Hypermethylation reduces expression of tumor-suppressor PLZF and regulates proliferation and apoptosis in non-small-cell lung cancers

Xiaotian Wang,*†,1 Lei Wang,‡,1 Shicheng Guo,*† Yang Bao,§ Yanyun Ma,*† Fengyang Yan,*† Kuan Xu,‡ Zhiyun Xu,§ Li Jin,*† Daru Lu,*† Jibin Xu,¶,2 and Jiu-Cun Wang*†,2

*State Key Laboratory of Genetic Engineering and †Ministry of Education Key Laboratory of Contemporary Anthropology, School of Life Sciences, Fudan University, Shanghai, China; ‡Department of Cardiothoracic Surgery, 455th Hospital of the People’s Liberation Army, Shanghai, China; §Yangzhou No.1 People’s Hospital, Yangzhou, China; ¶Fudan University Shanghai Cancer Center, Shanghai, China; and ‡Department of Cardiothoracic Surgery, Changhai Hospital of Shanghai, Second Military Medical University, Shanghai, China

ABSTRACT Deregulation of promyelocytic leukemia zinc finger protein (PLZF), a tumor suppressor gene, was reported in different types of solid tumors. This study for the first time explored the reduced expression of PLZF and its effects in non-small-cell lung cancer (NSCLC) carcinogenesis. PLZF was found to be down-regulated by 62.8% in 87.1% of 154 paired NSCLC samples by quantitative real-time PCR, and its expression was found to be associated with the sex of the patient (P=0.02). Further analysis showed that down-regulation of PLZF in 35.6% NSCLC samples (31 out of 87) was triggered by hypermethylation in the promoter region. This was validated by demethylation analysis using the A549 cell line. Dual-luciferase reporter assay indicated that CTCF binding to the promoter region could activate PLZF transcription. Overexpression of PLZF in both A549 and LTEP lung cancer cell lines was found to inhibit proliferation and increase apoptosis. Therefore, reduced expression of PLZF was found to be common in NSCLC. PLZF down-regulation was partially correlated with hypermethylation in the promoter region. Decreased levels of PLZF expression may contribute to the pathogenesis of NSCLC by promoting cell survival. Therefore, the restoration of PLZF expression may serve as a new strategy for NSCLC therapy.—Wang, X., Wang, L., Guo, S., Bao, Y., Ma, Y., Yan, F., Xu, K., Xu, Z., Jin, L., Lu, D., Xu, J., Wang, J.-C. Hypermethylation reduces expression of tumor-suppressor PLZF and regulates proliferation and apoptosis in non-small-cell lung cancers. FASEB J. 27, 4194–4203 (2013). www.fasebj.org

Key Words: BSP · MSP

Lung cancer is the leading type of cancer in men, comprising 17% of all new cancer cases and 23% of the total cancer deaths in 2008. In developing countries, lung cancer accounts for 11% of all female cancer deaths (1). In China, the incidence of lung cancer increased by 1.63% per year from 1988 to 2005, and lung cancer accounts for more deaths than any other type of cancer (2). Lung cancer can be classified as either small-cell or non-small-cell, and non-small-cell accounts for 80–85% of all cases. Although cigarette smoking is by far the most important risk factor for lung cancer, the accumulation of genetic and epigenetic alterations to oncogenes and tumor suppressor genes also contributes a great deal to tumorigenesis.

The promyelocytic leukemia zinc finger protein (PLZF), a tumor suppressor gene, also known as zinc finger and BTB domain containing 16 (ZBTB16), was initially identified via the reciprocal translocation t(11;17)(q23;q21), fusing it to the retinoic acid receptor α (RARα) gene in acute promyelocytic leukemia (APL) (3, 4). It is a transcriptional factor containing 9 Krüppel-type zinc-finger motifs at its carboxyl terminus that

Abbreviations: BSP, bisulfite-sequencing PCR; CI, cell index; FITC, fluorescein isothiocyanate; MBD-Seq, methyl-CpG binding domain protein sequencing; MM, malignant mesothelioma; MSP, methylation-specific PCR; NSCLC, non-small-cell lung cancer; PI, propidium iodide; PLZF, promyelocytic leukemia zinc finger protein; TF, transcription factor; TFBS, transcription factor binding site; TSA, trichostatin A; ZBTB16, zinc finger and BTB domain containing 16

1 These authors contributed equally to this work.
2 Correspondence: J.-C.W., National Ministry of Education Key Laboratory of Contemporary Anthropology, School of Life Sciences, Fudan University, Shanghai 200433, China. E-mail: jcwang@fudan.edu.cn; J.X., Department of Cardiothoracic Surgery, Changhai Hospital of Shanghai, Second Military Medical University, Shanghai 200433, China. E-mail: jibinx@yahoo.com
doi: 10.1096/fj.13-229070
This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.
bind to specific DNA sequences (5). There is a BTB/POZ domain at the amino terminus, which mediates self-dimerization and transcriptional repression through recruiting nuclear corepressors to the transcriptional complex (6). This domain is also responsible for correct nuclear localization and for some of the transcriptional function of PLZF (7).

