EXPRESSION LEVELS OF FANCD2 DNA REPAIR PROTEIN IN DRUG RESISTANT OVARIAN CANCER CELL LINES

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Declaration

I hereby declare that the work presented in this thesis is my own work, except as cited in the references, and has not been submitted for any other degree.

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ABSTRACT

Ovarian cancer (OC) is the fifth most common cancer among women in the UK, accounting for 4% of all new cases of cancer in females. Chemotherapy or radiotherapy or in combination are given the majority of cancer patients. The most effective cancer chemotherapeutics are the crosslinking agents such as nitrogen mustard and cisplatin. These drugs cause DNA damage by introducing DNA interstrand crosslinks and these are a challenging adduct for the DNA repair machinery to repair.

Frequently cancer cells develop resistance to anticancer chemotherapy which makes the clinical management of cancer difficult. As a result, when drug resistant occurs, a patient with cancer will often result in patient death. There is a plenty of evidence which suggests that alterations in the efficiency of DNA repair mechanisms in cancer cells are central to the development of drug resistance. This is a specific problem with human ovarian cancer which has a 35% five-year survival rate due in part because of the development of drug resistance by enhanced DNA repair capacity. DNA repair capacity in tumour cells may adjust the success of the treatment.

The aim of this work to determine responses to chemotherapeutics and compare DNA repair capacity increased in cisplatin resistant ovarian cancer cell lines with wild type ovarian cancer cells.

We have compared differences in FANCD2 foci induction between the A2780 resistant and A2780 parental cell lines to chemotherapeutic drug Cisplatin. Additionally, it was demonstrated that PEO1 cell lines have an elevated Mus81 expression profile over a 72-hour period post treatment with the cross-linking agent Cisplatin. As our results, there is no significant difference in foci numbers of FANCD2 biomarker between A2780 parental and A2780 resistant cell lines in response to Cisplatin.

1 INTRODUCTION

1.1 Ovarian Cancer

Ovarian cancer (OC) is a type of cancer appearing from the cells in and around the ovary and fallopian tube and the most treatable cancer types if it is diagnosed in early stages. The majority of OC will respond for the interim to surgery and chemotherapeutic agents. OC is the most lethal gynecologic malignancy in women as it frequently stands and recurs (Berek and Bast 2003). The most effective cancer therapeutics are alkylating agents which induce DNA interstrand crosslinks (ICL). It is discovered that in cancer cells acquired drug resistance may reduce the anticancer therapeutic efficacy (Adam-Zahir *et al.,* 2014). Acquiring resistance to Platin-based chemotherapeutic is the major obstacle in treatment and results in approximately 90% of ovarian cancer patients' deaths. It is discovered that almost 70% of patients with ovarian cancer responded well to initial Platin-based therapies, but this number declined to only 15- 20% survival rates at the end of through 5-year period (Agarwal and Kaye 2003).

1.2 Incidence

Ovarian cancer is the fifth most common cancer among females in the UK in 2013, accounting for 4% of all new cases of cancer in females. In the UK in 2013, there were 7,284 new cases of ovarian cancer in the UK, according to statistics published by Cancer Research UK.

1.2.1 Types

There are many different types of ovarian tumours classified by the types of cell and tissue. Approximately 85 to 95% of ovarian cancers come from epithelial cells. Epithelial ovarian cancer (EOC) is divided into its histologic subtypes: serous, mucinous, endometrioid, clear cell, transitional cell, or any combination of these. Serous histology is the most common, presenting 70% of EOC. Serous tumours are aggressive tumours that usually present at an

advanced stage, and they usually recur. Five to eight percent come from stromal cells or from germ cells. There are some rarer types of ovarian cancer that do not come from these cells (Erickson *et al.*, 2013).

1.2.2 Risk Factors

1.2.2.1 Age

Ovarian cancer incidence is interrelated to age, the rates of incidence are the highest in older females, peaking in their 60s and 70s. Incidence rates are at around 9 per 100,000 female population of women in their ages between 20 and 49. For women aged 50-69, rates are at 40 per 100,000 female population according to statistics published by UK Cancer Information Service (2009).

Figure 1.1: Age-specific Ovarian Cancer Rates

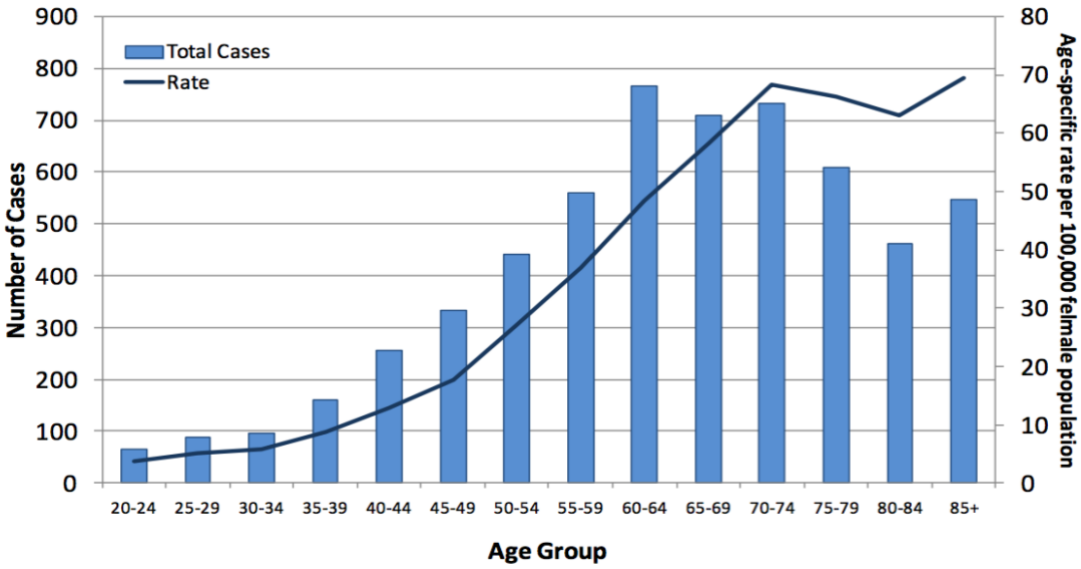

FIGURE 1.1: Age-specific incidence rates and numbers of cases diagnosed by five-year age group (England, 2009 Source: UK Cancer Information Service).

1.2.2.2 Family History

Approximately 10-20% of high grade of ovarian cancer cases are believed to be caused by an inherited mutation in a gene associated with OC gene, which is often the BRCA1 or BRCA2 genes. Women with inherited a ‘mutated’ copy of the BRCA1 or BRCA2 gene have a

much higher risk with 11-40% (Petrucelli, Daly and Feldman 2013) of developing ovarian cancer than the general population (Plo *et al.*, 2008).

1.2.3 Staging of Ovarian Cancer

Table 1.1: Ovarian Cancer Stages

Stage I	Growth limited to the ovaries.
Stage Ia	Growth limited to one ovary; no ascites. No tumour on the external surface; capsule intact.
Stage Ib	Growth limited to both ovaries; no ascites. No tumour on the external surfaces; capsules intact.
Stage Ic*	Tumour either Stage Ia or Ib but with a tumour on the surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension.
Stage IIa	Extension and/or metastases to the uterus and/or tubes.
Stage IIb	Extension to other pelvic tissues.
Stage IIc*	Tumour either Stage IIa or IIb, but with a tumour on the surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III.
Stage IIIa	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces.
Stage IIIb	Tumour of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces none exceeding 2 cm in diameter, Nodes are negative.
Stage IIIc	Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV.

Table 1.1: Stage-Grouping for Primary Carcinoma of the Ovary (table taken from FIGO Cancer Committee, 1986).

1.2.4 The Genetics of Ovarian Cancer

Tumour suppressor genes, oncogenes, and genes involved in DNA repair mechanisms; these three classes of genes which are key factors in tumour initiation. Ovarian

carcinogenesis, as in most cancers, involves alterations in these genes. Ovarian cancers display defects in many genes, such as RAS, MYC, TP53 and BRCA (Prowse *et al.,* 2003).

1.2.4.1 Key Tumour Suppressor Genes

TP53 functions as a cell cycle checkpoint protein by transactivating of genes which encode proteins with growth suppressing activities and it exerts its function during the G_1 phase of the cycle (Prives and Hall 1999; Appella and Anderson 2000). P53 is a protooncogene, the molecular mechanisms of p53 action in normal and malignant cells are not well known (Marks *et al.,* 1991). Mutations in P53 cause amino acid substitutions, seem to change the conformation of p53, which result in increased stability and higher steady-state levels of this normally short-lived protein (Finlay *et al.,* 1988). Loss of heterozygosity for genes on chromosome 17, due to chromosomal deletion, is a common event that occurs in almost 75% of ovarian cancers (Eccles *et al.,* 1990, Russel *et al.,* 1990). Since p53 is located on chromosome 17p and loss of heterozygosity may lead aberrant expression of p53 in epithelial ovarian cancer (Marks *et al.,* 1991).

BRCA1 and BRCA2 genes are members of a class of genes known as tumour suppressor genes, which play a role in homologous recombination (HR) (Wang *et al.,* 2000). They are essential proteins for a normal proliferative burst in early embryogenesis and are upregulated with the proliferation of breast epithelial cells during puberty, lactation, pregnancy (Rajan *et al.,* 1997). This role is complicated, given that in adult ovarian or breast epithelium, loss of BRCA1 or BRCA2 leads to tumourigenesis, it would seem mediated by cell proliferation (Wooster *et al.,* 1995). In addition, mutations in BRCA1 or BRCA2 gene leads to failure to repair damaged DNA. When damage occurs in critical checkpoint genes, such as p53, checkpoints cannot be activated and cells proliferate. It remains to be found out why germline mutations in BRCA1 and BRCA2 affect specific to breast and ovarian cancer (Welcsh *et al.,* 2000).

Figure 1.2: The BRCA1 network (image taken from (Narod *et al.*, 2004)

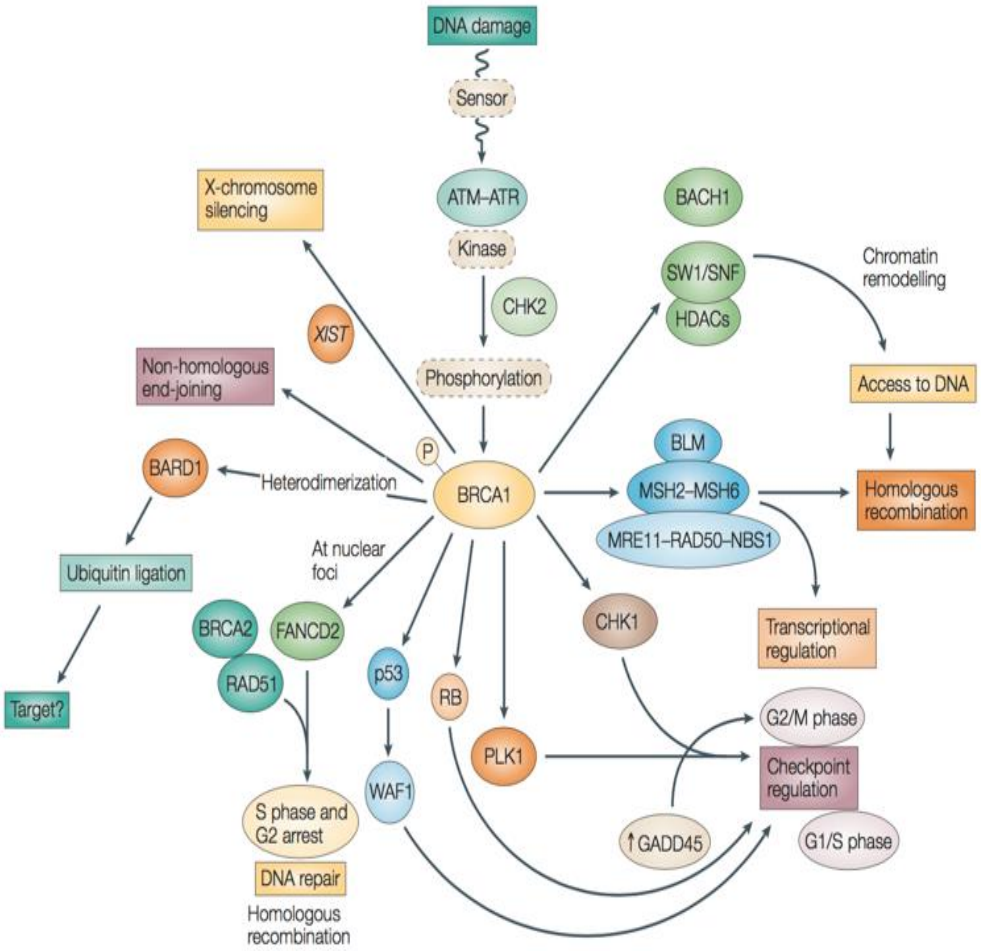

FIGURE 1.2. The BRCA1 network. BRCA1 regulate DNA repair, cell-cycle progression, ubiquitylation and transcriptional regulation. DNA damage triggers of BRCA1 activation. Ataxia telangiectasia mutated (ATM) and other kinases, are activated in response to DNA damage. Activated CHK2 by ATM prevents cell division by phosphorylating BRCA1 and p53. Downstream targets of BRCA1 activation include p53 and the retinoblastoma protein (RB). BRCA2 and RAD51 form a complex with FANCD2, which binds to BRCA1. This complex promotes S-phase or G2 arrest. BRCA1 forms a heterodimer with BARD1 to activate the ubiquitin-ligase function of BARD1. DNA repair by homologous recombination is mediated by the BRCA1-associated surveillance complex (comprised of BLM, MSH2–MSH6 and MRE11–RAD50–NBS1). This complex also regulates transcription. BRCA1 has been shown to interact with X-inactive specific transcript (*XIST*) to mediate X-chromosome silencing, and also to mediate non-homologous end joining during DNA repair. BRCA1 can form complexes with both BACH1 and SW1/SNF to mediate chromatin remodelling and homologous recombination. HDACs regulate the access of the SW1/SNF–BRCA1 complex to DNA. Finally, BRCA1 interacts with CHK1 and polo-like kinase 1 (PLK1) to regulate the G2/M and G1/S checkpoints, possibly via GADD45; thereby linking BRCA1 to the regulation of apoptosis.

