

ORIGINAL ARTICLE

Overexpression of Rev1 promotes the development of carcinogen-induced intestinal adenomas via accumulation of point mutation and suppression of apoptosis proportionally to the Rev1 expression level

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Abstract

Cancer development often involves mutagenic replication of damaged DNA by the error-prone translesion synthesis (TLS) pathway. Aberrant activation of this pathway plays a role in tumorigenesis by promoting genetic mutations. Rev1 controls the function of the TLS pathway, and Rev1 expression levels are associated with DNA damage induced cytotoxicity and mutagenicity. However, it remains unclear whether deregulated Rev1 expression triggers or promotes tumorigenesis *in vivo*. In this study, we generated a novel Rev1-overexpressing transgenic (Tg) mouse and characterized its susceptibility to tumorigenesis. Using a small intestinal tumor model induced by *N*-methyl-*N*-nitrosourea (MNU), we found that transgenic expression of Rev1 accelerated intestinal adenoma development in proportion to the Rev1 expression level; however, overexpression of Rev1 alone did not cause spontaneous development of intestinal adenomas. In Rev1 Tg mice, MNU-induced mutagenesis was elevated, whereas apoptosis was suppressed. The effects of hREV1 expression levels on the cytotoxicity and mutagenicity of MNU were confirmed in the human cancer cell line HT1080. These data indicate that dysregulation of cellular Rev1 levels leads to the accumulation of mutations and suppression of cell death, which accelerates the tumorigenic activities of DNA-damaging agents.

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Abbreviations

Hgb	hemoglobin
MNU	N-methyl-N-nitrosourea
TLS	translesion synthesis

Introduction

Cancer development involves accumulation of genetic changes that leads to the malignant transformation of normal cells. Mutations induced by DNA-damaging agents are closely associated with specialized translesion synthesis (TLS) DNA polymerases (1,2). Many TLS polymerases belong to the Y-family of DNA polymerases, which can replicate damaged nucleotides in an error-prone as well as error-free fashion (2,3). Four individual Y-family polymerases have been identified in vertebrates: Pol η , Pol ι , Pol κ and Rev1 (1–4). Mutations in genes encoding these polymerases may increase susceptibility to mutagenesis and cancer (5–9), as has been demonstrated for Pol η (10,11).

Rev1 is a key factor in error-prone TLS (12). Although Rev1 is a deoxycytidyltransferase that incorporates deoxycytosines opposite structurally diverse damaged nucleotides (13–18), its most important function in error-prone TLS is regulatory rather than catalytic (1,2,14,19). Deletion of Rev1 results in sensitivity to a wide range of DNA-damaging agents, such as UV light, hydrogen peroxide, cisplatin and X-rays (20–24). DNA damage-induced cytotoxicity and mutagenicity depend on the expression level of hREV1 (24–28).

Owing to its importance in the response to DNA damage, hREV1 has attracted considerable interest in the field of cancer research. Mutations in hREV1 have been detected in a minority of tumors (29), and single-nucleotide polymorphisms in the gene have been linked to various types of human cancer (30–34). As with other TLS polymerases, upregulation of hREV1 is associated with the pathogenesis of human glioma (35,36). These data imply that dysregulation of cellular Rev1 levels is a critical step in *in vivo* tumorigenesis. To date, however, this hypothesis has not been directly evaluated. In addition, details of the mechanism by which Rev1 affects carcinogenesis *in vivo* are not available. In this study, we investigated the effect of deregulated Rev1 expression on susceptibility to tumorigenesis in a new transgenic mouse model.

Materials and methods

Generation of Rev1 transgenic mice

A DNA fragment containing the mouse metallothionein promoter (MT-1), Rev1 cDNA, and a simian virus 40 polyadenylation (SV40 polyA) signal were used to generate transgenic mice by pronuclear injection of C57BL/6 zygotes. Transgenic founders were identified by PCR and Southern blotting of tail genomic DNA. Transgene-positive founders were bred with wild-type (Wt) C57BL/6N mice to establish independent lines. All mice were housed in compliance with the guidelines of the Institute of Laboratory Animal Science, Hiroshima University.

Real-time RT-PCR and quantitative real-time RT-PCR

To determine the relative expression of Rev1 in various tissues, total RNA was purified from various tissues and reverse transcribed into cDNA. To quantitate levels of Rev1 mRNA expression in the small intestine, quantitative real-time RT-PCR was carried out on an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems) using Absolute SYBR Green Mix. The expression level of Rev1 mRNA was normalized against *Gapdh*, and all reactions were performed in triplicate.

Mice and tumor induction

Male C57BL/6N mice were purchased from Charles River (Hino, Japan). Starting at 3 weeks of age, Rev1 Tg and normal C57BL/6N male mice were

treated with 25 mM ZnSO₄ in drinking water to induce transgenic Rev1 expression. Beginning at 6 weeks of age, the mice were injected intraperitoneally with N-methyl-N-nitrosourea (MNU) (SIGMA, Japan), 50 mg/kg of body weight, once per week for 2 weeks. Mice were observed daily until becoming moribund, and then sacrificed under anesthesia and necropsied. Concentration of hemoglobin (Hgb) in peripheral blood was measured using a PCE-310 hygrometer (ERMA). Tissue was collected from MNU-treated animals for weight measurements and histology.

Scoring and classification of intestinal tumors

For macroscopic assessment of intestinal adenoma, mice were sacrificed 2 months after MNU treatment, and the small intestine was isolated. The small intestine was flushed with phosphate-buffered saline, opened longitudinally and examined using a binocular operation microscope. Tumors were counted following alkaline phosphatase staining.

