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DEVELOPING NOVEL THERAPEUTIC APPROACHES IN CHEMORESISTANT OVARIAN CANCER PATIENTS

Volume II

A Thesis Submitted to the University of Dublin for the Degree of

Doctor of Philosophy (Ph.D.)

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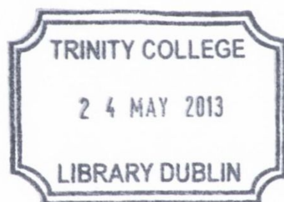
March 2012



Thesis 10061.2

Chapter 4

Gene Expression Profiling of Ovarian Cancer Cell Lines in Normoxia and Hypoxia

4.1 Introduction

4.1.1 Microarrays

Microarray technology was first developed in the 1980's as a way of providing high sensitivity immunoassays for diagnostic purposes [392]. Today there are microarray platforms available for analysis of DNA and protein samples. DNA microarrays consist of large numbers of DNA sequences which are immobilized onto a solid surface which allow for the analysis of thousands of genes simultaneously [393]. There are many different DNA microarray platforms available which have differences both in how they are fabricated and in their functions [394]. The normal workflow in a microarray experiment consists of RNA isolation from a source tissue, conversion of RNA to cDNA, labelling of cDNA and hybridization to the array, followed by array scanning and data analysis [393]. Good experimental design is essential for any microarray experiment, and one should consider many different factors including cost, number of replicates, the questions to be answered by the data and the characteristics of the source material [393].

4.1.2 Uses of DNA Microarrays

DNA microarrays have been employed for a wide variety of uses including gene profiling of cancer types, identification of biomarkers associated with disease diagnosis, prognosis or treatment and identification of signalling pathways within cells in response to stimuli such as drug treatments etc.

4.1.2.1 Gene Profiling

It has been noted that despite molecular heterogeneity in tumours, patients who receive a particular diagnosis are given a certain course of treatment [395]. However, molecular profiling of tumours may provide more useful clinical information on tumour classification and thus treatment course [395]. In B-cell lymphoma, the Revised European-American Lymphoma (REAL) classification is used, however, it does not ideally separate different tumour morphologies – a study by Alizadeh *et al.* showed using DNA microarray technology that the subgroups could be more accurately divided using molecular profiling [395].

Although some heterogeneity remained within the groups, the authors showed how DNA microarrays could improve upon previous classification based on morphology. In malignant melanoma, a cancer which generally does not display recurrent genetic changes, a previously unrecognized subgroup of the disease was identified using microarray technology [396]. Gene profiling of adrenocortical carcinoma, a rare cancer, was able to identify differences between benign and malignant neoplasms and identify key transcriptional events involved in its pathogenesis [397].

Epithelial ovarian cancer is a clinically heterogeneous disease, with a number of histological subtypes. Microarray technology has been extremely useful in the analysis of ovarian carcinomas. A study by Welsh *et al.* showed that gene expression profiles of normal ovarian tissue clustered separately from ovarian carcinomas due to differential expression of gene groups, and also that within the serous carcinoma group, there were a number of subgroups which clustered together [398]. A later study by Schwartz *et al.* showed that gene profiling could also differentiate between histological subtypes of ovarian cancer [399]. In addition, the group found that gene expression profiling could separate high grade from low grade tumours, indicating that differences in genetic makeup of the tumour could influence tumour aggressiveness. It has also been shown that clear cell ovarian carcinoma has quite a distinct gene signature relative to other types of ovarian cancer [399,400]. Thus DNA microarray profiling has provided a lot of information on molecular differences between normal ovarian tissue and different types of ovarian carcinoma, shedding light on genes linked to the different tumour biologies observed.

4.1.2.2 Diagnostic Biomarker Discovery

As the standard treatment for cancers often depend on or are linked to the tissue of origin, it is vital that a correct diagnosis is made. This is not always straightforward, particularly in cell types which are morphologically similar, or in the case of metastatic disease of an unknown primary. One example of where a correct diagnosis is essential is in a particular type of lung cancer. Patients presenting with a unilateral malignant pleural effusion may be suffering from malignant pleural mesothelioma or from metastatic adenocarcinoma of the lung [401]. These diseases have quite different treatments – mesothelioma requiring both surgery and

chemoradiation, and adenocarcinoma, chemotherapy. Histological diagnosis is not always easy due to lack of sufficient material [401], and an incorrect diagnosis could lead to certain patients suffering from side effects of unnecessary treatments, or patients not receiving the necessary measures. Gordon *et al.* used U95A Affymetrix arrays to screen over 200 specimens of both cancer types. Using relatively small quantities of starting material, they were able to identify panels of biomarkers which could discriminate between the two tumour types with sensitivity of 97%.

Prostate cancer is the second leading cause of cancer death in men [402]. Prostatic intraepithelial neoplasia (PIN) is a precursor for prostate cancer, and the presence of high grade PIN is a significant risk factor for later development of prostate cancer [402]. Identification of biomarkers which can diagnose prostate cancer and differentiate between high-grade and lower grades of PIN is essential for good patient care. Bull *et al.* developed custom microarrays based on a computer search for genes often overexpressed in cancer [402]. Using tissue samples from normal prostate, benign prostatic hyperplasia (BPH), PIN and prostate cancer they identified sets of genes overexpressed in PIN and prostate cancer relative to normal prostate, and also overexpressed in prostate cancer relative to BPH. These markers provided potential starting points for validation in prostate biopsy specimens as diagnostic biomarkers.

Ovarian cancer is normally diagnosed using radiological imaging, serum Ca125 levels and a thorough patient history. However, Ca125 has certain limitations as a diagnostic tool. It is often not raised in early stage ovarian cancers, and is raised in a number of benign conditions such as endometriosis [403]. Thus, identification of novel biomarkers is essential. Although all biomarkers to date have not shown tremendous potential, microarray analysis can continually provide novel targets. For example, microarray analysis of ovarian cancer cell lines as well as normal ovarian surface epithelium (NOSE) identified a potential biomarker of ovarian cancer, osteopontin [404]. A follow-up study validating the usefulness of osteopontin as a serum diagnostic biomarker found it could pick up early stage and

late stage ovarian cancers with sensitivities of 80.4% and 85.4% respectively [405]. Similarly, prostatin, another potential ovarian cancer biomarker was identified by microarray analysis of ovarian cancer cell lines and NOSE [406]. The group's validation of this biomarker in a large number of serum samples from patients with benign and malignant ovarian disease displayed its potential as a serum biomarker. In addition, microarray analysis has been used to identify gene signatures which can differentiate between metastatic ovarian carcinoma and colon carcinoma, two cancers which can be difficult to separate histologically and have completely different treatment regimens [407].

4.1.2.3 Prognostic Biomarker Discovery

Prognostic biomarkers have several uses in cancer treatment. They can identify patients who are more likely to have a poor outcome, and thus may indicate that more aggressive treatment is necessary. Also, they can identify patients who do not respond well to particular types of therapy and thus may benefit more by being spared harsh therapy regimens to no effect. Similarly to diagnostic biomarkers, microarray data has been vital in identifying novel prognostic biomarkers in many types of cancer. A study by Inoue *et al.* used microarrays to study tissue from patients with various stages of gastric cancer [408]. This group divided the tumours into two groups depending on the presence or extent of certain prognostic factors such as tumour size, metastasis etc. They then scored and averaged the gene expression intensity of genes associated with these prognostic factors for each group. Using these scores, they divided the patients into three groups associated with clinical response, thus demonstrating a use for microarray data in determining prognosis. Another study by Weigelt *et al.* identified a 70-gene profile able to predict later metastasis in young breast cancer patients [409]. They used microarrays to profile matched primary breast tumours and metastases and found that distant metastases of breast tumours display the same molecular profile and 70-gene signature as their primaries, and showed that this signature could predict the presence or absence of metastasis. Mammaprint®™ is a prognostic test based

on a 70-gene signature which classifies patients based on likelihood of metastasis [410]. The signature was initially identified in a set of 78 breast tumours, and subsequently validated in a number of studies [410]. Oncotype DX® is a similar breast cancer prognostic test based on expression of 21 genes which analyses the likelihood of recurrent disease in oestrogen receptor-positive, lymph node-negative patients [411]. It was formulated based on review of published data of breast cancer biomarkers which were then evaluated in three clinical association studies [411]. Tests such as these aid clinicians in decision making regarding appropriate treatment such as adjuvant chemotherapy [412] and demonstrate a tangible usefulness for microarray data in patient treatment.

In ovarian cancer, microarrays have also been employed to identify prognostic markers. In 2005, Okamoto *et al.* screened 8 serous adenocarcinomas as well as paclitaxel-resistant ovarian cancer cell lines using Affymetrix U133 arrays [413]. Using this data they identified 44 genes associated with resistance/sensitivity to chemotherapy. Following validation of the results, they selected one gene, indoleamine 2,3-dioxygenase (IDO) to evaluate at the protein level in a further 24 tumour specimens. They found that positive IDO expression in tumours was significantly associated with relapse and poorer outcome, thus identifying a prognostic predictor for ovarian cancer. Hartmann *et al.* gene expression profiled 79 ovarian cancer tumour samples obtained before chemotherapy [414]. They used a complex algorithm which they had previously established to analyse the gene expression data and compiled a list of 14 genes which they then applied to an independent sample cohort. This gene list was found to have an accuracy of 86% in predicting the outcome of late stage ovarian cancer patients following platinum-taxane chemotherapy. In addition, a study from our group also identified a gene expression profile distinguishing primary and recurrent ovarian cancers using Human Genome Survey Microarrays by Applied Biosystems [415]. Using an initial training set of five primary and five recurrent serous adenocarcinomas they identified a number of differentially expressed genes. They then profiled a set of matched

primary and recurrent samples from the same patients and identified a further list of genes, which interestingly belonged to the same gene families as those identified in the initial training set analysis. A subset of genes identified was then validated in a cohort of primary and recurrent adenocarcinomas. This study identified potentially important predictors of ovarian cancer recurrence – this is important as recurrent ovarian cancer adversely affects patient prognosis. Thus microarrays have been used in a variety of ways to identify markers associated with prognosis.

4.1.2.4 Therapeutic Biomarker Discovery

As many tumours are biologically and clinically heterogeneous, it would be reasonable to assume that one type of treatment would not work for all tumours. However, it has been normal to treat particular tumours with a set chemotherapy regimen for many cancers. DNA microarray technology has been employed to segregate tumour types, and to determine prognostic factors for different cancers as discussed above, thus it also can play a role in determining potential therapeutic targets. These can then be utilized and developed to provide more individualized therapy regimens for patients who will benefit most. Several therapeutic biomarkers have been identified using microarray technology. Schwartz *et al.* screened 70 cervical cancer tumours on Affymetrix U133+2 arrays and analysed tumour pathways associated with tumour metabolism [416]. They noted that genes involved in the PI3K/Akt signalling pathway were significantly up-regulated in more metabolically active tumours, and that tumours expressing high levels of Akt protein had a poorer clinical response than those with weak Akt expression. This is clinically significant, as *in vitro* studies have already shown PI3K inhibitors to increase sensitivity to radiation therapy in cervical cancer cell lines [417], thus it highlights a potential therapeutic role for these drugs in treatment of patients with up-regulation of the PI3K/Akt pathway. Yamamura *et al.* analysed a publically available dataset containing microarray data on a set of primary and metastatic (omental) ovarian cancer tumour specimens [418]. They found overexpression of genes associated with the transforming growth factor β (TGFβ) signalling pathway in

omental lesions compared with primary tumours. They also showed that protein levels of the TGFβ receptor 2 were up-regulated in omental lesions, validating the microarray data. They then used the TGFβ pathway inhibitor, A-83-01, to investigate its effects on the metastatic properties of the murine ovarian cancer cell line HM-1. They were able to show that when the inhibitor was applied, the invasion, motility and adhesion properties of the cells were reduced. Subsequent *in vivo* studies showed that A-83-01 was able to improve the survival time of mice with ovarian tumours, thus indicating its potential as an adjunct treatment for ovarian cancer.

Therefore, it can be seen that microarrays provide a minefield of data which can provide useful leads for researchers and provide novel data which can lead to improvements both in diagnosis and treatment of cancers.

4.1.3 Microarray Analysis of Hypoxia-Induced Changes

Microarray technology has also been employed to identify changes induced by hypoxia in a number of different diseases. Starmans *et al.* used microarrays to analyse gene expression following various exposures to hypoxia [419]. They looked at gene expression changes in colon cancer, breast cancer and prostate cancer cell lines and then picked gene lists based on genes which were either up- or down-regulated in at least two of the three cell lines as well as genes that were differentially expressed over time. They then cross-compared these lists to publically available mRNA data in primary breast cancer samples in order to identify genes which may hold prognostic value. They found that these up-regulated gene lists which were derived from *in vitro* samples were not prognostic when applied to the public databases, although interestingly down-regulated gene lists were highly prognostic although the authors noted that this may not be due to hypoxia *per se*, rather they considered that the down-regulated gene lists such as cell cycle genes were likely representative of other cellular phenomena such as cell proliferation.

Similarly, in a study of hepatocellular carcinoma, van Malenstein *et al.* exposed the human liver carcinoma cell line, HepG2, to hypoxia for up to three days followed by RNA extraction and analysis on Agilent microarrays [420]. They determined an *in vitro* hypoxic gene set of 265 up-regulated genes and compared their data with publically available data sets. They removed any genes whose expression did not correlate with *in vivo* data from the public data sets resulting in a smaller set of four up-regulated and three down-regulated genes. Expression of this gene set in the public data sets was able to predict progression free survival and overall survival, and identify patients with or without vascular invasion.

Chi *et al.* exposed a number of different normal human cell types to hypoxia and analysed the gene expression response on microarrays [421]. Genes which were more than 2.5-fold increased in epithelial cells exposed to hypoxia were selected for analysis in renal cell carcinoma, breast cancer and ovarian cancer. They identified repression of cell cycle genes in response to hypoxia although it was unclear whether this was directly due to hypoxia, or rather due to changes in the cells' energy requirements. They analysed the 'hypoxia response' in a set of 72 ovarian cancer samples and found that samples expressing the 'hypoxia response' genes had poorer progression free survival and overall survival.

4.1.4 Aim

The results from Chapter 3 indicated that exposure to hypoxia increased resistance to cisplatin in A2780 and A2780cis cells. The aim of this chapter was three-fold:

i) To identify genetic changes associated with hypoxia in ovarian cancer cell lines

ii) To determine whether these changes are associated with chemoresistance

iii) To evaluate their potential as biomarkers for chemoresistance in ovarian cancer

4.2 Methods

4.2.1 Sample Selection

From the results of the hypoxia matrix (Chapter 3) we selected the point on the matrix which provided the most consistent and significant changes in resistance. We chose the 'hypoxia naïve' samples which were in hypoxia for the entire duration of cisplatin treatment (72 hours). Twenty four arrays were processed in total. Three biological replicates from independent experiments were run for each of the following matrix conditions:

i) A2780 (Normoxia, untreated)

ii) A2780 (Hypoxia, untreated)

iii) A2780 (Normoxia, cisplatin treated)

iv) A2780 (Hypoxia, cisplatin treated)

v) A2780cis (Normoxia, untreated)

vi) A2780cis (Hypoxia, untreated)

vii) A2780cis (Normoxia, cisplatin treated)

viii) A2780cis (Hypoxia, cisplatin treated)

4.2.2 Sample Preparation

Total RNA was extracted from A2780 and A2780cis using the RNeasy mini kit as described in Chapter 2. RNA was quantified on the NanoDrop spectrophotometer and the quality was assessed using the Bioanalyzer before running the arrays. Samples were prepared for array analysis and run using the methods set out in Chapter 2. Arrays were run for three biological replicates for each cell line and condition. Data was analysed using Expression console software (Affymetrix, US).

4.2.3 Array Quality Control Analysis

All arrays were screened using quality control methods as set out by the quality assessment white paper. The arrays were visually checked for uniform intensity and for correct alignment. Other quality aspects were assessed using the algorithms set out in the quality control white paper [422]. Metrics were examined at probe level, probe set summarizations and individual probe levels. Three types of metrics are used for quality control – sample metrics, hybridization metrics and labelling metrics.

A. pm_mean is the mean intensity for all probes on the array before any intensity transformations. This allows the user to ascertain whether a chip is unusually dim or bright, which may have an effect on certain parameters e.g. may see unusually high median absolute deviation of residuals.

B. pos_vs_neg_auc is the area under the curve for a receiver operating characteristic plot comparing signals for positive controls to negative controls. This is used to measure how well the probe set can separate the signal from the positive controls to negative controls, assuming the negative controls are false positives and the positive controls are true positives. Values between 0.8 and 0.9 could normally be expected.

C. X_mean is the mean signal value for all probe sets analysed from category 'X'.

D. X_mad_residual_mean is the mean of the absolute deviation of the residuals from the median, for all probe sets from category 'X'. Different probes return different intensities when hybridized to the same target. The RMA algorithm is used to create models for responses – the residual is the difference between the actual value and the predicted 'model' value. This is used to determine the mean of all the absolute deviation values produced

and identifies if there are any problems with the chip e.g. if the median deviation of the residuals are unusually high.

E. X_rle_mean is the mean absolute relative log expression (RLE) of all probe sets analysed from category 'X'. This uses the signal estimate from a probe set on a particular chip and calculates the difference from the median signal of that probeset across all the chips. This should be low, reflecting low biological variability between samples.

F. all_probeset is all the probe sets analysed. It includes the majority of the probe sets which are used for the downstream statistical analysis and is the most representative measure of the quality of the data.

G. bac_spike is the set of probe sets which hybridize to the pre-labelled bacterial spike controls (BioB, BioC, BioD and Cre). It is used to identify any problems with hybridization and the chip. It generally shows more variability than other categories due to the limited number of probe sets and spikes.

H. polya_spike is the set of polyadenlylated RNA spikes (Lys, Phe, Thr and Dap). This identifies problems with the target preparation. These also have more variability due to the limited number of probe sets and spikes.

I. neg_control is the set of putative intron based probe sets from putative housekeeping genes. Four-probe probe sets were designed against intronic regions of probe sets which were shown to have constitutive expression over a large sample number on 3'IVT arrays. These are used to estimate the false positive rate for the pos_vs_neg_auc metric.

J. pos_control is the set of putative exon based probe sets from putative housekeeping genes. Four-probe probe sets were designed against exonic regions of probe sets which were shown to have constitutive expression over a large sample number on 3'IVT arrays. These are used to estimate the true positive rate for the pos_vs_neg_auc metric. This category, along with the

all_probeset category reflects the quality of the whole experiment and the nature of the details used for statistical analysis.

4.2.4 Array Data Analysis

All data was analysed using the Bioconductor libraries 'oligo', 'limma' and 'made4'. Limma is a widely used and highly regarded library for the statistical analysis of microarray data. Oligo is designed specifically for use with Affymetrix GeneChip microarrays while Made4 incorporates useful visualisation and analysis tools for further analysis. The Bioconductor package is a valuable computational resource for the analysis of high-throughput biology, most especially in the field of microarray analysis. It is a highly active collaborative project, written in R, which is an open source, interactive computer system for the visualisation and analysis of statistical data.

