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Application of the microfluidic-assisted replication track analysis to measure DNA repair in human and mouse cells

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ABSTRACT

Functional studies of the roles that DNA helicases play in human cells have benefited immensely from DNA fiber (or single molecule) technologies, which enable us to discern minute differences in behaviors of individual replication forks in genomic DNA *in vivo*. DNA fiber technologies are a group of methods that use different approaches to unravel and stretch genomic DNA to its contour length, and display it on a glass surface in order to immuno-stain nucleoside analog incorporation into DNA to reveal tracks (or tracts) of replication. We have previously adopted a microfluidic approach to DNA stretching and used it to analyze DNA replication. This method was introduced under the moniker *maRTA* or microfluidic-assisted Replication Track Analysis, and we have since used it to analyze roles of the RECQ helicases WRN and BLM, and other proteins in normal and perturbed replication. Here we describe a novel application of maRTA to detect and measure repair of DNA damage produced by three different agents relevant to etiology or therapy of cancer: methyl-methanesulfonate, UV irradiation, and mitomycin C. Moreover, we demonstrate the utility of this method by analyzing DNA repair in cells with reduced levels of WRN or of the base excision repair protein XRCC1.

1. Introduction

DNA helicases are essential molecular motors that separate strands of duplex DNA to enable DNA replication, repair, recombination, telomere maintenance, and transcription. The importance of helicases in the maintenance of a stable, efficiently operating genome is underscored by the fact that at least 15 out of 31 DNA helicases encoded in the human genome cause familial cancer-prone disease conditions, if mutated [1,2]. *In vivo* studies of molecular roles of DNA helicases in replication have greatly benefited from the development of DNA fiber assays. In these assays, genomic DNA is pulse-labeled *in vivo* with pyrimidine nucleoside analogs (i.e. BrdU, CldU, IdU), isolated, stretched, and deposited in an orderly fashion as individual, clearly separated molecules onto a glass surface. This enables visualization and robust quantitation of so-called replication tracks (or tracts) under a regular fluorescent microscope at a resolution of 1 μM = 2–4 Kb. Several different DNA fiber procedures have been developed. Some are known under specific brand names, e.g. DNA combing, SMARD, maRTA [3–10]. All of these procedures share DNA labeling and immunodetection protocols though differ in their approach to the critical step of stretching and immobilizing DNA molecules. Availability of antibodies that selectively detect CldU/BrdU or IdU/BrdU, offers versatility in designing well-controlled, informative experiments targeting different aspects of DNA replication. More recently, we added EdU to the repertoire of thymidine analog labels, enabling simultaneous detection of three colors of tracks [11] (also see Materials and Methods).

DNA fiber assays can measure such parameters of replication as replication fork rate of progression, frequency of replication origin firing, and density of replication firing events in replication domains. Perhaps yet more powerful is the application of DNA fiber assays to the study of perturbed replication known as replication stress, which is triggered by the presence of lesions in replicating DNA, replisome blockage, and insufficient or unbalanced

Abbreviations: BER, base excision repair; BrdU, bromo-deoxyuridine; CldU, chloro-deoxyuridine; CPD, cyclopyrimidine dimer; DSB, double-strand break; EdU, ethynyl-deoxyuridine; IdU, iodo-deoxyuridine; maRTA, microfluidic-assisted replication track analysis; MMC, mitomycin C; MMS, methyl-methane sulfonate; SSB, single-strand break; UV, ultra-violet; XRCC1, X-Ray Repair Cross-Complementing Protein 1.

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nucleotide, histone, or DNA-servicing enzyme pools. DNA fiber assays are excellent in detecting inappropriate fork stalling, slowing, or irreversible inactivation, as well as nascent strand resection – all of which become prevalent under conditions of replication stress. There are numerous examples of studies where DNA helicases, and RECQs in particular, were assigned specific roles in these aspects of normal and/or stressed replication based on the results of DNA fiber assays.

While the field of replication enjoys the ever wider availability of DNA fiber assays of various flavors, the related area of DNA repair research by and large lacks an equivalent technology. Since our introduction, in 2008, of a version of a DNA fiber assay called microfluidic-assisted replication track analysis, or maRTA, we have sought ways to apply it to the study of DNA repair processes that are not necessarily coupled with replication. We described our first application of maRTA to DNA repair in 2012 [12], and here we build upon our original research to expand the application of maRTA to measure BER, NER, and crosslink/bulky adduct repair.

2. Materials and methods

2.1. Cell lines and growth conditions

The SV40-transformed human fibroblast GM639 cell line and its pNeoA derivative GM639cc1 have been described before [13–16]. Primary fibroblast line Hi3 was described previously [17]. MCF10a, a spontaneously immortalized mammary epithelial cell line and HCC1937 *Brca1*−/− breast cancer cell line complemented with a *BRCA1* transgene are gifts of Drs. Piri Welcsh and Elizabeth Swisher (University of Washington), and Dr. Toshi Taniguchi (Fred Hutchinson Cancer Research Center).

GM639cc1 were grown in Dulbecco Modified Minimal Essential Medium (DMEM, Gibco or Hyclone) supplemented with L-glutamine, sodium pyruvate, 10% fetal bovine serum (FBS, Hyclone) and antibiotics, and MCF10a were grown in MGEM media (Lonza) supplemented with Single Quots (Lonza), 1% fetal bovine serum and antibiotics. HCC1937/*BRCA1* cells were grown in RPMI (Gibco or Hyclone) supplemented with 15% FBS and antibiotics. All cell lines were kept in a humidified 5% CO_2, 37 °C incubator.

