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Methylnitrosourea-induced Tumorigenesis in *MGMT* Gene Knockout Mice[1]

Kunihiko Sakumi, Akiko Shiraishi, Seiichiro Shimizu, Teruhisa Tsuzuki, Takatoshi Ishikawa, and Mutsuo Sekiguchi[2]

Department of Biochemistry, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-82 [K. S., A. S., T. T., M. S.]; Department of Pathology, Faculty of Medicine, The University of Tokyo, Tokyo 113 [S. S., T. I.]; and Department of Biology, Fukuoka Dental College, Fukuoka 814-01 [M. S.], Japan

ABSTRACT

Gene targeting was used to obtain mice defective in the *MGMT* gene, encoding O^6-methylguanine-DNA methyltransferase [Tsuzuki *et al.*, Carcinogenesis (Lond.), *17:* 1215–1220, 1996]. These $MGMT^{-/-}$ mice were most sensitive to the alkylating carcinogen, methylnitrosourea; when varied doses of methylnitrosourea were administered to 6-week-old mice and survivals at the 30th day were determined, LD_{50}s of $MGMT^{-/-}$ and $MGMT^{+/+}$ mice were 20 and 240 mg/kg of body weight, respectively. $MGMT^{+/-}$ mice were as resistant as $MGMT^{+/+}$ mice, but some difference in survival time was noted when the two genotypes of mice were exposed to a relatively high dose of methylnitrosourea. A large number of thymic lymphomas, as well as lung adenomas, occurred in $MGMT^{-/-}$ mice exposed to methylnitrosourea at a dose of 2.5 mg/kg of body weight. In case of exposure to the same dose of drug, no or few tumors occurred in the $MGMT^{+/+}$ and $MGMT^{+/-}$ mice. It appears that the DNA repair methyltransferase protein protected these mice from methylnitrosourea-induced tumorigenesis.

INTRODUCTION

Alkylation of DNA at the O^6 position of guanine is a critical event leading to induction of mutations and cancers (1, 2). Once O^6-methylguanine is formed, it can pair with thymine during DNA replication, with the result that there is a conversion of guanine-cytosine to adenine-thymine pairs in DNA (3). Such mutations are often present in tumors induced by alkylating agents (4). To counteract such effects, organisms possess a mechanism to repair O^6-methylguanine in DNA (5). An enzyme, O^6-methylguanine-DNA methyltransferase, catalyzes the transfer of a methyl group from O^6-methylguanine, as well as a minor methylated base, O^4-methylthymine, in DNA to the cysteine residue of its own molecule, thereby repairing the DNA lesions in a single-step reaction (6, 7). Because this reaction irreversibly inactivates the enzyme, the capacity for repair of the O^6-methylguanine adduct depends on the number of active enzyme molecules per cell.

The amounts of methyltransferase protein contained in the cell vary with the tissue, and it was reported that more tumors formed in tissues with less methyltransferase when animals were administered alkylnitrosourea (8). Some human tumor-derived cell lines are hypersensitive to alkylating agents, and these cell lines, termed Mer^- or Mex^-, have little or no methyltransferase activity (9). It was suspected that this methyltransferase deficiency might possibly explain the frequent occurrence of tumors, in certain cases, although methyltransferase deficiency is in most cases the consequence of cellular transformation (10).

To elucidate the roles of methyltransferase in carcinogenesis, appropriate animal models with altered levels of the enzyme activity are needed. Mice carrying extra copies of the bacterial *methyltransferase* gene were generated, and in the case of nitrosamine-induced hepatocarcinogenesis, such mice had a smaller number of tumors compared with normal mice treated in the same manner (11). Pronounced protective effects on MNU[3]-induced tumor formation in thymus and colon were observed in transgenic mice expressing cDNA for human methyltransferase (12, 13). Thus, the increasing levels of methyltransferase in tissues were shown to limit susceptibility of tissues to alkylating carcinogens. However, in these experiments involving transgenic mice, the suppressive effects on tumor formation were evident only when relatively high doses of alkylating agents were used. Moreover, as levels of expression of the transgenes vary with tissues, it is difficult to define the protective effects of the enzyme.

