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Inhibition of MUS81 by siRNA Reverses Resistance to Cisplatin in Human Ovarian Cancer Cell Lines.

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Running title:
Overcoming cisplatin resistance in ovarian cancer.

Key words: Ovarian cancer, drug resistance, interstrand cross link repair, MUS81 gene.

Abstract

Purpose: Drugs that induce DNA interstrand crosslinks form the mainstay of anticancer treatments for different cancers. These drugs are used to treat ovarian cancer which is the most prevalent gynaecological cancer. Five-year survival rates are approximately 40% and the development of drug resistant disease is an important factor in treatment failure.

Methods: In this study a comprehensive evaluation of the expression and function of the site-specific endonuclease MUS81 was conducted. Using quantitative real time PCR analysis and imaging flow cytometry we determined the mRNA and protein expression of MUS81 in three ovarian cancer cell lines and two immortalised human fibroblast cell lines which had been made resistant to cisplatin by chronic exposure. siRNA knockdown of MUS81 was employed to determine the effect on overall cell survival which was assessed using clonogenic assays.

Results: In the five cisplatin-resistant cell lines we observed increased MUS81 mRNA expression. In addition MUS81 protein expression in the form of discrete nuclear foci in cells was observed in all cell lines following cisplatin exposure, there being significantly more foci in cisplatin resistant cell lines. siRNA knockdown of MUS81 significantly reduced both mRNA and protein levels in two cell lines (SK-OV-3 and MRC5-SV1 – wild-type and resistant) and critically re-sensitised cisplatin resistant cells to wild-type level, determined by clonogenic assay.

Conclusion: MUS81 is central to the development of cisplatin resistance in ovarian cancer cell lines. Inhibition of MUS81 restored drug sensitivity to the cells. MUS81 may be a useful therapeutic target to overcome drug resistance in ovarian and other cancers.

Introduction

Chemotherapeutic drugs that introduce DNA interstrand crosslinks (ICLs) are central to the cytotoxicity induced in many chemotherapeutic regimens for the treatment of cancer. In fact this class of bifunctional alkylating agents are the mainstay of many successful anticancer treatments (Huang and Li, 2013). Indeed, the persistence of ICLs in the DNA of cancer cells following drug exposure is directly related to the cytotoxicity (cell killing) efficiency of ICL-mediated chemotherapy drugs (Palom *et al*., 2002, Clingen *et al*., 2007). ICLs are lesions that are composed of a covalent linkage between opposite strands of the DNA double helix. They result in DNA strand distortion whereby the ICL prevents strand dissociation thus inhibiting DNA synthesis, replication and transcription ultimately leading to cell death (Deans and West, 2011). ICLs are moreover a challenging lesion for the cellular DNA repair machinery to resolve and require the coordinated interaction of distinct DNA repair mechanisms (Hashimoto *et* al., 2016). While the precise mechanism of ICL repair is still to be fully elucidated, a fundamental understanding of the mechanism together with essential molecular components has been well-described. In brief, following the creation of an ICL by drug exposure and the resultant DNA strand distortion, proteins of the Fanconi Anaemia (FA) pathway are recruited to the lesion to conduct the initial incision events leading to ICL removal. Here the Fanconi-associated nuclease 1 (FAN1) with its inherent 5'-3' exonuclease activity and 5'FLAP endonuclease activity cleaves the ICL in a process called unhooking. As a result, the stalled replication fork caused by the initial ICL is converted into a DNA double strand break. Following the initial incision activity of the FAN1 nuclease other endonuclease which include MUS81-EME1 and the XPF-ERCC1 protein cut DNA on the 3' side of the ICL. The result here is a double strand break with potential loss of genetic information which if repaired by non-homologous end joining would potentially lead to frameshift insertion and deletion (INDEL) mutations. However, in cycling cells in the G2 and S phases, the lesion becomes substrate for repair by homologous recombination where a Holliday junction is created mediated by the Rad 51 protein. Following

strand resolution by the action of resolvases, the DNA is repaired in an error free manner (Deans and West, 2011; Huang and Li, 2013, Hashimoto *et al*., 2016).

Inherited defects in ICL repair factors are linked to human disorders associated with hypersensitivity to DNA damaging agents including chemotherapeutic drugs which induce ICLs, and also increased cancer incidence such as FA and Xeroderma Pigmentosum (XP). There are some 17 components of the FA group of proteins where clinical and cellular hypersensitivity to DNA damaging agents may be displayed to a greater of lesser extent (Palovcak *et al.*, 2017). In addition, there are seven complementation groups of XP whose proteins mediate nucleotide excision repair of large bulky adducts such as ultraviolet radiation induced dimer photoproducts and also ICLs (Knoch *et al*., 2012).

The development of resistance to anticancer chemotherapy can limit the effectiveness of treatment for many human adult solid cancers (*e.g.,* Carvalho *et al*., 2010). In many cases tumours intially respond well to chemotherapy treatment but can subsequently relapse with drug resistant disease. The mechanisms whereby the cancer cells acquire resistance can occur by multiple mechanisms and include, for example, increased drug efflux due to overexperssion of p-glycoprotein leading to multiple drug resistance (Wang *et al*., 2017); elevated damage tolerance following drug exposure (Johnson *et al*., 1997) and elevated expression of key DNA repair genes leading to increased repair of DNA damage following drug exposure (Adam-Zahir *et al*., 2014).

The development of cytotoxic chemotherapy drug resistance is of central importance in the clinical management of ovarian cancer. Ovarian cancer is the most prevalent gynacological malignancy in females with more than 7000 cases diagnosed in the United Kingdom each year (Cancer Research UK, 2020). Five year survival rates for stage 1 ovarian cancer approaches 90% but for stage 4 metastatic disease, this reduces to 5% (Altman *et al*., 2017, Cancer Research UK website, 2021). A mainstay for the treatment of late stage ovarian cancer is platimum-based cytotoxic chemotherapy where the introduction of DNA ICLs mediates the cancer cell kill. While this form of treatment provides good initial responses to treatment, patients often relapse with drug-resistant disease (Vaughan *et al*., 2011). There is an increasing body of evidence that elevated repair of key

DNA repair components during the DNA repair of ICLs at the homologous recombination site is central to the development of resistance to cytotoxic drugs which induce ICLs during ovarian cancer therapy. In fact in our previous publications we have demonstrated *in vitro* elevated expression of Rad 51 in ovarian cancer cell lines resistant to both cisplatin and nitrogen mustard. Rad 51 is a critical protein during HR repair of ICLs where it promotes an homology search aligning the homologus sister chromatid at the site of the strand break following incision of the ICL. Also we demonstrated increased γ-H2AX foci induction in cisplatin resistant cells indicating increased DNA strand break presumably related to the action of endonuclease factors during the excision of crosslinks (Adam-Zahir 2014). In addition it was demonstrated some 20 years ago that *in vitro* resistance to cisplatin in ovarian cancer cell lines was associated with elevated nucleotide excision repair, specifically enhanced activity of the XPF-ERCC1 site specific endonuclease (Ferry *et al*., 2000). Moreover, *in vivo* studies have demonstrated alterations in HR activity with increased expression of key HR proteins such as Rad51 in human ovarian cancer (e.g., Helleday, 2010).

In this present study we have examined the role of the MUS81 3' structure-specific endonuclease in the repair of ICLs in drug resistant ovarian cancer cell lines. As stated earlier, one of the integral proteins in the ICL repair process is MUS81. It associates with either EME1 or EME2, forming a 5' to 3'structure-specific endonuclease. MUS81 was first identified in fission yeast through its association with Cds1, a replication checkpoint kinase and in budding yeast where it associates with Rad54, a recombination repair protein (Boddy *et al*., 2000). It is involved in vital cellular processes such as meiosis (Agostinho *et al.*, 2013) and resolving recombination intermediates by cleaving nicked Holliday junctions (Chen *et al*, 2001). Also, Hanada *et al.*, 2007 found that stalled replication forks were unable to be recovered in the absence of MUS81; ultimately leading to the formation of chromosomal aberrations.

There is evidence that the function of MUS81 can mediate the response of cancer cell lines to anticancer drugs. For example, siRNA inhibition of MUS81 enhanced the sensitivity of breast cancer cell lines to the anticancer drug 5-fluorouracil (Qian *et al*., 2014). Also, MUS81 has been

shown to be associated with alterations in cell proliferation and cisplatin sensitivity in serous ovarian cancer *in vivo* (Xie *et al*., 2016). Finally, resistance to cisplatin in human hepatocellular carcinoma cell lines is increased by SiRNA knockdown of MUS81 (Wu *et al*., 2016)

Here we examine the role of MUS81 in resistance to cisplatin in five pairs of cell lines. Three pairs are derived from epithelial ovarian cancer in which a derivative of each wild-type cell line has been made resistant to cisplatin by chronic exposure to cisplatin (SK-OV-3, A2780 and PEA-1). Two pairs of the cell lines were derived from SV40 large-T antigen immortalised human diploid fibroblasts (MRC5-SV1 and NB1-hTERT) where again cisplatin resistance was developed by chronic drug exposure (Adam-Zahir *et al*., 2014). Increased MUS81 protein expression was observed in the cisplatin resistant derivative when compared to the sensitive wild-type counterpart. Elevated protein expression was detected in the form of MUS81-containing DNA damage foci during imaging flow cytometry (*e.g*., Parris *et al*., 2015). Moreover, this finding was corroborated by examination of mRNA levels using quantitative PCR. Here elevated levels of MUS81-specific mRNA were detected in the drug resistant cell lines compared to wild-type. Most importantly however, in two cell lines examined (SK-OV-3 and MRC5-SV1) siRNA inhibition of MUS81 resulted in a reversal of cisplatin resistance to wild-type levels.

It is concluded that elevated repair of ICLs during HR repair is an important mechanism whereby cancer cells (and in particular ovarian cancer) acquire resistance to anticancer crosslinking agents. This present study and together with previous investigations point to elevated expression of the MUS81 endonuclease contributing to drug resistance. We submit that MUS81 could provide a useful biochemical marker of induced drug resistance in ovarian (and other) cancers and may provide a target where selective inhibition of the endonuclease could reverse drug resistance in recurrent disease.

Materials and Methods

Cell Lines

Cell lines were purchased from the European Collection of Cell Cultures (ECACC) (Porton Down, Salisbury, Wiltshire, UK) or generated at Brunel University London (Uxbridge, Middlesex, UK). Cisplatin resistant cell lines were created by continuous exposure of the wild-type cell line to increasing doses of the *cis*-Diamine-dichloro-platinum (cisplatin). Details of the cell lines are listed in Table 1.

Table 1: Details of the cell lines used in this study

Cell Line	Cell Type	Origin	IC50 cisplatin concentration	Cell Culture Medium Type
SK-OV-3 Wild-type	Human Caucasian ovary adenocarcinoma	ECACC	6 µg/ml	RPMI-1640
SK-OV-3^{R}	Human Caucasian ovary adenocarcinoma	ECACC	23.50 µg/ml	RPMI-1640
PEA1 Wild-type	Human ovarian cancer; oestrogen receptor positive	ECACC	2 µg/ml	RPMI-1640
PEA1^{R}	Human ovarian cancer; oestrogen receptor positive	ECACC	4.50 µg/ml	RPMI-1640
A2780 Wild-type	Human ovarian carcinoma	ECACC	4.5 µg/ml	RPMI-1640
*A2780^{R}	Human ovarian carcinoma	ECACC	85.5 µg/ml	RPMI-1640
MRC5-SV1 Wild-type	Human foetal lung, SV40 transformed	ECACC	12 µg/ml	DMEM
MRC5-SV1^{R}	Human foetal lung, SV40 transformed	ECACC	25 µg/ml	DMEM
NB1-hTERT Wild-type	Human fibroblast, hTERT transformed	Brunel University London	13 µg/ml	DMEM
NB1-hTERT^{R}	Human fibroblast, hTERT transformed	Brunel University London	23 µg/ml	DMEM

*Resistant to cisplatin when purchased from ECACC.

