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MicroRNA 34c Gene Down-regulation via DNA Methylation Promotes Self-renewal and Epithelial-Mesenchymal Transition in Breast Tumor-initiating Cells*

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Background: The mechanisms for miRNA dysregulation in BT-ICs remain obscure.
Results: Single hypermethylated CpG site in the promoter region of *miR-34c* gene repressed *miR-34c* expression by reducing DNA binding activities of Sp1 and promoted self-renewal and EMT of BT-ICs.
Conclusion: Single hypermethylated CpG site in the promoter region contributes to the reduction of microRNA in BT-ICs.
Significance: Methylation regulates the expression of microRNA in BT-ICs.

Tumor-initiating cells (T-ICs), a subpopulation of cancer cells with stem cell-like properties, are related to tumor relapse and metastasis. Our previous studies identified a distinct profile of microRNA (miRNA) expression in breast T-ICs (BT-ICs), and the dysregulated miRNAs contribute to the self-renewal and tumorigenesis of these cells. However, the underlying mechanisms for miRNA dysregulation in BT-ICs remain obscure. In the present study, we demonstrated that the expression and function of *miR-34c* were reduced in the BT-ICs of MCF-7 and SK-3rd cells, a breast cancer cell line enriched for BT-ICs. Ectopic expression of *miR-34c* reduced the self-renewal of BT-ICs, inhibited epithelial-mesenchymal transition, and suppressed migration of the tumor cells via silencing target gene Notch4. Furthermore, we identified a single hypermethylated CpG site in the promoter region of *miR-34c* gene that contributed to transcriptional repression of *miR-34c* in BT-ICs by reducing DNA binding activities of Sp1. Therefore, *miR-34c* reduction in BT-ICs induced by a single hypermethylated CpG site in the promoter region promotes self-renewal and epithelial-mesenchymal transition of BT-ICs.

Recent studies indicate that cancer initiation and progression are related to a small population of tumor cells. Because these cells possess characteristics resembling normal stem cells, they are termed cancer stem cells or tumor-initiating cells (T-ICs)[4] (1). T-ICs are characterized by their self-renewal capacity, multilineage differentiation properties, and high tumorigenicity in immunodeficient mice (1–3). However, little is known regarding the mechanisms that regulate the biocharacteristics of T-ICs. Uncovering the molecular mechanisms that control the stem cell-like properties of T-ICs, termed "stemness," will facilitate our understanding of the etiology and metastasis of breast cancers (1–3).

MicroRNAs (miRNAs) are short 20–22-nucleotide RNA molecules that are negative regulators of gene expression in a variety of eukaryotic organisms (4). Recent findings suggest that miRNAs may play pivotal roles in the maintenance of T-IC stemness (5–8). We and others have identified a distinct profile of miRNA expression in T-ICs compared with differentiated cancer cells, and the dysregulated miRNAs, such as *let-7*, *miR-30*, *miR-200c*, and *miR-181*, have been shown to contribute to the self-renewal, differentiation, and tumorigenesis of these cells (5–8). However, the underlying mechanisms of miRNA dysregulation in T-ICs remain obscure.

The regulatory mechanisms of miRNA expression have been studied at both transcriptional and post-transcriptional levels (9, 10). Among them, hypermethylation of gene promoters is a common mechanism of miRNA silencing at the transcriptional

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[4] The abbreviations used are: T-IC, tumor-initiating cell; BT-IC, breast tumor-initiating cell; DAC, 5-aza-2′-deoxycytidine; EMT, epithelial-mesenchymal transition; miRNA, microRNA; qRT-PCR, quantitative RT-PCR.

level (11). It has been shown that DNA methylation plays an important role in regulating miRNA expression during tumorigenesis. Additionally, DNA methylation, as an epigenetic marker, may undergo dynamic changes during the differentiation of self-renewing stem cells (11–13). These findings suggest that DNA methylation may be important in regulating the expression of many miRNAs in breast tumor-initiating cells (BT-ICs).

miR-34c, a putative tumor-suppressor gene, has been reported to induce cell apoptosis and inhibit cell proliferation and invasion in a variety of tumor cells (14–16). More importantly, *miR-34c* has been shown to prevent T-ICs development in prostate and pancreatic cancer cells (17, 18). *miR-34c* expression is also reduced in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas, and the reduction has been strongly associated with the hypermethylation of its neighboring CpG island (11, 19, 20). Our previous study found that *miR-34c* expression is reduced in BT-ICs compared with differentiated breast cancer cells using an miRNA microarray analysis (5). However, whether this reduction is also associated with the hypermethylation of its neighboring CpG island is not clear.

The purpose of the present study was to demonstrate the function of *miR-34c* and the molecular mechanisms through which its expression was repressed in BT-ICs. This information may provide the basis for the potential of *miR-34c* as a novel therapeutic target for breast cancer.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—The MCF-7 and SK-3rd cells were maintained in DMEM (Invitrogen) that was supplemented with 10% FBS (Invitrogen). For mammosphere culture (5), cells (1,000 cells/ml) were cultured in serum-free DMEM-F12 medium (Invitrogen) supplemented with B27 (1:50, Invitrogen), 20 ng/ml EGF (BD Biosciences), 0.4% BSA (Sigma), and 4 mg/ml insulin (Sigma). Cells were treated with 2 μmol/liter 5-aza-2′-deoxycytidine (DAC) (Sigma) for 72 h, and the medium was replaced every 24 h. For cell differentiation (5), cells dissociated from spheres were plated (1×10^5 cell/ml) in 6-well plates precoated with collagen IV (BD Biosciences) in DMEM supplemented with 10% FBS without growth factors.

Methylated DNA Immunoprecipitation—Genomic DNA was isolated from cells using a DNeasy Tissue kit (Qiagen). About 1 μg of genomic DNA was randomly sheared by sonication to generate fragments between 300 and 1,000 bp. The sonicated DNA was treated with a Methylated DNA Capture kit (EPIGENTEK) to enrich the methylated DNA. Substitution of a normal mouse IgG for the antibody against methylated DNA served as a negative control. The enriched methylated DNA was measured by quantitative real time PCR with specific primers corresponding to *miR-34c* promoter regions (Table 1). -Fold enrichment in each immunoprecipitation was determined by the ratio to input DNA.

Analysis of Cell Surface Markers—After transfection with *miR-34c* mimics or Notch4 siRNA (si-Notch4), the BT-ICs were collected after trypsinization. Cells were washed in PBS with 1% BSA and stained with anti-CD44 (FITC-conjugated; BD Biosciences), anti-CD24 (Phycoerythrin-conjugated; BD Biosciences), anti-IgG (FITC-conjugated or Phycoerythrin-conjugated; BD) for 30 min on ice in the dark. After centrifugation at $300 \times g$ for 5 min at 4 °C, cells were washed once or twice with cold staining buffer before being subjected to flow cytometry. The ALDEFLUOR kit (Stem Cell Technologies) was used to test ALDH1 enzymatic activity following the manufacturer's instruction.

Genomic DNA Isolation and Bisulfite DNA Sequencing PCR Analysis—Genomic DNA was isolated from cells using a DNeasy Tissue kit. The DNA samples were treated with sodium bisulfite to convert cytosine to uracil using the Methyl Detector™ Bisulfite Modification kit (Active Motif North America) according to the manufacturer's instruction.

Bisulfite sequencing was performed as described previously (11), and the amplified bisulfite sequencing PCR products were cloned into the pGEM-T Easy vector (Promega). Ten clones from each sample were sequenced to determine the methylation status of each CpG site. The primer sequences are shown in Table 1.

