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DNA Repair in Murine Embryonic Stem Cells and Differentiated Cells

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Abstract

Embryonic stem (ES) cells are rapidly proliferating, self-renewing cells that have the capacity to differentiate into all three germ layers to form the embryo proper. Since these cells are critical for embryo formation, they must have robust prophylactic mechanisms to ensure that their genomic integrity is preserved. Indeed, several studies have suggested that ES cells are hypersensitive to DNA damaging agents and readily undergo apoptosis to eliminate damaged cells from the population. Other evidence suggests that DNA damage can cause premature differentiation in these cells. Several laboratories have also begun to investigate the role of DNA repair in the maintenance of ES cell genomic integrity. It does appear that ES cells differ in their capacity to repair damaged DNA compared to differentiated cells. This minireview focuses on repair mechanisms ES cells may use to help preserve genomic integrity and compares available data regarding these mechanisms with those utilized by differentiated cells.

Keywords

Embryonic stem (ES) cell; DNA Repair; Differentiation; Homologous recombination; Mismatch Repair; Nonhomologous end-joining; Nucleotide excision repair

Mutation Frequencies in Embryonic Stem Cells and Differentiated Cells

Embryonic stem (ES) cells are derived from cells of the blastocyst inner cell mass, are pluripotent, and have virtually unlimited self-renewal potential. Upon differentiation, these cells can contribute to the formation of cells of all types within an organism, including germ cells. To prevent an undue mutational burden at the level of both the individual and the species, it is important that they have robust mechanisms to ensure their genomic integrity. Mutations in differentiated somatic cells can lead to somatic diseases involving those cell lineages and cells with which they interact. In contrast, mutations in ES cells can result in catastrophic changes that can affect many different cell types in the organism and that may be passed on to its progeny.

There are distinct differences in mutation frequencies between ES cells and somatic cells, and hence in the maintenance of genomic integrity [1]. When the selectable *Aprt* gene is used as a reporter for mutagenesis in ES cells heterozygous at *Aprt*, they display spontaneous mutation frequencies that approach 10^{-6} . While this mutation frequency is somewhat lower than

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estimates using other model systems [2,3], ES cells display substantially lower frequencies than those in somatic cells. Mouse embryo fibroblasts (MEFs), for example, have mutation frequencies nearly 100 fold greater ($\sim 10^{-4}$) than their isogenic ES cell counterparts, and most of these events involve loss of heterozygosity (LOH) due to mitotic recombination. When spontaneous mutation frequencies at the *Hprt* gene were assessed in similar manner in ES cells and somatic cells, spontaneous mutation in ES cells was undetectable ($<10^{-8}$) whereas mutation frequency in MEFs was in the range of 10^{-5} . The *Hprt* gene is X-linked and therefore not susceptible to LOH as a consequence of mitotic recombination, which probably accounts for much of the difference in mutation frequency between *Aprt* and *Hprt*. In both cases, there was a dose-dependent elevation in mutation frequency when ES cells were exposed to ethyl methanesulfonate (EMS), a mutagenic alkylating agent. These data are consistent with the contention that ES cells have robust mechanisms to ensure the preservation of genetic stability. Spontaneous mutation in either cell type was generally the result of LOH at the *Aprt* locus (80%) vs. point mutation (20%); however, the spectrum of LOH induced mutations was very different between ES cells and MEFs. Whereas MEFs displayed mainly mitotic recombination to generate LOH, ES cells exhibited mainly nondisjunction, and to a lesser extent, mitotic recombination [1]. An independent study investigating LOH in ES cells reported a similar spectrum of events in ES cells [4].

While suppression of mutagenesis in ES cells appears to be one of the mechanisms that contributes to preservation of genomic integrity, it is not, by itself, sufficient. ES cells are hypersensitive to DNA damage and readily undergo apoptosis or differentiation which removes damaged cells from the pluripotent pool [5,6]. Loss of damaged self-renewing cells effectively maintains the proliferating cell population genetically pristine. Consistent with this observation, ES cells lack a functional G1 checkpoint, partly due to sequestration of p53 in the cytoplasm. A possible consequence of the absence of a G1 arrest is that cells with DNA damage can transit from G1 into S-phase where the damage can be exacerbated by proceeding through a round of replication [7–9]. Recently, it was reported that p53 facilitates differentiation by translocating to the nucleus and associating with the Nanog promoter and inhibiting its transcription, suggesting that the role of p53 is more important during differentiation than in responding to DNA damage in ES cells [10]. By supporting ES cell differentiation and consequent withdrawal of cells from the self-renewing population, this mechanism also helps maintain a pure population of cells. Several studies currently focus on the role that DNA repair plays in maintaining genomic stability in ES cells. Few studies, however, specifically compare the repair capacities between ES cells and somatic cells. The remainder of this review focuses on DNA repair in ES cells, and compares these processes to those of somatic cells when data for such comparisons exist.

