The *p16*-Specific Reactivation and Inhibition of Cell Migration Through Demethylation of CpG Islands by Engineered Transcription Factors

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Abstract

Methylation of CpG islands inactivates transcription of tumor suppressor genes including *p16* (*CDKN2A*). Inhibitors of DNA methylation and histone deacetylation are recognized as useful cancer therapeutic chemicals through reactivation of the expression of methylated genes. However, these inhibitors are not target gene-specific, so that they lead to serious side effects as regular cytotoxic chemotherapy agents. To explore the feasibility of methylated gene-specific reactivation by artificial transcription factors, we engineered a set of Sp1-like seven-finger zinc-finger proteins (7ZFPs) targeted to a 21-bp sequence of the *p16* promoter and found that these 7ZFPs could bind specifically to the target *p16* promoter probe. Then the *p16*-specific artificial transcription factors (p16ATFs) were made from these 7ZFPs and the transcription activator VP64. Results showed that transient transfection of some p16ATFs selectively up-regulated the endogenous *p16* expression in the *p16*-active 293T cells. Moreover, the transient transfection of the representative p16ATF-6I specifically reactivated *p16* expression in the *p16*-methylated H1299 and AGS cells pretreated with a nontoxic amount of 5'-aza-deoxycytidine (20 and 80 nM, respectively). In addition, stable transfection of the p16ATF induced demethylation of *p16* CpG island and trimethylation of histone H3K4, and inhibited recruitment of DNA methyltransferase 1 and trimethylation of H3K9 and H3K27 in the *p16* promoter in H1299 cells without 5'-aza-deoxycytidine pretreatment. Notably, inhibition of cell migration and invasion was observed in these *p16*-reactivated cells induced by transient and stable p16ATF transfection. These results demonstrate that p16ATF not only specifically reactivates *p16* expression through demethylation of CpG islands, but also restores methylated *p16* function.

Introduction

ALTERATIONS OF DNA methylation have been recognized as important mechanisms of cancer development. Hypermethylation typically occurs at CpG islands in the promoter region and associates with epigenetic gene inactivation. It may become a molecular target for cancer therapy (Jaenisch and Bird, 2003). Inactivation of *p16* (*CDKN2A*) by CpG methylation is one of the most frequent events in the development of many cancers (Herman *et al.*, 1995; Sun *et al.*, 2004; Jin *et al.*, 2009). Epithelial precancerous lesions containing methylated *p16* have a much higher risk of malignant transformation than the negative ones in follow-up studies (Hall *et al.*, 2008; Cao *et al.*, 2009). Inhibitors of DNA methylation and histone deacetylation are recognized as useful cancer therapeutic agents through reactivation of the methylated

genes. However, these inhibitors are not target gene-specific, so that they lead to serious side effects as common cytotoxic chemotherapy agents. Selective reactivation of methylation-silenced genes by artificial transcription factors (ATFs) may be an optimal strategy for treatment of human diseases (Beltran *et al.*, 2007).

Cys₂His₂-type zinc-finger proteins (ZFPs) can bind to specific DNA fragments based on its amino acid sequence within the α -helix of each zinc finger (Pavletich and Pabo, 1991; Moore *et al.*, 2001). A simple mode of DNA recognition by zinc-finger domains makes it possible to design artificial ZFPs with novel sequence specificities (Sera and Uranga, 2002; Carroll *et al.*, 2006; Sander *et al.*, 2007). However, the construction of engineered ZFPs was not always successful (Ramirez *et al.*, 2008), although the interaction model of zinc finger-DNA became increasingly clear. Regarding the

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specificity of a single DNA sequence in the genome and the fidelity of the ZFP-DNA recognition, which is lower than that of the DNA-DNA recognition, seven-zinc-finger proteins (7ZFPs) may achieve gene specificity in the human genome. Moreover, the use of a scaffold of natural DNA-binding ZFP expressed in mammalian cells may assure the accessibility of artificial ZFPs. It is well known that the transcription factor Sp1 binds to a 9-bp GC box in the promoter regions through a three-zinc-finger domain (Narayan *et al.*, 1997). Therefore, in the present study, the natural fragment of the three zinc fingers in Sp1 (Sp1-3ZF) and two designed two-zinc-finger domains based on the Sp1 finger 2 and 3 scaffolds were used to construct the 7ZFP candidates (Fig. 1A and B). We constructed a number of *p16*-specific ATFs (p16ATFs) through the fusion of 7ZFPs with the transcription activator VP64, and then studied their binding affinity to the *p16* promoter and the effects of transient and stable transfection of these p16ATFs on the reactivation of methylated *p16* through demethylation of CpG islands. The synergic effect between p16ATF and DNA methylation inhibitor 5'-aza-deoxycytidine (DAC), the changes of histone

modifications and recruitment of DNA methyltransferase 1 (DNMT1) in the *p16* promoter, and function restoration of methylated *p16* were also investigated.

