oncogenes and tumor suppressor genes are rarer than in colorectal cancer, although DNA methylation leads to the silencing of numerous genes. Infectious pathogens such as *Helicobacter pylori* and Epstein–Barr virus (EBV) have been implicated in the altered methylation seen in gastric cancer. For instance, methylation levels in the gastric mucosa of individuals with a history of *H. pylori* infection correlate with gastric cancer risk [49]. In addition, enlarged-fold gastritis, which is caused by *H. pylori*, is associated with an increased risk for gastric cancer, and the gastric mucosa in these patients show high levels of promoter methylation and hypomethylation of LINE1 [50]. The molecular mechanisms by which *H. pylori* and other pathogens induce DNA methylation remain unknown. It is known, however, that the chronic gastritis caused by *H. pylori* is associated with mucosal cell proliferation and cytokine expression. On the other hand, the expression levels of three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) are unaffected [51], indicating that other factors are likely involved in the methylation changes seen in gastric cancer.

It was also recently reported that there is a link between EBV infection and changes in DNA methylation in gastric cancer [29,48]. Unlike colorectal cancer with CIMP, gastric cancers with EBV infection rarely show MSI [29]. Gastric cancers with EBV also do not show mutations of *K-ras* or *p53*, but they do show methylation of multiple genes [29]. Similar EBV-associated aberrant methylation of multiple genes is also seen in nasopharyngeal cancers [52]. Several lines of evidence suggest that EBV induces LMP1-mediated expression of DNMT1 [53]. In nasopharyngeal carcinoma, for example, induction of DNMT1 by LMP1 is caused by activation of c-Jun NH2-terminal kinase (JNK)-activator protein (AP)-1 signaling [54]. EBV-associated aberrant methylation in cancer thus appears to be a good model with which to gain insight into the molecular mechanisms underlying the altered DNA methylation in cancer, and may facilitate the development of new therapies.

Epigenetic alterations caused by translocation in leukemia

The proteins involved in the epigenetic regulation of gene expression are often impaired in cancer. For example, the chromosomal translocations seen in leukemia often lead to epigenetic alterations of genes, although such alterations are rarely found in common solid tumors (FIGURE 4). In acute myeloid leukemia (AML), for

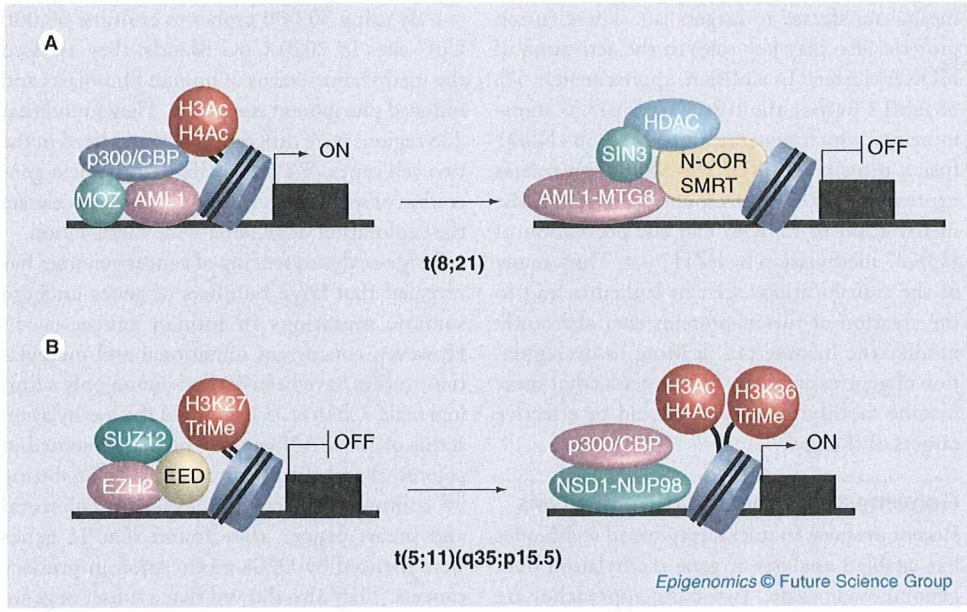

Figure 4. Model of aberrant transcription caused by translocations in leukemia.
(A) AML–MTG8 fusion protein in AML with t(8;21). AML-MTG8 lacks the p300 binding domain of AML1, which causes HAT activity to be replaced by HDAC activity within the complex. This in turn leads to silencing of genes involved in the differentiation of hematopoietic cells. **(B)** NUP98–NSD1 fusion protein in AML with t(5;11)(q35;p15.5). Histone H3K9 methylation by EZH2 prevents expression of genes such as HOXA9 in differentiated hematopoietic cells. NUP98-NSD1 transactivates HOXA9 via methylation of histone H3K36 and acetylation of histones H3 and H4.
HDAC: Histone deacetylase.

example, a protein complex that induces differentiation of hematopoietic cells is disrupted by translocations such as t(8;21), which generates AML1 (RUNX1)-MTG8 fusion [t(3;21)], leading to the expression of AML1-MTG8 fusion proteins [55]. These fusion proteins then act as dominant negative forms of the core transcription complex. AML1-MTG8 represses genes usually activated by AML1, including *FMS*, *p14ARF* and *C/EBPα* through recruitment of corepressor complexes, including HDAC [56–58]. AML1-MTG8 can also recruit DNMT1, thereby prolonging epigenetic silencing of target genes [59].

In normal hematopoiesis, expression of *HOXA7*, *HOXA9* and *HOXA10* promotes self-renewal. The downregulation of these genes coincides with terminal differentiation, and overexpression of HOXA loci is frequently observed in AML. Chromosomal translocations involve mixed lineage leukemia (MLL), a H3K4 histone methyltransferase that positively regulates gene expression. Leukemogenic MLL fusion proteins delete MLL SET domain lysine 4 methyltransferase activity and fuse MLL to a variety of translocation partners. For example, the MLL-CBP fusion protein affects genes by directly targeting histone acetyltransferase [60], while MLL-AF10 recruits hDotL1 histone methyltransferase to targets [61]. These fusion proteins also play key roles in the activation of HOXA clusters. In addition, approximately 5% of AMLs harbor the t(5;11)(q35;p15.5) translocation, which generates the NUP98–NSD1 fusion protein [62]. NUP98–NSD1 upregulates expression of the HOXA cluster through the methylation of H3K36 and the prevention of H3K27 methylation by EZH2 [63]. Thus, many of the translocations seen in leukemia lead to the creation of fusion proteins that aberrantly modify the histone tail, leading to dysregulation of gene expression. This suggests that these histone-modifying enzymes could be effective targets of therapy.

Genome-wide methylation analysis

Recent progress in microarray-based techniques has enabled analysis of gene methylation on a genome-wide scale. Two basic approaches are used to prepare microarray probes: methylation-sensitive enzyme digestion followed by adaptor ligation and PCR amplification [64], and immunoprecipitation of methylated DNA using an antibody specific for methylcytosine or methyl-CpG binding protein [65,66]. With microarray-based methylation analysis, the methylation profiles of thousands of genes become available. For instance, by applying a MCA microarray (MCAM) to hepatocellular cancer (HCC), Gao *et al.* assessed the methylation status of approximately 6500 CpG islands in HCC and liver cirrhosis [67]. They found that HCCs arising from liver cirrhosis had significantly more methylation than those arising from chronic hepatitis. In addition, Kuang *et al.* used MCAM to examine the methylation profile in acute lymphocytic leukemia. They found that 404 genes were hypermethylated in acute lymphocytic leukemia and that patients with methylation of multiple CpG islands had a poorer overall survival rate [68]. MCA can also be applied to bacterial artificial chromosome (BAC)-arrays (BAC array-based MCA [BAMCA]). For instance, Arai *et al.* examined the methylation profiles of precancerous regions and clear-cell renal cancers using BAMCA [69]. They found that clinicopathologically aggressive clear-cell renal cancers show methylation profiles that are distinct from less aggressive cancers. They suggested that alteration of DNA methylation during a precancerous stage may generate more malignant clear cell renal cancers and determine patient outcome. In addition, Deng *et al.* performed a massive methylation analysis using DNA prepared using a padlock probe [70]. By using 30,000 probes to examine 66,000 CpG sites in 2020 CpG islands, they assessed the methylation status of human fibroblasts and induced pluripotent stem cells. They found that 288 regions were differentially methylated in the two cell types. We anticipate that the next generation of sequencers will significantly accelerate the exploration of genome-wide methylation.

Large-scale sequencing of cancer genomes has revealed that large numbers of genes undergo somatic mutations in human cancer [71,72]. However, concurrent mutational and methylation studies have been carried out on only a limited scale. Chan *et al.* examined the methylation status of 189 CAN genes, which are mutated in colorectal and breast cancers [73]. By analyzing 36 common targets of mutation in colorectal and breast cancer, they found that 18 genes were silenced by DNA methylation in primary cancers. They also showed that a subset of genes targeted by both genetic and epigenetic events are useful predictors of a poor prognosis.

Future perspective

Recent studies have shown that there are multiple levels of genetic alterations (e.g., those affecting nucleotides or chromosomes) and epigenetic

alterations (e.g., DNA methylation, histone modification or alteration of chromatin structure) in cancer [74]. Moreover, the heterogeneity of these changes represents a major obstacle to full understanding of the mechanisms underlying cancer development. In this regard, there are three key questions that should be addressed in the future:

- What is the meaning of similar epigenetic alterations in different genome systems?
- What is the relationship between methylation of a specific gene and overall genome dynamics?
- Can individual cells within heterogeneous cell populations be studied for their epigenetic profile?

In addition, there are several potential clinical applications for the integrated analysis of genetic and epigenetic alterations in cancer. Genetic and epigenetic alterations in cancer can provide information useful for selecting appropriate treatments for patients diagnosed with cancer. Moreover, gene mutations and DNA methylation reportedly influence the sensitivity to chemotherapeutic drugs and could serve as molecular markers for predicting the responsiveness of tumors to chemotherapy. However, comprehensive analysis of the pharmacoepigenomics awaits the advent of genome-wide analysis of DNA methylation using microarrays and next-generation sequencers.

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Executive summary

CpG island methylator phenotype

- Cancer with simultaneous methylation of multiple CpG islands.

Genetic & epigenetic interactions in cancer

- Colorectal cancers with CpG island methylator phenotype show distinct genetic alterations, including microsatellite instability, BRAF and K-ras mutations and infrequent p53 mutations.

Chromosomal translocations & histone modifications

- Chromosomal translocations found in leukemia often cause aberrant histone modification by creating fusion genes that abolish normal histone-modifying activity.

Bibliography

Papers of special note have been highlighted as:

- • of interest
- •• of considerable interest

1 Reik W, Dean W, Walter J: Epigenetic reprogramming in mammalian development. *Science* 293, 1089–1093 (2001).

2 Jones PA, Baylin SB: The epigenomics of cancer. *Cell* 128, 683–692 (2007).

3 Ohm JE, McGarvey KM, Yu X *et al.*: A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat. Genet.* 39, 237–242 (2007).

4 Widschwendter M, Fiegl H, Egle D *et al.*: Epigenetic stem cell signature in cancer. *Nat. Genet.* 39, 157–158 (2007).

5 Toyota M, Issa JP: Epigenetic changes in solid and hematopoietic tumors. *Semin. Oncol.* 32, 521–530 (2005).

6 Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. *Cell* 87, 159–170 (1996).

7 Nakamura Y: The role of the adenomatous polyposis coli (APC) gene in human cancers. *Adv. Cancer Res.* 62, 65–87 (1993).

8 Polakis P: The many ways of Wnt in cancer. *Curr. Opin. Genet. Dev.* 17, 45–51 (2007).

9 Smith G, Carey FA, Beattie J *et al.*: Mutations in APC, Kirsten-ras, and p53 – alternative genetic pathways to colorectal cancer. *Proc. Natl Acad. Sci. USA* 99, 9433–9438 (2002).

10 Moslein G, Tester DJ, Lindor NM *et al.*: Microsatellite instability and mutation analysis of hMSH2 and hMLH1 in patients with sporadic, familial and hereditary colorectal cancer. *Hum. Mol. Genet.* 5, 1245–1252 (1996).

11 Feinberg AP, Vogelstein B: Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 301, 89–92 (1983).

12 Herman JG, Merlo A, Mao L *et al.*: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.* 55, 4525–4530 (1995).

13 Ahuja N, Mohan AL, Li Q *et al.*: Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res.* 57, 3370–3374 (1997).

14 Kane MF, Loda M, Gaida GM *et al.*: Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.* 57, 808–811 (1997).

15 Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG: Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.* 59, 793–797 (1999).