In hematopoietic systems, PLZF mRNA levels have been found to be down-regulated in NB-4 and HL-60 promyelocytic cell lines in response to retinoic acid-induced granulocytic differentiation and were very low in mature granulocytes (3, 8). PLZF, a transcriptional repressor, regulates cyclin A2, c-Myc, HoxD11, and other growth-related targets (9–11). During homeostasis, PLZF restricts proliferation and differentiation of human cord-blood-derived myeloid progenitors to maintain a balance between the progenitor and mature cell compartments (12). Enforced expression of PLZF in myeloid cell lines has been shown to inhibit proliferation and differentiation and promote apoptosis (9, 13, 14).

Deregulation of PLZF has been reported in some solid tumors. In one study, PLZF promoter methylation was confirmed in 35.2% of pancreatic cancer samples, and reduced PLZF protein expression was found in the majority of tested cancer samples (15). Several malignant mesothelioma (MM) cell lines showed loss of heterozygosity in the 11q region encompassing the PLZF gene, and its down-regulation was observed (16). PLZF expression was found to be substantially more down-regulated in high-risk melanomas (overall survival, ≤5 yr), than in low-risk melanomas (overall survival, >5 yr) (17). Overexpression of PLZF in human cervical cell lines could inhibit cell growth by inducing apoptosis and suppressing the promoter activity of human cyclin A2 (18). To characterize the role of tumor-suppressor PLZF in non-small-cell lung cancer (NSCLC), the most frequent primary malignant tumor in China, we conducted a gene expression assay and showed that PLZF was down-regulated in lung cancer tissues. In this study, reduced expression of PLZF was validated and found in the majority of NSCLC samples studied. We further showed that its down-regulation was partially determined by methylation, and low expression of PLZF may contribute to the tumorigenesis of NSCLC.

MATERIALS AND METHODS

Human tissues and cell lines

Fresh NSCLC samples and corresponding normal lung tissues were obtained from 154 patients who underwent pulmonary resection for primary NSCLC at Shanghai Institute of Cancer Research. All participants provided informed consent. A portion of each specimen was snap-frozen immediately after surgery and stored in liquid nitrogen until further processing for DNA or RNA extraction. The rest of each specimen was immediately put into RNAlater (Ambion, Austin, TX, USA) and stored at −20°C, in accordance with the manufacturer’s instructions. The demographic and clinical features of the 154 patients with NSCLC are summarized in Table 1. The research was conducted with official approval from the academic advisory board of Shanghai Hospital and the School of Life Sciences, Fudan University, Shanghai, China. Tissue microarrays (TMAs) of formalin-fixed, paraffin-embedded tumor and corresponding normal tissues were retrieved from 46 of the 154 patients. The TMAs were used for detection by immunohistochemistry assay and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Epithelial lung cancer cell lines A549 and NCI-H2999 and human embryonic kidney cell line H1299 were purchased from the Cell Bank of the Chinese Academy of Sciences. Cell lines NCI-H596, NCI-H460, NCI-H1703, and NCI-H226 were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained by the State Key Laboratory of Genetic Engineering of Fudan University. The normal human lung epithelial cell line Beas-2B was a gift from Dr. Yuehai Ke (Zhejiang University, Hangzhou, China). The pulmonary squamous carcinoma cell line LTEL was a gift from Second Military Medical University. Cells were cultured under recommended conditions.

DNA preparation, RNA extraction, and quantitative real-time PCR

Genomic DNA was isolated using AxyPrep Multisource Genomic DNA Miniprep kit (Axygen Scientific, Union City, CA, USA), according to the manufacturer’s protocol. Total RNA was isolated using TRizol (Sigma-Aldrich, St. Louis, MO, USA). First-strand cDNA was synthesized from 1 μg total RNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was carried out with an Applied Biosystems 7900 Prism real-time PCR machine and SYBR Premix Ex Taq (Takara, Dalian, Japan), in accordance with the manufacturer’s instructions. Yes-associated protein 1 (YAP1) was used as internal reference. Quantitative real-time PCR primers used for YAP1 and PLZF were listed in Table 2. The target gene expression in test samples was normalized to the corresponding YAP1 level and was reported as the fold difference relative to the YAP1 gene expression.

