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Integrated analysis of DNA methylation and mRNA expression profiling reveals candidate genes associated with cisplatin resistance in non-small cell lung cancer

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Abbreviations: 5-Aza-dC, 5-aza-2′-deoxycytidine; BSP, bisulphite sequencing; DNMT, DNA methyltransferase; GAS1, growth arrest-specific gene 1; HDAC, histone deacetylase; NSCLC, non-small cell lung cancer; PMR, percentage of methylation reference; TSA, trichostatin A

DNA methylation plays a critical role during the development of acquired chemoresistance. The aim of this study was to identify candidate DNA methylation drivers of cisplatin (DDP) resistance in non-small cell lung cancer (NSCLC). The A549/DDP cell line was established by continuous exposure of A549 cells to increasing concentrations of DDP. Gene expression and methylation profiling were determined by high-throughput microarrays. Relationship of methylation status and DDP response was validated in primary tumor cell culture and the Cancer Genome Atlas (TCGA) samples. Cell proliferation, apoptosis, cell cycle, and response to DDP were determined in vitro and in vivo. A total of 372 genes showed hypermethylation and downregulation in A549/DDP cells, and these genes were involved in most fundamental biological processes. Ten candidate genes (*S100P*, *GDA*, *WISP2*, *LOXL1*, *TIMP4*, *ICAM1*, *CLMP*, *HSP8*, *GAS1*, *BMP2*) were selected, and exhibited varying degrees of association with DDP resistance. Low dose combination of 5-aza-2′-deoxycytidine (5-Aza-dC) and trichostatin A (TSA) reversed drug resistance of A549/DDP cells in vitro and in vivo, along with demethylation and restoration of expression of candidate genes (*GAS1*, *TIMP4*, *ICAM1* and *WISP2*). Forced expression of GAS1 in A549/DDP cells by gene transfection contributed to increased sensitivity to DDP, proliferation inhibition, cell cycle arrest, apoptosis enhancement, and in vivo growth retardation. Together, our study demonstrated that a panel of candidate genes downregulated by DNA methylation induced DDP resistance in NSCLC, and showed that epigenetic therapy resensitized cells to DDP.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of overall lung cancers.[1] Platinum-based combination chemotherapy is currently the standard chemotherapy treatment for NSCLC, and cisplatin (DDP) is widely used.[2] However, the response to chemotherapy differs among cancer patients, and the efficacy of DDP treatment is often impaired by the emergence of resistance to the drug.[3] Therefore, a better understanding of the mechanisms of chemoresistance is required and strategies are needed to identify patients who are unlikely to benefit from the treatment.

DDP induces DNA interstrand and intrastrand crosslinks in tumor cells that subsequently inhibit cell replication and transcription. Chemoresistance to DDP is a complex phenotype that involves many cellular processes, including alterations in drug influx or efflux, apoptosis, cell cycle, DNA repair capacity, and other cellular pathways required for proper response to DNA damage.[4] Multiple cellular events, such as DNA methylation, single nucleotide polymorphisms, non-coding RNA, and stem cells, have been reported to be involved in the processes of acquired chemoresistance.[5-9]

DNA methylation is a major epigenetic modification that leads to gene silencing at the transcriptional level. These epigenetic alterations are heritable and play important roles in carcinogenesis and progression. Substantial DNA methylation either

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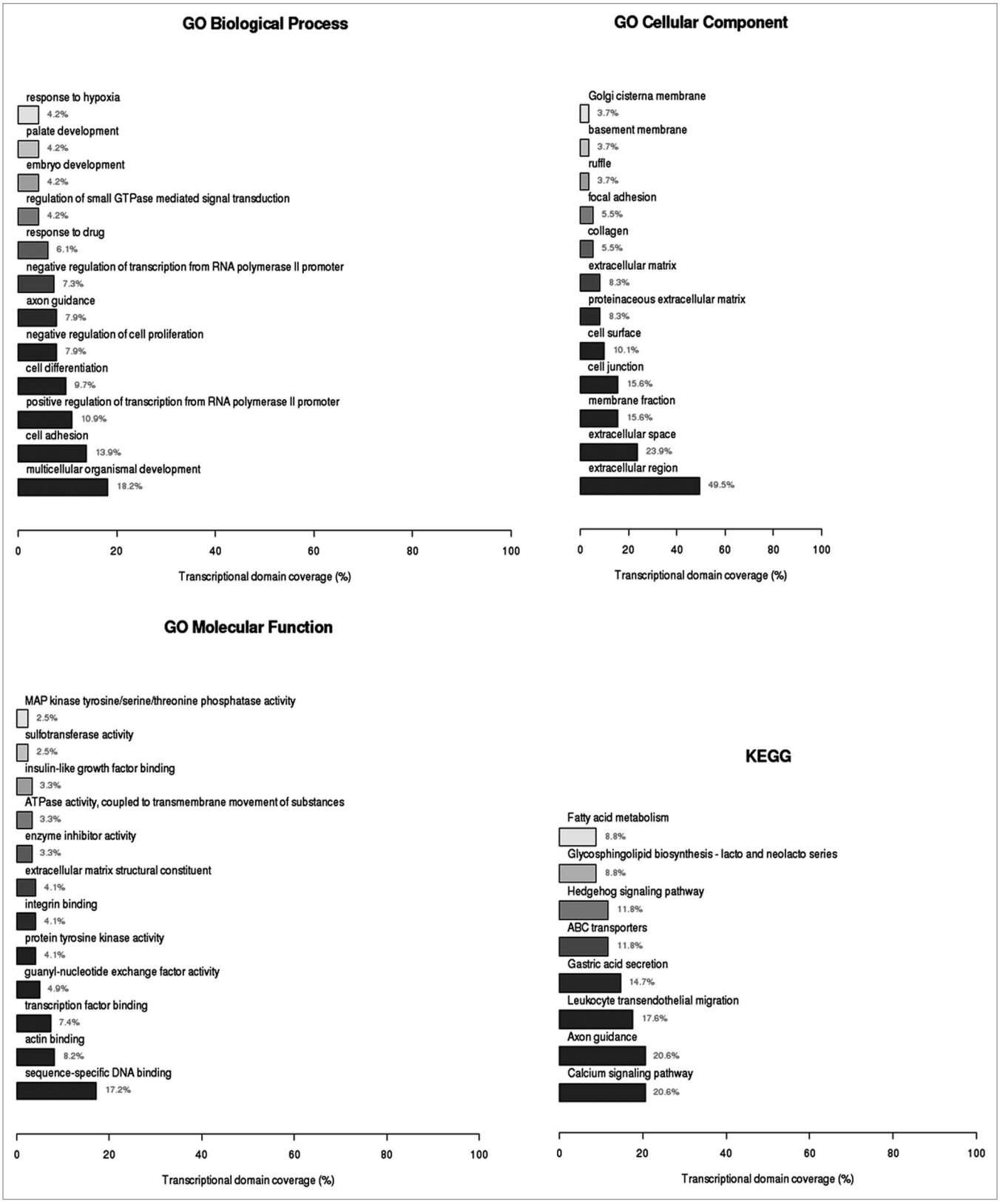

Figure 1. Function and pathway analysis of the 372 hypermethylated genes identified in the A549/DDP cell line. Genes were identified using the Illumina Infinium HumanMethylation450 BeadArray platform. Gene ontology (GO) analysis by three domains: Biological Process (**A**), Cellular Component (**B**) and Molecular Function (**C**). KEGG Pathway analysis (**D**).

alone or in combination with genetic changes during the acquisition of platinum resistance has been widely reported in various cell line models. Previous studies have reported changes in DNA methylation at promoter CpG islands and associated transcriptional gene silencing in DDP-resistant cancer cells.[10-13] However, data in NSCLC has been relatively insufficient.