Transfection with miR-34c Mimics and Notch4 siRNA—Mammospheric cells were collected after trypsinization and resuspended in mammosphere medium. The cells were then transfected with 100 nmol/liter Notch4 siRNA (GenePharma) or control siRNA and 20 nmol/liter *miR-34c* mimics using Lipofectamine 2000 (Invitrogen). After 3 days of transfection, the cells were transfected again with siRNA or miRNA. At 48 h after the last transfection, the cells were collected after trypsinization and were resuspended in mammosphere medium. The single cell suspension was plated in ultra low adherent wells of a 6-well plate (Corning) at 2,000 cells/well for the mammosphere assay. In addition, the cell lysates from the cells transfected with siRNA or *miR-34c* mimics were prepared in radioimmune precipitation assay buffer for the Western blot analysis. The radioimmune precipitation assay buffer contained 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, and 1× protease inhibitor mixture (5).

Quantitative RT-PCR (qRT-PCR)—The total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The reverse transcription was performed using transcriptase (Invitrogen), and the real-time PCR was performed in a LightCycler480 System using a SYBR Premix Ex Taq kit (Takara). The sequences of the qRT-PCR primers are shown in Table 1.

The mature form of the miRNAs was detected using the hairpin-it miRNAs qRT-PCR Quantitation assay (GenePharma) according to the manufacturer's instructions. U6 small nuclear RNA was used as an internal control.

Western Blotting and Electrophoretic Mobility Shift Assay (EMSA)—Western blot analysis was performed as described previously (5). The β-actin antibody (PeroTech), Notch4 antibody (Upstate), E-cadherin antibody (Santa Cruz Biotechnology), vimentin antibody (Santa Cruz Biotechnology), fibronectin antibody (Santa Cruz Biotechnology), slug (Santa Cruz Biotechnology), snail (Santa Cruz Biotechnology), zeb1 (Santa Cruz Biotechnology), and Sp1 (Santa Cruz Biotechnology) were all used according to the manufacturers' instructions.

TABLE 1
Sequences of RNA and DNA oligonucleotides

Name	Sense strand (5′-3′)	Antisense strand (5′-3′)
miRNA Duplexes		
miR-34c mimics	AGGCAGUGUAGUUAGCUGAUUGC	AAUCAGCUAACUACACUGCCUUU
NC	UCACAACCUCCUAGAAAGAGUAGA	UACUCUUUCUAGGAGGUUGUUAUU
miR-34c inhibitor	UCCGUCACAUCAAUCGACUAACG	
Inhibitor NC	UUGUACUACACAAAAGUACUG	
Primers for qRT-PCR		
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGT
miR-34b/*c* primary	GCTCTTTGTCCCTCCTGCTAGA	GTGGGCGGTCCCTGAAG
Primers for MeDIP-qPCR and BSP[a]		
miR-34c MeDIP-qPCR	CATCTTCTAGGCGTCTCCCTTG	AGCGGCCGAGTGACTGTG
miR-34c bis-seq	GTTTTAAGAATTTGGGTTTTTATTTTTTAG	CAAACTTCAATTCCCAACCCCAAAC
Primers for clone		
miR-34c-fragment-89 bp	CCGCTCGAGTTTGGGGCTGGGAACTGAAG	CCCAAGCTTTCAGGTTCCCCTCGCTTTTC
miR-34c-fragment-178 bp	CCGCTCGAGGGTTCCAAGGACGGTTGGTC	CCCAAGCTTTCAGGTTCCCCTCGCTTTTC
miR-34c-fragment-266 bp	CCGCTCGAGCCACAGCGCTTTCTCTCAGC	CCCAAGCTTTCAGGTTCCCCTCGCTTTTC
miR-34c-fragment-496 bp	CCGCTCGAGTACCCAAGCCCACCTCCATT	CCCAAGCTTTCAGGTTCCCCTCGCTTTTC
miR-34c-fragment-573 bp	CCGCTCGAGACAGAGCACAGAGGTGCAGATG	CCCAAGCTTTCAGGTTCCCCTCGCTTTTC
Mutant-CpG1	TCCCCCACCAGCCCCGTCTC	GAGACGGGGGCTGGTGGGGGA
Mutant-CpG2	GACGGTTGGTAGCCCCCGCCA	TGGCGGGGGGCTACCAACCGTC
Notch4 3′-UTR	CGGACTAGTAAGAATACATGGTAGGGAGGA	CCCAAGCTTATGGGTGACAGATTTAGGGT
Notch4 3′-UTR-mutant	GTCCCGAGAGGCCACAAATGGC	GCCATTTGTGGCCTCTCGGGAC
Oligonucleotides for EMSA		
Hot probe	ATCCCCTCCCCCACCCGCCCCGTCTC	GAGACGGGGCGGGTGGGGGAGGGGAT
Methyl-hot probe	ATCCCCTCCCCCACCmCGCCCCGTCTC	GAGACGGGGCGGGTGGGGGAGGGGAT
Mutant-hot probe	ATCCCCTCCCCCACCAGCCCCGTCTC	GAGACGGGGCTGGTGGGGGAGGGGAT

[a] BSP, bisulfite sequencing PCR.

The oligonucleotides used for EMSA are shown in Table 1. For the supershift experiments, Sp1 antibodies (Santa Cruz Biotechnology) were added to the reaction 20 min prior to the addition of the probe. Nuclear extracts were prepared using the Nuclear Extract kit (Active Motif North America). The EMSAs were performed using a LightShift Chemiluminescent EMSA kit (Pierce).

Construction of Luciferase Reporter Plasmids—Potential upstream promoter regions of *miR-34c* were amplified using PCR and cloned into the pGL3-basic vector as described previously (21). The C within the CpG dinucleotide was mutated into A using bridging-based two-round PCR (22). The 3′-UTR of the Notch4 gene was amplified using PCR and was cloned into the pMIR-REPORT vector. To create the mutated 3′-UTRs, point mutations were introduced at the *miR-34c*-matching nucleotides within the selected putative seeding sequence regions according to the following rules: A was changed to T and vice versa, and G was changed to C and vice versa. The sequences of the cloning primers are shown in Table 1.

Luciferase Assays—After transient transfection of the luciferase reporters (*miR-34* promoter fragments, pMIR-REPORT-3′-UTR/Notch4), luciferase activity was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer's protocol. For the Sp1-mediated *miR-34c* transactivation expression, the cells were co-transfected with luciferase reporters plus Sp1 expression vector (p-EZ-MO2-Sp1) or the control vector (p-EZ-MO2). The ratio of luciferase reporters and expression vector was 2:1.

Cell Migration Assay and Transwell Assay—Migration assays were performed in modified Boyden chambers with 8-μm pore filter inserts in 24-well plates (BD Transduction). Briefly, the lower chamber was filled with mammosphere medium containing 10% FBS. After transfection with *miR-34c* mimics and si-Notch4, the BT-ICs were collected after trypsinization, resuspended in 200 μl of DMEM-F12 medium, and transferred to the upper chamber (1×10^5 cells/well). After 8 h of incubation, the filter was gently removed from the chamber, and the cells on the upper surface were removed using a cotton swab. Next, the cells that had invaded to the lower surface areas were fixed, stained with crystal violet, and counted under a microscope in 15 randomly selected fields (9).