Such being the case, the acquisition of mice defective in their own *methyltransferase* gene would be helpful. The mouse *MGMT* gene, encoding the methyltransferase, was cloned, and the structure was elucidated (14, 15). We have now made use of targeted mouse gene and have generated mice deficient in methyltransferase activity (16). Using such mice, we carried out alkylation-induced tumorigenesis experiments.

MATERIALS AND METHODS

Gene-targeted Mice. Development of *MGMT* gene-disrupted mice was as described (16). In brief, the targeting vector contained an 8.0-kb genomic sequence interrupted in exon 2 by a *polII-neo*-poly(A) cassette, flanked by a pair of *herpes simplex virus thymidine kinase* genes. The construct was electroporated into CCE ES cells, and cells showing resistance to both G418 and ganciclovir were selected. Chimeras were produced by microinjecting these cells into 3.5-day-old C57BL/6J blastocysts and transplanting the embryos into the uteri of Jcl:MCH (ICR) pseudopregnant females. Chimeric males were tested for germ-line transmission of the agouti coat phenotype of 129/Sv-derived cells. By Southern blot hybridization, heterozygous F1 mice, $MGMT^{+/-}$, were identified, and pairs were crossed to acquire $MGMT^{-/-}$ mice. $MGMT^{+/+}$ and $MGMT^{-/-}$ mice were maintained by line-breeding, and $MGMT^{+/-}$ mice were produced by crosses of the homozygous mice.

Sensitivity to MNU. MNU was purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in PBS. Six-week-old mice were given an i.p. injection of 200 μl of MNU at defined concentrations. As controls, mice were given an i.p. injection of 200 μl of PBS. Mice were kept for defined periods, and survivors were scored.

Tumorigenesis Experiments. To examine MNU-induced tumorigenesis, mice were given a single i.p. injection of 2.5 mg of MNU/kg of body weight on the 14th day of birth. In certain cases, a higher dose of MNU (50 mg/kg of body weight) was given to 6-week-old $MGMT^{+/+}$ and $MGMT^{+/-}$ mice. Twenty to 28 weeks after injection, these mice were killed, and tissues were examined. Tissues were fixed in 10% buffered formalin and paraffin-embedded sections of the tissues, 3 μm in thickness, were stained with H&E.

RESULTS

Administration of MNU to Gene-targeted Mice. Mice carrying different numbers of intact *MGMT* alleles were generated by crosses of *MGMT* gene-targeted mice, and sensitivities to MNU were examined. Groups of mice with +/+, +/−, and −/− backgrounds, each consisting of about 50 animals (6 weeks old), were given a single i.p. injection of MNU (50 mg/kg of body weight). As shown in Fig. 1, there was a distinct difference in survival rate of the three genotypes of mice. Death of $MGMT^{-/-}$ mice occurred as early as 8 days after MNU administration, and all of the animals with this genotype died within 17 days, a finding essentially as described earlier (16). On the other hand, all of the $MGMT^{+/+}$ and $MGMT^{+/-}$ mice survived at least 70 days after the

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[2] To whom requests for reprints should be addressed, at Department of Biology, Fukuoka Dental College, Fukuoka 814-01, Japan.

[3] The abbreviations used are: MNU, methylnitrosourea; ES, embryonic stem.

