gerbils were inoculated with *HP* (ATCC 43504) at 5 wk of age. Eradication therapy was done at 55 wk of age by administering amoxicillin, clarithromycin, and lansoprazole by gavage (20). Gerbils that had *HP* after the eradication therapy were excluded from analysis. As a vehicle control, 0.5% of carboxymethyl cellulose was given by gavage. To suppress gastritis, gerbils were administered with 250 µg/mL cyclosporin A (CsA; Neoral, Novartis Pharma) in drinking water for 20 wk. The stomach was resected and cut along the greater curvature. From the posterior wall of the pyloric region (pyloric antrum), which contains the pyloric glands, gastric epithelial cells (GEC) were isolated by the gland isolation technique (21). The anterior wall of the pyloric region was further cut into two pieces: one for RNA and DNA extraction from a sample with mucus and mucosal and submucosal layers and the other for histologic analysis. Whole blood was obtained from the inferior vena cava. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Human clinical samples. Human gastric mucosae were obtained by endoscopic biopsy from 10 *HP*-negative (five

men and five women; average age 42.4 y, ranging from 29 to 56 y) and 10 *HP*-positive (four men and six women; average age 42.4 y, ranging from 23 to 53 y) healthy volunteers, whose *HP* status had been judged by a serum anti-*HP* antibody test (SBS). Gastric cancer samples were obtained from surgical specimens from 14 patients who underwent gastrectomy due to early gastric cancers (seven men and seven women; average age 65.9 y, ranging from 47 to 79 y). Sampling was conducted under the approval of Institutional Review Boards.

Nucleic acid extraction. From tissue sections, DNA was extracted by heating the dissected sections at 100°C for 20 min at pH 12, followed by phenol/chloroform extraction (22). From isolated glands, DNA was extracted by proteinase K digestion and the phenol/chloroform method. From the whole blood, DNA was extracted with a QuickGene DNA whole blood kit (Fujifilm). RNA was isolated with Isogen (Wako).

Quantitative PCR for gene expression analyses and HP detection. To analyze gene expression levels, cDNA was synthesized from 2 µg of DNase-treated RNA with an oligo-d(T)₁₂₋₁₈ primer. Real-time PCR using gene-specific primers (Supplementary Table S1) and SYBR Green Real-time PCR Master Mix (TOYOBO) was done, and the amplification curve of a sample was compared with curves of standard DNA samples with known copy numbers. Standard DNA samples were prepared by serial dilution of a PCR product or a plasmid containing a cloned PCR fragment after its quantification. Gene expression levels were normalized to that of *Gapdh*. To measure the amount of *HP*, real-time PCR using specific primers for the *jhpr3* gene of *HP* was carried out and normalized to the gerbil *Il4* gene (Supplementary Table S1).

Methylation-sensitive representational difference analysis. MS-RDA is a subtraction method that can identify differentially methylated loci between two genomes independent of genomic information (23) and was done using *HpaII* or *SacII* methylation-sensitive restriction enzyme as described previously (24). The final PCR product was cloned into pGEM T-Easy (Promega) and sequenced. If a DNA fragment had a CpG score ≥ 0.65 and G + C content $\geq 55\%$, the fragment was considered to be derived from a CGI. To identify homologous regions in mice and men, database searches were carried out at a GenBank web site.

Methylation analysis. Fully methylated and fully unmethylated controls were prepared by methylating genomic DNA with *SssI* methylase (New England Biolabs) and amplifying genomic DNA with $\phi 29$ DNA polymerase (GenomiPhi DNA Amplification Kit, GE Healthcare), respectively (25). One microgram of DNA digested with *BamHI* was treated with sodium bisulfite and suspended in 80 µL of Tris-EDTA (TE) buffer as described previously (22). In the case of paraffin-embedded samples, DNA was treated with sodium bisulfite without *BamHI* digestion and suspended in 20 µL of TE buffer. One microliter of aliquot was used as a template for methylation-specific PCR (MSP) and bisulfite sequencing. Conventional MSP and bisulfite sequencing were done with specific primer sets (Supplementary Table S2) as described previously (22). Quantitative MSP (qMSP) was done

Table 1. CGIs methylated in gerbil gastric cancer cell lines and *HP*-infected GECs