The MYC family of proto-oncogenes are transcription factors that their role in tumourigenesis fully understood. MYC gene amplification causes MYC overexpression that induces uncontrolled hyper-proliferation and occurs in approximately 35% of epithelial OCs. Another gene, the NMYC and STAT interactor (NMI), which related with NMYC, MYC, MAX, FOS, other transcription factors (Zhu *et al.,* 1999) and BRCA1 (Li *et al.,* 2002), is overexpressed in human leukaemias and other cancers (Bao and Zervos 1996).

1.2.4.2 Key Oncogenes

KRAS is the most frequently mutated oncogenes and it is known to be involved in OC (Forbes *et al.,* 2006). KRAS exerts its functions in the receptor tyrosine kinase pathway (Gemignani *et al.,* 2003). Activating mutations of KRAS are seen to be an early event in OC development (Gemignani *et al.,* 2003). Mutations in codons 12 and 13 have been detected in around 50% of mucinous OCs (Gemignani *et al.,* 2003). BRAF, is involved in the mitogen-activated protein kinase pathway, is a downstream effector of KRAS and has a major role in the transduction of cell growth signals (Cuatrecasas *et al.,* 1998). Overexpression of BRAF has been discovered in a variety of cancers, and mutations have been reported in 12% of OCs (Gemignani *et al.,* 2003; Russell and McCluggage 2004; Sieben *et al.,* 2004).

Src is an oncogene and dysregulation of Src and the other eight Src family kinase (SFK) members have been shown to be important in the development of many solid tumour types (Yeatman *et al.,* 2004). In normal tissues, Src is often found in an inactive form, but during cancer, development Src may become overexpressed or activated by mutations that increase its enzymatic activity. Src is a serine/threonine kinase and, when active, transduces signaling cascades through the STAT3/MYC, MAPK and PI3K pathways. Consequently, overexpression or mutation of Src is associated with a variety of tumourigenic phenotypes including angiogenesis, proliferation, invasion, motility and chemoresistance (Yeatman *et al.,* 2004; Duxbury *et al.,* 2004).

1.2.5 Ovarian Cancer Diagnose and Treatment

Ovarian cancer may not cause early signs or symptoms so most OC cases are diagnosed at advanced stages, resulting in poor diagnose. When signs or symptoms do emerge, the cancer is often advanced (Goff *et al.,* 2000).

Screening procedures such as vaginal ultrasound, cancer antigen 125 (CA-125) assay, and gynecologic assessment have had low predictive value in diagnosing ovarian cancer in women without special risk factors (Van *et al.,* 2011; Partridge *et al.,* 2009).

1.2.5.1 Diagnosis Methods

The procedures and tests are represented below may be used in the diagnosis and staging of ovarian epithelial, fallopian tube, or primary peritoneal cancer:

Physical exam and history.

Pelvic exam.

CA-125 assay.

Ultrasound exam (pelvic or transvaginal).

Computed tomography (CT) scan.

Positron emission tomography (PET) scan.

Magnetic resonance imaging (MRI).

Chest x-ray.

Biopsy.

CA-125 levels can be elevated in other malignancies and also CA-125 levels and histology are used to detect epithelial ovarian cancer (Berek *et al.,* 1986; Atack *et al.,* 1986).

1.2.5.2 Treatment

The present treatment for all advanced EOC is optimal cytoreductive surgery and chemotherapy that includes platinum and taxanes (Lokadasan *et al.,* 2016).

1.2.5.2.1 Chemotherapy

In spite of surgery and chemotherapy, almost 80% of patients with EOC will recur after first-line chemotherapy (Markman et al., 1991). There are two groups of recurrent ovarian cancer for optimising treatment. The platinum-sensitive group recurs after 6 months of completion of treatment. The platinum refractory and resistant group progresses during or recur within 6 months of treatment. 15-20% of tumours are resistant to primary chemotherapy (Lokadasan *et al.,* 2016).

Patients with recurrent ovarian cancer face the two challenges that are listed as follows: Chemoresistance with repeated platinum administration; Cumulative neurotoxicity if treatment is administered within one-year completion of the treatment. The chemotherapeutic agents have been used in platinum refractory cases – gemcitabine, liposomal doxorubicin, ifosfamide, topotecan, and docetaxel. The response rate is dramatically low about 10% or less and the survival is extremely short. Therefore, the intent of treatment for recurrent ovarian cancer is palliative and there is only the very little prospect of cure (Lokadasan *et al.,* 2016).

With the recent development of targeted agents, it is expected that the disease-free survival can be further extended with reduced toxicity (Lokadasan *et al.,* 2016).

1.2.5.2.2 Surgery

1.2.5.2.2.1 Surgery for Early Stage EOC

Comprehensive surgical staging of apparent “early stage” EOC involves an omental biopsy, total abdominal hysterectomy, peritoneal washing, bilateral salpingo-oophorectomy, bilateral pelvic and para-aortic lymphadenectomy to the renal vessels, and systematic peritoneal surface biopsies (Schilder *et al.,* 2002).

1.2.5.2.2.2 Surgery for Advanced-stage EOC

The stage is apparent in patients with the seriously metastatic intraperitoneal disease. The objective of surgery in advanced disease is tumour cytoreduction (debulking). Tumour

cytoreduction of advanced EOC has theoretical and clinical better outcomes. The most beneficial approach to the management of advanced EOC is an attempt at optimal cytoreduction followed by postoperative chemotherapy (Juretzka *et al.,* 2007).

1.2.5.2.3 Radiotherapy

"The effects of radiotherapy on ovarian reserve rely on factors including the patient's age, the dose received by ovaries, and simultaneous use of chemotherapy. Radiotherapy has its side effects such as ovarian failure and can damage the uterus, as well. This adverse impact is notably made by altering its muscular vascularization as well as decreasing uterine volume, both of which lead to of fetus growth during the pregnancy in regard to those females who underwent radiotherapy during their childhood" (Morice *et al.,* 2010). "The atrophy of endometrial glands and stroma are likely to be observed in radiotherapy of uterus and cervix receiving high doses of radiation. Moreover, normally ulceration and necrosis remain for several months, which may be replaced by dense collagen deposition. The cervix gets quite atrophic and loses its elasticity specifically in older patients. It is worth mentioning besides uterine and ovary damage, irradiation may increase disorders probability of placenta attachment such as placental accrete or placental percreta" (Cohen 2008).

1.2.5.2.4 Immunotherapy

Current immunotherapies for ovarian cancer divided into six broad categories: monoclonal antibodies; oncolytic viruses; checkpoint inhibitors and immune modulators; therapeutic vaccines; adoptive T cell transfer; and adjuvant immunotherapies (Chester *et al.,* 2015).

As the new therapeutic target for ovarian cancer, lipolysis-stimulated lipoprotein receptor (LSR) was identified which had one of the largest significant differences in protein level between normal ovarian epithelial and ovarian cancer cell lines. High expression of LSR in ovarian cancer was the poor prognostic factor. Anti-LSR monoclonal antibody (mAb) has a

significant tumour growth inhibition in antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) independent manner. Anti-human LSR mAb also inhibits LSR function and shows direct tumour growth inhibition inducing G0/G1 cell cycle arrest. Targeting LSR by mAb is a promising therapy for patients with LSR-positive ovarian cancer (Hiramatsu *et al.,* 2015).

1.2.5.2.5 Targeted Therapy

Targeted therapy is one of the newest type of cancer treatment that uses drugs or other substances to recognize and attack cancer cells while doing little damage to normal cells. These drugs or other substances attack the cancer cells' inner workings the programming that makes them different from normal and healthy cells. Each type of targeted therapy has different action mechanisms, but all change the way a cancer cell grows, divides repairs itself, or interacts with other cells (Lokadasan *et al.,* 2016).

Angiogenesis plays an essential role in the initiation and ovarian cancer progression (Abu-Jawdeh *et al.,* 1996). An increased expression of Vascular endothelial growth factor (VEGF) and patient with VEGF gene polymorphism are independent prognostic factors of ovarian cancer (Shen *et al.,* 2000). VEGF is expressed in higher levels in serous adenocarcinoma and clear cell tumours of the ovary. VEGF expression is showed to be higher in advanced stage tumours as compared to those at an early stage (Yamamoto *et al.,* 1997).

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody and its target is vascular endothelial growth factor (VEGF)-A, and is showed for the treatment of metastatic colorectal cancer, renal cell carcinoma, non-small cell lung cancer, and glioblastoma multiform (Escudier *et al.,* 2007; Friedman *et al.,* 2009; Hurwitz *et al.,* 2004; Sandler *et al.,* 2006). This antibody binds to VEGF-A and reduces the effects of all biologically active forms of VEGF-A, and then suppresses tumour growth and inhibits metastatic disease progression (Lin *et al.,* 1999). The advantage of VEGF antibodies in the treatment of ovarian carcinoma

was initially discovered in animal models, where VEGF blockade was shown to inhibit ascites formation and slow tumour growth (Byrne *et al.,* 2003). In addition, VEGF-targeting agents are thought to enhance the effects of chemotherapy by normalization of primitive tumour vasculature, leading to decreased interstitial fluid pressure, increased tumour oxygenation, and enhanced delivery of cytotoxic drugs (Jain 2005).

1.2.5.2.6 Therapies That Inhibit DNA Repair

Inhibition of DNA repair in a way that switches off a tumour's compensatory repair mechanisms and stimulates cell death. DNA repair inhibitors, especially small-molecule inhibitors, hold great promise for damaging tumour cells. Their specificity can be taken into account to target a single step or single protein of a DNA repair pathway. Achieving that goal moves us closer to truly personalized medicine. However, the development of such inhibitors is offset by several real-world challenges (Kelley *et al.,* 2014).

Because preserving the genome is paramount, there are plenty of different DNA repair mechanisms and also with variant plans. If one pathway is unable to repair a problem, another pathway can get involved (Plummer et al., 2010: Kaina et al., 2007). That is the principle of synthetic lethality, and PARP inhibition is the leader in that principle (Kelley *et al.,* 2014).

The poly(ADP-ribose) polymerases (PARPs) are a large family of multifunctional enzymes that it exerts its function in the repair of single strand breaks in DNA via the base excision repair (BER) (Amé *et al.,* 2004). BRCA1 or BRCA2 mutation, resulting in a lack of homologous recombination, makes cells sensitive to inhibition of PARP activity, which in turn leads to chromosomal instability, cell cycle arrest, and subsequent apoptosis. The most compelling evidence of the efficacy of PARP inhibitors in the treatment of cancer comes from studies that comprised patients with BRCA1 or BRCA2 mutations (Bryant *et al.,* 2005; Farmer *et al.,* 2005). PARP inhibition causes cell cycle arrest, chromosomal instability, and subsequent apoptosis, which leads to be attributable to the persistence of DNA lesions that are

normally repaired by homologous recombination (Farmer *et al.,* 2005). PARP inhibitors develop improvement in progression-free survival in women with relapsed platinum-sensitive ovarian cancer, as evidenced mainly by olaparib added to conventional treatment (Wiggans *et al.,* 2015).

1.2.6 Major DNA Repair Mechanisms

In human cells, DNA repair is an active process and responds to damage in DNA structure in order to prevent the mutation being passed on to daughter cells (Malumbres and Barbacid 2009) and maintain genomic integrity (Wang *et al.,* 2000). There are series of cell cycle checkpoints at every phase of the cell cycle that regulates DNA repair pathways (Hoeijmakers 2001). The human DNA repair pathways are;

- Base Excision Repair (BER)
- Nucleotide Excision Repair (NER)
- Strand break repair
- Homologous Recombination Repair (HR)

1.2.6.1 Base Excision Repair

The base excision repair (BER) pathway is a cellular mechanism that corrects single-base (nonhelix-distorting) damage caused by hydroxylation, deamination, alkylation, and ionizing radiation (IR) (Seeberg *et al.,* 1995). BER pathway has two subpathways; the activation of one or the other is predicated by first the cause and type of damage, second in the first repair step the type of abasic (apurinic, apyrimidinic) (AP) site generated (Luo *et al.,* 2010) and third the cell cycle phase in progress when the damage occurs (Fortini *et al.,* 2007). BER includes two types of pathways which are short-patch and long-patch pathways. Single base damages are repaired quickly by the short-patch pathway during the G1 phase; the long-patch pathway manages lengthier repairs during S or G2 when resynthesis of two to eight nucleotides surrounding the AP site is needed (Fortini *et al.,* 2007).

