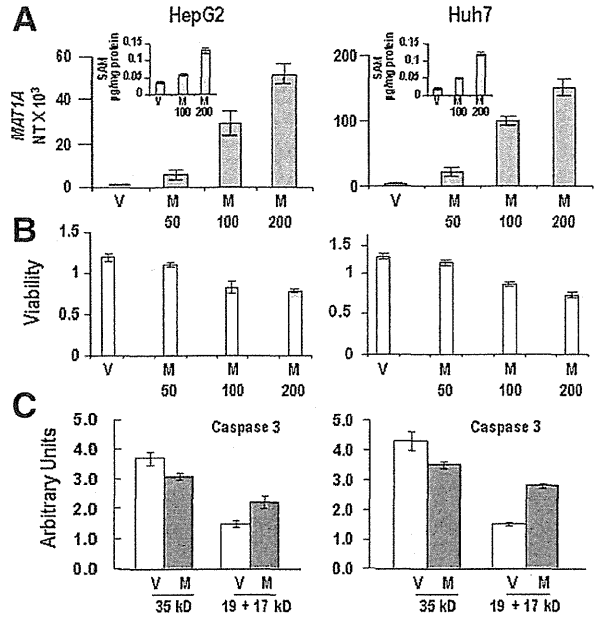


Fig. 7. Effect of *MAT1A* transfection of cell proliferation and gene expression. HepG2 and HuH7 cells were transiently transfected with *MAT1A* cDNA in pCMV6-XL vector. (A) *MAT1A* expression 72 and 96 hours in HepG2 and Huh7 cells, respectively, after transfection with empty vector (V) or the indicated amounts (ng) of *Mat1A* cDNA (M). Data are means ± SD of three independent experiments of N-fold differences in *MAT1A* expression relative to the *RNR-18* expression, and named N Target (NT). NT = $2^{-\Delta Ct}$, $\Delta Ct = [CT^{(target)} - CT^{(RNR\text{-}18)}]$. Insets: SAM levels. Tukey-Kramer test: *Mat1A* expression and SAM level, M versus V, $P < 0.001$. (B) Cell viability of HepG2 and Huh7 cells 72 and 96 hours after transfection, respectively. Tukey-Kramer test: M versus V $P < 0.001$ for M 100 and 200 ng. (C) Caspase 3 cleavage in HepG2 and Huh7 cells 72 and 96 hours after transfection, respectively, with empty vector (V) or 200 ng of *Mat1A* cDNA. Data are means ± SD of three independent experiments. Tukey-Kramer test: M versus V $P < 0.001$ for 35 and 17+19 kD fragments of both cell lines.

suppression of growth and induction of apoptosis of human HCC. Furthermore, because SAM level modulates IKK-$\beta$ activity of *in vitro*-growing cells[35] and counteracts NF-$\kappa$B activation in rat preneoplastic foci,[36] we evaluated the effects of *MAT1A* up-regulation on proliferation, apoptosis, and the IKK-NF-$\kappa$B cascade in human HCC cell lines transiently transfected with *MAT1A*. *MAT1A* transfection, which resulted in 95- and 70-fold increases in its expression at 96 hours, a 5 to 7-fold rise in SAM level (Fig. 7A), a significant reduction in cell proliferation (Fig. 7B), and increase in apoptosis, as shown by caspase 3 cleavage (Fig. 7C; Fig. S7) in HepG2 and Huh7 cells. This was associated with a significant down-regulation of *Cyclin D1, E2F1, IKK, NF-κB*, and the antiapoptotic *BCL2* and *XIAP* genes, and up-regulation of the proapoptotic *BaK* and *BAX* genes in HepG2 and Huh7 cells, 72 and 96 hours after transfection, respectively (Fig. S8).

## Discussion

Our results show a marked decrease in *Mat1A* expression and MATI/III activity, an increase in *Mat2A* expression and MATII activity, and a decrease in *Mat1A:Mat2A* expression ratio and the MATI/III: MATII activity ratio in DN and HCC of F344 rats, genetically susceptible to hepatocarcinogenesis. *Mat1A* and *Mat2A* expression and MAT isozymes underwent low/no changes in DN and HCC of genetically resistant BN rats. Changes in the MATs isozyme pattern, analogous to those of F344 HCC, occur in human HCC,[16,19,21] and have been associated with CCGG methylation of *MAT1A* promoter, and CCGG hypomethylation of *MAT2A* promoter.[19,21] Accordingly, we observed prevalent CpGs methylation and histone H4 deacetylation of the *Mat1A* promoter and prevalent CpGs hypomethylation and histone H4 acetylation in the *Mat2A* promoter of F344 HCC. Considering the ATG flanking regions of the two genes (+10 to −778 and +66 to −837 for *Mat1A* and *Mat2A*, respectively, analyzed by ERMA), ≈82% and ≈60% of CpGs were methylated in the *Mat1A* promoter of F344 and BN HCC, respectively. Methylation of 85%-86% of CpGs occurs in the ATG flanking region of the *Mat1A* promoter of mouse Hepa-1[37] and human Huh7 HCC cells.[20] In the *Mat2A* promoter, ≈68% and ≈20% of CpGs were unmethylated in F344 and BN HCC, respectively. Notably, ≈62% of CpGs in the ATP flanking region of human *MAT1A* were methylated in HCCP against ≈25% in HCCB. The *MAT2A* promoter was largely hypomethylated, with a lower difference between HCCP and HCCB. These changes roughly reproduce interstrain differences between rat HCCs differently prone to progress.