16 Devereux TR, Horikawa I, Anna CH *et al.*: DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (*hTERT*) gene. *Cancer Res.* 59, 6087–6090 (1999).

17 Montero AJ, Diaz-Montero CM, Mao L *et al.*: Epigenetic inactivation of EGFR by CpG island hypermethylation in cancer. *Cancer Biol. Ther.* 5, 1494–1501 (2006).

18 Toyota M, Shen L, Ohe-Toyota M *et al.*: Aberrant methylation of the Cyclooxygenase 2 CpG island in colorectal tumors. *Cancer Res.* 60, 4044–4048 (2000).

19 Toyota M, Ho C, Ahuja N *et al.*: Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res.* 59, 2307–2312 (1999).

20 Toyota M, Ahuja N, Ohe-Toyota M *et al.*: CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA* 96, 8681–8686 (1999).

21 Toyota M, Ohe-Toyota M, Ahuja N, Issa JP: Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc. Natl Acad. Sci. USA* 97, 710–715 (2000).

22 Shen L, Toyota M, Kondo Y *et al.*: Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc. Natl Acad. Sci. USA* 104, 18654–18659 (2007).

** **Comprehensive mutational and DNA methylation analysis was performed to show three types of colorectal cancers.**

23 Samowitz WS, Albertsen H, Herrick J *et al.*: Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology* 129, 837–845 (2005).

24 Weisenberger DJ, Siegmund KD, Campan M *et al.*: CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat. Genet.* 38, 787–793 (2006).

25 Kakar S, Deng G, Cun L, Sahai V, Kim YS: CpG island methylation is frequently present in tubulovillous and villous adenomas and correlates with size, site, and villous component. *Hum. Pathol.* 39, 30–36 (2008).

26 Kambara T, Simms LA, Whitehall VL *et al.*: BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 53, 1137–1144 (2004).

27 Barault L, Charon-Barra C, Jooste V *et al.*: Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. *Cancer Res.* 68, 8541–8546 (2008).

28 Terada K, Okochi-Takada E, Akashi-Tanaka S *et al.*: Association between frequent CpG island methylation and HER2 amplification in human breast cancers. *Carcinogenesis* 30, 466–471 (2009).

* **Correlation between CpG island methylator phenotype and amplification of HER-2 is shown in breast cancer.**

29 Kusano M, Toyota M, Suzuki H *et al.*: Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein–Barr virus. *Cancer* 106, 1467–1479 (2006).

30 Issa JP: Methylation and prognosis: of molecular clocks and hypermethylator phenotypes. *Clin. Cancer Res.* 9, 2879–2881 (2003).

31 Bakin AV, Curran T: Role of DNA 5-methylcytosine transferase in cell transformation by fos. *Science* 283, 387–390 (1999).

32 Gazin C, Wajapeyee N, Gobeil S, Virbasius CM, Green MR: An elaborate pathway required for Ras-mediated epigenetic silencing. *Nature* 449, 1073–1077 (2007).

33 Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR: Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell* 132, 363–374 (2008).

34 Samowitz WS, Slattery ML, Sweeney C *et al.*: APC mutations and other genetic and epigenetic changes in colon cancer. *Mol. Cancer Res.* 5, 165–170 (2007).

35 Sato H, Suzuki H, Toyota M *et al.*: Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. *Carcinogenesis* 28, 2459–2466 (2007).

36 Suzuki H, Watkins DN, Jair KW *et al.*: Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat. Genet.* 36, 417–422 (2004).

37 Cahill DP, Lengauer C, Yu J *et al.*: Mutations of mitotic checkpoint genes in human cancers. *Nature* 392, 300–303 (1998).

38 Rajagopalan H, Jallepalli PV, Rago C *et al.*: Inactivation of hCDC4 can cause chromosomal instability. *Nature* 428, 77–81 (2004).

39 Goel A, Nagasaka T, Arnold CN *et al.*: The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. *Gastroenterology* 132, 127–138 (2007).

40 Cheng YW, Pincas H, Bacolod MD *et al.*: CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. *Clin. Cancer Res.* 14, 6005–6013 (2008).

41 Derks S, Postma C, Carvalho B *et al.*: Integrated analysis of chromosomal, microsatellite and epigenetic instability in colorectal cancer identifies specific associations between promoter methylation of pivotal tumour suppressor and DNA repair genes and specific chromosomal alterations. *Carcinogenesis* 29, 434–439 (2008).

42 Estecio MR, Gharibyan V, Shen L *et al.*: LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. *PLoS ONE* 2, E399 (2007).

43 Rodriguez J, Frigola J, Vendrell E *et al.*: Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res.* 66, 8462–9468 (2006).

44 Yamada Y, Jackson-Grusby L, Linhart H *et al.*: Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc. Natl Acad. Sci. USA* 102, 13580–13585 (2005).

45 Nishida N, Nagasaka T, Nishimura T *et al.*: Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. *Hepatology* 47, 908–918 (2008).

46 Henken FE, Wilting SM, Overmeer RM *et al.*: Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. *Br. J. Cancer* 97, 1457–1464 (2007).

47 Yasunaga J, Taniguchi Y, Nosaka K *et al.*: Identification of aberrantly methylated genes in association with adult T-cell leukemia. *Cancer Res.* 64, 6002–6009 (2004).

48 Etoh T, Kanai Y, Ushijima S *et al.*: Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. *Am. J. Pathol.* 164, 689–699 (2004).

49 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.* 12, 989–995 (2006).

* **First paper describing the role played by *Helicobacter pylori* infection in the altered DNA methylation seen during gastric tumorigenesis.**

50 Yamamoto E, Toyota M, Suzuki H *et al.*: LINE-1 hypomethylation is associated with increased CpG island methylation in *Helicobacter pylori*-related enlarged-fold gastritis. *Cancer Epidemiol. Biomarkers Prev.* 17, 2555–2564 (2008).

51 Nakajima T, Yamashita S, Maekita T *et al.*: The presence of a methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. *Int. J. Cancer* 124, 905–910 (2009).

52 Zhou L, Jiang W, Ren C *et al.*: Frequent hypermethylation of RASSF1A and TSLC1, and high viral load of Epstein–Barr Virus DNA in nasopharyngeal carcinoma and matched tumor-adjacent tissues. *Neoplasia* 7, 809–815 (2005).

53 Tsai CN, Tsai CL, Tse KP, Chang HY, Chang YS: The Epstein–Barr virus oncogene product, latent membrane protein 1, induces the downregulation of E-cadherin gene expression via activation of DNA methyltransferases. *Proc. Natl Acad. Sci. USA* 99, 10084–10089 (2002).

54 Tsai CL, Li HP, Lu YJ *et al.*: Activation of DNA methyltransferase 1 by EBV LMP1 involves c-Jun NH(2)-terminal kinase signaling. *Cancer Res.* 66, 11668–11676 (2006).

55 Kurokawa M, Hirai H: Role of AML1/Runx1 in the pathogenesis of hematological malignancies. *Cancer Sci.* 94, 841–846 (2003).

56 Follows GA, Tagoh H, Lefevre P *et al.*: Epigenetic consequences of AML1-ETO action at the human c-FMS locus. *EMBO J.* 22, 2798–2809 (2003).

57 Linggi B, Muller-Tidow C, van de Locht L *et al.*: The t(8;21) fusion protein, AML1 ETO, specifically represses the transcription of the p14(ARF) tumor suppressor in acute myeloid leukemia. *Nat. Med.* 8, 743–750 (2002).

58 Pabst T, Mueller BU, Harakawa N *et al.*: AML1-ETO downregulates the granulocytic differentiation factor C/EBPα in t(8;21) myeloid leukemia. *Nat. Med.* 7, 444–451 (2001).

59 Liu S, Shen T, Huynh L *et al.*: Interplay of RUNX1/MTG8 and DNA methyltransferase 1 in acute myeloid leukemia. *Cancer Res.* 65, 1277–1284 (2005).

60 Wang J, Iwasaki H, Krivtsov A *et al.*: Conditional MLL-CBP targets GMP and models therapy-related myeloproliferative disease. *EMBO J.* 24, 368–381 (2005).

61 Okada Y, Feng Q, Lin Y *et al.*: hDOT1L links histone methylation to leukemogenesis. *Cell* 121, 167–178 (2005).

62 Cerveira N, Correia C, Doria S *et al.*: Frequency of NUP98-NSD1 fusion transcript in childhood acute myeloid leukaemia. *Leukemia* 17, 2244–2247 (2003).

63 Wang GG, Cai L, Pasillas MP, Kamps MP: NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis. *Nat. Cell Biol.* 9, 804–812 (2007).

64 Estecio MR, Yan PS, Ibrahim AE *et al.*: High-throughput methylation profiling by MCA coupled to CpG island microarray. *Genome Res.* 17, 1529–1536 (2007).

* **Reports on the application of methylated CpG island amplification to a promoter microarray, which allowed methylation analysis of thousands of genes.**

65 Jacinto FV, Ballestar E, Ropero S, Esteller M: Discovery of epigenetically silenced genes by methylated DNA immunoprecipitation in colon cancer cells. *Cancer Res.* 67, 11481–11486 (2007).

66 Rauch T, Li H, Wu X, Pfeifer GP: MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. *Cancer Res.* 66, 7939–7947 (2006).

67 Gao W, Kondo Y, Shen L *et al.*: Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas. *Carcinogenesis* 29, 1901–1910 (2008).

68 Kuang SQ, Tong WG, Yang H *et al.*: Genome-wide identification of aberrantly methylated promoter associated CpG islands in acute lymphocytic leukemia. *Leukemia* 22, 1529–1538 (2008).

69 Arai E, Ushijima S, Fujimoto H *et al.*: Genome-wide DNA methylation profiles in both precancerous conditions and clear cell renal cell carcinomas are correlated with malignant potential and patient outcome. *Carcinogenesis* 30, 214–221 (2009).

70 Deng J, Shoemaker R, Xie B *et al.*: Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nat. Biotechnol.* 27, 353–360 (2009).

71 Greenman C, Stephens P, Smith R *et al.*: Patterns of somatic mutation in human cancer genomes. *Nature* 446, 153–158 (2007).

72 Sjoblom T, Jones S, Wood LD *et al.*: The consensus coding sequences of human breast and colorectal cancers. *Science* 314, 268–274 (2006).

73 Chan TA, Glockner S, Yi JM *et al.*: Convergence of mutation and epigenetic alterations identifies common genes in cancer that predict for poor prognosis. *PLoS Med.* 5, E114 (2008).

** **Reports on the comprehensive analysis of genetic and epigenetic changes in colorectal and breast cancer.**

74 Heng HH, Bremer SW, Stevens JB *et al.*: Genetic and epigenetic heterogeneity in cancer: a genome-centric perspective. *J. Cell Physiol.* 220, 538–547 (2009).

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Sensitive and Specific Detection of Early Gastric Cancer with DNA Methylation Analysis of Gastric Washes

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CLINICAL-ALIMENTARY TRACT

Background & Aims: **Aberrant DNA methylation is an early and frequent process in gastric carcinogenesis and could be useful for detection of gastric neoplasia. We hypothesized that methylation analysis of DNA recovered from gastric washes could be used to detect gastric cancer.** ***Methods:*** **We studied 51 candidate genes in 7 gastric cancer cell lines and 24 samples (training set) and identified 6 for further studies. We examined the methylation status of these genes in a test set consisting of 131 gastric neoplasias at various stages. Finally, we validated the 6 candidate genes in a different population of 40 primary gastric cancer samples and 113 nonneoplastic gastric mucosa samples.** ***Results:*** **Six genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, *MLF1*) showed frequent differential methylation between gastric cancer and normal mucosa in the training, test, and validation sets. GDNF and MINT25 were most sensitive molecular markers of early stage gastric cancer, whereas PRDM5 and MLF1 were markers of a field defect. There was a close correlation ($r = 0.5–0.9$, $P = .03–.001$) between methylation levels in tumor biopsy and gastric washes. MINT25 methylation had the best sensitivity (90%), specificity (96%), and area under the receiver operating characteristic curve (0.961) in terms of tumor detection in gastric washes.** ***Conclusions:*** **These findings suggest MINT25 is a sensitive and specific marker for screening in gastric cancer. Additionally, we have developed a new method for gastric cancer detection by DNA methylation in gastric washes.**

Gastric cancer is the second-leading cause of cancer death in the world. Its prognosis is determined by clinical stage at diagnosis and treatment.[1–3] Diagnostic tools such as gastrointestinal (GI) endoscopy followed by pathologic analysis or fluoroscopy or both have proven useful; however, the mortality rate has remained high throughout the world. The sensitivity and specificity of GI endoscopy is high, but its diagnostic power depends on the technical skill of the endoscopist. Endoscopic biopsy is a topical procedure whereby only a small portion of abnormal tissue is removed. It can be difficult to determine which tissue layer to remove which occasionally leads to a misdiagnosis. Moreover, GI endoscopy is neither comfortable nor risk free for patients, and it is associated with frequent morbidity. Furthermore, gastric cancer is more prevalent among elderly patients, who are likely to be taking medications such as antiplatelet or anticoagulant drugs, which further complicates the procedure.