### Table 1. Demographic and clinical features of the 154 patients with NSCLC

<table>
<thead>
<tr>
<th>Pathological parameter</th>
<th>Tumors (n)</th>
<th>PLZF expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reduced</td>
<td>Preserved</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>77</td>
<td>68</td>
<td>10</td>
</tr>
<tr>
<td>&gt;60</td>
<td>77</td>
<td>66</td>
<td>10</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>33</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>Male</td>
<td>121</td>
<td>119</td>
<td>12</td>
</tr>
<tr>
<td>TNM clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA and IB</td>
<td>64</td>
<td>54</td>
<td>10</td>
</tr>
<tr>
<td>IIA and IIB</td>
<td>30</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>IIIA</td>
<td>54</td>
<td>48</td>
<td>6</td>
</tr>
<tr>
<td>IIIB and IV</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SqC</td>
<td>70</td>
<td>63</td>
<td>7</td>
</tr>
<tr>
<td>Ad</td>
<td>73</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>AS</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

SqC, squamous cell carcinoma; Ad, adenocarcinoma; AS, adenosquamous carcinoma; other, NSCLC other than SqC, Ad, or AS. *P < 0.05.
TABLE 2. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Sequence, 5’–3’</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>YAP1 F</td>
<td>VAP1 coding region</td>
<td>CACAGCGTCAGGATCTTTGAGC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>YAP1 R</td>
<td>VAP1 coding region</td>
<td>GGCATTGGTGGTGCTCGAGTCG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>PLZF F</td>
<td>PLZF coding region</td>
<td>TCACATACGGGAGGGCAGCC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>PLZF R</td>
<td>PLZF coding region</td>
<td>CTTGAGGCTGAACCTTGTGG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>MXD1 F</td>
<td>MXD1 coding region</td>
<td>AGACTGTTATGCCGGCTAGTTTAC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>MXD1 R</td>
<td>MXD2 coding region</td>
<td>AGATGAGCCCGCTATTTCTTCTC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>BSP F</td>
<td>PLZF promoter region</td>
<td>GGGAGAGAGAAAGATTTTTTTTTG</td>
<td>BSP</td>
</tr>
<tr>
<td>BSP R</td>
<td>PLZF promoter region</td>
<td>CAAATAATTACAAGCAATGAA</td>
<td>BSP</td>
</tr>
<tr>
<td>MSP methylation F</td>
<td>PLZF promoter region</td>
<td>GTTTCGAGTATGAGTGGGTG</td>
<td>MSP</td>
</tr>
<tr>
<td>MSP methylation R</td>
<td>PLZF promoter region</td>
<td>CAATATTACGCAATGCAAGC</td>
<td>MSP</td>
</tr>
<tr>
<td>MSP nonmethylation F</td>
<td>PLZF promoter region</td>
<td>TGTTTGAATAGATTTTATGG</td>
<td>MSP</td>
</tr>
<tr>
<td>MSP nonmethylation R</td>
<td>PLZF promoter region</td>
<td>CAATATTACACCAGGCAATGCAAA</td>
<td>MSP</td>
</tr>
<tr>
<td>PLZF BamHI I</td>
<td>PLZF CDS sequence</td>
<td>CGCCGTATGCAGTCTGACGAAATATG</td>
<td>PCR</td>
</tr>
<tr>
<td>PLZF Xho I</td>
<td>PLZF CDS sequence</td>
<td>GGGCCGCAGTATGCGAGATAGA</td>
<td>PCR</td>
</tr>
<tr>
<td>CTCF EcoR I</td>
<td>CTCF CDS sequence</td>
<td>CCGAATTCTGTAATGGCTAGGG</td>
<td>PCR</td>
</tr>
<tr>
<td>CTCF Xho I</td>
<td>CTCF CDS sequence</td>
<td>AGGCCTCAGTGTCACGCCTGAG</td>
<td>PCR</td>
</tr>
<tr>
<td>PLZF Mlu I</td>
<td>PLZF promoter region</td>
<td>GACAACGGTGAGAGACAGAAACT</td>
<td>PCR</td>
</tr>
<tr>
<td>PLZF Xho I</td>
<td>PLZF promoter region</td>
<td>ATATCGAGCAATTGTTTGGGGCGC</td>
<td>PCR</td>
</tr>
</tbody>
</table>

Immunohistochemistry

Protein expression of PLZF was detected using an immunohistochemistry assay using the monoclonal antibody against PLZF (sc-28319; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunohistochemistry and semiquantitative scoring were performed as described previously (19). All sections were scored on the basis of the percentage of cells that displayed immunoreactivity and on the intensity of the staining reaction. The samples were first grouped into four grades according to the staining intensity: 0, no staining; 1, weak staining; 2, medium staining; and 3, strong staining. The percentages of positive staining cells were ranked as 0 for 0–25%, 1 for 26–50%, 2 for 51–75%, and 3 for 76–100%, respectively. The final scores were the products of the proportion and intensity scores and fell within a range of 0–9. The sample was considered unstained if the final score was 0 or positive for staining if the final score were 1–9. Each cancer sample was considered to have preserved or a reduced PLZF expression if its final score was the same or less than that of its corresponding normal lung tissue sample. The staining intensity in normal bronchial tissues was considered as the strongest staining (Supplemental Fig. S1).