The mechanisms modulating MATs promoter methylation in hepatocarcinogenesis are still unknown. Interaction of DNA methyltransferases (DNMTs) with higher-order chromatin, inducing DNA methylation, may be reduced by histone acetylation.[38] *Dnmt1, Dnmt3a*, and *Dnmt3b* genes are equally overexpressed in HCC from both F344 and BN rats (data not presented). Further research should investigate the influence of interstrain variations in the mutual interplay between DNMTs, histone acetylation, and chromatin on *Mat1A* and *Mat2A* promoter methylation. Moreover, DNMT1 and DNMT3a interaction with histone

deacetylases may repress gene expression by way of a methylation-independent mechanism.[39]

Suppression of gene expression is frequently associated with methylated DNA and deacetylated histones, in a dense chromatin structure, whereas active transcription is associated with unmethylated DNA, hyperacetylated histones, in open chromatin.[40] According to recent results,[20] methylation of CCGG at −997 does not inhibit the *MAT1A* promoter, whereas CCGG methylation at +10 and +88 reduces promoter activity in Huh7 cells. However, no CCGG sequences are present in the first exon of rat *Mat1A* (NCBI AC_000084.1). Recent results indicate that binding to promoter of the transcription factor C/EBPβ, regulated by DNA methylation and histone deacetylation, determines *MAT1A* down-regulation in mouse, rat, and human.[37] Interestingly, C/*EBPβ* expression is ≈4-fold higher in F344 than BN HCC (data not shown), but C/EBPβ protein biding to promoter has not yet been evaluated. Nevertheless, in F344 HCC the highest histone H4 deacetylation was associated with the highest methylation of *Mat1A* promoter and low gene expression, whereas the highest histone H4 acetylation was associated with low methylation of the *Mat2A* promoter and gene up-regulation. These changes are very low/absent in BN HCC. Thus, our observations suggest, in accordance with previous results,[20,21] that changes in MATs promoter methylation and histone acetylation might be involved in *Mat1A:Mat2A* switch, and clearly indicate their association with the capacity of DN and HCC to progress.

Our results also support a role of posttranscriptional regulation of Mats expression in tumor progression and aggressiveness. A rise in AUF1 and HuR protein levels and of their interaction with *MAT1A* and *MAT2A* mRNAs, respectively, occurred in F344 rat and human HCC. Because the protein HuR promotes mRNA stabilization and AUF1 enhances mRNA decay,[22-24] our observations could at least in part explain the decrease in *MAT1A* and increase in *MAT2A* mRNA levels in these HCCs. AUF1 and HuR interaction with *Mat1A* and *Mat2A*, respectively, was very low in BN HCC. This should result in lower *Mat1A* mRNA decay and *Mat2A* mRNA stabilization in BN tumors, thus explaining the observed low changes in *Mat1A* and *Mat2A* expression with respect to normal liver.

A remarkable aspect of our results is the observation that genes responsible for genetic susceptibility to hepatocarcinogenesis control *Mat1A* and *Mat2A* methylation and posttranscriptional regulation, demonstrating, for the first time, that the regulation of CpG methylation of *Mat1A* and *Mat2A* promoter and posttranscriptional regulation of MATs can be connected to the genetic susceptibility of rats to HCC. This indicates that MATs epigenetic regulation is under genetic control and contributes to create a phenotype susceptible/resistant to hepatocarcinogenesis. In accordance with this assumption, our observations strongly suggest that *MAT1A:MAT2A* and MATI/III:MATII ratios are prognostic markers for HCC. Indeed: (1) the *MAT1A:MAT2A* expression ratio and the MATI/III:MATII activity ratio were directly correlated with proliferation rate and inversely correlated with apoptosis of HCC cells; (2) the direct correlation with global DNA methylation and genomic instability links these ratios to genomic alterations that are known to facilitate tumor progression[41]; (3) MATI/III:MATII ratio is strongly predictive of patient survival. Notably, according to our results, the MATI/III:MATII ratio influences the proliferation rate and global DNA methylation even in SL, suggesting that in human, as in rodent, early deregulation of the MATI/III:MATII ratio could influence the evolution of preneoplastic lesions to cancer.

The mechanisms involved in modulation of hepatocarcinogenesis by the *MATI/III:MATII* ratio are still incompletely known. *Mat1A* knockout (KO) mice undergo spontaneous oxidative stress and HCC development.[15] Conversely, HCC cells transfected with *MAT1A* exhibit lower proliferation and higher apoptosis and tumorigenesis in nude mice than untransfected cells[16,20] (and present results). *MAT1A* expression and the *MAT1A:MAT2A* ratio are major determinants of SAM content in rat and human hepatic cells that largely influences liver cell proliferation and tumorigenesis.[14,16] The decrease in expression of *IKK* in transfected cells, probably consequent to the increase in SAM level,[35] leads to *NF-κB* down-regulation, which could result in inhibition of the $G_1$ phase of cell cycle and $G_1$-S transition through restriction of Cyclin D1 and c-MYC expression, leading to *E2F1* down-regulation,[42] which was indeed found in *MAT1A* transfected cells. Furthermore, down-regulation of antiapoptotic *BCL2* and *XIAP* genes, and up-regulation of proapoptotic *BAX* and *BAK* genes, targeted by NF-κB,[43] may contribute to increased apoptosis. Interestingly, $G_1$/S transition and expression of NF-κB and its targets are significantly lower in preneoplastic and neoplastic lesions from BN rats and human HCCB than in F344 rats and HCCP.[2,7,8]

Various other mechanisms may be involved in the antiproliferative and proapoptotic effects of a high *MAT1A:MAT2A* ratio and SAM level. SAM has an antioxidant effect[15] and inhibits the activity of different growth-related genes and proteins,[16] including

*iNos* expression[44] and ERK1/2 activation.[16] Notably, SAM stabilizes APEX1 protein,[45] which should result in more efficient DNA repair and reduced GI. Thus, low MATI/III activity and SAM content in precancerous liver and HCC of susceptible rats should result in increased oxidative and nitrative stress, GI, overexpression of NF-κB and its targets, including c-*Myc, Cyclin D1, iNos*, and *VegfA*. All of these changes have indeed been observed in F344 HCC and HCCP, whereas they are low/absent in BN HCC and HCCB.[41]

In conclusion, our data indicate, for the first time, a posttranscriptional regulation of *MAT1A* and *MAT2A* by AUF1 and HuR in HCC. Notably, a low *MAT1A: MAT2A* ratio is a prognostic marker that contributes to determine a phenotype susceptible to HCC and patient survival. Finally, interference with cell cycle progression, through inhibition of IKK/NF-κB signaling, Cyclin D1, and E2F1, might represent one of the multiple mechanisms mediating the antiproliferative and proapoptotic effect of high SAM levels.