The need for less-invasive and more-efficient diagnostic tools has led to a search for gastric cancer antigens.[4,5] However, we now know that common biomarkers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are not found frequently enough to yield high specificity or sensitivity assays. Molecular markers that distinguish benign from clinically silent malignant disease are needed to reduce the number of unnecessary endoscopic biopsies and to increase power for early-stage detection of gastric dysplasia and early gastric cancer.

Cytosine DNA methylation is an important epigenetic change which leads to the recruitment of transcription repressors and chromatin changes. During the development and progression of gastric cancer, many genes are silenced by aberrant methylation of cytosine-phosphate-guanosine (CpG) islands (CGIs), which are CpG dinucleotide-rich areas located within the promoters of approximately 60% of human genes.[6] Aberrant DNA methylation occurs more frequently than mutations in gastric cancer.[7–13] Studies have detected cancer-specific DNA methylation in stool, blood plasma, urine, and pancreatic juice in several different cancers.[14,15] Furthermore, concordant promoter hyper-

Abbreviations used in this paper: **5-aza-dC, 5-aza-2′-deoxycytidine; ADJ, normal tissue adjacent to tumors; ANOVA, analysis of variance; bp, base pair; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CGI, CpG island; CpG, cytosine-phosphate-guanosine; EN, stomach mucosal tissue in endoscopically normal patient; EW, stomach wash sample in endoscopically normal patient; GI, gastrointestinal; PCR, polymerase chain reaction; ROC, receiver operating characteristic; T, tumor tissue in patient with gastric cancer; W, stomach wash sample in patient with gastric cancer.**

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methylation of multiple genes, which is described as the "CpG island methylator phenotype," has been found in both gastric and colorectal carcinomas.[16–21] Therefore, these epigenetic methylation markers could be useful for detecting gastric cancer. It has also been proposed that DNA methylation analysis could be useful to detect field cancerization in this disease.[22–24]

Because many mucosal cells can be found in stomach juice, the detection of molecular markers in stomach juice is a possible noninvasive approach to screening for gastric cancer. However, because of the secretion of hydrochloric acid from parietal cells in gastric mucosa, stomach juice is strongly acidic, with a pH < 3. It is difficult to use cells from gastric juice for molecular studies because of the DNA damage caused by this acidity.[25] One alternative to stomach juice is the use of gastric washes for molecular analysis. Endoscopists obtain washes for analysis by washing with a saline solution around the stomach mucosa during routine endoscopic examination. Moreover, given that cells exfoliate abundantly into the gastric washes and that undamaged DNA recovered from the washes can be assayed with sensitive and quantitative techniques, there is a strong biologic rationale to pursue this emerging technology.

Here, we identified 6 methylation markers for the detection of early gastric neoplasia. Applying this to DNA from gastric washes, we found a high sensitivity and specificity in detecting gastric cancer.

Materials and Methods

Cell Lines

Seven gastric cancer cell lines (Snu1, Snu5, AGS, MKN7, MKN74, MKN45, KatoIII) were obtained from the American Type Culture Collection (Manassas, VA) or the Japanese Collection of Research Bioresources (Tokyo, Japan). To analyze restoration of each gene expression, cell lines were incubated for 96 hours with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC), after which they were harvested and their RNA was extracted for further analysis.

Patient Characteristics and Sample Collection

Training and test sets of gastric tumor tissues were collected from Korean patients who underwent endoscopic or surgical resection of gastric dysplasia or cancer at Yonsei University Wonju Christian Hospital (Wonju, Korea) from January 2000 to December 2004. A total of 131 tumor tissue samples from 131 patients (Table 1*A*) were randomly collected and examined; 40 gastric dysplasias, 48 early gastric cancers, and 43 advanced gastric cancers. Among 78 surgically resected cancer patients, 64 matched adjacent gastric samples found not to contain cancer cells and ≥ 2 cm distant from the lesion were also used for methylation analysis. In addition to the tumor samples, nonneoplastic gastric mucosal samples from 22 age-matched patients who underwent surgery for peptic ulcer disease during the same period were also obtained for the analysis. Serial sections from paraffin-embedded tissue blocks were obtained and used for both histologic diagnosis and tissue DNA extraction in all cases. For all cases, the cancer epithelial compartments were needle microdissected from 10-μm thick sections. Microdissected areas were assessed by an expert pathologist (M-Y.C.) to estimate the percentage of gastric tumor cells in each preparation, and the microdissected areas of the gastric tumors were estimated to contain $> 70\%$ cellularity.

For the validation set (Table 1*B*), we collected tumor tissues (T), adjacent normal tissues (ADJ), and stomach wash samples (W) from October 2005 to September 2006 in a different population of patients (Japanese) with localized gastric cancer. All patients had never been treated for their cancer at the time of sample collection at St. Marianna University School of Medicine Hospital (Kanagawa, Japan). In addition, we collected stomach mucosal tissues (EN) and stomach wash samples (EW) from endoscopically normal patients who were undergoing endoscopy at the same hospital. No significant differences in age and sex were seen between the patients with gastric cancer and the endoscopically normal subjects. This study protocol was approved by the institutional review board of both Yonsei University Wonju Christian Hospital and St. Marianna University School of Medicine, and informed consent was obtained from each patient.

To obtain gastric washes, patients were required to swallow a liquid solution (100 mL of water containing 80 mg of dimethylpolysiloxane [Gascon: Kissei Pharmaceutical Co, Ltd, Matsumoto, Japan], 1 g of sodium bicarbonate, and 20,000 units of pronase [Pronase MS; Kaken Pharmaceutical Co, Ltd, Tokyo, Japan]) approximately 10 minutes before endoscopic examination. After local anesthesia had been administered, the endoscope was inserted into the stomach through the esophagus, and the effect of premedication with pronase on the visualization of the gastric mucosal wall was assessed. During endoscopic examination, the endoscopist washed the stomach wall with a washing solution of 5% Gascon in water. Wash solution was applied to the entire stomach wall, with no exclusive focus on areas that appeared abnormal. Gastric washes were aspirated through the suction channel of the endoscope into specimen collection containers (No. 16200BZZ00045; Nippon Sherwood, Tokyo, Japan). The specimen collection container was directly connected to the endoscope modulator, and the washes were vacuumed manually. The samples were immediately centrifuged, and the pellets were frozen at $-80°$C. DNA was extracted with the use of the standard phenol-chloroform method.

After the collection of gastric washes, biopsies were performed with biopsy forceps (Radial Jaw; Boston Sci-

Table 1. Clinicopathologic Characteristics of Patients and Controls Studied

	A. Test set			
	Nonneoplastic (n = 22)	Dysplasias (n = 40)	Early cancer (n = 48)	Advanced cancer (n = 43)
Age, mean ± SD, y[a]	61 ± 14	65 ± 8	63 ± 12	60 ± 11
Sex, n[a]				
Male	18	31	35	32
Female	4	9	13	11
Location, n				
Proximal[b]	1	4	14	22
Distal[b]	21	36	34	21
Endoscopic findings, n				
Protruded	—	5	2	—
Flat	—	33	11	—
Depressed	—	2	35	—
Bormann type 1	—	—	—	3
Bormann type 2	—	—	—	12
Bormann type 3	—	—	—	25
Bormann type 4	—	—	—	3
Histologic grades, n				
Low-grade dysplasia	—	19	—	—
High-grade dysplasia	—	21	—	—
Differentiation, n				
Well or moderate	—	—	25	10
Poorly or signet-ring cell type	—	—	23	33
Lauren classification, n				
Intestinal	—	—	40	30
Diffuse	—	—	8	13

	B. Validation set (gastric washes)	
	Nonneoplastic (n = 48)	Gastric cancer (n = 20)
Age, mean ± SD, y[b]	66 ± 20	65 ± 18
Sex, n[b]		
Male	20	13
Female	28	7
Location, n		
Proximal[b]	—	11
Distal[b]	—	9
Tumor size < 20 mm, n	—	4
Lauren classification, n		
Intestinal	—	10
Diffuse	—	10
UICC clinical stage, n		
Stage I	—	6
Stage II	—	4
Stage III	—	9
Stage IV	—	1

Note: UICC indicates International Union Against Cancer.
[a]Mean age and sex were not different among 4 groups.
[b]Proximal, cardia, fundus, and body; distal, angle, and antrum.

entific Corp, Natick, MA) under endoscopic guidance with a GIF-Q240 endoscope with the use of the EVIS LUCERA system (Olympus, Inc, Tokyo, Japan). Mucosal samples of the gastric body 5 mm in diameter were collected for biopsy. In the endoscopically normal subjects, endoscopic biopsy was performed at the corpus. In patients with gastric cancer, 2 biopsy samples were collected per site of cancer and adjacent tissue.

The concentration and quantity of all DNA extracted from biopsied tissues and gastric washes were measured with the NanoDrop spectrophotometer (ND-1000 Spectrophotometer; Nano Drop Technologies, Wilmington, DE).

Endoscopic and Histopathologic Analysis of Gastric Neoplasia

The endoscopic appearance of gastric dysplasia was classified on the basis of gastroscopic findings, as protruded-type (0–I), flat-type (IIa or IIb) or depressed-type (IIc, IIc + IIa, or III). Early gastric cancer was defined

by a depth of tumor invasion limited to the submucosal layer of the stomach regardless of the presence of lymph node involvement. Advanced gastric cancer was classified endoscopically with the Borrmann classification. All resected gastric neoplasias were diagnosed histologically by a pathologist (M.-Y.C.) according to the World Health Organization classification (Supplementary Figure 1).

Bisulfite Polymerase Chain Reaction and Pyrosequencing Analysis of DNA Methylation

Bisulfite treatment of gDNA was performed with an EpiTect bisulfite kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Bisulfite-treated DNA (1 μL) was used as a template in subsequent polymerase chain reaction (PCR). All of the primers and PCR conditions used for amplifying promoter CpG DNA fragments of candidate methylation marker genes are listed in Supplementary Table 1*A*. For most assays, we used touchdown PCR. All PCR assays included a denaturation step at 95°C for 30 seconds, followed by an annealing step at various temperatures for 30 seconds, and an extension step at 72°C for 30 seconds. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers (Supplementary Table 1*B*). Pyrosequencing was performed with PSQ HS 96 Gold single-nucleotide polymorphism reagents on a PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). The protocol for pyrosequencing has been described in detail previously.[26] Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given promoter. These adjacent sites usually show highly concordant methylation. Therefore, the mean percentage of methylation of detected sites was used as a representative value for each gene promoter.

K-ras and p53 Mutation Analysis with the Use of DNA from Tumor and Gastric Washes

Direct sequencing was conducted to identify mutations in codons 12 and 13 of the K-*ras* gene and in exons 2 through 11 of the p53 gene in T and W samples; primer sequences were obtained from a previous report, with minor modifications.[27] PCR products were directly sequenced in the M.D. Anderson Core Sequencing Facility with the use of the same primers.

Reverse Transcription PCR

First-strand cDNA was prepared by reverse transcription of 5-μg samples of total RNA with the use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative reverse transcription-PCR was carried out with the use of TaqMan Gene Expression Assays (GDNF, Hs00181185_m1; PRDM5, Hs00924598_m1; ADAM23, Hs01046804_m1; MLF1, Hs0023695_m1; RORA, Hs00536545_m1; and glyceraldehyde-3-phosphate dehydrogenase, Hs_00266705_gl; Applied Biosystems) with a 7500 Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions.[28] SDS2.1 software (Applied Biosystems) was used to do comparative Δ-Ct analysis. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control.