4.2.4.1 Data Normalization

Data normalization is carried out to correct for any differences in expression levels within and between chips, in order to facilitate comparison between chips. The Robust Multi-array Average (RMA) method was used to normalize and summarize the datasets. This is a log scale linear additive model which removes background intensity and normalizes probe level data across arrays [423]. Tukey's median polish is used to estimate expression values which are log base 2 transformed. Quantile normalization is used to make the distribution of probe intensities for each array in a set of arrays the same [424]. This accounts for any 'obscuring variation' in the data – variation which has been introduced in the sample prep etc., as opposed to true variation between biological samples [425]. Quantile normalization may be problematic if a probe has the same value across all arrays, however it does not seem to be a problem in reality [424]. As each gene of interest is probed with approximately 26 probes, the RMA method is used to summarize the probe intensities for each probe set [424]. The RMA method has certain advantages over

other methods of analysis e.g. average difference as it has been shown to produce a much smaller standard deviation (SD) for genes with low expression values and it is also better able to detect differentially expressed probe sets [425]. It has been shown to have better precision, more consistency and higher specificity and sensitivity than other models for data analysis [423].

4.2.4.2 Differential Gene Expression Analysis

Limma was used to test for statistical differences between gene expression levels across arrays. The fold change and standard errors are first estimated by fitting a linear model to each gene. This model summarises the data from each set of replicates to give a single value per condition, so that these values can be compared between groups, rather than between samples. This is then followed by the application of empirical Bayes smoothing to the standard errors. Pair-wise contrasts were then defined to compare the summarised samples to each other, thus allowing the algorithm to calculate fold change. Within Limma, the default method for ranking genes is the B statistic, which calculated the log odds of differential expression. The more well known adjusted p-value can be used to the same effect, and adjusted p-values will usually rank differentially expressed genes in the same order as the B-statistic. In this experiment, the adjusted p-value cut-off value of 0.05 was used to detect differentially expressed genes. Using the average fold-change as a means of ranking is generally not recommended because this ignores the variability between replicate arrays. In statistical analysis, the p-value controls Type I error i.e. the probability that a significant 'positive' result is really a true negative. A p-value <0.05 determines that in 100 significant results, 5 of them are predicted to be false. In small scale experiments this is perfectly adequate. However, in microarray experiments, thousands of genes are analysed simultaneously leading to an inflated false positive rate, known as the family-wise error rate (FWER). For this reason, an adjusted p-value was used in this study. The p-value was adjusted using the method of Benjamini and Hochberg [426]. Their false-discovery rate (FDR) is one method of controlling FWER which accounts for

both the presence and number of errors made. They define the FDR as 'the expected proportion of errors among the rejected hypotheses'.

4.2.4.3 Analysis of Target Gene Lists

Genes with a fold change of ≥2 and an adjusted p-value (FDR<0.05) were deemed significant for further analysis. In total, seven lists of genes were analysed.

i) A2780 v A2780cis (Normoxia, Untreated)

ii) A2780 (Normoxia, Untreated) v A2780 (Hypoxia, Untreated)

iii) A2780cis (Normoxia, Untreated) v A2780cis (Hypoxia, Untreated)

iv) A2780 (Normoxia, Untreated) v A2780 (Normoxia, Cisplatin Treated)

v) A2780 (Hypoxia, Untreated) v A2780 (Hypoxia, Cisplatin Treated)

vi) A2780cis (Normoxia, Untreated) v A2780cis (Normoxia, Cisplatin Treated)

vii) A2780cis (Hypoxia, Untreated) v A2780cis (Hypoxia, Cisplatin Treated)

Lists were analysed using the gene annotation database DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.7 [427,428]. DAVID is a web-based functional annotation tool which agglomerates gene annotation information from a number of public information sources such as Panther, Biocarta etc. It allows the user to access information such as gene ontology and function; identify related gene groups; and to visualise genes within pathway maps. It contains a pre-built Affymetrix chip background to allow better gene list comparisons for this microarray format. Following pathway analysis on DAVID, individual gene function and relevance was determined using PubMed and the online tool iHOP (information hyperlinked over Proteins) [429].

4.3 Results

Following the quality control and general clustering analysis, for clarity, results and discussion for Chapter 4 will be described together as three sections:

1. Comparison of A2780 and A2780cis
2. The Effect of Hypoxia on Gene Expression in A2780 and A2780cis
3. The Effect of Hypoxia on Genetic Response to Cisplatin Treatment in A2780 and A2780cis

4.3.1 Quality Control of Arrays

4.3.1.1 Visual Quality Control

All arrays passed initial visual inspections. The visual inspections checked for any scratches on the array surface, any 'patchy' staining, regional areas of high or low intensity or any overall differences in intensity between arrays. Expression of the B2 oligo positive control was also visually assessed (Figure 4.1). This control oligonucleotide hybridizes at various positions on the chip, including the checkerboard corners, at the edge and internally. This is necessary for correct alignment of the array's grid.

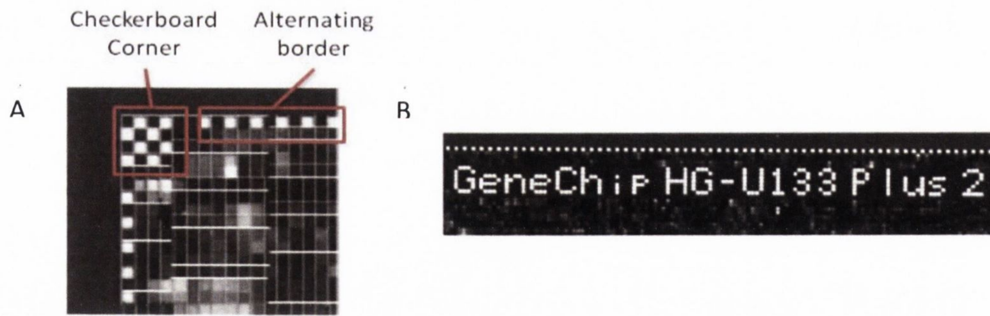
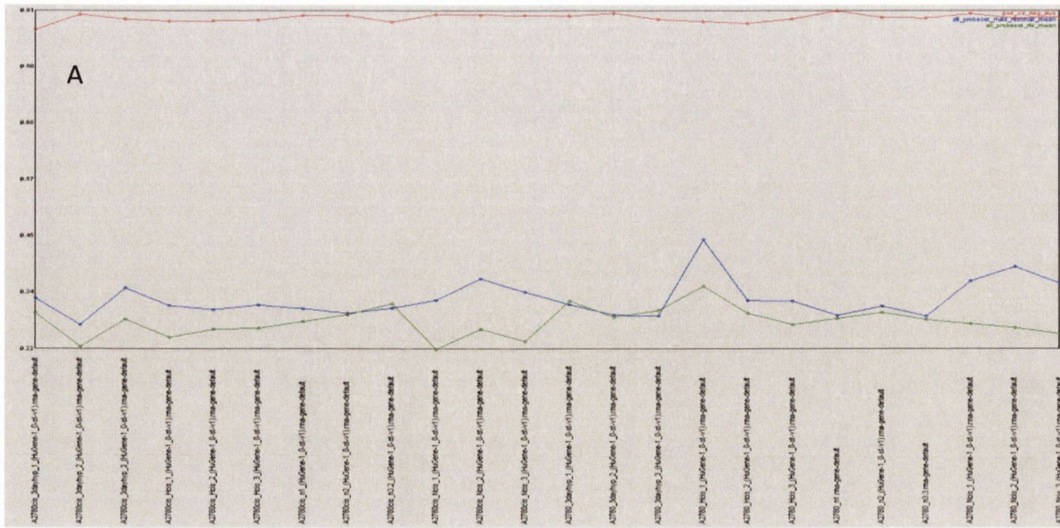


Figure 4.1. Location of B2 Oligo Positive Controls on GeneChip Arrays. All arrays were checked for intensity of B2 positive controls at regions within the chip, at the edge and checkerboard corners (A) and at the bottom of the chip (B). Good expression of these controls is necessary for correct alignment of the chip's grid.

4.3.1.2 Probe Metrics Quality Control

These quality control metrics involve computing summary statistics for each array in a set and then comparing all the arrays in the set together. It allows for identification of any outliers in the data set which may skew the results. Three levels of metrics were used – probe level, probe summarization and control probe signals. There was a very low level of variation observed for all the probe metrics. Some of the probe metric statistics are displayed in Figures 4.2 and 4.3. The area under the curve (AUC) for a receiver operating characteristic (ROC) plot to discriminate between positive and negative control signals was above 0.85 for all the arrays (Figure 4.2A). This indicates a high degree of separation between the positive and negative controls which are indicators of true positives and false positives respectively. Box plots displaying the relative log expression (RLE) signals for all the arrays (Figure 4.2B) identified no outliers within the data set. The median RLE for all arrays was approximately 0, indicating that there was no skew in the data following normalization. The mean absolute deviation of residuals for the positive controls, bac spikes and poly A spikes were very similar for all arrays (Figure 4.3A), indicating that there were no problems with target preparation, hybridization, washing etc. The hybridization controls displayed the expected rank order in relation to their respective concentrations (Figure 4.3B). Again, this provided assurance that sample preparation was carried out correctly. Therefore, overall the quality control metrics indicated that the microarray experiments were successful, and that there were no outlier arrays within the experiment.



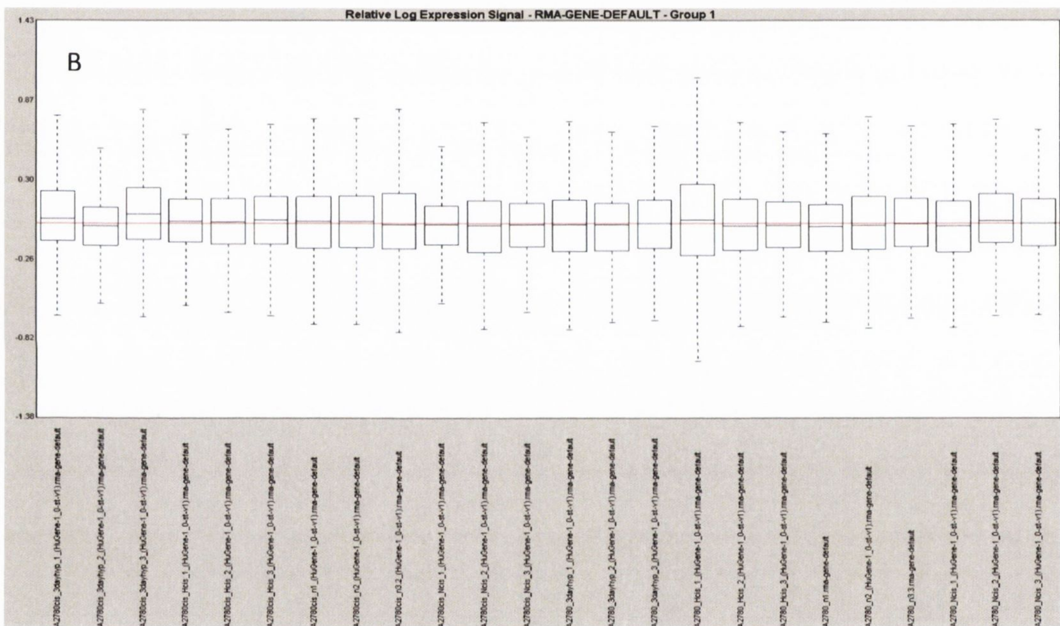


Figure 4.2. Probe Summarization Quality Control Metrics. The area under the curve for positive vs. negative controls was >0.85 for all the arrays (A, red line). This indicated good separation of signal from positive and negative controls which represent true positives and false positives respectively. The graph of the mad_residual_mean for all probes (blue line) is relatively constant, indicating the absence of outlying samples. The rle_mean for all probes (green line) is low, indicating low biological variability between samples. Relative log expression plots for all arrays (B) confirm that there are no outlying arrays within the dataset which may skew the results. In addition, the median relative log expression for all arrays was ~0, indicating the absence of skew in the data.

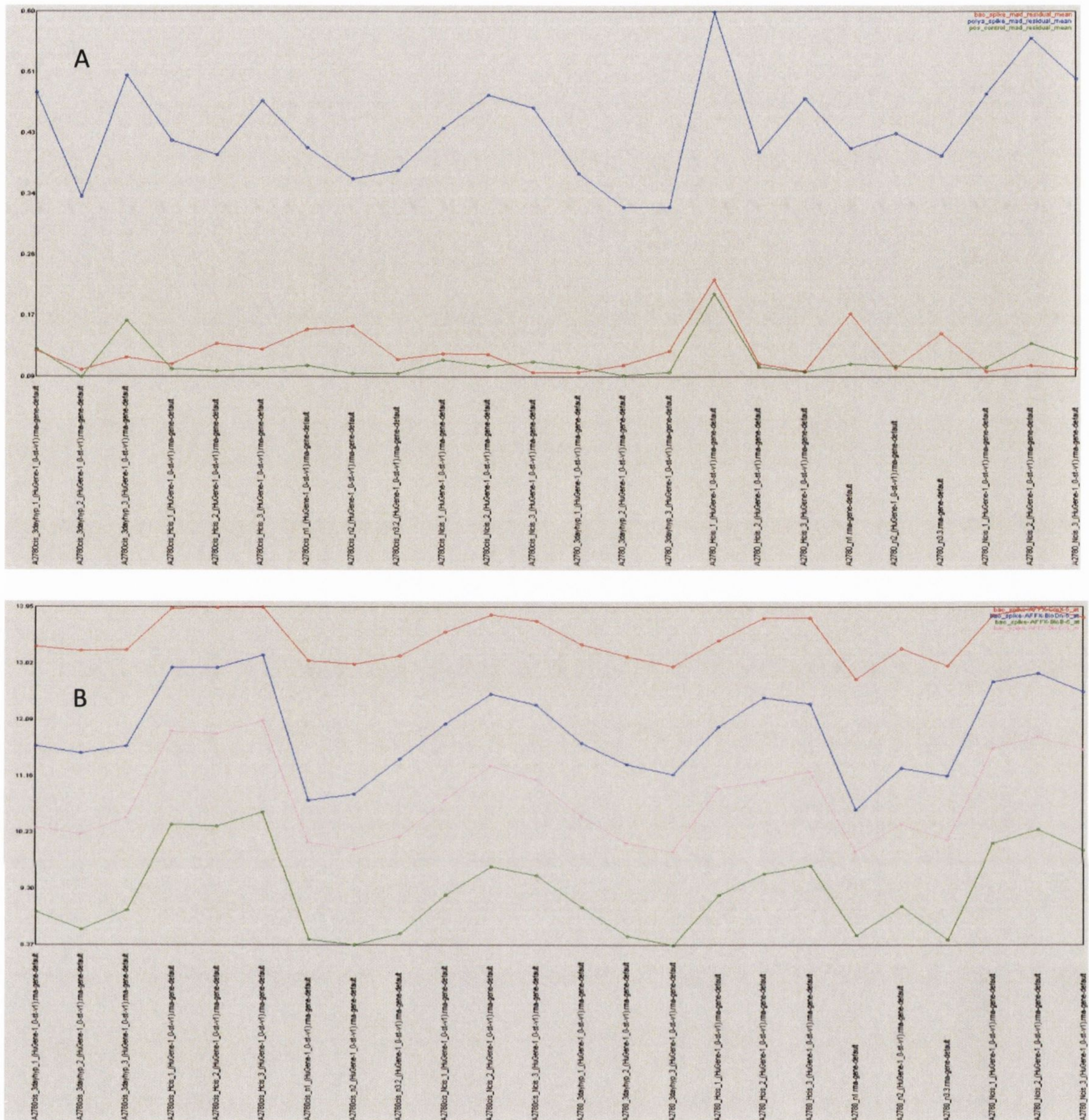


Figure 4.3. Control Signal Quality Control Metrics. The absolute deviation of residuals for positive controls (green line), bac spikes (red line) and poly A spikes (blue line) (A) were similar for all arrays. This is an indicator or overall data quality, and shows that there were no problems with target preparation, array hybridization, washing etc. The rank order of bac spikes (BioB (green) <BioC (pink) <BioD (blue) <Cre (red)) were as expected from their concentrations. This provided assurance of correct sample preparation.

4.3.2 Differential Gene Expression Analysis

4.3.2.1 Summary Characteristics Analysis

A Pearson's correlation was carried out to examine correlation of gene expression at two levels:

1. Between samples within a group
2. Between sample groups

A coefficient score of 1 indicates perfect correlation between the samples and is coloured red on the graph (Figure 4.4). Scores <1 indicate less correlation between samples and are coloured blue on the graph. The Pearson's correlation showed that the strongest correlations were seen between samples of the same group, which had coefficient scores of >0.97 for all samples, however, one sample in the untreated hypoxic A2780 group did not correlate as closely with the other two replicates. This sample was included in the analysis as it still displayed similar correlation coefficients with the other samples in the study as the other samples within its group. All A2780cis samples displayed low correlation with A2780 samples. Hypoxic samples correlated more closely than normoxic samples for each cell line, regardless of whether they were treated with cisplatin.

Hierarchical clustering analysis again demonstrated that the samples all clustered together according to group (Figure 4.5). Samples treated with cisplatin clustered more closely than samples which were untreated, in hypoxia and normoxia. All A2780cis samples clustered separately from A2780.