Unlike genetic mutations, DNA methylation is a reversible process. Therefore, although the mechanisms of demethylation and restoration of expression are not yet fully understood, epigenetic cancer therapy holds great promise for overcoming chemoresistance.[14] In this study, we identified a panel of candidate DNA methylation drivers of DDP resistance in NSCLC. Furthermore, we demonstrated that a combination therapy of epigenetic agents could resensitize cancer cells to DDP.

Results

Gene expression and methylation profiling

To identify possible candidate genes involved in DPP chemoresistance, we conducted gene expression and methylation profiling of the A549/DDP cell line, which was established by continuous exposure of A549 cells to increasing concentrations of DDP, using high-throughput microarrays. We filtered the genes according to the following criteria: expression log fold change A549 vs. A549/DDP > 1.0 and methylation delta_β A549 vs. A549/DDP < –0.7. These criteria identified a total of 372 genes, which covered 861 CpG sites (**Fig. S1**; **Table S1**). These hypermethylated and downregulated genes were considered candidate genes that may play a potential role in DDP resistance.

To identify possible cellular functions of these genes, we performed gene ontology (GO) analysis using three domains: biological process, which includes multicellular organismal development, cell adhesion, positive regulation of transcription from RNA polymerase II promoter, cell differentiation, negative regulation of cell proliferation, and response to drug (**Table S2**); cellular component, mostly involving the extracellular region (**Table S3**); and molecular function (**Table S4**), which is mainly related to sequence-specific DNA binding, actin binding, and transcription factor binding KEGG Pathway analysis included the calcium signaling pathway, ABC transporters and Hedgehog signaling pathway. Four key genes in the Hedgehog signaling pathway (encoding GAS1, BMP2, WNT5A, and WNT6) showed a significant difference between the paired cell lines ($P < 0.05$, **Table S5**; **Fig. 1**).

Validation of the gene expression and methylation status

To verify the results of the microarray, a self-assembling quantitative PCR array was employed to evaluate the differentially expressed genes in A549 and A549/DDP cells. In general, the expression of 62 candidate coding or non-coding genes from the PCR Array was consistent with results from the Agilent microarray. Moreover, 61.3% (38/62) of the candidate genes showed a >5-fold decrease in expression in A549/DDP cells compared with A549 cells (**Table S6**).

Based on the fold-change of gene expression, CpG status (low-CpG was excluded) and the literature, a total of 10 methylated genes (*S100P*, *GDA*, *WISP2*, *LOXL1*, *TIMP4*, *ICAM1*, *CLMP*, *HSP8*, *GAS1*, *BMP2*) detected in A549/DDP cells were selected for further analysis (**Table 1**). qMSP and BSP were used to evaluate their methylation status. All 10 candidate genes were confirmed to be hypermethylated in A549/DDP cells compared with A549 cells (**Fig. 2**). This data directly supports the results of the gene expression and methylation microarray.

Methylation status and DDP response

Next we evaluated the methylation status of the candidate genes in primary tumor samples. NSCLC samples were analyzed by primary tumor cell culture and drug susceptibility testing. Twenty NSCLC samples were identified as DDP sensitive (IC_{50} < 5 mg/L) and 20 samples were considered DDP resistant (IC_{50} > 10 mg/L). We tested the methylation status of the 10 candidate genes in these samples using qMSP. Eight genes (*S100P*, *GDA*, *WISP2*, *LOXL1*, *TIMP4*, *ICAM1*, *HSP8*, and *GAS1*) showed higher methylation levels in DDP-resistant samples compared with DDP-sensitive samples ($P < 0.05$, **Fig. 3A**).

We downloaded and analyzed publicly available data of lung adenocarcinoma and squamous cell carcinoma from the Cancer Genome Atlas Project (TCGA) Project (**Table S7**) and identified 71 patients with a history of cisplatin/carboplatin chemotherapy. Kaplan-Meier analysis showed that hypermethylation of *TIMP4* or *GAS1* was associated with adverse overall survival ($P = 0.024$ and $P = 0.02$, respectively), while *BMP2* methylation showed a protective role in survival ($P = 0.022$). Although no statistical significance was found, *WISP2* methylation tended to be associated with adverse overall survival ($P = 0.116$, **Fig. 3B**).

Epigenetic therapy in vitro

We next assessed the effects of epigenetic agents on A549/DDP cells. Epigenetic agents in experimental analysis were used at the minimum effective dose (**Fig. 4A**), and A549/DDP cells were cultured in RPMI-1640 medium containing 2 mg/L DDP in these experiments. Combinatorial treatments of 1 μM 5-Aza-dC and 100 nM TSA significantly inhibited cellular proliferation, induced G_1 arrest and increased apoptosis of A549/DDP cells compared with either single treatment or untreated cells (**Fig. 4B–E**).

DNA methyltransferase (DNMT) activity and histone deacetylase H3/H4 activity were inhibited by 1 μM 5-Aza-dC and 100 nM TSA, respectively, but combinatorial treatments had no synergistic inhibitory effects on DNMT and H3/H4 activity (**Fig. 4F**). This suggests that the synergistic anti-tumor effects of 5-Aza-dC and TSA might be due to the regulation of key gene expression. This speculation was further confirmed by the fact that hypermethylated status and downregulated expression of GAS1, TIMP4, ICAM1 and WISP2 genes in A549/DDP cells were all reversed after combinatorial treatments (**Fig. 4G and H**).

Epigenetic therapy in vivo

Epigenetic therapy effects were next evaluated in vivo. The doses of epigenetic agents were well tolerated by control mice without causing any serious toxicity, for instance hemorrhage, infection and death (**Fig. 5A**). As shown in **Figure 5B and C**, DDP, 5-Aza-dC or TSA alone did not result in significant suppression of tumor growth ($P = 0.467$, 0.316 and 0.105,

Table 1. Candidate genes selected based on the fold change value in gene expression, CpG status and literature

