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Quantification and genome-wide mapping of DNA double-strand breaks

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ABSTRACT

DNA double-strand breaks (DSBs) represent a major threat to the genetic integrity of the cell. Knowing both their genome-wide distribution and number is important for a better assessment of genotoxicity at a molecular level. Available methods may have underestimated the extent of DSBs as they are based on markers specific to those undergoing active repair or may not be adapted for the large diversity of naturally occurring DNA ends. We have established conditions for an efficient first step of DNA nick and gap repair (NGR) allowing specific determination of DSBs by end labeling with terminal transferase. We used DNA extracted from HeLa cells harboring an I-SceI cassette to induce a targeted nick or DSB and demonstrated by immunocapture of 3′-OH that a prior step of NGR allows specific determination of loci-specific or genome wide DSBs. This method can be applied to the global determination of DSBs using radioactive end labeling and can find several applications aimed at understanding the distribution and kinetics of DSBs formation and repair.

1. Introduction

The genotoxic character of DNA double-strand breaks (DSBs) is well known as they may lead to genomic rearrangements, cell death or cancer if left unrepaired. Most DSBs arise from endogenous mechanisms but may also be produced as a result of ionizing radiation or chemotherapy [1]. Although some endogenous DSBs are generated accidentally such as those resulting from DNA replication failure [2,3], many cellular processes require controlled DSBs formation such as transcription initiation [4], meiotic recombination [5,6], chromatin remodeling [7] or V(D)J recombination [8].

Establishing both the number and physical distribution of DSBs would allow for a better study of the repair kinetic and the consequences of the repair processes within gene related sequences or intergenic regions. Available methods to detect DSBs include indirect determination like immunofluorescence against markers such as γH2AFX or DSB repair proteins combined with microscopy, flow cytometry or chromatin immunoprecipitation [9–11]. These techniques probably underestimate the extent of DSBs as they use antibodies to detect proteins bound to DSB and are found as foci only at sites where active DSB repair proceeds. Non-canonical functions have however been recently associated with γH2AFX such as DNA single strand break (SSB) repair [12] and chromatin remodeling (reviewed by Turinetto and Giachino [13]). Direct techniques to detect DSBs include electrophoresis in neutral conditions such as single cell electrophoresis (comet) assay [14] and pulsed field gel electrophoresis. These techniques suffer from an obvious lack of sensitivity because DSBs must be present in a significant fraction of the cells and occur at several loci per cell in order to observe an increase in DNA mobility, whereas rare breaks within high molecular weight DNA can hardly be detected. Although direct labeling of 3′-OH DNA ends with enzymes and reporter molecules represent a sensitive method [15,16], specific and total labeling of DSBs has so far not been achieved.

Using terminal deoxynucleotidyl transferase (TdT), we describe a robust method for determination of DSBs that relies on an efficient first step of DNA nick and gap repair (NGR) that is suitable for site-specific analyses or genome-wide mapping. The specific mapping of DSBs represents a variation over our previously published technique (dDIP) that is based on the incorporation of a biotinylated nucleotide at 3′-OH DNA end followed by immunocapture of the labeled DNA [17], that we now term DNA break immunocapture (DBrIC). Global quantification of DSBs is also possible, this time

Abbreviations: DSB, DNA double-strand break; SSB, DNA single-strand break; TdT, terminal deoxynucleotidyl transferase; NGR, DNA nick and gap repair; dBrIC, DNA break immunocapture; qTUNEL, quantitative TUNEL; NGS, next-generation sequencing; %IP, percentage of immunoprecipitation (IP/input); DBrIC-Seq, DNA break immunocapture followed by next-generation sequencing.

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using TdT incorporation of a radiolabeled nucleotide followed by a simple and efficient purification step. In contrast to existing methods [15,16], such approaches proved to be very simple, reproducible and sensitive enough to detect and quantify any enzyme-induced breaks generating a free 3′-OH DNA end.

2. Materials and methods

2.1. Reagents

For the generation of the HeLa-I-SceI cell lines: SB-I-SceI-3L1 and pCMV(CAT)T7-SB plasmids (Sup. Fig. 1) were kindly provided by Dr. Astrid Roy-Engel (Tulane University). pBlueScript II SK (+) was purchased from Stratagene (Agilent, USA). GeneCellin (#GC1000) was from BioCellChallenge (Bulldog Bio Inc., USA) and HeLa cells from the ATCC (USA). Blasticidin (#R210-01) was from Invitrogen (Thermo Fisher Scientific, USA). For DNA purification in qTUNEL assays and DNA extractions: Lysis Buffer, Blood (#MD1392) and Alcohol Wash, Blood (#MD1412) were purchased from Promega (USA). Dynabeads® MyOne™ Silane (#37002D) and DynaMag™-2 Magnet (#12321D) were purchased from Invitrogen (Thermo Fisher Scientific, USA). Proteinase K (#BP1700-500) was purchased from Fisher Scientific (Thermo Fisher Scientific, USA). All DNA quantifications were done using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Invitrogen, USA) with the QuantiFluor® dsDNA System (Promega, USA). For DBrIC: DNA labeling was performed using TdT (#3333574001) from Roche (Sigma-Aldrich Canada, Roche, Canada) and Biotin-14-dATP (#19524016) from Invitrogen (Thermo Fisher Scientific, USA). β-Agarase I (#M0392) and Taq$^{\alpha}$I (#R0149) were obtained from New England Biolabs (USA). dsDNA Shearase™ Plus (#E2018-50) was from Zymo Research (USA). For immunocapture, Protein A/G Magnetic Beads (#B23202) were purchased from Bimake (USA) and anti-Biotin antibody (#ab6643) was from Abcam Inc. (USA). For qTUNEL labeling: dATP, [α-32P]- 3000Ci/mmol (#BLU012H500UC) was obtained from PerkinElmer (USA) and ddATP was from Roche (Sigma-Aldrich Canada, Canada). DNA fragmentation was performed using NEBNext® dsDNA Fragmentase® from New England Biolabs (USA). Radioactive quantification was performed using EcoLite(+)™ Liquid Scintillation Cocktail (MP Biomedicals, USA) and Beckman LS 6500 Scintillation System (Beckman Coulter, USA). For single-strand break repair, T4 DNA ligase (#M0202), T4 DNA polymerase (#M0203) and NEBuffer 2.1 (#B7202) were purchased from New England Biolabs (USA). For deep-sequencing: SPARK™ DNA Sample Prep Kit for Illumina® (#SPK0001-V08) was purchased from Enzymatics (D-MARK Biosciences, Canada).

