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Molecules and Cells

Epigenetic Up-Regulation of Leukemia Inhibitory Factor (LIF) Gene During the Progression to Breast Cancer

Jung Eun Shin[1,2], Su Hyung Park[1,2], and Yeun Kyu Jang[1,2,*]

The interleukin 6 family of cytokines including leukemia inhibitory factor (LIF) regulates the progression of several types of cancer. However, although LIF overexpression during breast cancer progression was observed in our previous report, the molecular mechanisms responsible for this deregulation remain largely unknown. Here we show that *LIF* expression is epigenetically up-regulated via DNA demethylation and changes in histone methylation status within its promoter region in the isogenic MCF10 model. Bisulfite sequencing revealed the CpG pairs within the promoter region are hypermethylated in normal breast epithelial cells, but extensively demethylated as breast cancer progresses. In agreement with the DNA methylation pattern, our chromatin immunoprecipitation showed that inactive epigenetic marks such as MeCP2 occupancy and histone H3-Lys9-dimethylation significantly decreased during the progression to breast cancer but an active histone mark was increased in an inverse manner. Also, the occupancy of the transcription factor Sp1, which has higher affinity for hypomethylated CpGs, increased. RNAi-mediated knockdown of *LIF* expression resulted in a significant reduction of cell growth and colony formation in breast cancer cells, suggesting the potential role of LIF-LIF receptor axis in autocrine stimulation of cancer cells. Collectively, our data suggest that the epigenetic up-regulation of the *LIF* gene likely play an important role in the development of breast cancer.

INTRODUCTION

The growth and metastatic behavior of primary tumors are largely mediated by autocrine and paracrine pathways in a cytokine-dependent manner (Kellokumpu-Lehtinen et al., 1996; Wysoczynski et al., 2007). Recent studies indicate that members of the interleukin 6 family of cytokines, including oncostatin M (OSM) and leukemia inhibitory factor (LIF), contribute to the proliferation and metastasis of several cancers (Estrov et al., 1995; Jorcyk et al., 2006; Kellokumpu-Lehtinen et al., 1996; Liu et al., 1998; Queen et al., 2005). A common receptor is shared between OSM and LIF, but these proteins have distinct effects on the biological activities of cancer cells (Wysoczynski et al., 2007). Exogenous LIF promotes the proliferation of several types of cancers, whereas OSM inhibits cancer cell growth (Garcia-Tunon et al., 2008; Grant et al., 2001). Furthermore, coexpression of LIF and its receptor (LIFR) is associated with breast cancer tumors, suggesting a potential role for this receptor in the regulation of breast tumor growth (Crichton et al., 1996; Dhingra et al., 1998). Exogenous LIF likewise induces increased expression of *LIF* and *LIFR* mRNA, suggesting that LIF may function as a growth factor in pancreatic carcinoma cells (Kamohara et al., 2007).

Methylation of DNA at the position 5 cytosine within a CpG dinucleotide is the predominant covalent modification in the eukaryotic genome. DNA methylation has been studied as a possible regulatory mechanism for the expression of a number of genes during the processes of cancer development. When DNA is modified at CpG sites in the promoter, transcription is inhibited due to interference with transcription initiation (Baylin and Jones, 2007). In several tumor types, the expression of a large repertoire of tumor suppressor genes has been found to be reduced by DNA methylation (Ballestar and Esteller, 2008; Baylin and Jones, 2007; Graff et al., 1997; Robertson, 2005). Tumor suppressor genes are among the pivotal genes known to be regulated by CpG methylation (Ballestar and Esteller, 2008; Chicoine et al., 2002; Murayama et al., 2006; Na et al., 2010; Park et al., 2007).

As tumors progress, cancer-related genes may be differentially expressed via such epigenetic regulation as DNA methylation in their promoter regions (Shvachko, 2009). Certain cancer cell types methylate the *BubR1,* 7-dehydrocholesterol reductase (*Dhcr7*), aquaporin-5 (*AQP5*), *RUNX3,* lysophosphatidic acid receptor-1 (lpa1), galectin-3, *CDX1, MUC5B*, PDZ-LIM domain-containing protein 2 (PDLIM2), *LOT1 (PLAGL1/ZAC1)*, *TNFSF7 (CD70)* genes, leading to their transcriptional suppression DNA hypomethylation at CpG islands within the promoters (Abdollahi et al., 2003; Ahmed et al., 2007; Kim et al., 2005; Motegi et al., 2005; Park et al., 2005; 2007; Perrais et al., 2001; Qu et al., 2010; Suh et al., 2002; Tsujiuchi et al., 2006; Yu et al., 2010). The upregulation of galectin-7, *CDX4*, and

[1]Department of Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea, [2]Yonsei Biomolecule Research Initiative, Yonsei University, Seoul 120-749, Korea
*Correspondence: ykjang@yonsei.ac.kr

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urokinase-type plasminogen activator (uPA), and *MMP-2* genes in several cancer types including breast cancer, ovarian cancer, and lymphoma cells is mediated by DNA hypomethylation (Chernov et al., 2009; Demers et al., 2009; Guo et al., 2002; Honda et al., 2006; Pakneshan et al., 2004). For transcription factors such as STAT1, NFAT and Oct-1, methylation at specific CpGs has been shown to directly inhibit protein binding and thus inhibit transcription in colon carcinoma cells and human T cells (McGough et al., 2008; Murayama et al., 2006). The Sp1 transcription factor has CG rich binding sites, indicating that Sp1 sites may be affected by DNA methylation both directly and indirectly. The methylation of adjacent CpG sites has been reported to affect Sp1/Sp3 binding and transcriptional activity in the p21 promoter (Clark et al., 1997; Zhu et al., 2003).