Detection of Helicobacter pylori *with DNA from Gastric Washes*

PCR was used to evaluate the presence of *ureA*, to show *H. pylori* infection, with the use of HPU1 and HPU2 primers.[29] PCR for *cagA* was performed to type the *H. pylori* strains, with the use of cagA1 and cagA2 primers.[30]

Selection of Candidate Genes in Gastric Cancer for Methylation Analysis

We first selected a total of 51 candidate genes. Eight of the 51 genes were identified as hypermethylated in multiple cancers by methylated CGI amplification and microarray[31] or methylated CGI amplification coupled with representational difference analysis (MCA − RDA).[32] In addition, we identified from a literature search 43 genes that were described as potential methylated tumor suppressor genes in gastric cancer cells or tissues.

Statistical Analysis

All statistical analyses were performed with SPSS for Windows, version 12 (SPSS, Inc, Chicago, IL) and PRISM software for Windows, version 4 (GraphPad Prism, Inc, San Diego, CA). Methylation level (in %) was analyzed as a continuous variable for comparison of each gene with clinicopathologic features; means and 95% confidence intervals were calculated. Comparisons of categorical variables were made using the chi-square test and Fisher's exact test when appropriate. Associations between continuous variables or levels of promoter methylation and clinicopathologic variables were evaluated with the use of analysis of variance (ANOVA) and Student's t test. In parallel, we computed the median DNA methylation value and range for each sample, and we defined the receiver operating characteristic (ROC) curve in SPSS software. The z score analysis was used to normalize the methylation levels of several genes in each sample. The z score for each gene was calculated as follows: z score = (methylation level of each sample − mean value of methylation level)/standard deviation of methylation level. In this analysis, a z score > 0 means that the methylation level is greater than the mean value for the population. We examined possible correlations between DNA promoter methylation levels and patient age in 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM*, and *MLF1*) found in normal-appearing mucosa, by calculating Spearman's nonparametric correlation coefficients (r and P, respectively). All reported P values were 2-sided, and $P < .05$ was considered statistically significant.

Table 2. Methylation Status of the 6 Differentially Methylated Markers According to Gastric Neoplastic Progression

Methylation, %	NGM (n = 22)	ADJ (n = 64)	GD (n = 40)	EGC (n = 48)	AGC (n = 43)	Cutoff[a]	*P* value[b]
MINT25							
Methylation, mean (95% CI)	5.9 (2.7–9)	16.7 (7.2–6.3)	67.4 (57.2–77.5)[c]	58.6 (45.3–71.8)[c]	60.3 (49.9–70.7)[c]	17.2	< .001
Frequency	6.7	23.3	89.7	77.8	84.4		
RORAa							
Methylation, mean (95% CI)	6.4 (5.3–7.6)	9.7 (7.4–12.0)[d]	36.8 (31.7–41.9)[c]	32.4 (26.1–38.7)[c]	17.6 (13.0–22.1)	11.6	< .001
Frequency	0	23	92.5	83.3	58.1		
GDNF							
Methylation, mean (95% CI)	9.7 (7.9–11.6)	16.6 (13.1–20.1)[d]	35.4 (29.2–41.5)[c]	39.8 (33.8–45.9)[c]	27 (22.0–32.0)[c]	17.9	< .001
Frequency	4.5	31.6	76.3	84.8	74.4		
ADAM23							
Methylation, mean (95% CI)	2.8 (1.2–4.4)	10.1 (4.9–15.4)[d]	38.7 (31.0–46.4)[c]	27.4 (18.0–36.7)[c]	19.3 (12.0–26.5)	10.2	< .001
Frequency	9.5	18.3	89.7	54.5	48.8		
PRDM5							
Methylation, mean (95% CI)	25.1 (18.4–31.7)	31.8 (24.9–38.8)	74.3 (64.5–84.1)[c]	59.4 (48.2–70.6)[c]	38.8 (29.3–48.2)	52.6	< .001
Frequency	0	25.5	85.3	63.6	33.3		
MLF1							
Methylation, mean (95% CI)	11 (8.2–13.7)	14.2 (11.8–16.6)	35.8 (29.2–42.3)[c]	26.5 (19.9–33.0)[c]	19.4 (14.9–23.9)	23.5	< .001
Frequency	4.5	16.7	67.5	50	41.9		

ADJ, normal-appearing gastric mucosa adjacent to gastric cancer; AGC, advanced gastric cancer; EGC, early gastric cancer; GD, gastric dysplasia; NGM, nonneoplastic gastric mucosa.
[a]The cutoff value for the 7 hypermethylated genes was determined according to the mean methylation level of each gene + 2 SDs.
[b]The methylation levels in the 5 groups were compared with ANOVA.
[c]Significantly different from nonneoplastic gastric mucosa in Tukey's multiple comparisons ($P < .05$).
[d]Significantly different from nonneoplastic gastric mucosa in *t* test ($P < .05$).

Results

Clinicopathologic Characteristics of Patients

Clinicopathologic characteristics of patients with gastric dysplasia, early gastric cancer, or advanced gastric cancer and age-matched controls without neoplasia in the training set and test set are listed in Table 1*A*. Table 1*B* lists the characteristics of patients in the validation set; in those, samples included Tumor tissue (T), normal-appearing tissue adjacent to tumors (ADJ), stomach mucosal tissue in endoscopically normal patients (EN), stomach wash samples in patients with gastric cancer (W), and stomach wash samples in endoscopically normal patients (EW). We observed no significant differences among these groups.

Methylation of Selected Markers in Gastric Neoplastic Progression with the Training and Test Sets

We determined the levels of methylation of all genes in 7 gastric cancer cell lines (AGS, KatoIII, MKN7, MKN45, MKN74, Snu1, and Snu5) and compared them with normal blood DNA obtained from 2 healthy persons. Methylation was determined by bisulfite-pyrosequencing, a quantitative method that yields percentage of methylated alleles in the studied DNA (examples in Supplementary Figure 2). The first criterion for marker selection among the candidate genes was hypermethylation (>15%) in > 3 of the 7 cancer cell lines but a lack of methylation (≤15%) in normal peripheral blood DNA. We found that 51 genes were hypermethylated in > 3 of the 7 cell lines (data not shown). Next, we compared the levels of methylation of the 51 genes in a test set of 13 gastric cancer tissue samples and 11 age- and sex-matched normal gastric mucosa samples (Supplementary Table 2). On the basis of differential methylation, we selected 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1*) as potential markers for the detection of gastric neoplasia.

Next, we tested these 6 potential markers with an independent set of gastric neoplasia samples (test set). The results are summarized in Table 2 and Figure 1. Methylation levels increased significantly in gastric neoplastic progression from nonneoplastic gastric mucosa tissue to normal adjacent tissue to tumors and to early gastric cancer samples for all 6 marker genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1*; $P < .001$, according to ANOVA). When considering progressive stages, 2 patterns were evident: (1) type 1 markers, showing consistently high levels of methylation in both gastric dysplasia and cancer (MINT25 and GDNF); (2) type 2 markers, showing high levels of methylation in early gastric cancer and gastric dysplasia but decreased levels in advanced gastric cancer (RORA, ADAM23, PRDM5, and MLF1). Of interest, use of the type 2 markers showed higher methylation levels in gastric dysplasia than in

advanced gastric cancer ($P < .001$), which is consistent with our studies in ulcerative colitis and colon cancer.[33]

Epigenetic Silencing of Selected in Gastric Cancer Cell Lines

To examine the expression profile of the candidate marker genes, we initially carried out real-time PCR with cDNA from normal colon and normal stomach tissues and 7 gastric cancer cells (MKN7, MKN74, MKN45, Snu1, Snu5, AGS, KatoIII). We detected expression of *GDNF*, *PRDM5*, *ADAM23*, *MLF1*, and *RORA* in both normal colon and stomach tissues; conversely, we detected weak expression of these genes in gastric cancer cells and also an inverse correlation of expression with DNA methylation level. MINT25 is an alternatively spliced form of the *CABIN1* gene and was therefore not studied. Treating those 7 cell lines with the DNA methyltransferase inhibitor 5-aza-dC restored silenced gene expression (Supplementary Figure 3).

DNA Methylation Levels of the 6 Genes in the Validation Set

To analyze the potential of these genes in screening for gastric cancer, we tested a validation set that also included gastric washes. We first measured DNA concentrations in T, N, W, EN, and EW samples. Gastric washes consistently yielded more DNA than did tissue biopsy samples from the same patients (Supplementary Figure 4). To test the quality of recovered nucleic acids, we ran the DNA on 1% agarose gels and found higher molecular weight (intact) DNA in gastric washes from some patients with cancer than from healthy controls (Supplementary Figure 5). DNA spectrophotometer analysis also showed better quality of DNA from washes from patients with cancer. Next, we stained gastric washes, and we could see both cancer cells and normal cells in the washes (cancer cells were stained by CEA). Many cells were already breaking down in the gastric washes. Finally, we tested by PCR the quality of DNA and found that it was more difficult to amplify DNA from controls than from patients with cancer, especially with fragments more than 200 base pairs (bp) (Supplementary Figure 5).

We next measured DNA methylation levels of the 6 genes (*MINT25*, *RORA*, *PRDM5*, *MLF1*, *ADAM23*, and *GDNF*) by pyrosequencing analysis in 153 validation set samples: T (20), ADJ (17), W (20), EN (48), and EW (48). The *z* scores of these 6 genes were significantly different in T and EN samples (Figure 1*B*). The difference in methylation densities between T and ADJ ($P < .0001$), as well as between W and EW ($P < .0001$), were highest in MINT25. The methylation levels of the genes tested in T samples did not differ significantly according to sex, age, or tumor stage except that methylation levels of *MLF1* and *ADAM23* showed a positive correlation with age (*MLF1*: $r = 0.5$, $P = .001$; *ADAM23*: $r = 0.3$, $P = .05$).

The DNA methylation densities of *PRDM5* and *MLF1* were significantly different between T and EN ($P < .0001$) and between W and EW ($P < .0001$) samples. However, the results did not show a significant difference between T and ADJ samples ($P = .16$). Moreover, DNA methylation densities in ADJ samples were significantly higher than methylation densities in EN samples ($P < .0001$), suggesting that these 2 genes are potential markers of an epigenetic field defect (Figure 1*B*).

Correlations in methylation levels between biopsy (T) and gastric wash (W) are shown in Figure 2. The methylation levels of all 6 genes were closely correlated by Spearman's analysis (*MINT25*: $r = 0.7$, $P = .001$; *RORA*: $r = 0.5$, $P = .03$; *PRDM5*: $r = 0.7$, $P < .001$; *MLF1*: $r = 0.9$, $P < .001$; *ADAM23*: $r = 0.7$, $P < .001$; *GDNF*: $r = 0.9$, $P < .001$). These results show that gastric washes closely mirrored gastric biopsy results. We therefore analyzed the sensitivity and specificity of the gastric wash methylation assays statistically with the use of single-gene and multigene panels. Each cutoff value was determined with ROC curves (Figure 3), and sensitivity and specificity were calculated. The best results were 90% sensitivity and 96% specificity with MINT25 alone and 95% sensitivity and 92% specificity with a combination of MINT25, ADAM23, and GDNF (Table 3). Six early-stage gastric cancer samples were included in validation sets. Methylation in gastric washes enabled detection of these cancers in 83.3% of cases (5/6) by MINT25, 66.7% (4/6) by GDNF, and 83.3% (5/6) by PRDM5. These results suggest that DNA from gastric washes can be an appropriate alternative to DNA from biopsied tissue for the determination of methylation status in gastric cancer and to screen for this deadly disease.

Genetic Analysis with Biopsy and Gastric Washes in Gastric Cancer

We checked for the presence of mutations in codons 12 and 13 of the K-*ras* gene and in exons 4 through 10 of the p53 gene in T and W samples. No K-*ras* mutations were detected in codon 12 or 13 in any sample. Mutations of p53 were found in 1 (5%) of 20 T samples and in 1 (5%) of 20 W samples. Both mutations were a 1-bp deletion in exon 10 (1006delG, heterozygous), and the 2 samples were from the same patient.

Detection of H. pylori

H. pylori requires urease protein to survive in the stomach flora because it needs protection from the acidic solution secreted by parietal cells. Therefore, we used *ureA* gene detection as a positive control for *H. pylori*. Twenty-six gastric wash samples were found to be *H. pylori* positive, and 42 were *H. pylori* negative. Of the 26 *H. pylori*–positive samples, 12 were from patients with cancer (12/20, 60%) and 14 were from cancer-free persons (14/48, 29%). In addition, 9 subjects were *cagA* positive in

W samples (9/20, 45%) and 6 were *cagA* positive in EW samples (6/48, 13%). We did not find any correlation between *ureA*- or *cagA*-positive detection and DNA methylation.