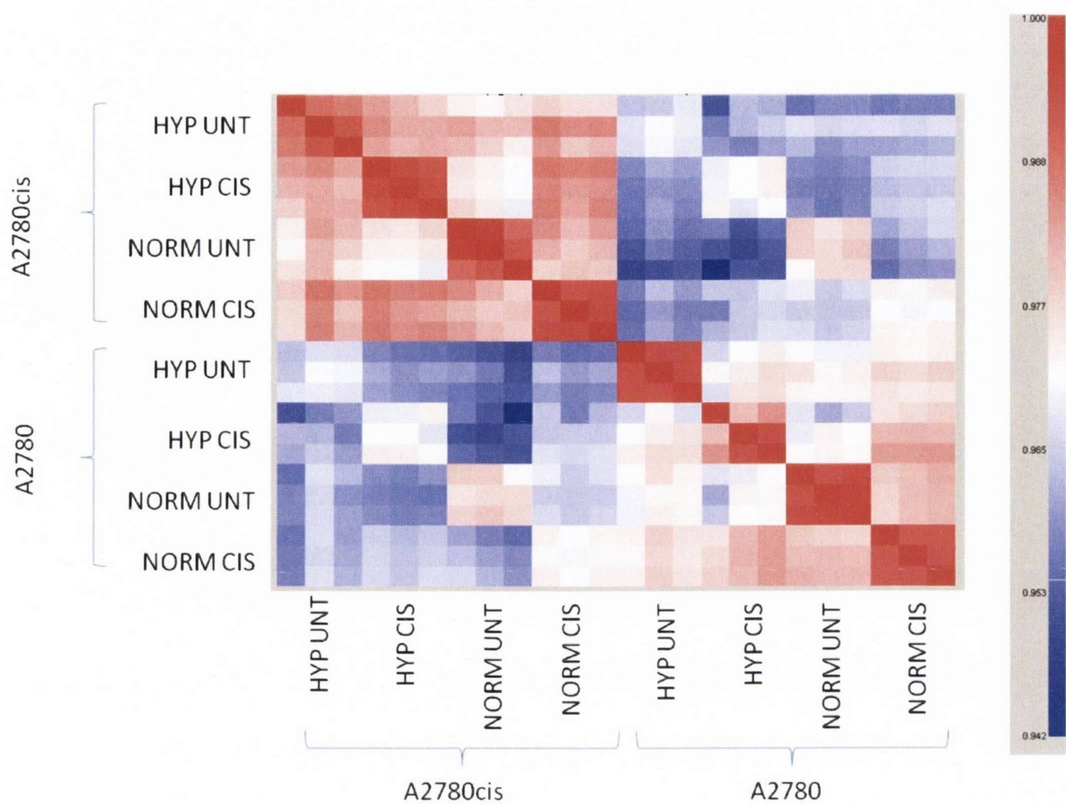


Figure 4.4. Pearson's Correlation of Samples Analysed on Affymetrix Arrays. Pearson's correlation was used to analyse correlation between sample groups based on their gene expression profiles. The strongest correlation was seen between samples of the same group represented by red blocks on the graph. The weakest correlations were generally seen between samples in the different cell lines.

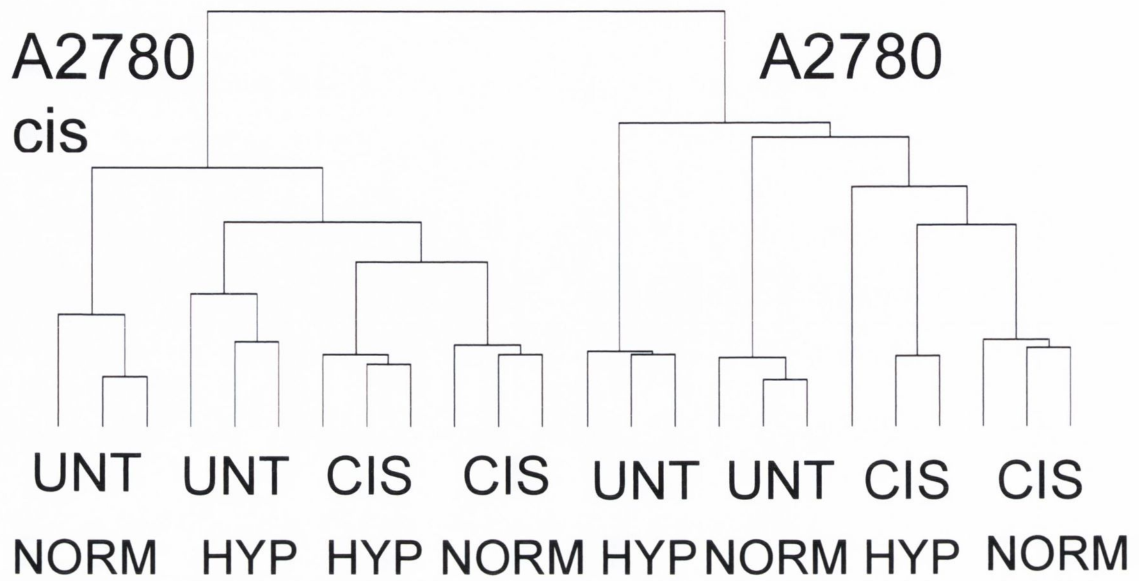


Figure 4.5. Clustering Denogram of Hierarchical Clustering Analysis of Gene Expression Profiles of A2780 and A2780cis Cells With/Without Exposure to Hypoxia and Treatment with Cisplatin. A2780cis samples clustered separately from A2780. Cisplatin treated samples clustered more closely than untreated samples, regardless of hypoxic exposure. Cells which were untreated but exposed to hypoxia clustered separately from normoxic untreated cells.

4.3.3 Results: Comparison of A2780 and A2780cis

The A2780/A2780cis model of cisplatin resistant ovarian cancer is often used in the literature, however, to my knowledge, there is no study which shows a whole-genome profile comparison of the two cell lines. A summary of the differential gene expression characteristics of the two cell lines is presented in Table 4.1. All data presented is for genes displaying a differential gene expression with a fold-change of ≥2 and an FDR<0.05.

Table 4.1. Differential Gene Expression Summary Characteristics of A2780 vs. A2780cis.

Total Number of Differentially Expressed genes	Up-regulated	Down-regulated
1202	511	691

Figure 4.6 outlines the chromosomal locations of the differentially expressed genes in A2780cis relative to A2780. Genes highlighted in yellow are up-regulated, while those in red are down-regulated. This is of interest as chromosomal changes are common in cancer – amplification of certain regions of the genome as well as translocation and loss of heterozygosity (LOH) have all been shown to be implicated in the pathogenesis of various cancers. A volcano plot (Figure 4.7A) allows for fast discrimination of differentially expressed genes with large and significant fold changes. Genes located on the top left or right of the plot identify those genes of interest. These included highly down-regulated genes such as MEF2C and ARHGAP28, and highly up-regulated genes such as PDGFC and FAM2C. A heat map (Figure 4.7B) allows large amounts of genetic data to be represented simply in graphic form. It allows for rapid identification of any patterns within the data.

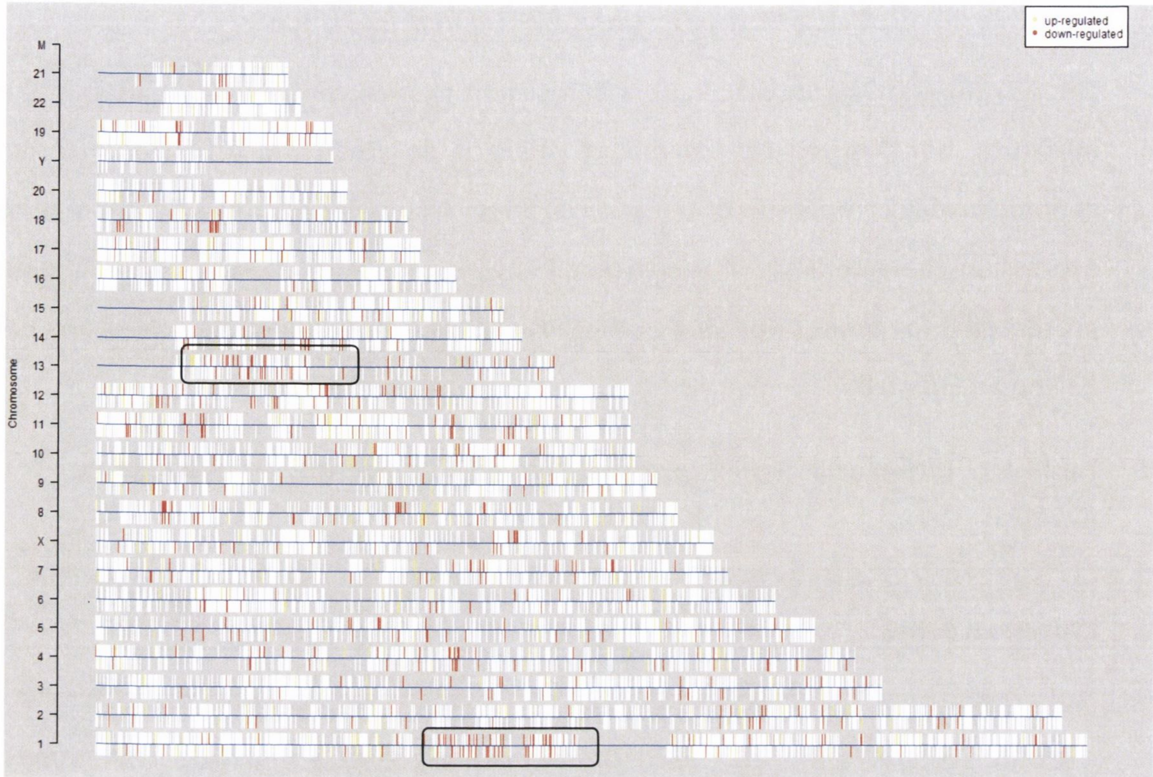
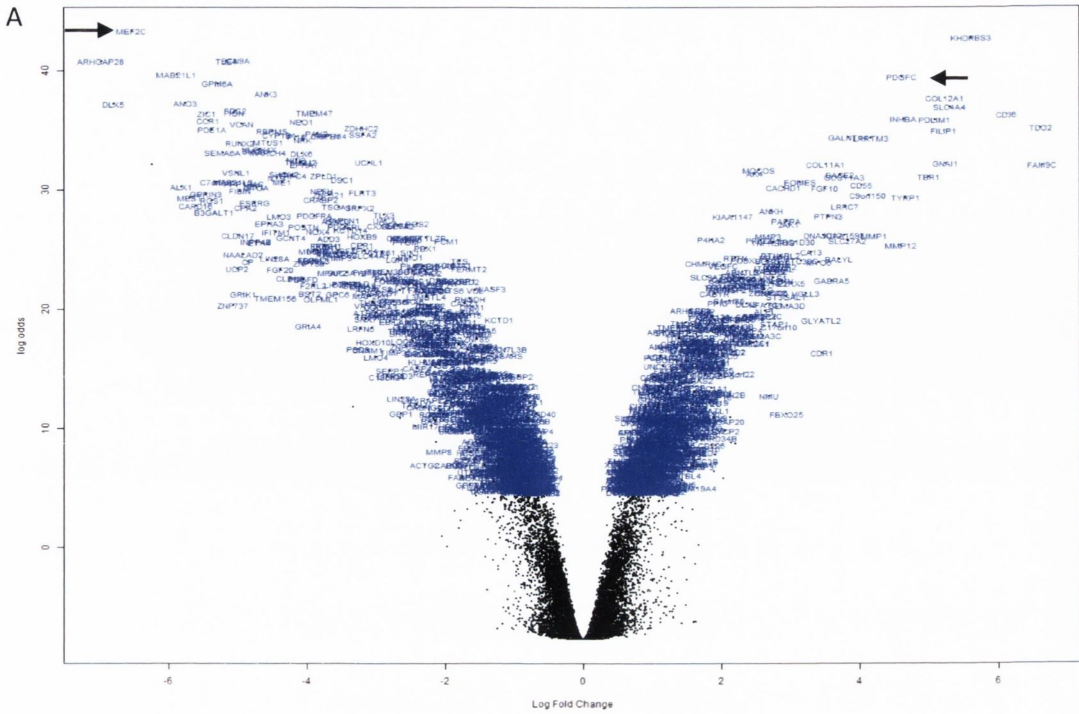


Figure 4.6. Chromosomal Location Plot of Differentially Expressed Genes in A2780cis Compared to A2780. Plot depicting the chromosomal location of up-regulated (yellow) and down-regulated (red) genes in A2780cis compared to A2780. The differentially expressed genes are generally spread evenly over the chromosomes, however some concentrated areas of down-regulated genes are noted on chromosome 1 and chromosome 13 (boxed regions). n = 3



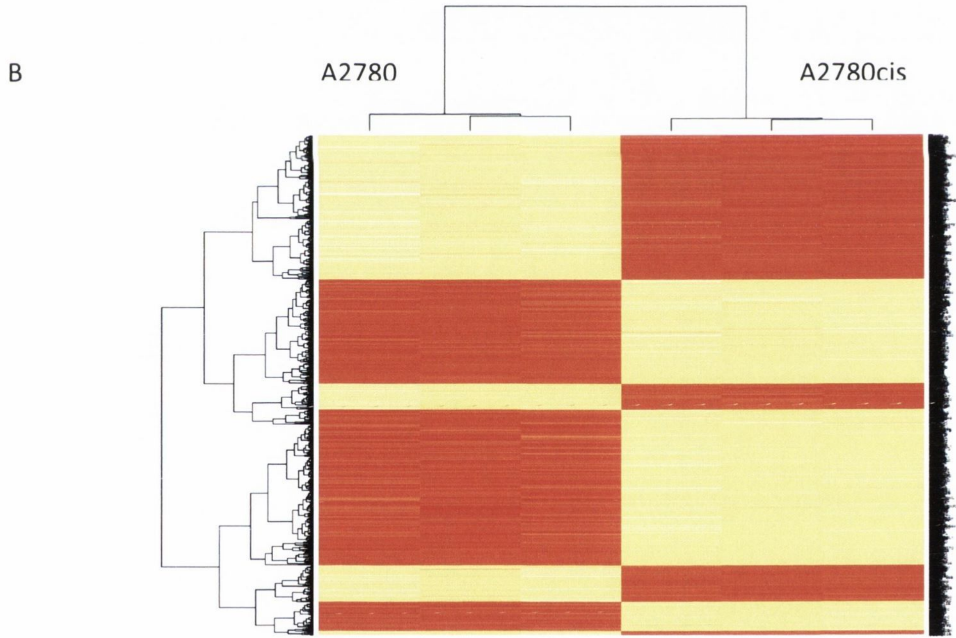


Figure 4.7. Overview of Gene Expression Changes in A2780 and A2780cis. Volcano plot (A) and Heat map (B) depicting overall gene expression changes in A2780cis relative to A2780. The volcano plot allows for fast identification of genes with large and significant fold changes such as MEF2C and PDGFC (arrows). n = 3

The two cell lines were compared using the DAVID database in order to identify significantly up- or down-regulated pathways which could contribute to the observed cisplatin resistance in A2780cis. Lists of the top five significantly up-regulated and down-regulated pathways and the genes affected are displayed in Tables 4.2 and 4.3. The pathways were identified by DAVID as part of the KEGG database. Genes present in more than one pathway are only represented once in the table. Genes picked for discussion are highlighted in bold.

Table 4.2. Top Five Significantly Up-Regulated Pathways in A2780cis Compared to A2780.

Pathway	**Genes**	**P-value**
Gap Junction	GNAI1, GUCY1A3, GUCY1B3,ITPR3, **PDGFC**, **PDGFA**, PrKCA, PrKCB, TUBB4	0.005
Pathways in Cancer	Fas, **Jak1**, **KITLG**, AR, ARNT2, CTNNA3, FGF1, FGF10, FGFR2, ITGA6, Jun, PPARγ, PLD1, VEGFC	0.01
Calcium Signalling	ATP2B4, CHRNA7, CACNA1H, CAMK4, CYSLTR2, GNAL, PTGER3, P2RX5, **ERBB3**	0.02
PPAR Signalling	CD36, ACSL1, CPT1A, FABP5, MMP1, SLC27a2	0.02
Long-term depression	PLA2G3	0.02

Table 4.3. Top Five Significantly Down-Regulated Pathways in A2780cis Compared to A2780.

Pathway	Genes	P-value
Focal Adhesion	FYN, SHC4, ACTN3, **CAV1**, CAV2, COL1A2, COL6A3, FLNC, HGF, IGF1R, ITGA5, ITGA8, LAMA1, PIK3CA, PDGFD, PDGFA, SPP1, **THBS1**, AKT3, VAV3, VCL	<0.0001
Arrythmogenic Right Ventricular Cardiomyopathy	CDH2, CACNG7, DSC2, DSG2, DMD, CACNA1C, JUP, SLC8a1, TCF7L1	<0.0001
Melanoma	CDKN2A, FGF18, FGF20, FGF5	0.001
Axon Guidance	EPHA3, EPHA7, NTNG1, PLXNC1, ROBO2, SEMA3E, SEMA6A, SEMA6D, SLIT2, UNC5C	0.006
Cell Adhesion Molecules	CDH2, CLDN17, CLDN8, CNTNAPA2, HLA-DPA1, HLA-DRB3, NEO1, NLGN4X, NEGR1, SDC2, VCAN	0.006

4.3.4 Discussion: Comparison of Gene Profile of A2780cis with A2780

The pathways which were found to be altered in A2780cis compared to A2780 were not surprising for a more aggressive, chemotherapy-resistant cell line. Up-regulation of biological signalling pathways as well as common cancer pathways may be expected in a cell line which has been induced to become resistant to treatment. In addition, down-regulation of adhesion molecules may signal the cells' movement towards a metastatic phenotype, with reduced gene expression for proteins which anchor the cells to their milieu. Indeed, in culture, A2780cis were seen to be less dependent on the culture surface, and many live cells grew within the media in suspension. We examined the differentially expressed genes in order to identify possible mechanisms by which the cells were more resistant to cisplatin.

Platelet-derived growth factor (PDGF) is a growth factor with many roles including regulation of smooth muscle and stimulation of angiogenesis and metastasis [430]. PDGFC is an isoform of PDGF identified in 2000 by Li *et al.* which binds to the PDGF receptor alpha (PDGFRα) [431]. It has been shown to be a potent transforming

agent *in vitro* [432] and has been linked to many disease pathologies, both cancerous and non-cancerous [433-436]. PDGFC has been shown to be associated with resistance to cisplatin in head and neck squamous cell carcinoma (HNCC) patients and knockdown of PDGFC was shown to increase sensitivity to cisplatin in HNCC cell lines [437]. Another PDGF isotype, PDGFA was shown to be overexpressed in A2780cis relative to A2780. PDGFA has been shown to act as a chemoattractant for recruitment of fibroblasts to tumour microenvironment [438]. Fibroblasts aid tumour growth and metastasis by secretion of paracrine growth factors and extra-cellular matrix (ECM) remodelling [439]. PDGFA has not yet been directly linked to cisplatin resistance in the literature, however it has been noted that regions of its promoter is a target for cisplatin binding [440].

Jak1 is a member of the janus kinase subfamily of cytoplasmic protein tyrosine kinases which play an important role in cytokine signalling [441]. Once activated by binding of a ligand, Jak's can phosphorylate and activate transcription factors known as signal transducers and activators of transcription (STATs) [441]. A previous microarray study has identified STAT1 as associated with cisplatin resistance in ovarian cancer [442]. Cisplatin resistance induced by prolactin in breast carcinoma was mediated through activation of the Jak pathway [443] and erythropoietin-induced cisplatin resistance in malignant melanoma was also shown to be mediated through Jak-STAT signalling [444]. Recently, Jak activation has been linked to cisplatin resistance as well as cell motility and enhanced cell migration in ovarian cancer [445] and down-regulation of Jak1 has been shown to abrogate cisplatin resistance induced by FGF-2 in an osteosarcoma model [446]. In addition, BRCA1 has been shown to activate Jak1 in a prostate cancer model [447], an interesting finding considering BRCA1 deficient ovarian cancers are generally sensitive to platinum agents [448]. Thus there is a collection of evidence supporting the role of Jak1 in cisplatin resistance.