1.2.6.2 Nucleotide Excision Repair (NER)

NER is a complex repair pathway and it functions to remove helix-distorting lesions caused by UV irradiation and chemical mutagens that crosslink adjacent purine bases and form intrastrand adducts (Fousteri and Mullenders 2008) and ICLs formed by chemotherapeutic agents such as Pt. Deficiencies in NER render cells sensitive to platinating agents, which cause the cell cycle arrest during the G2 phase. Nevertheless, intact NER activity contributes to chemoresistance because it can repair damage caused by cisplatin, carboplatin, and oxaliplatin (Neher *et al.*, 2011).

1.2.6.3 DNA Single Strand Break Repair

When only one of the two strands of a double helix has a defect, the other strand may be used as a template to manage the correction of the damaged strand. In order to repair damage to one of the two paired molecules of DNA, there are a number of excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand (Watson *et al.*, 2004). Damaged DNA is repaired by Nucleotide Excision Repair (NER) if it commonly consists of bulky, helix-distorting damage, such as pyrimidine dimerization caused by UV light (Reardon *et al.*, 2006). Mismatch repair systems are present in essentially all cells in order to correct errors that are not corrected by proofreading (Berg *et al.*, 2012).

1.2.6.4 DNA Double Strand Break Repair

Double-strand breaks, in which both strands of the double helix are severed, are particularly most toxic lesions to the cell because they can lead to genome rearrangements (Valerie and Povirk 2003). If repair is not possible, it can lead to cell death. Incorrect repairs may lead to chromosome rearrangements, loss of heterozygosity or loss of genetic information. These alterations may result in cell death or inheritable mutations and consequently, increase chances of developing cancer in the carriers of these alterations (Scott

and Pandita 2006). Activation of oncogenes or inactivation of tumour suppressor genes may be induced by this (Jackson 2002). Exogenous agents and chemotherapeutic agents (i.e. Cisplatin (Pt)) can induce DSBs.

1.2.6.5 Homologous Recombination

Homologous Recombination (HR), as it accurately corrects the most complicated and serious double-strand break (DSB) damage forms. HR controls predominantly during the S and G2 phases of the cell cycle so that it can find a large area of homology on a sister chromatid to use as a template for resynthesizing lost bases or damages (Chernikova *et al.*, 2012; Helleday *et al.*, 2010).

1.2.7 Ovarian Cancer Patients Become Resistant to Treatment by Chemotherapy

Become resistant to chemotherapeutic drugs tenders a serious limitation to the efficacy of the treatment of cancers (Boehm et al. 1997; Carvalho *et al.*, 2010). 90% of ovarian and breast cancer patients are acquiring resistance to treatment (Agarwal and Kaye 2003). The ways that have been suggested that lead to the development of drug resistance such as;

- Increased drug efflux
- Inactivation of drug
- Upregulated DNA repair
- Increased tolerance to DNA damage

P-glycoprotein (P-gp) is a membrane transporter protein encoded by the multidrug resistance (MDR1) gene increases drug efflux in tumour cells for preventing intracellular accumulation of chemotherapeutic agents by transporting the drug across the plasma membrane (Thiebaut *et al.*, 1987).

Development resistance may occur due to increased drug inactivation by cellular metabolites such as Glutathione (GSH) and also increase the level of DNA repair may also lead to the development of acquired resistance (Banath and Olive 2003; Siddik 2003). It is

found that there is an increased expression level of ERCC1 and ERCC2 and it is related to Pt resistance in human ovarian cancer patients (Dabholkar *et al.*, 1992).

1.2.7.1 Multiple Drug Resistance

Many anticancer drugs require metabolic activation, and thus cancer cells can also develop resistance through decreased drug activation. In patients with advanced ovarian cancer, treatment with platinum and taxane-based chemotherapy is applied post-operatively. One-way resistance to platinum can occur is through drug inactivation by methallothionein and thiol glutathione, which activate the detoxification system (Mehta *et al.*, 2009).

1.2.7.2 Drug Detoxification

It is possible that mutations or alterations in drug metabolizer enzymes may change these proteins' metabolic capabilities, such as increasing the breakdown of drugs and their secretion by the kidneys (Shen *et al.*, 2007). In this case, the drug would not maintain proper levels in the patient, and cancer would therefore be considered resistant to it. Another important example of drug activation and inactivation is observed in the GST superfamily, which is a group of detoxifying enzymes that function to protect cellular macromolecules from electrophilic compounds. GSTs assist in the development of drug resistance through direct detoxification and by inhibiting the mitogen-activated protein kinase (MAPK) pathway (Townsend *et al.*, 2003). An increase in the level of GST expression in cancer cells improves detoxification of the anticancer therapeutics, which results in less efficient cytotoxic damage of the cells (Monalitsas *et al.*, 1997). This increase is also related to resistance to apoptosis initiated by a variety of factors (Cumming *et al.*, 2001).

1.2.7.3 Changes in DNA Repair Capacity

The repair of damaged DNA has a certain role in anticancer drug resistance. In response to chemotherapy drugs that either directly or indirectly damage DNA, DNA damage

response (DDR) mechanisms can reverse the drug-induced damage. For example, platinum-containing chemotherapy drugs such as Cisplatin cause harmful DNA crosslinks, which is able to lead to apoptosis. However, resistance to platinum-based drugs often arises due to nucleotide excision repair and homologous recombination, the primary DNA repair mechanisms involved in reversing platinum damage (Bonanno *et al.,* 2014; Olaussen *et al.,* 2006; Selvakumaran *et al.,* 2003). Thus, the efficacy of DNA-damaging cytotoxic drugs depends on the failure of the cancer cell's DDR mechanisms.

1.3 Aims

The aim of this project was to determine whether drug resistance is associated with increased DNA repair as measured by FANCD2 and Mus81. DNA repair in normal cell lines was compared with resistant cell lines to evaluate this. To determine levels of FANCD2 and Mus81 proteins in Ovarian Cancer wild type and resistant cells, imaging flow cytometry was used.

2 MATERIALS AND METHODS

2.1.1 Cell Lines

A2780 is a human ovarian carcinoma cell line derived from tumour tissue from an untreated patient (Adam-Zahir *et al.,* 2014). Platinum-resistant cell lines derived from A2780 has been made resistant to Cisplatin. PEO1 is a human ovarian cancer cell line derived from patients with ovarian carcinoma and PEO1 was cisplatin sensitive (Sakai *et al.,* 2009).

2.1.2 Cell Culture

Cells (A2780, A2780cis and PEO1) were routinely cultured in RPMI 1640 (Scientific Laboratory Supplies, Nottingham, UK) which was supplemented with 100 U/ml penicillin and streptomycin (PAA Laboratories Ltd.), 10% foetal calf serum (SLS Ltd), 2mM L-glutamine and 100 units/mL (SLS Ltd). All cell lines were maintained as monolayers in RPMI 1640 at 37°C in a humidified atmosphere of 5% CO_2 in the air and were grown in 100mm Petri dishes (Sarstedt Ltd, Leicester, UK). All cell culture was carried out in a temperature-controlled tissue culture laboratory. Cell culturing process was carried out in a Heraeus Class II Laminar Flow hood.

2.1.3 Trypsinisation of Cells

Cells were harvested with trypsin when they reached about 80-90% confluency. When subculturing cells, the medium was first aspirated using a glass Pasteur pipette and cells were washed with 10 mL of PBS (Phosphate Buffered Saline (PBS) + 0.2% EDTA) (Severn Biotech Ltd, Kidderminster, UK). Approximately 1 mL of 0.25% Trypsin/EDTA (Fisher Scientific, Loughborough, Leicester, UK) was added to the plate and the cells were incubated in a humidified at 37°C, 5% CO_2 incubator for 5-10 minutes to allow cells to detach from the dish. Cells were then resuspended in 10 ml RPMI 1640 and were counted using the Countess (Invitrogen, Life Technologies, Paisley, UK). 10 μL of the cell and 10 μL of Trypan Blue were added in a sterile eppendorf for the cell count. Cell and Trypan blue mix were loaded

onto a glass and cell count performed with the Countess (Invitrogen, Life Technologies, Paisley, UK). Cells were seeded into plates at an approximate density of $2x10^6$ cells per plate for control, 6, 12, 24, 30 hours time points and also compensation samples and $1x10^6$ cells per plate for 48 and 72 hours time points.

2.1.4 Maintenance of Drug Resistance

After three passages of cells by trypsinisation, cells were exposed to 3μg/ml cisplatin (Sigma–Aldrich Ltd, Dorset, UK) for a period of 3-4 days after which the cells were replenished with fresh medium containing no drug.

2.1.5 Induction of DNA Damage in Cell Lines by 1 Hour Exposure to Cisplatin

12μg/ml cisplatin solution was created by dissolving cisplatin powder in DMSO and then in serum-free medium. Cells are exposed Pt for an hour. DNA damage induction was performed by using untreated control cells and cells exposed Pt for 1 hour. Drug included medium was aspirated from all samples after an hour. Cells were replenished with 10 ml complete medium containing no drug. Cells were incubated at 6, 12, 24, 30, 48 and 72 hours (hrs) post treatment. Cells were trypsinised at the different time points and then washed with 10 ml ice-cold PBS (Severn Biotech Ltd, Kidderminster, UK). Cells were collected using 1 mL of 0.25% Trypsin-EDTA and washed in ice-cold PBS (Severn Biotech Ltd, Kidderminster, UK) and then centrifuged at 1500 rpm for 5 min. They then were fixed in 1 ml ice-cold 50:50 (V:V) methanol:acetone and stored at -20°C until stained for imaging flow cytometry.

2.1.6 Immunocytochemistry and Antibody Staining

Fixed cells were rehydrated with a 5 minutes' incubation at room temperature (RT) in PBS. Cells were then permeabilised in PBS containing 0.5% Triton X-100 (Sigma-Aldrich, Dorset, UK) for 5 minutes on spinning wheel at 25 rpm at room temperature. After permeabilisation buffer was aspirated cells were resuspended in blocking buffer, comprised of

PBS containing 0.1% Triton X-100 and 10% Rabbit serum, for 1 hour on spinning wheel at 25 rpm at room temperature. Cells were incubated at 4°C overnight with 0.25 ml mouse monoclonal anti-FANCD2 antibody (clone 103, Abcam PLC, Cambridge, UK) at 1:1000 dilution in block buffer on spinning wheel at 25 rpm. Following three washes in wash buffer made up of PBS containing 0.1% Triton X-100, cells were incubated for 1hr at RT with Alexa Flour^{488} (AF^{488}) rabbit anti-mouse IgG (Life Technologies, Paisley, UK) at 1:1000 dilution in block buffer. Cells were washed again in wash buffer three times. Samples were then resuspended in 30 μL of Accumax flow cytometry buffer with 1 μM Draq5 (Biostatus Ltd., Leicestershire, UK). Draq5 was added to the sample prior to running on the ImageStream, providing DNA content and nuclear morphology features for the analysis. Two samples for fluorescence compensation were prepared with either antibodies or Draq5. These were fixed at 30 hours post treatment.

2.2 Imaging Flow Cytometry

Expression levels of key DNA repair proteins (Mus81, FANCD2) which are essential proteins involved in DNA interstrand crosslinks (ICL) repair was measured by Imaging flow cytometry. The Imagestream^{X} (Amnis Inc., Seattle, Washington, USA) system combines high-speed image capture with quantification of individual cells on their appearance in the flow. 10 000 images were captured using up to six different optical channels (Ch) 01 for brightfield (BF), and Ch05 for Draq5 to represent the nuclear staining of each cell. Following excitation with a 488 nm laser at a power setting of approximately 50- 100 mV, images were captured using a 40X objective to create a raw image file (rif).

Two samples for fluorescence compensation were prepared with either antibodies or Draq5 to detect the intensity of fluorescence in these samples. A compensation matrix was generated in order to separate fluorescence the emission spectra of dyes from the excitation light source as the long emission spectrum dyes causes overlap and leaks into other channels.

The sample fixed 30 hours post treatment with cisplatin was used to create a template file. This file was chosen due to being the highest induction of Mus81 and FANCD2 foci and therefore the brightest intensity of them. Templates were created for each A2780, A2780cis and PEO1 cell line.

The rif file created by the Inspire^{TM} software were analysed using the IDEAS software. The analysis comprises a multi-step process and it is initiated by pre-defined "building blocks" provided within the software. All unwanted images such as debris, doublet cells, and unfocused images were excluded from the population of cells being analysed. Histograms are generated to provide statistical information such as the population statistics, including means, medians, standard deviations, and standard statistical tests.

2.2.1 Cell Gating

The images provided with the Ideas software were gated for a number of parameters as described below.