Cell Culture

The cell lines were initially established in T25 or T75 cell culture flasks (Sarstedt, Leicester, UK) in RPMI 1640 or DMEM culture medium (Labtech International Ltd, East Sussex, UK) supplemented with 10% foetal bovine serum (Scientific Laboratory Supplies, Yorkshire, UK), 2.0 mM

L-Glutamine (Labtech International Ltd), 100 Uml^{-1} Penicillin and 100 μgml^{-1} Streptomycin (Labtech International Ltd). Cells were routinely incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell number and viability were determined as required using a Countess™ automated cell counter based upon the method of trypan blue exclusion (Invitrogen, Renfrewshire, UK). Cell lines were used over a restricted range of ten passages, during which cell viability was not less than 90% for any experiment.

Resistance to either cisplatin in the resistant derivative cell lines was maintained by exposing the cells while growing as monolayers at approximately 80% confluence, to the relevant drug for one passage every three passages.

Exposure of Cells to Chemotherapeutic Drugs

Cisplatin solutions (Sigma Aldrich Ltd, Gillingham, Dorset, UK) were prepared initially in dimethyl sulfoxide (Fisher Scientific, Loughborough, Leicestershire, UK) and final concentrations of drug solutions were prepared initially in the appropriate serum-free cell culture medium for the cell lines (Labtech International Ltd). Cisplatin was then further diluted in the relevant complete medium to the required concentrations.

Clonogenic Assay after Exposure of Cells to Chemotherapeutic Drugs

Approximately 500 000 cells were seeded into 5 cm dishes in 5 ml complete medium and incubated overnight. Cells were exposed to the drug solutions for 1 hour, after which the cells were washed in phosphate-buffered saline (PBS) solution (pH 7.4) (Severn Biotech Ltd, Worcestershire, UK) and then trypsinised with trypsin/EDTA solution (200 mg/L Versene [EDTA] with 170 000 U trypsin/L) (Lonza Biologics PLC, Slough, Berkshire, UK) for approximately 10 minutes at 37˚C. Cells were re-suspended in the appropriate complete medium, counted using a "Countess™" automated cell counter and seeded into three 10 cm tissue culture plates (Scientific Laboratory Supplies, Hessle, East Riding of Yorkshire, UK) for each drug dose. Seeding numbers of cells varied

between 2000 and 6000 cells per plate, depending on the drug concentration used and the cell cloning efficiency of each cell line.

Following cisplatin treatment cells were trypsinised, counted and reseeded into 10 cm dishes and allowed to form colonies over a 2-3 week period, whereupon colonies formed were fixed in room temperature industrial methylated spirits (Tennants Distribution Ltd, Manchester, UK), stained with methylene blue stain (ReAgent, Cheshire, UK) and allowed to air-dry. Colonies of 50 cells or more were counted. The average number of colonies from the three plates for that drug dose was then calculated and survival was expressed as a percentage of colonies grown in untreated control dishes.

MTT Assay after Exposure of Cells to Chemotherapeutic Drugs

The A2780 wild-type and cisplatin resistance derivative did not form discrete colonies that were easy to count in an accurate manner following a clonogenic assay. To determine cell viability after cisplatin exposure MTT assay was conducted on these cell lines instead. Cells were plated out into 96-well microplates at a density of 25 000 cells in 100 µL per well and exposed to cisplatin for 1 hour, then incubated (incubation conditions described previously) for 48 hours. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (50 mg) (Merck-Millipore Ltd, Watford, UK) was dissolved in 10 ml PBS and sterilised by filtration. 10 µL of this solution was added to each well containing cell suspension, and the contents mixed by gentle agitation of the plate, followed by incubation for 3 hours (incubation conditions detailed previously).
Colour development was achieved by the addition of 10 µL propan-2-ol with 0.04 N HCl and well contents were mixed by retro-pipetting. A Biotek® Instruments Inc. ELx808™ absorbance microplate reader (Biotek® Instruments Inc., Vermont, USA) was used to obtain absorbance values of each well at a test wavelength of 562 nm and a reference wavelength of 630 nm.

Cell Fixation for Immunocytochemistry and Imaging Flow Cytometry after Cisplatin Exposure

After cisplatin treatment for 1 hour, cells were washed with PBS (Severn Biotech Ltd) and trypsinised into suspension as described earlier. Cells were then washed in ice-cold PBS, pelleted by centrifugation, fixed in ice-cold methanol/acetone mixture (50% v/v) (Fisher Scientific) and stored at −20°C until the immunocytochemistry stage.

Cells were fixed at 6, 12, 24, 30, 48 and 72 hours post-cisplatin exposure. The control samples (not treated with drug) were fixed at the 6 hour time-point, and the compensation samples were fixed at the 30 hour time-point.

Identification of MUS81 foci Exposure of Cells to Chemotherapeutic Drugs

Cells were washed once in ice-cold PBS (Severn Biotech Ltd) then incubated with gentle agitation for 5 minutes at room temperature in PBS, followed by incubation with gentle agitation for 5 minutes at room temperature in permeabilisation buffer consisting of 0.5% Triton™ X-100 (Sigma-Aldrich) in PBS. Cells were then incubated with gentle agitation for 1 hour at room temperature in blocking buffer consisting of 10.0% rabbit serum (Labtech International Ltd) with 0.1% Triton™ X-100 in PBS. The blocking buffer was removed and the cells incubated with gentle agitation overnight at 4°C in primary antibody solution. The primary antibody solution consisted of an anti-MUS81 mouse monoclonal IgG2a antibody, clone MTA30 2G10/3 (Abcam, Cambridge, UK) diluted 1 in 1000 in blocking buffer. Primary antibody was removed by washing three times with wash buffer, consisting of 0.1% Triton™ X-100 in PBS. The secondary antibody solution consisted of an Alexa Fluor™488 rabbit anti-mouse IgG antibody (Invitrogen) diluted 1 in 1000 in blocking buffer. This was added to each sample, except the DRAQ5™ compensation samples, and incubated with gentle agitation for 1 hour at room temperature. During this incubation samples were protected from light by wrapping in aluminium foil. Secondary antibody was removed by washing three times with wash buffer. The cells were re-suspended in 100 µL Accumax™ solution (Global Cell Solutions, Virginia, USA) and left overnight at 4°C (no agitation). 1.0 µL of 5 mM DRAQ5™ solution (Biostatus Ltd, Leicestershire, UK)

was added to each sample, except the AlexaFluor®488 compensation samples. The samples were submitted for analysis by imaging flow cytometry.

Imaging Flow Cytometry after Exposure of Cells to Chemotherapeutic Drugs

Imaging flow cytometry was undertaken using the Imagestream^X Mark II system equipped with INSPIRE™ data acquisition software (Luminex Instruments, Thermofisher Ltd, UK) as previously described in Bourton *et al*., 2012 and Parris *et al*., 2015.

Image Compensation

Image compensation is described in detail previously (*e.g*., Bourton *et al.*, 2012; Parris *et al*., 2015). In brief, it was performed on the cell populations fixed 30 hours post-drug exposure where MUS81 protein expression, and hence staining intensity, was highest. Images of cells stained with either antibody or DRAQ5™ were collected with the 488 nm laser only and were used to generate the compensation matrix. The software compensation wizard generated a table of coefficients whereby detected light displayed by each image was placed into the proper channel (channel 2 for antibody staining and channel 5 for DRAQ5) on a pixel-by-pixel basis. The coefficients were normalised to 1 and each coefficient represented the leakage of fluorescent signal into juxtaposed channels. Calculated compensation values were applied to all subsequent analyses as appropriate.

Analysis of Cell Images - Calculation of Foci Number

Foci indicating the presence of the MUS81 protein were quantified in approximately 10,000 (1000 for the compensation samples) images of cells per time point captured using the INSPIRE™ imaging flow cytometry software. Foci were quantified in a similar manner as described previously (Parris *et al*., 2015), with the spot counting wizard in the IDEAS™ software permitting simplified foci quantitation. The wizard consists of sequential steps that identified all captured images of single cells that were in optical focus. Next, images of fifty cells from this population with varying numbers of clearly defined foci were selected to create a truth population. Images from the truth population

were used to produce a mask that identified only those foci located within the cell nuclei of each image. The finalised truth population was then saved as a template and applied to quantitate foci present in all acquired cell images.

Exposure of Cells to Chemotherapeutic Drugs and Cell Fixation for Quantitative Real-time PCR Analysis

Approximately 1×10^6 cells were seeded into 10 cm dishes in 10 ml complete medium and incubated overnight (incubation conditions described previously). Cells were exposed to cisplatin for 1 hour, after which the cells were trypsinised and counted as previously described. Cell pellets of approximately $1 \times 10^6 – 5 \times 10^6$ cells were snap-frozen by exposure to liquid nitrogen for 5 minutes and stored at −80°C. Cells were frozen at 24, 30 and 72 hours post-drug exposure. The control samples (not treated with drug) were fixed at the 24 hour time-point.

RNA Extraction from the Cells Fixed for Quantitative Real-time PCR Analysis

RNA was extracted from the frozen cell pellets using a GenElute™ Mammalian Total RNA Miniprep Kit and On-Column DNase I Digestion Set (Sigma-Aldrich), as per the manufacturer's instructions. In brief, cells were lysed, and RNase inactivated with the application of 250 µL lysis buffer/2-mercaptoethanol solution. Cell debris was removed by passing the lysed cells through a filtration column, and the resulting lysate was diluted 1:1 (v:v) with 70% ethanol (Fisher Scientific) in diethyl pyrocarbonate (DEPC)-treated water (Sigma-Aldrich). The diluted lysate was passed through a nucleic acid binding column and the bound RNA and DNA washed with proprietary wash buffer. Any DNA present on the column was degraded with DNase treatment, and the column washed three times with proprietary wash buffers. The RNA was eluted from the column and the resulting solution containing RNA stored on ice.

Verification of RNA Quality using Spectrophotometry & Agarose Gel Electrophoresis (qPCR)

The quality and quantity of the RNA was measured using a NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, Hertfordshire, UK). Quality was determined using the 260/280 value, and quantity was measured in nanograms/microlitre.
The integrity of the RNA was verified by gel electrophoresis using a 2% agarose gel.

Production of cDNA from RNA (q-PCR)

All the following reagents were supplied by Invitrogen: random hexamer primers; dNTP mix; first strand buffer; DTT; SuperScript® III reverse transcriptase. DEPC-treated water was supplied by Sigma-Aldrich and RNase Away® reagent by Life Technologies.
The RNA solutions were diluted with DEPC-treated water so that the reaction mixture contained at least 1 µg RNA. The reaction mixture for each RNA sample consisted of the following: 5 µl RNA solution containing at least 1 µg RNA; 1 µl random primers; 1 µl 10 mM dNTP mix; 6 µl DEPC-treated water. This mixture was incubated at 70°C for 10 minutes and the following reagents added to each sample: 4 µl first strand buffer; 2 µl 0.1 M DTT; 0.5 µl RNase Away® solution; 0.5 µl 100 U SuperScript® III reverse transcriptase enzyme; 1 µl DEPC-treated water. The mixture was incubated at the following temperatures and times: 42°C for 50 minutes, then 70°C for 15 minutes, then 25°C for 5 minutes. cDNA concentration was determined using the NanoDrop™ 2000c spectrophotometer and the cDNA stored at −80°C.

Verification of cDNA Quality (q-PCR)

The quality of the cDNA was verified by PCR followed by agarose gel electrophoresis. The reagents used were: ReadyMix™ REDTaq® PCR reaction mix with $MgCl_2$ (Sigma-Aldrich); nuclease-free water (ThermoFisher Scientific); primer pair for the GAPDH gene at 5 µM concentration with the following base sequences: forward primer GAAGGTGAAGGTCGGAGTC; reverse primer GAAGATGGTGATGGGATTTC (Sigma-Aldrich). The integrity of the PCR products (and hence the quality of the cDNA) was verified by gel electrophoresis using a 2% agarose gel.