There is interplay between DNA methylation and such histone modifications as acetylation such that the disruption of one of these two epigenetic mechanisms can affect the other. Hypermethylation of the CpG islands is the first event that triggers deacetylation of local histones, whereas lower levels of histone acetylation seem increase sensitivity to targeted DNA methylation. Though there is no consensus on which epigenetic mechanism initiates and steers this communication, a feedback loop may exist between histone modification and DNA methylation (Vaissiere et al., 2008). The recruitment of the methyl-CpG-binding protein (MeCP2) mediates the repression by DNA methylation (Kudo, 1998). DNA methylation has been recently linked to methylation of Lys9 of histone H3, which is a histone mark for transcriptional repression. Histone methylation can direct DNA methylation (Fuks et al., 2003a; 2003b).

Although accumulated evidence suggests that the *LIF-LIFR* axis may play a role in the growth and metastatic behavior of breast cancer cells, the molecular mechanism related to this process is largely unknown. Our previous report demonstrated that expression of the *LIF* gene was upregulated during breast cancer progression in the isogenic MCF10 model (Rhee et al., 2008). The isogenic MCF10 human breast cancer model, which includes seven different cell lines originally derived from the same parent cell line, provides an opportunity to study breast cancer initiation, development, and progression (Hurst et al., 2009; Marella et al., 2009; Rhee et al., 2008; Santner et al., 2001; Worsham et al., 2006). In this study, we tested if increased expression of the *LIF* gene during progression to breast cancer could be correlated with changes in the DNA methylation pattern of its promoter region. We identified the DNA methylation pattern of CpG pairs in the promoter and the first intron regions of *LIF* using this model. We found that DNA hypomethylation leads to epigenetic upregulation of the *LIF* gene and that the histone methylation status of the promoter is changed. Importantly, our data suggest that the upregulation of the *LIF* gene during progression to breast cancer in the isogenic MCF 10 model may occur via the increased binding of transcription factor Sp1 to the hypomethylated Sp1 binding site within the *LIF* promoter. The data presented here suggest that the proximal region of the *LIF* promoter contributes to its epigenetic regulation during progression to breast cancer.

MATERIALS AND METHODS

Cell lines and reagents

A series of MCF10 cell lines including MCF-10A (non-tumorigenic, non-metastatic), MCF-10AT1 (benign proliferation stage), MCF-101k (benign proliferation stage), MCF-10CA1a cl1 (invasive carcinoma stage), and MCF-10CA1d cl1 (invasive carcinoma stage) were obtained from the Barbara Ann Karmanos Cancer Institute (USA). The MCF-10DCIS.com (carcinoma *in situ* stage) cell line was purchased from Asterand, Inc. (USA). All cells were cultured as previously described (Rhee et al., 2008). 5-Aza-2′-deoxycytidine (5-aza-dC) was purchased from Sigma (USA).

RNA isolation and semi-quantitative RT-PCR analysis

Extraction of total cellular RNA and RT-PCR analysis was performed as previously described (Rhee et al., 2008). The oligonucleotide primers used for RT-PCR were described in Table S1. The expected sizes of amplified products and the number of amplification cycles for RT-PCR are as described in a previous report (Rhee et al., 2008).

Bisulfite sequencing

Bisulfite sequencing was done as mentioned in a previous report (Oh et al., 2009; Yu et al., 2010). Genomic DNA was isolated using the LaboPass™ Genomic DNA Extraction Kit (Cosmo, Korea), and 500 ng of the cytosine bases of genomic DNA were converted to uracil according to the protocol described in the EZ DNA Methylation Kit™ (Zymo Research Corporation, USA). To determine the location of the CpG islands in the promoter region of *LIF*, we used a program available online, Methyl Primer Express® Software v1.0 (https://products.appliedbiosystems.com/). The *LIF* promoter regions containing CpG islands were amplified from the bisulfite-modified DNA using four sets of primers (Supplementary Table S1).

PCR amplification was performed with Hot Star Taq DNA polymerase (Qiagen, USA). The cycling conditions involved an initial activation of Hot Star Taq DNA polymerase at 95°C for 5 min, 40 to 42 cycles at 95°C for 30 s, 50 to 54°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. The expected sizes of amplified products were 420 bp for the upper strand of the promoter region and 571 bp for the bottom strand, 495 bp for the upper strand of the intron region and 442 bp for the bottom strand. The PCR products were cloned into pUCm-T using the TA cloning system (RBC, Taiwan, Banqiao). After *Escherichia coli* were transformed, 7 to 12 subclones were selected and sequenced using M13F primers. After bisulfite genomic sequencing, BiQ Analyzer software (http://biqanalyzer.bioinf.mpi-sb.mpg.de/) and the CpG view program (http://dna.leeds.ac.uk/cpgviewer/) were used to align these sequences with the original sequences.

Chromatin immunoprecipitation

MCF-10A and MCF-10DCIS.com cells were used for cross-linking and isolation of chromatin fraction. Chromatin immunoprecipitation (ChIP) assays were performed on the supernatants using a Chromatin IP kit (#9002; Cell Signaling Technology, USA). DNA-protein complexes were immunoprecipitated with control IgG or with the target-specific antibodies anti-H3 (#2650, Cell Signaling Technology), anti-H3K4 diMe (ab1220, Abcam, USA), anti-H3K9 diMe (ab3256, Abcam), anti-Sp1 (#17-601, Millipore), and anti-MeCP2 (ab2828, Abcam). Immunoprecipitated DNA was amplified for semiquantitative PCR with these oligonucleotide primers described in Supplementary Table 1S.

