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Mismatch and Base Excision Repair Proficiency in Murine Embryonic Stem Cells

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Abstract

Accumulation of mutations in embryonic stem (ES) cells would be detrimental to an embryo derived from these cells, and would adversely affect multiple organ systems and tissue types. ES cells have evolved multiple mechanisms to preserve genomic integrity that extend beyond those found in differentiated cell types. The present study queried whether mismatch repair (MMR) and base-excision repair (BER) may play a role in the maintenance of murine ES cell genomes. The MMR proteins Msh2 and Msh6 are highly elevated in mouse ES cells compared with mouse embryo fibroblasts (MEFs), as are Pms2 and Mlh1, albeit to a lesser extent. Cells transfected with an MMR reporter plasmid showed that MMR repair capacity is low in MEFs, but highly active in wildtype ES cells. As expected, an ES cell line defective in MMR was severalfold less effective in repair level than wildtype ES cells. Like proteins that participate in MMR, the level of proteins involved in BER was elevated in ES cells compared with MEFs. When BER activity was examined biochemically using a uracil-containing oligonucleotide template, repair activity was higher in ES cells compared with MEFs. The data are consistent with the suggestion that ES cells have multiple mechanisms, including highly active MMR and BER that preserve genetic integrity and minimize the accumulation of mutations.

Keywords

mismatch repair; base excision repair; embryonic stem; ES; DNA repair

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Conflict of interest

The authors declare that there are no conflicts of interest.

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1. Introduction

Cells are continuously exposed to DNA damaging agents that derive from endogenous sources, such as normal cellular metabolism byproducts, and from exogenous sources, such as radiation and chemical exposure. Both endogenous and exogenous challenge can contribute to genomic instability as well as aging [1]. To reduce or avoid the consequences of such insults, cells have evolved multiple mechanisms, including the involvement of several DNA repair pathways, to minimize DNA damage and the subsequent accumulation of mutations.

The mismatch repair (MMR) pathway repairs mismatches between bases on complementary DNA strands due to errors in nucleotide incorporation and introduction of short insertions and deletions (indels) due to polymerase slippage during replication (reviewed in [2]). Failure to accurately repair such errors can result in the accumulation of mutations and microsatellite instability, characteristic of hereditary nonpolyposis colon cancer (HNPCC), where components of the mismatch repair machinery are inactivated by loss of function mutations or promoter hypermethylation [3–6].

The base excision repair (BER) pathway repairs DNA damage resulting from damaged bases, generally caused by oxidation, alkylation, or deamination, as well as DNA damage in the form of single strand breaks (reviewed in [7]). Repair is generally divided into two subtypes: short patch repair, where a single nucleotide is removed and replaced, and long patch repair in which a stretch of several nucleotides is removed from the damaged strand followed by DNA synthesis and ligation. The former is partially dependent upon DNA polymerase β to complete repair, while the latter is dependent upon PCNA and is independent of DNA polymerase β [8].

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst and are capable of forming a complete embryo. If mutations occur in ES cells or during a very early stage of development, the genetic damage could affect multiple tissues and can also be passed on to future generations. To avoid the accumulation of mutations and their deleterious effects, ES cells must have multiple robust mechanisms to maintain genomic stability and keep mutations to a minimum. Consistent with this notion, mouse ES cells display a spontaneous mutation frequency that is about a 100-fold lower than that of mouse embryo fibroblasts (MEFs) [9]. Compared with MEFs, ES cells are also hypersensitive to DNA damaging agents and, consequently, are more prone to undergo cell death upon challenge. This hypersensitivity to DNA damage effectively removes cells with damaged DNA from the self-renewing stem cell pool and maintains this pool of cells pristine [10–12]. Other studies indicate that ES cells can also undergo differentiation upon nuclear translocation of p53, which is normally localized in the cytoplasm of these cells [13–14]. Nuclear p53 protein for example, can associate with the Nanog promoter and result in the down-regulation of Nanog transcription [15]. As a consequence, ES cells may activate a differentiation program in response to certain types of DNA damage, providing an alternative mechanism by which self-renewing cells with DNA damage can be removed from the stem cell population [16]. Indeed, it has been reported that p53 localizes in the nucleus of ES cells following DNA damage [17]; however, such nuclear localization has not been universally observed [18].