Cell motility was assessed using a wound healing assay. Briefly, after transfection with *miR-34c* mimics and si-Notch4, the BT-ICs were seeded onto 6-well plates to near confluence. The cells were then carefully wounded using a 20-μl sterile pipette tip, and the cellular debris was removed by washing with PBS. The wounded monolayers were then incubated in DMEM-F12 medium for 24 h and photographed under a light microscope (9).

RESULTS

miR-34c Expression Was Reduced in BT-ICs—BT-ICs were generated using a suspension culture, and differentiated breast cancer cells were generated using an adherent culture. We used an miRNA microarray analysis to compare the miRNA expression levels between the BT-ICs and the differentiated breast cancer cells in our previous study, and we observed that *miR-34c* expression was reduced in the BT-ICs (5). To verify this reduction, we performed qRT-PCR in the present study. Our results revealed that primary *miR-34b*/*c* (Fig. 1*A*) and mature *miR-34c* (Fig. 1*B*) levels were down-regulated in the BT-ICs compared with the differentiated breast cancer cells. *miR-34b* expression was also reduced in the BT-ICs (Fig. 1*C*). This result was not surprising because *miR-34b* was in the same cluster as *miR-34c*.

To investigate the function of *miR-34c*, a pMIR-REPORT luciferase reporter vector with a *miR-34c*-targeted sequence

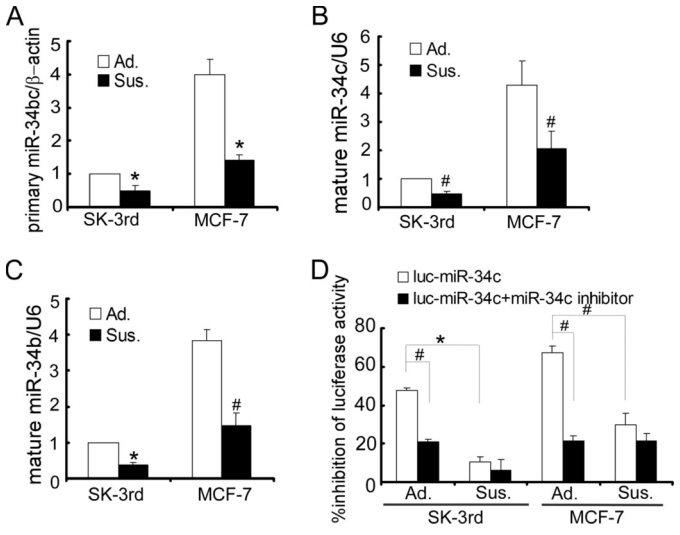

FIGURE 1. ***miR-34c* expression was reduced in BT-ICs.** *A*, expression of primary *miR-34b/c* was reduced in BT-ICs (*Sus.*) compared with differentiated breast cancer cells (*Ad.*) using qRT-PCR analysis. *B* and *C*, expression of mature *miR-34c* and *miR-34b* was reduced in BT-ICs (*Sus.*) compared with differentiated breast cancer cells (*Ad.*) using qRT-PCR analysis. *D*, *miR-34c* function, as assessed using luciferase assays, was reduced in BT-ICs (*Sus.*) compared with differentiated breast cancer cells (*Ad.*). Data are shown as relative mRNA or microRNA levels normalized to β-actin or U6. *, $p < 0.05$; #, $p < 0.01$; *error bars* correspond to mean ± S.D.

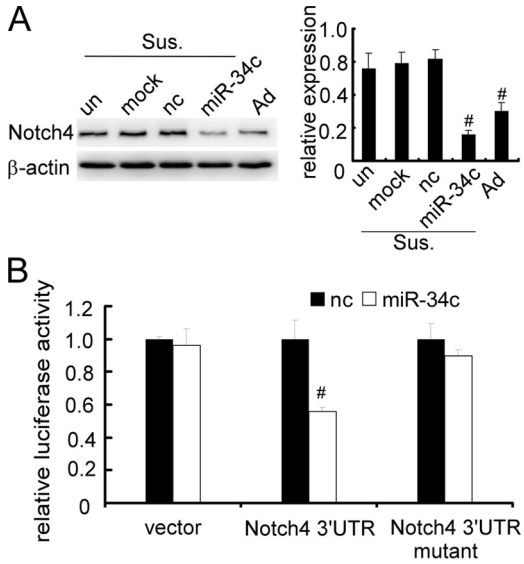

FIGURE 2. **Notch4 was negatively regulated by *miR-34c* in BT-ICs.** *A*, Notch4 protein levels were reduced after transfection with *miR-34c* mimics in BT-ICs compared with controls. In addition, Notch4 protein levels were also lower in differentiated breast cancer cells compared with BT-ICs, as assayed using Western blotting. *Histogram* shows the gray scale quantitative analysis for Western blotting using Gel-pro software. *B*, *miR-34c* mimics significantly inhibited the luciferase activity of the 3′-UTR of the Notch4 gene and no longer inhibited activity when the binding site was mutated, as assayed using a dual luciferase reporter assay. #, $p < 0.01$; *error bars* correspond to mean ± S.D.

cloned into its 3′-UTR was used. The luciferase activity was suppressed by 67.2% and 47.6% compared with the reporter without the *miR-34c*-targeted sequence in the differentiated MCF-7 and SK-3rd breast cancer cells, respectively, whereas only a 30.9% or 10.1% suppression was observed in the BT-ICs of the MCF-7 and SK-3rd cells, respectively (Fig. 1*D*). Co-transfection of the BT-ICs or differentiated breast cancer cells with a *miR-34c* inhibitor (antisense oligonucleotide) significantly reduced the suppression of the luciferase activity by endogenous *miR-34c* (Fig. 1*D*).

Notch4 Was Negatively Regulated by miR-34c in BT-ICs—Because Notch4 is a target of *miR-34c* (18, 23), we analyzed the Notch4 protein levels in the differentiated breast cancer cells and BT-ICs. As expected, the Notch4 protein levels were lower in the differentiated breast cancer cells compared with the BT-ICs (Fig. 2*A*). Furthermore, Notch4 protein levels in BT-ICs were decreased after transfection with *miR-34c* mimics compared with a control miRNA mimic (Fig. 2*A*). To demonstrate further that Notch4 was negatively regulated by *miR-34c*, we generated luciferase reporters containing the full-length 3′-UTR of the Notch4 gene along with its corresponding mutant counterpart at the *miR-34c* target site. These reporter plasmids were transfected into BT-ICs with *miR-34c* mimics or the control. Following 24 h of incubation, the cells were subjected to luciferase assays. As shown in Fig. 2*B*, the *miR-34c* mimics significantly reduced the luciferase activity of the reporter with Notch4 gene 3′-UTR. However, the luciferase activity was no longer inhibited when the binding site of *miR-34c* in the Notch4 gene 3′-UTR was mutated.

Restoration of miR-34c Reduced the Level of Mammosphere Formation and Phenotypes of BT-IC, Migration, and Epithelial-Mesenchymal Transition (EMT)—To determine whether the reduction in *miR-34c* in the BT-ICs contributes to the maintenance of self-renewal and BT-IC phenotypes, we first studied the effect of *miR-34c* restoration on BT-IC self-renewal using a mammosphere assay. The results revealed that after transfection with *miR-34c* mimics, the BT-ICs formed ~3-fold fewer mammospheres than the control cells. Mammosphere formation was also delayed, and the mammospheres were smaller after transfection with the *miR-34c* mimics. Silencing Notch4 expression with siRNAs also reduced mammosphere formation (Fig. 3*A*). ALDH1$^+$ and CD44$^+$CD24$^{-/low}$ have been used as markers to identify BT-ICs. We evaluated the cell population of ALDH1$^+$ and CD44$^+$CD24$^{-/low}$ after transfection with the *miR-34c* mimics and Notch4 siRNAs. Results showed that ALDH1$^+$ and CD44$^+$CD24$^{-/low}$ cells have significantly reduced by about 3-fold after transfection with the *miR-34c* mimics or Notch4 siRNA (Fig. 3, *B* and *C*). These results suggest that *miR-34c* suppresses the self-renewal capacity of BT-ICs and thus reduces the number of BT-ICs via targeting Notch4.