Detection of mutations in the Ctnnb1 (β -catenin) gene

DNA was extracted from frozen intestinal adenoma using a DNA extraction kit. PCR primers were designed to amplify the region of the *Ctnnb1* gene encoding the consensus sequence for GSK-3 β phosphorylation. The PCR products were purified and sequenced.

Quantitation of apoptosis *in vivo*

At the indicated time points after injection, at least three mice were sacrificed. The small intestine was removed from each mouse, flushed with phosphate-buffered saline, and fixed in 10% formalin. Histological sections were prepared, and apoptosis was scored in intestinal crypts as reported previously (37). At least 50 half-crypts were scored. TUNEL assays were performed using the *In Situ* Apoptosis Detection Kit (TaKaRa, Japan). Slides were counterstained with hematoxylin.

Cell culture and the establishment of stable cell lines

The human fibrosarcoma cell line HT1080 was purchased from the ATCC (no. CCL121). Once resuscitated, the line was authenticated through monitoring of cell morphology and karyotype. Cells were grown under standard conditions in MEM supplemented with fetal bovine serum and antibiotics. Cells were transfected with FuGENE HD transfection reagent (Roche, Japan). HT1080 T-REx cells that express hREV1 protein were generated by transfection with pcDNA4/TO (Thermo Fisher Scientific K. K., Japan) carrying the hREV1 gene. Zeocin-resistant colonies were selected and screened for hREV1 protein by Western blotting. To induce hREV1, cells were incubated with 1 μ g/ml tetracycline 24 h before experiments started.

Colony-formation assays

Colony-formation assays were performed by seeding 300 cells in each of three 100 mm dishes. Twenty-four hours after seeding, cells were cultured with or without tetracycline to induce hREV1 expression for 24 h, and the indicated amounts of MNU were then added to the dishes. The medium was replaced 1 h later, and the cells were incubated for 8 days. Colonies were fixed, stained with crystal violet and counted.

HPRT assays

HPRT mutants were selected, and mutation frequencies were determined according to published protocols. Cells were pre-selected for functional HPRT by expanding the cultures for 2 days in medium supplemented with 1 \times HAT, and then cultured for an additional 3 days. Growing cells were treated with or without MNU. The cells were maintained in logarithmic growth phase by replating until they underwent 4–7 population doublings for mutation expression. Mutant selection was performed by replating cells at 3 \times 10⁵ cells/10 cm dish into medium containing 40 μ M 6-thioguanine. Colony-forming efficiency at the time of selection was determined by plating 300 cells/10 cm dish without 6-thioguanine (three dishes for each condition).

Statistical analyses

Statistically significant differences and exact p values of comparisons between survival curves were determined using the Logrank (Mantel-Cox)

test. In all other cases, t-tests (for two sets of data) or ANOVA analysis (for three or more sets of data) were performed using the StatMate III and GraphPad 6 software packages.

Results

Generation and characterization of Rev1 Tg mice

We generated Rev1 transgenic mice (Rev1 Tg) to determine whether overexpression of Rev1 would result in tumorigenesis. The metallothionein promoter 1 (MT-1) was used to achieve inducible Rev1 expression (Supplementary Figure 1a, available at *Carcinogenesis* Online). Four founder mice were identified, three of which passed the transgene on to their offspring (Supplementary Figure 1b, available at *Carcinogenesis* Online). All of the resultant Rev1 Tg mice were apparently normal regarding development, gross morphology, behavior and fertility, even when receiving ZnSO₄ in drinking water; hence, data reported

hereafter were obtained from mice treated with ZnSO₄ as described in Materials and Methods. Expression of the transgene was examined in multiple organs obtained from the Rev1 Tg T5 line, which expressed the highest level of the transgene. In male mice, which were used in the following experiments, Rev1 was expressed at low levels in the liver and kidney, but at dramatically higher levels in the thymus, spleen and lymph nodes (Figure 1a). The F₁ generation of the Rev1 Tg T5 line was inter-crossed to yield a Mendelian distribution of transgenic Rev1 Wt (Tg⁻/Tg⁻), Tg⁺/Tg⁻ and Tg⁺/Tg⁺ progeny. Pups homozygous (Tg⁺/Tg⁺) for the Rev1 transgene were selected for establishment of the colony designated as Rev1 Tg (Homo), whereas hemizygous (Tg⁺/Tg⁻) pups were used to establish the Rev1 Tg (Hemi) colony. MNU causes intestinal tumors in mice (38), making intestinal adenomas an attractive model in which to study MNU tumorigenesis. Expression of Rev1 in the small intestine was approximately twice as high in Rev1 Tg (Homo) as in Rev1