Gene Symbol	Gene Name	Genomic Coordinates	Log.FC.A549.vs.DDP[1]	FC.A549.vs DDP[2]	The main function
S100P	S100 calcium binding protein P	chr4:6698819–6698878	10.9517	11465.41	Cell cycle progression and differentiation[23,24]
GDA	guanine deaminase	chr9:74764241–74764903	10.10189	21100.65	Microtubule assembly[37]
WISP2	WNT1 inducible signaling pathway protein 2	chr20:43356347–43356406	9.287682	852.17	Downstream of the WNT1 signaling pathway that is relevant to malignant transformation[25,26]
LOXL1	lysyl oxidase-like 1	chr15:74241855–74241914	7.908342	411.57	Formation of crosslinks in collagens and elastin[27,28]
TIMP4	Tissue inhibitor of metallopeptidase 4	chr3:12194787–12194728	7.575363	200.16	Remodeling of the Extracellular matrix[29]
ICAM1	intercellular adhesion molecule 1	chr19:10396298–10396358	7.333666	119.84	cell adhesion[30,31]
CLMP	CXADR-like membrane protein	chr11:122943288–122943229	7.327258	2328.20	A component of epithelial tight junctions[38]
HSPB8	heat shock 22kDa protein 8	chr12:118100934–118101264	7.275802	1704.34	regulation of cell proliferation, apoptosis, and carcinogenesis[32]
GAS1	growth arrest-specific 1	chr9:89559953–89559894	6.892795	175.46	Growth suppression[33,34]
BMP2	bone morphogenetic protein 2	chr20:6760810–6760869	5.316618	55.52	Cell growth, differentiation, migration and invasion[35]

[1]Data from Agilent SurePrint G3 Human Gene Expression microarray; [2]Data from customized PCR array; FC, fold change.

respectively). However, pre-treatment with 5-Aza-dC or TSA followed by DDP caused significant tumor inhibition (>50% compared with control, $P = 0.010$ and 0.005, respectively), while pre-treatment with 5-Aza-dC and TSA fully inhibited tumor growth compared with other groups ($P < 0.001$). Together this data suggests that epigenetic pre-treatment reactivated the genes potentially related to DDP chemosensitivity.

The methylation and expression profiles of *GAS1*, *TIMP4*, *ICAM1* and *WISP2* were determined in each experimental group. As expected, methylation levels were lowest and expression levels were highest in combinatorial pre-treatment groups (**Fig. 5D and E**).

GAS1 function in DDP chemoresistance

To examine the association between candidate gene expression and DDP chemosensitivity, A549/DDP cells were stably transfected with the N-eGFP-GAS1 vector. Real-time-PCR and western blot analyses confirmed that the expression of GAS1 was significantly increased in A549/DDP cells ($P < 0.001$), but still lower than the level in parental A549 cells (**Fig. 6A and B**).

Next, MTT assay was used to determine chemosensitivity of cells overexpressing GAS1 to DDP. Compared with mock-transfected ones, the IC_{50} values for DDP in GAS1-transfected A549/DDP cells was significantly reduced by 66.8% (10.45 ± 0.95 mg/L vs. 3.27 ± 0.48 mg/L, respectively) ($P < 0.001$, **Fig. 6C**). Colony formation assay results showed that upregulation of GAS1 led decreased proliferation of A549/DDP cells ($P < 0.001$, **Fig. 6D**). Flow cytometry assay showed that enforced expression of GAS1 in A549/DDP cells resulted in cell cycle arrest in G_1 phase ($P = 0.011$, **Fig. 6E**) and a dramatic increase of apoptosis ($P = 0.001$, **Fig. 6F**). Morphological features of apoptotic cells and necrosis were also observed in GAS1-transfected A549/DDP cells (**Fig. 6G**).

A549/DDP cells transfected with GAS1-expressing or mock plasmid were injected subcutaneously into nude mice, followed by DDP treatment. As shown in **Figure 6H**, tumors derived from GAS1-transfected A549/DDP cells grew more slowly compared with controls. All mice were sacrificed 2 wk after the transplantation, and the average size of tumors derived from GAS1-transfected A549/DDP cells was significantly reduced by 53.3% ($P = 0.041$, **Fig. 6I**). These results suggested that GAS1 downregulation by methylation was associated with DDP chemoresistance.

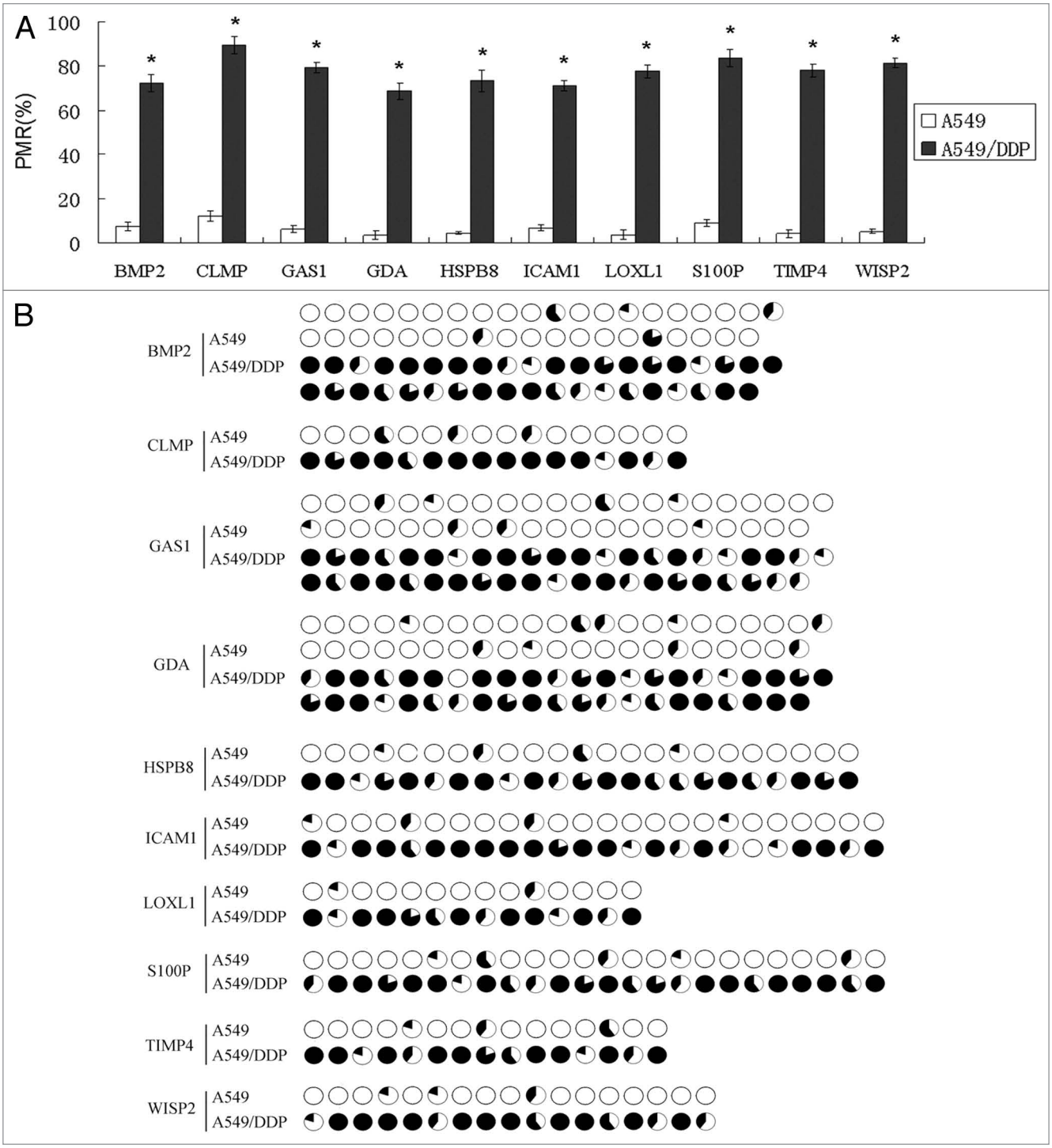

Figure 2. Validation of the methylation status by qMSP (**A**) and BSP (**B**). All 10 candidate genes were confirmed to be hypermethylated in A549/DDP cells compared with A549 cells. The amount of methylated DNA was determined by the threshold cycle number (Ct) for each sample and assessed as the percentage of methylation reference (PMR). Each pie in BSP results represented a CpG site; methylation (black) and unmethylation (white) ratios were calculated according to the five clones.