Because EMT often accompanies the self-renewal of tumor cells (24), we examined several EMT markers in BT-ICs after transfection with the *miR-34c* mimics and Notch4 siRNA using Western blotting. The results revealed an increased expression of an epithelial marker, E-cadherin, and decreased expression of mesenchymal markers, fibronectin and vimentin, after transfection with *miR-34c* mimics and Notch4 siRNA (Fig. 3*D*). Meanwhile, several EMT-related transcriptional factors were assessed, and results showed that snail, slug, and zeb1 were all down-regulated after transfection with *miR-34c* mimics and Notch4 siRNA (Fig. 3*E*).

Furthermore, by wound healing assay, mammospheric cancer cells transfected with *miR-34c* or Nothch4 siRNAs migrated more slowly compared with control cells (Fig. 3*F*). By Transwell

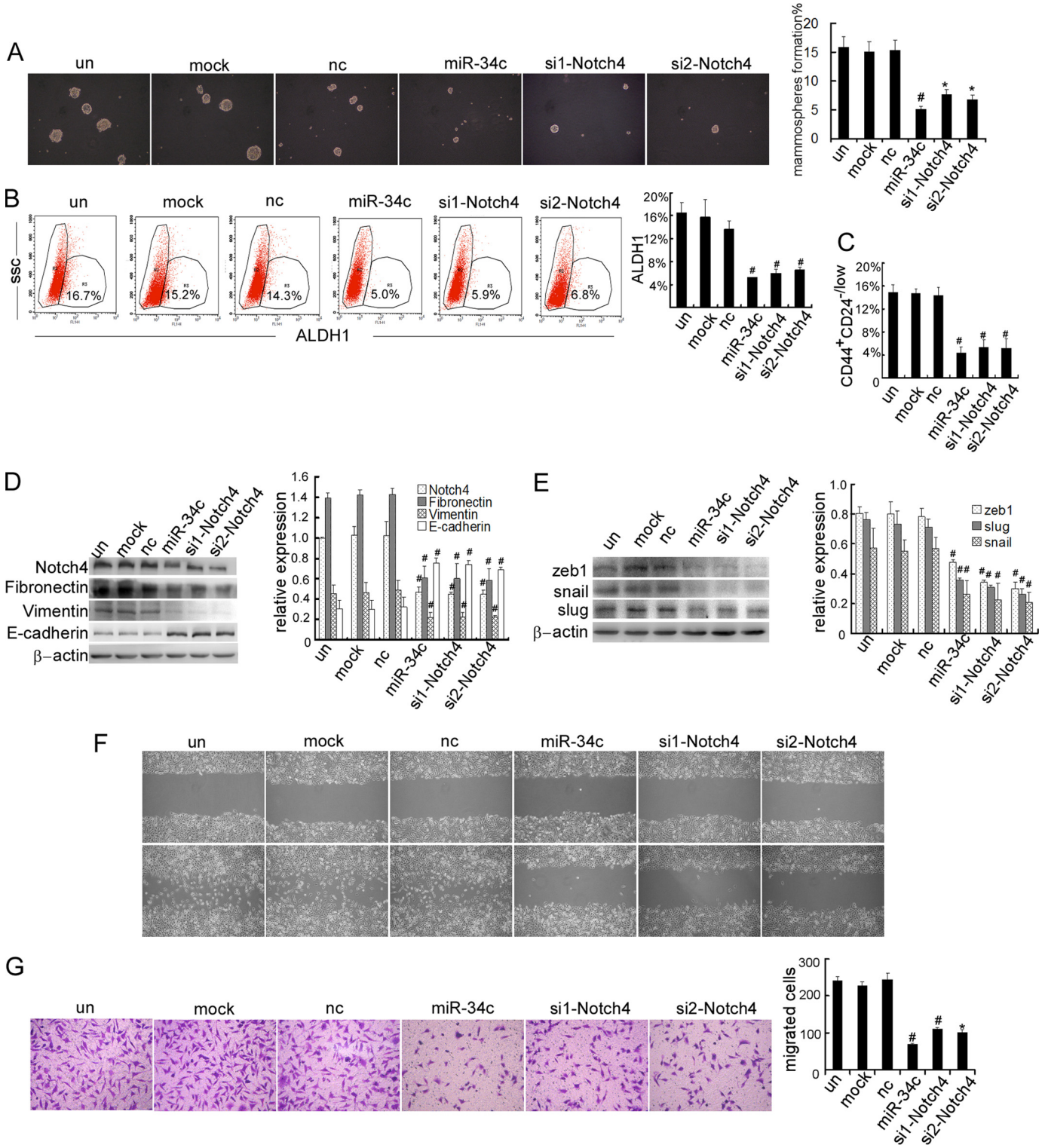

FIGURE 3. **Ectopic expression of *miR-34c* in BT-ICs reduced self-renewal, migration, and EMT.** *A*, mammosphere formation was reduced after transfection of BT-ICs with *miR-34c* mimics and Notch4 siRNA. *B* and *C*, proportion of $ALDH^+$ and $CD44^+CD24^{-/low}$ cells was reduced after transfection with *miR-34c* mimics and Notch4 siRNA, as analyzed using FACS. *D*, E-cadherin was up-regulated, and fibronectin and vimentin were down-regulated in BT-ICs after transfection with *miR-34c* mimics and Notch4 siRNA, as analyzed using Western blotting. *Histogram* shows the gray scale quantitative analysis for Western blotting using Gel-pro software. *E*, zeb1, slug, and snail were down-regulated in BT-ICs after transfection with *miR-34c* mimics and Notch4 siRNA, as analyzed using Western blotting. *Histogram* shows the grayscale quantitative analysis for Western blotting using Gel-pro software. *F* and *G*, cell migration was significantly reduced in BT-ICs after transfection with *miR-34c* mimics and Notch4 siRNA, as assessed using wound healing assays and Transwell assays using Boyden chambers. *, $p < 0.05$; #, $p < 0.01$, compared with untransfected cells; *error bars* correspond to mean ± S.D.