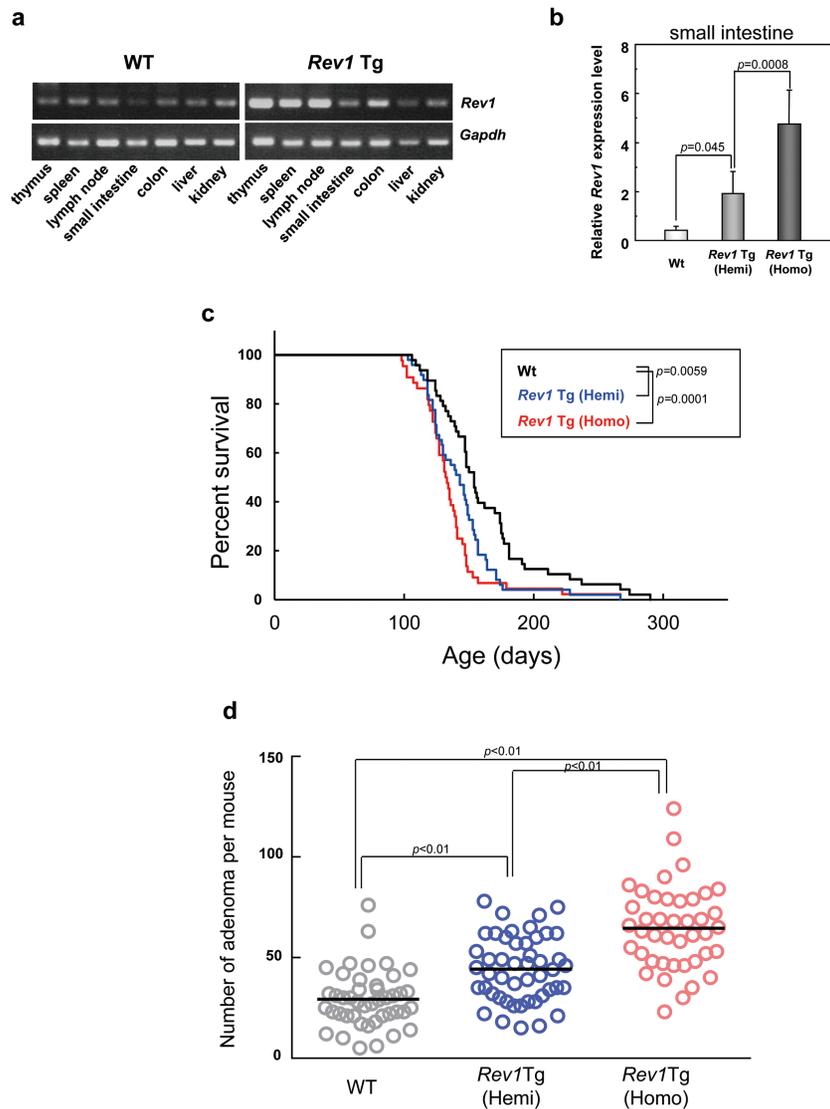


Figure 1. MNU-induced small intestinal tumorigenesis in Rev1 Tg mice. (a) Rev1 expression levels of wild-type and Rev1 Tg (Hemi) male mice were measured by semi-quantitative RT-PCR in indicated tissues. *Gapdh* was used as a loading control. (b) Relative Rev1 expression level in small intestines. Significant differences were observed between Wt and Rev1 Tg (Hemi) mice ($P = 0.046$), and between Wt and Rev1 Tg (Homo) mice ($P = 0.0008$). (c) Survival curves of wild-type (Wt), Rev1 Tg (Hemi) and Rev1 Tg (Homo) male mice [Wt; black line, Rev1 Tg (Hemi); blue line, and Rev1 Tg (Homo); red line] after repeated intraperitoneal injection of MNU (2×50 mg/kg of body weight at 6 and 7 weeks of age). Significant differences were observed between Wt and Rev1 Tg (Hemi) mice ($P = 0.0059$), and between Wt and Rev1 Tg (Homo) mice ($P = 0.0001$). (d) Numbers of adenomas per mouse in Wt (gray), Hemi (blue) and Homo (red) mice. Each circle indicates the result from each mouse.

Tg (Hemi) mice (Figure 1b), confirming that intestinal adenomas were a suitable model for MNU tumorigenesis in Rev1 Tg mice. Rev1 Tg (Homo) and Rev1 Tg (Hemi) mice were used in subsequent experiments to determine whether overexpression of Rev1 could contribute to small intestine tumorigenesis.

Accelerated MNU-induced intestinal tumorigenesis in Rev1 Tg mice

To determine whether overexpression of Rev1 influences spontaneous tumor initiation and progression, we monitored cohorts of Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice over their lifespan (>2 years). No significant effect on overall survival or tumor incidence was observed in the Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice, suggesting that overexpression of Rev1 by itself is not sufficient to stimulate tumorigenesis (data not shown).

To determine the effect of the Rev1 transgene on tumorigenesis induced by MNU, we monitored MNU-treated Rev1 Tg (Homo), Rev1 Tg (Hemi) and Wt males until they became moribund. As shown in Figure 1c, the lifespans of MNU-treated Rev1 Tg (Homo) and Rev1 Tg (Hemi) mice were slightly but significantly shorter than those of Wt mice treated with a similar dose of MNU. Mice were sacrificed after they became moribund, and adenomas in the intestine were counted and measured. The average number of intestinal adenomas after MNU treatment, as well as the mean adenoma diameter, increased in the following order: Wt mice, Rev1 Tg (Hemi) and Rev1 Tg (Homo), in accordance with the Rev1 expression level (Table 1; Figure 1c and d). These observations indicate that the development of intestinal adenomas after MNU treatment was accelerated by Rev1 overexpression in proportion to the Rev1 expression level. By contrast, metastatic invasion of the intestinal adenocarcinoma, as determined by histologic examination, did not differ significantly among the three groups, although the Rev1 Tg (Homo) and (Hemi) mice exhibited slightly higher incidences of adenocarcinoma than Wt mice (Table 1). Thymic lymphomas were also detected after MNU treatment, and Rev1 overexpression was associated with earlier onset and higher incidence of this malignancy (Supplementary Table 1, available at *Carcinogenesis Online*).

