The Evaluation of Platinum-Sensitivity in a Cell Line Model of High Grade Serous Ovarian Cancer and the Induction of Resistance from a Chemo-sensitive Cell Line through the Repopulation of Cells Following Short-term Cisplatin Treatment

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Abstract

Introduction

Resistance to platinum-based therapy develops in most patients treated for High Grade Serous Ovarian Cancer (HGSOC), a fact that largely accounts for this disease's elevated mortality. It was previously shown by Cooke and colleagues (Oncogene, 2010) that platinum (Pt) resistance results from the expansion of sub-clonal populations of resistant cells present prior to treatment. Current *in vitro* models of Pt-resistance may be confounded by a lack of fidelity to the genetic signature of HGSOC, or by the methods used to elicit the onset of resistance which may lack clinical relevance. This project aims to address whether Pt-resistance can be evolved from a population of Pt-sensitive cells exposed to cisplatin (CDDP) in a clinically relevant manner using patient-derived cell lines such as PEO1. The central aim was to uncover what heterogeneity might exist within this cell line and to determine if *in vitro* derived Pt-resistance can result from the selection of pre-existent subsets of cells as occurs *in vivo*.

Methods

The Pt-sensitivity of the cell lines used, was established by exposing cells in culture to CDDP for 1 hr. Live cell number and percent viability were assessed 72 hours after drug removal using Guava microcytometry. Long-term assessment of Pt toxicity was provided by use of the clonogenic survival assay. To achieve Pt-resistance *in vitro*, a culture of PEO1 was exposed to 10 μM CDDP for 1 hour and allowed to repopulate. These cells were passaged and used to establish a novel cell line (PEO1X) with 20-fold diminished Pt-sensitivity confirmed via the clonogenic survival assay. PEO1X cells were assayed for histopathological and cell-fate markers by immunohistochemistry and compared to PEO1. Migratory capacity was assessed via the

Boyden chamber method, while cell cycle status 72 hours after CDDP exposure was interrogated using propidium iodide staining. Doubling time was also determined, and a partial genetic signature was established by sequencing a panel of 33 cancer-related genes.

Results

PEO1 exhibits morphological asymmetry, with a dichotomy between epithelial and mesenchymal phenotypes. This is further evidenced by the heterogeneous expression of markers such as E-Cadherin, Vimentin and CA125. Although similar to PEO1 in proliferation rate, PEO1X cells possess obvious differences in morphology, being smaller and more homogenously rounded in appearance with altered expression of markers such as E-Cadherin, Vimentin, CA125 and CD133. Unlike PEO1, the cell cycle status of PEO1X is barely altered 72 hrs following CDDP exposure even at 10 μM. PEO1X also possesses a more migratory phenotype than PEO1 with significantly more cells transiting through the pores of the Boyden chamber membrane in 30 hrs. Sequencing revealed heterogeneity in the status of P53 in PEO1 with PEO1X being slightly enriched in P53 WT expressing cells. Interestingly, the gene NF1, encoding the tumor-suppressor neurofibromin was found to contain an indel mutation in PEO1 and PEO1X while containing a different loss-of-function point mutation in PEO4 and being WT in PEO6 despite all being established from the same patient.

Conclusion

The cell line PEO1 contains a sub-population of Pt-resistant cells that can be selected for, *in vitro*, by clinically relevant CDDP treatment. These cells are distinct from those having emerged clinically in the original patient.

Résumé

Introduction

La résistance au traitement à base de platine se développe chez la plupart des patients traités pour le cancer de l'ovaire séreux de haut grade (HGSOC), un fait qui explique en grande partie la mortalité élevée de cette maladie. Cooke et ses collègues (Oncogene, 2010) ont montré précédemment que la résistance au platine (Pt) résulte de l'expansion des populations sous-clonales de cellules résistantes présentes avant le traitement. Les modèles en-vitro actuels de résistance au Pt peuvent être confondus par un manque de fidélité à la signature génétique du HGSOC ou par les méthodes utilisées pour provoquer l'apparition d'une résistance pouvant être dépourvue de pertinence clinique. Ce projet vise à déterminer si la résistance aux Pt peut être développée à partir d'une population de cellules sensibles au Pt exposées au cisplatine (CDDP) d'une manière cliniquement pertinente en utilisant des lignes cellulaires dérivées de patients telles que PEO1. L'objectif central était de découvrir quelle hétérogénéité pourrait exister dans cette lignée cellulaire et de déterminer si la résistance à la Pt dérivée en-vitro pouvait résulter de la sélection d'une fraction de cellules préexistants, comme cela se produit en-vivo.

Méthodes

La sensibilité au platine des lignes cellulaires utilisées a été établie en exposant les cellules en culture au CDDP pendant 1 heure. Le nombre de cellules vivantes et le pourcentage de viabilité ont été évalués 72 heures après le retrait de la drogue en utilisant une microcytométre Guava Muse. L'évaluation à long terme de la toxicité du Pt a été réalisée avec l'aide du test de survie clonogénique. Pour atteindre la résistance au Pt en-vitro, une culture de PEO1 a été exposée à 10

μM de CDDP pendant 1 heure et laissée se repeupler. Ces cellules ont été passé en culture et utilisées pour établir une nouvelle ligne cellulaire (PEO1X) avec une sensibilité à la Pt diminuée de 20 fois, ce qui a été confirmée par le test de survie clonogénique. Les cellules PEO1X ont été testées pour les marqueurs histopathologiques et de l'état EMT par l'immunohistochimie et comparées à PEO1. La capacité de migration a été évaluée par la méthode de la chambre Boyden, tandis que le statut du cycle-cellulaire 72 heures après l'exposition au CDDP a été interrogé en utilisant une coloration à l'iodure de propidium. Le temps de doublage a également été déterminé et une signature génétique partielle a été établie en séquençant un panel de 33 gènes liés au cancer.

Résultats

PEO1 exige une asymétrie morphologique, avec une dichotomie entre les phénotypes épithéliaux et mésenchymateux. Ceci est encore démontré par l'expression hétérogène de marqueurs tels que E-Cadherin, Vimentin et CA125. Bien que similaires au taux de prolifération de PEO1, les cellules PEO1X présentent des différences de morphologie évidentes, étant plus petites et ayant un aspect plus homogène avec une expression modifiée de marqueurs tels que E-Cadhérine, Vimentine, CA125 et CD133. Contrairement à le PEO1, le cycle cellulaire de PEO1X est à peine modifié 72 heures après l'exposition au CDDP, même à 10 μM. PEO1X possède également un phénotype plus migrateur que le PEO1 avec beaucoup plus de cellules transitant la membrane de la chambre Boyden après 30 heures. Le séquençage a révélé une hétérogénéité dans l'état de P53 dans le PEO1, avec le PEO1X étant légèrement enrichi dans les cellules exprimant P53 WT. Il est intéressant de noter que le gène NF1, codant pour la protéine Neurofibromine (un suppresseur de tumeur), contient une mutation indel dans PEO1 et PEO1X tout en contenant une mutation de point différente de PEO4 et en étant WT dans PEO6.

Conclusion

La ligne cellulaire PEO1 contient une sous-population de cellules résistantes au Pt qui peuvent être sélectionnées en-vitro par un traitement avec CDDP aux paramètres pertinents cliniquement. Ces cellules sont distinctes de celles ayant émergé cliniquement chez le patient d'origine.

List of Abbreviations

Pt: Platinum

CDDP: *cis*-diamminedichloridoplatinum(II), *cis*-$[Pt(NH_3)_2Cl_2]$ AKA Cisplatin

HGSOC: High Grade Serous Ovarian Carcinoma

EOC: Epithelial Ovarian Cancer

TCGA: The Cancer Genome Atlas

ATCC: American Type Culture Collection

OSE: Ovarian Surface Epithelium

FTSEC: Fallopian Tube Secretory Epithelial Cells

STIC: Serous Tubal Intra-epithelial Carcinoma

WHO: World Health Organization

ChIp: Chromatin Immunoprecipitation

ATAC: Assay for Transposase-Accessible Chromatin

CIC: Cortical Inclusion Cyst

FDA: Food and Drug Administration

EMA: European Medicines Agency

VEGF: Vascular Endothelial Growth Factor

MAPK: Mitogen-Activated Protein Kinase

LOF: Loss Of Function (Mutation)

Indel: Insertion-Deletion (Mutation)

WT: Wild-Type (Allele)

SEER: Surveillance, Epidemiology, and End Results (Program of NIH)

NIH: National Institutes of Health (USA)

GWAS: Genome-Wide Association Studies

SNP: Single-Nucleotide Polymorphisms

OC: Oral Contraceptives

HR: Homologous Recombination (of DNA double-strand breaks)

PH3: Phospho-Histone H3

DSB: Double-Strand Break

PFS: Progression-Free Survival

OS: Overall Survival

MRI: Magnetic Resonance Imaging

PET: Positron-Emission Tomography

CT: Computed Tomography

NACT: Neo-Adjuvant Chemo-Therapy

PFI: Progression-Free-Interval

RTK: Receptor Tyrosine Kinase

NER: Nucleotide Excision Repair

MMR: Mismatch Repair

ROS: Reactive Oxygen Species

CAF: Cancer Associated Fibroblast

TAM: Tumor Associated Macrophage

IARC: International Agency for Research on Cancer

LOH: Loss of Heterozygosity

CNA: Copy Number Alteration

GEMM: Genetically Engineered Mouse Models

PDX: Patient-Derived Xenograft

SCID (mice): Severe Combined Immunodeficient

NSG (mice): NOD-SCID IL2R-gamma^{null}

MCS: Multi-Cellular Structure

EMT: Epithelial-to-Mesenchymal Transition

VAF: Variant Allele Frequency

CSC: Cancer Stem Cell

EV: Extracellular Vesicle

NCI: National Cancer Institute (American)

Literature Review

1.1 Ovarian Cancer Prevalence and Mortality

In the present-day ovarian cancer continues to represent a salient public health concern which, in spite of its infrequent incidence, remains the deadliest form of gynecological malignancy. According to the WHO, on average each year an estimated total of 225,500 cases of ovarian cancer will be diagnosed, and 140,200 patients will succumb to this disease, representing the 7th most common form of cancer and the 8th leading cause of cancer-related death among women worldwide [1,2]. These figures, taken together, serve to underline the status of ovarian cancer as a significant source of morbidity and mortality in the global population. In "Western" nations, ovarian cancer is the fifth most frequent cause of cancer-related death in women [3]. The Surveillance, Epidemiology, and End Results (SEER) program of the American National Cancer Institute (NCI) records an annual incidence of 11.6 cases/ 100,000 women per year according to the latest statistical cohort with an estimated 224,940 women living with the disease in 2015 [4]. In Canada, the Canadian Cancer Society predicts an average of 2,800 cases diagnosed and 1,800 deaths/ year (2017) [5]. Whereas the survival rates for a number of solid tumors have improved significantly in last 50 years, a recent meta-analysis drawing upon survival data from numerous countries concluded that the 5-year overall survival from ovarian cancer had remained virtually unchanged since about 1980 [6]. According to the most recent figures published by the SEER (2008-2014) the current 5-year survival rate in the US is approximately 47.4% [4].

1.2 Ovarian Cancer Classification and Histopathological Subtyping

Although the term "ovarian cancer" implies a unitary disease, from the perspective of the pathologist it was apparent as early as the 1930s that it was more appropriate to classify ovarian neoplasms as multiple distinct entities through the lens of histopathology [7]. This culminated in the 1973 WHO guidelines which signified the first systematic attempt to delineate the many

ovarian cancer subtypes [7]. Histologically, about 90% of ovarian tumors are deemed to have occurred through the transformation of epithelial cells as opposed to those originating from germ cells or sex-cord-stromal tissues [8]. These are thus designated as Epithelial Ovarian Cancers (EOC). That nomenclature itself applies to a broad category of disease with a whole range of taxonomy therein contained. This includes the four well defined histological subtypes which have served as the basis for EOC diagnosis over the past few decades. These are referred to as: serous, mucinous, clear-cell and endometrioid; appellations deriving from their morphology and tissue architecture as observed through microscopy. Furthermore, the assignment of a tumor grade, based on the apparent degree of cytological aberration, allows for an additional degree of stratification for serous and endometrioid EOCs [3]. Despite sharing a similar histological appearance, high-grade and low-grade serous carcinomas of the ovary are now considered to be two entirely different neoplasms; with distinct modes of carcinogenesis, molecular-genetic features and sites of origin [9]. Clear-cell carcinomas, although not usually assigned a grade, are considered more similar to high grade lesions [10].

While the overwhelming majority of cases observed clinically belong to either of the four major histotypes, a number of rarer types have been noted. These include malignant transitional cell (Brenner) tumors as well as cases of mixed histology such as seromucinous carcinoma and carcinosarcoma, along with undifferentiated carcinoma [10]. Furthermore, most ovarian borderline tumors are typically treated as EOC in the clinic; these abnormal growths of low malignant potential can encompass a number of different histologies [9,10].

Although referred to as ovarian cancer, it has long been seen that the histology of these tumors resembles non-ovarian tissues. For example, endometrioid ovarian carcinoma, as its name suggests, features a glandular architecture similar to the endometrium, while mucinous tumors

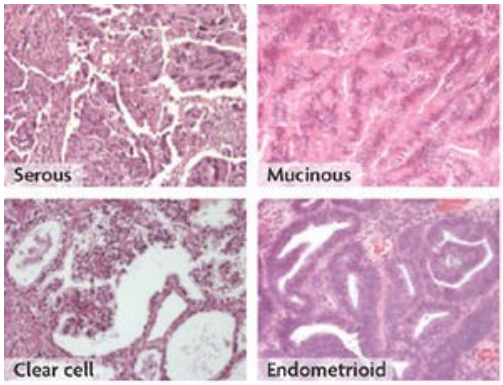
can resemble either endocervical glands or the gastrointestinal epithelium [11]. Recent studies have confirmed the extra-ovarian origins of the majority of mucinous tumors as well as the clear-cell and endometrioid subtypes, which derive in most cases from metastatic intestinal tumors and endometriotic lesions respectively [12,13]. The origin of the serous subtype is still debated, but in the case of high-grade serous neoplasms there is now little doubt that the majority derive from the epithelium of the fallopian tube.

Recent efforts to study EOC from the molecular and genetic perspective have led to a paradigm shift in the classification of this disease through the introduction of the dualistic model of ovarian carcinogenesis. This model was first proposed by Kurman and Shih in 2004 and has since garnered widespread acceptance, being officially recognized in 2014 by the WHO in their updated classification guidelines for tumors of the female reproductive organs (Illustration 1: p. 18) [9,14]. This model segregates EOC into two broad categories called Type 1 and Type 2. Type 1 tumors normally develop in a step-wise progression from pre-malignant or borderline lesions in a manner common to many other epithelial cancers [10]. From the genetic perspective these tumors have frequent oncogenic alterations to many cellular signaling pathways such as RAS-MAPK and PI3K-AKT but are otherwise genomically stable and P53 wild type [10]. From the perspective of the clinician, these tumors typically present as large, unilateral, cystic neoplasms that grow in an indolent fashion [10]. When confined to the ovary they have an excellent prognosis [10]. This category includes low-grade serous, clear-cell, mucinous and transitional cell (Brenner) subtypes [10]. In contrast, the Type 2 category is marked by a far more aggressive disease behavior. Tumors develop rapidly and are usually widely disseminated at the time of presentation, resulting in a poor overall prognosis [10]. From a genetic perspective these tumors are characterized by P53 mutations and genomic instability due to defects in DNA repair [10]. The prototypical Type II

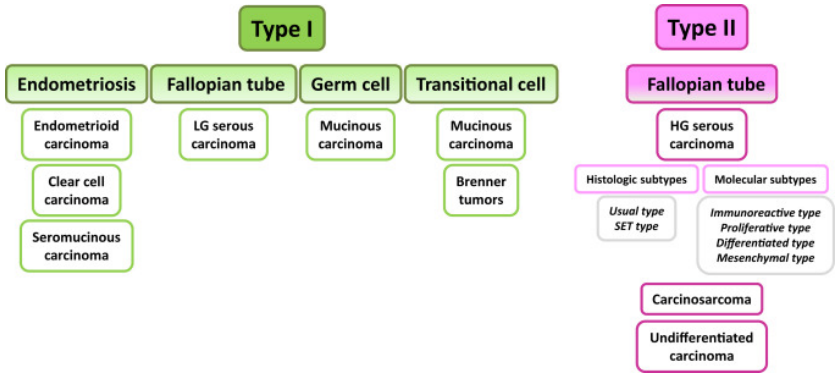
neoplasm, High Grade Serous Ovarian Carcinoma (HGSOC), is by far the dominant subtype diagnosed clinically and accounts for 70-80% of deaths from all forms of ovarian cancer [10,15]. In summary, ovarian cancer is a broad designation for a myriad of distinct diseases sharing an anatomical site upon presentation. Given its preponderance in the clinical setting and its enduringly grim prognosis, the bulk of the scientific effort in this field has coalesced around the study of high grade serous ovarian cancer with the goal of improving patient outcomes [15].

Illustration 1: Ovarian Cancer Histology and Classification

A)



B)



A): Representative images of the four main histological subtypes of EOC visualized by microscopy of tissue sections stained with H&E. Image adapted from Bast, Hennessey & Mills, *Nature Reviews Cancer* **9**, 415–428 (2009). [16]

B) Diagram depicting the updated version of the dualistic classification scheme for ovarian neoplasms developed by Kurman and Shih. Image adapted from Kurman & Shih, *AJPath* 186 (4), 733-747 (2016). [10]

1.3 HGSOC Histopathology

From the perspective of the pathologist visualizing stained sections of tumor under the microscope, HGSOC typically features solid masses of cells with slit-like fenestrations [9]. In more differentiated areas, the tumors often have a papillary, glandular or cribriform architecture that is said to resemble the surface epithelium of the fallopian tube [9,11]. Regions of solid growth are frequently accompanied by areas of extensive necrosis [9]. In certain cases, HGSOC may present with areas that display a solid growth pattern that simulates endometrioid or transitional cell carcinoma [10]. Although morphologically distinct, these tumors show an immunoreactivity identical to typical HGSOC and are thus not considered as a separate entity [10]. Researchers have recently named this type of HGSOC as the SET ('Solid, pseudo-Endometrioid and/or Transitional cell carcinoma-like') variant [17]. It was found that these tumors frequently associate with BRCA1 mutations and contain a greater number of tumor-infiltrating lymphocytes compared to typical HGSOC [17]. From the cytological perspective, HGSOC is characterized by high-grade nuclear atypia; with large, hyperchromatic and pleomorphic nuclei with the potential for multinucleation [9]. The nucleoli are usually prominent and might appear large and even eosinophilic [9]. There is usually a high mitotic index with an abundance of visible mitotic figures that may be of abnormal appearance [9]. Psammoma bodies, which are areas of calcification typically associated with papillary tumors, are also typically present [9]. A number of immunological markers are used to differentiate HGSOC from other subtypes of EOC. Unlike low-grade serous tumors, HGSOC is almost invariably P53-mutant and will usually stain with a strong diffuse nuclear-positivity in nearly all cells [9]. This however is dependent on the type of mutation present. Missence mutations in TP53 typically correlate with positive staining due to the mutant protein accumulating due to a lost capacity for degradation by HDM2 [9]. If, however,

the gene contains a nonsense mutation, then the resultant truncated form of the protein might not be detectable by the antibody [9]. In that case the staining would be almost totally negative. Compared to the alternative forms of EOC, HGSOC is frequently found to stain positive for WT1 and PTEN, as well as CDKN2A (a.k.a. P16) [9]. The proliferation index, assessed through the number cells positive for Ki-67, would be higher compared to low grade serous lesions [9]. In relation to mucinous carcinoma, the epithelial marker CK7 is positive in HGSOC but CK20 is usually negative [11]. Compared to ovarian clear-cell carcinoma, HGSOC is HNF1β negative and ARID1A positive [18]. In common with most other forms of EOC is the expression of PAX8, a marker of tissues of Mullerian origin including the fallopian tube [19]. The expression of the Estrogen Receptor (ER) is detectable in about 80% of cases, whereas the Progesterone Receptor (PR) is only found to be positive in around 30% of HGSOC patients [9].

1.4 Ovarian Cancer Epidemiology and Risk Factors

The distribution of ovarian cancer incidence worldwide is not even, with great variance based on geography, ethnicity and the level of economic development. The peak age-adjusted incidence rates are in northern and central/eastern Europe; with intermediate rates seen in North America, Western Europe and Australia; and lower rates in Asia and Africa [20]. Recent trends seem to show a stable or slight reduction in age-standardized rates in most high-income countries, whereas they appear to be rising in many low and middle-income countries [20]. As such, there is far less disparity in incidence compared to 30 years ago [20]. In countries with a multi-ethnic population, the incidence may depend on ethnicity. For example, in the United States the disease is more frequent among Caucasian women compared to Hispanic, Asian or African-American women [4]. Like most other epithelial cancers, EOC tends to be diagnosed more frequently as a function of increasing age. As such, the number of cases occurring each year is

expected to increase as global life-expectancies continue to improve [20]. In the United Stayes the median age of diagnosis is at 63 years of age [4]. Epithelial ovarian cancer subtypes are infrequently seen in pre-menopausal women (≤ 45 years of age) while ovarian germ cell tumors occur mainly in younger women [20]. The total lifetime risk of developing ovarian cancer has been estimated at 1.3% for American women, however there are a number of known risk-factors that may modify the risk in individuals [4,20]. For example, there is a substantial heritable component of risk due to genetic factors. The risk for women with an affected first-degree relative is threefold greater than that of women without any affected relatives [21]. Familial cases are usually due to germline mutations in the tumor-suppressor genes BRCA1 and BRCA2 which also contribute increased risk of developing breast cancer in these same families [20]. A recent study found that 3.6% of ovarian cancer patients have germline mutations in BRCA1 while 3.3% have germline mutations in BRCA2 [22]. Overall it was estimated that germline BRCA1 and BRCA2 mutations contribute to the development of 10–20% of EOCs [23]. Compared to the normal population, BRCA1 mutation carriers have an estimated 44% risk of developing ovarian cancer by age 70, while this risk is up to 27% for BRCA2 mutant individuals [24]. The cancers occurring in these women are usually high-grade serous carcinoma which manifests at an earlier age than in sporadic cases [20]. The contribution of high-penetrance alleles of BRCA1/2 can only account for a small part of the heritable component of ovarian cancer [23]. Many other genes bearing low penetrance mutations are thought to play an important role. For instance, women with mutations in the genes BRIP1, RAD1C and RAD1D have estimated lifetime risks of developing ovarian cancer of 5.8%, 5.2% and 12%, respectively [25,26]. Other gene variations that have been linked with greater risk include BARD1, CHEK2, MRE11A, RAD50, PALB2 and ATM [26,27]. A common link between all the genes previously listed is their role in Homologous Recombination

(HR) mediated DNA repair which is known to play a role particularly in the pathophysiology of HGSOC (reviewed further below). Women with Lynch syndrome bearing mutations in genes involved in DNA mismatch repair also have a greater risk of developing ovarian cancer, usually of the clear-cell and endometrioid subtypes [28]. Genome-Wide Association Studies (GWAS) have also been used to search for Single-Nucleotide Polymorphisms (SNPs) correlating with greater risk of developing ovarian cancer [23]. Several of these loci have been identified and, while each is associated with only a miniscule increase in absolute risk, the combination of multiple alleles has been demonstrated to considerably impact on an individual's polygenic risk score [29]. Endometriosis is known to predispose individuals towards developing EOC, particularly the clear-cell and endometrioid subtypes which are known to derive from endometriotic lesions [13,20].

Many modifiable or lifestyle factors have also been viewed as influencing an individual's risk of developing ovarian cancer. Generally, ovarian cancer has been associated with women having experienced a greater number of ovulatory cycles in their lifetime [20]. As such, factors tending to reduce a woman's ability to ovulate have been linked with a reduced risk of developing this disease. For example, both the early occurrence of menarche and an older age at menopause have been connected with a possible increased risk, although in the case of the age at menarche, the evidence is not very clear [30,31]. Likewise, women who have given birth have a lower risk than nulliparous women, with a risk reduction of 10-20% associated with each additional birth [31]. Studies have also found that women who breastfeed have lower risk compared to those who do not, although other reports failed to validate this correlation [31,32]. The use of hormone-containing Oral Contraceptives (OC) has been robustly associated with a reduced risk of developing ovarian cancer; with users or former users having an up to 30% lower risk compared to never-users [20,33]. This apparent protective effect was more apparent in long-term

users and extended to all major subtypes of EOC [31,33]. One study claimed that the use of these compounds might have prevented up to 200,000 worldwide cases of EOC over the past several decades [33]. Procedures to reduce fertility including tubal ligation have been shown to reduce the risk of developing certain forms of EOC while the use of estrogen hormone therapy during menopause is related to a greater risk in these women (also subtype dependent) [31,34,35]. Other potential risk factors include: obesity, diabetes, smoking and usage of perineal talc [20]. Unfortunately, many of the modifiable factors that have been associated with ovarian cancer are not easily amenable to change, while others, including pregnancy and OC use, cannot be recommended as a cancer prevention strategy [20]. Moreover, a recent study of an Australian cohort concluded that only about 7-11% of ovarian cancer cases could be attributed to these modifiable factors [36].

1.5 The Origin of HGSOC

The precise cell and tissue of origin for HGSOC has long been a matter of contention. This is because, unlike low-grade serous tumors which are known to arise from pre-existing lesions such as serous cystadenomas or serous borderline tumors, it had proven difficult to locate an established precancerous component in the case of HGSOC [37]. In fact, HGSOC remains the only epithelial cancer without a recognized pre-cancerous lesion [11]. Because most HGSOC patients, even at an early stage, feature the cancerous involvement of the ovary, it was natural to assume that the disease originated in that location. The tissue of origin was reputed to be the ovarian surface epithelium (OSE), a simple, uncommitted layer of flat-to-cuboidal epithelium that is derived from the coelomic mesoderm and related to the mesothelial covering of the peritoneal cavity [11,38]. As early as 1971, Fathalla put forward the “incessant ovulation” hypothesis that would attain widespread acceptance in subsequent years [37,39]. His theory contended that the

constant cycle of repair and regeneration experienced by the OSE due to ovulation might contribute to carcinogenesis by creating the kind of pro-inflammatory and pro-oxidative microenvironment that is conducive to acquiring DNA damage [37-39]. The inability to adequately repair such damage experienced by the OSE cells was thought to be at the root of HGSOC carcinogenesis [37]. Indeed, it has since become known that patients harboring an inherent deficiency in DNA repair, particularly in that pathway implicated in the HR-mediated repair of Double Strand Breaks (DSBs) due to germline mutations in BRCA1/2, are at much greater risk of developing HGSOC [37]. Under this theory, the total number of ovulatory cycles experienced by a woman would be directly related to her risk of acquiring HGSOC [37,39]. It was therefore viewed as confirmatory when numerous studies concluded that factors that suppressed ovulation, such as pregnancy, breastfeeding and the use of hormone containing oral contraceptives, reduced an individual's risk of developing the disease [37,38,40]. Furthermore, it was observed that ovulatory repair resulted in the tendency for certain sections of the OSE to invaginate and become trapped beneath the surface of the ovary in the form of structures called Cortical Inclusion Cysts (CICs) [38]. Within the ovary, CICs are exposed to several hormones with properties promoting growth and differentiation, resulting in the transition to a state of metaplasia in the OSE lining of these CICs [38]. In cells harboring pre-existent mutations or DNA damage, this would create the ideal scenario for neoplastic transformation [38]. The theory of ovarian origin for HGSOC nevertheless remained problematic in a few key areas. Histologically, HGSOC is said to more closely resemble tissues developmentally derived from the Mullerian duct during embryogenesis [11,40,41] . It was not known how the coelomic cells of the OSE could differentiate into a Mullerian-like tissue during carcinogenesis [11]. It was postulated that the relatively undifferentiated nature of the OSE would allow it to more readily undergo metaplasia to resemble a Mullerian phenotype [40,41].

One study would demonstrate that the ectopic activation of the gene HOXA9 in cultured mouse OSE was sufficient to induce the formation of tumors histologically resembling serous carcinoma [42]. Although this study provided a potential mechanistic framework for an OSE origin of HGSOC, doubt nevertheless remained due to the enduring absence of any identifiable precursor lesion or in-situ carcinoma in the ovaries of these patients [11,40]. At the time many speculated that it was due to their destruction during the process of tumorigenesis [43]. In a research 1999 paper, Dubeau cast doubt on the dogma that HGSOC originated from the OSE, and instead argued for source derived from the Mullerian epithelium [44]. Even still, the belief that HGSOC could originate from a tissue of extra-ovarian origin was not widely held until the introduction of risk-reducing salpingo-oophorectomy in patients with inherited BRCA mutations [10]. In 2001, the group of Piek et al. from the Netherlands described the presence of small dysplastic lesions similar to high-grade serous carcinoma within the fallopian tubes of suspected BRCA mutation carriers [40,45]. These lesions were morphologically characterized by the presence of stratified, disorganized, and enlarged epithelial cells with highly atypical nuclei [40]. The examination of samples from a cohort of non-mutant individuals failed to locate any such lesions [37,45]. This discovery was aided by the introduction of a new histological technique for sampling the fallopian tube in which the entire fallopian tube, with particular attention to the fimbria, was sectioned [10]. Previously, studies examining the ovary for precursor lesions failed to completely examine the fallopian tube [7]. These lesions, which later became known as Serous Tubular Intra-Epithelial Carcinoma (STIC), featured the virtual absence of ciliated cells with a shift in favor of the secretory population [38]. Later studies established that these lesions were far more common at the ciliated end of the fallopian tube; in the part directly adjacent to the ovary [38,46]. By immunohistochemistry these lesions were found to stain strongly for P53 compared to the

surrounding epithelium which suggested that these cells were P53 mutant [11,47]. They also over-expressed γH2AX, a marker of DNA double strand breaks [11,47]. It was found that 38% of BRCA mutant women having undergone salpingo-oophorectomy harbored these lesions (STIC) in their fallopian tubes but not in their ovaries [46]. The existence of these microscopic intra-epithelial carcinomas suggested that the secretory epithelial cells of the distal (fimbriated) end of the fallopian tube (FTSEC) were the preferred site of origin for HGSOC, at least in women bearing BRCA1/2 mutations. This was strongly supported by a study by Kuhn et al. showing that STICs possessed the identical P53 mutation to that present within the concurrent HGSOC in women with this disease [48]. Furthermore, it was shown that STICs contained shortened telomeres compared to the co-existent cancer within the same patient [49]. Telomere shortening has become established as a hallmark of the early stages of carcinogenesis [10]. While STICs were well known for occurring in women with BRCA mutations, it was not known if they could contribute to carcinogenesis in sporadic cases of HGSOC. A 2007 study by Kindelberger et al. showed that STICs were found in 52% of patients with sporadic advanced-stage HGSOC [47]. STICs have also been reported in the fallopian tubes of women undergoing hysterectomy and bilateral salpingo-oophorectomy for non-prophylactic reasons [10,50]. Recent genetic studies have also established that HGSOC and paired STICs have many other shared genetic alterations including changes in gene copy number [23]. One study established that, in HGSOCs featuring CCNE1 amplification, a similar copy number change was present in the STICs isolated from the same patient [51]. The tubal origin for HGSOC has also been reinforced by a study that used gene expression profiling to show that the pattern of gene expression in HGSOC more closely resembled that of the normal fallopian tube epithelium rather than that of the OSE [52]. Transformation of cultured human fallopian tube epithelium *in vitro* also results in cells that resemble the morphology,

immunophenotype and gene expression profile of human HGSOC [53]. In addition, a novel mouse model that features induced mutations in the same genes commonly affected in human patients, also develops serous carcinoma from the fallopian tube [54]. The reason why the overwhelming majority of STICs are found to occur at the fimbriated end of the fallopian tube has remained an enduring mystery [43]. Some recent studies have claimed to show that the fimbriae are enriched in cells with "stem like" properties [55]. It has thus been argued that the fimbriae represent a developmental "transition zone" analogous to that present in the cervix which is prone to malignant transformation [43]. Notwithstanding the convincing evidence that the fallopian tube is the major site of origin for HGSOC, it remains established that ovulation is a consistent risk factor in epidemiological studies. To explain this, it has been proposed that the proximity of the fimbriae to the ovarian surface might subject it to many of the same pro-inflammatory mediators and ROS thought to contribute to the development of genotoxic stress in the OSE following ovulation [38]. Yet despite even the most diligent examination, it is consistently observed that a significant percentage of cases of HGSOC present without involvement of the fallopian tube [15]. This has led some to suggest that there still might still be precursor cells in the ovary that underlie such cases [15]. A new unifying theory contends that such cases arise from the early implantation of FTSECs in the OSE through a process called "endosalpingiosis" [43]. Under such a scenario, the fallopian tube epithelium might become incorporated into the CICs that have been the proposed site of origin for HGSOC in the ovary [41,43]. As has already been highlighted, the microenvironment of the ovary is more favorable for neoplastic transformation than that of the fallopian tube [38,43]. Although endosalpingiosis has been demonstrated in mouse ovaries, the same process has never been observed in humans [43], and thus the precise progenitor for a substantial percentage of HGSOC cases remains obscure.

Illustration 2: The Proposed Site of Origin for each EOC Subtype

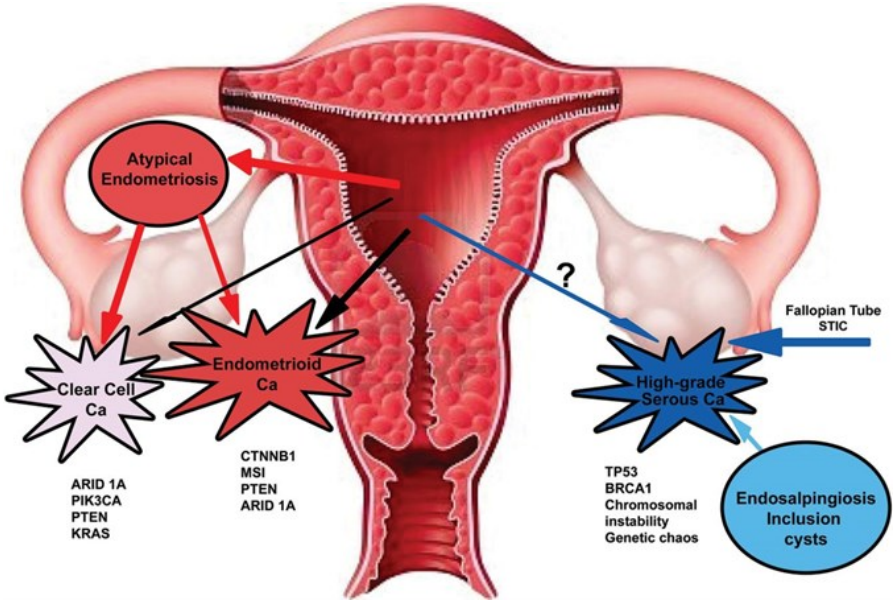


Illustration outlining the proposed site(s) and mechanisms of origin for the main histological subtypes of EOC (does not include mucinous) along the characteristic genetic traits of each malignancy. Both Clear-Cell and Endometrioid carcinoma of the ovary have been linked to endometriosis and thus an extraovarian origin from the uterine epithelium. These tumors frequently display many oncogenic alterations, such as those involving RAS, PI3K-AKT, PTEN and ARID1A. By contrast HGSOC has two proposed sites of origin: the OSE and the secretory epithelial cells of the distal fallopian tube (FTSEC). This malignancy is characterized by genomic instability, but with few recurrent mutations other those involving P53 and BRCA1/2.

Image adapted from: Prat, Ann Oncol; **23** (suppl_10):111-117 (2012) [56]

1.6 HGSOC Symptomatology, Diagnosis and Staging

One of the principal factors influencing the elevated mortality experienced by HGSOC patients is the inability to diagnose the disease at an early, localized stage. Only about 13% of cases of serous ovarian carcinoma are diagnosed at stage I or stage II [57]. By contrast the vast majority of cases are usually diagnosed at the stage of distant metastasis which greatly impacts an individual's prognosis [4]. The 10-year survival of patients diagnosed with early stage HGSOC is 55%, compared to only 15% for those having presented with advanced-stage disease [57].

There are currently no effective screening strategies for the early detection of ovarian cancer [8]. A recent trial evaluating the utility of transvaginal ultrasonography in combination with serum CA125 levels demonstrated some promise in terms of early detection but failed to improve patient outcomes [58,59]. Genetic tests might be useful to detect heritable BRCA mutations in patients with a known family history of breast and ovarian cancer [8]. In such cases the at-risk individual might elect to undergo risk-reducing prophylactic surgery such as bilateral salpingo-oophorectomy, typically upon completion of their childrearing or at least by the age of 40 [23]. This technique is effective in preventing the emergence of ovarian cancer in 85-90% of cases [60].

Because the symptoms associated with HGSOC are often diverse and non-specific, there is usually little likelihood that a patient will encounter the appropriate medical specialist in time for an early diagnosis to be made [8]. Symptoms are typically gastrointestinal and include abdominal pain, bloating, nausea, constipation, anorexia, diarrhea and acid reflux [8,9]. Other symptoms include fatigue, back pain, tenesmus as well as elevated urinary frequency [8,9]. At an advanced stage, respiratory symptoms might be present such as cough and dyspnea [9]. Unfortunately, by the time a patient becomes symptomatic their disease is found to already be at an advanced stage in

between 75-80% of cases [9]. By contrast, other forms of EOC such as clear-cell carcinoma typically become symptomatic at a far earlier stage [8].

If a diagnosis of EOC is suspected, the patient will typically be subjected to a pelvic and rectovaginal examination along with radiographic imaging such as transvaginal or abdominal ultrasonography, CT, MRI or PET [8]. Blood levels of CA125 might also be measured which, in combination with other tests might be of diagnostic value [8]. Imaging typically reveals large, complex, hyper-vascular pelvic masses and omental/peritoneal nodules [9]. The serum CA125 levels are often elevated, especially in advanced cases, with average values around 500-1000 U/ml [61]. Advanced disease will typically feature extensive peritoneal carcinomatosis, involving most of the major abdominal organs, and may be associated with the accumulation of large volumes of ascites [11].

To aid in diagnosis, laparoscopic surgery is usually performed to obtain a tumor sample for biopsy and to aid in the staging of the disease [8]. The staging system is based on the degree of dissemination of the disease at diagnosis. At stage I, the cancer is still confined to the ovaries or fallopian tubes [62]. By stage II, the disease has already spread to other pelvic organs such as the uterus [62]. Stage III involves spread beyond the pelvis to organs or tissues within the peritoneal cavity or to the retroperitoneal lymph nodes [62]. Stage IV results from spread beyond the peritoneal cavity, including to the lungs, liver and spleen but also encompassing the involvement of inguinal and other extra-abdominal lymph nodes [62]. The end stage of the disease is characterized by malignant bowel obstruction due to the formation of fibrous adhesions between loops of the bowel by the metastatic tumors [16]. This impedes the patient from normal alimentation, leading to cachexia, malnutrition and, eventually, death from factors which may include intercurrent infection [16].

1.7 HGSOC Dissemination

High grade serous ovarian carcinoma is notable because it does not require the blood or lymph in order to metastasize. In order for other epithelial cancers to spread, the tumor cells must typically undergo a sequence of cellular transformations to traverse the basement membrane, migrate to and invade the vasculature, survive in suspension, extravasate and re-establish themselves as a colony at a distant site. Many cancers possess a preferred site of implantation that is thought to be especially conducive for the growth of the metastasis. This phenomenon was first noticed more than 100 years ago by English pathologist Stephen Paget who postulated the famous "seed and soil" theory that has now achieved widespread acceptance [63]. His original theory contended that the organ-biased pattern of implantation exhibited by breast cancer was due to favorable interactions between metastatic tumor cells (the "seed") and the micro-environment of the organ in question (the "soil") [64]. This theory has recently been applied to a number of different malignancies and been supported by studies that have sought to elucidate the mechanistic basis of the interactions between the metastatic tumor cells and the stroma at their favored secondary site [64].

By contrast, HGSOC typically spreads by direct extension to adjacent organs within the peritoneal cavity or by the detachment of cells from the primary tumor [11]. For a tumor growing on the surface of the ovary or fallopian tube, there are no anatomical barriers restricting the spread of the tumor cells throughout this fluid-filled space between the body's visceral organs [16]. Once the cells have exfoliated from the primary tumors site, either singly or in clusters, they become suspended in the peritoneal fluid and are spread by a passive process that follows the physiological flow of this fluid around the peritoneal cavity [11]. These cells can then implant and seed distant organs or tissues with nests of cancerous cells which develop rapidly into secondary

tumor nodules. Although virtually every organ or structure within the peritoneal cavity may be involved, HGSOC cells are known to exhibit a particular predilection for the omentum [11]. In fact, 80% of patients with HGSOC present with omental metastases [65]. Composed largely of energy-dense adipocytes, this large fat-pad extends from the stomach and covers the intestines. It has been hypothesized that the preference of HGSOC for the omentum stems from a cellular metabolic requirement for fatty-acid based catabolism (β-oxidation) [65]. It has been shown that adipocytes produce pro-inflammatory cytokines such as IL-8 which promote the homing and invasion of tumor cells [65]. In the same study the co-culturing adipocytes with ovarian cancer cells was seen to promote greater lipolysis in adipocytes and β-oxidation in the cancer cell [65]. This co-culturing also led to increased proliferation of ovarian cancer cells *in vitro* and rapid growth of transplanted tumors *in vivo* [65].

While HGSOC spreads readily within the peritoneal cavity, its metastatic growths are only superficially invasive for those organs affected [11]. Secondary tumor implants typically invade through the mesothelial cell layer but no further, leaving the deeper lamina largely intact [11]. Spread outside the peritoneal cavity is uncommon, although certain pelvic and/or para-aortic lymph nodes can sometimes be involved [66]. Hematogenous spread is thought to be largely precluded, based on the observation that patients treated with peritoneovenous shunts who, having received billions of tumor cells into the circulation, mostly failed to develop distant metastasis even two years after the procedure [67]. There is the potential, however, for metastasis to the liver, while in the most advanced stage of HGSOC, tumor cells may also cross the diaphragmatic barrier and enter the pleural space where they can cause pleural effusions or even implant in the parenchyma of the lung [8]. Patients with late stage disease frequently develop ascites which feature a prominent cellular component and are thus referred to as “malignant

ascites". HGSOC cells might participate in the formation of these ascites either by blocking the lymphatic drainage or by secreting vasoactive and angiogenic factors which promote vascular permeability [11]. A lingering mystery is the role of multicellular structures in the pathogenesis of HGSOC. These structures frequently appear in the form of spheroids or aggregates of suspended tumor cells and are commonly isolated from the ascites of patients with advanced disease. They have been proposed to be a fundamental unit of metastatic spread in addition to being a chemo-resistant niche to survive therapy [11]. Importantly, the formation of multicellular structures might allow the cells to survive in anchorage independent conditions by preventing anoikis [11].

1.8 Treatment for HGSOC: Surgery

The primary recourse initiated in patients with HGSOC is a procedure called surgical cytoreduction or "debulking". The goal of this surgical approach is to achieve macroscopic total resection of all the disseminated tumor masses contained within the peritoneal cavity of the patient [8]. The surgery is typically the responsibility of a gynecological oncologist, although these are not always available, which often negatively impacts treatment quality and patient outcomes [8]. The extent and difficulty of the procedure is directly proportional to the disease stage, with advanced patients having a diminished likelihood of operative success given the widespread nature of the many metastatic foci hindering complete cytoreduction [68]. The aggressive surgical technique involves the *en bloc* removal of all gross tumor tissue, the reproductive organs, and the sigmoid colon along with complete peritonectomy and omentectomy [8,11]. Systematic dissection of the pelvic and para-aortic lymph nodes is also usually performed depending on the stage of the patient and degree of nodal involvement [8]. A successful surgical outcome is defined as one resulting in the absence of any macroscopic residual disease [8]. In practice, however, the optimal degree of cytoreduction is identified as one resulting in less than 1 cm residual cancer [8].

Anything above this is considered to be a suboptimal result. The level of primary cytoreduction achieved is perhaps the most important prognostic factor influencing the eventual fate of the patient [9]. In cases where total resection has been accomplished the long-term outlook is comparatively favorable with some patients even being cured after subsequent chemotherapy [8]. The outcomes for patients with "optimal" cytoreduction are substantially worse than those with no residual disease but nevertheless better than those with suboptimal cytoreduction [8]. Underscoring the distinctive pathobiology of HGSOC is the fact that it is one of the few epithelial cancers for whom the removal of metastatic tumors has been found to improve overall survival [11].