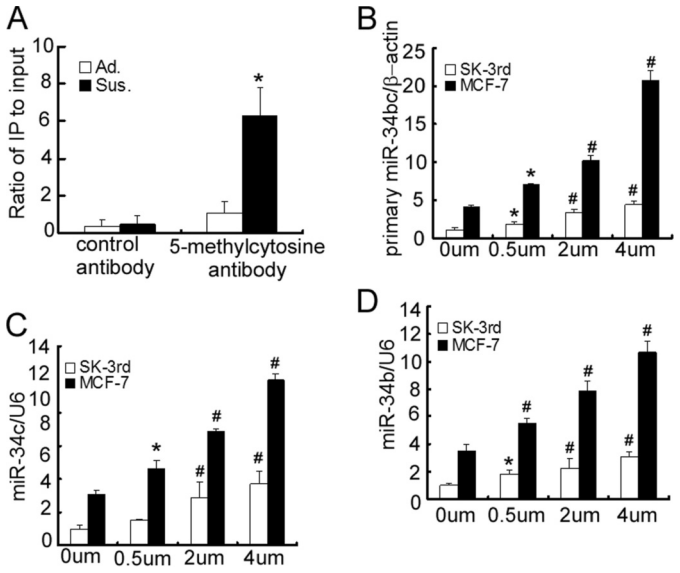

FIGURE 4. **Reduction of *miR-34c* in BT-ICs was associated with the hypermethylation status of its neighboring CpG island.** *A*, *miR-34c*-neighboring CpG islands were hypermethylated to a greater extent in the BT-ICs (*Sus.*) compared with differentiated breast cancer cells (*Ad.*), as assessed using MeDIP-qPCR. *B–D*, expression levels of primary *miR-34b/c*, mature *miR-34c*, and mature *miR-34b* in BT-ICs were up-regulated upon DAC treatment in a dose-dependent manner. Data are shown as relative mRNA or miRNA levels normalized to β-actin or U6. *, $p < 0.05$; #, $p < 0.01$; *error bars* correspond to mean ± S.D.

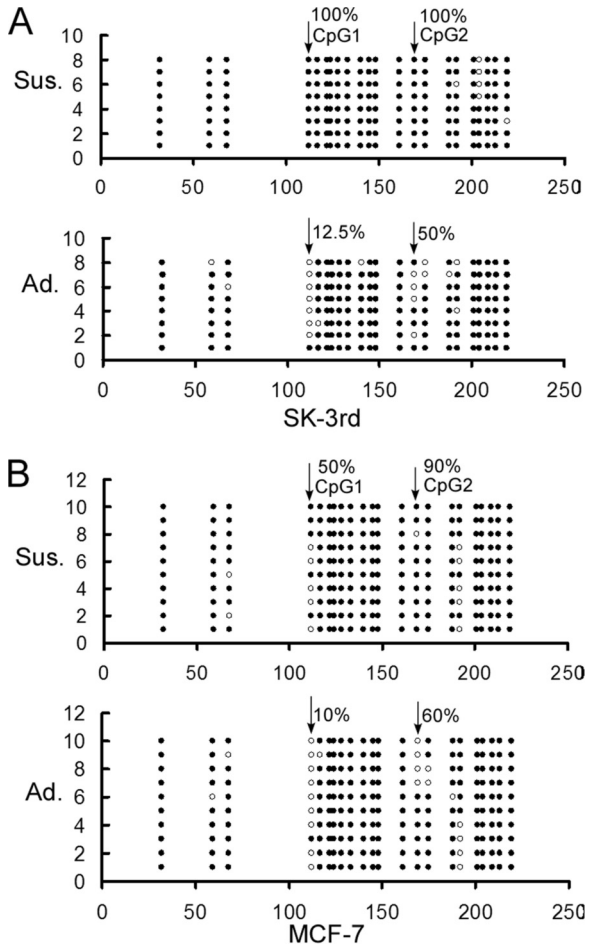

FIGURE 5. **CpG1 and CpG2 in the *miR-34c*-neighboring CpG islands were hypermethylated in BT-ICs.** *A*, in the SK-3rd cell line, CpG1 and CpG2 in the *miR-34c*-neighboring CpG islands were fully methylated in all of the BT-ICs clones, whereas only 25 and 50% of CpG1 and CpG2, respectively, were methylated in the differentiated breast cancer cells. *B*, in the MCF-7 cell line, CpG1 and CpG2 were 50 and 90% methylated in the BT-ICs compared with 10 and 60% methylation, respectively, in the differentiated breast cancer cells, as assessed using bisulfate sequencing PCR in the SK-3rd and MCF-7 cell lines. Each *line* represents the methylation status of a single clone, and the *open* and *filled circles* represent the unmethylated and methylated CpG sites, respectively.

migration assays, we found that transfection with *miR-34c* mimics or Notch4 siRNAs reduced the number of migrated cancer cells by 71.6, 54.5, and 58.2%, respectively (Fig. 3*G*). These results suggest that *miR-34c* mimics and Notch4 siRNA significantly reduce the level of cell migration.

miR-34c Reduction in BT-ICs Was Associated with Hypermethylation of Its Neighboring CpG Island—We further evaluated the mechanism responsible for the reduction of *miR-34c* expression in BT-ICs. First, we compared the methylation status of the *miR-34* promoter region between the BT-ICs and differentiated breast cancer cells using MeDIP-qPCR. Interestingly, the neighboring CpG island of *miR-34c* was hypermethylated in the BT-ICs but was not in the differentiated breast cancer cells (Fig. 4*A*). Next, we analyzed the expression level of *miR-34c* in the BT-ICs treated with increasing concentrations of DAC, a strong inducer of DNA demethylation. As shown in Fig. 4, *B–D*, DAC treatment dose-dependently increased the expression of *miR-34c* and *miR-34b*.

miR-34c-neighboring CpG Islands Were Hypermethylated in BT-ICs—To verify our results obtained from MeDIP-qPCR, we used bisulfite sequencing PCR to assess the methylation status of the *miR-34c*-neighboring CpG islands in the BT-ICs and differentiated breast cancer cells. We found that two specific CpG sites, CpG1 and CpG2, were hypermethylated in the BT-ICs but were not in the differentiated breast cancer cells. As shown in Fig. 5*A*, in the SK-3rd line, CpG1 and CpG2 were fully methylated in all of the BT-ICs clones, whereas only 25 and 50% of the CpG1 and CpG2, respectively, were methylated in differentiated breast cancer cells. Similar results were obtained in the MCF-7 line because CpG1 and CpG2 were 50 and 90% methylated, respectively, in the BT-ICs compared with only 10 and 60%, respectively, in the differentiated breast cancer cells (Fig. 5*B*). These results coincided with the expression level of *miR-34c* in the BT-ICs and differentiated breast cancer cells. Therefore, hypermethylation of CpG1 and CpG2 may be essential to the silenced status of *miR-34c* in BT-ICs.

CpG1 Was Located in Crucial Regulatory Element of miR-34c Promoter—To evaluate the role of CpG1 and CpG2 in *miR-34c* transcription, five different fragments of *miR-34c* promoters were designed based on the positions of CpG1 and CpG2 in the reported *miR-34c* promoter region (11). These constructs were transfected into BT-ICs for the analysis of the promoter activity, and all of the constructs containing CpG1 exhibited a significant level of promoter activity. The luciferase activity of fragment *miR-34c*-266-Luc was significantly increased compared with that of fragment *miR-34c*-178-Luc (Fig. 6*A*), suggesting the presence of crucial regulatory elements between −178 bp and −266 bp of the promoter region. Consistently, CpG1 was located in this region. These results suggest that CpG1 may be an important regulatory element of the miR-34c

