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Cooperatively transcriptional and epigenetic regulation of sonic hedgehog overexpression drives malignant potential of breast cancer

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Sonic hedgehog (Shh), a ligand of Hedgehog signaling pathway, is considered an important oncogene and an exciting potential therapeutic target in several cancers. Comprehensive understanding of the regulation mechanism of Shh in cancer cells is necessary to find an effective approach to selectively block its tumorigenic function. We and others previously demonstrated that nuclear factor-kappa B (NF-κB) activation and promoter hypomethylation contributed to the overexpression of Shh. However, the relationship between transcriptional and epigenetic regulation of Shh, and their roles in the malignant phenotype of cancer cells are still not clearly elucidated. In the present study, our data showed that the level of Shh was higher in breast cancer tissues with positive NF-κB nuclear staining and promoter hypomethylation. In addition, survival analysis revealed that Shh overexpression, but not hypomethylation and NF-κB nuclear staining, was a poor prognosis indicator for breast cancers. Moreover, *in vitro* data demonstrated that both NF-κB activation and hypomethylation in promoter region were positively associated with the overexpression of Shh. Mechanistically, the hypomethylation in Shh promoter could facilitate NF-κB binding to its site, and subsequently cooperate to induce transcription of Shh. Furthermore, the biological function data indicated that overexpressed Shh enhanced the self-renewal capacity and migration ability of breast cancer cells, which could be augmented by promoter demethylation and NF-κB activation. Overall, our findings reveal multiple and cooperative mechanisms of Shh upregulation in cancer cells, and the roles of Shh in tumor malignant behavior, thus suggesting a new strategy for therapeutic interventions to reduce Shh in tumors and improve patients' prognosis.

Sonic hedgehog (Shh) was first identified as a ligand of Hedgehog signaling pathway that is crucial to the growth and patterning in a wide variety of tissues during embryonic development.[1,2] In the past decade, Shh has been reported to play an essential role in the development of multiple cancers.[3,4] In addition, overexpression of Shh has been found to be indicator of poor outcome in several cancers, including oral squamous cell carcinoma,[5] breast cancer,[6] gastric cancer,[7] gallbladder carcinoma,[8] glioma,[9] bladder cancer,[10] prostate cancer[11] and colon cancer.[12] Furthermore, it is well demonstrated that the ligand-dependent activation of the Shh pathway plays critical roles in developing cancer stem cells (CSC) and leads to angiogenesis, migration, invasion and metastasis.[13,14] These findings suggest that Shh may be a viable therapeutic target for treatment of cancer.

Understanding the mechanism of its gene regulation is crucial as Shh emerges as an important target in cancer therapy. It has been proposed that the transcription nuclear factor-kappa B (NF-κB) plays a regulatory role in Shh expression.[15,16] The NF-κB pathway is the means of oncogenic signaling that orchestrates inflammatory responses, cellular proliferation, angiogenesis, migration, invasion, differentiation and self-renewal by human cancer cells.[17,18] It is activated by diverse stimuli that include cellular stress, pro-inflammatory cytokines and growth factors.[18] These stimuli can prompt the active NF-κB to translocate into the nucleus where it binds with NF-κB-specific DNA binding sites to transcriptionally activate its target genes. Kasperczyk *et al.* report that NF-κB regulates Shh expression, which contributes to NF-κB-mediated proliferation and apoptosis resistance in pancreatic cancer.[16] Our group and others have previously shown that the level of Shh protein is positively related to the activation of NF-κB in multiple cancers, including breast cancer.[19–21] These studies demonstrate that NF-κB might be a transcription activator of *Shh* gene.

Another plausible mechanism resulting in overexpression of Shh is epigenetic regulation. It has been reported that aberrant hypomethylation within the promoter is correlated with increased expression of Shh in several tumors.[16,22] In addition, we previously demonstrated that 5-azacytidine, a DNA methyltransferase inhibitor, could reduce the methylation of Shh promoter and increase the expression of Shh protein in

breast cancer cells.[16] Interestingly, the NF-κB binding site is included in the hypomethylation area of Shh promoter, which suggests a possible correlation between transcriptional and epigenetic regulation of Shh expression.

The aims of the present study were to investigate whether there is a correlation between transcriptional and epigenetic regulation of Shh expression, and how the transcriptional and epigenetic regulations affect the malignant phenotype, particularly in cancer stem cell phenotype, of breast cancer.

Materials and Method

Patients and clinical information. A total of 106 patients with breast cancer were consecutively recruited from 2006 to 2010 at the First Affiliated Hospital of China Medical University. All patients were followed up with telephone calls, letter interviews or clinic visitations every quarter during the first 3 years of the study and semi-annually thereafter. Breast cancer death was regarded as the follow-up end, and the cut-off time was 104 months from the first diagnosis. A total of 14 cases failed to follow up (the deaths unrelated to breast cancer were regarded as "failed to follow up"). As such, there were 92 cases in this prognosis analysis. Clinicopathological information on the patients regarding age, tumor size, histological type, stage and lymph node metastasis were obtained from patient records, and are summarized in Table S1. Ethical oversight and approval were obtained from the Institutional Review Board of the First Affiliated Hospital of China Medical University.

Immunohistochemistry. Four-micron thick sections were prepared from the paraffin-embedded tissues. Following antigen retrieval and blocking, the sections were immunostained using antibodies against Shh (1:150 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and NF-κB (p65) (1:100 dilution; Cell Signaling, Danvers, MA, USA) with detection using the avidin–biotin complex method (DAKO, Produktionsvej, Glostrup, Denmark) visualized by DAB. Slides were lightly counterstained with hematoxylin. Evaluation of both the intensity of immunohistochemical staining and the proportion of positively stained epithelial cells were previously described.[23] Briefly, the intensity of immunostaining (1 = weak, 2 = moderate and 3 = intense) and the percentage of positive cells (0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75%.) were assessed in at least 5 high power fields (×400 magnification). The scores of each sample were multiplied to give a final score of 0, 1, 2, 3, 4, 6, 8, 9 or 12, and the tissues were finally determined as negative if score <4 the mean of immunoreactivity score (I.S.); and positive expression if score ≥4 the mean of I.S.

