

## Accumulation of the Oxidative Base Lesion 8-Hydroxyguanine in DNA of Tumor-Prone Mice Defective in Both the *Myh* and *Ogg1* DNA Glycosylases

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### Abstract

The OGG1 and MYH DNA glycosylases prevent the accumulation of DNA 8-hydroxyguanine. In *Myh*<sup>-/-</sup> mice, there was no time-dependent accumulation of DNA 8-hydroxyguanine in brain, small intestine, lung, spleen, or kidney. Liver was an exception to this general pattern. Inactivation of both MYH and OGG1 caused an age-associated accumulation of DNA 8-hydroxyguanine in lung and small intestine. The effects of abrogated OGG1 and MYH on hepatic DNA 8-hydroxyguanine levels were additive. Because there is an increased incidence of lung and small intestine cancer in *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice, these findings support a causal role for unrepaired oxidized DNA bases in cancer development.

### Introduction

Oxidative DNA damage is one of the most common threats to genome stability, and several DNA repair enzymes protect DNA from the miscoding properties of oxidized bases. In *Escherichia coli*, the functional cooperation of three proteins minimizes the mutagenicity of the miscoding oxidized base 8-hydroxyguanine (8-oxoG; Refs. 1, 2). The MutM DNA glycosylase removes 8-oxoG from 8-oxoG:C bp. The MutY DNA glycosylase removes adenine incorporated opposite 8-oxoG during replication. The preferential insertion of a cytosine opposite 8-oxoG during subsequent repair synthesis reconstitutes a substrate for repair by MutM. Evidence for this base excision repair cycle is provided by the 100-fold increase in spontaneous mutation (principally GC→TA transversion) in *mutM mutY* double mutants (3). The increase is synergistic, and inactivation of each gene alone confers only a modest mutator phenotype. A third component of the protective process, the MutT-encoded hydrolase, prevents incorporation of 8-oxoG into DNA by removing 8-oxo-dGTP from the deoxynucleoside triphosphate pool (4). In contrast to MutM and MutY, inactivation of MutT alone is sufficient to produce a severe mutator phenotype (>100-fold increase), particularly in AT→CG transversions. Each of these protective factors has a counterpart in mammalian cells. Independent disruption of the murine MutM, MutY, or MutT homologues (OGG1, MYH, and MTH) confers, at most, only a minor increase in spontaneous mutation rate. In *Ogg1*<sup>-/-</sup> mice (5, 6) and in *Myh*<sup>-/-</sup> ES cells (7), this increase is ~2-fold. In *Mth*<sup>-/-</sup> mice, although there is a change in the mutational spectrum, in particular an increase in A:T to C:G transversions and frameshifts, there is no significant increase in overall rate (8), apparently due to the presence of a related back-up hydrolase (9).

Age-dependent accumulation of DNA 8-oxodG occurs in hepato-

cytes but not in splenocytes, spermatocytes, or renal cells of *Ogg1*<sup>-/-</sup> mice (10). No information is available on the levels of this oxidized base in the DNA of tissues of *Myh*-defective mice. We therefore compared the levels of DNA 8-oxodG in several organs of *Myh*<sup>-/-</sup> mice to those of doubly defective *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> animals. Our data demonstrate that, as with OGG1, inactivation of MYH does not cause significant age-dependent accumulation of DNA 8-oxodG in mouse tissues, with the notable exception of liver. When both MYH and OGG1 are inactivated, 8-oxodG gradually accumulated in the DNA of lung and small intestine. Because there is an increased incidence of lung or small intestine cancer in double *Myh/Ogg1* knockout mice (11), our results are consistent with a model in which the accumulation of unrepaired oxidized bases in DNA contributes to neoplastic transformation.