Knock-down by lentiviral shRNA system

We selected two validated 21mer targets to knock down *LIF* expression from MISSION shRNA Plasmid DNA (SHCLNDNM_002309, Sigma). Subcloning of shRNA oligos, viral packaging, and introduction of lentivirus into host cells were done according to the Addgene pLKO.1 protocol (Addgene, USA).

Measurement of cell viability by MTT assay and colony formation assay

The Lentivirus infection system included the gene for puromycin resistance and was used to virally infect MCF-10DCIS.com cells with one of the human *LIF*-specific shRNAs *LIF* shRNA1 or *LIF* shRNA2, or the pLKO.1 control vector, or scrambled shRNA. After antibiotic selection, 2000 cells from each construct were seeded in wells of a 96-well plate where 100 μl of 0.1% thiazolyl blue tetrazolium bromide (M2128, Sigma) dissolved in PBS was added. The cells were incubated at 37°C for 3 h. Then, after it was removed, 100 μl dimethyl sulfoxide (Duchefa Biochemie, The Netherlands) was added. The absorbance was analyzed by the Magellan5 program (Tecan, USA) at 540 nm. Colony formation assay was performed as previously described (Rhee et al., 2008).

RESULTS

Upregulation of the LIF gene is correlated with development of breast cancer cells

We previously detected that the *LIF* gene is upregulated during progression to breast cancer in the isogenic MCF10 model (Rhee et al., 2008). Here, semiquantitative RT-PCR confirmed that the carcinoma cell lines exhibited significantly increased levels of *LIF* transcripts relative to normal and benign tumor cell lines (Fig. 1A). However, the expression of the tumor suppressor TP73 was downregulated following the transition to the noninvasive carcinoma stage (Fig. 1A). These results are consistent with our previous report, suggesting that the upregulation of the cytokine *LIF* may be involved in development of breast cancer cells.

Treatment of non-tumorigenic breast cells with hypomethylating agent caused neo-expression of LIF at mRNA levels

The known hypomethylating agent 5-aza-dC was used to treat the normal MCF-10A, MCF-10AT1 (benign proliferation stage), and noninvasive carcinoma MCF-DCIS.com cell lines to establish a relationship between DNA hypomethylation and the induction of *LIF* expression. This agent acts as an epigenetic modifier to cause DNA demethylation and gene activation by inhibiting the DNA methyltransferase activity of such Dnmt enzymes as Dnmt3A, 3B and Dnmt1. After treatment with 1 μM 5-aza-dC for 96 h, the mRNA expression of the *LIF* gene was moderately increased in normal MCF-10A cell lines, but there was no little change in the MCF-10AT1 and MCF-DCIS.com cell lines (Fig. 1B). This result suggests that low level of *LIF* expression observed in the MCF-10A cell line might be in part caused by DNA hypermethylation.

DNA hypomethylation in the LIF promoter correlates with its upregulation during the development of breast cancer

Since our CpG microarray experiments (unpublished data) demonstrated a differential DNA methylation pattern between normal MCF-10A cells and its derivatives, we asked if the methylation pattern of CpG islands within the *LIF* promoter may be involved in upregulation of the *LIF* gene during progression to breast cancer. A recent report suggested that the CpG methylation pattern might be different between the upper and bottom strands, raising the possibility that CpG methylation may be strand-specific (Metivier et al., 2008). Based on this concept, we analyzed the DNA methylation patterns for 56 CpGs on both strands in the *LIF* promoter and the first intron region, which is highly enriched for CpG islands (Fig. 2; Supplementary Fig. S1). Genetically-related five MCF10 breast epithelial cell

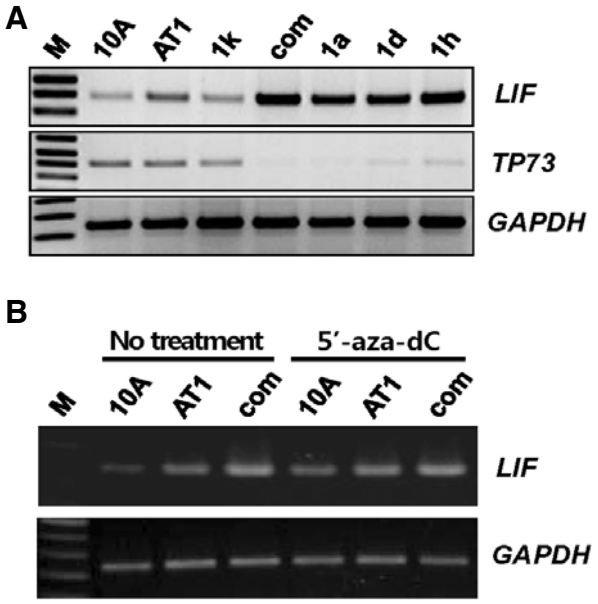

Fig. 1. LIF expression during breast cancer progression and the effect of 5-aza-dC treatment on its expression in the isogenic MCF10 model. (A) Semi-quantitative RT-PCR determined that the expression of *LIF* mRNA is upregulated during progression to breast cancer in MCF10 cell lines, while TP73 mRNA expression is decreased. The TP73 gene encodes a p53-related tumor suppressor whose expression would be expected to be downregulated during carcinogenesis [25]. (B) Treatment with 1 μM of 5-aza-dC, a Dnmt inhibitor, for 96 h followed by 5 μg RNA for cDNA synthesis reversed downregulation of *LIF* expression as examined by semi-quantitative RT-PCR. The rows are: M, 1-kb DNA ladder; 10A, untransformed MCF-10A; AT1, benign MCF-10AT1; 1k, benign proliferation MCF-10AT1kcl2; com, carcinoma *in situ* MCF-10DCIS.com; 1a, invasive MCF-10CA1acl1; 1d, invasive MCF-10CA1d cl1, and 1h, invasive carcinoma MCF-10CA1h cl2. GAPDH was amplified as an internal control.

