Mouse embryonic stem cells are hypersensitive to apoptosis triggered by the DNA damage O⁶-methylguanine due to high E2F1 regulated mismatch repair

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Exposure of stem cells to genotoxins may lead to embryonic lethality or teratogenic effects. This can be prevented by efficient DNA repair or by eliminating genetically damaged cells. Using undifferentiated mouse embryonic stem (ES) cells as a pluripotent model system, we compared ES cells with differentiated cells, with regard to apoptosis induction by alkylating agents forming the highly mutagenic and killing DNA adduct O⁶-methylguanine. Upon treatment with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), ES cells undergo apoptosis at much higher frequency than differentiated cells, although they express a high level of the repair protein O⁶-methylguanine-DNA methyltransferase (MGMT). Apoptosis induced by MNNG is due to O⁶-methylguanine DNA adducts, since inhibition of MGMT sensitized ES cells. The high sensitivity of ES cells to O⁶-methylating agents is due to high expression of the mismatch repair proteins MSH2 and MSH6 (MutS α), which declines during differentiation. High MutS α expression in ES cells was related to a high hyperphosphorylated retinoblastoma (ppRb) level and E2F1 activity that upregulates MSH2, causing, in turn, stabilization of MSH6. Non-repaired O⁶-methylguanine adducts were shown to cause DNA double-stranded breaks, stabilization of p53 and upregulation of Fas/CD95/Apo-1 at significantly higher level in ES cells than in fibroblasts. The high apoptotic response of ES cells to O⁶-methylguanine adducts may contribute to reduction of the mutational load in the progenitor population.

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Embryonic stem (ES) cells are pluripotent cells and a highly valuable tool for generating transgenic animals. They are also being considered for use in organ replacement and regeneration therapy. During embryogenesis, ES cells form the inner cell mass of the blastocyst and lead to, upon differentiation, germ layer formation. Critical steps during embryogenesis are stem cell proliferation and differentiation. Essential in these early steps of organism development is the maintenance of genomic integrity. In view of this, it is important to know the manner in which ES cells respond to genotoxic insults that damage DNA. Thus, ES cells might be protected against genotoxic exposures due to a high capacity for repairing their DNA. Alternatively, they may be highly sensitive in that they eliminate low levels of DNA damage by provoking death of the affected cell. Here, we tested these alternative hypotheses by comparing undifferentiated ES cells of mice with their differentiated counterparts and mouse fibroblasts (MFs).

An important source of DNA damage originates from endogenous and exogenous alkylating species.¹ Therefore, we chose as DNA damaging insult the powerful methylating

genotoxin *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), which is a representative of a large group of highly relevant environmental, food and tobacco smoke carcinogens.^{1–3} Moreover, it represents a group of alkylating anticancer drugs.⁴ MNNG is highly mutagenic, forming among 12 other DNA lesions, the mispairing DNA damage O⁶-methylguanine (O⁶MeG). Because of this DNA lesion, methylating agents give rise to mutations,⁵ sister chromatid exchanges (SCEs) and chromosomal aberrations,⁶ and are powerful tumor initiators.⁷ They are also cytotoxic due to apoptosis triggered by O⁶MeG in cells that are inefficient in repairing this specific DNA damage.⁸

O⁶MeG is repaired via a methyl transfer reaction by the DNA repair protein O⁶-methylguanine-DNA methyltransferase (MGMT).⁹ MGMT is a key player in the defence against the cytotoxic, mutagenic and carcinogenic effects of O⁶MeG that has been studied in cell, cancer and animal models.¹⁰ When O⁶MeG is not repaired by MGMT, it will mispair with thymine during DNA replication¹¹ and, therefore, in the absence of mismatch repair (MMR), and a second round of

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Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B-cell leukemia/lymphoma 2; Cdk2, cyclin-dependent kinase 2; Chip, chromatin immunoprecipitation assay; DSBs, DNA double-stranded breaks; EMSA, electrophoretic mobility shift assay; ES, embryonic stem; Fas, apoptosis stimulating fragment; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts; MF, mouse fibroblast; MGMT, O⁶-methylguanine-DNA methyltransferase; MMR, mismatch repair; MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; NER, nucleotide excision repair; O⁶BG, O⁶-benzylguanine; O⁶MeG, O⁶-methylguanine; ppRb, hypophosphorylated retinoblastoma; ppRb, hyperphosphorylated retinoblastoma; RA, retinoic acid; Rb, retinoblastoma; SCEs, sister chromatid exchanges

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replication, induces GC to AT point mutations.¹² In MMR competent cells, the O⁶MeG/thymine mismatch is bound by MutS α ,¹³ a heterodimer comprised of the proteins MSH2 and MSH6.¹⁴ The MMR protein binding has been shown to be required for O⁶MeG-triggered apoptosis.¹⁵ In line with this, cells lacking either MSH2 or MSH6 are highly resistant to O⁶-methylating agents because they tolerate O⁶MeG lesions.^{16,17} MSH2 knockout mice are predisposed to cancer, and cell lines derived from them show microsatellite instability and tolerance to methylating agents.¹⁸ Also, ES cells manipulated to express a 10-fold lower level of MSH2 acquired tolerance to methylating agents and a mutator phenotype.¹⁹

