CRISPR/Cas9-Mediated Knockdown Of *BRCA1/2* Restores Response To Olaparib In Pancreatic Cancer Cell Lines

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Research Article

Keywords: Pancreatic ductal adenocarcinoma, olaparib, BRCA, CRISPR/Cas9-mediated knock-in

Posted Date: March 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1439273/v1

Abstract

Background: Pancreatic cancer is one of the most aggressive diseases with a very poor outcome. Olaparib, a PARP inhibitor, as maintenance therapy showed benefits in patients with metastatic pancreatic adenocarcinoma bearing germline *BRCA1/2* mutations and that did not undergo progression during at least 16 weeks of a prior platinum-based chemotherapy regimen. However, germline *BRCA* mutation has been described in only 4 to 7% of patients with pancreatic adenocarcinoma.

Methods: A CRISPR/Cas9-mediated system was used to knock-in the c.763G>T (p.Glu255*) and c.2133C>A (p.Cys711*) mutations in cell lines to obtain truncated BRCA1 and BRCA2 proteins, respectively. A CRISPR/Cas9 ribonucleoprotein (RNP) was assembled for each mutation and transfected into two PDAC cell lines (T3M4 and Capan-2) and into a breast cancer cell lines (MCF7) as control. Expected mutations were detected using ddPCR assay.

Results: Allelic frequencies of 85% for MCF7, 65% for T3M4 and 20% for Capan-2 were found for both *BRCA* mutations, proving the transfection efficiency of our CRISPR/Cas9 systems. Calculated olaparib IC_{50} were significantly reduced for all cell lines harbored *BRCA1* or *BRCA2* mutations compared to wild-type *BRCA1/2* cells ($P < 0.01$). Furthermore, we find that olaparib induces more apoptosis after 72h treatment in *BRCA* knockdown cells than in wild-type cells.

Conclusions: The different CRISPR/Cas9 systems allow the *in vitro* induction of deleterious *BRCA1/2* mutations by knock-in in different pancreatic cancer cell lines and increase their sensitivity to olaparib. This strategy might offer a new insight for the management of patients with pancreatic cancer and open new perspective based on the use of CRISPR/Cas9 strategy *in vivo*.

Background

Pancreatic Cancer has a poor prognosis and a 5-year survival under 10%. Pancreatic ductal adenocarcinomas (PDAC), occurring in exocrine glands, represent 85% of all pancreatic cancer (1). Surgery is considered as the only curative therapy but 80% of patients with PDAC are diagnosed at advanced stage and cannot undergo surgery (2). Overall survival has been slightly improved in patients with advanced PDAC, with different validated regimens such as FOLFIRINOX protocol (5-Fluorouracil, Leucovorin, Irinotecan and Oxaliplatin) (3, 4), gemcitabine-plus-*nab*-paclitaxel (5) and gemcitabine in monotherapy (6). These regimens are often associated with high-grade toxicities and are sometimes ineffective given the complex and heterogeneous molecular mechanism of PDAC (7).

More recently, the results of the phase III POLO trial (Pancreas Cancer Olaparib Ongoing, NCT02184195) have been published. This double-blind, placebo-controlled, and randomized study showed the efficacy of olaparib, a poly (ADP-ribose) polymerase (PARP) inhibitor (PARPi), as a maintenance therapy in patients with a germline *BRCA (breast cancer susceptibility gene)*-mutated metastatic pancreatic cancer and without tumor progression during at least 16 weeks of a first-line platinum-based chemotherapy. Median progression-free survival (PFS) was significantly improved with olaparib compared to placebo (7.4 months

versus 3.8 months; hazard ratio 0.53; 95% CI [0.35–0.82]; *p* = 0,004) and the 2-year PFS was 22.1% for patients in the olaparib group compared to 9.6% for patients in the placebo group (8). Quality of life in the olaparib group was also comparable to placebo group and associated to few toxicities (8, 9). After this trial, olaparib has been approved in december,2019 by the U.S. Food and Drug Administration (FDA) for this indication (10).

Olaparib, approved for the management of *BRCA*-mutated ovarian (11), metastatic breast cancer (12), and prostate cancer (13) is the first targeted therapy used in monotherapy to show real benefits for the management of patients with metastatic PDAC (8). Presence of a deleterious *BRCA*-mutation is critical for the prescription of olaparib (14) since its efficiency is based on the concept of synthetic lethality discovered in 2005 by Bryant *et al.* (15) and Farmer *et al.* (16). This concept requires 2 defective genes or proteins to cause cell death. PARP proteins are involved in DNA single-stranded breaks (SSBs) repair through the base excision repair (BER) pathway. When PARP proteins are blocked by PARPi, DNA lesions can still be repaired by the homologous recombination repair (HRR) pathway that is also able to repair DNA double-strand breaks (DSBs) and where BRCA1 and BRCA2 proteins are involved. However, deleterious *BRCA1/2* mutations lead to a homologous recombination deficiency (HRD). Thus, inhibition of PARP proteins by PARPi and deleterious *BRCA1/2* mutations are both required to induce cytotoxicity (14, 17, 18). Another possible etiology of HRD is the mutation of other homologous recombination (HR) genes, such as *ATM*, *PALB2* or *RAD51* genes family (19) and this deficiency can lead to tumors sensitivity to PARPi (20).

Only 10% of PDAC are considered as familial (21) and germline *BRCA* mutations have been described in 4 to 7% of patients with pancreatic cancer (22). In this study, we induced *BRCA1* and *BRCA2* mutations in PDAC cell-lines using a clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 (Cas9)-mediated knock-in (KI) technology and evaluated whether this strategy had an impact on olaparib sensitivity.