lines were used to compare methylation patterns and confirm the effects of 5-aza-dC treatment. We found that the DNA methylation patterns from both the upper and bottom strands were almost the same, so they were not strand-specific. The normal MCF-10A cell line had 9 of 16 CpG pairs (CpG 1-9) in the promoter region between nucleotides -631 and -335 with heavy methylation, but these were hypomethylated in the other MCF10 variants (Figs. 2A and 2B). In the intron region of the *LIF* gene, however, most CpGs remained unmethylated in the normal MCF-10A cells, and the DNA methylation status of the 38 CpG pairs in the intron region between +126 and +359 on both upper and bottom strand had little change even in the other MCF10 variants (Supplementary Fig. S1). This result suggests massive demethylation at CpG sites within the *LIF* promoter during the progression to breast cancer in the isogenic MCF10 model. Slight methylation of 6 of the 16 CpG pairs (CpG 10-14, 16) in this region was observed in the normal MCF-10A cells, but the methyl groups were completely removed following the transition from normal to the tumor stage. Moreover, the 5-aza-dC treated MCF-10A cell line had the DNA methylation level of total CpGs sites slightly reduced from 32.4% to 30.5% on the upper strand and from 48.1% to 41.3% on the bottom strand (see Figs. 2A and 2B). This suggests that the transient treatment with 5-aza-dC was insufficient to fully demethylate the CpG sites of the *LIF*