Here, we show that undifferentiated pluripotent ES cells are more sensitive to O⁶MeG-triggered cell death than their differentiated counterparts. The pronounced sensitivity of ES cells to methylating agents is due to a strong induction of apoptosis in response to O⁶MeG lesions. The mechanism behind this has been elucidated. The data show a high level of MutS α -dependent MMR, which is regulated by retinoblastoma (ppRb) protein, along with E2F1 and nuclear B-cell leukemia/lymphoma 2 (Bcl-2). Finally, we report on the apoptotic pathway employed by ES cells to remove cells from the population containing pre-mutagenic O⁶MeG lesions.

Results

Sensitivity of ES cells versus fibroblasts. To determine whether ES cells are sensitive to O⁶MeG-induced cellular toxicity, we compared the pluripotent ES (R1) cell line with Swiss Albino 3T3 (SA 3T3) MFs. Following two methods were employed: clonogenic survival and apoptosis induction. For apoptosis, mouse embryonic fibroblasts (MEF) were included for comparison. As shown in Figure 1a, ES (R1) cells are more sensitive to the methylating agent MNNG than SA 3T3 cells. When pretreating the cells with the MGMT inhibitor O⁶benzylguanine (O⁶BG),²⁰ a large increase in cellular sensitivity was observed for the ES (R1) line, while only a marginal effect was seen for SA 3T3 cells (Figure 1a). To determine whether this cellular sensitivity is due to the induction of apoptosis, the apoptotic response of the cells was determined. Again ES (R1) cells exhibited significantly more apoptosis after MNNG treatment than SA 3T3 and MEF cells (Figure 1b). This difference in the apoptotic response is even more apparent after inhibiting MGMT with O⁶BG. Thus, ES (R1) cells showed a clearly higher apoptosis level already at a low dose (1 μ M) of MNNG, while SA 3T3 cells were only slightly more sensitive and no difference was observed for MEF (Figure 1b). Representative histograms for ES (R1) cells, SA 3T3 and MEF not treated and treated with MNNG (5 μ M) are shown in Figure 1c. The difference in cellular distribution clearly shows that fibroblasts are not responding, while ES cells undergo apoptosis (Figure 1c).

MGMT and mismatch repair status of ES cells versus MF. Two major factors determining the cellular sensitivity and apoptotic response that originates from O⁶MeG lesions are MGMT and MMR. The ES (R1) line and the SA 3T3 cell line both showed comparable high MGMT activity (Figure 2a)

of 316 and 268 fmol/mg protein, respectively. Regarding MMR, Western blot analysis revealed that the expression of MSH2 and MSH6 is much higher in ES (R1) cells than in SA 3T3 cells (Figure 2b). As ES (R1) cells are much smaller than SA 3T3 cells, we also compared the expression of MSH2 and MSH6 per 1×10^5 cells. Even under these conditions, ES (R1) cells showed a higher MSH2 and MSH6 level than SA 3T3 cells (Figure 2c). High level of MSH2/MSH6 protein expression does not necessarily imply a higher MutS α DNA binding activity. Therefore, MutS α DNA binding was determined by electromobility shift assay (EMSA). As shown in Figure 2d, ES (R1) cells displayed strong MutS α binding compared to SA 3T3 cells. The data indicate that high sensitivity of ES cells to MNNG is related to their high MSH2 (MutS α) expression level.

To determine whether an increase in the MSH2 level in SA 3T3 cells will enhance their sensitivity to MNNG, we created SA 3T3 cells that stably express MSH2 (the expression level is shown in Figure 2e). As shown in Figure 2f, all MSH2 overexpressing clones are more sensitive to MNNG than the control SA 3T3 cell line. These results support the conclusion that high level of MSH2/MSH6 expression is responsible for the hypersensitivity of ES cells to methylating agents.

Control of MSH2 protein expression in multiple ES and fibroblast cell lines and influence on apoptosis. Next we addressed the question of whether the high expression of MSH2 found in the ES (R1) cell line is unique for this line. Western blot analysis of four ES lines and five MF cell lines showed that all ES lines exhibit a significantly higher MSH2 protein level compared to the MF lines (Figure 3a). Obviously, high MSH2 expression is a general property of ES cells.

How is the high MSH2 protein expression achieved in ES cells? MSH2 gene expression is under the control of E2F1²¹ and can be controlled via the inhibiting effect that Bcl-2 has on the kinase activity of cyclin-dependent kinase 2 (Cdk2).²² Based on this, we determined the nuclear Bcl-2 level. In all the MF cell lines tested, a higher nuclear localized Bcl-2 level was found than in the ES lines (Figure 3a). Contrary to this, the ES lines all showed the hyperphosphorylated (inactive) form of retinoblastoma (ppRb) protein that was less expressed in fibroblasts (Figure 3a). There was no clear difference between the total Rb protein level if fibroblasts and ES cells were compared (Figure 3a). All nine cell lines analysed showed the same E2F1 protein level (Figure 3a).