In addition to the above-mentioned mechanisms for preserving genomic integrity, there are additional means by which ES cells deal with DNA damage. They have very robust stress defense pathways [19], and human ES cells also have a level of BER activity that is much higher than that of differentiated cell types when tested by the alkaline comet assay [20]. Furthermore, murine ES cells preferentially utilize high fidelity homology-mediated repair

pathways to repair DNA double-strand breaks rather than the error-prone and mutagenic non-homologous end joining pathway that predominates in somatic cells [21–22].

This present study has examined the inherent capacity of murine ES cells to repair damaged DNA templates using the MMR and BER pathways and to assess the proposition that ES cells are more efficient at repairing such damage compared with MEFs, their differentiated counterparts.

2. Materials and Methods

2.1 Cells and Transfections

ES cell lines derived from blastocysts from a cross between mouse strains 129/Sv and C3H (designated as 3C4 or wildtype) and an ES cell line from MSH2 mutant mice designated MSH2 G674A or mutant were grown as previously described [21–22]. Isogenic 129/SvXC3H wildtype MEFs were cultured in DMEM supplemented with 10% fetal bovine serum, 2mM SG-200, 1X non-essential amino acids, and 100µg/mL penicillin and 100U/mL streptomycin (all from Fisher Scientific, Pittsburgh, PA, USA) and used at passages 1–2 for all experiments. All cells were maintained at 37°C and 7.5% CO₂. ES cells were transfected with xfect stem (Clontech, Mountain View, CA, USA) according to the manufacturer's recommendations with the following modifications: cells were seeded on gelatin-coated plates 24 hours prior to transfection at 60% confluence. Transfection reagent was kept on the cells for 24 hours prior to media change. MEF feeders were not added to the transfected cells at any time. For transfection of MEFs, passage 1 MEFs were plated and allowed to adhere at 60% confluence prior to transfection with dreamfect gold (OZ Biosciences, Marseille, FR) reagent according to manufacturer's recommendations.

2.2 Western Blot

Adherent cells were dislodged from the culture dish with 0.25% trypsin and harvested by centrifugation (850xG). Cells were lysed in ice cold RIPA buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 1% SDS, 0.5% deoxycholic acid) and boiled in 2X protein loading buffer (20% glycerol, 120mM Tris, pH 6.8, 4% SDS, 0.01% bromophenol blue). Lysates containing 20 µg or 60 µg total cellular extract were loaded on 6 or 10% polyacrylamide gels and subjected to SDS-PAGE, using electrophoretic settings of 250V for 45 minutes in a standard Tris-Glycine buffer system. Proteins were transferred to PVDF membranes and blocked with 3% milk-PBS-T. Antibodies to Ape1 (Cell Signaling, Danvers, MA, USA), Msh2 (clone FE11/Ab-2; EMD Biosciences, San Diego, CA, USA), DNA Ligase III, MSH6 (7, 44; BD Biosciences, San Jose, CA, USA), Oct4, Mlh1, PcnA, Pms2, Xrcc1 (H-134, N-20, PC10, C-20, H-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Parp1, Ung2 (E102, n/a; Abcam, Cambridge, MA, USA), and β-actin (AC-15; Sigma, St. Louis, MO, USA) were incubated overnight at 4°C. Blots were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase prior to incubation with Supersignal west pico chemiluminescent reagent (Fisher Scientific, Pittsburgh, PA, USA) and exposure to X-ray film.