Methods

Cell lines and cell culture

Human PDAC cell line Capan-2 (Cat #HTB-80) and Human breast cancer cell line MCF7 (Cat #HTB-22), *BRCA1* and *BRCA2* WT, were obtained from the American Type Culture Collection (Manassas, VA, USA). Human pancreatic ductal adenocarcinoma (PDAC) cell line T3M4, *BRCA1* and *BRCA2* wild type (WT), was a gift from Dr. J. Werner (University of Heidelberg, Heidelberg, Germany). Cells were cultured in antibiotic-free RPMI 1640 (Gibco, Carlsbad, CA, USA) and incubated at 37°C in a 5% CO_2 humidified atmosphere. Medium was supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and was changed every 3–4 days. T3M4, Capan-2 and MCF7 were periodically tested for *Mycoplasma* contamination using the VenorH GeM Mycoplasma Detection Kit (Minerva Biolabs GmbH, Berlin, Germany).

CRISPR/Cas9-mediated knock-in (KI)

CRISPR RNAs (crRNAs) and homology directed repair (HDR) template sequence design

Deleterious mutations were selected to obtain truncated BRCA1 and BRCA2 proteins. Design of CRISPR RNAs (crRNAs) and donor templates was performed using the CRISPR LIFEPIPE® tool (Life&Soft, Plessis-Robinson, France) and results were confirmed using the Dharmacon CRISPR Design Tool and the Edit-R homology directed repair (HDR) Donor Designer (Dharmacon, Lafayette, CO, USA).

Preparation of ribonucleoprotein (RNP) complex with Cas9 protein, single-guide RNAs (sgRNAs) and single-stranded oligodeoxynucleotides (ssODNs)

All components of ribonucleoprotein (RNP) complex were purchased from Dharmacon: Edit-R Cas9 Nuclease protein NLS (Cat #CAS11200), Edit-R synthetic trans-activating CRISPR RNA (tracrRNA) oligos (Cat #U-002005), Edit-R crRNA oligos (designed for mutations of interest) and Edit-R HDR Donor template (single-stranded oligodeoxynucleotides (ssODNs), designed for specific knock-in (KI) of mutations of interest). tracrRNA and crRNA stock solutions were prepared according to manufacturer's protocol « Dharmacon™ Edit-R™ synthetic guide RNA resuspension protocol ». Briefly, RNA pellets were resuspended in nuclease-free 10 mM Tris pH 7.4 (Dharmacon, Cat #B-006000-100) for a 10 µM stock solution and aliquoted to not exceed five freeze-thaw cycles. Concentrations were verified using Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and Qubit® RNA HS Assay Kit (Life Technologies). Five nanomoles of donor oligos were resuspended in 50 µL Tris buffer to obtain a 100 µM stock solution. Concentrations were assessed using Qubit 3.0 fluorometer (Life Technologies) and Qubit® ssDNA HS Assay Kit (Life Technologies).

Transfection

Capan-2, MCF7 and T3M4 were respectively seeded into 96-wells plates at a density of 8.10^3, 1.10^4 and 2.10^5 cells per well in triplicate and were maintained in complete medium to reach 70% of confluence on the next day. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere overnight. Transfection of RNP complex into Capan-2, MCF7 and T3M4 cells was next performed following the protocol entitled «Transfection of ssDNA donor oligonucleotides for HDR-mediated gene modifications using the Dharmacon™ Edit-R™ system» and using a lipid-based transfection reagent called DharmaFECT Duo (Dharmacon). Briefly, a 2.5 µM Cas9 protein working solution, a 1 µM donor oligo working solution, a 2 µM sgRNA transfection complex (with crRNA:tracrRNA) and a 6 µg/mL DharmaFECT Duo working solution were prepared from the stock solutions. RNP complex was then assembled with Cas9 protein at 25 nM, donor oligo at 10 nM and sgRNA at 50 nM. The final transfection mixture was consisted of RNP complex, transfection reagent and serum-free medium and was incubated 18 hours under usual culture conditions. Two controls were also prepared: a gene editing control (RNP complex without donor oligo) and a negative control (untransfected cells).

Droplet Digital PCR (ddPCR)

CRISPR/Cas9-mediated induction of mutations was controlled using droplet digital PCR (ddPCR). Briefly, total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol for cultured cells. All DNA were quantified using Qubit 3.0 fluorometer (Life Technologies) and Qubit® dsDNA HS Assay Kit (Life Technologies).

Bespoken probes were designed to respectively detect *BRCA1* c.763G > T p.(E255*) (Bio-Rad, UniqueAssayID dHsaMDS901307380) and *BRCA2* c.2133C > A p.(C711*) mutations (Bio-Rad, UniqueAssayID dHsaMDS403398316). Reaction mix was prepared using 30 ng of DNA, 11 µL of 2x ddPCR supermix for Probes (no dUTP) (Bio-Rad, Hercules, CA, USA), 1 µL of ddPCR Mutation Assay (that contains primers and probes) and nuclease-free water to a final volume of 22 µL. DNA was replaced by 1 µL nuclease-free water for negative control. The ddPCR mix was loaded on a 96-well reaction plate (DG32™ Cartridge, Bio-Rad) and placed into the QX200 Automated Droplet Generator (Bio-Rad) for droplets generation. Droplets were then transferred to a 96-wells PCR plate followed by PCR using the C1000 Touch™ Thermal Cycler (Bio-Rad) with the following conditions: 1 cycle for 10 minutes at 95°C, 40 two-steps cycles (30 seconds at 94°C and 60 seconds at 55°C for *BRCA1* probe and 52,5°C for *BRCA2* probe; ramp rate 2°C/second), 1 cycle for 10 minutes at 98°C and a 4°C hold. The plate was finally analyzed using the QX200 Digital PCR Reader (Bio-Rad). DNA quantity (copies/µL) was determined using QuantaSoft analysis software version 1.7.4.0917 (BioRad) and only samples with a number of droplets greater than 10,000 were analyzed according to manufacturer's recommendations.