2.2. Construction of the HeLa-I-SceI model

SB-I-SceI-3L1 is a pUC57 plasmid containing a cassette harboring the I-SceI recognition sequence and the blasticidin resistance gene under the human elongation factor-1 alpha promoter. The cassette is flanked by Inverted Repeats/Direct Repeats (IR/DR) used by the Sleeping beauty transposase for insertion of the transposon (Sup. Fig. 1A). pCMV(CAT)T7-SB plasmid contains the Sleeping beauty transposase under the control of the human cytomegalovirus promoter (Sup. Fig. 1B). Both plasmids were stably transfected into HeLa cells using Genecellin and pBlueScript II SK (+) as carrier, according to manufacturer's instructions. Following selection with blasticidin for one week, cells were trypsinated and plated in 96 well plates using a BD FACSAria™ III cell sorter to individualize the cells and start a clonal cell line harboring the incorporated insert. Clonal cell lines were maintained under blasticidin selection. Incorporation was assessed by qPCR using primers targeting on the insert.

2.3. DNA extraction

Ten million HeLa-I-SceI cells were trypsinated, washed once with 1 x PBS and lysed for 4 h at 55 °C in 500 μL of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl; pH 8.0, supplemented with 1% Triton X-100, 0.2 mg/ml proteinase K, 10 mM DTT and 0.1 mg/ml RNase A just before use. Five hundred microliters of Lysis Buffer (Promega) was added, followed by 200 μL of Dynabeads® MyOne™ Silane and 500 μL of 100% isopropyl alcohol. DNA was allowed to bind to the magnetic beads for 5 min by rotation at room temperature. DNA bound to the beads was washed once using 1 mL of Lysis Buffer using the magnet and twice with 1 mL of Alcohol Wash. DNA-bead pellet was allowed to dry for 5 min at room temperature. DNA was eluted with 500 μL of elution buffer (10 mM Tris-HCl; pH 9.0, 0.1 mM EDTA) pre-warmed at 65 °C.

2.4. DNA digestions and fragmentations

DNA digestion methods where as outlined in legend to figures. For FastPrep mechanical fragmentation, 0.5 mm glass beads were added to 100 μL genomic DNA and fragmented for 1 min at 6 500 RPM. For sonication, DNA was fragmented using a Misonix sonicator S-4000 for 6 × 30 s at amplitude 25, with 10 s lapse between each pulse. I-SceI and Nt.BbvCI were used to create a unique DSB and to nick the DNA, respectively, with 3 U per μg DNA for 4 h at 37 °C followed by heat inactivation for 20 min at 80 °C. DNA for qTUNEL was digested with 0.5 U of dsDNA Shearase per μg DNA for 20 min at 42 °C and heat inactivated for 5 min at 65 °C.

2.5. Nick and gap repair

DNA nick and gap repair (NGR) was performed in two steps at a final concentration of 40 ng/μL DNA, 1 x NEBuffer 2.1, 0.1 mM dNTP, 1 μM ATP and 400 U T4 DNA ligase per μg DNA. Nick sealing was allowed to proceed for 10 min at 12 °C and one unit of T4 DNA polymerase per μg DNA was added to fill the remaining gaps for 15 min at 12 °C. Reactions were terminated by heat inactivation for 20 min at 75 °C. Subsequent labeling reactions for either DBrIC or qTUNEL were performed directly, without any prior DNA purification step.

2.6. DNA break immunocapture

Three prime hydroxyl DNA ends were labeled for 15 min at 37 °C at a final concentration of 20 ng/μL DNA, 1 x TdT reaction buffer, 5 mM $CoCl_2$, 0.1 mM dATP, 6.25 μM Biotin-14-dATP and 500 U of TdT per μg DNA. TdT was heat-inactivated for 10 min at 65 °C. Labeled DNA was purified with either ethanol precipitation or embedded in agarose plugs, followed by 3 × 20 min washes in 1 x TE on a rotating wheel. Plugs were then digested with β-Agarase in 1 x TE buffer. Plug washes have the advantage of limiting unincorporated nucleotide carryover and DNA loss through ethanol precipitation. Purified labeled DNA was fragmented with either Taq$^{\alpha}$I (66.7 U per μg DNA) or dsDNA Shearase (1.75 U per μg DNA) in appropriate buffer. Immunoprecipitation was carried out following the manufacturer's protocol with minor modifications. Thirty microliters of Protein A/G Magnetic Beads and 1.2 μg of anti-Biotin antibody per μg of starting DNA were used in 2 to 4 vols of the initial beads. Antigens were allowed to bind antibodies on beads for 1 h at room temperature on a rotating wheel in 1 x IP buffer (1 x PBS, 0.02% Tween-20). Four washes were performed in 1 x IP buffer using the magnet and immunocaptured DNA was eluted in one initial bead volume of ultrapure water at 80 °C for 20 min using a thermomixer.

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