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# Development of a novel approach, the epigenome-based outlier approach, to identify tumor-suppressor genes silenced by aberrant DNA methylation

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ABSTRACT

Identification of tumor-suppressor genes (TSGs) silenced by aberrant methylation of promoter CpG islands (CGIs) is important, but hampered by a large number of genes methylated as passengers of carcinogenesis. To overcome this issue, we here took advantage of the fact that the vast majority of genes methylated in cancers lack, in normal cells, RNA polymerase II (Pol II) and have trimethylation of histone H3 lysine 27 (H3K27me3) in their promoter CGIs. First, we demonstrated that three of six known TSGs in breast cancer and two of three in colon cancer had Pol II and lacked H3K27me3 in normal cells, being outliers to the general rule. *BRCA1*, *HOXA5*, *MLH1*, and *RASSF1A* had high Pol II, but were expressed only at low levels in normal cells, and were unlikely to be identified as outliers by their expression statuses in normal cells. Then, using epigenome statuses (Pol II binding and H3K27me3) in normal cells, we made a genome-wide search for outliers in breast cancers, and identified 14 outlier promoter CGIs. Among these, *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* were confirmed to be methylated in primary breast cancer samples. Knockdown of *DZIP1* in breast cancer cell lines led to increases of their growth, suggesting it to be a novel TSG. The outliers based on their epigenome statuses contained unique TSGs, including *DZIP1*, compared with those identified by the expression microarray data. These results showed that the epigenome-based outlier approach is capable of identifying a different set of TSGs, compared to the expression-based outlier approach.



## 1. Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs), especially that of nucleosome-free regions (NFRs), causes silencing of their downstream genes [1], and is known as an alternative mechanism to point mutations and chromosomal deletion for inactivation of tumor-suppressor genes (TSGs) [2–6]. Now, TSGs involved in various biological pathways such as cell proliferation, cell adhesion, and DNA repair are known to be silenced by aberrant DNA methylation in human cancers [7,8]. To identify novel TSGs silenced by aberrant DNA methylation, two major approaches for genome-wide screening have been reported, namely (1) genome-wide screening of methylated genes and (2) screening of genes whose expression is induced by a DNA demethylating agent (chemical genomic screening) [9–13].

A genome-wide screening of methylated genes usually isolates several hundreds to one thousand genes whose promoter CGIs are methylated in cancers [12,14]. Unfortunately, most of these genes are not expressed or expressed at only low levels in normal cells [14]. Since genes with low transcription tend to be methylated [15], most of the genes identified by genome-wide screening of methylated genes are considered to be methylated as a consequence of carcinogenesis. In chemical genomic screening, cell lines are treated with a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), and genes whose expression is induced are screened by expression microarray analysis. However, since 5-aza-dC forms DNA adducts [16] and induces double-strand breaks [17], treatment with 5-aza-dC leads to up-regulation of not only methylation-silenced genes but also numerous genes involved in responses to DNA damage, including p53-activated genes [18–21].

It was recently shown that the vast majority of genes aberrantly methylated in cancers have specific epigenetic statuses in normal cells, namely the lack of RNA polymerase II (Pol II) and the presence of trimethylation of histone H3 lysine 27 (H3K27me3) [14,22]. H3K27me3 is known to be most influential on the susceptibility of genes to become methylated among various histone

*Abbreviations:* CGIs, CpG islands; ChIP, chromatin immunoprecipitation; FHC, normal fetal human colon epithelial cell line; HMECs, normal human mammary epithelial cells; H3K27me3, trimethylation of histone H3 lysine 27; NFR, nucleosome-free region; Pol II, RNA polymerase II; TSG, tumor-suppressor gene.

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modifications [14]. At the same time, approximately 5% of the genes methylated in cancers do not follow the general rule [14], constituting a group of "outliers". Since a TSG is functioning in normal cells, it is expected to have Pol II in normal cells and belong to the outliers. Even if silencing of a TSG is a very rare event due to the Pol II binding, a cell with its silencing will become dominant due to the resultant growth advantage, and the TSG appears as if it is susceptible to methylation. Indeed, by searching outliers with high expression in normal cells, TSGs have been successfully identified [11,23,24]. However, some TSGs, including *CDKN1A*, are known to have no or low expression in normal cells and are activated by extracellular stimuli [25,26].

In this study, we aimed to show that a significant fraction of known TSGs silenced by aberrant DNA methylation are outliers. Then, we will show that a different set of TSGs could be identified by searching for outliers with high Pol II and without H3K27me3 (epigenome-based outlier approach), compared to those identified by searching for outliers with high expression (expression-based outlier approach).

## 2. Materials and methods

### 2.1. Cell lines and primary breast cancer samples

Human breast cancer cell lines (BT-20, BT-474, HCC38, HCC1428, HCC1937, Hs 578T, MCF7, MDA-MB-231, MDA-MB-436, MDA-MB-468, SK-BR-3, T-47D, and ZR-75-1), normal fetal human colon epithelial cell line (FHC), and 293TN cell line were purchased from the American Type Culture Collection (Rockville, MD). Normal human mammary epithelial cells (HMECs) were purchased from Cambrex (East Rutherford, NL). Forty primary breast cancer samples were obtained from patients who underwent mastectomy or breast conserving surgery with informed consent. Resected primary samples were frozen and stored at −80 °C until the extraction of genomic DNA. The analysis was approved by the Institutional Review Boards.

### 2.2. Chromatin immunoprecipitation (ChIP)

Eight μg of chromatin extracted from HMECs was incubated with 0.7 μg of antibodies against Pol II (ab5095; Abcam, Cambridge, UK) or H3K27me3 (07-449; Millipore, Billerica, MA) overnight at 4 °C. Then the immunocomplex was collected with Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway). Recovered DNA was dissolved in 33 μl of 1 × TE (10 mM Tris-HCl and 1 mM EDTA) [14], and one μl of DNA was used for ChIP-quantitative PCR (ChIP-qPCR) using primer sets listed in Supplementary Table 1. The specificity of the ChIP assay was assessed by using primers for control regions whose histone modification statuses were already known [14] (Supplementary Fig. 1).