Crystal Violet assay

Capan-2, MCF7 and T3M4 cells were respectively seeded into 96-wells plates at a density of 5.10^3, 1.10^4, and 2.10^5 cells per well and were maintained in 200 µL of complete medium for 24 hours. Cells were then exposed to 2, 5, 7, 10, 15, 25, 30, 40, and 50 µM concentrations of olaparib (Clinisciences, Nanterre, France) for 72 hours. Olaparib solution stock was previously reconstituted in DMSO (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Control cells were exposed to medium without olaparib or to 50 µM DMSO. After incubation, cells were fixed with 100 µL of 70% ethanol for 10 minutes. Next, 100 µL of 0.2% Crystal Violet (Sigma-Aldrich Corp., St. Louis, MO, USA) in 20% ethanol was added into each well for 15 minutes, followed by four washes of distilled water and elution solution consisting of 100 µL of 0.1% acetic acid in 50% ethanol was added *per* well. Optical density was determined using a microplate reader (Multiskan Ascent; Thermo Fisher Scientific) at 540 nm. The half-maximal inhibitory concentration (IC_{50}) values of olaparib were determined by a non-linear regression ([inhibitor] vs. normalized response) using GraphPad Prism 9® (GraphPad Software, La Jolla, CA, USA).

Apoptosis detection assay

MCF7 and T3M4 cells were seeded into 6-wells plates at a density of 1.10^4 cells/mL per well and Capan-2 cells at a density of 1.10^5 cells/mL per well. Cells were maintained in complete medium for 24 hours and then were exposed to 40 nM of olaparib or medium. After 72 hours, 1.10^5 cells were stained with annexin V/propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's

instructions. Cell apoptosis was quantified within 1 hour by flow cytometry using a Accuri C6Plus system (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

All experiments were performed in three independent tests of triplicates. Experimental data were analyzed by using one-way ANOVA or paired *t* test and differences were considered statistically significant when *P* value was less than 0.05. All graphs and statistical analysis were performed using GraphPad Prism 9.

Results

Knockdown (KD) of BRCA1 and BRCA2 using CRISPR/Cas9 ribonucleoprotein (RNP) Knock-in (KI).

To achieve the knockdown (KD) of *BRCA1* (NM 007294.4) and *BRCA2* (NM 000059.3), a nonsense mutation for both gene was selected: *BRCA1* c.763G > T p.(Glu255*) (rs80357009) located in exon 9 and *BRCA2* c.2133C > A p.(Cys711*) (rs535547513) located in exon 11. Each mutation was described as deleterious and pathogenic in the UMD BRCA share™ (23) and ClinVar database. sgRNAs in rank 1 (called sgRNA n°1) and in rank 2 (called sgRNA n°2) for each mutation were kept among the 5 proposed by the CRISPR LIFEPIPE® tool (Table 1). We chose the two best crRNAs, those with a GC content within the range of 40%-60% as recommended (24). CRISPR/Cas9 RNP systems were assembled with one or two sgRNA for each mutation and with a single-stranded DNA (ssDNA) donor of 61 nucleotides. The donor works as a template for HDR and allows the introduction of a stop codon: “TAG” for *BRCA1* and “TGA” for *BRCA2*. The recommended donor sequence for *BRCA1* c.763G > T (E255*) mutation was 5’-GATTTGAACACCACTGAGAAGCGTGCAGCT**t**agAGGCATCCAGAAAAGTATCAGGGTAGT-3’ and for *BRCA2* c.2133C > A (C711*) was 5’-TTTATTACCCCAGAAGCTGATTCTCTGTCAtg**a**CTCCAGGAAGGACAGTGTGAAAATGAT-3’.

Table 1
All guides RNA (gRNAs) designed by the CRISPR LIFEPIPE® tool and the Dharmacon CRISPR Design Tool.

Mutation	Rank	gRNA sequence	PAM	Cutoff site	Strand	CrisprScan score	GC %
BRCA1 763G > T	1	CTGAGAAGCGTGCAGCTGAG [43,094,766 – 43,094,785]	AGG	43094768	-	64	60
	2	CTCAGCTGCACGCTTCTCAG [43,094,766 – 43,094,785]	TGG	43094782	+	64	60
	3	AACTTGCATGTGGAGCCATG [43,094,710 – 43,094,729]	TGG	43094712	-	50	50
	4	GCATGAGTATTTGTGCCACA [43,094,692 – 43,094,711]	TGG	43094708	+	39	45
	5	GTTCTCATGCTGTAATGAGC [43,094,670 – 43,094,689]	TGG	43094686	+	16	45
BRCA2 2133C > A	1	TGATTCTCTGTCATGCCTGC [32,336,473 – 32,336,492]	AGG	32336489	+	25	50
	2	TCTCTGTCATGCCTGCAGGA [32,336,477 – 32,336,496]	AGG	32336493	+	31	55
	3	CATGACAGAGAATCAGCTTC [32,336,468 – 32,336,487]	TGG	32336470	-	27	45
	4	ATGACAGAGAATCAGCTTCT [32,336,467 – 32,336,486]	GGG	32336469	-	44	40
	5	TGACAGAGAATCAGCTTCTG [32,336,466 – 32,336,485]	GGG	32336468	-	53	45

For each selected on-target mutation, 5 sequences of gRNAs are proposed with their PAM sequences, cutoff sites, DNA strand, and GC content. The CrisprScan Score are also given for each gRNA. This score is correlated with the gRNA activity (25) and goes from 0 to 100.