Determination of the Optimum Housekeeping gene for qPCR using the geNorm™ Kit

Two reference genes were identified using the geNorm™ Human Reference 12-Gene Selection Kit (Primerdesign Ltd, Eastleigh, Hampshire, UK) as per the manufacturer's instructions. The kit included primer sets for the following reference genes: TOP1; YWHAZ; EIF4A2; RPL13A; SDHA; ACTB; 18s; GAPDH; UBC; ATP5B; B2M; CYC1. Additional reagents used were: Fast SYBR® Green Master Mix (Applied Biosystems/Thermo Fisher Scientific); nuclease-free water (Severn Biotech Ltd); DEPC-treated water (Sigma-Aldrich). In brief, cDNA samples from cell lines SK-OV-3 (wild-type), cisplatin resistant SK-OV-3^{R}, wild-type MRC-5-SV1 and cisplatin resistant MRC-5 SV1^{R} were volumetrically adjusted with DEPC-treated water such that each had a concentration in the range 5 ng/µl - 10 ng/µl in a volume of at least 132 µl. A reaction mixture was created using the following: 1 µl proprietary primer mix; 10 µl Fast SYBR® Green Master Mix; 4 µl proprietary RNase/DNase-free water. This mixture was added to one well of a 96-well plate (Applied Biosystems/Thermo Fisher Scientific) and 5 µl of the diluted cDNA solution added. RNase/DNase-free water was used in place of cDNA solution for the control samples. The samples were prepared in triplicate and submitted for amplification to a QuantStudio 7 Flex real-time PCR instrument (Applied Biosystems/Thermo Fisher Scientific) using 40 cycles of the following conditions: enzyme activation (2 minutes at 95°C), then denaturation (5 seconds at 95°C), then data collection (20 seconds at 60°C) followed by a melt curve stage.

The resulting data was analysed with the qbase^{PLUS} software, which gave the average expression stability value for each reference gene. The two most stable reference genes with the least variation between cDNA samples were found to be YWHAZ and SDHA. Therefore, these two genes were used as internal reference standards for all subsequent q-PCR analyses.

Establishing the Percentage Efficiency of the MUS81, YWHAZ & SDHA Primers (QPCR)

The percentage efficiency of the MUS81, YWHAZ and SDHA primer pairs from the geNorm™ Human Reference 12-Gene Selection Kit (details as above) was determined as follows. The reagents

used were: primers for the MUS81 gene at 5 µM concentration with the following base sequences: forward primer ACGCAGGAGCCATCAAGA; reverse primer TGGCAGGGGTGCTGTATC (Sigma-Aldrich); primers for the YWHAZ and SDHA genes at 6 µM (sequences not supplied by manufacturer); Fast SYBR® Green Master Mix (details as above); nuclease-free water (details as above); cDNA sample made previously from the SK-OV-3 cell line (concentration 475.4 ng/µl). The cDNA sample was diluted with nuclease-free water to yield cDNA concentrations within the PCR reaction samples of: 10 ng/µl; 25 ng/µl; 50 ng/µl; 75 ng/µl; 100 ng/µl and 125 ng/µl. The reagents were combined in the following quantities to amplify one cDNA sample: 10 µl Fast SYBR® Green Master Mix; 8 µl nuclease-free water; 0.5 µl each of the MUS81 forward and reverse primer solutions or 1 µl (6 µM primer concentration) of the YWHAZ/SDHA primer solutions; 1 µl cDNA solution or nuclease-free water (control samples). Samples were prepared in triplicate in 96-well plates (as before) and PCR amplification performed using a qbase^{PLUS} Flex Real-Time PCR instrument using 40 cycles of the following conditions: enzyme activation (2 minutes at 95°C), then denaturation (5 seconds at 95°C), then data collection (20 seconds at 60°C). The resulting data was analysed using the qbase^{PLUS} Real-Time PCR software. This permitted the creation of standard curves for each primer pair where the mass of cDNA was plotted against the corresponding C_T value. The resultant slope of each graph approximated to a value of −3 and the R^2 value approximated to 1. From this the percentage efficiency values for each primer pair were calculated as follows: MUS81 89.38%; YWHAZ 104.91%; SDHA 95.66%. To conclude: the percentage efficiency values were sufficiently close to one another and approximate to 100% to permit the use of the primers in the QPCR method.

Determination of MUS81 Gene Expression using Reference Genes YWHAZ & SDHA (QPCR)

The expression of the MUS81 gene in each cell line was performed using the cDNA of each cell line (preparation details as above). The reagents used were: primers for the MUS81, YWHAZ and SDHA genes (details in the previous section); Fast SYBR® Green Master Mix (details in the previous section); nuclease-free water (details in the previous section). cDNA samples were diluted with

nuclease-free water such that each sample had a concentration range of 5 ng/µl to 10 ng/µl with a tolerance of 2 ng/µl between samples. The reagents were combined in the following quantities to amplify one cDNA sample: 10 µl Fast SYBR® Green Master Mix; 4 µl nuclease-free water; 0.5 µl each of the MUS81 forward and reverse primer solutions or 1 µl of the YWHAZ/SDHA primer solutions; 5 µl cDNA solution or nuclease-free water (control samples). Samples were prepared in triplicate in 96-well plates and amplification performed as described previously. The resulting data was analysed using the qbase^{PLUS} Real-Time PCR software, where the Relative Quantification (RQ) value was calculated for each sample.

Optimisation of Cell Density in 96-well Plates (siRNA Knockdown)

To determine optimum cell density, cells from cell lines SK-OV-3^{R} and MRC-5 SV1^{R} were established in duplicate in 96-well plates in the following concentrations (number of cells/well in 100 µl cell suspension): 10 000; 15 000; 20 000; 25 000; 30 000; 35 000 and the percentage confluence was estimated each day over a five-day period. It was concluded that the optimum cell number/well of a 96-well plate were 10 000 (for cell line SK-OV-3^{R}) and 25 000 (for cell line MRC-5 SV1^{R}).

Determination of Transfection Efficiency using 96-well Plates (siRNA Knockdown)

The transfection efficiency of siRNA in the wild-type SK-OV-3, cisplatin resistant SK-OV-3^{R}, wild-type MRC-5 SV1 and MRC-5 SV1^{R} cell lines was established as follows with all reagents supplied by Dharmacon/GE Healthcare, Amersham, Buckinghamshire, UK. The optimum number of cells for each cell line (established previously) was seeded into each well of a 96-well plate and cultured under conditions described previously for 24 hours, the complete medium removed, and the cells washed once with 100 µl of either additive-free RPMI-1640 cell culture medium (SK-OV-3 cell lines) or DMEM cell culture medium (MRC-5 SV1 cell lines). The 5× siRNA buffer was diluted to a 1× siRNA buffer with the addition of RNase-free water. The siGLO™ Green Transfection Indicator siRNA 100 µM stock solution was diluted with the 1× siRNA buffer to create a 5 µM solution, and further diluted with the appropriate additive-free cell culture medium such that the cells/well would be

exposed to 0.5 µl in a total volume of 10 µl. The transfection reagent was diluted with the appropriate additive-free cell culture medium such that the cells/well would be subjected to: no transfection reagent (control samples); 0.05 µl; 0.28 µl or 0.5 µl volumes of the transfection reagent in a total volume of 10 µl. The 10 µl diluted siGLO™ Green Transfection Indicator siRNA solution and the 10 µl diluted transfection reagent solution for each well of cells were mixed and incubated at room temperature for 20 minutes, before being transferred to the appropriate sample on the 96-well plate. The samples were routinely incubated for either 22 hours or 46 hours, then the transfection efficiency estimated using a FLoid Cell Imaging Station (Life Technologies). It was found that the optimum transfection efficiency was achieved using a volume of 0.28 µl transfection reagent/sample which resulted in an at least 80% transfection rate for the cell lines.

Reduction of MUS81 Gene Expression using the siRNA Knockdown Technique

Wild-type SK-OV-3, cisplatin resistant SK-OV-3^{R}, wild-type MRC-5 SV1 and cisplatin resistant MRC-5 SV1^{R} cells were plated out into 6-well plates at optimum densities (established previously). The transfection reagents (all supplied by Dharmacon) were prepared as follows. The 5× siRNA buffer was diluted to a 1× siRNA buffer with the addition of RNase-free water. The ON-TARGETplus™ SMARTpool Human MUS81 and ON-TARGETplus™ Control Pool non-targeting siRNA 100 µM stock solutions were diluted with the 1× siRNA buffer to create 5 µM solutions, then further diluted (10 µl 5 µM siRNA solution + 190 µl additive-free cell culture medium per plate well of cells). The transfection reagent was diluted (5.6 µl transfection reagent + 194.4 µl additive-free cell culture medium per plate well of cells). The diluted siRNA was combined with the diluted transfection reagent and 1600 µl of the appropriate antibiotic-free complete medium and incubated at room temperature for 20 minutes.

The antibiotic-free complete medium was removed from the cells by aspiration and the siRNA/transfection reagent/antibiotic-free complete medium solution added. The cells were incubated (conditions described previously) for 3 days if a single knockdown was being performed,

before being processed for either MUS81 protein expression or for a clonogenic assay. A double knockdown was achieved by incubating the cells for 2 days in the first batch of siRNA/transfection reagent/antibiotic-free complete medium solution, then removing and replenishing it with a fresh batch of siRNA/transfection reagent/antibiotic-free complete medium solution, before processing the cells for either MUS81 protein expression or for a clonogenic assay after a further 2 days incubation.

Determination of MUS81 mRNA and Protein Expression Post (Double) siRNA Knockdown after Cisplatin Exposure using qPCR and/or Imaging Flow Cytometry

The aim of these experiments was to determine the MUS81 expression using either qPCR or imaging flow cytometry on cells that had undergone a double knockdown event before being exposed to cisplatin for 1 hour. In both cases, the cells underwent the double knockdown of the MUS81 gene (method described above). Two days after the addition of the second batch of siRNA/transfection reagent/antibiotic-free complete medium solution, the cells were incubated for 1 hour in 312 µg/ml cisplatin. Cisplatin-containing medium was removed and replaced with the appropriate complete medium, the cells were incubated for 24 or 48 hours before being fixed for either qPCR or imaging flow cytometry to determine MUS81 foci number (MUS81 protein expression).

Determination of Cell Survival using Clonogenic Assays Post (Double) siRNA Knockdown of MUS81 Gene with Cisplatin Exposure

The aim of these experiments was to determine cell survival using a clonogenic assay on cells that had undergone a double knockdown event before being exposed to cisplatin for 1 hour. Cells were plated out for the clonogenic assay (as described above) 24 hours after being exposed to 12 µg/ml cisplatin for 1 hour, at a density of 2000 cells/10 cm plate. The cells were incubated for two or three weeks, before being fixed, stained and counted as described above.

Results

Determination of Cisplatin-Resistance in cell lines.

In this study five pairs of cell lines were examined for the expression and function of MUS81 and its contribution to cisplatin resistance. The cells lines are described in the Materials and Methods section. For the immortalised fibroblast cells lines MRC5-SV1 and NB1-hTERT, the levels of resistance to cisplatin have been described previously and have been shown to display a 2 fold resistance (MRC5-SV1) and 3 fold resistance (NB1-hTERT) to cisplatin when the dose required to reduce survival to 50% of untreated controls (IC50) in a clonogenic, colony forming assay were compared (*e.g.,* Adam-Zahir *et al*., 2014).

The response of the SK-OV-3 ovarian cancer cell line following a 1-hour exposure to increasing doses of cisplatin in a clonogenic assay is demonstrated in Figure 1A. The wild-type SK-OV-3 cell line displayed an IC50 to cisplatin of 6 µg/ml. The SK-OV-3^{R} derivative displayed an IC50 of 23.5 µg/ml cisplatin. This represents an increase in resistance of 3.92-fold.

For the A2780 cell line pair, that data are shown in Figure 1B. Here the survival was determined using an MTT assay (described in the Materials and Methods section) because these cells failed to produce adherent colonies in a clonogenic assay. For the wild-type cell lines the IC50 was determined as 4.5 µg/ml. The cisplatin resistant derivative cell line (A2780^{R}), the IC50 was determined as 85.5 µg/ml. This represents a 19-fold increase in resistance.

Finally in the PEA-1 ovarian cancer cell line pair (Figure 1 C), the wild-type cells exhibited a cisplatin IC50 of 2 µg/ml whereas the resistant cell line had doubled the IC50 to 4 µg/ml. Taken together, five pairs of cell lines (two pairs from immortalised human fibroblasts – MRC5-SV1 and NB1-hTERT) and three pairs from ovarian epithelial cancers (SK-OV-3, A2780 and PEA-1) in which resistance to cisplatin was displayed.