Kit ligand (KITLG), also known as stem cell factor, binds to the proto-oncogene c-kit, a tyrosine kinase receptor which can bind a number of ligands including PDGF [449].

KITLG is a mitogenic and angiogenic molecule involved in carcinogenesis [450] and has been implicated in cisplatin resistance. Co-treatment of ovarian cancer cells with KITLG and cisplatin increased cisplatin resistance, whereas inhibition of KITLG using a neutralizing antibody enhanced the cells' sensitivity to cisplatin [8]. In addition, a study by Zhang *et al.* in 2008 identified a sub-population of ovarian cancer cells with stem-like properties which over-expressed KITLG and were resistant to cisplatin and paclitaxel [451].

ERBB3 (Her3) is a member of the epidermal growth factor receptor (EGFR) family [452]. It has been shown to be over-expressed in a subset of breast tumours [453], oral squamous cell carcinomas [454], malignant melanoma [455] and ovarian carcinoma [456]. ERBB3 has no intrinsic enzymatic activity, and forms a heterodimer with ERBB2 (Her2) resulting in signal transduction [457]. ERBB2 over-expression has been linked to chemoresistance and poor survival in ovarian cancer [458], and expression of ERBB3 has been associated with cisplatin resistance in lung cancer models [459]. In fact, co-expression of ERBB2 and ERBB3 has been linked to enhanced chemoresistance to a number of drugs in breast cancer [460]. In addition, a study by Chan *et al.* investigated the role of the epidermal growth factor in drug resistance using both *in vitro* and *in vivo* methods [461]. They showed that transfection of cells with a type of EGFR deficient in tyrosine kinase signalling ability resulted in reduced colony forming ability and increased sensitivity to cisplatin relative to cells with competent EGFR.

A number of down-regulated genes were identified which have linked to chemoresistance in A2780cis. CAV1 (caveolin 1) is an integral membrane protein which is a marker of caveolae, invaginations in the plasma membrane [462]. A study by Koleske *et al.* in 1995 demonstrated a reduction in caveolin expression in a transformed fibroblast cell line [463] and transfection of breast carcinoma cells with full-length CAV1 was shown to reduce the cells' proliferation and colony forming ability [464]. Up-regulation of CAV1 has been shown to be associated with multi-drug resistance in a number of cancer types [465,466] however low expression of

CAV1 has been linked to cisplatin resistance in oral squamous cell carcinoma [467] and overexpression of CAV1 has been shown to increase cisplatin sensitivity in breast cancer [468]. In ovarian carcinoma, CAV1 expression has been shown to be reduced relative to normal ovarian epithelium and is a putative tumour suppressor candidate [469,470]. In addition, high expression of CAV1 in prostate cancer has been linked to longer progression-free survival (PFS) times [471] however no relationship has yet been shown between CAV1 expression and PFS in ovarian cancer [469,472].

Thrombospondin-1 (THBS1) is a glycoprotein which facilitates cell adhesion and regulates cell proliferation in a cell type-dependent manner [473]. THBS1 expression has been shown to be down-regulated in breast cancer [474] and re-expression of THBS1 in breast cancer cells has been associated with reduction in proliferation and angiogenesis [475]. In bladder cancer, low THBS1 expression has been linked to increased recurrence of disease and poorer overall survival [476]. THBS1 has been shown to be expressed in a large proportion of ovarian carcinomas, and high THBS1 expression was shown to be associated with improved survival and inversely related to p53 expression [477,478]. However, other studies have identified high THBS1 expression to be associated with poorer prognosis [479,480], thus it is unclear how THBS1 expression affects survival in ovarian cancer. A THBS1 mimetic, ABT-510 has been shown to reduce ascites, tumour growth and metastasis in orthotopic mouse models of ovarian cancer [481] and has been shown to increase the cytotoxic ability of cisplatin and paclitaxel [482]. In addition, up-regulation of THBS1 by cellular pre-treatment with 5-fluorouracil, another cytotoxic agent, has been shown to increase cisplatin sensitivity in head and neck squamous cell carcinoma [483]. Therefore it is clear that THBS1 does play a role in cisplatin resistance.

Some concentrated regions of gene down-regulation were identified on chromosome 1 and chromosome 13. Loss of heterozygosity (LOH) refers to the loss of function of a gene allele where the other allele was previously inactivated, and in

cancer is usually associated with loss of tumour suppressor function. LOH at the p53 locus has previously been associated with chemoresistance in osteosarcoma [484]. In addition, changes in copy number of certain genes have been linked to resistance to certain cytotoxic drugs e.g. depletion of topoisomerase IIα has been associated with reduced sensitivity to topoisomerase inhibitors in breast carcinoma [485]. Similarly, in ovarian cancer, loss of expression of methylation-controlled J protein (MCJ) has been linked to reduced cisplatin sensitivity [486], while in gastric cancer, loss of PTEN (phosphatase and tensin homologue) was associated with increased cisplatin resistance [487]. LOH has previously been identified in ovarian cancer on several chromosomes including chromosomes 1 and 13. Zborovskaya *et al.* identified regions of LOH on chromosome 1 in both benign and invasive ovarian tumours [488]. Other studies of ovarian cancer have also identified LOH on chromosome 1 [489,490] and chromosome 13 [491,492] as observed in this study. A study by Prasad *et al.* demonstrated monosomy of chromosome 13 in A2780cis, thus rendering the cell line susceptible to LOH [493]. LOH of chromosome 13q14 has been linked to chemoresistance in leukaemia [494], while deletion of 13q has been identified as a prognostic marker in myeloid leukaemia patients [495]. LOH of chromosome 1 has not yet been related to chemoresistance in the literature.

In addition, DNA hypermethylation resulting in gene silencing has also been associated with cancer progression [22] and could provide an alternative mechanism for gene down-regulation in A2780cis. Changes in chromosome copy number, including amplification and deletion can be detected using comparative genomic hybridization (CGH). CGH analysis of many tumours has identified karyotypic abnormalities associated with chemoresistance such as loss of chromosome 17 and regions of chromosome 2 in lung cancer [496], loss of chromosome 11 in ovarian cancer [497], and decrease in copy numbers of topoisomerase enzymes in multiple drug-resistant cell lines [498].

Thus there are a number of different mechanisms by which cisplatin resistance may be occurring in A2780cis involving a number of different pathways within cells

including down-regulation of cell adhesion and up-regulation of cellular signalling. Only a small number of genes which may be involved in this process have been discussed, and there were many other genes identified which have been linked to cisplatin resistance. Functional analysis of these targets may identify potential therapeutic biomarkers for ovarian cancer.

4.3.5 Results: The Effect of Hypoxia on the Transcription Profile of A2780 and A2780cis

In order to determine the influence of hypoxia on the gene expression of A2780 cells and A2780cis pathway analysis was carried out on the gene lists generated by the Bioconductor analysis for each cell line, and subsequently compared the gene expression differences between the two cell lines. In particular, altered genes which were common for the two cell lines were examined, to see if these genes could account for the increased resistance to cisplatin observed in hypoxia for the two cell lines. In addition, the changes induced by hypoxia in A2780's were compared to the first analysis (A2780 v A2780cis) to see if any of the gene changes induced by hypoxia were the same as gene changes induced by prolonged cisplatin treatment. The gene changes induced by hypoxia for both cell lines are summarized in Table 4.4.

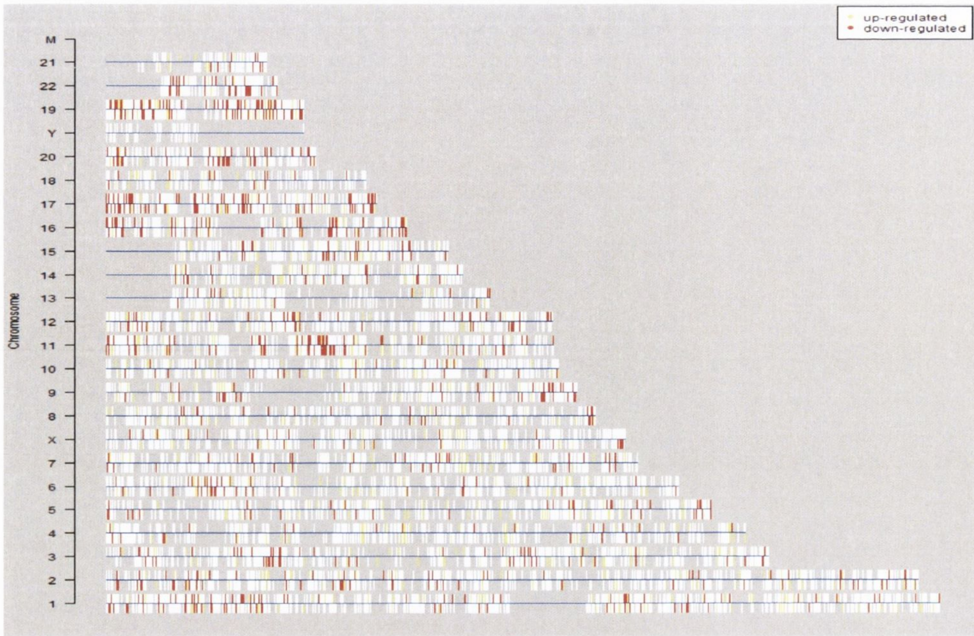
Table 4.4. Summary of the Gene Expression Changes Induced by Hypoxia in A2780 and A2780cis.

Cell Line	Total Number Differentially Expressed Genes	Up-regulated	Down-regulated
A2780	2675	1130	1545
A2780cis	1611	885	726

Less changes were induced in A2780cis cells compared to A2780 but for both cell lines, similar numbers of genes were up- and down-regulated. Chromosomal location plots (Figure 4.8), volcano plots (Figure 4.9A, 4.10A) and heat maps (Figure 4.9B, 4.10B) graphically summarize the data. The top up- and down-regulated

pathways from DAVID analysis are summarized in Tables 4.5 – 4.8. If genes were part of more than one pathway, they are represented once in the table. Genes which are later discussed are highlighted in bold.

A



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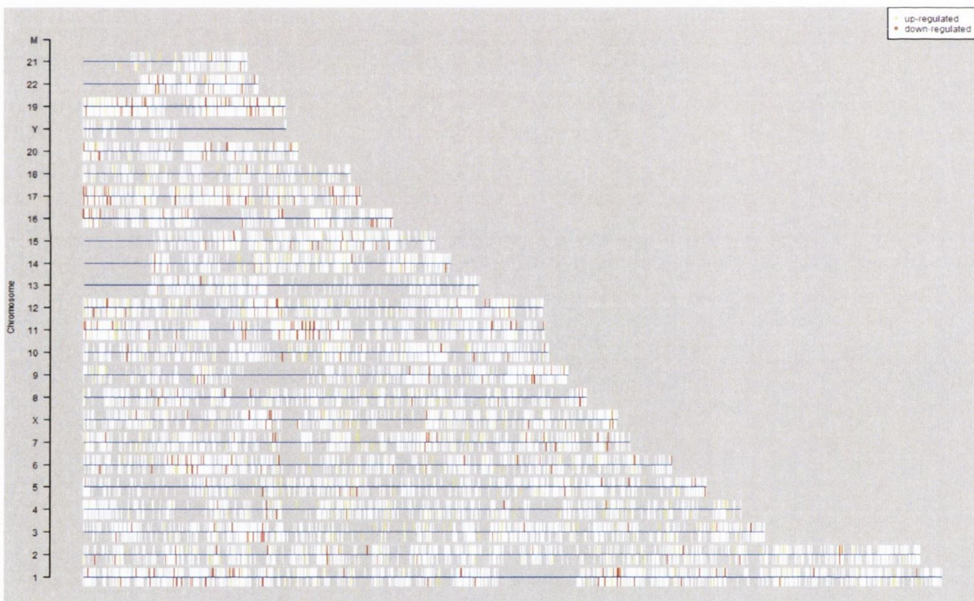


Figure 4.8. Chromosomal Location Plots of Genes Differentially Expressed by Hypoxia in A2780 and A2780cis cells. Plots show the chromosomal positions of genes differentially expressed in A2780 (A) and A2780cis (B) in response to hypoxic exposure. Up-regulated genes are represented in yellow, down-regulated in red, while genes whose expression was unchanged in A2780cis compared to A2780 are represented in white. More changes in gene expression are observed in A2780 with chromosomes 16, 17 and 19 demonstrating a lot of down-regulation of gene expression. Gene expression changes are more evenly distributed across the chromosomes in A2780cis. n = 3

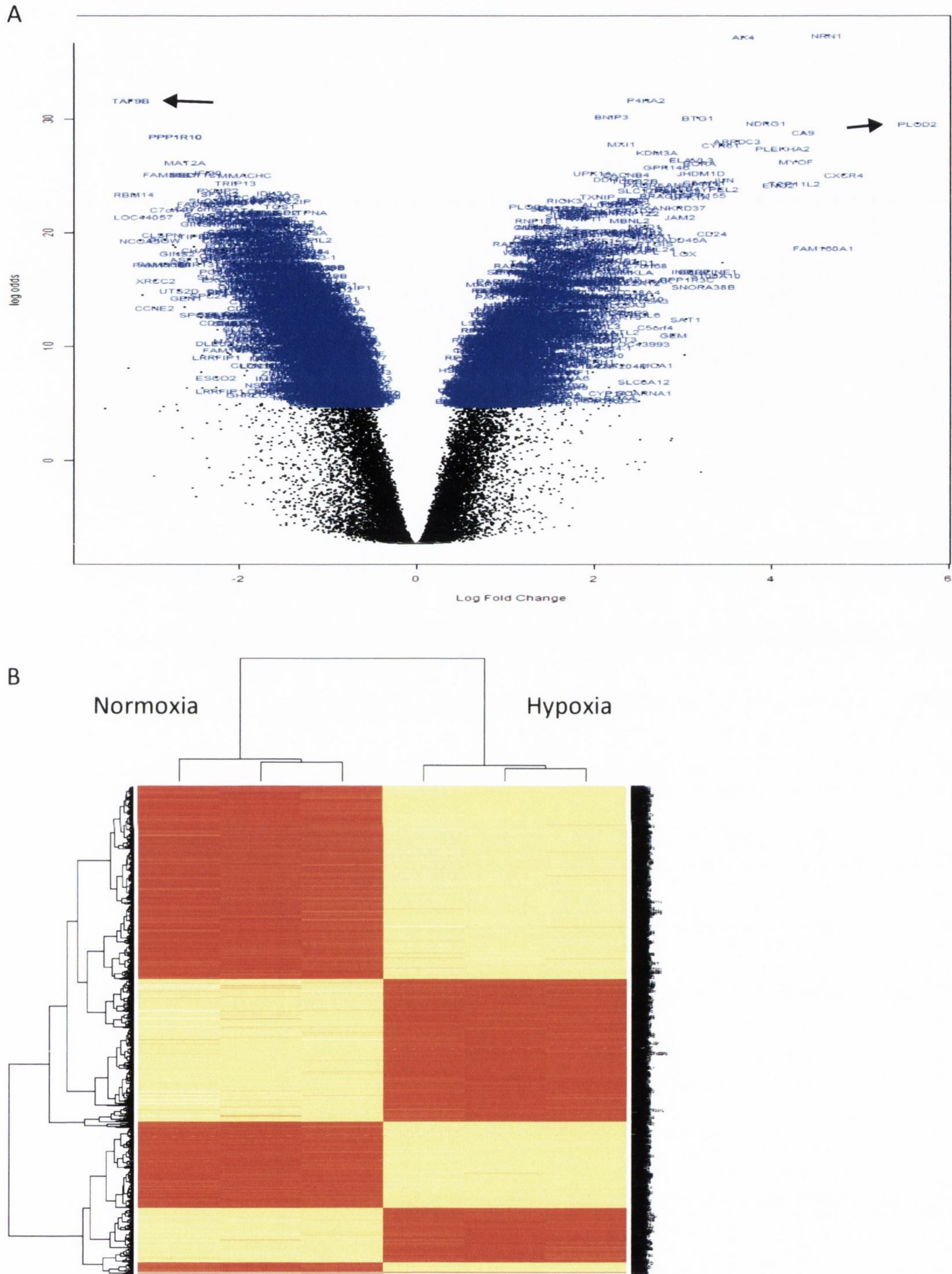
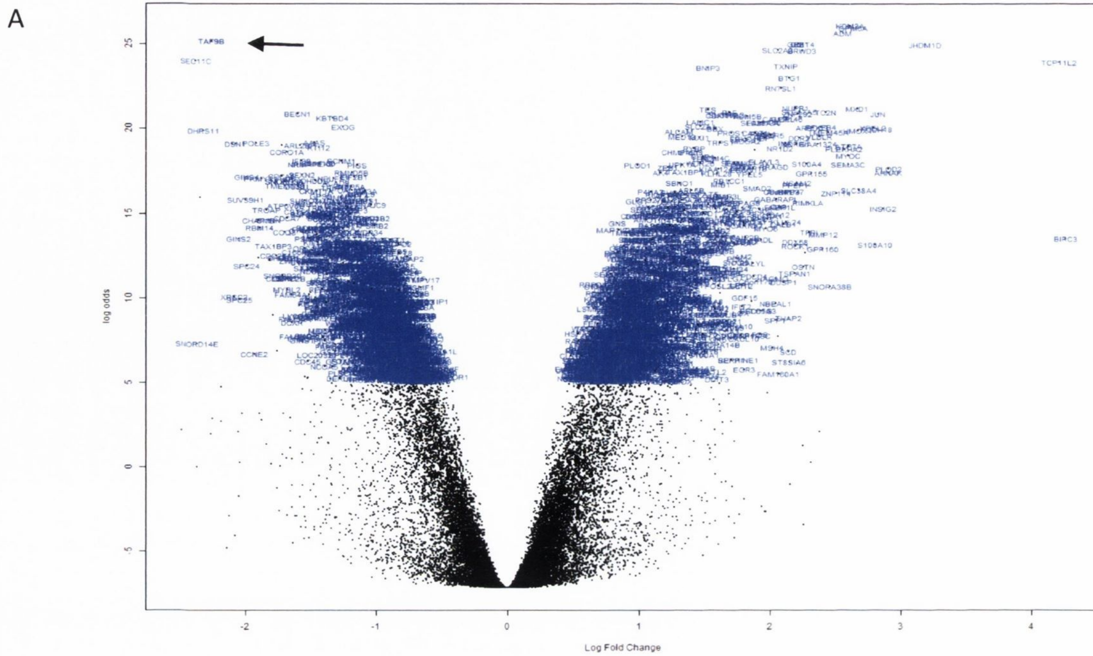


Figure 4.9. Volcano Plot and Heat Map of Gene Expression Changes in A2780 Cells in Response to Hypoxia. Volcano plot (A) identifies genes which have large fold-changes and significance such as TAF9B and PLOD2 (arrows) while the heat map (B) allows for fast visualization of the pattern of differences between gene expression in normoxia and hypoxia. n = 3

A



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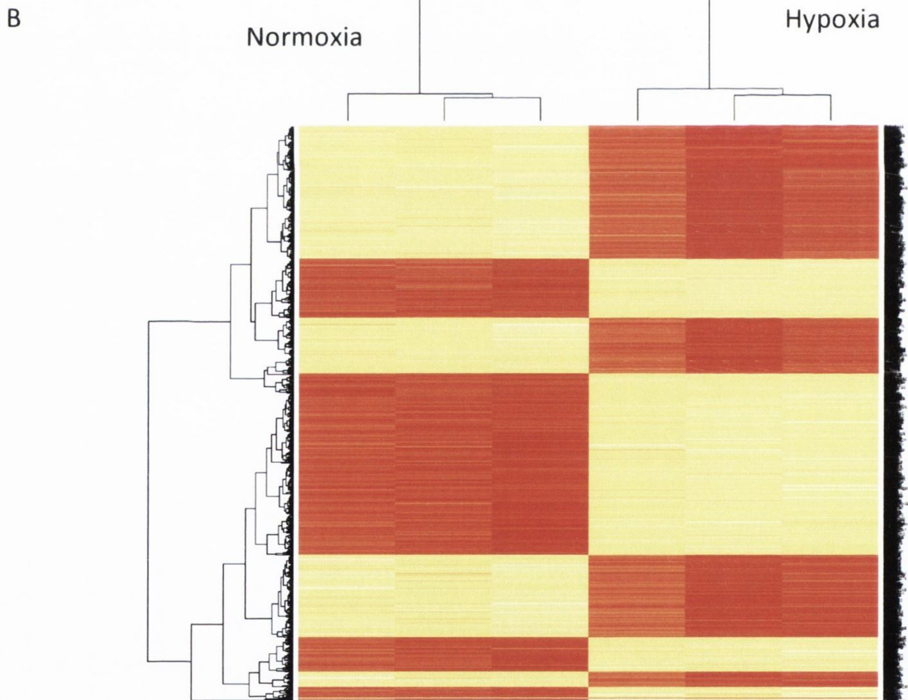


Figure 4.10. Volcano Plot and Heat Map Gene Expression Changes in A2780cis Cells in Response to Hypoxia. Volcano plot (A) provides fast identification of those genes whose differential gene expression values (fold-changes) are both large and highly significant such as TAF9B (arrow). Heat map (B) allows for easy identification of patterns in differential gene expression. n = 3

Table 4.5. Top Five Up-Regulated Pathways in A2780 Cells Exposed to Hypoxia.