DNA extraction, bisulfite treatment and methylation-specific PCR. The different samples genomic DNA were extracted from cell lines and tissues using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and QIAamp DNA FFPE Tissue Kit (Qiagen), respectively. Bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Kit (Zymo Research. Irvine, CA, USA) according to manufacturer's recommendations. PCR primers were designed to amplify denatured DNA, and included at least two CG dinucleotides. Primer sequences and PCR condition were consistent with previous reports.[16] Unmethylated and methylated products were 179 and 169 bp long, respectively. After PCR, products were separated on a 2% agarose gel, and stained with ethidium bromide. Bisulfite treatment and methylation-specific PCR (MS-PCR) assays were performed in duplicate for all samples. Each experiment was done at least three times. Results were confirmed by sequencing the methylated samples.

Cell lines and drug treatment. Human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-436 and Bcap37 were maintained in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS (GIBCO) with 100 units/mL streptomycin and 100 units/mL penicillin in a humidified atmosphere with 5% CO_2. All cell lines used were between passages 3 and 8 for each experiment and were demonstrated to be free of mycoplasma using a Mycoplasma PCR Detection Kit (Sigma, St. Louis, MO, USA).

5-azacytidine (5-Aza; Sigma-Aldrich) and PDTC (specific NF-κB inhibitor, Sigma-Aldrich, St. Louis, MO, USA) were freshly prepared in culture medium before use. A vehicle control consisting of culture medium alone was included in the analysis. MDA-MB-231 and Bcap37 cells were cultured for 24 h, then treated with 20 μM 5-Aza or PDTC for 24–72 h. Cells were collected by centrifugation, then genomic DNA, RNA and proteins were extracted and analyzed by MS-PCR, quantitative RT-PCR and western blot, respectively.

Western blot analysis. Western blot was performed as described previously.[16] Briefly, the protein was extracted with lysis buffer for 1 h at 4°C. The supernatants were centrifuged, and total protein was harvested. Aliquots containing 20 μg of proteins were separated on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes. After blocking, the blots were, respectively, incubated with primary antibody directed against Shh (1:500, Santa Cruz), NF-κB (1:500, Cell Signaling) or β-actin (1:1000, Cell Signaling) overnight at 4°C and followed by each corresponding second antibody at room temperature for 1 h at 37°C. Then the results were developed by ECL (Pierce, Rockford, IL, USA). The experiments were repeated independently three times.

Quantitative RT-PCR analysis. Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen) as described in the product insert. The RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo, Waltham, MA, USA) and PCR was done using iQ SYBR Green Supermix and the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Primers used were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Reverse primer 5′-CCC TCA ACG ACC ACT TTG TCA-3′ and forward primer 5′-TTC CTC TTG TGC TCT TGC TGG-3′; *Shh* reverse primer 5′-CAC CGA GCA GTG GAT ATG TG-3′ and forward primer 5′-AGT GGC CAG GAG TGA AAC TG-3′.

Transient transfection. Human Shh full-length cDNA were cloned into the pCMV expression vectors so that the Shh was expressed. The pCMV-Shh (1 μg/μL) was transiently transfected into MDA-MB-231 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection efficiency was verified by western blotting.

Cloning. To generate Shh luciferase reporter gene constructs, a series of deletion constructs of promoter region were obtained by PCR products generated by restriction site containing primers. The sequence of primers are shown in Table S2. The resulting fragments were cloned upstream of the luciferase gene into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA). All plasmids were controlled by sequencing.

Luciferase assays. The Dual-Luciferase Reporter Assay System (Promega) was used to determine luciferase activities according to the manufacturer's instructions. Cells in 96-well plates were transfected with Shh-firefly luciferase vector and *Renilla* luciferase vector under control of the ubiquitin pro-

moter per well using Lipofactamine 2000. After 24 h, cells were treated as indicated and lysed with Passive Lysis Buffer (Promega). Measurements were performed with a Berthold luminometer (Berthold Technologies, Bad Wildbad, Germany), and firefly luciferase values were normalized to *Renilla* luciferase values.

ChIP. A ChIP assay kit was used according to the Upstate Biotechnology ChIP protocol. Briefly, 5×10^6 cells were treated with 5-Aza (20 μM) and/or TNFα (20 ng/mL) for the indicated time periods. Protein-DNA complexes were immunoprecipitated overnight at 4°C with antibodies against NF-κB p65. Antibody complexes were pulled down for 4 h with 60 μL Protein A agarose/salmon sperm DNA. Unbound chromatin in the no-antibody sample was used as input. DNA from both unbound and eluted chromatin was purified using the PCR Purification Kit (Roche, Bromma, Sweden). The immunoprecipitated DNA was quantified by real-time quantitative PCR. Amplification was performed with the default PCR setting using the following primers: 5′-ATT CCA GCC CCT GTC TGG GT-3′ (forward) and 5′-GTG CGT GTG CGC TCT CTC T-3′ (reverse). Input DNA was used as the endogenous control.

Nuclear factor-kappa B DNA binding assay. To determine NF-κB activation, we performed a DNA binding assay using the TransAM NF-κB kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions and as previously described.[24] Briefly, MDA-MB-231 cells were treated with 5-Aza (20 μM) or TNF-α (20 ng/mL) for 24 h. Nuclear protein extracts were prepared using a nuclear extraction kit (Invitrogen) according to the manufacturer's instructions. Protein concentration was determined using the BCA Protein Assay Kit (Pierce), and 20 μg of nuclear extract was added to the plates precoated with an NF-κB consensus oligonucleotide sequence and incubated at room temperature for 1 h with continuous shaking. The plates were washed and incubated with a p65 primary antibody, followed by a secondary antibody, then substrate solution was added. Next, the plates were read at a reference wavelength of 655 nm.