2.3 Quantitative PCR

Total RNA was isolated from 5x10⁵ MEFs and ES cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and mRNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA). Two micrograms of total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Carlsbad, CA, USA). An aliquot of the cDNA was amplified for 40 cycles using an ABI Prism 7900HT Sequence Detection System with gene-specific primers designed using the Primer Express software (Applied Biosystems, Carlsbad, CA, USA). Sybr Green dye was used for signal detection.

All analyses were carried out in triplicate, and non-template controls and dissociation curves were used to ensure specific template amplification. Target gene expression level was calculated as a ratio of the mRNA level of the transcript being measured relative to a control mRNA level for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same cDNA preparation. Graphs are the averages $\pm$ S.E.M., normalized to MEFs. Primer sequences used were: MSH2-(5'-CATTGTGGATTGCATCCTTGC-3' and 5'-TGACCTGAGGATGGAAGCAGTC-3'); MSH6-(5'-AGGCAAAGGATCTCAACGGAG-3' and 5'-CCAAACCAAATCACCTGGTGA-3'); and GAPDH-(5'-CTCCACTCACGGCAAATTCAA-3' and 5'-GATGACAAGCTTCCCATTCTCG-3').

2.4 Mismatch Repair Assay

Plasmid p111 contains a wildtype EGFP gene that normally expresses GFP protein. The reporter plasmid p189 is identical to p111 except for a mutation at position 58 in the EGFP reading frame that generates a stop codon (TGG to TAG) that prevents expression of functional GFP. Both plasmids were a kind gift from Lu-Zhe Sun (U. Texas Health Science Center) and were prepared as described with minor modifications [23]. Briefly, to create the MMR reporter plasmid, p189 was linearized with BstXI enzyme and extracted with phenol-chloroform. The p111 plasmid was nicked with Nb.Bpu101 enzyme (Recognition sequence 5'-CCTNAGC-3'/3'-GGANT[^]CG-5'; Fermentas, Inc., Glen Burnie, MD, USA), extracted with phenol-chloroform and precipitated with isopropanol. Nicked p111 plasmid was resuspended in TE buffer and treated with Exonuclease III (Fermentas, Inc., Glen Burnie, MD, USA) for 30 minutes at 37°C to create single-stranded p111. To anneal the construct, linearized p189 and single-stranded p111 were mixed in a ratio of 1:1.5 and heated at 90°C in a heat block for 5 minutes prior to slow overnight cooling to room temperature. The MMR plasmid was treated with plasmid-safe DNase (PSAD; Epicentre Biotechnologies, Madison, WI, USA) for 25 minutes at 37°C to degrade remaining single-stranded DNA, followed by heat inactivation of the PSAD at 70°C for 30 minutes. An aliquot of DNA from each step was subjected to gel electrophoresis to confirm that digestion or annealing was complete. The resultant mismatch repair reporter plasmid contains a nick and a TGG-ATC mismatch producing a stop codon on the same strand. Cells were transfected with the mismatch repair plasmid or p189 (serving as an empty vector) and co-transfected with pDsRed-Express (Clontech, Mountain View, CA, USA) and allowed to repair for 48 hours prior to harvesting. Cells were resuspended in PBS and analyzed by flow cytometry using a BD LSRII (BD Biosciences, San Jose, CA, USA) flow cytometer. The instrument was aligned with AlignFlow 2.5µm beads (Molecular Probes, Carlsbad, CA, USA) for the 488 nm laser. Data were collected and analyzed live with FACSDiVa software (BD Biosciences, San Jose, CA, USA). Cells were excited with the 488 nm line of an argon-ion laser using GFP and DsRed fluorochromes. Log fluorescence was recorded for GFP using a 530/30 band pass filter and for DsRed using a 585/42 band pass filter. In all experiments, ten thousand events were collected, and the ratio of GFP positive divided by DsRed positive cells was used for data processing. Data presented are the average of at three experiments. Error bars represent S.E.M.

