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Human DNA methyltransferase gene-transformed yeasts display an inducible flocculation inhibited by 5-aza-2′-deoxycytidine

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ABSTRACT

Mammalian DNA methyltransferases (DNMTs) play an important role in establishing and maintaining the proper regulation of epigenetic information. However, it remains unclear whether mammalian DNMTs can be functionally expressed in yeasts, which probably lack endogenous DNMTs. We cotransformed the budding yeast *Saccharomyces cerevisiae* with the human *DNMT1* gene, which encodes a methylation maintenance enzyme, and the *DNMT3A/3B* genes, which encode *de novo* methylation enzymes, in an expression vector also containing the *GAL1* promoter, which is induced by galactose, and examined the effects of the DNMT inhibitor 5-aza-2′-deoxycytidine (5AZ) on cell growth. Transformed yeast strains grown in galactose- and glucose-containing media showed growth inhibition, and their growth rate was unaffected by 5AZ. Conversely, 5AZ, but not 2′-deoxycytidine, dose-dependently interfered with the flocculation exhibited by *DNMT*-gene transformants grown in glucose-containing medium. Further investigation of the properties of this flocculation indicated that it may be dependent on the expression of a Flocculin-encoding gene, *FLO1*. Taken together, these findings suggest that *DNMT*-gene transformed yeast strains functionally express these enzymes and represent a useful tool for *in vivo* screening for DNMT inhibitors.

1. Introduction

The methylation of DNA, an epigenetic modification, is of central importance for normal cellular development, and changes in DNA methylation may affect the development of cancer [1]. The methylation of cytosine residues in nuclear DNA, a common form of DNA methylation found predominantly in cytosine–phosphate–guanine (CpG) dinucleotides, is an important epigenetic event [2]. Three catalytically active DNA methyltransferases (DNMTs) are involved in the establishment and maintenance of DNA methylation patterns in mammalian cells. DNMT1, which preferentially methylates hemi-methylated DNA, is critical for maintaining DNA methylation patterns [3]. Conversely, DNMT3A and DNMT3B, members of the DNMT3 family of *de novo* DNA methyltransferases, are involved in establishing DNA methylation patterns during normal embryonic and germ cell development and mainly act on cytosines within unmethylated CpGs [4,5].

5-Aza-2′-deoxycytidine (decitabine; 5AZ), an analog of the natural nucleoside 2′-deoxycytidine, is known to inhibit DNA methylation and is approved for the treatment of myelodysplastic syndrome [6,7]. 5AZ exerts an inhibitory effect on the activity of DNMTs, consequently promoting the reactivation of tumor suppressor genes silenced by DNA methylation [8]. 5AZ taken into cells is phosphorylated and incorporated into the DNA by DNA polymerase [9,10]. Subsequently, DNMTs become trapped by covalently binding to 5AZ, resulting in the depletion of DNMTs [11]. Therefore, generation of the phenotypes associated with 5AZ may facilitate the identification of novel DNMT inhibitors.

In most eukaryotes, DNA methylation occurs exclusively at cytosine residues. Reportedly, 80% of CpG dinucleotides in mammalian genomes are methylated [12]. In contrast, no *DNMT*-like genes are found in the genome of the budding yeast *Saccharomyces cerevisiae*, a model organism for eukaryotic cells [13]. The ease of genetic manipulation of *S. cerevisiae* predisposes it for use as a platform for the functional analysis of mammalian DNMTs. The development of a yeast cell-based system for detecting DNA methylation levels may provide an *in vivo* screening system to identify novel DNMT inhibitors. In this study, we created yeast strains

Abbreviations: 5AZ, 5-aza-2′-deoxycytidine; ACT1, β-actin; CpG, cytosine–phosphate–guanine; DNMT, DNA methyltransferase; OD_{600}, optical density at 600 nm; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction.

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transformed with plasmids encoding the human *DNMT1* and *DNMT3A*/*3B* cDNAs and revealed that these transformants displayed an inducible flocculation phenotype that could be inhibited by 5AZ. Furthermore, we discovered that significantly high mRNA transcript levels of *FLO1*, a Flocculin-encoding gene that causes nonsexual flocculation in yeast [14], are present in the transformants in a glucose-based medium, whereas the upregulation of *FLO1* mRNA is repressed by 5AZ. Here, for the first time, we report the creation of engineered yeasts that have gained the ability to respond to 5AZ.

2. Materials and methods

2.1. Strains, culture conditions, and plasmids

S. cerevisiae YPH250 (*MAT*a *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2–801 ade2–101 ura3–52*) was obtained from the Yeast Genetic Stock Center (University of California at Berkeley, CA, USA). Yeast cells were cultivated at 30 °C in a synthetic medium with a 0.67% yeast nitrogen base containing the appropriate amino acids and carbon sources (final concentration 2%). For 5AZ treatment, yeast cells were incubated with specified amounts of 5AZ. To construct human DNMT1, DNMT3A, and DNMT3B expression plasmids, the open-reading frame (ORF) regions of each gene were amplified by polymerase chain reaction (PCR) and inserted into the multiple cloning site of the expression vectors pYES2/CT or pYES3/CT (Invitrogen, Carlsbad, CA, USA). Construction of the expression plasmids for each DNMT proceeded as follows. The DNMT1 expression plasmid, pY2CThD1, was constructed by cloning a fragment encoding the *DNMT1* ORF, amplified from *DNMT1* cDNA (BC144093), into the pYES2/CT vector by PCR using the primers 5′-CCATCGATAAAAAAATGCCGGCGCGTACCGCCCCAGC-3′ and 5′-GGGAATTCCTAGTCCTTAGCAGCTTCCT-3′. The DNMT3A expression plasmid, pY3CThD3A, was constructed by cloning a fragment encoding the *DNMT3A* ORF, amplified from *DNMT3A* cDNA (BC043617), into the pYES3/CT vector by PCR using the primers 5′-CCATCGATAAAAAAATGCCCGCCATGCCCTCCAGCGG-3′ and 5′-GGGAATTCTTACACACACGCAAAATACT-3′. The DNMT3B expression plasmid, pY3CThD3B, was constructed by cloning a fragment encoding the *DNMT3B* ORF, amplified from *DNMT3B* cDNA (BC111933), into the pYES3/CT vector by PCR using the primers 5′-CCATCGATAAAAAAATGAAGGGAGACACCAGGCATCT-3′ and 5′-GGGAATTCTCACATGCAAAGTAGTCCTT-3′. The constructed plasmids were verified by DNA sequencing.