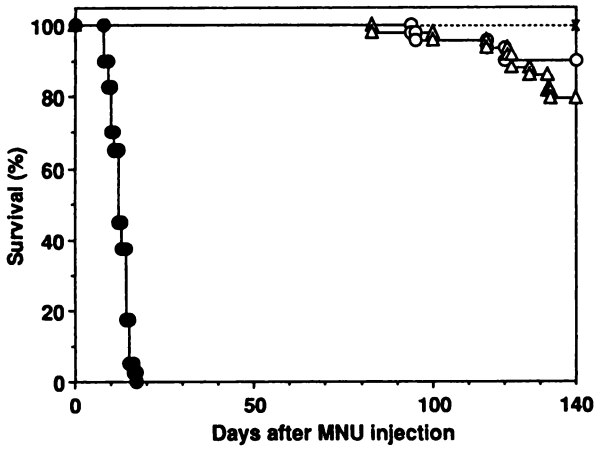

Fig. 1. Survival curves of mice after MNU administration. Forty-nine $MGMT^{+/+}$, 49 $MGMT^{+/-}$, and 40 $MGMT^{-/-}$ mice were given MNU (50 mg/kg of body weight) i.p. at postnatal day 42 (±1). ○, $MGMT^{+/+}$; △, $MGMT^{+/-}$; ●, $MGMT^{-/-}$. - - - - -, all PBS-injected $MGMT^{+/+}$, $MGMT^{+/-}$, and $MGMT^{-/-}$ mice survived.

Table 1 *Frequencies of tumors in $MGMT^{+/+}$ and $MGMT^{+/-}$ mice with or without MNU administration*

MGMT genotype	Treatment	Sex	No. of mice (%)		
			Total no. of mice examined	No. of mice with thymic lymphoma	No. of mice with lung adenoma
+/+	Saline	M[a]	5	0	0
		F	4	0	0
	MNU	M	25[b]	7 (28)	7 (28)
		F	24[b]	3 (13)	11 (46)
+/−	Saline	M	6	0	0
		F	6	0	0
	MNU	M	28[c]	11 (39)	15 (54)
		F	21[c]	10 (48)	9 (49)

[a] M, male; F, female.
[b] Four males and one female, which died by the 20th week after MNU injection, were included.
[c] Four males and six females, which died by the 20th week after MNU injection, were included.

treatment, after which about 90% of $MGMT^{+/+}$ and 80% of $MGMT^{+/-}$ mice survived until the 140th day. As controls, PBS was injected into the three types of mice (+/+, 9 mice; +/−, 12 mice; −/−, 21 mice), and all survived for over 3 months.

The mice were killed 20 weeks after MNU administration, and organs were examined for tumor formation (Table 1). MNU-administered mice, either +/+ or +/− genotype, carried a number of tumors, whereas no tumor was found in control mice given no drug. Although a smaller number of thymic lymphomas were formed in $MGMT^{+/+}$ mice as compared with $MGMT^{+/-}$ mice, the difference was not statistically significant. In the case of lung adenoma, no difference was observed in the two genotypes of mice.

Tumor Formation in MGMT-deficient Mice. To determine the sublethal doses of MNU which lead to tumor formation, different doses of MNU were administered to the $MGMT^{+/+}$ and $MGMT^{-/-}$ mice. Fig. 2 shows the survival rates on the 30th day after MNU administration. LD_{50}s of $MGMT^{-/-}$ and $MGMT^{+/+}$ mice was 20 and 240 mg/kg of body weight, respectively. On the basis of these observations, we did the following tumorigenesis experiments.

Mice with three different *MGMT* genotypes (about 50 each) were given a single i.p. injection of MNU (2.5 mg/kg of body weight) 14 days postnatally. Of 52 $MGMT^{-/-}$ mice, 3 died within 28 weeks after MNU administration, with thymic lymphoma. All of the remaining animals were killed 28 weeks after MNU injection, and organs were examined. Our observations are summarized in Table 2.

Thymic lymphomas were present in 7 of 49 $MGMT^{-/-}$ mice, and in most cases (6 of 7), there was lymphoma infiltration to other tissues, including lung, spleen, liver, kidney, ovary, lymph nodes, pancreas, and heart. Fig. 3*A* shows a typical lymphoma, found in an $MGMT^{-/-}$ mouse. The tumor covered both lung and heart with effusion in the thoracic cavity and weighed 0.8 g; that is, it 26 times heavier than that of the normal mouse thymus. As shown in Fig. 3*B*, the tumor is composed of diffuse proliferation of lymphoma cells, some of which seem to express apoptotic morphology ("starry sky" appearance). Lymphoma cell infiltrations were evident in the liver (Fig. 3*C*). There was no evidence of lymphoma in any of the $MGMT^{+/+}$ and $MGMT^{+/-}$ mice examined.