2.2.1.1 Single cells

Figure 2.1: Identification of single cells

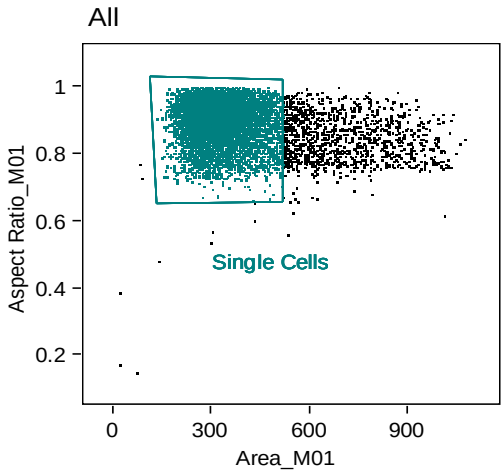

Figure 2.1: Identification of single cells. Single cells are gated within the entire population using the polygon tool. The area marked in blue indicates single cells. This is done to eliminate debris as well as doublet cells in order to use only single cells are used in the analysis.

2.2.1.2 Focused Cell

Figure 2.2: Identification of focused cells

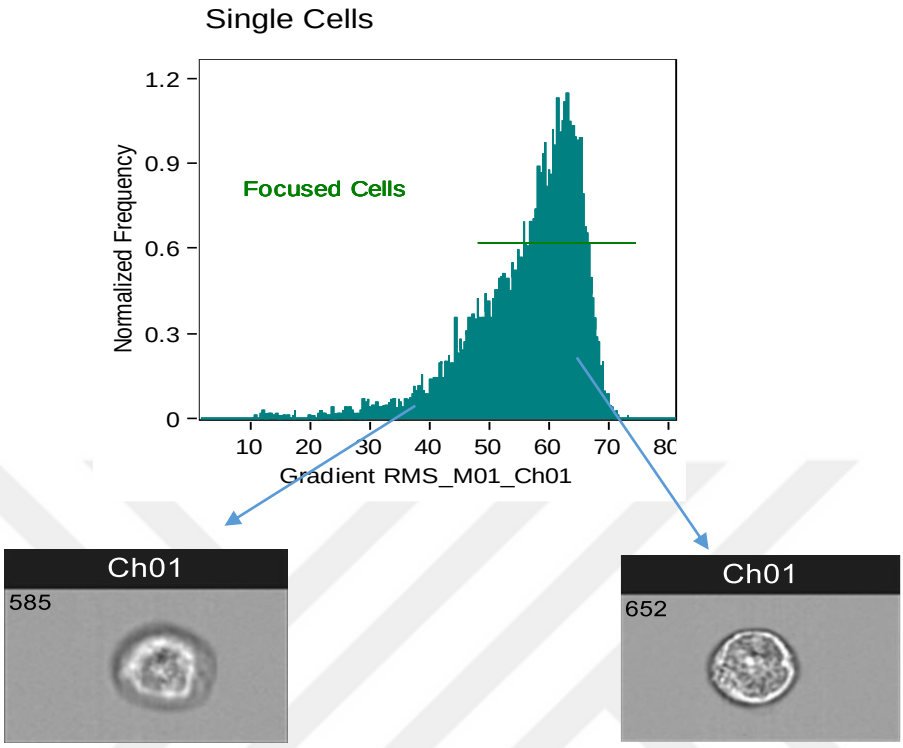

Figure 2.2: Identification of focused cells. Focused cells are gated using the Line region tool. The line region in green indicates focussed cells. Image of the cell out of focus and (on the left) Image of cell in focus (on the right).

2.2.1.3 Truth Population

A series of masks were created to determine the number of FANCD2 and Mus81 foci within the nucleus of each cell. To eliminate false or cytoplasmic staining, a "truth population" of cells with low and high numbers of FANCD2 and Mus81 foci were created by manually identifying cells with high and low foci numbers. A nuclear morphology mask was created to determine overall FANCD2 and Mus81 intensity in the nucleus and therefore overall levels of DNA damage. Images are shown in BF (Ch01), PE for FANCD2 staining (Ch02) and Draq5 for nuclear staining (Ch05). Cells are shown masked (Figure 2.3A) showing localisation of FANCD2 foci, also cells are shown unmasked in Figure 2.3B.

Figure 2.3: Demonstration of foci count

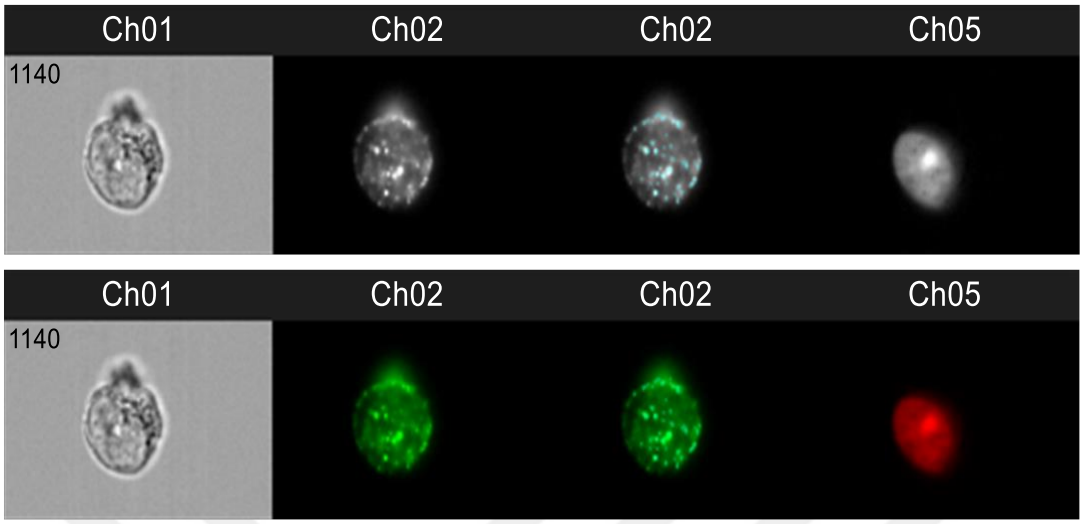

Figure 2.3 Black and white image of cells (upper line) with spot counting mark (light blue). Applied to the channel 2 image demonstrating how foci are counted. FANCD2 and Mus81 foci were identified and masked. A nuclear morphology mask is created using the Draq5 stained images from Ch05. Also, a spot mask is applied to the images seen in Ch02 (FANC2 and Mus81 foci). Light blue dots can be seen overlaying the foci demonstrating the identification of foci. Coloured image of cells (lower line) with spot counting mark (light green). Applied to the channel 2 image demonstrating how foci are counted.

To confirm the accuracy of the mask created, a histogram was plotted using only the truth population. The data analysis file (daf) that was created and was saved as a template file. This allows you to apply the same Spot masks and Intensity masks across all the time points that were collected for each cell line.

Figure 2.4: Foci distribution in cell line

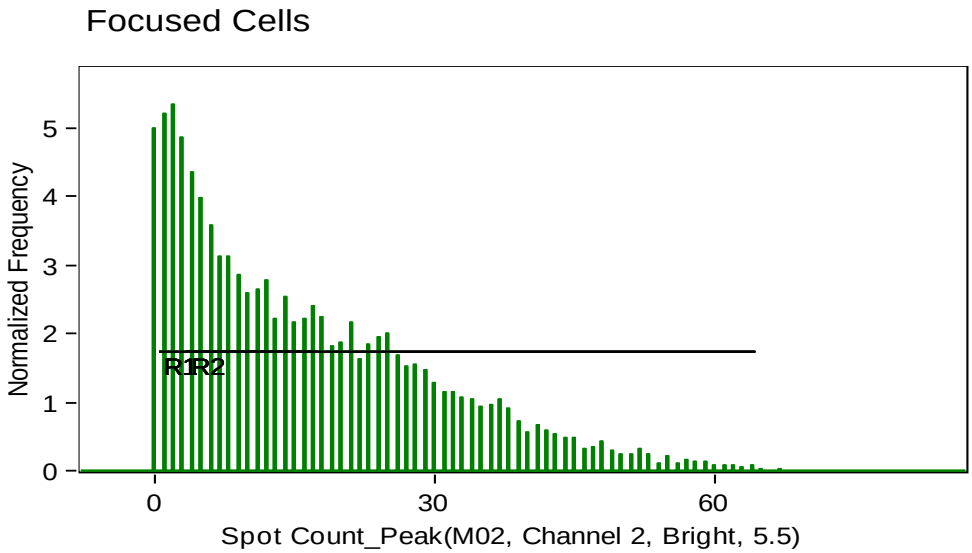

Figure 2.4: Foci Distribution in cell line over the 72-hour period post treatment to Cisplatin.

Figure 2.5: High and low foci count

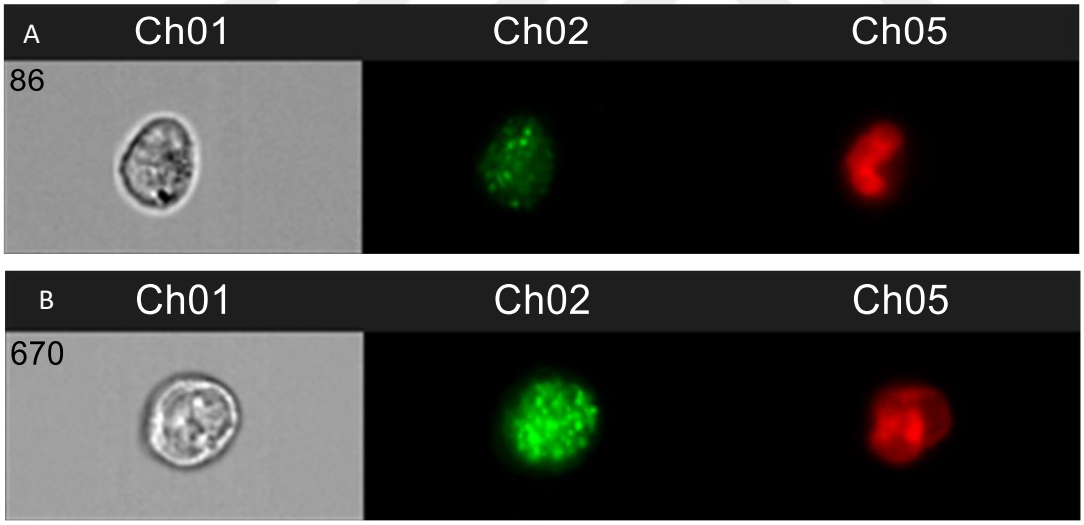

Figure 2.5A - shows a population of PEO1 cell lines with low Mus81 foci numbers and Figure 2.5B shows a population of PEO1 cell lines with high Mus81 foci numbers. 50 cells were chosen to identify the low and high numbers of foci accounted for different types of staining patterns such as foci shape and size, the intensity of the staining and the level of background. Channel 2 image demonstrating how average foci are counted by identifying low and high foci numbers. Ch01 shows BF images and green dots in Ch02 represents AF488 staining of Mus81 foci and Ch05 for Draq5 to represent the nuclear staining.

3 RESULTS

3.1 MUS81 foci induction in the ovarian cancer cell line PEO1

In this study, imaging flow cytometry was employed to acquire the necessary practical skills for future analysis of FANCD2 foci expression in ovarian cancer cell lines. The induction of the DNA repair endonuclease Mus81 in the PEO1 cell lines was determined by counting 5000 cells with the following exposure to 6,12,24,30,48,72 hrs Pt treatment.

Crossover junction endonuclease MUS81 is an enzyme that in humans are encoded by the MUS81 gene. Mus81-associated endonuclease resolves Holliday junctions into linear duplexes by cutting across the junction exclusively on strands of like polarity. In addition, Mus81 protein abundance rises in cells following exposure to agents that block DNA replication (Chen *et al.,* 2011). Mus81 is actually a member of the FA pathway. Mus81 operates outside of the FA pathway with respect to ICL repair (Larin *et al.,* 2014).

Figure 3.1: Representative examples of high and low Mus81 foci in the PEO1` ovarian cancer cell line

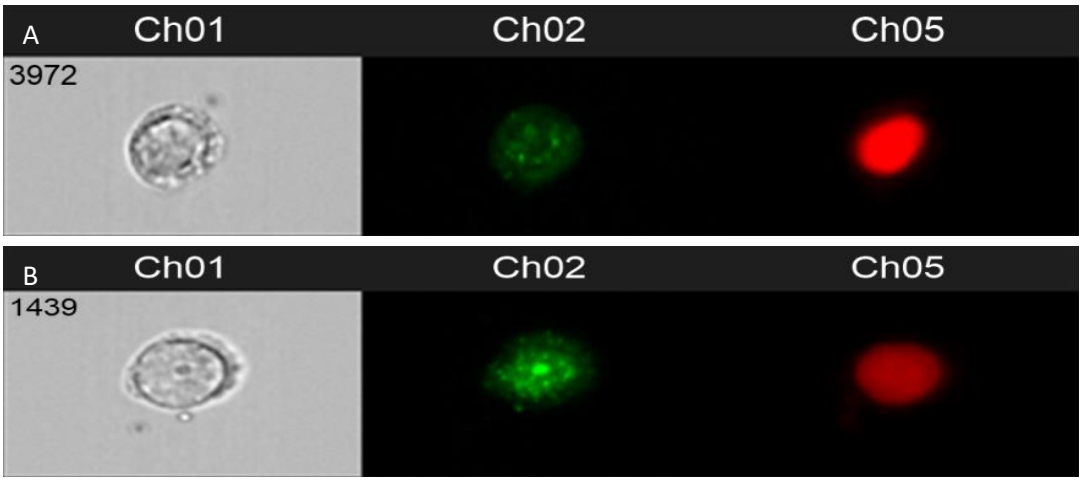

Figure 3.1A shows a population of PEO1 cell lines with low Mus81 foci numbers and Figure 3.1B shows a population of PEO1 cell lines with high Mus81 foci numbers. 50 cells were chosen to identify the low and high numbers of foci accounted for different types of staining patterns such as foci shape and size, the intensity of the staining and the level of background. Channel 2 image demonstrating how average foci are counted by identifying low and high foci numbers. Ch01 shows BF images and green dots in Ch02 represents AF488 staining of Mus81 foci and Ch05 for Draq5 to represent the nuclear staining.