### Materials and Methods

**Generation of Double Knockout *Myh/Ogg1* and Genotyping.** The generation of *Ogg1*<sup>-/-</sup> (5) and *Myh*<sup>-/-</sup> (11) mice has been described previously. To obtain the double knockout *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup>, *Myh*<sup>-/-</sup>, and *Ogg1*<sup>-/-</sup>, mice were crossed, and the littermates *Myh*<sup>+/-</sup>/*Ogg1*<sup>+/-</sup> were intercrossed. The offspring obtained from inbreeding was consistent with Mendelian segregation. All mice were genotyped by PCR analysis of DNA isolated from tail tips. A combination of primer pairs was used to detect wild-type and mutant alleles. The primers pair OGG-1A (5'-CCCCAGGTTGTAGTATGAGTG-3') and OGG-1B (5'-ATGGACATCACAGGCAC-3') generates a 266-bp fragment containing the wild-type *Ogg1* allele, whereas the amplification products of the primers pair NeoA (5'-ACAAGATGGATTGCACGC-3') and NeoB (5'-CGTCCTGCAGTTCATTCA-3') is a 367-bp fragment containing the mutant *Ogg1* allele. The primers pair Y2 (5'-CAAGTGCTGGGATCAAAGGTG-3') and Y89 (5'-GCTCCTTCTGTAGCCGACG-3') generates a 262-bp fragment (wild-type *Myh* allele), whereas the primers pair Y2 and N9 (5'-TCCTCGTCTTTACGGTATCG-3') identifies the mutant *Myh* allele as a 367-bp fragment. The PCR reactions were carried out in 50 μl of buffer containing 0.25–1 μg of DNA, 50 pmol of each primer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleoside triphosphate, and 2.5 units of AmpliTaq DNA polymerase (Applied Biosystems). The PCR profile for the *Myh* gene was 94°C for 1 min, 58°C for 2 min, and 72°C for 2 min for 35 cycles, whereas the PCR profile for the *Ogg1* gene was 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min for 40 cycles.

**Preparation of Liver, Spleen, Kidney, Brain, Small Intestine, and Lung DNA.** Similar numbers of males and females have been used to prepare DNA for 8-oxodG measurements. Mice were killed by cervical dislocation, and excised organs were washed with ice-cold PBS. Liver was diced and washed with hypotonic KCl. Washed tissues were snap-frozen in liquid nitrogen. Before DNA extraction, thawed tissues were finely minced in lysis buffer [10 mM Tris HCl (pH 8.0), 10 mM EDTA, 10 mM NaCl, and 0.5% SDS]. DNA was extracted by a high-salt protein precipitation method. Briefly, following lysis tissues were digested with RNase at 37°C for 1 h and protease (Qiagen) at 37°C overnight. Proteins were precipitated by adding NaCl to 1.5 M, and DNA in the supernatant was collected by addition of 2 vol of ethanol.

**Measurements of 8-OxodG.** DNA 8-oxodG was measured by high-performance liquid chromatography with electrochemical detection as described

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previously (12). Briefly, DNA was resuspended in Tris-EDTA, incubated with RNases A and T1 at 37°C for 1 h, and precipitated again with ethanol. Enzymatic digestion was then performed at 37°C using nuclease P1 (Boehringer Mannheim) for 2 h and alkaline phosphatase (Boehringer Mannheim) for 1 h. Enzymes were precipitated by addition of  $\text{CHCl}_3$ , and the upper liquid layer stored at  $-80^\circ\text{C}$  under  $\text{N}_2$  for subsequent analysis 8-oxodG. The DNA hydrolysate was analyzed by high-performance liquid chromatography with electrochemical detection (Coulchem I; ESA, Inc., Chelmsford, MA) using a C18  $250 \times 46$  mm  $5\text{-}\mu\text{m}$  Uptisphere column (Interchim) equipped with a C18 guard column. The eluent was 50 mM ammonium acetate (pH 5.5), containing 9% methanol, at a flow rate of 0.7 ml/min. The potentials applied were 150 and 400 mV for E1 and E2, respectively. The retention time of 8-oxodG was  $\sim 23$  min. Deoxyguanosine was measured in the same run of corresponding 8-oxodG with a UV detector (model SPD-2A; Shimadzu, Kyoto, Japan) at 256 nm; the retention time was  $\sim 17$  min.

## Results

The level of DNA 8-oxodG was measured in several organs (brain, kidney, spleen, liver, lung and small intestine) as a function of both the age and the genotype of the mice. No significant age-dependent changes in DNA 8-oxodG were found in any of the tissues from wild-type animals between 1.5 and 10 months of age (Figs. 1A–C, 2A; and 3, A and B). These results are in good agreement with the

observations of Osterod *et al.* (10) in that these oxidized bases did not accumulate in hepatocytes, splenocytes, spermatocytes, or kidney cells of wild-type animals. No significant age-dependent accumulation of DNA 8-oxodG was observed in brain, kidney, spleen, lung or small intestine of *Myh*<sup>-/-</sup> mice (Figs. 1D–F and 3, C and D). The single exception was liver of *Myh*<sup>-/-</sup> mice in which there was an approximately linear increase ( $R^2 = 0.447$ ;  $P = 0.013$ ) in DNA 8-oxodG levels with time (Fig. 2B). Inactivation of MYH was associated with an  $\sim 4$ -fold increase in liver DNA 8-oxodG ( $0.2$  versus  $0.78$  8-oxodG  $\times 10^6$  dG) over a 10-month period (Fig. 2B). This increase is of a similar magnitude to that previously reported for untreated *Ogg1*<sup>-/-</sup> mice (10, 13).