Given that development of intestinal adenoma causes anemia (39), we also measured the Hgb concentration in peripheral blood (Supplementary Figure 2, available at *Carcinogenesis Online*). Since both number and size of adenomas contribute to the onset, incidence and severity of anemia, the parameter 'total adenoma area, mm²' was calculated for each animal as the sum of the areas of all adenomas. A negative correlation was observed between the total adenoma area and Hgb level (Supplementary Figure 2c, available at *Carcinogenesis Online*). The average Hgb level decreased in the following order: Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo), in accordance with intestinal adenoma number and size (Supplementary Figure 2a, available at *Carcinogenesis Online*). Mice with anemia (Hgb level < 5g/dl) were detected much earlier among Rev1 Tg (Homo) and Rev1 Tg (Hemi) compared with Wt mice, and the incidence of anemia increased in

the following order: Wt mice, Rev1 Tg (Hemi) and Rev1 Tg (Homo) (Supplementary Figure 2b, available at *Carcinogenesis Online*).

Rev1 overexpression accelerates both initiation and progression of MNU-induced intestinal tumors in an expression level-dependent manner

To assess the role of overexpressed Rev1 in the development of intestinal tumors, Rev1 Tg (Homo), Rev1 Tg (Hemi) and Wt mice were sacrificed and examined 1 or 2 months after MNU treatment. As shown in Figure 2a and b, the number of adenomas increased in the following order: Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo). Intestines of Rev1 Tg (Homo) mice contained significantly more tumors than those of Rev1 Tg (Hemi) and Wt mice 1 month after MNU treatment, suggesting that Rev1 overexpression accelerates the initiation of tumorigenesis. The difference in tumor number between Rev1 Tg (Homo) mice and Rev1 Tg (Hemi) or Wt mice was less pronounced at the 2-month time point due to a saturation effect in Rev1 Tg (Homo) mice. Rev1 Tg (Hemi) mice exhibited an intermediate phenotype at the 2-month time-point only (Figure 2b and Supplementary Figure 3 and Table 2, available at *Carcinogenesis Online*). No significant difference between Rev1 Tg (Hemi) and Wt mice were present at the 1-month time-point. This observation indicates that the relationship between tumor induction and level of Rev1 expression may not be linear in the first weeks after MNU treatment. Like tumor number, average adenoma diameter increased in the following order: Wt, Rev1 Tg (Hemi), Rev1 Tg (Homo). No significant differences were observed at the 1-month time point; however, 2 months after treatment, adenomas in Rev1 Tg (Homo) mice were significantly larger than those in Wt and Rev1 Tg (Hemi) mice (Figure 2c; Supplementary Figure 4 and Table 2, available at *Carcinogenesis Online*). These results imply that Rev1 overexpression accelerates both initiation and progression of tumor formation after MNU treatment.

Rev1 overexpression accelerates mutagenesis, as well as tumorigenesis, after MNU treatment

To determine the mechanism by which Rev1 overexpression promotes MNU-induced carcinogenesis, as well as its effect on mutagenesis, we characterized *Cttnb1* (β -catenin) mutations by sequencing *Cttnb1* exon 3. *Cttnb1* exon 3 encodes the GSK-3 β phosphorylation consensus motif, which plays an important role in the stability and function of its protein. The mutant sequences are summarized in Table 2. The mutation frequency in the *Cttnb1* gene exon 3 was highest in Rev1 Tg (Homo), followed by Rev1 Tg (Hemi) and Wt (Table 2).

All of the mutations detected in *Cttnb1* exon 3 were located in codons 32, 33, 34, 37 and 41 (Table 2). Mutations in codons 34 and 41 of *Cttnb1* were previously identified as hotspots for chemically induced adenoma in mouse (40), but the mutations at codon 32 might represent a mutation specific to adenomas in Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice. Furthermore, to analyze the MNU-induced MF *in vivo*, we performed TCR mutant assays in splenocytes isolated from Wt and Rev1 Tg (Homo) mice.

Table 1. Comparison of tumor number, tumor size and adenocarcinoma incidence

Genotype	No. of mice	Average number of tumors per mouse	Average size of tumor (mm)	Incidence of adenocarcinoma (%)
Wt	45	29.0 \pm 14.08	1.54 \pm 0.44	0.31
Rev1 Tg (Hemi)	48	44.3 \pm 16.36	1.53 \pm 0.37	0.38
Rev1 Tg (Homo)	43	63.6 \pm 21.35	1.69 \pm 0.31	0.40

*P < 0.01.