1.9 Treatment for HGSOC: Cytotoxic Chemotherapy

Following successful cytoreductive surgery, virtually all patients with HGSOC are recommended to undergo adjuvant chemotherapy [8]. This contrasts with other, primarily low-grade, subtypes of ovarian cancer where the extent of treatment is dictated by the disease stage, with many patients diagnosed with localized (stage I) disease deemed not to require any further treatment after surgery [8]. The type of chemotherapy regimen received by the patient is the same irrespective of the EOC subtype involved [69]. Historically, ovarian cancer was one of the first malignancies to be successfully treated with cytotoxic chemotherapy [70]. The first class of chemotherapeutic drugs to be developed were the alkylating agents which were introduced in the 1950s [71]. These function as antineoplastic agents by their capacity to cause DNA damage through the addition of bulky alkyl groups to guanine nucleotide bases, thereby inhibiting DNA synthesis. Many such agents were previously used in the treatment of ovarian cancer including melphalan, thiotepa and cyclophosphamide [70]. They were soon joined in the clinical setting by other types of cytotoxic agents including methotrexate, 5-fluorouracil, doxorubicin, and

hexamethylmelamine [71]. Although many of these drugs demonstrated good single-agent activities in the treatment of ovarian cancer, it was promptly ascertained that the most effective strategy would be to employ these agents in combination [71,72]. This was based on the theory that multiple drugs, each with different mechanisms of action, would behave synergistically and reduce the risk of the disease acquiring chemoresistance [71]. In the 1970s, many such combinations were in use for the treatment of advanced ovarian cancer, with the most popular regimens consisting of cyclophosphamide and doxorubicin; along with cyclophosphamide, methotrexate and 5-fluorouracil [71]. Since the late 1970s interest has settled around the use of platinating agents for the treatment of this disease, so-much-so that that the therapeutic standard-of-care for ovarian cancer in recent decades has been referred to as platinum-based therapy. The first such drug to be approved for clinical use was called Cisplatin (CDDP). Its introduction followed the results of trials that validated its effectiveness in the context of recurrent disease resistant to alkylating agents and doxorubicin [71]. It was soon incorporated into primary chemotherapeutic regimens either singly or in combination with cyclophosphamide, doxorubicin or hexamethylmelamine among others [71]. The late 1980s saw the introduction of a new platinating agent in the form of carboplatin. A set of trials, completed in 1992, concluded that carboplatin demonstrated comparable effectiveness to cisplatin in the treatment of ovarian cancer in combination with cyclophosphamide but possessed a far more favorable toxicity profile [73,74]. These results prompted the majority of clinicians in developed nations to begin replacing cisplatin-based regimens with those involving carboplatin [71]. The late 1980s also saw the introduction of a new class of drug, the taxanes, which promptly began testing in the context of ovarian cancer. These drugs, of which paclitaxel is the prototype, were first isolated from the bark of the pacific yew tree (*Taxus brevifolia)* and function by inhibiting tubulin

depolymerization [75]. This stabilization of the microtubular cytoskeleton results in dysregulation of the cell cycle, culminating in mitotic failure and cell death [75]. Early reports indicated that paclitaxel could achieve objective responses in women with advanced, Pt-resistant ovarian cancer [71,76,77]. In 1996, the results of a landmark clinical trial evaluating the effectiveness of cisplatin-paclitaxel combination therapy versus cisplatin-cyclophosphamide were revealed. They indicated that cisplatin-paclitaxel combination therapy was capable of significantly improving objective response rates, progression free survival and overall survival compared to the then standard regimen of cisplatin-cyclophosphamide [78]. In the interest of averting the toxic side-effects of cisplatin, subsequent trials have confirmed similar results using carboplatin instead of cisplatin along with paclitaxel [79-82]. This combination has, for the last 20 years, been the standard of care for the treatment of ovarian cancer [71]. Although known to behave synergistically *in vitro*[83], it is still debated if this effect is relevant *in vivo* [71]. Clinical trials have failed to show an improvement in outcome for cisplatin/carboplatin-paclitaxel, compared to cisplatin/carboplatin monotherapy (with increased single-agent dose), although the combination was associated with reduced toxicity [79,84]. Attempts to add a third drug to the current doublet have all failed to demonstrate any significant improvements in patient outcomes [71]. Efforts in recent years have centered rather on optimizing the current platinum-taxane treatment regimen. The usage of a dose-dense treatment schedule for paclitaxel (weekly) in combination with carboplatin (every 3 weeks) was associated with improvements in outcomes for Japanese women [85]. A similar trial in Western women (MITO7), failed to show any differences in outcome for a dose-dense schedule, although the aims and design of the study were different and thus complicate the interpretation of the results [86].

Another approach currently being explored is intraperitoneal delivery of the chemotherapeutic agents. The rationale behind this method is that because ovarian cancer is almost always confined to the peritoneal cavity, the delivery of the drugs directly to this environment might allow for much greater local drug concentrations to be achieved [71]. Studies have indeed shown that intraperitoneal delivery can achieve 20-fold increase in cisplatin concentration and 1000-fold increase in the local concentration of paclitaxel [87,88]. This increase in local concentration would be highly dependent upon the size of the residual disease present as the depth of penetration of cisplatin and other agents into tumor tissue is thought to be limited to only a few millimeters from the surface of the peritoneal cavity [71]. Three trials have shown improvements in progression-free and overall survival for patients treated with intraperitoneal cisplatin with intravenous paclitaxel [89-91] . These results convinced the National Cancer Institute of the United States of America (NCI), in 2006, to notify physicians that intraperitoneal cisplatin treatment improves patient survival [92]. Nevertheless, intraperitoneal chemotherapy has not been widely adopted in the clinical setting due to elevated toxicity and poor patient tolerability [68]. The administration of carboplatin via the intraperitoneal route in currently being explored. One major trial (GOG 252) reported that it was better tolerated than intraperitoneal cisplatin but failed to show any meaningful survival benefit of the intraperitoneal approach unlike the previous trials (many confounding factors) [92-94]. The current intravenous treatment protocol consists of 75 mg/m^2 cisplatin infusion, plus 135 mg/m^2 paclitaxel infused over 24 hours every 3 weeks for a total of 6 cycles [71]. The usage of cisplatin requires aggressive rehydration to prevent nephrotoxicity [71]. For carboplatin the dose is Area Under the Curve (AUC) 6 along with paclitaxel 175 mg/m^2 infused over 3 hours with the same treatment schedule and number of cycles [71]. Patients that are too ill to undergo initial surgical cytoreduction or whose disease is too

extensive to allow for complete resection may choose to receive neoadjuvant chemotherapy (NACT) [8]. These individuals are administered the first three cycles of chemotherapy which is then followed by an interval whereupon they undergo surgical cytoreduction and then the remaining three rounds of chemotherapy [8]. Two randomized trials have concluded that NACT is not inferior to initial surgery in terms of progression free survival and overall survival [95,96]. Many observation studies, however, have reported that survival is significantly worse for patients receiving NACT in regular clinical practice [97-99].

1.10 HGSOC Relapse and Treatment Resistance

Although the 70% of ovarian cancer patients initially respond favorably to the first application of platinum-based chemotherapy [16], it is estimated that ≥ 80% of these will eventually relapse at some stage [8]. For the subset of patients whose disease is judged to be refractory to the front-line chemotherapy, alternative or second-line drug combinations may be utilized in order to try and elicit an objective response [71]. After successful completion of chemotherapy, the patient is typically assessed radiologically or using CA125 as a biomarker of disease activity level [68]. In at least half of patients, residual cancer cannot be detected using imaging studies and serum markers after 5 months of treatment [16]. During remission, the patient is typically followed-up every 2-4 months with a physical examination or optional radiographic imaging and serum CA125 bioassay [8]. Recurrence is generally asymptomatic at first and is frequently detected by an increase in CA125 levels [8]. The doubling of such levels above the upper limit of normal is considered to be the threshold for diagnosing recurrence [68]. Rarely, a CT scan might detect an asymptomatic recurrence, or the relapse might present with symptoms and a clinically detectable mass [68]. Although CA125 has proven useful for detecting early recurrence, treatment is not typically re-introduced in the absence of clinical symptoms [8]. One study

established that early re-treatment was not associated with any improvements in patient outcome [100]. If relapse consists of a discrete, highly localized tumor mass (rare in the case of HGSOC), then a second cytoreductive surgery may be performed, although a recent meta-analysis of patient data found no benefit to this approach [8,101]. Otherwise, surgery is only used to palliate the effects of intestinal obstruction associated with an isolated site of disease [68]. Typically, patients are re-treated with the standard platinum based-chemotherapeutic regimen [8]. The decision to re-use platinum is complicated by the presence of persistent side-effects from previous treatment such as neuropathy and pancytopenias as well as the potential for life-threatening platinum hypersensitivity reactions [8]. Approximately 50% of patients possess recurrent disease that is still responsive to re-treatment with platinum, albeit with diminishing returns as the progression-free interval (PFI) invariably decreases with each successive platinum therapy [8]. In patients relapsing with a disease that is platinum-resistant, a variety of alternative treatment modalities may be pursued, including the use of pegylated liposomal doxorubicin, topotecan, gemcitabine, etoposide and vinorelbine [8] . The average response rate to this kind of salvage therapy in only about 10-15% with a median progression free survival of 3-4 months [8] . Ultimately, 80-90% of patients diagnosed with advanced-stage disease will develop treatment resistance, which inevitably heralds eventual mortality [15].

1.11 Novel Chemotherapeutics for Treatment of HGSOC

The ideal form of cancer chemotherapy would involve targeting only those pathways known to be abnormally activated in the context of the cancer cell while sparing the cytotoxic effect from the body's normal cells. This is the philosophy behind targeted therapy which has ushered in a new era in the way many cancers are treated. Sadly, in relation to ovarian cancer, this approach has not been especially fruitful as of yet, with only a few new treatments reaching the

clinic and yielding only marginal improvements in outcome. This is partly related to the molecular biology of HGSOC which does not often present with many oncogenic alterations that can be easily targeted with small-molecular inhibitors [15]. That being said, a number of avenues are currently being explored in order to develop novel therapeutics for this disease. One of the more promising relates to a class of drugs known as PARP inhibitors. HGSOC is characterized by widespread genomic instability and the majority of patients possess some deficiency in DNA repair pathways (germline or somatic), particularly that involving the repair of DNA double-strand breaks by Homologous Recombination (HR). The proteins encoded by BRCA1 and BRCA2 are involved in this pathway along with many others. In patients with a deficiency in HR, the cancer cells are over-reliant on the Poly (ADP-Ribose) Polymerase (PARP) mediated Base Excision Repair (BER) of single-strand breaks to resolve spontaneous DNA damage [102]. As such, drugs targeting PARP would be expected to display significant anti-tumor activity in these patients due to synthetic lethality [102]. This term denotes the phenomenon whereby the loss of function in one gene can be tolerated by a cell but can become lethal when combined with the loss of an additional gene product or pathway (Illustration 3: p. 43) [103]. Drugs exploiting this concept are considered desirable because they would have a much greater specificity for tumor cells due to the inherent requirement for a further underlying mutation in order to be effective [103]. The first PARP inhibitor to be tested in patients with HGSOC was Olaparib [102]. Its use has primarily been envisaged as a treatment for recurrent disease or as a maintenance therapy to prolong the progression free interval [8]. Early phase I and randomized clinical trials of Olaparib showed an impressive clinical response in patients with recurrent HGSOC with BRCA mutations [68]. In one phase I trial, a 28 % radiologic response was observed in patients treated with Olaparib 200 mg twice daily [104]. A subsequent phase II trial established that Olaparib was significantly

more effective in relapsed BRCA mutant patients than pegylated liposomal doxorubicin [105]. Another phase II trial using a 400 mg dose twice daily in both BRCA mutant and WT recurrent HGSOC showed objective response rates of 50 % in the BRCA WT cohort and 60 % in the BRCA mutant cohort for those whose disease was Pt-sensitive [106]. In patients with Pt-resistant disease the response rates were 33 % in the mutation-positive cohort, but in only 4 % in the BRCA WT cohort [106]. A trial testing Olaparib as a maintenance therapy for patients with relapsed disease showed a significant increase in progression free survival compared to placebo but without any increase in overall survival [107]. This study also demonstrated that some patients without BRCA mutations could also benefit from this type of treatment [23,108]. In 2014 Olaparib was approved by the European Medicines Agency (EMA) for use as a maintenance therapy in cases of Pt-sensitive recurrent disease in patients with BRCA mutations [8]. Its use was also approved by the FDA as a monotherapy for patients with germline BRCA mutations who have already undergone 3 prior applications of chemotherapy regardless of Pt sensitivity [8]. A phase III study (SOLO2) confirmed the efficacy of Olaparib as a maintenance therapy in BRCA mutant individuals with relapsed Pt-sensitive disease, though the effect on the non-mutant population was not evaluated [109]. Olaparib has also been tested as a supplement to traditional Pt-based therapy in the context of Pt-sensitive relapsed disease [102]. One phase II trial established that progression free survival was longer in the cohort receiving Olaparib after Carboplatin-Paclitaxel therapy but with the effect being greater for BRCA mutant patients [110]. There was, however, no difference in overall survival and toxicity was greater in the group receiving Olaparib [102,110]. Recently, the use of two other PARP inhibitors: Rucaparib and Niraparib was approved by the FDA for use in patients with relapsed ovarian cancer irrespective of BRCA mutation status or Pt-sensitivity [111]. This was based on the outcome of two Phase-III clinical trials showing the

effectiveness of these drugs in prolonging progression free survival in both BRCA mutant and WT individuals when administered as a maintenance therapy in patients with Pt-sensitive, recurrent disease [112,113].

Another targeted therapy being investigated involves targeting the tumor micro-environment through the use of anti-angiogenic agents such as Bevacizumab [68]. This humanized monoclonal antibody targets the cytokine VEGF-A which directs the recruitment of blood vessels to the tumor, something that is required for its growth beyond a certain size [68]. Two important clinical trials (ICON7 and GOG 218), showed an increase in progression free survival with the addition of Bevacizumab to the standard Carboplatin-Paclitaxel regimen as maintenance therapy [114,115]. This prompted the approval of this drug in Europe for use as a maintenance therapy [8]. Two other trials in the context of Pt-sensitive[116] and Pt-resistant [117] disease have shown that the addition of Bevacizumab to cytotoxic chemotherapy improves progression free survival. The use of Bevacizumab in the context of advanced EOC carries the risk of significant adverse effects including gastrointestinal perforation or fistula, hypertension, proteinuria, neutropenia and wound disruption [8,69]. Other anti-angiogenic therapies aim to inhibit the VEGF receptor and other related receptor tyrosine kinases (RTK) [8]. One such agent is Pazopanib. In one trial, the use of this agent as a maintenance therapy significantly prolonged the progression free survival, albeit without increasing overall survival and with a significant toxicity profile [118]. Other anti-angiogenic agents are currently being tested including Nintedanib, Trebananib, Sunitinib, Cabozantinib and Cediranib [8]. The most promising of these is probably Cediranib which has been shown to exert significant single-agent activity in the context of both Pt-sensitive and resistant relapsed disease [8,119]. It has also been shown to increase PFS when used in combination with cytotoxic chemotherapy or as a maintenance therapy [120]. One phase II study even explored a

combination treatment with Olaparib in patients with Pt-sensitive, relapsed ovarian cancer [121]. The combination showed a significant increase in progression free survival compared to Olaparib monotherapy, though this effect was only seen in BRCA WT individuals [122]. BRCA germline-mutant patients, whose response to Olaparib alone is greater than in WT individuals, had no additional improvement with the addition of Cediranib [23,122]. Another potential targeted therapy is the inhibition of AKT signaling. One recent phase Ib/II study reported promising clinical activity of an oral AKT inhibitor (Afuresertib) in combination with carboplatin and paclitaxel in platinum-resistant ovarian cancer [15,123].

Illustration 3: The Concept of Synthetic Lethality

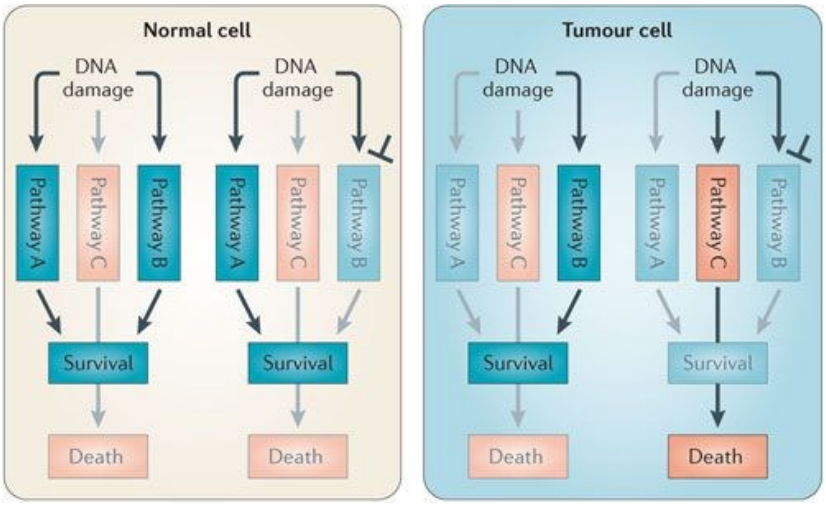


Diagram illustrating the concept of synthetic lethality with respect to tumor cell biology and with a view to its importance for guiding the development of novel chemotherapeutics for selectively targeting tumor cells. In a normal cell the disruption of one pathway contributing to cell survival is non-lethal due to compensation via another pathway with functional redundancy. In a cancer cell, there is already somatic disruption of one of the two pathways such that disruption of the other would be lethal in the cancer cell but not in a normal cell.
Image adapted from: Curtin, *Nature Reviews Cancer* **12**, 801–817 (2012). [124]

1.12 The Origins and Mechanistic Basis of Platinum Chemotherapy

The cytotoxic activity of platinum was discovered quite serendipitously more than 50 years ago in the laboratory of bio-physicist Dr. Barnett Rosenberg at Michigan State University [125]. In 1965, after applying an electrical field to a bacterial growth chamber containing E. Coli, it was noticed that the morphology of the organisms had drastically altered. Instead of appearing as the typical short rods, the bacteria had in fact transformed into long filaments 300 times the normal size [125]. Rather than being an effect of the electric field, it was determined that the causative agent was actually an electrolysis product emanating from the instrument's platinum electrodes [126]. As it turned out, the isolated platinum coordination complex had first been described as early as 1845 and was known as Peyronne's chloride with the chemical name cis-diammine-dichloroplatinum (CDDP) which has since become known as cisplatin [125,126]. The idea to use cisplatin as a chemotherapeutic drug in the treatment of cancer came as the result of experiments conducted in 1968 in which tumor-bearing mice treated with this compound demonstrated a striking regression of tumor burden [127]. Testing on human patients began in 1971 and, after a short period of clinical trials, the drug was approved for medical use by the FDA in 1978; barely 10 years after its initial discovery [125]. In the 40 years since, platinating agents have been a mainstay in the treatment of all forms of ovarian cancer along with many other malignancies including those of the testes, cervix, head and neck, and non-small cell carcinoma of the lung [128]. Cisplatin and its many derivatives share a similar basic chemical structure. In the case of cisplatin, the platinum (II) atom is surrounded on one side (Cis- isomer) by two ammine groups, forming a strong polar bond with the central metallic atom, and on the other by two chloride residues which function as "leaving groups" (Illustration 4, p. 49) [129]. When cisplatin is dissolved in a solution containing a high concentration of chloride ions, the leaving groups

remain stably associated to the parent molecule [129]. Intracellularly, however, the low concentration of Cl^{2-} favors the aquation of cisplatin, with the displacement of the two chloride leaving groups by water molecules [130] (Illustration 4: p. 49). The displacement of these two leaving groups leads to the formation of an unstable and highly reactive species, allowing cisplatin to form high-affinity covalent interactions with DNA [128]. Platinum agents have been shown to enter the cell primarily through the high affinity copper transporter CTR1 with a minor contribution from passive diffusion [128]. This was first elucidated in yeast and in mouse cells in which the mutation or deletion of CTR1 led to reduced platinum uptake and decreased sensitivity *in vitro* [131]. Having entered the cell, the aquated cisplatin molecule binds to DNA with a predilection for the nucleophilic N-7 sites on purine bases [130]. It has been seen that cisplatin binds much more frequently to guanine residues compared to adenine [129]. It is thought that this difference is primarily due to the protonation of guanine which contributes to a greater localization of electron density at the N7 binding site in relation to adenine [129]. The binding of cisplatin leads to the formation of protein-DNA complexes as well as 1-2 or 1-3 intra and inter-strand adducts which are known as "crosslinks" [129,130]. The crosslinks formed are primarily (90%) 1,2-intrastrand d(GpG) adducts, with lesser amounts of the 1,2-intrastrand d(ApG), 1,3-intrastrand d(GpXpG) and interstrand adducts [129] (Illustration 4: p. 49). The presence of these cisplatin modifications contributes to DNA damage by distorting the structure of the DNA double helix. The 1,2- intrastrand crosslinks bend the DNA duplex significantly towards the major-groove, thereby causing a wide, shallow exposure of the minor-groove surface which serves as a point of recognition for many DNA-binding proteins [128]. Many of these proteins are involved in DNA damage repair which proceeds through a number of distinct pathways.

Conversely, it has been found that certain HMG (High Mobility Group) proteins bind to the distorted DNA and protect the platinum-DNA adducts from DNA repair enzymes [132].

The predominant pathway involved in the repair of platinum-DNA adducts is Nucleotide Excision Repair (NER) [130]. Proteins of the Mismatch Repair (MMR) pathway are also thought to be involved in the recognition of cisplatin-induced DNA damage [130]. If the cell possesses the required repair machinery and if the platinum-induced damage is limited in extent, then the likely response would consist of cell-cycle arrest including a prolonged G2-phase blockade [125,130]. This affords the cell sufficient time to resolve the DNA damage before proceeding while averting the potential for mitotic catastrophe [130]. The presence of DNA-platinum adducts can interfere with a number of cellular processes, including DNA replication and gene expression, that require strand separation in order to proceed [125]. If the damage cannot be repaired, then the cell must resort to undergoing programmed cell death [130]. Several kinds of kinases localize to the sites of DNA damage and become activated, triggering a signal transduction cascade that culminates in the activation of the machinery enacting programmed cell-death [130]. These include the kinases ATM and ATR which phosphorylate other kinases including CHEK1 which in turn phosphorylates the protein P53 at serine-20 leading to its stabilization [130]. This transcription factor and tumor suppressor is a master-regulator of the pathways mediating apoptotic programmed cell death. Normally, P53 activation leads to cell death via the mitochondrial pathway of apoptosis which depends upon the activation of caspase 9 [129]. Although undoubtedly important in the context of a normal cell, P53 regulated pathways are unlikely to be essential in the context of HGSOC due to the ubiquity of P53 mutations present in patients with the disease. Several other signaling pathways participate in the transduction of a cisplatin-induced response in the cell and likely contribute to its toxicity, especially in the absence of functional P53. These

include members of the MAPK family P38 and JNK [128]. The kinase C-ABL has also been shown to play an important role in the signaling of cisplatin-induced DNA damage [128]. C-ABL translocates to the nucleus upon DNA damage and participates in the activation of JNK signaling through a mechanism dependent on MMR recognition of DNA damage foci [133]. C-ABL also interacts with pro-apoptotic P53-family member P73 which participates in the initiation of apoptosis [134].

Although the major cytotoxic effect of cisplatin is likely caused by DNA damage, it is not the only mechanism by which platinum causes cellular lethality [129]. This was proven in experiments using enucleated cells which were also shown to be sensitive to cisplatin cytotoxicity [135,136]. In the cytoplasm, cisplatin is able to induce oxidative stress through binding to proteins containing nucleophilic thiol groups [130]. These include reduced glutathione (GSH), methionine, metallothioneins and other cysteine-containing proteins. This action of cisplatin in the cytoplasm depletes the cellular anti-oxidant pool and tips the redox balance towards oxidative stress [130]. The formation of Reactive Oxygen Species (ROS) by cisplatin depends both on the concentration and the duration of exposure [129]. The excessive production of ROS can lead to the activation of both the intrinsic (mitochondrial) and extrinsic pathways of apoptosis, in a manner independent of DNA damage [129]. Aquated cisplatin can also directly target the mitochondrial membrane through the uncoupling of oxidative phosphorylation, leading to mitochondrial dysfunction, inhibition of ATP synthesis and aberrant calcium release which disrupts the activity of many cellular signaling pathways [137]. Cisplatin can also cause cells to undergo programmed necrosis [128], although this effect is seen mainly *in vitro* and occurs at concentrations higher than would be achievable clinically.

Carboplatin has a much more stable cyclobutene-1, 1-dicarboxylate (CBDCA) leaving group which confers a much slower kinetic of DNA binding compared to cisplatin [129]. Although the DNA-adducts formed by carboplatin are essentially the same as its parent compound, a 20-40-fold higher concentration is required to achieve the same degree of DNA damage [125]. Fortunately, carboplatin causes much less systemic toxicity compared to cisplatin, allowing it to be administered in higher doses. Typically, the concentration of carboplatin required to be administered intravenously to a patient is four-fold greater than cisplatin to achieve a similar tumor response [129]. Carboplatin also has a much more stable retention in the patient compared to cisplatin, with a half-life of 30 hours compared to 1.5-3.6 hours for cisplatin [129]. This stability ensures that up to 90% of the infused carboplatin will be excreted in the urine [129].

1.13 Platinating Agents: Mechanisms of Resistance

Cancer cells can become resistant to the effects of platinum through a number of distinct mechanisms. Most of these have been elucidated through the use of *in vitro* models and their relevance to those processes occurring in human patients is doubtful. On the one hand, cells can prevent platinum from exerting its damaging effect on genomic DNA by preventing its intracellular accumulation, or by sequestering and inactivating it in the cytoplasm [130]. The level of intracellular platinum accumulation is predominantly controlled through the rate of influx through the plasma membrane [130]. Platinum influx into the cell is controlled by the transmembrane copper transporter CTR1 [131]. It has been shown that exposure to clinically relevant concentrations of CDDP results in diminished drug sensitivity through the downregulation of CTR1 [138]. In human ovarian cancer cell lines, platinum was seen to trigger internalization of the receptor by macropinocytosis followed by its proteasomal degradation [138].

In human ovarian cancer cell lines having acquired Pt-resistance *in vitro*, the expression of CTR1 is diminished and the rate of influx of both copper and cisplatin is significantly reduced [139]. Furthermore, it was demonstrated that the level of expression of CTR1 in human ovarian tumors was substantially correlated with disease outcome and response to chemotherapy, with patients expressing less CTR1 having decreased overall survival [140]. The sensitivity to platinum depends on the levels of cellular copper such that cells pretreated with copper lose sensitivity while those treated with copper chelators become more sensitive [140,141].

Illustration 4: The Formation of Pt-DNA Adducts by Cisplatin

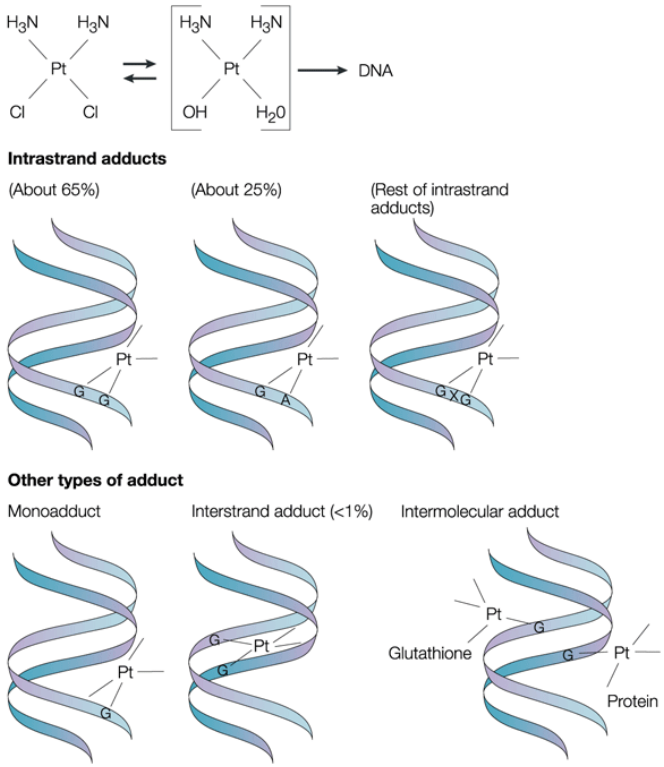


Figure illustrating the mechanism of aquation of cisplatin (CDDP) along the with the types of Pt-DNA adducts formed (crosslinks) and the observed frequency of each.

Image adapted from Masters & Köberle. *Nature Reviews Cancer* **3**, 517–525 (2003) [142]

Like many chemotherapeutic drugs, cancer cells can efflux cisplatin through an array of non-specific membrane ATPase pumps of the ABC and MDR families [130]. Many *in vitro* models of CDDP-resistance have been shown to be dependent on the activity of these efflux pumps [130]. One study on clinical samples showed that the level of expression of P-type copper ATPase ATP7B correlated with response to chemotherapy in patients with ovarian cancer [143]. Moreover, it has been reported the gene encoding MDR1 is upregulated, because of promoter translocation or fusion events, in about 8% of cases of recurrent HGSOC [144]. Nevertheless, the strategy aiming to pharmacologically inhibit the activity of these efflux pumps has not been proven to be effective in elevating cisplatin intracellular accumulation and restoring the drug-sensitivity of tumor cells [130,145]. While the depletion of the cytoplasmic pool of reduced thiol-group containing proteins contributes to the cytotoxic effect of platinating agents, these same nucleophilic species can also serve to sequester and neutralize the reactive aquated platinum species and thereby limit its toxicity [130]. The cellular antioxidant glutathione has, in particular, been implicated in this process and elevated levels of both GSH and the enzyme GST have been observed in the context of Pt-resistance [130]. The platinum resistant, patient-derived HGSOC cell line PEO4 was shown to have higher levels of reduced glutathione and 2.9-fold greater GST activity compared to the Pt sensitive cell line PEO1 isolated from the same patient before the onset of chemoresistance [146].

In the presence of Pt-induced DNA damage, cancer cells can acquire resistance through increased capacity for DNA repair or an elevated tolerance for DNA damage [130]. Because NER is predominantly responsible for the resolution of Pt-DNA adducts, gains in the proficiency of this pathway would likely contribute to Pt-resistance [130]. The endonuclease ERCC1 is one of the rate-limiting participants in the NER pathway. Its level of expression has been identified as a biomarker of patient survival/ response to therapy for a number of cancers treated with

platinating agents, including ovarian cancer [130,147]. Although the MMR pathway cannot repair Pt-DNA adducts, its proteins participate in the recognition of Pt-DNA damage foci and trigger pro-apoptotic signaling to mediate Pt-sensitivity [130]. Accordingly, MMR proteins including MSH2 and MLH1 are often found to be mutated or under-expressed in scenarios of Pt-resistance [130]. As a result, methylation dependent silencing of MLH1 has been viewed as a biomarker of negative prognostic significance in patients with ovarian cancer [148]. In HGSOC tumors deficient in HR-mediated repair of DNA double strand breaks due to BRCA mutation, the onset of resistance is often accompanied by reversion mutations that restore functional HR [130]. This was shown in the context of the sequentially derived HGSOC cell line series of PEO1, PEO4 and PEO6 in which PEO1 is BRCA2 mutant while in PEO4 and PEO6 there is a reversion mutation and functional HR [149,150].

Replicative bypass by translesion synthesis is one mechanism that tumor cells might employ in order to replicate DNA in the presence of Pt-induced DNA damage [130]. A number of specialized DNA polymerases are implicated in this process including REV3 and PolH [130]. REV3 overexpression in glioma cells confers greater CDDP-resistance *in vitro* and high levels in clinical samples correlate with a high tumor grade [151]. The inability to undergo apoptosis through dysregulation of pro- and anti-apoptotic BCL-2 family proteins has also been associated with Pt-resistance [130]. Overexpression of the anti-apoptotic protein BCL-XL *in vitro* conferred diminished Pt-sensitivity to ovarian cancer cell line A2780 and high levels of this protein in ovarian cancer patient samples correlated with poor prognosis [152].

One further unusual mechanism that may contribute to Pt-resistance is autophagy. In an *in vitro* derived model of CDDP-resistance using the ovarian cancer cell line SKOV3, it was shown that resistance is associated with an increase in the level of P62 [153]. This protein participates in

the trafficking of ubiquitinated proteins to autophagosomes to ensure their degradation. Knockdown of this protein or the pharmacological inhibition of autophagy was sufficient to resensitize these cells to cisplatin [153]. In a model of clinically acquired Pt resistance using the HGSOC cell lines PEO4/PEO6, PEA2 and PEO23, the group of Stronach and colleagues showed that resistance resulted from a novel mechanism involving the deacetylation of STAT1 by HDAC4 [154]. Aberrant activation of pro-survival signaling including PI3K-AKT has also been implicated in Pt-resistance [130]. For example, with the same model system, the group of Stronach and colleagues also demonstrated another resistance mechanism featuring the Pt-mediated nuclear localization of AKT and its activation by DNA damage-induced kinase DNA-PK in the context of the resistant cell lines [155].

In addition to the many cell-intrinsic resistance mechanisms outlined, the interaction of tumor cells with other cells in the microenvironment may lead to the acquisition of Pt-resistance in a conditional manner [156]. For example, Cancer Associated Fibroblasts (CAFs), adipocytes and Tumor Associated Macrophages (TAMs) have been shown to mediate microenvironmental interactions with ovarian cancer cells to promote Pt resistance [156]. HGSOC associated CAFs were shown to export reduced glutathione (GSH) and cysteine which was taken up by co-cultured EOC cells *in vitro* to decrease Pt sensitivity [157]. Stromal cells have also been shown to produce inflammatory mediators and cytokines such as IL-6, IL-8, VEGF and IL-10 which can activate pro-survival signaling in HGSOC cells such as AKT, NFκB and STAT3 [156]. Another unique mechanism involved the transfer of Extracellular Vesicles (EVs) containing miRNAs such as MiR-21 by adipocytes [158]. This miRNA was shown to target APAF-1 which may mediate drug resistance by antagonizing the initiation of apoptosis [158].

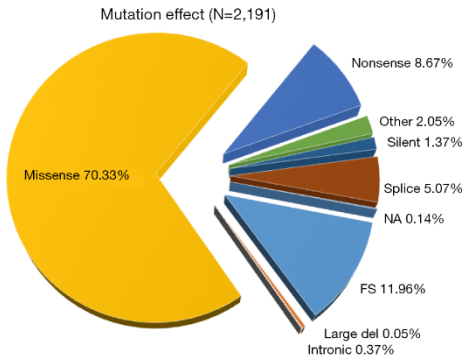
1.14 The Genetics of HGSOC

A major milestone in the understanding of HGSOC occurred as the result of a 2011 study by the TCGA network which sought to evaluate the disease's genetic features through whole exome sequencing of samples from 316 patients [159]. This revealed that the genomic landscape of HGSOC is characterized by profound genomic instability with few recurrent gene mutations other than TP53 [159]. This was in contrast to other Type-1 EOCs which are characterized by frequent oncogenic mutations to genes such as BRAF, KRAS, PTEN, CTNNB1 and PIK3CA while being P53 WT [23]. In the case of HGSOC, it was found that upwards of 96 % of the samples contained somatic TP53 mutations, seeming to suggest that this mutation is a defining feature of HGSOC and likely to be required for disease initiation [23]. Retrospective studies established that the small percentage of P53 WT samples from the aforementioned study derived from patients whose disease was likely misdiagnosed as HGSOC [160]. Thus, it is possible to conclude that virtually 100% of cases of HGSOC feature TP53 mutations. Because P53 is commonly found to be mutated even in the precursor lesions of HGSOC (STIC), it is likely that this event is one of the earliest events in the sequence of carcinogenesis for HGSOC [23]. Further studies have shed light on the precise nature and function of the P53 mutations present in patients with HGSOC. One study, using data from the International Agency for Research on Cancer (IARC) P53 database, reported that 70.4% of TP53 mutations were in fact missense mutations which encode for a protein with an amino-acid substitution (Illustration 5: p. 54) [161]. There was a much smaller contribution from frameshift, nonsense and splice mutations which affect 12%, 8.67 and 5.1% of patients respectively [161]. These mutations encode for truncated or malformed proteins which are equally likely to confer a total abolition of function. The missense mutations can result in three distinct phenotypes depending on their effects on P53 protein function. There is the potential for

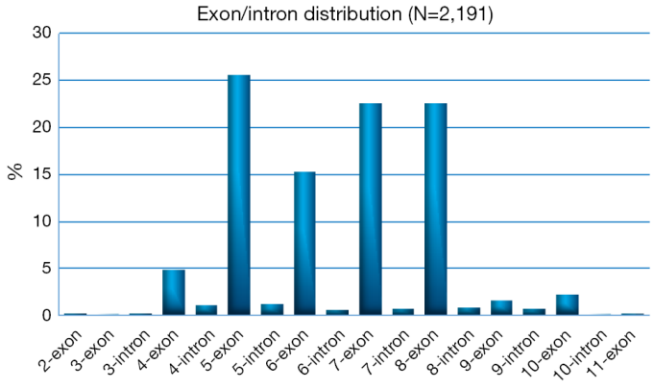
either a loss of function, dominant negative or a gain of function mutation [161]. The P53 protein consists of multiple structural domains which each specify one aspect of the protein's functional behavior. Around 80% of the total mutations are located in the central DNA binding domain of the P53 protein which likely result in a loss of function due to the inability to serve as a transcription factor to modulate the expression of target genes (Illustration 5: p. 54) [161].

Illustration 5: Type and Frequency of TP53 mutations in EOC

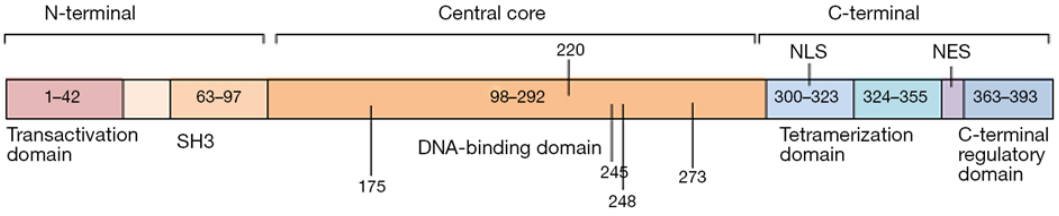
A)



B)



C)



A): Frequency of each individual type of mutation affecting TP53 in EOC

B): Frequency of each mutation affecting TP53 in EOC by location on the gene

C) Structure of the TP53 gene illustrating the order and relative size of each of its protein functional domains

Images adapted from: Zhang et al. *Translational Cancer Res* 5, (6) 650-663 (2016) [161]

Because P53 functions as a tetramer, many missense mutations may also result in the formation of a dominant-negative protein which can inhibit tetramerization even in the presence of residual P53 WT protein [161]. Mutant p53 protein is far more stable in the cell than its WT counterpart due to the inability to interact with its inhibitor HDM2 which normally ensures its timely degradation by the proteasome [161]. This increased stability, and thus the higher protein levels, of mutant P53 may enable it to possess an additional, oncogenic gain of function activity [161]. Studies have shown that the type and location of a patient's TP53 mutation have implications on the individual's prognosis [162,163]. Besides TP53, few other gene mutations are common between patients with HGSOC. The TCGA study found BRCA1 mutations in about 12.5% of patients (9% of germline mutations and 3.5% somatic mutations), BRCA2 was mutant in about 11.5% of cases (8% germline mutations and 3.3% somatic mutations), along with a smaller number of mutations involving CSMD3 in 6%, NF1 in 4%, CDK12 in 3%, GABRA6 in 2% and RB1 in 2% of patient samples [159].

By contrast, there appears to be a much more prominent role for gene copy-number variation in HGSOC due to genomic instability, resulting in the amplification or loss of many genes. The most frequent focal amplifications involve the genes CCNE1, MYC and MECOM with each being involved in more than 20% of the samples analyzed [159]. An analysis integrating mutational frequency, copy number alterations, and changes in gene expression has provided evidence of the main pathways involved in HGSOC pathogenesis. This method revealed the Homologous Recombination pathway of DNA repair is defective in 51% of cases [159]. This involved mutations to BRCA1 and BRCA2 (germline and somatic) in 20% of patients, with a further 11% having BRCA1 silencing by DNA hypermethylation [159]. In patients with hereditary BRCA1/2 mutations, the other allele almost always displays Loss Of Heterozygosity (LOH) [23]. One study

determined that 91% of patients with BRCA1 germline mutation displayed locus specific LOH compared to 72% of non-carriers [164]. Another group estimated that 100% of patients with germline BRCA1 mutations have LOH along with 76 % of those with inherited mutations in BRCA2 [165]. Moreover, the type of mutation present may be significant, especially in the case of BRCA2 for which patients with truncating mutations in the RAD15 binding domain had a survival advantage over individuals with other mutations in the same gene [166]. Other genes encoding proteins in the HR pathway are also recurrently affected in HGSOC, including PTEN, RAD51C, ATM and ATR, as well as many of the Fanconi anemia genes [159]. The involvement of these genes would suggest that defects in HR DNA repair play a major role in the etiology of HGSOC. Intriguingly, cases involving CCNE1 amplification segregated from those with BRCA amplification, suggesting that there could be two distinct pathways driving the pathogenesis of HGSOC with a heterogeneity among patients [159]. It is notable that BRCA mutant patients possessed a significant overall survival advantage over those with CCNE1 amplification [159]. In general, it has been seen that patients with BRCA mutations initially respond better to chemotherapy and therefore have a better 5-year survival than non-mutant individuals [167]. This survival advantage, however, is not maintained in the long-term in the case of BRCA1, but with a small persistent advantage in the case of BRCA2 [167]. Other pathways frequently altered include: RB1 (67%), PI3K/Ras (45%) and NOTCH (22%) [159]. The TCGA study also identified activation of the FOXM1 transcription factor pathway as another important hallmark of HGSOC, with 87% of patients presenting with overactivation of the transcription network downstream of this protein [159]. Importantly, FOXM1 is normally suppressed by P53 in the context of DNA damage, suggesting that P53 mutation might contribute to the overactivity of this pathway [159].

Many studies have highlighted the important role of PI3K-AKT pathway in HGSOC [23]. The TCGA study linked amplifications of PIK3CA, PIK3CB and PIK3K4 with decreased overall survival [159]. Another study found that, in patients with advanced HGSOC, *PI3KCA* mutations were found in 5% of samples, along with amplification of PI3KCA and AKT2 in 12% and 10% of samples respectively [168]. It has also been argued that most genetic studies underestimate the percentage of cases with the loss of expression of PTEN [169]. Using an immunohistochemical analysis by tissue microarray, one study showed that around 50-75% of cases are characterized by PTEN loss or under-expression [169]. Also, GAB2, an adaptor protein between the Ras-MAPK and PI3K-AKT signaling pathways, was shown by one study to be recurrently amplified in 44% of cases [170]. Analysis of haploinsufficiency has suggested that the autophagic pathway is also significantly disrupted through gene deletion [171]. One study reported that the genes BENC1 and LC3 were mono-allelically deleted in 94% of patients with HGSOC [171]. In addition, it was shown that HGSOC cell lines were hypersensitive to treatment *in vitro* with autophagy inhibitors [171]. The downregulation of the Let-7 family of micro-RNA is also thought to play an important role in the etiology of HGSOC, leading to the translational overexpression of many proteins including the DNA binding factor HMGA2 [172]. This protein is an important regulator of chromatin conformation and functions as a structural factor regulating the assembly of the enhanceosome complex, thus participating in the expression of many genes. HMGA2 was found to be overexpressed in 64% of HGSOC tumors by immunohistochemistry and its expression correlated with a less-differentiated phenotype and poor patient prognosis [172,173]. Although genomic instability is a feature of HGSOC, only a few recurrent recombination events have been identified to result in the creation of fusion genes [23]. One such event is the formation of the inter-chromosomal fusion gene CDKN2D-WDFY2 which was detected in 20% of cases of HGSOC

[174]. CDKN2D (A.K.A P19) is a cell-cycle specific regulator of AKT signaling and the fusion protein was demonstrated to be sufficient to activate the PI3K-AKT pathway in transfected cells [174].

1.15 HGSOC Gene Expression Profiling and Molecular Subtypes

Although referred to as a singular class of malignancy, a recent line of evidence from studies employing gene expression profiling has revealed that HGSOC is actually characterized by a whole spectrum of molecular diversity [23]. One such influential study, by the group of Tothill et al., succeeded in delineating four distinct molecular subtypes of HGSOC with significant correlations to patient outcome [175]. Using 285 predominantly high-grade serous tumor samples, their analysis of differential gene expression segregated the pooled data into six robust clusters which were accorded the names C1-C6 [175]. Of these, clusters C3 and C6 were deemed unlikely to represent HGSOC [175]. Cluster C1 is marked by its association with a reactive stromal signature and upregulation of genes associated with extracellular matrix production/ remodeling, cell adhesion, cell signaling and angiogenesis [23,175]. At the histopathological level, this subtype is distinguished by extensive myofibroblast infiltration (desmoplasia) [23,175]. It was discovered that this signature was associated with a poor overall prognosis [175]. By contrast the C2 was termed "immunoreactive" because of its association with higher numbers of tumor-infiltrating $CD3^+$ T-lymphocytes and a gene expression signature defined by the upregulation of genes involved in immune cell activation [23,175]. This subtype was found to have a greater overall survival [175]. C4 demonstrated a low stromal response with certain parallels in gene expression to C2 but with elevated CA125 [23,175]. It was also associated with a better prognosis [175]. C5 exhibited a signature that featured many genes involved in mesenchymal development, including certain HOX genes, high-mobility group members, as well as WNT/ catenin and cadherin signaling pathways [23,175].