Double Strand Break Repair

Double strand breaks (DSBs) in DNA are the most toxic type of DNA lesion a cell encounters [11]. Repair of DSBs is expected to be important for ES cells, since there is a high basal level of γ -H2AX staining, a common marker of DSBs (Figure 1). In contrast, unchallenged MEFs display no detectable staining with γ -H2AX. Treatment with etoposide, a topoisomerase II poison that generates DSBs, markedly increases γ -H2AX staining in both cell types. The possible causes of the high level of basal staining in ES cells could be the result of replication fork collapse or reactive oxygen species (ROS) from oxidative metabolism. The latter, however, is unlikely since Saretzki et al. (12) demonstrated that ES cells can be grown in hyperoxic conditions (40% O₂) with little effect on cell proliferation compared with cells grown under normoxic culture conditions. When MEFs were grown in hyperoxic conditions, they underwent fewer than half the number of population doublings compared with those grown in normoxic conditions. This study also suggests that ES cells repair DSBs far more quickly than

mouse 3T3 cells following exposure to IR. Which type of DSB repair, however, was not addressed [12].

There are two major pathways for DNA DSB repair. These are: homologous recombination-mediated repair (HRR) and nonhomologous end-joining (NHEJ). In HRR, repair of DSBs involves the use of a template containing hundreds of base pairs of sequence homology, usually the sister chromatid or homologous chromosome, resulting in faithful, error-free repair. This pathway is active predominantly in the late S to G2 phases of the cell cycle, where sister chromatids are available to serve as templates [13–15]. Many of the proteins involved in this pathway belong to the RAD52 epistasis group and are conserved from yeast to mammals [16]. Mice in which a subset of genes involved in HRR has been knocked out result in early embryonic lethality, suggesting a critical role for these genes in early development [17]. The other major DSB repair pathway is NHEJ which requires little or no sequence homology for efficient repair and can be error-free or error-prone, depending on the type of ends present at the site of the DSB. Direct religation of compatible ends results in error-free repair. If end processing is required prior to religation, however, nucleotides can be deleted or added at the site of the repaired lesion. In contrast to HRR, NHEJ occurs more commonly in the G1 and early S phases of the cell cycle, when a sister chromatid is not available to serve as a template for repair. It also plays a critical role in the generation of immunoglobulin diversity through V (D)J recombination [18, reviewed in 19]. Mammalian somatic cells preferentially use NHEJ whereas HRR is the predominant mechanism for DSB repair in the yeast *S. Cerevisiae* [20].

Considering the need for high fidelity DNA repair of DSBs in ES cells, it is reasonable to speculate that ES cells utilize HRR rather than NHEJ as the principle mechanism of repair. These cells are proficient in homologous recombination as evidenced by their use in gene targeting experiments. The proportion of cells that are correctly targeted, however, is generally low. When a DSB is introduced into the targeting vector or at the target locus, targeting efficiencies increase significantly consistent with functional HRR in ES cells [21–23]. Furthermore, the inherent properties of ES cells are very different from those of differentiated cells. For example, they lack a G1 checkpoint and have very short cell cycle G1 and G2 phases, while spending about 75% of their cycle time in S-phase [24]. The protracted proportion of time spent in S-phase might promote the preferential use of HR rather than NHEJ, since many of the proteins involved in homologous recombination also participate in DNA replication and are regulated by E2F. In addition, because of the very brief G1 phase, the majority of ES cell genomes would have sister chromatids that would be available for efficient recombination-mediated repair.