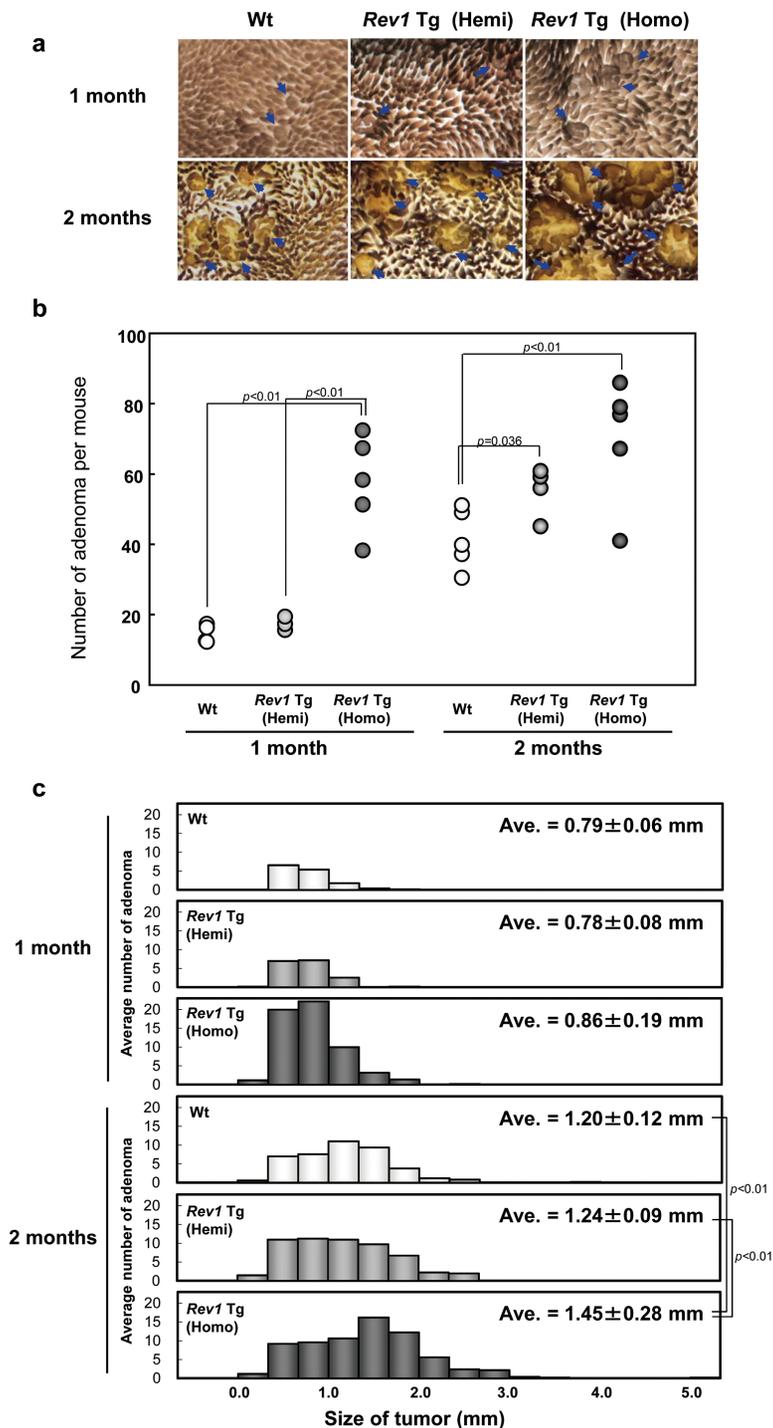


Figure 2. Initiation and progression of tumor formation 1 and 2 months after MNU treatment. (a) Images of intestines from Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice 1 or 2 months after MNU treatment. Magnification: $\times 10$. Arrows indicate adenomas. (b) Numbers of adenoma tumors in the intestinal tracts of Wt and Rev1 Tg mice. Each circle indicates the result from one mouse [Wt; white circle, Rev1 Tg (Hemi); gray circle, and Rev1 Tg (Homo); black circle]. $P = 0.018$ was calculated by the t-test. (c) Histogram of intestinal adenoma tumor size in Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice after MNU treatment. In each histogram, the number of adenomas for each size interval is presented as average from the mice used. In all cases five mice were used, with the exception of Rev1 Tg (Hemi) at 2 months, where four mice were used due to technical error.

Even though TCR mutant frequency in splenocytes is expected to be closely associated to thymic lymphoma incidence and is not directly relevant to initiation of intestinal adenoma, the test was used as a general indicator of *in vivo* mutagenicity. Overexpression of Rev1 resulted in MNU-induced TCR mutant

frequencies much higher than that in the control (i.e., Wt) (Supplementary Figure 5, available at *Carcinogenesis Online*).

These observations strongly suggest that Rev1 overexpression leads to elevated mutagenesis, which may accelerate the development of intestinal adenomas.

Table 2. Mutation frequency and patterns in exon 3 of the *Cttnb1* gene in MNU-induced intestinal adenomas

Mutation frequency	Wt		Rev 1 Tg (Hemi)		Rev1 Tg (Homo)			
	23/36	(63.9%)	14/19	(73.7%)	32/36	(88.9%)*		
	Codon	Base change	No	%	No	%	No	%
	32	GAT → AAT	1	(2.8)	3	(15.8)	7	(19.4)
	33	TCT → TTT	1	(2.8)	0	(0.0)	1	(2.8)
Mutation pattern	34	GGA → GAA	10	(27.8)	5	(26.3)	16	(44.4)
	37	TCT → TTT	1	(2.8)	0	(0.0)	2	(5.6)
	41	ACC → ATC	10	(27.8)	6	(31.6)	6	(16.7)

All mutation G:C to A:T.

*Significantly different from the value in Wt mice $P = 0.026$.