Imaging flow cytometry to determine MUS81 foci expression

To determine if there was an alteration in the number of MUS81 DNA repair foci (protein levels) in the nuclei of wild-type and resistant cells following cisplatin exposure, imaging flow cytometry was

used to examine MUS81 protein expression levels at the individual cell level. Imaging flow cytometry was conducted and images of at least 10,000 cells were captured in two independent experiments. The average foci levels per cell for each of the five cell lines are presented in Figure 2A-E. MUS81 foci per cell for each cell line were determined in untreated cells and following exposure to an IC50 concentration of cisplatin at 6, 12, 24, 30, 48 and 72 hours post cisplatin exposure. For all five cell lines, there are significantly more foci in the cisplatin resistant derivatives compared to the wild-type counterparts. The only exception here is at the 6-hour time point in the A2780 ovarian cancer cell line where there are slightly more foci in the wild-type cells compared to the resistant. Using analysis of variance (ANOVA) to compare the difference in distribution of MUS81 foci between the wild-type and resistant cells, it was determined that there are significantly more foci in the cisplatin resistant cell lines variants compared to the match wild-type. The P values are shown in Table 2.

Table 2. ANOVA analysis demonstrating a significant increase in MUS81 foci in the five cisplatin resistant cell lines.

Cell line comparison MUS81 foci distribution	ANOVA P value (significance)
SK-OV-3 wild-type vs SK-OV-3^{R}	P = 0.0167
PEA-1 wild-type vs PEA-1^{R}	P = 6.34×10^{-6}
A2780 wild-type vs A2780^{R}	P = 0.0228
MRC5-SV1 wild-type vs MRC5-SV1^{R}	P = 5.17×10^{-5}
NB1-hTERT wild-type vs NB1-hTERT^{R}	P = 3.6×10^{-5}

Representative examples showing the appearance and increasing number of MUS81 foci in the nuclei of cells is shown in Figure 3**.**

q-PCR Determination of MUS81 mRNA Levels in Wild-Type and Cisplatin resistant ovarian cancer and immortalised human fibroblast cell lines.

As described above, the levels of MUS81 mRNA expression were determined by Q-PCR. Expression in each pair of cell lines (wild-type and cisplatin-resistant) are shown in Figure 4A-E. mRNA levels were measured in the five pairs of cell lines (wild-type and drug resistant) at zero, 24, 30 and 72 hours following exposure to cisplatin. mRNA level were determined in two independent experiments. In all cell line pairs there was an elevation in the level of mRNA in all cells lines at the 24, 30 and 72 hour time points after exposure to cisplatin. Increased level of MUS81 mRNA were most pronounced in all

cell lines at the 30 and 72 hour time points. In addition, the increase was more pronounced in the cisplatin resistant derivative of each cell line pair.

Reduction in MUS81 mRNA Expression Following siRNA Double Knockdown

Using Q-PCR techniques previously described we measured the expression of MUS81 mRNA following a double siRNA knockdown as described in the Materials and Methods section. Level of mRNA were compared in the ovarian cancer wild-type and drug-resistant cell lines, in each case following treatment with the ON-TARGETplus™ SMARTpool Human MUS81 and ON-TARGETplus™ non-targeting siRNA pool (scrambled siRNA). The double knockdown occurred over a period of five days with the second application of target and scrambled siRNA applied day days after the first application. Experiments were carried out in triplicate and the data are shown in Figure 5 A and B. We assumed that the level of RNA expression in cell samples receiving the scrambled target was a nominal 1.0 in terms of relative quantification. Levels of MUS81 mRNA in on-target siRNA was expressed as a fraction of that in non-targeted samples. It can be observed in Figure 5 A and B in both wild-type and cisplatin-resistant cells (SK-OV-3^{R} and MRC5-SV1^{R}) there is a significant reduction in mRNA levels when a MUS81-specific siRNA was applied. Using a Student's unpaired T-test, in all samples the reduction in MUS81 mRNA was significantly reduced ($P< 0.0005$) in all samples tested. Having demonstrated a significant reduction in MUS81 mRNA expression we proceeded to examine the effect of knockdown on MUS81 foci numbers in the nuclei of SK-OV-3 and MRC5-Sv1 cells. In addition we determined if a reduction in Mus81 expression in cells resulted in a restoration of drug sensitivity in drug resistant cells, measured by clonogenic assay.

Expression of MUS81 Foci in Cell Nuclei Following siRNA Knockdown after Cisplatin Exposure

To determine the expression of MUS81 protein in the form of nuclear foci, cells were stained for MUS81 using the immnocytochemical method described above, following siRNA 'double' knockdown and exposure to cisplatin. As proof principle of the effect of a reduction in MUS81 expression on foci formation, analysis was carried out in two of the five pairs of cell lines described above, namely wild-type and cisplatin resistant SK-OV-3 and wild-type and cisplatin resistant MRC5-SV1 cells. Following e double knockdown over a four-day period and a further one day exposure to an IC50 concentration of cisplatin, both wild-type and resistant cells were subjected to imaging flow cytometry and enumeration of MUS81 foci (Figure 6). For the SK-OV-3 cisplatin resistant cell line there was an average of 17.59 +/-0 0.032 SE, MUS81 foci per cell when a non-targeted, scrambled siRNA was applied. However, when a MUS81 on-target siRNA was used there was a reduction in the number of MUS81 foci to 7.368 +/- 0.041 SE. For the MRC5-SV1 cisplatin resistant immortalised human fibroblast cell line a similar reduction in MUS81 foci was observed following MUS81 siRNA knockdown. For the scrambled siRNA a mean of 11.08 +/- 0.031 SE foci per cell was seem. However, using an on-target siRNA approach, there was a reduction in the number of foci per cell to 5.185 +/- 0.021. These data indicate that the double knockdown approach was effective in reducing the expression of MUS81 protein on a cell-by-cell basis and this reduction was manifest in fewer MUS81 DNA repair foci forming in cisplatin treated SK-OV-3 and MRC5-SV1 cisplatin resistant cells

Cell Survival of SK-OV-3 and MRC5-SV1 Cells after MUS81 Knockdown

To determine the effect on clonogenic cell survival of the SK-OV-3 and MRC5-SV1 cisplatin resistant cells following siRNA MUS81 knockdown, cells were subjected to either a scrambled or on-target MUS81 double knockdown (over a period of four days) after which cells were exposed to an IC50 concentration of cisplatin (12 µg/ml) for one hr. Survival in the siRNA on target and scrambled knockdown cells was compared to that of untreated control cells and the data can be seen in Figure 7. For SK-OV-3, in scrambled cells survival was significantly reduced from 100% in untreated to 91.82% +/-1.52 SE (P = 0.0057, Students unpaired T-test). However, in on target siRNA cells, survival

was further and significantly reduced to 47.55% +/- 5.16 SE (P = 0.00053, Students unpaired T-test). In the MRC5-SV1 cell a similar pattern of cell survival was observed after exposure to an IC50 concentration of cisplatin. In cells exposed to scrambled siRNA survival was non-significantly reduced to 90.91% +/- 4.86 SE (P = 0.135, Students unpaired T-test). However, in the on target siRNA cells survival was reduced to 47.96 +/- 5.01 (Students unpaired T-test, P= 0.00048). The levels of mRNA and protein (in the form of MUS81 foci) resulted in a significant reduction in cell survival after cisplatin exposure in the two resistant cells lines indicating a re-sensitisation of the drug resistant cells after MUS81 knockdown.

Discussion

In this study we have examined the role of the site-specific endonuclease MUS81 in the repair of DNA interstrand crosslinks induced by the bi-functional alkylating agent cisplatin. We have comprehensively examined the role of MUS81 in five cell lines in which cisplatin resistance was induced by chronic exposure to increasing concentrations of cisplatin. Three of the cell lines (SK-OV-3, PEA1 and A2780) were derived from epithelial human ovarian cancers, however, the drug resistant derivatives were first described in this study. Two additional cell lines MRC5-SV1 and NB1-hTERT were also included in the study in which resistance to cisplatin and nitrogen mustard has been previously documented (Adam-Zahir *et al*., 2014). In this study we demonstrated in 5 cell lines in which resistance to cisplatin had been developed there was: 1). A significant increase in MUS81 protein expression determined by an elevation in the number of MUS81 foci in the nuclei of drug resistant compared to the wild-type-cells. 2) An increase in mRNA levels for MUS81 in drug resistant cells compared to wild-type following a 1 hour exposure to an IC50 of cisplatin at 24, 30 and 72 hours post exposure. 3) A significant reduction of MUS81 mRNA expression in wild-type and cisplatin resistant SK-OV-3 and MRC5-SV1 after a double exposure knockdown with on-target MUS81 siRNA. 4) MUS81 knockdown resulted in a reduction of MUS81 protein expression in SK-OV-3 and MRC5-SV1 cells (in the form of DNA damage foci) and 5), the reduction in MUS81 foci was associated with a re-sensitisation of the drug resistant SK-OV-3 and MRC5-SV1 cells to wild-type levels.

This investigation adds to several previously published *in vitro* studies which appear to establish that over-expression of MUS81 in cancers of different histological origin can be associated with resistance to a variety of anticancer therapies. For example, inhibition of MUS81 by siRNA in breast cancer cells increased cellular sensitivity to the anticancer drug 5-fluorouracil (Qian *et al*., 2014). Also, inhibition of MUS81 was seen to sensitise ovarian cancer (A2780 and SK-OV-3 ovarian cancer cell lines) cells to X-ray and Olaparib treatment both *in vitro* and *in vivo,* by activation of the CHK1 and cyclin B signalling pathways (Zhong *et al*., 2019). In addition, a further investigation into the role of MUS81 in cellular response to anticancer treatment revealed that MUS81 lentivirus siRNA

mediated knockdown in the colon cancer cell lines HCT116 and LS180 resulted in elevated cellular sensitivity to therapeutic drugs including cisplatin. This sensitisation was again mediated via the CHK1 signalling pathway followed by an S phase cell cycle arrest and an increase in cellular apoptosis (Wu *et al*., 2016, 2017).

Given that there is emerging evidence of a central role of MUS81 in the response of cancer cells to a variety of anticancer chemotherapeutic drugs, it would be appropriate to consider if there are methods to inhibit the function of the MUS81 endonuclease in the clinical/oncological setting, potentially using small molecular inhibitors or other approaches. Recent computational modelling approaches has identified potential inhibitors of the winged helix domain of the MUS81 endonuclease (Ngo, Vu and Phung 2020). The tyrosine kinase inhibitor nilotinib which is a BCR-ABL kinase inhibitor used in the treatment of chronic mylogenous leukaemia (Blay and von Mehren, 2011) has been modelled as a potential candidate. In addition, the antiviral simeprevir, used in the clinical management of hepatitis C (Izquierdo, *et al*., 2014) has also demonstrated theoretical inhibitory potential against MUS81. Subsequent *in vitro* and *in vivo* testing will establish the experimental and clinical effectiveness of these candidate drugs for MUS81 inhibition as a mechanism for sensitising drug resistant cancer cells.

Given the evidence that both experimentally and clinically, alterations in DNA repair capacity mediated by enhanced expression of DNA repair proteins my lead to resistance to anti-cancer therapy in a variety of cancers, and in particular ovarian cancer, the strategy of inhibiting DNA repair pathways as a viable approach in cancer treatment is gaining significant momentum. For example, the expanding clinical use of polyADPribose polymerase (PARP) inhibitors to induce synthetic lethality in *BRCA1* negative cancers (e.g., breast and ovarian cancers) is an example of modulation of DNA repair to enhance clinical response (Slade, 2020). Also, small molecule inhibitors of the Ataxia telangiectasia (ATM) Ataxia Telangiectasia and Rad 3 related (ATR) cell cycle checkpoint proteins are currently in early phase clinical trial (Weber and Ryan, 2020) and will further add to the repertoire of

strategies to modulate or inhibit DNA repair processes in cancer cells for front line therapy or as second line treatment vis-à-vis drug resistance cancer.

In summary, in this report we have conducted an *in vitro* investigation into the role of the site specific endonuclease MUS81 in repair of cisplatin induced DNA damage in wild-type and drug resistance ovarian cancer cell lines. We provide compelling evidence that inhibition of MUS81 might be a suitable target for inhibition for the treatment of drug resistant cancers. Future investigations will examine the role of small molecule inhibitors of MUS81 (e.g., nilotinib and simeprevir) on drug resistant ovarian cancer cell lines.