Discussion

DNA methylation at CpG islands within or near promoter regions has been demonstrated as an important epigenetic regulatory mechanism of gene expression. Hypermethylation could lead to an alteration of the chromatin framework, thereby directly repressing transcription and leading to the downregulation or silencing of tumor suppressor genes, thus contributing to cancer initiation and progression.[15] Previous studies have demonstrated an association between abnormal methylation and cancer

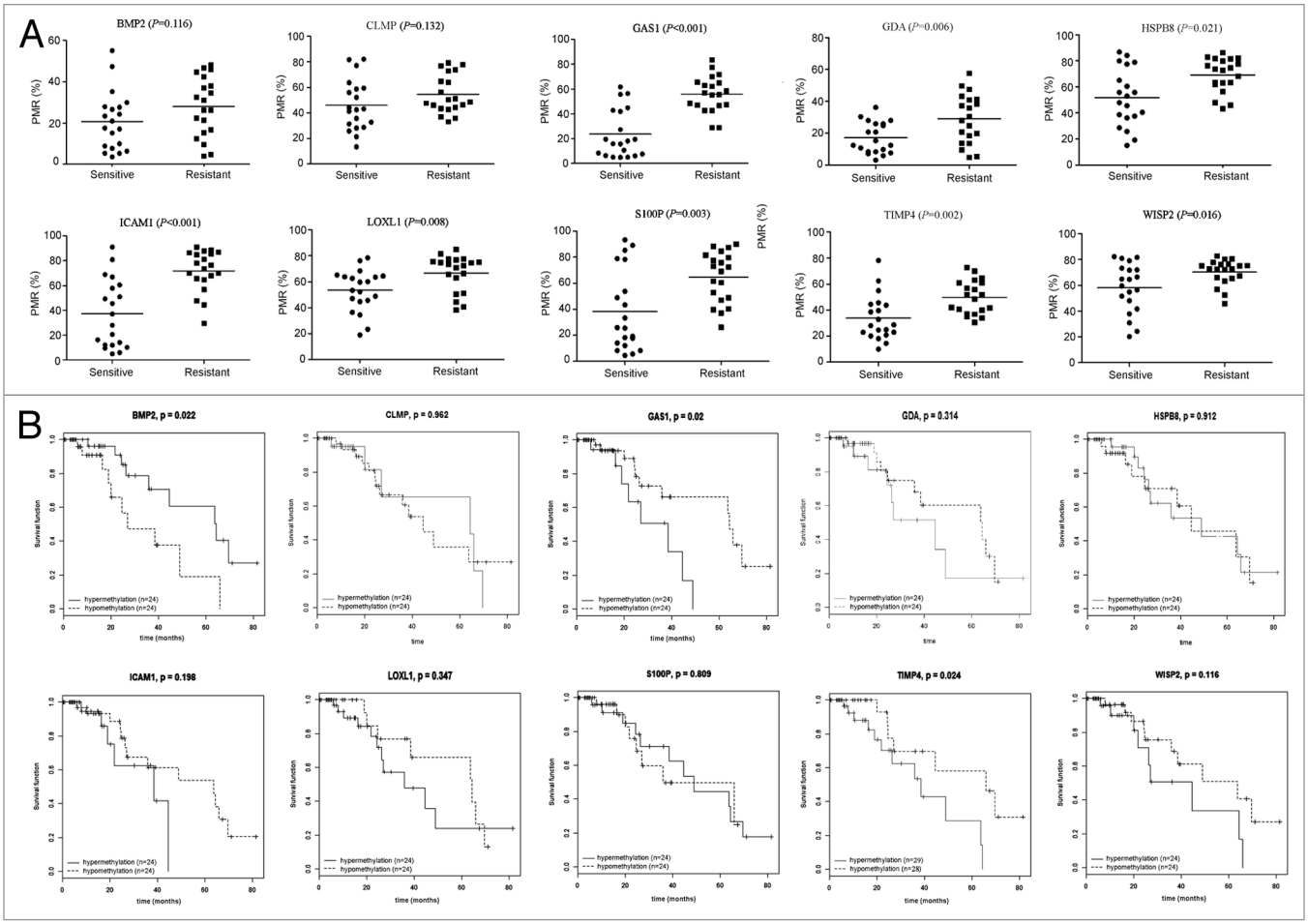

Figure 3. Methylation status and DDP response. (**A**) By primary tumor cell culture and drug susceptibility testing, 20 NSCLC samples were considered DDP sensitive (IC_{50} < 5 mg/L) and 20 samples were considered DDP resistant (IC_{50} > 10 mg/L). Methylation status of 10 candidate genes was tested by qMSP; (**B**) Kaplan-Meier analysis of candidate gene methylation and overall survival in patients who received platinum-based chemotherapy using TCGA data. Hyper- or hypo-methylation was defined by the median of methylation index β-values.

diagnosis or prognosis in various cancers.[16] In addition, DNA methylation plays a critical role in the development of acquired chemoresistance via transcriptional inactivation of several key genes. Clinically, colorectal cancers with hMLH1 methylation are less aggressive, but nonreactive to 5-Fu.[17] The correlations between CHFR methylation and sensitivity to microtubule inhibitors,[18] and MGMT gene methylation and response to alkylating agents in patients with glioblastoma[19] have also been confirmed.

In this study, integrated analysis of DNA methylation and gene expression in a genome-wide profiling revealed specific methylated and silenced genes associated with DDP resistance in a lung adenocarcinoma cell line. GO analysis showed these 372 genes are involved in nearly all the fundamental biological processes, such as multicellular organismal development, cell adhesion, positive regulation of transcription from RNA polymerase II promoter, cell differentiation, negative regulation of cell proliferation, and drug response. KEGG pathway analysis revealed functions in the calcium signaling pathway, ABC transporters and Hedgehog signaling pathway. Except for a few genes (such as *S100P*), our results were not fully consistent with previously reports, which identified p16, hMLH1, RASSF1A, and IGFBP3 methylation, Wnt, JNK/p38 MAPK, p53 and their signaling pathways in DDP resistance.[20-22] The differences may be due to different cell lines and microarray analysis approaches used. Furthermore, as chemoresistance processes involve multiple major cellular pathways, it is likely that the underlying mechanisms are broad and complex, and thus each of these studies only provides a narrow glimpse of an intricate regulatory network.