### 2.3. Analyses of microarray data

DNA methylation data of 9838 promoter CGIs in HMECs, BT-474, MCF7, MDA-MB-231, MDA-MB-468, and ZR-75-1 were obtained from our previous study using methylated DNA immunoprecipitation (MeDIP)-CGI microarray analysis [27]. Pol II binding and H3K27me3 data of 9838 promoter CGIs in HMECs were obtained from our previous study using ChIP-CGI microarray analysis [14]. Pol II binding and H3K27me3 levels of each CGI were evaluated using the average of the signal ratio [Cy5 signal (bound)/Cy3 signal (input)] of the probes within a NFR. CGIs with a mean signal ratio larger than that at the 80th percentile of the total probes were considered as CGIs with high Pol II binding or H3K27me3, and those with an average signal ratio smaller than that at the 20th percentile of the total probes were considered as CGIs with low Pol II binding or H3K27me3. Other CGIs were considered as genes with intermediate Pol II binding or H3K27me3 levels. Gene expression data in HMECs were obtained from our previous study using the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA), and genes were classified into those with high, moderate, and low expression levels, as described previously [14].

### 2.4. Quantitative methylation-specific PCR (qMSP)

Genomic DNA was digested with *Bam*HI, and one μg of digested DNA was used for bisulfite modification [28]. qMSP was performed using primer sets specific to methylated target loci and to the *Alu* repeat sequence (Supplementary Table 2). Modified DNA was dissolved in 40 μl of 1 × TE, and one μl was used for qMSP. The number of DNA molecules in a sample was obtained by comparing its amplification curve with those of standard samples with known numbers of DNA molecules. DNA methylation levels were calculated as the percentage of the methylation reference (PMR) = [(number of molecules methylated at a target CGI in a sample)/(number of *Alu* repeat sequences in the sample)]/[(number of molecules methylated at the target CGI in a fully methylated DNA)/(number of *Alu* repeat sequences in the fully methylated DNA)] × 100 [29,30]. Genomic DNA treated with *SssI* methylase (New England Biolabs, Beverly, MA) was used as a fully methylated DNA. Since the copy number of the target CGI was normalized to the copy number of the *Alu* repeat sequence, PMR could reach more than 100% when the locus containing the target CGI had an increased copy number.

### 2.5. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was synthesized from one μg of total RNA using SuperScript III reverse transcriptase and an oligo $(dT)_{12-18}$ primer (Life Technologies, Carlsbad, CA). qRT-PCR was performed using primer sets listed in Supplementary Table 3, as described previously [31]. The number of cDNA molecules in a sample was obtained by comparing its amplification curve with those of standard samples with known numbers of cDNA molecules, and the number of target cDNA molecules was normalized to those of human *GAPDH* cDNA molecules.

### 2.6. 5-Aza-dC and trichostatin A (TSA) treatment

T-47D cells were seeded on day 0, and exposed to a designated dose of freshly prepared 5-aza-dC (SIGMA-ALDRICH, St. Louis, MO) for 24 h on days 1 and 3, and treated with TSA (SIGMA-ALDRICH) on day 4. The cells were harvested on day 5, and total RNA was extracted.

### 2.7. Knockdown of DZIP1 and cell growth assay

*DZIP1* was knocked down by two different short hairpin RNAs (shRNA1 and shRNA2; Supplementary Table 4) designed by using siDirect version 2.0 [32], and shRNA for firefly luciferase was used as the control. The sense and antisense oligonucleotides containing each shRNA sequence were annealed, and then ligated into *Bam*HI/*Eco*RI sites of pGreenPuro™ shRNA Cloning and Expression Lentivector (System Biosciences, Mountain View, CA). The shRNA expression construct was co-transfected with pPACK Packaging Plasmid Mix (System Biosciences) into 293TN cells using Lipofectamine™ with Plus™ Reagent (Life Technologies). Medium containing pseudovirus was collected 48 h after transfection, and stored at −80 °C until use. HCC1937 and MDA-MB-436 cells were infected with pseudovirus according to the manufacturer's protocol.

Cell growth was analyzed by seeding cells at a density of $5 \times 10^4$ cells/6 cm plate on day 0, and counting the cell numbers on days 1, 2, 3, 4, 5, and 8 using TC™ Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA).

## 3. Results

### 3.1. Confirmation of known TSGs as outliers

To confirm that TSGs are outliers to the general rule of genes methylated in cancer cells, we first analyzed six known TSGs silenced in breast cancer by aberrant DNA methylation, *BRCA1*, *CDKN2A* (*p16*), *HOXA5*, *MASPIN*, *RASSF1A*, and *RBP1* [33–38]. The Pol II binding and H3K27me3 levels of these six genes were analyzed in HMECs (Fig. 1A). Among the six TSGs, *BRCA1*, *HOXA5*, and *MASPIN* had high Pol II binding and almost undetectable levels of H3K27me3 in the HMECs, demonstrating that these genes are outliers. In contrast, *RASSF1A* had low Pol II binding and high H3K27me3 levels, showing that *RASSF1A* was methylated according to the rule. *CDKN2A* and *RBP1* had intermediate levels of Pol II binding and H3K27me3. We also analyzed expression levels of the six TSGs in normal cells. Among the three outliers, *MASPIN* was expressed at high levels (active Pol II), and *BRCA1* and *HOXA5* were expressed at low levels (stalled Pol II) (Fig. 1C). In the case of three TSGs known to be methylation-silenced in colon cancer, *MLH1* and *RASSF1A* [7] had high levels of Pol II binding and low expression levels (stalled Pol II) (Fig. 1B and D). These results indicated that a significant fraction of TSGs are "outliers" to the general rule of genes methylated in cancer cells, and that the outliers could be classified into those with active Pol II and with stalled Pol II.