To verify the efficiency of our CRISPR/Cas9 RNP and its capacity to induce the desired on-target mutations, we conducted a ddPCR analysis. Allelic frequencies were reported in Table 2. In all tested cell lines, the on-target *BRCA* mutations were found for transfected cells with the CRISPR/Cas9 RNPs and not for untransfected cells (WT) and gene editing controls cells (controls). Regarding MCF7 cells (not tested on PDAC cell lines), we tested the four sgRNA alone and in duo. The combination of two sgRNAs for the CRISPR/Cas9 RNP-assembly shows greater efficiency than sgRNAs alone (> 90% *vs.* < 90%). For both mutations, transfection is more effective in MCF7 and T3M4 cells than in Capan-2 cells (up to 90%,

between 50 and 80%, and about 20%, respectively). To note the experiments were carried out on the pool of transfected cells and not on clones. A cloning by limiting dilution for the three cell lines and for both mutations was attempted but failed. Indeed, after two months of culture in regular growth medium, no *BRCA1* or *BRCA2*-mutated clones survived. Moreover, the pool of transfected cells must be used up to passage 3 maximum because the level of *BRCA* mutated cells decreases by approximately 25% with each passage (Figure 1). The same phenomenon was observed with freezing; less than 4% of transfected cells carried one of the targeted *BRCA* mutation after thawing (table 2). No experiment carried out on the Capan-2 cells due to a lower cell transfection percentage. Besides all cells keep the *BRCA2* mutation longer than the *BRCA1* mutation. Hence for each experiment, a new CRISPR/Cas9 RNP system was assembled and transfected into all cell lines to ensure an elevated percentage of mutated cells and a maximal reproducibility.

Table 2
Allelic frequencies (AF) obtained by ddPCR analysis.

Cell line	gRNA	AF after transfection (%)	AF after thawing (%)
MCF7	*BRCA1* gRNA n°1	59.40 ± 5.38	/
	BRCA1 gRNA n°2	73.20 ± 2.14	/
	BRCA1 gRNA n°1 + n°2	90.68 ± 0,73	4.60 ± 0.42
	BRCA2 gRNA n°1	77.78 ± 2.64	/
	BRCA2 gRNA n°2	82.38 ± 1.68	/
	BRCA2 gRNA n°1 + n°2	96.58 ± 0.38	3.65 ± 0.21
Capan-2	*BRCA1* gRNA n°1 + n°2	24.85 ± 0.49	0.14 ± 0.01
	BRCA2 gRNA n°1 + n°2	19.85 ± 2.38	0.5 ± 0.02
T3M4	*BRCA1* gRNA n°1 + n°2	54.80 ± 0.57	0.98 ± 0.34
	BRCA2 gRNA n°1 + n°2	77.85 ± 1.63	3.04 ± 0.14

AF are given for each pool of cells just after transfection by each CRISPR/Cas9 RNP designed and after one freeze-thaw cycle.

Off-target prediction of BRCA KD cell lines.

Potential off-target sites were first predicted by the CRISPR LIFEPIPE® tool during the design of sgRNA and results were confirmed by the CrispRGold tool. No off-target sites were predicted with the CRISPR LIFEPIPE® tool for all sgRNAs designed. In contrast, CrispRGold predicted 3 potential off-target sites for the both sgRNAs for the mutation *BRCA1* G763T (see Additional file 1: Table S1). Only 2 potential off-target sites were identified for the first sgRNA of the mutation *BRCA2* c.(2133C > A) p.(Cys711*) but 13 for the second sgRNAs of the mutation *BRCA2* c.(2133C > A) p.(Cys711*). All potential off-targets were intronic or intergenic. The specificity score of sgRNAs was on average of 6 whereas a specificity score of 12 is

necessary to undoubtedly avoid off-target sites. However, no sgRNAs with a specificity score up or equal at 12 can be established for the two desired on-target mutations.

Increased sensitivity of BRCA KD cell lines to olaparib.

The sensitivities of *BRCA1* and *BRCA2* KD cells relative to control and WT cells to olaparib were analyzed with a crystal violet assay. Increasing doses of olaparib were tested and viability was evaluated 96 hours later. DMSO at the concentrations used for olaparib dilutions had no impact on cell viability. Olaparib IC50 were regrouped in Table 3 and Fig. 2. No difference was observed between WT and control cells for all cell lines ($P > 0.05$). Induction of *BRCA1* c.763G > T p.(Glu255*) mutation and *BRCA2* c.(2133C > A) p.(Cys711*) mutation in MCF7 cells increased the cells sensitivity to olaparib. The olaparib IC50 was reduced from 31.73 ± 5.53 µM to 11.12 ± 4.28 µM for MCF7 *BRCA1* KD cells ($P < 0.01$) and 5.92 ± 3.65 µM for MCF7 *BRCA2* KD cells ($P < 0.01$). All *BRCA* KD PDAC cells were also significantly most sensitive to olaparib ($P < 0.01$) than WT *BRCA* cell lines. Among non-modified cell lines, Capan-2 cells were the most resistant to olaparib, whereas T3M4 cells showed greater sensitivity. In contrast, Capan-2 cells harboring *BRCA1* and *BRCA2* mutations were 3 times more sensitive to olaparib than WT cells, while T3M4 cells harboring *BRCA1* and *BRCA2* mutations presented an IC50 1,5 times smaller than WT cells.