Soft agar colony formation assay. A density of 500 cells/mL MDA-MB-231 cells were seeded onto a 24-well plate with 0.4 mL of 0. 3% low melting agarose (Sigma). After the different treatment, including 5-Aza (20 μM), pCMV-Shh (1 μg/μL), TNF-α (20 ng/mL) and cyclopamine (10 μM), these cells were further cultured for 10 days. Colonies with more than 50 cells were counted, and the colony formation rate was calculated according to the following equation: Colony formation rate (%) = (Number of colony formation/Number of seeded cells) × 100%.

Wound-healing migration assay. Wound healing migration assay was performed as described previously.[25] Briefly, Bcap37 cells were starved to inactivate cell proliferation and then wounded by pipette tips. DMEM containing 5% FBS was added with different agents, including 5-Aza (20 μM), pCMV-Shh (1 μg/μL), TNF-α (20 ng/mL) and cyclopamine (10 μM). Images of the cells were taken after 24 h incubation. Migrated distance was quantified manually. Three independent experiments were performed.

Cell proliferation assay. The *in vitro* cell proliferation effects of Shh signaling were determined by MTT assay. The MDA-MB-231 cells (1×10^5 cells/mL) were seeded into 96-well culture plates. After overnight incubation, the cells were treated with different agents, including 5-Aza (20 μM), pCMV-Shh (1 μg/μL), TNF-α (20 ng/mL) and cyclopamine (10 μM), for 48 h. Then 10 μL MTT solution (2.5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37°C. The medium with MTT was aspirated, followed by the addition of 100 μL DMSO. The optical density of each well was measured at 570 nm using a Biotek Synergy HT Reader (BioTek Instruments, Winooski, VT).

Statistical analysis. Statistical analysis of group differences was performed using Pearson's χ^2-test and ANOVA. With regard to survival analysis, we analyzed 92 breast cancer patients using Kaplan–Meier analyses. $P < 0.05$ was regarded as statistically significant. All statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

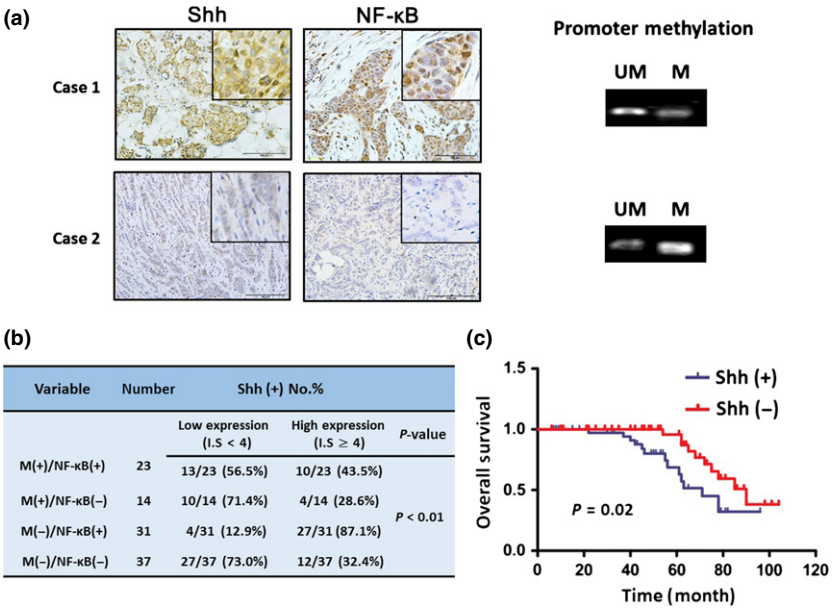
Results

Correlation among Sonic hedgehog expression, nuclear factor-kappa B expression, promoter hypomethylation and patients outcome. To explore the correlation among Shh, NF-κB and promoter methylation in breast cancer, we detected the expression of Shh and NF-κB using the immunohistochemistry method, and measured the promoter methylation status of Shh using MS-PCR. Our data showed that the level of Shh was higher in tissues with positive NF-κB nuclear staining and promoter hypomethylation, whereas the level of Shh was lower in tissues with negative NF-κB nuclear staining and promoter hypermethylation (see Fig 1a). In detail, the rate of positive Shh expression was 87.1% in tissues with positive NF-κB nuclear staining and promoter hypomethylation, but 28.6% in tissues with negative NF-κB nuclear staining and promoter hypermethylation (see Fig 1b). Statistical analysis data indicated a significant difference among methylation (−)/NF-κB (+), methylation (+)/NF-κB (−), methylation (−)/NF-κB (−) and methylation (+)/NF-κB (+) in Shh expression level (see Fig. 1b), suggesting a crucial role of the hypomethylation along with NF-κB activation in the regulation of Shh expression.

To elucidate the clinical significance of Shh, NF-κB and promoter methylation in breast cancer, survival analysis was performed using the Kaplan–Meier method. We found that Shh promoter methylation status and NF-κB nuclear expression cannot predict patients' overall survival (data not shown). Further survival analysis revealed that the cases with Shh-positive expression experienced worse clinical outcome compared with those with Shh-negative expression (see Fig. 1c), supporting the oncogenic role of Shh in breast cancer.

Correlation of NF-κB nuclear expression, promoter methylaion status with Sonic hedgehog expression in breast cell lines. To confirm the correlation among NF-κB nuclear expression, promoter methylation status with Shh expression, several breast cell lines, MCF-7, Bcap37, MDA-MB-436 and MDA-MB-231, were used in our experiments. As shown in Figure 2a, MCF-7 cells expressed Shh at a relatively higher level, Bcap37 and MDA-MB-231 cells displayed a moderate expression, but MDA-MB-436 cells expressed Shh at a lower level. Interestingly, a similar pattern was shown in NF-κB nuclear expression, suggesting a positive correlation between NF-κB and Shh. As the transcription factor, NF-κB regulates target gene expression in mRNA level. Thus, we also detected the expression of Shh in mRNA level using quantitative RT-PCR. Consistent with protein expression data, Shh was expressed at a higher level in MCF-7 cells, but not in Bcap37, MDA-MB-436 and MDA-MB-231 cells (see Fig. 2b), demonstrating that the crucial regulation of Shh expression is presented in the transcription level.