To determine whether the different MSH2 expression levels have an impact on the induction of O⁶MeG-triggered apoptosis, the cells were treated with MNNG either in the presence or absence of the MGMT inhibitor O⁶BG (Figure 3b). In all cases, the ES cell lines showed a stronger apoptotic response than the fibroblast cell lines, which was most obvious when MGMT was depleted (Figure 3b). In an effort to give a quantitative relationship between MSH2, ppRb, total retinoblastoma (Rb) and apoptosis, the correlation between protein expression and apoptotic response of the cell lines upon MNNG treatment was determined. MSH2 (Figure 4a) and ppRb (Figure 4b) showed a significant correlation with apoptosis, while total Rb protein (Figure 4c) did not. This

Figure 1 Cellular toxicity and apoptosis induced by O⁶MeG in ES cells, MEF and a fibroblast cell line. (a) Colony survival of ES (R1) and SA 3T3 cells in the presence and absence of the MGMT inhibitor O⁶BG (10 μM) after MNNG treatment. (b) Dose response of MNNG-induced apoptosis in ES (R1), MEF and SA 3T3 cells in the presence and absence of the MGMT inhibitor O⁶BG (10 μM) at time point 72 h after treatment. (c) Cell cycle distribution of untreated and MNNG-treated (5 μM) ES (R1), MEF and SA 3T3 cells in the presence of the MGMT inhibitor O⁶BG (10 μM). Flow cytometric analyses were performed 72 h after MNNG treatment and histograms were analysed using Modfit LT

supports the conclusion that the high MSH2 expression observed in ES cells is related to their high E2F1 level.

The ppRb levels observed in ES cells should give rise to a higher level of E2F1 binding to the promoter of MSH2. To determine whether this is the case, we subjected ES (R1) and SA 3T3 cells to the chromatin immunoprecipitation assay (ChIP). β-Actin was used as negative control. The results clearly show that there is more E2F1 bound to the promoter region of the MSH2 gene in ES (R1) cells than in SA 3T3 cells (Figure 4d).

MSH2 and MSH6 expression in ES cells and their differentiated progeny, and its influence on apoptosis. The argument can be made that fibroblasts cannot be compared to undifferentiated ES cells (they do not originate from these specific ES cells). To address this issue,

we differentiated a panel of ES cells by omitting leukemia inhibitory factor (LIF) from the medium and adding retinoic acid (RA). This differentiates ES cells to fibroblast and neuronal cell types.²³ The apoptosis response of ES (R1) cells and the RA differentiated cells (R1 RA) are given in Figure 5a. The results show, in the presence of O⁶BG, a higher sensitivity of ES (R1) cells compared to R1 RA throughout the dose range used. Western blot analysis of the panel of undifferentiated and differentiated ES cells showed that undifferentiated ES cells exhibit a higher MSH2 and MSH6 protein level (Figure 5b). Furthermore, as expected, the gene expression (as determined by RT-PCR) of MSH2, but not of MSH6, was higher in undifferentiated ES cells than in the differentiated progeny (Figure 5c). To determine the influence of differentiation on the apoptotic response following O⁶MeG induction, undifferentiated ES cells and

Figure 2 Activity of MGMT and MutS α in ES cells and fibroblasts. **(a)** MGMT activity in ES (R1) and SA 3T3 cells. **(b)** Expression levels of the MutS α heterodimer proteins MSH2 and MSH6 in ES (R1) and SA 3T3 cells after 5 μ M MNNG treatment. A 30 μ g weight of protein was loaded in the gel. **(c)** Expression level of the MutS α heterodimer proteins MSH2 and MSH6 for equal cell number in ES (R1) and SA 3T3 cells. Protein from 1×10^5 cells was loaded in the gel. **(d)** EMSA in MeWo (positive control), DLD1 (negative control), ES (R1) and SA 3T3 cells showing MutS α binding to a GT mismatch containing oligonucleotide. **(e)** Western blot analysis of SA 3T3 MSH2 transfected cells showing three positive clones. **(f)** Colony survival of SA 3T3 and MSH2 transfected SA 3T3 cells in the presence of the MGMT inhibitor O⁶BG (10 μ M) after MNNG treatment

their differentiated progeny were treated with MNNG after depleting MGMT with O⁶BG. The differentiated progeny showed a clearly reduced level of apoptosis, ranging from 20 to 30% of the level induced by MNNG in ES cells (Figure 5d).