Materials and Methods

Construction of the *p16*-specific 7ZFP vectors

The 21-bp *p16* promoter fragment including an Sp1-binding site (5'-GAG GAA GGA AAC GGG GCG GGG-3'; Fig. 1A) was used as the target DNA to design 7ZFPs. These 7ZFPs were engineered with the natural Sp1-3ZF module as the N-terminal domain and two kinds of Sp1-like artificial two-zinc-finger modules (*mut1*-6 and *mutA*-I) as the middle domain and C-terminal domain (Fig. 1B). The Sp1-3ZF module was amplified from the human *Sp1* mRNA in the HeLa cell line by reversed transcription PCR (RT-PCR) with the forward primer containing a *Kpn*I and an *Nco*I restriction site (5'-c ggt acc gcc acc atg gat cct ggc aaa aag-3') and the reverse primer containing an *Xho*I and an *Age*I restriction site (5'-gg ctc gag acc ggt gtg ggt ctt gat atg-3'). Two kinds of Sp1-like modules, *mut1*-6 and *mutA*-I, were designed

FIG. 1. Construction and detection of affinity of 7ZFPs. (A) Illustration of the *p16* promoter. CpG sites in the sequence are labeled as gray bars; the long bold line represents the fragment of the *p16* promoter for the reporter assay, and the short bold lines represent locations of the 7ZFP-binding target or probes for EMSA. (B) The 21-bp 7ZFP target *p16* sequence and the corresponding zinc-finger modules within the designed 7ZFPs. (C) Workflow of the synthesis of the two-zinc-finger modules by overlap PCR using various oligo DNAs (see Supplementary Tables S1 and S2). (D) Analysis of binding activity and specificity of 7ZFP to the target *p16* fragment with competitive EMSA. (E) Comparison of binding affinity and specificity of 7ZFP to the unmethylated "probe-1" and methylated "Mprobe-1" in competitive EMSA.

according to the published natural or artificial zinc-finger sequences and the mode of DNA-ZFP recognition (Sera and Uranga, 2002). According to the amino acid sequence of a designed module (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/hum), these oligos containing the coding sequence of key amino acids targeted to the triplet sequences were synthesized and overlapped complementarily at last 15bp with each other (Supplementary Table S2). The two-zinc-finger modules were amplified from these overlapped oligos using Primer-F containing an *Xma*I site, and Primer-R containing *Age*I and *Xho*I sites (Fig. 1C, Supplementary Tables S2 and S3). The Sp1-3ZF module (the finger F1–F3), Sp1-like modules *mut1*–6 (the finger F4 and F5), and *mutA*–I (the finger F6 and F7) were ligated subsequently by two restriction enzymes, *Xma*I and *Age*I. The 7ZFPs were cloned into the optimized pET-28a vector for induction of prokaryotic expression. The 7ZFP coding sequences were fused with the sequences coding the transcription activator VP64 (VP16, DALDDFDLMDLGS; $\times 4$) and nucleic localization signal (NLS, DPKKKRKV; $\times 3$), and then inserted into pcDNA3.1-*myc*/His-a or pEGFP-N2 vector to construct p16-specific transcription activation factors p16ATFs (see Fig. 2A) (Beerli et al., 1998).

Induction of 7ZFP expression and purification

The BL21 bacteria transformed with these pET28a-7ZFP expression vectors were cultured in LB medium overnight. After the density of the culture was adjusted to 0.5 OD_{600nm} with fresh medium, 7ZFP expression was induced by 0.1 mM IPTG (Genview, USA) for 24 hr at 17°C. The bacteria were pelleted by centrifugation, resuspended in 2 mL of cold PBS/0.1% NP-40 per 10 mL of bacteria culture, and lysed by ultrasonication followed by centrifugation. The soluble 7ZFPs in the sonic supernatant were purified by Ni-NTA-Sepharose beads (GE Healthcare, Germany) and used in an electrophoresis mobility shift assay (EMSA).

Western blot

The primary monoclonal antibody against myc-Tag (GenScript, A00172; China), P16 (Abcam, ab50282; Cambridge, UK), GAPDH (ProteinTech, 60004-1; China), or green fluorescent protein (GFP) (ProteinTech, 50430-2-AP; China) was applied at 1:5,000 and 1:1,000 dilutions. The signals were visualized using the Enhanced Chemiluminescence kit (Pierce, USA).