This group was also found to have an inferior overall survival [175]. A subsequent study by the TCGA group built upon these results, using a refined methodology to identify 4 overlapping subtypes dubbed: immunoreactive, differentiated, proliferative and mesenchymal [159]. This study, however, failed to identify any significant correlations to patient outcomes [23]. Conversely, a recent re-analysis of the TCGA dataset, using a more sophisticated protocol managed to elaborate on the molecular signature of the 4 previously identified subtypes and to demonstrate prognostic significance for each [176]. The mesenchymal and proliferative subtypes possessed the worst overall survival while the immunoreactive featured a better prognosis, with the differentiated being intermediate on the scale [23,176]. These results relating to prognosis were later independently confirmed by another group [177]. Recently, due to persistent heterogeneity in the differentiated subtype, it was decided to declare a further group known as anti-mesenchymal due to the downregulation of genes involved in the Mesenchymal subtype [178] . This new subtype was associated with a better prognosis [23,178]. The popularity of these molecular subtypes has instigated efforts to introduce a new histopathological classification system based on characteristics relating to each of the molecular subtypes [23,179].

Needless to say, a great many groups are publishing reports on the prognostic significance of certain gene signatures [23]. In some cases, these might involve hundreds of genes, while in others only a few. In one instance, the expression of certain genes involved in DNA repair pathways has been associated with a favorable prognosis after platinum chemotherapy [180]. Another identified the expression of HIF-1α and its associated response genes as predictive of poor overall survival [181]. Yet another, linked genes encoding extracellular matrix proteins involved in collagen remodeling with poor overall patient survival and high metastatic potential [182]. Efforts have also sought to define signatures predicting the extent of optimal debulking after surgery or the

response to platinum chemo-therapy or PARP inhibitors [181,183,184] . On the whole, while this approach bears much promise and has been well validated in a scientific setting, the utility in clinical practice has never been proven [8]. Furthermore, the introduction of gene expression profiling to the clinic is likely to be hampered by the degree of technical complexity and the considerable cost associated with such technology [23]. If nothing more, this line of inquiry has succeeded in refining our understanding of heterogeneity and molecular diversity at the heart of HGSOC.

1.16 *In Vitro* Models of HGSOC

The use of cancer cell lines to model HGSOC *in vitro* is perhaps the most frequently employed method of interrogating the disease experimentally. It is thus of paramount importance to ensure that the cell lines in current usage accurately represent the disease's fundamental biology, while maintaining a practical ease of use and accessibility to all researchers for the purpose of standardization. These cell lines should each possess a well-annotated source of origin, with available information relating to the clinical history of the donor patient and the precise manner in which they were initially established. Unfortunately, until recently, most of the cell lines in widespread usage were poorly characterized, with obscure origins and uncertain histopathology [185]. These included many of the top cell lines by publication figures including: SK-OV-3, A2780, OVCAR-3, CAOV-3 and IGROV-1 [185]. These cell lines, including the two most commonly used ovarian cancer cell lines SK-OV-3 and A2780, were popular because they rapidly and reproducibly form discrete tumors when injected orthotopically or ectopically into nude mice, while also being easily manipulated using transfection techniques [186]. The publication by the TCGA, in 2011, of a comprehensive genomic characterization of HGSOC would finally permit a determination on the suitability of the then popular cell lines to be made on the basis of

their genetic similarity to the disease [159]. To this end, Domcke et al. compared the genomic features of the cell lines purported to represent HGSOC with the data derived from the TCGA study of primary tumor samples [185]. This yielded a number of shocking results, in that virtually all the cell lines commonly used in the literature as models of HGSOC were deemed to, in fact, poorly recapitulate the genetic features of the disease. This was especially true for the two most popular cell lines SK-OV-3 and A2780 which were not assigned a histological subtype by the originator, but which together contributed to about 60% of all publications using ovarian cancer cell lines [185]. While almost 100% of HGSOC cases are characterized by TP53 mutation, these two cell lines are P53 WT and contain very few gene copy number alterations [185]. A variant of SK-OV-3 has since been identified that contains a P53 deletion, but this is far more likely to have emerged in culture [186]. These cell lines feature many of the mutations typically found in the other histotypes of EOC including ARID1A, BRAF, PIK3CA and PTEN [185]. Based on this evidence it is possible to classify both A2780 and SK-OV-3 as being derived from type-1 non-serous tumors and thus unrelated to HGSOC [185]. IGROV-1, another popular cell line quoted as being from HGSOC, was P53 mutant but displayed a hypermutant phenotype which is not among the features of this disease [185]. Clustering analysis grouped IGROV-1 among the endometriosis-derived subtypes (Clear-cell and endometrioid) [185]. Other cell lines such as OVCAR-3 possessed a similar mutational profile to HGSOC but had CNAs (Copy Number Alterations) that diverged from the mean of the tumor samples from the TCGA [185]. These cell lines were probably derived from cases of HGSOC but do not perfectly recapitulate the genomic features of the disease. The cell lines ascertained to best fit the genomic picture of HGSOC were Kuramochi, OVSAHO, SNU119, COV362 and OVCAR-4 [185]. It is notable that none of these cell lines were in widespread use and that Kuramochi had previously been described as being

derived from an undifferentiated neoplasm [185]. Recently the group of Coscia et al. used a proteomic signature to stratify putative HGSOC cell lines into three distinct groups (called groups I-III) [187]. Although the majority of cell lines with a high genetic fidelity to HGSOC were classified as group-I and bore a more epithelial proteome, there were two cell lines recapitulating the genetic features of the disease (59M and TYKNU) which clustered in group-III with a more mesenchymal proteome [187]. Notably there was a striking concordance between the proteomic signature of group-I cell lines and a set of HGSOC patient samples. This signature also clustered closely with cultured fallopian tube epithelial cells. On the other hand, group-III cell lines resembled the signature of immortalized OSE cells. These findings suggest that there is heterogeneity in the proteome of HGSOC which is likely based on disparate sites of origin (OSE vs. FTSEC) and that this diversity is mirrored in HGSOC cell lines [187].

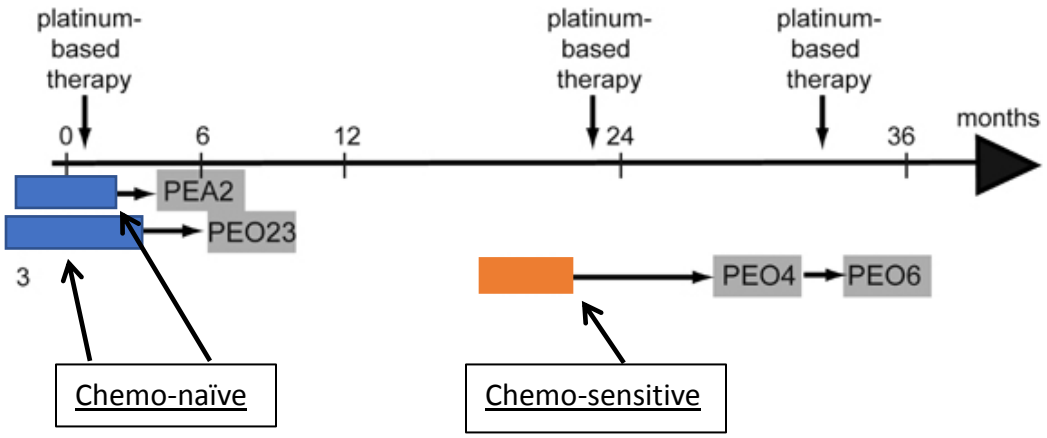
There is a striking lack of patient-derived cell lines of chemo-resistant HGSOC. The vast majority of cell-lines used to represent Pt-resistant disease have been generated *in vitro* by exposing cells to platinum continuously for extended durations of time (usually many months). Although this method has often succeeded in generating cells with many-fold greater resistance to platinum, the type of drug exposure used to elicit the resistance phenotype is unrealistic and fails to resemble what is achievable clinically. As a result, the mechanisms involved in mediating the resistance that is created are likely to be totally different from those acting within the physiological environment of the patient. An ideal scenario would be to establish multiple cell lines longitudinally from the same patient so that the molecular and genetic changes that accompany the onset of resistance can be identified. Presently, few such models of HGSOC are known to exist with the notable exception of a set of matched cell lines established in the UK during the 1980s [188]. These cell lines, known as PEO1/PEO4/PEO6; PEO14/PEO23;

PEA1/PEA2, were each established from an individual patient before and after the onset of chemoresistance (Illustration 6, p. 64). PEO14 and PEA1 were established in a chemo-naïve state before the induction of chemotherapy and their counterparts PEO23 and PEA2 were isolated upon relapse with treatment resistant disease after 7 and 5 months respectively [188]. PEO1 originated as a first relapse of a patient treated 22 months prior with cisplatin, 5-fluorouracil and chlorambucil [188]. At that stage, the disease was still sensitive to retreatment, but upon a second relapse after 10 months the disease was found to be resistant, with the patient failing to respond following three months of an elevated-dose regimen of cisplatin [188]. The second relapse resulted in the generation of PEO4, while PEO6 was generated just 3 months later before the patient succumbed to the disease [188]. Although these cell lines were not assessed in the paper by Domke et al., a subsequent paper by Beaufort et al. assigned them all to be putative HGSOC based on genomic features [189]. This paper also established concordance between the morphology of the cell line *in vitro* and its molecular subtype as defined by Tothill et al. (See above). Cells displaying a spindle morphology clustered with the C1 (stromal) tumours, those with rounded morphology with the C5-mesenchymal subtype, and the epithelial-like cell lines with the C4 subtype [189].

1.17 HGSOC *In vivo* Models and Novel Culturing Techniques

There are currently few *in vivo* model systems for the study of HGSOC that closely resemble the human disease. Efforts to develop a new generation of accurate murine models have employed contrasting approaches. First of all, there are the Genetically Engineered Mouse Models (GEMM) that are designed to mirror the same sequence of genetic defects that contribute to human carcinogenesis. Upon induction, these mice begin to develop tumors either from the Ovarian Surface Epithelium (OSE) or the fallopian tube that may recapitulate many of the

Illustration 6: Origins and Lineage of the Three Series of Longitudinally Matched HGSOC Cell Lines



This diagram illustrates the lineage and the relationship between each of the cell lines comprising the three longitudinally matched HGSOC cell line series which were established in the United Kingdom during the 1980s. They were first described by Dr. Simon Langdon of the University of Edinburgh. Both PEO14 and PEA1 originated from a patient who was chemo-naïve. In the case of each of these patients, their disease returned after a short interval following chemotherapy and was found to be treatment resistant (from which was generated PEO23 and PEA2). PEO1 originated as a chemosensitive primary relapse 20 months after initial treatment. Following a further remission, the disease returned and was no longer responsive to treatment. From this patient were generated two further cell lines PEO4 and PEO6, with PEO6 having been isolated after a final, unsuccessful attempt to treat the patient.
Image adapted from: Cooke et al. *Oncogene* (2010) 29, 4905–4913 [149]

features found in the human disease [23]. The advantage to this approach is that it allows for the investigation of the disease at its earliest stages, something that cannot be replicated with another methodology [190]. Until recently, the creation of these murine models was hampered by a lack of tissue specific promoters for the putative site of origin for majority of HGSOC, which has been traced to the distal fallopian tube [190]. Historically, these models have targeted the OSE using the conditional expression of oncogenes such KRAS or deletion of tumor suppressors such as TP53, RB, BRCA1/2 and PTEN [23]. This approach, however, has yielded tumors that do not match the histology, marker expression and disease course exhibited by patients with the disease [23]. These failures have stimulated the development of a new generation of mouse models with greater

emphasis on the fallopian tube as the site of origin for HGSOC carcinogenesis [23]. Recently Kim et al. demonstrated a model using the conditional deletion of DICER and PTEN using the Mullerian specific promotor Amhr2 in loxP-Cre mice [191]. These animals develop high-grade serous adenocarcinoma from the fallopian tube with many histological and molecular similarities to human HGSOC [23,191]. The disease course in these mice also parallels that of the human disease [191] This model, however, failed to demonstrate emergence from the precursor lesions (STIC) that are thought to represent the earliest stage of HGSOC progression [23]. Another model by Perets and colleagues employs a triple deletion of BRCA, TP53 and PTEN, all mutations found recurrently in patients, under the control of the Pax8 promotor [54]. These transgenic mice developed disease resembling many of the clinical-pathological features HGSOC from the secretory epithelial cells of the distal fallopian tube (FTSEC) [23,54]. Moreover, the mice presented in early onset with precursor lesions in the same locations as the STICs observed in humans [54]. Sherman-Baust et al. have also created a model that uses the expression of the SV40 Large T-antigen under the control of the Mullerian-specific Ovgp-1 promotor [192]. In this case, spontaneous neoplastic lesions are seen to originate in the fallopian tube and endometrium [192]. This model has furthermore demonstrated a progression from a precursor lesion resembling the STIC to a highly invasive disease similar to HGSOC [23,192]. Finally, there is the model generated by Zhai and colleagues involving the deletion of the four tumor suppressor genes BRCA1, TRP53, RB1, and NF1 also using the Ovgp-1 promotor [193]. This model established the essential role of PTEN in the evolution of HGSOC in this model system, as animals without PTEN deletion failed to develop tumors even in the presence of mutations to the other three genes [193]. Animals in which all four genes were inactivated presented with STICs that progress to HGSOC or carcinosarcoma, with the presence of widespread metastatic disease in many mice

[193]. NF1 deletion was not necessary for this process but the disease arose quicker in animals with all four genes deleted [193]. The existence of these new and improved GEMM models offers a promising avenue for addressing many inquiries relating to the early stages of HGSOC pathogenesis as well as providing an opportunity to identify new therapeutic targets and test experimental treatments [23].

Beyond GEMM there are also *in vivo* models that involve the transplantation of human cancer cells into immunodeficient mice (xenografts). Traditionally these have involved the transplantation of cells from human cell lines into athymic (nude) mice either subcutaneously or orthotopically such as into the peritoneal cavity or within the ovarian capsule or fallopian tube [23]. Because the majority of cell lines previously used to represent HGSOC poorly recapitulate the characteristic features of the disease at the genetic level, a new effort is under way to develop transplantation models using cell lines with high genetic fidelity to HGSOC [185,186]. Unfortunately, many such cell lines grow poorly when transplanted intraperitoneally in nude mice, requiring more Severe Combined Immunodeficient (SCID) or NOD-SCID IL2R-gamma^{null} (NSG) mice to be used [186,194], something that is not considered desirable in view of the important contribution of immune cells to the HGSOC micro-environment.

An alternative approach that has been highlighted recently involves the transplantation of minced tumor fragments freshly isolated from human patients into orthotopic sites in immunodeficient mice [190]. Many studies have evaluated the efficacy and suitability of this method, also known as the Patient-Derived Xenograft (PDX), and have generally described a high degree of resemblance to the disease observed within the specific patient, including the preservation of intra-tumor phenotypic heterogeneity [23,195]. The efficiency of transplantation has been seen to depend upon the degree of immunodeficiency of the recipient mouse and the site of

injection [23]. One study has shown that tumors that engraft more readily are associated with a poorer prognosis for the patient from whom they were derived [196]. In the future, these patient-derived xenografts might be used clinically to test novel therapies and to predict the response of the patient to treatment [23]. Nonetheless, some deficiencies have been underlined regarding these models. For one, they represent only the late stage of the disease due to the injection of the cells or tissue directly within the peritoneal cavity [190]. On the other hand, variation exists between the models using SCID versus NSG mice. In the SCID mice the human stroma is rapidly replaced by mouse stroma whereas for NSG mice the human stroma is maintained in the long-term [190].

New 3D organotypic cultures have also been developed that better mimic the *in vivo* growth environment compared with growth on plastic [23]. One novel method developed by Kenny et al. involves growing ovarian cancer cells on a multilayered substrate consisting of fibroblasts, mesothelial cells and extracellular matrix [197]. Cells can also be grown in 3-dimensions on a layer of Matrigel™ or using intact peritoneal explants from human or animal sources [198]. Because HGSOC cells have a propensity *in vivo* to form spheroids and aggregates that grow in suspension, culture systems can be employed that favor the formation of such structures *in vitro*, such as a low-adherence culture surface, spinning flasks or through the use of the hanging drop method [198]. Certain HGSOC cell lines, such as PEO6. have been seen to spontaneously form spheroids even in high-adherence conditions [199].

1.18 Tumor Heterogeneity and the Clonal Evolution Hypothesis

While it has attained popularity rather recently, it is not commonly acknowledged that tumor heterogeneity is far from a modern concept. In fact, the notion that cancerous tumors might consist of distinct populations of cells was something familiar to many of the early pioneers in the field of pathology [200]. The origin of this idea can be traced as far back as 1833 when the

renowned German physiologist Johannes Muller and his disciples first applied the technique of microscopy to the study of tumor tissue [200]. This led to the observation that individual tumors harbored a wealth of heterogeneity in terms of the morphology of the cells therein contained. This perspective was further promulgated by one of Muller's students; none less than the renowned pathologist Rudolf Virchow, widely considered to be the founder of modern pathology [200]. Virchow was the first to argue that tumors emerged from transformation of normal cells and would provide some of the earliest descriptions of cellular heterogeneity within tumors [200]. In 1890 David Von Hansemann would coin the term anaplasia in reference to the nuclear and mitotic atypia resulting from his observations of tumor morphological heterogeneity [201]. At first, the roots of these morphological distinctions between the cells comprising a tumor were decidedly unclear. One early indication came in the early 20th century, from the work of Theodor Boveri, who determined that cancer cells with different chromosomal alterations were phenotypically dissimilar [200]. These experiments also succeeded in establishing that aberrant mitoses and defects in chromosomes were the basis for carcinogenesis [200]. Although tumor heterogeneity continued to be observed and catalogued over the years, it gradually came to be viewed as inconsequential in light of the acknowledged monoclonal origin of cancer [202]. This all began to change in 1976 with the publication of a famous perspective from the geneticist Peter Nowell, who argued in favor of conceptualizing cancer through the lens of Darwinian natural selection [200,203] . This was the beginning of what became known as the clonal evolution hypothesis which is now arguably the dominant theory for explaining the existence of cellular heterogeneity within individual cancer patients. The crux of Nowell's argument was that because cancer cells were genetically unstable, they would accumulate mutations at an accelerated rate compared to somatic cells in the normal physiological context [203]. These genetic changes, arising

in a single cell within the tumor, would then specify for properties capable of conferring an adaptive advantage relative to the other tumor cells. The specific microenvironment of the tumor would thus select for those phenotypes better able to contribute to the task of tumorigenesis while ensuring the extinction of those deemed deleterious [203]. Cells bearing a fitness advantage would pass on these heritable traits to their progeny which would persist in the form of a distinct subpopulation or sub-clone within the tumor. Thus, the stochastic genetic heterogeneity inherent to a tumor population would serve as the substrate for natural selection to drive the evolutionary process [203]. This genetic heterogeneity can arise due to mutagenesis, numerical or structural chromosomal instability or from epigenetic changes [204]. At any given moment the level of tumor heterogeneity would reflect the underlying clonal dynamics, with a likelihood of observing multiple genetically distinct sub-clones in constant competition for the limited resources available within the tumor environment [204]. As the tumor progresses, any inherent heterogeneity might become more or less pronounced due to the relative dominance of a particular population under the current conditions [205]. Accordingly, tumor evolution might proceed according to either a linear or branched mode. In the linear model, heterogeneity would be abolished in each successive "generation" owing to the capacity of newer populations to outcompete and displace the old [205]. In a branched mode of evolution, multiple populations may co-exist and thus evolve in parallel due to the inability of one to outcompete the others [204,205]. This would lead to the existence of more heterogeneous tumor over time [205]. Many solid tumors have been seen to represent the branched pattern while the linear pattern is most often seen in certain hematological malignancies [205]. Tumor evolution might also be characterized by a gradualistic or punctuated pattern [204]. Tumor gradualism implies a sequential accumulation of additional genetic changes at a steady rate over time [204]. By contrast, punctuated equilibrium features long periods of relative

stasis interrupted by evolutionary "bursts" which usually involve the rapid acquisition of novel properties through large-scale genomic alterations, such as would occur in response to a sudden change in the environment [204]. An example of such a macro-evolutionary event within the framework of cancer is illustrated by the well-documented phenomenon of chromothripsis; when a single catastrophic event leads to the generation of thousands of clustered rearrangements affecting one or multiple chromosomes [204]. Within the same patient, tumor heterogeneity can vary as a function of position and time from initiation [201]. The capacity to metastasize may be asymmetrically distributed among the various sub-clones within the primary tumor [201]. In breast cancer it was shown that metastasis can occur from a minor sub-clone within a heterogeneous primary tumor [206]. The tumor present at the metastatic site contained a less divergent mutational frequency, suggesting the occurrence of a process akin to evolutionary bottlenecking [201,206]. If a single sub-clone from the primary tumor is able to seed multiple metastatic sites, then there is an expectation for low inter-metastatic heterogeneity [204]. If polyclonal (polyphyletic) seeding occurs such as when multiple sub-clones have acquired the capacity for metastasis, then the metastatic lesions may in fact partially recapitulate the heterogeneity of the primary tumor [205]. In this scenario the seeding of different locations by a variety of sub-clones may result in more pronounced inter-metastatic diversity [204]. The degree of heterogeneity present at any metastatic focus and its genetic similarity to the primary tumor may reflect whether it occurred early in disease progression or as a later event [205]. For a secondary lesion at a distant site, the local microenvironment may modulate the course of tumor evolution in a manner different than at the primary site, resulting in the selection of novel characteristics and a greater divergence from the site of origin [205]. The presence of similar selective pressures between different metastases may contribute to convergent inter-metastatic evolution even in the context of polyphyletic spread [204].

The advent of next-generation sequencing technologies has enabled the degree of intra-tumor genetic heterogeneity to be quantified in-depth for the first time [204].

In some cases, the heterogeneous distribution of mutations within a tumor can be used to reconstruct its evolutionary history [204]. In the case of the same mutation being found ubiquitously in every cell at all sites, it is more likely to represent an early event in tumorigenesis, thereby belonging to the "trunk" of a tumor's evolutionary history [201]. While, in the case of mutations being found heterogeneously, they probably took place only after the separation of distinct sub-clonal populations and therefore form part of the tumor's evolutionary "branches" [201]. The relative abundance of these sub-clonal mutations can be used to infer the hierarchical relationship among sub-clones in the tumor's phylogenetic tree [204]. Recently, evidence has begun to cast doubt on the notion that clonal evolution need be driven by Darwinian selective forces [204]. It has been shown, in a number of cases, that the longitudinal pattern of intra-tumor heterogeneity present is more consistent with a model of neutral growth, without positive selection of any individual sub-population [204]. Likewise, the dogma of perpetual competition between different sub-clones has been challenged by observation of functional cooperativity in a number of model systems mediated through paracrine signaling by one population to support the growth or metastatic capacity of another [204]. The clinical implications of intra-tumor heterogeneity for shaping the disease response to chemotherapy has recently been viewed as being of paramount importance. In the presence of tumor heterogeneity, the evolution of chemoresistance may result following two distinct pathways [205]. On the one hand, intrinsically resistant sub-clones may already be present within the tumor prior to treatment [205]. In this case they would likely be found at a low overall frequency due to the fitness disadvantage associated with maintaining the resistant phenotype under normal conditions when it is not needed [207]. Their

presence, therefore would be masked by more dominant, chemo-sensitive sub-clones. When, however, the majority of the treatment-sensitive cells are ablated by chemotherapy, the surviving resistant sub-clones are no longer subjected to competition for resources and are thus readily able to clonally expand, resulting in a relapsed tumor enriched in more resistant cells [204,205]. Alternatively, if the tumor does not harbor pre-existent chemo-resistant sub-populations, then the resistant phenotype can be evolved de-novo through the accumulation of additional genetic changes in a single cell under the selective pressure of chemotherapy [205]. In this case the particular genetic event conferring chemo-resistance would be more likely to occur in a cell belonging to the dominant sub-clone within the tumor, simply by virtue of its greater share of the tumor population [149].

1.19 Evidence for Genetic Heterogeneity and Clonal Evolution in HGSOC

Many recent studies have sought to quantify the extent of intra-tumor heterogeneity in HGSOC in order to clarify its relation to clonal evolution and the emergence of chemoresistance. One of the first such studies, published in 2010 by the group of Cooke and colleagues, undertook a genomic analysis of a set of paired, patient-derived HGSOC cell lines established before and after the onset to chemoresistance [149]. These 3 aforementioned cell line series (PEO1/4/6, PEO14/23 and PEA1/2) were each established from patient ascites in England during the early 1980s and were first described by Langdon et al. in 1988 [188]. In their 2010 paper, Cooke et al. reported a striking genomic divergence between the sensitive and resistant cell lines established from the same patient [149]. In fact, these paired cell lines shared few derivative chromosomes and contained mutually exclusive endo-reduplication and loss of heterozygosity events [149]. These differences led the authors to conclude that the cell lines, although each isolated from the same patient, could not have evolved as a linear progression [149]. Indeed, their findings suggest that

these populations of cells are only distantly related, having diverged from a common ancestor cell early in the course of tumor evolution, thus evolving in parallel rather than as a direct progression [149]. The authors were therefore able to argue that their study supported the notion of the chemo-resistant clones being already present at an early stage before treatment, and which thereafter underwent clonal expansion upon relapse [149]. Subsequent studies have shed more light on the array of genetic heterogeneity present within the same patient and its spacio-temporal distribution over the course of tumor evolution. In 2011, a study by Malek et al. demonstrated widespread copy number variation between localized and metastatic disease in 9 patient samples [208]. The genes subject to alteration were found to particularly involve those implicated in cytokine signaling and the Jak/STAT pathway [208]. In 2013 Bashashati and colleagues found widespread intratumoral variation involving mutation status, copy number and gene expression profile between 31 spatially and temporally separated HGSOC tumor specimens from 6 different patients [209]. Interestingly, a number of putative "driver" mutations involving genes such as PIK3CA, CTNNB1, PDGFRB, and NF1 were found to have sub-clonal distribution in individual patients, indicating that they had occurred independently in distinct subpopulations of cells after having diverged during the course of tumor evolution [209]. On the other hand, mutations affecting TP53 were found to be conserved throughout the patient, including in precursor lesions, suggesting that this event is one of the earliest in HGSOC carcinogenesis [209]. In a similar fashion Hoogstraat et al., using samples from treatment-naive disease, showed a number of site-specific genomic alterations between omental and peritoneal metastases compared to lesions present in the ovaries [210]. This study even showed the presence, in one patient, of distinct TP53 mutations between different sites, implying either that these mutations occurred following metastasis or that disease was polyclonal and emerged from the independent transformation of two distinct

cells [210]. Subsequently, Schwarz et al. used deep sequencing to quantify intra-patient heterogeneity using 135 spatially and temporally separated samples from 14 individuals receiving platinum-based chemotherapy [211]. Using a bioinformatic technique they were able to approximate the evolutionary trajectory taking place within each patient. In line with previous studies, they observed a robust but variable degree of heterogeneity present prior to treatment [211]. The amount of heterogeneity was not seen to influence an individual's prognosis [211]. The nature of the genetic events between individual sites indicated the involvement of a pattern of metastasis-to-metastasis spread to drive disease propagation [211]. It was also shown that patients undergoing neoadjuvant chemotherapy had a corresponding minor alteration in disease heterogeneity [211]. Critically, it was shown that a high degree of clonal expansion upon relapse was associated with treatment resistance through a clear reduction in progression-free and overall survival [211]. It was even demonstrated that the resistant sub-clones that expanded upon relapse were detectable at low frequency in the pre-treatment sample [211]. The group of Choi et al. analyzed heterogeneity in the cells isolated from patient ascites and discovered abundant sub-clonal variation affecting many cancer-related genes [212]. Phylogenetic reconstruction revealed that the ascitic cells had originated from multiple regionally distributed solid tumor masses [212]. Furthermore, a study by Mcpherson et al. examined multiple intraperitoneal lesions in individual patients and concluded that, although the majority of lesions were clonally pure with evidence of unidirectional seeding from the primary site, in a few cases there was evidence of polyclonal mixing to drive reseeding between distant foci [213].

1.20 Adaptive Chemotherapy

The recognition that chemo-resistance results from the outgrowth of minor resistant sub-clones present prior to treatment has fueled the development of novel therapeutic strategies based

around the concept of managing tumor heterogeneity. Typical chemotherapeutic regimens treat cancer as a homogeneous mass of cells and aim to use the maximum tolerable dose of the drug to kill the greatest possible number of tumor cells [207]. Meanwhile, the pre-existence of chemo-resistant sub-populations is masked by their low overall frequency, which is maintained through a competitive inhibition imposed by the more dominant, sensitive clones [207]. As aforementioned, there is an evolutionary cost associated with upholding the resistant phenotype under normal conditions when it is not required. This typically takes the form of an additional metabolic demand on the cells, necessitating a diversion of cellular resources away from tasks such as proliferation and invasion [214]. The resistant cell's greater energy needs, in an environment of competition for limited space and scarce nutrients, results in a passive restriction on its population size compared to the better adapted chemo-sensitive cells [214]. When, however, treatment ablates the bulk of the chemo-sensitive cells, the surviving sub-clones are freed from the limitations of competition and are thus able to proliferate and expand at an accelerated pace in a resource-rich environment to cause treatment-resistant relapse [204,214]. This phenomenon has been termed "competitive release" and it may provide an explanation for the inability of many novel chemotherapeutic agents to increase the overall survival of patients in spite of improvements in progression-free survival [204]. Among the new treatment strategies devised to address this problem is a technique called adaptive therapy. The theoretical underpinnings behind this novel concept in cancer therapeutics were formulated by Gatenby and colleagues in 2009 [207]. Instead of achieving maximum cytoreduction, this approach aims to maximize the time to progression by maintaining a stable population of tumor cells while averting the emergence of chemoresistance [207]. By contrast to traditional chemotherapeutic regimens using the maximum tolerated dose administered in short cycles, adaptive therapy envisages the use of limited

"bursts" of treatment with only the minimum dose needed to halt tumor growth [207]. The explicit goal is to maintain a residual population of treatment-sensitive cells capable of restraining the proliferation of the resistant sub-clones when therapy is withdrawn [207]. The optimal implementation of this method proceeds according to two stages [214]. An early inductive stage utilizing an intensive treatment schedule is required to halt the exponential growth of the tumor [214]. As the tumor begins to stabilize it is possible to gradually transition to lower doses and more infrequent treatments over time [214]. A key tenet of adaptive therapy is the need to constantly adjust the treatment parameters in order to compensate for the tumor response [207]. This requires frequent and precise disease monitoring, using sensitive imaging techniques or effective biomarkers, to assess the disease response [207]. Gatenby and colleagues have tested their theoretical approach using basic pre-clinical models of breast and ovarian cancer involving human cell lines orthotopically xenografted in immunodeficient mice which are then treated with carboplatin or paclitaxel [207,214]. This has validated the capacity of adaptive therapy to significantly prolong progression-free survival compared to a standard treatment regimen. In their demonstration using breast cancer cell lines MCF-7 and MDA-MB-231/luc, the progressive decline in tumor size that was observed in 60-80% of animals would eventually permit them to increase treatment-free intervals to as long as several weeks [214]. Imaging of the tumors present in such animals revealed an increased vascular density and less abundant necrosis [214]. The authors speculate that the vascular normalization that results from tumor stabilization may have contributed to ongoing tumor control with lower doses [214]. Beyond the work of this group, it was also recently demonstrated in melanoma that resistance to vemurafenib can be forestalled by using variable and intermittent treatments not unlike those prescribed by adaptive therapy [215].

Project Purpose, Hypothesis and Objectives

2.1 Project Purpose and Hypothesis

The long-term survival of patients with ovarian cancer has been virtually unimproved over the past 40 years. The existence of intra-patient tumor heterogeneity is thought to be among the most salient factors influencing treatment failure in High Grade Serous Ovarian Cancer (HGSOC). It is thus of paramount importance to obtain a greater understanding of the scope and character of the cellular heterogeneity contained within HGSOC in order to provide a basis for new therapeutic strategies aimed at managing or overcoming this heterogeneity in patients. HGSOC is distinguished by profound genomic instability due to defects in HR-mediated DNA repair and the loss of tumor-suppressor P53 [159]. This factor may serve to underlie the existence of genetic diversity among the clonal tumor cells, thereby providing a fertile substrate to fuel the clonal dynamics that drive disease behavior. The unique microenvironment of the peritoneal cavity may also contribute to specifying the selective process guiding the evolution of the distributed disease foci that are present in advanced disease. Recent studies employing next-generation sequencing technologies have confirmed the existence of multiple genetically distinct sub-clones in samples derived from patients with HGSOC. This heterogeneity was observed to include a notable spatial and temporal dimension. Two influential publications by Schwarz et al. and Cooke et al. have revealed the crucial role of tumor heterogeneity in determining treatment outcomes in HGSOC [149,211]. Both groups have independently established that treatment resistance is associated with the expansion of a minor sub-clone already present prior to chemotherapy. The study of Cooke et al. utilized a cell line model of HGSOC consisting of a series of paired cell lines established longitudinally from the same patient before and after the onset of chemoresistance. Unlike the majority of cell lines used to represent HGSOC, the cell line series PEO1/4/6, PEO14/23 and PEA1/2 have well annotated clinical histories and have been validated to accurately recapitulate

the genetic features of the disease [189]. This study found that the cell lines, despite being isolated from the same patient, could not have been linearly related and therefore likely evolved in parallel rather than as a direct progression [149]. Because these cell lines are examples of clinically acquired Pt-resistance, they present a valuable opportunity to uncover the mechanisms behind the development of chemoresistance *in vivo*. As has previously been alluded to, the majority of model systems used to study Pt-resistance have been generated *in vitro* through the continuous exposure of cells to increasing drug concentrations over a period of several months. This treatment regimen is more accurately depicted as a process of desensitization and does not resemble the method of exposure occurring in patients. Accordingly, the biological mechanisms of the resistance obtained would almost certainly be different from those having originated in an *in vivo* environment. Early reports aiming to uncover the processes underlying Pt-resistance in the PEO1/4/6 series of cell lines, focused on the role of glutathione which was seen to be elevated in its reduced form in PEO4 cells compared to those of PEO1 [146]. Lately, however, the group of Stronach and colleagues have discovered two novel pathways that contribute to Pt-resistance in these cell lines. In the first instance HDAC4 was found to de-acetylate the signaling molecule STAT1 in the resistant cell lines but not the Pt-sensitive cell lines from the same series [154]. Secondly, cisplatin was shown to activate the kinase DNA-PK in resistant cell lines which resulted in the phosphorylation of AKT and the stimulation of pro-survival signaling [155]. It should be noted that the cell line PEO1 was previously used to derive a model of Pt-resistance *in vitro*, but the procedure involved long-term cisplatin exposure over a duration of many months [216]. The resultant entity, termed PEO1-cddp, was shown to exhibit a different mechanism of Pt-resistance which involved an altered response to extracellular ligands and the auto-activation of ERK-MAPK signaling [216]. The gene expression profile of this cell line was revealed to be

different from that of PEO4 or PEO6 which both emerged in the original patient [154]. More recent efforts to develop *in vitro* analogues of Pt-resistance have made use of methods with greater clinical relevance including the so-called "pulse method" which applies treatment with high doses of CDDP for a short duration in multiple cycles [217,218]. The resistance mechanisms having ensued from such a treatment protocol have not yet been adequately assessed and it is not known if they mirror those occurring *in vivo*. Although recent studies have characterized HGSOC cell lines at the genetic level, the notion of heterogeneity has never been adequately addressed. In general, the implicit assumption is that cell lines are more-or-less homogenous systems. As such, heterogeneity within cell lines used to represent HGSOC has not been well defined, and if so, its appearance has been deemed incidental or of trivial importance.

The overall purpose for this project was to assess what heterogeneity might be contained within chemo-sensitive, patient derived HGSOC cell lines. From the study of Cooke et al., it is known that the cell lines PEO1/4/6 and PEO14/23 were isolated from heterogeneous tumors [149]. Also, from this study, it was established that the resistant cells that emerged upon relapse were already present prior to treatment and were thus selected from a pool of pre-existent clonal diversity. If a portion of this heterogeneity is retained in the cell lines that were established prior to treatment, then it might be possible for these cell lines to contain intrinsically resistant sub-populations of cells analogous to those having emerged *in vivo*. If this is true, then the treatment of these cell lines using clinically achievable doses and exposure-times of cisplatin should enrich for these resistant sub-clones, thereby creating a Pt-resistant cell line with similar characteristics to those of the matched resistant cell line established from the same patient. The usage of a paired cell line model offers a unique opportunity to interrogate if the resistance mechanisms evolved *in vitro* are the same as those having occurred clinically. It was hypothesized that

chemo-sensitive, patient-derived HGSOC cell lines harbor minor resistant subpopulations, analogous to those present within the original patient, which are capable of clonal expansion after *in vitro* challenge with a clinically achievable regimen of platinum-based therapy.

2.2 Specific Aims and Objectives

1. To ascertain, in quantitative terms, the Pt-sensitivity of each cell line to be used using a protocol that accurately reflects the parameters of exposure experienced by patients in a clinical setting.

The first stage of the project involves the creation of a protocol for treating cells *in vitro* with platinating agents in order to determine the level of sensitivity exhibited by each cell line. In patients, HGSOC habitually displays a profound sensitivity to Pt-based therapy, with an objective response rate approaching 70% [16]. Disease recurrence, however, with the loss of drug sensitivity is the principal cause of patient mortality. The typical measure used to define drug sensitivity is the IC50 which is considered to be the concentration of the drug required to decrease the proliferation of cells *in vitro* by 50%. The assays typically used to establish the IC50 are standard proliferation assays such as MTT or SRB. Both these methods involve the quantification of a colorimetric substrate that is proportional to the number of live cells present within a given treatment group. The IC50 is the dose resulting in the reduction of the absorbance by 50%. Although these methods offer the benefit of simplicity and convenience, with a suitability for high-throughput screening, they entail a number of challenges for use with drugs such as cisplatin. First of all, the design of the experiment is not conducive towards the careful modulation of drug exposure time. In the typical MTT assay, the cells are exposed to the drug for the full duration of the experiment which can often be as much as 72 hours. This duration of

exposure is not desirable since it is much longer than what can be tolerated by a patient. Moreover, the half-life of the drug in the body is limited due to bioconversion and excretion. These colorimetric assays are also incapable of distinguishing the effects of growth inhibition from those caused by cell death. The propensity to undergo cell death following exposure is an important hallmark of Pt-sensitivity that is not directly interrogated by standard proliferation assays. The goal of developing this alternative approach to assessing Pt-sensitivity was to be able to limit the exposure of the cells to just 1 hour while including the capacity to measure the amount of ensuing cell death. Another important factor to be considered is the dosage delivered which should be within the pharmacological range. For this project, the upper limit to be considered pharmacological was identified as 10 μM. This was based on the results of a study by Nagai and colleagues that measured the average steady-state plasma concentration of unchanged CDDP to be 2.56 μg/ ml (≈ 8.5 μM) after a 2-hour infusion with 100 mg/ m^2 CDDP (usually considered as the maximum tolerable dose) [219]. The computation of IC50s to cisplatin for each cell line will provide a valuable means of verifying if the cell lines isolated from chemo-resistant disease are indeed less sensitive to the drug *in vitro*. At the same time, it will serve to provide a baseline and target for comparison to the cells obtained after repopulation following CDDP exposure. It should be noted that the Pt-sensitivities of the cell lines to be used including PEO1/4/6 as well as PEO14/23 were previously reported elsewhere but were obtained through the use of the SRB assay [154]. It was decided to verify these results using a different approach with greater clinical relevance. Although most patients in the present day are also treated with paclitaxel, it was decided to exclude this drug from the project because none of the original patients were administered this drug [188].

2. To generate an *in vitro* model of Pt-resistance from a chemo-sensitive HGSOC cell line through the repopulation of cells following exposure to a clinically relevant regimen of CDDP.

It was previously hypothesized that chemo-sensitive, patient derived HGSOC cell lines contain minor sub-populations of intrinsically resistant cells. If this is indeed the case, then the treatment of these cell lines with clinically achievable doses and exposure times of CDDP should be sufficient to select for these resistant cells through ablation of the bulk of the non-resistant subsets. The period subsequent to drug removal should be marked by a massive induction of cell death affecting the great majority of the cells present. The percentage of mortality that occurs is crucial for concluding if a selective process has actually been accomplished. If too few cells have been removed, then the concentration of the drug was likely inadequate to kill the necessary percentage of sensitive cells. In such a scenario, the repopulation occurring will likely be from the remaining Pt-sensitive cells and the resultant entity would still be sensitive to the drug. Therefore, it is of vital importance to make use of only the greatest dose of the drug achievable clinically. If the dose is too great, then even the putatively "resistant" cells would be unlikely to survive. This is because the concept of drug resistance is essentially relative. Clinically, the disease is deemed "resistant" if the patient fails to respond to the maximum tolerable dose of the drug that can be administered. *In vitro*, where any conceivable dose can be used, the notion of resistance becomes altogether more difficult to define. With the biological means available to them, cells are only capable of developing diminished drug sensitivity to a certain extent, after which they will invariably die. This is why it remains important to limit the study solely to clinically relevant doses; so that cells can be said to have gained a diminished sensitivity solely to the doses that would have been encountered within the patient. If the cells *in vitro* fail to

exhibit a sufficient degree of cell death to even the greatest pharmacological dose, then they would be presumed to already be "resistant" in the clinical sense of the term. If Pt-resistant clones are already present within an otherwise sensitive cell line, then they would necessarily be found at a very low overall frequency (Or else the cell line would not be Pt-sensitive; hence the need to determine the IC50 beforehand). The selection of these cells would result in repopulation taking place from only a limited number of discrete foci, which have the potential to be derived from just a single surviving cell or a limited number thereof. The repopulating cells should be proliferative and bear a healthy appearance; without any outward signs of Pt-induced toxicity. It is also imperative to minimize the overall extent of exposure: a single high dose should be sufficient to enable selection while minimizing the potential for acquiring *de-novo* pathways of resistance via the accumulation of additional mutations. This is always a pertinent concern when it comes to HGSOC, in which the cells usually have a high degree of genomic instability, making the acquisition of further genetic changes more likely.

3. To assess the level of phenotypic and morphological heterogeneity normally present within chemo-sensitive, patient derived HGSOC cell lines

Heterogeneity within HGSOC cell lines is not usually subjected to a detailed assessment. As has previously been outlined, the presence of heterogeneity is a prerequisite for clonal evolution. If different populations of cells co-exist within HGSOC cell lines, they may possess different phenotypes which might be detected through morphological differences or through the expression of distinct immunophenotypic markers. In order to diagnose if one particular group of cells is selected by platinum chemotherapy it is necessary to characterize the spectrum of heterogeneity that was already present prior to treatment. If it is possible to delineate different types of cells in the original cell line, then the existence of a particular phenotype in the

repopulating cells may indicate that a particular type of cell has been enriched as a result of treatment.

4. To compare and contrast features of the repopulating cells obtained following Pt treatment to both the parental cell line and the matched chemo-resistant cell line(s) established longitudinally from the same patient.

Once repopulation has been successfully accomplished after CDDP treatment, the resultant cells can be passaged in culture to yield a novel cell that may be evaluated for its similarity to either the parental cell line or to the matched resistant cell line(s) from the same patient. According to the hypothesis, the repopulating cells should be more closely related to the chemo-resistant cells that emerged from the original patient. In the first instance, it must be established if the repopulating cells have indeed acquired diminished sensitivity to CDDP. The determination of the IC50 will confirm if these cells are indeed more resistant than the parental cell line, in the same manner as the cells responsible for chemo-resistance *in vivo*. Subsequently, if the cells have indeed become insensitive to pharmacological doses of CDDP, they can be subjected to an array of tests intended to reveal the extent of the biological changes that accompany the attainment of Pt-resistance. This includes an initial characterization of the morphology and growth patterns of the cells under normal conditions. Despite their common origin, cells from the same cell line series such as PEO1/4/6 display substantially different behavior in culture. In this laboratory it was previously recognized that that the cell line PEO6 has the capacity to spontaneously form a floating component consisting of multicellular spheroids and aggregates even in conditions of high-adherence [199]. On the other hand, PEO4 routinely forms foci that demonstrate a pattern of 3-dimensional growth, while PEO1 is largely a monolayer even at high confluency. A gain of such a novel growth pattern on the part of the repopulating cells derived from PEO1 would be

indicative that these cells are indeed more closely related to PEO4 or PEO6. This laboratory has also reported that the doubling times of PEO4 and PEO6 are significantly longer than that of PEO1. An increase in doubling time relative to the parental cell line might also support the hypothesis. Immunohistochemistry might also be employed to show if the expression of certain phenotypic markers is altered in the repopulating cells compared to the parental cell line. This laboratory has also demonstrated a change in the expression of a number of markers within the same cell line series corresponding with disease progression. The adoption of a similar immunophenotype by the repopulating cells to the cell lines established from chemo-resistant disease would also support the hypothesis. The spectrum markers to be assayed will include histopathological markers of HGSOC, EMT markers, and markers of so-called "cancer stem cells". Other tests may include functional assays such as migration and invasion. Another project from this laboratory has discovered that migration and invasion actually decrease with more advanced disease stage. A similar observation in the case of the repopulating cells would provide further verification for the hypothesis. The most definitive measure that can be applied would involve the use of genetic testing. Prior studies, including that of Cooke et al. [149], have showed obvious genetic differences between cell lines isolated longitudinally from the same patient. For example, PEO1 was observed to be BRCA2 mutant whereas PEO4 and PEO6 contained a reversion mutation restoring the function of the gene [150]. The genomic structure was shown to be considerably more "normal" in the resistant cell lines which had HR functionality [149]. If the repopulating cells were to indeed present with a similar mutational or genomic-structural profile to the matched chemo-resistant cell line(s) then this would provide the ultimate confirmation for the hypothesis contending that HGSOC cell lines harbor resistant sub-clones analogous to those that emerged *in vivo*.