Mechanism of O⁶MeG-induced apoptosis in ES cells. To verify that apoptosis observed in ES (R1) cells conforms to

the mechanism observed in other cell systems in response to alkylating agents,^{24,25} the following marker proteins were assayed: phosphorylated H2AX, p53, p21, Fas/CD95/Apo-1, Bcl-2, Bcl-2-associated X protein (Bax), cytochrome *c*, active fragments of caspase-7 and caspase-3. As shown in Figure 6, in the nucleus of ES (R1) cells histone H2AX becomes phosphorylated after MNNG treatment, which was

Figure 3 Mechanism of MSH2 protein expression in ES cells and effect on the apoptotic response. (a) High MSH2 protein expression was observed in the nucleus of all assayed (four) ES cell lines but not in the mouse fibroblast (MF 1–5) cell lines. Nuclear localization of Bcl-2, ppRb protein, total Rb protein level and the transcription factor E2F1 in the cell lines tested. ERK2 was used for loading control. (b) Apoptotic response in the ES and fibroblast cell lines in the presence and absence of the MGMT inhibitor O⁶BG (10 μ M) at 72 h after treatment with 5 μ M MNNG

Figure 4 Correlations between protein expression and apoptotic response relates to E2F1 binding to the MSH2 promoter. (a) Relative MSH2 nuclear protein level as a function of the apoptotic response upon MNNG. (b) Relative ppRb nuclear protein level as a function of the apoptotic response. (c) Relative total retinoblastoma (Rb) nuclear protein level compared to apoptotic response. (d) Chip analysis: Exponentially growing ES (R1) and SA 3T3 cells were subjected to Chip analysis. Immunoprecipitation (IP) was performed using an E2F1-specific antibody. PCR was done using specific primers for the promoter of MSH2 and, as negative control, for the β -actin 5' UTR

detectable 24 and 48 h after treatment. In fibroblasts (SA 3T3 cells), H2AX phosphorylation occurred at a clearly lower extent (Figure 6a). Stabilization of p53 was also observed in

ES (R1) cells, which paralleled H2AX phosphorylation; it was not found in SA 3T3 cells (Figure 6a). Concomitant to p53 stabilization, p21 was found to be upregulated in ES cells in

Figure 5 Down-regulation of MSH2 during differentiation and its effect on apoptosis. (a) Dose response of MNNG-induced apoptosis in ES (R1) and RA differentiated cells (R1 RA) in the presence of the MGMT inhibitor O⁶BG (10 μM) at time point 72 h after treatment. (b) MSH2 and MSH6 protein expression in whole cell extracts of undifferentiated ES cells and RA differentiated cells. (c) MSH2 and MSH6 gene expression in undifferentiated ES cells and RA differentiated cells. (d) Decrease in apoptosis after RA differentiation of ES cells compared to undifferentiated parental ES cells

response to MNNG (Figure 6b), indicating that p53 in ES cells was transcriptionally active. In ES (R1) cells, the death receptor Fas/CD95/Apo-1 was expressed at a higher level than in SA 3T3 fibroblasts after MNNG treatment (Figure 6c). Further, caspase-7 and caspase-3 became activated again to a higher extent in ES cells than in fibroblasts (Figure 6d and f). No cytochrome *c* release was observed in the cytoplasm of both ES (R1) and SA 3T3 cells after MNNG treatment (Figure 6d), indicating that mitochondria-triggered apoptosis is not involved. We should point out that lack of a cytochrome *c* signal in the Western blot is not due to low specificity of the antibody used. This was verified in a control experiment in which cytochrome *c* release was mediated by lysis of mitochondria in the cytoplasmic fraction of cells, which gave a clear signal (Figure 6e). Interestingly, an increase in Bcl-2 protein was observed in ES (R1) cells and, albeit at lower level, in SA 3T3 cells upon treatment with MNNG (Figure 6f and g). No significant change in Bax was observed (Figure 6g; induction factors calculated by comparison with ERK2). Due to the higher Bcl-2 level after MNNG exposure, the Bcl-2/Bax fraction increased in ES cells (Figure 6h), which is in line with the lack of cytochrome *c* release from mitochondria. Overall, data suggest that O⁶MeG lesions trigger the death receptor rather than the mitochondrial pathway of apoptosis in ES cells.

Discussion

Many environmental mutagens, notably *N*-nitrosamines, as well as endogenously formed alkylating species induce

the formation of O⁶MeG in DNA, which is considered a major source for mutations and cancer. Since pluripotent stem cells give rise to all types of tissue, it is of utmost importance that especially these cells are protected against genotoxic insults that cause mutations. Protection against genotoxins could occur because of highly effective DNA repair or, alternatively, because of an increased ability to eliminate damaged cells by (programmed) cell death. In order to elucidate whether ES cells are indeed protected against DNA damaging agents, we compared undifferentiated ES cells with differentiated cell types and studied how they respond to the methylating genotoxin MNNG that induces the highly mutagenic DNA adduct O⁶MeG. After treatment with MNNG, ES (R1) cells showed a significantly greater cell killing response, due to apoptosis, than SA 3T3 fibroblasts. Increased sensitivity of ES cells to MNNG could be due to a low expression level of the DNA repair protein MGMT that specifically repairs the O⁶MeG lesion. Surprisingly, mouse ES cells expressed high MGMT. From the high MGMT expression level, we would anticipate that ES cells are highly resistant to MNNG-induced apoptosis. This, however, was not the case. ES cells were clearly more sensitive than SA 3T3 cells. After inactivation of MGMT by the specific inhibitor O⁶BG, ES (R1) cells showed a dramatic increase in apoptosis even at a very low dose of MNNG (1 μM), while SA 3T3 cells showed no significant response over the entire dose range. This shows that non-repaired O⁶MeG lesions are a powerful trigger of apoptosis in ES cells but not to the same extent in fibroblasts. Obviously, ES cells are highly effective in repairing O⁶MeG DNA adducts