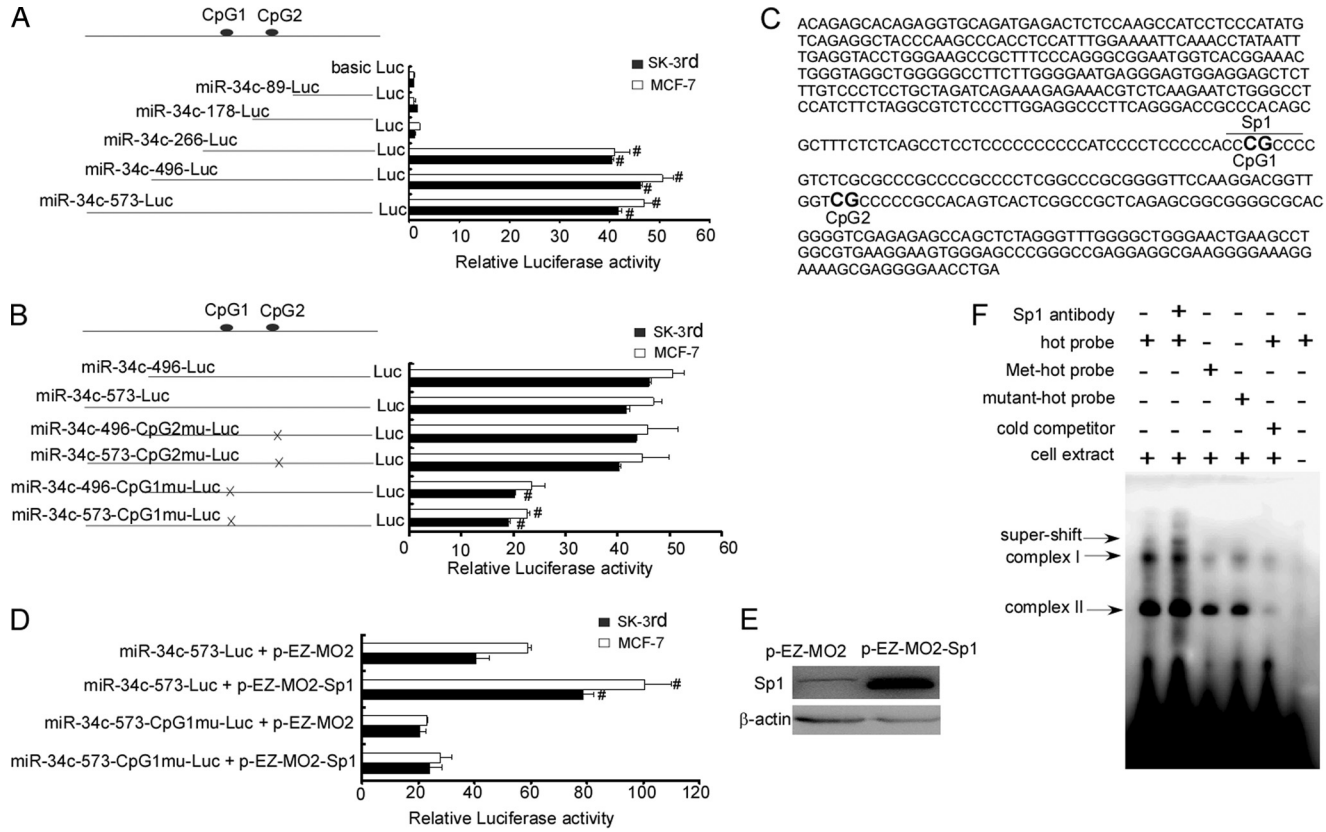

FIGURE 6. **CpG1 was located in a crucial regulatory element of the *miR-34c* promoter, and methylated CpG1 decreased binding activity of Sp1 to this site.** *A*, promoter activities of different fragments, based on the position of CpG1 and CpG2 sites in the reported *miR-34c* promoter region, were assayed using a dual luciferase reporter assay. All of the constructs containing CpG1 exhibited a significant level of promoter activity, and the luciferase activity increased significantly from fragment *miR-34c*-178-Luc to fragment *miR-34c*-266-Luc. #, $p < 0.01$, compared with the pGL3-basic vector; *error bars* correspond to mean ± S.D. *B*, changes in the putative promoter activities after mutation of CpG1 and CpG2 in fragments *miR-34c*-496-Luc and *miR-34c*-573-Luc were assayed using a dual luciferase reporter assay. When CpG1 was mutated, the promoter activity was significantly reduced, whereas there was no significant reduction when CpG2 was mutated. #, $p < 0.01$, compared with unmutated *miR-34c*-496-Luc and *miR-34c*-573-Luc; *error bars* correspond to mean ± S.D. *C*, DNA sequence of *miR-34* promoter was demonstrated. The CG in *bold* represents CpG1 and CpG2. The predicted Sp1 binding site was also indicated. *D*, co-transfection of Sp1 expression vector (p-EZ-MO2-Sp1) with *miR-34c*-573-Luc or *miR-34c*-573-CpG1mu-Luc into BT-ICs showed that the promoter activity of *miR-34c*-573-Luc was significantly up-regulated, whereas that of *miR-34c*-573-CpG1mu1-Luc was not affected. *E*, Sp1 expression was up-regulated after transfection of the Sp1 expression vector, as analyzed using Western blotting. *F*, methylated CpG1 decreased the binding activity of Sp1, as assessed using EMSAs.

promoter. To evaluate further the influence of CpG1 and CpG2 on miR-34 transcription, we used site-directed mutagenesis to mutate each of the cytosines within CpG1 and CpG2 to an adenine. After transient transfection of these fragments, the cells were subjected to luciferase assays. Mutation in these sites resulted in different levels of reduced luciferase activity (Fig. 6*B*). When CpG1 was mutated, the promoter activity was significantly reduced in the BT-ICs, whereas there was no significant reduction in the promoter activity when CpG2 was mutated. Similarly, mutation in CpG1 and CpG2 in the fragments of *miR-34c*-496-Luc and *miR-34c*-573-Luc led to similar results.

Methylated CpG1 Decreased Binding Activity of Sp1 to miR-34c Promoter—To investigate further the influence of the methylation status of CpG1 on *miR-34c* transcription, bioinformatics was used to analyze the binding sites for transcription factors around CpG1. We observed a Sp1 binding motif around CpG1 in the *miR-34c* promoter region (Fig. 6*C*). Thus, we co-transfected Sp1 expression vector (p-EZ-MO2-Sp1) with *miR-34c*-573-Luc or *miR-34c*-573-CpG1mu1-Luc into BT-ICs to investigate the role of CpG1 in Sp1-mediated *miR-34c* transactivation. The promoter activity of *miR-34c*-573-Luc was up-regulated significantly after overexpression of Sp1; however, the promoter activity of *miR-34c*-573-CpG1mu1-Luc was not affected (Fig. 6*D*). These results suggested that CpG1 was crucial in Sp1-mediated *miR-34c* transactivation. To determine whether CpG1 methylation may influence the binding activity of Sp1, EMSA was performed using synthesized oligonucleotides containing either a nonmethylated or methylated cytosine of CpG1. As shown in Fig. 6*F*, two DNA-protein complexes (I and II) were formed, which were specifically eliminated by unlabeled probes of a 100-fold excess and were decreased by probes containing CpG1 mutation. Complex I could be supershifted by anti-Sp1 antibodies, but the presence of methylated CpG1 reduced the binding activity. Although complex II was not supershifted by anti-Sp1 antibodies, its binding activity was also affected by the presence of methylated CpG1. These results suggest that CpG1 methylation alters the accessibility of Sp1 to the regulatory element of the *miR-34c* promoter and thus interferes with its binding activity.