In this study, our gene microarray results were verified by a low-throughput, self-assembling quantitative PCR array. Ten genes (*S100P*, *GDA*, *WISP2*, *LOXL1*, *TIMP4*, *ICAM1*, *CLMP*, *HSP8*, *GAS1*, *BMP2*) were initially screened for analysis, based on fold-change of gene expression, CpG status and literature. These genes were involved in the regulation of cell growth, cell cycle, differentiation, apoptosis, adhesion, migration and tumor microenvironment.[23-36] Except *GDA37* and *CLMP*,[38] the other genes have been proven to have important significance in tumor development, and many have critical roles in drug resistance.[39-42] For example, S100P, a 95 amino acid protein, acts as an autocrine

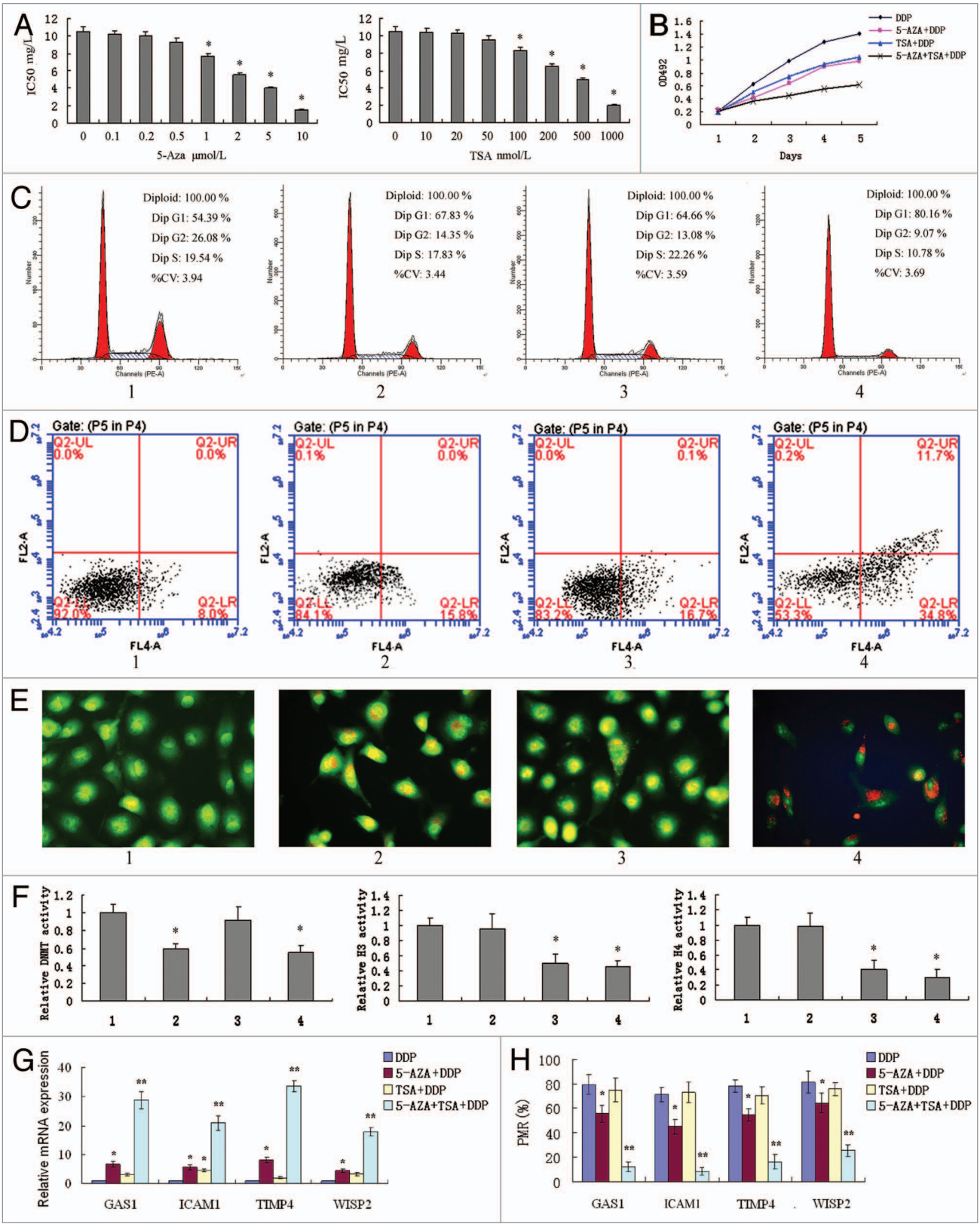

Figure 4. In vitro effects of the combinatorial treatments with 5-Aza-dC and TSA in the A549/DDP cell line. (**A**) Minimum effective dose of 5-Aza-dC and TSA determined by MTT; (**B**) cell proliferation determined by MTT; (**C**) cell cycle determined by flow cytometry; (**D**) apoptosis determined by flow cytometry; (**E**) apoptosis determined by fluorescence microscope; (**F**) DNMT activity and histone deacetylase H3/H4 activity; (**G**) mRNA expression of four candidate genes; (**H**) methylation status of four candidate genes. 1, A549/DDP cells; 2, A549/DDP cells treated with 1 μM 5-Aza-dC; 3, A549/DDP cells treated with 100 nM TSA; 4, A549/DDP cells treated with 1 μM 5-Aza-dC and 100 nM TSA. Cells were cultured in RPMI-1640 medium containing 2 mg/L DDP in these experiments. *$P < 0.05$ vs control; **$P < 0.05$ vs. other 3 groups.