Electrophoresis mobility shift assay

EMSA was carried out according to the protocol for the Light Shift Chemiluminescent EMSA Kit (Pierce). Five nanomoles of the biotin-labeled probe-1 (or probe-2) was used in the regular EMSA (Fig. 1A, Supplementary Table S4). In the competition assay, 50-, 100-, 200-, and 500-fold excesses of the corresponding unlabeled probes were pre-incubated with the tested proteins for 30 min prior to the addition of the labeled probes. *M.SssI* was used to prepare methylated probe-1 (Mprobe-1) according to the manufacturer's instructions. The probe-2 matched to the p16 5' untranslated region (UTR) and the p16-unrelated probe-3 matched to the *E-cadherin* promoter were also used in the competitive assay.

Dual-luciferase reporter assay

The p16 promoter [–597 nucleotides (nt) to +155 nt] was amplified with the primer set p16-F2/R2 (Supplementary Table S4) and cloned into pGL3-Basic vector (GenBank accession no. U47295; Promega, Madison, WI) by the *Kpn*I and *Xho*I sites, and was named as pGL-p16p. Transient transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) after the human cell line 293T was plated into a 24-well culture plate at a density of 5×10^4 cells/well (three wells per plasmid) and cultured for 24 hr to 80% confluence. Three different plasmids were cotransfected: pGL-p16p, p16ATFs, and pRL-SV40. Statistics were performed using Student's unpaired two-tailed *t* test.

Transfection of mammalian cell lines with the p16ATFs

Human cell lines 293T, H1299, and AGS were obtained from laboratories at Beijing Cancer Hospital/Institute and were cultured in RPMI 1640 or F12 medium with 10% fetal bovine serum at 37°C with 5% CO₂. p16ATFs were transfected into 293T, H1299, and AGS cell lines that were plated into a 24-well culture plate using Lipofectamine 2000. After 48 hr, the cells were collected to analyze the expression of p16 mRNA and protein by quantitative RT-PCR and western blot, respectively. p16ATFs in pEGFP-N2 vector were used in the H1299 cell stable transfection assay. After transfection, the pooled transfected cell line was obtained after treatment with 450 μ g/mL G418 for 3 weeks and sorted for GFP-positive cells by cytoflowmeter (BD, FACSAria, USA).

DAC pretreatment

The H129 and AGS cell lines were pretreated with DAC (optimized final concentration, 20 and 80 nM, respectively; Sigma, St. Louis, MO) for 48 hr and then used in the transient p16ATF transfection experiment.

Cell proliferation assay

A 100- μ L cell suspension (10,000 cells/mL) was dispensed in each well on two 96-well plates, with six wells for each treatment. The cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8; Dojindo, China). In brief, 10 μ L of CCK-8 solution was added to each well of the plate, the plate was then incubated for 1 hr in a 37°C incubator, and then the absorbance at 450 nm was measured using a Bio-Rad microplate reader. The cells were measured daily for 5 continuous days. The average absorbance was calculated.

Scratch wound assay

Cells were seeded onto the six-well plate and cultured at 37°C until cells reached 100% confluence to form a monolayer. Then the monolayer cells were rinsed three times with the culture medium, and three parallels were scratched with a 20- μ L micropipette tip and rinsed another two times. The images were acquired from the same region that matched the reference points, after a 24-hr incubation.

Transwell migration and Matrigel invasion tests

A 150- μ L cell suspension (1×10^5 cells/mL) in the serum-free medium was plated onto the top of a Transwell insert, whereas serum-containing media (10%) were placed in the

well below. After a 24-hr incubation at 37°C in 5% CO₂, the cells in the upside of the insert were erased, and the cells that moved to the lower side were fixed with 2% formalin and stained by crystal violet. For the invasion test, the Transwell insert was laid with 80 µL of Matrigel (diluted with RPMI 1640 medium at 1:2) (BD Bioscience, San Jose, CA) to solidify before the cell suspension plated, and stained at 36 hr. Triplicate Transwell inserts were used for each treatment.

Cell-cycle analysis

The cells were harvested at 48 hr after transfection, resuspended in PBS, and fixed with absolute ethanol to a final concentration of 75%. Then the cells were pelleted and resuspended in PBS containing 100 µg/mL RNase A and 50 µg/mL propidium iodide and incubated at 37°C for 1 hr in the dark. Then the samples were analyzed by cytoflowmetry.

RT-PCR

The total RNA of transfected cells was extracted by TRIzol (Invitrogen) and reverse-transcribed using a first-cDNA synthesis kit (Transgen Co., Beijing, China). The quantitative RT-PCR for *p16*, *Arf*, and five other control genes, including three genes (*Cdk6*, *Ets2*, and *Ezh2*) containing the Sp1-binding site and two genes (*Cdk4* and *Suz12*) without the Sp1-binding site, were analyzed using primer sets shown in Supplementary Table S4. GAPDH was used as reference. The quantitative RT-PCR reactions were amplified with Power SYBR Green PCR Master Mix (Fermentas, Glen Burnie, MD) and an ABI-7500 or ABI-7500 Fast Instrument.