Clone name	GenBank accession no.	Genomic location deduced from analyses using human or mouse genome database	Nucleotide position in human or mouse sequences
HE6	AB429514	Exon 2 of <i>Ntrk2</i> gene*	16,449,514–16,449,840 bp in NT_023935.17 (human chr. 9)
HG2	AB429515	Exon 1 of <i>Gpr37</i> gene*	49,589,571–49,589,704 bp in NT_007933.14 (human chr. 7)
SA9	AB429516	Exon 1 of <i>Nol4</i> gene*	13,292,105–13,292,430 bp in NT_010966.13 (human chr. 18)
SB1	AB429517	Intergenic region between <i>Sp4</i> and <i>Sp8</i> genes*	20,698,454–20,698,697 bp in NT_007819.16 (human chr. 7)
SB5	AB429513	Not identified	Not identified
SC3	AB429518	Promoter region of <i>Rnf152</i> gene*	7,352,575–7,352,875 bp in NT_025028.13 (human chr. 18)
SD2	AB429519	Promoter region of <i>Nptx2</i> gene*	23,480,374–23,480,422 bp in NT_007933.14 (human chr. 7)
SE3	AB429520	Intron 1 of <i>Slc35f1</i> gene*	39,311,942–39,312,270 bp in NT_001838990.2 (human chr. 6)
SF12	AB429521	Intergenic region between <i>Cntn1</i> and <i>Pdzr4</i> genes	53,513,634–53,513,936 bp in NT_039621.7 (mouse chr. 15)
SH6	AB429522	Intergenic region between <i>Sox1</i> and <i>Loc729095</i> gene*	213,253–213,298 bp in NT_027140.6 (human chr. 13)

*Conserved regions identified in the human database.

by real-time PCR using primers specific to DNA molecules methylated at a locus and to a repeat sequence. Methylation levels were expressed as a percentage of the methylated reference, which was obtained as [(number of methylated fragments of a target CGI in sample) / (number of repeat sequences in sample)] / [(number of methylated fragments of a target CGI in *SssI*-treated DNA) / (number of repeat sequences in *SssI*-treated DNA)] × 100. As a repeat sequence, the B2 repeat was used for gerbil DNA (ref. 26; Supplementary Table S2 and Supplementary Fig. S1) and the *Alu* repeat was used for human DNA (27).

Statistical analysis. Statistical analyses were conducted with SPSS 13.0J (SPSS Japan, Inc.). To evaluate significant difference between two independent groups of sample data, the Mann-Whitney *U* test was used. Spearman's rank correlation coefficient (*r*) was used to measure correlation.

Results

Identification of CGIs specifically methylated by *HP* infection in GECs of Mongolian gerbils. To identify CGIs methylated in GECs of gerbils with *HP* infection, we adopted the strategy of a genome-wide screening in cancers and high-sensitivity analysis in GECs. The genome-wide screening was done by MS-RDA using a pool of two gerbil gastric cancer cell lines (MGC1 and MGC2) as the driver and GECs of noninfected gerbils as the tester. The final products of two series of MS-RDA using *HpaII* and *SacII* were cloned and 180 DNA fragments were sequenced. One hundred three of them were

nonredundant, and 56 of them contained a sequence likely to have originated from a CGI. Due to the lack of information on the gerbil genome, we first analyzed the methylation statuses of CpG sites within the DNA fragments isolated using MSP. MSP primers were successfully designed for 27 of the 56 DNA fragments, and we analyzed the two gastric cancer cell lines, five samples of GECs from gerbils infected with *HP* for 50 weeks, and five samples of GECs from age-matched gerbils without infection. Ten (HE6, HG2, SA9, SB1, SB5, SC3, SD2, SE3, SF12, and SH6) of the 27 DNA fragments were methylated in the cell lines and GECs of *HP*-infected gerbils, but not in any GECs of gerbils without infection (Table 1; Fig. 1). The others were methylated only in the cell lines or methylated even in GECs of gerbils without infection.

Methylation in primary gastric cancers was analyzed for three randomly selected CGIs (HE6, SA9, and SB5). The methylation levels of HE6 and SB5 in eight primary cancer samples were similar to or below the mean methylation levels in GECs with *HP* infection for 50 weeks. In contrast, the methylation level of SA9 in most cancer samples was 2.1- to 19.1-fold higher than the mean methylation level in GECs from *HP*-infected gerbils (Supplementary Fig. S2). These results suggested that *HP* infection induced aberrant methylation of multiple but specific CGIs in gerbil GECs, and that methylation of some of these CGIs was associated with growth advantage of the cells.

Methylation of the corresponding CGIs in human samples. To examine whether or not these CGIs are also methylated in humans by *HP* infection, conserved regions of the

10 gerbil CGIs in humans were searched for. Eight of the 10 CGIs were found to be conserved between gerbils and humans (marked in Table 1), and five were located in the vicinities of genes (Fig. 2A, left). When the methylation levels of these five CGIs were quantified in human gastric mucosal biopsies, all of them had 5- to 48-fold higher methylation levels in individuals with *HP* infection ($n = 10$) than in those without ($n = 10$; right). Their methylation levels had close correlation with each other (correlation coefficient = 0.70–0.88; Supplementary Table S3).