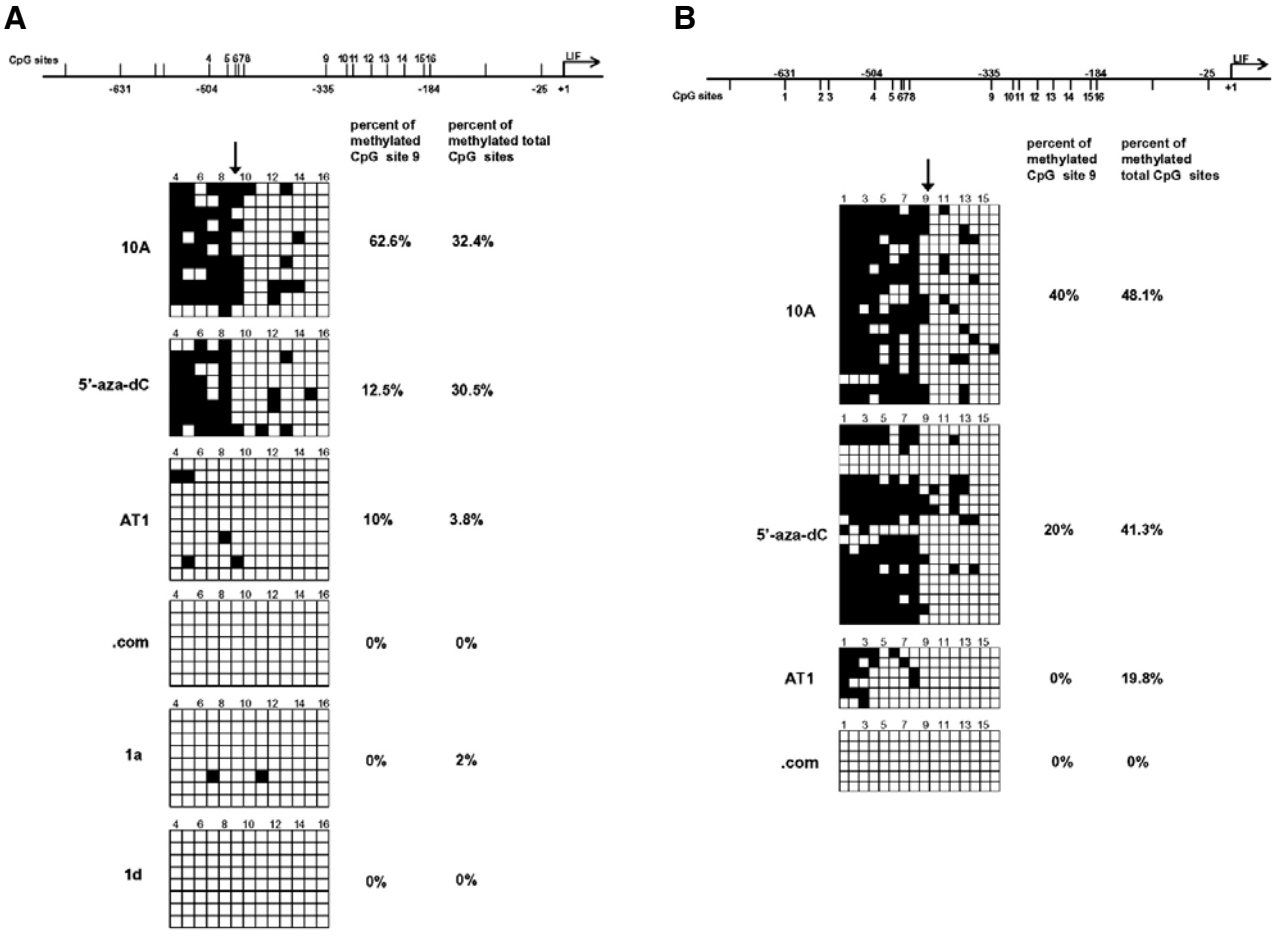

Fig. 2. Fine mapping of DNA methylation within the 5′-flanking region of *LIF* gene by bisulfite sequencing. (A) Schematic diagram of the *LIF* gene indicates the transcription start site (TSS) as +1, exon 1 as a small box, the relative positions of the CpG dinucleotides with vertical lines. The 13 CpGs from 4 to 16 on the upper, antisense strand in the promoter region of *LIF* are shown with vertical lines (Top panel). The CpG methylation on the upper of the *LIF* promoter region in each plasmid clone was determined by bisulfite sequencing analysis, with black boxes indicating methylated CpG sites and white boxes indicating unmethylated. The CpG number is indicated above each square and the vertical arrow indicates CpG site 9 (Bottom panel). (B) The 16 CpGs from 1 to 16 on the bottom, sense strand in the promoter region of *LIF* are shown with vertical lines (Top panel). The CpG methylation was determined and displayed as mentioned in (A).

promoter of the normal MCF-10A cells, which is consistent the RT-PCR data that show a partial recovery of *LIF* mRNA in 5-aza-dC-treated MCF-10A cells (Fig. 1B). However, the ninth CpG site had methylation that was reduced from 62.6% to 12.5% on the upper strand and 40% to 20% on the bottom. Thus, although change in DNA methylation at *LIF* promoter may be in part caused by altered expression of epigenetic modifiers such as DNMT1 or DNMT3, these results may suggest that inducing *LIF* expression is at least associated with changes in DNA methylation pattern during the progression to breast cancer in the isogenic MCF10 model.

The occupancy profiles of MeCP2, histone methylation marks, and transcription factor Sp1 are correlated with methylation pattern of CpGs within the LIF promoter

Since DNA hypomethylation in the *LIF* promoter correlates with its upregulation during the progression to breast cancer, we hypothesized that MeCP2, a protein that binds methylated CpG sites, may have a higher affinity for the *LIF* promoter of MCF-10A cells than for 5-aza-dC-treated MCF-10A or MCF-10DCIS.com cells. Chromatin immunoprecipitation (ChIP) was assayed through the use of three different PCR primer sets that amplified DNA fragments containing putative Sp1 binding sites (Figs. 3A and 3B). The ChIP data revealed that the occupancy of MeCP2 over the three promoter regions considerably decreased in 5-aza-dC-treated MCF-10A and *in situ* carcinoma MCF-10DCIS.com cells compared to untreated MCF-10A cells (Fig. 3). The results suggest that MeCP2 may play a role in epigenetic silencing of the *LIF* gene in untransformed MCF-10A cells via binding to the hypermethylated DNA within the promoter region.

We next examined histone methylation patterns within the *LIF* promoter to understand the influence of DNA methylation on histone methylation and vice versa (Fuks et al., 2003a; Vaissiere et al., 2008). Our results were similar to the data of MeCP2 occupancy within the *LIF* promoter. The level of histone H3-Lys9-dimethylation, which is a hallmark for transcriptional repression, was significantly decreased in 5-aza-dC-treated MCF-10A cells and MCF-10DCIS.com cells compared to untreated MCF-10A cells. In contrast, the level of H3-Lys4-dimethylation, which is a hallmark for transcriptional activation, was increased in 5-aza-dC treated MCF-10A and MCF-10DCIS.com cells