Figure 6 Expression of apoptotic marker proteins in ES cells and MFs. For all Western blots shown, cells were treated with the MGMT inhibitor O⁶BG (10 μ M). Thereafter, treatment was performed with MNNG (5 μ M). Cells were harvested at different times after MNNG treatment and subjected to Western blot analysis. ERK2 served as loading control. Induction factors (I/F) are indicated where appropriate. (a) Expression of γ H2AX and p53 in the nuclear fraction. (b) Expression of p21 in total cell extracts. (c) Expression of Fas in the membrane fraction. (d) Levels of the active form of caspase-3 and cytochrome c in the cytoplasmic fraction. (e) Positive control added for cytochrome c release from the mitochondria into the cytoplasm. For positive control, cytoplasmic extracts of cells were prepared still containing mitochondria. Mitochondria were then lysed in the extract to simulate the release of cytochrome c as it would occur during apoptosis. (f) Expression of the active form of caspase-7 as well as Bcl-2 protein levels in the cytoplasmic fraction. (g) Expression of Bax in the cytoplasmic fraction. (h) Calculation of Bcl-2/Bax fraction after MNNG treatment

and, at the same time, display a high killing response if O⁶MeG is not repaired.

What is the reason for the strong apoptotic response observed in ES cells? Reproductive cell death and apoptosis triggered by O⁶MeG lesions require MMR.^{14,15} In this process, the heterodimer MutS α , comprised of MSH2 and MSH6, is critically involved because of its binding to O⁶MeG adducts mispaired with thymine.^{13,26} It has been proposed that unsuccessful removal of thymine by MutS α leads to a futile DNA repair cycle that either directly or indirectly, via the formation of DNA double-stranded breaks (DSBs), gives rise to apoptosis.^{24,27} If this does not occur, cells will survive and O⁶MeG adducts will give rise to mutations.¹² Interestingly, MMR is upregulated in ES cells since all ES cell lines showed a significantly higher MSH2 and MSH6 protein level than differentiated cells. This resulted in a higher G–T DNA binding

activity of MutS α . Overall, the data indicate that high sensitivity of ES cells to MNNG is due to high MutS α -mediated MMR activity.

The high MutS α level in ES cells implicates that ES cells harboring O⁶MeG lesions in their genome will be eliminated by MMR-mediated apoptosis before mutations are formed (Figure 7a). The data also showed that the MSH2 expression level significantly correlated with the level of apoptosis induced by MNNG. This indicates that, despite high MGMT repair capacity, MMR determines whether cells harboring O⁶MeG lesions will be eliminated or not. This conclusion gained support from transfection experiments we performed with SA 3T3 cells whose resistance to MNNG could be overcome by ectopic overexpression of MSH2. Since MMR also corrects spontaneously occurring G–T mismatches arising, for example, from deamination of 5-methylcytosine

Figure 7 Strategy of ES cells to clean up their genome from pre-mutagenic O⁶MeG adducts and control on MSH2 expression in pluripotent and differentiated cell types. (a) Although MGMT is highly expressed in ES cells, they will eliminate cells still harboring O⁶MeG in their genome by apoptosis. In differentiated cells, this elimination gets lost. They will therefore survive at the expense of mutations. (b) A model of regulation of high MSH2 protein expression in undifferentiated ES cells. Rb protein is in the hyperphosphorylated state and therefore cannot bind E2F1, which transcribes the MSH2 gene. This will lead to effective MMR and, in the case of O⁶MeG stress, will signal apoptosis. (c) A model for low MSH2 protein expression in differentiated MFs. Bcl-2 localized in the nucleus prevents hyperphosphorylation of Rb by Cdk2. Therefore, Rb binds E2F1 and transcription of MSH2 stops. This will lead to loss of MMR and, in the case of O⁶MeG, a methylating agent-resistant phenotype

in DNA,²⁸ one might expect ES cells to show a lower spontaneous mutation frequency than somatic cells. This has indeed been found to be the case.²⁹

What regulates MMR in ES cells? As outlined in Figure 7b, MSH2 gene expression is governed by the transcription factor E2F1,²¹ which is regulated by Rb. Previously it was demonstrated that Bcl-2 localized in the nucleus is able to inhibit the transcription activity of E2F1. This involves hypophosphorylation of the Rb protein (pRb),²² which occurs via a mechanism, where Bcl-2 downregulates Cdk2 activity by a direct interaction with the catalytic subunit of Cdk2²² (see Figure 7c). Upon pRb binding to E2F1, the expression of MSH2 ends.²² To see whether this proposed mechanism of MSH2 regulation applies to ES cells, we compared MSH2, ppRb, Bcl-2 and E2F1 protein levels in four ES cell lines with five fibroblast cell lines. All MF lines showed a higher nuclear Bcl-2 level than the ES cell lines. Conversely, all ES lines showed a higher ppRb level than fibroblasts, while E2F1 levels were similar in all cell lines. The Chip assay clearly showed that ES (R1) cells have more E2F1 bound to the

promoter of MSH2 than SA 3T3 cells. From this and the fact that ES cells exhibit a higher MSH2 expression than MFs, we conclude that the high MSH2 expression level is due to high E2F1 activity.