The methylation levels of the five CGIs were then analyzed in primary human gastric cancers. *NTRK2*, *GPR37*, *NOLA*, and *NPTX2* had methylation in seven, three, four, and five, respectively, of 14 cancers analyzed, using the average methylation level of mucosal biopsies of *HP*-infected healthy volunteers as a threshold. There was no case with methylation of *RNF152* (Fig. 2B). These results showed that some of these CGIs were also methylated in human gastric cancers.

Induction of DNA methylation by chronic *HP* infection.

Using the 10 CGIs isolated by MS-RDA, the effect of *HP* infection on methylation induction was analyzed at 1, 5, 10,

and 50 weeks after *HP* infection (Fig. 3A). The methylation levels of HG2, SB5, and SD2 started to increase at 5 weeks after infection. At 10 weeks, CGIs other than SE3 and SH6 showed significantly higher methylation levels than those of the noninfected gerbils (3.2- to 85.0-fold). At 50 weeks, all the CGIs showed significantly higher methylation levels (14.3- to 215-fold; Fig. 3B; Supplementary Fig. S3). These results suggested that chronic *HP* infection, not acute *HP* infection, was responsible for methylation induction.

The presence of dense methylation (methylation of a majority of CpG sites on a single DNA molecule) was confirmed by bisulfite sequencing of HE6 and SA9 in GECs of two gerbils with *HP* infection and two without. Densely methylated DNA molecules were detected only in *HP*-infected gerbils (Fig. 3C). The vast majority of DNA molecules were either largely unmethylated or largely methylated, and the fraction of methylated DNA molecules was in accordance with methylation levels measured by qMSP. The methylation levels of the 10 CGIs closely correlated with each other (average correlation coefficient = 0.87; range 0.70–0.95; Fig. 3D; Supplementary Table S4).

Decrease in methylation levels after *HP* eradication. *HP* was eradicated at 50 weeks after infection, and the methylation levels of the 10 CGIs were measured in GECs of the gerbils before and 1, 10, and 20 weeks after the eradication (Fig. 3A). Complete absence of *HP* was confirmed by PCR of *HP* genomic DNA (Fig. 4C). At 1 week after eradication, no decrease in methylation was observed (Fig. 3B; Supplementary Fig. S3). At 10 weeks after eradication, in contrast, the methylation levels of the 10 CGIs decreased to 9% to 32% of those before the eradication (significant for 9 of the 10 CGIs, except for SH6). An additional 10 weeks (20 weeks after eradication) did not lead to a further decrease in methylation levels. Importantly, the methylation levels after the decrease due to eradication were still significantly ($P < 0.01$ for two CGIs, and $P < 0.05$ for seven CGIs) higher than those in gerbils without any *HP* infection in their life.

Close association between methylation induction and inflammation, and not *HP* itself. *HP* infection is known to induce severe inflammation in gastric mucosae in gerbils, as in humans. Histologic analysis revealed that infiltration of polymorphonuclear cells and mononuclear cells started at 5 to 10 weeks after *HP* infection, and it became severe at 50 weeks (Fig. 4A; Supplementary Fig. S4). After eradication, a decrease in infiltration was not clear at 1 week, but was marked by 10 and 20 weeks (Fig. 4A). These histologic findings were paralleled by expression of inflammatory cell markers [*Cd3g*, *Cd14*, *Ela2*, and *Ms4a1* (*Cd20*) for T cell, macrophage, neutrophil, and B cell, respectively] in gastric tissues containing both mucosal and submucosal layers (Fig. 4B). Although *Ms4a1* expression decreased after eradication, gerbils without eradication (continuous infection) also showed a similar decrease, indicating that the decrease in *Ms4a1* expression (B-cell infiltration) was independent of *HP* eradication.

To explore the components of inflammation associated with methylation induction, the expression of inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*,

