Impact of the DNA polymerase Theta on the DNA replication program

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A B S T R A C T

The physiological function of the human DNA polymerase θ (pol θ) is still unclear despite its in vitro translesion synthesis capacity during DNA damage repair process. However this DNA polymerase is always present along the cell cycle in the absence of replication stress and DNA damage. Is there a different molecular function? We present the genomic data of replication timing in depleted pol θ cells (GSE49693) and in cells overexpressing pol θ (GSE53070) indicating that Pol θ holds a novel role in the absence of external stress as a critical determinant of the replication timing program in human cells.

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Direct link to deposited data

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49693.
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53070.

Experimental design, materials and methods

Cell transfection

Pol θ depleted cells

RKO cells were transfected with siRNA targeting POL θ by two independent pools of siRNA:

– POLQ-1 siRNA: ON-TARGET plus smart pool of 4 POLQ siRNA from Dharmacon: UCAGGGAGGGCGACUA, GAAUUAUCUUCAACUA, CAAUUUACAGUACGGAAA, UGAUAGAUUAGCCUAGUA

– siRNA POLQ-2: ON-TARGET plus smart pool of 4 POLQ siRNA from Dharmacon: UCAGGGAGGGCGACUA, GAAUUAUCUUCAACUA, CAAUUUACAGUACGGAAA, UGAUAGAUUAGCCUAGUA

RKO cells were transfected with siRNA Luciferase as control (5′-CGUACGCGGAAUACUUCGAdTdT-3′ from Sigma-Aldrich, Saint-Louis, USA).

Cells overexpressing Pol θ

MRC5-SV fibroblasts were transfected with the pol θ overexpression vector. The design and construction of the pol θ expression vector as well as the overexpressing Pol θ cells and their isogenic controls were previously described [1].

Transfections were performed by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA) at the final concentration of 50 nM following the manufacturer’s suggestions. Transfection medium was changed after 8 h to complete medium.

Growth protocol

RKO cells: The human RKO cell line was purchased from ATCC and grown in DMEM with GlutaMAX I, high-glucose, sodium pyruvate (Gibco, Life Technologie), supplemented with 10% fetal bovine serum (Lonza), penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹) (Gibco) at 37 °C, 5% CO2 and 5% O2 (standard culture conditions).

MRC5-SV: The Human MRC5-SV cell line was purchased from ATCC and grown in Modified Eagle Medium with GlutaMAX™ I, High glucose,
Sodium Pyruvate (Gibco, Life technologies), supplemented with 10% Fetal Bovine Serum (Lonza), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco) at 37 °C, 5% CO2 and 5% O2 (standard culture conditions).

**BrDU treatment**

Cells were incubated with BrdU (50 μM) for 1 hour, collected, washed three times in PBS, then fixed in 75% ethanol, and stored at −20 °C. Fixed cells were first re-suspended in PBS with RNAse (0.5 mg/ml) and then with propidium iodide (50 μg/ml) a 30 min incubation at room temperature before the cell sorting.

**Cell sorting**

80,000 cells were sorted in two fractions S1 and S2 using INFLUX 500 (Cytopeia purchased by BD Biosciences) corresponding to early and late fractions respectively (Fig. 1).

**DNA extraction**

A proteinase K treatment (0.2 mg/ml) was performed in both fractions in lysis buffer (50 mM Tris pH = 8; 10 mM EDTA; 300 mM NaCl) during 2 h at 65 °C. DNA is protected from light. Then, tRNA was added (50 ng/μl) and a phenol-chloroform extraction followed by an ethanol precipitation was performed. The pellet is resuspended in 100 μl of Tris buffer (10 mM). Then the two fractions were sonicated in order to obtain fragments between 500 and 1000 bp. DNA is denatured at 95 °C 5 min and then kept on ice during 10 min.