We further asked whether the high MutS α expression levels found in undifferentiated cells are still detectable in their differentiated progeny. Three different ES lines were allowed to differentiate *in vitro* to fibroblast and neuronal cell types. After differentiation of the ES cells, the protein levels of both MSH2 and MSH6 clearly decreased. This was also found on MSH2 gene level. We should note that the MSH6 gene expression level remained similar to undifferentiated ES cells. Nevertheless, this has impact on MutS α heterodimer level because MSH2 stabilises the MSH6 protein.³⁰ Therefore, as MSH2 protein decreases, so will MSH6 protein. This was indeed observed. The differentiated fibroblast- and neuronal-like cells, expressing low MutS α , showed a significant (~75%) decrease in apoptosis compared to the parental ES cells upon treatment with MNNG. This shows that loss of high MutS α activity of ES cells during their differentiation has an

impact on the response of cells to non-repaired O⁶MeG lesions. Thus, the ability of O⁶MeG lesions to trigger cell death decreases as ES cells become differentiated.

Apoptosis induced by O⁶MeG was shown to require DNA replication²⁴ and MutS α -mediated formation of DSBs²⁷ that trigger either the mitochondrial or the death receptor apoptotic pathway.³¹ Here, we show that upon the induction of O⁶MeG and under MGMT-depleted conditions, H2AX becomes phosphorylated in ES (R1) cells, which is indicative of the formation of DSBs.³² Further, ES cells showed a higher level of nuclear p53 in response to MNNG, compared to fibroblasts. p53 is a transcriptional regulator of the apoptosis stimulating fragment (Fas) receptor (Fas/CD95/Apo-1).³³ Again, ES cells exhibited a higher O⁶MeG-triggered Fas receptor level than fibroblasts. Furthermore, in ES (R1) cells caspase-3 and caspase-7 became activated upon MNNG treatment. The mitochondrial damage pathway appears not to be activated since the Bcl-2/Bax fraction increased, which is an indication of protection against mitochondrial-mediated apoptosis.³⁴ Also, no cytochrome *c* release was observed. Overall, the data suggests that in ES cells non-repaired O⁶MeG lesions trigger apoptotic death via the Fas (CD95, Apo-1) death pathway.

The MutS α -mediated hypersensitivity of ES cells to O⁶MeG DNA lesions does not necessarily influence its sensitivity to other DNA damaging agents. Although cisplatin-induced DNA adducts can be recognized by MMR, it has been shown that MSH2-deficient and proficient ES cells exhibit similarly survival levels following cisplatin treatment.³⁵ Furthermore, ES cells were shown to be hypersensitive to ionizing radiation, which has been ascribed to a lack of G1 arrest in ES cells compared to differentiated cells.³⁶ It has also been reported that ES cells have a defect in repairing photolesions by nucleotide excision repair (NER),³⁷ and are therefore more sensitive to UV-C light than MEF.

Our data on MGMT and MMR in ES cells suggest that ES cells protect themselves from O⁶MeG born mutations on two fronts: firstly, by expressing high levels of MGMT and, secondly, by a high level of MutS α that is essentially involved in triggering apoptosis in response to this pre-mutagenic DNA damage (Figure 7a). It therefore appears that ES cells have evolved a fine-tuned system aimed at protecting them from mutations provoked by O⁶MeG DNA adducts induced endogenously or exogenously by methylating genotoxins that are ubiquitously present in the environment. The efficient elimination of cells harboring O⁶MeG lesions by apoptosis prevents the fixation and spread of mutations to the progeny of the stem cells, thereby maintaining a genetically unmodified stem cell population. This protection by elimination of damaged cells by apoptosis gets lost once the ES cells start to differentiate.

Materials and Methods

Cell lines and culture conditions. The ES cell lines R1 (bought by Dr. E Bockamp from Dr. A Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Ontario, Canada), E14 (gift to Dr. E Bockamp from Dr. T Moroy, Institute für Zellbiologie (Tumorforschung), IFZ, Universitätsklinikum, Essen, Essen, Germany), W9.5 (gift to Dr. E Bockamp from Dr. JR Mann, Division of Biology, Beckman Research Institute of the City of Hope, Duarte, California, USA), I7 and CY7 (gift from Dr. E Bockamp) were used in this study. ES lines were cultivated in

DMEM containing 15% fetal bovine serum (FBS) (Biocrom AG), 0.1 mM 2-mercaptoethanol, 4 mM L-glutamine, 5 ml MEM non-essential amino acids (Gibco), 0.2 U/ml penicillin, 0.2 μ g/ml streptomycin and 2000 U/ml LIF (Chemicon International). SA 3T3 and five additional lines designated MF1–5 (MFs obtained from different sources) were grown in DMEM containing 10% FBS. Low-passage number MEF were grown in DMEM containing 10% FBS.