Lung adenomas were frequent in the MNU-treated $MGMT^{-/-}$ mice, but there were only a few in $MGMT^{+/+}$ and $MGMT^{+/-}$ animals treated in the same manner. Of 11 $MGMT^{-/-}$ mice carrying adenomas, 9 had one tumor and 2 had three and four tumors. Fig. 3, *D* and *E*, shows findings of a lung adenoma in $MGMT^{-/-}$ mouse.

Thus, it is clear that mice with a defect in the *MGMT* gene are most susceptible to MNU-induced tumorigenesis. There is no apparent difference in susceptibilities to MNU of $MGMT^{+/+}$ and $MGMT^{+/-}$ mice, under our experimental setup.

DISCUSSION

Mouse lines deficient in the *methyltransferase* gene were established by gene targeting (16). Tissues from these gene-targeted mice contained essentially no methyltransferase protein, and administration of MNU to these mice led to early death, whereas in normal mice treated in the same manner, there were no untoward effects. In the methyltransferase-deficient mice given MNU treatment, the bone marrow became hypocellular,

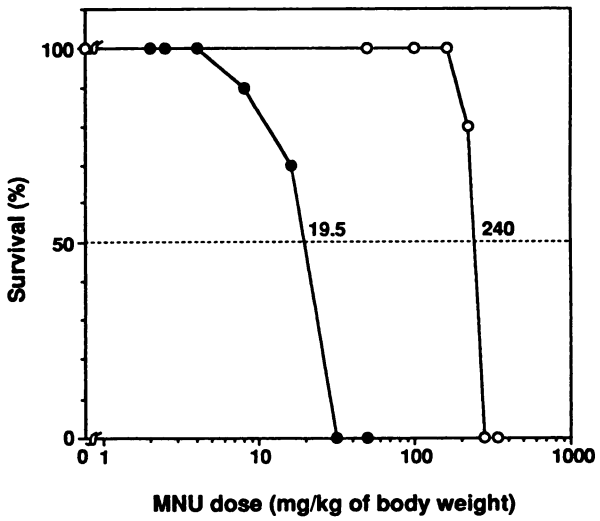

Fig. 2. Survival of mice given different doses of MNU. Groups of $MGMT^{+/+}$ and $MGMT^{-/-}$ mice (at least 10 per group) were given different amounts of MNU i.p. on postnatal day 42 (±1). Survival rates at the 30th day after injection were plotted. ○, $MGMT^{+/+}$; ●, $MGMT^{-/-}$.

Table 2 *Tumor induction by MNU in mice with different MGMT alleles*

MGMT genotype	Sex	Total no. of mice examined	No. of mice with thymic lymphoma	No. of mice with lung adenoma	No. of lung adenomas per animal
+/+	M[a]	29	0	0	0
	F	23	0	1 (4%)	<0.1
+/−	M	22	0	1 (5%)	<0.1
	F	21	0	1 (5%)	<0.1
−/−	M	25[b]	2 (8%)	7 (28%)	0.5
	F	27[b]	5 (19%)	4 (15%)	0.1

[a] M, male; F, female.
[b] One male and two females, which died by the 28th week after MNU injection were included.