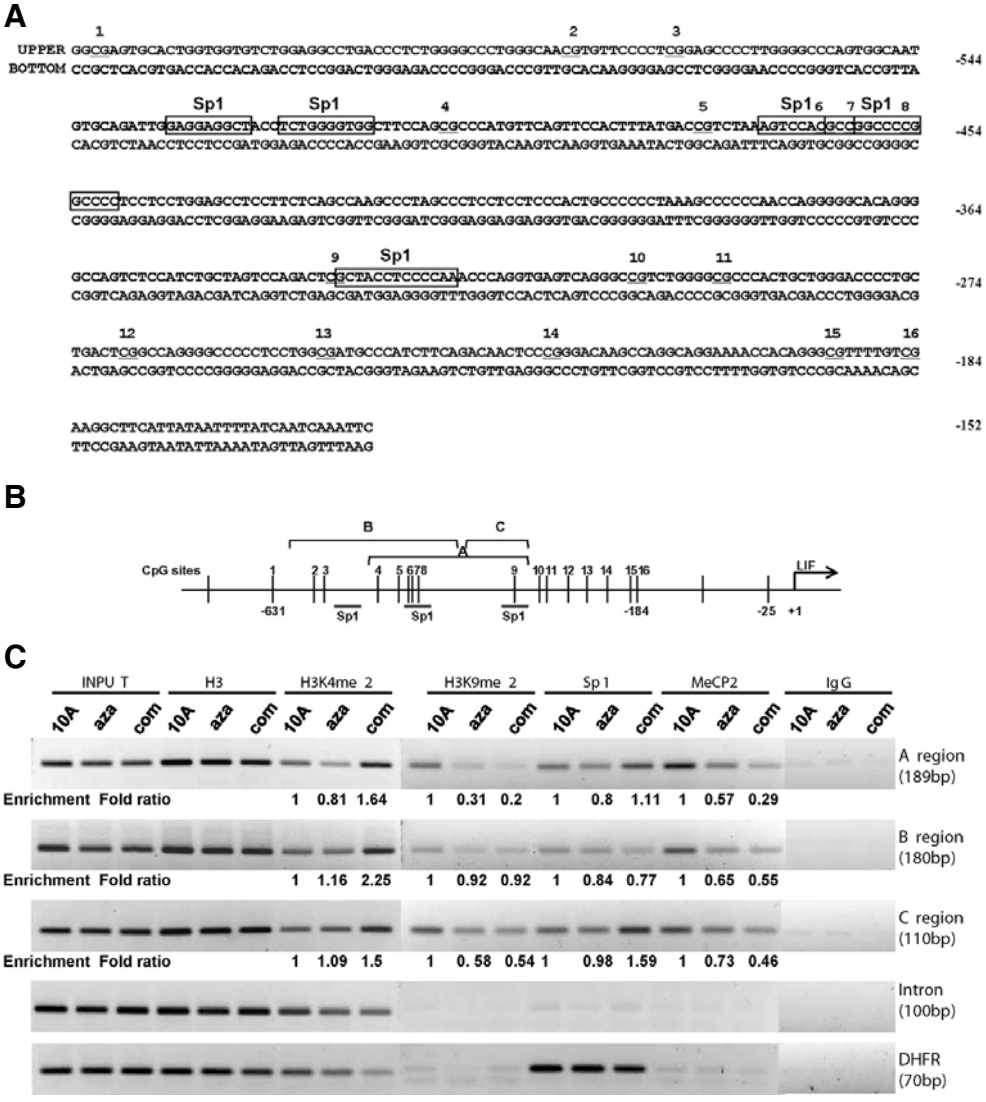

Fig. 3. ChIP analysis of the promoter region of the *LIF* gene to examine the occupancy of MeCP2, histone methylation marks, and Sp1. (A) The upper and bottom strand sequences in the promoter region spanning nucleotides -633 to -152 relative to the transcription start site with 16 CpGs (GenBank AC004264.1) are illustrated as underlined, with five of the putative Sp1 binding in black boxes. (B) The *LIF* gene structure is schematically diagrammed with the transcription starting site indicated as +1 and the PCR region containing each CpG as half box lines. Each CpG site is indicated above the vertical lines that show the CpG position relative to TSS. The Sp1 binding sites are indicated as bottom line bars. (C) ChIP analysis of MCF-10A cells with or without 5-aza-dC and MCF-10DCIS.com cells using the nonspecific antibodies H3, H3K4me2, H3K9me2, anti-Sp1 (Sp1), MeCP2, and IgG antibodies. Compare 10A vs aza or com for H3-K9 in the A region and C region in (C). The PCR-amplified regions and band sizes are indicated on the right side of each panel. The enrichment fold ratio relative to the band intensity of INPUT (PCR products amplified from whole chromosomal DNAs extracted without addition of antibody) was used to calculate the occupancy of each protein within the *LIF* promoter. The promoter region of DHFR was amplified as a positive control (bottom row) and the first intron region of *LIF* gene (fourth row) as a negative control.

compared to untreated MCF-10A cells in an inverse manner. Thus, our data indicate that changes in the histone methylation profile correlate with both the DNA methylation profile and MeCP2 occupancy.

The effect of methylation patterns of CpG sites on Sp1/Sp3 binding and activity in the p21 promoter was previously demonstrated (Clark et al., 1997; Zhu et al., 2003). We tested for the involvement of the transcription factor Sp1 in upregulation of *LIF* via binding to the potential consensus sequence containing the hypomethylated CpG pairs. The fourth to ninth CpG pairs in the *LIF* promoter are located within a potential binding site of Sp1 (Mancini et al., 1999). A ChIP assay with an anti-Sp1 antibody tested the influence of DNA methylation on Sp1 binding to the *LIF* promoter region, and showed that the Sp1 occupancy within the *LIF* promoter region containing the ninth CpG pair had a slight but consistent increase in the localized carcinoma MCF-10DCIS.com cells compared to the untransformed MCF-10A cells (Fig. 3C). The Sp1 enrichment fold ratio remained unchanged in hypomethylating drug-treated MCF-10A cells relative to untreated cells (Fig. 3C).

Taken together, our data suggest that the upregulation of *LIF* gene in 5-aza-dC treated MCF-10A and localized carcinoma MCF-10DCIS.com cells may occur through some decrease in the level of histone H3-Lys9-dimethylation and MeCP2 binding and/or through recruitment of Sp1 to hypomethylated CpG sites.

RNAi-mediated knockdown of LIF results in breast cancer cells with decreased cell growth and decreased colony forming ability

Most evidence about *LIF* suggests that it stimulates the proliferation of most carcinoma cell lines, including the estrogen-dependent MCF-7 and the estrogen-independent SK-BR30 breast cancer cell lines (Douglas et al., 1997; 1998; Kellokumpu-Lehtinen et al., 1996). To understand if a *LIF* deficiency affects the growth or survival of MCF10 cells, we performed knockdown experiments in noninvasive carcinoma MCF-10DCIS.com cells, which have high levels of *LIF* expression. We used two human *LIF*-specific shRNAs, *LIF* shRNA1 and *LIF* shRNA2.