Analysis of p16 methylation

The methylation status of *p16* CpG island was detected by the 150/151-bp methylation-specific PCR (MSP), 392-bp denatured high-performance liquid chromatography (DHPLC), or clone sequencing as described (Herman *et al.*, 1996; Luo *et al.*, 2006). In brief, the bisulfite-modified genomic DNA was amplified using the methylated/unmethylated *p16*-specific primer sets in the MSP assay or using the CpG-free universal primer set in the DHPLC and sequencing assays (Supplementary Table 4S). All the PCR products were amplified with HotStarTaq DNA polymerase (QIAGEN GmbH, Hilden, Germany).

Confocal microscope examination

Cells grown on the cover glass were fixed in 4% polyformaldehyde for 10 min at room temperature, treated with 1% Triton X-100 in PBS for 10 min, blocked with 5% nonfat milk for 1 hr, and then hybridized by mouse monoclonal antibody against P16 (1:100; Abcam, UK) overnight at 4°C. The rhodamine-labeled second antibody was incubated for 1 hr at room temperature, followed by nucleus dyeing with DAPI. The fluorescence images were acquired by using a Leica inverted fluorescence microscope under an oil objective. The intensity of rhodamine fluorescence of 200 GFP-positive or -negative cells was determined and used to represent levels of P16 protein expression in cells with different transfections. Wilcoxon tests were performed to examine the difference between cells transfected with p16ATF and vector control. Mouse IgG antibody was used as a negative control for each cell.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed and analyzed essentially as described (Shang *et al.*, 2000; Li *et al.*, 2010). The antibodies used were rabbit anti-trimethylated H3K4 (H3K4me3; no. 9132; Cell Signaling, Beverly, MA), rabbit anti-trimethylated H3K9 (H3K9me3; no. 07-442; Millipore, Billerica, MA), rabbit anti-trimethylated H3K27 (H3K27me3; no. 07-449; Millipore), goat anti-Myc (AB9132; Abcam, UK), rabbit anti-GFP (AB290; Abcam, Hong Kong), and mouse anti-Dnmt1 (AB13537; Abcam, Hong Kong). Anti-Myc and anti-GFP antibody was used to precipitate exogenous p16ATF protein. Each ChIP experiment was performed in triplicate and repeated at least three times. The primer sets used are listed in Supplementary Table S4. PCR reactions were amplified with HiFi polymerase (Transgen, China) at 95°C for 5 min, (95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec) × 35 cycles, followed by 72°C for 10 min. Quantitative PCR reactions were amplified with Power SYBR Green PCR Master Mix (Fermentas, USA) and ABI-7500 Fast Instrument.

Results

Analysis of affinity and specificity of 7ZFPs to p16 promoter

To obtain the *p16* promoter-specific 7ZFPs with a high binding affinity, we designed and constructed coding sequences of 28 kinds of 7ZFPs targeted to a 21-bp fragment within the *p16* core promoter [−170 nt to −150 nt; transcription start site (TSS), +1 nt; Fig. 1A–C]. The affinity and specificity of these designed 7ZFPs were detected by EMSA using the purified prokaryotic expression products. It showed that 18 of 28 tested 7ZFPs (64%) could bind to the biotin-labeled target DNA probe-1 (Supplementary Table S4). The *p16*-binding activity of these 7ZFPs was inhibited concentration-dependently by the unlabeled probe-1, but by neither the probe-2 matched to another Sp1-binding site within the *p16* 5'-UTR nor the *p16*-unrelated probe-3 control (Fig. 1D). These results indicate that most of these selected 7ZFPs could specifically bind to their target DNA *in vitro*.

Interestingly, we found that the 7ZFPs had a higher binding affinity to the methylated probe-1 (Mprobe-1) than to the unmethylated probe-1. The binding of 7ZFP to the unmethylated probe-1 was almost completely competed by the unlabeled Mprobe-1, whereas the binding to the Mprobe-1 was only weakly competed by the unlabeled and unmethylated probe-1 (Fig. 1E).

