DNA REPAIR 7 (2008) 1471–1483

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journal homepage: www.elsevier.com/locate/dnarepair

Mouse but not human embryonic stem cells are deficient in rejoining of ionizing radiation-induced DNA double-strand breaks

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ARTICLE INFO

Article history:
Received 23 April 2008
Accepted 8 May 2008
Published on line 3 July 2008

Keywords:
Mouse embryonic stem cells
Double-strand break repair
γH2AX
DNA-PK

ABSTRACT

Mouse embryonic stem (mES) cells will give rise to all of the cells of the adult mouse, but they failed to rejoin half of the DNA double-strand breaks (dsb) produced by high doses of ionizing radiation. A deficiency in DNA-PK$_{cs}$ appears to be responsible since mES cells expressed <10% of the level of mouse embryo fibroblasts (MEFs) although Ku70/80 protein levels were higher than MEFs. However, the low level of DNA-PK$_{cs}$ found in wild-type cells appeared sufficient to allow rejoining of dsb after doses <20 Gy even in G1 phase cells. Inhibition of DNA-PK$_{cs}$ with wortmannin and NU7026 still sensitized mES cells to radiation confirming the importance of the residual DNA-PK$_{cs}$ at low doses. In contrast to wild-type cells, mES cells lacking H2AX, a histone protein involved in the DNA damage response, were radiosensitive but they rejoined double-strand breaks more rapidly. Consistent with more rapid dsb rejoining, H2AX$^{-/-}$ mES cells also expressed 6 times more DNA-PK$_{cs}$ than wild-type mES cells. Similar results were obtained for ATM$^{-/-}$ mES cells. Differentiation of mES cells led to an increase in DNA-PK$_{cs}$, an increase in dsb rejoining rate, and a decrease in Ku70/80. Unlike mouse ES, human ES cells were proficient in rejoining of dsb and expressed high levels of DNA-PK$_{cs}$. These results confirm the importance of homologous recombination in the accurate repair of double-strand breaks in mES cells, they help explain the chromosome abnormalities associated with deficiencies in H2AX and ATM, and they add to the growing list of differences in the way rodent and human cells deal with DNA damage.

1. Introduction

Mouse embryonic stem (mES) cells were isolated more than 25 years ago [1]. Since then, the ability to use gene targeting approaches in mES cells together with chimera formation to develop gene knockout mice has provided an invaluable experimental tool to study the role of individual genes. The subsequent isolation and culture of human embryonic stem cells was hailed as a landmark accomplishment in 1998 [2], providing new opportunities for studying human development and cell based therapies for many diseases [3]. It is therefore surprising that there is relatively little information concerning the DNA repair pathways that operate in either human or mouse embryonic stem cells [4].

DNA double-strand breaks (dsb) stimulate activation of the ATM kinase which phosphorylates H2AX, a minor

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doi:10.1016/j.dnarep.2008.05.005

nucleosomal histone protein [5]. Complexes containing the phosphorylated form, called γH2AX, form at sites of dsb; this signals the presence of dsb and enhances the accuracy of their repair [6,7]. H2AX knockout mice share many features in common with ATM knockout mice in that they are radiosensitive, growth retarded, and exhibit chromosomal abnormalities [8,9]. We expected that H2AX$^{-/-}$ mES cells, like ATM deficient cells, would exhibit a partial defect in repair of dsb; in ATM cells, this defect is characterized by normal dsb rejoining for the first few hours after irradiation, but greater residual dsb relative to normal cells [10,11].

In preliminary experiments that characterized the rate of dsb rejoining in mES cells, we found that wild-type mES cells showed a significant deficiency in both the rate and extent of rejoining of radiation-induced dsb whereas H2AX$^{-/-}$ mES cells rejoined dsb more rapidly. This result was initially surprising, given that mES cells will differentiate to give rise to the adult mouse so the need to maintain a "pristine" genome is vital. Two major pathways are involved in the repair of radiation-induced dsb. Non-homologous end-joining (NHEJ) is the predominant dsb repair pathway in most mammalian cells; essential proteins in this pathway include the Ku70/80 heterodimer that binds to DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PK$_{cs}$). Loss of DNA-PK$_{cs}$ or Ku proteins results in a deficiency in both the rate and extent of rejoining of dsb [12,13]. Mouse ES cells that lack DNA-PKcs, have been reported to be insensitive to X-rays [14] and etoposide [15] supporting the idea that NHEJ is not a major pathway in mES cells. The second pathway, homologous recombination, is believed to be the preferred pathway for mES cells that spend much of their time in S phase [4]. The relative contribution of homologous recombination and NHEJ changes during development; mouse embryos lacking the recombination protein RAD54 are hypersensitive to ionizing radiation whereas adult mice are not affected by loss of this protein [16]. As homologous recombination is considered to be error-free relative to NHEJ, relying on recombination is an important strategy that mES cells can employ to enhance the accuracy of dsb repair. If the rate of dsb rejoining is faster in H2AX$^{-/-}$ cells, perhaps these cells are able to up-regulate the NHEJ pathway.