Dissecting the role of HRR in embryonic stem cells has proven difficult, since the absence of many of these proteins in genetically modified mice results in early embryonic lethality [17]. This observation suggests that these proteins may be critical for the continued propagation of stem cells, through their DNA repair or other yet undetermined functions. Rad51 is a key player in HRR and is involved in the strand invasion process [25]. Mice that are nullizygous for Rad51 are inviable and die early in development. Doubly targeted ES cells lacking Rad51 cannot be obtained, and cells derived from Rad51 null blastocysts do not proliferate, suggesting that Rad51 plays a key role either in cell proliferation or in the repair of DSBs generated by endogenous processes [26,27]. Deletion of Rad51 on a p53 deficient background yielded similar results, although development proceeded to 8.5dpc [27]. Consistent with the contention that HRR is a major repair pathway in ES cells, Rad51 protein levels are about 20-fold higher in ES cells than in MEFs where NHEJ predominates (Figure 2).

The process of NHEJ can be error-free or error-prone, depending the type of DNA ends at the site of the break. Mice lacking components of the NHEJ pathway generally display an increased SCID-like phenotype due to an inability to complete V(D)J recombination. They also display

increased sensitivity to IR with varying severity [28–31]. For example, Ku70 and Ku80 knockout mice are viable, whereas DNA Ligase IV and XRCC4 knockout mice are late embryonic lethal (e14.5) due to massive p53-mediated neuronal apoptosis and defective lymphogenesis [32–34]. The similar phenotype seen with each of these latter proteins is likely due to their required interaction for functionality [35]. The XRCC4 protein level is similar in ES cells and MEFs (figure 2) suggesting that NHEJ may operate equivalently in the two cell types, Functional analyses, however, are required to determine which of the two major DSB repair pathway plays a greater role in repairing DNA lesions in ES cells.

There are limited studies that compare DSB repair capacity and repair mechanisms between ES cells and differentiated somatic cells. One study using two mutant neomycin resistant markers targeted to unlinked loci, reported that ES cells repaired DSBs by HRR 81% of the time and by NHEJ 19% of the time. When differentiated into embryoid bodies (EBs), which can form all three germ layers, HRR accounted for 71% of repair events, NHEJ 7%, and single-strand annealing (SSA), a mutagenic subpathway of HRR, 19%. Further differentiation of EBs to myeloid-like cells resulted in 100% of DSBs being repaired by HRR, a surprising result [36]. Another study, using a GFP reporter system DR-GFP [37], suggested that about 75% of DNA DSBs in ES cells were faithfully repaired by HRR, while only 15% were imprecisely repaired by NHEJ [38].

The NHEJ pathway does function in ES cells as evidenced by the repair of two DSBs in cis spaced about 9kb apart in chromosomal regions that lack sequence homology. The ability for ES cells to repair these lesions was very low, with repair frequencies ranging between 6×10^{-4} and 6×10^{-6} . Repaired sequences showed large-scale genomic rearrangements consisting of large deletions (up to several thousand base pairs) and insertions but no translocations [39]. Investigating the role of NHEJ in differentiated MEFs using another reporter suggested that this process, either error-free or error-prone, can occur with high frequency, but only in the absence of p53 [40]. No studies have been reported on NHEJ-mediated repair in ES cells with a p53 null background, but NHEJ frequently occurs in wildtype differentiated cells [41,42]. Not all published data are consistent regarding the contribution of HRR in DSB repair in ES cells [43]. One study used an approach in which the DSBs were initiated not only by the I-SCE1 endonuclease, as in the reports above, but also by RAG1 and RAG2 that are proteins important for the generation of immunoglobulin diversity. Using the RAG system alone, this study reported that in wildtype ES cells 92% of all repair events were the result of NHEJ, while in Ku70 $-/-$ cells, HRR predominated at 53%. When using I-SCE1 to induce the DSB, however, the level of HRR repair was similar to that of NHEJ and SSA, suggesting that the enzymes inducing the DSB may determine the pathway by which it is repaired. The strategy utilized in this study may have been biased to NHEJ since a recent report suggested that the RAG complex usually shepherds RAG-dependent DSBs directly into the NHEJ pathway in a DNA-PK $_{cs}$ or Ku-dependent manner [44]. In aggregate, available data suggest that HRR plays an important role in maintaining ES cell genetic stability. Contradictions, however, do exist, perhaps because of the different strategies and systems employed. Further study is definitely required to resolve the discrepancies.