2.5 *In vitro* analysis of base excision repair (U:G mismatch repair incorporation assay)

Nuclear extracts were prepared as previously described [24], and an *in-vitro* BER assay that measures the short patch BER efficiency of cell extracts was performed [25]. Briefly, a 30-mer oligonucleotide 5'-GAGCCGGCACTGGUCCCCAGCTGATATCGC-3' containing a uracil at position 14 (underlined) was annealed to the oligonucleotide 5'-GCGATATCAGCTGGGCGCCAGTGCCGGCTC-3'. The corresponding faithful duplex containing a CG base pair, rather than the UG mismatch, was used as control. The DNA duplex was incubated in a reaction mixture (25 µL) with an equivalent amount (6 µg) of

nuclear extract from MEF or ES cells at 37°C for 30 min. The reaction mixture contained 40 mM phosphocreatine di(Tris) salt, 5 mM MgCl₂, 1 mM dithiothreitol, 2 mM ATP, 20 μM each of dATP, dGTP, dTTP and dCTP, 1 μCi [α -³²P] dCTP, 2.5 μg creatine phosphokinase, 50 mM NaCl, and 0.5 μg 30 bp duplex oligonucleotide (UG or CG substrate). Reactions were stopped by proteinase K treatment (1 μL of 10 mg/ml proteinase K per reaction) at 37°C for 10 min. Samples were loaded onto a 15% polyacrylamide gel and electrophoresed at 150 V for 60 min. The BER activity is determined by the incorporation of the [α -³²P] dCTP, in replacing the uracil in the 30 bp DNA duplex. The radiolabeled 30 bp fragment was visualized and quantified with a Typhoon PhosphoImager using ImageQuant software (Molecular Dynamics/GE Healthcare, Piscataway, NJ, USA).

2.6 *In vitro* analysis of base excision repair (U:G mismatch repair incision assay)

The overall procedure for completing the DNA glycosylase assay has been described previously [26]. Oligonucleotides described in section 2.5 were 5' end labeled with [γ -³²P] ATP using a 5' end labeling kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Unincorporated radiolabeled nucleotides were separated from the labeled oligonucleotides using Sephadex G-50 spin columns (GE Healthcare, Piscataway, NJ, USA). Labeled oligonucleotides were annealed to their complement unlabeled oligonucleotides at a concentration of 1:2 (labeled to unlabeled). Ten micrograms of nuclear extract from 129/C3H MEFs or ES cells were incubated for 30 minutes at 37°C in a reaction buffer containing 20 ng of indicated oligonucleotide duplex, 45 mM Hepes-KOH, pH 7.8, 70 mM KCl, 1 mM dithiothreitol, and 2 mM EDTA. For a negative control, reactions were prepared without nuclear extract. Reactions were stopped by the addition of 15 μL formamide loading dye (0.1% bromophenol blue, 0.1% xylene cyanol, in 100% formamide) and incubating the mixture at 90°C for 5 minutes. Products were subjected to denaturing electrophoresis on 20% polyacrylamide gels containing 7 M Urea, 89 mM Tris-borate pH 8.3, and 2 mM EDTA and imaged and quantitated using a Typhoon PhosphoImager using ImageQuant software (Molecular Dynamics/GE Healthcare, Piscataway, NJ, USA).

3. Results

3.1 The abundance of mismatch repair proteins and transcripts is elevated in ES cells but not in MEFs

To begin to address the intrinsic MMR capacity of ES cells and MEFs, the relative abundance of proteins involved in mismatch repair in unchallenged cells was measured by Western blot (figure 1A). Both Msh2 and Msh6, the predominant proteins in mismatch recognition, were expressed at a very high level in wildtype ES cells, compared with MEFs and were consistent with other studies [10, 27]. Similarly, the levels of the accessory proteins Pms2 and Mlh1 were also elevated in ES cells, but not to the same extent. As a control for subsequent studies that examine MMR activity, we also assessed the level of these proteins in an Msh2-mutant ES cell line. This cell line, which is homozygous for a glycine to alanine substitution at position 674 (G674A) in the ATP binding domain of Msh2, renders the Msh2 protein incapable of supporting DNA repair but retains the capacity for apoptotic signaling [28]. This Msh2 mutant ES cell line expresses MMR proteins at levels equivalent to those found in the wildtype ES cells. To ensure that the ES cells analyzed had remained undifferentiated, they were probed for Oct4 expression as a marker for undifferentiated self-renewing cells.