Pathway	Genes	P-value
MAPK signalling	DDIT3, **RASGRP1**, RASGRF1, **BDNF**, CACNB4, **DUSP1**, DUSP16, DUSP3, FGF1, FGF11, FGF23, FLNC, **GADD45A**, GADD45B, JUN, MAPT, TAB2, MAP3K2, PDGFA, PLA2G3, PPM1A, STK3, TNF, AKT3, FOS	0.001
Focal adhesion	**BIRC3**, CAV1, CAV2, COL6A3, **IGF1R**, ITGA10, ITGB3, LAMA1, LAMB1, PDGFD, SPP1, ROCK1, THSB1, TNXA, **VEGFA**	0.002
Renal Cell Carcinoma	GAB1, EGLN1, EGLN3, EPAS1, SLC2A1	0.01
Starch and Sucrose Metabolism	GBE1, HK2, PGM1, PGM2L1, PYGL, PYGM	0.04
Complement and Coagulation Cascade	**CD55**, BDKRB1, C5AR1, FGG, MBL2, PROS1, SERPINE1, C3	0.04

Table 4.6. Top Five Down-Regulated Pathways in A2780 Cells Exposed to Hypoxia.

Pathway	Genes	P-value
DNA replication	FEN1, MCM2-7, POLA1, POLA2, POLE2, POLE3, POLD3, PRIM1, PCNA, RFC3, RFC5, RNASEH1, RNASEH2A	<0.001
Cell cycle	CDC45, E2F1, E2F2, E2F4, ANAPC13, ANAPC4, ANAPC5, ATR, **CDC25A**, CDC6, **CCND1**, **CCND3**, **CCNE1**, **CCNE2**, **CDKN2A**, ESPL1, PLK1, PKMYT1, RBL1, TFDP1	<0.001
Pyrimidine metabolism	CTPS, CAD, ITPA, PNP, POLR1A, POLR1B, POLR1C, POLR2L, POLR3B, POLR3H, RRM2, UMPS	<0.001
Base Excision Repair	OGG1, APEX2, MBD4, UNG, TDG	<0.001
Homologous Recombination	BLM, RAD51, RAD51L3, RAD54L, BRCA2, XRCC2, EME1, TOPO3A	<0.001

Table 4.7. Top Five Up-Regulated Pathways in A2780cis Cells Exposed to Hypoxia.

Pathway	Genes	P-value
Focal adhesion	XIAP, BIRC2, **BIRC3**, ACTN3, CAV1, CAV2, **IGF1R**, ITGA5, ITGB1, ITGB3, JUN, COL11A1, LAMC1, MET, PIK3CA, PDGFA, PDGFRA, SPP1, ROCK1, **VEGFA**, TNC	<0.001
Axon guidance	EPHA6, **L1CAM**, SRGAP1, CXCR4, GNAI1, SEMA3A, SEMA3C, SEMA3D, NFAT5, SLIT2, KRAS	0.002
TGF beta signalling	E2F5, SMAD2, INHBA, BMPR2, INHBE, ID4, LTBP1, RBL2, ZFYVE16	0.007
MAPK signalling	DDIT3, TAOK1, CACNA2D1, CACNB4, CACNG7, **DUSP1**, DUSP16, DUSP2, FGF1, MAPT, MAP3K2, TAB2, PPM1A, PPM1B, FOS	0.02
Toll like receptor signalling	TRAF3, CCL5, CXCL10, CXCL11, PIK3CA, TLR3	0.02

Table 4.8. Top Five Down-Regulated Pathways in A2780cis Cells Exposed to Hypoxia.

Pathway	Genes	P-value
DNA replication	MCM3-5, FEN1, POLA2, POLE2, POLE3, POLD3, PRIM1, PRIM2, PCNA, RFC3, RFC5, RNASEH2A	<0.001
Cell cycle	CDC45, SKP2, ANAPC13, ANAPC5, CDC20, **CDC25A**, CDC6, **CCND3**, **CCNE2**, CDK2, ESPL1, PLK1, PKMYT1	<0.001
Oxidative phosphorylation	ATP5D, ATP6V0E2, ATP6V0C, ATP6VOB, ATP6V1B2, COX3, COX17, NDUFA3, NDUFB10, NDUFS3, NDUFV1, ND2, ND4, COX8A, COX6B1	<0.001
Pyrimidine metabolism	PNP, POLR2E, POLR2L, RRM1, TK1	0.001
Base excision repair	APEX2	0.004

4.3.5.1 Identification of Common Gene Expression Changes in A2780 and A2780cis in Response to Hypoxia

The common patterns of gene expression in response to hypoxia were further examined in both cell lines. In total, 914 genes were commonly dysregulated in A2780 and A2780cis cells exposed to hypoxia. Of these, 431 were up-regulated and 483 were down-regulated. A break-down of the differential gene expression patterns is provided in Figure 4.11.

Pathway analysis was carried out on the commonly dysregulated genes in order to identify genes which may account for the increased cisplatin resistance observed in the two cell lines in hypoxia. A summary of the pathway analysis is provided in Tables 4.9 and 4.10. Genes which were chosen for discussion are highlighted in bold.

Figure 4.11. Summary of Common Differential Gene Changes in A2780 and A2780cis Cells Exposed to Hypoxia. Venn Diagram showing summary of gene expression patterns in A2780 (pink) and A2780cis (green). In total, 914 genes were commonly dysregulated in response to hypoxia in the two cell lines. Of these, 431 were up-regulated and 483 were down-regulated. n = 3

Table 4.9. Significantly Enriched Pathways from Commonly Up-Regulated Genes in A2780 and A2780cis in Response to Hypoxia.

Pathway	Genes	P-value
MAP kinase signalling	DDIT3, CACNB4, **DUSP1**, DUSP16, FGF1, JUN, MAPT, MAP3K2, TAB2, PDGFA, FOS, PPM1A	0.02
Focal adhesion	**BIRC3**, CAV1, CAV2, **IGF1R**, ITGB3, SPP1, ROCK1, **VEGFA**	0.02

Table 4.10. Top Five Significantly Enriched Pathways from Commonly Down-Regulated Genes in A2780 and A2780cis in Response to Hypoxia.

Pathway	Genes	P-value
DNA replication	FEN1, MCM3 – 5, POLA2, POLE2, POLE3, POLD3, PRIM1, PCNA, RFC3, RFC5, RNASEH2A	<0.001
Cell Cycle	CDC45, ANAPC13, ANAPC5, **CDC25A**, CDC6, CCND3, **CCNE2**, ESPL1, PLK1, PKMYT1	<0.001
Base Excision Repair	APEX2	0.001
Mismatch Repair	EXO	0.001
Oocyte Meiosis	CALM1, PPP1CA	0.006

4.3.5.2 Evaluation of Common Gene Expression Differences in A2780cis and Hypoxic A2780 Cells

The similarities in gene expression changes between A2780cis cells and A2780 cells which had been made hypoxic were examined to investigate whether the gene changes being induced by hypoxia were similar to those induced by repeated cisplatin exposure. This may identify common mechanisms of cisplatin resistance. The gene expression changes are summarized in Figure 4.12. We found that only 128 genes were commonly dysregulated in both conditions, representing only a very small fraction of the total number of gene changes observed. Hypoxia induced a far greater number of changes in gene expression than repeated cisplatin exposure – in total exposure of A2780 cells to hypoxia resulted in differential expression of over 2,500 genes. In comparison, A2780 cells which had been made cisplatin resistant

through repeated cisplatin exposure (A2780cis) had only approx. 1,200 differentially expressed genes. This indicates that hypoxia affects a far greater number of cellular processes than cisplatin alone.

As so few genes were commonly dysregulated, pathway analysis on DAVID identified only two commonly up-regulated pathways (Table 4.11) and one commonly down-regulated pathway (Table 4.12) although this was non-significant. The entire list of dysregulated genes was then searched in order to find potential links with cisplatin resistance. In total, five genes (three up-regulated, two down-regulated) were found that have been associated with cisplatin resistance in the literature (Table 4.13).

Figure 4.12. Comparison of Gene Expression Changes Induced by Hypoxia and Repeated Cisplatin Exposure in A2780. A2780cis (A2780 cells repeatedly exposed to cisplatin, pink) differentially expressed a total of 1202 genes in comparison to A2780. A2780 exposed to hypoxia (green) differentially expressed a total of 2,675 genes compared to A2780 in normoxia. Of these, 71 genes were up-regulated in common, while 57 were down-regulated. n = 3

Table 4.11. Commonly Up-Regulated Pathways in A2780 Exposed to Hypoxia and A2780cis.

Pathway	Genes	P-value
MAP kinase signalling	CACNB4, FGF1, JUN, MAPT, PLA2G3, PDGFA	0.01
Arginine and Proline Metabolism	ARG2, P4HA2, SAT1	0.03

Table 4.12. Commonly Down-Regulated Pathways in A2780 Exposed to Hypoxia and A2780cis.

Pathway	Genes	P-value
DNA Replication	RFC3, RNASEH1	0.07

Table 4.13. Commonly Dysregulated Genes in A2780 Exposed to Hypoxia and A2780cis which are Linked to Cisplatin Resistance in the Literature.

Gene	Annotation	Dysregulation
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	Up-regulated
USP2	ubiquitin specific peptidase 2	Up-regulated
CXADR	coxsackie virus and adenovirus receptor	Up-regulated
TIMP3	TIMP metallopeptidase inhibitor 3	Down-regulated
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	Down-regulated

4.3.6 Discussion: General Gene Expression Response to Hypoxia in A2780 and A2780cis

Many genes and pathways which have been well documented to be involved in the cellular response to hypoxia were identified in A2780 and A2780cis. Some common markers of hypoxia were dysregulated in both A2780 and A2780cis in response to hypoxia.

Although HIF-1α was not up-regulated (>2-fold, FDR<0.05) in either cell line in response to hypoxia, marked up-regulation of some surrogate markers of hypoxia was observed. In A2780 exposed to hypoxia, the glucose transporter, GLUT-1 (Slc2a1) was increased 2.61 fold, NDRG1 was increased 15.47 fold, carbonic anhydrase 9 was increased 20.42 fold and HIF-2α was increased 2.61 fold. In A2780cis, NDRG1 was increased 4.18 fold and carbonic anhydrase was increased 4.18 fold. This provided confidence that the effects observed were truly due to a hypoxic response. Although it may seem unusual that HIF-1α mRNA was not altered following hypoxic exposure, it is possible for HIF-1α to be transcribed under normal oxygen conditions, and it has been shown that HIF-1α is regulated at the protein level [499]. A previous study in a murine hepatoma cell line found that the mRNA transcript level of HIF-1α was not altered following hypoxic exposure, however, the DNA binding ability of HIF-1α was markedly increased [499]. In addition, a study in A2780 cells by Huang *et al.* showed that the hypoxia mimetic $CoCl_2$ did not alter mRNA levels of HIF-1α, yet increased the protein levels [500]. We have shown in Chapter 2 that hypoxia increased the protein levels of HIF-1α – while we saw virtually absent bands on Western blot for HIF-1α in normoxia in A2780 and A2780cis, we saw HIF-1α protein expression from 4 hours hypoxia exposure.

The mitogen-activated protein kinase (MAPK) signalling pathway is a downstream signalling pathway of activated Ras proteins and is associated with subsequent activation of mitogen/extracellular signal regulated kinases (MEK) and extracellular regulated kinases (ERK) leading to regulation of cell proliferation and survival [22]. We found up-regulation of many members of this pathway including RAS guanyl

releasing protein 1 (RASGRP1), a protein essential for Ras activation [501] and its overexpression has been implicated in carcinogenesis in keratinocytes [502] and resistance to MEK inhibitors in leukaemia [503].

Brain-derived neurotrophic factor (BDNF) is a growth factor produced by neurons and its expression has been shown to be induced in response to hypoxia [504,505] and it provides protection from hypoxia-induced cell death [506,507]. It has been shown to influence cell proliferation and promote cell survival in neuroblastoma [508], stimulate invasion in pancreatic adenocarcinoma [509], promote cell survival in breast cancer [510]. In addition, it has been implicated in the pathogenesis of prostate carcinoma [511], lung cancer [512], stomach cancer [513] and hepatocellular carcinoma [514]. It has also been shown to confer protection against cisplatin in neuroblastoma [515] and [516].

GADD45A (growth arrest and DNA damage inducible protein) is a protein induced by cellular stresses such as hypoxia and DNA-damaging agents. It is activated by p53 [517] and has many functions including induction of growth arrest, DNA repair, apoptosis, maintenance of genome stability and regulation of cell signalling [518]. Previous microarray data has shown it to be induced by hypoxia [519]. Its overexpression has been implicated in the pathogenesis of pancreatic ductal adenocarcinoma [520], oesophageal squamous cell carcinoma [521]. In addition, it has been identified as a both a promoter and inhibitor of breast carcinogenesis depending on the other signalling pathway alteration [522] and indeed other studies have identified GADD45A as a pro-apoptotic mediator [523,524]. *In vivo* experiments have shown that GADD45A-null mice are more prone to ovarian cancers as well as vascular tumours and in males, hepatocellular carcinomas [525].

Up-regulation of genes involved in focal adhesion was also observed. BIRC3 (baculoviral IAP repeat domain containing 3) is a member of the inhibitor of apoptosis family of proteins. Its expression can be induced by tumour necrosis factor alpha (TNFα) [526] and the human papillomavirus (HPV) via NFκB activation

[527]. Its expression has been associated with cisplatin resistance in prostate cancer cell lines [528]. BIRC3 has previously been shown to be induced by hypoxia [529,530] and has been linked to increased proliferation and reduced apoptosis in gastric cancer [531]. It has been shown in breast cancer cell lines that oestrogen treatment up-regulated BIRC3 mRNA and protein leading to protection from cell death by TNFα treatment [532]. In ovarian cancer, up-regulation of BIRC3 has been shown to protect cells from apoptosis [533,534]. BIRC3 has been linked to resistance to cisplatin in lung cancer [535] and has been identified as a possible therapeutic target for oral squamous cell carcinoma [536], breast carcinoma [532] and prostate cancer [537].

Vascular endothelial growth factor A (VEGFA) is a member of the VEGF family of angiogenic growth factors. VEGF induces angiogenesis through a variety of actions including stimulation of endothelial cell proliferation, inducing secretion of proteases which allow for cell migration, stimulation of vascular leakiness and promotion of survival of nascent epithelial cells [538]. Its production is stimulated by hypoxia [539], certain hormones such as oestrogen and testosterone [540] and by cytokines [541]. Expression of VEGFA has been observed in many tumour types such as leukaemia [542], breast cancer [543] and prostate cancer [544]. In ovarian cancer, VEGFA expression in tumour tissue has been shown to be higher than in normal ovary [545] and higher levels of VEGFA have been observed in serum samples from ovarian cancer patients than in those from patients presenting with benign ovarian disease [546]. It has been identified as a potentially useful marker of disease persistence and survival following first-line chemotherapy [547]. VEGFA is currently being exploited as a therapeutic target through monoclonal antibody therapy and its receptor through tyrosine-kinase receptor inhibitors [122]. Bevacizumab is a monoclonal antibody directed against VEGFA which is being used in combination with chemotherapy in a number of different cancers [122]. Early trials of bevacizumab in persistent or recurrent ovarian cancer revealed the drug to have significant activity, and although it was associated with some haematologic

toxicity, it was generally well tolerated by patients [548], and a recent large scale phase III trial of bevacizumab in combination with carboplatin in first line chemotherapy for epithelial ovarian cancer revealed significantly increased progression free survival and overall survival in patients receiving bevacizumab [549].

L1CAM (L1 cell adhesion molecule) is a cell surface antigen initially identified in neurons [550]. It has been implicated in the pathogenesis of many cancers. *In vivo* studies in lung cancer have demonstrated L1CAM to be involved in tumour metastasis [551]. In ovarian cancer, L1CAM expression has been associated with increased tumour aggressiveness, poorer survival and chemoresistance [552]. Its expression is HIF-1α-inducible, and together with angiopoietin-like 4 (ANGPTL4) – which we also found to be over-expressed in hypoxia – has been shown to mediate vascular metastasis of breast cancer [553,554]. It has been linked to cisplatin resistance in cholangiocarcinoma [555], ovarian carcinoma [556] and renal cell carcinoma It has also been identified as a potential therapeutic target – in mouse studies, combination therapy of ovarian tumours with a combination of anti-L1CAM antibodies and paclitaxel increased tumour response compared with paclitaxel alone [557].