Mismatch Repair

The mismatch repair (MMR) pathway corrects mispaired nucleotides in DNA resulting from replication errors, recombination intermediates, or base mutations caused by DNA damaging agents [reviewed in 45]. Mutations within genes in this repair pathway generally lead to hereditary nonpolyposis colorectal cancer (HNPCC) [46,47]. Loss of mismatch repair genes in mice can result in spontaneous lymphomas and colorectal tumors upon challenge with DNA damaging agents, and may produce sterility, and microsatellite instability, depending on which

component of the pathway is targeted [17]. One of the key proteins in MMR is Msh2, which will be the focus of this discussion.

The contributions of mismatch repair in preserving the genetic stability of ES cells are still emerging. ES cells deficient in Msh2 are more resistant to killing (i.e. less prone to undergo apoptosis) following exposure to fluoropyrimidine compounds, a trend also noted in human cells [48]. Spontaneous mutation frequencies in Msh2 deficient ES cells are about 30-fold higher than in wildtype ES cells, increasing from $\sim 5 \times 10^{-6}$ to $\sim 1.5 \times 10^{-4}$ [49]. Also, exposing wildtype ES cells to low dose UVC irradiation resulted in a mutation frequency of $\sim 4.2 \times 10^{-5}$ for wildtype cells compared with $\sim 2.2 \times 10^{-4}$ for the Msh2 null ES cells, an approximately six-fold increase [49]. Thus, Msh2, a component of the MMR pathway is functional in ES cells and helps prevent the accumulation of mutations, as it does in some other cell types. In cells of the colon of Msh2 knockout mice, for example, the mutation frequency is about ten times higher than that observed in wildtype mice [50]. A ten-fold increase was also seen in the thymus and a two-fold increase reported in the heart [51].

Mismatch repair capacity has been compared between undifferentiated ES cells, ES cells whose differentiation was induced with retinoic acid, 3T3 cells, and MEFs [52]. Treatment of ES cells with the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) produced a two fold higher induction of apoptosis compared with other cell types. Western blots revealed significantly elevated levels of Msh2 and Msh6 in ES cells compared with 3T3 cells. When Msh2 was overexpressed in 3T3 cells, the cells became increasingly sensitive to MNNG, indicating that this sensitivity is dependent, in part, on the mismatch repair system. The mechanism by which the high level of Msh2 in ES cells was suggested to exert its effect is a consequence of hyperphosphorylated RB, which results in more E2f1 binding to the Msh2 promoter. When ES cells were first induced to differentiate and then treated with MNNG, the cells had reduced levels of Msh2 and displayed a 20% to 30% lower frequency of apoptosis compared to their undifferentiated counterparts [52]. Thus it appears that levels of Msh2 protein can direct cells to either repair their DNA or to undergo apoptosis and that the apoptotic responses of ES cells to exogenous challenge are partly dependent on their high levels/activity of mismatch repair proteins. Consistent with this model, Msh2 deficient ES cells and ES cells expressing ten-fold less Msh2 than have increased tolerance to MNNG [53]. Further support for a role for high levels of Msh2 in the induction of apoptosis derives from experiments which demonstrate that loss of one or both Msh2 alleles in ES cells reduces the apoptotic response following exposure to low level ionizing radiation [54]. Mismatch repair proteins also appear to play a role in apoptosis in other cell types. Mouse embryo fibroblasts deficient in Msh2 have reduced apoptotic responses following treatment with MNNG compared to wildtype cells [55]. Similarly, apoptosis in the small intestine was reduced after MNNG, temozolamide, or cisplatin treatment of Msh2 null mice, compared to wildtype mice [56]. Thus, MMR is important in maintaining genomic stability in both ES cells and differentiated cells. However, it appears to play an equally or more critical role in promoting apoptosis in ES cells in response to stress due to ES cell hypersensitivity to DNA damage.

Nucleotide excision repair

Defects in nucleotide excision repair (NER) result in the human disorders Xeroderma Pigmentosum, Cockayne's Syndrome, and Trichothiodystrophy. Mice defective for genes involved in the NER pathway are viable, with the exception of Xpd, a component of the basal transcription machinery [17]. The NER pathway repairs predominantly DNA containing nucleotides with bulky adducts or the photoproducts produced by UV irradiation. ES and MEFs have very different capacities to repair damage incurred by UV light. As early as 1976, Pedersen and Cleaver showed that cells of the blastocyst inner cell mass, from which ES cell are isolated, underwent minimal unscheduled DNA synthesis (UDS) following UV irradiation compared