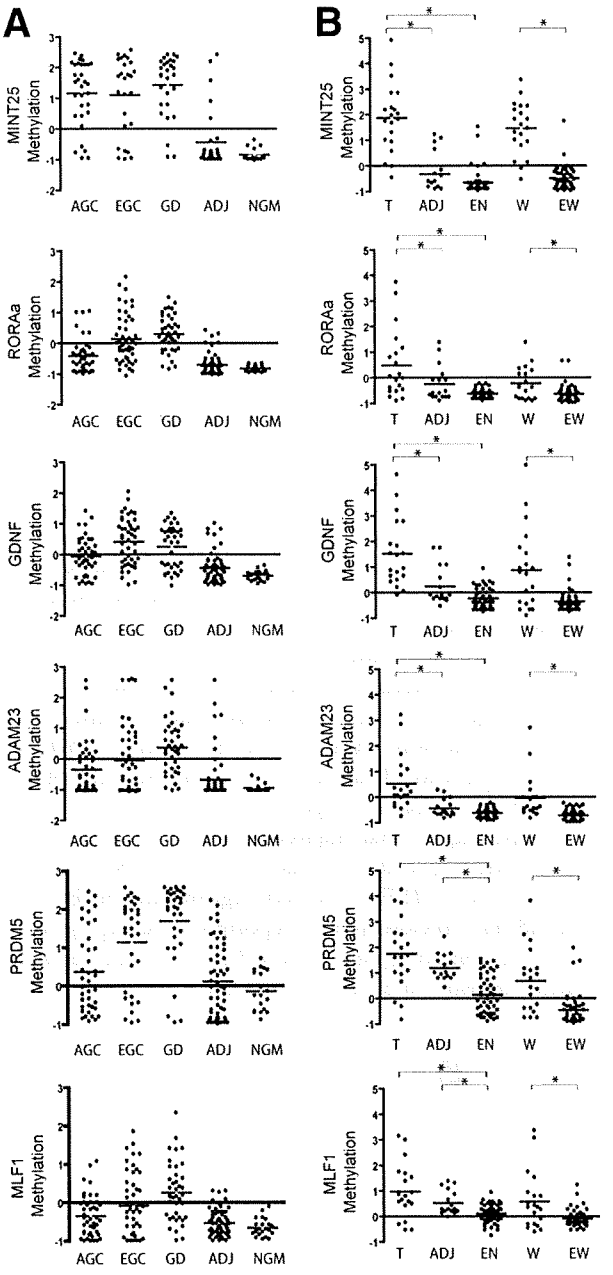

Figure 1. Methylation in gastric cancer. Methylation levels of 6 genes (*MINT25*, *RORAa*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1*) was measured by bisulfite pyrosequencing and normalized by the *z* score method. (*A*) Results of individual genes in the test set. (*B*) Results of individual genes in the validation set. EGC, early gastric cancer; AGC, advanced gastric cancer; GD, gastric dysplasia; NGM, nonneoplastic gastric mucosa; T, tumor tissue in patient with gastric cancer; ADJ, normal adjacent tissue to tumors; W, stomach wash sample in patient with gastric cancer; EN, stomach mucosal tissue in endoscopically normal patient; EW, stomach wash sample in endoscopically normal patient (*$P < .05$).

Discussion

Gastric cancer is still a lethal disease around the world. Early detection yields the opportunity for less-invasive curative treatment and may improve prognosis. Some detection tools are currently being used such as fluoroscopy, endoscopy, and tumor markers; however, these tools lack sensitivity and may require invasive techniques.[34] Alternatively, serum DNA methylation can be used as a marker; however, it provides only a narrow range of sensitivity.[35] Using stool DNA is not useful for gastric cancer detection because of DNA damage because of stomach acidity and the length of the GI tract. Here, we have identified sensitive markers of early gastric cancer, and we developed a new method of gastric cancer detection with the use of methylation analysis of gastric washes.

The use of stomach juice as a molecular diagnostic or prediction tool has been previously shown to be unfeasible because DNA is easily denatured by gastric acidity. Therefore, it is important to obtain genomic DNA from fresh cells not affected by stomach acidity. Our data show that gastric washes can yield enough DNA from shed epithelium to be used for the screening and detection of gastric cancer and that methylation analysis in this compartment confers a high sensitivity and specificity.

We found a close correlation between methylation levels in biopsy and wash samples. Our data suggest that cancer cells from the mucosal layer are easily exfoliated into gastric washes, possibly because of loosening cell-to-cell junctions, whereas the exfoliation of normal mucosal cells is limited. In addition, the success of the technique may relate to the fact that, normally, DNA recovered from gastric washes is relatively degraded. In patients with cancer, a significant proportion of the DNA derives from exfoliated cells, is of larger molecular weight, is less degraded through the apoptotic process, and is easier to amplify by PCR; therefore; its methylation reflects well that of the tumors (Supplementary Figures 5 and 6). Therefore, we can obtain a larger fraction of cancer cells than normal cells in the washes, even if the area of the cancer site is smaller than that of the normal mucosa. Indeed, our approach was successful, although the washes were not specifically directed at diseased parts of the stomach. These data raise the hope that gastric washes without requiring an endoscope may also be successful in cancer detection, an approach that should be tested in future trials.

We identified 6 frequently methylated genes in gastric neoplasia that can serve as biomarkers for the disease. Methylation changes of these markers during gastric carcinogenesis are gene and tumor stage dependent. Of these genes, *MINT25* and *GDNF* were stable biomarkers, because they were highly methylated in gastric tumor samples irrespective of tumor stage. The 6 genes were already reported to be densely methylated in gastric cancer except for *GDNF*.[36,37] For 5 genes (*AMAD23*, *PRDM5*,

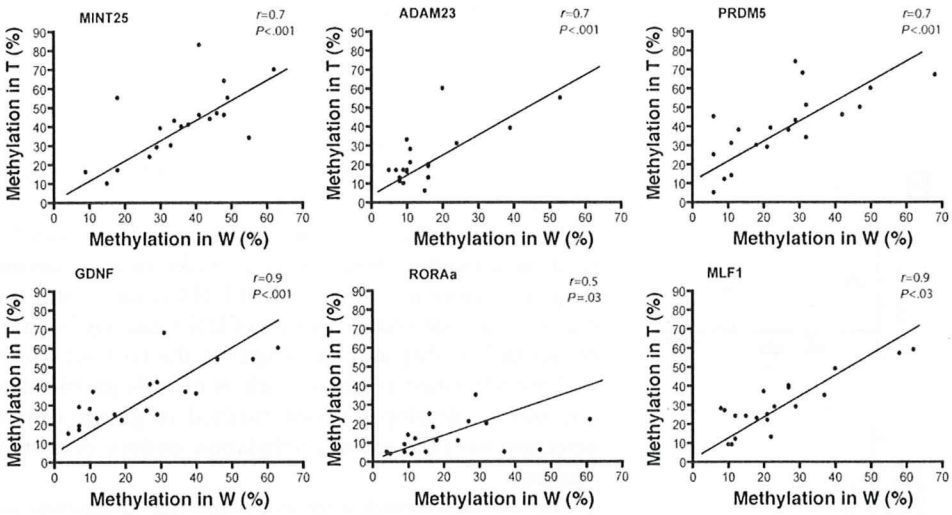

Figure 2. Correlation of methylation levels between tumor and gastric washes samples. Shown are Spearman correlation coefficients *r* and *P* values. Lines show linear regression models.

GDNF, *RORA*, *MLF1*), we showed correlations between expression and methylation. For *MINT25*, this was not shown, because it corresponds to an alternate promoter of the *CABIN1* gene. Our data do not address whether these genes are functionally involved in gastric neoplasia, but this criterion is not necessary for cancer detection.

Of the genes we studied, *MINT25* had the best sensitivity (90%), specificity (96%), and area under the ROC curve (0.961) in terms of tumor detection. A combination of individual genes in methylation panels could increase the performance of these markers: *MINT25* + *ADAM23* + *GDNF* (sensitivity, 95%; specificity, 92%; area under the ROC curve, 0.965; positive predictive value, 0.83; negative predictive value, 0.98). The panel of *MINT25* + *ADAM23* + *GDNF* had greater sensitivity than did *MINT25* alone (Table 3); thus, it may be better for screening. It will be important to validate gene combinations in separate data sets, however.

In this study, we found surprising differences in DNA methylation between different stages of gastric cancer. Dysplasia and early cancer have clearly higher methylation than normal stomach. By contrast, advanced gastric cancer shows significantly lower methylation than dysplasia and early cancer for all genes except *MINT25*. This paradoxical situation is strikingly similar to what we previously observed in ulcerative colitis–associated colon neoplasia.[33] Rather than a decrease in methylation with disease progression, we propose that the data are consistent with separate pathways to carcinogenesis. One pathway involves intense methylation and dysplasia/carcinoma progression. We hypothesize that a distinct, more aggressive pathway characterized by lower methylation evolves rapidly to advanced cancer with little time (if any) spent at the dysplasia stage.

Comparing normal adjacent tissue to tumors (ADJ) from endoscopically normal patients (EN), 2 genes (*PRDM5* and *MLF1*) showed significant differences. *PRDM5* has previously been reported to be highly methylated in primary gastric cancer.[38] In our data, its methylation appears to be a very early event. It appears likely

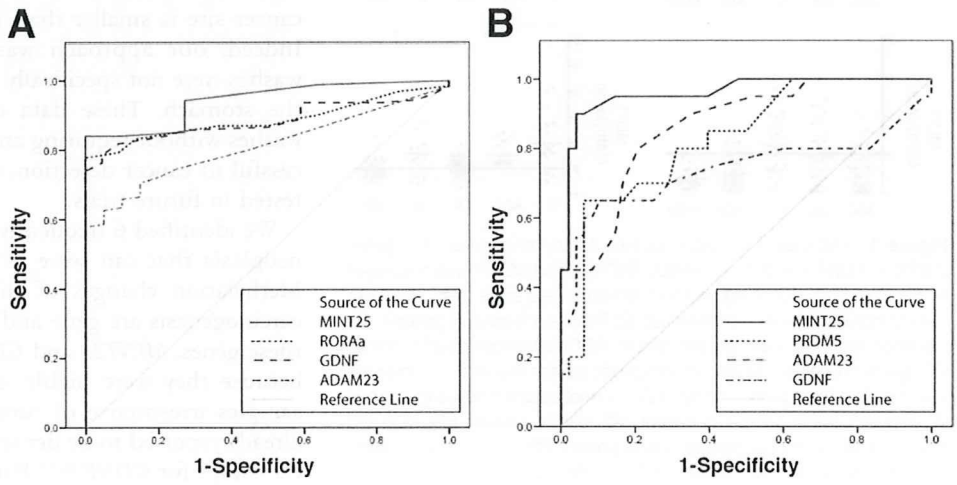

Figure 3. Receiver operating characteristic (ROC) curves for methylation-based detection of gastric cancer. The *z* score of DNA methylation in test (*A*) and validation (*B*) sets were plotted with the use of the top 4 candidate genes in each (test set: *MINT25*, *RORAa*, *GDNF*, *ADAM23*; validation set: *MINT25*, *ADAM23*, *PRDM5*, *GDNF*).