Up-regulation of endogenous p16 expression in mammalian cells by p16ATFs

To investigate whether these 7ZFPs could be used to engineer p16ATFs, we selected three active 7ZFPs with the *p16*-binding affinity (1F, 2F, and 6I) and four inactive 7ZFPs without the binding affinity (2A, 1G, 2G, and 6G) in the above EMSA as the representative proteins (Supplementary Table S1), and fused them with a 3×NLS and the transcription activator VP64 in the mammalian expression vector pcDNA3.1-myc/His A (Fig. 2A). In the *p16* promoter luciferase reporter assay, the promoter activity of three active 7ZFP-derived p16ATFs was significantly higher than that of four inactive 7ZFP-derived p16ATFs in the *p16*-active 293T cells transiently transfected with these p16ATFs (Fig. 2B).

FIG. 2. Up-regulation of endogenous *p16* expression by p16ATF. (A) Construction of p16ATF using the coding sequence of 7ZFP, VP64, and 3NLS. (B) The reporter activities of the *p16* promoter induced with various p16ATFs in 293T cells. (C, D) Analysis of *p16* expression in the *p16*-active human 293T cells transiently transfected with p16ATF or control vector by western blot (using Myc antibody) and quantitative RT-PCR, respectively. (E) Analysis of binding of p16ATF to the *p16* promoter by ChIP assay. (F) Quantification of P16 protein (median) in the 293T cells transiently transfected with p16ATF in the pEGFP-N2 vector. (G) Confocal fluorescence images of p16ATF/Vector-GFP (green), P16 (red), and nucleus (blue). The p16ATF-GFP-positive cell (arrows) was also the P16 strongly expressed cell.

Thus, the most active p16ATF-6I was used as the representative in the following studies.

Results of western blot and quantitative RT-PCR showed that transient transfection of p16ATF-6I did increase endogenous *p16* expression in 293T cells (Fig. 2C and D). Results of

the ChIP assay showed that the representative p16ATF-6I specifically bound to the *p16* promoter (Fig. 2E). Moreover, confocal examination showed that the P16 protein level was increased in the GFP-positive cells transiently transfected with the p16ATF-6I in the pEGFP-N2 vector, but not in the

cells transfected with the empty control vector (Fig. 2F and G). To study whether the effect of the p16ATF-6I is *p16*-specific, we analyzed transcription levels of *Arf* at the same locus (sharing the same exon-2 and exon-3 with *p16*) and did not find a significant change of *Arf* transcription (Supplementary Fig. S1a). This result suggests that p16ATF-6I could selectively up-regulate endogenous *p16* expression.

Furthermore, we also analyzed transcription changes of five *p16* expression-related genes, including activators (*Cdk4*, *Cdk6*, and *Ets2*) and repressors (*Ezh2* and *Suz12*), whether containing an Sp1-binding site (*Cdk6*, *Ets2*, and *Ezh2*) or not (*Cdk4* and *Suz12*) in their promoter regions. We saw that the mRNA level of the activator *Ets2* was slightly increased. In contrast, the transcription level of the repressor *Suz12* was significantly decreased (Supplementary Fig. S1a). However, the p16ATF-6I bound to the *Ets2* or *Suz12* promoter was not observed in the ChIP assay (Supplementary Fig. S1b). These results indicate that these genes were affected by the ATF indirectly.

Transient transfection of p16ATF-6I selectively reactivates methylated *p16* expression in the DAC-pretreated cell lines

To investigate whether these p16ATFs are able to activate the methylated *p16*, the most active p16ATF-6I and inactive p16ATF-2G were transiently transfected into the *p16*-methylated cell lines H1299 and AGS, respectively. Neither *p16* transcription nor demethylation of *p16* CpG island was detected in these cell lines at 48–72 hr after the transfection (data not shown). It is interesting that when H1299 cells were pretreated with DNA methylation inhibitor DAC at the optimized nontoxic concentration (20 nM) for 48 hr, p16ATF-6I significantly increased transcription of *p16* in these cells at 48 hr after the subsequent transfection (Fig. 3A). Reactivation of methylated *p16* by p16ATF-6I was also observed in the

80 nM DAC-pretreated AGS cells (Fig. 3C). Transcription of *Arf* was not changed in the p16ATF-6I transfected H1299 or AGS cells (Fig. 3B and D).

To study the stability of *p16* reactivation, we dynamically detected *p16* mRNA level in the AGS cells, and found that at day 9 after transfection, the enhancement of *p16* transcription by p16ATF-6I and 2G was reached to the maximum level, and then gradually decreased along with the cell passages. At experimental day 20, *p16* mRNA was not detectable in the DAC-pretreated cells transfected with vector control, but still detectable in the DAC-pretreated cell with the p16ATF-6I transfection (Fig. 4).