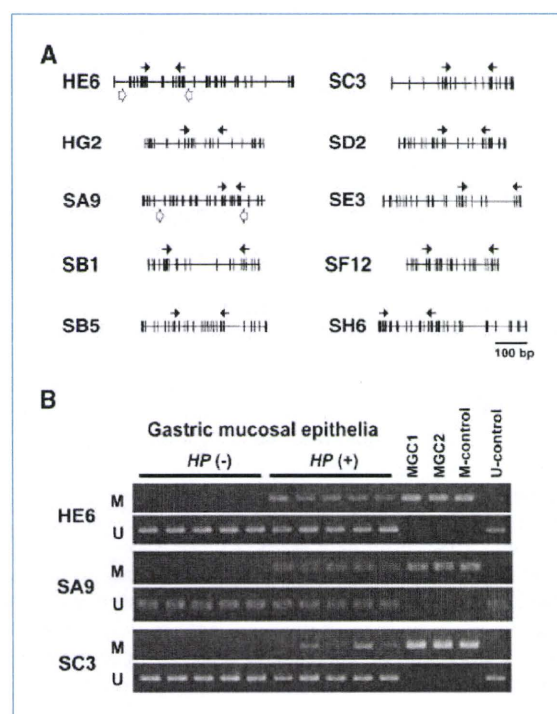


Figure 1. Isolation of CGIs that were aberrantly methylated in gerbil gastric cancers and GECs. A, a CpG map of the fragment isolated by MS-RDA. Vertical lines, individual CpG sites; arrows, positions of MSP primers; open arrows (HE6 and SA9), positions of bisulfite sequencing primers. B, representative results of MSP analyses in GECs from gerbils with and without *HP* infection for 50 wk and gastric cancer cell lines. M, MSP using a primer pair specific to methylated DNA; U, MSP using a primer pair specific to unmethylated DNA; M-control, genomic DNA treated with *SssI* methylase; U-control, DNA amplified with GenomiPhi.

Il6, *Il7*, *Nos2* (*iNos*), and *Tnf* (*Tnf-α*) was also quantified (Fig. 4B). A marked increase after *HP* infection and a decrease after eradication were observed for *Cxcl2*, *Il1b*, *Nos2*, and *Tnf*, paralleling inflammatory cell markers (Fig. 4B). The *Cox2*, *Ifng*, *Il2*, *Il4*, and *Il6* expression did not parallel the methylation levels after *HP* eradication, and the *Il7* expression showed a paradoxical increase compared with the group of continuous infection (Fig. 4B). Regarding the amount of *HP* in gastric mucosae, it had no association with methylation levels (Fig. 4C).

There remained a possibility that inflammatory cells had methylation of the CGIs analyzed, and that their contamination into GECs led to an apparent increase in methylation

levels. To exclude this possibility, we analyzed the methylation levels of the 10 CGIs in DNA extracted from the whole blood of *HP*-infected gerbils. With the exception of SB1 and SB5, which showed relatively high methylation levels in the blood, 8 of the 10 CGIs showed almost no methylation (Supplementary Fig. S5). This excluded the possibility that methylation detected in the GECs was due to methylation in inflammatory cells contaminating the GECs.

Suppression of methylation induction by suppression of inflammation. To conclude that inflammation is indispensable for methylation induction, we suppressed *HP*-induced inflammation by administration of CsA, which blocks T-cell activation through inhibition of the calcineurin signal