Materials and Methods

3.1 Cell Lines and Growth Conditions

The cell lines utilized were either obtained directly from Dr. Simon Langdon of the University of Edinburgh (In the case of PEO1/4/6) or obtained commercially through the ATCC (PEO14). STR sequencing analysis (2017) confirmed the identity and purity of all the cell lines used. When not in use, the cells were stored by cryopreservation in liquid nitrogen while immersed in Crystor® media. Before transfer to the liquid nitrogen tank the cells were pre-frozen at -80°C in a Mister Frosty ™ freezing container (containing isopropanol). Cells were grown under standard conditions in T75 or T25 cm^2 plastic cell culture flasks (ThermoFisher™ Nunc™ EasYFlask™ with treated surface) at 37°C in a humidified incubator with 5% CO_2. The medium was composed of 0.1% insulin, 1% Sodium Pyruvate, 2% Glutamax, 10 mM HEPES, 1% Penicillin-Streptomycin, 86% RPMI-1640, along with either 10% fetal bovine serum (FBS) or 5% FBS and 5% bovine serum. All assays were typically performed in medium with 10% FBS for the purpose of standardization. PEO6 was found to require the medium with 10% FBS under normal growing conditions. For all other cell lines, the 5% medium could be substituted without consequence. The medium was normally changed every 48-72 hours or more often as needed.

3.2 Cisplatin Treatment Methodology and Measurement of Acute Toxicity

Cells were plated in triplicate or quadruplicate (sextuplicate for the early Pt-sensitivity experiments) at a density of 200,000 cells/well in either a 6 or 8-well culture plate (in 2 ml media per well). A total of 4 or 5 conditions were defined; composed of 3-4 treatment doses plus a vehicle control, with each being assigned a set of between 3-6 wells (n= 3, 4 or 6). For the cell lines PEO1 and PEO14, 48 hours was allotted for the cells to attach and multiply prior to treatment. For cells with longer doubling times such as PEO4 and PEO6 this period was usually extended to 72 hours.

CDDP powder was dissolved in either isotonic saline or the equivalent volume of PBS to create a stock solution of 3,333.33 μM. This was serially diluted in culture media to create solutions with the correct treatment doses of the drug. For treatment, the media in each well was replaced with media containing either the drug or a vehicle (saline or PBS). The duration of treatment was typically 1 hour unless otherwise specified. After the completion of treatment, the drug-containing media was replaced with fresh media and the cells were left in culture for another 72 hours (or as otherwise specified) for the drug to take effect. After such time, the floating and adherent cells from each replicate were collected and assayed for cell quantity and percent viability by Guava® Viacount® micro-cytometry. This proprietary technique determines cell viability through differential permeability of the plasma membrane to two DNA binding dyes. The nuclear dye stains all nucleated cells, while the viability dye stains just the dying cells. In this fashion, the assay is able to distinguish viable, dying, and dead cells while excluding debris. This technique was validated against Trypan Blue staining using the cell line PEO14 (Bio-Rad TC-20 instrument). This established its enhanced sensitivity for detecting CDDP-mediated toxicity at supra-pharmacological doses of the drug. The average number of live cells and the average percent viability for the vehicle control plus each treatment dose were plotted using a bar graph and subjected to statistical analysis.

3.3 Procedure for the Clonogenic Survival Assay

For cells subjected to the clonogenic survival assay, the treatment procedure was the same as that denoted above until the point in which the cells were counted and assessed for viability using the Guava® Viacount® assay. Using the data obtained from this assay, the volume corresponding to 1,000 live cells was re-plated from each replicate at a low density in a new well of a 6 or 8-well plate containing 2 ml of fresh growth media. The number of cells plated in each

well occasionally had to be amended for those cell lines with a low colony-forming efficiency such as PEO6. For PEO6, as much as 4,000 live cells had to be used per replicate to obtain a sufficient quantity of colonies. The clonogenic cells were allowed to grow and form colonies until such time as the colonies in the vehicle-treated condition were of an appropriate size (greater than 50 cells on average). The required time depended on the intrinsic doubling-time of the particular cell line. For PEO14, PEO1 and its derivatives, the time required was only 10 days. For PEO4 and PEO6, a period of 14-15 days was usually necessary. Additional media had to occasionally be added, typically after 7 days, to compensate for evaporation. After having allowed for colony formation, the plates were fixed in 4% PFA for 30-45 minutes and stained with crystal violet for 20 minutes. The gross appearance of the plates was photographed using a digital camera to be used for illustrative purposes. The number of colonies above a threshold size was quantified manually for each replicate using an inverted microscope. The threshold value was typically set at 50 cells/colony. For the cell lines PEO14 and PEO6, a lower threshold of 30 cells/colony was used due to the inability of the cells to form colonies of a larger size, even in the control condition. Once the number of suitable colonies in each well per condition were quantified, the numbers were averaged and normalized against the number of the vehicle control. These values were processed with Calcusyn (Biosoft©, Cambridge, UK) software which calculated a CDDP IC50 (in μM) for each cell line. A minimum of three treatment doses were needed for the software formula to yield an accurate final result. The average of two independent experiments was used in the determination of the final IC50 value for each cell line.

3.4 Procedure for Cell Cycle Analysis after CDDP Exposure

For cell cycle analysis, the same procedure for treating the cells was followed as that employed previously (See above for description). The same 4 conditions were used for each cell

line in order to aid comparison: Vehicle control, 2.5, 5, and 10 μM CDDP. 72 hours subsequent to treatment, after the cells had been collected and assessed for quantity and percent viability, the cells from each replicate of every condition were retained in 1 ml of PBS and fixed with 250 μL of 4% PFA. After fixation was complete the cells were resuspended in 0.5 ml fresh PBS and the volume corresponding to 200000 total cells (both live and dead according to Viacount®) was transferred to a centrifuge tube. After centrifugation, the supernatant was removed, and the pellet of cells was resuspended in 200 μl of propidium iodide solution. The cells were stained for 30 minutes at room temperature or overnight at 4° C. The DNA content of the stained cells was then analyzed using the cell cycle protocol of the Guava® Muse® micro-cytometer. The program classified events (cells) according to size (forward scatter) and a relative DNA content index (based on staining intensity). The parameters sorting events as live cells (vs debris) and as belonging to each phase of the cell cycle (G1, S and G2/M phase) were manually set for each run according to the segregation of the event clouds by DNA content (Most were actually overlapping). Because of the arbitrary nature of these criteria, an earnest effort was applied at standardization between runs from the same experiment, although this was not always possible due to some possible variance in the degree of staining. Sub-G1 (hypodiploid) DNA content percentage was defined as pertaining to events of the appropriate size but lacking in staining intensity (those events outside and lower on the scale compared to the G1 event cloud). Many events in the replicates belonging to the CDDP treatment doses contained a significant proportion of events with hyperploid DNA content index which was defined as greater than 2X the G1 DNA content index. Unfortunately, the program was unable to quantify the percentage of hyperploid cells independently from the percentage of sub-G1 cells and thus, the values for each

cannot be directly compared. Ultimately the percentage of hyperploid cells was excluded from consideration during the analysis of the data and is therefore not reported in the results.

3.5 Procedure for Boyden Chamber Migration Assay

To test migration, it was decided to use the Boyden chamber assay. This method uses a cylindrical insert which is placed within the well of a cell culture plate. The insert features a membrane at its lower surface with 8 μm pores through which cells may migrate in response to chemotactic factors in the surrounding well. For all cell lines used, 200000 cells were placed in each insert of a Boyden chamber device containing 1.5 ml of media with 0.1% fetal bovine serum. The cells had been incubated in the 0.1% FBS containing medium for 24 hours prior to the commencement of the experiment. The surrounding well (outside the insert) was filled with 2.5 ml of normal media comprised of 10 % FBS. The cells were allowed to migrate for 30 hours while in an incubator at 37°C. Following this period, the cells that had migrated onto the opposite side of the membrane were fixed with 4% PFA for 30 min. The cells remaining in the upper chamber were removed using a cotton swab. The bottom surface of the membrane and lower chamber were then stained with the fluorescent dyes ALEXA Fluor 594-Phalloidin (F-Actin cytoskeleton in red) for 20 minutes and Sytox ® green (nuclei in green) for 10 minutes. The plates were stored at 4°C with PBS and then imaged with a fluorescence microscope (Leica DMI-8) at 20X magnification. Twenty images were taken of each well using an overlay of the red and green emittance spectra. The number of green-stained nuclei on the reverse side of the membrane was quantified in each image and summed across the 20 images to give a total for each well. The average number per well was obtained for each cell line (3 wells per condition: N =3) and the results subjected to statistical analysis (student's t-test). The data was displayed in the form of a bar graph.

3.6 Protocol for the Repopulation of PEO1 after Cisplatin Treatment

In order to induce repopulation, about 1 million cells were plated in a T25 cm^2 cell culture flask and allowed 72 hours to attach and proliferate. The media was then replaced with an equivalent volume containing 10 μM CDDP and incubated with the drug for 1 hour at 37°C. The drug was subsequently removed, and fresh media was introduced. The treated cells were left under normal culturing conditions and allowed to repopulate for approximately two weeks with regular changes of media. Progress was followed using inverted microscopy. After the repopulating cells had attained a sufficient degree of confluency, they were re-plated in a larger 100 mm dish. To verify if the repopulating cells were less sensitive to CDDP, they were retreated with 10 μM CDDP again for 1 hour. After a further week in culture, the cells once again exhibited full confluency and were transferred to a T75 cm^2 cell culture flask. Approximately 1 month after the commencement of the experiment, it was decided to preserve the repopulating cells for future use. The cells were counted, divided into equal aliquots and suspended in Cryostor® freezing media, after which they were frozen; first at -80°C for 3 days, then in liquid nitrogen.

3.7 Procedure for Immunohistochemistry of Cell Lines

50,000 cells were placed into each well of an 8-well, glass-bottom chamber slide (Lab-Tek™) in 300 μl of normal culture medium. The cells were grown at 37°C until a satisfactory degree of confluency was reached. The slides were then fixed with 4% PFA for 1 hour, after which they were stored at 4°C in PBS. Each of the 8 wells was assessed either a different marker or assigned as a negative control to verify the specificity of the secondary antibodies (anti-mouse and anti-rabbit). To perform IHC, the growth-chamber portion of the slides was removed,

leaving just the glass-bottom containing the fixed cells. The slides were first permeabilized using 0.5% TRITON X-100 for 30 min. They were then treated to washes using a sequence of 2X PBS-T (0.1 % Tween) for 3 minutes each followed by 1X PBS also for 3 minutes. The same washing procedure was used throughout the experimental protocol. After permeabilization, each of the wells of the slide were subjected to blocking using Normal Horse Serum (NHS)[Vector® ImmPRESS™] for either 30 minutes or 1 hour at 4°C in a humidified chamber. The individual wells were then incubated with the correct primary antibody for either 30 minutes or overnight at 4°C. The duration was dependent on the antibody specificity and probability of non-specific binding. The negative control conditions were left with NHS instead of the primary antibody. The slides were then given another round of washes before quenching the endogenous peroxidase in a solution of 3% H_2O_2 for 30 min. After more washes, each well was exposed to the appropriate secondary antibody (either anti-mouse or anti-rabbit HRP conjugated [Vector® ImmPRESS™]) for 30 minutes at 4°C. Following another round of washes, the wells were each treated with DAB (Vector® ImmPACT™) for 5 min. The slides were then washed in PBS and the reaction was verified using microscopy. Hematoxylin was used for counterstaining. Optimal results were obtained using a ½ dilution of a commercial formulation of Mayer's hematoxylin (Vector® Hematoxylin QS) for 5 seconds/slide. Slides were then washed in running tap water for 15 minutes. The slides were then mounted with a cover-slip (Fisher Chemical ™ Permount™ mounting medium) after dehydration in ethanol and removal of the rubber gasket using xylene. Photography was done using a microscope with a camera at 20X magnification.

3.8 Procedure for Evaluating the Doubling Time of the Cell Lines

To evaluate the doubling time of the cell lines, it was necessary to plate 200,000 cells each in 15 wells belonging to 3 six-well plates. A total of 5 conditions were defined, with each being

assigned 3 wells (N=3). The cells were allowed 72 hours to attach and proliferate before the commencement of data collection, after which the adherent cells within three of the wells were collected and counted using Guava® Viacount® micro-cytometry. The juncture at which the first three samples were processed was defined as the 0-hour of the experiment. On each of the following days, a further set of three wells were processed and counted at timepoints defined according to 24, 48, 72 and 96 hours following the start of the experiment. The media of the remaining wells was changed every 48 hours (at 0-hour and 48-hour timepoints). The number of live cells was plotted as a function of time in the program GraphPad Prism 5. A non-linear regression was used to fit the data with the defined criterion of exponential growth. In this fashion the program calculated a doubling time for the data-set.

3.9 Protocol for Mutational Profiling of the Cell Lines Through Partial Exome Sequencing

The mutational profile of each cell line was interrogated using partial exome sequencing of a panel of 33 putative oncogenes and tumor-suppressors. The panel in known as CLINV4 and it is in routine clinical use within the MUHC healthcare network. This method utilizes a Roche™ NimblGen™ SeqCap® library which employs a capture-based target enrichment technology allowing for the selective amplification of specific genomic loci for subsequent sequencing. The ClinV4 panel featured probes conferring coverage of 240 loci belonging to 33 cancer-related genes (see below for list). This technique allows the quantification of Variant Allele Frequency (VAF) with a lower detection limit of 5 %. Cells were taken from liquid nitrogen and pelleted in a 1.5 ml Eppendorf tube containing PBS. The supernatant was removed, and the pelleted cells were transported at -20°C. The sequencing was performed at the McGill Molecular Pathology Unit of the Jewish General Hospital. The machine utilized was an Illumina™ MiSeq® system. The data was interpreted with the aid of Dr. Leon Van Kempen (formerly) of the McGill Dept. of

Pathology. Data was displayed as a table listing the genetic variant of interest along with its associated VAF (Provided by Dr. Van Kempen for Table 1.).

List of Genes Covered by the CLINV4 Panel		
1. MPL	2. MET	3. AKT1
4. NRAS	5. BRAF	6. MAP2K1
7. DNMT3A	8. FGFR1	9. TP53
10. ALK	11. JAK2	12. NF1
13. CTNNB1	14. CDKN2A	15. SMAD4
16. PIK3CA	17. GNAQ	18. GNA11
19. FGFR3	20. ABL1	21. CALR
22. PDGFRA	23. PTEN	24. CEBPA
25. KIT	26. FGFR2	27. SRC
28. FBXW7	29. HRAS	30. EGFR
31. NPM1	32. KRAS	33. FLT3

3.10 Methods of Statistical Analysis

Statistical analysis was always performed using the program GraphPad Prism 5. For experiments with more than 2 groups, a one-way ANOVA was performed followed by Newman-Keuls post-tests to determine significance between individual pairs of group means. The threshold for statistical significance was set at $p < 0.05$ and denoted on the graphs using an asterisk (*). Multiple asterisks were used to represent further reductions in p-value (**: $p < 0.01$ and ***: $p < 0.001$) For experiments with only two groups, a single-tail student's t-test was performed.

Results

4.1 Elucidating the Cisplatin Sensitivity of HGSOC Cell Lines: A Preamble

The initial stage of this project was tasked with determining, in quantifiable terms, the cisplatin sensitivity of the HGSOC cell lines which were to be used for the attempted *in vitro* selection of minor resistant subpopulations. These were namely the cell lines PEO14 and PEO1, both of which originated prior to the onset of chemoresistance in the clinical setting. Prior publications have reported cisplatin IC50s for each of these cell lines but using methods that poorly represent the treatment modalities experienced by human patients with the disease. These involved the use of high-throughput growth-inhibition assays (MTT or SRB) where the duration of exposure is not subject to modulation, resulting in drug exposure times that typically last upwards of 72 hours. Because this approach lacks clinical relevance, a key aim for this project was to develop a protocol for ascertaining Pt sensitivity using a drug exposure time of just 1 hour. At first, it was decided to direct a greater focus on the cell line PEO14 due to the fact that it was isolated from a chemo-naïve disease state. This was considered desirable in view of the need to preserve, to the greatest extent, the inherent heterogeneity of the disease prior to treatment. Moreover, the patient from which PEO14 was generated experienced treatment resistant relapse only 7 months following the induction of therapy (thereby yielding PEO23) [188]. This may suggest that the chemo-resistant clones that were eventually selected (i.e. PEO23) were originally present in a greater overall frequency for chemo-resistance to have emerged so rapidly. If this proportion was somehow captured and maintained in the creation of the cell line PEO14, then one could perhaps imagine that it would be easier to select any remaining chemo-resistant subpopulations through *in vitro* Pt treatment. Alternatively, in the case of PEO1, which was created from a therapy-responsive relapse occurring 22 months after initial treatment, the pre-existent tumor heterogeneity may be altered or diminished [188]. On the other hand, the previous

treatment may have served to enrich for the more resistant subsets compared to their original fraction in the pre-treatment disease.

4.2 Quantifying the Cisplatin Sensitivity of the Cell Line PEO14: Testing the Initial Experimental Approach

At first, it was decided to test the validity of the approach used to assess Pt mediated toxicity by exposing PEO14 to supra-pharmacological doses of CDDP for 1 hour, with toxicity measured after 72 hours. Two methods for measuring CDDP-induced cell death were utilized; Guava Viacount was envisaged as the standard method, however in this case it was deemed necessary to compare its readout to that of another well-known cell viability assay, namely that utilizing Trypan blue permeability. The percentage of Trypan blue positive cells was assessed using the Bio-Rad TC-20 automated cell counting device. The results, using the elevated doses of 10, 20 and 40 μM CDDP, were somewhat surprising. While it was possible to distinguish a dose dependent decrease in the number of live cells obtained compared to the control, this decrease was only statistically significant at the highest dose of 40 μM (n=6) (Figure S1. A, B: p 179). In terms of the percent viability of the treated cells there was no obvious dose response visible between the vehicle-treated control and the 10 and 20 μM treated conditions. The only dose to yield any discernable reduction was again the highest dose of 40 μM and this difference was again statistically significant (Figure S1. A, B: p 179) These same results were obtained using both means of assessing cell viability. The Guava Viacount method appeared to exhibit a greater sensitivity for diagnosing cell death compared to the more commonly used Trypan blue method. The overall trend of the data obtained using both methods was, however, the same, with the only significant reduction for both the live cell number and percent survival obtained at the highest dose of 40 μM. One point of concern, detected using both the Guava Viacount and Trypan blue

assays, was the rather low viability measured for the vehicle treated cells. Normally it might be expected that these cells would be healthier, having not been exposed to the drug in question. Via Trypan blue the percent survival of the vehicle treated cells was close to 80%, but this was no better than the lower treatment doses of 10 and 20 μM (Figure S1. A: p 179). In the case of Guava Viacount the viability was much worse, being hardly above 50% while again being no greater than the lowest treatment doses (Figure S1. B: p 179). One other visible trend was the reduction in live cell number that was independent of any differences in percent survival, in that the number of live cells detected was seen to be lower, compared to the control, at doses where the viability was virtually unchanged (10 and 20 μM) (Figure S1. A, B: p 179). This indicates that in this cell line, at these doses and with a 1-hour exposure time, the main toxic effect of cisplatin was in terms of growth inhibition and not cell death. This was considered to be unusual because even these doses were much greater than would be tolerable clinically and the cell line was supposedly chemo-sensitive. Overall it was not possible to glean a definitive assessment of the Pt-sensitivity of the cell line PEO14 using this experimental approach.

4.3 Quantifying the Cisplatin Sensitivity of the Cell Line PEO1: Experimental Approach Fails to Yield Sufficient Toxicity

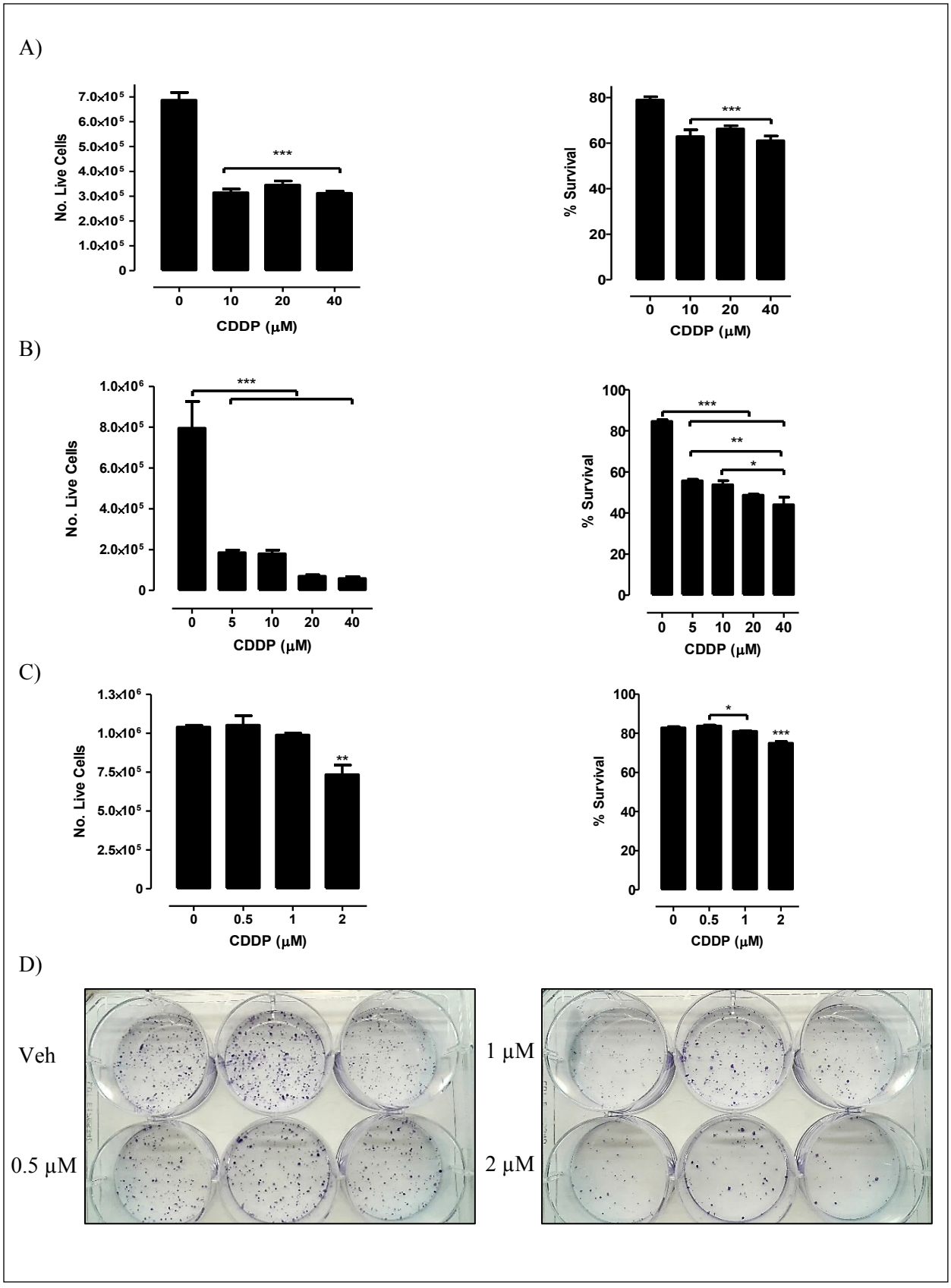
Next, it was decided to confirm if these findings from PEO14 could be extended to the other cell line in question, which was PEO1. It was realized that the poor percent survival obtained in the vehicle control condition of PEO14 may have complicated the interpretation of the results from this cell line (Figure S1. A: p 179). Furthermore, the essential agreement between both methods used to measure cell number and viability in the previous experiment allowed the simplification of the protocol in favor of one of the two approaches. It was decided to proceed with the use of the Guava Viacount assay due to its greater sensitivity, and ease of use across

large sample sets. Also, the number of replicates was decreased to 4 (n= 4), due to difficulties encountered in the previous experiment in processing all 6 replicates in a timely and efficient fashion. Otherwise, the methodology was identical to that used for PEO14 (including the doses employed). This time, the results were different but no less surprising. There was a large and significant difference between the vehicle control and each of the three treatment doses, both in terms of live cell number and percent survival (Figure 1. A: p 104). The anticipated dose-response, however, was not realized. There was no appreciable difference between any of the treatment doses despite twofold increases in drug concentration between each successive condition (Figure 1. A: p 104). This effect was observed in both sets of data measuring the live cell number and the percent survival (Figure 1. A: p 104). The baseline percent viability of the vehicle treated control condition was seen to be better in PEO1 compared to PEO14 with an average of around 80% live cells collected (Figure 1. A: p 104). The trend obtained from the data seemed to indicate that a kind of plateau state had already been reached in terms of the Pt toxicity affecting PEO1 with the three chosen treatment doses. If true, this would necessitate the use of lower doses of CDDP to yield the desired dose-response in the case of this cell line. A major concern was that the magnitude of the decrease in viability between the control and the largest dose of CDDP (40 μM) was only around 20% and the percent survival never decreased below 50% (Figure 1. A: p 104). This was in spite of the fact that all treatment doses were supra-pharmacological. While the use of lower doses might be sufficient to obtain a dose response and perhaps an IC50 (in terms of the number of live cells present), this would totally ignore the lethal effect of cisplatin which seemingly cannot be detected with the present approach. Ultimately, an appropriate measure of the Pt-sensitivity of the cell line PEO1 is unlikely to be established using only the previously delineated experimental approach.

4.4 Quantifying the Cisplatin Sensitivity of the Cell Line PEO1: Altering the Methodology by Lengthening Drug-Exposure Time

As has been emphasized, the preceding experiments had revealed deficiencies in the initially adopted experimental approach for producing reliable dose dependent decreases in cellular viability. It was thus resolved to introduce amendments to the treatment protocol which were intended to provide a clearer picture of the Pt-sensitivity of the HGSOC cell lines under question. In addition to the dose of the drug, other variables may also impinge on the degree of cellular toxicity resulting from exposure to CDDP. These primarily involve the duration of drug exposure and the period of time subsequent to drug removal in which CDDP has time to exert an effect. The 1-hour exposure time of the previous experiments was a conservative approximate of the time in which the drug is available in the disease microenvironment of human patients. It was estimated that a 3-hour exposure to CDDP was on the upper limit of what can be considered clinically relevant in this *in vitro* context. Because the quantity of Pt entering the cell is time dependent, a longer exposure time would conceivably result in a greater degree of toxicity and hence more cell death than would occur from only a 1-hour treatment. On the other hand, the time between when the drug is removed and the when the cells are collected for assessment may also be crucial for modulating the percentage of cell death that is eventually measured. The main mechanism of action of cisplatin is related to its ability to cause DNA damage. The cell death resulting from this effect would more likely be observed over a longer duration than 72 hours post exposure. The 72-hour period that was chosen in past experiments was based on the concerns associated with keeping the cells in culture for too long without being able to change media. While CDDP may limit the proliferation of the treated cells, the vehicle control is under no such constraints and the number of cells will likely continue to increase exponentially until

confluency is reached. It was observed that 72 hours (plus the 48 hours before treatment) was about the time required for confluency to be reached in the wells of the culture dishes used (at least for PEO1). This concern would probably be less pressing when utilizing cells with a slower proliferation rate than PEO1. In order to elevate the percentage of cell death measured in the treated samples, it was deemed valuable to test the effect of 24-hour CDDP exposure on the dose response occurring in the PEO1. Using a similar range of doses to previous experiments, the cisplatin plus control conditions were incubated with the drug or vehicle for 24 hours, followed by another 72 hours before the cells were collected and processed (total of 96 hours; n= 4). In the case of PEO1, this resulted in much greater toxicity in terms of the reduction in percent survival relative to control in each treatment dose (Figure 1. B: p 104). There was also a more obvious difference in the viability between treatment doses, with a significant reduction at the highest dose of 40 μM compared to the lower doses of 5 and 10 μM (Figure 1. B: p 104). The magnitude of this difference was however not especially impressive with only a 10% difference in percent survival between 5 μM and 40μM CDDP (Compared to 30% between the vehicle and the 5 μM dose) (Figure 1. B: p 104). The difference in the number of live cells at all doses relative to the control was especially pronounced with the additional 24 hours of growth in the vehicle-treated condition. There was, however, no significant change in this variable between the individual doses (Figure 1. B: p 104). This experiment was indeed successful in proving that a lengthened time of drug-exposure was sufficient to elevate the frequency of cell death beyond 50%. The use of this approach, however, entailed an unacceptable departure from clinical relevance both in terms of the time of drug-exposure and the magnitude of the doses involved.


A)
No. Live Cells
7.0x10⁵
6.0x10⁵
5.0x10⁵
4.0x10⁵
3.0x10⁵
2.0x10⁵
1.0x10⁵
0
***
0
10
20
40
CDDP (μM)
% Survival
80
60
40
20
0
***
0
10
20
40
CDDP (μM)
B)
No. Live Cells
1.0x10⁶
8.0x10⁵
6.0x10⁵
4.0x10⁵
2.0x10⁵
0
***
0
5
10
20
40
CDDP (μM)
% Survival
100
80
60
40
20
0
***
**
*
0
5
10
20
40
CDDP (μM)
C)
No. Live Cells
1.3x10⁶
1.0x10⁶
7.5x10⁵
5.0x10⁵
2.5x10⁵
0
**
0
0.5
1
2
CDDP (μM)
% Survival
100
80
60
40
20
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CDDP (μM)
D)
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1 μM
2 μM

E)

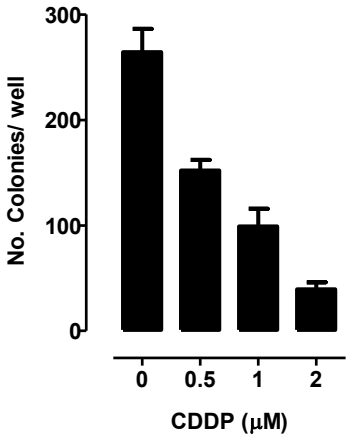


Figure 1: *Evaluating the Cisplatin Sensitivity of the HGSOC Cell Line PEO1*

A): The number of live cells and the corresponding percent survival of PEO1 cells treated 72 hours prior with supra-pharmacological doses of CDDP for 1 hour. (n = 6) After 72 hours there was a significant reduction at every dose in both the live cell number and percent survival. There were, however, no significant differences between any of the treatment doses.

B): Acute toxicity data from PEO1 cells exposed to supra-pharmacological doses of CDDP for 24 hours. Data was obtained 72 hours after drug removal (total of 96 hours after drug administration). (n = 4). With 24-hour exposure there was a much more evident reduction in both parameters. In terms of percent survival there was a significant difference between each dose.

C): Acute toxicity data from PEO1 cells exposed to low doses of CDDP for 1 hour. Data collected 72 hours after drug removal. (n =3) Data correspond to cells plated in following clonogenic survival assay. Using lower doses and 1-hour exposure, there is very little toxicity evident 72 hours after drug removal. The only significant change is in terms of a reduction in both the live cell number and percent survival at 2 μM CDDP and a reduction in percent survival at 1 μM CDDP.

D): Images taken of the plates resulting from a clonogenic survival assay performed with the cell line PEO1. 1,000 treated cells PEO1 per replicate per condition were re-plated in 2 ml fresh media and allowed to form colonies for 10 days. Colonies were fixed and stained with Crystal violet.

E): Quantification of the average number of colonies per well from each treatment condition of the PEO1 cells plated and allowed to grow for 10 days in a clonogenic survival assay (data correspond to the images presented above). Data from two independent experiments was processed with Calcusyn software to yield IC50 of 0.56 μM.

Statistical analysis = 1-way ANOVA + Newman-Keuls post-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

4.5 Quantifying the Cisplatin Sensitivity of the Cell Lines PEO4 and PEO6: Altered Methodology Does Not Validate Diminished Sensitivity of Chemo-Resistant Cell Lines

In order to further highlight the effect of a lengthened drug exposure time on the toxicity incurred by HGSOC cell lines, it was decided to pursue treatment of the nominally chemo-resistant cell lines PEO4 and PEO6. Using exactly the same methodology as the preceding experiment (involving PEO1), cells from both cell lines were exposed to the same range of doses of CDDP for 24 hours, followed by 72 hours without the drug before the samples were processed (total 96 hours; n=4). In the case of both of the cell lines, the data obtained pointed to an unexpected result. For both of the supposedly "resistant" cell lines, the percent viability figures at the same doses were either no better or even worse than those recorded from PEO1 (Figure S2. A: p 181, S3. A: p 182). In both cases the percent survival of the treated cells dropped to about 20% at the 40µM dose (Figure S2. A: p 181, S3. A: p 182). When the effect on live cell number is considered, it cannot be clearly established if these two cell lines are in fact less sensitive to CDDP than PEO1. This is because the number of live cells in the vehicle condition is much lower than that obtained for PEO1. Therefore, due to the slower proliferation rate of these cells, the relative difference between the treated conditions and the control is reduced. If one considers only the effect on proliferation, then PEO4 and PEO6 may be considered actually more resistant because there is less growth inhibition occurring. This would however totally ignore the capacity of CDDP to cause cell death, to which PEO4 and PEO6 appear to be more sensitive compared to PEO1. It is notable that the MTT assay is only able to measure live cell number and cannot define how much cell death is occurring. Thus, the previous studies may have erred in designating PEO4 and PEO6 as being more resistant than PEO1 because they only considered the effect of CDDP on growth inhibition without factoring in the intrinsic differences in

proliferation rate between each cell line. These results, however, involved both clinically unachievable doses and exposure times, which may account for the failure to demonstrate any "resistance" under these conditions. It is more likely that these HGSOC cell lines, both generated from chemo-resistant disease, only display less sensitivity to the doses and durations to which they would have been exposed *in vivo*. This fact underlines the need for a new approach for assessing CDDP-mediated toxicity that is sensitive enough to reveal responses occurring under clinically relevant treatment parameters.

4.6 Elucidating the Cisplatin Sensitivity of HGSOC Cell Lines: The Adoption of a New Approach

It had become clear that the major limitation of the initial experimental approach was the limited time frame allotted for Pt-mediated toxicity to become evident. The 72 hours was simply insufficient to distinguish the toxic effect of cisplatin using clinically relevant doses and exposure times. It was necessary, therefore, to adopt a completely different technique for assessing drug toxicity that would interrogate not just the short-term viability of the treated cells but also their long-term proliferative potential. These criteria would influence the selection of the clonogenic survival assay as the principal tool for elucidating the sensitivity of the cell lines under consideration. The potential advantage of this method was that it would provide an indisputable measure of the long-term effects of cisplatin while also allowing for the short-term toxicity to be assessed. Using the same *in vitro* treatment protocol, 72 hours after drug removal the cells would be collected for assessment of the quantity of live cells and their percent survival as before. Except this time, instead of subsequently discarding the samples, 1000 live cells (as determined by the Guava Viacount assay) were isolated from each individual replicate and re-plated at low density in an empty well of another 6 or 8-well culture dish (containing 2 ml of

10% FBS media). Over the course of a week or more, each individual cell would have the opportunity to give rise to its own colony of cells. The cells sensitive to the effects of CDDP would either be incapable of forming any sort of colony or would create what is referred to as an abortive colony composed of a handful of unhealthy or abnormal cells. The resulting number of proliferative colonies would therefore be proportional to the treatment dose used. Plotting graphically the average number of colonies above a threshold size allows for the identification of the dose yielding a 50% reduction in colony formation compared to control. This dose can be taken as the IC50 for a particular cell line, therefore yielding a quantitative measure of its sensitivity to the drug. An advantage of using this method for uncovering the IC50 is that it takes into consideration the long-term health of the cells rather than the immediate effects on proliferation. It also allows the greater freedom to modulate drug exposure relative to MTT but at the price of increased time and complexity. In fact, the clonogenic survival assay is held by some to be the gold standard for assessing drug cytotoxicity [220].

4.7 Quantifying the Cisplatin Sensitivity of the Cell Line PEO1: Use of New Methodology Confirms Cisplatin Sensitivity

In the first instance, it was intended to subject PEO1 cells to a clonogenic survival assay using the same range of supra-pharmacological doses utilized in previous experiments with a 1-hour exposure time. The result was that virtually no colonies were formed in any of the treatment doses (data not shown). This was despite the fact that 1000 viable cells had been plated for each replicate. This confirmed the suspicion that despite the cells being technically “alive” (assessed through the criterion of membrane permeability) at 72 hours following CDDP exposure, the majority of these cells were likely either in the process of dying or were incapable of any further cell division due to DNA or other cellular damage. Consequently, it was realized that a lower

range of doses needed to be used if a dose dependent decrease in the number of colonies was to be obtained. Ultimately it was necessary to settle on a range of pharmacological doses between 0.5 and 2 μM CDDP, because few if any colonies could be formed after treatment with higher doses. At these low drug concentrations, and with 1-hour exposure, there was a minimal effect discernable 72 hours post-exposure using Guava Viacount micro-cytometry (Figure 1. C: p 104). Only a small but significant reduction in the number of live cells was detectable at the highest dose of 2 μM (Figure 1. C: p 104). Percent survival was also minimally affected, declining by around 4% at 1 μM and 8% at 2 μM, although these differences were determined to be statistically significant (Figure 1. C: p 104). When these cells were subjected to a clonogenic survival assay, after 7-10 days, the number of colonies with 50 or more cells did indeed decrease in a dose dependent fashion. This was readily apparent from the gross appearance of the stained plates, but it was also borne out through quantification of the colonies present per condition under the microscope (Figure 1. D, E: p 104). The average IC50 calculated by the program Calcusyn for this cell line was 0.56 μM CDDP (Two independent experiments). It was thus discovered, through the use of the clonogenic survival assay, that the cell line PEO1 is indeed sensitive to pharmacological doses of CDDP. This was the result expected all along and it confirms the reports from previous studies.

4.8 Quantifying the Cisplatin Sensitivity of the Cell Lines PEO14, PEO4 and PEO6: Validation of the New Approach in Diagnosing Reduced Drug Sensitivity

Now that a procedure for establishing Pt-sensitivity had finally been validated, it was deemed a requirement to define the Pt IC50 values of the other HGSOC cell lines involved in this project. Beginning with PEO14, it was found to be sensitive to a similar range of doses as PEO1 (Figure S1 B, C: p 179). The calculated IC50 was 1.07 μM CDDP (average of two independent

experiments). This cell line was far less efficient at colony formation and both the average number and size of the colonies in the control was far less then that occurring for PEO1. This would necessitate a lower threshold colony size to be used compared to PEO1 (20 cells vs. 50 cells), while also requiring more cells to be plated per well (2000 cells/well vs. 1000 cells/well). This occurrence is in keeping with the results of earlier experiments that seemed to show that PEO14 had a diminished viability under normal conditions. It is not clear if this is a general feature of this cell line or just the result of a sub-optimal batch of cells that was utilized. The putatively chemo-resistant cell line PEO4 required a more elevated range of pharmacological doses in order for a dose response to be observed by clonogenic survival (Figure S2. C: p 181). Ultimately, an average platinum IC50 of 6.79 μM CDDP was calculated for this cell line (average of two independent experiments) (Figure S2. D: p 181). This number is many-fold higher than that determined for PEO1, confirming that PEO4 is truly more resistant compared to its counterpart established earlier from the same patient. To verify if this was also the case for PEO6, the final cell line (longitudinally) of the series, the same technique and range of doses was employed to that utilized for PEO4. It transpired that PEO6 was also less-sensitive to cisplatin compared to PEO1 and to a similar degree as PEO4. The average IC50 computed for this cell line using the clonogenic survival assay was 7.31 μM CDDP (average of two independent experiments) which is similar to PEO4 and much greater than PEO1(Figure S3. D: p 183). The successful implementation of the clonogenic survival assay has therefore allowed for the first aim of this project to be satisfied. The CDDP-sensitivity of all the cell lines under scrutiny was evaluated quantitatively and an IC50 value was defined for each. In the process, many important insights were gleaned about the nature of platinum-mediated cytotoxicity which was observed to unfold over a duration longer than 72 hours. Accordingly, the use of only a short-term approach

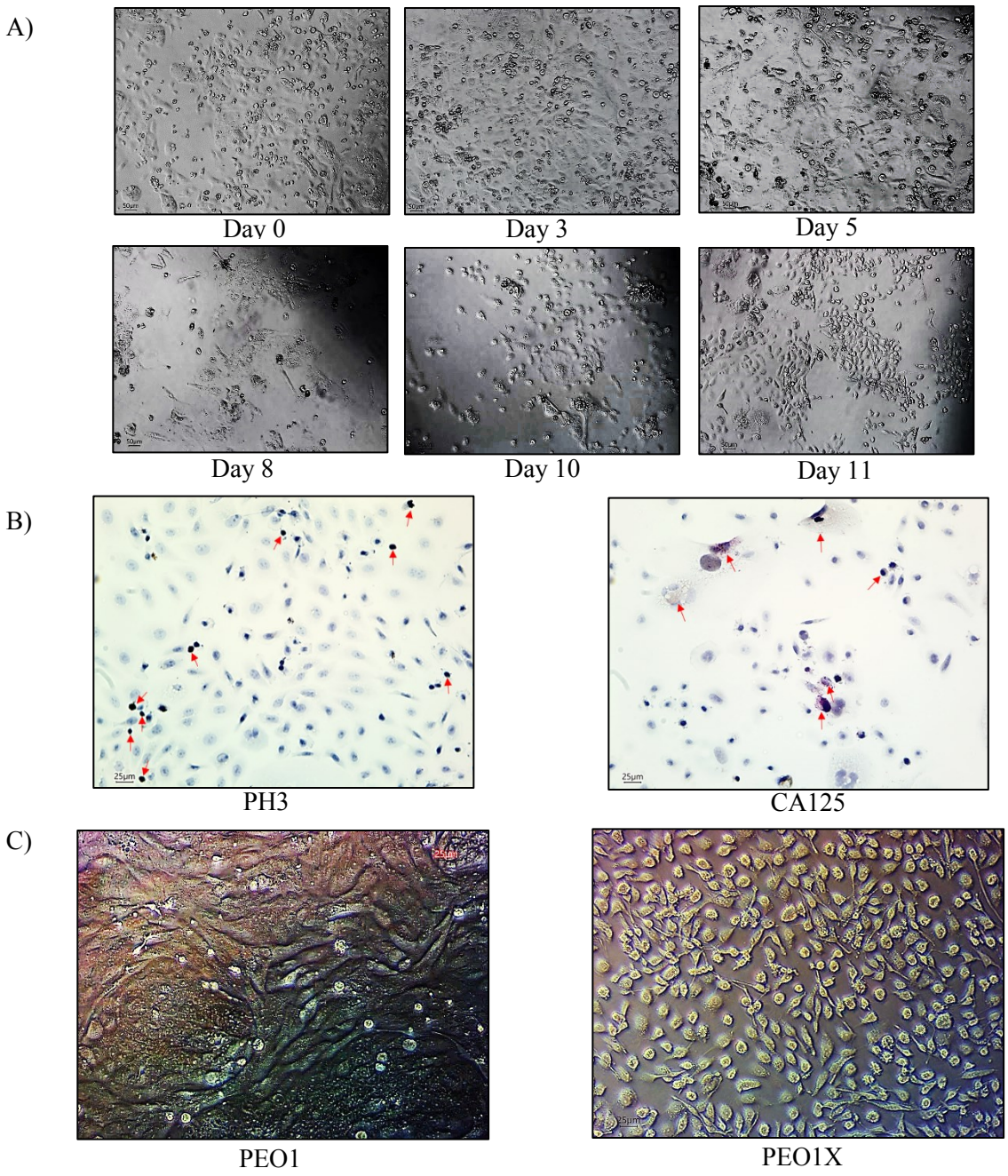


Figure 2: *The Derivation of the Novel Cell Line PEO1X Through Repopulation of PEO1 After CDDP Treatment and Assessment of its Morphological Features*

A): Images taken sequentially of the repopulation of PEO1 following 1-hour treatment with 10 µM CDDP. Images captured at 10x with inverted microscope.