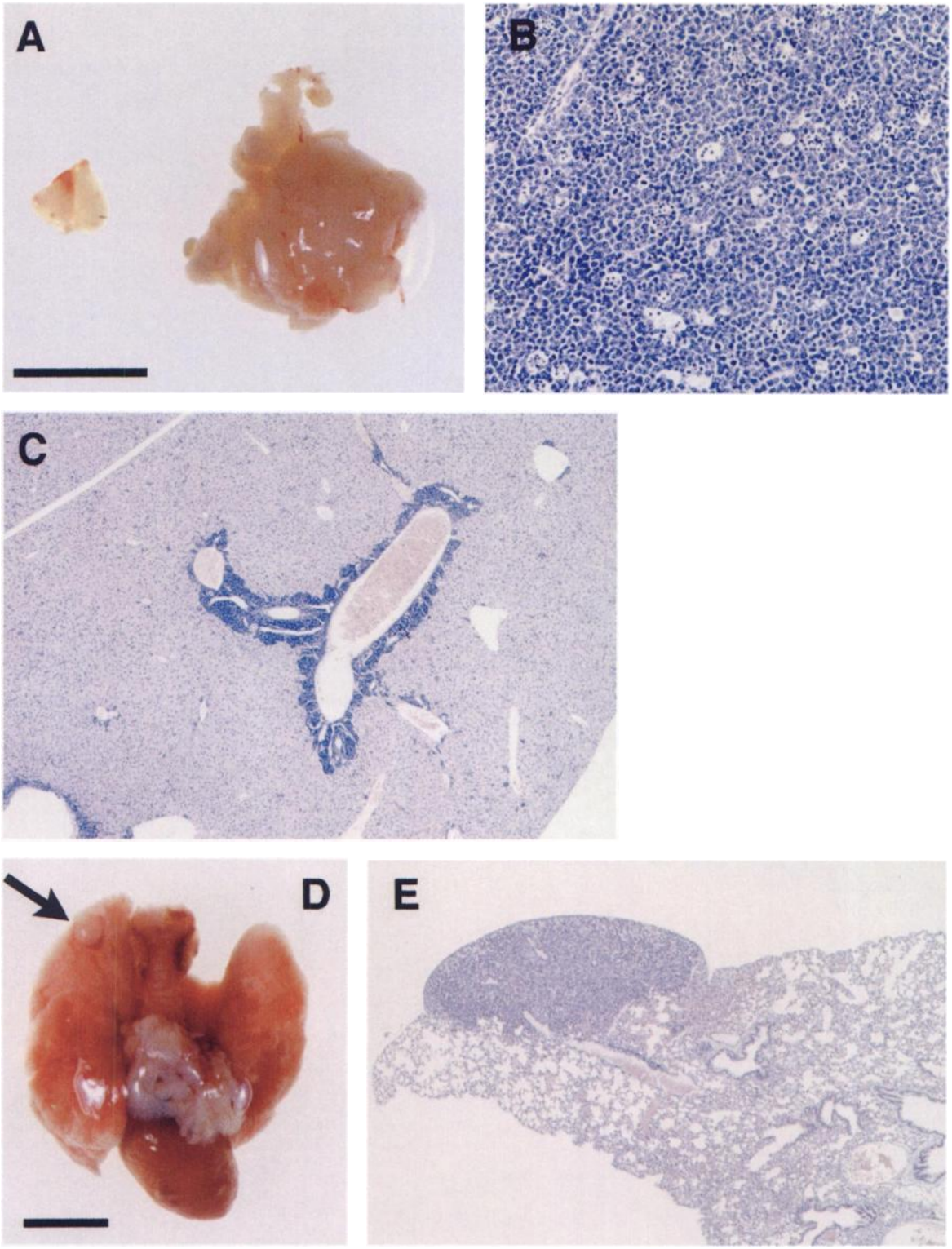

Fig. 3. Thymic lymphoma and lung adenoma formed in MNU-administered $MGMT^{-/-}$ mice. Twenty-eight weeks after MNU (2.5 mg/kg of body weight) injection, the mice were killed. *A, left*, a normal thymus; *right*, thymic lymphoma. *Scale bar*, 10 mm. *B*, histology of thymic lymphoma (H&E, ×50). *C*, histology showing lymphoma cell infiltration into liver (H&E, ×10). *D*, lung adenoma (*arrow*). *Scale bar*, 5 mm. *E*, histology of lung adenoma (H&E, ×10).

and there was a drastic decrease in the number of leukocytes and platelets, thereby indicating an impaired reproductive capacity of hematopoietic stem cells. We considered the possibility that methyltransferase protected these mice from the pancytopenia caused by the alkylating agent.