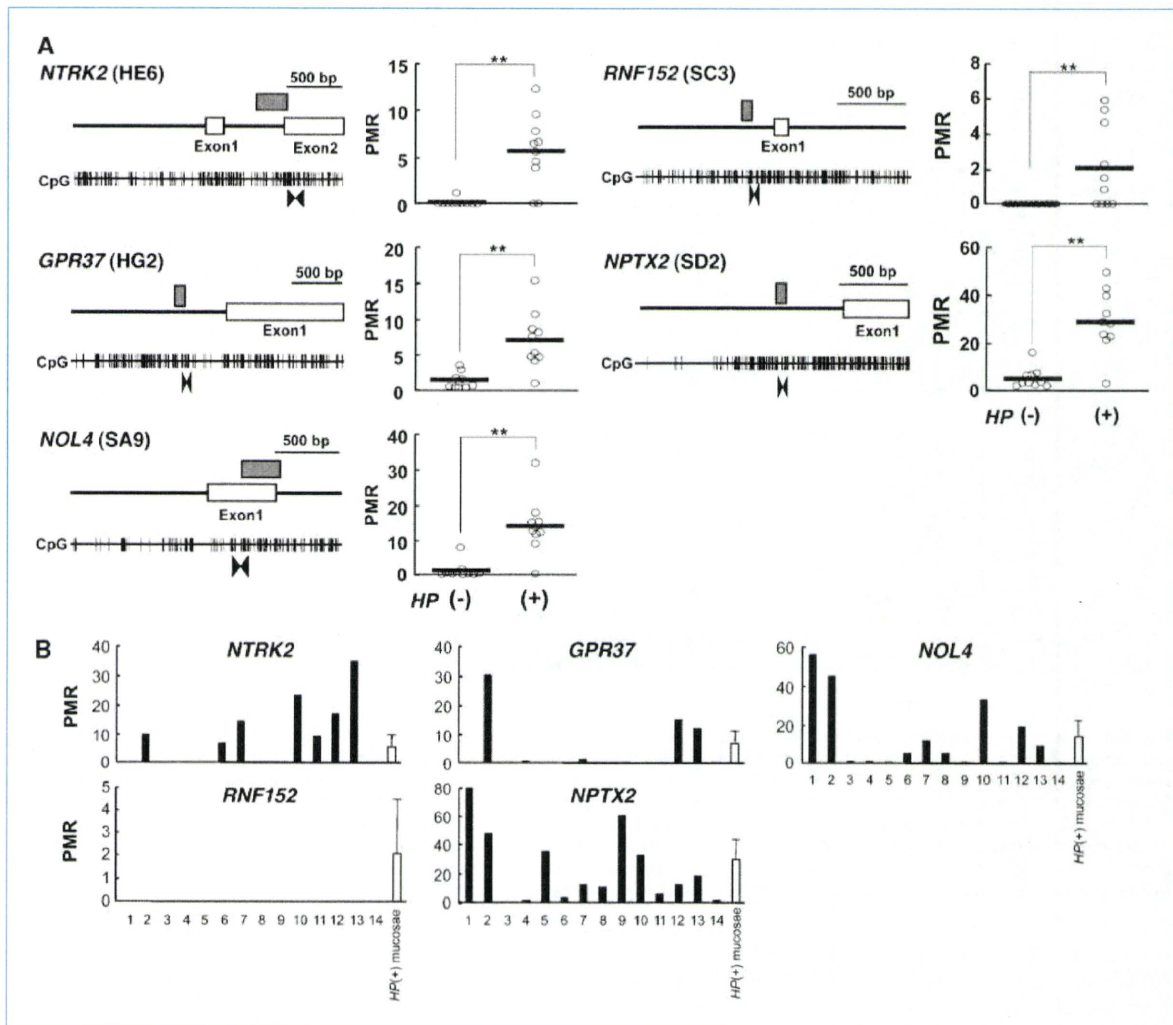


Figure 2. Methylation of homologous regions in human gastric mucosae. A, methylation levels in human gastric mucosal biopsies. Left, genomic structures and the regions analyzed by qMSP. Vertical lines, individual CpG sites; gray box, regions with homology between gerbil and man; open boxes, exons; faced arrowheads, positions of primers for qMSP. Right, result of qMSP analyses. Methylation levels were quantified in 10 healthy volunteers without *HP* infection and 10 with *HP* infection. Bold horizontal bars, average. **, $P < 0.01$. B, methylation levels in primary gastric cancers. Fourteen primary gastric cancer samples and a pool of 10 mucosal biopsies of *HP*-infected healthy volunteers were analyzed. For the gastric mucosae, their mean methylation level and SD are shown. PMR, percentage of the methylated reference.