Stable transfection of p16ATFs specifically induces *p16* reactivation and demethylation of CpG island

To investigate the long-term effects of p16ATF expression on the epigenetic repression of *p16* expression, we analyzed the *p16* expression status in the pooled H1299 cells stably transfected with p16ATF-6I and 2G. Western blot and quantitative RT-PCR assays showed that *p16* was significantly reactivated in H1299 cells stably transfected with the p16ATF-6I (Fig. 5A and B). Fully demethylated CpG islands in 82% of *p16* alleles were detected in the p16ATF-6I transfected cells by DHPLC and confirmed by bisulfite sequencing (Fig. 5C and D). A weak *p16* reactivation and demethylation could also be induced by the p16ATF-2G. Under fluorescence confocal microscopy, it was observed that cells expressing p16ATF-6I expressed P16 proteins simultaneously (Fig. 5E). The amount of P16 protein in the p16ATF-6I stably transfected GFP-positive cells was significantly higher than that in these cells transfected with p16ATF-2G or control vector (median, 11.3 vs. 4.5 or 3.6; $p < 0.001$; Fig. 5E, left column). Dynamic analysis of the proportion of the demethylated *p16* CpG islands and *p16* reactivation in the p16ATF stably transfected cells showed

FIG. 3. Transcription derepression of methylated *p16* by transient transfection of representative p16ATFs in DAC-pretreated cells. (A, B) *p16* and *Arf* mRNA levels in each group of H1299 cells at 48 hr after transfection, determined with quantitative RT-PCR. (C, D) *p16* and *Arf* mRNA levels in each group of AGS cells at 48 hr after transfection, determined with quantitative RT-PCR.

FIG. 4. Dynamic restoration of *p16* repression in the AGS cells pretreated with DAC for 2 days and subsequently transfected with p16ATF transiently 1 day after DAC treatment (defined as experimental day 1). The *p16* mRNA level was detected with quantitative RT-PCR at 48 hr after the transfection on experimental day 3, and so on.

that *p16* demethylation and reactivation were simultaneously and gradually increased along with the cell passages (Supplementary Fig. S2).

To investigate the possible mechanism for reactivation and demethylation of *p16*, we analyzed the binding status of DNMT1 to *p16* CpG island and found a significant decrease of DNMT1 binding to the *p16* promoter in the p16ATF-6I stably transfected cells (Fig. 6A). In addition, formations of histone H3K27me3 and H3K9me3 were significantly decreased in the p16ATF-6I transfected cells (Fig. 6B and C). In contrast, formation of histone H3K4me3, an active transcription marker, was significantly increased in these cells (Fig. 6D).

Transient and stable transfection of p16ATFs inhibits cancer cell migration

To investigate the functional significance of the reactivation of *p16* expression in the p16ATF transiently transfected cells, we compared their migration ability using the scratch wound tests, and found that the p16ATF transfection inhibited migration of the DAC-pretreated H1299 cells (Supplementary Fig. S3). Results of Transwell tests also showed the marked inhibition of migration of the DAC-pretreated H1299 and AGS cells with the transient p16ATF-6I transfection (Fig. 7A and B).

We further analyzed characteristics of morphological appearance, proliferation, cell cycle, and migration of the p16ATF stably transfected H1299 cells, and found that more elongated cells were observed in the p16ATF-6I and p16ATF-2G transfected cells (Supplementary Fig. S4). A significant difference of cell proliferation was not induced by the p16ATFs (Supplementary Fig. S5). However, the cell migration and invasion ability of the p16ATF-6I transfected cells was much lower than that of the control cells transfected with the empty vector in the scratch wound, Transwell, and Matrigel tests (Fig. 7C and D, Supplementary Fig. S6).

Discussion

Artificial ZFPs are becoming novel and powerful biotechnological tools for gene-specific regulation and manipulation (Beerli et al., 1998; Tan et al., 2003; Sera, 2009). The

engineered ZFPs composed of several Cys₂His₂ zinc-finger scaffolds perform the DNA binding activity and play different biological functions when fused with a transcriptional regulation domain or cleavage domain of an endonuclease (Li et al., 2007; Shamim et al., 2011). Here we explored the approach to selectively reactivate methylation-silenced genes using engineered ATFs composed of designed ZFPs and the transcription activator domain VP64. We demonstrated for the first time, to our knowledge, that the p16ATF made from 7ZFP with the *p16* promoter-specific affinity could reactivate methylated *p16* expression through demethylation of *p16* CpG islands and restore its function.