Next, we also detected the methylation status of Shh promoter in MCF-7, Bcap37, MDA-MB-436 and MDA-MB-231



Variable	Number	Shh (+) No.%		
		Low expression (I.S < 4)	High expression (I.S ≥ 4)	*P*-value
M(+)/NF-κB(+)	23	13/23 (56.5%)	10/23 (43.5%)	
M(+)/NF-κB(–)	14	10/14 (71.4%)	4/14 (28.6%)	
M(–)/NF-κB(+)	31	4/31 (12.9%)	27/31 (87.1%)	*P* < 0.01
M(–)/NF-κB(–)	37	27/37 (73.0%)	12/37 (32.4%)	

Fig. 1. Sonic hedgehog (Shh) expression, nuclear factor-kappa B (NF-κB) expression and promoter hypomethylation in breast cancer. (a) The expression of Shh and NF-κB, and methylation status of *Shh* promoter in representative breast cancer tissues. M, methylation; UM, unmethylation. Figures magnified 200× or 400×. (b) The correlation among Shh, NF-κB and promoter methylation in breast cancer. A sample is defined as Shh or NF-κB + if it has an IS ≥4. (c) Overall survival according to expression of Shh in breast cancer.

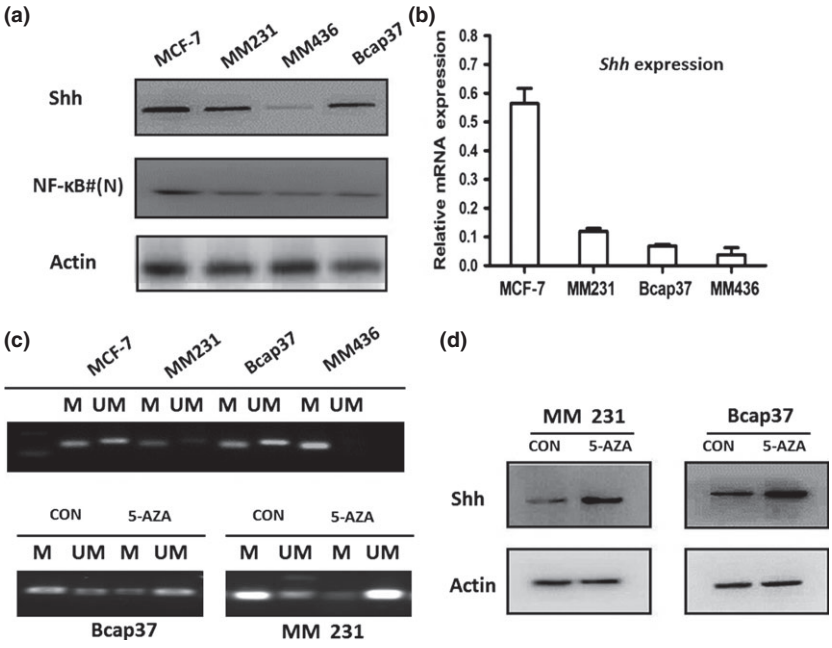


Fig. 2. The correlation of nuclear factor-kappa B (NF-κB) expression and promoter methylation status with Sonic hedgehog (Shh) expression in breast cell lines. (a) The expression of Shh and nuclear NF-κB in breast cancer cell lines. (b) The expression of Shh mRNA in breast cancer cell lines. MDA-MB-231 (MM231); MDA-MB-436 (MM436). (c) Methylation status of Shh promoter in breast cell lines and the effect of 5-azacytidine (5-Aza) treatment. MM231 and Bcap37 cells were treated with 20 μM 5-Aza for 72 h. The genomic DNA was extracted for methylation-specific (MS)-PCR. M, methylated; UM, non-methylated. (d) Expression of Shh in breast cell lines after 5-Aza treatment.

cells. In agreement with our previous data,[16] Shh promoter was methylated weakly in MCF-7 cells. However, the promoter region was enhancedly methylated in MDA-MB-436, Bcap37 and MDA-MB-231 cells (see Fig. 2c). Moreover, we also investigated the effect of 5-Aza (20 μM), a DNA methyltransferase inhibitor, on the methylation status and protein expression of Shh in MDA-MB-231 and Bcap37 cells, which showed a moderate level of Shh expression and promoter methylation. Our data revealed that 5-Aza treatment resulted in a decrease in methylation in MDA-MB-231 and Bcap37 cells (Fig. 2c). Meanwhile, 5-Aza treatment also lead to an increased expression of Shh in both cell lines (Fig. 2d). These results strongly suggested that promoter methylation might be another important regulation mechanism of Shh in breast cancer.

Hypomethylation and nuclear factor-kappa B cooperate to induce transcription of Sonic hedgehog gene. To elucidate the characteristics of Shh promoter region, a series of pGL3-Shh-promter-luciferase vectors were constructed, and the activities of reporter genes were measured after being transfected in MDA-MB-231 cells for 24 h. Our results indicated that pGL3-Shh-P1 reporter and pGL3-Shh-P2 reporter displayed similar activity, suggesting that the transcription factor Sp1 binding site has no significant effect on activity of the Shh promoter (Fig. 3a). In contrast, the deletion of the NF-κB binding site (pGL3-Shh-P3 reporter) resulted in an obvious decrease (Fig. 3a), indicating that the NF-κB binding site is crucial to maintain transcription activity of the Shh promoter.