The genes and pathways which were down-regulated in response to hypoxia were similar in A2780 and A2780cis. Cell cycle genes were down-regulated in both cell lines. This is unsurprising, as hypoxia has been observed to down-regulate cells involved in the cell cycle in many different cell types, inducing cell cycle arrest [558]. Hypoxia has been shown to down-regulate cyclin D1 through activation of p38 in prostate carcinoma cell lines [559], while in ovarian carcinoma, hypoxia has been shown to reduce levels of cyclin D1 and D2 as well as decreasing levels of cyclin E [560], all genes which we found to be under-expressed in hypoxia. We also observed reduction of cell cycle dependent phosphatase CDC25A, which is the master regulator of cell cycle transition from G_1 to S phase [561]. Loss of CDC25A in hypoxia has also been observed in colon cancer cells [558,561].

In addition, down-regulation of the cyclin-dependent kinase inhibitor CDKN2A was observed in hypoxia in A2780 cells. CDKN2A (p16) is a tumour suppressor which inhibits HIF-1α activity [562]. It also down-regulates expression of VEGF through its interaction with HIF-1α [563]. In ovarian cancer, studies have shown that the p16 gene can be methylated in up to 50% of cases [2] and that p16 protein expression is reduced with increasing grade of disease [564]. Other studies have shown high p16 protein expression in moderately- and poorly-differentiated ovarian carcinomas [565,566]. CDKN2A expression has been suggested as a predictive biomarker of hypoxic cell-sensitizing agents such as nimorazole in oesophageal squamous cell carcinoma [567] and p16 expression has been associated with sensitivity to cisplatin in several different tumour types [568-570].

Loss of proteins involved in DNA replication was also observed in A2780 and A2780cis. There was reduced expression of many members of the mini-chromosome maintenance family of proteins (MCMs). MCMs are components of a helicase enzyme involved in DNA replication and cell proliferation, and they inhibit HIF-1α activity [571]. Hypoxia has been previously shown to down-regulate MCM expression [572,573], thus indicating a potential mechanism by which cells potentiate the hypoxic response. MCM expression has been shown to be increased in increasing grades of ovarian carcinoma [574,575] and high MCM3 expression has been linked to poorer survival times [576]. In addition MCM2 expression has been linked to cisplatin resistance [577].

Concentrated regions of gene down-regulation were observed on chromosomes 11, 16, 17 and 19 in A2780 exposed to hypoxia. Chromosome 16 has been shown to have regions of deletion and hypermethylation in ovarian cancer [578]. Similarly, LOH has been observed on chromosome 17 [579,580] and 19 [581] in ovarian tumours. Hypoxia has been associated with chromosomal aberrations in cancer, and particular variants of the HIF-1α protein have been linked to LOH in lung cancer [582]. In addition LOH has also been linked to hypoxia in prostate cancer [583], and

breast carcinoma cells cultured in hypoxic conditions were shown to have complex karyotypic abnormalities [584].

LOH of chromosome 11 has been associated with Ras-mediated cellular transformation, indicating the presence of tumour suppressor genes [585]. In ovarian cancer, deletion of 11p13 has been associated with multi-drug resistance and loss of CD44 expression [586]. Similarly, loss of regions of chromosome 11 in head and neck squamous cell carcinoma is associated with increased risk of recurrent disease [587], resistance to chemo- and radiotherapy in leukaemia [588], and in chronic lymphocytic leukaemia (CLL) is linked to more aggressive disease and early disease progression [589]. LOH of chromosomes 11q24 and 17q21 in ovarian cancer has been linked to poor survival [590], and chromosome 11 has been noted as a source of tumour suppressor genes for ovarian cancer such as RPL27A, which was down-regulated in A2780's exposed to hypoxia in our study [591].

LOH on chromosome 16 has been associated with recurrence and more aggressive tumour pathology in Wilms tumour [592]. Similarly, in endometrial cancer, LOH of chromosome 16q is associated with increased tumour grade and poorer prognosis [593], while in prostate cancer, LOH on chromosome 16q is associated with invasion and metastasis [594]. CGH analysis of ovarian tumours has identified loss of chromosome 16 in serous carcinomas [595] and BRCA2 mutated cancers [596]. There is currently no information on LOH of chromosome 16 in relation to hypoxia in the literature.

LOH of chromosome 17 in ovarian cancer has been associated with loss of tumour suppressor genes in both cancer tissues and cell line models [579]. A study of BRCA1-mutated ovarian cancers found LOH of the entire chromosome 17 in 12 of 14 cases, while two cases had LOH of 17q [580]. A cisplatin-resistant osteosarcoma cell line has been shown to have genomic instability of chromosome 17 [597]. Chromosome 17 has also been implicated in acquired cisplatin resistance in prostate

carcinoma [598] and neuroblastoma [599]. Hypoxia has not yet been shown to directly induce chromosomal alterations to chromosome 17.

Chromosome 19 was also shown to contain regions of concentrated gene down-regulation. Allelic loss at chromosome 19q12 has been shown to be predictive of poor prognosis in borderline mucinous ovarian tumours [581]. In a study of 20 ovarian tumours of mixed histology, LOH of chromosome 19 was observed in approximately half of tumours, in a region encoding DNA repair genes such as XRCC1 and Akt2, both of which were down-regulated by hypoxia in A2780 cells in our study [600]. LOH of chromosome 19 has not yet been linked to hypoxia or cisplatin resistance in the literature.

Overall, the patterns of gene expression changes we observed were in accordance with the evidence in the literature. The genes observed which have been previously linked to cisplatin resistance were up-regulated in response to hypoxia. Down-regulated genes were generally not related to cisplatin response or were indicative of cisplatin sensitivity. However, when choosing regions of ovarian tumours to process for histochemical and pathological analysis, regions of necrosis – hypoxic regions – are generally avoided, therefore, gene expression from any one tumour region may not be representative of the entire tumour.

Pathway analysis was carried out on commonly expressed genes in A2780 and A2780cis in response to hypoxia in order to firstly narrow down the large number of dysregulated genes in either cell line alone, and secondly in order to search for any potentially stronger biomarker candidates – i.e. candidate genes in more than one cell line. Two significantly up-regulated pathways were identified – MAP kinase signalling and focal adhesion. DUSP1 (dual specificity phosphatase 1) is an enzyme responsible for dephosphorylating MAP kinases and required for relief of cellular genotoxic stress [601,602]. It has been shown to be hypoxia-inducible and it has been shown to have a number of roles including dephosphorylation and inactivation of JNK (c-Jun N terminal kinase) [603], inhibition of chemotaxis of immune cells to

the tumour microenvironment [604], tumour metastasis [605,606] and regulation of VEGF expression and microvessel density [607]. It has been shown to have anti-proliferative effects in breast cancer [608] and its expression has been negatively correlated with tumour differentiation in lung cancer [609]. In studies of ovarian cancer, the role of DUSP1 is a little unclear. Positive DUSP1 expression has been associated with reduced progression free survival [610], however another study has associated DUSP1 expression with reduced malignant potential [611] and a third study was unable to associate DUSP1 with any clinical outcome [612]. However, expression of DUSP1 has been shown to mediate resistance to cisplatin in ovarian cancer [613] and in lung cancer cells [614].

The insulin like growth factor 1 receptor (IGF1R) is a transmembrane tyrosine kinase receptor which is activated by binding of its ligands, insulin-like growth factor 1 and 2. The IGFs act as tissue growth factors and their binding to IGF1R results in the stimulation of several signalling pathways within the cell including the PI3K/Akt pathway, mTOR and Ras/MAPK pathways [615]. IGF1 has been shown to increase ovarian cancer cell proliferation [616] and inhibition of the IGF1R attenuates this response [617,618]. The expression of IGF1R has been shown to be up-regulated in recurrent ovarian cancer compared to primary [619]. Transfection of normal ovarian epithelial cells with IGF1R resulted in significantly increased proliferation, reduced expression of Fas, a receptor involved in apoptosis and increased colony forming ability [620]. Injection of transfected cells into mice resulted in formation of tumours, indicating increased tumourigenicity of the IGF1R-transfected cells. BRCA1 suppresses IGF1R activity [621], thus, lack of this suppression in BRCA1-deficient tumours may contribute to their pathophysiology. Up-regulation of the IGF1R signalling pathway has been implicated in cisplatin resistance in cancer. Eckstein *et al.* demonstrated up-regulation of IGF1R and PI3K pathways in cisplatin-resistant ovarian cancer cells [622]. IGF1R has also been implicated in cisplatin resistance in oesophageal carcinoma [623]. The IGF1R has been identified as a potential therapeutic target in ovarian cancer and others. An antibody against the IGF1R was

shown to inhibit cell proliferation and survival in *in vitro* studies and, in combination with the cytotoxic agent gemcitabine, stimulated tumour regression in orthotopic tumour models [624]. Further *in vitro* and *in vivo* studies using the ovarian cancer model A2780 showed antibody therapy to reduce tumour cell proliferation and inhibit xenograft tumour growth [625]. A novel small molecule inhibitor of the IGF1R, BMS-554417, has been shown to reduce cell proliferation and tumour growth through inhibition of the Akt signalling pathway and G_0-G_1 arrest [626].

The commonly dysregulated pathways in A2780 cells exposed to hypoxia and A2780cis cells were compared in order to determine if similar pathways were involved in hypoxia-induced resistance and cisplatin-induced resistance. The genes contained within the pathways found had no association to cisplatin resistance in the literature. The entire list of commonly dysregulated genes identified five genes which have links to cisplatin resistance.

CD55 regulates activation of the complement system by accelerating the degradation of certain enzymes within the pathway [627] and up-regulation of CD55 expression has been linked to cisplatin resistance in oral squamous cell carcinoma [628]. Its expression has been linked to the pathogenesis of several cancer types. Expression of CD55 at the invasive front of rectal cancer cells was associated with increased tumour recurrence and metastasis [629]. A study of lung cancer, CD55 inhibition with anti-CD55 antibody was shown to ameliorate the effects of herceptin antibody therapy [630]. Its up-regulation in breast cancer has been shown to protect against complement-mediated cytotoxicity [631] and inhibition of its expression with siRNA sensitizes cancer cells to complement-mediated attack *in vitro* [632,633]. Hypoxia has previously been shown to induce CD55 expression *in vitro* in both epithelial and non-epithelial cell lines [634] and *in vivo* [635]. In ovarian cancer, expression of CD55 has been observed in up to three quarters of ovarian tumours [636], and CD55 monoclonal antibody therapy has been suggested as a potentially useful therapy for occult micrometastases which cannot be picked up on imaging following cytoreductive surgery [637].

USP22 (ubiquitin specific peptidase 22) is an enzyme which is involved in regulation of protein degradation via the proteasome and is expressed in many different human tissues including ovary [638]. USP22 has been identified as a putative cancer stem cell marker whose function lies in deubiquitination of histones, transcriptional activation and in progression of the cell cycle – cells depleted of USP22 arrested in G_1 [639]. In addition to deubiquitination of histones, USP22 has also been shown to regulate activity of TRF1, a telomere-associated protein [640]. Recent studies have shown USP22 to act through a number of signalling pathways such as INK4a/ARF, Akt [641] and Jak-Stat [642]. Overexpression of USP22 in colorectal carcinoma has been linked to advanced stage of disease, poorer survival and correlated with expression of other biomarkers such as c-myc [643]. In breast cancer, overexpression of USP22 was positively correlated to lymph node metastasis and poorer outcome [553], while in gastric cancer, co-expression of USP22 with BMI-1 was also linked to poorer outcome [644]. This is the first study to identify USP22 as induced by hypoxia.

The coxsackie virus and adenovirus receptor (CXADR) was identified in 1997 [645]. Its functional utility in cancer treatment was demonstrated by a study which used the receptor to facilitate adenoviral-mediated transfer of Fas ligand to lymphocytes, thus stimulating apoptosis [646]. Indeed, adenovirus-mediated overexpression of the CXADR was shown to increase transgene expression [647]. In ovarian cancer cell line studies, it was shown that a minimum level of CXADR expression is necessary for adenoviral gene transfer [648], and that the level of CXADR expression is linked to the susceptibility of cells to adenovirus-mediated transfection [649]. A study of CXADR in ovarian tumours showed it to be expressed in the majority of tumours, with stronger expression in well-differentiated carcinomas [650]. Transfection of the ovarian carcinoma cell line, SKOV3, with CXADR resulted in increased cell adhesion and reduced colony formation thus presenting a potential role in metastasis prevention [651], however a further study of ovarian cancer CXADR expression linked over-expression with poorer progression free survival and overall

survival [652]. Increased levels of CXADR have been observed in a cisplatin-resistant laryngeal carcinoma cell line [653]. A previous study found reduced levels of CXADR in gastric, colon and prostate carcinoma cell lines following hypoxic exposure [654], however, we found up-regulation of CXADR following hypoxia in both A2780 and A2780cis. Our study used 0.5% O_2 for 72 hours, whereas the previous study used 1% O_2 for 24 hours – this may indicate a time-dependence or severity of hypoxia-dependence on the effects observed. While there may be an initial drop in CXADR expression on exposure to hypoxia, this may be reversed during prolonged hypoxia – although a time-course of CXADR expression would need to be carried out to confirm this.

TIMP3 (metallopeptidase inhibitor 3) is a member of a family of proteins which deactivate tissue metallopeptidases – enzymes employed by tumour cells to break down the extracellular matrix - and is repressed by EGFR activation [655,656]. A previous study also found TIMP3 to be down-regulated following hypoxic exposure [657]. Hypermethylation of TIMP3 has been observed in many cancer types including ovarian [658]. Previous cDNA microarray analysis has identified it as down-regulated in lung cancer [659], uveal melanoma [660] mengioma [661]. Methylation of TIMP3 in papillary thyroid cancer was associated with extrathyroidal invasion, metastasis to lymph nodes and multi-focal tumours [662]. Its methylation as part of a panel of three markers was recognised as a diagnostic tool to separate patients with Barret's oesophagus at risk of progression to oesophageal adenocarcinoma [663]. Its potential as a diagnostic biomarker was identified by Leung *et al.*, who identified methylation of TIMP3 in serum samples from late stage gastric cancer patients [664]. Transfection of a breast cancer cell line with recombinant TIMP3 resulted in reduced proliferation and reduced metastatic potential, thus indicating its potential as a therapeutic target [665]. A study of paired ovarian tumour samples, pre- and post-chemotherapy, identified TIMP3 as one of the down-regulated genes following chemotherapy and a potential marker of chemoresistance [666]. Adenoviral-mediated transfection of TIMP3 into cervical

cancer cell lines resulted in increased apoptosis, reduced proliferation and had synergistic effects when co-incubated with cisplatin and *in vitro* studies showed TIMP3 to inhibit growth of tumour xenografts, an effect that was ameliorated when combined with cisplatin therapy [667].

4.3.7 Summary

Thus, our studies of the effect of hypoxia on gene expression in A2780 and A2780cis identified several findings:

1. Hypoxia induces a large number of changes in gene expression in both A2780 and A2780cis, although more changes are induced in A2780
2. A large proportion of genes are commonly dysregulated in both A2780 and A2780cis in response to hypoxia
3. Few genes are commonly dysregulated in A2780 cells exposed to hypoxia and normoxic A2780cis
4. Most gene expression differences in hypoxia which were associated with cisplatin resistance were up-regulated
5. A number of potential biomarkers of cisplatin resistance in relation to hypoxia were identified including BDNF, BIRC3, VEGFA and IGF1R (up-regulated) and CDC25A, CDKN2A and TIMP3 (down-regulated).

4.3.8 Results: The Effect of Hypoxia on Response to Cisplatin in A2780 and A2780cis

4.3.8.1 Comparison of Untreated A2780 Cells with Cisplatin Treated A2780 Cells in Normoxia and Hypoxia

The changes in gene expression in A2780 following treatment with cisplatin were compared between normoxia and hypoxia. Differential gene expression changes are summarized in Table 4.14. Data is displayed for genes with a fold change of ≥2 and FDR <0.05. More genes were differentially expressed in A2780 cells which were treated with cisplatin in hypoxia. Similar proportions of genes were up- and down-regulated in A2780 treated with cisplatin in normoxia and hypoxia. Chromosomal location plots (Figure 4.13) display the location of gene expression differences on the chromosomes. A volcano plot and heat map (Figures 4.14 and 4.15) graphically display differences in gene expression patterns between both groups.

Table 4.14. Summary of Differential Gene Expression Characteristics for A2780 Cells Treated with Cisplatin for 72 hours in Normoxia or Hypoxia.