### 3.2. Screening of outliers in breast cancer based on their epigenome statuses

Confirming that three of six known TSGs in breast cancer and two of three known TSGs in colon cancer are outliers, we pro-

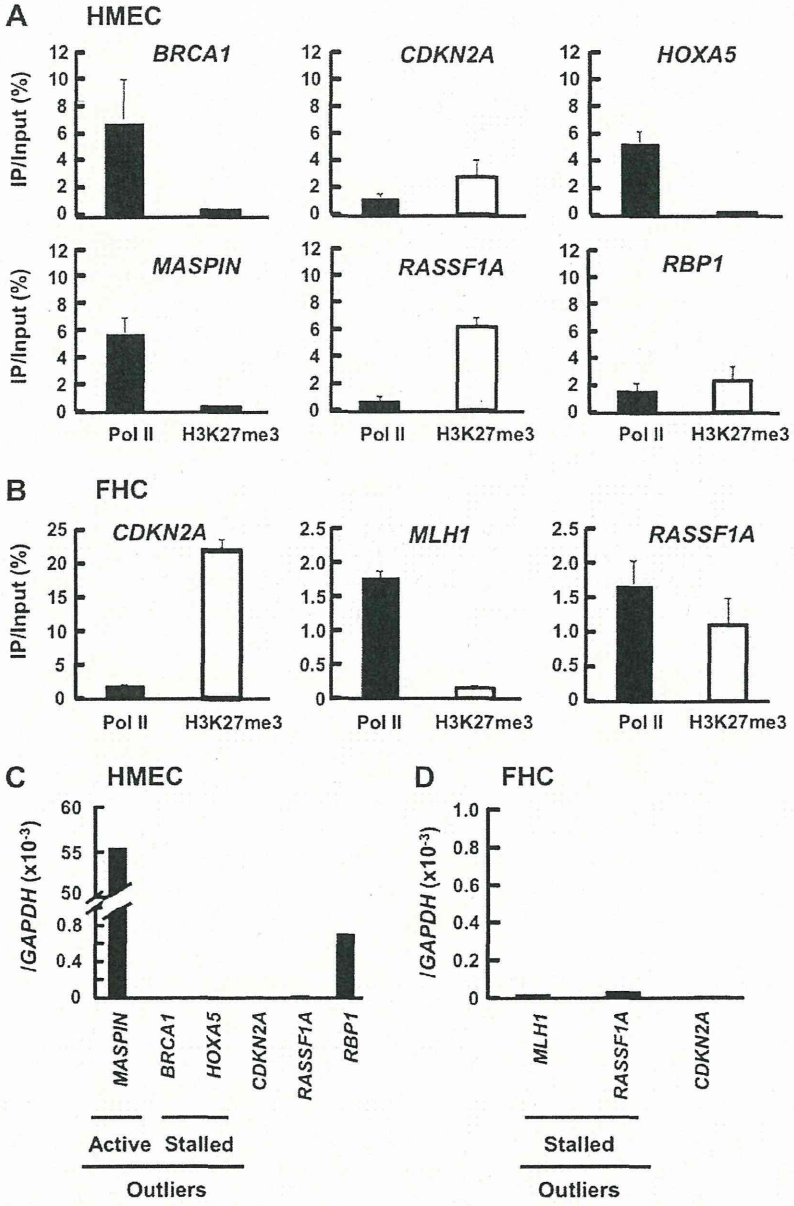


**Fig. 1.** Confirmation of known TSGs as outliers to the general rule. (A) Pol II binding and H3K27me3 levels in HMECs of the six TSGs in breast cancer analyzed by ChIP-qPCR. Three of the six TSGs showed relatively high Pol II binding levels and low H3K27me3 levels in the HMECs, showing that they are outliers to the general rule. The mean ± SE values of three independent experiments are shown. (B) Pol II binding and H3K27me3 levels in FHC of the three TSGs in colon cancer analyzed by ChIP-qPCR. Two of the three TSGs showed high Pol II binding and low H3K27me3 levels, being outliers. (C) and (D) Expression levels of the known TSGs in normal cells. Expression levels of TSGs in HMECs (C) and FHC (D) were analyzed by qRT-PCR. Among the five outliers in breast and colon cancers, only *MASPIN* was expressed at high levels in HMECs, and the other four outliers were expressed at low levels. Active; active Pol II, Stalled; stalled Pol II.

ceeded to identify novel TSGs in breast cancer by making a genome-wide search for outliers. Based on DNA methylation statuses of two different lots of normal breast epithelial cells (HMECs) and five breast cancer cell lines obtained by MeDIP-CGI microarray analyses, we previously identified 280 promoter CGIs unmethylated in both lots of the HMECs and methylated in two or more of the breast cancer cell lines as methylation-susceptible promoter CGIs in breast cancer [27]. To identify outliers based on the epigenome statuses, Pol II binding and H3K27me3 statuses in the HMECs [14] were utilized. Among the 280 promoter CGIs susceptible to methylation induction during breast carcinogenesis, 37 CGIs had high Pol II binding (Fig. 2A), and 14 of the 37 CGIs had low H3K27me3 levels in the HMECs (Fig. 2B). Since two promoter CGIs were shared by two genes, respectively, 16 genes downstream of these 14 promoter CGIs were considered as outliers in breast cancers (Table 1).