B): Images taken (20X) of repopulating cells fixed at 15 days post-treatment and double stained for phospho-histone H3 (brown) and CA125 (purple). Red arrows indicate positive cells.

C): Images of confluent live cells taken at 20x magnification with and inverted microscope that illustrate the differences in morphology and growth pattern between PEO1 and PEO1X.

is unlikely to reveal an accurate "picture" of the sensitivity of cells to CDDP. Moreover, a drug exposure time of 24 hours or more is simply too long and induces a large amount of acute cell death that may not be caused by DNA damage.

4.9 Repopulation of the HGSOC Cell Line PEO1 Following Clinically-Achievable Cisplatin Treatment: The Derivation of a Novel Entity PEO1X

The principal aim of this project was to determine if chemo-sensitive HGSOC cell lines harbor minor resistant sub-clones that can be selected for by clinically achievable, *in vitro* platinum chemotherapy. In order to address this question, it was necessary to settle on an *in vitro* treatment regimen with sufficient toxicity to eliminate the greater proportion of sensitive cells while sparing those with greater intrinsic resistance to CDDP. The importance of selecting a pharmacological dose along with a short duration of exposure was highlighted in the previous platinum-sensitivity assays where it was shown that the cell lines derived from *in vivo* Pt-resistance could not withstand supra-pharmacological doses and longer exposure times than 1 hour *in vitro*. Because 10 μM CDDP was acknowledged as the *in vitro* analogue to the maximum tolerable dose administered clinically [see above p. 82 and ref. [219]], it was decided that this threshold should not be exceeded for fear that the cells should suffer too great a harm such that repopulation would be precluded. Even more important was the need to minimize the extent of re-treatment in order to exclude the prospect of any additional genetic changes arising from continuous exposure to the genotoxic stress of cisplatin. Ideally, a single, strong dose of the drug would be sufficient to bring about the kind of response envisioned by the hypothesis. The particular dose in question, however, was yet to be determined. It was decided to test for this quantity using the cell line PEO1 which was exposed to either 2.5, 5 or 10 μM CDDP for 1 hour and allowed to repopulate. These cells were followed over time using microscopy to evaluate the

eventual response to each dose. Ultimately, it was observed that both the 2.5 and 5 μM doses caused an insufficient degree of cell death for the repopulation to occur from a discrete population of cells (data not shown). Between 5- and 10-days post-treatment, a response in terms of cell death was evident, with cells displaying obvious signs of damage and others simply detaching from the surface. For both doses, however, the cells returning after 14 days were too numerous and too evidently similar to those present before treatment to conclude if the repopulation had in fact resulted from the expansion of a novel subset of cells. On the other hand, the PEO1 cells treated with the 10 μM dose demonstrated a far more drastic reaction. By about 8 days post-treatment, the overwhelming majority of the cells had been removed, with little remaining in the culture dish except for a handful of distorted, vacuolated and unhealthy-looking cells (Figure 2. A: p 111). Then, around 10 days post-treatment, small pockets of healthy-looking cells began to emerge (Figure 2. A: p 111). In contrast to the few other remaining cells, these cells were small and rounded with a reduced cytoplasmic volume. Moreover, the morphology of these cells was strikingly homogeneous in appearance, and their outgrowth emerged from discrete foci within the well. By 11 days these repopulating cells had increased markedly in number, seeming to display a high proliferative capacity (Figure 2. A: p 111). In fact, these distinct foci of repopulation gave the impression of colonies such as would descend from a single cell during a clonogenic survival assay. Within the culture vessel, multiple, spatially segregated colonies were present, with each being composed of similar-looking cells that nevertheless appeared different from the PEO1 cells from which they arose. It was abundantly clear that the emergence of these proliferative colonies from PEO1 after 10 μM CDDP treatment was the desired outcome specified through the objective. Unfortunately, however, these repopulating colonies could not be maintained further in culture (due to the risk

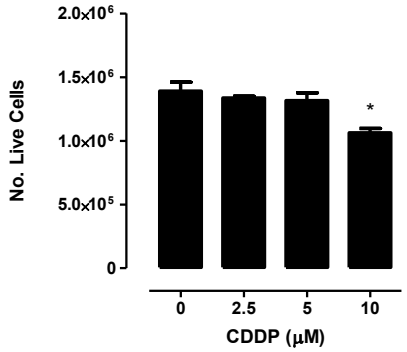
of contamination) and were thus fixed in PFA after 14 days. Repopulating foci fixed and stained for Phospho-Histone H3 displayed a significant number of positive cells, thereby confirming that these cells possessed a high proliferative capacity (Figure 2. B: p 111). Likewise, these "colonies" of repopulating cells were found to be negative for CA125, a biomarker used clinically to assess the disease activity level (Figure 2. B: p 111). To discover if this phenomenon could be replicated the experiment was repeated on a larger scale, with the resulting cells retained for further evaluation. Ultimately, a similar course of repopulation was perceived in this instance, except that in this case the repopulating colonies were permitted to proliferate until confluency in the flask was reached. Upon re-plating in a larger dish, it was decided to attempt one further treatment with 10 μM CDDP in order to verify if the cells had gained resistance to this dose of the drug. Despite retreatment, the cells demonstrated no visible response or outward signs of toxicity after 7 days. It was, therefore decided to designate these cells as a distinct entity with the name of PEO1X. These cells were passaged in culture before being frozen and preserved for later characterization.

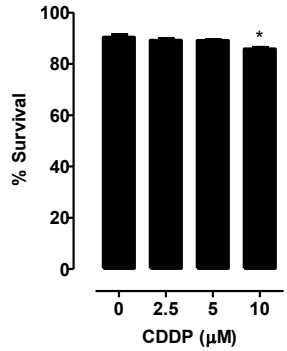
4.10 Quantifying the Cisplatin Sensitivity of the Cell Line PEO1X: Diminished Sensitivity is Confirmed

Having obtained repopulation from a chemo-sensitive HGSOC cell line after two rounds of clinically relevant CDDP treatment, it was necessary to validate if these cells were now in fact less sensitive to the drug compared to the parental cell line. According to the hypothesis, chemo-sensitive HGSOC cell lines such as PEO1 should harbor minor sub-populations of resistant cells that can be selected for by *in vitro* exposure to CDDP. In order to confirm if the cells that were selected from PEO1 were indeed more resistant, it was decided to quantify their sensitivity to the

Figure 3

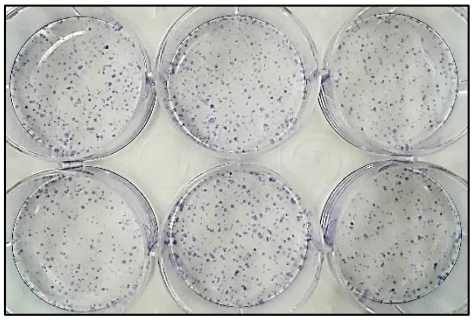
A)



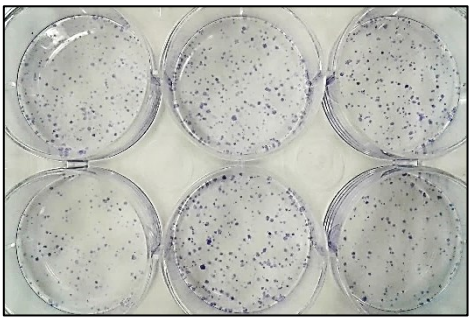


B)

Veh



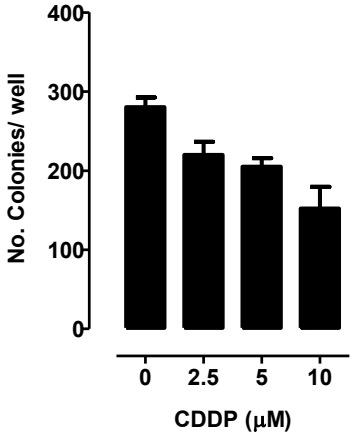
5 μM



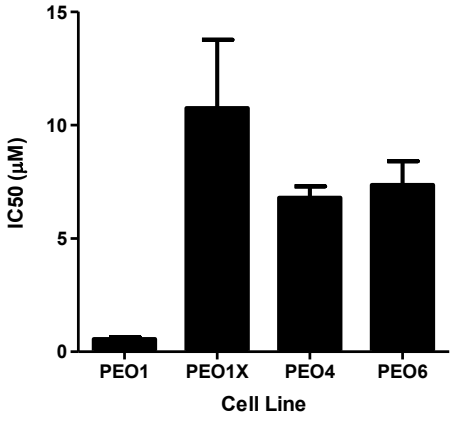
2.5 μM

10 μM

C)

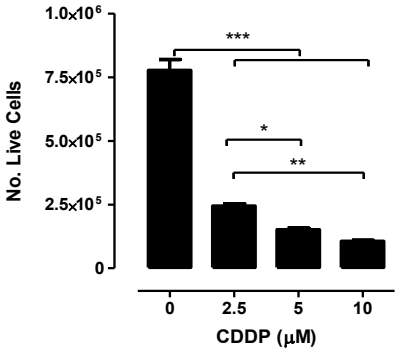


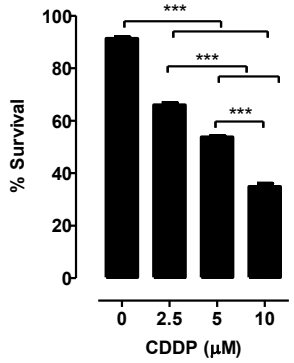
D)



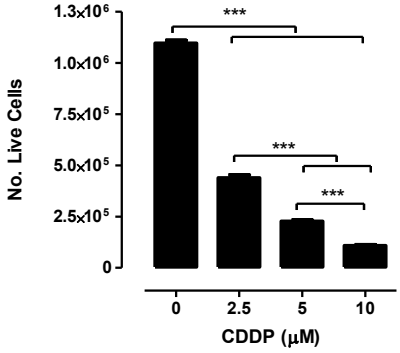
E)

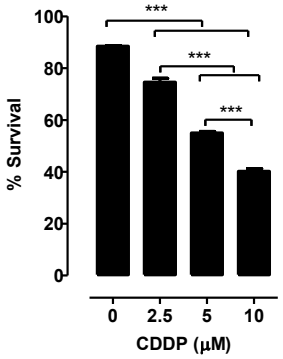
PEO1



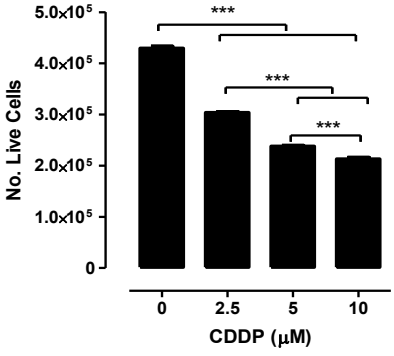


PEO1X





PEO4



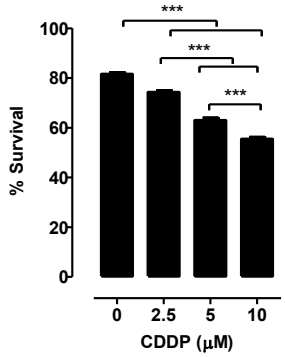


Figure 3: *Evaluating the Cisplatin Sensitivity of the Novel Cell Line PEO1X*

A): Acute toxicity data from PEO1X cells exposed to pharmacological doses of CDDP for 1 hour. Data collected 72 hrs after drug removal. (n =3) Data correspond to cells plated in clonogenic survival assay below. Even with a higher range of pharmacological doses, there is little toxicity evident after 72 hours, except for a small but significant reduction in both the live cell number and percent survival at the highest dose of 10 μM CDDP.

B): Images taken of the plates resulting from a clonogenic survival assay performed with the cell line PEO1X. 1,000 treated cells PEO1X per replicate per condition were re-plated in 2 ml fresh media and allowed to form colonies for 10 days. Colonies were fixed and stained with Crystal violet. In contrast to PEO1, there is not much evident decrease in the number of colonies with increasing dose of CDDP.

C): Quantification of the average number of colonies per well from each treatment condition of the PEO1X cells plated and allowed to grow for 10 days in a clonogenic survival assay (data correspond to the images presented above). Data from two independent experiments was processed with Calcusyn software to yield IC50 of 10.75 μM, which is 20-fold greater than that of PEO1.

D): Graphical comparison of the average cisplatin IC50 values calculated for each cell line of the series of PEO1/4/6 plus the novel cell line PEO1X. IC50s were determined from the quantification of colonies from an average of two independent clonogenic survival assays per cell line. PEO1X demonstrates greater overall cisplatin resistance compared to PEO1 but also PEO4 and PEO6.

E): Cells from PEO1, PEO1X and PEO4 were treated with the same pharmacological doses of CDDP for 24 hrs. Toxicity was measured in terms of live cell no. and % survival 48 hrs after drug removal (72 hrs after administration). The decrease in the no. of live cells was transformed and processed with Calcusyn in order to calculate an IC50 for each cell line. The IC50 for PEO1 was 0.867 μM while that of PEO1X was 1.82 μM and for PEO4 it was 8.92 μM. (n =3). PEO1X is much less resistant to 24-hour exposure compared to the 1-hour exposure. PEO4 is much more resistant than PEO1X using this methodology which highlights a difference between these two populations of resistant cells. Statistical analysis = 1-way ANOVA + Newman-Keuls post-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

drug using the same methodology previously used to ascertain the IC50s for PEO1, PEO4 and PEO6. It was also stated in the hypothesis that the resistant cells selected *in vitro* should be similar or analogous to those that emerged *in vivo*. This would mean that the PEO1X cells isolated from PEO1 should present with similar features and characteristics to either PEO4 and PEO6, which are known (from the study by Cooke and colleagues [149]) to have been present from an early stage before treatment in the original patient. Thus, if the repopulating cells (PEO1X) are indeed related to PEO4 or PEO6 then they should demonstrate a similar degree of resistance to cisplatin. From the earlier experiments, it is known that the IC50s for both PEO4 and PEO6 are many-fold greater than for PEO1. It was suspected that the PEO1X cells might be less sensitive to cisplatin due to the minimal response that was observed to a second 10 μM dose of the drug. After the cells had been passaged and cryopreserved, a sample of cells was then re-grown so that it could be used to discover the IC50 for this novel cell line. The PEO1X cells were plated in triplicate (n=3) in 6-well plates and treated with either a vehicle (saline), or 2.5, 5, and 10 μM CDDP for 1 hour. After 72 hours the cells were collected, and the acute toxicity was assessed by using Guava Viacount micro-cytometry to measure the number of live cells and the percent survival for each condition (Figure 3. A: p 115). Subsequently, 1000 cells from each replicate were plated in a clonogenic survival assay and allowed to form colonies for 10 days (the time necessary for the colonies to attain a sufficient size) (Figure 3. B: p 115). Quantification of the average number of resultant colonies for each dose led to the determination of an average CDDP IC50 for PEO1X of 10.75 μM (average of two independent experiments) (Figure 3. C: p 115). This figure is about twenty-times that calculated for the parental cell line PEO1, therefore confirming that these cells are in fact resistant to all pharmacological doses of CDDP (with a 1-hour exposure) (Figure 3. D: p 116). The CDDP IC50 of PEO1X is also greater

than those of both PEO4 and PEO6 (6.79 and 7.31 μM respectively) (Figure 3. D: p 116). This difference in sensitivity, although noteworthy, is not drastic enough that one would consider it beyond the realm of comparison. It is therefore possible to assert that the treatment of PEO1 cells with CDDP *in vitro*, using a clinically relevant dose and exposure time, was sufficient to select for cells with a similar degree of resistance to those having emerged from the original patient.

4.11 Quantifying the Cisplatin Sensitivity of the Cell Line PEO1X: The Evolved Resistance is Contingent on Time of Exposure

Techniques aiming to condition the evolution of Pt-resistance in cell lines have typically involved the chronic exposure of cells to gradually increasing doses of the drug. Because this method of treatment is not clinically achievable, the resistance mechanisms resulting from this process would likely be different than those which arise in vivo. One of the main limitations of cell lines created using this technique is that the degree of resistance developed is not contingent on the time of exposure to the drug, which is a major limiting factor clinically due to its considerable toxicity. Since PEO1X was created through exposure of PEO1 cells to CDDP for just 1 hour, it was expected that the resistance developed would be only be applicable to clinically relevant durations of exposure. To verify if this was in fact true, PEO1X cells were exposed to pharmacological doses of the drug in vitro for 24 hours and toxicity was assessed 72 hours after treatment commencement (48 hours after drug removal) using Guava Viacount® microcytometry. It was decided to overlook the long-term toxicity due to the improbability of obtaining sufficient colony formation with the 24-hour drug exposure. Instead, the IC50 was calculated using the average number of live cells obtained from each condition 72 hours after treatment commencement. In this instance, the data gleaned from PEO1X revealed a much more evident response, with a significant step-wise reduction in both the live cell number and percent

viability for each treatment dose (Figure 3. E: p 116). At these same doses, there had been very little effect when the cells were exposed for only 1-hour, except at the highest dose of 10μM (Figure 3. A: p 115). When the data is transformed to calculate the IC50, the figure returned is 1.82 μM which is more than 5-fold less than the number obtained using 1-hour exposure (which albeit used a more long-term approach with the clonogenic survival assay). When the identical conditions are applied to the parental cell line PEO1, the results that are generated are not too dissimilar to those derived from PEO1X (Figure 3. E: p 116). The IC50 yielded for PEO1 in this scenario is 0.867 μM which is not much different than that which was calculated using 1-hour exposure (0.56 μM using the clonogenic survival assay). When PEO4 is subjected to the same experimental conditions, it appears to display a much less sensitivity with a resultant IC50 of 8.93 which is actually greater than that calculated earlier for 1-hour exposure (Figure 3. E: p 116). The CDDP-sensitivity of PEO1X calculated using this method is only about half that of PEO1 compared to the 20-fold greater resistance calculated for the experiments using 1-hour exposure (and the clonogenic survival assay). This confirms that PEO1X is resistant to only pharmacological doses and clinically-achievable exposure-times of the drug cisplatin. It is unclear if this finding supports the hypothesis because, in theory, the cells selected *in vitro* should only be resistant to the conditions of treatment to which the original patient would have been exposed. On the other hand, the corresponding resistant cell line PEO4, selected *in vivo*, displayed a much greater resistance to the 24-hour exposure than PEO1X. This would point to a fundamental dissimilarity between these two types of cells which disagrees with the hypothesis.

4.12 Morphological Characterization of PEO1X: An Altered Appearance Divergent from the Phenotype Evolved *In vivo*

The most obvious criteria for differentiating between PEO1 and its derivative PEO1X would be on the basis of their morphology and *in vitro* growth characteristics. PEO1 exhibits a marked dichotomy at the level of its morphology. In a state of high confluency, the cells of PEO1 segregate into distinct morphological subtypes. One portion of the cells display the classic epithelioid morphology with flat, "cobblestone-like" appearance and defined cellular borders. These cells are distributed in clusters resembling "islets" on the growth surface and are surrounded by a network of cells presenting a defined mesenchymal morphology (Figure 2. C: p 111). These have a classic spindle shaped appearance resembling a fibroblast with elongated cytoplasmic processes and indistinct cellular borders. Although this pattern may be indicative of the presence of two different sub-populations of cells, it is far more likely to result from the existence of a phenotypic plasticity within this cell line, with cells able to assume either morphology depending on the particular conditions. This deduction stems from the observations that in clonogenic colonies, ostensibly derived from a single cell, both phenotypes may be present (Figure S4: p 185). Over the course of progression within the same cell line series as PEO1, the morphology and growth characteristics are strikingly different. In PEO4, the cells have a much more uniformly epithelioid morphology, but the principal defining feature of this cell line is its pattern of growth. Unlike PEO1 which tends to rapidly distribute into a complete monolayer, the growth of PEO4 emanates from small clusters which gradually enlarge and then overlap as the culture develops, resulting in an overall "patchy" look, even in more advanced cultures (Figure S5. A: p 186). The most distinctive trait of PEO4, however, is its capacity to form foci of three-dimensional growth. In these foci, the polarity of cell division is altered such

that the cells no longer form a monolayer but grow on top of one another (Figure S5. A: p 186). This phenomenon is also present in PEO6, however in the case of this cell line, the cells evince a tendency towards growth in suspension despite adherent conditions. PEO6 spontaneously forms multicellular spheroids and less organized aggregates of floating cells that resemble the multicellular structures present in the ascites of patients with advanced HGSOC [199] (Figure S5. B: p 186).

PEO1X, while being derived from the repopulation of PEO1 after *in vitro* exposure to CDDP, manifests with a difference at the level of its morphology. Unlike PEO1, the morphology of PEO1X is remarkably homogeneous (Figure 2. C: p 111). This may be consistent with the possibility of its origin from a putative minor resistant sub-population within PEO1. Its morphology, however, is not similar to either PEO4 or PEO6 which are predominantly epithelioid in appearance. PEO1X is composed primarily of small, rounded cells with a large nucleus relative to the size of the cytoplasm (Figure 2. C: p 111). It appears to present with something of an intermediate phenotype between epithelial and mesenchymal. The cells do not feature the defined cellular borders of epithelioid cells even at high confluency but neither to they frequently display the typical spindle shaped, "mesenchymal-like" morphology. Depending on the particular passage, some cultures of PEO1X do contain cells with a mesenchymal-type appearance. This might indicate that there is also a certain morphological plasticity in PEO1X. Furthermore, this novel cell line does not show the tendency for three-dimensional growth or growth in suspension which are characteristic of both PEO4 and PEO6. This disagrees with the tenet of the hypothesis which maintains that the resistant populations selected *in vitro* from a chemo-sensitive HGSOC cell line should be more similar to those having arisen *in vivo*.

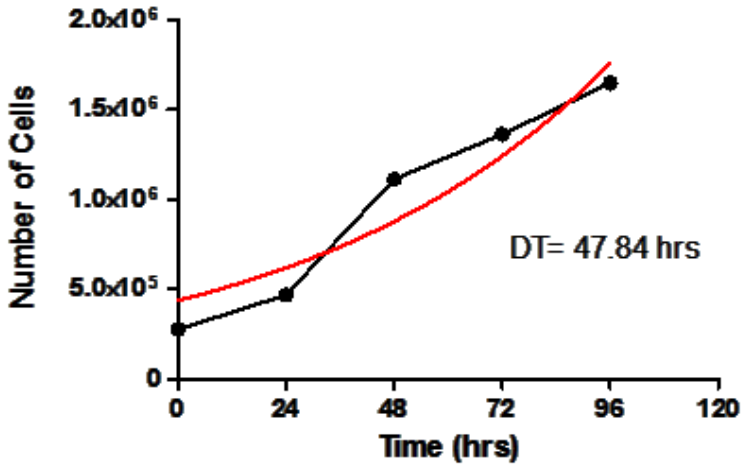


Figure 4: *Calculating the Doubling Time of the Novel Cell Line PEO1X*

PEO1X cells were grown in 6-well plates starting 72 hours prior to the commencement of data collection, with a total of three replicates per condition (n =3). The doubling time of PEO1X was determined through harvesting and counting the total number of cells present in three wells every 24 hours (Using Guava Viacount). The values were averaged and plotted with a non-linear regression with robust fit (GraphPad Prism Software). The doubling time calculated by the program was 47.84 hours. This indicates that the doubling time of PEO1X is not significantly increased relative to the parental cell line PEO1 which has a doubling time of 44.92 hours (data not shown).

4.13 Determining the Doubling Time of PEO1X: The Proliferation Rate is Unchanged

Previous experiments undertaken in this laboratory have elucidated the doubling times of each cell line from the series PEO1/4/6 and PEO14/23 (data not shown). It was discovered that the chemo-resistant cells from these series invariably exhibited a longer doubling time than their matched sensitive counterparts from the same patient. The fact that resistance to chemotherapeutic drugs should be associated with a more quiescent phenotype is hardly surprising, since these drugs possess a greater affinity for cells with a high proliferation rate. The replication of the cell's genetic material during mitosis affords a greater accessibility to the DNA strands which are the primary targets for drugs such as cisplatin. Cells that divide slower or less frequently would be expected to possess a diminished sensitivity to cisplatin due to a reduced capacity of the drug to cause DNA damage in these cells. This is especially true when the duration of exposure is limited, as is the case for many cytotoxic agents including platinum. If the novel cell line PEO1X, which is also resistant *in vitro* to cisplatin, presented with an elongated doubling time compared to the parental cell line, then this might provide an indication as to a possible resistance mechanism. More saliently, the demonstration of an extended doubling time similar to PEO4 or PEO6 could support the notion that the cells selected for *in vitro* by platinum treatment are at least similar to those having emerged *in vivo*. Early observations, however, had tended to indicate that the repopulating cells that gave rise to PEO1X were highly proliferative. This was shown by virtue of immunohistochemistry for Phospho-Histone H3 (PH3) which is a marker of cells transiting the M-phase of the cell cycle. The repopulating colonies that appeared after single-dose CDDP treatment contained a significant percentage of PH3 positive cells which would support the assertion that the cells are highly proliferative (Figure 2. B: p 111). This view was confirmed through experimentation which

permitted the calculation of a doubling time of 47.84 hours for PEO1X (Figure 4: p 123). This value is hardly changed from that already established for PEO1 which has a doubling time of 44.92 hours (data not shown). It is also far less than the doubling time of PEO4 which was determined to be 75.67 hours (data not shown). Based on this information alone, it would appear unlikely that PEO1X is similar to either PEO4 or PEO6. This would disagree with the hypothesis, which had stated that the minor resistant sub-clones selected for *in vitro* by platinum treatment should be similar to those having emerged from the original patient.

4.14 Determining the Migratory Capacity of PEO1X: A Gain of Motility not Shared with Matched Resistant Cells Evolved *In Vivo*

Another biological property that can be used to distinguish chemo-resistant HGSOC cells from their sensitive counterparts is motility and migratory capacity. Usually in cancer, the capacity for migration is associated with the process of metastasis, in which a subset of tumor cells will acquire the capacity to penetrate the basement membrane and infiltrate the surrounding stroma, where they subsequently invade the lymphatic or capillary networks in order to disseminate to distant sites. The biological program resulting in the acquisition of migratory properties has been referred to as Epithelial-to-Mesenchymal Transition (EMT). EMT specifies a shift in cellular phenotype from being essentially epithelial, where the cells maintain polarity and undergo laminar growth while remaining tightly associated with surrounding cells, to one resembling mesenchymal cells such as fibroblasts which are characterized by their motility and cell-matrix interactions [221]. Canonically, EMT is controlled by transcription factors such as SNAIL, SLUG, Twist, Goosecoid, FOXC2 and Zeb1 which regulate the downregulation of E-cadherin in favor of other cadherins (N or P-cadherin) and integrins which permit interactions with stromal cells and Extracellular Matrix (ECM) [221,222]. In addition to its role in

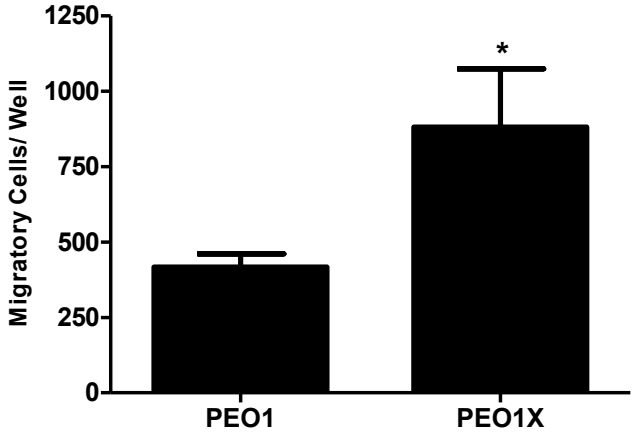


Figure 5: *Comparing the Migratory Capacity of PEO1X to its Parental Cell Line PEO1*
200,000 cells of each cell line were plated in a Boyden chamber with media containing 0.1% FBS. The lower chamber contained media with 10% FBS. After 30 hours the remaining cells in the upper chamber were removed. The migratory cells in the lower chamber were fixed and stained with ALEXA Fluor 594-Phalloidin and Sytox ® Green. The number of cells (nuclei) in 20 fields/well at 20x magnification were counted with a fluorescent microscope and summed together to give an average per well. (n=3). The number of migratory cells per well was found to be significantly greater in PEO1X relative to PEO1.
Statistical analysis = Single tailed student's T-test *: $p < 0.05$,

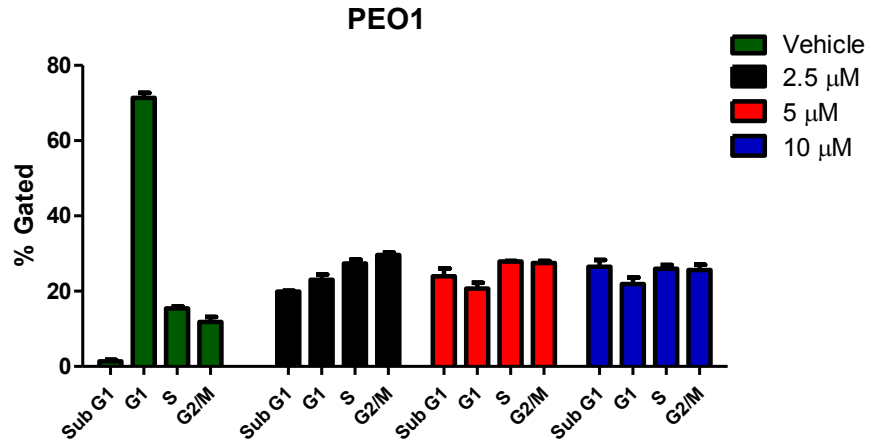
metastasis, EMT has also been implicated in the establishment of drug-resistance, having been shown to inhibit the apoptotic pathway and correlate with the enrichment of stem-like features [221]. Many previous studies using *in vitro* models of Pt-resistance developed from ovarian cancer cell lines have demonstrated that the resistance developed is associated with EMT and the gain of a more mesenchymal phenotype [223-226]. This association has also been observed in certain epithelial ovarian carcinoma patient samples of platinum resistant disease [227]. The role of EMT in HGSOC has nevertheless remained controversial. Unlike most malignancies HGSOC does not typically undergo hematogenous spread, usually remaining confined to the peritoneal cavity [11]. The process of dissemination is instead facilitated by a more passive mechanism involving the shedding of tumor cells from the primary site and their distribution by the physiological circulation of the peritoneal fluid [11]. Nor does HGSOC deeply invade the organs or tissues whereupon it establishes secondary lesions [11]. The need, therefore, for motility and invasiveness in HGSOC is therefore limited. Moreover, many of the studies correlating Pt-resistance with EMT employed models of resistance that are not clinically relevant and involved cell lines that do not adequately resemble HGSOC. One recent study has found that EOC (including HGSOC) cell lines with a more epithelial status are *inherently* more resistant to cisplatin through a mechanism that involves NF-κB activation [228]. Work in our laboratory has also supported the notion that the development of chemo-resistance *in vivo* is associated with a more epithelial and less migratory phenotype. It was found that both PEO4 and PEO6 exhibited a significantly lower migratory and invasive capacity compared to PEO1 [229]. PEO1X, although derived from PEO1 by treatment with CDDP, was the result of an attempt to select for any intrinsically resistant cells that might exist at a low abundance within this cell line. The methods used to elicit the resistant phenotype were different than those employed in previous studies; being more clinically relevant

through the use of only two 10 μM doses of CDDP with only 1-hour exposure time. Early observations had noted that PEO1X displayed a morphology with less epithelial features than PEO1 (Figure 2. C: p 111). It was decided to test if this was associated with any change in migratory capacity relative to the parental cell line. The method employed was the Boyden chamber migration assay. Cells from each cell line were plated in a Boyden chamber above a membrane containing 8 μm pores. The surrounding well contained media with 10% FBS which served as a chemoattractant to stimulate migration across the membrane. After 30 hours with which to migrate, the cells on the underside of the membrane were fixed and stained with fluorescent dyes. The number of migratory cells was quantified in each well per condition (n=3). The results indicated that the PEO1X cells were significantly more migratory than PEO1 (Figure 5: p 126). This is the opposite trend to what is observed with PEO4 and PEO6 which both arose in the original patient [229]. The result, however, agrees with many of the previous cell line studies demonstrating that acquired Pt-resistance *in vitro* is associated with a more migratory, "mesenchymal" phenotype [223-226]. Conversely, this finding further conflicts with the hypothesis which states that the resistant cells selected from a chemo-sensitive HGSOC cell line *in vitro* should be more similar to those that emerged *in vivo*.

4.15 Determining the Effect of Cisplatin Exposure on the Cell-Cycle Status of PEO1X: Validation of the Resistance Evolved *In Vitro* but with Discordant Details to That Emerging *In Vivo*

The effect of platinating agents can be evaluated through their impact on the capacity of cells to successfully transit the cell cycle. In a population of sensitive cells, after platinum exposure, the surviving cells would be expected to be either permanently or transiently arrested at either the G1 or G2/M phases of the cell cycle. This is underlined by the fact that in these stages are

A)



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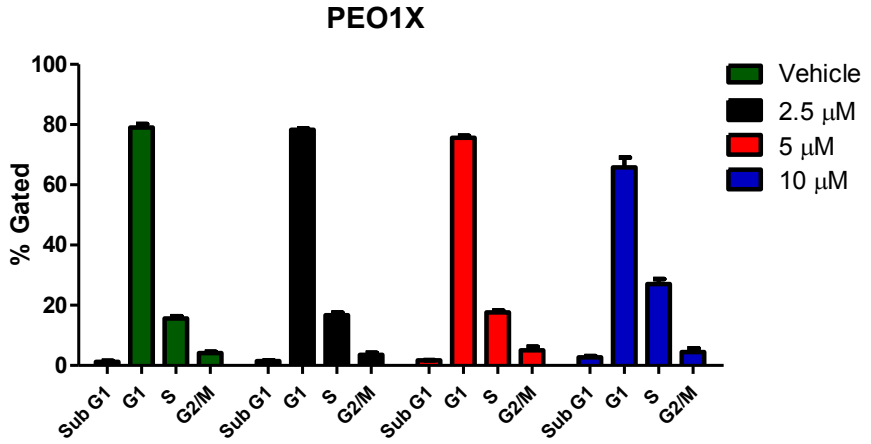


Figure 6: *Determining the effect of Cisplatin Exposure on the Cell Cycle Status of PEO1X*
Cells treated with CDDP for 1 hour or with vehicle were fixed and stained with propidium iodide. Staining was measured using Guava micro-cytometry. Cells were separated from debris using a size index. Cells with greater than 4N DNA content were excluded from consideration. Cell cycle stage was defined roughly using DNA content index and peak location as a guide. Where possible the same criteria were used across a given experiment. (n=3)
A): In PEO1, the cell cycle status is significantly disrupted 72-hours following CDDP exposure at every treatment dose. There are fewer cells in G1 and more in both S and G2/M phases. The percentage of cells with sub-G1 DNA content is also increased relative to control at each dose of the drug and is proportional with each increase in dosage.
B): In PEO1X, there is very little disruption to the cell-cycle status 72 hours after CDDP exposure for any of the pharmacological doses used. The percentage of cells with sub-G1 DNA content is also not substantially increased at any treatment dose relative to control.

found the cell cycle "check-points", that together ensure a cell is in a suitable state to correctly complete the mitotic process before proceeding. If an anomaly is detected, which usually consists of some form of DNA damage, then the cell cycle is arrested at these check-points to allow sufficient time for the damage to be repaired. If the damage is too extensive, then the cell will be required to undergo programmed cell death, usually through apoptosis. In response to exogenous genotoxic insult, cells may also become arrested in the S- phase of the cell cycle when DNA replication is interrupted after having already commenced. The particular phase in which the cell cycle is arrested after platinum treatment depends on what phase was being transited when the drug was introduced [230]. Cells in G1 phase display the greatest sensitivity to drug just prior to the onset of DNA synthesis, being unable to overcome the G1/ S checkpoint and therefore arresting in the G1 phase of the cell cycle [230]. Cisplatin can also increase the duration of S-phase and cause arrest in G2 phase in a dose-dependent manner [230] . Cells that have become resistant to platinum would likely only experience transient cell cycle arrest due to a capacity to either exclude the drug, resolve the DNA damage or simply ignore it. In each case, with sufficient time after platinum exposure, the cell cycle distribution of these resistant cells would likely return to normal (comparable to untreated state). Most assays for assessing cell cycle status use the DNA content as an approximate for each particular stage. Under these criteria late-S phase, G2 and M phases would have double the DNA content of G1 phase while S-phase would be an intermediate between these two groups. Cells with sub-G1 DNA content are assumed to be apoptotic due to mitotic catastrophe or DNA fragmentation leading to the formation of enucleated apoptotic bodies. For normal proliferating cells, the cell cycle distribution is naturally canted in favor of G1 phase with a lesser percentage of cells found in both S and G2/M phases.

In a comparison between PEO1 and PEO1X in terms of their cell cycle status 72 hours post exposure to CDDP at 3 pharmacological doses (Vehicle plus 2.5, 5 and 10 μM) (n= 3) for 1 hour, PEO1X would be expected to demonstrate a reduced impact on its cell cycle distribution compared to control. This was indeed borne out by the data collected from these two cell lines. For PEO1, the vehicle treated cells present with a normal distribution, with the majority of cells in G1 and comparatively few in both S and G2/M phases (Figure 6. A: p 129). There was also a negligible quantity of Sub-G1 (apoptotic) cells obtained for the control. This likely reflects the high confluency attained by the vehicle-treated cells at 72 hours post treatment, with the majority of cells not transiting the cell cycle. By contrast, even at the lowest treatment dose, the cell cycle distribution of PEO1 is dramatically affected, with an increase in Sub-G1 cells and a concomitant decrease in the percentage of G1, along with an elevation of the percentages of cells potentially arrested in both S and G2/M phases (Figure 6. A: p 129). This trend was preserved throughout all 3 treatment doses with little difference among them except for an increase in the percentage of cells with Sub-G1 DNA content with increasing dosage (Figure 6. A: p 129). For PEO1X on the other hand, there was little observable effect at any treatment dose relative to the vehicle control (Figure 6. B: p 129). Even at the highest dose of 10 μM CDDP, the percentage of sub-G1 was minimally increased and the cell cycle distribution was only marginally affected (Figure 6. B: p 129). In any case the majority of cells were still found in G1 which is similar to control; likely reflecting the capacity of these cells to fully transit the cell cycle even after exposure to CDDP. This confirms what was observed in the earlier assessment of cisplatin sensitivity for this cell line, where the number of live cells obtained 72 hours following CDDP exposure is not greatly diminished except at the highest dose of 10μM (Figure 3. A: p 114). When the chemo-resistant cell lines PEO4 and PEO6 are assayed under identical conditions, the

results are comparable to those obtained for PEO1X in that the cell cycle status is not much altered 72 hours after CDDP exposure (Figure S6. A, B: p 187). There are, however, small differences in the baseline (vehicle treated) cell cycle distributions between these cell lines and PEO1X that may be indicative of intrinsic differences at the level of proliferation between these cells. In fact, the baseline cell cycle distribution of PEO1X is much more similar to that of PEO1 with each possessing ≥ 80% of cells in G1 and comparably few in each of the other phases (Figure 6: p 129). By contrast, for both PEO4 and PEO6 there are less cells in G1 and more in S and G2/M phases (Figure S6: p 187). This probably relates to the longer doubling time (see above) of these cell lines compared to PEO1 and PEO1X (Figure 4: p 123). In PEO4 and PEO6 there are likely more cells transiting the cell cycle at 72 hours post-treatment because of the slower proliferation rate, which ensures that confluency is not reached by this point relative to PEO1 and PEO1X. Thus, it is clear from these results that the diminished sensitivity of PEO1X to CDDP is reflected in the minimal alteration of its cell cycle status 72 hours post-exposure (Figure 6. B: p 129). This is consistent with what was also observed for the cell lines PEO4 and PEO6, established longitudinally from the same patient (Figure S6: p 187). In this respect the results support the hypothesis predicting the essential similarity of such cells, however the subtle differences observable at baseline likely signify an intrinsic disparity between these cells at the level of their proliferative dynamics.

4.16 Revealing the Mutational Profile of PEO1X: The Lineage Becomes Clear

In order to ascertain either the fundamental difference or similarity between PEO1 and its derivative PEO1X, the ultimate and definitive criteria is genetics. The seminal study by Cooke and colleagues [149] revealed substantial differences in mutational status and genomic structure between PEO1 and their resistant siblings PEO4 and PEO6 which nevertheless shared a common

clonal origin in the same patient. These divergent genetic features were used to argue that, in the original patient, these cells could not have evolved as a linear progression, but rather in parallel from a common ancestor early in the progression of the disease [149]. By the terms of the hypothesis, the resistant cells having been selected *in vitro* through treatment with CDDP (PEO1X) should either be related or similar to those which emerged in the original patient (PEO4 or PEO6). In order to differentiate PEO1 and PEO1X at the genetic level it was decided to use a sequencing approach with the resolution to detect minor genetic variants within a large sample set. This kind of technique would allow for the assessment of genomic heterogeneity within PEO1 and would furthermore indicate if there was a minor population within the parental cell line that was undergoing clonal expansion in the resistance induced *in vitro* (PEO1X). The mutational status of the PEO1/4/6 cell line series has not been reported on in a comprehensive or detailed fashion in previous publications. This is likely due to the nature of HGSOC which specifies for very few actual gene mutations other than those affecting TP53 [159]. Even so, a study by Sakai et al. showed that while PEO1 contained an inactivating mutation in the gene BRCA2, both PEO4 and PEO6 contained separate reversion mutations that restored the WT gene functionality [150]. Separate studies have also showed thatPEO1 can spontaneously develop a distinct BRCA2 reversion mutation *in vitro,* a phenomenon purported to be common to many BRCA mutant cell lines [154,231]. It was decided to settle on a methodology using partial exome sequencing of a panel of 33 commonly mutated, cancer-related genes. This approach would enable for a determination of Variant Allele Frequency (VAF) for each sequenced gene. The panel, known as CLINV4, which is in clinical use for diagnostic purposes covered mainly oncogenes and tumor-suppressors and unfortunately did not include genes involved in DNA repair pathways. The main challenge would be to determine if it were possible to distinguish

Sample	*TP53* c.731G>A p.(Gly244Asp)	*NF1* c.478A>T p.(Arg160Trp)	*NF1* c.7340_7343delins p.(Glu2447_Glu2248delinsValLeuThr)
PEO1	80%	not detected	98%
PE01-X	69%	not detected	99%
PEO4	98%	99%	not detected
PEO6	100%	not detected	not detected

Table 1: *Ascertaining the Mutational Profile of PEO1X Compared to Other Cell Lines in the Same Series*

Each cell line was sequenced using CLINV4 panel which offers coverage of 240 loci belonging to 33 frequently mutated cancer-related genes. Variant Allele Frequency (VAF) of each allele variant was quantified. This revealed that PEO1 is heterogeneous for P53 mutation status with 20% WT alleles. In PEO1X the frequency of WT alleles is increased. The tumor-suppressor gene NF1 contains an indel mutation in PEO1 and PEO1X. In PEO4 this is no longer present but instead there is a point mutation conferring a putative Loss of function. In PEO6 the gene is entirely WT. No other significant differences were detected. (Sequencing provided by Molecular Pathology Lab of MUHC Jewish General Hospital and interpreted with the aid of Dr. Leon Van Kempen)

PEO1, from PEO4 and PEO6 using this very limited panel of genes. The results would support the hypothesis if they were to show that PEO1X is closer to PEO4 and PEO6 at the genetic level than it is to PEO1. Ultimately, the results would point to some intriguing differences between the cell lines in spite of the limited number of genes used. Firstly, it was revealed that PEO1 is heterogeneous in terms of its P53 mutation status. Only 80% of alleles in the sample contained a point mutation whereas the remainder were WT (Table 1: p 134). This indicates that a certain percentage of the cells in PEO1 likely retained a certain degree of normal P53 function. This is highly unusual as the mutation of P53 is known to be one of the earliest events in HGSOC carcinogenesis, being found even in the STIC precursor lesions. This could perhaps indicate that there was a secondary reversion mutation that occurred *in vitro* that restored the WT sequence in these cells. In PEO4 and PEO6, the same P53 mutation is also found but at a frequency of virtually 100% (Table 1: p 134). Conversely, in PEO1X the opposite trend is observed with a decrease in the frequency of P53 mutant alleles to a percentage of 69 % (Table 1: p 134). It is not clear if this decrease is significant, however it is altogether plain that this cell line does not reflect the characteristics of PEO4 and PEO6, which are both almost entirely P53 mutant. In order to confirm this finding, it was decided to sequence an independent replicate of the repopulating cells deriving from PEO1 that was generated in parallel with PEO1X using the same methodology. In these cells (which were not characterized further) the frequency of P53 mutation was close to 100% (Table S1: p 198). It may be, therefore, that there is no positive selection for WT P53 in the specification of the resistant phenotype and thus the greater frequency of this allele observed in PEO1X might just be incidental. Apart from TP53, the only other significant result was obtained for the gene NF1. This gene encodes the protein known as Neurofibromin which functions as GAP (GTPase Activating Protein) to negatively regulate the

activation state of the protein RAS [232]. This important signal transduction factor regulates the activity of many downstream signaling cascades including ERK/MAPK and PI3K-AKT [232]. From the TCGA study (2011), it is known that the gene NF1 is either deleted or mutated in 12% of patients with HGSOC [159]. From the analysis of current sequencing data, it was seen that NF1 contained a deletion in the cell line PEO1 (Table 1: p 134). This deletion was not present in either PEO4 or PEO6. Instead, PEO4 contained a distinct point mutation conferring a putative loss of function (LOF) (Table 1: p 134). PEO6, which longitudinally is the most advanced of the three cell lines, contained only wild type alleles (Table 1: p 134). PEO1X, which is derived from PEO1, contained the same indel mutation as its parental cell line (Table 1: p 134). These results, for a single gene, are highly significant in that they can reveal the evolutionary relationship between all four cell lines. The fact that PEO1, PEO4 and PEO6 all contain a different allelic variant of the gene NF1 confirms that the specific mutations occurred only after the three populations had diverged evolutionarily, and hence that each evolved in parallel rather than as a direct progression. The fact that PEO1X contains the same deletion as the parental cell line likely suggests that it is directly descended from PEO1. These results, although limited in scope, prove unequivocally that PEO1X is not related to either PEO4 or PEO6. This disproves a crucial tenet of the hypothesis which holds that the resistant cells selected *in vitro* by treatment of a chemo-sensitive HGSOC cell line should be analogous or related to those which arose *in vivo*. On the other hand, it was validated that PEO1 is actually genetically heterogeneous at least in terms of P53 status, although it is not certain if this reflects the existence of distinct populations of cells. There might in fact be a marginal selection for P53 WT encoding cells in PEO1X which is unexpected since it is known that WT P53 confers sensitivity to platinating agents [130].

