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Global DNA hypomethylation coupled to cellular transformation and metastatic ability

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ABSTRACT

Global DNA hypomethylation and DNA hypermethylation of promoter regions are frequently detected in human cancers. Although many studies have suggested a contribution to carcinogenesis, it is still unclear whether the aberrant DNA hypomethylation observed in tumors is a consequence or a cause of cancer. Here, we show that the enforced expression of Stella (also known as PGC7 and Dppa3) induced not only global DNA demethylation but also transformation of NIH3T3 cells. Furthermore, overexpression of Stella enhanced the metastatic ability of B16 melanoma cells, presumably through the induction of metastasis-related genes. These results provide new insights into the function of global DNA hypomethylation in carcinogenesis.

1. Introduction

DNA methylation is important for epigenetic gene regulation and implicated in many biological processes, such as early embryonic development, cell differentiation, and zygotic and somatic cell reprogramming [1,2]. In addition, the pathogenesis of many types of disease, including cancer, has been suggested to be affected by DNA methylation status [2,3]. Circumstantial evidence concerning the DNA methylation status in malignant tumor cells is available; however, most is derived from clinical samples of established tumors. Little direct evidence of the precise pathogenic role of DNA methylation has been reported [4].

DNA methyltransferase 1 (DNMT1) mediates inheritance of DNA methylation patterns by daughter cells from parental cells [5]. It has been reported that Np95 is required for the recruitment of DNMT1 to hemi-methylated DNA generated at the replication fork during the S-phase [6], and that Np95 binds to hemi-methylated DNA through its SET and RING finger-associated (SRA) domain [7]. We have investigated the function of Stella, a maternal factor essential for early embryogenesis. Stella preserves the DNA methylation status in zygotes by binding to dimethylated lysine 9 of histone H3 (H3K9me2) [8–10]. Recently, we reported that overexpression of Stella induced global DNA hypomethylation through binding to Np95 and thus the subsequent recruitment of DNMT1 to hemi-methylated DNA [11].

In the present study, we found that enforced expression of Stella in NIH3T3 cells not only induced global DNA demethylation but also induced transformation of the cells. In addition, we found that the metastatic activity of B16 melanoma cells was increased by the expression of Stella. Although this unique experimental sys-

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tem is an artificial one, Stella-induced global DNA hypomethylation is a useful model that could facilitate investigation of the pathogenic role of DNA hypomethylation in cancer.

2. Materials and methods

2.1. Plasmids

Stella and Ha-Ras cDNAs were inserted into the pMY-IRES-EGFP retroviral vector or pWPI lentiviral vector [12]. For generation of shRNA constructs, oligonucleotides were cloned into the pLVTH vector. Oligonucleotide sequences are shown in Supplementary Table 1.

2.2. Cell culture and viral infections

NIH3T3 cells, B16F10 cells, 293T cells, and Plat-E packaging cells [13] were maintained in Dulbecco's modified Eagle's Minimum Essential Medium supplemented with 100 U/mL penicillin, streptomycin sulfate, and 10% fetal bovine serum (FBS). Retroviral and lentiviral infections were carried out as previously reported [11].

2.3. Soft agar assay

NIH3T3 cells, Ha-Ras-expressing NIH3T3 cells (45 days after infection), Stella-expressing NIH3T3 cells (45 days after infection), and EGFP-expressing NIH3T3 cells (45 days after infection) (3×10^3) were resuspended into single-cell suspensions in 3 mL of medium. The cell suspensions were mixed with 1 mL of 1.5% pre-warmed soft agar (BD Biosciences) and plated into 30-mm plates in triplicate. The colonies formed on the soft agar matrix were counted under a light microscope after 2 weeks.

2.4. Global DNA methylation analysis

Global DNA methylation status was measured as described previously [11].

2.5. In vivo growth assay

NIH3T3 cells, Ha-Ras-expressing NIH3T3 cells (45 days after infection), and Stella-expressing NIH3T3 cells (45 days after infection), (5×10^5) were injected subcutaneously into nude mice. After 2 weeks, the tumors were harvested and each tumor was weighed.