Cell Line	Total Number Differentially Expressed Genes	Up-regulated	Down-regulated
A2780 Normoxia	1521	702	819
A2780 Hypoxia	2099	1037	1062

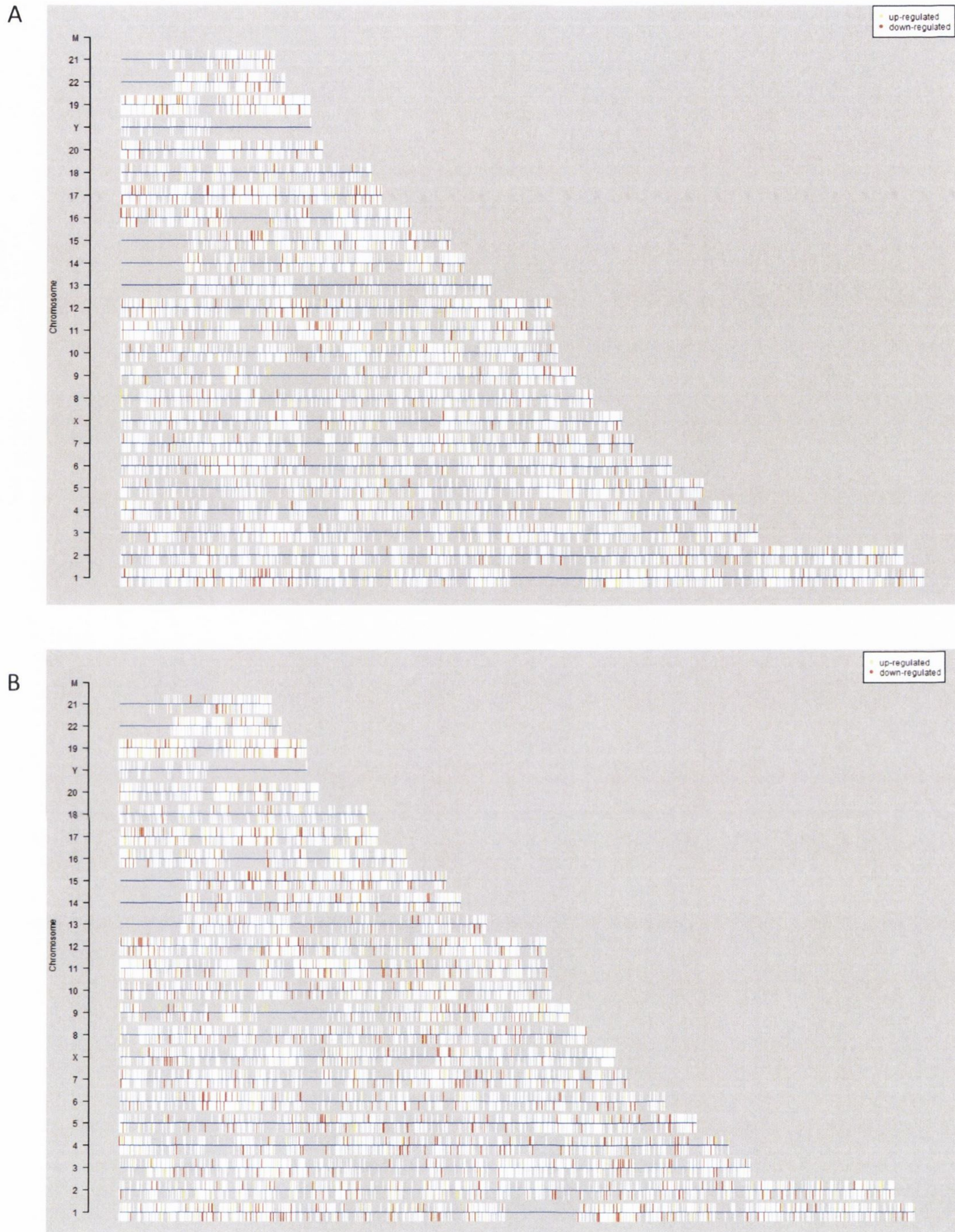


Figure 4.13. Chromosomal Location Plots for Differentially Expressed Genes in A2780 Cells in Response to Cisplatin Treatment in Normoxia and Hypoxia. Chromosomal location plots for A2780 cells treated with cisplatin in normoxia (A) and hypoxia (B). Genes up-regulated in response to cisplatin treatment are represented in yellow, down-regulated in red and unchanged are represented in white. Up- and down-regulated genes were spread evenly across the chromosomes. n = 3

A

B

Untreated

Cisplatin Treated

Figure 4.14. Differences in Gene Expression Patterns Between Untreated and Cisplatin Treated A2780 Cells in Normoxia. Volcano Plot (A) and heat map (B) displaying differences in gene expression patterns between normoxic A2780 which are untreated and normoxic A2780 treated with cisplatin. n = 3

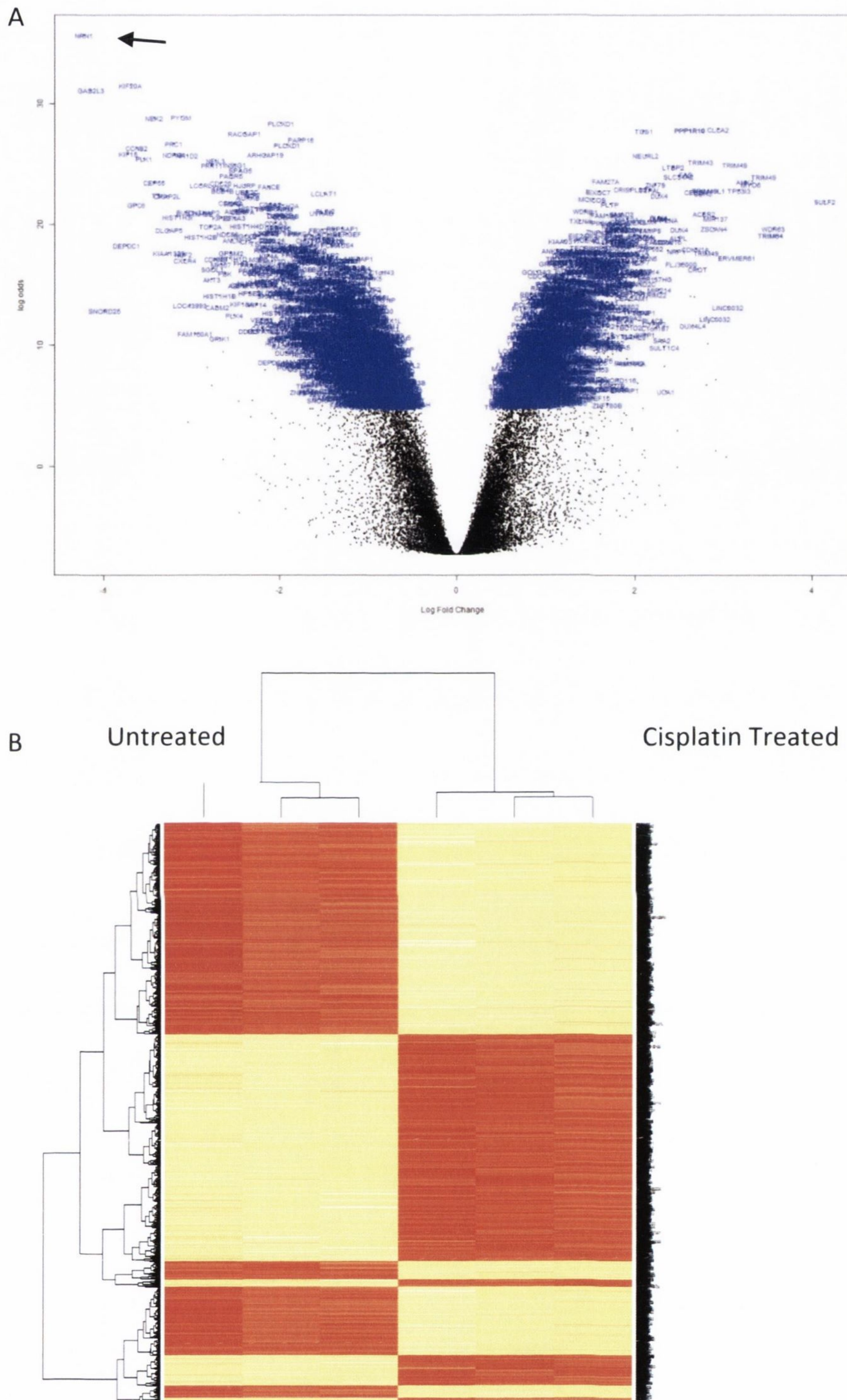


Figure 4.15. Differences in Gene Expression Patterns between Untreated and Cisplatin Treated A2780 Cells in Hypoxia. Volcano plot (A) and heat map (B) displaying differences in gene expression patterns. Volcano plot identifies genes which display a high magnitude of differential expression such as NRN1 (arrow) while heat map identifies clusters of genes which are differentially expressed. n = 3

Analysis of the gene expression on DAVID identified a number of pathways up- and down-regulated following cisplatin treatment. The top five pathways up- and down-regulated by cisplatin treatment in normoxia are summarized in Tables 4.15 and 4.16. The top pathways up- and down-regulated by cisplatin treatment in hypoxia are summarized in Tables 4.17 and 4.18. Genes which appear in more than one pathway are represented once on the tables.

Table 4.15. Top Four Significantly Up-Regulated Pathways in A2780 Cells Treated with Cisplatin in Normoxia.

Pathway	Genes	P-value
p53 signalling	**Fas**, MDM2, CCNG2, **p21**, GADD45A, RRM2B, SESN1, ATM, TP53I3, ZMAT3	<0.001
Lysosome	CD63, GM2A, GNPTAB, **CTSL1**, FUCA1, IUDA, **LAMP2**, **LAMP3**, MAN2B1, MANBA, SCARB2, SLC17a5	<0.001
Ribosome	RPL13, RPL13A, RPL24, RPL30, RPS18, RPS24, RPS27, RPS27L, RPS29, RPL32	<0.001
Other glycan degradation	FUCA1, MAN2B2	0.006

Table 4.16. Top Four Significantly Down-Regulated Pathways in A2780 Cells Treated with Cisplatin in Normoxia.

Pathway	Genes	P-value
DNA Replication	DNA2, FEN1, MCM2-7, **POLA1**, **POLA2**, **POLD1**, **POLE2**, **POLE3**, **POLD3**, **PRIM1**, **PRIM2**, RFC2, RFC3, RFC5, RNASEH1, RNASEH2A,	<0.001
Cell cycle	CDC45, E2F2, **MAD2L1**, SKP2, ANAPC1, ANAPC5, BUB1, BUB1B, **CDK1**, CDC20, CDC25, CDC6, **CCNA2**, **CCNB2**, **CCNE1**, **CCNE2**, **CDK2**, CDKN2A, ESPL1, GAD45G, PLK1, PKMYT1	<0.001
Pyrimidine metabolism	DHODH, PNP, POLR3D, POLR3G, RRM1, RRM2, TK1, UCK1	<0.001
Oocyte meiosis	FBXO5, AURKA, SGOL1	<0.001

Table 4.17. Top Four Up-Regulated Pathways in A2780 Cells Treated with Cisplatin in Hypoxia.

Pathway	Genes	P-value
p53 signalling	CD82, **Fas**, MDM2, APAF1, CASP9, **CCND3**, **CDKN1A**, LRDD, **PMAIP1**, SESN1, SFN, TP53I3, ZMAT3	<0.001
Apoptosis	**BCL2L1**, CAPN1, CASP6, IKBKB, PIK3R1, PIK3R3, PKA, RIPK1	<0.001
ABC Transporters	ABCA1, ABCA10, ABCA5, ABCC10, ABCD1, ABCD3, TAP1	0.01
Chronic myeloid leukemia	SHC4, SHC1, SMAD3	0.01

Table 4.18. Top Four Down-Regulated Pathways in A2780 Treated with Cisplatin in Hypoxia.

Pathway	Genes	P-value
Cell Cycle	CHEK1, DBF4, E2F5, MAD2L1, RAD21, SKP2, TTK, WEE1, BUB1, BUB1B, CDK1, CDC20, CDC25, CDC6, **CCNA1**, **CCNB1**, **CCNB2**, **CDK2**, CDKN1B, GADD45B, ORC1L, ORC6L, PTTG1, PLK1, STAG2, TGFB2	<0.001
Oocyte meiosis	FBXO5, AURKA, MAPK3, IP3R3, IGF1R, PPP3CC, SGOL1	<0.001
DNA replication	**POLE2**, **PRIM1**, **PRIM2**, RFC1-5	<0.001
MAPK signalling	DDIT3, RASGRP1, RASGRF1, ATF4, CACNB4, DUSP1, DUSP16, FGF23, JUN, MAPT, MAP3K2, NR4A1, PLA2G3, PDGFA, PKCA, PKCG, STK3, STMN1, ZAK, TGFB2, TNF, AKT3, FOS	0.002

Pathway analysis revealed that similar pathways are stimulated when cells are treated with cisplatin in hypoxia and normoxia. To further clarify the role of the genes identified, the break-down of gene expression in the two conditions was examined. Figure 4.16 is a Venn Diagram which displays the numbers of genes which are up- and down-regulated following cisplatin treatment in normoxia and

hypoxia. Of all the genes differentially expressed in the two lists, 338 are common. Of these, 137 are up-regulated, while 201 are down-regulated.

Figure 4.16. Comparison of Differntial Gene Expression Changes in A2780 Cells Treated with Cisplatin in Normoxia and Hypoxia. Venn Diagram summarizing the comparison in gene expression between A2780 treated with cisplatin in normoxia (pink) and hypoxia (green). In total, 338 genes were in common between the two lists. Of these, 137 were up-regulated while 201 were down-regulated. n = 3

Genes which were only over-expressed or under-expressed in cells treated with cisplatin in hypoxia were examined in order to identify genes which may account for the increased cisplatin resistance observed in hypoxic cells in Chapter 3. Pathway analysis was then carried out on the genes which were up-and down-regulated in A2780 treated in hypoxia only. The significantly over-represented pathways are summarized in Tables 4.19 and 4.20. Genes occurring in more than one pathway are represented once in the table.

Table 4.19. Pathway Analysis of Up-Regulated Genes in A2780 Cells Treated with Cisplatin in Hypoxia Only.

Pathway	Genes	P-value
Apoptosis	**BCL2L1**, APAF1, CAPN1, CASP6, CASP9, IKBKB, PIK3R1,PIK3R3, PRKACA, RIPK1	0.001
ABC Transporters	ABCA1, ABCA10, ABCA5, ABCC10, ABCD1, ABCD3, TAP1	0.002
Amyotrophic Lateral Sclerosis	CCS, GRIN2C, GPX1, MAP2K3	0.005
Small Cell Lung Cancer	TRAF4, ITGA3	0.02
p53 signalling	CD82, CCND3, LRDD, **PMAIP1**, SFN	0.02
Pancreatic Cancer	SMAD3, RALGDS	0.02
Chronic Myeloid Leukemia	SHC4, SHC1	0.03

Table 4.20. Pathway Analysis of Down-Regulated Genes in A2780 Cells Treated with Cisplatin in Hypoxia Only.

Pathway	Genes	P-value
MAPK signalling	DDIT3, RASGRP1, RASGRF1, ATF4, CACNB4, DUSP1, DUSP16, FGF23, GADD45B, JUN, MAPT, MAP3K2, NR4A1, PLA2G3, PDGFA, PKCA, PKCG, PPP3CC, STK3, STMN1, ZAK, TGFB2, TNF, AKT3, FOS	<0.001
Cell Cycle	CHEK1, DBF4, E2F5, RAD21, WEE1, CDC20, CDC25, **CCNB1**, CDKN1B, ORC6, PTTG1, STAG2	0.002
Steroid Biosynthesis	DHCR7, CYP51A1, EBP, SC4MOL, SC5DL	0.005
ErbB Signalling	NCK1, NRG4, ERBB4, ERBB3	0.01
Nitrogen Metabolism	ASNS, CA9, CA14, CTH, CA5B	0.02
Systemic Lupus Erythematosus	HIST1H2AC, HIST1H2AB, HIST1H2AG, HIST1H2BH, HIST1H2BI, HIST1H2AD, HIST1H4A, HIST2H2AB, HLA-DMA	0.02
Axon Guidance	EPHA6, EPHA7, CXCR4, CFL2, LRRC4C, SEMA3C, SEMA3E, UNC5C	0.02
Colorectal Cancer	FZD2, IGF1R, MSH2, TCF7L1	0.03
Gap Junction	ITPR3, PDGFD, PRKG1, TUBB2B	0.04

From the pathway analysis, a list of genes which have been directly linked to cisplatin resistance in the literature are displayed in Table 4.21. Genes which were picked for discussion are highlighted in bold.

Table 4.21. Dysregulated genes in the Hypoxic Response to Cisplatin in A2780 which have been Directly Linked to Cisplatin Resistance.

Gene Symbol	Annotation	Reference	Fold-Change	FDR
BCL2L1	BCL2-like 1	[668]	2.17	<0.001
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	[669]	2.13	<0.001
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	[670]	3.29	<0.001
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	[671]	3.51	<0.001
ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	[498]	2.18	<0.001
GPX1	glutathione peroxidase 1	[672]	3.46	<0.001
CCND3	cyclin D3	[673]	2.33	<0.001
SFN	stratifin	[674]	3.97	<0.001
DDIT3	DNA-damage-inducible transcript 3	[675]	-5.12	<0.001
NR4A1	nuclear receptor subfamily 4, group A, member 1	[676]	-2.75	<0.001
TNF	tumour necrosis factor	[677]	-2.87	<0.001
E2F5	E2F transcription factor 5, p130-binding	[678]	-2.08	<0.001
UNC5C	unc-5 homolog C (C. elegans)	[679]	-2.62	<0.001
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	[680]	-2.03	<0.001

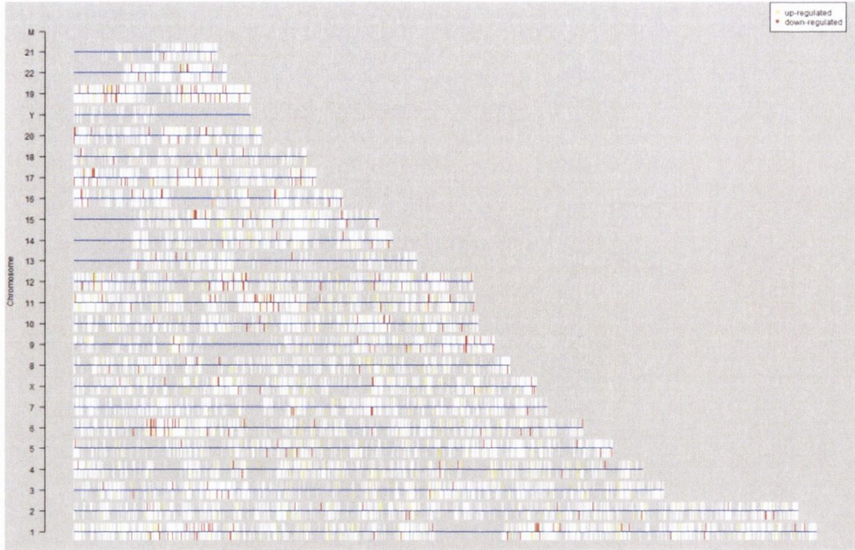
4.3.8.2 Comparison of Untreated A2780cis Cells with Cisplatin Treated A2780cis Cells in Normoxia with Hypoxia

The effect of cisplatin treatment on A2780cis was examined in order to identify the changes induced by cisplatin in normoxia and hypoxia and to identify any common changes between the two conditions. A summary of the differential gene expression characteristics of A2780cis treated with cisplatin in normoxia and hypoxia are presented in Table 4.22. Chromosomal location plots, volcano plots and heat maps provide graphical representation of the data (Figures 4.17, 4.18 and 4.19). The top up- and down-regulated pathways in response to cisplatin treatment are summarized in Tables 4.23 and 4.24 (normoxia) and Tables 4.25 and 4.26 (hypoxia). A summary of the 'hypoxia only' response to cisplatin is provided in Figure 4.21 and Tables 4.27 and 4.28.

Table 4.22. Summary of Differential Gene Expression Characteristics of A2780cis Treated with Cisplatin in Normoxia and Hypoxia.