To better understand the basis for the very high abundance of MMR protein in ES cells, we analyzed the mRNA levels for most of these elevated proteins. The level of Msh2 transcript was about four-fold higher in the ES cells than in the MEFs (figure 1B). The level of Msh6

transcripts was even higher, displaying about a six-fold elevation over that in MEFs. The greater disparities between levels of MMR proteins in ES cells and MEFs compared with differences in levels of their respective mRNAs, argues for mechanisms beyond increased transcription to generate the elevated MMR protein levels in ES cells.

3.2 Mismatch repair is significantly more active in ES cells than in MEFs

To measure actual MMR capacity, we generated a MMR reporter plasmid (diagrammed in Figure 2A and 2B), which contains a premature stop codon-containing mismatch early in the EGFP reading frame as well as a nick on the same strand. Introducing this reporter in cells will not produce any GFP positive cells unless the mismatched strand containing the stop codon is accurately repaired. A lack of repair or inaccurate repair results in the generation of premature stop-codon containing or mutant GFP transcripts and a lack of fluorescence. However, accurate repair of the reporter results in reestablishment of a functional GFP protein and is a measure of faithful MMR. For specific information on how the MMR reporter was prepared, see section 2.4. Since the nicked strand is also the strand that contains the stop codon, this strand is favored for repair of the mismatch (reviewed in [29]).

The MMR reporter plasmid DNA or control circular p189 plasmid DNA that harbors a mutant EGFP but no base mismatch, were transfected into MEFs, or into wildtype (3C4) ES cells, or the Msh2 mutant ES cells, along with a DsRed vector to control for transfection efficiency. Transfected cells were given time to repair and subsequently analyzed live by flow cytometry (Figure 2C). Data are displayed as the percent of GFP positive cells divided by the percent DsRed positive cells and normalized to the control vector. Using this method, the data resulting from differences in the transfection efficiencies between the different cell types will not favor one cell type over another. When examined in this manner, wildtype ES cells displayed robust MMR (Figure 2D), with a greater than 30-fold higher repair capacity than the control vector, consistent with data obtained by western blot. When the homozygous Msh2 mutant ES cells were examined in the same manner, repair capacity was only about 7-fold higher than control and four- to five-fold lower than the wildtype ES cells. By contrast, DNA repair by MMR was only about 2-fold higher in MEFs transfected with the reporter plasmid than with the control.

3.3 Base-excision repair activity *in vitro* is higher in ES cells than in MEFs

To assess the potential of ES cells and their differentiated MEF counterparts to carry out BER, cell extracts from both cell types were probed for BER expression by western blot. Reminiscent of MMR, the abundance of all BER proteins examined in ES cells was highly elevated (figure 3A). For example, Ape1 and Xrcc1 were barely detectable in MEFs, even when the amount of MEF lysate used was three times that used to examine protein levels in ES cells. DNA Ligase III, Parp-1, and UNG2 were undetectable in MEFs. PCNA was about 3-fold elevated in ES cells compared with MEFs after normalizing for protein loading. Again, Oct4 was used to distinguish undifferentiated ES cells. In addition, proteins DNA polymerase β , MBD4, and Thymine DNA glycosylase were also expressed at higher levels in ES cells compared with MEFs (supplementary figure 1).