In *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice, the effects of each genetic defect on the age-associated accumulation of hepatic DNA 8-oxodG were additive. The level of 8-oxodG in the liver DNA of the doubly defective mice increased linearly between 4 and 14 months of age. In this 10-month period, hepatic DNA 8-oxodG levels increased  $\sim 6$ -fold ( $R^2 = 0.695$ ;  $P = 0.000$ ; Fig. 2C). Interestingly, DNA 8-oxodG also accumulated in the lung and small intestine of *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice (Fig. 3, E and F). The accumulation of DNA 8-oxodG in lung was approximately linear over the period from 4 to 16 months and overall was  $\sim 3$ -fold ( $R^2 = 0.569$ ;  $P = 0.005$ ) by 16 months. Over the same period, there

Fig. 1. Steady-state levels of 8-hydroxydeoxyguanosine (8-oxodG) in brain, kidney, and spleen DNA of wild-type and base excision repair-defective mice. The amount of 8-oxodG residues was measured by high-performance liquid chromatography with electrochemical detection (see "Materials and Methods") in genomic DNA of wild-type (WT,  $\blacklozenge$ , A–C), *Myh*<sup>-/-</sup> ( $\blacksquare$ , D–F), and *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> ( $\bullet$ , G–I) mice. The time intervals for DNA 8-oxodG measurements were 1.5–10 months for wild-type mice and 3–16 months for *Myh*<sup>-/-</sup> and *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice. Each point represents a single mouse. The best line fitting the data are shown together with the corresponding  $R^2$  values (coefficient of determination) and the  $P$  for each  $R^2$ .

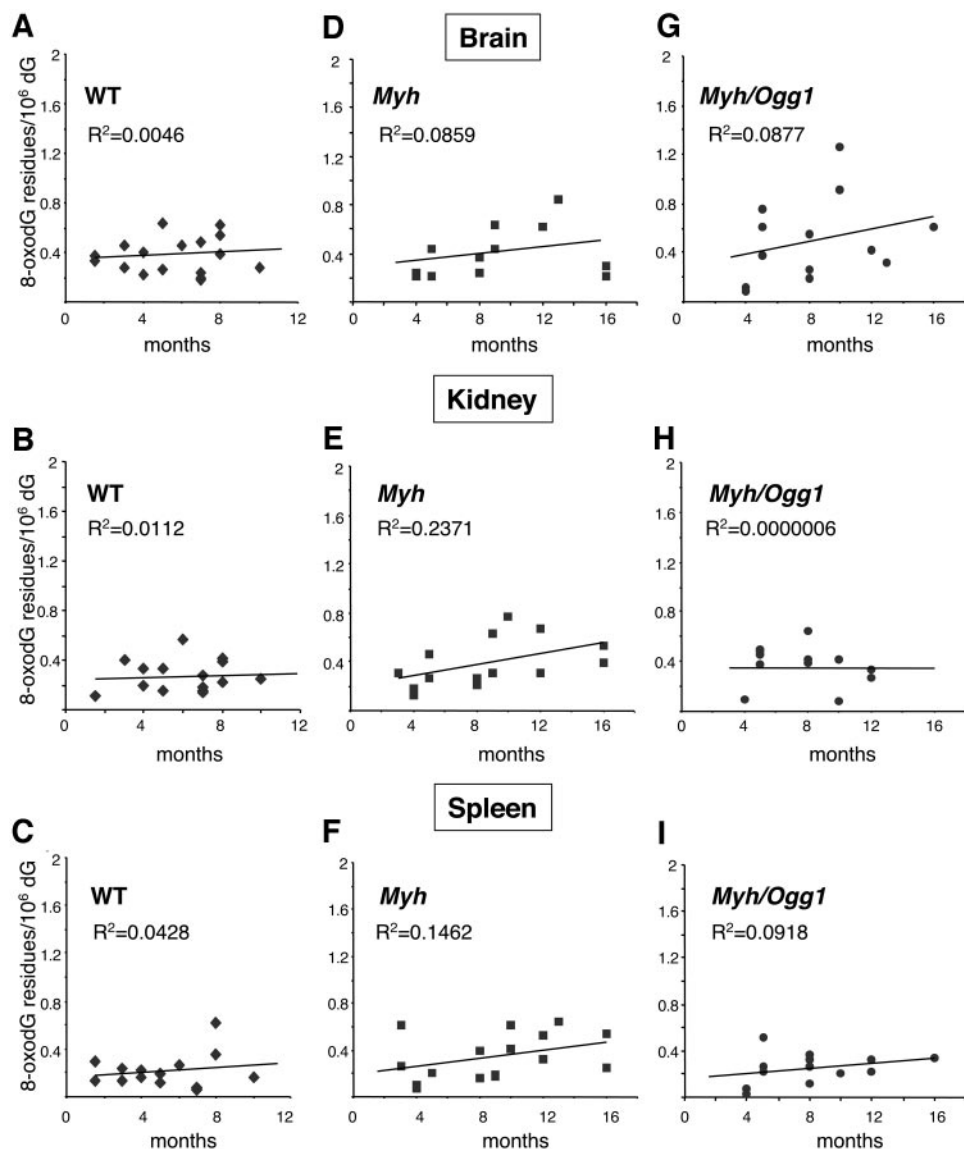
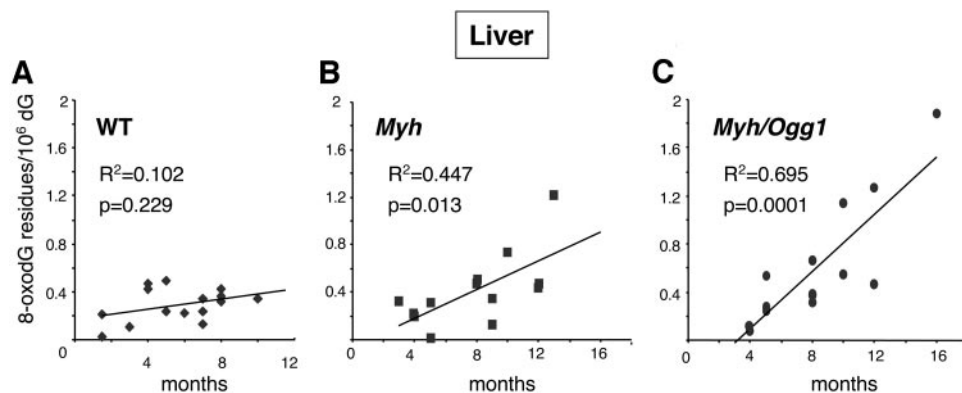