Table 3
Calculated IC50 (µM) of olaparib for MCF7, Capan-2 and T3M4 cell lines.

Cell lines	**IC50 (µM)**		
	MCF7	**Capan-2**	**T3M4**
WT	31.73 ± 5.53	> 50	13.67 ± 0.29
Control	28.29 ± 5.65 ns	> 50 ns	12.74 ± 1.24 ns
BRCA1 KD	11.12 ± 4.28 **	14.19 ± 1.58 ***	9.04 ± 0.42 **
BRCA2 KD	5.92 ± 3.65 **	17.51 ± 1.36 ***	8.26 ± 1.12 ***

Data are represented as the mean values and standard derivation (SD) of three independent experiments for wild-type (WT) cells, control cells, *BRCA1* knockdown (KD) cells, and *BRCA2* KD cells. *** $P < 0.001$, ** $P < 0.01$, and ns not significant (ANOVA).

Increased apoptosis after treatment by olaparib for BRCA-KD cells.

The sensitivity of *BRCA* KD cells to olaparib was investigated by detecting apoptotic cells labeled with Propidium Iodide (PI) and Annexin V. Cells were treated during 72h with olaparib at 40 µM, a dose above the IC50 of the 3 models. All *BRCA* KD cells shown an increased apoptosis after treatment by olaparib at 40 µM concentration compared to WT cells (Table 4 and Fig. 3). No difference was observed in the percentage of apoptotic cells after treatment with 0 µM and 40 µM of olaparib concentration for WT and control cells ($P > 0.05$).

Table 4
Percentage of apoptotic cells of MCF7, Capan-2 and T3M4 cell lines.

Cell lines	Apoptotic cells (%)					
	MCF7		Capan-2		T3M4	
Concentration of olaparib (µM)	0	40	0	40	0	40
WT	8.76 ± 1.66	9.76 ± 1.23 ns	9.09 ± 2.06	9.97 ± 1.98 ns	9.15 ± 0.91	12.49 ± 1.869 ns
Control	17.36 ± 0.66	15.92 ± 2.96 ns	5.72 ± 0.45	11.25 ± 2.08 ns	6.57 ± 2.70	13.33 ± 0.88 ns
BRCA KD	7.58 ± 1.02	12.81 ± 0.37 **	7.20 ± 1.26	17.63 ± 0.46 **	6.65 ± 1.27	16.00 ± 1.28 **

Population positive for annexin V-FITC and both for annexin V-FITC and propidium iodide was gated for apoptosis analysis after 72h treatment with olaparib at concentrations 0 and 40 µM concentration. At least 1.10^4 cells/event were evaluated for each analysis. Data are represented as the mean values and standard error of mean (SEM) of three independent experiments. ** $P < 0.01$, and ns not significant (Student's *t*-test).

Discussion

The PARPi olaparib is widely used for the treatment of patients with breast cancer (26) *and its efficacy has been demonstrated on breast cancer cell line such as MCF7* (27). Thus, the MCF7 breast cancer cell-line was used as control. *Pathogenic or deleterious mutations in BRCA1 or BRCA2 are good response predictors to olaparib. However, only 4 to 7% of patients with pancreatic cancer harbor a germline BRCA mutation* (22), *which ultimately represents few patients. In this study, we were able to successfully knockdown BRCA1 or BRCA2 using the CRISPR/Cas9-mediated KI technology in two PDAC cell lines (Capan-2 and T3M4). We first optimized and controlled the effective delivery of our CRISPR/Cas9 system in breast cancer MCF7 cells, this technology being already used on these cells* (28) *and as expected the olaparib sensitivity was higher for MCF7 BRCA KD cells compared to MCF7 WT cells.*

Our CRISPR/Cas9 system is an all-in-one HDR complex, comprising Cas9-RNP (recombinant Cas9 protein complexed with two sgRNAs transcribed in vitro) and donor template DNA (ssODNs) with phosphorothioate modifications at extremities. This all-in-one strategy increases the HDR efficiency due to the presence of donor template at the time of DSB generation (29). *Utilization of ssODNs for donor template with phosphorothioate modifications at extremities also improves the efficiency of HDR. The design of our ssDNA donors proposed by tools was symmetric but it has been shown that asymmetric donor design may favored for HDR* (30). *It would be interesting to experiment more if an asymmetric donor design can improve HDR efficiency in this study. The donor design is thus critical for the success of CRISPR/Cas9 experiment. For our all sgRNAs, the DSBs induced by Cas9 and the donor template insertion-site are separated by less than ten nucleotides and all presented a PAM motif, as recommended* (31). *The*

nature of nucleotides at position -4 from the PAM sequence also influences the editing precision. In our study, the two crRNAs for the BRCA1 mutation harbored a "T" in position -4 and for the BRCA2 mutation a "C" and "A" and the presence of a "T" or "A" at position -4 predict efficient insertions at the regions of interest. In contrast, a "G" is synonymous with a more imprecise target (32)*, which concerns none of our sgRNAs. Some chemicals as nocodazole or ABT751 allowing cell cycle synchronization can be used to increase HDR, which is restricted to the late S and G2 phases. However, these molecules have a potential toxicity in vivo* (29)*. Hence, we chose not to use them during the CRISPR/Cas9-system optimization on cell lines in order to transpose the protocol more quickly to in vivo.*