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Conflicts of Interest/Competing interests
There are no conflicts of interest or competing interests declared by any author.

Availability of data and material
Raw image files for the imaging flow cytometry used in the measurement of MUS81 foci/protein are available on Figshare (DOI: XXXXXXXXXXX). Raw data for Q-PCR experiments can be made available on request.

Code availability
Not applicable

Author contribution

Author	Contribution
Bourton	Bulk of laboratory experimentation
Adam-Zahir	Bulk of laboratory experimentation
Plowman	Co-investigator, clinical lead, contribution to manuscript writing
Foster	Some laboratory experimentation, contribution to manuscript writing
Al-Ali	Some laboratory experimentation, contribution to manuscript writing
Parris	Principal investigator, study lead, main manuscript author

Figure legends

Figure 1 A-C: shows the clonogenic cell survival (SK-OV-3 and PEA-1) and MTT assay survival (A2780) of three ovarian cancer cell lines following a 1-hour exposure to cisplatin. A: SK-OV-3 wild-type and cisplatin resistant. B: A2780 wild-type and resistant. C; PEA-1 wild-type and cisplatin resistant. Data are derived from three independent experiments and error bars of the graphs show standard error of the mean survival at each concentration of cisplatin.

Figure 2 A-E: The number of MUS81 foci (protein expression) in five pairs of cell lines is shown in Figures 2 A-E. Data are derived by imaging flow cytometry whereby images of at least 10,000 cells were captured and foci counted using a spot counting masking feature in the imaging flow cytometry analysis software (Ideas™). Figure 2A – foci in SK-OV-3 wild-type and cisplatin resistant cells following exposure to an IC50 concentration of cisplatin (12 µg/ml). Foci we counted at 6, 12, 24, 30, 48 and 72 hours post cisplatin exposure. ANOVA analysis revealed that there were significantly more foci in the cisplatin resistant cell line compared to the wild-type cells ($P < 0.05$). Figure 2B – PEA-1 ovarian cancer cell line, with significantly more foci in the drug resistant cells (ANOVA < 0.001). Figure 2 C – A2780 ovarian cancer cell line, with significantly more foci in the drug resistant cells (ANOVA < 0.001). Figure 2 D – MRC5-SV1 immortalised human fibroblast cell line, with significantly more foci in the drug resistant cells (ANOVA < 0.0001). Figure 2 E – NB1-hTERT human immortalised fibroblast cell line, with significantly more foci in the drug resistant cells (ANOVA < 0.05).

Figure 3
A representative example of cells displaying different number of MUS81 foci in the nuclei of cell post cisplatin treatment. The first column shows the bright field image of the cells. The Alexa Fluor™ ^{488} staining of the cells is shown with and without the enumeration mask applied and overlaying the MUS81 foci. The Draq 5™ staining determines the boundaries of the nuclear region of the cells.

Figure 4
mRNA expression determined by qPCR analysis in all five cell lines in wild-type and cisplatin resistant cells, following exposure to an IC50 concentration of drug. MUS81 mRNA expression was measured at 0, 24, 30 and 72 hours following cisplatin exposure.

Figure 5
Figure 5 demonstrates the effect of a double siRNA knockdown on mRNA expression in two of the cells lines for wild-type and drug resistant cells. Following treatment with the ON-TARGETplus™ SMARTpool Human MUS81 and ON-TARGETplus™ non-targeting siRA pool (scrambled siRNA), mRNA was measured by Q-PCR. mRNA expression levels were significantly (student's unpaired T-test) reduced following treatment with MUS81 targeting siRNA compared to scrambled non-targeting siRNA. Data was derived from three independent experiments. Error bars depict standard error of the mean.

Figure 6
Figure 6 A and 6B show the effect of siRNA knockdown on the number of MUS81 foci on two pairs of cell lines (as representative examples). Figure 6 A shows that the distribution of MUS81 foci in the nuclei of SK-OV-3 cisplatin resistant cells of at least 10,000 counted cells is reduced when the ON-TARGETplus™ SMARTpool Human MUS81 siRNA is used (green line, average of 7.368 +/- 0.032 SE) compared to a scrambled siRNA (red line, average of 17.59 +/- 0.041 SE). $P < 0.001$, Student's unpaired *t*-test. Figure 6 B shows that the distribution of MUS81 foci in the nuclei of MRC5-SV1 cisplatin resistant cells of at least 10,000 cells is reduced when the ON-TARGETplus™ SMARTpool

Human MUS81 siRNA is applied (green line, average of 5.185 +/- 0.021 SE) compared to a scrambled siRNA (red line, average of 11.08 +/- 0.031 SE). $P < 0.005$, Student's unpaired T-test.

Figure 7

Figure 7 A and B show the clonogenic cell survival of SK-OV-3^R cells (A) and MRC5-$SV1^R$ cells (B) following one hr exposure to cisplatin (12 µg/ml). Untreated cells are shown with 100% clonogenic cell survival. In both cell lines treated with a scrambled siRNA there is a modest (approximately 10%) reduction in cell survival. However, with an ON target siRNA, survival is reduced to near wild-type levels.

Figure 1

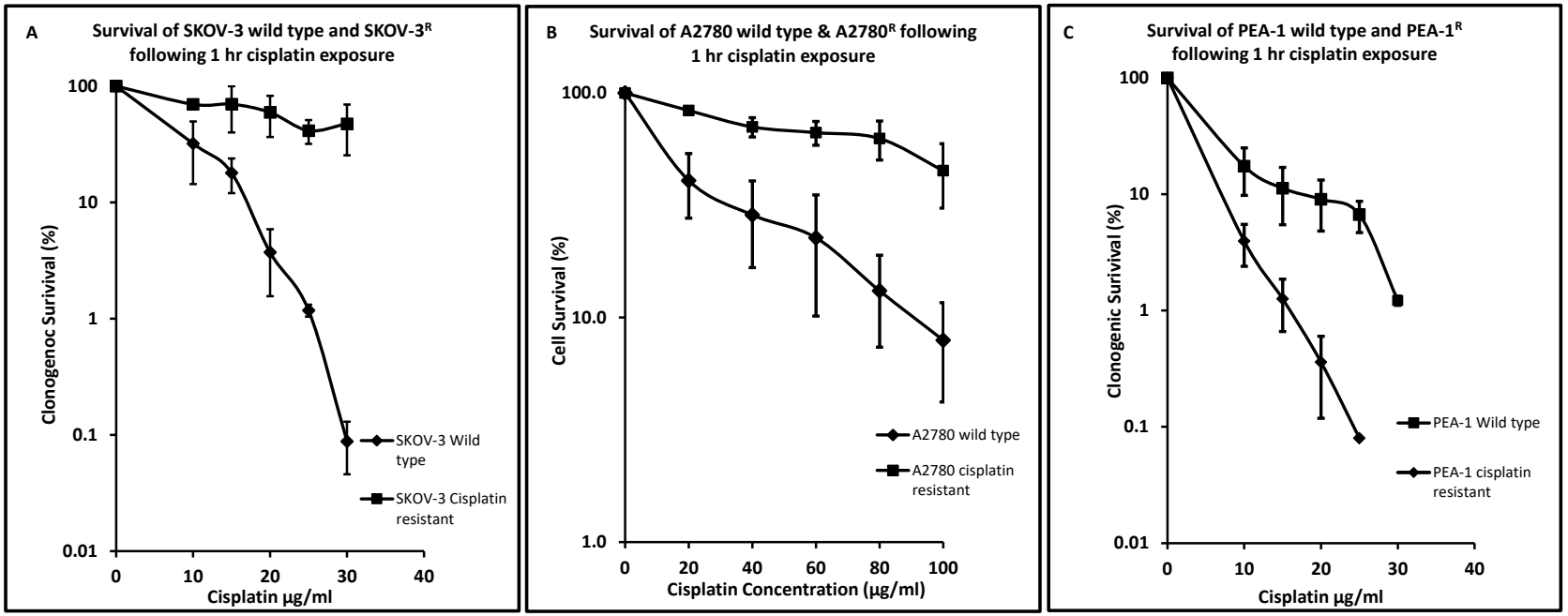
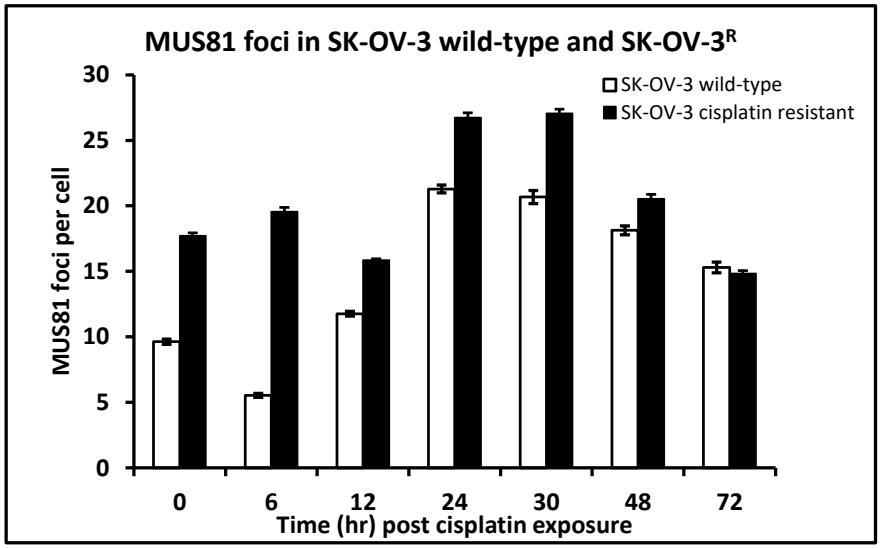
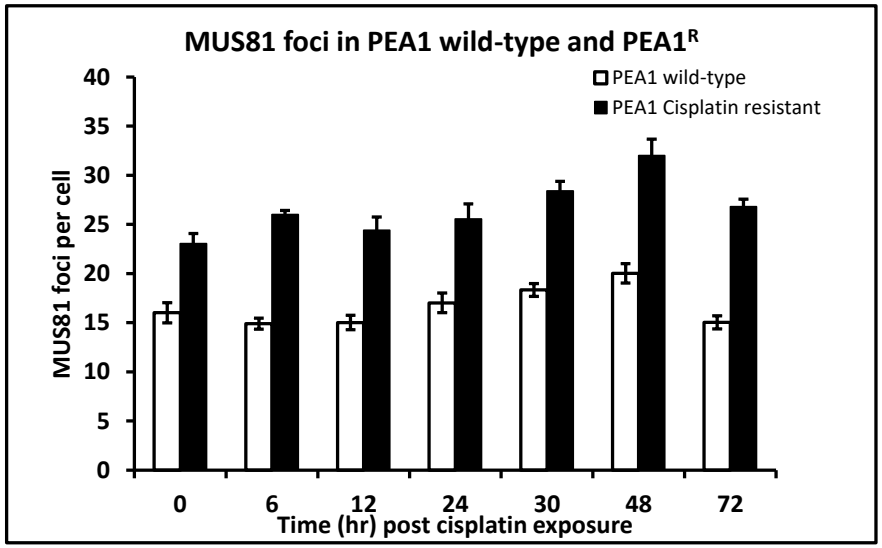
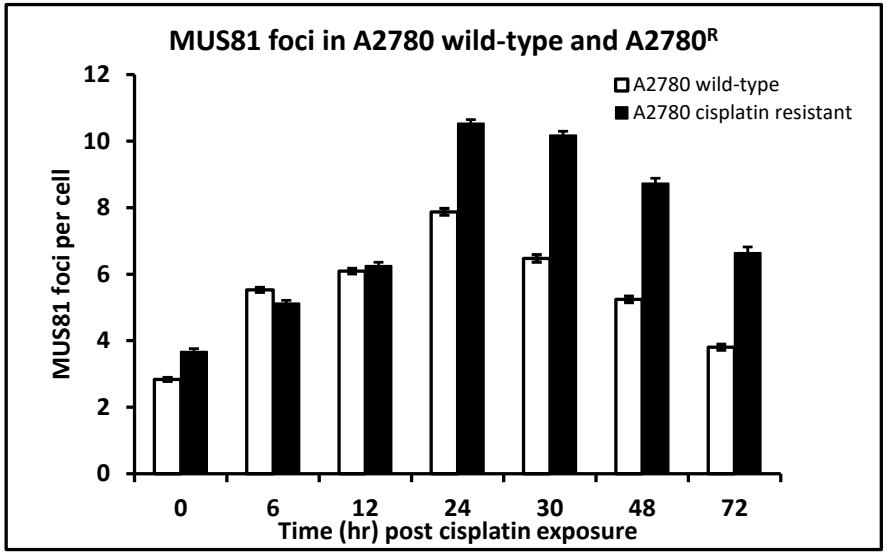
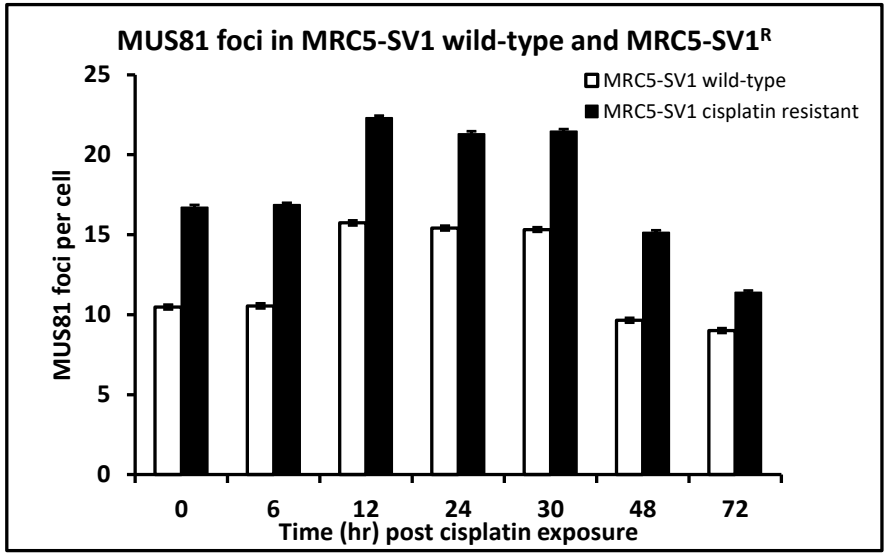