Figure 3.2: Average foci numbers for the PEO1` ovarian cancer line treated with Cisplatin

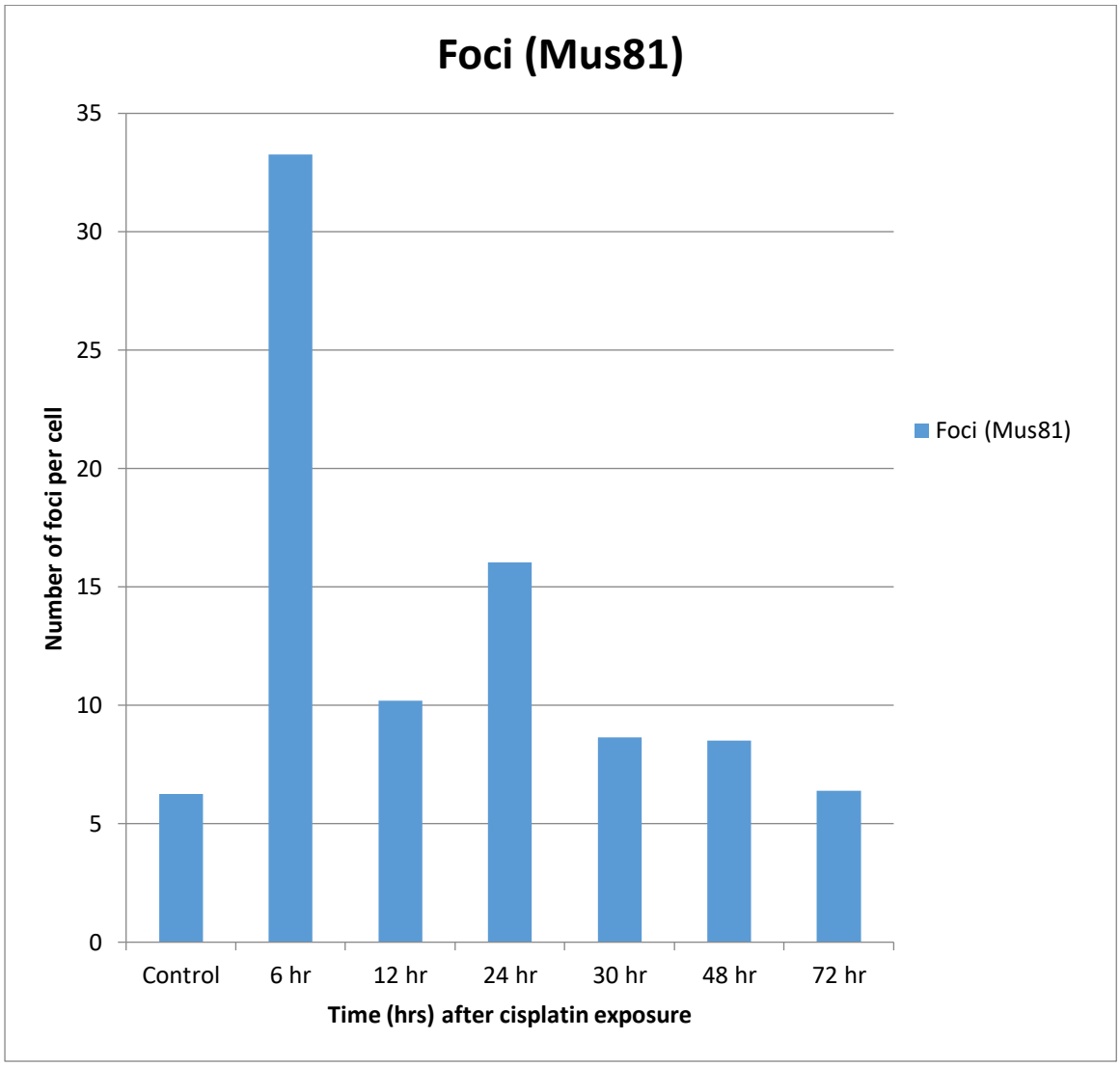

Figure 3.2: Average foci numbers for cells treated with Cisplatin. The geo mean a number of Mus81 foci seen in PEO1 cell line over a 72 hr period post treatment to cisplatin measured using in situ immunofluorescence. It can be observed that in untreated control cells there is an average of 6.246 foci per cell. This increases dramatically at 6hrs post exposure to cisplatin were on average 33.26 foci per cells are observed. This declines rapidly after 6hrs and by 30 hours post exposure, the number of foci per cell has decreased to near control levels indicating repair of DNA cross-links.

Figure 3.3: Foci distribution in the PEO1 cell line

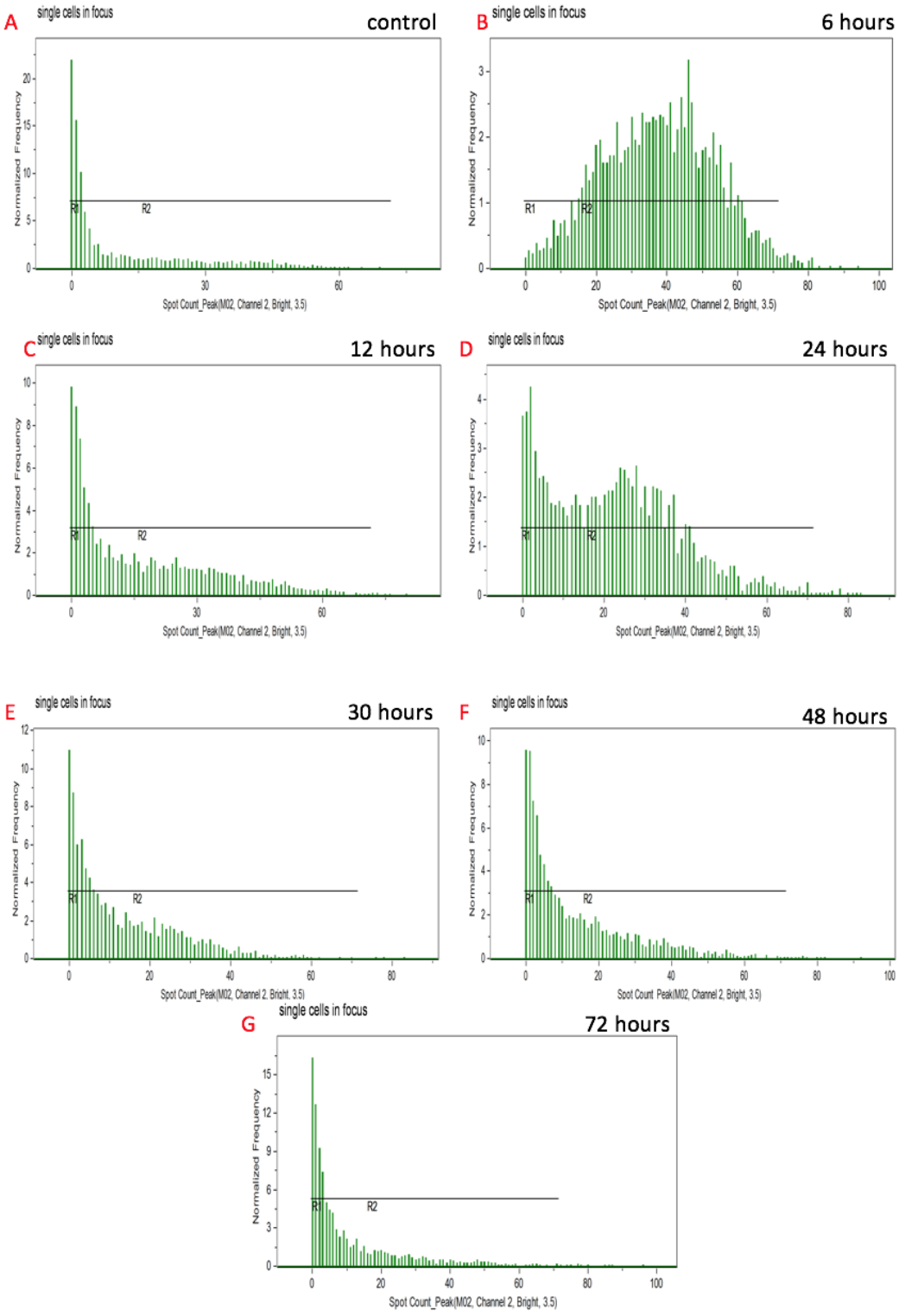

Figure 3.3: Foci Distribution in the PEO1 cell line over the 72-hour period post treatment to Cisplatin. The above figures show the foci distribution seen in PEO1 cell line. A dramatic induction in Mus81 foci induction was seen at 6 hours post treatment to Cisplatin. At 72 hrs post treatment, is similar to untreated control.

3.2 Comparison of FANCD2 foci in A2780 W/T and Resistant

To determine if drug resistance was associated with FANCD2 expression, foci numbers were quantified at different time points over a maximum of 72 hr after 1 hr of exposure to Pt (fig. 3.4).

The strand distortion caused by an ICL is recognised by proteins of Fanconi anemia (FA) and FANCD2 functions in the FA pathway. Fanconi anemia group D2 protein is a protein that in humans are encoded by the FANCD2 gene. FANCD2 is involved in cellular decision-making in response to DNA damage (Berger et al., 1993).

Figure 3.4: Images of FANCD2 foci with A2780cis and A2780 parental cell lines

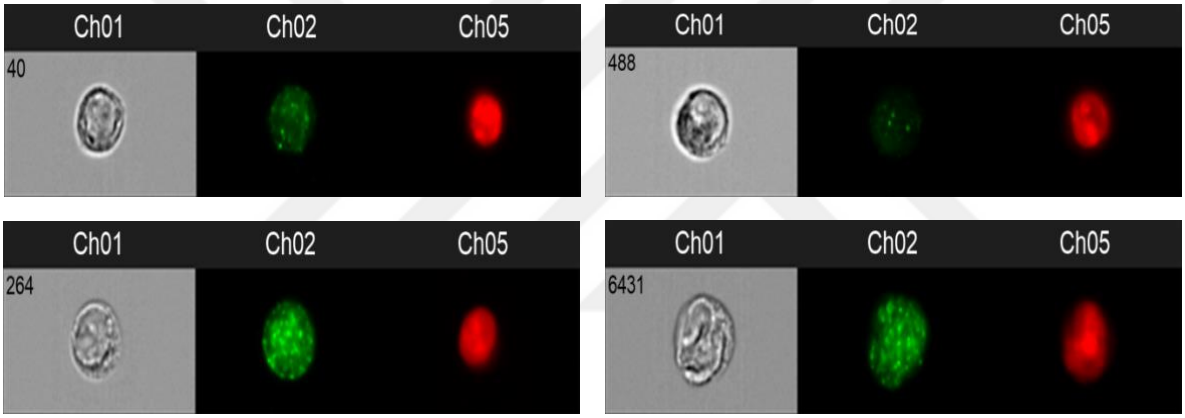

Figure 3.4: Images of FANCD2 foci with A2780cis (on the left) and A2780 parental (on the right) cell lines. Images are shown on upper line demonstrates A2780 parental and A2780cis cell lines with low foci numbers. Images are shown on lower line demonstrates A2780 parental and A2780cis cell lines with high foci numbers.