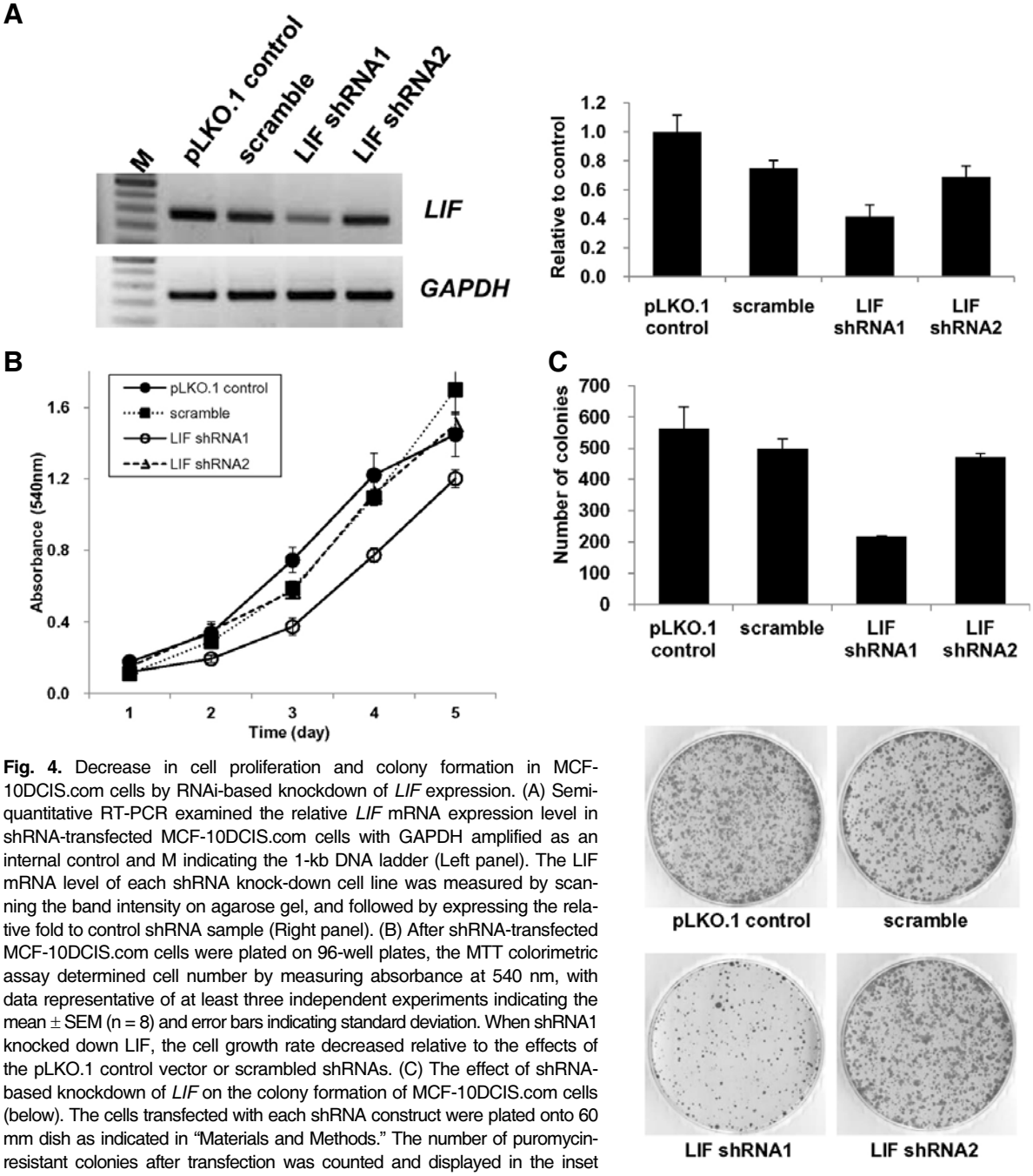

Fig. 4. Decrease in cell proliferation and colony formation in MCF-10DCIS.com cells by RNAi-based knockdown of *LIF* expression. (A) Semi-quantitative RT-PCR examined the relative *LIF* mRNA expression level in shRNA-transfected MCF-10DCIS.com cells with GAPDH amplified as an internal control and M indicating the 1-kb DNA ladder (Left panel). The LIF mRNA level of each shRNA knock-down cell line was measured by scanning the band intensity on agarose gel, and followed by expressing the relative fold to control shRNA sample (Right panel). (B) After shRNA-transfected MCF-10DCIS.com cells were plated on 96-well plates, the MTT colorimetric assay determined cell number by measuring absorbance at 540 nm, with data representative of at least three independent experiments indicating the mean ± SEM (n = 8) and error bars indicating standard deviation. When shRNA1 knocked down LIF, the cell growth rate decreased relative to the effects of the pLKO.1 control vector or scrambled shRNAs. (C) The effect of shRNA-based knockdown of *LIF* on the colony formation of MCF-10DCIS.com cells (below). The cells transfected with each shRNA construct were plated onto 60 mm dish as indicated in "Materials and Methods." The number of puromycin-resistant colonies after transfection was counted and displayed in the inset (upper). All results are representative of three independent experiments.