The activity of engineered ATFs *in vivo* is dependent on their successful accessibility to endogenous target promoter sites within chromatin. It is well known that most genes containing CpG islands around TSS contain GC box(es), and hence are regulated by the transcription factor Sp1. To obtain a p16ATF with specific accessibility to a methylated *p16*, we selected a natural Sp1-binding site (GGG GCG GGG) and its 5'-flank sequence (GAG GAA GGA AAC) within the *p16* core promoter that is located near the linker DNA sequence in *p16*-active cells as the binding target to design 7ZFP candidates (Lu et al., 2012). It is also reported that Sp1 is favored to bind to the methylated DNA targets (Jane et al., 1993). Thus, it is reasonable to expect that the Sp1-like 7ZFPs made from Sp1 scaffold may be favorable to bind the methylated DNA target. As expected, results of the *in vitro* competitive EMSA in the present study showed that the designed 7ZFPs had a higher affinity to the methylated target probe than to the unmethylated probe. These 7ZFPs could be used to construct *p16*-specific transcription factors to reactivate methylated *p16*.

DNA demethylation of methylated CpG sites within the *maspin* gene by ATF was reported recently in NCI-H157 cells (Beltran and Blancafort, 2011), although ATFs were used previously to reactivate methylated *H19* or *maspin* genes *in vitro* (Jouvenot et al., 2003; Beltran et al., 2007). Because normal NCI-H157 cells (without ATF treatment) can express a low level of *maspin*, it is uncertain whether the reported *maspin* up-regulation resulted from the demethylation of these CpG sites. In addition, there are only 13 CpG sites within the 300-bp region around its TSS (−150 nt to +150 nt; the ratio of observed CpG/expected CpG=0.37). Thus, *maspin* is generally not considered to contain a typical CpG island (the ratio of observed CpG/expected CpG ≥0.65). In the present study, we demonstrated that p16ATF could not only up-regulate endogenous *p16* expression in the *p16*-active 293T cells, but also specifically promote methylated *p16* expression in the DAC-pretreated AGS and H1299 cells. Furthermore, we found that the stable transfection of p16ATFs alone could induce the methylated *p16* gene expression and demethylation of *p16* CpG islands.

DNA methylation is considered a deep-epigenetic silencing event and is associated with a compact chromatin structure. We hypothesize that the p16ATFs approach the methylated *p16* promoter at first, then gradually alter the recruitment status of histone and DNA methyltransferases to the chromatin, and finally reactivate *p16* expression. As expected, both a decrease of DNA maintenance methylation enzyme DNMT1 recruitment and formation of histone H3K27me3 and H3K9me3, and an increase of transcription active marker H3K4me3 were observed in

FIG. 5. Reactivation and demethylation of *p16* by p16ATFs. (**A**, **B**) Analysis of derepression of *p16* expression in the *p16*-inactive human H1299 cells stably transfected with two representative p16ATFs (6I and 2G; passage 11) and vector control by western blot and quantitative RT-PCR, respectively. (**C**, **D**) Detection of demethylated *p16* alleles in the p16ATF stably transfected H1299 cells by DHPLC and bisulfite clone sequencing, respectively. Genomic DNA of the *p16*-hemimethylated cell line HCT116 was used as the positive control of unmethylated and methylated *p16* alleles by DHPLC. (**E**) Results of derepression of *p16* expression in H1299 cells stably transfected with two p16ATF-6I and 2G in the confocal assay. The amount (median) of P16 in the p16ATF transfected cells was significantly higher than that in the vector control transfected cells, as illustrated on the left column.

the *p16* promoter in the p16ATF stably transfected cells, which may account for the reactivation of *p16* and demethylation of this CpG island. It is reported that H3K4me3 may be a potential inhibitor of DNMT3a for *de novo* DNA methylation (Li *et al.*, 2011). The present

study suggests that H3K4me3 may also be involved in DNA demethylation.

Polycomb group (PcG) proteins and other factors play important roles in the regulation of *p16* expression (Gil and Peters, 2006; Deng *et al.*, 2010; Li *et al.*, 2010). In the 293T cells

FIG. 6. Inhibition of DNMT1 binding and inactive histone modifications (H3K27me3 and H3K9me3) and induction of the active histone modification (H3K4me3) within the *p16* promoter in H1299 cell stably transfected with p16ATFs. (A) Result of DNMT1 ChIP assay. (B) Result of H3K27me3 ChIP assay. (C) Result of H3K9me3 ChIP assay. (D) Result of H3K4me3 ChIP assay. The amplicon for the *p16* ChIP assay is the same as illustrated in Fig. 2E.

transiently transfected with p16ATF-6I, we found that the transcription level of the *p16* activator *Ets2* gene was slightly increased and that of the repressor *Suz12* gene was significantly decreased. As the p16ATF did not bind to the *Ets2* and *Suz12* promoters, we considered that the expression changes of *Ets2* and *Suz12* might be indirect feedback effects due to the up-regulation of *p16*, because p16ATF binding may decrease the accessibility of the *p16* promoter to the PcG proteins and recruit more coactivators to the *p16* promoter.