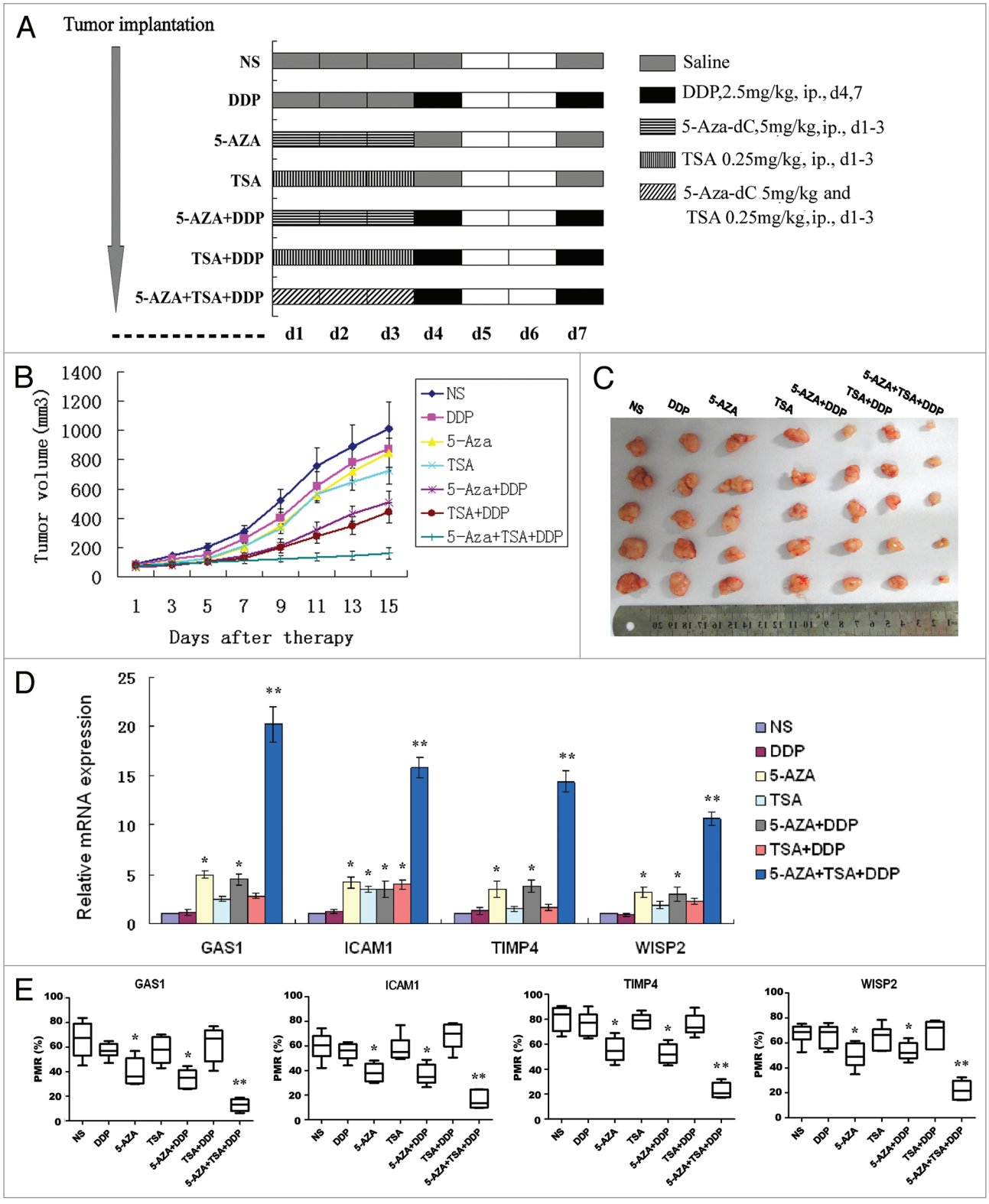

Figure 5. Epigenetic therapy in vivo. A549/DDP cells (2×10^6/100 μL PBS) were subcutaneously inoculated into the right flank of BALB/c nu/nu mice and animals were randomly divided into 7 treatment groups as described in Methods (**A**). The tumor size was monitored every other day (**B**). Mice were sacrificed and the tumors were isolated after two weeks (**C**). The mRNA expression (**D**) and methylation (**E**) profiling of GAS1, TIMP4, ICAM1 and WISP2 were determined in each group. *$P < 0.05$ vs. NS group; **$P < 0.05$ vs. other 6 groups.

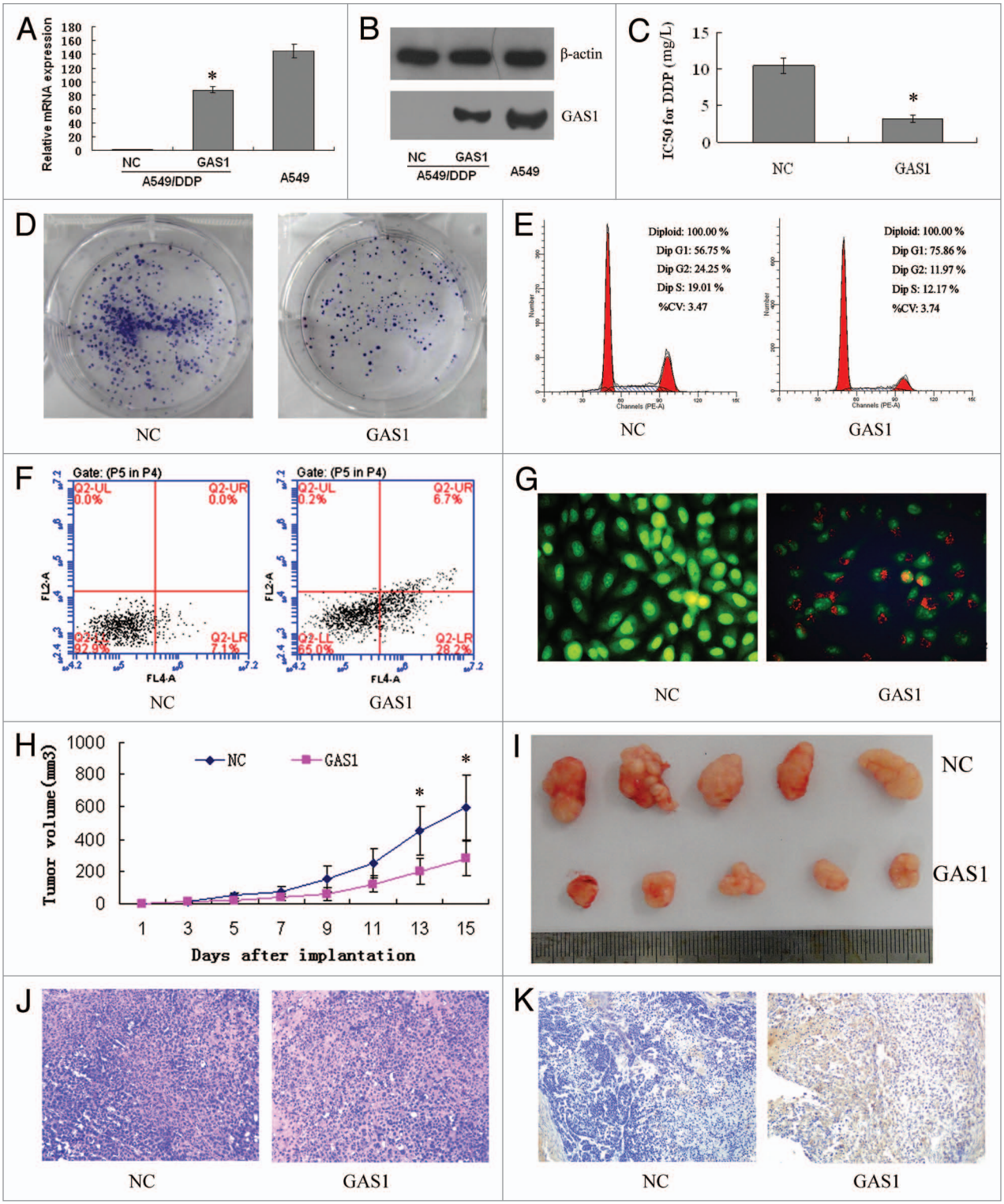

Figure 6. Verification of GAS1 function. A549/DDP cells were stably transfected with N-eGFP-NC or N-eGFP-GAS1 vector, and GAS1 expression levels were detected via real-time PCR (**A**) and western blot (**B**) 48 h post-transfection. (**C**) MTT assay revealed that cells with upregulated GAS1 expression were more sensitive to DDP. (**D**) Colony formation assay revealed suppression of cellular proliferation with upregulated GAS1 expression. (**E**) Flow cytometric analysis of cell cycle indicated that upregulation of GAS1 significantly induced G1 phase arrest. Flow cytometric analysis (**F**) and fluorescence microscope (**G**) indicated that upregulation of GAS1 significantly induced apoptosis. (**H**) Growth curve of tumors derived from GAS1-transfected A549/DDP cells and NC cells. Each point represents the mean ± SD of 5 mice. (**I**) Representative photographs of tumors formed 2 wk after the subcutaneous transplantation. Transplanted tumors with H&E staining (**J**), and immunohistochemistry of GAS1 (**K**).

growth and survival factor. Decreased *S100P* expression was observed in at least five platinum-resistant cell lines, including one uterine cervix cancer, one liver cancer, one colon cancer and two bladder cancer cell lines.[12] All 10 candidate genes were confirmed to be hypermethylated in A549/DDP cells compared with parental A549 cells. Relationships of methylation status and DDP response were further validated in primary tumor cells and samples from TCGA patients who underwent platinum-based chemotherapy, and four genes (*GAS1*, *TIMP4*, *ICAM1,* and *WISP2*) were selected for subsequent analysis. Our results confirm that hypermethylation of these genes involved in DDP resistance in NSCLC. Further research is needed to explore the mechanisms underlying their contribution to chemoresistance.