Gene-targeted mice have been used in the present study to observe the putative protective role of methyltransferase in alkylation carcinogenesis. We first determined the levels of MNU that allow for normal growth of methyltransferase-deficient mice and selected 2.5 mg of MNU/kg of body weight as the proper dose to be given to mice in long-term tumorigenesis experiments. The so-treated $MGMT^{-/-}$ mice had numerous thymic lymphomas, as well as lung adenomas, whereas no lymphomas and only a few adenomas were formed in $MGMT^{+/+}$ and $MGMT^{+/-}$ mice. These observations can be taken as evidence that, among various methylated bases of DNA formed by the

action of alkylating agent, O^6-methylguanine and O^4-methylthymine are the primary lesions responsible for induction of tumors. The cellular methyltransferase enzyme plays a vital role in preventing mutation caused by these methylated bases and reduces, in turn, the frequency of malignant transformation of cells.

Mouse ES cell lines defective in the *methyltransferase* gene were established, and using these cell lines, $MGMT^{+/-}$ cells were seen to contain about half the amount of methyltransferase protein carried by $MGMT^{+/+}$ cells, whereas $MGMT^{-/-}$ cells contained no protein.[4] When the colony-forming abilities of these cells after exposure to alkylating agents were determined, the LD_{37}s of $MGMT^{+/+}$, $MGMT^{+/-}$, and $MGMT^{-/-}$ cells were 11.3, 7.3, and 0.11 μM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, respectively. This relationship may indicate the sensitivities of the three types of mice to MNU. However, in the present study, there was little or no difference between $MGMT^{+/-}$ and $MGMT^{+/+}$ mice in terms of both lethality and tumorigenic effects of MNU. To observe such subtle difference in the two types of mice, we might need more defined experimental protocols.

Because methyltransferase-deficient cells and animals are hypersensitive to alkylating agents, O^6-methylguanine and O^4-methylthymine may also be responsible for the cell killing evoked by the alkylation of DNA. The toxicity of such lesions was attributed to the inappropriate processing of mismatch repair because mutations in the mismatch recognition genes in *Escherichia coli dam*$^-$ strains confer protection against the toxicity of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (17, 18). Involvement of the methylated bases in mammalian cell lethality was deduced from the observation that nicks persisted in the DNA of methyltransferase-deficient human cells that had been exposed to alkylating agents (19). Recent studies indicated that an acquired resistance (methylation tolerance) of mammalian methyltransferase-deficient cell lines is associated with the loss of capacity for mismatch repair (20, 21). It has been proposed that the accumulation of alkylated bases in chromosomal DNA may provoke abortive mismatch repair, thereby leading to cell death. Mice defective in one of the mismatch-repair genes have been developed (22, 23), and it is now possible to construct mice defective in both methyltransferase and mismatch repair.

It has been proposed that one early step in the progression of human tumors is the elevation in the rate of spontaneous mutation, and this argument is based on findings that the progression of many human tumors is accompanied by an accumulation of a large number of mutations (24–26). Thus, if changes in spontaneous mutation rates are indeed involved in carcinogenesis, it is important to define pathways that influence spontaneous mutation rates in mammalian cells. There are reports describing the formation of O^6-methylguanine and O^4-methylthymine in the DNA of normally growing cells (27–30). By making use of methyltransferase-deficient mice, one could evaluate the extent of an endogenous alkylation-induced DNA lesion that would lead to mutation, in the absence of repair. The availability of mutant mice lacking methyltransferase would pave the way toward events related to spontaneous carcinogenesis.

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