Figure 3.5: Average foci numbers for the A2780 parental and A2780cis` ovarian cancer lines treated with Cisplatin

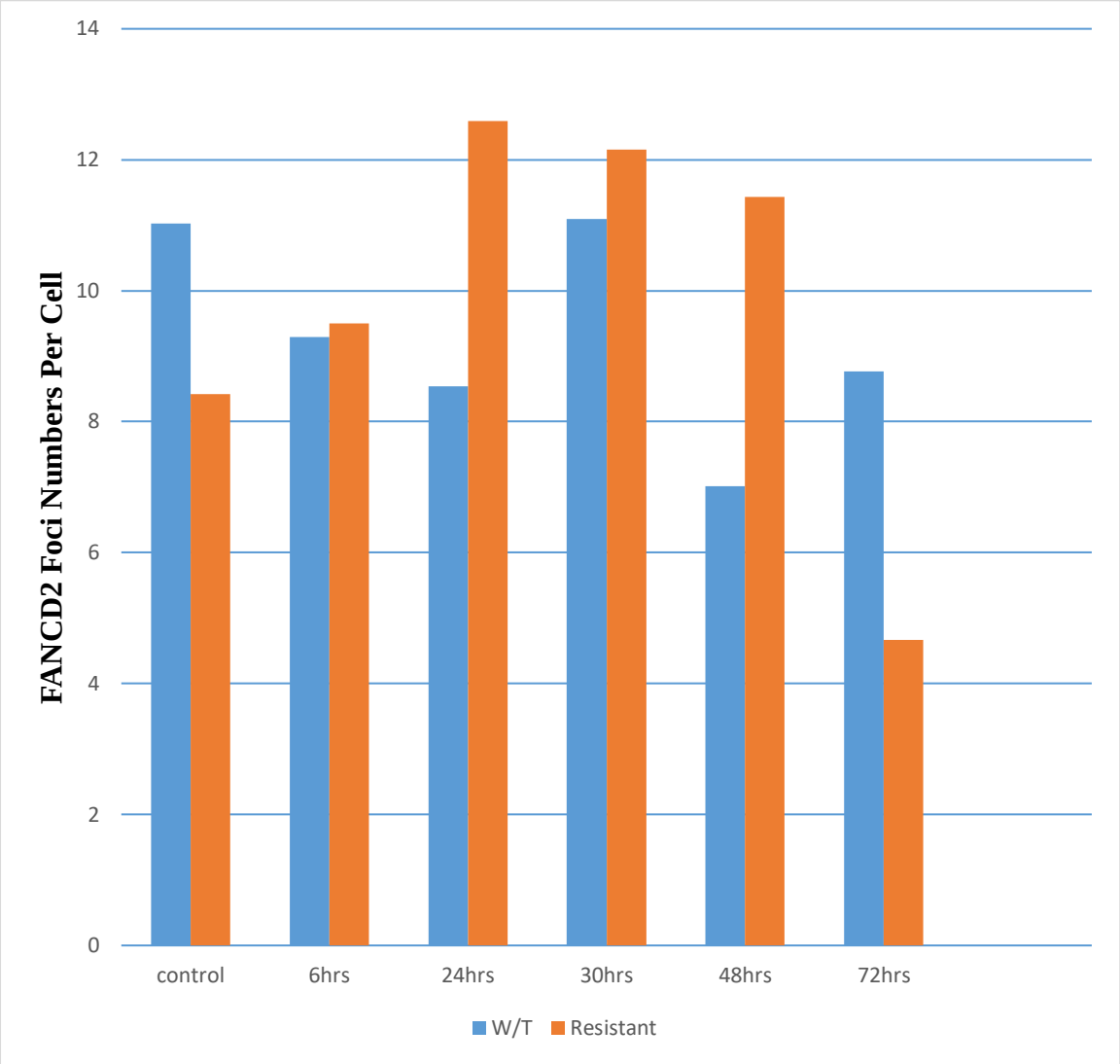

Figure 3.5: The geo mean number of FANCD2 foci seen in A2780 W/T and A2780cis cell lines over a 72-hour period post treatment to cisplatin measured using in situ immunofluorescence. The A2780cis cell line indicates increased FANCD2 foci formation at 24 hr in comparison to the A2780 parental cell line. The A2780 cell line shows a similar repair profile to the resistant cell line with peak foci formation. Chi-square analysis was used to compare the distribution of foci in resistant and parental cell lines over a 72-hour period post treatment to the chemotherapeutic agent Pt. This was performed over the whole time course of the experiment with a p value <0.05 being considered as significant. There is no significant difference between parental and drug-resistant cell lines with a p value 0.196.

Figure 3.6: Foci Distribution in the A2780cis cell line over the 72-hour period post treatment to Cisplatin

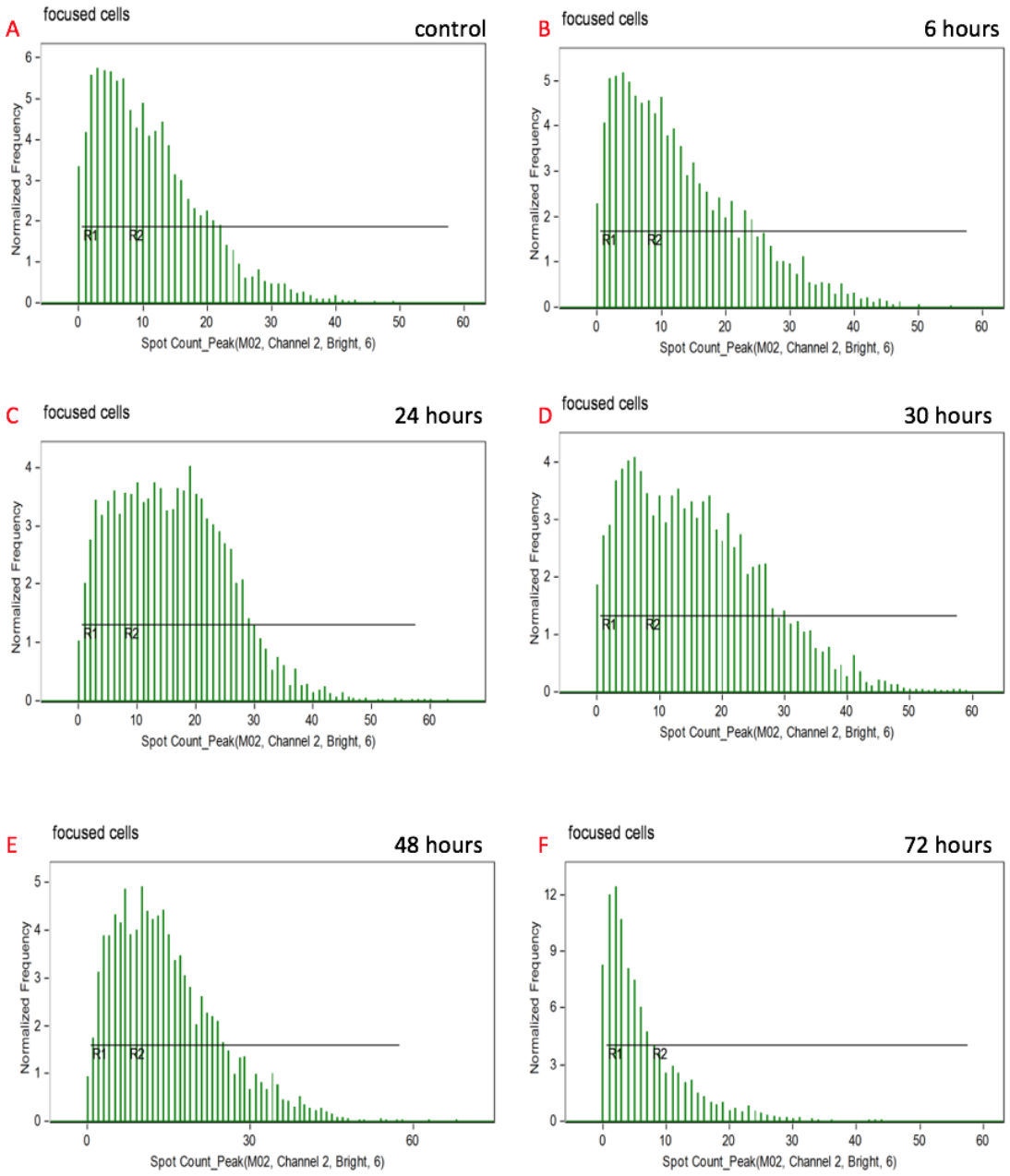

Figure 3.6: Foci Distribution in the A2780cis cell line over the 72-hour period post treatment to Cisplatin. The above figures show the foci distribution seen in A2780cis cell line. The A2780cis cell line indicates a peak of FANCD2 foci formation at 24 hr. A dramatic induction in Mus81 foci induction was seen at 6 hours post treatment to Cisplatin. At 72 hrs post treatment, is similar to untreated control.

Figure 3.7: Foci Distribution in the A2780 parental cell line over the 72-hour period post treatment to Cisplatin

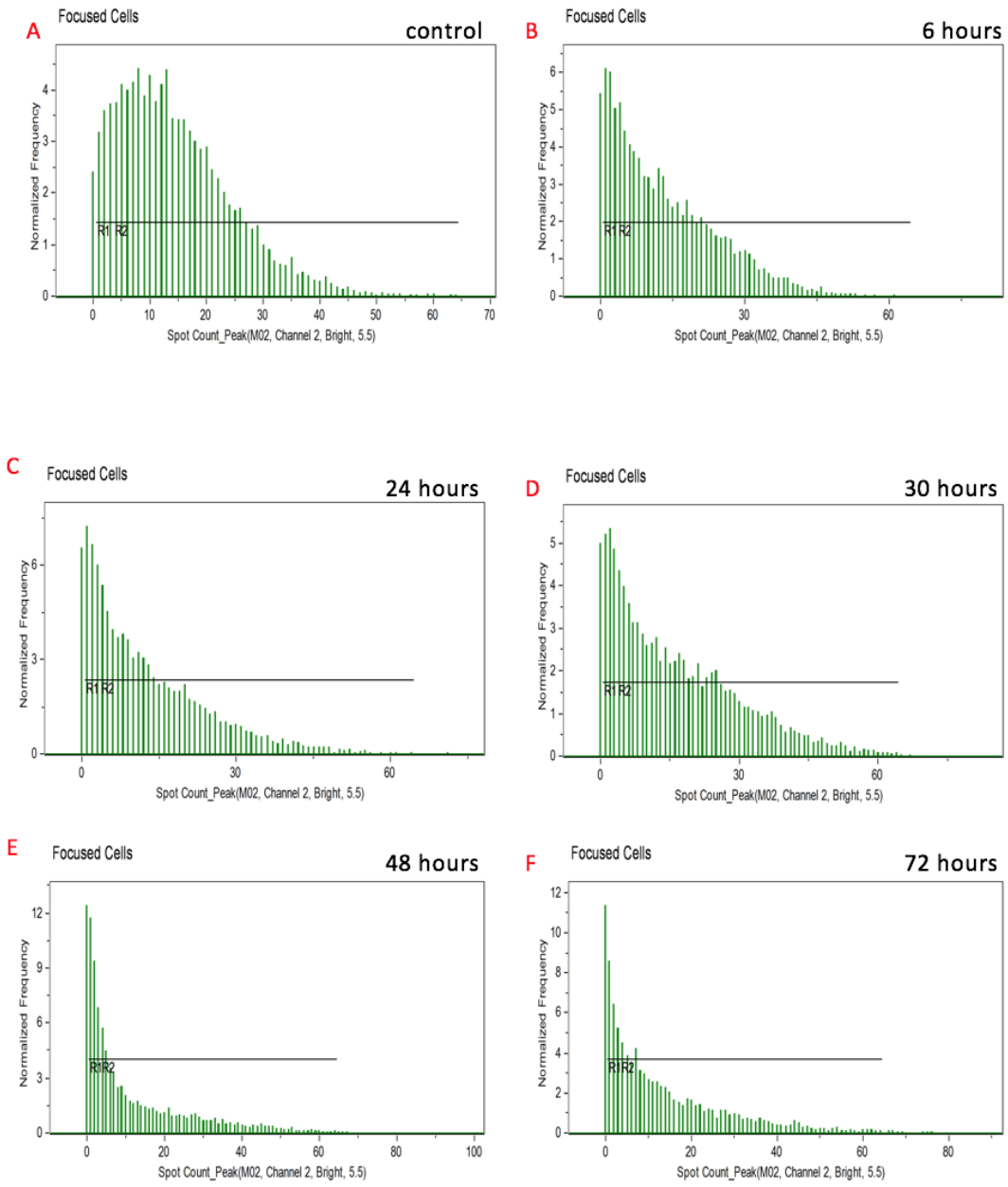

Figure 3.7: Foci Distribution in the A2780 parental cell line over the 72-hour period post treatment to Cisplatin. The above figures show the foci distribution seen in the A2780 parental cell line. The A2780cis cell line indicates a peak of FANCD2 foci formation at 24-hour. A dramatic induction in Mus81 foci induction was seen at 6 hours post treatment to Cisplatin. At 72-hour post treatment, is similar to untreated control.

4 Discussion

In this project differences in FANCD2 foci induction between the A2780 resistant and the A2780 parental cell lines were compared to identify resistant cellular phenotypes to chemotherapeutic drug Cisplatin. Additionally, it was demonstrated that Mus81 foci induction in the PEO1 cell lines over a 72-hour period post treatment with the cross-linking agent Cisplatin (Pt). Imaging flow cytometry was used to detect of FANCD2 and Mus81 foci post exposure with chemotherapeutic agent Pt. Three different cell lines were applied in this project:

- A2780 (derived from untreated ovarian cancer patient)
- A2780cis (Pt resistant: made resistant in vitro by chronic drug exposure)
- PEO1 (Pt sensitive: ovarian cancer cell line)

The cell lines were utilised in this project are; A2780 parental cell line which is a human ovarian carcinoma cell line derived from tumour tissue from an untreated patient, A2780cis Platinum-resistant cell line which derived from A2780 has been made resistant to Cisplatin and also PEO1 Platinum-sensitive cell line which is a human ovarian cancer cell line derived from patients with ovarian carcinoma (Sakai *et al.,* 2009).