4.17 Illustrating the Immunophenotype of PEO1X: Obvious Alterations from PEO1 but with Little Relation to either PEO4 or PEO6

Another means of illustrating any potential differences between PEO1 and PEO1X would be in terms of their expression of phenotypic markers. A variety of markers, detectable using immunohistochemistry, are employed clinically by pathologists to aid in the diagnosis of HGSOC. In addition, a great number of markers have recently been highlighted for their association with stem-like features and have been used as the basis for identifying and isolating putative populations of Cancer Stem Cells (CSCs). Prior research conducted in this laboratory has attempted to elucidate the immunophenotypic features of the PEO1/4/6 cell line series in order to discover the changes that accompany disease progression and the onset of chemo-resistance. It was decided to contrast the immunophenotype of PEO1X with its parental cell line PEO1 as well as with PEO4 and PEO6, the two matched chemo-resistant cell lines established from the same patient. In order to accomplish this, cells from each cell line were grown in 8-well, glass-bottomed chamber slides and then fixed in PFA before being used for immunohistochemistry. The antigenic markers assayed included many histopathological markers of HGSOC identity, markers of EMT as well as markers commonly used to identify CSCs.

Staining for P53 showed that both PEO1 and PEO1X demonstrate nuclear positivity in the vast majority of cells present (Figure 7. A: p 140). The presence of P53 immunoreactivity in a cell is usually indicative of a mutant form of the protein that cannot be efficiently degraded. Both PEO1 and PEO1X are known to be P53 mutant from the earlier exome sequencing experiment (Table 1: p 134). That experiment, however, revealed that there is a fraction of cells in both PEO1 and PEO1X that express the WT allele of the protein. It was decided to attempt a confirmation, using IHC, of the results showing increased P53 WT expression in PEO1X

compared to PEO1. Using a semi-quantitative test, the fraction of P53 negative (no-staining) cells in 9 separate fields (normalized based on the total number of cells in one representative field) was manually counted and then averaged to obtain a final score per cell line. The results supported the earlier finding that there was a greater proportion of cells encoding WT P53 in PEO1X relative to PEO1 (Figure S7: p 188). The nuclear marker WT1 (Wilms Tumor 1) usually stains positive in patient samples of HGSOC and it has thus been used clinically as a diagnostic biomarker [9]. This marker actually stains negative in PEO1 and also in PEO1X (Figure 7. B: p 140). It is, however, expressed in both PEO4 and PEO6 which represent more advanced disease (Figure S8. B: p 189). The marker CA125 is a membrane glycoprotein that is used clinically as a biomarker to assess the response of a patient to treatment and to diagnose recurrence. In PEO1 this marker stains heterogeneously positive in clusters that might correspond with the pockets of cells with epithelioid morphology previously described (Figure 7. C: p 140). In PEO1X, which does not feature the epithelioid component, the expression of this marker is reduced, and its distribution is more sporadic, being mostly confined to individual cells (Figure 7. C: p 140). In both PEO4 and PEO6, the expression of CA125 is much increased, being found, at varying intensity, in the majority of cells present (Figure S8. C: p 189).

ARID1A is a gene encoding a chromatin remodeling factor that is commonly mutated in clear cell and endometrioid carcinomas. Its presence, as determined through immunohistochemistry, is thus more indicative of HGSOC (or something other than clear cell or endometrioid EOC). This marker is mostly nuclear-positive in all three of PEO1, PEO4 and PEO6 (Figure 7. D: p 141, S8. D: p 190). In PEO1X, it is also observed to be positive (Figure 7. D: p 141).

PAX8 is a transcription factor that is used a lineage marker for tissues derived from the Mullerian duct, including the fallopian tube epithelium from which HGSOC traces its origin. By

immunohistochemistry all four of the cell lines stain largely positive for this nuclear marker (Figure 7. E: p 141, S8. E: p 190).

The trans-membrane protein E-Cadherin is a prominent component of the intercellular junctions between epithelial cells. In PEO1 this marker stains membrane-positive in the clusters of cells with epithelioid morphology but not in the surrounding network of spindle shaped, mesenchymal-like cells (Figure 7. F: p 188). In PEO1X the expression of E-cadherin, similar to that of CA125, is much reduced and its distribution also appears more random rather than concentrated (Figure 7. F: p 188). In PEO4 and PEO6, this marker stains positive in a greater proportion of cells than in both PEO1 and PEO1X (Figure S8. F: p 190). This supports the notion that in advanced disease, the cells become more epithelial.

In the case of vimentin, which is a cytoskeletal protein associated with a more mesenchymal phenotype, its expression in PEO1 is confined to the spindle-shaped component and is not present in the "islets" of epithelioid cells (Figure 7. G: p 142). In PEO1X, its expression appears to be more widespread, but the pattern of staining within each individual cell is seemingly altered (Figure 7. G: p 142). In PEO1, vimentin expression is visible in the elongated processes of the spindle-shaped cells (Figure 7. G: p 142). In PEO1X, because the cells have a more rounded morphology, the staining looks to be more perinuclear (Figure 7. G: p 142). Conversely, in PEO4 and PEO6 this marker is virtually absent which again upholds the conclusion that they are more epithelial rather than mesenchymal (Figure S8. G: p 191).

The final category of markers to be evaluated were the so-called cancer stem cell markers whose expression correlates with a greater tumorigenic capacity in *in vitro* tumor-forming assays and *in vivo* transplantation experiments. A previous study by Baba and colleagues reported to show that the $CD133^{+}$ fraction of PEO1 had increased tumorigenic capacity [233]. Moreover, the

Figure 7

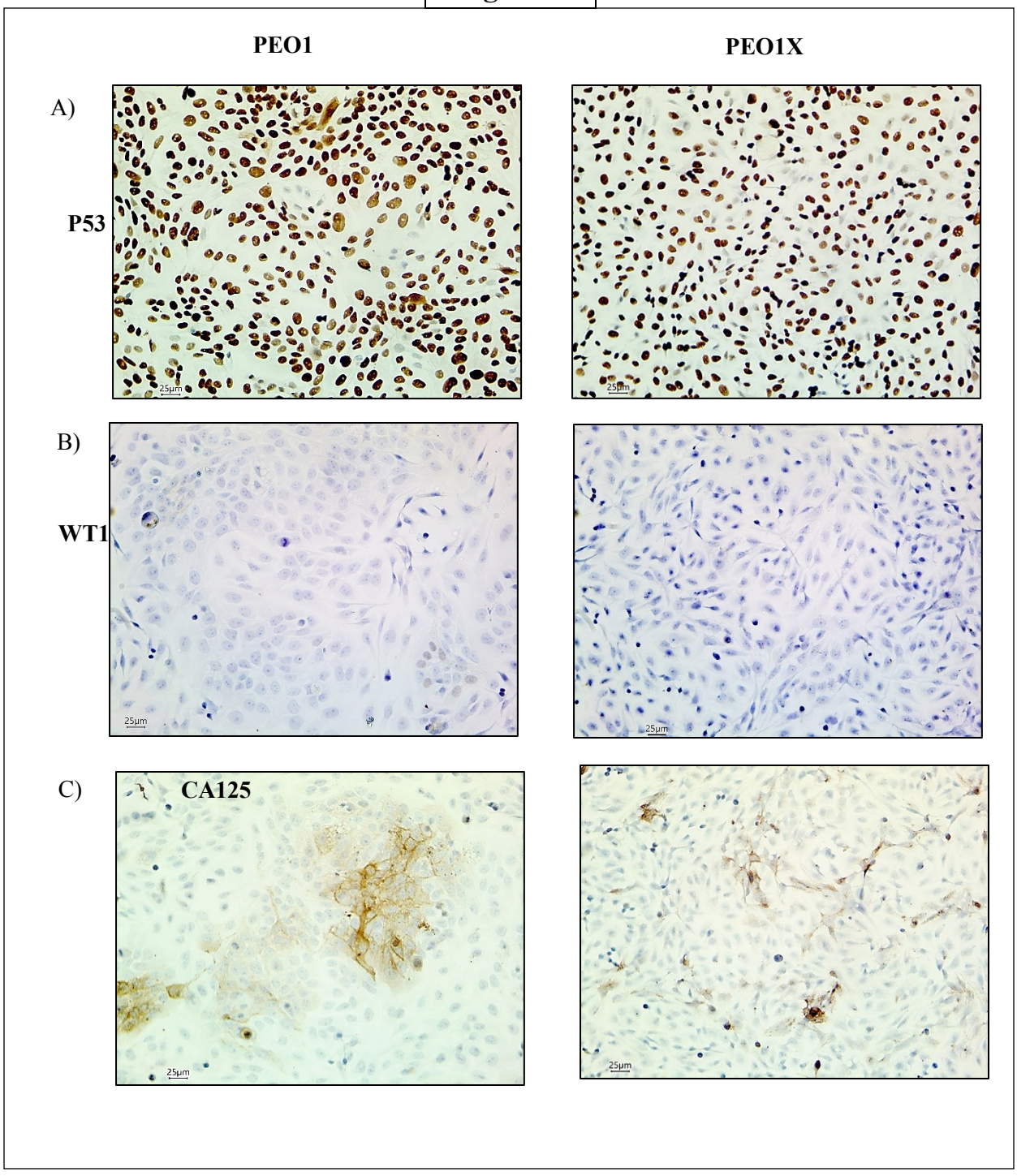


Figure 7 (cont.)

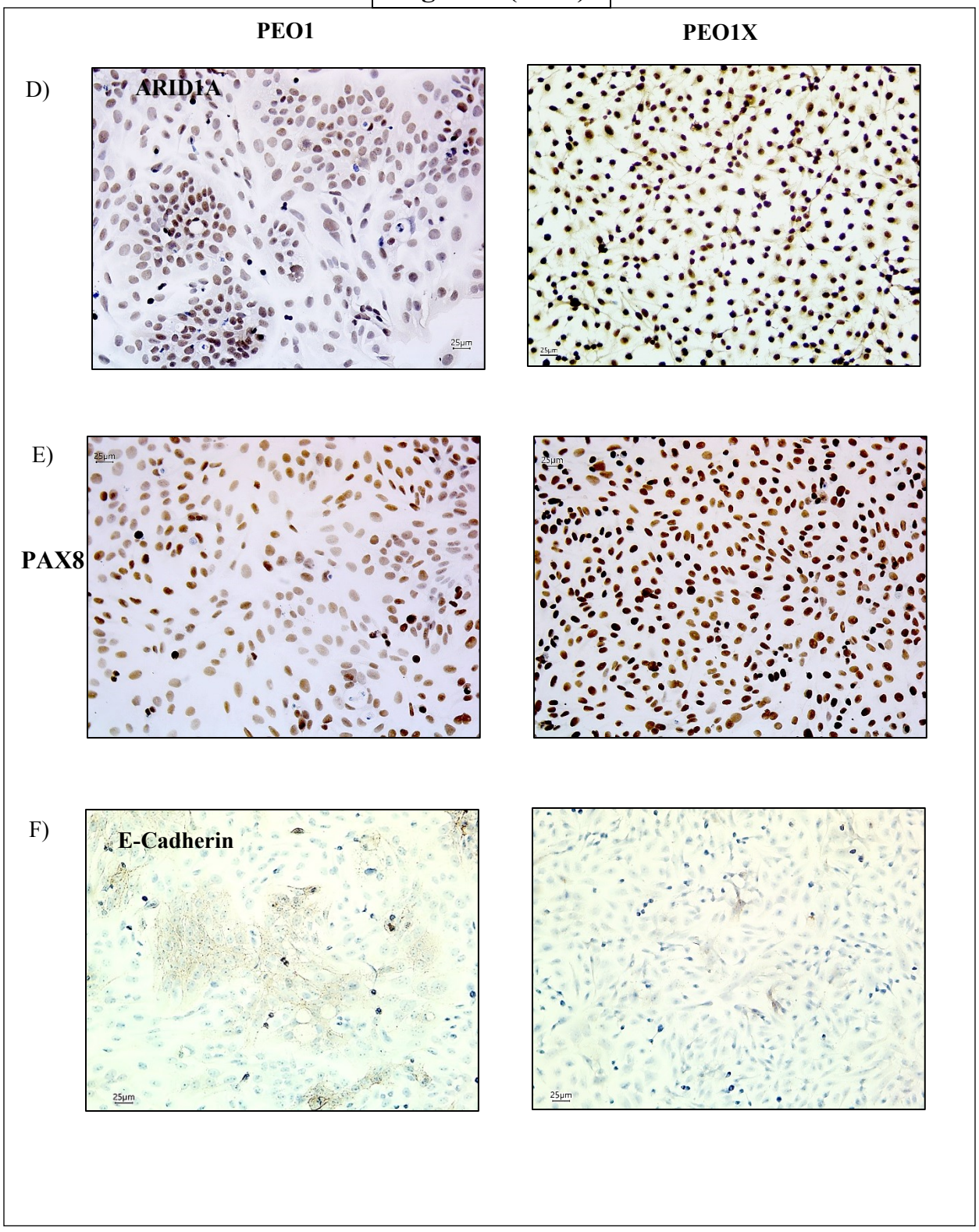


Figure 7 (cont.)

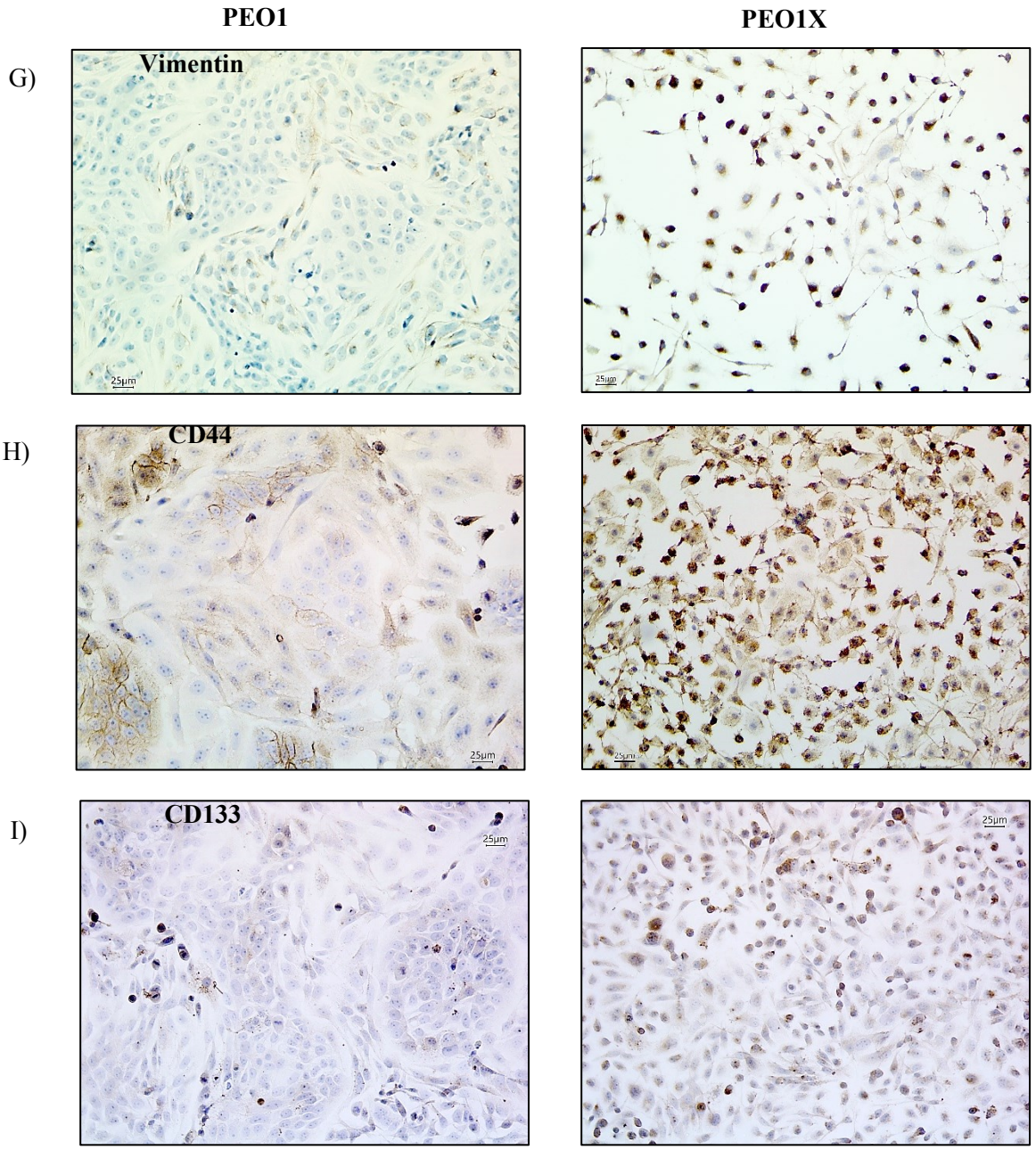


Figure 7.: *Evaluating the Immunophenotype of PEO1X in Comparison to PEO1*
Images taken of cells grown in 8-well chamber slides fixed in PFA. Antibody binding visualized by brown DAB reaction (Vector ImmPress peroxidase conjugate). Nuclear contra-staining is with hematoxylin. Images taken at 20X magnification (Scale bar is 25 µm). PEO1 and PEO1X have similar marker expression except that PEO1X displays less E-cadherin and more Vimentin. PEO1X also exhibits less CA125 staining and more positivity for the stem cell marker CD133.

group of Abubaker et al. showed that the short-term, single exposure of ovarian cancer cell lines to CDDP *in vitro* was sufficient to increase the expression of many putative CSC markers [234]. In order to confirm if this phenomenon were also true for PEO1X, it was decided to interrogate the expression of CSC markers in this novel population of cells. The membrane glycoprotein CD44 has frequently been associated with cancer stem cells including in the context of ovarian cancer [235]. By immunohistochemistry, this marker was revealed to be widely expressed in PEO1 with varying degrees of membrane-positivity (Figure 7. H: p 142). In its descendant, PEO1X, this marker was also largely positive (Figure 7. H: p 142). In both PEO4 and PEO6, CD44 stains more heterogeneously than in PEO1, with less expression in PEO6 relative to PEO4 (Figure S8. H: p 191). For CD133, which is reputed to be marker of the "CSC-like" population within PEO1 [233], its expression is barely discernable by IHC in this cell line (Figure 7. I: p 142). By contrast, in PEO1X it stains more visibly in many more cells than in the parental cell line (Figure 7. I: p 142). This marker also demonstrates a more obvious heterogeneous pattern of expression in the resistant cell lines PEO4 and PEO6 compared to the minimal expression which is visible in PEO1 (Figure S8. I: p 191). In summary, the phenotype of PEO1X is significantly altered in comparison to its parental cell line but in a manner that does not converge with that belonging to either cell line deriving from the chemo-resistant disease that emerged *in vivo*. PEO1X demonstrates a more mesenchymal cell fate, thereby corresponding with the results of previous experiments, while both PEO4 and PEO6 are plainly more epithelial. The expression of other markers such as WT1 and CA125 exhibits a divergent pattern of expression in PEO1X relative to PEO4 and PEO6. This dissimilarity makes it unlikely that PEO1X is in any way related to either population of resistant cells that emerged *in vivo*, thereby further refuting what was maintained in the hypothesis. Finally, although CD44 is abundantly expressed in both PEO1 and PEO1X,

the expression of CD133 in PEO1X appears to be increased relative to PEO1. This may imply that PEO1X is enriched in cells with increased tumorigenicity and stem-cell properties.

4.18 Repopulation of PEO1 After Combinatorial Drug Treatment: Resistance of the Resultant Entity PEO1.3X is not Replicated

Although platinating agents have been a mainstay of the chemotherapeutic approach to treating ovarian cancer since their inception, they have nearly always featured in a combination with a secondary cytotoxic agent with a distinct mechanism of action. The goal is ostensibly to broaden the spectrum of toxicity experienced by each targeted cancer cell in order to maximize the degree of lethality, while also minimizing the capacity of affected cells to develop adaptive or compensatory mechanisms. This should, in theory, serve to forestall the onset of chemo-resistance. Although the current standard of care consists of platinum-taxane combination therapy, prior to its adoption in the 1990s a number of non-standard platinum-based regimens were routinely administered at the discretion of the attending oncologist. For instance, the literature mentions that the patient from whom was generated the HGSOC cell line PEO1, had been treated with a drug combination consisting of cisplatin, 5-fluorouracil and chlorambucil [188]. Although unconventional this therapy would seem to initially have been effective, as the patient experienced a remission of around 22 months before relapsing [188]. The second application of particular regimen would presage the onset of chemoresistance which occurred after a 10-month interval [188]. Because only a single agent (CDDP) was used in the derivation of PEO1X, it was thought that this might ignore the potential of the other 2 drugs to influence the selection of the resistant cells that would eventually repopulate. It was postulated that treatment of PEO1 with the full combination of agents administered to the original patient would further refine the resultant selection towards a kind of cell better resembling those which arose in the patient.

Unfortunately, information regarding the specific doses of the drugs or the order of their infusion was not seemingly recorded in the primary sources. Thus, an analogous *in vitro* treatment would have to be contrived de-novo. Ultimately, the three drugs were combined in a cocktail with a ratio of 5 μM 5-FU to 10 μM chlorambucil to 10 μM CDDP and dispensed to a culture of PEO1 cells for a total of 1 hour. Much the same as the cisplatin monotherapy, toxicity took a few days to manifest but subsequently the response was profound, with an overwhelming percentage of cells succumbing by 10 days post-treatment (Figure S9. A: p 192). As before, repopulation began to occur from a small number of isolated foci, resulting in the appearance of a number of proliferative "colonies" of cells with a healthier appearance (Figure S9. A: p 192). These were eventually grown out and passaged to create another novel cell line which was assigned the name PEO1.3X. The assumption was that these cells would prove similar either to PEO1X or to PEO4 or PEO6. In reality, however, neither prediction proved to be accurate. When it came time to determining the Pt-sensitivity of PEO1.3X, it was found that these repopulating cells had not actually lost any sensitivity to the drug. The average CDDP IC50 calculated (two independent experiments) was 0.70 μM which is not significantly increased relative to the parental cell line (Figure S10 C: p 193). In contrast to PEO1X, the morphology of these cells is more evidently spindle-shaped and mesenchymal-like, without the epithelioid component found in PEO1 (Figure S9 B: p 192). This negligible gain in resistance can be further illustrated through the effect of CDDP treatment on the cell cycle status of PEO1.3X. Unlike PEO1X which demonstrates very little perturbation even at 10 μM CDDP, PEO1.3X is significantly affected at all treatment doses (Figure S12: p 195). There is a profound reduction in cells in G1 with a concomitant increase in the percentage of cells arrested in both S and G2/M phases (Figure S12: p 195). In addition, while there is a miniscule quantity of cells with hypodiploid DNA content at any dose in

PEO1X, in the case of PEO1.3X the percentage of such cells is significant in all treatment doses (Figure S12: p 195). The doubling time of PEO1.3X was found be similar to both PEO1 and PEO1X at 42.80 hours (Figure S11: p 194) compared to 47.84 hours for PEO1X (Figure 4: p 123) and 44.92 hours for PEO1. The immunophenotype shares features with both PEO1 and PEO1X. Like both of its related cell lines, PEO1.3X lacks the expression of WT1 and stains ubiquitously for P53 and ARID1A (Figure S13 A, B, D: p 196). In terms of the stem cell markers, PEO1.3X features abundant CD44 similar to PEO1 and no perceptible expression of CD133 (Figure S13. H, I: p 197. In keeping with its more mesenchymal-like phenotype, PEO1.3X displays minimal staining for E- cadherin, while the expression of CA125 is also virtually absent relative to PEO1 (Figure S13 C, F: p 196). Furthermore, the marker Vimentin is also more prominently expressed in PEO1.3X compared to PEO1 and especially compared to both PEO4 and PEO6 (Figure S13. G: p 197). At the genetic level PEO1.3X also appears to be more closely related to PEO1 due to the retention of the NF1 indel mutation that is present in PEO1 but not in PEO4 or PEO6 (Table S1: p 198.). The frequency of P53 mutation in PEO1.3X is actually greater than in PEO1, with 99.89% of alleles being mutant in PEO1.3X compared to approximately 80% in PEO1 and 69% in PEO1X (Table S1: p 198).

Discussion

5.1 On the Goals of the Project and the Choice of Cell Lines

In the broadest sense, this project sought to study the heterogeneity present within a selection of HGSOC cell lines in order to determine if a subset of the clonal diversity found in a patient's intraperitoneal disease was maintained in the *in vitro* environment. More specifically, it aimed to evaluate the potential for the existence of minor, intrinsically-resistant subpopulations within these cell lines. The assumption was that these cells could be selected for *in vitro* using a clinically relevant regimen of platinum-based therapy, analogous to that undergone by the original patient. The initial hypothesis was that the cells repopulating after *in vitro* Pt treatment should be similar or analogous to those which emerged clinically upon relapse of the patient from which the cell lines were initially derived. The principal tool for interrogating this question would be a set of paired, patient-derived, HGSOC cell lines which were established longitudinally from the same patient before and after the onset of chemo-resistance. If resistance could be induced from the chemo-sensitive cell line of the series *in vitro*, its characteristics could then easily be compared to the matched chemo-resistant cell line from the same set. The core assumption behind the formulation of this hypothesis was that a certain degree of cellular heterogeneity from the original disease was preserved within these cell lines, such that the resistant populations would be present at low overall frequency, comparable to their abundance *in vivo*. Given that the majority would consider cancer cell lines to be homogeneous entities, this premise may be viewed as somewhat naïve or even outlandish by those accustomed to dealing with more complex systems for modeling cancer. In fact, the whole rationale for the recent development of Patient-Derived Xenografts (PDXs) and sophisticated, three-dimensional *in vitro* cell culturing techniques was to better recapitulate the spectrum of cellular diversity present within human tumors. Nevertheless, in contrast to the majority of cell lines which were

previously used to model HGSOC, the cell lines of the PEO1/4/6 and PEO14/23 series have well annotated clinical histories and have been confirmed to represent the disease at the genetic level [189]. In addition, these cell lines, although created in the early 1980s, have only recently attained some measure of popularity and are still incompletely characterized, especially from the perspective of heterogeneity. Added to this is the fact that the samples of PEO1/4/6 that were used were obtained directly from the primary source (the laboratory of Dr. Simon Langdon at the University of Edinburgh) rather than having been purchased commercially. Presumably, the cells were from a relatively low passage number, and some heterogeneity was still present from when the cells lines were initially established (although it would be impossible to say for certain). Some earlier anecdotal evidence gathered in this laboratory supported the notion of heterogeneity within the cell line PEO1 through the observation of cells with distinct morphologies and marker expression patterns (unpublished data). All of this information factored into the selection of the hypothesis. It was thought that if ever there was a chance of witnessing this kind of cellular heterogeneity within an HGSOC cell line, the PEO1/4/6 or PEO14/23 series of cell lines would represent ideal candidates for testing this theory and for granting affirmation to this hypothesis.

5.2 On the Results of the Cisplatin Sensitivity Assays and the Methodology Employed

A crucial early aim for this project was to develop a means of defining the CDDP sensitivity of the cell lines to be used so that the sensitivity of the repopulating cells could be compared to that of their parental cell line. This would require the institution of a protocol for *in vitro* Pt treatment and for diagnosing the resultant toxicity. Making use of the laboratory equipment available, it was decided to employ the Guava Viacount assay to measure the cellular response to the drug in terms of cell number and percent viability. At first the approach adopted involved the use of a range of supra-pharmacological doses of CDDP to which the cells were exposed for only

1 hour, with the effect being assayed around 72 hours after drug removal. The result was that for both PEO1 and PEO14 the response of the cells in terms of both the live cell number and percent survival deviated from the expected dose dependent decrease that normally typifies the effect of cytotoxic drugs. In PEO14 the only significant decrease in either variable was observed at the highest dose of 40 μM, whereas for PEO1 there was no substantive differences between any of the treatment doses. This indicated a defect in the approach that was being used to quantify sensitivity to the drug. It eventually became clear that the range of doses selected was simply too high and that the allotted period of 72 hours post-treatment offered insufficient time for the toxicity to manifest, especially in terms of cell death. At the same time, it was confirmed that a 24-hour time of exposure to the drug was too long, causing the nominally resistant cell lines PEO4 and PEO6 to exhibit an equivalent or greater degree of toxicity compared to the supposedly sensitive PEO1 (with supra-pharmacological doses).

What was needed therefore, was a way of ascertaining a more long-term measure of toxicity to pharmacological doses of the drug, which would allow the cells to be exposed for only 1 hour. Because maintaining the cells in the same culture plates for more than 96 hours proved impractical (due to confluency attained by the vehicle-treated condition and the inability to change the medium without discarding a substantial percentage of the dead cells in suspension), it was plain that a completely new methodology would need to be employed. For this reason, it was eventually decided to settle in favor of the technique called the clonogenic survival assay which assesses the long-term capacity of a drug to inhibit the colony formation ability of treated cells. The use of this technique further highlighted the contrast between the short and long-term effects of CDDP. For instance, in the case of PEO1, cells treated with even a large, clinically unachievable dose of 40 μM CDDP displayed only a moderate reduction of about 30% in terms

of percent survival, relative to control, when cells were treated for 1 hour and the viability assessed 72 hours later by Guava Viacount. By contrast, these same cells, when used to perform a clonogenic survival assay, displayed a 50% reduction in colony forming capacity at a drug concentration of less than 1 μM. In fact, the most obvious impact of CDDP discernable after 72 hours was in terms of growth-inhibition which can be recognized by a reduction in the number of live cells without any corresponding drop in percent survival. Moreover, because the cells that were plated in the clonogenic assay were first assessed at 72 hours post-treatment with Guava Viacount microcytometry, it was possible to also ascertain the short-term toxicity resulting from these relatively small doses. Rarely was there ever any significant decrease in either parameter assayed at the doses later deemed capable of halving colony formation for the same cells. It warrants further emphasis that the cells used in the clonogenic survival assay were plated from an equivalent number of *live* cells per condition. This leads to the conclusion that the cells which were deemed alive using the Guava Viacount method were either in the process of dying or had otherwise accrued sufficient damage so as to obviate the capacity of these cells to proliferate. Thus, the clonogenic survival assay, in the manner employed in this work, represents a more comprehensive means of measuring drug toxicity beyond the short-term evaluation of cell viability and growth inhibition. Prior publications have reported on the CDDP sensitivity of the cell lines here utilized, but their assessments were based on different techniques, which mostly included high-throughput proliferation assays such as the MTT and SRB assays which can only quantify short-term growth inhibition. Also, these prior studies did not attempt to modulate the duration of CDDP exposure and, consequently, the cells were allowed to remain in the presence of the drug for as long as 72 hours. This approach lacks clinical relevance and may introduce additional modes of toxicity that would not normally be operative *in vivo*. Nevertheless, the

actual values for the IC50s calculated were in general agreement with those recorded in the literature and which were obtained using different methods. This project possibly represents the first instance in which the CDDP sensitivities of this group of cell lines was verified using the clonogenic survival assay, which is considered to be the gold standard for conducting drug toxicity screens [220]. In spite of this, the values which were put forward are likely only rough estimates, rather than precise calculations of the IC50s for these cells. This was due to the technical complexity and labor-intensive nature of this method, which only allowed for the minimum number of doses and replicates to be used in any given experiment, something that negatively influences the accuracy of the results. The main advantage of colorimetric techniques such as MTT is that they are relatively quick and easy to perform, and the design of the assay (in a 96-well plate) allows for a large number of conditions (with many replicates each) to be tested at the same time. Also, with the experimental design used in this project, there was the prospect of the drug having an independent effect on cell-adhesion which would have hindered colony formation without necessarily being a sign of irreversible cytotoxicity. Furthermore, a few cell lines such as PEO14 and PEO6 were not naturally conducive to forming colonies, necessarily leading to a continual adaptation of the protocol to suit each cell line. This was necessary to ensure that the number of colonies was in a range that could accurately be quantified. Ultimately, despite these limitations, it can be concluded that the values for the CDDP-sensitivites of PEO14 and PEO1/4/6, although not totally accurate, were acceptable for the purpose of comparison with the repopulating cells obtained later on.

5.3 On the Generation of PEO1X and its Significance

The central pillar of this thesis involved the creation of a novel Pt-resistant cell line called PEO1X through exposure of chemo-sensitive PEO1 cells to CDDP in culture. The hypothesis

posited that the exposure of a chemo-sensitive HGSOC cells *in vitro* to a clinically relevant regimen of CDDP would be followed by the expansion of Pt-resistant sub-populations, thereby selecting for the emergence of the resistant phenotype after only a minimal course of treatment. The last point is particularly important, as many resistant cell lines over the years have been produced through the exposure of cancer cell lines to chemotherapeutic drugs in culture, but few can reasonably claim that these cells are selected from minor populations of resistant cells that were present initially within the parental cell line. This is because the methodology usually involved culturing the cells in the presence of gradually increasing concentrations of the drug over a chronic duration. This approach, although proven effective in many cases, is totally opposed to the modality in which cells would encounter the drug in the context of a human patient. This kind of process typically requires many months of continuous exposure such that the cells gradually build up a tolerance to the drug. The end result is usually a population of cells that are capable of enduring even drastically elevated and supra-pharmacological doses of the drug upon re-exposure. The evolution of the resistant phenotype in this manner likely involves the progressive acquisition of de-novo alterations to cellular biology under the chronic selective pressure of the drug. This mechanism stands in opposition to the actual process of clonal selection which is known to occur in HGSOC. Also, in contrast to clinically acquired Pt-resistance, the resistance developed in this manner is not permanent and requires periodic or continuous re-exposure for this property to be maintained. It was thus imperative that the extent of treatment be minimized in order to prevent the evolution of resistance in this manner. At the same time, in order for the selection of pre-existent subsets of resistant cells to occur, a certain amount of heterogeneity must initially be present within this cell line. For this project, this was the main question that needed to be answered with respect to cell lines such as PEO1. In should

be recognized that a Pt-resistant cell line was previously created from PEO1 through *in vitro* exposure to CDDP. Called PEO1-cddp, it however was the result of continuous exposure of the original cell line to increasing concentrations of the drug (from 25 nM to 1 μM) for a period of 4 months [216]. Recently a certain number of studies in the field of HGSOC have claimed to show the acquisition of Pt-resistance in a cell line model through a selective process, this has never been conclusively proven. Thankfully, the trend in recent times has been towards a more realistic means of inducing the development of Pt-resistance *in vitro* through the application of the so-called "pulse" method [217,218]. This involves the use of multiple rounds of treatment, with a limited duration of drug exposure, that are interrupted by periods allowing for the cells to recover and for repopulation to occur. A variant of this type of approach was used in this project except that after the first round of treatment the cells seemed to have already become resistant, thus rendering retreatment superfluous. It was therefore decided to halt the course of treatment after only two rounds of exposure to 10 μM CDDP for 1 hour each. The two doses were administered approximately two weeks apart and the cells had already repopulated to an impressive degree during that time. The choice of the particular dose used was intended to replicate the highest tolerable pharmacological dose of CDDP, so as to kill the maximum number of sensitive cells. This would ensure that the ensuing repopulation would be composed of purely resistant subsets. At this elevated dose, the response of PEO1 cells proved to be just as dramatic as anticipated. In the days following the initial treatment, the cells increasingly began to exhibit the obvious signs of Pt-mediated toxicity such as swollen/enlarged cytoplasmic volume, vacuolation, distorted morphology, etc. The bulk of the dying cells would progressively detach from the surface, leaving only a dwindling number of remaining cells visible within the culture flask. It should be noted that this kind of response was only observed in the case of PEO1. Although a similar

methodology was applied to the cell line PEO14, the response obtained was not anywhere near as robust as that which affected PEO1. It would seem that PEO1 has a greater propensity to undergo cell death in response to CDDP treatment compared to PEO14. This may be due to the fact that PEO1 possesses a BRCA2 mutation which limits the capacity of the cells to repair the DNA double strand breaks, which are typically induced by CDDP treatment. A similar hypersensitivity has been observed in the case of HGSOC patients with inherited BRCA mutations, who are known to demonstrate greater initial response rates to platinum-based chemotherapy and greater 5-year survival compared to WT patients. The persistence of the P53 WT genotype in a certain segment of PEO1, which was uncovered by the genetic testing, may also play some role in mediating the elevated Pt-induced lethality observed in this cell line. Following the initial phase of widespread cell death, which occurred for about 1 week after the treatment of PEO1, repopulation took place from a limited number of isolated foci. These foci resembled the colonies that arise from the implantation of a single cell in a clonogenic assay. While, it is not known if these repopulating "colonies" are each descended from a single cell, their limited number and isolated situation suggest that they derive from the outgrowth of only a handful of cells within the original cell line. Their distinct appearance, which is altered from that which normally characterizes PEO1, implies the emergence of a different entity compared to PEO1. The fact that the repopulating cells are outwardly healthy-looking and proliferative may indicate that they were capable of evading drug-induced damage, which might be a sign that these cells were intrinsically resistant to CDDP. All of this would seem to support the hypothesis which asserts that chemo-sensitive HGSOC cell lines contain minor resistant sub-populations that can be selected for by *in vitro* Pt treatment. Unfortunately, similar results were not forthcoming in the case of PEO14; despite numerous attempts, a similar sort of repopulation

could not be replicated in the case of this cell line. This would suggest that perhaps the postulated minor resistant sub-populations are not in fact contained within every HGSOC cell line, but only a select few. Nevertheless, this project presents possibly the first clear evidence of a Pt-resistant cell line being created following such an abbreviated course of treatment *in vitro*. Although it cannot be stated definitively that the resultant PEO1X cells originated as a minor population within PEO1, the likelihood of the drug being able to induce a de-novo adaptation after such minimal time of exposure is altogether minute. Conversely, if this were the case, then one would expect the derivation of PEO1X to be something of a one-off phenomenon, whereas actually it was possible to independently replicate the type of repopulation leading to PEO1X on numerous occasions. In the future one might imagine the use of such novel technologies as single cell RNA-sequencing to identify if multiple distinct populations, at the level of gene expression, exist within PEO1, and if any of these might correspond to the gene expression signature of PEO1X.

5.4 On the Revelation that PEO1X is Resistant to Clinically Achievable Cisplatin Treatment *In Vitro*

The revelation that the repopulating cells, termed PEO1X, had lost a substantial degree of their sensitivity to cisplatin after only two rounds of exposure to what amounts to clinically achievable treatment parameters, was perhaps the most significant finding of this project. These cells, after minimal passaging in culture, would be found to demonstrate upwards of 20-fold greater resistance to 1-hour CDDP treatment relative to their parental cell line PEO1. The average IC50 to CDDP, which was calculated from the data gleaned from two independent clonogenic survival experiments, was determined to be around 10.75 μM which is greater than the 10 μM dose to which they were originally exposed prior to their emergence by repopulation.

This signifies that PEO1X is virtually resistant to even the upper limit of clinically relevant CDDP treatment *in vitro*. The extent of the *in vitro* resistance attained is even greater than that exhibited by the matched cell lines derived from the same patient at the stage of clinically-diagnosed chemoresistance. It should be noted that, in the original patient, the disease was treated with more than just cisplatin (a combination of cisplatin, 5-fluorouracil and chlorambucil), so that the resistance which developed was distributed between each of these drugs rather than just cisplatin. Part of the rationale for using cisplatin in a combination therapy is to enable treatment with lower doses of this agent relative to a monotherapy, which was often necessary due to its elevated toxicity profile. This might explain why the cell lines PEO4 and PEO6 express a greater sensitivity to CDDP compared to PEO1X, in which the resistance was conditioned to CDDP without exposure to either 5-FU or chlorambucil. Other factors might account for the disparity in the degree of Pt-resistance attained by PEO1X compared to both PEO4 and PEO6. Particularly the fact that, in the case of PEO4 and PEO6, the resistance resulted from a selective process modulated by a complex *in vivo* environment within the peritoneal cavity. This environment can doubtless sustain many distinct niches capable of nurturing the clonal diversity of the developing disease. Some of these may be conducive for the maintenance of the resistant phenotype, which would therefore be attributable to a confluence of both genetic and environmental factors. For instance, it is known that the many stromal cell types in the HGSOC microenvironment such as fibroblasts, adipocytes and immune cells can release a variety of soluble mediators which can impinge on the behavior of the tumor cells. While the genetic contribution to chemo-resistance is cell-intrinsic and is thus preserved *in vitro*, the contribution of the microenvironment is inherently conditional and cannot be replicated *in vitro*.

Another issue might be the potential for the resistant phenotype to be gradually diluted by adaptation to the Pt-free conditions *in vitro*. It could be that a certain amount of the Pt-resistance developed after repeated exposure is due to a temporary adaptation on the part of the cells that is not dependent on any change in genotype. This would involve, for example, certain epigenetic changes, alterations in gene expression, or in signaling pathways that can be more rapidly acquired than alterations to genomic sequence or copy-number. After the selective pressure of the drug is removed, then the cells would likely slowly begin to transition back to the normal, default state because any changes are not permanently instituted. In addition, the maintenance of the resistance phenotype probably entails an energetic cost. The cell lines PEO4 and PEO6 have undoubtedly spent the equivalent of many years in culture since the time of their isolation. By contrast, PEO1X was newly derived after exposure (albeit briefly) to CDDP and has only spent at maximum what amounts to a few weeks in culture. It is therefore not difficult to imagine that the phenotype is still "fresh" in PEO1X but not in either PEO4 and PEO6. It is not known exactly how "durable" the Pt-resistance will be in PEO1, but in multiple experiments with different passage numbers separated by several months, the magnitude to the resistance was not diminished. It may be the case, however, if the mechanism of resistance in PEO1X is not genetically determined, that the diminished CDDP-sensitivity might begin to "wear off" in these cells. Albeit, this would likely require many months of continuous culturing under normal conditions in order to take place.

Another important discovery was the assessment that PEO1X is much less resistant to 24-hour exposure compared to only 1-hour exposure. It is important to consider that most of the cell lines which have been made resistant *in vitro* (especially those raised on long-term, continuous exposure to increasing doses) are insensitive regardless of duration. This finding signifies that

the type of resistance evolved is more closely related to that which occurs *in vivo*. It may also indicate the facility with which resistance develops to only limited exposure compared to more extended exposure. Typically, in studies of this type, it is not considered if resistance to platinum is dependent on the duration of exposure. Thus, most investigators would not be inclined to consider a cell line resistant if it were not resistant to 24 hours or more of exposure to the drug. This would overlook a potential model for representing a kind of platinum resistance that is more similar to what is derived clinically. This study might even represent one of the only attempts to distinguishing Pt-resistance in-vitro depending on the time of exposure. That being said, it was later determined that PEO4 is much less sensitive to a longer duration of exposure, which would contradict the claim that PEO1X embodies greater relevance to the resistance emerging in vivo. This especially true when considering the effect of the of the longer doubling time of PEO4, which naturally confers a greater resistance to longer-durations of treatment relative to PEO1X.