### 3.3. DNA methylation of outlier promoter CGIs in primary breast cancer samples

DNA methylation levels were quantified by qMSP for 13 of the 14 promoter CGIs (except for a CGI upstream of two core histone genes) in 13 breast cancer cell lines, including the five breast cancer cell lines used for identification of methylation-susceptible promoter CGIs, and HMECs (Fig. 3A and Supplementary Fig. 2). All the 13 outlier promoter CGIs showed low methylation in the HMECs, and five (*DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3*) of them showed

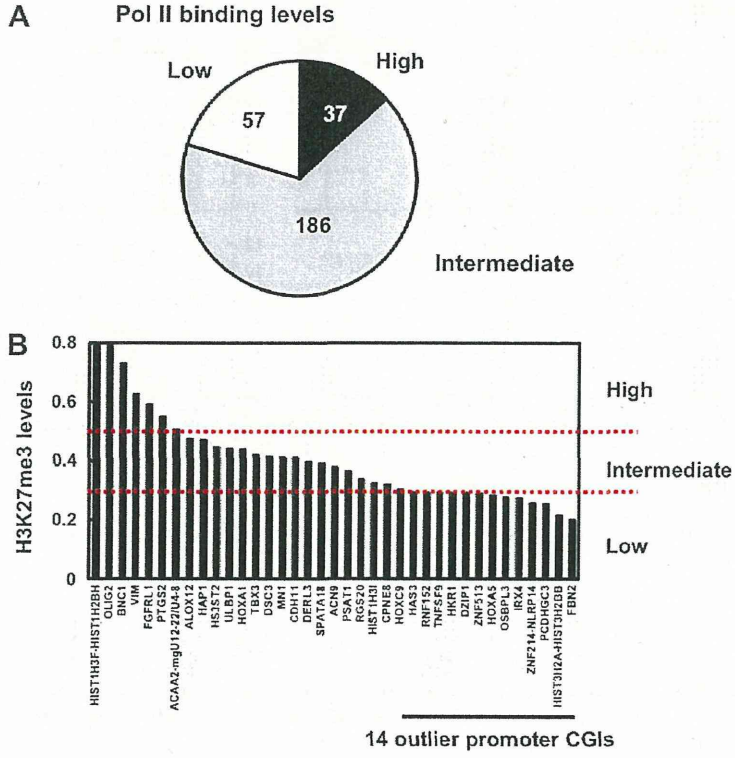


**Fig. 2.** Genome-wide screening of outliers based on the epigenome. (A) Pol II binding levels of the 280 methylation-susceptible promoter CGIs in the HMECs. Thirty-seven CGIs (13% of susceptible CGIs) showed high Pol II binding levels in the HMECs. (B) H3K27me3 levels of the 37 CGIs with high Pol II binding in the HMECs. Fourteen promoter CGIs with high Pol II binding levels showed low H3K27me3 levels in the HMECs, being outliers.

**Table 1**
List of outlier promoter CGIs in breast cancer.

| | Gene symbol | Gene name | Chr | DNA methylation | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Normal | | Cancer | | | | |
| | | | | HMEC#1 | HMEC#2 | BT-474 | MCF7 | MDA-MB-231 | MDA-MB-468 | ZR-75-1 |
| 1 | *DZIP1* | DAZ interacting protein 1 | 13 | UM | UM | UM | HM | UM | HM | MM |
| 2 | *FBN2*[a] | Fibrillin 2 | 5 | UM | UM | UM | MM | HM | HM | UM |
| 3 | *HAS3* | Hyaluronan synthase 3 | 16 | UM | UM | MM | HM | MM | MM | HM |
| 4 | *HIST3H2A-HIST3H2BB*[b] | Histone cluster 3, H2a/histone cluster 3, H2bb | 1 | UM | UM | UM | UM | HM | HM | UM |
| 5 | *HKR1* | GLI-Kruppel zinc family member | 19 | UM | UM | UM | UM | HM | UM | HM |
| 6 | *HOXA5*[a] | Homeobox A5 | 7 | UM | UM | UM | UM | HM | HM | HM |
| 7 | *HOXC9* | Homeobox C9 | 12 | UM | UM | UM | UM | HM | HM | UM |
| 8 | *IRX4* | Iroquois homeobox 4 | 5 | UM | UM | UM | UM | HM | HM | UM |
| 9 | *OSBPL3* | Oxysterol binding protein-like 3 | 7 | UM | UM | HM | HM | UM | UM | UM |
| 10 | *PCDHGC3* | Protocadherin gamma subfamily C, 3 | 5 | UM | UM | UM | UM | HM | HM | UM |
| 11 | *RNF152* | Ring finger protein 152 | 18 | UM | UM | UM | UM | HM | HM | UM |
| 12 | *TNFSF9* | Tumor necrosis factor (ligand) superfamily, member 9 | 19 | UM | UM | HM | UM | UM | HM | UM |
| 13 | *ZNF214-NLRP14*[b] | Zinc finger protein 214/NLR family, pyrin domain containing 14 | 11 | UM | UM | UM | HM | UM | HM | UM |
| 14 | *ZNF513* | Zinc finger protein 513 | 2 | UM | UM | UM | HM | HM | UM | HM |

UM, unmethylated; MM, moderately methylated; HM, highly methylated; chr, chromosome number.
[a] Known tumor-suppressor gene.
[b] Promoter CGIs shared by two genes.

high methylation levels (PMR higher than 100%) in at least one cancer cell line (Fig. 3A).

Then, using 40 primary breast cancer samples, the methylation levels of these five outlier promoter CGIs were analyzed. Methylation of *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* was present in primary breast cancer samples, while that of *OSBPL3* was not observed (Fig. 3B). These results indicated that *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* are outliers methylated not only in cell lines but also in primary breast cancers. Among the four outliers, *HOXA5* and *FBN2* were known as TSGs silenced by aberrant DNA methylation in