The Imagestream^X (Amnis Inc., Seattle, Washington, USA) was employed to measure expression levels of key DNA repair proteins (Mus81, FANCD2). which appear as DNA damage foci. These experiments were conducted in order to determine whether drug resistance is associated with increased expression of the DNA repair protein Mus81 and FANCD2. DNA interstrand crosslinks (ICL) repair in normal cell lines was compared with resistant cell lines over the 72-hour period post treatment to Pt. Imaging flow cytometry system allows the capture images of cells in real time during the flow of cell through the fluidics system allowing the capture of up to 1000 cells per second. The system allows the assessment of cell with the given quantification in flow and gives morphology fluorescence

localisation, and population statistics. The Imagestream provides 6 different optical channels and images were captured using up to this six different optical channels. Only three channel were used in this experiment which is Ch01 for brightfield (BF) to give an idea for individual cell morphology, Ch02 to represent the antibody staining (Mus81 and FANCD2) and Ch05 for Draq5 to represent the nuclear staining of each cell. 10 000 single cells were acquired with a 488 nm laser at a power setting of approximately 50- 100 mV using a 40X objective to create a raw image file (rif). These files were used to create data analysis file (daf) to analysis data by using the Ideas^{TM} software.

Treatment failure due to drug resistance is a major issue in clinical management in many cancers such as ovarian cancer. Therefore, examining the DNA repair of DNA interstrand crosslinks may reveal the role of elevated DNA repair in the role of treatment failure and drug resistance in ovarian cancer (Boehm *et al.,* 1997; Carvalho *et al.,* 2010). Antibodies that target specific DNA repair proteins can be useful to investigate understanding of DNA interstrand crosslinks repair mechanisms (Agarwal and Kaye 2003). This was highlighted by Adam-Zahir, (2014) who found that there was a significant increase in expression levels of DNA repair biomarkers Rad51 and gamma-H2AX in human cell lines resistant to the chemotherapeutic agents nitrogen mustard and Pt. Therefore, finding a relationship between expression levels of the DNA repair biomarker FANCD2 and the DNA ICL repair mechanism that could provide an understanding of induced drug resistance would aid in improving patient outcome before undergoing therapy.

As our result, the A2780 cell line shows a similar repair profile to the resistant cell line with peak foci formation. Chi-square analysis was carried to compare the distribution of foci in resistant and parental cell lines over a 72-hour period post treatment to the chemotherapeutic agent Pt. This was achieved across the whole time course of the experiment with a p value <0.05 being considered as significant. There is no significant difference

between parental and drug-resistant cell lines with a p value 0.196.

Repair of DNA crosslinks is regulated by the FA genes in a genome stability pathway. Once FANCD2 and FANCI are ubiquitinated that leads to the formation of active repair complexes on chromatin, thus FA pathway is activated. The FA pathway activation is formed not only by DNA crosslinks but also by treatment. BRCA2 functions as a mediator of homologous recombination (HR) and inhibits the ATPase activity of RAD51 and by catalyzing the formation of RAD51 nucleofilaments in order to repair double strand breaks (Heyer *et al.,* 2010).

FA and HR pathways are linked genetically and biochemically. FANCD2 and BRCA2 cooperate and co-localize in DNA damage-induced foci (Hussain *et al.,* 2004). Several HR proteins, including BRCA2 itself, are encoded by FA genes (Howlett *et al.,* 2002; Welcsh and King 2007). The upstream FA proteins and BRCA2/FANCD1 participate in a common pathway for tumor suppression, termed the Fanconi Anemia/BRCA pathway. FA proteins may recruit DNA polymerases to restart the fork but the mechanism is still not well-known (Moldovan *et al.,* 2012).

In patients with metastatic ovarian cancer, most relapse and eventually die due to the development of drug resistance. In act, 5-year survival for ovarian cancer is currently approximately 35% (CR-UK statistics). Drug resistance is thought to be a major issue by causing treatment failure and death in more than 90% of patients with metastatic disease. Drug resistance can develop due to pharmacokinetic, tumour micro-environmental and cancer-cell-specific abnormalities. A number of resistance mechanisms have been identified *in vitro*. Overall, a better understanding of the FA pathway and its regulation of DNA repair could provide an understanding of induced drug resistance this would allow improvement in therapy for cancer patients displaying resistance to anticancer chemotherapy (Agarwal and Kaye 2003).

FA pathway has shown a complex interaction of nucleolytic incision, and HR repair steps initiated from an ubiquitin signaling pathway (Kim *et al.,* 2012). FANCD2-Ub is an essential gateway to the ICL repair process, connecting upstream signaling with downstream enzymatic repair steps. The biochemical and genetic analyses of the pathway have also provided a rationale for platinum-based chemotherapies in cancer treatment. (Kim *et al.,* 2012). With that FANCD2 monoubiquitination and FANCD2 nuclear foci, the formation has been indicated to be prognostic in drug resistance (Chen *et al.,* 2007; Taniguchi *et al.,* 2003).

mTOR plays a critical role in anti-cancer drug resistance (Guo *et al.,* 2013) and a correlation of mTOR-associated genes with FANCD2 gene expression has been proven in alveolar rhabdomyosarcoma patients (Singh *et al.,* 2014). In mTOR signaling, the FANCD2 expression is differentially controlled by NF-κB in normal cells versus cancer cells. In normal cells (e.g. mouse hematopoietic stem and progenitor cells, human B lymphoblasts, mouse embryonic fibroblasts), it is shown that mTOR inhibition leads to an increased NF-κB activation, which in turn suppresses FANCD2 expression (Guo *et al.,* 2013), whereas, in cancer cells (e.g. T-ALL), mTOR inhibition leads to a decreased NF-κB activation and FANCD2 expression (Guo *et al.,* 2014).

Despite the improvement in cancer chemotherapy and targeted therapy, alkylating agents such as Pt still has a major role in treatment. But its action is limited as patients acquire resistance to cisplatin-induced DNA damage (Boehm et al., 1997; Carvalho 2010). Finding an increase in the level of proteins involved in HR such as FANCD2 can offer personalised treatment to patients with acquired resistance to Pt. It is interesting to note that Rudland *et al.,* (2010) showed how a cohort of breast tumours exhibiting reduced cytoplasmic FANCD2 expression was correlated with poor survival outcomes. A combination of FA protein inhibitors and the platinum drug may be more effective than either drug alone.

Tumour cell lines are widely used to study the biology of cancer and to examine the factors influencing the response of tumours to therapeutic agents and regimens (Rockwell 1980). It can be conceded that utilising actual tumour samples might provide a more realistic reflection of the role of altered DNA repair in the development of drug resistance and recurrent ovarian cancer. Experiments can be replicated. I cannot generalise from the results of a single experiment (as was the case in this investigation) and it could not be possible to repeat this experiment due to the lack of time. Therefore, more replicate experiments should be considered in future investigations. In this experiment only one pair of cell line applied which was not enough to determine the relation between FANCD2 and DNA crosslink. For further work would involve

- Increase the number of cell lines
- Include cell lines that have been made resistant to other chemotherapeutic drugs
- Assess other biomarkers representing the FA pathway
- Use actual tumour samples from untreated and recurrent, drug resistance ovarian cancer

References

Abu-Jawdeh GM, Faix JD and Niloff J et al (1996). Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms. Lab Investig J Tech Methods Pathol 74(6) 1105–1115.

Adam Zahir S, Plowman PN, Bourton EC, Sharif F, Parrıs CN. Increased γ-H2AX and Rad51 DNA repair biomarkers expression in human cell lines resistant to the chemotherapeutic agents nitrogen mustard and cisplatin. Chemotherapy 2014; 60:310-320.

Agarwal, R. and S. B. Kaye (2003). "Ovarian cancer: strategies for overcoming resistance to chemotherapy." Nat Rev Cancer 3(7): 502-516.

Amé JC, Spenlehauer C, de Murcia G. The PARP superfamily (2004). Bioessays 26, 882–893.

Appella E, and Anderson CW (2000). Pathol. Biol. (Paris), 48, 227–245.

Atack DB, Nisker JA, Allen HH, et al (1986). CA 125 surveillance and second-look laparotomy in ovarian carcinoma. Am J Obstet Gynecol 154 (2): 287-9, 1986.

Banath, J. P., and P. L. Olive (2003). "Expression of phosphorylated histone H2AX as a surrogate of cell killing by drugs that create DNA double-strand breaks." Cancer Res 63(15): 4347-4350.

Bao J, Zervos AS (1996). Isolation and characterization of Nmi, a novel partner of Myc proteins. Oncogene. 1996;12:2171–2176.

Berek JS, Bast RC Jr. Epithelial Ovarian Cancer. In: Kufe DW, Pollock RE, Weichselbaum RR, et al., editors. Holland-Frei Cancer Medicine. 6th edition. Hamilton (ON): BC Decker; 2003. Available at: http://www.ncbi.nlm.nih.gov/books/NBK12433/

Berek JS, Knapp RC, Malkasian GD, et al (1986). CA 125 serum levels correlated with second-look operations among ovarian cancer patients. Obstet Gynecol 67 (5): 685-9, 1986.

Berg, M; Tymoczko, J; Stryer, L (2012). Biochemistry 7th edition. New York: W.H. Freeman and Company. p. 840. ISBN 9781429229364.

Berger, R., Le-Coniat, M. & Gendron, M.C. Fanconi anemia. Chromosome breakage and cell cycle studies. Cancer genet. *Cytogenet.* 68, 13−16 (1993).

Boehm, T., J. Folkman, T. Browder and M. S. O'Reilly (1997). "Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance." Nature 390(6658): 404- 407.

Bonanno L., Favaretto A., Rosell R. Platinum drugs and DNA repair mechanism in lung cancer. Anticancer Res. 2014;34:493–502.

Bryant, H. E. et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 434, 913–917 (2005).

Byrne, A., Ross, L., Holash, J., Nakanishi, M., Hu, L., Hofmann, J. et al. (2003) Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res 9: 5721–5728.

Carvalho, H., L. M. Garrido, R. L. Furlan, G. Padilla, M. Agnoletto, T. Guecheva, J. A. Henriques, J. Saffi and C. F. Menck (2010). "DNA damage induced by the anthracycline cosmomycin D in DNA repair-deficient cells." Cancer Chemother Pharmacol 65(5): 989-994.

Chen CC, Taniguchi T, D'Andrea A. The Fanconi anemia (FA) pathway confers glioma resistance to DNA alkylating agents. J Mol Med (Berl) 2007;85:497–509.

Chen XB, Melchionna R, Denis CM, Gaillard PH, Blasina A, Van de Weyer I, Boddy MN, Russell P, Vialard J, McGowan CH (Nov 2001). "Human Mus81-associated endonuclease cleaves Holliday junctions in vitro". Molecular Cell. 8 (5): 1117–27.

Chernikova SB, Game JC, Brown JM. Inhibiting homologous recombination for cancer therapy. Cancer Biol. Ther. 2012; 13(2):61–68. [PubMed: 22336907]

Cancer Research UK, http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/ovarian-cancer/incidence, Accessed 09.2016.

Chester C., Dorigo O., Berek J.S., Kohrt H. Immunotherapeutic approaches to ovarian cancer treatment. J. Immunother. Cancer. 2015;3:7. doi: 10.1186/s40425-015-0051-7.

Cohen LE. Cancer treatment and the ovary: the effects of chemotherapy and radiation. Ann N Y Acad Sci 2008; 1135: 123-125.

Cuatrecasas M, Erill N, Musulen E, Costa I, Matias-Guiu X, Prat J. K-ras mutations in nonmucinous ovarian epithelial tumors: a molecular analysis and clinicopathologic study of 144 patients. Cancer. 1998;82:1088–1095.

Cumming R.C., Lightfoot J., Beard K., Youssoufian H., O'Brien P.J., Buchwald M. Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1. Nat. Med. 2001;7:814–820. doi: 10.1038/89937

Dabholkar, M., F. Bostick-Bruton, C. Weber, V. A. Bohr, C. Egwuagu and E. Reed (1992). "ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients." J Natl Cancer Inst 84(19): 1512-1517.

Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Inhibition of SRC tyrosine kinase impairs inherent and acquired gemcitabine resistance in human pancreatic adenocarcinoma cells. Clin Cancer Res 2004; 10: 2307–2318.

Eccles, D. M., Cranston, G., Steel, C. M., et al. Allele losses on chromosome 17 in human epithelial ovarian carcinoma. Oncogene. 5: 1599-1601. 1990.

Erickson BK., Conner MG. et al. The fallopian tube in the origin of ovarian cancer. Am J Obstet Gynecol. (2013); 209(5): 409-414.

Escudier, B., Pluzanska, A., Koralewski, P., Ravaud, A., Bracarda, S., Szczylik, C. et al. (2007). Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double- blind phase III trial. Lancet 370: 2103–2111.

Farmer, H. et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 434, 917–921 (2005).

FIGO Cancer Committee, Staging Anouncement. Gynecologic Oncology 25, 383-385 (1986).

Finlay, C. A., Hinds, P. W., Tan, T-H., et al. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-line. Mol. Cell. Biol., 8: 531-539, 1988.

Forbes S, Clements J, Dawson E., et al. 2006. Cosmic 2005. Br J Cancer 94:318–322.

Fortini P, Dogliotti E. Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways. DNA Repair. 2007; 6(4):398–409. [PubMed: 17129767]

Fousteri, M. and L. H. Mullenders (2008). "Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects." Cell Res 18(1): 73-84.

Friedman, H., Prados, M., Wen, P., Mikkelsen, T., Schiff, D., Abrey, L. et al. (2009). Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. J Clin Oncol 27: 4733–4740.