Colony cellular survival. Colony assays were performed on log-phase growing cells as described previously.³⁸ Shortly, cells were seeded in duplicate at appropriate cell numbers in 60 mm Petri dishes to yield approximately 100 surviving colonies. After 12 h, when cells were attached, cells were exposed to graded doses of MNNG. After 10–14 days cultures were fixed (in acetic acid: methanol: H₂O 1 : 1 : 8), stained (in 0.01% amido black) and colonies containing 50–100 cells were counted. Survival curves represent averages of three experiments.

Quantification of apoptosis. Apoptotic response after MNNG treatment, in the presence and absence of MGMT, was measured using the flow cytometric method of sub-G1 determination. Cells were fixed in ethanol (70%) and stained with propidium iodide (16.5 μ g/ml) in PBS after RNase (0.03 μ g/ml) digestion. Samples were analysed on a FACS Calibur (Becton Dickinson) using the CellQuest Software. Cell cycle distributions were analysed using ModFit LT (Verity Software House Inc.).

Preparation of protein extracts

Total cell extracts. Cell pellets were snap frozen and stored at -80°C . After thawing, the cell pellets were resuspended in total cell extract buffer (20 mM Tris, 1 mM EDTA, 1 mM β -mercaptoethanol, 5% glycerine, 1 μ M Na₃VO₄, 0.5 mM PMFS, 10 mM DTT, pH 8.5). The cells were then lysed by sonication and cell debris was removed by centrifugation at 10 000 r.p.m. for 10 min. The protein concentration were determined as described.³⁹ For Western blot analysis of proteins from equal cell numbers, cells were first counted before being lysed by sonication.

Fractionated cell extracts. Cell pellets of treated and untreated samples were suspended in fractionation buffer A (10 mM HEPES-KOH, pH 7.4, 0.1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 μ M Na₃VO₄, 0.5 mM PMFS, 10 mM DTT). The cells were lysed by freeze/thaw/vortexing. The lysate was then centrifuged at 10 000 r.p.m. for 10 min and the supernatant contained the cytoplasmic proteins isolated. The pellet, containing the nuclei, organelles and membranes, were then suspended in fractionation buffer B (20 mM Tris, 1 mM EDTA, 1 mM β -mercaptoethanol, 5% glycerine, 1 μ M Na₃VO₄, 0.5 mM PMFS, 10 mM DTT, pH 8.5). This suspension was then homogenized by sonication. The suspension was centrifuged at 10 000 r.p.m. for 10 min. The supernatant contains the nuclear proteins and the pellet the membrane fragments. This membrane pellet was suspended in fractionation buffer B containing 1% Triton X-100. For the cytoplasmic fraction containing cytochrome *c*, cells were lysed in fractionation buffer A by freeze/thaw/vortexing. The lysate was then centrifuged at 700 g for 10 min to remove the nuclei. The supernatant contained the cytoplasmic extract with mitochondria, which was loaded on the SDS-PAGE. The protein concentration were determined as described.³⁹

Nuclear extracts for EMSA. Fresh cells were suspended in 1 ml of lysis buffer I (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5 mM PMSF and 2 mM DTT) and incubated for 10 min on ice. After the addition of Nonidet P-40 (final concentration 0.5%) the solution was vortexed, incubated on ice for 5 min and centrifuged (400 g, 5 min). Thereafter, the pellet was again washed with lysis buffer I. The pellets were then resuspended in two volumes of lysis buffer II (20 mM HEPES-KOH, pH 7.4, 600 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 2 mM DDT) and incubated for 30 min on ice. After centrifugation, the supernatant was diluted by the addition of one volume of lysis buffer III (20 mM HEPES-KOH, pH 7.4, 0.2 mM EDTA, 0.5 mM PMSF, 2 mM DDT). Glycerol was added to a final concentration of 20% and aliquots were shock-frozen in liquid nitrogen and stored at -80°C .

Cell extracts for MGMT activity assay. Cells were harvested and homogenized by sonication in buffer containing 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1 mM β -mercaptoethanol, 5% glycerol and the protease inhibitor PMSF (0.1 mM). The extract was centrifuged at 10 000 r.p.m. (10 min) in the cold in order to remove debris and the supernatant was snap frozen in aliquots using liquid nitrogen and stored at -80°C until use. The protein concentration were determined as described.³⁹

MGMT activity assay. The MGMT activity in ES (R1) and SA 3T3 cells was determined. It is based on a radioactive assay, where tritium-labelled methyl groups

are transferred from the O⁶-position of guanine to protein in the cell extract.⁴⁰ Data are expressed as femtomoles of radioactivity transferred from ³H-labelled DNA to protein per milligram of protein within the sample.