It is reported that aberrant methylation of *p16* is initiated after gene silencing (Hinshelwood *et al.*, 2009). To investigate whether demethylation of CpG island is the prerequisite condition for further reactivation of methylated *p16*, we analyzed the proportion of the demethylated *p16* and *p16* reactivation in the p16ATF stably transfected cells dynamically, and found that *p16* demethylation and reactivation were increased simultaneously and gradually along with the cell passages. This result implies that demethylation maybe a necessary step for reactivation of methylated *p16* by p16ATFs. As transient p16ATF transfection alone does not

lead to demethylation of *p16* CpG islands, the process of stable p16ATF transfection inducing *p16* demethylation may be a passive demethylation through inhibition of re-methylation of the daughter DNA strands by DNMT1 at the replication forks, as observed in *Xenopus* oocytes by others (Matsuo *et al.*, 1998; Stünkel *et al.*, 2001). The decreased binding of DNMT1 to *p16* CpG island in the p16ATF transfected H1299 cells supports this hypothesis.

Although transient p16ATF transfection did up-regulate endogenous *p16* expression in the *p16*-active 293T cells, the reactivation and demethylation of *p16* gene were not observed in the p16ATF transiently transfected *p16*-methylated H1299 cells. These results illustrate that the methylation-inactivation of genes is so solid that short-term binding of transcription factors could not rapidly reverse the epigenetic repression of this gene.

A synergic effect of the inhibitor of DNA methylation on ATF to reactivate *maspin* was reported recently (Beltran *et al.*, 2008; Beltran and Blancafort, 2011). In the present study, we also found that the transient transfection of p16ATF-6I

FIG. 7. Transient and stable transfection of p16ATF-6I inhibits cell migration and invasion in Transwell tests. (A, B) Images of DAC-pretreated H1299 and AGS cells transiently transfected with the p16ATF-6I and empty control vectors, respectively. (C, D) Transwell-migration and Matrigel-invasion images of H1299 cells stably transfected with the p16ATF-6I (100 $\times$). Color images available online at www.liebertpub.com/hum

significantly increased the transcription level of *p16* in the H1299 and AGS cells pretreated with DNA methylation inhibitor DAC at nontoxic concentration (20–80 nM), which is much lower than the regularly used concentration (500–5,000 nM) to reactivate methylated genes or damage DNMT1 (Bender *et al.*, 1998; Ghoshal *et al.*, 2005). Such a phenomenon could not be observed for the *Arf* gene located upstream of the *p16* locus. Together, these phenomena illustrate that low-dose DAC pretreatment can improve the ATFs' efficiency to reactivate methylated genes and that DNA methylation obstructs efficient reactivation of gene expression by administration of only transcription factors. Most importantly, these results also suggest that ATFs could be used not only to enhance the demethylation effect of DAC, but also to enable DAC to reactivate methylated genes specifically and persistently for a longer time.

It is well recognized that *p16* is one of the most important tumor suppressor genes and P16 controls G1→S phase transition in the cell cycle, which may play important roles in normal cell senescence and inhibition of cancer cell proliferation. The expression level of P16 is very low in normal tissues. We observed that a higher proportion of p16ATF transfected cells was suspended in the culture medium compared with the control vector transfected cells, and that *p16* expression was detected earlier in the suspended cells than in the adherent cells (data not shown). However, the growth of the adherent cells was not affected by the p16ATF-6I stable transfection. These results suggest that up-regulation of *p16* expression by the p16ATF might result in cell senescence, but not a visible change of cell proliferation nor the cell cycle (data not shown). This may also account for why a sharp increase of *p16* expression is not obtained in the p16ATF transfected cells.

Although it is reported that *p16* inactivation by deletion or methylation is positively associated with metastasis of many cancers (Luo *et al.*, 2006; Ignatov *et al.*, 2008; Su *et al.*, 2010), whether P16 directly inhibits cancer metastasis has not been reported before. In the present study, we found that reactivation of *p16* expression by p16ATFs significantly and consistently inhibited cancer cell migration *in vitro*. It is well known that elongation of cells may inhibit cell migration (Ridley *et al.*, 2003). We found that more elongated cells could be observed in the p16ATF stably transfected H1299 cells. Together, these phenomena imply that *p16* may function as a cell migration/metastasis inhibitor gene. An *in vivo* validation study is expected to confirm the biological function of P16 protein in cancer metastasis.

In conclusion, this study showed that the representative p16ATF could specifically reactivate methylated *p16* expression through demethylation of CpG islands, enable DAC *p16*-specific targeting, and restore function of *p16* gene to inhibit cell migration. These suggest the potential applications of p16ATFs in transcriptional therapy of methylated *p16* related diseases.

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Author Disclosure Statement

The authors declare no conflict of interest.

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