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Development of a novel method to create double-strand break repair fingerprints using next-generation sequencing

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ABSTRACT

Efficient DNA double-strand break (DSB) repair is a critical determinant of cell survival in response to DNA damaging agents, and it plays a key role in the maintenance of genomic integrity. Homologous recombination (HR) and non-homologous end-joining (NHEJ) represent the two major pathways by which DSBs are repaired in mammalian cells. We now understand that HR and NHEJ repair are composed of multiple sub-pathways, some of which still remain poorly understood. As such, there is great interest in the development of novel assays to interrogate these key pathways, which could lead to the development of novel therapeutics, and a better understanding of how DSBs are repaired. Furthermore, assays which can measure repair specifically at endogenous chromosomal loci are of particular interest, because of an emerging understanding that chromatin interactions heavily influence DSB repair pathway choice. Here, we present the design and validation of a novel, next-generation sequencing-based approach to study DSB repair at chromosomal loci in cells. We demonstrate that NHEJ repair "fingerprints" can be identified using our assay, which are dependent on the status of key DSB repair proteins. In addition, we have validated that our system can be used to detect dynamic shifts in DSB repair activity in response to specific perturbations. This approach represents a unique alternative to many currently available DSB repair assays, which typical rely on the expression of reporter genes as an indirect read-out for repair. As such, we believe this tool will be useful for DNA repair researchers to study NHEJ repair in a high-throughput and sensitive manner, with the capacity to detect subtle changes in DSB repair patterns that was not possible previously.

1. Introduction

Homologous recombination (HR) and non-homologous end-joining (NHEJ) represent the two major DSB repair pathways in cells [1–4]. While HR utilizes homologous DNA sequences as a template for repair, NHEJ processes and re-ligates the ends of the breaks [5]. The NHEJ pathway is considered more error prone than HR and occurs more frequently in cells. NHEJ is the predominant pathway in the G0/G1-phases of the cell cycle, while HR increases during S/G2, when a sister chromatid becomes available as a template for repair. Emerging evidence indicates that many sub-pathways exist within NHEJ and HR repair. In particular, NHEJ repair mainly is comprised of canonical NHEJ and non-canonical NHEJ repair. The latter process is been given many names, including back-up NHEJ (bNHEJ), alternative NHEJ (aNHEJ), and microhomology-mediated NHEJ (MMEJ; [6]). The former pathway results in minimal processing of the DSB ends [7], while the latter process typically results in deletions with local sequence microhomology [8–11]. Canonical NHEJ proteins include DNA-PKcs, XRCC4, and Ligase IV [7]. Alternative NHEJ is a poorly defined pathway but appears to require MRE11 [12], and PARP-1 [13]. Ligase III and XRCC1 have also been implicated in alternative NHEJ [14,15], although more recent studies have questioned the requirement of these proteins in this pathway [16–18]. Collectively, the non-canonical NHEJ repair processes share a common theme of higher rates of insertions, deletions,

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and microhomology usage. As such, we have termed this pathway mutagenic NHEJ repair, in order to distinguish cNHEJ repair from bNHEJ, aNHEJ, MMEJ, which often are used interchangeably [19].

Fluorescence-based assays have become an important tool to assess DSB repair in cells. The DR-GFP assay is a commonly employed HR assay, which uses the I-SceI endonuclease to induce a site-specific DSB in a cell [20]. We recently developed a novel NHEJ repair assay, termed End Joining-Red Fluorescent Protein (EJ-RFP), which can be combined with DR-GFP to measure both DSB repair pathways simultaneously [19]. We integrated this system into a number of cell lines, including U2OS DR-GFP cells (referred to as U2OS EJ-DR cells). We also developed a novel, ligand-dependent I-SceI system for DSB induction in cells, which regulates DNA cleavage by controlling the nucleocytoplasmic localization of the protein [19]. Many other elegant reporter assays and inducible cleavage systems have been described previously, which have been used to gain critical insights into DSB repair regulation (examples in [9,12,21–24]). In particular, Samson and colleagues recently developed a novel, plasmid-based system in which multiple DNA repair pathways, including NHEJ and HR repair, can be interrogated simultaneously, which can be applied even to primary cell cultures [25]. However, many of these systems, including our own previously described EJ-RFP assay, are limited by the requirement to use a fluorescent protein- or luciferase-based readout as an indirect measure of DSB repair activity. While DNA damage foci formation does not suffer from this limitation, it nonetheless relies on the binding of proteins to induced DSBs as an indirect measure of repair activity. As such, there is great interest in developing novel assays which can assay DSB repair at chromosomal loci which is independent of secondary reporters. Furthermore, assays which can measure DSB repair activity in a high-throughput manner with fine resolution would be advantageous to study multiple conditions simultaneously, and to assess subtle changes in repair patterns. In parallel, next-generation sequencing technologies have emerged as a tool for massively parallel sequencing of DNA [26]. Recent studies revealed that these tools can be utilized to assess genomic translocation junctions [27,28] and the outcomes of DNA repair [25,29].