EMSA. For gel retardation assays, 29-nt oligomers with the sequence 5'-GGGCTCGAGCTGCAGCTGCTAGTAGATCT-3' were annealed to oligomers with the sequence 5'-GGGAGATCTACTAGNAGCTGCAGCTCGAG-3' (N = C or T) and labelled with γ -³²PdATP using PNK. An aliquot of 4 μ g nuclear proteins for human extracts or 10 μ g nuclear proteins for mouse extracts was incubated in 10% glycerol, 10 mM HEPES-KOH, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 5 mM Tris-HCl, 0.5 mM DTT, 0.5 mM EDTA, 0.5 μ g BSA and 2 μ g poly(dI-dC) with 25 fmol radioactively labelled duplex DNA for 45 min at 16°C, after which the DNA-protein complexes were separated on 4% polyacrylamide gels using 0.25 $\times$ TBE buffer.

Western blot analysis. Western blot analysis was performed as described.²⁵ Antibodies used were anti-P53 and anti-cytochrome c (Oncogene), anti-Fas, anti-Bcl-2, anti-Bax and ERK2 (Santa Cruz Biotechnology Inc.), anti-caspase-3, anti-caspase-7 and anti-phospho-Rb (ser807/811) (Cell Signaling technology), anti-Rb (Pharmingen), MSH2 (Calbiochem) and MSH6 (Transduction Laboratories).

RA differentiation of ES cells to fibroblast-like cells. The differentiation of ES cells to fibroblast-like cells were performed by using RA. Shortly, ES cell cultures were treated with 10⁻⁶ mol/l RA for 6–12 days in the absence of LIF. After this time period, the majority of cells were fibroblast-like cells, while the minority were neuron like.

Preparation of RNA and RT-PCR. Total RNA was extracted by using a commercial RNA isolation kit (Machery-Nagel); 2 μ g of RNA were transcribed into cDNA by Superscript II (Invitrogen) in a volume of 50 μ l; 3 μ l cDNA was subjected to PCR, which was performed using specific primers for the MSH2 (up, 5-TCAACTGAAAGGCGTCTCCACATT-3; low, 5-CGGCTCGGCTTCGTACATC-3) and MSH6 (up, 5-GTGTGCGCCAGAAATTGTGTACC-3; low, 5-GGCTATCG CTGTCCCATCAAAAGT-3) and the Red-Taq Ready Mix (Sigma-Aldrich). PCR program was as follows: 1.5 min, 94°C, followed by 25 cycles of denaturation, 45 s, 94°C; annealing was, 45 s, 58°C; elongation, 1 min, 72°C, followed by 10 min at 72°C. The total amplification product was subjected to a 1% tris-borate-EDTA agarose gel.

Transfection of SA 3T3 cells with MSH2. MSH2 transfectants were generated by transfection of SA 3T3 cells with a mammalian expression vector (pHAg3c) harboring the mouse MSH2 gene, which has been described previously.³⁵ The transfection method employed was the Effectene transfection kit (QIAGEN, Hilden, Germany). In brief, ~2 μ g of pHAg3c were transfected and then the transfected cells were selected for with 2.2 μ g/ml puromycin until clones formed. Puromycin-resistant clones were picked in 24-well plates and tested for MSH2 expression using Western blot analysis. Transfectant clones were routinely cultured in medium containing 2.2 μ g/ml puromycin that was omitted during the experiments.

Chip. Cellular genomic DNA and proteins were crosslinked by addition of 37% formaldehyde (1% final concentration) to the medium for 10 min in a 10 ml dish. The reaction was stopped by addition of 1.25 M glycine (0.125 M final concentration). After 5 min incubation, medium was removed, cells were washed twice with PBS, collected and resuspended in 1 ml PBS containing 1 μ l PMSF. Genomic DNA was fragmented by sonication to a fragment size between 500 and 1500 bp. Sodium lauroyl sulfate was added to a final concentration of 0.5%. After 20 min mixing, membrane fragments were removed by centrifugation (10 min 10 000 g). Equal amounts of fragmented DNA were subjected to immunoprecipitation (IP) using an E2F1-specific antibody (sc-193, Santa Cruz Biotechnology) and protein G-sepharose. Immunoprecipitated proteins crosslinked to DNA were washed four times in 1 ml wash buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA 8.0, 20 mM Tris-HCl (pH 8.0, protease inhibitors). After the final washing step, the immunoprecipitate was resuspended in 1 ml washing buffer containing 500 mM NaCl. After an additional centrifugation step, the immunoprecipitate was resuspended in 400 μ l elution buffer (1% SDS, 100 mM NaHCO₃) and 500 μ g/ml Proteinase K and RNase I were added and incubated 30 min at 37°C. Crosslinks were reversed by heating at 65°C for overnight. The probe was adjusted to 200 mM NaCl, proteins were removed by phenol-chloroform extraction, DNA was recovered by ethanol precipitation and resuspended in aqua bidest. PCR was performed using

specific primers for the MSH2 promoter region and, as negative control, β -actin. For MSH2, the primers were up 5'-CGGCTTGCCAAAGTGTCTATTGG-3' and low 5'-CAAGTGACCGGAAACGTAAGAGTG-3'. For β -actin, the primers were up 5'-GG GTGGCTTTTGGGAGGGTGA-3' and low 5'-TGAAGCGCAGCAGATTGGTTG G-3'. PCRs were repeated by using variable cycle numbers and different amounts of templates to ensure that results were within the linear range of PCR.

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