Neo-synthesized DNA was immunoprecipitated with BrdU antibodies (10 μg. Anti BrdU Pure, BD Biosciences, # 347580) by an immunoprecipitated indirect method. Neo-synthesized DNA was incubated overnight at 4 °C with 10 μg of BrdU antibody, then incubated during 2 h with magnetic beads prepared as mentioned by the manufacturer (Adamtech; ChIP adambeads #04342). Beads were washed with different buffers (IP buffer: Tris pH = 8 10 mM; EDTA 1 mM; NaCl 150 mM, Triton ×100 0.5%; BufferB: Tris pH = 8 20 mM; EDTA 2 mM; NaCl 250 mM; Triton ×100 0.25%). Beads are resuspended in Tris buffer (10 mM). The reversion step was then performed with SDS (1%) and 0.5 mg of protease K at 65 °C overnight. Neo-synthesized DNA is purified by a pheno-chloroform treatment and an ethanol precipitation. Pellet is resuspended in Tris buffer (pH = 8, 10 mM). To control the quality of enrichment of early and late fractions in S1 and S2, qPCR was performed (Fig. 2A, B, C, D) with CAV2 oligonucleotides as early control (GGCTTGGAGTTTCAACAGGA and CGCAGGTACGAGTCACAACA) and with bgGRM8 oligonucleotides as late control (GGGAAGGAATGCAAGACAA and AATTTGGCTGCTTAGCATGG) and normalized with amplicon from neo-synthesized mitochondrial DNA (AACCGCTA RGTATTTCGTACA and GGTTGATTGCTGTACTTGCTTG).

**WGA amplification and labeling**

Microarray hybridization requires a minimum amount of 500 ng of DNA. To obtain sufficient specific immunoprecipitated neo-synthesized
DNA, whole genome amplification was conducted (WGA, Sigma). To be sure that this step does not introduce bias, a qPCR after WGA was performed to confirm the specific enrichment in each fraction S1 and S2 (Fig. 2E, F, G, H). After amplification, early and late neo-synthesized DNA fractions were labeled with Cy3 and Cy5 ULS molecules (Genomic DNA labeling Kit, Agilent) as recommended by the manufacturer.

**Hybridization protocol**

The hybridization was performed according to the manufacturer’s instructions on 4 × 180 K human microarray (SurePrint G3 Human CGH Microarray Kit, 4 × 180 K, AGILENT Technologies, genome reference Hg18) that covers the whole genome with one probe every 13 Kb (11 Kb in RefSeq sequences).

**Scan protocol**

Microarrays were scanned with an Agilent’s High-Resolution C Scanner using a resolution of 2 μm and the autofocus option.

**Data processing**

Feature extraction was performed with the Feature Extraction 9.1 software (Agilent technologies). For each experiment, the raw data sets were automatically normalized by the Feature extraction software using the CGH_1105_oct12 FE protocol browser. Analysis was performed with the Agilent Genomic Workbench 5.0 software. The log²-ratio timing profiles (Fig. 3) were smoothed using the Triangular Moving Average option of the Agilent Genomic Workbench 5.0 software with the Triangular algorithm and 500-kb windows.

The algorithm from CGH applications of the Agilent Genomic Workbench 5.0 software was used in order to determine the replication domains (early and late domains) in Pol θ-depleted RKO cells and in Pol θ-overexpressing MRC5-SV cells. The aberration detection algorithms associated with the Z-score and with a threshold of 1.8 define the boundaries and magnitudes of the regions of DNA loss or gain corresponding to the late and early replicating domains respectively (Regions with a positive log-ratio is replicated early and late when the log-ratio is negative).

**Basic analysis**

A comparison was conducted between early and late domains between each cell lines. As mentioned by Hiratani et al. [2], two major changes can be characterized. The intersection between CTRL siRNA late and POLQ-1 siRNA early intervals will reveal segments that are replicated earlier due to the depletion of Pol θ. Conversely with CTRL siRNA early versus POLQ-1 siRNA late domains, intervals that are replicated later will be characterized. Similarly, the intersection between MRC5-CTRL late and MRC5-overexpressing Pol θ early intervals will reveal segments that have become earlier by the overexpression of Pol θ, and conversely with MRC5-CTRL early versus MRC5-overexpressing Pol θ late domains, intervals that are replicated late will be characterized. The intersection was performed with the Intersection GALAXY tools (Operate on genomic intervals, https://main.g2.bx.psu.edu/).

**Discussion**

Here we put in evidence a new function of Pol θ because a change in expression of this protein disturbs the replication timing program.
These data and other evidences are published recently and demonstrate the molecular role of Pol θ in DNA replication [3]. This novel Pol θ function could be particularly important within the context of cancer because in colon, breast and lung cancers, a high pol θ expression was observed and associated with poor clinical outcome of patients [1,4–7].

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