Table 3. Performance of Gene Markers for the Detection of Gastric Neoplasia

A. Test set (cancer versus normal)

Marker	Area (SE)	Cutoff (*z* score)	Neoplasm		Normal		Sensitivity (%)	Specificity (%)	PPV	NPV
			Positive	Total	Positive	Total				
MINT25	0.943 (0.025)	−0.4541	75	88	1	15	84.1	90.9	.99	.52
RORAa	0.887 (0.026)	−0.7135	114	131	8	22	83.2	86.4	.93	.45
GDNF	0.884 (0.027)	−0.4911	104	127	2	22	81.9	90.9	.98	.47
ADAM23	0.812 (0.036)	−0.7876	83	126	2	20	65.9	90.0	.98	.30
PRDM5	0.754 (0.042)	0.5094	68	106	1	19	64.2	94.7	.99	.32
MLF1	0.727 (0.040)	−0.4170	81	131	4	22	61.8	81.8	.95	.26

B. Validation set (gastric washes in cancer patients versus controls)

Variable	Area (SE)	Cutoff (*z* score)	W		EW		Sensitivity (%)	Specificity (%)	PPV	NPV
			Positive	Total	Positive	Total				
MINT25	0.961 (0.025)	0.0571	18	20	2	48	90.0	95.8	.90	.96
RORAa	0.707 (0.076)	−0.4213	11	19	7	48	60.0	85.4	.61	.84
GDNF	0.740 (0.083)	0.0285	13	20	5	47	65.0	89.6	.72	.86
ADAM23	0.864 (0.047)	−0.4949	13	19	8	48	70.0	83.3	.62	.87
PRDM5	0.827 (0.054)	0.0939	13	20	3	48	65.0	93.7	.81	.87
MLF1	0.678 (0.089)	0.2411	12	20	7	48	60.0	85.4	.63	.84
MINT25 + PRDM5 + ADAM23	0.963 (0.020)	−0.6015	18	20	4	48	90.0	91.7	.82	.96
MINT25 + ADAM23 + GDNF	0.965 (0.020)	−0.8141	19	20	4	48	95.0	91.7	.83	.98

EW, gastric wash in cancer-free controls; NPV, negative predictive value; PPV, positive predictive value; SE indicates standard error; W, gastric wash in patients with cancer.

that these 2 genes are associated with field cancerization,[22] and it would be interesting to determine prospectively whether this methylation can be found in "at risk" populations before cancer development.

DNA methylation analysis can be a useful biomarker of cancer, but it is important to consider detection methods. Pyrosequencing is a cost- and time-effective assay that provides quantitative screening. This allows one to set cutoff points, which makes accurate comparisons possible, and overcomes some of the problems associated with very sensitive bisulfite DNA amplification methods. Its applicability however is limited to situations in which the tissue/DNA to be studied has a high fraction of tumor cells.

In summary, we identified 6 methylation markers for detection of early gastric neoplasia, 2 of which could be useful as markers of the field cancerization. Moreover, we have developed a new method for gastric cancer detection by DNA methylation analysis in gastric washes. This technology should now be tested in prospective studies for evaluation and detection of gastric cancer.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.02.085.

References

1. Hohenberger P, Gretschel S. Gastric cancer. Lancet 2003;362: 305–315.
2. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–543.
3. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
4. Denk H, Tappeiner G, Davidovits A, Holzner HJ. The carcinoembryonic antigen (CEA) in carcinomata of the stomach. Virchows Arch A Pathol Pathol Anat 1973;360:339–347.
5. Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic Cell Genet 1979;5:957–971.
6. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM. Predicting aberrant CpG island methylation. Proc Natl Acad Sci U S A 2003; 100:12253–12258.
7. Ushijima T, Sasako M. Focus on gastric cancer. Cancer Cell 2004;5:121–125.
8. Horii A, Nakatsuru S, Miyoshi Y, et al. The APC gene, responsible for familial adenomatous polyposis, is mutated in human gastric cancer. Cancer Res 1992;52:3231–3233.
9. Nanus DM, Kelsen DP, Mentle IR, Altorki N, Albino AP. Infrequent point mutations of ras oncogenes in gastric cancers. Gastroenterology 1990;98:955–960.
10. Maesawa C, Tamura G, Suzuki Y, et al. The sequential accumulation of genetic alterations characteristic of the colorectal adenoma-carcinoma sequence does not occur between gastric adenoma and adenocarcinoma. J Pathol 1995;176:249–258.
11. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer 2003;3:253–266.
12. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004;4:988–993.
13. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223–231.

14. Belinsky SA, Klinge DM, Dekker JD, et al. Gene promoter methylation in plasma and sputum increases with lung cancer risk. Clin Cancer Res 2005;11:6505–6511.
15. Itzkowitz SH, Jandorf L, Brand R, et al. Improved fecal DNA test for colorectal cancer screening. Clin Gastroenterol Hepatol 2007; 5:111–117.
16. Suzuki H, Toyota M, Sato H, Sonoda T, Sakauchi F, Mori M. Roles and causes of abnormal DNA methylation in gastrointestinal cancers. Asian Pac J Cancer Prev 2006;7:177–185.
17. Kusano M, Toyota M, Suzuki H, et al. Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. Cancer 2006;106:1467–1479.
18. Issa JP, Shen L, Toyota M. CIMP, at last. Gastroenterology 2005; 129:1121–1124.
19. Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. Proc Natl Acad Sci U S A 2000;97:710–715.
20. Toyota M, Ahuja N, Suzuki H, et al. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res 1999;59:5438–5442.
21. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–8686.
22. Shen L, Kondo Y, Rosner GL, et al. MGMT promoter methylation and field defect in sporadic colorectal cancer. J Natl Cancer Inst 2005;97:1330–1338.
23. Dakubo GD, Jakupciak JP, Birch-Machin MA, Parr RL. Clinical implications and utility of field cancerization. Cancer Cell Int 2007;7:2.
24. Kim SK, Jang HR, Kim JH, et al. The epigenetic silencing of LIMS2 in gastric cancer and its inhibitory effect on cell migration. Biochem Biophys Res Commun 2006;349:1032–1040.
25. Ferrini AM, Mannoni V, Pontieri E, Pourshaban M. Longer resistance of some DNA traits from BT176 maize to gastric juice from gastrointestinal affected patients. Int J Immunopathol Pharmacol 2007;20:111–118.
26. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 2003;35:146–150.
27. Rhei E, Bogomolniy F, Federici MG, et al. Molecular genetic characterization of BRCA1- and BRCA2-linked hereditary ovarian cancers. Cancer Res 1998;58:3193–3196.
28. Qin T, Youssef EM, Jelinek J, et al. Effect of cytarabine and decitabine in combination in human leukemic cell lines. Clin Cancer Res 2007;13:4225–4232.
29. Clayton CL, Kleanthous H, Coates PJ, Morgan DD, Tabaqchali S. Sensitive detection of Helicobacter pylori by using polymerase chain reaction. J Clin Microbiol 1992;30:192–200.
30. Smith SI, Oyedeji KS, Arigbabu AO, et al. Comparison of three PCR methods for detection of Helicobacter pylori DNA and detection of cagA gene in gastric biopsy specimens. World J Gastroenterol 2004;10:1958–1960.
31. Estecio MR, Yan PS, Ibrahim AE, et al. High-throughput methylation profiling by MCA coupled to CpG island microarray. Genome Res 2007;17:1529–1536.
32. Chung W, Kwabi-Addo B, Ittmann M, et al. Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. PLoS ONE 2008;3:e2079.
33. Konishi K, Shen L, Wang S, Meltzer SJ, Harpaz N, Issa JP. Rare CpG island methylator phenotype in ulcerative colitis-associated neoplasias. Gastroenterology 2007;132:1254–1260.
34. Posner MR, Mayer RJ. The use of serologic tumor markers in gastrointestinal malignancies. Hematol Oncol Clin North Am 1994;8:533–553.
35. Koike H, Ichikawa D, Ikoma H, Otsuji E, Kitamura K, Yamagishi H. Comparison of methylation-specific polymerase chain reaction (MSP) with reverse transcriptase-polymerase chain reaction (RT-PCR) in peripheral blood of gastric cancer patients. J Surg Oncol 2004;87:182–186.
36. Takada H, Imoto I, Tsuda H, et al. ADAM23, a possible tumor suppressor gene, is frequently silenced in gastric cancers by homozygous deletion or aberrant promoter hypermethylation. Oncogene 2005;24:8051–8060.
37. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M, Ushijima T. Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2′-deoxycytidine treatment and oligonucleotide microarray. Cancer Sci 2006;97:64–71.
38. Watanabe Y, Toyota M, Kondo Y, et al. PRDM5 identified as a target of epigenetic silencing in colorectal and gastric cancer. Clin Cancer Res 2007;13:4786–4794.

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Conflicts of interest

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Cell Stem Cell
Brief Report

Rest Promotes the Early Differentiation of Mouse ESCs but Is Not Required for Their Maintenance

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The functional significance of *Rest* in the maintenance of ESC pluripotency remains controversial. We herein showed that *Rest* is not necessary for the maintenance of mouse ESCs, and instead suggested that the Rest transcriptional repressor connects to the Oct3/4-Sox2-Nanog core regulatory circuitry during early ESC differentiation.

The pluripotency of ESCs is maintained by coordinated expression of a core regulatory circuit of genes that includes Oct3/4, Sox2, and Nanog. Rest (also called Nrsf) is abundantly expressed in ESCs and is a target of the Oct3/4-Sox2-Nanog regulatory network. However, the functional significance of Rest in the maintenance of pluripotency remains controversial. We have generated *Rest* conditional knockout and *Rest*-inducible ESC lines. Conditional ablation of *Rest* showed that it is not required for maintenance of pluripotency, but it is involved in the suppression of self-renewal genes during early differentiation of ESCs. In addition, forced expression of *REST* in ESCs results in rapid differentiation. These results indicate that *Rest* is not necessary for the maintenance of mouse ESCs, and instead suggest that the Rest transcriptional repressor connects to the Oct3/4-Sox2-Nanog core regulatory circuitry during early ESC differentiation.

The transcriptional repressor Rest is a zinc finger protein that binds to a conserved 23 bp motif known as RE1 (repressor element 1, also called NRSE) in a number of genes encoding the fundamental neuronal traits (Chong et al., 1995; Schoenherr and Anderson, 1995). Rest is expressed throughout early development where it represses the expression of neural genes, such as *Syp* and *Syt4* (Schoenherr et al., 1996). Rest is also expressed in ESCs and it has also been shown to be one of target genes of the regulatory circuitry of the pluripotent state in ESCs (Johnson et al., 2008; Sun et al., 2005). However, the functional significance of Rest in the maintenance of pluripotency in ESCs still remains controversial (Buckley et al., 2009; Jørgensen et al., 2009a; Singh et al., 2008). A previous study with a heterozygous *Rest* ESC line combined with an siRNA knockdown indicated that Rest maintains pluripotency through the induction of self-renewal genes, such as *Oct3/4*, *Nanog*, and *Sox2* (Singh et al., 2008). In contrast, Jørgensen et al. generated a *Rest* null ESC line and reported that such *Rest* null ESCs revealed no substantial change in either the Oct3/4 protein levels or alkaline phosphatase activity in comparison to matched wild-type controls (Jørgensen et al., 2009a, 2009b).

In order to elucidate the role of *Rest* in the maintenance of pluripotency, we first generated an ESC line and mice that contained the conditional knockout alleles of *Rest*. The first *Rest* allele in the ESCs (V6.5) was replaced with the KO vector carrying the floxed last exon of *Rest*, which encodes the coRest binding site that is essential for the generation of the silencing complex (Andrés et al., 1999; Grimes et al., 2000), followed by *ires-Gfp* to monitor the transcription of the modified allele ($Rest^{3lox/+}$; Figure 1A). The transient expression of *Cre recombinase* generated a *Rest* floxed ESC line that lacks a drug selection cassette ($Rest^{2lox/+}$). Analyzing the GFP expression allowed us to confirm that *Rest* is expressed in ESCs (Figure 1B).

$Rest^{-/-}$ ESCs were next generated with the floxed *Rest* ESC line together with a plasmid expressing *Cre recombinase* (Figure 1A). After the excision of the floxed *Rest* gene by the transient transfection of *Cre* ($Rest^{+/-\ (1lox)}$), the second *Rest* allele was also replaced with the floxed allele ($Rest^{3lox/-}$). The transient transfection of *Cre* into $Rest^{3lox/-}$ ESCs resulted in the establishment of $Rest^{-/-}$ ESCs that were isogenic to the parental ESCs without any genetic modification except for the *Rest* alleles.

After the recombination of the *Rest* alleles, the lack of a *Rest* transcript in such $Rest^{-/-}$ ESCs was confirmed by a northern blot analysis (Figure 1B; Figure S1A available online). Consistent with the recombination, a FACS analysis revealed a lack of any GFP signal in the $Rest^{-/-}$ ESCs (Figure 1B). In addition, a western blot analysis revealed the lack of any Rest protein in such $Rest^{-/-}$ ESCs (Figure 1B). *Syt4* possesses RE1 and it is expressed while relying solely on dissociation of the Rest repressor complex from the RE1 site for maximal expression (Ballas et al., 2005). The expression of *Syt4* significantly increased in the $Rest^{-/-}$ ESCs, thus indicating that the Rest-targeted gene is derepressed in $Rest^{-/-}$ ESCs (Figure S1B).