Treatment of cells with 5-Aza-dC and trichostatin A (TSA)

5-Aza-dC (Sigma-Aldrich) and TSA (Sigma-Aldrich) were used for demethylation assay. Human NSCLC A549 cells were plated at a density of 10^4 cells/cm² in 60-mm Petri dishes on d 0. The demethylation agent, 5-aza-dC, was added to fresh medium on d 0 and d 1–4 to maintain the concentration of 1.5 μM. TSA was added on d 0. The demethylation agent, 5-aza-dC, was added to fresh media on d 0. The cells were harvested on d 4 for extraction of RNA and DNA. Control cells were incubated without 5-aza-dC or TSA.

Bisulfite treatment and methylation-specific PCR (MSP) and bisulfite-sequencing PCR (BSP) analyses

The bisulfite conversion and PCR analyses were performed as described previously (20). BSP and MSP primers used for PLZF were listed in Table 2. The Tm values of BSP and MSP were 58°C, and there were 40 PCR cycles. Mixtures of genomic DNA from A549, H1299, and H226 cell lines served as positive controls. DNA from the normal human lung epithelial cell line Beas-2B was used as a negative control.

Dual-luciferase reporter gene assay

Primers used for constructing plasmids are listed in Table 2. The full-length CTCF was cloned to pCMV-Myc vector (Clontech, Mountain View, CA). The fragment in PLZF promoter region (chr11:113,929,586–113,929,905), which was tested in BSP assay, was cloned to PGL3-Basic vector (Promega, Madison, WI, USA) to make a reporter construct, PLZF-promoter-Luc. Both of the construct inserts were verified by sequencing. H293t cells plated on 48-well plate were transfected with CTCF expression vector/control vector, PLZF-promoter-Luc, and pRL-SV40 vector (Promega) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). The luciferase activity was measured with the dual-luciferase reporter assay system (Promega).

Plasmids and transfection

Full-length cDNA PLZF expression vector in PCMV-SPORTS6 (4944546) was obtained from the Source BioScience Life-Science (Nottingham, UK). This vector was directionally subcloned into pEGFP-C1 plasmid (Clontech) after the PLZF cDNA was amplified using primers containing the sequence flanking BamHI and XhoI sites. Primers are listed in Table 2. The construct insert was verified by sequencing. Transfection of A549 cells using Fugene HD (Roche Applied Science, Mannheim, Germany) was performed according to the manufacturer’s instructions.

DAPI staining

A549 cells (4×10^5/well) were seeded overnight in a 6-well plate and then transfected with PCMV-SPORTS6-PLZF vector or PCMV-SPORTS6 empty vector. After 48 h, the medium was poured off and A549 cells were washed with 1× PBS. Cells were then fixed in 10% formaldehyde for 20 min at room temperature. The plates were rinsed 3 times with PBS. The cells were treated with 0.5% of Triton-X-100 for permeabilization at room temperature for 15 min. The plates were then rinsed 3 times with PBS. DAPI staining solution (100 ng/ml)
Western blot analysis

Cells were seeded overnight in a 6-well plate and were transfected with PCMV-SPORTS6-PLZF vector or PCMV-SPORTS6 empty vector. Cells were harvested after 48 h, and lysates were prepared with nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk and incubated with antibodies of PLZF (sc-28319; Santa Cruz Biotechnology) and GAPDH (no. 2118s; Cell Signaling Technology, Danvers, MA).

Cell viability assay

Cell viability was measured by the xCELLiGence system (Roche Applied Science), which monitors cell growth in response to treatment in real time. Cells grow on top of electrodes, so that the impedance varies on the basis of the number of cells attached and the quality of cell-electrode interactions. As previously reported, the dimensionless parameter cell index (CI) was used to quantify cell status, including the number of cells, cell viability, the degree of adhesion, and cell morphology based on cell-electrode impedance (21, 22). Lung cancer cell lines A549 and LTEP (1×10⁵/well) were seeded overnight and then transfected with PCMV-SPORTS6-PLZF or PCMV-SPORTS6 empty vectors. Cell growth was monitored continuously.

Cell cycle analysis

Lung cancer cell lines A549 and LTEP (4×10⁵/well) were seeded overnight in a 6-well plate and then transfected with PCMV-SPORTS6-PLZF vector or PCMV-SPORTS6 empty vector. Cells were washed with PBS and harvested with trypsin (Life Technologies) 48 h after the transfection. The cells were spun down at 1000 g and the pellet was washed with cold PBS. After a second centrifugation, the cells were resuspended in 1 ml of cold 70% ethanol with gentle vortexing and fixed overnight at −20°C. Cells were stained with 50 μg/ml propidium iodide (Sigma-Aldrich) and treated with RNaseA (Shenggong, Shanghai, China) for 30 min at room temperature. They were then analyzed by flow cytometry and a FACScan cell analyzer (BD Biosciences, San Jose, CA, USA) equipped with CellQuest software (BD Biosciences). They were also analyzed using ModFit LT (Verity Software House, Topsham, ME, USA).