To examine this hypothesis, wild-type, H2AX$^{-/-}$, ATM$^{-/-}$ and DNA-PK$_{cs}$$^{-/-}$ mES cells were examined for radiosensitivity, dsb rejoining capacity, DNA-PK$_{cs}$ and Ku70/80 expression, radiation response through the cell cycle, and radiosensitivity after exposure to DNA-PK inhibitors. Results indicate a significant deficiency in DNA-PK$_{cs}$ but not Ku70/80 in undifferentiated mES. However, H2AX$^{-/-}$ and ATM$^{-/-}$ mES cells appear to compensate for their loss by increasing the expression of DNA-PK$_{cs}$.

2. Materials and methods

2.1. Cell lines and X-irradiation

Undifferentiated R1 murine embryonic stem cells were derived from 129/Sv ES cells and originally obtained from Dr. A. Nagy at the University of Toronto [17]. Cells were used between passages 8–14. Confirmatory experiments were performed using a second wild-type mES cell line, C1$^{+/+}$ of the same genetic background [18]. H2AX$^{-/-}$, ATM$^{-/-}$ and DNA-PK$_{cs}$$^{-/-}$ mES were generated in 129/Sv/Ev ES cells [19] and were kindly provided by Dr. Fred Alt, Harvard [14,20]. Mouse embryonic stem cells were maintained as previously described [21] in Dulbecco's Modified Eagle's medium containing 15% fetal calf serum (Hyclone) supplemented with factors that included 10 ng/ml leukemia inhibitor factor, 200 mM L-glutamine, 10 mM non-essential amino acids, 100 mM sodium pyruvate and 100 μM monothioglycerol (all reagents from Sigma) [21]. All cells were grown at 37 °C under ambient air and 5% CO_2. Mouse ES cells were maintained in exponentially growth in the absence of feeders by subcultivation three times per week on gelatin coated dishes seeded at 5×10^4 cells/60 mm dish. The differentiation status of the mES cells was confirmed using intact cells stained with antibodies against SSEA1, a cell surface marker of the undifferentiated mES phenotype [22] and by immunoblotting of nuclear proteins using antibodies to lamin A/C that are not expressed in undifferentiated stem cells [23]. To promote differentiation of mES cells, LIF was omitted from the medium and cells were grown in spinner culture vessels for 2 weeks. The undifferentiated status of human ES cells was confirmed by flow cytometry using SSEA3 antibodies [24]. Mouse embryo fibroblasts (MEFs) were purchased from Stem Cell Technologies and obtained from day 12 mouse embryos from CD1 mice; MEFs were cultured in the same medium as mES cells but lacking LIF. Chinese hamster V79 lung fibroblasts, SiHa human cervical carcinoma cells, and primary human skin fibroblasts (HSF) were maintained in exponential growth by bi-weekly sub-cultivation in Dulbecco's minimal essential medium containing 10% FBS (GIBCo).

The human ES cell lines used for these studies included two Wisconsin lines (H1, H9) [2] and one Canadian line (CA1) derived by Dr. A Nagy. The cells were used between passages 37–67 and were maintained in either feeder-based [2] or defined [25] culture conditions. For feeder-based culture, cells were maintained on mitotically inactivated mouse embryonic fibroblasts (StemCell Technologies) in medium consisting of DMEM/F12 supplemented with 20% Knockout Serum Replacer (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 0.1 mM non-essential amino acids, 1 mM glutamine and 4 ng/ml FGF2, and passaged enzymatically with 1 mg/ml collagenase (Invitrogen) every 7 days. For defined culture, human ES cells were maintained on dishes coated with Matrigel (BD Biosciences) in mTeSR1 medium (StemCell Technologies) and passaged either enzymatically using 1 mg/ml dispase (Invitrogen) for 7 days, or harvested via TrypLE (Invitrogen) and Rho-associated kinase inhibitor (Calbiochem) [26] and cultured for 7 days.

Cells attached to tissue culture dishes were exposed to 300 kV X-rays on ice at a dose rate of 4.8 Gy/min. Cells were then returned to the incubator for up to 24 h after radiation to allow time for repair. After exposure for 5 min to 0.1% trypsin in citrate saline, single cells were plated for clonogenicity measurements, used to analyze dsb rejoining with the comet assay, or fixed in 70% ethanol for subsequent antibody staining and flow cytometry analysis of DNA content and γH2AX.

2.2. Clonogenicity assays

For clonogenic survival assays, trypsinized mES cells were irradiated in suspension and seeded onto 60 mm gelatin coated dishes containing 5 ml LIF supplemented Dulbecco's medium as described above. One week later, colonies were stained with malachite green and counted. Surviving fraction is expressed relative to the plating efficiency of the untreated cells. Three or more experiments were performed.

2.3. Neutral comet assay

The neutral comet assay was used as previously described to measure induction and rejoining of dsb [27]. Briefly, cells were embedded in 0.75% low gelling temperature agarose on microscope slides, and samples were submersed in lysis solution containing 2% sarkosyl, 0.1 M