XRCC1 null heterozygote and wild type mouse fibroblasts were generated from ear tissue of adult mice described previously [12,18]. Mice were euthanized by CO_2 inhalation. Ear tissue was sterilized with Povidone-Iodine swabsticks (PDI), rinsed with 70% ethanol and PBS containing penicillin/streptomycin and 2X Fungizone (Life Technologies), then minced in PBS containing 1 mg/ml collagenase/dispase (Roche Applied Science). Tissue fragments were rotated at 37° C in a 5% O_2 incubator for 45 min, then overnight at 37° C after adding 5 ml of media. Tissue was further dissociated by pipetting, and then filtered through a 100 mm mesh nylon filter (BD Biosciences) prior to centrifugation and resuspension in fresh growth media. Mouse cells were grown in Dulbecco Modified Eagle's Medium (DMEM) with 4.5 g/L glucose and pyruvate (BioWhittaker), supplemented with 2 mM L-glutamine, 10% (v/v) Fetal Clone III serum (Hyclone), penicillin G (100 U/ml) and streptomycin sulfate (100 mg/ml; BioWhittaker) and 2X non-essential amino acids (BioWhittaker) in a humidified 37 °C, 7% CO_2 incubator in 5% O_2.

2.2. Drugs and other reagents

Stock solution of 5-iododeoxyuridine (IdU, Sigma-Aldrich) was 2.5 mM in water, 5-chlorodeoxyuridine (CldU, Sigma-Aldrich) was 10 mM in water, 5-ethynyldeoxyuridine (EdU, Life Technologies) was at 10 mM in DMSO. IdU and CldU were used at a final concentration of 50 μM and EdU was used at 10 μM. Stock of mitomycin C (EMD Chemicals) was at 10 mM in DMSO. Methyl-methanesulfonate was purchased from Sigma-Aldrich and diluted to 1% in growth media prior to use. T4 PDG endonuclease was purchased from NEB and S1 nuclease from Thermo Scientific. All reagent stocks and enzymes were stored at −20 °C.

2.3. UV irradiation

UV irradiations were performed with a UVG-4 portable UV lamp (UVP), and UV doses were calibrated using a shortwave UV measuring meter J-225 (UVP). Cells were irradiated in PBS or DMEM media without the pH indicator.

2.4. WRN depletion

shRNA-mediated depletion of WRN was performed as previously described [14,15] and the WRN protein level was quantified in Western blots with α-WRN antibody 195C (Cat. No. W0393 Sigma) and α-CHK1 (Cat. No. sc-8408 Santa Cruz) as loading control. Proteins were visualized on Western blots by ECL (ThermoScientific) and quantified using Storm Phosphorimager (Molecular Dynamics) or FluorChem Imager (Alpha Inotech).

2.5. maRTA

2.5.1. DNA isolation for maRTA and treatments to convert lesions to double-strand breaks

Embedding cells in agarose, cell lysis, and release of DNA from agarose (with β-agarase, NEB) were as described in detail previously [8]. β-agarase treatments were overnight at 42 °C. A typical DNA sample was isolated from 200,000 cells and had a final volume of 400–500 μL (in 1x β-agarase buffer). To convert MMS lesions to double-strand breaks, an overnight incubation at 56 °C during the step of cell lysis in agarose plugs, which is performed during our DNA isolation procedure, was sufficient for mouse fibroblasts. For human cells, an additional step of heating DNA preps for 4 h at 56 °C was carried out after DNA was released from agarose.

For T4 PDG endonuclease treatments, 10 μL of genomic DNA (aliquoted with a wide bore tip) was assembled into a reaction mixture with 2 μL of 10x PDG buffer (supplied with the enzyme), deionized water and 2 units of PDG to make 20 μL final volume. Amount of PDG may require initial titration. Control reactions had DNA and 1x PDG buffer only. Reactions were incubated at 37 °C for 30 min, then placed on ice. If S1 nuclease digestion was included, reaction mixes from the PDG step were adjusted with modified 5x S1 buffer (200 mM Na acetate pH 4.5, 1 M NaCl, 10 mM $ZnSO_4$; this buffer takes into account that reaction mixes already contain 100 mM NaCl) to make buffer conditions compatible with S1, and incubated with 1/128 to 1/16 units of the enzyme for 30 min at 37 °C. Reactions were returned to ice, adjusted to 1 mM EDTA and incubated at 65 °C for 10 min to inactivate the enzymes. 10 μL of digests were loaded onto a 0.75% agarose gel to assess the degree of digestion. Mock-treated genomic DNA should migrate as a single, high molecular weight band, and enzymatic digestion produces an enzyme dose-dependent smearing beneath this band. Optimal digestion is within the range of enzyme doses that retain the high molecular weight band virtually unaffected and produce little to no smearing.

For S1 only digestion, 10 μL of single genomic DNA or 7 μL each if two DNAs were used, were mixed with 4 μL of 5X S1 buffer supplied with the enzyme, and 1/32 to 1/2 units of S1, then incubated at 37 °C for 30 min and quenched as above. To check for digestion, DNAs were resolved in 0.75% agarose as above. The optimal range of S1 is typically 1/8–1/2 units per reaction. All digested DNAs