To investigate whether NF-κB regulates transcription through NF-κB response elements, cells were transfected with

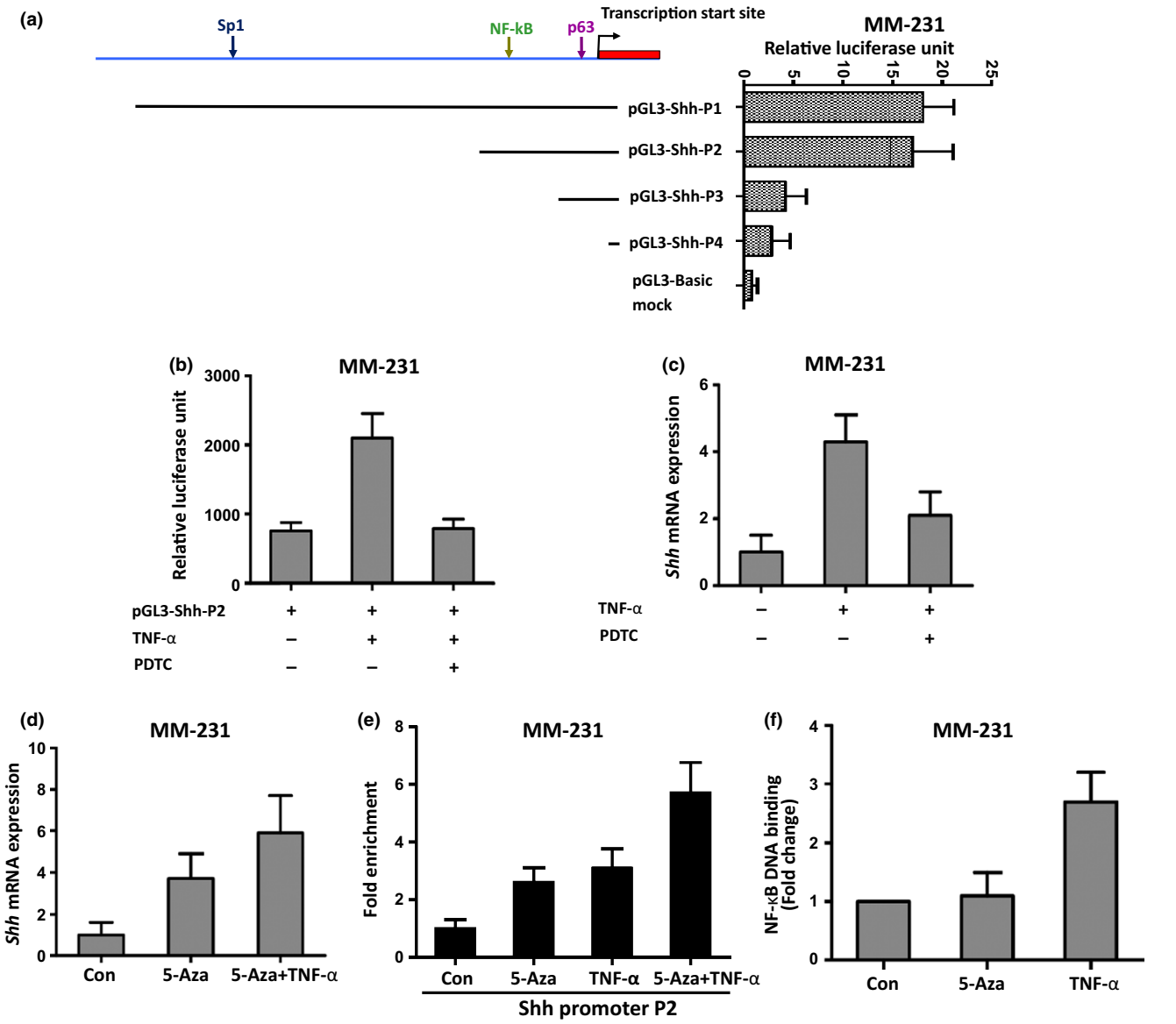


Fig. 3. The effects of promoter methylation and nuclear factor-kappa B (NF-κB) on the transcription of Sonic hedgehog (*Shh*) gene in MDA-MB-231 cells. (a) Position and promoter activities of the *Shh* deletion pGL3 vector. Schematic representation of the promoter/luciferase constructs. Promoter activities of the cloned 5′-untranscribed sequences of the *Shh* gene and deletion constructs. The promoter activities were measured using the Dual-Luciferase Reporter Assay System (Promega) after transfection into MDA-MB-231(MM231) cells. The bars represent average luciferase activities compared with the control pGL3 vector. (b) The promoter activity of Shh-P2 reporter after being treated with TNF-α or/and PDTC in MM231 cells. (c) The effect of TNF-α or/and PDTC on Shh mRNA expression in MM231 cells. The mRNA were analyzed for *Shh* levels by quantitative PCR using specific primers as described in the Materials and Methods. GAPDH was used as control. (d) The effect of TNF-α or/and 5-Aza on Shh mRNA expression in MM231 cells. (e) ChIP assays confirmed the binding of NF-κB(p65) to the promoter regions upstream of *Shh* gene. Promoter 2 located in −83 to +80 upstream of *Shh* gene. (f) The effects of 5-Aza and TNF-α on NF-κB DNA binding activity. MM231 cells were stimulated with TNF-α (20 ng/mL) or 5-Aza (20 μM) for 24 h. Nuclear extracts from MM231 cells were assayed for NF-κB p65 activation.

a luciferase construct containing pGL3-Shh-P2 and treated with TNF-α, an NF-κB activator and/or PDTC, a specific NF-κB inhibitor. In MDA-MB-231 cells, TNF-α alone induced transcription, but this could be completely reversed by the addition of PDTC (Fig. 3b). To confirm the relevance of these results, we studied the effect of TNF-α and/or PDTC on the transcription of Shh gene. As shown in Figure 3c, the expression of Shh was increased after treatment with TNF-α, but the increase could be blocked by PDTC. These results demonstrate that NF-κB plays an important role in the regulation of Shh. Simultaneously, we also assessed the effect of 5-Aza on the transcription activity of Shh. As shown in Figure 3d, treatment with 5-Aza could induce the transcription of Shh in MDA-MB-231, but this could be further enhanced by the addition of TNF-α. The above results were further confirmed by the ChIP experiments. The results revealed an increase in p65 binding to the *Shh* promoter in cells treated with 5-Aza and TNF-α alone. When 5-Aza and TNF-α were combined, an obvious enhancement in p65 binding was observed (Fig. 3e). In addition, we further detected the NF-κB activation in MDA-MB-231 cells after treatment with 5-Aza or TNF-α. The data showed that TNF-α could induce an NF-κB activation in

MDA-MB-231 cells (Fig. 3f). In contrast, NF-κB activity could not be induced by 5-Aza treatment (Fig. 3f), suggesting the impossibility of directly NF-κB activation by 5-Aza. Taken together, our results demonstrate that the transcription of Shh is regulated by cooperation between hypomethylation and NF-κB in breast cancer.