In vitro detection of apoptosis

Lung cancer cell lines of A549 and LTEP (4×10⁵/well) were seeded overnight in a 6-well plate and then transfected with PCMV-SPORTS6-PLZF vector or PCMV-SPORTS6 empty vector. Cells were washed with PBS and harvested with trypsin express (Life Technologies) 48 h after the transfection. Floating and attached cells were collected and detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Calbiochem, Darmstadt, Germany), and analyzed by flow cytometry in accordance with the manufacturer’s instructions. Detection of sub-G₁ apoptotic cells was performed by collecting floating and attached cells for cell cycle analysis. The cells were analyzed using CellQuest software (BD Biosciences).

Statistical analysis

Statistical analysis was performed using the paired-samples t test, χ² test, corrected χ² test, and Fisher exact test by SPSS (SPSS, Chicago, IL, USA). Values of P < 0.05 were considered statistically significant. GraphPad Prism5 (GraphPad, San Diego, CA, USA) was used to make the figures.

RESULTS

Levels of PLZF expression in NSCLC tissues and association with the sex of the patient

PLZF mRNA expression was evaluated in 154 pairs of NSCLC samples (Table 1) by quantitative real-time PCR, and it was found to be down-regulated by 62.8% relative to normal controls (relative mRNA levels <0.75) in 87.1% (135/154) of cancer tissues studied (95% CI: 50.2–75.3%, P<0.01; Fig. 1A). PLZF was also found to be significantly down-regulated in all 6 lung cancer cell lines (Fig. 1B). Association analysis was performed between PLZF expression and clinical features of all patients. As shown in Table 1, a significant association was found between PLZF expression and the sex of the patient (P=0.02). However, no significant association was found between PLZF expression and age, TNM clinical stage, histological type, or any other clinical feature. To confirm the relationship between the expression of mRNA and protein, immunohistochemical analysis was performed to evaluate PLZF protein expression in tissues. Out of the 46 pairs of NSCLC and normal tissues, protein expression was found to be lower in the cancerous tissues than in the normal counterparts 29 times (63.0%; Fig. 1C). The mean histoscores of the tumor tissues and corresponding normal tissues were 3.04 vs. 4.76 (P<0.01; Fig. 1D).

Effects of hypermethylation of the PLZF promoter on PLZF expression

DNA methylation is often implicated in the deregulation of a variety of genes. A CpG island was found in the PLZF promoter region using information from the University of California–Santa Cruz (UCSC; Santa Cruz, CA, USA) database (http://genome.ucsc.edu/cgi-bin/hgGateway). The genome-wide methylation profile of NSCLC and normal lung tissues established by methyl-CpG binding domain protein sequencing (MBD-Seq) indicated a hypermethylation signal in this region (Fig. 2A and unpublished data). In this way, the DNA methylation status of this region (chr11: 113, 929, 586–113, 4197) was determined using a BSP-based assay on 3 lung cancer cell lines and 1 normal lung epithelial cell line. As shown in Fig. 2B, E, the average methylation rate in 3 lung cancer cell lines (70.8%) was significantly higher than in the normal lung epithelial cell line Beas-2B (18.3%) (P<0.01), indicating that the pro-
moter region was hypermethylated in lung cancer cell lines. To confirm the correlation between PLZF promotor methylation status and PLZF expression, demethylation treatment was performed on A549 with the DNA methyltransferase inhibitor 5-Aza-dC and the histone deacetylase inhibitor, TSA. As shown in Fig. 2C, E, most of the methylated sites were demethylated after 4 d of treatment with 5-Aza-dC and TSA. Another qRT-PCR assay revealed that PLZF expression in the 5-Aza-dC-treated A549 cells was greatly up-regulated relative to that in untreated control cells (Fig. 2F). In addition, MSP confirmed the hypermethylation of the promotor region in 31 of the 87 NSCLC tissues (35.6%) and in only 11 of the 87 corresponding normal lung tissues (12.6%; Fig. 2G and Table 3).