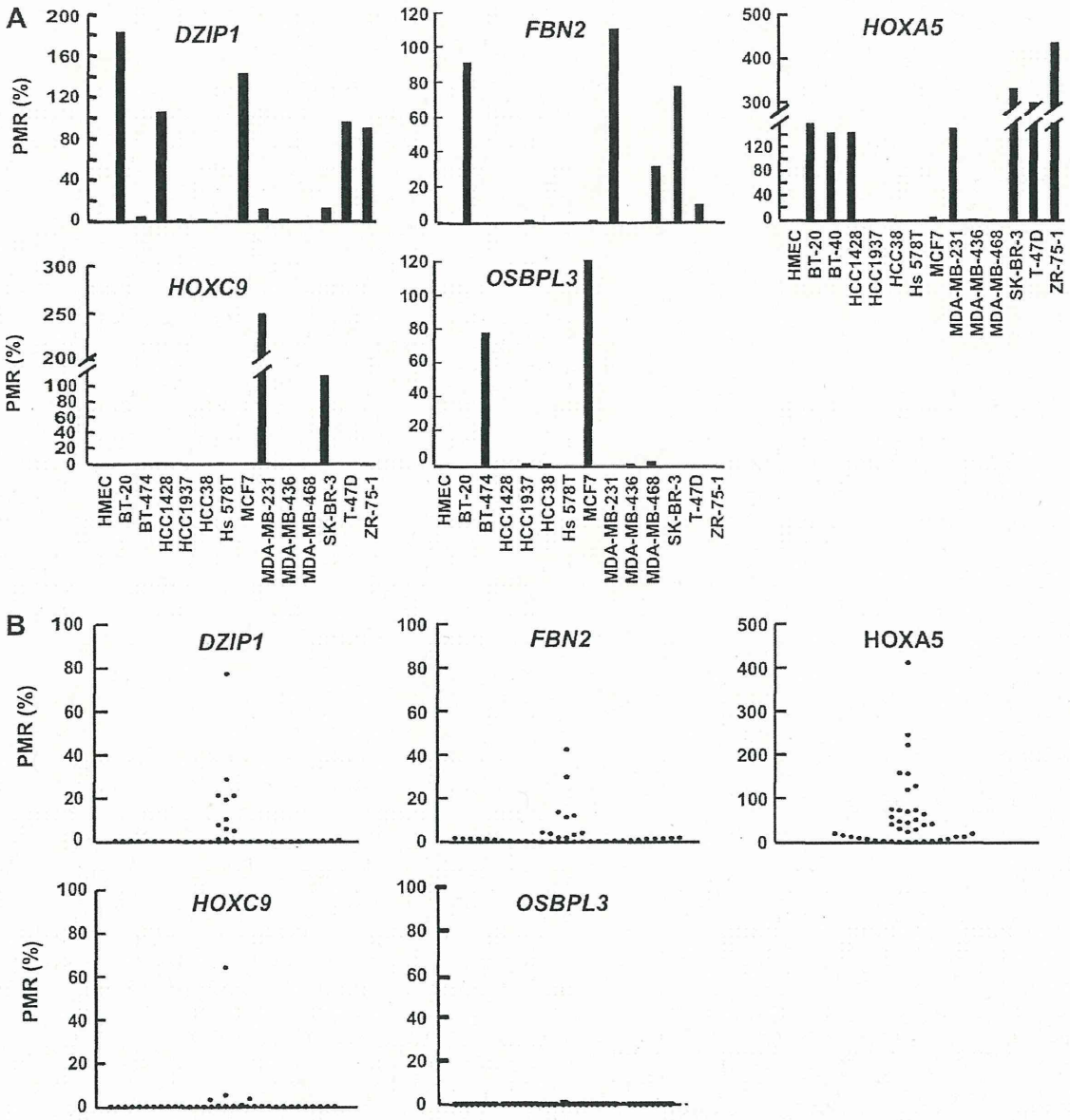


**Fig. 3.** DNA methylation levels of the outlier promoter CGIs in breast cancer cell lines and primary breast cancer samples. (A) Methylation levels of the outlier promoter CGIs in the HMECs and 13 breast cancer cell lines analyzed by qMSP. Five outliers, *DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3*, showed high methylation levels (PMR higher than 100%) in one or more breast cancer cell line. Since the copy number of the target CGI was normalized to the copy number of the *Alu* repeat sequence, it was possible that PMR reached more than 100% when the locus containing the target CGI had an increased copy number. (B) DNA methylation levels of *DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3* in primary breast cancer samples. Methylation of each CGI was analyzed in 40 primary breast cancer samples by qMSP. *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* showed aberrant methylation in primary breast cancer samples.

breast cancer and renal cell carcinoma, respectively [37,39]. These results indicated that searching for outliers might be an efficient way to identify TSGs.

### 3.4. Silencing of *DZIP1* by aberrant methylation of its promoter CGI

Excluding known TSGs, *HOXA5* and *FBN2*, we focused on *DZIP1* and *HOXC9*. First, an association between aberrant DNA methylation and loss of expression was analyzed in normal cells and cancer cell lines (Fig. 4A). *DZIP1* was expressed both in normal cells and in cancer cell lines without its aberrant methylation, except for BT-474 and SK-BR-3, and was not expressed in cancer cell lines with its aberrant methylation. In contrast, *HOXC9* was not expressed or had only very low expression levels both in normal cells and in all cancer cell lines, regardless of their methylation levels. Since a knockdown experiment could not be performed for *HOXC9*, it was excluded from further analyses.

Methylation-silencing of *DZIP1* was confirmed by observing its re-expression after treatment with 5-aza-dC and TSA using T-47D cells that had methylated promoter CGIs among the 13 breast cancer cell lines and did not express *DZIP1*. First, T-47D cells were treated with 0, 10, and 20 μM of 5-aza-dC to obtain a dose that inhibited cell growth to 50–70% of non-treated cells. The growth of T-47D cells was inhibited to 50% at 20 μM (Supplementary Fig. 3). The presence of demethylated DNA molecules was confirmed at this dose. A combination of 5-aza-dC with TSA induced *DZIP1* re-expression while TSA only did not (Fig. 4B). These results indicated that *DZIP1* was silenced by aberrant DNA methylation.