Cooperation of hypomethylation, nuclear factor-kappa B and Sonic hedgehog driving malignant potential of breast cancer cells. It is well known that the activation of Shh signaling would result in a malignant potential of cancer cells.[3,4] Therefore, we next investigated whether hypomethylation, NF-κB and Shh cooperate to drive the malignant potential of breast cancer cells. As shown in Figure 4a, the overexpression of Shh led to an increase in the colony formation rate of MDA-MB-231 cells. Similarly, there was a significant increase in the number of colony-forming cells after treatment with TNF-α or 5-Aza. In addition, triple combination resulted in a significant increase in the colony formation rate, indicating an enhanced self-renewal capacity of these breast cancer cells. Interestingly, pretreatment with cyclopamine, a specific inhibitor of Shh signaling, led to an obvious decrease in the number of colony-forming cells, suggesting that the induction of Shh expression by 5-Aza and NF-κB is critical in the self-renewal of breast cancer cells. Moreover, the migration ability of Bcap37 cells was also measured. In agreement with the data obtained from the colony formation assay, the migration cells were increased after being treated with overexpressed Shh, TNF-α or 5-Aza (see Fig. 4b). Meanwhile, the number of invasion cells was significantly increased after the combination, but obviously reversed by cyclopamine (see Fig. 4b). Furthermore, we also assessed the effect of Shh signaling on cell proliferation. As shown in Figure S1, overexpression of Shh resulted in an increase in the cell viability of MDA-MB-231 cells, whereas pretreatment with cyclopamine reversed Shh-mediated cell proliferation. In contrast to colony formation and migration results, a slight reduction of cell viability was shown after treatment with TNF-α or 5-Aza. Taken together, our data showed that hypomethylation, NF-κB and Shh cooperate to drive self-renewal capacity and migration ability of breast cancer cells.

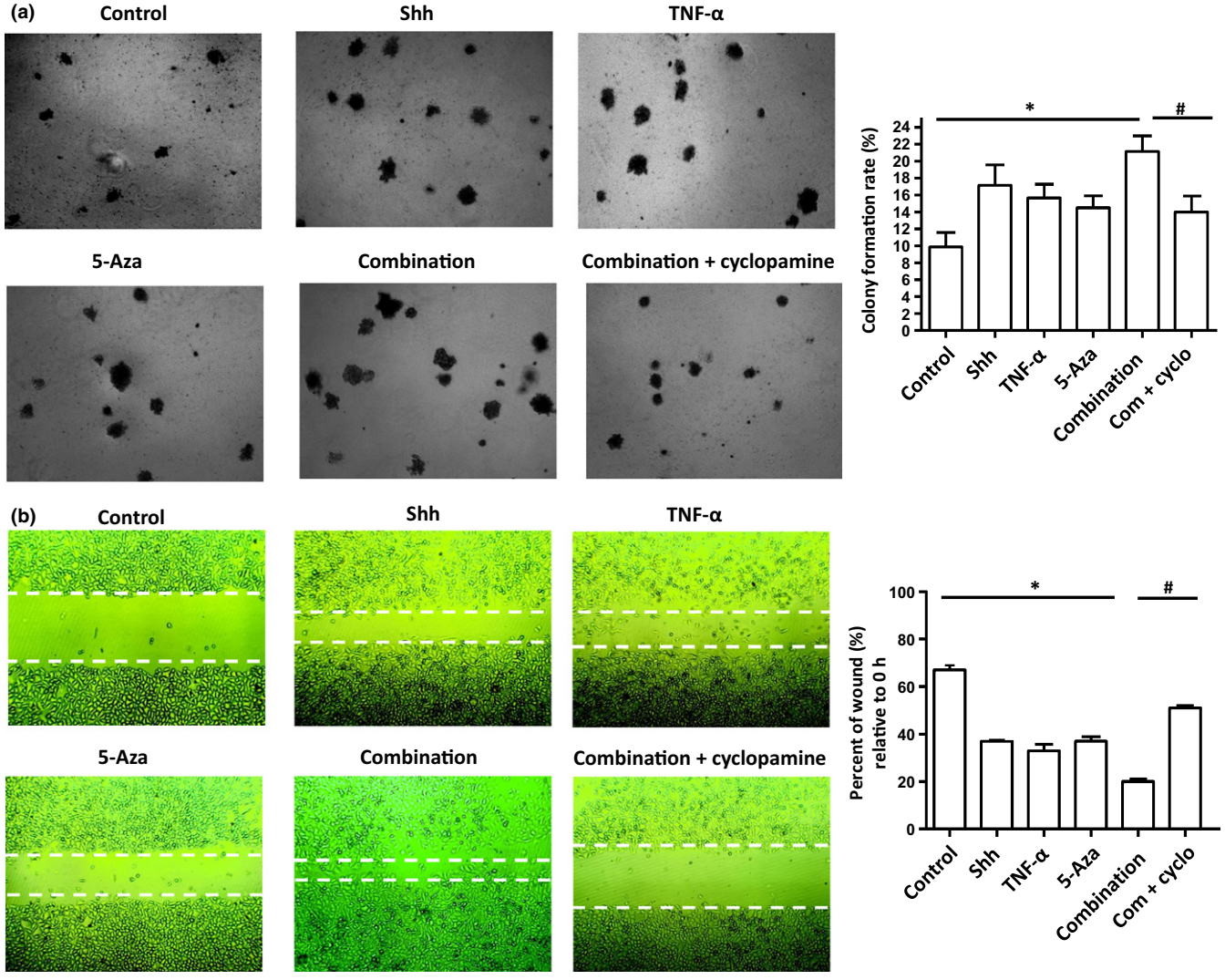


Fig. 4. The effects of Sonic hedgehog (Shh) overexpression, TNF-α, 5-Aza, cyclopamine or combination on colony formation and migration of breast cancer cells. (a) MM231 colony formation was measured after different agents (5-Aza, pCMV-Shh, TNF-α and cyclopamine) were treated for 72 h. (b) Bcap37 migration was measured by wound-healing migration assay after different agents (5-Aza, pCMV-Shh, TNF-α and cyclopamine) were treated for 24 h. All error bars are SEM. $^{*}P < 0.05$ compare with control group; $^{\#}P < 0.05$ compare with combination group.