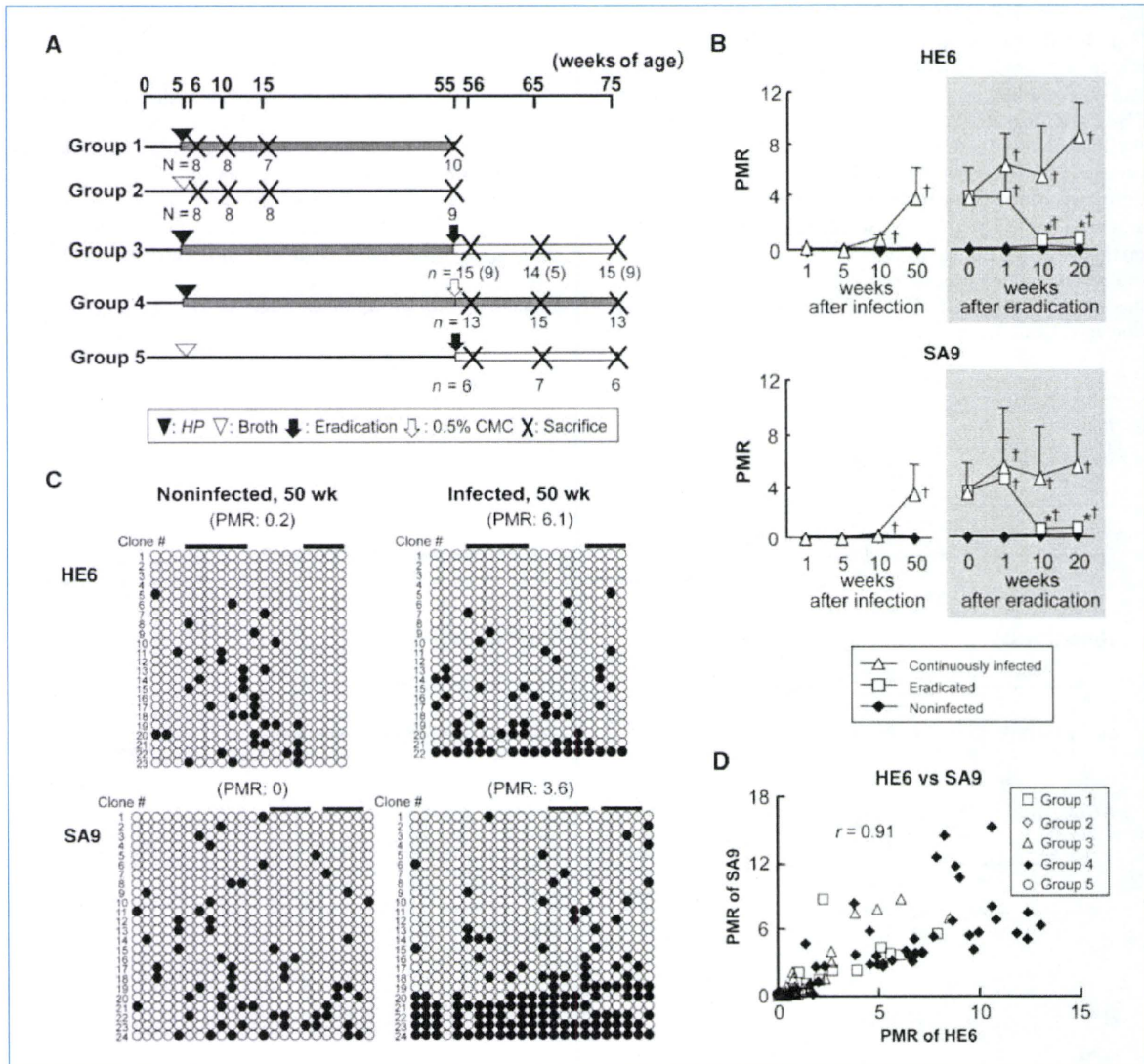


Figure 3. Temporal profiles of DNA methylation levels after *HP* infection and its eradication. A, experimental design for *HP* infection and eradication. The numbers of gerbils that were successfully eradicated of *HP* are indicated in parentheses. B, temporal profiles of methylation levels. Methylation levels are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the methylation level before the eradication. C, the presence of dense methylation in the GECs of gerbils with *HP* infection. Bisulfite sequencing of HE6 and SA9 was done in GECs of a gerbil infected with *HP* for 50 wk and an age-matched control gerbil. The fractions of clones with dense methylation were in accordance with methylation levels (percentages of the methylated reference given in parentheses). Bars, CpG sites on which qMSP primers were designed. Similar patterns were observed for another pair of noninfected and infected gerbils (data not shown). D, scattered plot of methylation levels of HE6 versus those of SA9. The values of all 149 gerbils whose methylation was analyzed in this study were plotted. r , correlation coefficient.

(ref. 28; Fig. 5A). Macroscopically, administration of CsA to *HP*-infected gerbils markedly suppressed erosion and the formation of nodules. Histologically, it suppressed induction of hyperplasia almost completely, but infiltration of mononuclear and polymorphonuclear cells remained (Fig. 5B). Importantly, the number of *HP* colonized in the stomach was not affected by the CsA treatment (Supplementary Fig. S6). The expression levels of inflammatory cell markers (*Cd3g*, *Cd14*, and *Ela2*) were not reduced, indicating that the

number of inflammatory cells normalized against other cells was not affected. However, the expression of three inflammation-related genes (*Cxcl2*, *Il1b*, and *Nos2*), whose expression paralleled methylation induction in the temporal analysis, was significantly reduced by the CsA treatment (Fig. 5C).

The DNA methylation levels of the 10 CGIs were markedly reduced in GECs of CsA-treated gerbils (0% to 28% of methylation levels of GECs from *HP*-infected gerbil without the

CsA treatment; Fig. 5D; Supplementary Fig. S7). These results showed that the CsA treatment suppressed inflammatory responses but not *HP* colonization, and that the suppression of inflammatory responses markedly repressed methylation induction.

Expression analysis of genes with promoter methylation in *HP*-infected GECs. HG2, SC3, and SD2 were located in the

promoter regions of *Gpr37*, *Rnf152*, and *Nptx2*, respectively. Promoter CGIs are generally resistant to DNA methylation (29), and only when genes are transcribed at low levels are they susceptible to DNA methylation (30–32). To confirm the low expression and the effect of methylation on gene expression, we analyzed their expression levels in GECs isolated from gerbils with and without *HP* infection (10 and 50 weeks