It was not verified if the resistant phenotype evolved in PEO1X can confer cross-resistance to other chemotherapeutic drugs. This would greatly depend upon the mechanism at work, and if it is particular to the effects of CDDP or broader in its specificity, being therefore able to influence the cellular response to multiple agents. A recent paper reported on a series of *in vitro* derived, cell line models of Pt-resistance that also possessed cross-resistance to paclitaxel and doxorubicin [217]. This paper, however, used a methodology to establish drug-sensitivity different to that employed in this project. Moreover, it is not known if the cell lines derived from *in vivo* acquired Pt-resistance such as PEO4 and PEO6 are cross-resistant to paclitaxel or doxorubicin. It should be noted that the original donor patient was not treated with either of these two drugs but with a combination of cisplatin, 5-fluorouracil and chlorambucil. In it the future, it would be

more worthwhile to test if either PEO1X or PEO4/6 is resistant to the combination of drugs to which the original patient was exposed.

5.5 On the Results of the Experiments Interrogating Cell-Cycle Status after Cisplatin Exposure

A further validation of the resistance newly gained by PEO1X comes from the data defining the cell cycle status of cells treated 72 hours prior with CDDP (1-hour exposure). While the parental cell line PEO1 presents with a significant disruption of the normal cell cycle distribution (relative to Vehicle Control) even at the lowest treatment dose of 2.5 μM, PEO1X demonstrates hardly any disturbance at a much higher dose of 10 μM. When compared to the data collected under identical conditions for PEO4 and PEO6, it is evident that PEO1X displays a similar response (or lack thereof) to these two resistant cell lines. As in PEO1X, the distribution in the phases of the cell cycle was not significantly altered in any of the pharmacological doses of CDDP that were used. Another indication as to the resistance of PEO1X, was in terms of the percentage of cells with sub-G1 DNA content that were obtained 72 hours post-treatment. Considered as an analogue of cells undergoing the apoptotic process, in PEO1X the percentage of hypodiploid cells increased in proportion to the dose, but their abundance was only ever a fraction of that present in PEO1 at the same dose. These data confirm that, unlike PEO1, PEO1X can transit the cell cycle even shortly following exposure to CDDP. The results agree with the earlier Pt-sensitivity data which showed a minimal amount of growth inhibition 72 hours post-treatment, even at a dose of 10 μM. These results signify that, contrary to PEO1 where treatment with even low doses of CDDP is sufficient to cause cell cycle arrest, in PEO1X the cells are either able to resolve the Pt-induced cell cycle arrest within 72 hours or they simply fail to experience any arrest. Based on the number of live cells present (without any change in viability)

at these same doses in the Pt-sensitivity assays, it would be logical to conclude that PEO1X suffers no significant impediment in its ability to transit the cell cycle after pharmacological CDDP treatment.

The results also illustrate some notable differences between PEO1X and PEO4/6 which corroborate the previously ascertained differences in doubling time between these cell lines. PEO1X, with a significantly shorter doubling time, is able to transit the cell cycle much quicker, so that the cells attain maximum confluency much sooner. This explains the particular cell cycle distribution which was observed with this cell line, where the great majority of the cells are in G1 and much fewer are in the other phases. In PEO4 and PEO6, the slower doubling time ensures that there will be more cells registered in S and G2/M phases of the cell cycle compared to PEO1 and PEO1X. For reasons that are not clear, there appear to be more cells with sub-G1 DNA content in PEO4 and PEO6 compared to PEO1X, even in the control condition. This likely points to some intrinsic differences in the growth patterns of these cells that result in a somewhat elevated tendency to undergo apoptosis in PEO4/6 at baseline. Although, the absolute percentage of hypodiploid cells is still quite small and is not dramatically altered, like in PEO1, with increasing dose. If this experiment were to be performed again it would be advisable to select an earlier timepoint such as at 24- or 48-hours post-treatment to exclude the possibility of confluency being reached in PEO1X. This would also enable the discernment of a possible transient cell cycle alteration that may occur immediately following CDDP exposure, and which would normally resolve by 72 hours post-treatment. It would also be worthwhile to have a better control condition than the vehicle-treated condition, such as a sample taken before treatment at 0 hours, where the cells are more likely to be in the exponential phase of growth compared to the vehicle-treated cells. Even if the results yielded with this experimental approach are sufficiently

accurate to validate the diminished Pt-sensitivity of PEO1X, they should still be viewed critically due to the limitations of this particular method. Foremost of these is the fact that the DNA content index used by the program is a relative measure based on the staining intensity, which may not always be consistent between assays even with the same cell line. Also, the gating for each phase of the cell cycle is not automatically determined and must be inputted manually. This results in an inherent degree of arbitrariness in the definition of these limits, with standardization usually being impossible even within the same assay. It would be preferable to make use of a more sophisticated and precise means of testing for cell cycle status if this experiment were to be repeated in the future. Alternatively, the protocol could be further optimized to ensure a greater quality and consistency of staining, which would potentially better help to resolve the different stages of the cell cycle based on DNA content index. While these results help to satisfy the hypothetical premise that the cells obtained via repopulation of PEO1 are resistant to CDDP, they also highlight fundamental differences compared to PEO4 and PEO6. The different cell cycle distribution speaks to a slower rate of proliferation in PEO4 and PEO6 which is inconsistent with the hypothesis.

5.6 On the Morphology and Growth Characteristics of PEO1X

After the determination that PEO1X was indeed resistant to CDDP, the remainder of the project was devoted to characterizing these novel cells while comparing their features against those of their parental cell line PEO1 and the matched chemo-resistant pair of PEO4 and PEO6. The aim was to elucidate the areas that were altered with the onset of resistance, while contrasting the phenotype with that exhibited by cells that had become chemo-resistant *in vivo*. According to the hypothesis, the phenotype of the repopulating cells arising after CDDP exposure should be more convergent with the cells selected for expansion in the original patient.

As will be argued, this did not prove to be the case, and it quickly became evident that PEO1X was a unique entity, separate from PEO1 and divergent from both PEO4 and PEO6. The first area where this was noticed was in terms of morphology. While PEO1 is dichotomous in terms of morphology, containing a mixed population of both epithelioid and mesenchymal-like cells, PEO1X is remarkably homogeneous, with a uniformly small and rounded appearance. While normally the transition towards a more homogenous phenotype is indicative of a selective process, in this case it proved otherwise. In PEO1, the two patterns of morphology are likely the result of an intrinsic plasticity in terms of this feature, an assumption derived from the fact that each is observable within colonies derived from a single cell. It remains unclear as to what role, if any, this plasticity plays in PEO1 and how it is specified. Its abolition in PEO1X, however, is not paralleled by a gain in resemblance to either PEO4 and PEO6 which are both more predominantly epithelioid in terms of morphology. These cell lines also possess growth characteristics that are not replicated in PEO1X such as the ability to grow in three-dimensional foci and to spontaneously form multicellular spheroids in suspension. From this perspective as least, it can be established with surety that PEO1X is similar neither to PEO1 nor to PEO4 and PEO6. With this concluded, it remains uncertain as to how one would classify the morphology of PEO1X in terms of the continuum from epithelial-to-mesenchymal. It would be more appropriate to describe this sort of morphology as a kind of intermediate between the two extremes. Although not addressed directly in this project, the distinctive morphology of PEO1X highlights the potential role of the EMT program in specifying the identity of these cells. This possibility will be more fully discussed later on, but it for now it suffices to say that PEO1X is likely influenced to a certain extent by the EMT process. However, it would be impossible to conclude definitively based on morphology alone. Many prior studies have also documented changes in

morphology on the part of cells which have been made resistant to platinating agents in culture. This project adds to the growing consensus regarding this commonly observed phenomenon. It has not been satisfactorily addressed if this shift in morphology is simply correlative with the onset of Pt-resistance or if there is a more mechanistic role for these changes in the adaptation to a more resistant phenotype. If one views morphology as being essentially downstream of EMT, then it could be envisaged to perform RNAi against the transcription factors regulating EMT, such as SNAIL or TWIST, in PEO1X (or another appropriate cell line with *in vitro* derived Pt-resistance). If this is sufficient to re-sensitize the cells to CDDP, then it would be possible to affirm a mechanistic role for this process in the biology of the Pt-resistant cell.

5.7 On the Doubling Time of PEO1X

Inasmuch as a cell's doubling time is a fundamental characteristic, the results of this measure quantified for PEO1X speak to an important point of dissonance with the hypothesis. Prior experimentation in this laboratory had revealed that the cell lines PEO4 and PEO6 displayed significantly longer doubling times relative to their chemo-sensitive counterpart PEO1. From these experiments arose the notion that, along disease progression and with the transition towards chemo-resistance, the less proliferative the cells become. In many ways this is hardly surprising, having been foretold in the theoretical framework of the clonal evolution hypothesis. This model predicts that among the clonal diversity of the primary tumor before treatment, the intrinsically resistant sub-clones reside at a low overall frequency due to a fitness disadvantage that is associated with the maintenance of the resistant phenotype under conditions where it is not required. Part of this supposed evolutionary cost would be manifested in terms of a lower intrinsic proliferation rate relative to the more chemo-sensitive bulk of the tumor, something that would account for the low abundance of these cells prior to treatment. Only in the absence of

competition following treatment and the removal of the more populous tumor bulk are these cells free to expand their share of the population, thus leading to eventual relapse with chemo-resistant disease. Cells that are more quiescent are inherently less sensitive to genotoxic agents. This is because of the relative specificity of these drugs for targeting rapidly dividing cancer cells. The more quiescent chemo-resistant sub-clones would be more likely to persist as the majority of the chemo-sensitive populations perish. This paradox was the impetus for the formulation of the novel therapeutic strategy of adaptive chemotherapy which aims to avert the development of chemo-resistance by exploiting the natural evolutionary dynamics within the tumor, including the lower proliferation rate of the chemo-resistant cells. Bearing this in mind, it was natural to expect that the repopulating cells which expanded after *in vitro* treatment of the cell line PEO1 would have a longer doubling time than their parental cell line. This assumption, however, was proved to be incorrect in that the doubling time of PEO1X not significantly longer than that of PEO1. This finding raises a number of perplexing questions which cast doubt on the origins of PEO1X. For example, it had been argued that PEO1X had originally existed as a minor population within PEO1 that was selected after *in vitro* CDDP treatment. If PEO1X has a similar doubling time to PEO1 then it would be difficult to imagine how it could possibly remain at such a low abundance relative to the other more sensitive cells. The concept of a "passive inhibition" would therefore not likely apply to this scenario. This finding does not discount this possibility completely but raises the potential for other more obscure mechanisms for why PEO1X is not a more prominent component of PEO1, if indeed these cells were ever present as a minor population before treatment. This finding also suggests that the mechanism of resistance in PEO1X is different from that featured in PEO4 and PEO6. Typically, the reason which is brought forward for why a resistant cell would have a low proliferation rate is that there is an

energetic cost being paid by these cells which diverts cellular resources away from cell-division towards maintaining this phenotype. The common example which has been discussed involves the expression of ATP-dependent efflux pumps such as those of the ABC or MDR family, which act to prevent the accumulation of platinum within a cell. The energetic requirements of these pumps have been theorized to be sufficient to reduce the proliferation rate of the cells in which they are abundantly expressed. If true, then this mechanism is not likely to be at work in PEO1X for which the doubling time is barely elevated. One may conclude that whatever the mechanism of resistance, it likely is "passive" enough not to overly impede a cells proliferation rate. This cannot be said for either PEO4 or PEO6, for which the doubling time is much prolonged compared to both PEO1 and PEO1X.

The issue of doubling time is not often examined in studies describing *in vitro* derived models of Pt-resistance. It is therefore not known if the findings regarding this characteristic point to a novel attribute of PEO1X or are a common feature among the variety of induced Pt-resistant cells encountered in the literature. This is probably the first instance where it can be ascertained definitively that the characteristic of doubling time differs between a type of Pt-resistant cell created *in vitro* relative to a matched population of resistant cells that arose *in vivo* from the original patient from whom the cell line series originated.

5.8 On the Increased Migratory Capacity of PEO1X

The motility or migratory capacity of a particular cell can be viewed as an analogue of its status in terms of phenotype along the continuum of epithelial to mesenchymal. The role of EMT in controlling the acquisition of mesenchymal-type features was mentioned earlier in the discussion pertaining to morphology. Cells demonstrating a more mesenchymal phenotype would be expected to migrate more efficiently compared to those that are more epithelial in

nature. In cancer, migration and invasion are integral components of the metastatic process whereby cells disseminate and colonize disparate sites around the body. In HGSOC the tumor cells rarely spread beyond the peritoneal cavity and their distribution is facilitated by passive forces such as the physiological flow of the peritoneal fluid. The importance of EMT to the metastatic process in ovarian cancer is thus more debatable than in other cancers. The data from studies undertaking the molecular sub-typing of HGSOC has, however, revealed that patients possessing a disease with a more mesenchymal gene expression profile are at a greater risk of poor prognosis. Recently, the evidence gathered by another student in this laboratory has indicated that along disease progression, and with the onset of chemoresistance, the cells become less motile and invasive [229]. This was determined using the same cell line model of HGSOC which was employed in this project, namely the PEO1/4/6 series of longitudinally matched cell lines. If the hypothesis were to be verified, then a similar result should be obtained for the resistant cells selected *in vitro* from PEO1. In fact, the opposite was observed, in that PEO1X proved to be more migratory than its parental cell line. In many ways this is not surprising; many previous studies have correlated the gain of resistance *in vitro* by cell line models with the enrichment of mesenchymal features, including increased motility [223-226]. The findings regarding migration further reinforce the earlier observations relating to morphology, which concluded that PEO1X was less epithelial in appearance compared to PEO1, along with both PEO4 and PEO6. Although significant in terms of the characterization of this novel cell line, the results are not sufficient to confirm the involvement of EMT in contributing to the identity of PEO1X. This would need to be addressed more directly such as through the interrogation of EMT marker expression by Western blot. It has also not been resolved if the attainment of these mesenchymal features by PEO1X is causally related to their decreased Pt-sensitivity or if it is simply a

correlation. These results further reinforce the notion that, in spite of all the efforts to maintain a clinically relevant treatment methodology, the Pt-resistance that evolved *in vitro* is simply of a different character than that which emerges in patients with the disease. For whatever reason, the more mesenchymal phenotype is selected *in vitro*, whereas *in vivo* the more epithelial fate is preserved. The preference for one phenotype in the conditioning of a chemo-resistant population may be a function of the particular drug treatment used. Alternatively, it may depend on the type of malignancy, with HGSOC being perhaps the exception rather than the norm. On the other hand, the selection of a particular phenotype might be entirely stochastic, in which case there would be no preference in terms of the selection for epithelial or mesenchymal features in the resistant cells. One recent paper screening a panel of ovarian cancer cell lines concluded that the cell lines with more epithelial patterns of gene expression had greater intrinsic resistance to cisplatin [228]. Although this might be true between different cell lines, it is not known if this is also the case between cells within a single cell line (PEO1 contains cells with both epithelial and mesenchymal morphologies) or within the heterogeneous tumor of a single patient.

5.9 On the Genetics of PEO1X and the Implications of the Mutational Data from All Four Cell Lines

The mutational data gathered from PEO1X using the CLINV4 panel of exome sequencing is sufficient to refute the notion that these repopulating cells are cells closer in relation to the resistant cells which emerged in the original patient (PEO4 and PEO6). This determination was based on the results obtained from a single gene. This tumor suppressor gene called NF1 has been found to be recurrently mutated or deleted in 12 % of cases of HGSOC by the TCGA [159]. The gene was found to have three distinct allelic variants in each of PEO1/4/6. In PEO1 this gene contained an insertion/deletion mutation, while in PEO4 it was found to possess a point mutation

resulting in an amino acid substitution, putatively resulting in a loss of protein function. This same gene was however not altered in PEO6 which corresponds chronologically to the most advanced stage of the disease. Because it is incredibly unlikely that a cancer cell would be capable of sequentially repairing two distinct mutations affecting the same gene along the course of progression, these results would therefore indicate that these mutations arose independently, in genetically distinct populations of cells only after they had diverged during the course of tumor evolution. In other words, in the original patient from whom these cell lines originated, this gene could be said to have sub-clonal variation. This sub-clonal variation in the NF1 mutational profile confirms the findings from the paper of Cooke et al. [149] which concluded that these cell lines could not be linearly related, but had rather evolved in parallel. On the other hand, these mutations might have arisen in PEO1 and PEO4 *in vitro* after the establishment of the cell lines. This, however, would be a remote possibility due to the fact that these mutations are found in nearly all the cells which were sequenced. If a spontaneous mutation had occurred in a single cell *in vitro*, one would not expect that it would be capable of being enriched to such a level unless there were a powerful selective force mandating its expansion at the expense of every other WT cell. Under the same conditions *in vitro,* PEO6 is WT for NF1 which would counter the argument that NF1 mutation is required for growth *in vitro*. A prior study has demonstrated that NF1 expression is downregulated at the mRNA and protein level in PEO1 and PEO4 [236]. This study also identified that PEO4 possessed a mutation that resulted in the production of a truncated mRNA (splice mutant) which confirms the results presented here, which predicted a LOF of the point mutation detected [236]. This study was not able to show the mutation in PEO1 which was identified here, likely due to a lower resolution of the method used by these authors. The cell line PEO6 was not tested in the study in question, so it is not known if the NF1 protein

is expressed normally in this cell line compared to PEO1 and PEO4 [236]. Even in the absence of a mutation, the expression of the NF1 gene may be prevented by epigenetic or post-transcriptional means. NF1 mutations have been found to possess sub-clonal distribution in HGSOC patient samples by studies using deep sequencing technologies to quantify the heterogeneity present in pre-treatment disease [209,210,212,213]. Because NF1 mutations have sub-clonal variation, they are likely to represent a relatively late event in the disease evolution compared with the mutation of TP53, in which the same mutation is found across all three cell lines. It is unlikely that this genetic alteration is required for the emergence of the disease but the fact that NF1 is mutated independently in different populations suggest that there is a fitness advantage for cells in which this gene is mutated. Likewise, the fact that NF1 was not mutated in PEO6 suggests that this mutation is not selected for in the more advanced stages of the disease. The fact that PEO1X contains the same indel mutation as PEO1 rules out the possibility of PEO1X being closely related to either PEO4 or PEO6 which, had they been present initially within PEO1, would likely have been selected by *in vitro* Pt treatment. The fact that the mutation is the same as that found in PEO1, which itself arose only after the divergence of PEO1/4/6, makes it almost certain than PEO1X is a derivative of PEO1. It cannot be determined based on these data if PEO1X was already present prior to treatment as a minor population within PEO1 or alternatively if it evolved as a direct consequence of CDDP exposure. The results from TP53 are more difficult to interpret because they disagree with a well-established feature of HGSOC. It is not clear why PEO1 would be heterogeneous for TP53 mutational status when this mutation is perhaps one of the earliest known events in HGSOC carcinogenesis. It may be possible that there was a reversion mutation that arose *in vitro* which restored the WT sequence of the gene and was subsequently retained. The results conflict as to whether this trait is enriched in PEO1X. In

PEO1X, the frequency of TP53 mutation declines to 69%, while in an independently derived replicate of the repopulating cells selected from PEO1 (same methodology as for PEO1X), it was subsequently determined that this frequency had increased to 99%. It may be that the selection of this trait in the repopulating cells is not directional but simply stochastic, in the sense that it has no influence on the development of resistance. Because this panel covers only a miniscule percentage of the genome, it was not possible to detect if there are any genetic differences between PEO1 and PEO1X. To answer such a question would require the use of either whole exome or complete genome sequencing. If a particular allelic variant were to be prominently featured in PEO1X then it might be possible to identify if this variant is present at a low frequency within PEO1. This would support the notion that PEO1X was selected from a minor population within PEO1. This, however, would be an unlikely occurrence due to the inherently low mutational rate of HGSOC. A potentially more fruitful approach would be to test for gene copy number variation or the presence of unique chromosomal translocation events using an approach such as FISH or array-CGH (Comparative Genomic Hybridization). It would also be desirable to test for any epigenetic differences between PEO1 and PEO1X, especially in the absence of any obvious genomic alterations. One can also elect to conduct gene expression profiling using cDNA-microarray or RNA-seq technologies to show any differences at the level of the mRNA.

5.10 On the Immunophenotype of PEO1X and its Relation to PEO1 as well as PEO4 and PEO6

The immunohistochemical data for PEO1X helped to reinforce many of the findings of previous experiments, but largely failed to uncover any novel insight as to the phenotype of these cells. Also, because only a handful of candidate markers were tested instead of a wider unbiased

screen, there is almost certainly a wide array of phenotypic changes that were excluded from this analysis. From the standard panel HGSOC histopathological markers it would be possible to conclude that PEO1X displays most of the known hallmarks of HGSOC in like manner to PEO1. This is true for both PAX8 and P53, which both stain mainly positive in both cell lines. In the case of WT1, a nuclear antigen that normally stains positive in tissue samples from patients with HGSOC, its expression is virtually absent from both PEO1 and PEO1X, while being positive in PEO4 and PEO6. At least on the basis of this marker, it can be said that the phenotype of PEO1X follows more closely that of the parental cell line rather than the resistant cells which emerged *in vivo*. In addition, while CA125 expression increases along disease progression from PEO1 to PEO6, in PEO1X there is far less abundant CA125 staining compared to PEO1. In line with the results from the exome sequencing and based on a rough quantitation, it would seem that there are more P53-negative staining cells in PEO1X. The assessment of epithelial/ mesenchymal markers E-cadherin and vimentin helped to confirm the findings of previous experiments which showed a loss of epithelial identity in PEO1X and a more widespread distribution of mesenchymal features. This is seen in terms of the reduced expression of E-cadherin in PEO1X, which was found in the pockets of epithelioid cells in PEO1. The results from vimentin were surprising in that, despite the lack of the characteristic spindle-shaped morphology, which is present in PEO1, there appears to be a greater proportion of cells expressing this marker in PEO1X compared to PEO1. Again, the results from PEO4 and PEO6 were just the opposite, with a greater prevalence of E-cadherin staining and very little discernable vimentin expression in these two cell lines. This further supports the view that PEO1X is divergent to PEO4/6 in terms of phenotype rather than being similar.

Another avenue of inquiry that was pursued was the potential of PEO1X to be enriched in markers of Cancer Stem Cells (CSCs). The two that were employed, CD44 and CD133, were chosen because prior studies aiming to identify the CSC population in ovarian cancer had shown a marked increase in tumorigenic capacity upon transplantation of both the $CD44^+$ and the $CD133^+$ components [233,235]. The problem with PEO1 is that CD44 is widely expressed in this cell line, so that it would prove difficult to conclude if there was an increase in PEO1X. Thus, it could not be determined if PEO1X is enriched in CSC-like features based on this marker alone. In any case the staining from this marker is similar in PEO1X compared to PEO1. Conversely, in terms of CD133 expression, there appears to be an increase in PEO1X relative to PEO1. In summary, it may be that PEO1X is enriched in cells with CSC-like potential ($CD44^+$, $CD133^+$). This would be in accordance with the results of a study by Abubaker and colleagues that showed enhanced expression of CSC markers after transient exposure to chemotherapeutic drugs such as cisplatin [234]. It will be necessary to verify the relative expression of these markers in PEO1 and PEO1X using a more reliable and quantitative technique such as flow cytometry. Ultimately, the only way to settle this question conclusively would be to use xenotransplantation of both cell lines orthotopically into immunodeficient mice in order to establish if PEO1X has greater tumor initiating capacity.

5.11 Derivation of PEO1.3X and the Significance of its Lack of Cisplatin Resistance

When it became clear that PEO1X did not bear a similar set of features to the chemo-resistant cells which emerged in the original patient, it was decided to attempt to derive a new batch of repopulating cells using the same three-drug combination which the patient was reported to have received. PEO1 was exposed to a cocktail of the three drugs together for 1-hour in total; with 10 μM CDDP, 5 μM 5-FU and 10 μM chlorambucil. Although repopulation took place as expected,

with the resulting cells being called PEO1.3X, these cells were not any more resistant to CDDP than their parental cell line. This raises a number of important questions and invalidates many previously held notions about the nature of the resistance evolved *in vitro*. For instance, it was assumed that the cells having survived and proliferated after a high dose of *in vitro* cytotoxic chemotherapy would inevitably have an inherent resistance to the drug. While it cannot be excluded that PEO1.3X is more resistant to the two drugs other than cisplatin, the question is trivial given that cisplatin is the linchpin of the regimen, and therefore these cells would likely never be exposed to the other two drugs in its absence. It may be that the repopulating cells that gave rise to PEO1.3X gained only transient resistance to the drug cocktail such that its removal was sufficient to re-sensitize them to cisplatin. The other salient question was why the addition of the other two drugs was sufficient to obviate the development of resistance relative to cisplatin alone? If PEO1X exists as an inherently Pt-resistant component of PEO1, why was it not selected again in the presence of the combinatorial treatment? It may be that PEO1X is not cross-resistant to the other drugs and that their addition was enough to prevent the repopulation of these particular cells. The maintained sensitivity of PEO1.3X speaks to the fundamental wisdom of the combinatorial approach to chemotherapy; that it is much more difficult to develop resistance to multiple drugs when exposed at the same time or in close proximity. Because, in this day and age, platinating agents are rarely used in the absence of a taxane, it would be appropriate to develop *in vitro* models of resistance that are simultaneously insensitive to both agents in combination. Because the mechanisms of these drugs are so different, the resultant resistant cells which emerge would likely need to employ more exotic and multi-dimensional means of evading drug-toxicity. These would be important to elucidate given the elevated frequency of resistance occurring in the clinical setting. In any case, the failure to develop resistance in PEO1.3X in

response to the three-agent combination does not necessarily prove that this is impossible *in vitro*. With a more refined protocol, multiple treatments and reduction in the doses used for each agent, it might be conceivable to eventually cause resistance to come about. The problem, as with CDDP alone, is how to ensure that the resistance evolves in a similar manner to that occurring *in vivo*; via the selection of intrinsically resistant sub-populations that were already present, rather than developing the phenotype de-novo. The usage of drug-combinations makes this a much more remote possibility. From thence comes the principal limitation of cell line models; there is simply not enough heterogeneity retained from the original disease for selection to be comparable *in vitro*. The results of this project further clarify this predicament, to the extent that it could justifiably be asserted that no matter how realistic the treatment modality implemented *in vitro*, it would remain insufficient to ensure the development of resistant cells comparable to those having arisen *in vivo*.

5.12 A Brief Summary of the Project, an Assessment of What was Accomplished and Future Implications

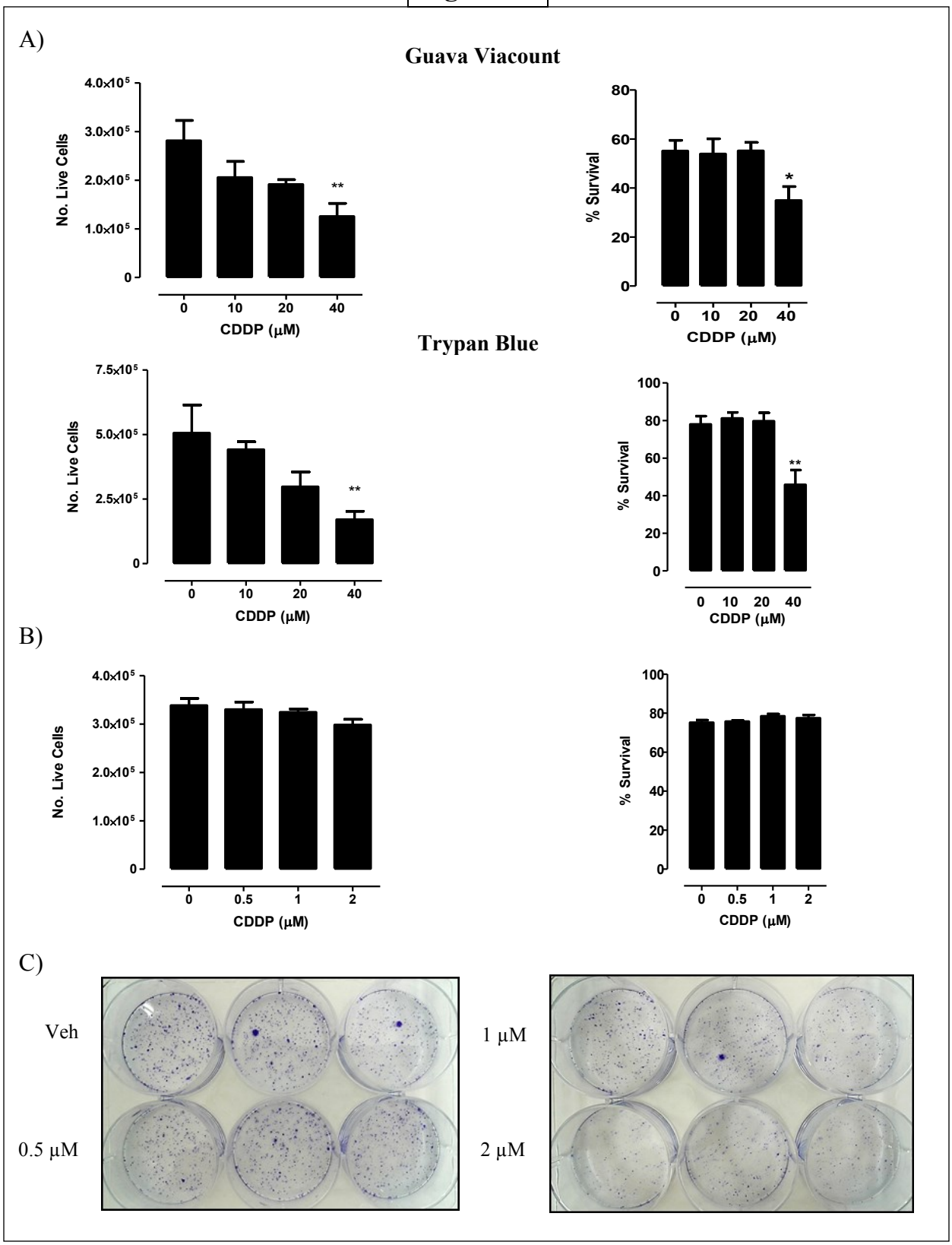
In summary, this project has shown that it is possible to induce the development of Pt-resistance *in vitro* from a chemo-sensitive HGSOC cell line using clinically achievable treatment parameters and minimal re-treatment. The resulting repopulating cells, called PEO1X, have 20-fold diminished sensitivity to cisplatin (when exposed for 1 hour) compared to their parental cell line PEO1 and are capable of transiting the cell cycle with minimal disruption following exposure to CDDP. However, the hypothesis was only partly satisfied, and after characterization of PEO1X it soon became clear that the phenotype of these cells differed substantially from the matched resistant cell lines established longitudinally from the same patient as PEO1. PEO1X presents with an altered morphology with a loss of epithelial features and a gain of mesenchymal

properties such as motility. The doubling time of these cells is similar to PEO1 and, from the perspective of genetics, PEO1X appears to be derived from PEO1 due to the presence of an indel mutation in the gene NF1 that is found in the parental cell line, but not in PEO4 and PEO6. It was also shown that PEO1 is heterogeneous from the perspective of morphology, immunophenotype and genetics (P53 status). An effort to develop resistance from PEO1 in response to the same three-drug combination administered to the patient eventually ended in failure. It could not be ascertained if PEO1X was derived from a minor, intrinsically resistant sub-population within PEO1 as hypothesized. Furthermore, the mechanism of resistance was not established as part of this project. The results of this project have certain implications for the scientific community and for clinical practice in the area of oncology. Firstly, the possible existence of heterogeneity (genetic or otherwise), even within supposedly uniform systems such as cancer cell lines, should not be dismissed and should be assessed whenever possible. The advent of new technologies such as single-cell RNA sequencing will allow researchers to evaluate population-level heterogeneity at an unprecedented depth in the near future. In addition, the importance of replicating *in vitro*, as near as possible, the methodologies used to treat patients cannot be overstated. It might well prove to be a much simpler task to develop *in vitro* models of Pt-resistance if scientists were to consider the issue of using clinically relevant drug exposure-times. The usage of drug-sensitivity assays that factor into account a more long-term measure of Pt-mediated toxicity would also be deemed desirable based on the findings of this project. More than anything, this project has highlighted the supreme adaptability cancer cells in terms of responding to the exogenous insults imposed by cytotoxic chemotherapy. After a maximum of two rounds of exposure to CDDP (1 hour each), a totally distinct entity in the form of PEO1X emerged that was many times more resistant than the cells from which it was derived.

Because the treatment methodology closely replicated the clinical approach to chemotherapy, this phenomenon highlights the need for novel therapeutic strategies, such as adaptive chemotherapy, which acknowledge the heterogeneity and evolutionary dynamics of malignancies such as HGSOC.

Supplementary Figures

Figure S1


A)
Guava Viacount
No. Live Cells
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
**
0 10 20 40
CDDP (μM)
% Survival
80
60
40
20
0
*
0 10 20 40
CDDP (μM)
Trypan Blue
No. Live Cells
7.5x10^5
5.0x10^5
2.5x10^5
0
**
0 10 20 40
CDDP (μM)
% Survival
100
80
60
40
20
0
**
0 10 20 40
CDDP (μM)
B)
No. Live Cells
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
0 0.5 1 2
CDDP (μM)
% Survival
100
80
60
40
20
0
0 0.5 1 2
CDDP (μM)
C)
Veh
1 μM
0.5 μM
2 μM

D)

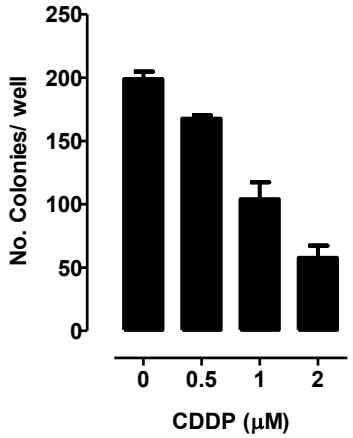


Figure S1: *Evaluating the Cisplatin Sensitivity of the HGSOC Cell Line PEO14*

A): Comparison of the acute toxicity data from PEO14 cells exposed to supra-pharmacological doses of CDDP for 1 hour using two different cell viability assays: Guava Viacount and Trypan Blue permeability (BioRad TC20). Data was obtained 72 hours after drug removal. (n = 6). Data show a similar trend between Guava Viacount and Trypan Blue permeability. In each case, there is only a significant decrease in both parameters at the highest treatment dose of 40 μM CDDP. In both assays the viability of the control is poor but it worse for the Guava Viacount assay.

B): Acute toxicity data from PEO14 cells exposed to low doses of CDDP for 1 hour. Data collected 72 hours after drug removal. (n =3) Data correspond to cells plated in clonogenic survival assay below. At these low doses and with 1-hour exposure there is no significant decrease in either parameter for any of the doses of CDDP administered. Viability of the control was still low.

C): Images taken of the plates resulting from a clonogenic survival assay performed with the cell line PEO14. 2,000 treated cells PEO14 per replicate per condition were re-plated in 2 ml fresh media and allowed to form colonies for 11 days. Colonies were fixed and stained with Crystal violet. There is a visible dose-dependent reduction in the number of colonies present for each condition

D): Quantification of the average number of colonies per well from each treatment condition of the PEO14 cells plated and allowed to grow for 11 days in a clonogenic survival assay (data correspond to the images presented above). Data from two independent experiments was processed with Calcusyn software to yield IC50 of 1.065 μM. This indicates that PEO14 is Pt-sensitive. Statistical analysis = 1-way ANOVA + Newman-Keuls post-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

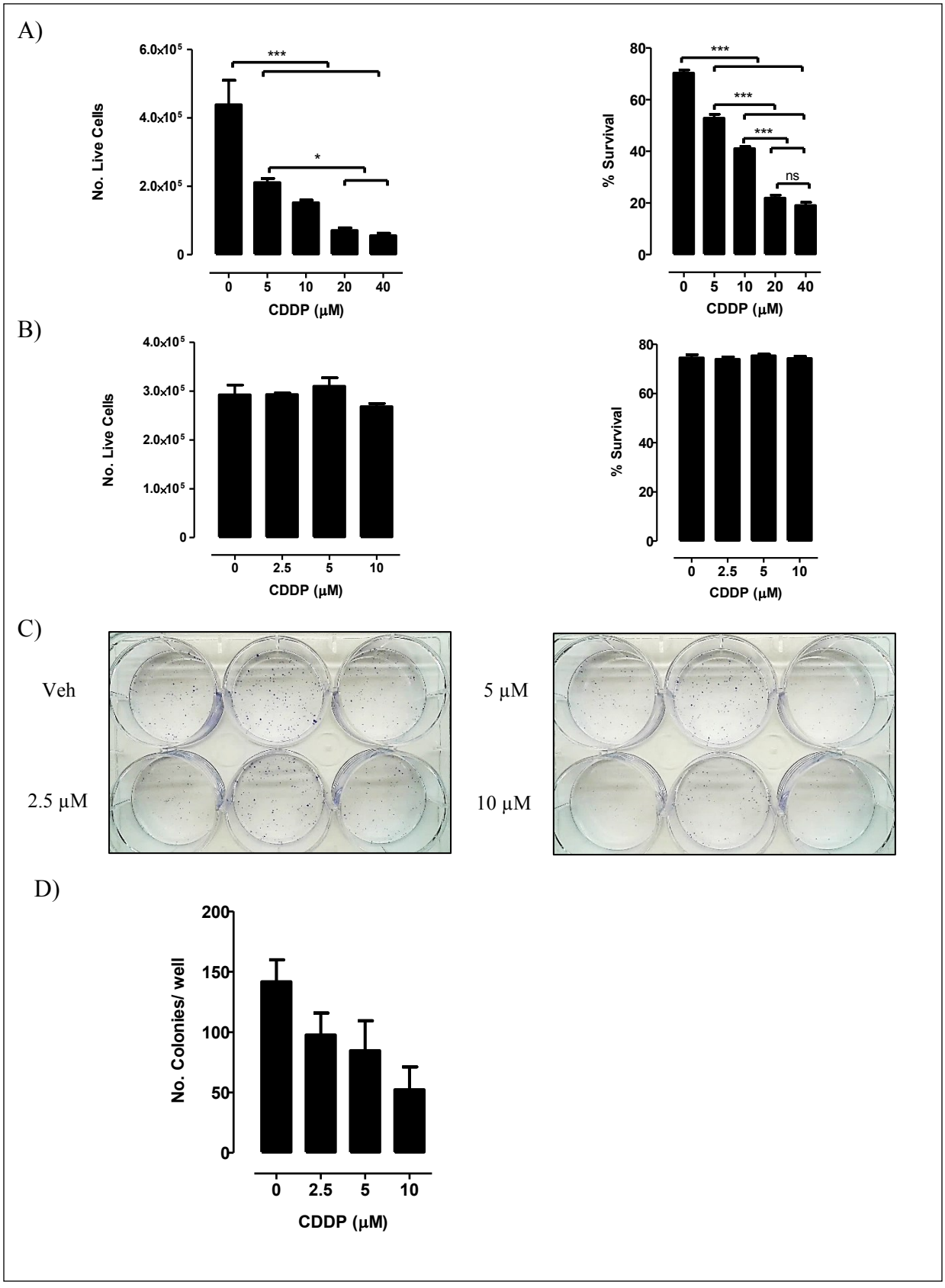

A)
No. Live Cells
6.0x10^5
4.0x10^5
2.0x10^5
0
***
*
0 5 10 20 40
CDDP (μM)
% Survival
80
60
40
20
0
***
***
***
ns
0 5 10 20 40
CDDP (μM)
B)
No. Live Cells
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
0 2.5 5 10
CDDP (μM)
% Survival
80
60
40
20
0
0 2.5 5 10
CDDP (μM)
C)
Veh
5 μM
2.5 μM
10 μM
D)
No. Colonies/ well
200
150
100
50
0
0 2.5 5 10
CDDP (μM)

Figure S2: *Evaluating the Cisplatin Sensitivity of the HGSOC Cell Line PEO4*

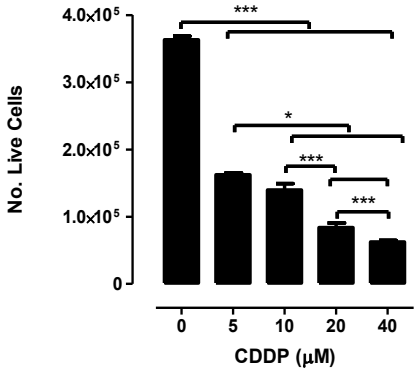
A): Acute toxicity data from PEO4 cells exposed to supra-pharmacological doses of CDDP for 24 hours. Data was obtained 72 hours after drug removal (total of 96 hours after administration). (n = 4) The results are little different than those obtained for PEO1 under the same conditions. In both parameters there was a large and significant difference relative to the control for each treatment dose. There are also significant differences in percent survival between doses with the exception of between 20 and 40 μM CDDP. For the live cell number there are fewer cells in the control and thus the difference relative to the treatment doses is less than for PEO1.

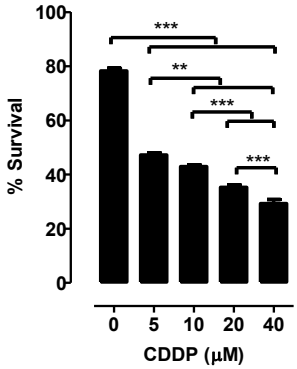
B): Acute toxicity data from PEO4 cells exposed to low doses of CDDP for 1 hour. Data collected 72 hours after drug removal. (n =3) Data correspond to cells plated in clonogenic survival assay below. There is no toxicity evident in terms of either parameter at any of the doses of CDDP used.

C): Images taken of the plates resulting from a clonogenic survival assay performed with the cell line PEO4. 1,000 treated cells PEO4 per replicate per condition were re-plated in 2 ml fresh media and allowed to form colonies for 14 days. Colonies were fixed and stained with Crystal violet. The dose-dependent decrease in the number of colonies is less evident for this cell line. There are fewer colonies than for PEO1, PEO1X and PEO14.

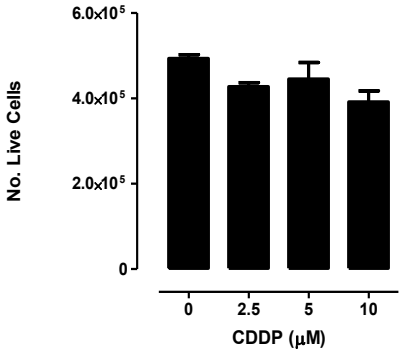
D): Quantification of the average number of colonies per well from each treatment condition of the PEO4 cells plated and allowed to grow for 14 days in a clonogenic survival assay (data correspond to the images presented above). Data from two independent experiments was processed with Calcusyn software to yield IC50 of 6.79 μM. PEO4 is thus much less Pt-sensitive compared to PEO1. Statistical analysis = 1-way ANOVA + Newman-Keuls post-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

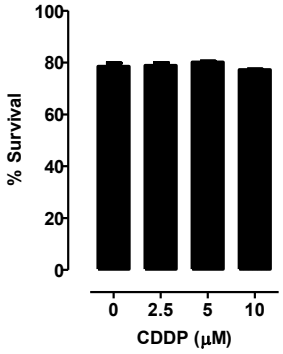
A)





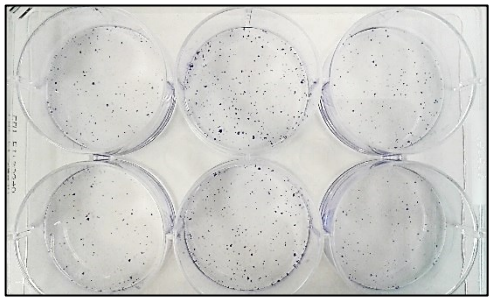
B)



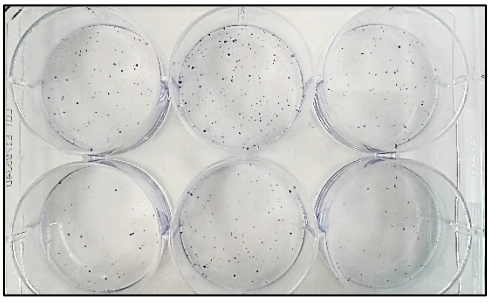


C)

Veh



5 μM



2.5 μM

10 μM

D)

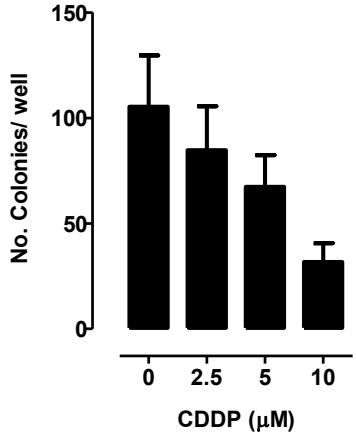


Figure S3: *Evaluating the Cisplatin Sensitivity of the HGSOC Cell Line PEO6*

A): Acute toxicity data from PEO6 cells exposed to supra-pharmacological doses of CDDP for 24 hours. Data was obtained 72 hours after drug removal (total of 96 hours after administration). (n = 4). In PEO6 the results are similar to those obtained for PEO1 and PEO4 under the same conditions. In both parameters there was a large and significant difference relative to the control for each treatment dose. This time there was significant difference between each condition for both parameters.

