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 (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 mammospheres 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 × 10⁵ 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 USPASY (EMSA) —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                 | AGCCAGCCAGAGAUCAGGAGACGACAGUCGUCAGUCAGUC | AAUCAGCUAACUAACACUGCCUUU                  |
| NC                             | UCCUGCAGCAUACUAGUGCCUGAC                 | UACCUCUUCAAGGGAGUUGUUAUU                  |
| miR-34c inhibitor              | UCCUGCAGCAUACUAGUGCCUGAC                 | UACCUCUUCAAGGGAGUUGUUAUU                  |
| Inhibitor NC                   | UGGUAGCAACAAAGAAAGUCG                    |                                             |
| Primers for qRT-PCR            |                                          |                                             |
| GAPDH                          | ACCACACTTCATCCATCCAC                    | TCCACACACTGTGTGCTGT                      |
| miR-34c primary                | GCCCTTTGTCCTCCCTCTCTCAAG                | GTGGCGCGGTCCTCCATAG                      |
| Primers for MeDIP-qPCR and BSP |                                          |                                             |
| miR-34c MeDIP-qPCR             | GGTGGTTGGTAGCCCCCGCCA                  | CAAAATCTCAATTTCCCACCCCCCAACAC            |
| miR-34c bis-seq                | GGTGGTTGGTAGCCCCCGCCA                  | CAAAATCTCAATTTCCCACCCCCCAACAC            |
| Primers for clone             |                                          |                                             |
| miR-34c-fragment-89 bp         | CCGCTGATGTTGCCCCCGGAAGCTGAAG            | CCGAGTCGTTCCGCTTGGTTGGTTGGTTGGTTGGTTGG   |
| miR-34c-fragment-178 bp        | CCGCTGATGTTGCCCCCGGAAGCTGAAG            | CCGAGTCGTTCCGCTTGGTTGGTTGGTTGGTTGGTTGG   |
| miR-34c-fragment-266 bp        | CCGCTGATGTTGCCCCCGGAAGCTGAAG            | CCGAGTCGTTCCGCTTGGTTGGTTGGTTGGTTGGTTGG   |
| miR-34c-fragment-496 bp        | CCGCTGATGTTGCCCCCGGAAGCTGAAG            | CCGAGTCGTTCCGCTTGGTTGGTTGGTTGGTTGGTTGG   |
| miR-34c-fragment-573 bp        | CCGCTGATGTTGCCCCCGGAAGCTGAAG            | CCGAGTCGTTCCGCTTGGTTGGTTGGTTGGTTGGTTGG   |
| Mutant-CpG1                    | CCCACAGGCGCCGCGCTTC                    | GAGACGGGGCTCTGAGGGGG                     |
| Mutant-CpG2                    | GACGGTGTGACGGCCGCCGCA                   | TGGCGGGGGCTACCAACCGTC                   |
| Notch4 3’-UTR                  | CGAGCTAGAAGAGAGACAGAGAGTAGAGGGAGAGA    | CCAAGATCGATTAGTTAGACGATTTAGGG           |
| Notch4 3’-UTR-mutant           | GTCCCAGAGGCCAACAAATAG                  | GCCATTGGCGCGCTTCCAGGG                   |
| Oligonucleotides for EMSA     |                                          |                                             |
| Hot probe                      | ATCCCCCCCCCCCCCCCGCCCGGGGTC            | GAGACGGGGGGGGGCTGGGGGGGGGGGGGGGGGGAT     |
| Methyl-hot probe               | ATCCCCCCCCCCCCCCCGCCCGGGGTC            | GAGACGGGGGGGGGCTGGGGGGGGGGGGGGGGGGAT     |
| Mutant-hot probe               | ATCCCCCCCCCCCCCCCGCCCGGGGTC            | GAGACGGGGGGGGGCTGGGGGGGGGGGGGGGGGGAT     |

* 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-34c 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 × 10⁵ 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 (Fig. 1A) and mature miR-34c (Fig. 1B) 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. 1C). 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...
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. 1D). 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. 1D).

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. 2A). Furthermore, Notch4 protein levels in BT-ICs were decreased after transfection with miR-34c mimics compared with a control miRNA mimic (Fig. 2A). 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. 2B, 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. 3A). 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. 3B 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 siRNAs 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. 3D). 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. 3E).

Furthermore, by wound healing assay, mammospheric cancer cells transfected with miR-34c or Notch4 siRNAs migrated more slowly compared with control cells (Fig. 3F). 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+/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.
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. 3G). 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. 4A). Next, we analyzed the expression level of miR-34 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. 5A, in the SK-3rd line, CpG1 and CpG2 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. 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 bisulfite 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.

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. 6A), 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
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. 6B). When CpG1 was mutated, the promoter activity was significantly reduced, whereas there was no significant reduction 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. 6C). Thus, we cotransfected Sp1 expression vector (p-EZ-MO2-Sp1) with miR-34c-573-Luc or miR-34c-573-CpG1mu-Luc into BT-ICs to investigate the role of CpG1 in Sp1-mediated miR-34c transcription. The promoter activity of miR-34c-573-Luc was upregulated significantly after overexpression of Sp1; however, the promoter activity of miR-34c-573-CpG1mu1-Luc was not affected (Fig. 6D). 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. 6E, 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 addi-
tion, 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 asso-
ciated with EMT (26), whereas down-regulating Notch signal-
ing using siRNA inhibits EMT of gemcitabine-resistant pancre-
atic cancer cells, which is ascribed to reduced expression of
zeb1, snail, slug, vimentin, and NF-kb (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 find-
ings insinuate a possible correlation of p53 and Notch4 signal-
ing 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 dif-
erentiated 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 (32), \(p53\) (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 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 influ-
ce 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),
11\(\beta\)HSD2 (41), killer cell immunoglobulin-like receptor (KIR)
(42), cell death-inducing DFF45-like effector A (\(CIDE-A\)) (40),
and \(\alpha\)-adrenergic receptors (\(\alpha\)ARs) (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 11\(\beta\)HSD2 promoters because meth-
ylation 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 modu-
Irating 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.

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