Fig. 2. Levels of 8-hydroxydeoxyguanosine (8-oxodG) in liver DNA of wild-type and base excision repair-defective mice. Levels of 8-oxodG residues/ $10^6$  dG were measured in genomic DNA of wild-type (WT,  $\blacklozenge$ , A), *Myh*<sup>-/-</sup> ( $\blacksquare$ , B), and *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> ( $\bullet$ , C) mice. The time intervals for DNA 8-oxodG measurements were 1.5–10 months for wild-type mice and 3–16 months for *Myh*<sup>-/-</sup> and *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice. There was no significant difference in the levels of DNA 8-oxodG between male and female animals. The best line fitting the data are shown together with the corresponding  $R^2$  and  $P$  values.



was a similar increase in the level of 8-oxodG in DNA of the small intestine of *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice. Although the increase was of a similar magnitude, the final levels of DNA 8-oxodG were higher because the level of background DNA oxidation was  $\sim$ 2-fold higher in this tissue independent of the genotype of the mouse (Fig. 3B–F). In contrast to the results obtained in lung and small intestine, no significant age-dependent increases in genomic levels of 8-oxodG were detected in brain, kidney, or spleen of *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice (Fig. 1G–I).

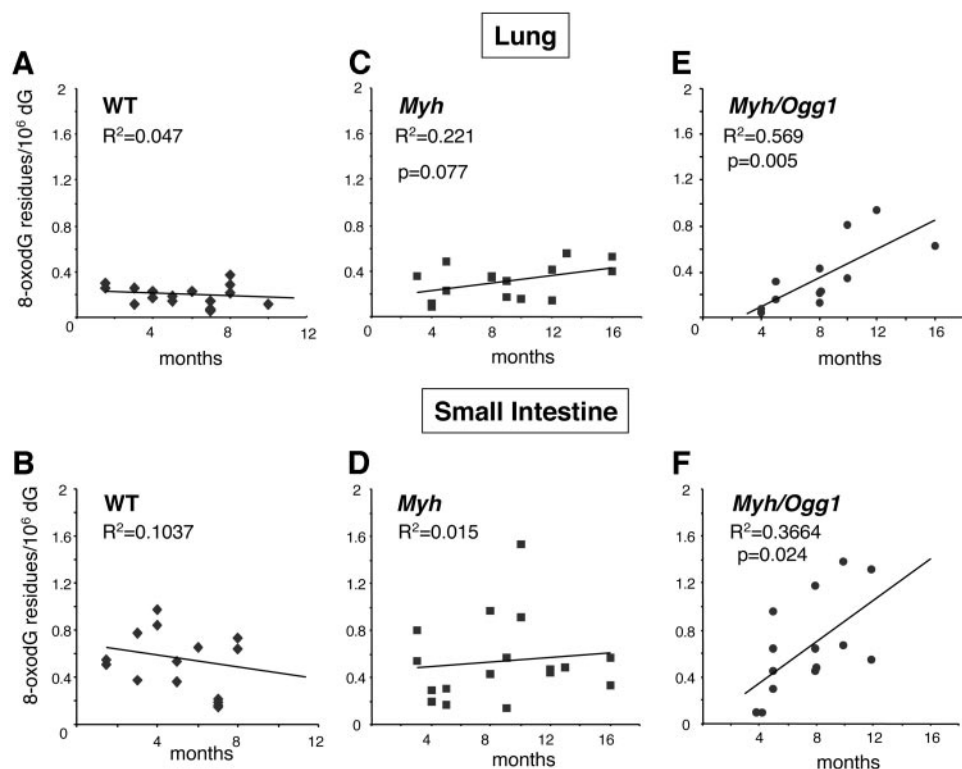
## Discussion

Our data provide the first direct evidence that DNA 8-oxodG accumulates in several mouse organs in which both the MYH and OGG1 DNA glycosylases are inactive. This accumulation occurs in the absence of exposure to exogenous oxidants. This synergistic increase in the steady-state level of DNA oxidation in the liver, lung, and small intestine of double knockout animals is reminiscent of the synergistic effect on spontaneous mutation of combined *mutM* and *mutY* mutations in *E. coli*. This parallelism is consistent with the

similar mode of action of the bacterial and mammalian DNA glycosylases.

The complexity of the mammalian organism is, however, reflected in the age dependency and tissue specificity of DNA 8-oxodG accumulation. Age-dependent accumulation of DNA 8-oxodG was not observed in all organs of the double knockout animals. There was no significant change in the steady-state level of the oxidized purine in brain, kidney, or spleen. The reason for the selective accumulation in lung and small intestine DNA is unclear. It is striking, however, that it is these organs in which there is an increased cancer incidence in the doubly defective *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice (11). These findings suggest that oxidized DNA purines can play a causative role in the development of cancer. Liver is the only organ in which inactivation of a single gene, either *Ogg1* (10) or *Myh* (present article), is associated with an age-dependent accumulation of DNA 8-oxodG. This may reflect a high level of oxidative metabolism or the role of this organ in detoxification. However, this accumulation of the lesion in liver DNA is not associated with carcinogenesis in *Ogg1*<sup>-/-</sup> or *Myh*<sup>-/-</sup> mice (5, 6, 11).

Fig. 3. Levels of DNA 8-hydroxydeoxyguanosine (8-oxodG) in lung and small intestine of wild-type and base excision repair-defective mice. Levels of 8-oxodG residues/ $10^6$  dG were measured in genomic DNA of wild-type (WT,  $\blacklozenge$ , A and B), *Myh*<sup>-/-</sup> ( $\blacksquare$ , C and D), and *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> ( $\bullet$ , E and F) mice. The time intervals for DNA 8-oxodG measurements were 1.5–10 months for wild-type mice and 3–16 months for *Myh*<sup>-/-</sup> and *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice. There was no significant difference in the levels of DNA 8-oxodG between male and female animals. The best line fitting the data are shown together with the corresponding  $R^2$  and  $P$  values.



The absence of significant age-associated accumulation of oxidized DNA purines in the majority of tissues of *Myh*<sup>-/-</sup>/*Ogg1*<sup>-/-</sup> mice suggests that there may be other DNA repair systems to deal with this type of DNA damage. Candidates include the recently characterized homologue of the bacterial Fpg/Nei DNA glycosylase, Neil 1, which shares with OGG1 the ability to remove 8-oxoG from 8-oxoG:C bp (14). In addition, DNA mismatch repair is known to play a role in reducing the burden of DNA 8-oxoG and associated mutations (15, 16). In view of the tissue selectivity of DNA 8-oxoG accumulation, it may be that these alternative repair systems are limiting in certain organs. In this connection, it is noteworthy that inactivation of mismatch repair or MTH1 in mice is associated with small intestinal tumors (17) and lung tumors, respectively (18).

In humans, inherited *MYH* variants are associated with colorectal carcinomas (19, 20), and deficiencies in 8-oxoG repair might be a risk factor for lung cancer (21). Possibly, additional deficiencies in mismatch repair or other base excision repair genes might enhance tumorigenesis in these tissues.

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