The number of cells with the on-target mutations is overall higher for T3M4 cells than for Capan-2 cells (> 50% and < 50% respectively) despite an identical CRISPR/Cas9 system for all cell lines. Besides the transfection capacity specific to each cell type, this difference can be explained by the fact that HDR activity is restricted to late S and G2 phases of cell cycle. Indeed, the Capan-2 cell doubling time is three times longer than the T3M4 cell doubling time, thus cell cycles were staggered and not at the same phase at the CRISPR/Cas9 transfection-time. Furthermore, T3M4 cells harbor the TP53 mutation c.215C>G p.Pro72Arg. TP53 plays a significant role in the cell cycle checkpoint control of G1/S phases and the Arg72 form induces lower G1 arrest than the Pro72 form (33)*, which can also explain the difference observed between Capan-2 and T3M4 cells. In an ideal setting to increase the transfection efficiency, the CRISPR/Cas9 transfection and preparation protocol should be developed and adapted for each cell lines, but we chose to use only one to simplify and attempt to do so universalize.*

We observed a decrease of BRCA mutated cells according with cells passaging or after thawing for all models. On one side, cells which were not transfected with the CRISPR/Cas9 survived more longer than BRCA KD cells, due to the consequences of genetic instability generated by the loss of BRCA1/2 (blocking cell proliferation or apoptosis) (34)*. This finally leads to a pool population only composed of no BRCA KD clones. Another phenomenon has also been observed in vivo inducing resistance to treatment: patients may present a BRCA recovery upon progression while at diagnosis they owned a somatic disruption of BRCA1/2* (35)*. Chemotherapy regimens composed of DNA damaging agents or PARPi can also lead to secondary mutations restoring BRCA1/2 or induce a selective pressure on BRCA1/2 restored cells* (36)*. These kinds of reversion mutations could be prevented by a pharmacological inhibition of the DNA end-joining repair pathways, as suggested by Tobalina et al.* (37)*, although drug development is still ongoing* (38)*. Therefore, the CRISPR/Cas9 transfection can be optimized to obtain better clonal evolution with 100% of cells exhibiting a biallelic deletion of BRCA1/2 and resulting in a total-loss-of-function mutation before treatment with PARPi. On the other side, the non-persistence of BRCA mutations over time or after thawing of our cells may occur due to a transient expression of our RNP complex, a DNA-free CRISPR/Cas9 mediated gene editing without genome integration. Utilization of plasmid as delivery method for CRISPR/Cas9 might allow to override this phenomenon due to a better stability. However, transfection of plasmid often leads to random genome integration and host immunogenic activation in vivo, and generate potentially more off-target effects than RNP complex due to a persistent expression of Cas9 in cells* (39).

The main limitation of CRISPR/Cas9 technology is the high probability of off-target effects with a frequency > 50% (40)*. However, reducing off-targets effects while maintaining editing efficacy remains a challenge. Several strategies for limiting off-targets effects are now proposed, such as optimization of Cas9, sgRNAs, and guides designs* (41)*. CRISPR/Cas9 design tools make it possible to obtain the best designs and can highlight hypothetical off-target sites. In our study, no off-target effect was predicted with the* CRISPR LIFEPIPE® tool. In contrast, the CrispRGold tool predicted various off-target effects for each sgRNAs but mainly are intergenic or intronic. In cultured cells, this category of off-targets are not to be taken into account, at least for the functional studies (42). Utilization of RNP complex for the delivery of Cas9 protein and gRNAs, as in our study, appears to be an excellent delivery method for the CRISPR/Cas9 system with decreased off-target effects and high on-target mutations (40,41).

Olaparib, a PARPi, is known to be effective on cancer cells which presented a deficiency in HRR, including BRCA mutated cells. Indeed, PARPi induce accumulation of SSBs which lead to DSBs, resulting to an accumulation of DNA damage in HRD tumors, and therefore tumor-cell death (43)*. Therapeutic efficacy of olaparib has now been proven as maintenance treatment in metastatic pancreatic cancer which harbored a germline BRCA mutation* (8)*. As expected, in our study, all BRCA KD cell lines presented an increased sensitivity at olaparib. IC50 values of BRCA KD cells were significantly lower than IC50 values of WT cells ($P < 0.01$), especially for the two PDAC cell lines. Our in vitro results are also in agreement with clinical results. Apoptosis analysis results of the present study have shown an increased apoptosis of pancreatic cells after treatment with 40µM of olaparib, and these differences were found to be statistically significant for BRCA KD cells ($P < 0.01$). In fact olaparib is known to induce apoptosis of cancer cells* (44,45)*. In this respect, our in vitro results suggest that BRCA KD cells may have clinically relevant response to olaparib treatment.*

Conclusions

In conclusion, we developed a CRISPR/Cas9-mediated KI technology to create PDAC cell lines bearing a premature termination codon in BRCA1 or BRCA2 gene. The cell lines-BRCA KD were more sensitive to olaparib than cell lines-BRCA WT. In addition, the Cas9-RNP used has short-term activity after transfection into cells due to a rapid degradation by cellular proteases, allowing a reduced number of off-target effects and a transient genome editing. Therefore, this system might be easily transposed in vivo. Thanks to the promise of restoring a PARPi sensitivity mutation, this technology will offer an attractive therapeutic alternative for pancreatic cancer.