2.6. Wound healing assay

B16-F10, EGFP-expressing B16-F10, and Stella-expressing B16-F10 cells were grown to confluence in six-well culture plates. Cell layers were scraped with a sterile pipette tip and incubated at 37 °C with 5% CO_2. Migration from the edge of the injured monolayer was quantified by measurement of the distance between the wound edges and the recovered edges.

2.7. Metastasis assay

B16-F10, EGFP-expressing B16-F10, and Stella-expressing B16-F10 cells (2×10^5) were suspended in 200 μL of PBS and injected in the lateral tail vein of C57BL/6 mice. Lungs were harvested 14 days post-injection and fixed in formalin. Metastases were counted and subjected to statistical analysis (one-way ANOVA).

2.8. Microarray array analysis

Microarray analysis was performed in NIH3T3 cells including two independent Stella-expressing NIH3T3 clones and a non-clonal Stella-expressing NIH3T3 cell population, and in B16-F10 cells including three independent Stella-expressing B16-F10 clones. Total RNA was extracted from cells using an RNeasy Total RNA Mini Kit (Qiagen). RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturer's instructions. Signal values and detection calls for all samples were determined using MAS5.0 (Affymetrix). Further analyses were performed with the Subio Platform version 1.15 plug-in software (Amami). To normalize the variations in staining intensity among chips, the signal values for all probes on a given chip were divided by the 75th percentile value for expression of all genes on the chip. To eliminate changes within the range of background noise, we used probes the raw signal values of which were >50 in at least any one sample. The microarray data have been deposited in the Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/) and given the series accession number GSE68837.

2.9. Genome-wide profiling of promoter methylation by the Microarray-based Integrated Analysis of Methylation (MIAMI) method

Genome-wide profiling and promoter methylation status of Stella-expressing NIH3T3 cells were evaluated using Microarray-based Integrated Analysis of Methylation (MIAMI) method [14]. Briefly, genomic DNA was digested with methylation-sensitive *Hpa*II or the methylation-insensitive isoschizomer *Msp*I. The digested genomes were ligated with adaptor, and amplified by PCR with primers designed against the adapter sequences. The samples were then further digested with *Hpa*II and *Msp*I, and amplified again with the same primers. The amplified products were then labeled with Cy3 or Cy5 and co-hybridized to a microarray. After hybridization, the microarray was scanned, and the obtained fluorescence intensities were quantified and normalized. The same pooled samples were treated first with *Msp*I instead of *Hpa*II and analyzed on a duplicate array to correct for false-positives caused by single nucleotide polymorphisms or incomplete digestion. Unchanged, DNA hyper-, and hypo-methylation of each genomic regions are indicated by "0", "+1", and "−1" in column AB, respectively (Supplementary Table 2).

2.10. RT-PCR

Total RNAs prepared from cells were treated with DNase I, and subjected to RT-PCR using the ThermoScript RT-PCR system (Invitrogen) and random hexamers as primers for cDNA synthesis. The primers used in this study are shown in Supplementary Table 1. PCR were carried out following condition: 5 min at 95 °C followed by 24 and 26 cycles (for *IAP* and *Line 1*), 35 cycles of (for *Arhgap20*, *Smagp*, and *Notch3*), or 30 cycles (for *Gapdh*) of PCR consisting of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C.

2.11. Bisulfite sequence analysis

B16 melanoma cells and Stella-expressing clones were treated with bisulfite using an EZ DNA Methylation-Direct Kit (Zymo Research). Sequences of the PCR primers are listed in Supplementary Table 1. PCR amplification of *Arhgap20*, *Smagp*, and *Notch3* promoter regions was carried out using EpiTaq HS (Takara) under the following conditions: 2 min at 95 °C followed by 35 cycles of PCR consisting of 20 s at 98 °C, 30 s at 56 °C, and 30 s at 72 °C. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen), cloned into the pGEM-T Easy Vector (Promega), and then