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Formation and nucleolytic processing of Cas9-induced DNA breaks in human cells quantified by droplet digital PCR

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ABSTRACT

Cas9 endonuclease from *S. pyogenes* is widely used to induce controlled double strand breaks (DSB) at desired genomic loci for gene editing. Here, we describe a droplet digital PCR (ddPCR) method to precisely quantify the kinetic of formation and 5′-end nucleolytic processing of Cas9-induced DSB in different human cells lines. Notably, DSB processing is a finely regulated process, which dictates the choice between non-homologous end joining (NHEJ) and homology directed repair (HDR). This step of DSB repair is also a relevant point to be taken into consideration to improve Cas9-mediated technology. Indeed, by this protocol, we show that processing of Cas9-induced DSB is impaired by CTIP or BRCA1 depletion, while it is accelerated after down-regulation of DNA-PKcs and 53BP1, two DSB repair key factors. In conclusion, the method we describe here can be used to study DSB repair mechanisms, with direct utility for molecularly optimising the knock-out/in outcomes in genome manipulation.

1. Introduction

In mammalian cells, DSB lesions are faster repaired through NHEJ, often resulting in small insertions or deletions [1,2]. This has been exploited by the CRISPR-Cas9 technology to generate gene knock-out. Alternatively, in the S and G2 phases of the cell cycle, DSB can be extensively 5′-end nucleolytic processed (a phenomenon called DSB resection) by several endo- and exo-nucleases, in cooperation with helicases, triggering HDR [3]. Notably, from yeast to mammals KU complex and 53BP1 physically antagonize the DSB resection process [3,4]. Concurrently, an intricate network of regulation suppresses DSB resection in G1 cell cycle phase and in post-mitotic cells, thus preventing HDR. Moreover, chromatin context influences the efficiency of induced DSB formation and processing [5]. Once resection starts, DSB cannot be any longer repaired by NHEJ, thus being a critical step to regulate NHEJ and HDR outcomes. Remarkably, misregulation of the balance between NHEJ and HDR triggers genome rearrangements and instability [3]. Indeed mutations in genes encoding factors involved in DSB processing (*e.g.* MRE11, BRCA1, CTIP) have been found in several cancer-prone inherited syndromes. As such, some of those factors are already studied as attractive targets in cancer therapy [6,7]. Importantly, to edit a desired locus in the genome with a donor template (*e.g.* gene sequence substitution or single-codon modifications), resection at Cas9-induced DSB (Cas9-DSB) is compulsory. Therefore, all the regulations and cellular aspects (such as cell cycle phase and chromatin context of the target gene), that influence this crucial step of DSB repair, have to be taken in to account to foresee the success of gene editing protocol. In this scenario, a method to precisely quantify Cas9-DSB formation and processing may be of great utility. In particular, in the literature an open question is how Cas9-DSBs are resected, also considering the relative prolonged binding of the Cas9 protein on the substrate once DNA is cleaved *in vitro* [8,9].

2. Material and methods

2.1. Cell culture, plasmids and treatments

U-2OS and HEK293T cells were grown in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco) and 1% Penicillin/Streptomycin (Euroclone). U-2OS-SEC (Stably Expressing Cas9, under the tetracycline-inducible element) and HEK293-SEC (Stably Expressing Cas9, under the tetracycline-inducible element) were available from Professor John Rouse (https://mrcppureagents.dundee.ac.uk, see also [10]), and were grown in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco), 1% Penicillin/Streptomycin (Euroclone) supplemented with hygromicin B 100 μg/ml and blasticidin 15 μg/ml. U-2OS shSCRAMBLE and sh53BP1 were grown in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco) and 1% Penicillin/Streptomycin (Euroclone) supplemented with puromycin 1 μg/ml. In U-

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2OS-SEC and HEK293-SEC, SpCas9 was induced with 1 μg/ml doxycycline for 24 h before transfection. Plasmid all-in-one vectors px330A-1x2 and px330S-2, carrying *Spcas9* gene and the sgRNAs cloning site, were obtained from Dr. Takashi Yamamoto *via* Addgene (plasmids #58766 and # 58778 respectively [11]. sh53BP1 plasmid was purchased from Sigma-Aldrich (Mission shRNA, NM_005657). siRNA against luciferase (5′-CGUACGCGGAAUACUUCGATT-3′), CTIP (5′-UCCACAACAUAAUCCUAAU-3′) or BRCA1 (5′-CAGCUACCCUUCCA UCAUA-3′) were used for transfection with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. NU7441 compound (a DNA-PKcs inhibitor) was purchased from Selleckchem and was used at a concentration of 5 μM for 24 h after cell transfection with the all-in-one vectors. For resection experiments in Cas9-inducible stable cell lines, sgRNAs targeting DSB1 and DSB2 were synthesized and purchased from ThermoFisher and used for transfection with Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. For experiment with cell nucleofection with the all-in-one vectors, 10^6 U-2OS or HEK293T cells were electroporated with Amaxa Nucleofector II, according to the manufacturer's instructions using the X-001 program and Q-001 respectively.

2.2. Human genomic DNA extraction

U-2OS and HEK293T cells and their derivatives were grown on 6-well plates after transfection. At the indicated time points, cells where trypsinized, washed in PBS and genomic DNA was extracted by NucleoSpin™ Tissue kit (Macherey-Nagel), according to the manufacturer's instructions. The day after, 15 μl of genomic DNA (DNA concentration is around 100 ng/μl) were digested or mock with 20 units of *Bsr*GI or *Bam*HI restriction enzymes (New England BioLabs) for 5 h at 37 °C. Digested or mock DNA was purified and 5 μl were used for the ddPCR reaction.