DISCUSSION

In the present study, we demonstrated that reduced *miR-34c* in BT-ICs of two different breast cancer lines contributes to the

self-renewal capacity and EMT of these cells. Previously, a direct link between EMT and the stem-cell like properties was appreciated in breast cancer cells (25), and inhibition of the EMT process has been shown to eliminate T-ICs (24). In addition, signaling of Notch family, which are direct targets of *miR-34c*, has been shown to play an important role in the EMT process. Activating Notch signaling in endothelial cells is associated with EMT (26), whereas down-regulating Notch signaling using siRNA inhibits EMT of gemcitabine-resistant pancreatic cancer cells, which is ascribed to reduced expression of zeb1, snail, slug, vimentin, and NF-κB (27). Further, our present study suggested that Notch4 overexpression due to *miR-34c* reduction is crucial to maintain EMT in BT-ICs of two different breast cancer lines, whereas ectopic *miR-34c* expression may reduce BT-IC self-renewal through the inhibition of EMT. On the other hand, p53, which was a known regulator of *miR-34*, has also been shown to play an important role in the EMT process. Inactivated p53 or mutated p53 has been shown to induce the process of EMT (28), accompanied by an increased population of stem-cell like cancer cells. Together, these findings insinuate a possible correlation of p53 and Notch4 signaling linked by miR-34c in the process of EMT in T-ICs, whereas further studies are needed to elucidate their interrelationship. Because MCF-7 cells contain few BT-ICs (29) whereas SK-3rd cells are enriched for BT-ICs (5), consistent findings in these two cancer lines suggest that reduced *miR-34c* maintains self-renewal, and EMT of BT-ICs may be generally applicable to breast cancers with various proportions of BT-ICs. Therefore, *miR-34c* may hold significant promise as a novel molecular therapy for human breast cancers via targeting to BT-ICs.

Another important finding in our current study was that *miR-34c* reduction in BT-ICs was due to hypermethylation of its neighboring CpG islands. In BT-ICs, CpG1 and CpG2 were significantly hypermethylated compared with those in the differentiated breast cancer cells. When CpG1 was mutated, the promoter activity of miR-34c was reduced significantly, whereas there was no significant reduction in this promoter activity when CpG2 was mutated. Therefore, CpG1 is probably located in a crucial regulatory element of the *miR-34c* promoter region. However, because these sites cannot be recognized by recently identified specific methylases, unlike HpaII (CCGG) or HhaI sites (GCGC) (30), we could not evaluate the effect of the methylation status of CpG1 and CpG2 in *miR-34c* transcription using luciferase reporter assays *in vivo*. In fact, accumulating evidence has indicated that changes in the methylation status of a single CpG site in promoter regions are sufficient to affect gene expression (31–35). The hypermethylation of a single CpG site within the promoter region of the herpes simplex virus *tk* gene has been shown to down-regulate *tk* gene expression (31). In contrast, demethylation of a single CpG site in the *EBV* latency C promoter efficiently up-regulates EBV expression (32). In human cells, *interleukin2* (33), *p53* (34), and *XAF1* (35) have all been reported to be regulated by the methylation status of a specific CpG site in their promoter regions. Taken together, these results indicate that changes in the methylation status of a specific CpG site, rather than of the total promoter region, are sufficient to influence gene expression.

One possible explanation for methylation-induced gene silencing is a direct hindrance to the binding of transcription factors (36). Many transcription factors are unable to interact with their cognate sites when a specific CpG is methylated in the promoter region, such as AP-2 (37), HIF-1 (38), c-Myc (39), and Sp1 (36, 40). These transcription factors are known to bind to motifs containing CpG dinucleotides, and the binding fails when the CpGs are methylated. Bioinformatic analysis reveals that CpG1 is located in a Sp1 binding site. However, the influence of methylation on Sp1 binding activities seems to depend on promoters. Sp1 binding activity is reduced as a result of DNA hypermethylation in the promoters of $p21^{Cip1}$ (36), *11βHSD2* (41), killer cell immunoglobulin-like receptor (*KIR*) (42), cell death-inducing DFF45-like effector A (*CIDE-A*) (40), and α_1-adrenergic receptors (*α1ARs*) (43). In contrast, DNA hypermethylation does not influence the Sp1 binding activities to *TLR2* (44) and *CLDN4* (45) promoters. Our present study suggests that *miR-34c* promoter belongs to the first category, similar to the $p21^{Cip1}$ and *11βHSD2* promoters because methylation of CpG1 significantly reduced the Sp1 binding activity (which was contained in complex I) in the BT-ICs nuclear extracts. Although we could not confirm the exact components of complex II, its binding activity was also influenced by the methylation status of CpG1. Therefore, the methylation status of CpG1 influences *miR-34c* transcription by decreasing the binding activity of transcription factors.

In summary, we have shown that *miR-34c* expression is reduced in BT-ICs. Restoration of *miR-34c* reduced the self-renewal and EMT of BT-ICs, which was accompanied by reduced migration. We also observed that *miR-34c* reduction in BT-ICs was correlated with the hypermethylation of CpG1 in its neighboring CpG island. Moreover, Notch4 is the target for *miR-34c* involvement in BT-IC self-renewal and EMT. By modulating the self-renewal and EMT of BT-ICs, *miR-34c* may hold significant promise as a novel molecular therapy for breast cancers. Our data also imply that modulating the status DNA methylation in *miR-34c*, an essential epigenetic mechanism, may also serve as a novel therapeutic strategy for breast cancers by targeting tumor-initiating cells.

REFERENCES

1. Dalerba, P., Cho, R. W., and Clarke, M. F. (2007) *Annu. Rev. Med.* **58,** 267–284
2. Wicha, M. S. (2006) *Clin. Cancer Res.* **12,** 5606–5607
3. Norton, L. (2011) *Oncology* **25,** 30, 32
4. Ren, J., Jin, P., Wang, E., Marincola, F. M., and Stroncek, D. F. (2009) *J. Transl. Med.* **7,** 20
5. Yu, F., Yao, H., Zhu, P., Zhang, X., Pan, Q., Gong, C., Huang, Y., Hu, X., Su, F., Lieberman, J., and Song, E. (2007) *Cell* **131,** 1109–1123
6. Yu, F., Deng, H., Yao, H., Liu, Q., Su, F., and Song, E. (2010) *Oncogene* **29,** 4194–4204
7. Shimono, Y., Zabala, M., Cho, R. W., Lobo, N., Dalerba, P., Qian, D., Diehn, M., Liu, H., Panula, S. P., Chiao, E., Dirbas, F. M., Somlo, G., Pera, R. A., Lao, K., and Clarke, M. F. (2009) *Cell* **138,** 592–603
8. Meng, F., Glaser, S. S., Francis, H., Demorrow, S., Han, Y., Passarini, J. D., Stokes, A., Cleary, J. P., Liu, X., Venter, J., Kumar, P., Priester, S., Hubble, L., Stoloch, D., Sharma, J., Liu, C. G., and Alpini, G. (2011) *J. Cell. Mol. Med.*, doi: 10.1111/j.1582-4934.2011.01282.x
9. Kong, W., Yang, H., He, L., Zhao, J. J., Coppola, D., Dalton, W. S., and Cheng, J. Q. (2008) *Mol. Cell. Biol.* **28,** 6773–6784
10. Davis, B. N., Hilyard, A. C., Lagna, G., and Hata, A. (2008) *Nature* **454,**