The next step in this work is the in vivo translatability, consider future clinical application. The vectorization in a maximum of nucleus and the addressing only in the pancreas despite a systemic introduction are the main limitations to the use of CRISPR/Cas9 technology in vivo. The delivery vehicle must be stable and versatile to allow a transient expression of the editing machinery and avoid unintentional off-target mutation within the host genome (46)*. Immunogenicity and dose response must be taken into account to achieve a maximal HDR efficiency and a minimal cytotoxicity without immune response* (47)*. The Cas9-RNP can circumvent these problems, but its direct delivery simultaneously with the donor DNA remains a*

challenge in vivo. Non-viral vehicles seem to be the most suitable format for the delivering of the Cas9-RNP together with the donor DNA (46)*. Among potential vehicles, nanoparticles such as the CRISPR-Gold system are good candidates for the delivery of CRISPR/Cas9 machinery. Their efficacy and safety of use in vivo have already been demonstrated* (47–49)*. Identifying off-target effects at the genomic level remains the last challenge before the clinical use of the CRISPR/Cas9 system. Different off-target detecting methods exist, but GUIDE-seq appears to be the best genome-wide detection assay* (50)*. All these recommendations will thus allow a future clinal transposition of our CRISPR/Cas9 system.*

Abbreviations

CRISPR/Cas9 Clustered regularly interspaced short palindromic repeats/associated protein 9

crRNAs CRISPR RNAs

ddPCR Droplet digital PCR

gRNAs guides RNA

HDR Homology directed repair

HR Homologous recombination

HRD Homologous recombination deficiency

HRR Homologous recombination repair

KI Knock-in

KD Knockdown

PARP Poly (ADP-ribose) polymerase

PARPi PARP inhibitor

PDAC Pancreatic ductal adenocarcinoma

PI Propidium iodide

RNP Ribonucleoprotein

sgRNAs single-guide RNAs

SSBs single-stranded breaks

ssDNA single-stranded DNA

ssODNs single-stranded oligodeoxynucleotides

tracrRNA trans-activating CRISPR RNA

WT Wild-type

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

No funding.

Authors' contributions

AW, MH, PG and AH contributed to the experiment design and the manuscript draft. AW, JDA, MR, JDE and AF performed the experiments. AW and JDA analyzed the data and conducted the statistical analysis. AW wrote the manuscript. JLM and AH revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

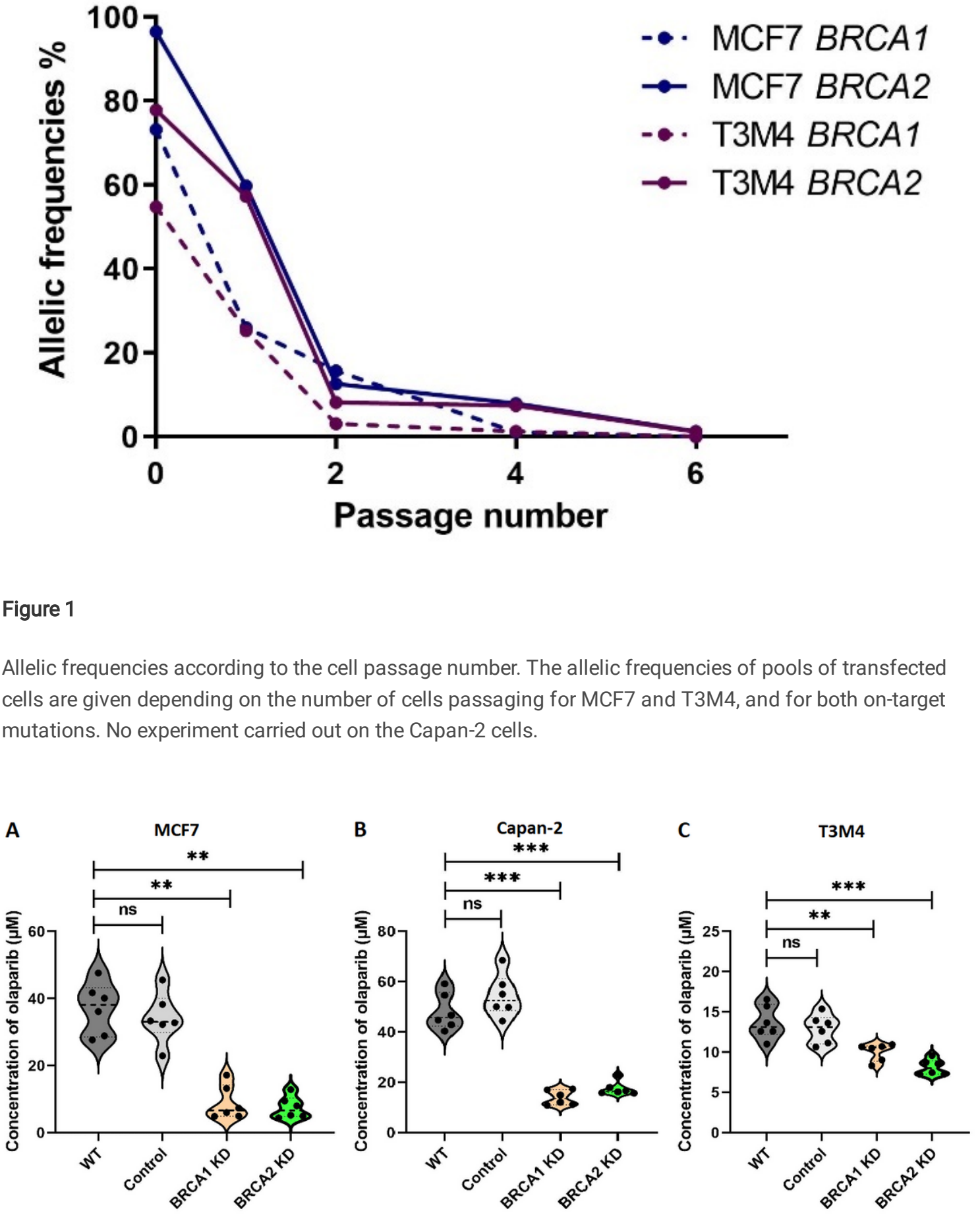

Figure 1

Allelic frequencies according to the cell passage number. The allelic frequencies of pools of transfected cells are given depending on the number of cells passaging for MCF7 and T3M4, and for both on-target mutations. No experiment carried out on the Capan-2 cells.