Consistent with the findings by Jørgensen et al. (2009a, 2009b), the growth and morphology of the $Rest^{-/-}$ ESCs were indistinguishable from those of wild-type V6.5 ESCs under the self-renewal conditions (under the presence of LIF and MEF). Furthermore, when the expression of the pluripotent genes was compared, the expression of *Nanog*, *Oct3/4*, and *Sox2* in $Rest^{-/-}$ ESCs were not altered

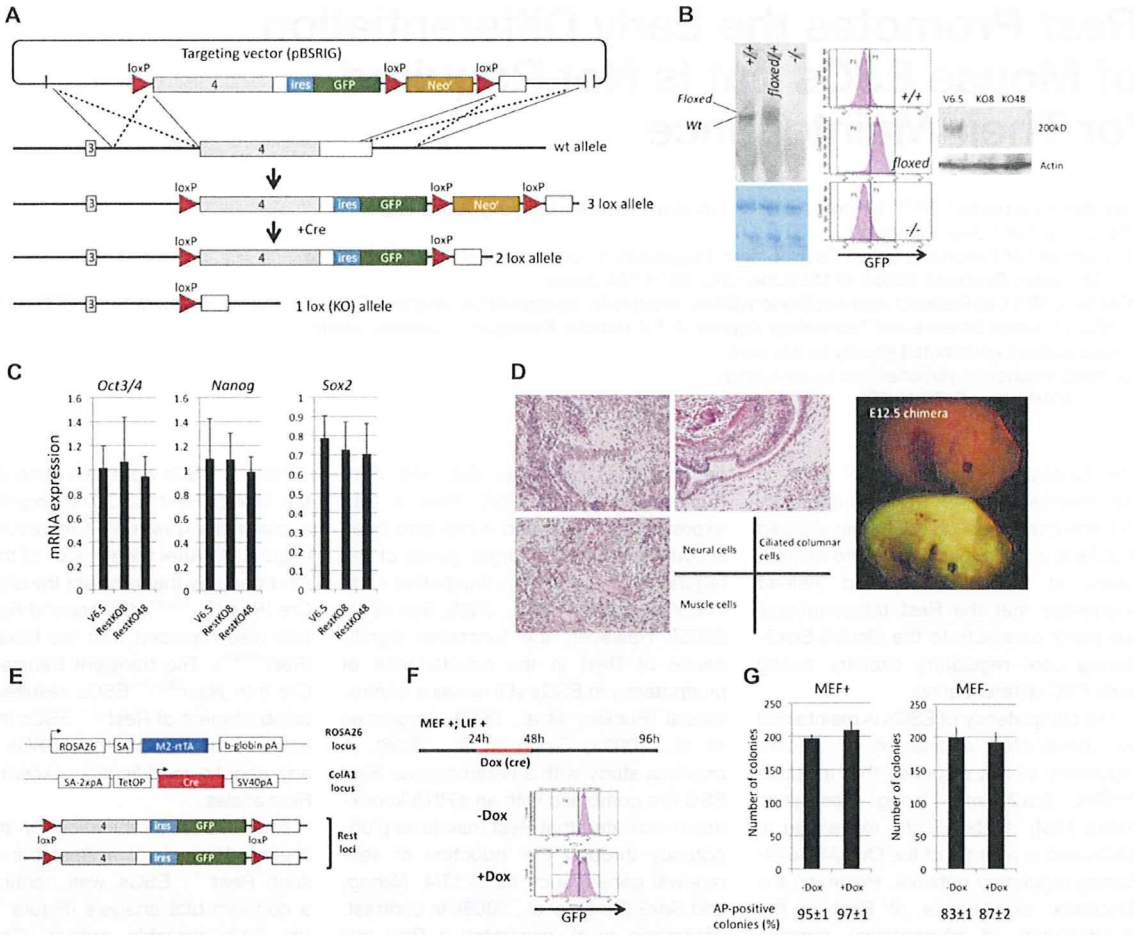

Figure 1. *Rest* Is Not Required for the Maintenance of ESC Pluripotency

(A) A schematic drawing of the *Rest*-conditional knockout vector and targeted *Rest* allele.

(B) A northern blot analysis reveals a lack of *Rest* transcripts. GFP fluorescence is observed to have disappeared in the $Rest^{-/-}$ ESCs. A western blot analysis shows the absence of any Rest protein in two independent knockout ESC lines, RestKO8 and RestKO48.

(C) Transcript levels of pluripotent genes in $Rest^{-/-}$ ESCs. No significant changes in the expression of *Oct3/4*, *Nanog*, and *Sox2* are detectable in the $Rest^{-/-}$ ESCs relative to the control ESCs. Transcript levels were normalized to *β-actin* levels. The data are presented as the average values with SD of six independent samples.

(D) $Rest^{-/-}$ teratomas differentiate into three different germ layers, including neural cells, ciliated columnar cells, and muscle cells. E12.5 chimeric mice were generated by injecting $Rest^{-/-}$ ESCs into blastocysts.

(E) A schematic drawing of the conditional *Rest* knockout ESC line containing doxycycline-inducible *Cre* alleles.

(F) An experimental protocol. Conditional *Rest* knockout ESCs were treated with doxycycline (2 μg/ml) for 24 hr starting at 24 hr and then were harvested at 96 hr after the passage. A FACS analysis revealed the presence of GFP-negative cells, thus indicating the occurrence of *Rest* ablation at 96 hr after passage.

(G) The conditional deletion of the *Rest* gene does not suppress the development of alkaline phosphatase (AP)-positive ESC colonies under the presence or absence of feeder cells. *Rest*-floxed *Cre*-inducible ESCs were exposed to doxycycline and then were fixed after 3 days of exposure. The total number of colonies and the percent positivity for AP are indicated. The data are presented as the mean ± SD of three independent 35 mm wells.

in comparison to those in the control ESCs (Figure 1C). To further examine the pluripotency of $Rest^{-/-}$ ESCs, $Rest^{-/-}$ ESCs were next injected into the subcutaneous tissue of nude mice. $Rest^{-/-}$ ESCs could generate teratomas with evidence of differentiation into three different germ layers (Figure 1D). To fully evaluate the differentiation ability of the $Rest^{-/-}$ ESCs, GFP-labeled $Rest^{-/-}$ ESCs were injected into blastocysts followed by transplantation into the uteri of pseudo-pregnant mice to generate chimeric embryos (Yamada et al., 2004). Eventually, this generated E12.5 chimeric mice with the widespread contribution of GFP-positive cells into the three germ layers (Figure 1D; Figure S1C).

In order to rule out the possibility that the adaptive responses, which occurred as a result of multiple cell passages, reduced the requirement of Rest-mediated maintenance of ESCs, the initial response of the gene expression was examined after the conditional ablation of the *Rest* genes. For this purpose, an ESC line was derived from transgenic embryo that harbors a doxycycline-inducible *Cre* transgene together with *Rest*-floxed alleles (Figure 1E; *Rest 2lox/2lox*;

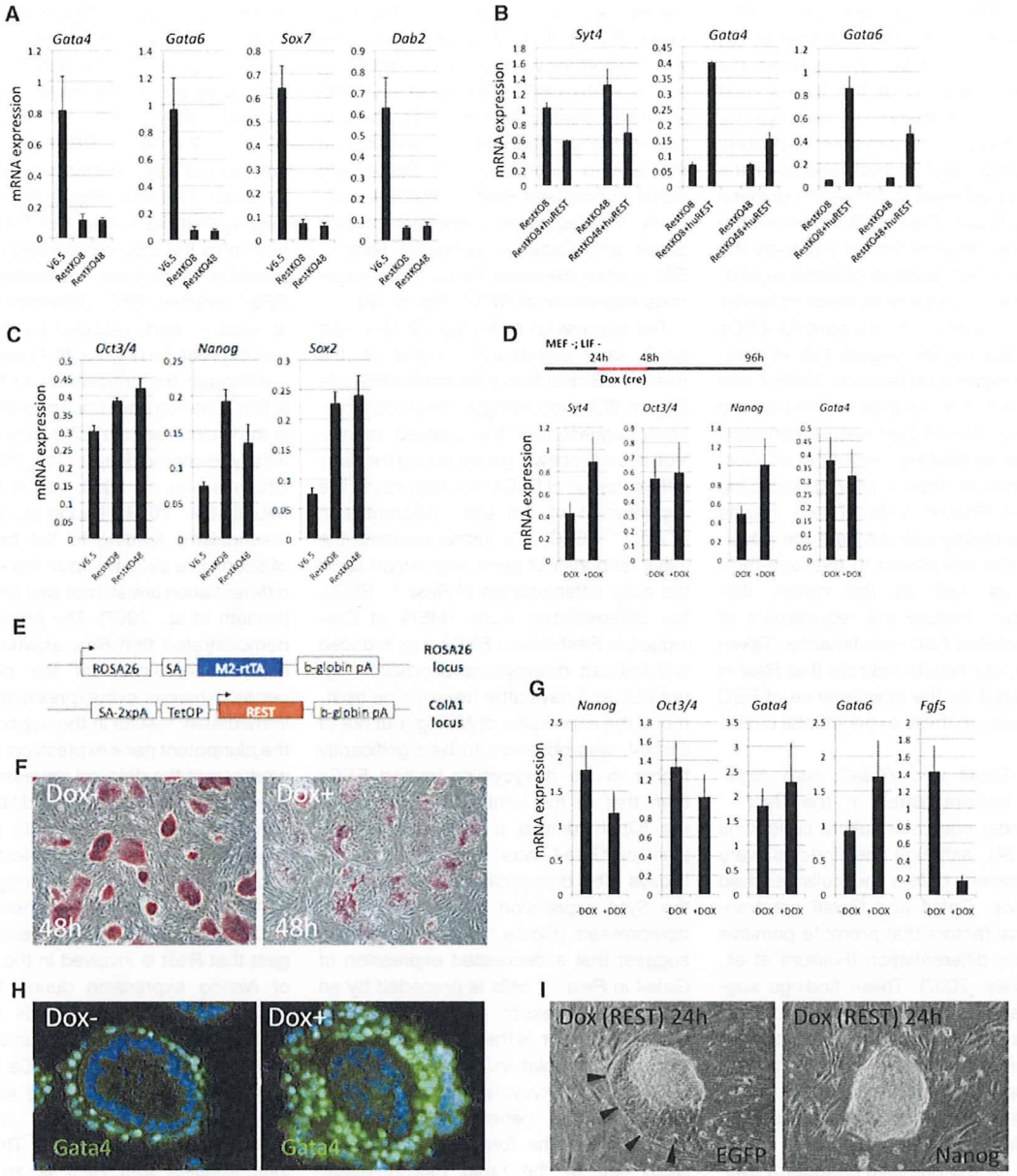

Figure 2. *Rest* Promotes Primitive Endoderm Differentiation in ESCs

(A) Under confluent culture conditions, the expression of *Gata4* and *Gata6* were significantly lower in the $Rest^{-/-}$ ESCs in comparison to the control isogenic ESCs (V6.5). The expression of *Sox7* and *Dab2*, which are both markers for the primitive endoderm, are suppressed in $Rest^{-/-}$ ESCs. Transcript levels were normalized to *β-actin* levels. The data are presented as the average values with SD of six independent samples.

(B) The exogenous expression of *REST* rescued the suppression of *Gata4* and *Gata6* in $Rest^{-/-}$ ESCs. Mean ± SD of three independent samples.

(C) The expression of pluripotent genes in the embryoid body (EB) cells. The expression of *Occt3/4*, *Nanog*, and *Sox2* are upregulated in $Rest^{-/-}$ EB cells relative to the control EB cells. The data are presented as the mean ± SD of six independent samples.

(D) Conditional *Rest* knockout ESCs were cultured under differentiation culture conditions and treated with doxycycline (2 μg/ml) for 24 hr starting at 24 hr. The cells were harvested at 96 hr after the passage. The expression of *Syt4*, *Oct3/4*, *Nanog*, and *Gata4* after the conditional deletion of *Rest* under the differentiation culture condition. Note that the expression of *Nanog* and *Syt4*, but not of *Oct3/4*, were upregulated in the doxycycline-treated cells. The data are presented as the mean ± SD of six independent samples.

(E) A schematic drawing of the doxycycline-inducible *REST* ESC line.

(F) 48 hr of the induction of *REST* causes the ESC differentiation into epithelium-like colonies with a decreased AP activity.