B): Acute toxicity data from PEO6 cells exposed to low doses of CDDP for 1 hour. Data collected 72 hours after drug removal. (n =3) Data correspond to cells plated in clonogenic survival assay below. As with PEO4, there is no toxicity evident 72 hours after exposure to any of the doses of CDDP used.

C): Images taken of the plates resulting from a clonogenic survival assay performed with the cell line PEO6. 4,000 treated cells PEO6 per replicate per condition were re-plated in 2 ml fresh media and allowed to form colonies for 14 days. Colonies were fixed and stained with Crystal violet. There are few colonies compared to PEO1, even in the control, despite the 4000 cells plated per well. There is subtle dose-dependent decrease in the number of colonies visible.

D): Quantification of the average number of colonies per well from each treatment condition of the PEO6 cells plated and allowed to grow for 14 days in a clonogenic survival assay (data correspond to the images presented above). Data from two independent experiments was processed with Calcusyn software to yield IC50 of 7.31 μM. PEO6 is much less Pt-sensitive compared to PEO1, with a similar level of resistance to PEO4.

Statistical analysis = 1-way ANOVA + Newman-Keuls post-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

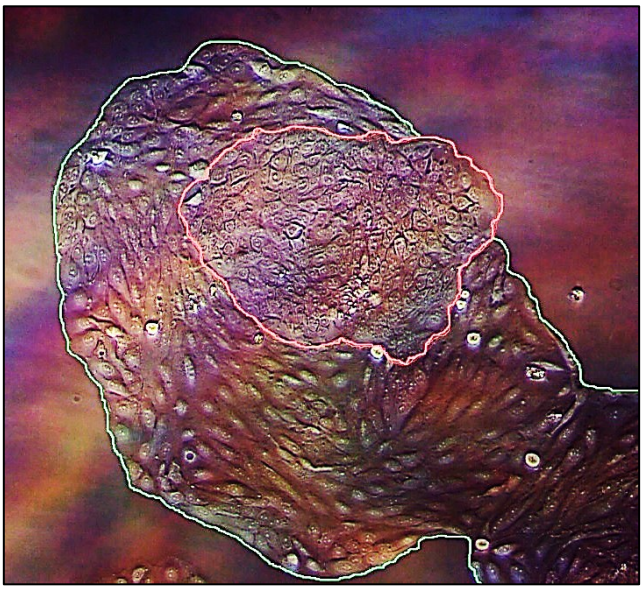
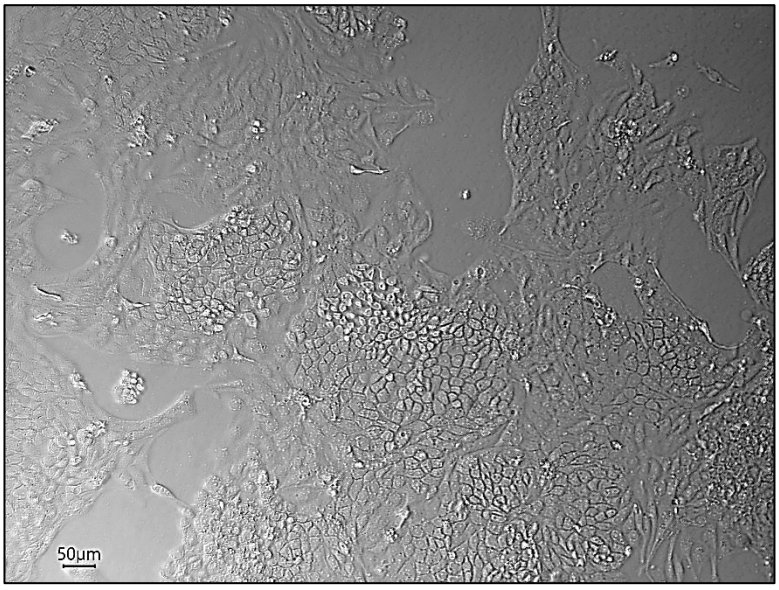


Figure S4.: *The Clonal Origins of Phenotypic Diversity in PEO1*

Vehicle treated PEO1 cells plated in a clonogenic survival assay at a density of 1000 cells/ well form colonies derived from a single cell. These colonies contain cells with both epithelial and mesenchymal morphologies. In most cases there is a small "nest" of epithelial-like cells surrounded by a network of cells that resemble fibroblasts with an elongated morphology and loose inter-cellular attachments. The Red area indicates cells with epithelioid morphology while the Green area encompasses cells with mesenchymal morphology.

A)



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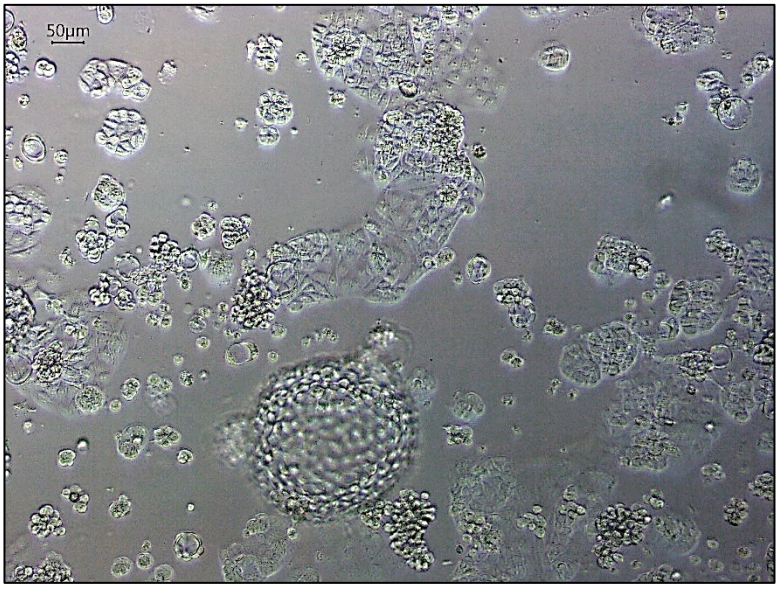
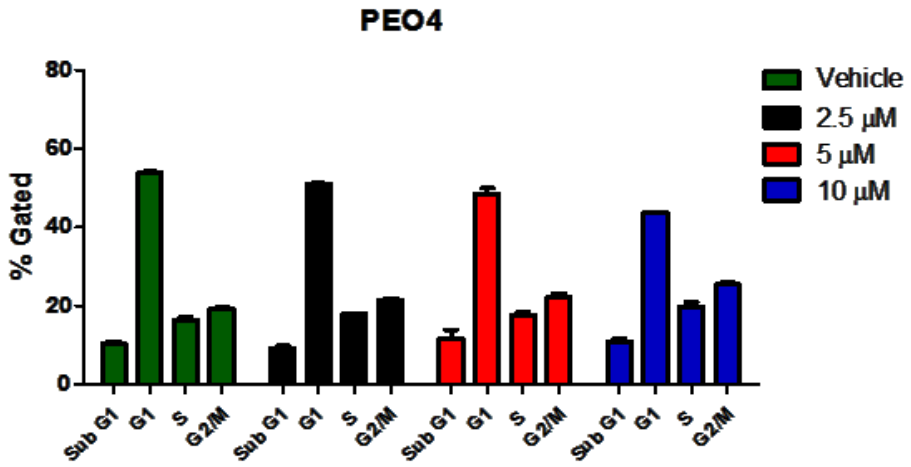


Figure S5: *Morphology and Growth Characteristics of the Cell Lines PEO4 and PEO6*
Images of live confluent cells taken with an inverted microscope at 10X that illustrate the morphology and growth characteristics of the cell lines PEO4 (A) and PEO6 (B). Scale bar is 50 µm in size.

A)



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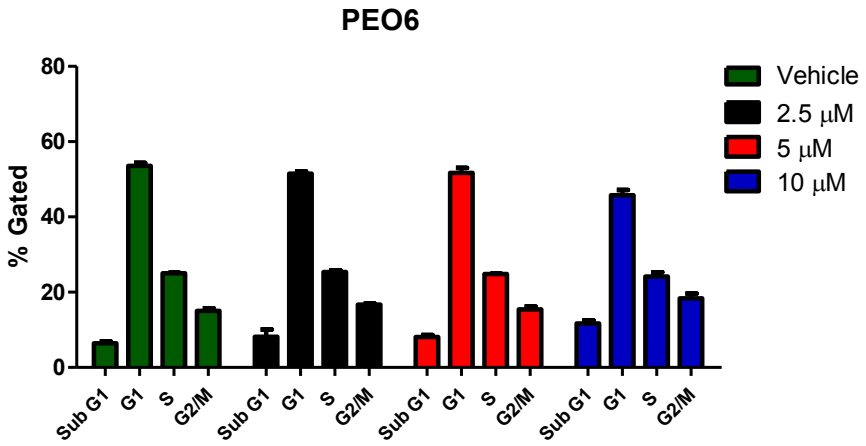


Figure S6: *The Effect of Cisplatin Exposure on the Cell Cycle Status of PEO4 and PEO6*
Under the identical conditions as those listed in the preceding figure, the cell cycle status for both PEO4 (A) and PEO6 (B) was approximated 72 hours after 1-hour cisplatin exposure and are displayed above in graphical form for each cell line. (n= 3)
A) In PEO4 there is little visible impact on the cell cycle status at any dose 72 hours after CDDP exposure. The number of cells with sub-G1 DNA content is high even in the control and does not increase substantively at any treatment dose of CDDP. The percentage of cells in G1 for the control is less than for PEO1 and PEO1X.
B) For PEO6, the results are similar to those obtained for PEO4; there is no obvious perturbation in the cell cycle at any dose of CDDP. The percentage of cells with sub-G1 DNA content is also high in the control, but it increases further in the 10 µM dose of CDDP. Also, there are less cells in G1 compared to both PEO1 and PEO1X.

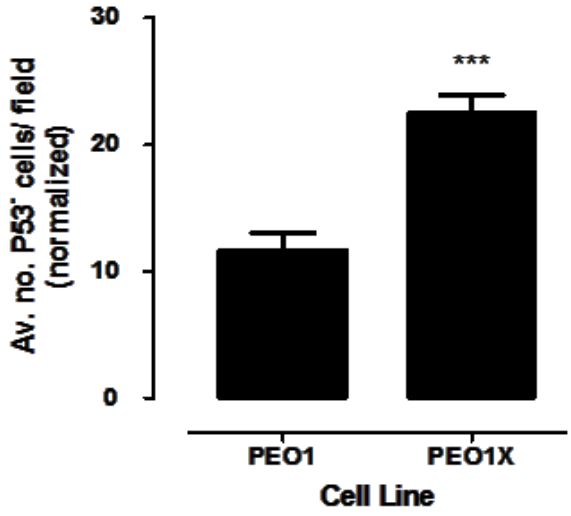


Figure S7: *Semi-quantitation of P53 Protein Expression by Immunohistochemistry between PEO1 and PEO1X*

In order to verify the results of the earlier genetic screen, the expression of P53 was assessed semi-quantitatively by IHC between PEO1 and PEO1X. The number of P53-negative cells was quantified in 9 random fields per slide and averaged. To compensate for the greater number of total cells in each field of PEO1X, the results were normalized according to the ratio between the number of total cells in one random field for each cell line. (n =9). There were significantly more P53-negative cells in PEO1X, even when adjusted to the approximate number of cells per field.

Statistical analysis = single-tailed student's T-test. ***: $p < 0.001$

Figure S8

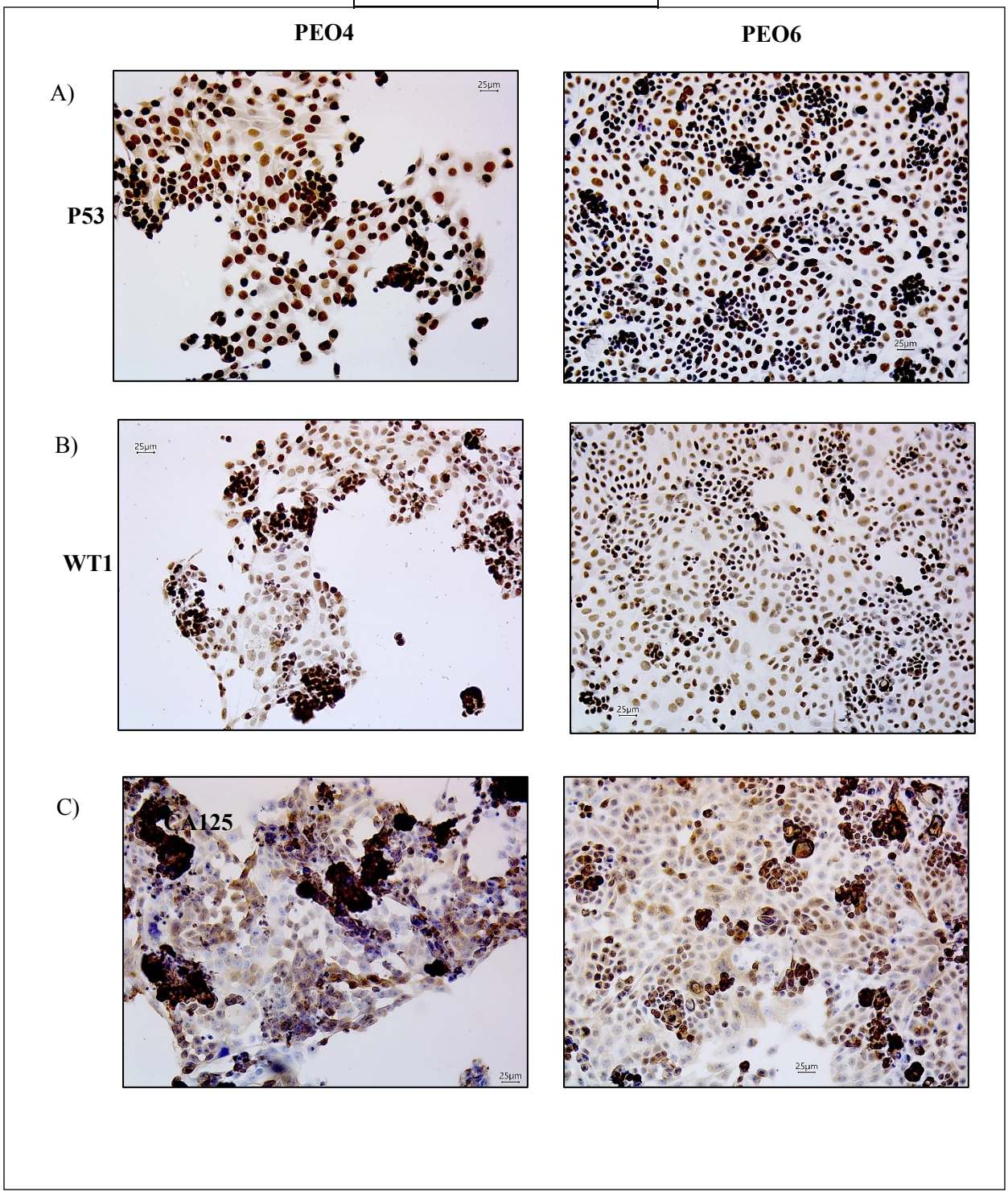


Figure S8 (cont.)

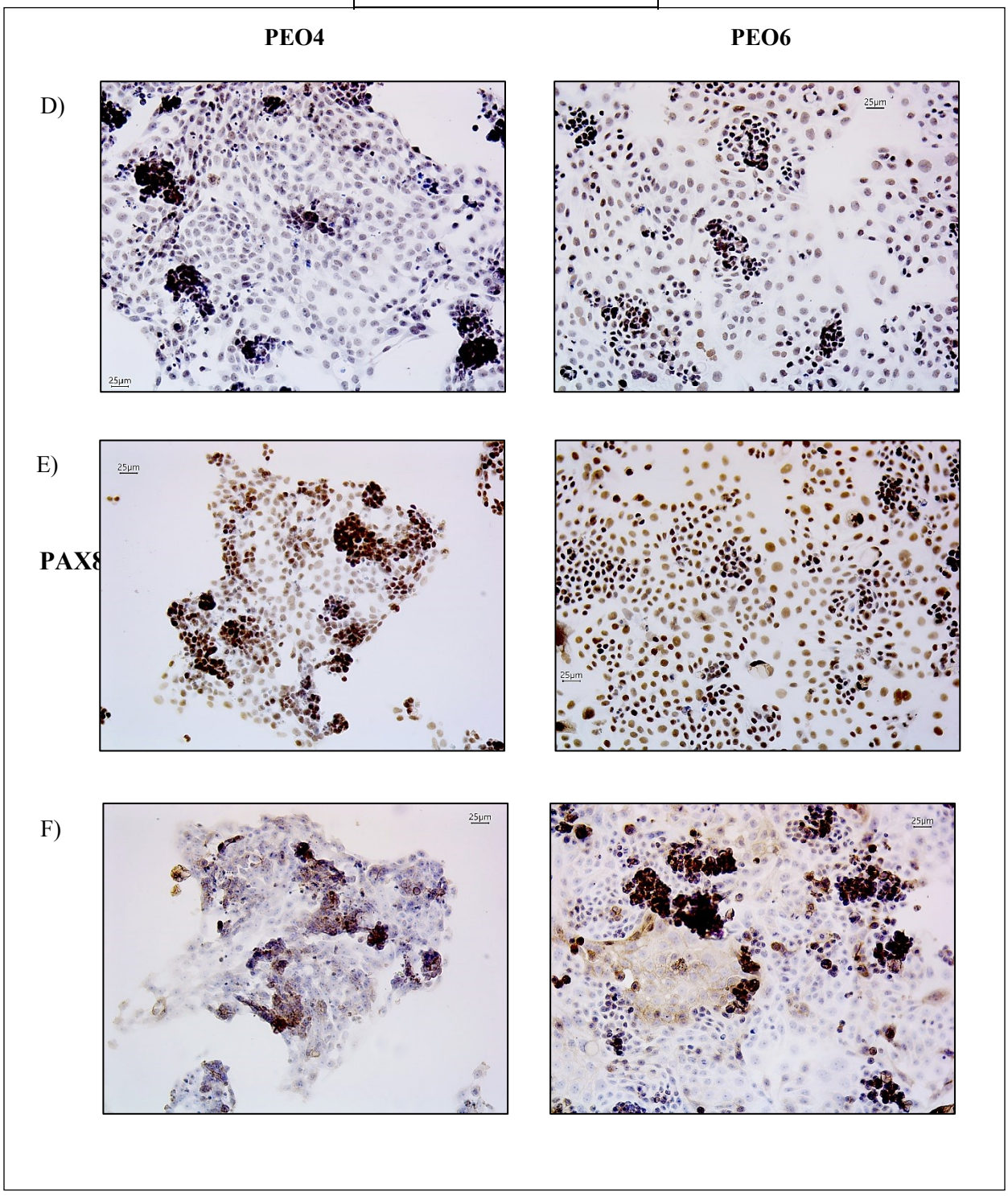


Figure S8 (cont.)

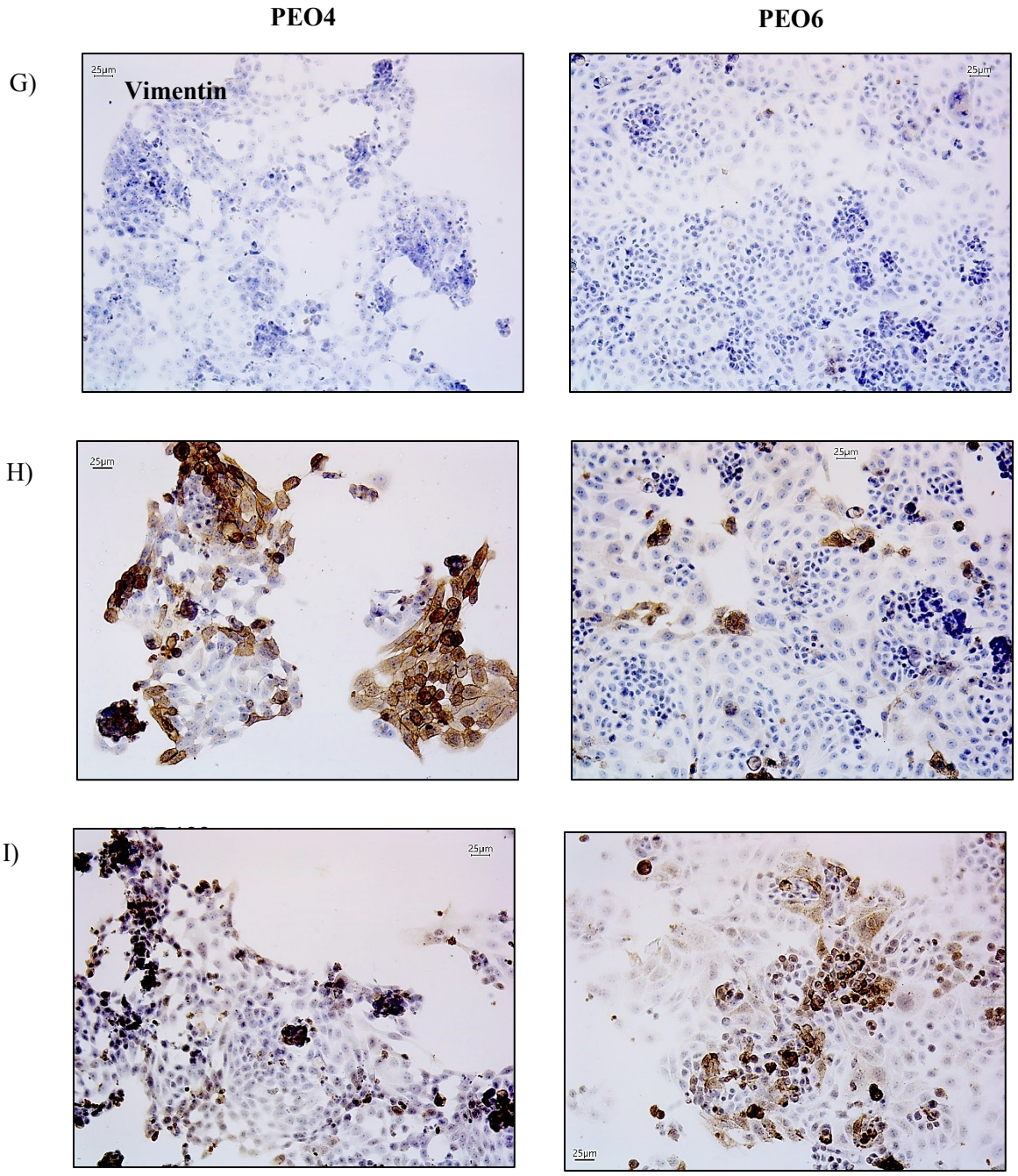
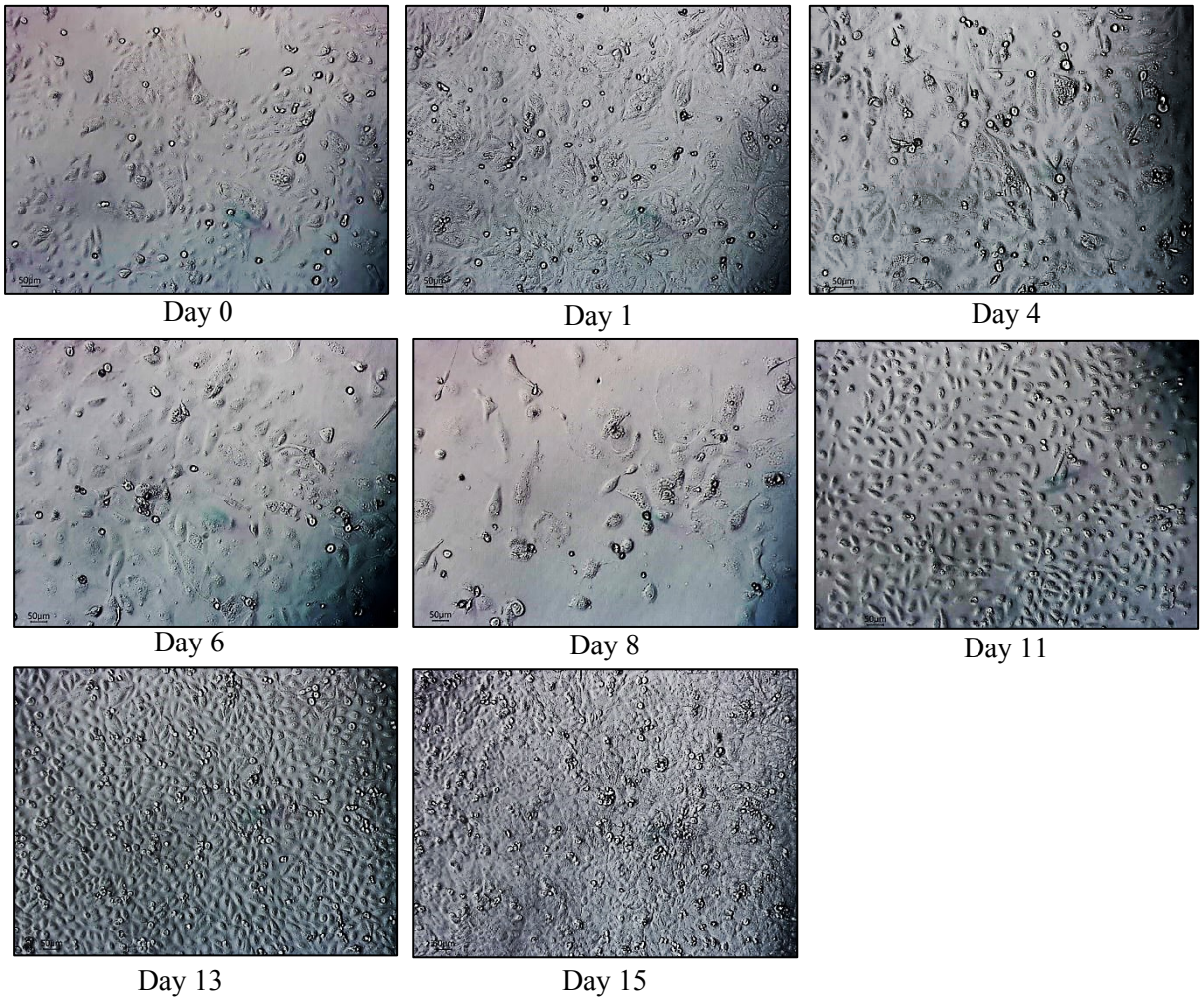


Figure S8.: *Evaluating the Immunophenotype of PEO4 and PEO6*
Immunohistochemistry of PEO4 and PEO6 using the same series of markers used previously for PEO1 and PEO1X. Antibodies, dilutions and incubation times are comparable between cell lines. Nuclear contra-staining with hematoxylin. Images were taken at 20X magnification. Scale bar is 25 µm. Compared to both PEO1 and PEO1X, PEO4 and PEO6 are WT1 positive and Vimentin negative. In both cell lines there is also more expression of both E-cadherin and CA125 and CD133. CD44 is expressed more heterogeneously in both cell lines compared to either PEO1 or PEO1X.

A)



B)

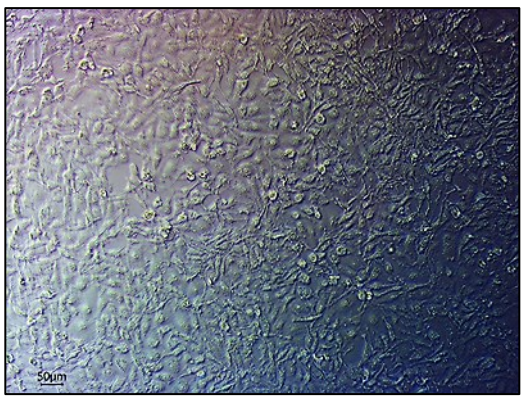


Figure S9.: *The Derivation of a Novel Cell Line PEO1.3X Through Repopulation of PEO1 after Multi-drug Treatment and Assessment of its Morphological Features*

A): Images of live cells taken sequentially of the repopulation of PEO1 following 1-hour treatment with 10 µM CDDP, 5 µM 5-FU and 10 µM Chlorambucil. Images captured at 10x with inverted microscope.

B): Images of confluent live cells taken at 10x magnification with and inverted microscope that illustrate morphology PEO1.3X.

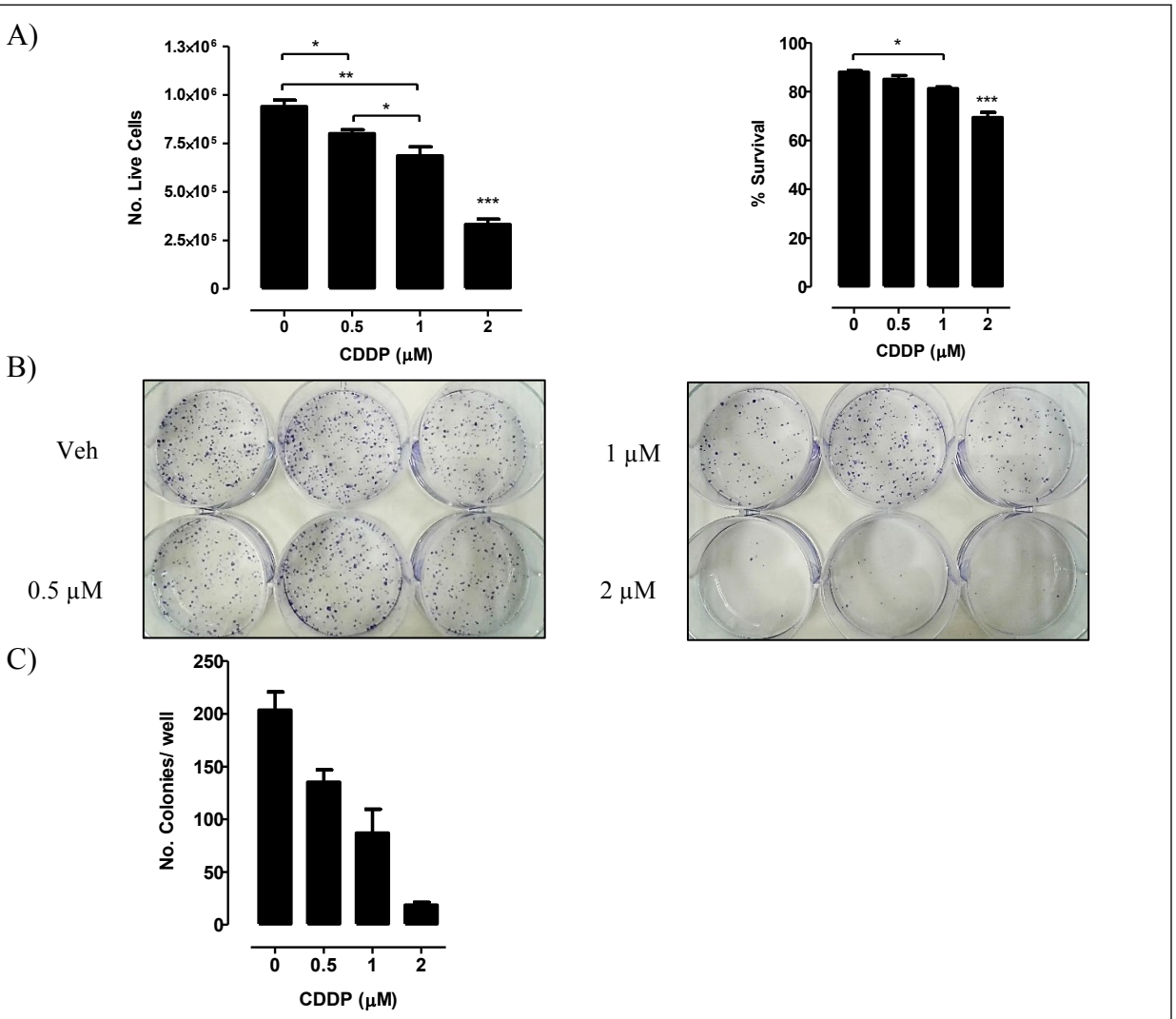


Figure S10: *Evaluating the Cisplatin Sensitivity of the Novel Cell Line PEO1.3X*

A): Acute toxicity data from PEO1.3X cells exposed to pharmacological doses of CDDP for 1 hour. Data collected 72 hrs after drug removal. (n =3) Data correspond to cells plated in clonogenic survival assay below. There is a significant decrease relative to the control in terms of live cell number for each dose, with a significant difference between each dose. In terms of percent survival, there is a significant decrease at both 1 and 2 μM CDDP.

B): Images taken of the plates resulting from a clonogenic survival assay performed with the cell line PEO1.3X. 1,000 treated cells PEO1.3X per replicate per condition were re-plated in 2 ml fresh media and allowed to form colonies for 10 days. Colonies were fixed and stained with Crystal violet. There is a very obvious dose-dependent decrease in the number of colonies with very few at 2 μM CDDP.

C): Quantification of the average number of colonies per well from each treatment condition of the PEO1.3X cells plated and allowed to grow for 10 days in a clonogenic survival assay (data correspond to the images presented above). Data from two independent experiments was processed with Calcusyn software to yield IC50 of 0.77μM. PEO1.3X is Pt-sensitive, with little change relative to PEO1. Statistical analysis = 1-way ANOVA + Newman-Keuls post-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

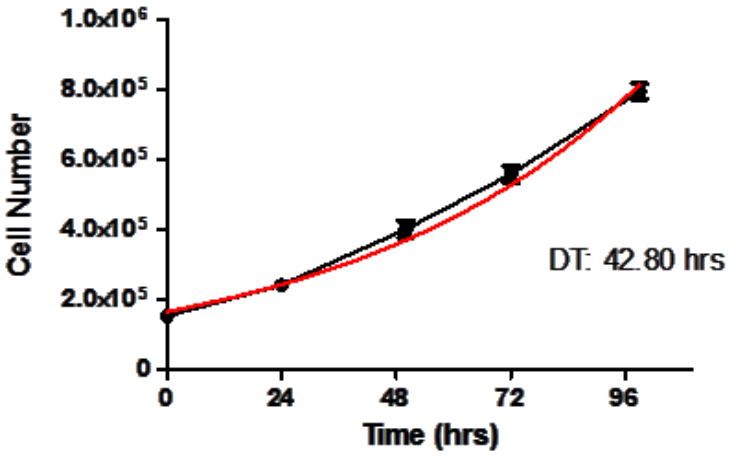


Figure S11: *Calculating the Doubling Time of the Novel Cell Line PEO1.3X*

PEO1X cells were grown in 6-well plates starting 48 hours prior to the commencement of data collection, with a total of three replicates per condition (n =3). The doubling time of PEO1.3X was determined through harvesting and counting the total number of cells present in three wells every 24 hours (Using Guava Viacount). The values were averaged and plotted with a non-linear regression with robust fit (GraphPad Prism Software). The doubling time calculated by the program was 42.80 hours. This value is virtually the same as that calculated for PEO1 (44.92 hours) and slightly less than that obtained for PEO1X (47.84 hours).

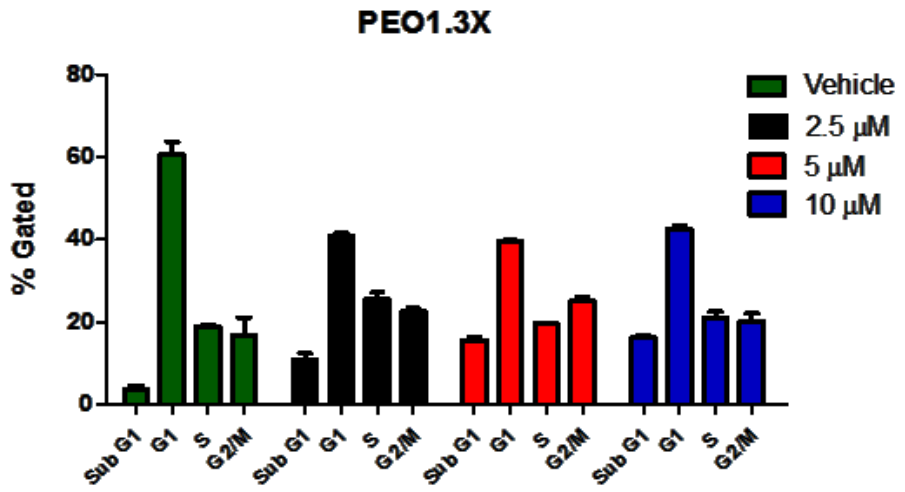


Figure S12: *Determining the Effect of Cisplatin Exposure on the Cell Cycle Status of PEO1.3X*

PEO1.3X cells treated with CDDP for 1 hour or with vehicle were fixed and stained with propidium iodide. Staining was measured using Guava micro-cytometry. Cells were separated from debris using a size index. Cells with greater than 4N DNA content were excluded from consideration. Cell cycle stage was defined roughly using DNA content index and peak location as a guide. Where possible the same criteria were used across a given experiment. (n=3) As with PEO1, there was a significant disruption in the cell cycle statues 72-hours following CDDP exposure at every treatment dose. There are fewer cells in G1 and more in both S and G2/M phases. The percentage of cells with sub-G1 DNA content is also increased relative to control at each dose of the drug and is proportional with each increase in dosage.

Figure S13

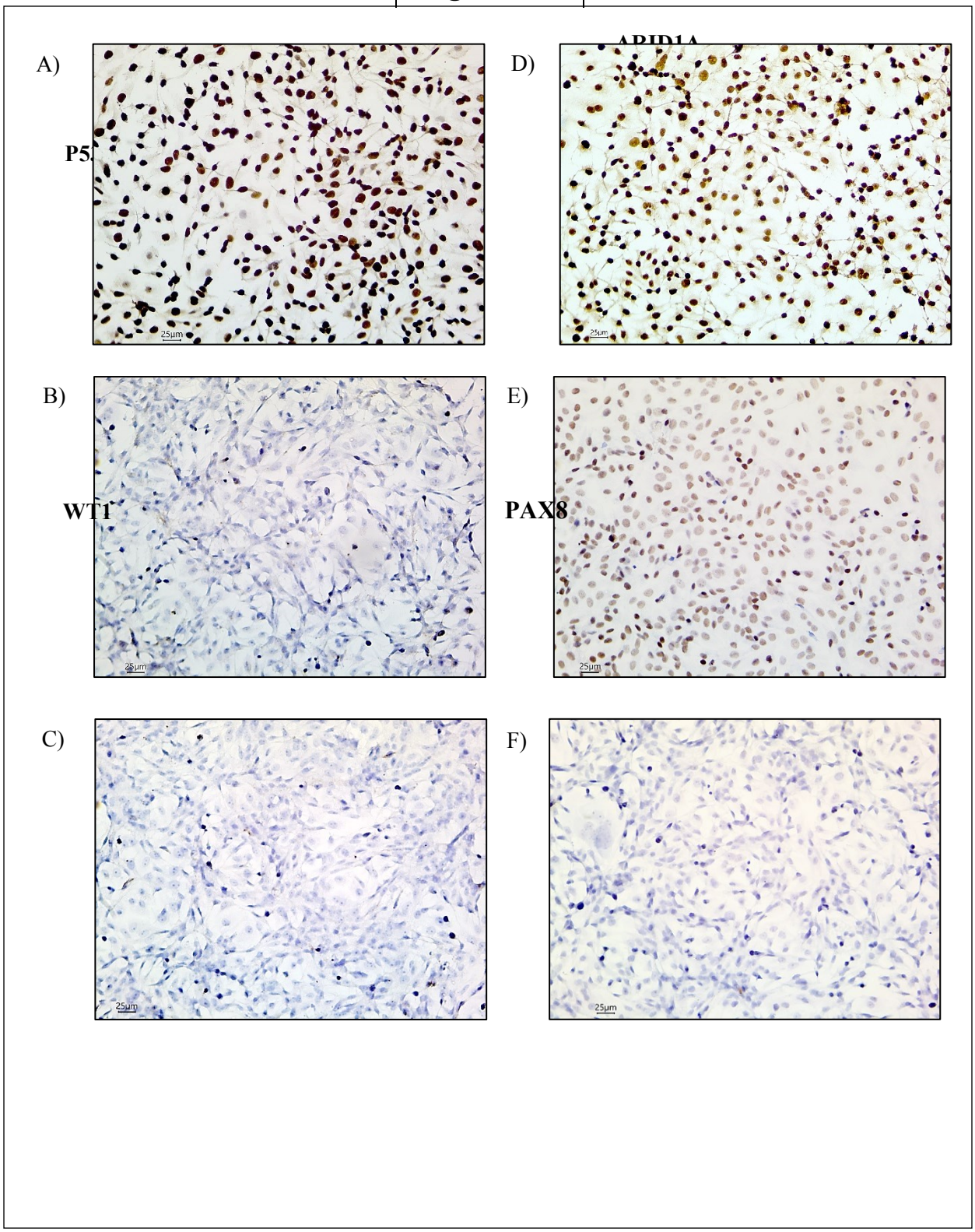
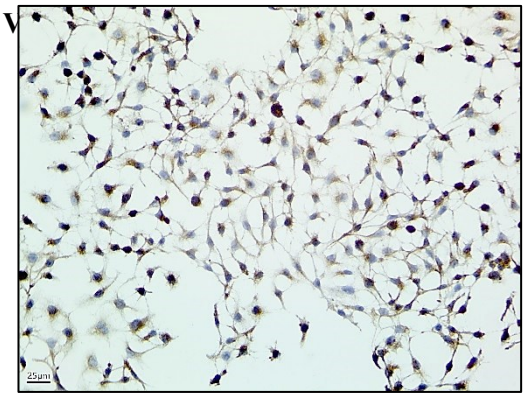
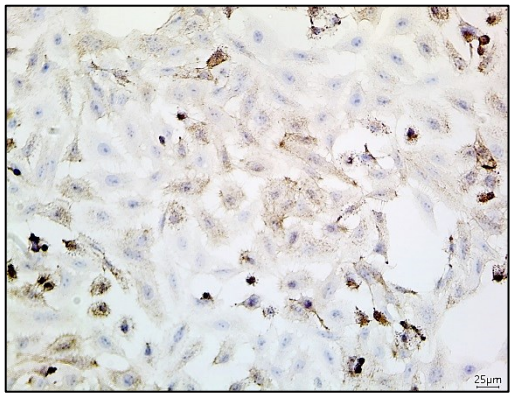


Figure S13 (cont.)

G)



H)



I)

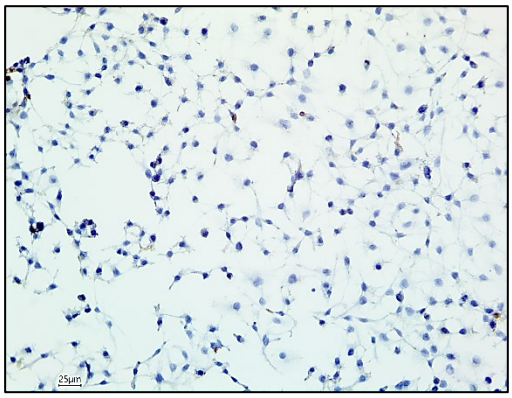


Figure S13.: *Evaluating the Immunophenotype of PEO1.3X*

Immunohistochemistry of using the same series of markers used previously for PEO1, PEO1X, PEO4 and PEO6. Antibodies, dilutions and incubation times are comparable to those used for the other cell lines. Nuclear contra-staining with hematoxylin. Images were taken at 20X magnification. The scale bar is 25 μm. Compared to PEO1, PEO1.3X has less E-cadherin, CA125 and more Vimentin. It is also P53 positive, ARID1A positive and WT1 negative. Like PEO1, it expresses abundant CD44, but little CD133. The pattern of marker expression is similar to PEO1X except for CD133, of which there is more in PEO1X.

Sample	*TP53* c.731G>A p.(G244D)	*NF1* c.478A>T p.(R160W)	*NF1* c.7341_7342insCT,c.7342G>T p.E2448X,p.E2448delinsLKfs
PEOXX	98.99	not detected	99.39, 99.21
PEO1.3X	99.89	not detected	99.68, 99.77

Table S1: *Ascertaining the Mutation Profile of PEO1.3X and a Further Sample of the Repopulating Cells Derived from PEO1*

Samples of PEO1.3X and a further independent replicate of the repopulating cells derived from PEO1 (called PEO1XX) following CDDP treatment (Same methodology as for PEO1X but with uncertain Pt-sensitivity) were sequenced using the same CLINV4 panel as used previously. The numbers displayed are of the VAF (Variant Allele Frequency) for each mutation. PEO1XX has a greater percentage of P53-mutant alleles than both PEO1 and PEO1X. It does, however, contain the same indel mutation in NF1 as PEO1. PEO1.3X also has nearly 100% of P53 mutant alleles and the same indel mutation in NF1 as in PEO1. All other mutations in the sample set were not displayed due to a lack of differences being observed between the cell lines (PEO1/4/6) which was diagnosed in the previous assay and interpreted by Dr. L Van Kempen. Data were intended to verify results of previous sequencing. (Sequencing provided by Molecular Pathology Lab of MUHC Jewish General Hospital.)

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