A
Survival of SKOV-3 wild type and SKOV-3R following 1 hr cisplatin exposure
Clonogenoc Surivival (%)
100
10
1
0.1
0.01
0
10
20
30
40
Cisplatin µg/ml
SKOV-3 Wild type
SKOV-3 Cisplatin resistant
B
Survival of A2780 wild type & A2780R following 1 hr cisplatin exposure
Cell Survival (%)
100.0
10.0
1.0
0
20
40
60
80
100
Cisplatin Concentration (µg/ml)
A2780 wild type
A2780 cisplatin resistant
C
Survival of PEA-1 wild type and PEA-1R following 1 hr cisplatin exposure
Clonogenic Surivival (%)
100
10
1
0.1
0.01
0
10
20
30
40
Cisplatin µg/ml
PEA-1 Wild type
PEA-1 cisplatin resistant

Figure 2


MUS81 foci in SK-OV-3 wild-type and SK-OV-3R
SK-OV-3 wild-type
SK-OV-3 cisplatin resistant
MUS81 foci per cell
0
5
10
15
20
25
30
0
6
12
24
30
48
72
Time (hr) post cisplatin exposure


MUS81 foci in PEA1 wild-type and PEA1R
PEA1 wild-type
PEA1 Cisplatin resistant
MUS81 foci per cell
0
5
10
15
20
25
30
35
40
0
6
12
24
30
48
72
Time (hr) post cisplatin exposure


MUS81 foci in A2780 wild-type and A2780R
A2780 wild-type
A2780 cisplatin resistant
MUS81 foci per cell
0
2
4
6
8
10
12
0
6
12
24
30
48
72
Time (hr) post cisplatin exposure


MUS81 foci in MRC5-SV1 wild-type and MRC5-SV1R
MRC5-SV1 wild-type
MRC5-SV1 cisplatin resistant
MUS81 foci per cell
0
5
10
15
20
25
0
6
12
24
30
48
72
Time (hr) post cisplatin exposure

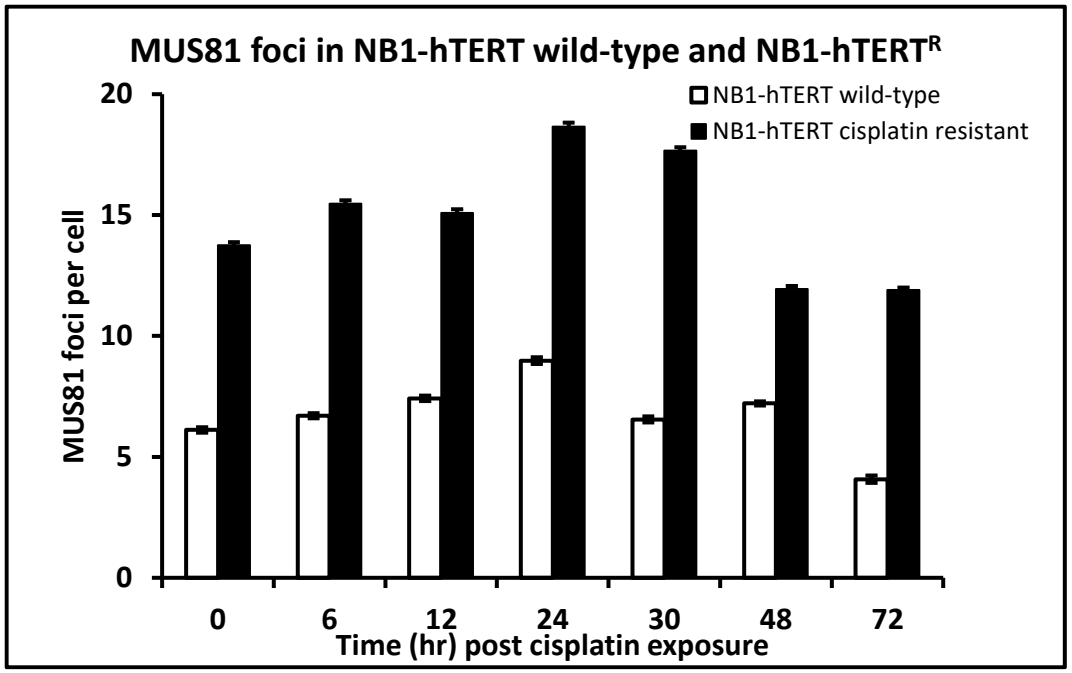

MUS81 foci in NB1-hTERT wild-type and NB1-hTERT^R
NB1-hTERT wild-type
NB1-hTERT cisplatin resistant
MUS81 foci per cell
20
15
10
5
0
0
6
12
24
30
48
72
Time (hr) post cisplatin exposure

Figure 3

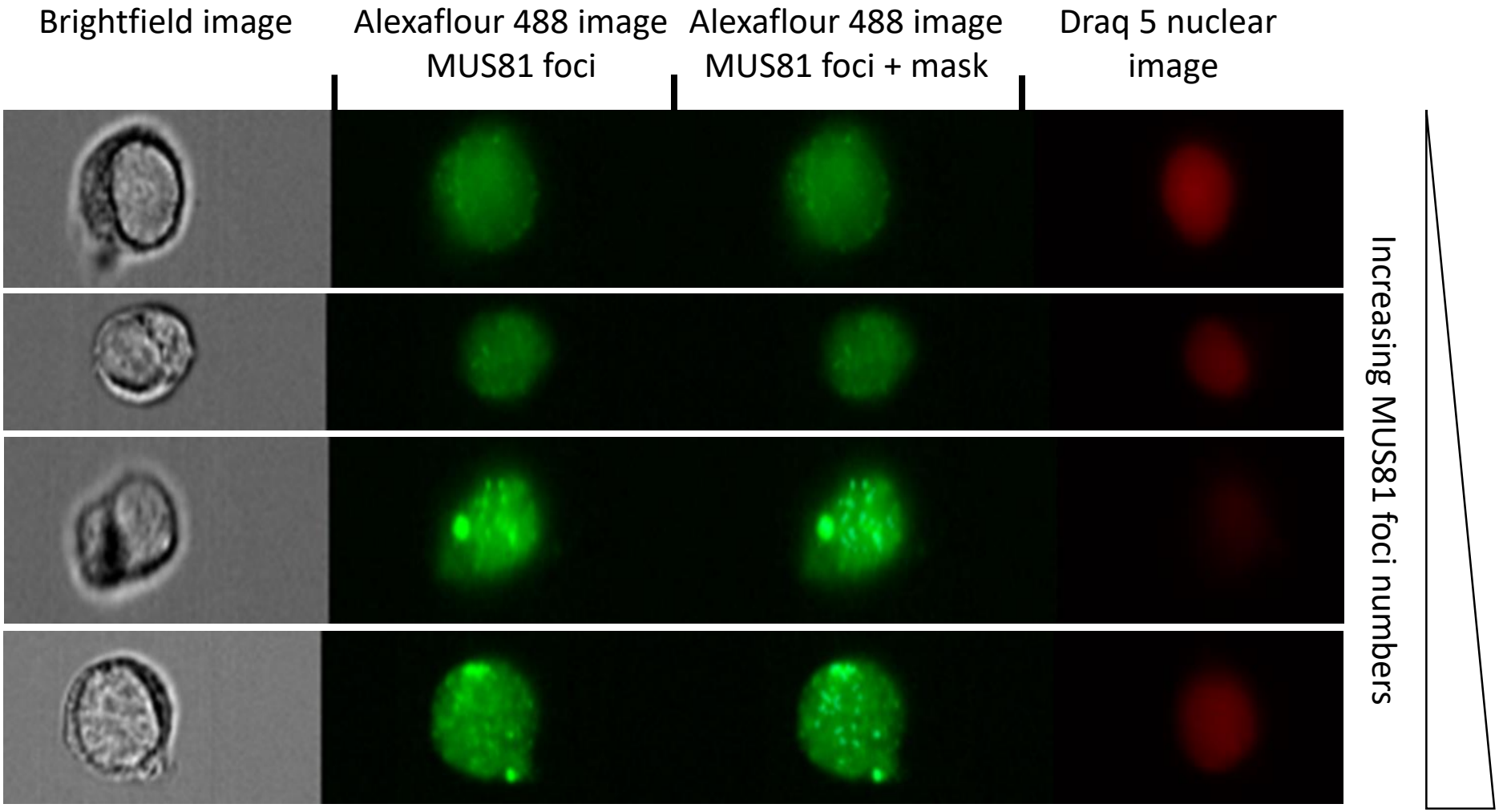
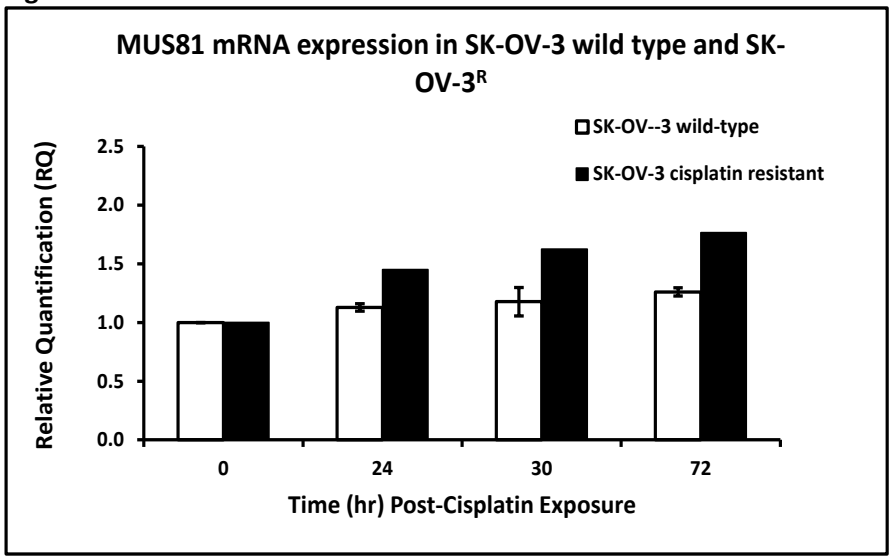
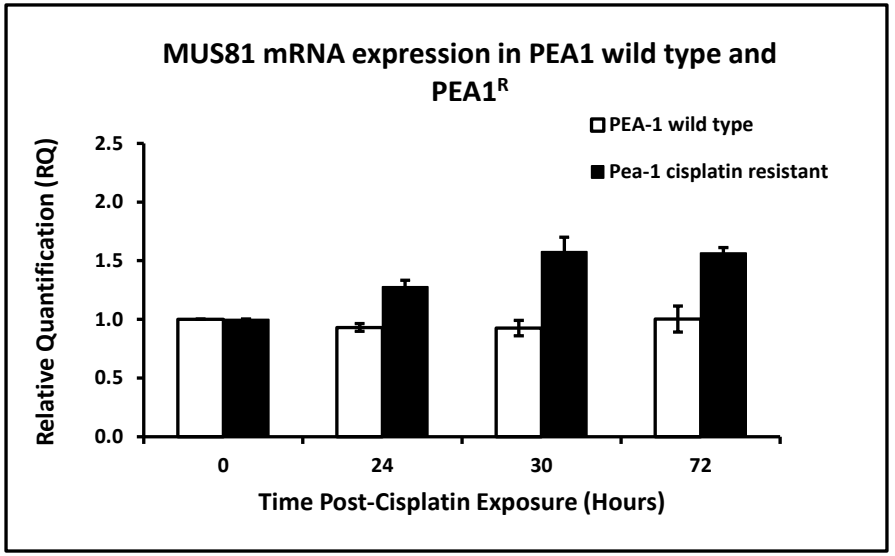
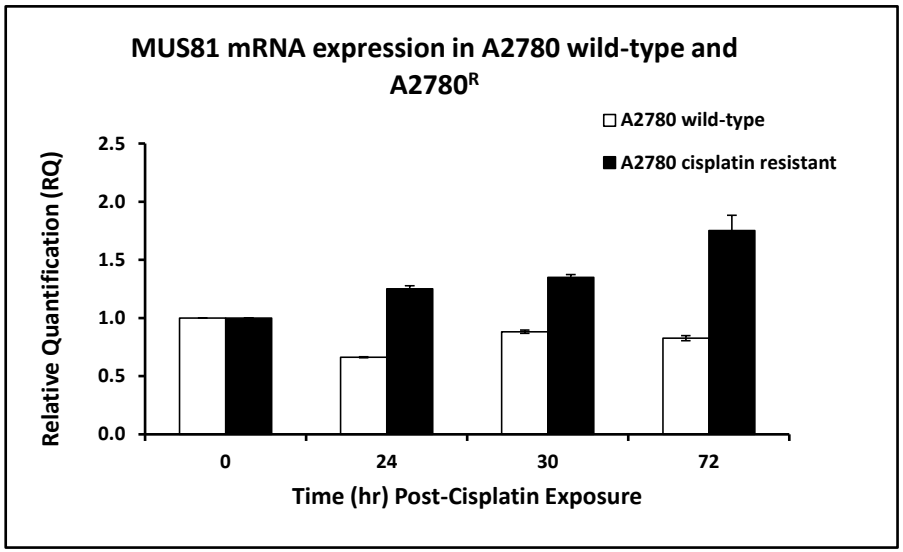
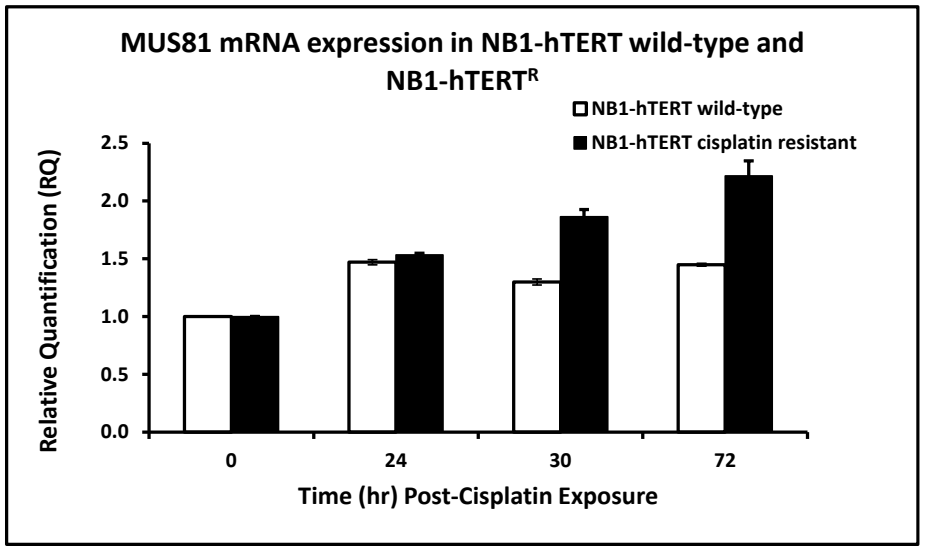