(G) The forced expression of *REST* in ESCs leads to decreased expression of *Nanog*, *Oct3/4*, and *Fgf5*, whereas it results in increased expression of *Gata6*. The data are presented as the mean ± SD of six independent samples.

(H) In vitro differentiation of *REST*-inducible ESCs into EBs under the absence or presence of doxycycline. The exogenous *REST* expression results in an increased number of Gata4-positive cells at the periphery of EBs.

Rosa26::*rtTA*; *Col1a1*::*tetO-Cre* ESCs; Beard et al., 2006; Hochedlinger et al., 2005). This new ESC line enabled the conditional deletion of the floxed *Rest* genes in the presence of doxycycline. After 3 days of doxycycline exposure, the recombination in both alleles of the *Rest* was confirmed in 70%–80% of these ESCs by FACS (Figure 1F). However, the conditional deletion did not suppress the formation of AP-positive colonies regardless of the presence or absence of feeder cells in comparison to the parental ESCs without doxycycline (Figure 1G). In addition, the expression levels of *Oct3/4* and *Nanog* did not change, whereas the expression level of *Syt4* was derepressed while demonstrating evidence of *Rest* recombination shortly after doxycycline treatment (Figure S1D). These results therefore clearly rule out both the possibility of the adaptation in the long-term culture as well as the notion that feeder cells reduce the requirement of Rest-mediated ESC maintenance. Taken together, our results indicate that *Rest* is not required for the maintenance of ESC pluripotency in these experimental conditions.

Both *Gata4* and *Gata6* were significantly downregulated in the $Rest^{-/-}$ ESCs under confluent culture conditions (Figure 2A), although the findings were not prominent before the cells reached confluence. *Gata4* and *Gata6* are transcriptional factors that promote primitive endoderm differentiation (Fujikura et al., 2002; Niwa, 2007). These findings suggest that the genetic deletion of *Rest* prevents ESCs from differentiating toward the primitive endoderm. The notion of the suppression of primitive endoderm differentiation is confirmed by the decreased expression of both *Sox7* and *Dab2*, markers for the primitive endoderm (Shimoda et al., 2007; Yang et al., 2002), in $Rest^{-/-}$ ESCs (Figure 2A). Consistent with this notion, embryoid bodies (EBs) generated from $Rest^{-/-}$ ESCs revealed a decreased number of Gata4-expressing cells in the periphery of EBs on the histological sections in comparison to the control EBs (13.1 ± 15.0/EB and 30.4 ± 9.02/EB in RestKO8 EBs and V6.5 EBs, respectively, $p < 0.006$ by Student's t test) (Figure S2A). Rescue experiments were performed with a plasmid containing human *REST* cDNA (Grimes et al., 2000) to further investigate the direct association of the genetic deletion of *Rest* and the altered expression of *Gata4* and *Gata6* in confluent $Rest^{-/-}$ ESCs. Importantly, the decreased expression of both *Gata4* and *Gata6* in confluent $Rest^{-/-}$ ESCs were derepressed by the exogenous expression of *REST* (Figure 2B).

The expression of *Nanog*, *Oct3/4*, and *Sox2* were significantly higher in the $Rest^{-/-}$ EB cells than in the control EB cells (Figure 2C). Accordingly, these observations suggest that the delayed repression of self-renewal genes during the early differentiation of ESCs may thus cause the suppression of the early differentiation of $Rest^{-/-}$ ESCs. To further examine the initial response of gene expression upon the early differentiation of $Rest^{-/-}$ ESCs, the differentiation (-LIF, -MEF) of *Cre*-inducible *Rest*-floxed ESCs was induced with/without doxycycline exposure (Figure 2D). At 3 days after doxycycline treatment, the expression of *Nanog*, but not of *Oct3/4*, was observed to be significantly higher in the doxycycline-treated ESCs than that of the nontreated ESCs (Figure 2D). In contrast, a decreased expression of *Gata4* was not detectable at 3 days after doxycycline treatment when the *Syt4* expression had already been derepressed (Figure 2D). These results suggest that a decreased expression of *Gata4* in $Rest^{-/-}$ cells is preceded by an increased expression of *Nanog* and that *Gata4* repression is therefore a secondary effect of *Rest* ablation.

Finally, a doxycycline-inducible *REST* ESC line was generated (Figure 2E; Figure S2B). The forced expression of *REST* led to the rapid morphological changes of ESC colonies into an epithelium-like shape, which was accompanied by decreased AP activity (Figure 2F). In line with such morphological changes, ESCs with exogenous *REST* expressed significantly lower levels of self-renewal genes. The expression of *Gata6* was higher, whereas the expression of an epiblast marker, *Fgf5*, was significantly lower in such ESCs (Figure 2G). Furthermore, an increased number of Gata4-expressing cells in the periphery of EBs was observed in the exogenous *REST*-induced EBs (79.2 ± 19.6/EB and 50.7 ± 17.6/EB in *REST*-induced EBs and control EBs, respectively, $p < 0.004$ by Student's t test) (Figure 2H), thus suggesting that the forced *REST* expression promotes the ESC differentiation into the primitive endoderm. Importantly, the *REST*-induced ESC differentiation was, at least in part, rescued by the *Nanog* overexpression (Figure 2I; Figure S2D).

Although the critical role of the Oct3/4-Sox2-Nanog core transcription circuitry in the maintenance of ESC pluripotency is widely accepted (Boyer et al., 2005, 2006; Chambers et al., 2003; Loh et al., 2006; Mitsui et al., 2003; Niwa et al., 2000), the mechanisms leading to the breakdown of such core circuitry upon the early ESC differentiation are still not well understood (Kunath et al., 2007). The present study demonstrated that *Rest* ablation causes delayed repression of the pluripotent genes, whereas overexpression of *REST* immediately results in the suppression of the pluripotent gene expression. It is noteworthy that the delayed repression of the pluripotent genes by the conditional ablation of *Rest* was predominantly observed in *Nanog*. Given the fact that Rest is a transcriptional repressor and *Nanog* harbors RE1 in its promoter (Johnson et al., 2008), the current results therefore suggest that Rest is involved in the silencing of *Nanog* expression during the early differentiation of ESCs. This notion is also supported by the observation that ectopic *REST* in $Rest^{-/-}$ ESCs predominantly repressed the *Nanog* expression relative to the expression in original $Rest^{-/-}$ ESCs (Figure S2C). These findings suggest that Rest is an external factor connecting to the Oct3/4-Sox2-Nanog regulatory network core circuitry to influence the initial differentiation of ESCs. It is interesting to note that Rest is abundantly expressed in ESCs and it is a target of the Oct3/4-Sox2-Nanog regulatory network core circuitry (Johnson et al., 2008). It is possible that the negative feedback loop through Rest may play

(I) The *Nanog* overexpression dampens the *REST*-mediated ESC differentiation. *REST* was induced in *Nanog*-overexpressing and *EGFP*-overexpressing ESC colonies by the doxycycline exposure. The 24 hr exposure of doxycyline led to the rapid differentiation in *EGFP*-overexpressing ESCs (arrowheads), whereas *Nanog*-overexpressing ESCs retained an undifferentiated morphology. After the 48 hr exposure of doxycyline, 16 out of 25 *EGFP*-overexpressing colonies (68%) started to differentiate, whereas none of *Nanog*-overexpressing colonies (0/21, 0%) revealed the evidence of differentiation (see also Figure S2D).

a role in the stable transcriptional circuitry and in the rapid response upon the early differentiation of ESCs.

The current findings also suggest that Rest promotes the early ESC differentiation. Epiblast and the primitive endoderm are two distinct cell types in the inner cell mass (ICM) of the blastocyst. Genetic evidence indicates that the *Nanog* and *Gata* family transcription factors play a role in the segregation of epiblast and primitive endoderm within ICM (Chambers et al., 2003; Koutsourakis et al., 1999; Mitsui et al., 2003; Soudais et al., 1995). Indeed, Nanog and Gata6 are expressed in the ICM in a mutually exclusive manner (Chazaud et al., 2006), thus indicating the reciprocal control of the gene expression. The current study found that the conditional ablation of *Rest* results in the delayed repression of *Nanog* during the early differentiation of ESCs, whereas *REST* overexpression causes an increased expression of *Gata6*, which is accompanied by the rapid differentiation. In addition, the expression of *Fgf5*, an epiblast marker, was significantly downregulated by the *REST* overexpression. These results suggest that Rest may be involved in the segregation of epiblast and primitive endoderm through modifying the *Nanog* expression.

In summary, the conditional ablation of the *Rest* gene revealed that *Rest* is not absolutely required for the maintenance of ESC pluripotency. These results also indicate that *Rest* plays a role in the suppression of the pluripotent gene expression upon the early differentiation of ESCs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.stem.2009.12.003.

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REFERENCES

Andrés, M.E., Burger, C., Peral-Rubio, M.J., Battaglioli, E., Anderson, M.E., Grimes, J., Dallman, J., Ballas, N., and Mandel, G. (1999). CoREST: A functional corepressor required for regulation of neural-specific gene expression. Proc. Natl. Acad. Sci. USA *96*, 9873–9878.

Ballas, N., Grunseich, C., Lu, D.D., Speh, J.C., and Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell *121*, 645–657.

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., and Jaenisch, R. (2006). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis *44*, 23–28.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947–956.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349–353.

Buckley, N.J., Johnson, R., Sun, Y.M., and Stanton, L.W. (2009). Is REST a regulator of pluripotency? Nature *457*, E5–E6.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643–655.

Chazaud, C., Yamanaka, Y., Pawson, T., and Rossant, J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. Dev. Cell *10*, 615–624.

Chong, J.A., Tapia-Ramírez, J., Kim, S., Toledo-Aral, J.J., Zheng, Y., Boutros, M.C., Altshuller, Y.M., Frohman, M.A., Kraner, S.D., and Mandel, G. (1995). REST: A mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell *80*, 949–957.

Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J., and Niwa, H. (2002). Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev. *16*, 784–789.

Grimes, J.A., Nielsen, S.J., Battaglioli, E., Miska, E.A., Speh, J.C., Berry, D.L., Atouf, F., Holdener, B.C., Mandel, G., and Kouzarides, T. (2000). The co-repressor mSin3A is a functional component of the REST-CoREST repressor complex. J. Biol. Chem. *275*, 9461–9467.

Hochedlinger, K., Yamada, Y., Beard, C., and Jaenisch, R. (2005). Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell *121*, 465–477.

Johnson, R., Teh, C.H., Kunarso, G., Wong, K.Y., Srinivasan, G., Cooper, M.L., Volta, M., Chan, S.S., Lipovich, L., Pollard, S.M., et al. (2008). REST regulates distinct transcriptional networks in embryonic and neural stem cells. PLoS Biol. *6*, e256.

Jørgensen, H.F., Chen, Z.F., Merkenschlager, M., and Fisher, A.G. (2009a). Is REST required for ESC pluripotency? Nature *457*, E4–E5.

Jørgensen, H.F., Terry, A., Beretta, C., Pereira, C.F., Leleu, M., Chen, Z.F., Kelly, C., Merkenschlager, M., and Fisher, A.G. (2009b). REST selectively represses a subset of RE1-containing neuronal genes in mouse embryonic stem cells. Development *136*, 715–721.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extra-embryonic development. Development *126*, 723–732.

Kunath, T., Saba-El-Leil, M.K., Almousailleakh, M., Wray, J., Meloche, S., and Smith, A. (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development *134*, 2895–2902.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat. Genet. *38*, 431–440.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631–642.

Niwa, H. (2007). How is pluripotency determined and maintained? Development *134*, 635–646.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372–376.

Schoenherr, C.J., and Anderson, D.J. (1995). The neuron-restrictive silencer factor (NRSF): A coordinate repressor of multiple neuron-specific genes. Science *267*, 1360–1363.

Schoenherr, C.J., Paquette, A.J., and Anderson, D.J. (1996). Identification of potential target genes for the neuron-restrictive silencer factor. Proc. Natl. Acad. Sci. USA *93*, 9881–9886.

Shimoda, M., Kanai-Azuma, M., Hara, K., Miyazaki, S., Kanai, Y., Monden, M., and Miyazaki, J. (2007). Sox17 plays a substantial role in late-stage differentiation of the extraembryonic endoderm in vitro. J. Cell Sci. *120*, 3859–3869.

Singh, S.K., Kagalwala, M.N., Parker-Thornburg, J., Adams, H., and Majumder, S. (2008). REST maintains self-renewal and pluripotency of embryonic stem cells. Nature *453*, 223–227.