56–61
11. Toyota, M., Suzuki, H., Sasaki, Y., Maruyama, R., Imai, K., Shinomura, Y., and Tokino, T. (2008) *Cancer Res.* **68,** 4123–4132
12. Wilting, S. M., van Boerdonk, R. A., Henken, F. E., Meijer, C. J., Diosdado, B., Meijer, G. A., le Sage, C., Agami, R., Snijders, P. J., and Steenbergen, R. D. (2010) *Mol. Cancer* **9,** 167
13. Lehmann, U., Hasemeier, B., Christgen, M., Müller, M., Römermann, D., Länger, F., and Kreipe, H. (2008) *J. Pathol.* **214,** 17–24
14. Rokhlin, O. W., Scheinker, V. S., Taghiyev, A. F., Bumcrot, D., Glover, R. A., and Cohen, M. B. (2008) *Cancer Biol. Ther.* **7,** 1288–1296
15. He, C., Xiong, J., Xu, X., Lu, W., Liu, L., Xiao, D., and Wang, D. (2009) *Biochem. Biophys. Res. Commun.* **388,** 35–40
16. Cai, K. M., Bao, X. L., Kong, X. H., Jinag, W., Mao, M. R., Chu, J. S., Huang, Y. J., and Zhao, X. J. (2010) *Int. J. Mol. Med.* **25,** 565–571
17. Liu, C., Kelnar, K., Liu, B., Chen, X., Calhoun-Davis, T., Li, H., Patrawala, L., Yan, H., Jeter, C., Honorio, S., Wiggins, J. F., Bader, A. G., Fagin, R., Brown, D., and Tang, D. G. (2011) *Nat. Med.* **17,** 211–215
18. Ji, Q., Hao, X., Zhang, M., Tang, W., Yang, M., Li, L., Xiang, D., Desano, J. T., Bommer, G. T., Fan, D., Fearon, E. R., Lawrence, T. S., and Xu, L. (2009) *PloS One* **4,** e6816
19. Vogt, M., Munding, J., Grüner, M., Liffers, S. T., Verdoodt, B., Hauk, J., Steinstraesser, L., Tannapfel, A., and Hermeking, H. (2011) *Virchows Arch.* **458,** 313–322
20. Corney, D. C., Hwang, C. I., Matoso, A., Vogt, M., Flesken-Nikitin, A., Godwin, A. K., Kamat, A. A., Sood, A. K., Ellenson, L. H., Hermeking, H., and Nikitin, A. Y. (2010) *Clin. Cancer Res.* **16,** 1119–1128
21. Sato, H., Suzuki, H., Toyota, M., Nojima, M., Maruyama, R., Sasaki, S., Takagi, H., Sogabe, Y., Sasaki, Y., Idogawa, M., Sonoda, T., Mori, M., Imai, K., Tokino, T., and Shinomura, Y. (2007) *Carcinogenesis* **28,** 2459–2466
22. Xu, J., De Zhu, J., Ni, M., Wan, F., and Gu, J. R. (2002) *Cell Res.* **12,** 177–197
23. Ji, Q., Hao, X., Meng, Y., Zhang, M., Desano, J., Fan, D., and Xu, L. (2008) *BMC Cancer* **8,** 266
24. Kong, D., Banerjee, S., Ahmad, A., Li, Y., Wang, Z., Sethi, S., and Sarkar, F. H. (2010) *PloS One* **5,** e12445
25. Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., and Weinberg, R. A. (2008) *Cell* **133,** 704–715
26. Noseda, M., McLean, G., Niessen, K., Chang, L., Pollet, I., Montpetit, R., Shahidi, R., Dorovini-Zis, K., Li, L., Beckstead, B., Durand, R. E., Hoodless, P. A., and Karsan, A. (2004) *Circ. Res.* **94,** 910–917
27. Wang, Z., Li, Y., Kong, D., Banerjee, S., Ahmad, A., Azmi, A. S., Ali, S., Abbruzzese, J. L., Gallick, G. E., and Sarkar, F. H. (2009) *Cancer Res.* **69,** 2400–2407
28. Chang, C. J., Chao, C. H., Xia, W., Yang, J. Y., Xiong, Y., Li, C. W., Yu, W. H., Rehman, S. K., Hsu, J. L., Lee, H. H., Liu, M., Chen, C. T., Yu, D., and Hung, M. C. (2011) *Nat. Cell Biol.* **13,** 317–323
29. Klopp, A. H., Lacerda, L., Gupta, A., Debeb, B. G., Solley, T., Li, L., Spaeth, E., Xu, W., Zhang, X., Lewis, M. T., Reuben, J. M., Krishnamurthy, S., Ferrari, M., Gaspar, R., Buchholz, T. A., Cristofanilli, M., Marini, F., Andreeff, M., and Woodward, W. A. (2010) *PloS One* **5,** e12180
30. Zhang, J., Yu, J., Gu, J., Gao, B. M., Zhao, Y. J., Wang, P., Zhang, H. Y., and De Zhu, J. (2004) *Cell Res.* **14,** 283–294
31. Ben-Hattar, J., and Jiricny, J. (1988) *Gene* **65,** 219–227
32. Robertson, K. D., Hayward, S. D., Ling, P. D., Samid, D., and Ambinder, R. F. (1995) *Mol. Cell. Biol.* **15,** 6150–6159
33. Murayama, A., Sakura, K., Nakama, M., Yasuzawa-Tanaka, K., Fujita, E., Tateishi, Y., Wang, Y., Ushijima, T., Baba, T., Shibuya, K., Shibuya, A., Kawabe, Y., and Yanagisawa, J. (2006) *EMBO J.* **25,** 1081–1092
34. Torng, P. L., Lin, C. W., Chan, M. W., Yang, H. W., Huang, S. C., and Lin, C. T. (2009) *Mol. Cancer* **8,** 120
35. Zou, B., Chim, C. S., Zeng, H., Leung, S. Y., Yang, Y., Tu, S. P., Lin, M. C., Wang, J., He, H., Jiang, S. H., Sun, Y. W., Yu, L. F., Yuen, S. T., Kung, H. F., and Wong, B. C. (2006) *Gastroenterology* **131,** 1835–1843
36. Zhu, W. G., Srinivasan, K., Dai, Z., Duan, W., Druhan, L. J., Ding, H., Yee, L., Villalona-Calero, M. A., Plass, C., and Otterson, G. A. (2003) *Mol. Cell. Biol.* **23,** 4056–4065
37. Comb, M., and Goodman, H. M. (1990) *Nucleic Acids Res.* **18,** 3975–3982
38. Wenger, R. H., Kvietikova, I., Rolfs, A., Camenisch, G., and Gassmann, M. (1998) *Eur. J. Biochem.* **253,** 771–777
39. Prendergast, G. C., and Ziff, E. B. (1991) *Science* **251,** 186–189
40. Ozsolak, F., Poling, L. L., Wang, Z., Liu, H., Liu, X. S., Roeder, R. G., Zhang, X., Song, J. S., and Fisher, D. E. (2008) *Genes Dev.* **22,** 3172–3183
41. Friso, S., Pizzolo, F., Choi, S. W., Guarini, P., Castagna, A., Ravagnani, V., Carletto, A., Pattini, P., Corrocher, R., and Olivieri, O. (2008) *Atherosclerosis* **199,** 323–327
42. Liu, Y., Kuick, R., Hanash, S., and Richardson, B. (2009) *Clin. Immunol.* **130,** 213–224
43. Michelotti, G. A., Brinkley, D. M., Morris, D. P., Smith, M. P., Louie, R. J., and Schwinn, D. A. (2007) *FASEB J.* **21,** 1979–1993
44. Furuta, T., Shuto, T., Shimasaki, S., Ohira, Y., Suico, M. A., Gruenert, D. C., and Kai, H. (2008) *BMC Mol. Biol.* **9,** 39
45. Honda, H., Pazin, M. J., Ji, H., Wernyj, R. P., and Morin, P. J. (2006) *J. Biol. Chem.* **281,** 21433–21444