As DDP resistance was associated with downregulation of key genes by DNA methylation in NSCLC, we speculated whether epigenetic therapy could reverse DDP resistance. DNMT inhibitors can effectively induce DNA demethylation and phenotypic changes related to the reactivation of epigenetically silenced genes. These findings were later adapted to the targeting of epigenetic mutations in cancer and brought about the fundamental concept of epigenetic cancer therapy.[43] In addition to DNMT inhibitors,[44] histone deacetylase (HDAC) inhibitors have also contributed to shape epigenetic cancer therapy.[45] In solid tumors, additional DNMT and HDAC inhibitors are currently being evaluated in both preclinical studies and clinical trials. However, a direct link between DNA demethylation and clinical responses has not yet been demonstrated, and currently there are no established DNA methylation biomarkers that accurately predict patient response. It is important to note that histone deacetylation has been shown to synergistically interact with DNA methylation in the epigenetic silencing of cancer genes.[46] This observation has also provided the scientific rationale for various clinical trials with combinations of DNMT and HDAC inhibitors. Somewhat surprisingly, these studies have yet to reveal synergistic clinical effects, which might again be related to complexities in the drugs' modes of action that are only beginning to become elucidated.

Therefore, epigenetic cancer therapy combined with chemotherapy may be a direction worth exploring. Combining 5-Aza-dC with cytotoxic drugs has been found to increase sensitivity of neuroblastoma cells to chemotherapeutic drugs.[47] In a phase II clinical trial, low-dose decitabine altered DNA methylation of genes and cancer pathways, restoring sensitivity to carboplatin in patients with heavily pretreated ovarian cancer and resulting in a high response rate and prolonged progression-free survival.[48] In this study, pretreatment of low-dose 5-Aza-dC and TSA significantly increased DDP chemosensitivity of resistant cells both in vitro and in vivo. Re-expression of key genes, such as *GAS1*, *TIMP4*, *ICAM1,* and *WISP2*, was considered to account for the underlying mechanisms. However, global demethylation does not only affect cancer-specific genes but also affects some developmental genes.[49] Moreover, epigenetic side effects could severely curtail the specificity of therapy.[50] Dose limitation may be an effective way to reduce side effects, while a combination of low-dose epigenetic agents improves the efficacy. The combined modes, dose of epigenetic agents, and cytotoxic drugs still need further study to achieve optimal therapeutic effect.

To directly verify candidate DNA methylation drivers of DDP resistance in NSCLC, the effect of GAS1 transfection on A549/DDP cells chemosensitivity was examined. Forced expression of GAS1 in A549/DDP cells by gene transfection contributed to increased sensitivity to DDP, proliferation inhibition, cell cycle arrest, apoptosis enhancement, and in vivo growth retardation. Thus, GAS1 downregulation by methylation had a close relationship with DDP chemoresistance. GAS1, a glycosylphosphatidyl inositol-linked protein, was originally identified as a protein involved in the contact inhibition of fibroblasts.[51] As a tumor suppressor gene, it is directly related to cell cycle arrest in the G_0 to S phase transition.[52] A previous study also indicated that GAS1 was an epirubicin resistance-related gene in gastric cancer cells because upregulation of GAS1 reversed the chemoresistance of gastric cancer. GAS1 is considered to enhance hedgehog signaling activity,[53] activate p38MAPK[54] and prevent the activation of Akt,[55,56] all of which are crucial mediators of survival and cellular proliferation pathways.

Taken together, we identified a panel of candidate genes downregulated by DNA methylation and demonstrated that these genes induced DDP resistance in NSCLC. Epigenetic therapy resensitized cells to DDP both in vitro and in vivo, along with the demethylation and restoration of expression of candidate genes. Finally, we proved that overexpression of GAS1 directly affected the DDP chemosensitivity of resistant cells. Our study enhanced the understanding of DDP resistance mechanisms and contributed to developments for treatment for NSCLC.

Materials and Methods

Cell culture

The A549 human lung adenocarcinoma cell line was purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Cell Resource Center. The DDP-resistant A549/DDP variant cell line was established by continuous exposure to increasing concentrations of DDP for a 10-mo period following the methodology described previously (**Fig. S2**).[13,57] A549 and A549/DDP cell lines were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal calf serum (Gibco) in a humid atmosphere containing 5% CO_2 at 37 °C. The A549/DDP cells medium additionally contained 2 mg/L DDP to maintain the cells' drug-resistant phenotype. Cells in the logarithmic phase of growth were used for all experiments. For treatment with 5-aza-2′-deoxycytidine (5-Aza-dC, Sigma), a specific DNA methyltransferase inhibitor, or trichostatin A (TSA, Sigma), a specific histone deacetylase inhibitor, the cell culture medium was changed every 24 h.

Methylome analysis

DNA methylation was assessed using the Illumina Infinium HumanMethylation450 BeadArray platform, as previously described.[58] DNA methylation index (MI) is reported as β-values, calculated from mean methylated (M) and unmethylated (U) signal intensities for each locus for each sample using the formula (β = M/[M + U]).

Gene expression microarrays

The cDNA from cell lines was labeled with Cy3 dye and hybridized to Agilent SurePrint G3 Human Gene Expression microarrays (8?60K) to assess gene expression according to the manufacturer's protocol (Agilent Technologies). Fluorescence images of the hybridized arrays were generated using the Agilent DNA Microarray Scanner, and the intensities were extracted with Agilent Feature Extraction software ver.10.7.3.1. The fold change was log2 transformed.

PCR array

This study used a customized PCR array from CT Bioscience[59] to compare the expression profile of a selected group of genes in A549 and A549/DDP cells. Sixty-two potential genes based on methylation and expression microarray were selected as the target mRNAs. *GAPDH*, β2-MG, *ACTB*, *RPL27*, *HPRT1,* and *OAZ1* were used as housekeeping genes for normalization. After RNA isolation, 1 μg of total RNA was used for reverse transcription using an RT kit (CT Bioscience, Cat # CTB101) in a 20 μL volume. The PCR array employs SYBR Green I-based real-time PCR to quantify gene expression level. Gene specific primers are pre-deposited into wells of a 96-well PCR plate in the array.