Discussion

Gene expression is tightly regulated by both genetic and epigenetic mechanisms. It has been well elucidated that cis and trans-acting elements are basic requirements for gene expression regulation, and that promoter methylation can interfere with gene transcription through regulating DNA-transcription factor interactions.[26] In our previous study, we found that overexpression of cancer-related gene Shh in human breast cancer might be caused by the hypomethylation in the promoter region and the activation of transcription factor NF-κB.[16] Meanwhile, the question has been raised as to whether two regulation mechanisms cooperate to regulate the expression of Shh in breast cancer. The present study revealed that both the hypomethylation in promoter and the activation of NF-κB directly contributed to the regulation of Shh gene in breast cancer, and the demethylation in NF-κB binding element by 5-Aza would be facilitate to the binding of NF-κB to its cis-element, then led to an enhancement of transcription of Shh. Using the genetic and epigenetic approach, our study, for the first time, clearly elucidates the cooperative regulation mechanisms of Shh gene.

DNA hyper-/hypomethylation in the promoter of genes is one of the powerful epigenetic modifications that regulates gene transcription.[26] DNA methylation profiles of many genes have been linked with cancer initiation and progression.[27] For tumor suppressor genes, CpG methylation silences genes by sterically impeding binding of transcription factors that recognize sequences containing CG or by recruiting repressor proteins that bind specifically to methyl-CpGs. In contrast, CpG demethylation results in transcription activation of oncogenes. In the present study, the demethylation by 5-Aza in breast cancer cells led to the transcription activation of Shh gene, which is consistent with our and other previous group reports.[16,21] Interestingly, we also demonstrated that the demethylation facilitates the binding of NF-κB to the promoter region of Shh, subsequently promote transcription, which could be explained by the following facts. First, the promoter activity and expression of Shh was enhanced by NF-κB activator TNF-α, but was completely blocked by the addition of NF-κB inhibitor PDTC (Fig. 3b,c), suggesting the crucial role of NF-κB in the transcriptional regulation of Shh. Second, the expression of Shh and binding of p65 to Shh promoter was increased by DNA methyltransferase inhibitor 5-Aza, and further enhanced by the addition of TNF-α (Fig. 3d,e), which demonstrated a synergistic regulation model of Shh transcription by demethylation and NF-κB activation. Third, the NF-κB activity could be induced by TNF-α but not by 5-Aza, suggesting that the synergistic regulation of Shh by 5-Aza was mediated by demethylation but not by direct activation of NF-κB. In fact, a similar regulation approach has been reported in other cancer-related genes. James *et al.*[28] found that the overexpression of MAGEA11, a cancer germline antigen, is regulated by promoter hypomethylation cooperating nucleosome occupancy. Therefore, Shh regulation is highly instructive for understanding mechanisms regulating oncogenes in human cancer.

The Shh signaling pathway plays a key role in tumor biology, including cell proliferation, differentiation, apoptosis and migration, and in the regulation of CSC self-renewal and tumorigenic potential,[3,4] suggesting that Shh signaling could be a promising therapeutic target in breast cancer. In the present study, we found that the overexpression of Shh resulted in an enhanced self-renewal capability and an increased migration potential in breast cancer cells, suggesting a key role of Shh in tumor progression. Interestingly, our data also showed that the demethylation by 5-Aza and the activation of NF-κB by TNF-α led to a malignant phenotype of breast cancer cells, and the combination with Shh overexpression strengthens this alteration, supporting a synergistic role of methylation, NF-κB and Shh in tumor progression. In view of the limited efficacy of Shh inhibitors,[29] due to compensated oncogenic mechanisms, in the clinical study, the finding provides a new strategy for the combination therapy of breast cancer.

Aberrant methylation changes in certain cancer-related genes were also suggested to be a biomarker for prognosis of tumor patients. For a series of cancer-related genes, including BMP family genes,[30] LINE-1[31] and SPP1,[32] it has been reported that the aberrant hypomethylation of their promoters is related to a poor outcome of tumor patients. Here, we found that, in contrast to Shh expression, both the methylation status of Shh promoter and the activation of NF-κB were not associated with patients' prognosis. Consistent with the tissue results, our *in vitro* data indicated that overexpression of Shh, but not demethylation and activation of NF-κB, contributes to the cell proliferation (see Fig. S1). The discrepancy between Shh and its regulation factors in clinical value and biological function of breast cancer could be elucidated by the following facts. Whether demethylation or NF-κB activation could contribute to a series of gene alterations, which subsequently mediates complicated biological events.[18,33] Hence, it is possible that Shh and its regulation factors exhibit a inconsistent biological function.

In summary, the present study reveals that the cooperation between promoter hypomethylation and NF-κB activation results in the overexpression of Shh, which might contribute to the acquisition of malignant tumor behavior in breast cancer.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Casey B, Hackett BP. Left-right axis malformations in man and mouse. *Curr Opin Genet Dev* 2000; **10**: 257–61.
2 Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 2001; **15**: 3059–87.
3 Chari NS, McDonnell TJ. The sonic hedgehog signaling network in development and neoplasia. *Adv Anat Pathol* 2007; **14**: 344–52.
4 Jiang J, Hui CC. Hedgehog signaling in development and cancer. *Dev Cell* 2008; **15**: 801–12.
5 Fan HX, Wang S, Zhao H *et al.* Sonic hedgehog signaling may promote invasion and metastasis of oral squamous cell carcinoma by activating MMP-9 and E-cadherin expression. *Med Oncol* 2014; **31**: 41.
6 Bièche I, Lerebours F, Tozlu S, Espie M, Marty M, Lidereau R. Molecular profiling of inflammatory breast cancer: identification of a poor-prognosis gene expression signature. *Clin Cancer Res* 2004; **10**: 6789–95.

