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Pluripotent cells display enhanced resistance to mutagenesis

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ABSTRACT

Pluripotent cells have been reported to exhibit lower frequencies of point mutations and higher levels of DNA repair than differentiated cells. This predicts that pluripotent cells are less susceptible to mutagenic exposures than differentiated cells. To test this prediction, we used a *lacI* mutation-reporter transgene system to assess the frequency of point mutations in multiple lines of mouse pluripotent embryonic stem cells and induced pluripotent cells, as well as in multiple lines of differentiated fibroblast cells, before and after exposure to a moderate dose of the mutagen, methyl methanesulfonate. We also measured levels of key enzymes in the base excision repair (BER) pathway in each cell line before and after exposure to the mutagen. Our results confirm that pluripotent cells normally maintain lower frequencies of point mutations than differentiated cells, and show that differentiated cells exhibit a large increase in mutation frequency following a moderate mutagenic exposure, whereas pluripotent cells subjected to the same exposure show no increase in mutations. This result likely reflects the higher levels of BER proteins detectable in pluripotent cells prior to exposure and supports our thesis that maintenance of enhanced genetic integrity is a fundamental characteristic of pluripotent cells.

1. Introduction

Pluripotent cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), maintain an epigenetic state similar to that found in germ cells and early embryonic (inner cell mass or epiblast) cells (Murphey et al., 2009; Kalkan and Smith, 2014). The early embryo derives from gametes that are known to maintain enhanced genetic integrity relative to somatic cells (Murphey et al., 2013) as predicted by the Disposable Soma Theory (Kirkwood, 1977). Just as enhanced maintenance of the male and female germline genomes is advantageous for faithful propagation of pristine genetic information between generations, enhanced maintenance of genetic integrity in early embryonic cells ensures optimal propagation of high fidelity genetic information to each developing cell lineage within each generation. Thus, pluripotent cells normally maintain enhanced genetic integrity relative to differentiated cell types (Chen et al., 2016; Rouhani et al., 2016), and this reflects elevated activity of a variety of DNA repair pathways in pluripotent cells including base excision repair (BER) (Tichy et al., 2011).

We previously showed that enhanced maintenance of genetic integrity results from the epigenetic reprogramming process that follows somatic cell nuclear transfer (cloning) (Murphey et al., 2009), as well as that which accompanies reprogramming of differentiated somatic cells to form iPSCs (Chen et al., 2016). In addition, we described a direct link between the pluripotency and genetic integrity gene networks at the genomic level in human and mouse ESCs and iPSCs (Cooper et al., 2014). A predicted functional ramification of this elevated DNA repair activity and enhanced maintenance of genetic integrity in pluripotent cells is that these cells should be less susceptible than differentiated cells to mutagenic effects. Surprisingly, only a few studies have examined susceptibility of pluripotent cell types to mutagenesis (Momcilović et al., 2009; Bañuelos et al., 2008; de Waard et al., 2008; Maynard et al., 2008; Krutá et al., 2014).

Many previous studies examining mutagenesis in pluripotent cells have utilized UV radiation to induce DNA damage (Bártová et al., 2011; Martins-Taylor and Xu, 2012; Momcilović et al., 2009; Bañuelos et al., 2008; de Waard et al., 2008; Maynard et al., 2008). Damage of this sort is normally repaired by NER (Goodsell, 2001), but NER often activates p53-mediated apoptosis in stem cells (Qin et al., 2007), which can obscure mutagenic effects by eliminating the most severely affected cells. UV radiation can also induce long-lasting oxidative stress (Clutton et al., 1996) that can further complicate any direct assessment of a strictly DNA-repair based response to this exposure.

Our goal was to directly assess the relative susceptibility of pluripotent and differentiated cell types to a similar mutagenic exposure based primarily on differential DNA repair capacities in each cell type. In particular, we sought to examine the effects of a moderate mutagenic exposure which enhances generation of alkylated bases, mimicking damage

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accrued during long-term culture. Therefore we chose to use the mutagen methyl methanesulfonate (MMS) which induces DNA alkylation damage on both guanine (to 7-methylguanine) and adenine (to 3-methyladenine) bases, causing mismatched base pairing and replication fork stalling (Begley and Samson, 2004) that is normally repaired by the BER pathway (Xu et al., 2008).

We have previously demonstrated that the *lacI* mutation-reporter transgene system (Kohler et al., 1991) can be used to assess mutagenesis induced by MMS (Sanchez et al., 2015). In the study described here we exposed mouse pluripotent ESCs and iPSCs, as well as differentiated mouse embryonic fibroblasts (MEFs) and adult mouse tail tip fibroblasts (TTFs) to a similar, moderate dose of MMS and assessed the frequency and spectrum of point mutations as well as levels of BER-related proteins before and after this exposure in each cell type. Our results confirm that pluripotent cells are markedly more resistant to MMS-induced mutagenesis than differentiated cell types, and that this correlates with elevated levels of BER pathway proteins in pluripotent cell types relative to those in differentiated cell types. This supports our central thesis that ongoing upregulation of the genetic integrity gene network leads to enhanced maintenance of genetic integrity in pluripotent cells (Cooper et al., 2014), and provides insight into basic biological distinctions between pluripotent and differentiated cell types that impact genetic integrity in a manner that is directly relevant to the potential clinical use of these cell types.