Gemignani ML, Schlaerth AC, Bogomolniy F, Barakat RR, Lin O, Soslow R, Venkatraman E, Boyd J. Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma. Gynecol Oncol. 2003;90:378–381.

Goff BA, Mandel L, Muntz HG, et al.: Ovarian carcinoma diagnosis. Cancer 89 (10): 2068-75, 2000.

Guo F, Li J, Du W, Zhang S, O'Connor M, et al. mTOR regulates DNA damage response through NF-κB-mediated FANCD2 pathway in hematopoietic cells. Leukemia. 2013;27:2040–2046.

Guo F, Li J, Zhang S, Du W, Amarachintha S, et al. mTOR kinase inhibitor sensitizes T-cell lymphoblastic leukemia for chemotherapy-induced DNA damage via suppressing FANCD2 expression. Leukemia. 2014;28:203–206.

Helleday T. Homologous recombination in cancer development, treatment and development of drug resistance. Carcinogenesis. 2010; 31(6):955–960. [PubMed: 20351092]

Heyer WD, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. Annual review of genetics. 2010; 44:113–139.

Hiramatsu K, Serada S, Enomoto T, Nakagawa S, Morimoto A, Fujimoto M, Yokoyama T, Takahashi Y et al: Anti-human LSR monoclonal antibody inhibits tumour growth of ovarian cancer directly. Cancer Res Aug 2015 (75) (15 Supplement) 4382; DOI: 10.1158/1538-7445.AM2015-4382.

Hoeijmakers, J. H. (2001). "Genome maintenance mechanisms for preventing cancer." Nature 411(6835): 366-374.

Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, De Die-Smulders C, Persky N, Grompe M, Joenje H, Pals G, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. Science New York, NY. 2002; 297:606–609.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W. et al. (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350: 2335–2342.

Hussain S, Wilson JB, Medhurst AL, Hejna J, Witt E, Ananth S, Davies A, Masson JY, Moses R, West SC, et al. Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. Human molecular genetics. 2004; 13:1241–1248. [PubMed: 15115758]

Jackson, S. P. (2002). "Sensing and repairing DNA double-strand breaks." Carcinogenesis 23(5): 687-696.

Jain, R. (2005) Antiangiogenic therapy for cancer: current and emerging concepts. Oncology (Williston Park) 19(Suppl. 3): S7–S16.

Juretzka MM, Abu-Rustum NR, Sonoda Y, et al.: The impact of video-assisted thoracic surgery (VATS) in patients with suspected advanced ovarian malignancies and pleural

effusions. Gynecol Oncol 2007, 104:670–674.

Kelley MR, Logsdon D, Fishel ML. Targeting DNA repair pathways for cancer treatment: what's new? Future Oncol. 2014 May; 10(7): 1215–1237.

Kim H, D'Andrea AD. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway (2012). Available online:http://www.genesdev.org/cgi/doi/10.1101/gad.195248.112.

Larin M., Gallo D., Tamblyn L. et al., (2014). Fanconi anemia signaling and Mus81 cooperate to safeguard development and crosslink repair. Nucleic Acids Res. 2; 42(15): 9807–9820.

Li H, Lee TH, Avraham H. A novel tricomplex of BRCA1, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer. J Biol Chem. 2002;277:20965–20973.

Lin, Y.S., Nguyen, C., Mendoza, J.L., Escandon, E., Fei, D., Meng, Y.G. et al. (1999) Preclinical pharmacokinetics, interspecies scaling, and tissue distribution of a humanized monoclonal antibody against vascular endothelial growth factor. J Pharmacol Exp Ther 288: 371–378.

Lokadasan R., James F. V., Narayanan G., Prabhakaran K. Targeted agents in epithelial ovarian cancer: review on emerging therapies and future developments. ecancer 2016, 10:626 DOI: 10.3332/ecancer.2016.626

Luo M, He H, Kelley MR, Georgiadis MM. Redox regulation of DNA repair: implications for human health and cancer therapeutic development. Antioxid. Redox Signal. 2010; 12(11):1247– 1269.

Malumbres, M. and M. Barbacid (2009). "Cell cycle, CDKs and cancer: a changing paradigm." Nat Rev Cancer 9(3): 153-166.

Manolitsas TP, Englefield P, Eccles DM, Campbell IG. No association of a 306 bp insertion polymorphism in the progesterone receptor gene with ovarian and breast cancer. Br. J. Cancer. 1997;75:1397–1399. doi: 10.1038/bjc.1997.238.

Markman M, Rothman R and Hakes T et al (1991) Second-line platinum therapy in patients with ovarian cancer previously treated with cisplatin J Clin Oncol Off J Am Soc Clin Oncol 9(3) 389–393.

Marks JR, Davidoff AM, Kerns BJ, et al. Overexpression and Mutation of p53 in Epithelial Ovarian Cancer. Cancer Res, 1991;51:2979-2984.

Mehta K., Bates S.E., Siddik Z.H. Drug Resistance in Cancer Cells. Springer; New York, NY, USA: 2009. pp. 95–114.

Moldovan G.L., D'Andrea A.D. To the rescue: the Fanconi anemia genome stability pathway salvages replication forks. Cancer Cell. 2012;22:5–6.

Morice P , Uzan C, Gouy S, Pautier P , Lhomme C, Balleyguier C, et al. Effects of radiotherapy (external and/or internal) and chemotherapy on female fertility. Bull Acad Natl Med 2010; 194: 481-492.

Narod SA., Foulkes WD. BRCA1 and BRCA2: 1994 and Beyond (2004). Nature Reviews.

Neher TM, Bodenmiller D, Fitch RW, Jalal SI, Turchi JJ. Novel irreversible small molecule inhibitors of replication protein A display single-agent activity and synergize with cisplatin. Mol. Cancer Ther. 2011; 10(10):1796–1806. [PubMed: 21846830]

Olaussen K., Dunant A., Fouret P., Brambilla E., Andre F., Haddad V., Taranchon E., Filipits M., Pirker R., Helmut P., et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N. Engl. J. Med. 2006;355:983–991. doi: 10.1056/NEJMoa060570.

Partridge E, Kreimer AR, Greenlee RT, et al.: Results from four rounds of ovarian cancer screening in a randomized trial. Obstet Gynecol 113 (4): 775-82, 2009.

Petrucelli N, Daly MB, Feldman GL. BRCA1 and BRCA2 Hereditary Breast and Ovarian Cancer. 1998 Sep 4 [Updated 2013 Sep 26]. In: Pagon RA, Adam MP, Ardinger HH, et al.,

editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2016. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1247/

Plo, I., C. Laulier, L. Gauthier, F. Lebrun, F. Calvo and B. S. Lopez (2008). "AKT1 inhibits homologous recombination by inducing cytoplasmic retention of BRCA1 and RAD51." Cancer Res 68(22): 9404-9412.

Plummer R. Perspective on the pipeline of drugs being developed with modulation of DNA damage as a target. Clin. Cancer Res. 2010; 16(18):4527–4531. [PubMed: 20823148]

Prives C and Hall PA. (1999). J. Pathol., 187, 112–126. | Article | PubMed | ISI | ChemPort |

Prowse, A., Frolov, A., and Godwin, A.K. (2003). The genetics of ovarian can- cer. In American Cancer Society Atlas of Clinical Oncology, R.F. Ozols, ed. (Hamilton, Ontario: B.C. Decker Inc.), pp. 49–82.

Rajan JV, et al. Developmental expression of Brca2 colocalizes with Brca1 and is associated with proliferation and differentiation in multiple tissues. Dev. Biol., 184 (1997), pp. 385–401.

Reardon, J; Sancar, A (2006). "Purification and Characterization of Escherichia coli and Human Nucleotide Excision Repair Enzyme Systems". Methods in Enzymology 408: 189–213. doi:10.1016/S0076-6879(06)08012-8. PMID 16793370.

Rockwell S (1980) In vivo-in vitro tumour cell lines: characteristics and limitations as models for human cancer. The British Journal of Cancer Supplement 4: 118–122.

Rudland PS, Platt-Higgins AM, Davies LM, de Silva Rudland S, Wilson JB, Aladwani A, Winstanley JH, Barraclough DL, Barraclough R, West CR, Jones NJ. Significance of the Fanconi anemia FANCD2 protein in sporadic and metastatic human breast cancer. Am J Pathol. 2010;176(6):2935–2947.

Russell SE, McCluggage WG. A multistep model for ovarian tumorigenesis: the value of mutation analysis in the KRAS and BRAF genes. J Pathol. 2004;203:617–619.

Russell. S. E. H., Hickey. G. I., Lowry. W. S., et al. Allele loss from chromosome 17 in ovarian cancer. Oncogene, 5: 1581-1583, 1990

Sakai W, Swisher EM, Jacquemont C, et al. Functional Restoration of BRCA2 Protein by Secondary BRCA2 Mutations in BRCA2-Mutated Ovarian Carcinoma. Cancer Research. 2009;69:6381–6386.

Sandler, A., Gray, R., Perry, M., Brahmer, J., Schiller, J., Dowlati, A. et al. (2006) Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell cancer. N Engl J Med 355: 2542–2550.

Schilder JM, Thompson AM, DePriest PD, et al.: Outcome of reproductive age women with stage IA or IC invasive epithelial ovarian cancer treated with fertility- sparing therapy. Gynecol Oncol 2002, 87:1–7.

Scott, S. P. and T. K. Pandita (2006). "The cellular control of DNA double-strand breaks." Journal of Cellular Biochemistry 99(6): 1463-1475.

Seeberg, E., L. Eide and M. Bjoras (1995). "The base excision repair pathway." Trends Biochem Sci 20(10): 391-397.

Selvakumaran M., Pisarcik D., Bao R., Yeung A., Hamilton T. Enhanced cisplatin cytotoxicity by disturbing the nucleotide excision repair pathway in ovarian cancer cell lines. Cancer Res. 2003;63:1311–1316.

Shen GH,Ghazizadeh M and Kawanami O et al (2000). Prognostic significance of vascular endothelial growth factor expression in human ovarian carcinoma. Br J Cancer 83(2) 196–203 PMID: 10901370 PMCID: 2363477 4

Shen H., He M., Liu H., Wrighton S., Wang L., Guo B., Li C. Comparative metabolic capabilities and inhibitory profiles of CYP2D6.1, CYP2D6.10, and CYP2D6.17. Drug Metab. Dispos. 2007;35:1292–1300.

Siddik, Z. H. (2003). "Cisplatin: mode of cytotoxic action and molecular basis of resistance." Oncogene 22(47): 7265-7279.

Sieben NL, Macropoulos P, Roemen GM, Kolkman-Uljee SM, Jan Fleuren G, Houmadi R, Diss T, Warren B, Al Adnani M, De Goeij AP, Krausz T, Flanagan AM. In ovarian neoplasms, BRAF, but not KRAS, mutations are restricted to low-grade serous tumours. J Pathol. 2004;202:336–340.

Singh M, Leasure JM, Chronowski C, Geier B, Bondra K, et al. FANCD2 is a Potential Therapeutic Target and Biomarker in Alveolar Rhabdomyosarcoma Harboring the PAX3/FOXO1 Fusion Gene. Clin Cancer Res. 2014;20:3884–3895.

Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, et al. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med. 2003;9:568–574.

Thiebaut, F., T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan and M. C. Willingham (1987). "Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues." Proc Natl Acad Sci U S A 84(21): 7735-7738.

Townsend D.M., Tew K.D. The role of glutathione-S-transferase in anticancer drug resistance. Oncogene. 2003;22:7369–7375. doi: 10.1038/sj.onc.1206940.

Valerie, K. and L. F. Povirk (2003). "Regulation and mechanisms of mammalian double-strand break repair." Oncogene 22(37): 5792-5812.

Van Nagell JR Jr, Miller RW, DeSimone CP, et al. Long-term survival of women with epithelial ovarian cancer detected by ultrasonographic screening. Obstet Gynecol 118 (6): 1212-21, 2011.

Wang, Y., D. Cortez, P. Yazdi, N. Neff, S. J. Elledge and J. Qin (2000). "BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures." Genes Dev 14(8): 927-939.

Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losick R. (2004). Molecular Biology of the Gene. Pearson Benjamin Cummings; CSHL Press. 5th ed., chapters 9 and 10.

Welcsh P. L., Owens K. N., King M., Insights into the functions of BRCA1 and BRCA2. Trends in Genetics, (2000), pp. 69–74. http://dx.doi.org/10.1016/S0168-9525(99)01930-7

Wiggans, Alison J; Cass, Gemma KS; Bryant, Andrew; Lawrie, Theresa A; Morrison, Jo; Morrison, Jo (2015). "Poly(ADP-ribose) polymerase (PARP) inhibitors for the treatment of ovarian cancer". Reviews. doi:10.1002/14651858.CD007929.pub3.

Wooster P, et al. Identification of the breast cancer susceptibility gene BRCA2. Nature, 378 (1995), pp. 789–792.

Yamamoto S. et al. Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. Br J Cancer 76, 1221–1227 (1997).

Yeatman TJ. A renaissance for SRC. Nat Rev Cancer 2004; 4: 470–480.

Zhu M, John S, Berg M, Leonard WJ. Functional association of Nmi with Stat5 and Stat1 in IL-2- and IFNgamma-mediated signaling. Cell. 1999;96:121–130.