Rev1 overexpression resulted in reduced apoptosis in the intestinal crypts of Rev1 Tg (Hemi) and (Homo) mice after MNU treatment

To explore the mechanism by which Rev1 overexpression drives stimulation of MNU-induced adenoma formation in the small intestine, we analyzed the prevalence of apoptosis in the intestinal crypt compartment following exposure to MNU. Apoptotic cells were identified using morphological criteria, including cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation (Figure 3a). Crypt compartments of Wt and Rev1 Tg (Homo) mice without MNU treatment contained similar numbers of apoptotic cells. However, MNU induced a measurable increase in intestinal apoptosis, with peak induction at 6 h after treatment; moreover, induction was much more pronounced in Wt than in Rev1 Tg (Homo) mice (Figure 3b). The frequency of apoptosis 6 h after MNU treatment decreased in the following order: Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo), in accordance with the Rev1 expression level (Figure 3c). Analysis of Bcl2 and Bax expression confirmed that MNU-induced apoptotic signaling was lower in Rev1 Tg (Homo) than in Wt mice (Supplementary Figure 6, available at *Carcinogenesis Online*). These findings suggest that Rev1 controls resistance to apoptosis in the intestinal crypt epithelium after MNU treatment, in proportion to the Rev1 expression level, and that resistance to MNU-induced apoptosis might cause accumulation of mutated intestinal cells.

hREV1 overexpression is associated with elevated tolerance to DNA damage with a concomitant increase in mutation frequency

To assess the effects of Rev1 overexpression on cellular responses to MNU-induced DNA damage, we transfected the human HT1080 cell line with a Tet-ON system in which hREV1 is induced by the addition of tetracycline. Forty-eight clones were isolated and expanded, and hREV1 protein levels were determined by Western blotting. As shown in Figure 4a and Supplementary Figure 7a, available at *Carcinogenesis Online*, two clones, HT1080-6TR-hREV1-C6 and -C7, expressed substantially higher levels of hREV1 than parental untransfected HT1080 cells and HT1080-6TR cells.

Clonogenic assays were used to determine whether overexpression of hREV1 could modulate cellular sensitivity to MNU. In both HT1080-6TR-hREV1-C6 and -C7, addition of tetracycline caused cells to become resistant to MNU treatment (Figure 4b). Thus, overexpression of hREV1 rendered cells resistant to MNU. Knockdown of hREV1 made cells much more sensitive to MNU than cells treated with control siRNA (Figure 4d and e; Supplementary Figure 7b, available at *Carcinogenesis Online*). Notably, we observed a strong inverse relationship between hREV1 protein levels and cellular sensitivity to MNU exposure.

To determine whether overexpression of hREV1 can affect MNU-induced mutagenesis, we measured mutation frequency in the *HPRT* gene by counting the number of 6-thioguanine-resistant colonies. In the HT1080-6TR-hREV1-C6 cell line, spontaneous mutation frequency did not change significantly as a result of the addition of tetracycline (Figure 4c). However, overexpression of hREV1 increased the level of *HPRT* mutations following MNU treatment. By contrast, hREV1 knockdown decreased the frequency of MNU-induced mutation in *HPRT* (Figure 4f). These results indicate that the hREV1 expression level modulates the cytotoxicity and mutagenicity of MNU in HT1080 cells. Furthermore, overexpression of hREV1 lowered the frequency of MNU-induced sister chromatid exchange (SCE) (Supplementary Figure 8, available at *Carcinogenesis Online*).

Discussion

This study was the first to demonstrate a causal relationship between Rev1 overexpression *in vivo* and chemically induced mutagenesis and tumorigenesis. Rev1, a member of the Y-family of DNA polymerases that function in translesion DNA synthesis (TLS), is a key factor in error-prone TLS. The deletion and overexpression of Rev1 is responsible for the cytotoxicity and mutagenicity of the various DNA-damaging agents (21,24-27). Furthermore, upregulation of hREV1 is associated with the pathogenesis of human glioma (35). However, the *in vivo* role of Rev1 remains poorly understood. The findings reported here show that Rev1 overexpression causes elevated mutagenesis and tumorigenicity after MNU treatment, in a manner that depends on the Rev1 expression level, and that Rev1 plays a critical role in chemically induced tumorigenesis.

Mutation analyses showed that all mutations characterized in MNU-induced intestinal tumors were G:C-to-A:T transitions. Such transitions might be generated by a O⁶-methylguanine (m6G) lesion, the major DNA adduct produced by MNU. Rev1 addresses m6G lesions by inserting C nucleotides opposite such lesions, resulting in error-free repair. Accordingly, it remains unclear which pathway provokes the G:C-to-A:T transition in the context of MNU-induced m6G lesions.

Biochemical and genetic data obtained in yeast show that Pol δ can insert a nucleotide (T more efficiently than C) opposite a m6G lesion site, and that Pol ζ then extends from the inserted nucleotide (41). The combination of Pol ζ and Pol δ promotes m6G bypass more efficiently than either polymerase alone, and might result in a G:C-to-A:T transition. Rev1 plays a crucial role in the association of Pol ζ with a subunit of Pol δ (42,43). Furthermore, Rev1 enhances extension by Pol ζ in a nonenzymatic manner (41,44). These results imply that the overexpression of Rev1 accelerates the activity of the Pol δ -Pol ζ pathway in the presence of m6G lesions, thereby increasing MNU-induced mutagenesis.