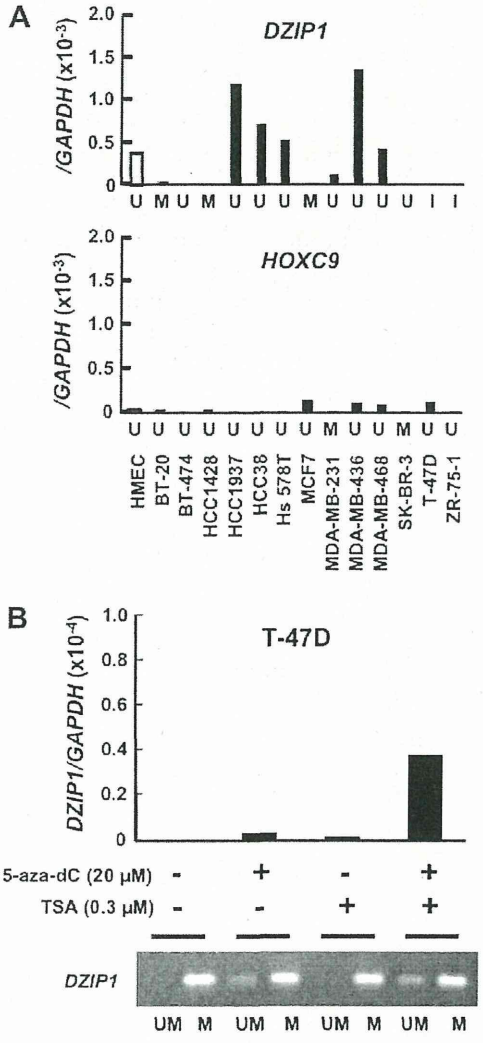


**Fig. 4.** Evaluation of methylation-silencing of two outliers, *DZIP1* and *HOXC9*. (A) An association between DNA methylation and loss of expression (analyzed by qRT-PCR). *DZIP1* was expressed in cell lines without its aberrant methylation and was silenced in cell lines with its aberrant methylation. In contrast, *HOXC9* was not expressed in any cell lines regardless of its low methylation. M, PMR > 100%; I, PMR 20–100%; U, PMR < 20%. (B) Re-expression of *DZIP1* by 5-aza-dC and TSA treatment. Expression levels were analyzed by qRT-PCR, and methylation statuses were analyzed by MSP. Demethylation of *DZIP1* was induced in T-47D cells by treatment with 20 μM of 5-aza-dC, and re-expression of *DZIP1* was observed after 5-aza-dC and TSA treatment. UM, unmethylated DNA; M, methylated DNA.

### 3.5. Tumor-suppressive function of DZIP1

To analyze whether *DZIP1* has tumor-suppressive function in breast cancer cells, *DZIP1* was knocked down by two different shRNAs (*DZIP1* shRNA1 and shRNA2) in two breast cancer cell lines that had the highest expression levels of *DZIP1*. Treatment of HCC1937 cells with shRNA1 and shRNA2 reduced the expression level of *DZIP1* mRNA to 20% and 60%, respectively, of cells with control shRNA. Treatment of MDA-MB-436 cells with shRNA1 and shRNA2 reduced the *DZIP1* expression levels to 40% of cells with control shRNA (Fig. 5A). HCC1937 cells with shRNA1 showed approximately 2-fold higher cell growth rate than HCC1937 cells with control shRNA. Likewise, MDA-MB-436 cells with shRNA1 or shRNA2 showed 1.5 to 2-fold higher cell growth rates than MDA-MB-436 cells with control shRNA (Fig. 5B). These results showed that *DZIP1* is a candidate novel TSG in breast cancer.

### 3.6. Comparison between epigenome-based and expression-based outlier approaches

To analyze the difference between the epigenome-based outlier approach and the expression-based outlier approach, we analyzed whether or not promoter CGIs identified by these approaches overlapped. First, to identify outliers expressed at high levels in normal cells, the expression status in the HMECs [14] was utilized. Among the 280 promoter CGIs susceptible to methylation induction during breast carcinogenesis, 21 promoter CGIs had downstream genes expressed at high levels in the HMECs (Supplementary Table 5).

Their overlap with the promoter CGIs identified by the epigenome-based outlier approach was then analyzed. Since two of the 14 promoter CGIs identified by the epigenome-based outlier approach were shared by two genes (Table 1), the remaining 12 promoter CGIs (and thus genes) were used for the analysis. Among the 12 promoter CGIs identified by the epigenome-based approach, only four promoter CGIs overlapped with those identified by the expression-based outlier approach (Fig. 6). Three and six TSGs were included in the 12 and 21 promoter CGIs, respectively, identified by the epigenome-based approach and the expression-based approach, respectively. While one TSG overlapped between the two approaches, *DZIP1* could be identified only by the epigenome-based approach. These results indicated that the epigenome-based approach could identify a different set of TSGs from the expression-based approach.

## 4. Discussion

We here showed (i) that a significant fraction of known TSGs silenced by aberrant DNA methylation in breast and colon cancer were outliers, and could be classified into TSGs with active Pol II and those with stalled Pol II, and (ii) that a different set of TSGs could be identified by an epigenome-based outlier approach or by an expression-based outlier approach. The epigenome-based outlier approach is established for the first time in this study. It is also applicable to obtain information on individual methylated genes whether they are TSGs or not. Epigenome information in normal cells is now being compiled by an international collaborative effort [40], and the epigenome-based outlier approach is expected to become more useful when this effort is completed.

Most genes identified by the epigenome-based and/or expression-based outlier approaches were unique. Among the five known TSGs that were outliers, only *MASPIN* had high expression, and the other four genes (*BRCA1*, *HOXA5*, *MLH1*, and *RASSF1A*) had stalled Pol II, indicating that they can be identified only by the epigenome-based approach. Among the 29 outliers identified by the genome-wide analyses, eight and 17 were uniquely identified by the epigenome-based and expression-based approaches, respectively. Genes identified only by the epigenome-based approach had stalled Pol II and thus low expression levels. Genes identified only by the expression-based approach were considered to have high levels of Pol II in gene bodies but not in promoter regions, which we analyzed in this study. It is reported that genes with active Pol II tends to have lower Pol II levels in promoter regions than those with stalled Pol II [41].

Using the epigenome-based outlier approach, *DZIP1* was identified as a candidate novel TSG. *DZIP1* is known to be involved in the regulation of hedgehog (Hh) signaling pathway [42,43]. Hh signaling pathway is activated in several types of human cancers, such as breast, esophageal, gastric, pancreatic, lung, and prostate cancers [44–51]. Although it is unknown how *DZIP1* is involved in the dysregulation of the Hh signaling pathway in mammals, in zebrafish embryo, a loss-of-function mutation of *Dzip1* is known to lead to ectopic expression of the downstream genes of the Hh signaling