Figure 2

Cytotoxicities of Olaparib. Violin plots of IC50 (μM) for non-transfected cell lines (wild-type (WT) cells), transfected cell lines with a CRISPR/Cas9 complex without donor sequences (control) and transfected cell lines with a CRISPR/Cas9 RNP and donors (BRCA1 or BRCA2 knockdown (KD) cells), for MCF7 cell line (A), Capan-2 cell line (B) and T3M4 cell line (C). *** $P < 0.001$, ** $P < 0.01$, and ns not significant (ANOVA).

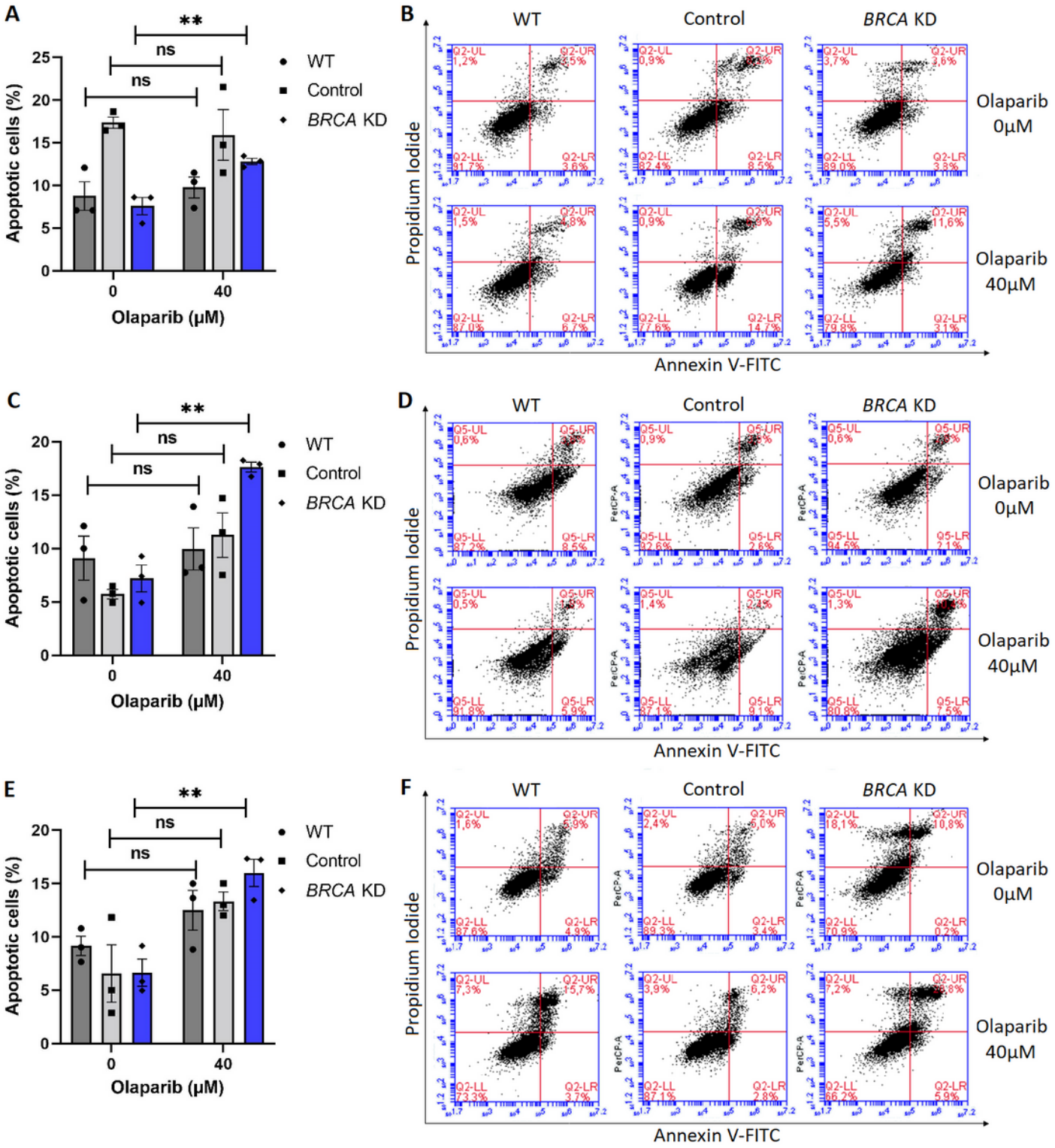

Figure 3

Effect of olaparib on apoptosis of MCF7 (A-B), Capan-2 (C-D), and T3M4 (E-F) cells. Wild-type (WT) cells, control cells, and BRCA knockdown (KD) cells were treated during 72h with olaparib at concentrations 0 and 40 µM. Apoptotic cells were detected through annexin V-FITC(AV)/propidium iodide (PI) staining and analyzed by FACS. At least 1.10^4 cells/event were evaluated for each analysis. (A-C-E) Histograms depict the percentage of apoptotic cells. Data are represented as the mean values and SEM of three independent experiments. ** $P < 0.01$, and ns not significant (Student's t-test). (B-D-F) Dot plots of one representative experiment divided into four quadrants indicate necrotic (UL: AV-/PI+), late apoptotic (UR: AV+/PI+), early apoptotic (LR: AV+/PI-) and viable (LL: AV-/PI-) cells.

Supplementary Files

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