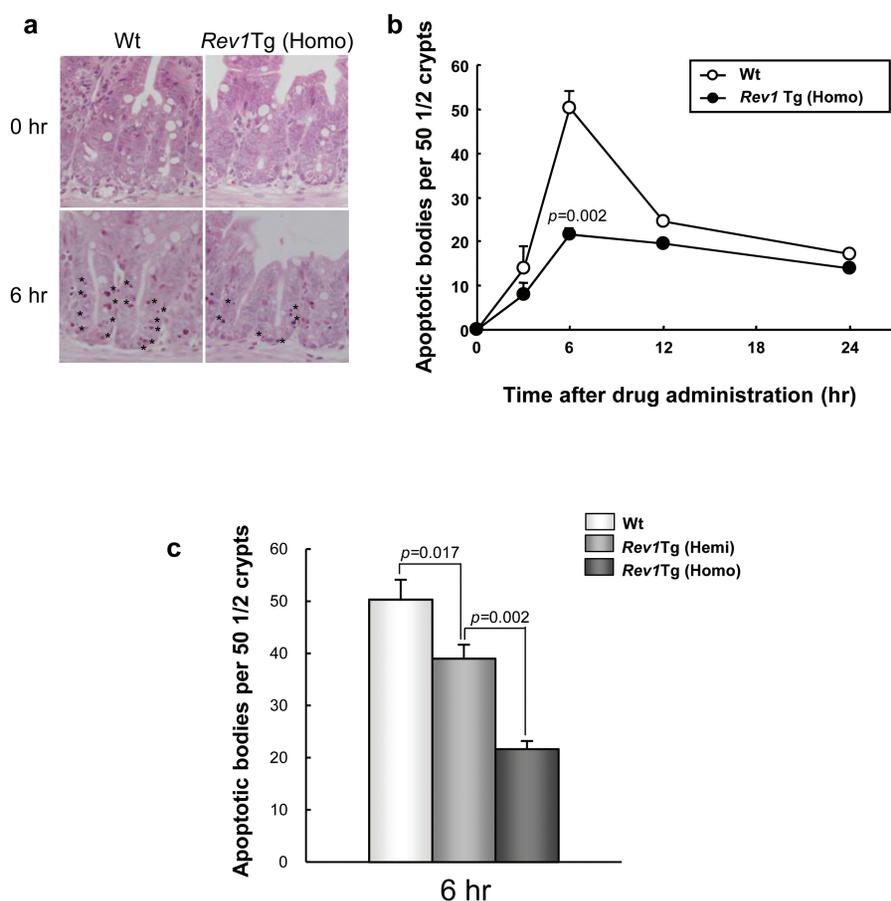


Figure 3. Apoptotic response in the intestinal crypts of Wt and Rev1 Tg mice after MNU treatment. (a) Hematoxylin and eosin staining of Wt and Rev1 Tg (Homo) mice intestines. Asterisks represent apoptotic cells. Magnification, $\times 200$. (b) Number of apoptotic bodies per 50 half-crypts in Wt and Rev1 Tg (Homo) mice at the indicated time points after MNU treatment (Wt, open circles; and Rev1 Tg (Homo), closed circles). Each data point represents the mean and standard deviation from three mice. The rate of apoptosis at 6 h differed significantly ($P = 0.002$) between Wt and Rev1 Tg (Homo) mice. (c) Prevalence of apoptotic bodies per 50 half-crypts in Wt, Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice at 6 h after MNU treatment [Wt, white bars; Rev1 Tg (Hemi), gray bars; and Rev1 Tg (Homo), dark gray bars]. Each bar represents the mean and standard deviation from three mice. The rates of apoptosis differed significantly between Wt and Rev1 Tg (Hemi) mice and between Rev1 Tg (Hemi) and Rev1 Tg (Homo) mice ($P = 0.017$ and $P = 0.002$, respectively).

We observed an MNU-induced mutation in codon 32 of *Ctnnb1* exon 3 at a higher frequency in Rev1 Tg than in Wt mice. This codon was previously reported as a hotspot in rats, but not in mice, suggesting that it may represent a specific hotspot in Rev1 Tg mice (40,45). Thus, when Rev1 is overexpressed, replication of the MNU-induced m6G lesion at codon 32 of *Ctnnb1* exon 3 by Pol δ and Pol ζ is more efficient than error-free translesion DNA synthesis by Rev1 or other polymerases such as Pol η . However, further investigations are required to test this hypothesis.

The results reported here show that overexpressed Rev1 suppresses apoptosis and increases the mutation frequency in the *Ctnnb1* exon 3 region after MNU treatment. The surviving fraction of mutated cells was higher under Rev1 overexpression, resulting in acceleration of carcinogenesis. These observations suggest that both mutagenic TLS and error-free TLS by dCMP insertion are promoted by overexpression of Rev1, allowing completion of stalled replication and inhibition of apoptosis. Consistent with this hypothesis, an increase in hREV1 expression in HT1080 cells increased both MNU resistance and MNU-induced mutagenicity. Furthermore, overexpression of hREV1 lowered the frequency of MNU-induced sister chromatid exchange (SCE) (Supplementary Figure 8, available at *Carcinogenesis* Online). The reduced sensitivity and elevated mutagenicity of MNU in hREV1-overexpressing cells could be attributed to a higher level of error-free and mutagenic TLS. By contrast, suppression of hREV1 expression in

HT1080 cells increased MNU cytotoxicity and decreased MNU mutagenicity. Strong associations were observed between these parameters, indicating that the REV1 level modulates MNU cytotoxicity and mutagenicity. Further investigations are needed to clarify the molecular mechanisms underlying Rev1-mediated regulation of apoptosis induced by DNA-damaging agents.

Previous work showed that Rev1^{B/B} cells, which contain a deletion in the N-terminal BRCT domain of Rev1, are sensitive to exogenous DNA-damaging factors and exhibit lower levels of ultraviolet C (UVC)-induced mutagenesis (9). However, the same study showed that Rev1^{B/B}XPC^{KO} mice are more vulnerable to skin carcinogenesis than Xpc^{KO} mice, despite the reduction in mutagenesis due to the Rev1^{B/B} alleles (46). The authors hypothesized that this paradoxical phenotype was due to the induction of inflammatory hyperplasia, implying that Rev1 plays a role in tumor promotion. Thus, maintenance of optimal Rev1 levels is important for tumor initiation and promotion, and thereby controls carcinogenesis.