2.2. Western blotting analysis

Transformed yeast cells were grown to the late logarithmic phase (approximate optical density at 600 nm [OD_{600}] = 4) in synthetic glucose-containing medium lacking uracil and tryptophan, then the cells were collected by centrifugation, washed, and resuspended at an OD_{600} of 0.4 in synthetic galactose-containing medium supplemented with appropriate requirements. Yeast cells harboring plasmids were cultivated for 0, 16 and 24 h. After centrifugation, cell pellets were rinsed once with 0.9% NaCl. Pellets were suspended in phosphate-buffered saline containing a protease inhibitor cocktail for yeast (Sigma–Aldrich Corporation, St. Louis, MO, USA). The suspension was vortexed with zirconium beads at 4 °C for 5 min, then centrifuged at 3500×*g* for 10 min to remove cell debris. To examine the expression of DNMT1, DNMT3A, DNMT3B, and β-actin (ACT1), we conducted Western blotting analysis using rabbit polyclonal anti-DNMT1 (ab19905; Abcam, Cambridge, UK), rabbit polyclonal anti-DNMT3A (SC-20703; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), mouse monoclonal anti-DNMT3B (IMG-184A; Imgenex Corporation, San Diego, CA, USA), and mouse monoclonal anti-β-actin (Abcam) antibodies. β-Actin was used as a loading control. Horseradish peroxidase-conjugated anti-rabbit/mouse Immunoglobulin G antibodies from Jackson Immunoresearch Labs (West Grove, PA, USA) were used as the secondary antibodies. The signals were visualized using Immunostar LD (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.3. Measurement of growth rate and flocculation

Transformed yeast cells were cultured in synthetic glucose- or galactose-containing minimal medium, with or without 100 μM 5AZ. Cell growth was monitored by measuring OD_{600}. To measure flocculation ability, culture tubes allowed to settle for 5 min were photographed from beneath and the diameters of both floc (F) and tube (T) were measured. Relative flocculation activity was calculated using the following equation:

$$\text{Relative flocculation activity} = 100 \times (F/T)$$

2.4. Semi-quantitative reverse-transcription polymerase chain reaction

Yeast cells grown to the late logarithmic phase in the glucose-containing medium were harvested. Total RNA was prepared by the glass bead method using an RNeasy Kit (Qiagen N.V., Venlo, Limburg, The Netherlands) with RNase-free DNase treatment, according to the manufacturer's instructions. Total RNA (0.5 μg) was subjected to reverse-transcription (RT)-PCR using the SuperScript® One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). The following primer pairs were used: *FLO1*, 5′-CTCATCGCTATATGTTTTTGG-3′ (forward) and 5′-CGAGTAAACAACCTTCATTGG-3′ (reverse); *FLO11*, 5′-GTCACGACGGCTATTCCAACCACAGTTATTACC-3′ (forward) and 5′-GAATACAACTGGAAGAGCGAGTAGCAACCAC-3′ (reverse); and *ACT1*, 5′-ATTCTGAGGTTGCTGCTTTGG-3′ (forward) and 5′-GAAGATTGAGCAGCGGTTTGC-3′ (reverse).

2.5. Statistical analysis

Statistical tests comparing multiple groups were performed using one-way analysis of variance followed by Dunnett's *post hoc* test. Data are expressed as means ± the standard error of the mean.

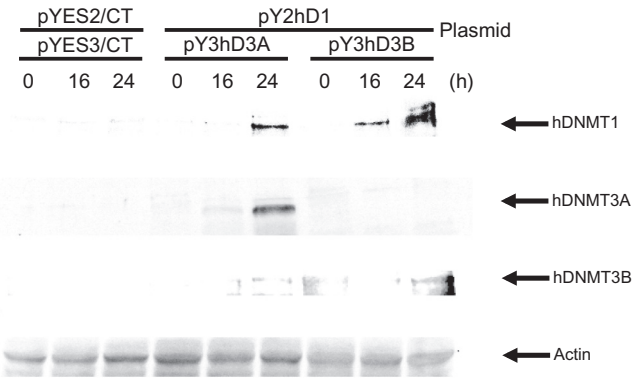

Fig. 1. Levels of expression of DNA methyltransferases. Cells were grown to the late logarithmic phase (an optical density at 600 nm [OD_{600}] of 3–4) in liquid synthetic glucose-containing medium lacking uracil and tryptophan. Cells were collected by centrifugation, washed, and adjusted to an OD_{600} of 0.4 with synthetic galactose-containing medium lacking uracil and tryptophan. The cells were further cultured for 16 or 24 h. The extracts were prepared and analyzed for DNA methyltransferase (DNMT) 1, DNMT3A, DNMT3B, and β-actin proteins by Western blotting. The results are representative of three independent experiments.