CTCF functioned as a transcriptional activator to PLZF

We searched for locus information in the Encode Project (23). All 1296 Chip-seq experiments of the transcription factor binding sites (TFBSs), which involved 200 transcription factors (TFs), were downloaded from the UCSC database (24). Bedtools was used to perform an intersection analysis between targeted sequences and TFBSs (25). The PLZF fragment (chr11:113,929,586–113,929,905) tested in BSP and MSP assay was found to be located at the CTCF (CCCTC binding factor) binding region in 15 cell lines, such as Caco-2, HCM, HMEC, and HMF but not in A549 cell lines (Supplemental Table S1). To verify that CTCF, as a TF, binds to the promotor region of PLZF to regulate the transcription, a dual-luciferase reporter assay was performed with CTCF expression vector and a reporter construct (PLZF-promoter-Luc) containing PLZF promoter sequence (chr11:113,929,586–113,929,905). As shown in Fig. 3, CTCF specifically activated this reporter gene by ~7-fold (P=0.02). It indicated that CTCF could activate PLZF transcription by binding to the unmethylated promotor region.

Subcellular location of PLZF in lung cancer cells

PLZF overexpression was induced in A549 cells by transient transfection of PLZF expression vector (pEGFP-C1-PLZF). As a result, PLZF was expressed in both nucleus and cytoplasm 48 h after transfection (Fig. 4B), although most of it was located in the nucleus, observable in a speckled pattern (Fig. 4A), as in a previous report (8). There are two isoforms of PLZF. The major one has 673 aa and molecular mass of 74 kDa. The other one has 550 aa and molecular mass of 61 kDa. Both isoforms were expressed in A549 cells, but only the major isoform was expressed in LTEP cells (Fig. 4C), H293t cells (Supplemental Fig. S2B), and Beas-2B cells (Supplemental Fig. S2F).

Effects of overexpression of PLZF on cell proliferation and apoptosis

To gain insight into the potential role of down-regulation of PLZF in NSCLC, the function of PLZF on proliferation was assessed in lung cancer cell lines. As shown in Fig. 5A, overexpression of PLZF (PLZF expression vector, pCMV-SPORTS6-PLZF) in lung cancer cell lines A549 and LTEP gradually induced a persistent decline in CI relative to the control (empty vector, pCMV-SPORTS6), which was monitored by

Figure 1. Decreased expression of PLZF in NSCLC. A) mRNA expression levels of PLZF were analyzed in 154 pairs of NSCLC tissues. Red dots represent female patients; black dots represent male patients. The y axis is plotted on a logarithmic scale. ***P < 0.001. B) mRNA expression levels of PLZF in 6 lung cancer cell lines and 1 normal cell line. C) Protein expression of PLZF in NSCLC tissues, as indicated by immunohistochemical analysis (×400). a) Reduced expression in SqC. b) Preserved expression in corresponding normal lung tissues. Arrows indicate cells expressing PLZF. D) Histoscores of PLZF as indicated by immunohistochemical analysis (n=46). Data represent means ± SD from ≥3 independent experiments. Relative mRNA expression of PLZF in corresponding normal lung tissues and the Beas-2B cells was normalized to 1.
the xCELLiGence system. This suggested that overexpression of PLZF in lung cancer cells could lead to reduced cell growth. Cell cycle examined by FACS analysis revealed that 10.1% more cells accumulated in the G0/G1 compartment of the cell cycle with overexpression of PLZF in LTEP cells (P < 0.04). Meanwhile, 7.7% less cells in S phase in A549 cells (P < 0.09) and 8.6% less cells in S phase in LTEP cells (P < 0.03) were found as PLZF was overexpressed (Fig. 5B), indicating that overexpression of PLZF in lung cancer cells could attenuate cell growth. Apoptosis was examined by FACS analysis after double staining with annexin V-FITC and propidium iodide (PI). PLZF-overexpressing cells showed a higher rate of apoptosis than control cells. As revealed in Fig. 5C, differences in apoptosis status were found between the cells that did and did not overexpress PLZF in both cell lines. In detail, 11.1% of the A549 cells treated with PLZF for 48 h underwent early apoptosis, while only 7.1% of the cells with control vector underwent early apoptosis (P < 0.06). 8.6% of the LTEP cells treated with PLZF underwent early apoptosis, while only 4.4% of the cells with control vector underwent early apoptosis (P < 0.02). Apoptosis was also examined by FACS analysis after staining with PI. At 48 h after transfection, significant difference in sub-G1 proportion appeared between cells treated with PLZF