2.3. Droplet digital PCR assay

The ddPCR reaction was assembled as follows: 5 μl of genomic DNA (approximately 50 ng), 1X ddPCR™ Supermix for Probes (no dUTP, Bio-Rad), 900 nM for each pair of primers, 250 nM for each probe (HEX and FAM, TaqMan probes) and dH_2O to 20 μl per sample. We produced droplets pipetting 20 μl of the PCR reaction mix into single well of a universal DG8™ cartridge for droplets generation (Bio-Rad). 70 μl of droplet generation oil were also added in each well next to the ones containing the samples. Cartridges were covered with DG8™ droplet generator gaskets (Bio-Rad) and then placed into the droplet generator (QX200™, Bio-Rad). After droplet generation, 40 μl of emulsion were transferred from the right well of the cartridge to a 96-well ddPCR plate (BioRad). Before PCR reaction, 96-well PCR plates were sealed with peelable foil heat seals at the PCR plate sealer machine (PX1™, Bio-Rad).

PCR (T100™ thermocycler, BioRad) was run using a ramp rate of 2.5 °C/s between each step. First, Taq polymerase was activated at 95 °C for 5 min and then 39 cycles of 95 °C for 30 s and 58.7 °C for 1 min were made. At the end of the cycles, one additional cycle at 4 °C for 5 min and one at 90 °C for 5 min were made, then temperature was held at 12 °C. After the PCR, FAM and HEX fluorescence was read at the droplet reader (QX200™, BioRad) using QuantaSoft™ software (BioRad). For each sample the number of droplets generated were on an average of 15 000. The number of copies/μl of each target locus was determined setting an empirical baseline threshold identical in all the samples.

For the calculation of Cas9 cleavage efficiency (*CE*), we made the ratio (*r*) between the number of copies of the locus across the Cas9 sites (HEX1 and HEX2 probes) and a control locus on Chr. XXII (FAM probe) in cells transfected with or without sgRNAs. We then calculated:

$$R = r_{+sgRNA}/r_{-sgRNA}$$

and the final *CE* value with the following equation:

$$\%\ Cas9\ cut\ efficiency\ (CE) = (1 - R)*100\%$$

For the measurement of ssDNA generated by the resection process (% ssDNA, *SS* value), we calculated the ratio (*r′*) between the number of copies nearby the DSB loci (335 bp from DSB1 and 364 bp from DSB2, recognized by HEX3 and HEX4 probes respectively) and a control non-target locus (NT locus) on Chr. XXII (FAM probe), with or without sgRNA, digested or mock with *Bsr*GI or *Bam*HI restriction enzymes. The absolute percentage of ssDNA was then calculated with the following equation:

$$\%\ ssDNA\ (SS) = [(r'_{digested}/r'_{mock})_{+sgRNA} - (r'_{digested}/r'_{mock})_{-sgRNA}] *100\%$$

The final percentage of DSB resected (*RES* value) was calculated with the formula:

$$RES = SS/CE$$

All the sequences of primers and probes used are listed in Table S1.

2.4. Real Time PCR assay

Real Time PCR analysis was performed as previously described [12]. In brief, 4 μl of digested and undigested DNA was used as template in a 20 μl reaction containing 10 μl 2x TaqMan Master mix (Genespin), 250 nM of each probe and 450 nM of each primer. % ssDNA (*SS*), % Cas9 cut efficiency (*CE*) and % DSB resected (*RES*) were calculated using the formula described in [13].

2.5. Immunoblot analysis

For total protein extract preparation, cells were lysed in 1% SDS sample buffer and protein samples, after loading normalization, were analyzed by SDS-PAGE. Antibodies used in this study were the following: anti-FLAG (clone M2, Sigma-Aldrich), anti-53BP1 (Cell Signaling), anti-GAPDH (Santa Cruz Biotechnology), anti-BRCA1 (Santa Cruz Biotechnology), anti-CTIP (a kind gift from Dr. Pablo Huertas), anti-Actin (Sigma-Aldrich).

3. Results and discussion

DSB resection is a key process to trigger HDR, favouring knock-in outcomes in gene editing. Alternatively, NHEJ pathway promotes limited processing of the break, with addition/removal of one or few bases (indels), often resulting in gene knock-out (Fig. 1A). Aiming to verify and accurately quantify resection at Cas9-DSB, we developed a ddPCR-based protocol. The rational of ddPCR is based on the partitioning of DNA input molecules into thousands of nanoliter-sized uniform droplets that result in an end-point absolute quantification of DNA targets after a standard PCR reaction [14]. Indeed, a ddPCR-based method has been recently described to accurately and precisely measure Cas9-induced cut efficiency in human cell lines [15]. We started from a protocol originally developed in the model organism *S. cerevisiae* to analyse DNA end resection of DSB induced by the homothallic nuclease HO [16,17]. More recently, this was adapted to analyse DSBs induced by the restriction enzyme *Asi*SI (*Asi*SI-DSB) in U-2OS cells by real-time PCR (RT-PCR) [12,18,19]. Here the protocol has been further modified to quantify the cut efficiency and the 5′-end resection of Cas9-DSB through a novel and versatile ddPCR-based method (ddPCR assay at DSB, or ddPaD). Among the several advantages of the ddPCR over the RT-PCR, there are the high sensitivity and reproducibility of the results, coupled with the use of internal references, without the need of standard curves [20]. Therefore, despite the droplet fluorescence-reading step, overall ddPCR may allow to save time and reagents if compared to RT-PCR (see Material and methods).

In order to set up the method, we chose two *Asi*SI cut sites on Chromosome I, which were already proven to be resected by RT-PCR in