7 Saze Z, Terashima M, Kogure M, Ohsuka F, Suzuki H, Gotoh M. Activation of the sonic hedgehog pathway and its prognostic impact in patients with gastric cancer. *Dig Surg* 2012; **29**: 115–23.
8 Li J, Wu T, Lu J *et al.* Immunohistochemical evidence of the prognostic value of hedgehog pathway components in primary gallbladder carcinoma. *Surg Today* 2012; **42**: 770–5.
9 Li Q, Zhang Y, Zhan H *et al.* The Hedgehog signalling pathway and its prognostic impact in human gliomas. *ANZ J Surg* 2011; **81**: 440–5.
10 He HC, Chen JH, Chen XB *et al.* Expression of hedgehog pathway components is associated with bladder cancer progression and clinical outcome. *Pathol Oncol Res* 2012; **18**: 349–55.
11 Kim TJ, Lee JY, Hwang TK, Kang CS, Choi YJ. Hedgehog signaling protein expression and its association with prognostic parameters in prostate cancer: a retrospective study from the view point of new 2010 anatomic stage/prognostic groups. *J Surg Oncol* 2011; **104**: 472–9.
12 Xu M, Li X, Liu T, Leng A, Zhang G. Prognostic value of hedgehog signaling pathway in patients with colon cancer. *Med Oncol* 2012; **29**: 1010–6.
13 Ruiz i Altaba A, Sánchez P, Dahmane N. Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat Rev Cancer* 2002; **2**: 361–72.
14 Bailey JM, Singh PK, Hollingsworth MA. Cancer metastasis facilitated by developmental pathways: sonic hedgehog, Notch, and bone morphogenic proteins. *J Cell Biochem* 2007; **102**: 829–39.
15 Nakashima H, Nakamura M, Yamaguchi H *et al.* Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res* 2006; **66**: 7041–9.
16 Cui W, Wang LH, Wen YY *et al.* Expression and regulation mechanisms of Sonic Hedgehog in breast cancer. *Cancer Sci* 2010; **101**: 927–33.
17 Shostak K, Chariot A. NF-κB, stem cells and breast cancer: the links get stronger. *Breast Cancer Res* 2011; **13**: 214.
18 Prasad S, Ravindran J, Aggarwal BB. NF-kappaB and cancer: how intimate is this relationship. *Mol Cell Biochem* 2010; **336**: 25–37.
19 Kasperczyk H, Baumann B, Debatin KM, Fulda S. Characterization of sonic hedgehog as a novel NF-kappaB target gene that promotes NF-kappaB-mediated apoptosis resistance and tumor growth in vivo. *FASEB J* 2009; **23**: 21–33.
20 Yamasaki A, Kameda C, Xu R *et al.* Nuclear factor kappaB-activated monocytes contribute to pancreatic cancer progression through the production of Shh. *Cancer Immunol Immunother* 2010; **59**: 675–86.
21 Wang TP, Hsu SH, Feng HC, Huang RF. Folate deprivation enhances invasiveness of human colon cancer cells mediated by activation of sonic hedgehog signaling through promoter hypomethylation and cross action with transcription nuclear factor-kappa B pathway. *Carcinogenesis* 2012; **33**: 1158–68.
22 Wang LH, Choi YL, Hua XY *et al.* Increased expression of sonic hedgehog and altered methylation of its promoter region in gastric cancer and its related lesions. *Mod Pathol* 2006; **19**: 675–83.
23 Wang LH, Kim SH, Lee JH *et al.* Inactivation of SMAD4 tumor suppressor gene during gastric carcinoma progression. *Clin Cancer Res* 2007; **13**: 102–10.
24 Giri PK, Jing-Song F, Shanmugam MK *et al.* NMR structure of *Carcinoscorpius rotundicauda* thioredoxin-related protein 16 and its role in regulating transcription factor NF-κB activity. *J Biol Chem* 2012; **287**: 29417–28.
25 Wang LH, Chen GL, Chen K *et al.* Dual targeting of retinoid X receptor and histone deacetylase with DW22 as a novel antitumor approach. *Oncotarget* 2015; **6**: 9740–55.
26 Rodríguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med* 2011; **17**: 330–9.
27 Berdasco M, Esteller M. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. *Dev Cell* 2010; **19**: 698–711.
28 James SR, Cedeno CD, Sharma A *et al.* DNA methylation and nucleosome occupancy regulate the cancer germline antigen gene MAGEA11. *Epigenetics* 2013; **8**: 849–63.
29 Ning H, Mitsui H, Wang CQ *et al.* Identification of anaplastic lymphoma kinase as a potential therapeutic target in Basal Cell Carcinoma. *Oncotarget* 2013; **4**: 2237–48.
30 Hsu YT, Gu F, Huang YW *et al.* Promoter hypomethylation of EpCAM-regulated bone morphogenetic protein gene family in recurrent endometrial cancer. *Clin Cancer Res* 2013; **19**: 6272–85.
31 Baba Y, Watanabe M, Murata A *et al.* LINE-1 hypomethylation, DNA copy number alterations, and CDK6 amplification in esophageal squamous cell carcinoma. *Clin Cancer Res* 2014; **20**: 1114–24.
32 Haller F, Zhang JD, Moskalev EA *et al.* Combined DNA methylation and gene expression profiling in gastrointestinal stromal tumors reveals hypomethylation of SPP1 as an independent prognostic factor. *Int J Cancer* 2015; **136**: 1013–23.
33 Stefansson OA, Esteller M. Epigenetic modifications in breast cancer and their role in personalized medicine. *Am J Pathol* 2013; **183**: 1052–63.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. The effects of Shh overexpression, TNF-α, 5-Aza, cyclopamine on cell proliferation of breast cancer. MDA-MB-231 cell proliferation was measured by MTT method after different agents (5-Aza, pCMV-Shh, TNF-α and cyclopamine) were treated for 48 h.

Table S1. Clinicopathological parameters in breast cancer patients.

Table S2. Primer sequence of Shh promoter construct.