The findings of this study demonstrate the effect of Rev1 overexpression on a mouse model of tumorigenesis. Overexpression of Rev1 promotes mutagenic TLS to safeguard replication on damaged templates at the expense of mutagenesis, thereby accelerating tumorigenesis. Although the mechanisms responsible for hREV1 overexpression in human carcinogenesis are not well understood, control of Rev1 levels is clearly necessary for genomic stability and tumor suppression.

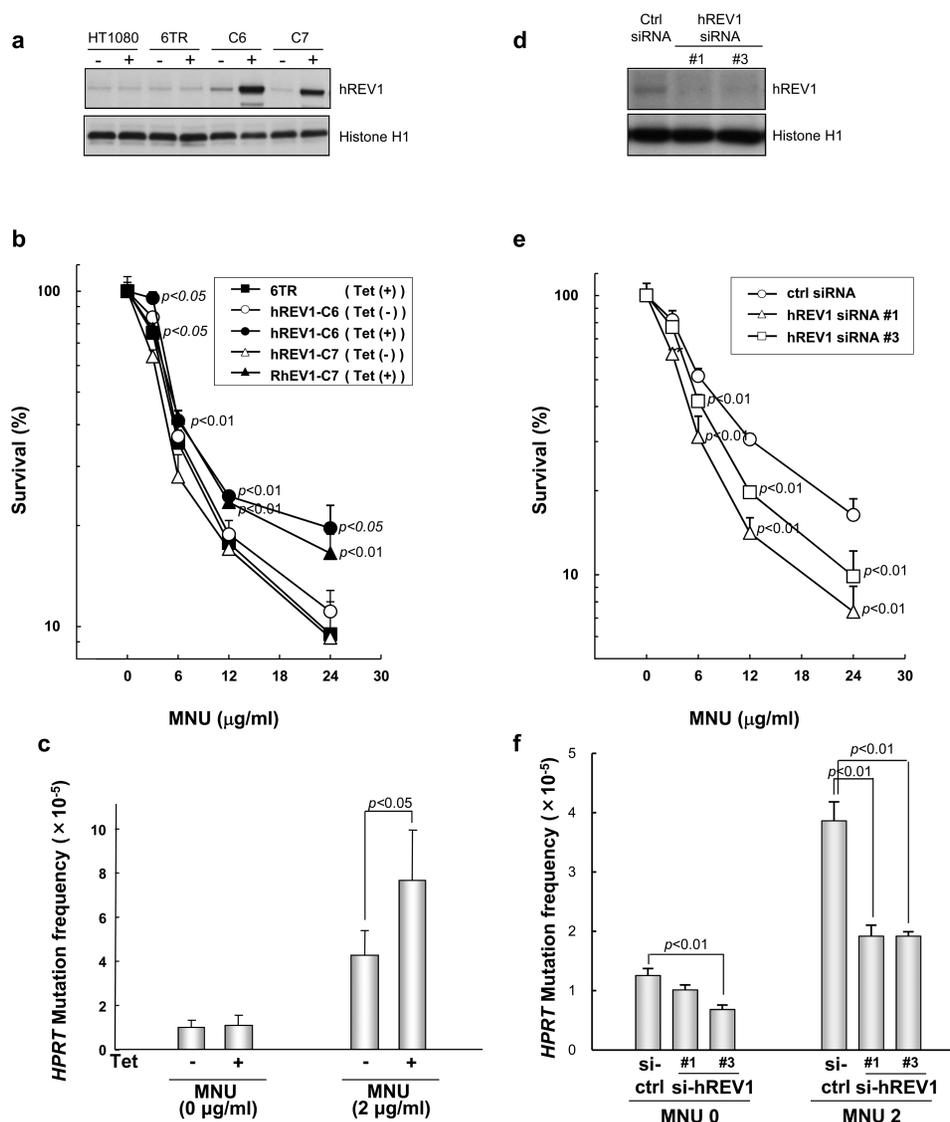


Figure 4. Association between hREV1 expression levels and cytotoxicity and mutagenicity of MNU in HT1080 cell lines. (a) hREV1 expression levels in parental HT1080 cells and the HT1080-6TR, -C6 and -C7 sublines, as determined by Western blotting; histone H1 was used as an internal control. Full length plot was exhibited in Supplementary Figure 7a, available at *Carcinogenesis* Online. (b) Cytotoxic effects of MNU treatment in the hREV1-overexpressing HT1080-hREV1-C6, -C7 cell lines. Cell survival was determined by colony-formation assays. (c) HPRT mutation frequency in HT1080-hREV1-C6 cells after MNU treatment. (d) hREV1 expression level in HT1080-6TR cells transfected with si-Ctrl or si-hREV1. Full-length plot was exhibited in Supplementary Figure 7b, available at *Carcinogenesis* Online. (e) Sensitivity of HT1080-6TR cells transfected with si-Ctrl or si-hREV1 to the cytotoxic effects of MNU treatment. Cell survival was determined by colony-formation assays. (f) HPRT mutation frequency in HT1080-6TR cells transfected with si-Ctrl or si-hREV1 after MNU treatment.

Rev1 Tg mice represent a promising tool for developing a deeper understanding of the role of Rev1 in tumorigenesis, as well as for elucidating the mechanism of tolerance to anticancer agents used therapeutically.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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