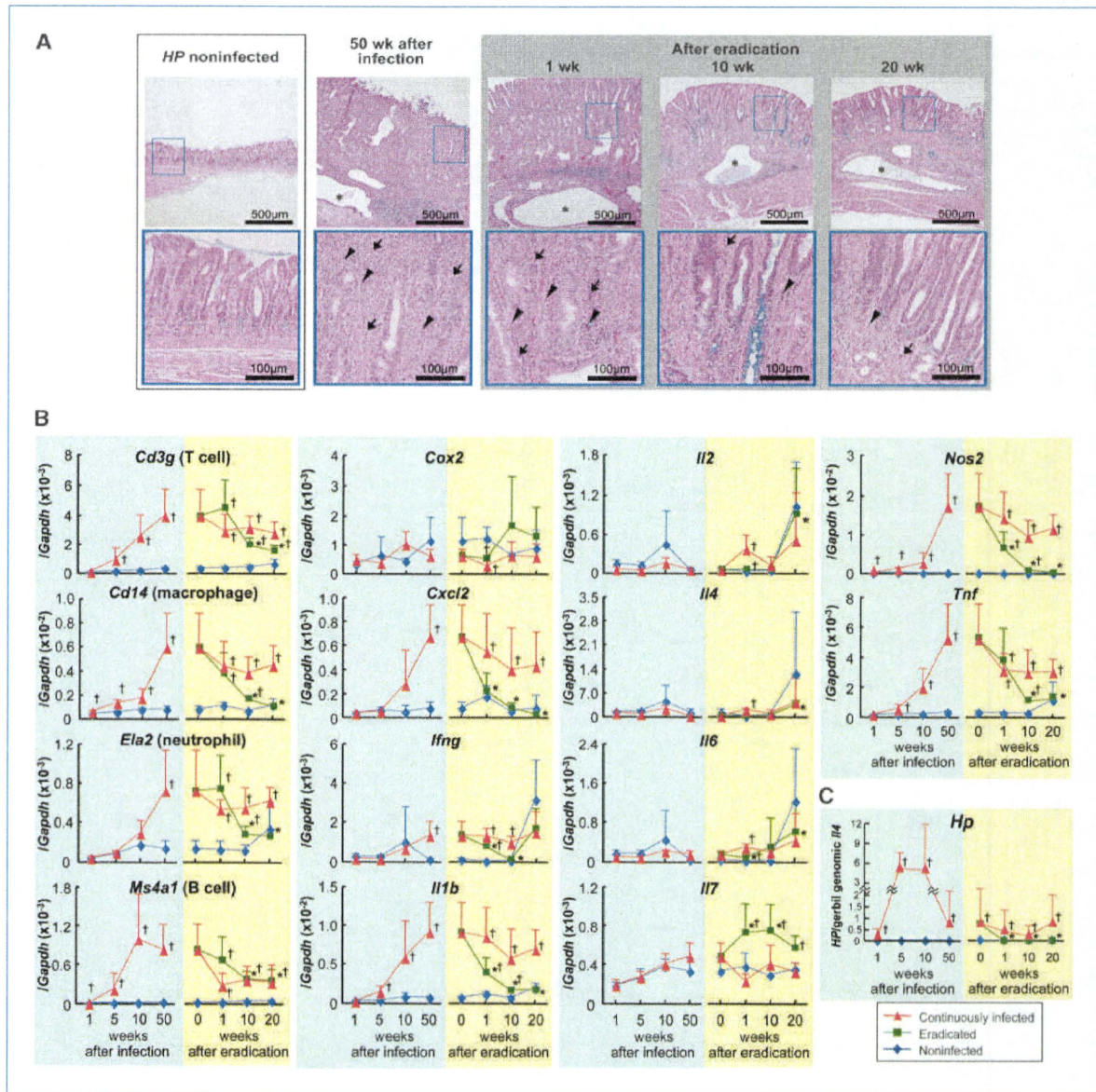


Figure 4. Changes in inflammation after *HP* infection and its eradication. **A**, histologic changes in gastric mucosa before and after *HP* eradication. Sections were stained with hematoxylin, eosin, and Alcian blue. Infiltration of numerous mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) did not change at 1 wk after eradication but markedly decreased at 10 and 20 wk. However, the presence of fibrosis and heterotopic proliferative glands (*) did not differ. **B**, temporal profiles of expression of inflammatory cell markers and inflammation-related genes. Red, green, and blue lines, gerbils with continued infection, gerbils with eradication, and those without any *HP* infection, respectively. **C**, numbers of *HP* in the gerbil stomach. Real-time PCR of *HP*-specific DNA using DNA extracted from gastric tissues containing mucus was done. Values are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the expression level before eradication.