with other cells of the blastocyst *in vitro* [57]. When ES cells are exposed to high dose UVC, severely damaged ES cells are rapidly eliminated by apoptosis [58]. There appears to be little or no removal of cyclobutane pyrimidine dimers (CPDs) in either strand of either the p53 or Hprt genes for up to 24 hours after exposure to 10 J/m² UVC light [58]. This finding is intriguing since rodent cells generally do not repair cyclobutane pyrimidine dimers (CPDs) in inactive regions of DNA but do so readily in actively transcribed genes [59]. The repair capacity of a second UV photoproduct, the 6-4 photoproduct (6-4PP) in ES cells appears to reach a maximum after 4 hours following exposure, but only ~30% of lesions are repaired [58]. This finding is dissimilar to other observations in MEFs, Chinese hamster ovary (CHO) cells, and murine cardiomyocytes, since most of the lesions are repaired over this same time period [58,60]. In comparing ES cells with MEFs, Van Sloun *et al.* [58] demonstrated that at low doses of UV, replication repair levels were similar, but at doses of greater than 5 J/m², the ES cell repair machinery appears to become saturated and repair remains incomplete. This apparent saturation occurs in ES cells at a dose that is three fold lower than in MEFs. This observation is inconsistent with reports that NER is generally lower in terminally differentiated cells compared to their progenitors and appears to be dependent on cell cycle processes [61], and would suggest that NER is more functional in ES cells. Not surprisingly, when spontaneous and induced mutation frequencies were compared between wildtype and NER-deficient (ERCC1 null ES cells), the wildtype cells had significantly lower levels of mutation [58]. These observations indicate that NER is proficient in ES cells; however NER appears to plateau at later time points, perhaps to facilitate or as a consequence of an apoptotic response.

Summary

Embryonic stem cells are highly specialized and programmed to differentiate into cells of all types and lineages at specific stages of embryogenesis. To prevent the accumulation of mutations in daughter cells, ES cells must have robust mechanisms to protect their DNA from endogenous as well as exogenous sources of damage. One such mechanism may be through the induction of apoptosis in response to DNA damage, to remove damaged cells from the self-renewing ES cell pool. The lack of a G1 checkpoint may accomplish this, by allowing DNA damage to become exacerbated during subsequent replication, thereby increasing the cell's mutational burden and promoting apoptosis. A complementary mechanism is the propensity of ES cells to have effectual processes for DNA repair. Indeed, repair of DSBs appears to occur quickly in ES cells; however, there are conflicting reports regarding which DSB repair pathway predominates. Based on available data, it is reasonable to suspect that HRR might predominate in ES cells due to their inherent cell cycle properties and the inability to obtain ES cells that are deficient in many of the members of the HRR pathway. In contrast, differentiated somatic cells, likely use NHEJ as the predominant DSB repair mechanism. Mismatch repair also appears to have important roles in ES cells, perhaps acting as a switch to determine whether or not a cell undergoes DNA repair or apoptosis. The high endogenous levels of Msh2 protein in ES cells tend to promote apoptosis whereas low levels favor DNA repair, as it does in differentiated cells. Even low MSH2 protein levels can diminish the amount of spontaneous mutation in ES cells compared with MSH2 null ES cells, suggesting that mismatch repair plays a fundamental role in regulating the level of mutagenesis in this cell type. It is likely that NER functions a similar way, since ES cells appear unable to repair UV damage once the NER activity has plateaued, so that cells tend to undergo apoptosis. Thus, ES cells are inherently different than differentiated, somatic cells in their capacity to repair DNA, indicating that pathways that lead to repair of damaged DNA, and alternatively to apoptosis may be complementary mechanisms by which ES cells preserve the integrity of their genomes.

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Figure 1. γ -H2AX staining in ES cells and Mouse Embryo Fibroblasts (MEFs)

129/Sv derived ES cells (A, B) or passage 3 MEFs (C, D) were grown on coverslips in the absence (A, C) or presence (B, D) of 10 μ m etoposide for 4 or 6 hours. Cells were fixed and stained with γ -H2ax and nuclei were counterstained with draq5. Note the basal staining of γ -h2ax in ES cells (A).

Scale bar represents 20 μ m.

Figure 2. Endogenous protein expression levels of Rad51 and XRCC4 in MEFs and ES cells
SDS-PAGE was performed using 20 μ g of 129/Sv ES cell or passage 3 MEF whole cell extracts and probed with the given antibodies.