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Methylated</th>
<th>Unmethylated</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>31</td>
<td>54</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Normal</td>
<td>11</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Hypermethylation analysis of PLZF in NSCLC cell lines and clinical samples. A) A fragment with 21 CGs (in red) was selected from the CpG island located in the promoter region of PLZF. B) Methylation status of the fragment was examined by BSP in lung cancer cell lines A549, H1299, and H226 and in normal lung cell line Beas-2B. C) Methylation status of the fragment was assessed using BSP in A549 cells with and without demethylation treatment. D) Densitometric analysis of methylation rate of the tested fragment in different cell lines based on BSP results. Methylation rates in A549, H1299, H226, and Beas-2B were 52.4, 58.6, 91.4, and 18.3%, respectively. *P < 0.01, ***P < 0.001. E) Densitometric analysis of methylation rate of the tested fragment in A549 cells with and without demethylation treatment based on BSP results. **P < 0.01. F) PLZF mRNA expression in A549 cells with and without demethylation treatment. Data represent means ± sd from at least 3 independent experiments. *P < 0.05. G) Methylation status in pairs of NSCLC tissues by MSP. Mixture of genomic DNA from A549, H1299, and H226 cell lines was used as positive control. DNA from the normal human lung epithelial cell line Beas-2B was used as a negative control. M, PCR for CpG-methylated DNA; U, PCR for CpG-unmethylated DNA. Relative mRNA expression of PLZF in A549 control cells was normalized to 1.
and control vectors. In A549 cell line, the proportion of cells in sub-G1 phase almost doubled in PLZF-overexpressing cells relative to the control vector culture (P<0.05). In LTEP cell line, the proportion of cells in sub-G1 phase rose by 44% (P<0.01) when PLZF was overexpressed (Fig. 5D).

DISCUSSION

PLZF acts as a tumor suppressor in a variety of cancers, including leukemia, malignant melanoma, malignant mesothelioma, prostate cancer, and pancreatic cancer (9, 15–18, 26). Deregulation of PLZF is associated with tumorigenesis in these cancers. In the present study, PLZF expression was found to be significantly downregulated at both the mRNA and protein levels in NSCLC. Promoter methylation was found to account for the low PLZF expression. Altered expression of PLZF affected its biological functions associated with tumorigenesis, such as proliferation, cell cycle, and apoptosis. These results supported the conclusion that PLZF acts as a tumor suppressor in NSCLC.

The accumulation of genetic and epigenetic alterations to proto-oncogenes and tumor suppressor genes can lead to tumor transformation. Changes in DNA methylation patterns are frequently associated with decreases in tumor suppressor gene expression. In the present study, we found a hypermethylation signal in the promoter region of PLZF in NSCLC based on genome-wide methylation profiles of NSCLC by MBD-Seq. This was confirmed in 3 lung cancer cell lines and in 31 of 87 (35.6%) NSCLC tissues. A demethylation assay validated the contribution of methylation to its expression. TFs bind to promoter region to mediate the transcription process.

Hypermethylation in promoter region was found to affect the binding of TFs, causing aberrant gene expression. CTCF is a transcriptional regulator protein with 11 highly conserved zinc finger (ZF) domains. It binds to numerous sites throughout the genome that have insulator and chromatin barrier activity, including promoter regions. In vitro studies have suggested that CTCF protects the retinoblastoma (Rb) gene promoter, a classic CpG island, from DNA methylation. When this control region is abnormally methylated, it can induce epigenetic silencing of the promoter (27). In addition, it has been confirmed that experimentally directed methylation could prevent binding of CTCF and other factors in vitro (28, 29). The PLZF promoter region detected in this study was hypermethylated in A549 cells, and it was not found to be bound by CTCF (Supplemental Table S1). The result of the dual-luciferase reporter gene assay showed that CTCF specifically activated the reporter gene containing PLZF promoter sequence. This suggested that CTCF could bind to this promoter region to activate the transcription of PLZF and that methylation in this region might

Figure 3. Dual-luciferase reporter assay of CTCF and PLZF promoter sequence. CTCF activated the reporter gene. Data represent means ± sd from ≥3 independent experiments with 3 replicates in each experiment. Statistical analysis was performed using the paired-samples t test. *P < 0.05.

Figure 4. PLZF was located in nucleus in a speckled pattern. A) PLZF-GFP was expressed in the nucleus with a speckled pattern in A549 cells. B) PLZF was detected in both nucleoprotein and plasmosin. Arrows indicate the two isoforms of PLZF. C) PLZF was detected in whole-cell lysis of LTEP cells.

4200 Vol. 27 October 2013 WANG ET AL. The FASEB Journal · www.fasebj.org
prevent binding of CTCF and lead to repressed transcription.

Vincent et al. (15) have also found PLZF to be hypermethylated and underexpressed in pancreatic cancers, in which PLZF promoter methylation was confirmed in 35.2% of pancreatic cancer samples. However, the targeted sequence in their MSP assay did not overlap with the sequences analyzed in our study. Other methylation sites or fragments might account for the low levels of PLZF expression observed in our study. Genetic alterations in PLZF were also found to lead to the down-regulation. Cheung et al. (16) identified overlapping focal deletions in 11q23.2 in several MM cell lines, with deletions consistently encompassing the PLZF gene, and hemizygous deletion of PLZF was validated in half of 22 tested MM cell lines by real-time quantitative PCR analysis of genomic DNA. Methylation at sites other than those here targeted in MSP assay or deletion of the gene might account for the PLZF down-regulation in the remaining NSCLC tissues.