To examine BER activity in MEFs and ES cells, we first used nuclear extracts to perform an *in-vitro* short-patch base excision repair assay (Figure 3B). Relative BER activity in the cells was assessed by subtracting the signal pixel intensity from the incorporation of ^{32}P -dCMP (from $[\alpha]$ ^{32}P -dCTP) of the CG template from that of the UG template (indicative of repair). These values were normalized to those obtained for MEFs. The MEFs were able to carry out repair by BER, despite the low expression of proteins required for this repair activity, and displayed limited incorporation into the control CG template. The ES cells, with higher BER protein levels also displayed higher BER activity than the MEFs based on incorporation into

the UG template. However, they also showed elevated background incorporation into the control CG template, which lowered overall repair measurements.

To validate the BER incorporation assay, we also employed a DNA incision assay using the same oligonucleotide duplexes as with the incorporation assay. However, in this assay, the sense strand was end labeled with ^{32}P at its 5' end prior to the incubation step with nuclear extracts. Incision of the radiolabeled strand at the site containing a uracil will yield a 13-mer product when reactions are electrophoresed on denaturing PAGE gels. Pixel intensities of the incision products were normalized to the lanes containing no extract but only the respective DNA oligonucleotide. When the normal CG template was used in this manner, both the MEF and ES cell reactions displayed little incision activity compared with the control lane containing DNA but no nuclear extract. When the UG template was utilized, extracts from MEFs produced an incision product at a level of more than 2-fold greater than control without any extract. ES cell extracts, on the other hand, generated a product which was over 12-fold greater than control levels.

4. Discussion

The canonical role of MMR is to repair DNA base pair mismatches as well as single and dinucleotide insertions and deletions to minimize the accumulation of mutations. Not surprisingly, ES cells derived from knockout mice that lack Msh2 display significantly higher mutation rates compared with wildtype cells and are more resistant to killing by 6-thioguanine, possibly due to the role Msh2 plays in apoptosis [26]. The elevated levels of MMR proteins we observed in ES cells, therefore, may explain in part why wildtype ES cells undergo apoptosis in response to induced O⁶-methylguanine adducts [10] while ES cells that express ten times less Msh2 protein are resistant to death following treatment with methylating agents [30–31].

The question remains as to the mechanism by which ES cells induce such high expression of proteins involved in MMR. Since promoters of many MMR-encoding genes contain binding sites for the E2F transcription factor [32–33], it is likely that E2F-regulated transcription plays a key role in producing the elevated levels of mRNA encoding MMR proteins seen in ES cells. This observation is consistent with several reports showing that the S-phase, where E2F promoters more often appear to be accessible, comprises a far greater proportion of the cell cycle in ES cells than in more differentiated somatic cells [34–35]. However, additional mechanisms must be in place in ES cells in order to explain the elevated protein expression, as transcription alone cannot account for the level of protein expression observed.

We now show that MMR proteins are not only elevated in ES cells, but that their abundance correlates with a higher capacity to carry out MMR under basal conditions. To exclude the possibility that some other mechanism, such as BER, contributed to the apparent high levels of MMR activity in ES cells observed with our reporter, a mutant ES cell line with impaired Msh2 activity was used as a control. Since Msh2 is a critical component of recognition complexes containing either Msh6 (MutS α) or Msh3 (MutS β), the ES cell line harboring the Msh2 G674A mutation will have no detectable MMR activity. Thus, some other mechanism, the most likely candidate being BER, contributes about 20% of the total fluorescence generated by reconstitution of functional GFP in our MMR assay. This finding, however, does not alter the overall conclusions of the experiment.

There are a limited number of reports which thoroughly examine BER activity in ES cells. One study examined BER activity in human ES cells after exposure to different DNA damaging agents [20]. Following oxidative damage due to exposure to H₂O₂ and analysis by comet assay, repair of induced damage was significantly higher in the ES cells compared