Brightfield image
Alexaflour 488 image MUS81 foci
Alexaflour 488 image MUS81 foci + mask
Draq 5 nuclear image
Increasing MUS81 foci numbers

Figure 4


MUS81 mRNA expression in SK-OV-3 wild type and SK-OV-3R
SK-OV--3 wild-type
SK-OV-3 cisplatin resistant
Relative Quantification (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
0
24
30
72
Time (hr) Post-Cisplatin Exposure


MUS81 mRNA expression in PEA1 wild type and PEA1R
PEA-1 wild type
Pea-1 cisplatin resistant
Relative Quantification (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
0
24
30
72
Time Post-Cisplatin Exposure (Hours)


MUS81 mRNA expression in A2780 wild-type and A2780R
A2780 wild-type
A2780 cisplatin resistant
Relative Quantification (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
0
24
30
72
Time (hr) Post-Cisplatin Exposure


MUS81 mRNA expression in NB1-hTERT wild-type and NB1-hTERTR
NB1-hTERT wild-type
NB1-hTERT cisplatin resistant
Relative Quantification (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
0
24
30
72
Time (hr) Post-Cisplatin Exposure

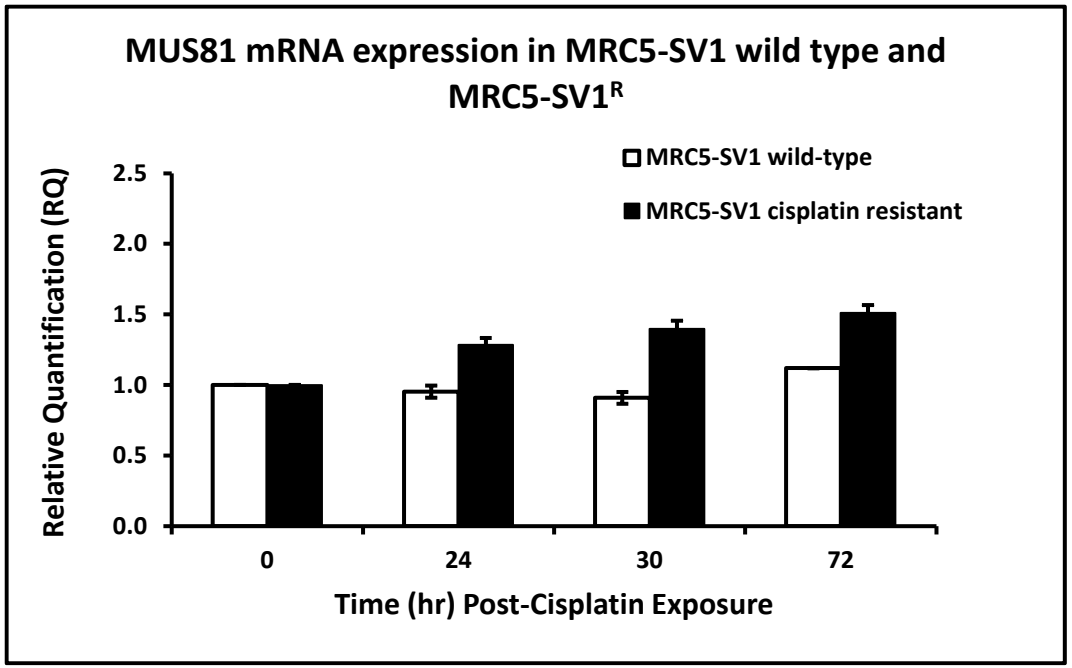
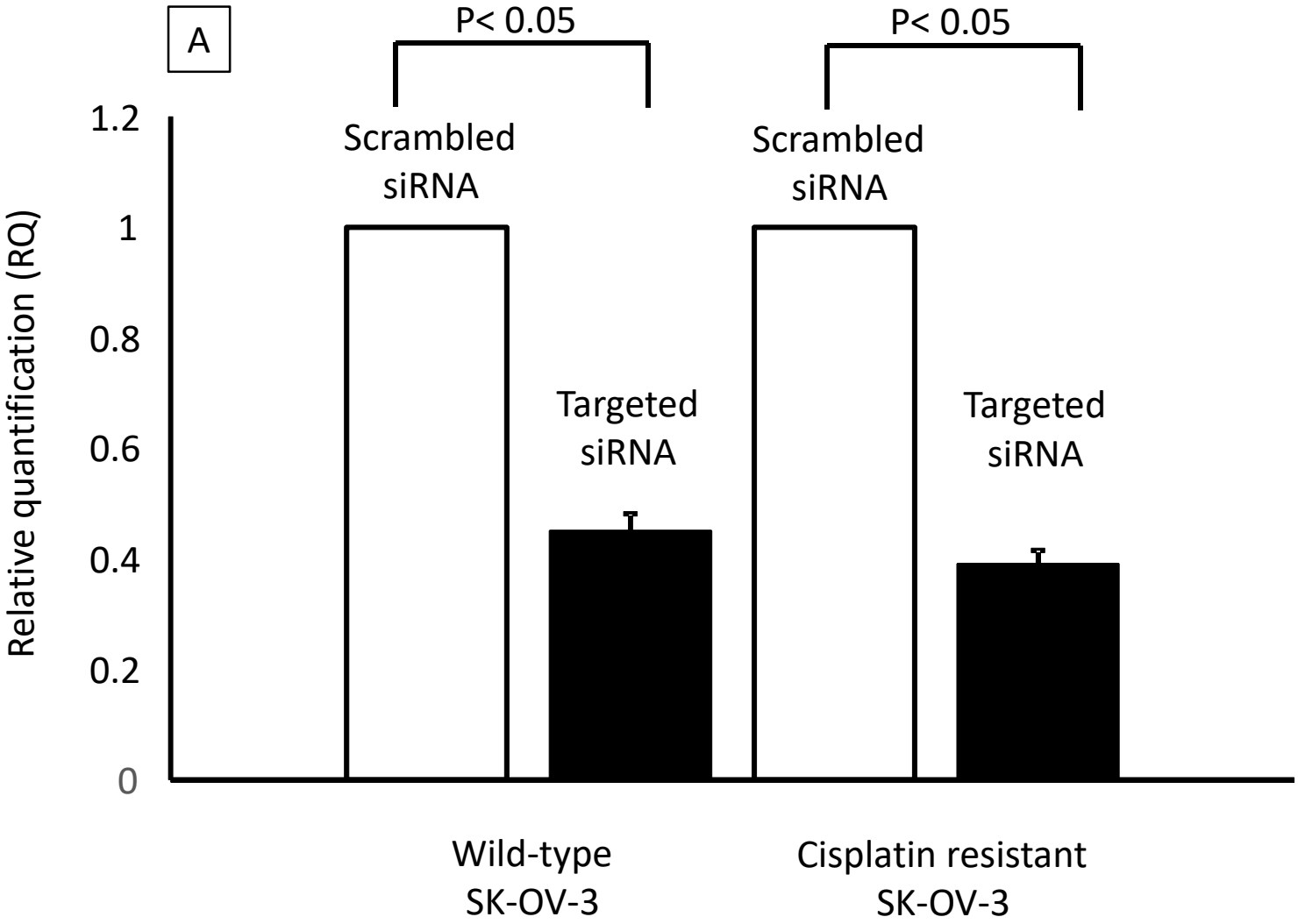