Here, we report the design and validation of novel approach to analyze induced DSB breakpoints using next-generation sequencing. Our approach can be utilized to rapidly assess the rates of insertions, deletions and patterns of microhomology usage at a given DSB of interest. In this manner, a "fingerprint" of NHEJ repair events can be created for a site-specific DSB such as one induced by I-SceI. We have validated that our approach can detect the expected shifts in DSB repair activity using a collection of small interfering RNAs (siRNAs) and drugs targeting NHEJ repair and other DSB repair pathways, and also in the setting of serum starvation. Using our platform, we also describe unexpected findings regarding the patterns of insertions, deletions and microhomology usage under these conditions. We have created a web-based sequence alignment and analysis program specifically designed for our platform, and we have made it publicly available. Our novel approach will be useful as an alternative approach to measure NHEJ repair a chromosomal loci, which does not depend on the expression of reporter genes. Furthermore, it can analyze large numbers of sequences (e.g., millions of alleles) with multiplexing, and thus it will be well-suited to assess subtle NHEJ repair patterns at levels of resolution and throughput which were not previously possible.

2. Materials and methods

2.1. Cell lines and culture conditions

U2OS EJ-DR and U2OS EJ-DRs cells (i.e., U2OS EJ-DR cells which contain the ligand-inducible I-SceI) have been described previously [19]. These cell lines were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine containing 10% tetracyline-free (tet-free) fetal bovine serum (FBS; Clontech Laboratories and Atlanta Biologics). All cells were maintained at 37 °C with 5% CO_2. Tet-free serum was required to prevent *DsRed* gene expression in the EJ-RFP system from residual tetracycline found in most commercially available FBS preparations. For studies involving ddSceGR, cells were cultured in charcoal-stripped FBS (Invitrogen Corporation), to minimize the levels of endogenous glucocorticoids present in untreated FBS preparations. For the experiments involving serum-deprivation, cells were washed two times in DMEM containing reduced FBS concentrations to remove residual serum, followed by replacement of the culture medium with DMEM containing 0.1% FBS. Ligand-induced DNA cleavage by ddSceGR was performed by adding the Shield1 and Triamcinolone Acetonide (TA) ligands at concentrations of 0.5–1 μM and 100 nM, respectively, to the cell cultures. Unless otherwise indicated, ligands were incubated in the cells for 24 h, followed by one or two washes with DMEM containing 10% FBS without ligands. For DNA-PKcs inhibition, the small molecule DNA-PKcs inhibitor, NU7441 (Tocris Biosciences), was added at a concentration of 1 μM to cells for 24 h, during the period of DSB induction, to U2OS EJ-DRs cells.

2.2. DSB repair assays

For DSB repair experiments involving ddSceGR, the Shield1 and TA ligands were added to cell cultures in order to induce DSBs as described above. NHEJ and HR repair activity was assessed by quantification of the percentages of DsRed+ and GFP+ cells, respectively, using a FACScan flow cytometer (Becton, Dickinson and Company; BD) at the indicated times. Standard compensation techniques were used when GFP and DsRed were analyzed simultaneously in order to minimize spectral overlap. DsRed+, GFP+ and parental cells were used as controls for optimization and the data were analyzed using FloJo (Tree Star Inc.). Experiments were performed in either triplicate or quadruplicate, and error bars represent standard errors of the mean (SEM).

2.3. Next-generation sequencing analyses

For next-generation sequencing of the I-SceI breakpoint in U2OS EJ-DR cells, genomic DNA first was extracted using the Wizard Genomic DNA kit (Promega Corporation). The I-SceI site and flanking sequence at the Sce-TetR locus was amplified by PCR using the primers: 5′-GTTACAATGATATACACTGTTTGA-3′ (forward) and 5′-GACTTAGTAAAGCACATCTAAAAC-3′ (reverse) with Platinum Taq DNA polymerase as per the manufacturer's specifications (Life Technologies Corporation). The PCR amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen Corporation), and then subjected to next-generation sequencing on the Pacific Biosciences (PacBio) Single Molecule, Real-Time (SMRT) DNA Sequencing System (Pacific Biosciences) as per the manufacturer's protocols, at the Yale Center for Genomic Analysis (YCGA), and as described previously [30]. Briefly, SMRT bell libraries were created with the Pacific Biosciences template preparation kit and sequencing protocol for 0.25–3 kb libraries. Next, SMRT bell templates were bound to the polymerases with the DNA/polymerase binding kit and corresponding primers. Complexes containing polymerase and templates then were bound to magnetic beads and the sequencing was performed on the PacBio RS system. Sub-read filtering was performed with the PacBio SMRT analysis software package. A single SMRT cell was used for each sample. In selected cases, samples were first PCR barcoded and pooled into a single SMRT cell, and the data was de-convoluted as described previously using PacBio software [31]. PCR barcodes which are compatible