with two fibroblast cell lines. Quantification of 8-oxyguanine lesions in unchallenged ES cells also found this marker of DNA damage to be lower in the ES cells than in a fibroblast line, suggesting that human ES cells suppress the accumulation of such damage. Our current investigation assessed both the expression of several proteins involved in the BER process, as well as the cells' intrinsic repair capacity to correctly remove and/or replace a uracil moiety from a short double-stranded oligonucleotide template. Elevated BER protein expression in the murine ES cells correlated with an increased ability to repair the oligonucleotide template, regardless of the method used. A confounding factor in the incorporation assay, however, was the higher than expected incorporation of radiolabeled dCTP into the control oligonucleotide in the ES cells. This background incorporation resulted in an overall decrease in the apparent relative efficiency of BER as measured in these cells. A possible explanation is that ES cells have somewhat higher nuclease activity, resulting in partial degradation of the template and subsequent incorporation of ³²P-dCTP. Consistent with this interpretation, we have previously observed somewhat higher nuclease activity on linearized plasmids in ES cells (supplementary figure 2), which may contribute to the incorporation observed with the BER control template. Nevertheless, the DNA incision assay confirmed our DNA incorporation assay demonstrating that ES cells are more proficient at removing a uracil from DNA than are MEFs.

Embryonic stem cells must have multiple mechanisms to preserve genomic integrity since a low mutational burden is critical to the proper development of an organism and the continued health and propagation of a species. Our data demonstrate that murine ES cells utilize MMR and BER pathways under basal conditions with greater efficacy than MEFs, which may contribute to the prevention of the accumulation of endogenous DNA damage and resultant unwanted mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Quantitation of cellular MMR proteins and mRNAs

A) Wildtype ES cells, ES cells with mutant Msh2, and wildtype MEFs were harvested and whole cell lysates were probed by Western blot for basal expression of proteins involved in MMR. Oct4 served as a marker of undifferentiated cells and β -actin was used as a loading control. B) RNA was isolated from wildtype MEFs and ES cells and subjected to quantitative PCR analysis for Msh2 and Msh6. Data is displayed as the ratio of Msh mRNA to GAPDH mRNA and normalized to that observed in MEFs.

Figure 2. Mismatch Repair Capacity of ES Cells and MEFs

A) Schematic of the steps involved in preparation of the MMR plasmid reporter template. Nb.Bpu101= nicking enzyme, ExoIII = Exonuclease III, BstXI = restriction enzyme, PSAD = Plasmid Safe DNase. PSAD selectively digests DNA that is not structured as plasmid DNA. The resulting reporter is a nicked double-stranded plasmid containing a mismatch. B) Agarose gel electrophoresis of DNA samples isolated at different steps of the MMR repair plasmid preparation. C) Representative flow cytometry scatter plots of MEFs and ES cells that had been transiently transfected with control vector (p189) or the MMR repair reporter vector and co-transfected with pDsRed Express to account for differences in transfection efficiency. At 48 hours after transfection, cells were harvested by trypsinization and subjected to bivariate flow cytometry. Axes represent mean fluorescent intensity on a 5-log scale. The X axis represents GFP positive cells and Y axis represents DsRed positive cells. A minimum of 10,000 cells were sorted per trial, only 5000 are displayed. D) Quantification of MMR activity in ES cells and MEFs. Data represent the ratio of GFP-positive/DsRed-positive cells normalized to background generated by the control vector in each of the cell types.

Figure 3. Western blot of BER proteins and measurement of BER activity in ES cells and MEFs
 A) Whole cell lysates of wildtype MEFs and ES cells were probed by Western blotting using antibodies to the indicated BER proteins. Oct4 was used as a marker to of undifferentiated ES cells and β-actin served as a loading control. Note: The MEF lane was intentionally loaded with three times as much lysate as the ES cell lane, which was confirmed by the β-actin loading control. B) In Vitro Short-Patch BER Assay comparing ^{32}P -dCTP uptake into mispaired (UG) and faithfully paired (CG) DNA substrates in ES cell and MEF nuclear extracts. C) DNA Glycosylase Incision Assay measuring the cleavage of $[\gamma\text{-}^{32}\text{P}]$ 5' end labeled mispaired (UG) and faithfully paired (CG) DNA substrates in ES cell and MEF nuclear extracts.