It has been reported previously that PLZF acts as a growth inhibitory and proapoptotic factor during early embryogenesis in the limb bud. Inactivation of PLZF in mice produced patterning defects, affecting all the skeletal structures of the limb (11). PLZF overexpression has been shown to promote apoptosis in different types of human cancer cells, including Jurkat T-cell leukemia cells, mesothelioma cells, and cervical carcinoma cells (14, 16, 18). In the present study, overexpression of PLZF in two lung cancer cell lines, A549 and LTEP, inhibited cell proliferation, indicating that the down-regulation of PLZF in NSCLC tissues can promote cell growth. Further analysis showed that PLZF also induced increased apoptosis in both cell lines, indicating that low levels of PLZF expression in NSCLC tissues could inhibit apoptosis, contributing to the tumorigenesis.

The role of PLZF in tumorigenesis of NSCLC might be different from its role in other cancers. In a hematopoietic cell model, continued PLZF expression was associated with cell cycle arrest in G1/S phase and repression of c-Myc and cyclin A2 (CCNA2) (9, 10). Pre-B-cell leukemia transcription factor 1 (Pbx1) has been reported as a PLZF target gene in both prostate cancer cells and melanoma cells (26, 30). However, our quantitative PT-PCR analysis did not detect changes in the expression of these genes in lung cancer cells transfected with PLZF (data not shown). In addition, overexpression of PLZF in lung cancer cells induced reduction of number of cells in the S phase. Other researchers have also failed to find a connection between expression of PLZF and that of cyclin A2 in melanoma cells or c-Myc in spermatogonial stem cells (31, 32). In addition, ectopic expression of PLZF in malignant mesothelioma cells was not found to modulate cyclin A2 expression nor result in cell cycle arrest.

**Figure 5.** Overexpression of PLZF inhibited cell proliferation and increased cell apoptosis. A) Two lung cancer cell lines, A549 and LTP, transfected with pCMV-SPORTS6-PLZF vector both showed less growth than those transfected with the control vector. B) LTEP cells with overexpression of PLZF showed accumulation of cells in G0/G1 phase. Both cell lines showed repressed S-phase progression. C) Left panel: overexpression of PLZF increased apoptosis, as indicated by annexin V-FITC and propidium iodide (PI) double staining. Right panel: densitometric analysis of the top segment. D) Left panel: overexpression of PLZF causes cells to accumulate in the sub-G1 phase. Right panel: densitometric analysis of the top segment. M1 represents sub-G1 phase. Data represent means ± sd from ≥3 independent experiments with 3 replicates in each experiment. Statistical analysis was performed using the paired-samples t test. *P < 0.05, **P < 0.01.
(16). This suggests that, in solid tumors, PLZF might regulate cell cycle progression through cyclins other than CCNA2. We have examined more than 20 genes that had been reported to be the possible target genes for PLZF, including CCNA2, Pbx1, CDK6, CDK10, ID2, TIMP, BID, PTEN, Birc5, MXD1, and so on. It was found that PLZF overexpression could lead to up-regulation of MXD1 mRNA expression both in A549 cells (P<0.09) and in LTED cells (P=0.05; Supplemental Fig. S2). MXD1 encodes protein that belongs to a distinct subfamily of MAX-interacting proteins. MXD1 and MYC compete for binding to MAX (33). MXD1 inhibits cellular proliferation by repressing S-phase progression in resting fibroblasts (34, 35). Thus, it might be plausible that PLZF inhibited cell proliferation by regulating MXD1 expression.

Frequent down-regulation of PLZF may play an important role in NSCLC tumorigenesis by promoting cell survival. Meanwhile, because the transfection efficiency of the normal human lung epithelial cell line Beas-2B was low (Supplemental Fig. S2), and the cellular proliferation and apoptosis had not been changed when PLZF was overexpressed by transfection, H293t, a human embryonic kidney cell line, was also used to confirm the effects of overexpression of PLZF on normal cells. The results indicated that overexpression of PLZF did not affect proliferation, cell cycle progression, and apoptosis in H293t cells (Supplemental Fig. S2). It seems that lung cancer cells were more sensitive to PLZF overexpression than normal cells. The restoration of PLZF expression may serve as a new strategy for NSCLC therapy.

The authors thank Prof. Jian Yu (State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai Cancer Institute, Shanghai, China) for providing MBD-Seq data of NSCLC and normal lung tissues. This study was partially supported by the grants from the National Science Foundation of China (NSFC; 81172228), and National S&T Major Special Project (2011ZX09102-010-01). J.-C.W. and L.J. were supported by the grants from the National Science Foundation of China (81270120, 31271338).

REFERENCES


4202  Vol. 27  October 2013  The FASEB Journal · www.fasebj.org  WANG ET AL.


Received for publication February 5, 2013.

Accepted for publication June 18, 2013.