2. Materials and methods

2.1. Cell lines

All cell lines used in this study were derived from mice carrying a *lacI* mutation-reporter transgene (Kohler et al., 1991). The ESCs were derived from C57Bl/6–129sv (B6/129) F1 hybrid embryos hemizygous for the *lacI* mutation-reporter transgene as described (Chen et al., 2016). The B6/129 TTFs and iPSCs also carried a *Rosa* (*4F2A*) transgene, which consists of a polycistronic transgene encoding SOX2, OCT4, KLF4, and cMYC under control of a doxycycline-inducible *Tet0* promoter to efficiently generate iPSC lines in vitro (Carey et al., 2010; Chen et al., 2016).

MEFs were derived from fetuses as described (Takahashi and Yamanaka, 2006). Embryonic stem cells were generated from embryos homozygous for the *lacI* mutation-reporter transgene as described (Chen et al., 2016). Induced pluripotent stem cells (iPSCs) were generated from adult TTFs from a $lacI^{tg/+}$/$Rosa(4F2As^{tg/tg}$ hybrid as described (Chen et al., 2016; and Carey et al., 2010). A total of eight mouse cell lines (four pluripotent – two iPSC and two ESC lines; and four somatic/differentiated – two MEF and two TTF lines) were used in these experiments, all of which carried the *lacI* mutation-reporter transgene. All ESC and iPSC lines used in this study were shown to have normal karyotypes, demonstrated expression of standard pluripotency markers including SOX2, OCT4, NANOG, and SSEA-1, were alkaline phosphatase positive, and gave rise to cell types representative of all three germ layers in teratomas formed upon injection into SCID mice as described (Chen et al., 2016). Doubling times for these cell lines were established in a previous study and were found to be 9–12 h among the pluripotent cell lines and 10–15 h among the differentiated cell lines (Chen et al., 2016). In addition, all cell lines were in growth phase when exposed to the mutagen (see below).

2.2. Dose-response and EC_{50} calculation

To ensure an equal effect of MMS on all cell types, we first performed dose-response experiments (Crump et al., 1976) to calculate the median effective concentration (EC_{50}) for each cell line, or the concentration at which 50% of exposed cells survive when compared to survival of unexposed control cells (Sebaugh, 2011). The DNA alkylating agent, MMS, was used as the mutagen for all experiments described in this study. Cells were plated 24 h prior to treatment with MMS. MMS was added (for experimentals) or not (for controls) to the appropriate (pluripotent or differentiated cell) serum-free media as described (Poersch et al., 2007) and diluted to obtain a series of doses of MMS. The EC_{50} for each cell line was calculated based on a trendline fit in Microsoft Excel describing the percentage of remaining cells after exposure to MMS relative to cells remaining in unexposed controls. Experiments to determine each EC_{50} were performed in triplicate for each cell line.

2.3. Exposure of cells to MMS for mutation frequency and protein analyses

Aliquots of each cell line were exposed to the corresponding EC_{50} dose of MMS previously calculated for that cell line (Fig. S1), or with media only to generate unexposed controls for each cell line, for 24 h. DNA and protein samples were collected in triplicate from both MMS-exposed and unexposed samples of each cell line immediately after the exposure period.

2.4. Analysis of mutation frequencies

The Big Blue Mouse® mutagenesis (*lacI* mutation-reporter transgene) assay system (Stratagene/Agilent, Santa Clara, CA) was used to determine the mutation frequency and spectrum detected in each differentiated and pluripotent cell line as previously described (Murphey et al., 2009). Putative mutant plaques, characterized by a blue color phenotype, were confirmed by replating, and the *lacI* gene was then amplified by PCR and outsourced to the University of Texas at Austin DNA Sequencing Lab to confirm the presence of a mutation and to determine the type and location of each point mutation detected. Mutation frequencies shown in Table 1 were calculated as the number of confirmed, independent (non-clonal) mutant plaques divided by the total number of plaques (pfu) screened from each sample.

Mutation frequencies were analyzed by a Poisson model with parameter estimates obtained by the method of maximum likelihood (Agresti, 2002). Because of the low expected frequencies, exact p-values were calculated by the exact conditional test for Poisson variables to compare differences among mutation frequencies, using the Exactci package implemented in R (Fay, 2010; and Hirji, 2006). $p \leq 0.05$ was considered statistically significant as previously described (Chen et al., 2016; Murphey et al., 2009, 2013).

2.5. Western blot analysis of base excision repair proteins

Western blotting was performed as described by Mull et al. (2014). Primary antibodies against either APEX1 (Santa Cruz Biotechnology, Santa Cruz, CA), LIGIII (GeneTex, Irvine, CA), DNA Polymerase β (Novus Biologicals, Littleton, CO), or γ-tubulin (Abcam, Cambridge, England), were followed by secondary antibodies including either anti-mouse (Life Technologies) or anti-rabbit (Santa Cruz Biotechnology) antibodies, and chemifluorescence was detected by use of the ECL Advance Western Blotting Detection Kit (Fisher, Austin, TX).

3. Results

3.1. EC_{50} values

Median effective concentrations of MMS were optimized for each cell line based on determination of the relevant EC_{50} doses. Results of replicate dose-response experiments to determine the EC_{50} dose for each cell line are shown in Fig S1 in the Supplementary Information and the specific EC_{50} dose for each line is shown in Table 1. The relatively similar EC_{50} doses of MMS determined for the different cell types used in this study suggest that the level of cellular toxicity induced by this exposure did not vary as a function of the pluripotent or differentiated state of these cells.