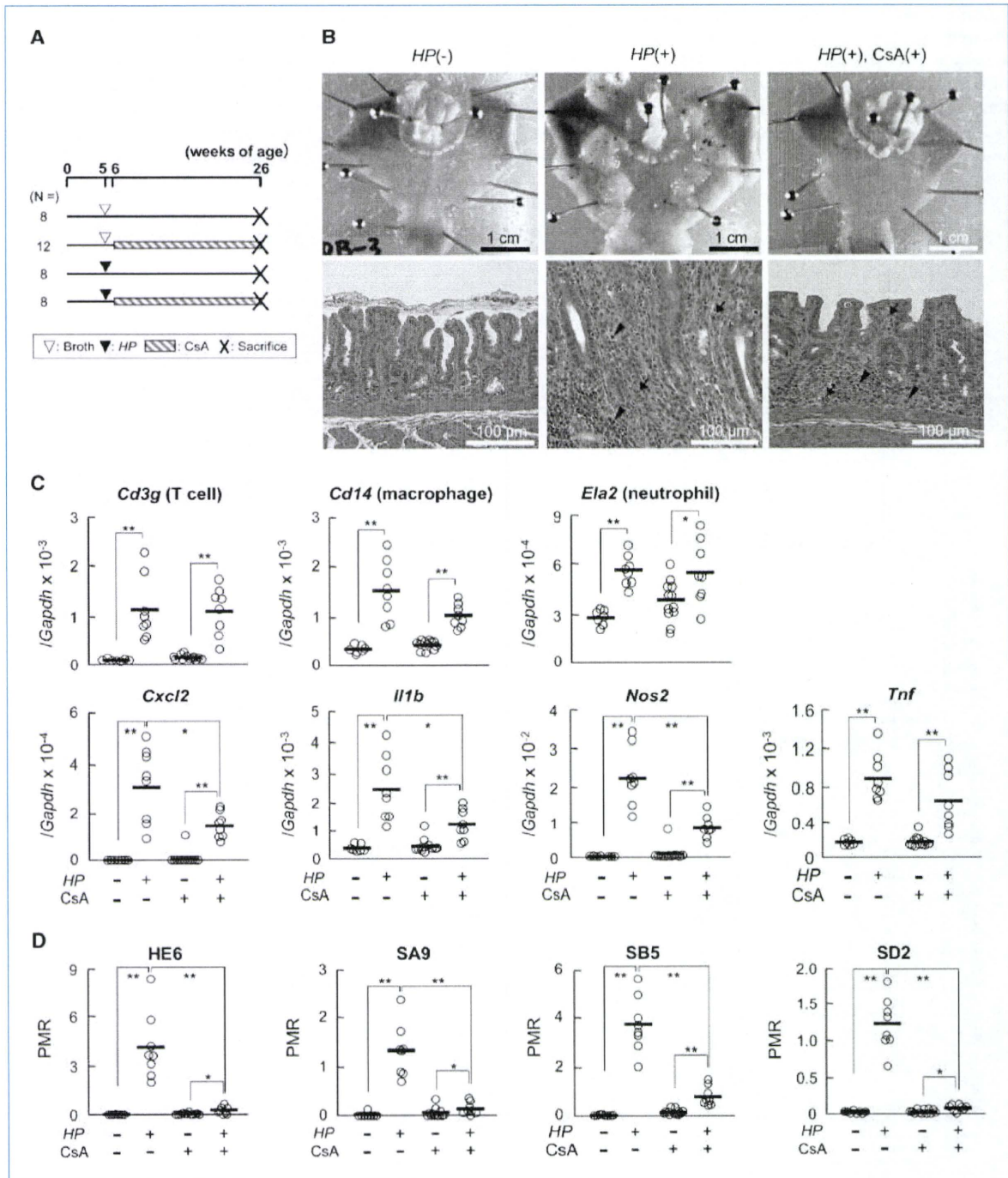


Figure 5. Suppression of inflammation and methylation induction by CsA treatment. **A**, experimental design for CsA treatment and *HP* infection. **B**, macroscopic (top) and histologic (bottom) analyses of gastric mucosae. Hyperplastic changes in pyloric area were prominent in *HP*-infected gerbils without the CsA treatment and were markedly suppressed by the CsA treatment. Infiltration of mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) was also severe in *HP*-infected gerbils without the CsA treatment and was repressed in CsA-treated animals. Gastric mucosae of *HP*-negative gerbils with CsA treatment showed no abnormal changes (data not shown). **C**, expression of inflammatory cell markers and inflammation-related genes. The expression of inflammatory cell markers normalized to *Gapdh* expression was not reduced. However, the expression of three inflammation-related genes (*Cxcl2*, *Il1b*, and *Nos2*) was significantly reduced by the CsA treatment. **D**, methylation levels in GECs. The CsA treatment markedly suppressed methylation induction by *HP* infection. Bold horizontal bar, average. *, $P < 0.05$; **, $P < 0.01$.