The lentiviral shRNA1 effectively inhibited *LIF* while shRNA2 did not, as determined by semiquantitative RT-PCR. The results were compared to pLKO.1vector, which is a negative control vector containing a nonhairpin insert, and to a scrambled shRNA (Fig. 4A). In agreement with the knockdown results, we found that when *LIF* expression was reduced by *LIF* shRNA1, MTT assay results indicate there was a significant decrease in the growth rate of the cells compared to those treated with the control vector or scrambled shRNA. Cells transfected with *LIF* shRNA2 had growth rates comparable to those treated with control or scrambled shRNA (Fig. 4B). The data suggest that MCF-10DCIS.com cells transfected with scrambled shRNA or *LIF* shRNA2 secreted sufficient LIF protein for their growth. We counted colonies after 14 days of culture in puromycin selection medium and found that knockdown by *LIF* shRNA1 resulted in a 2- to 2.5-fold reduction in colony numbers relative to cells treated with the control vector, scrambled shRNA, or shRNA2 (Fig. 4C). Therefore, our data support the idea that the *LIF-LIFR* axis may have roles in supporting the proliferation, growth, and survival of breast cancer cells.

DISCUSSION

The epigenetic regulatory mechanism of *LIF* gene expression

during development of breast cancer is poorly understood. Normal breast epithelial and isogenic benign tumor cell lines express low levels of *LIF*, but isogenic carcinoma cell lines considerably upregulate its expression (Fig. 1; Rhee et al., 2008). We focused on involvement of epigenetic control systems such as DNA methylation or histone modifications in *LIF* overexpression during breast cancer progression. The most significant finding was that the upregulation of *LIF* expression was correlated with the DNA methylation status of the *LIF* promoter and the occupancy profile of MeCP2 and histone methylation marks. In addition, knock-down of *LIF* expression significantly reduced cell growth and colony formation in breast cancer cells. Together, our data suggest that epigenetic modifications of the *LIF* gene promoter likely play an important role in the development of breast cancer.

Accumulating evidence supports the role of such epigenetic silencing mechanisms as methylation and inactive histone modifications in the transcriptional inactivation of tumor suppressor genes during cancer developmental processes (Ballestar and Esteller, 2008; Baylin and Jones, 2007;Robertson, 2005). In contrast, tumor progression may involve the epigenetic activation of oncogene-like genes, as evidenced by the upregulation of galectin-7, *CLDN4, uPA*, and *MMP-2* genes in several cancer cells via DNA hypomethylation in their promoter regions (Chernov et al., 2009; Demers et al., 2009; Guo et al., 2002; Honda et al., 2006; Pakneshan et al., 2004; Shvachko, 2009). This epigenetic activation involves the reversal of inactive epigenetic marks including histone deacetylation by histone acetylation and DNA methylation by DNA hypomethylation. The epigenetic interplay between DNA methylation and inactive histone methylation provides evidence that MeCP2 associates with histone H3 Lys9 methyltransferase and may deliver this inactive mark to a DNA methylated gene (Fuks et al., 2003b). In addition, the interplay between DNA methylation and histone H3-Lys27-methylation was suggested in the recent report that demonstrated epigenetic analysis of the invasion promoting MMPs in cancer cells (Chernov et al., 2009). In agreement, our data demonstrated that inactive epigenetic marks such as DNA methylation, MeCP2 occupancy, and histone H3-Lys9-dimethylation significantly decreased during the progression to breast cancer but the active hallmark of H3-Lys4-dimethylation was increased in an inverse manner (Figs. 2 and 3). Thus, the data here suggest that epigenetic regulation via DNA methylation and histone methylation is involved in transcriptional activation of *LIF* gene during the progression to breast cancer.

Transcription factors including AP-2, cAMP-responsive element binding factor (CREB), retinoblastoma binding protein 1 (RBP1), and SP1 have CG-rich binding sequences within their DNA recognition elements (Comb and Goodman, 1990; Iguchi-Ariga and Schaffner, 1998; Ohtani-Fujita et al., 1993). Methylation of CpG dinucleotides located within DNA recognition elements may interfere with the binding of transcription factors, which inhibits transcription (Siegfried et al., 1999). Methylated CpG dinucleotides interfere with SP1 binding and further the reduced binding of Sp1 may be involved in suppression of transcriptional activity of the genes such as *AQP5*, and *CLDN4* (Clark et al., 1997; Honda et al., 2006; Kudo, 1998; Mancini et al., 1999; Motegi et al., 2005; Zhu et al., 2003). In agreement with these findings, we observed that the normal breast epithelial cells MCF-10A had a heavily methylated potential SP1-binding site, the ninth CG of the *LIF* promoter, while benign and carcinoma MCF10 variants did not. These methylation patterns correlated with the expression patterns of the *LIF* gene during development of breast cancer in the isogenic MCF10 model. The Sp1 binding increased more in the *in situ* carcinoma MCF-10 DCIS.com cells than in normal breast cells as measured by our ChIP assay. However, the occupancy of Sp1 was little different between the 5-aza-dC treated MCF-10A cells and untreated cells. In contrast to the *DHFR* promoter, Sp1 occupancy maintained a low level in the *LIF* promoter. This raises the possibility that the transcription of the *LIF* gene may involve other transcription factors such as AP-2 and CREB, which share common CpG pairs in their binding consensus sequences (Comb and Goodman, 1990; Iguchi-Ariga and Schaffner, 1998).

In conclusion, this report is the first that we know of to demonstrate that the upregulation of the *LIF* gene during the progression to breast cancer in the isogenic MCF10 model occurs via DNA hypomethylation of CpG pairs, changes in histone methylation status, changes in MeCP2 occupancy, and increased Sp1 binding affinity within the *LIF* promoter region. The epigenetic activation of *LIF* gene by the *LIF-LIFR* axis may contribute to increased cell growth and colony formation of MCF10 carcinoma variants through autocrine stimulation. Future studies to assess the value of aberrant hypomethylation of the *LIF* gene promoter as biomarkers to predict the progression of breast cancer malignancies are warranted.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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