MUS81 mRNA expression in MRC5-SV1 wild type and MRC5-SV1R
MRC5-SV1 wild-type
MRC5-SV1 cisplatin resistant
Relative Quantification (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
0
24
30
72
Time (hr) Post-Cisplatin Exposure

Figure 5

Reduction of MUS81 mRNA expression in SK-OV-3 cells by siRNA



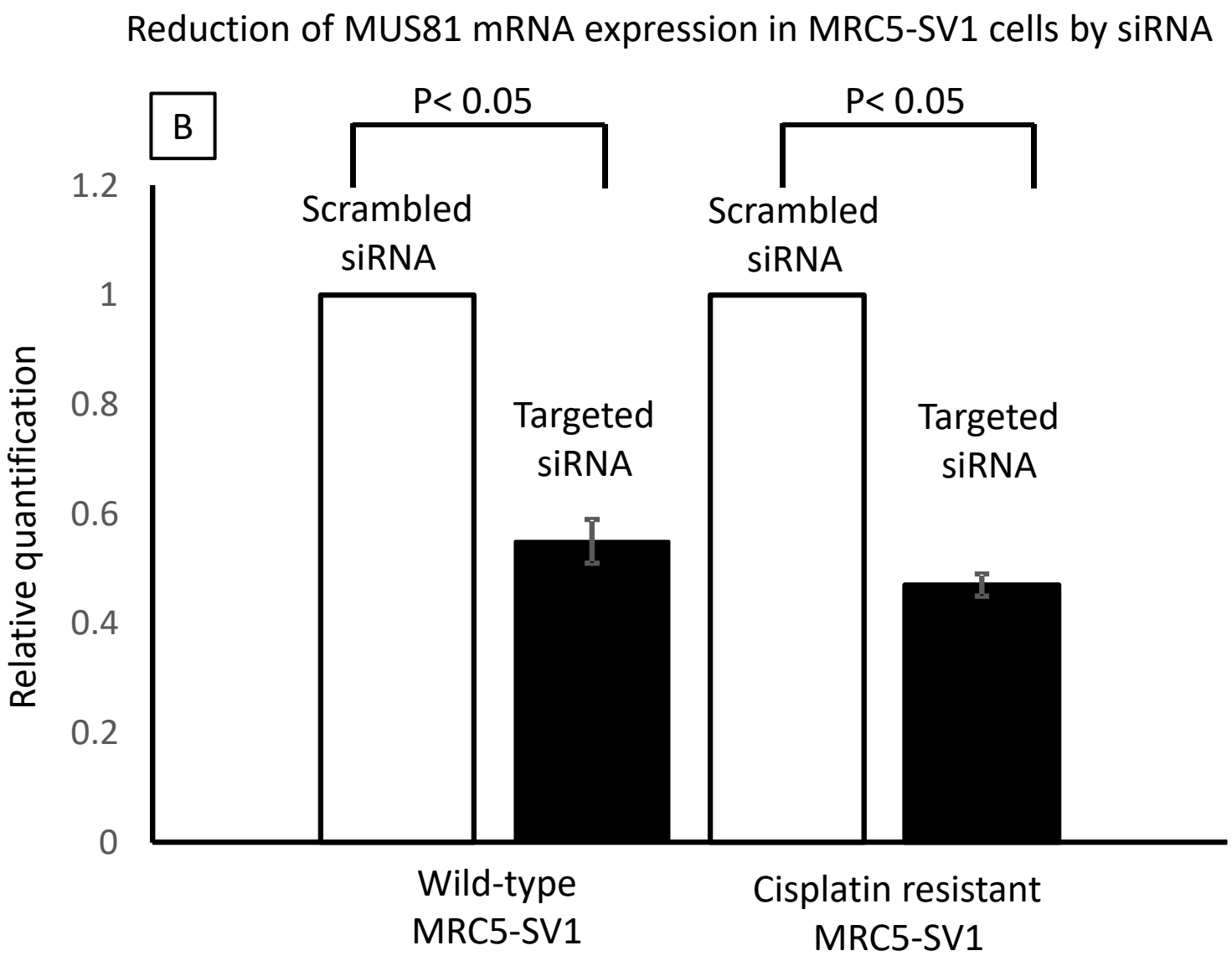
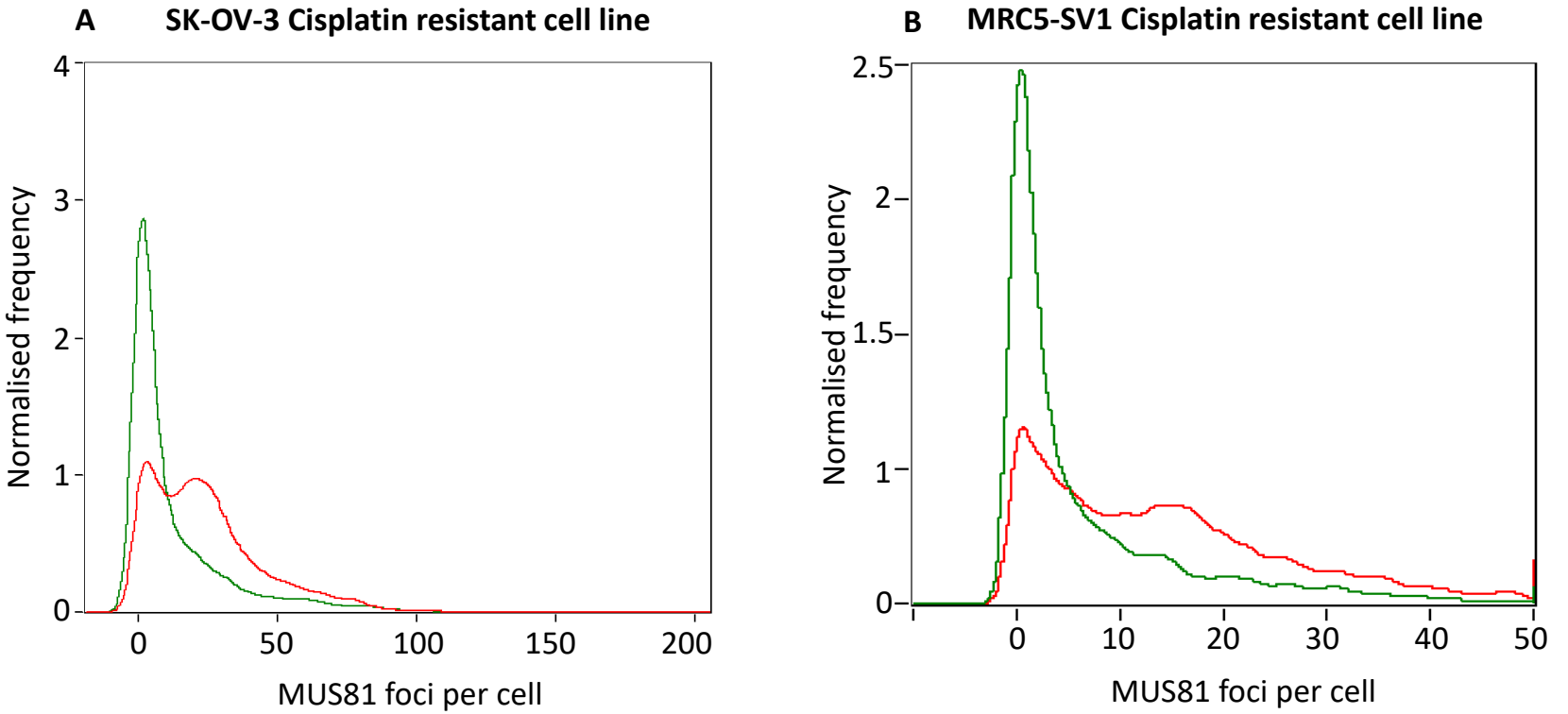

Reduction of MUS81 mRNA expression in MRC5-SV1 cells by siRNA
B
P< 0.05
P< 0.05
Scrambled siRNA
Scrambled siRNA
Targeted siRNA
Targeted siRNA
Relative quantification
1.2
1
0.8
0.6
0.4
0.2
0
Wild-type MRC5-SV1
Cisplatin resistant MRC5-SV1

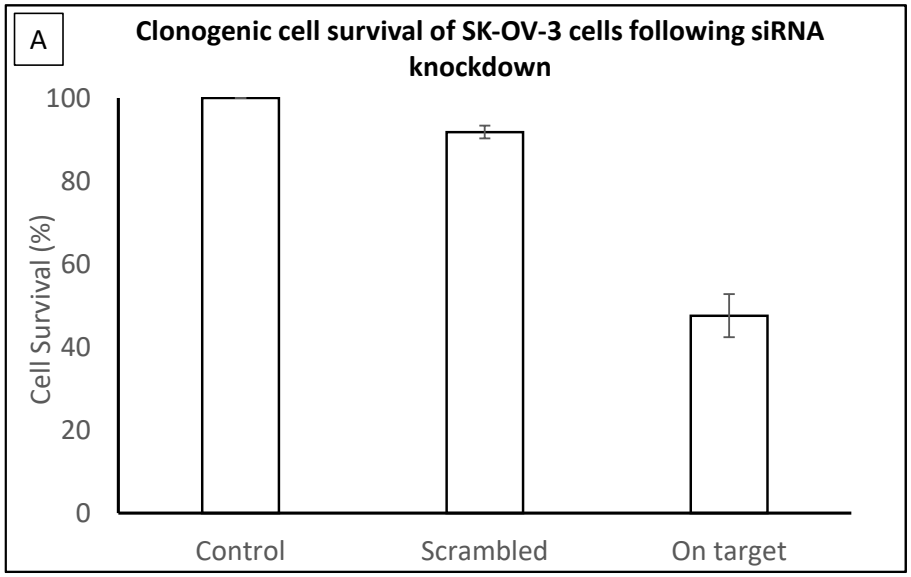
Figure 6

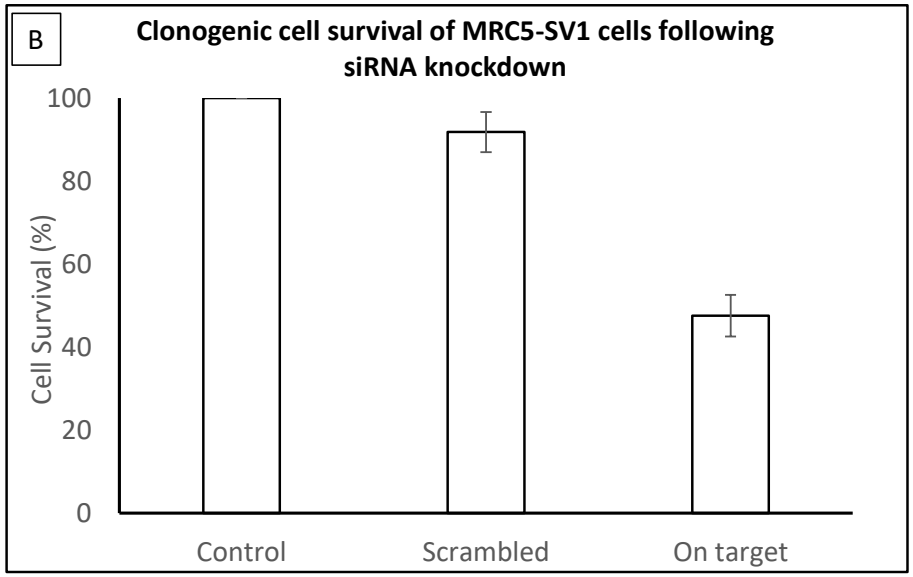


On target siRNA MUS81 foci/cell average = 7.368 +/- 0.032 SE
Scrambled siRNA MUS81 foci/cell average = 17.59 +/- 0.041 SE

On target siRNA MUS81 foci/cell average = 5.185 +/-0.021 SE
Scrambled siRNA MUS81 foci/cell average = 11.08 +/- 0.031 SE

Figure 7


A
Clonogenic cell survival of SK-OV-3 cells following siRNA knockdown
Cell Survival (%)
100
80
60
40
20
0
Control
Scrambled
On target


B
Clonogenic cell survival of MRC5-SV1 cells following siRNA knockdown
Cell Survival (%)
100
80
60
40
20
0
Control
Scrambled
On target