Culture and DDP treatment of NSCLC tissues

To measure the sensitivity of the primary NSCLC samples to DDP, fresh tumors were minced, passed through a nylon mesh and enzyme disaggregated with collagenase type II and hyaluronidase in Dulbecco's modified Eagle's medium nutrient mixture F-12/Ham media (Sigma Aldrich) with antibiotics. Cells were resuspended in 96-well microtiter plates followed by exposure to various concentrations of DDP as described previously.[13]

Validation in the cancer genome atlas samples

To verify associations of methylation observed in microarray with overall survival in NSCLC patients, we downloaded and analyzed data publicly available from the Cancer Genome Atlas Project (TCGA; http://tcga-data.nci.nih.gov/). Patients who received platinum-based chemotherapy were selected. Survival curves were calculated using the Kaplan-Meier method and compared by log-rank testing. Hyper- or hypo-methylation was defined by the median of methylation index β-values.

Real-time quantitative PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription reaction was performed using 2 μg of total RNA with a first strand cDNA kit (Takara). PCR amplification was performed for 20 s at 95 °C, followed by 40 cycles at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s in ABI 7300 Thermocycler (Applied Biosystems), using the SYBR Premix Ex Taq kit (Takara). The specific primer sequences for each gene were listed in **Table S8**. Data analysis was done using the $2^{-\Delta\Delta CT}$ method for relative quantiðcation, and all samples were normalized to GAPDH.

Detection of methylation status

After genomic DNA extraction and spectrophotometric quantization, 1 μg of genomic DNA was bisulfite-treated with the EZ-DNA methylation Gold Kit (Zymo Research). Quantitative methylation-specific PCR (qMSP) was then performed in an ABI 7300 Thermocycler, using the SYBR Premix Ex Taq kit (Takara) as described previously.[60] The specific primer sequences for each gene were designed by Methyl Primer Express® Software v1.0 and are listed in **Table S8**. β-actin (ACTB) was used to normalize for the input DNA. The amount of methylated DNA was determined by the threshold cycle number (Ct) for each sample and assessed as the percentage of methylation reference (PMR) using the formula: $[(\text{gene/ ACTB})^{\text{sample}}: (\text{gene/ ACTB})^{\text{SssI-treated genomic DNA}}] \times 100$. Samples with ≥4% of PMR were regarded as hypermethylated. For bisulphite sequencing (BSP), the bisulphite DNA was amplified by PCR with BSP primers and PCR products were cloned into the pUC57 vector (Genscript). Five clones were selected and sequenced for each sample.

MTT assay for cell growth inhibition

A549/DDP cells (cultured in RPMI-1640 medium containing 2 mg/L DDP) were seeded at a density of 2×10^3 cells in each well of the 96-well plates. 5-Aza-dC (1 μM) and/or TSA (100 nM) were added to the wells for 24, 48 and 72 h. MTT (5 g/L, 20 μL/well) was added to each well and incubated at 37 °C for 4 h. DMSO was then added (150 μL/well) to each well to dissolve any crystals and the plates were agitated for 10 min. Absorbance values at 492 nm were detected by the microplate reader.

Cell viability assay

A549/DDP cells (cultured in RPMI-1640 medium without DDP) were seeded into 96-well plates (2×10^3 cells/well) 24 h after drug treatment or transfection and allowed to attach overnight. Freshly prepared DDP was then added at different final concentrations for 48 h. Cell viability was assessed via a standard MTT assay and IC_{50} was calculated. All assays were performed in quintuplicate and repeated at least three times.

Transfection of cells

GAS1 gene-expressing plasmid (N-eGFP-GAS1) and the mock N-eGFP-negative control (N-eGFP-NC) were purchased from GenePharma. All plasmid DNA was extracted using a Plasmid Mini Kit (Qiagen). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. G418 (600 mg/L, Sigma) was used to screen the stably transfected cells. GAS1 expression was confirmed by real-time PCR and western blotting.

Flow cytometric analysis

Cells were harvested directly or 48 h after drug treatment or transfection and washed with ice-cold phosphate-buffered saline (PBS). The PI/RNase staining kits (Multisciences) and annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (KeyGEN Biotech) were used to detect cell cycle and apoptosis in a FACScan instrument (Becton Dickinson), respectively.

Fluorescence microscope

For drug treatment, A549/DDP cells (cultured in RPMI-1640 medium containing 2 mg/L DDP) were seeded at 5×10^4 in each well of 24-well plates, and 5-Aza-dC (1 μM) and/or TSA (100 nM) were added to the wells for 48 h. For functional analysis, A549/DDP cells transfected with GAS1 gene-expressing or mock plasmid were seeded in 24-well plates and cultured in RPMI 1640 media without DDP for 48 h. Then cells were harvested and suspended in PBS containing fluorescence dye

AO/EB (both AO and EB were at the concentration of 100 mg/L in PBS), following by preparation for slides. The morphology of the cells was observed under a fluorescence microscope (IX71, Olympus) and photographed.

Colony formation assay

A total of 1000 cells were seeded into six-well plates with 2 mL culture medium. After culturing in RPMI 1640 media supplemented with 10% FBS at 37 °C and 5% CO_2 for 14 d, cells were washed twice with PBS, fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. The cloning efficiency (%) = (the number of clones/the number of seed cells) × 100%.

In vivo experiments

The animal study protocol was approved by the Animal Experimentation Ethics Committee of the Jinling Hospital. For epigenetic therapy, A549/DDP cells (2 × 10^6/100 μL PBS) were subcutaneously inoculated into the right flank of BALB/c nu/nu mice. When the average tumor size reached approximately 50 mm^3, animals were randomly divided into 7 groups, with five in each group, and subjected to the corresponding treatment. The epigenetic therapy used in this study was 5-Aza-dC 5 mg/kg and/or TSA 0.25 mg/kg, ip, d1–3. DDP chemotherapy regimen was 2.5 mg/kg, ip, twice per week. Combinatorial treatments were performed by pre-treatment with 5-Aza-dC and/or TSA for 3 d and then DDP was added on the 4th and 7th day. The experimental groups were as follows: (1) normal saline (NS); (2) DDP alone; (3) 5-Aza-dC alone; (4) TSA alone; (5) pre-treatment with 5-Aza-dC followed by DDP; (6) pre-treatment with TSA followed by DDP; (6) pre-treatment with 5-Aza-dC and TSA followed by DDP. Tumor volume was estimated every other day by the formula: 0.5 × length × $width^2$.

For GAS1 function, A549/DDP cells (2 × 10^6/100 μL PBS) transfected with GAS1 gene-expressing or mock plasmid were injected subcutaneously into nude mice on the right flank (n = 5 mice per group). When the average tumor size reached approximately 50 mm^3, DDP was given via intraperitoneal injection at a dose of 2.5 mg/kg, two times a week. All mice were sacrificed after 2 wk. Tumor tissues were excised, paraffin-embedded, and formalin-fixed, and H&E staining was performed. Immunostaining analysis for GAS1 protein was performed according to a previous report.[36]

Statistical analysis

The SPSS 16.0 software system (SPSS) was used for statistical analysis. Data are expressed as the mean ± standard error. The differences between groups were analyzed using a Student t test when only two groups were compared or one-way analysis of variance when more than two groups were compared. All tests performed were two-sided. $P < 0.05$ was taken as statistical significance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/28601

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