Cell Line	Total Number of Dysregulated Genes	Up-regulated	Down-regulated
A2780cis Normoxia	1282	710	572
A2780cis Hypoxia	928	292	636

A



B

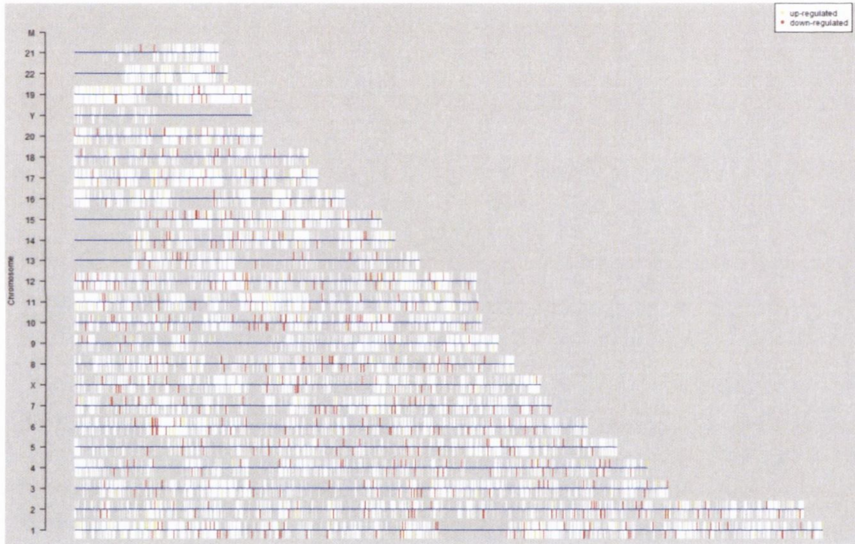
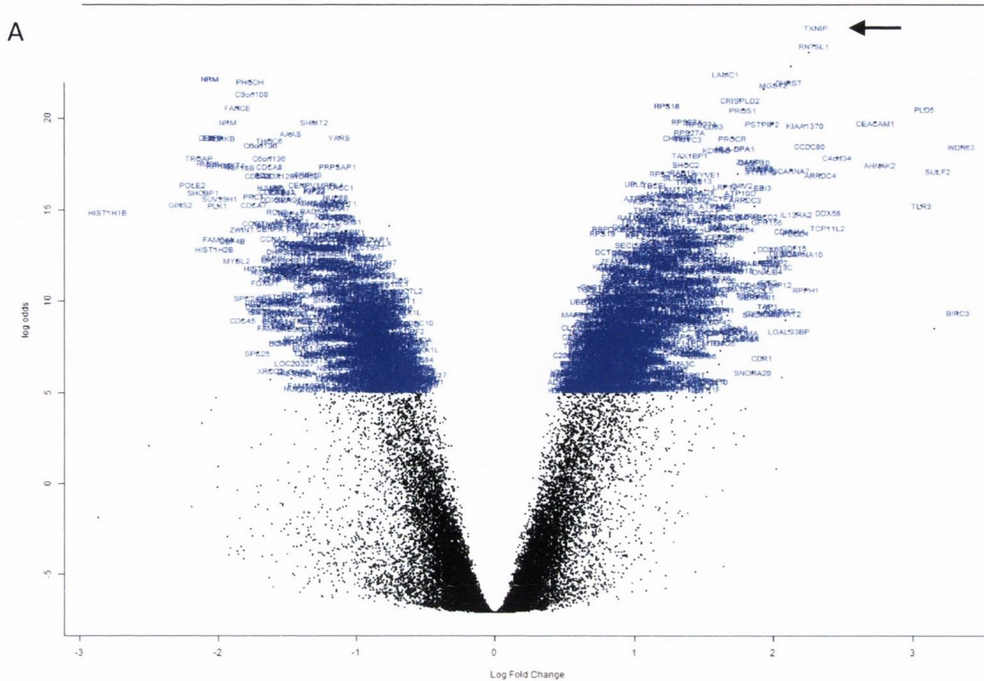


Figure 4.17. Chromosomal Location Plots for Differentially Expressed Genes in A2780cis Cells in Response to Cisplatin Treatment in Normoxia and Hypoxia. Chromosomal location plots demonstrate evenly distributed gene expression changes in normoxia (A) and hypoxia (B). Genes up-regulated in response to cisplatin are represented in yellow, down-regulated in red, and genes whose expression was unchanged in response to cisplatin are represented in white. n = 3



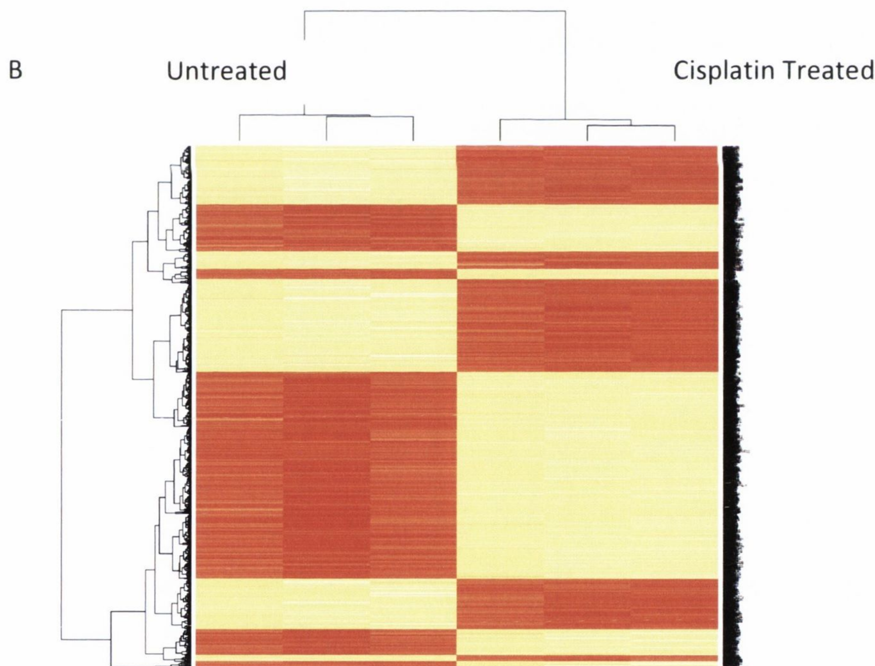
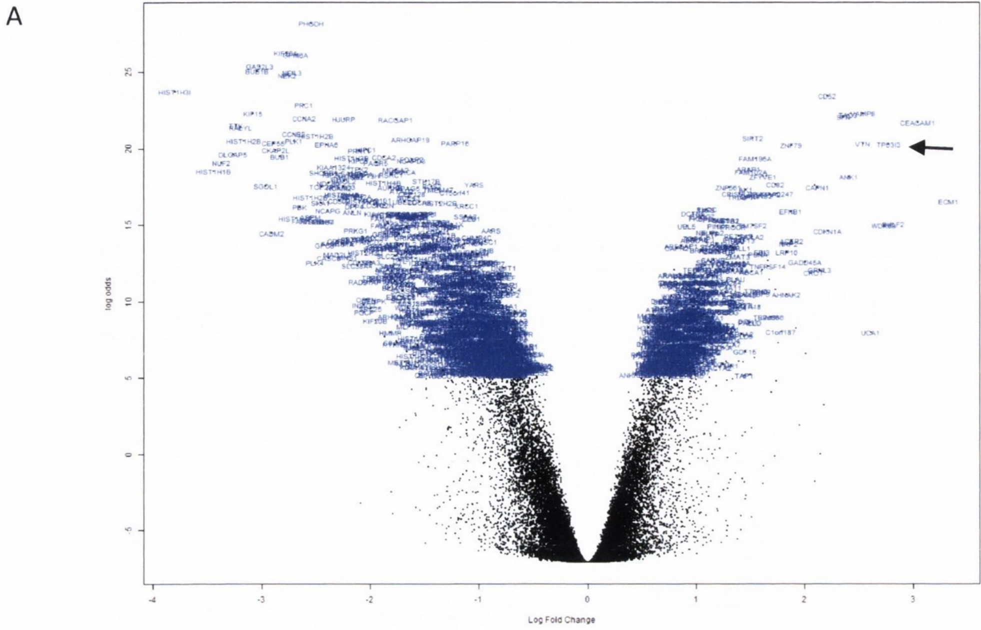


Figure 4.18. Volcano Plot and Heat Map Depicting Gene Expression Changes in Response to Cisplatin in A2780cis in Normoxia. Volcano plot identifies genes which were highly significantly dysregulated such as TXNIP (arrow, A), while heat map provides rapid visual presentation of patterns of gene expression differences (B). n = 3

A



B

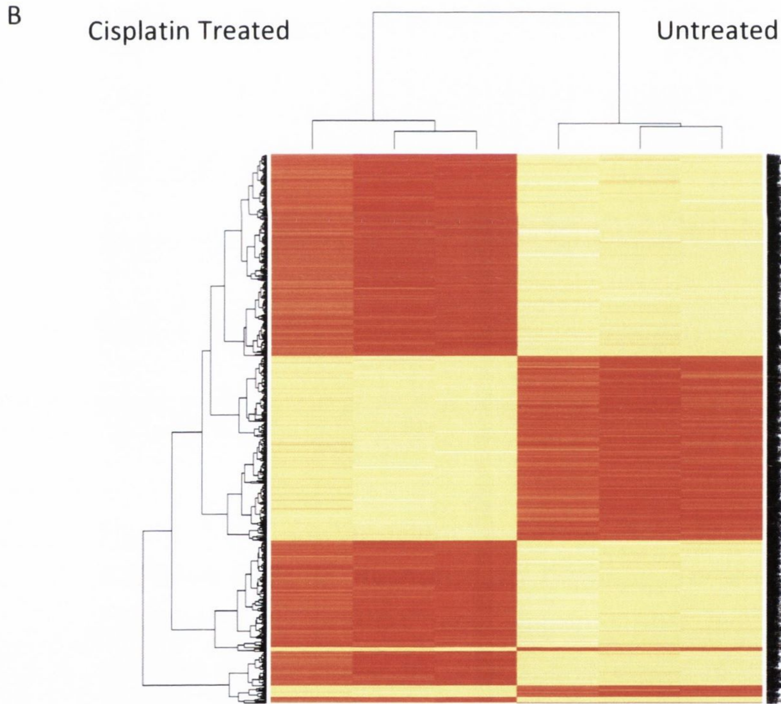


Figure 4.19. Volcano Plot and Heat Map Depictingl Gene Expression Changes in Response to Cisplatin in A2780cis in Hypoxia. Volcano plot (A) identifies genes which are highly significantly altered in response to cisplatin such as CEACAM1 (arrow) while heat map (B) provides rapid visualization of patterns of gene expression differences in cisplatin treated A2780cis. n = 3

Table 4.23. Top Five Significantly Up-Regulated Pathways in A2780cis Treated with Cisplatin in Normoxia.

Pathway	Genes	P-value
p53 signalling	CD82, MDM2, PERP, APAF1, CCNG2, **CDKN1A**, GADD45A, RRM2B, SESN1, SFN, ATM, THBS1, TNFRSF10B, TP53I3, ZMAT3	<0.001
Apoptosis	XIAP, BIRC3, CASP10, IL1R1, IRAK4, PIK3R3, PPP3CA, TNFSF10, TNFRSF10D	<0.001
Ribosome	FAU, RPL11, RPL13A, RPL27, RPL24, RPL30, 18S, RPS21, RPS24, RPL32, RPS29	<0.001
Lysosome	CD63, GNPTAB, CTSL1, CTSO, FUCA1, HGSNAT, IDS, **LAMP3**, MANBA, SCARB2, SLC17A5, SMPD1	0.002
Systemic Lupus Erythematosus	H3F3A, C1R, C1S, HIST1H2AD, HIST1H2BK, HIST1H4A, HLA-DMA, HLA-DPA, C3	0.02

Table 4.24. Top Five Significantly Down-Regulated Pathways in A2780cis Treated with Cisplatin in Normoxia.

Pathway	Genes	P-value
Cell cycle	CDC45, **E2F1**, SKP2, TTK, ANAPC1, ANAPC5, BUB1, BUB1B, **CDK1**, CDC20, CDC25A, **CCNA2**, **CCNB2**, **CCNE2**, **CDK2**, ESPL1, MCM2 – 5, MCM7, ORC1L, PLK1, PKMYT1	<0.001
DNA Replication	**POLD1**, **POLE3**, **POLD3**, **PRIM1**, **PRIM2**, RFC2, RFC3, RFC5, RNASEH2A	<0.001
Oocyte meiosis	FBXO5, SGOL1	<0.001
Mismatch Repair	EXO1	<0.001
Homologous Recombination	RAD51, RAD54L, XRCC2, EME1	<0.001

Table 4.25. Top Three Significantly Up-Regulated Pathways in A2780cis treated with Cisplatin in Hypoxia.

Pathway	Genes	P-value
p53 signalling	CD82, MDM2, APAF1, **CDKN1A**, GADD45A, LRDD, SESN1, SFN, TP53I3, ZAMAT3	<0.001
Bladder Cancer	PGF, HRAS	0.03
ABC Transporters	ABCA1, ABCD1, ABCG4, TAP1	0.03

Table 4.26. Top Three Significantly Down-Regulated Pathways in A2780cis Treated with Cisplatin in Hypoxia.

Pathway	Genes	P-value
Cell Cycle	E2F5, MAD2L1, SKP2, TTK, ANAPC1, BUB1, BUB1B, **CDK1**, CDC20, CDC25C, CCNA2, CCNB1, CCNB2, **CDK2**, ESPL1, MCM3, MCM4, MCM6, ORC6L, PTTG, PLK1	<0.001
DNA Replication	DNA2, **POLA1**, **POLE2**, **PRIM1**, **PRIM2**, RFC1, RFC3, RFC5	<0.001
Oocyte Meiosis	FBXO43, FBXO5, **MAD2L1**, AURKA, IGF1R, SGOL1	<0.001

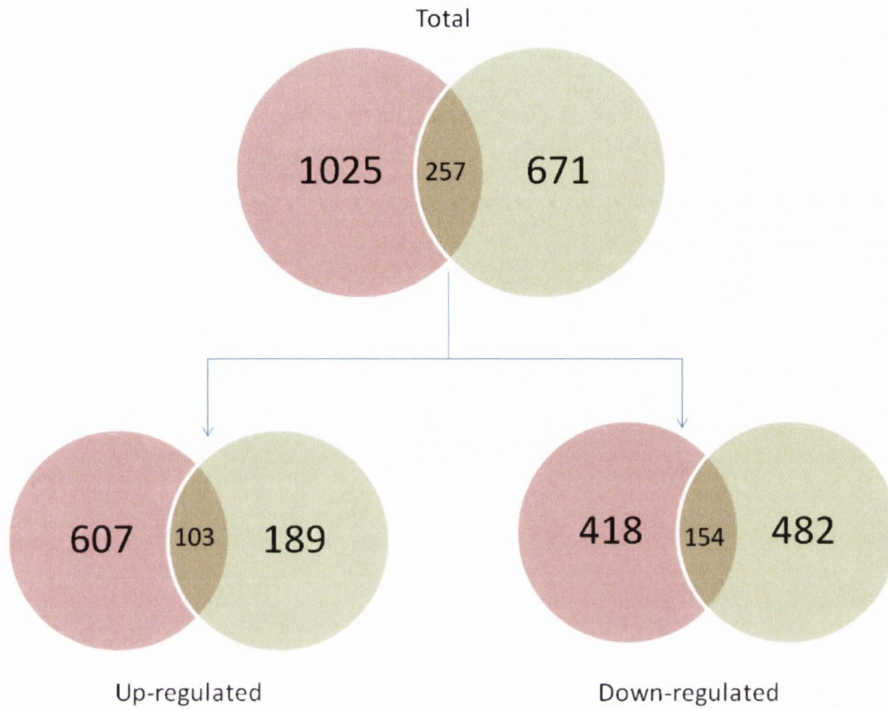


Figure 4.20. Comparison of Gene Expression Changes in A2780cis Treated with Cisplatin in Normoxia and Hypoxia. In total, 257 genes were commonly dysregulated in A2780cis treated with cisplatin in normoxia (pink) and hypoxia (green). Of these, 103 were up-regulated and 154 were down-regulated. n =3

Table 4.27. Pathway Analysis of Up-Regulated Genes in the A2780cis Hypoxic Response to Cisplatin.

Pathway	Genes	P-value
Focal Adhesion	ITGA3, PGF, PXN, PARVA, HRAS, VTN	0.04

Table 4.28. Pathway Analysis of Down-Regulated Genes in the A2780cis Hypoxic Response to Cisplatin.

Pathway	Genes	P-value
Systemic Lupus Erythematosus	GRIN2B, C5, HIST1H2AC, HIST1H2AE, HIST1H2BI, HIST1H2BN, HIST1H4A, HIST2H2AB, HIST2H2AC, HIST2H2BF	<0.001
Valine, Leucine and Isoleucine degradation	ACAT1, BCAT1, ALDH6A1, BCKDHA, HADH, PCCA	0.004
Homologous Recombination	BLM, RAD51L1, RAD54B, BRCA2	0.03
Oocyte meiosis	FBXO43, MAD2L1, ARUKA, CDC25C, **CCNB1**, IGF1R, PTTG	0.05

Table 4.29. Dysregulated Genes in A2780cis Hypoxic Only Response to Cisplatin Linked to Cisplatin Resistance in the Literature.

Gene Symbol	Annotation	Reference	Fold-Change	FDR
LRDD	Leucine rich and death domain containing	[681]	3.48	<0.001
S100A4	S100 calcium binding protein A4	[682]	2.36	<0.001
RAD51C	RAD51 homolog C (S. cerevisiae)	[683]	2.12	<0.001
PCNA	proliferating cell nuclear antigen	[684]	2.97	<0.001
NOTCH1	Notch 1	[685]	2.04	<0.001
SFN	Stratifin	[686]	5.14	<0.001
CDK1	cyclin-dependent kinase 1	[687]	-4.94	<0.001
RGS2	regulator of G-protein signaling 2, 24kDa	[688]	-2.69	<0.001
JUN	jun proto-oncogene	[689]	-2.37	<0.001
FHIT	fragile histidine triad gene	[690]	-2.82	<0.001
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	[335]	-4.91	<0.001
EGR1	early growth response 1	[691]	-2.19	<0.001
CCNB1	cyclin B1	[692]	-2.30	<0.001
GPC3	glypican 3	[693]	-5.35	<0.001
E2F5	E2F transcription factor 5, p130-binding	[678]	-2.06	<0.001
UPRT	uracil phosphoribosyltransferase (FUR1) homolog (S. cerevisiae)	[694]	-2.14	<0.001

The gene lists which were generated in the 'hypoxic only' response to cisplatin in A2780 and A2780cis were compared in order to identify any commonly dysregulated genes or pathways – dysregulation in two cell lines may prove these targets to be stronger biomarker candidates. In total, 30 genes were commonly up-regulated in the two cell lines' 'hypoxic only' response to cisplatin, while 95 genes were commonly down-regulated. The pathway analysis on these lists is displayed in Tables 4.30 and 4.31. Only one pathway was significantly up-regulated based on the 30 commonly up-regulated genes – p53 signalling. Four separate pathways were significantly down-regulated and are summarized in Table 4.31. Genes which were contained within more than one pathway are represented once on the table.

Table 4.30. Significant Commonly Up-Regulated Pathways in the A2780 and A2780cis 'Hypoxic Only' Response to Cisplatin.

Pathway	Genes	P-value
p53 signalling	CD82, APAF1, SFN	<0.001

Table 4.31. Significant Commonly Down-Regulated Pathways in the A2780 and A2780cis 'Hypoxic Only' Response to Cisplatin.

Pathway	Genes	P-value
Systemic Lupus Erythmatosus	HIST1H2AC, HIST1H2AE, HIST1H2BI,HIST1H4L, HIST2H2AB	0.02

A literature search was carried out to identify those genes identified on pathway analysis which have been previously linked to cisplatin resistance. One gene identified on the pathway analysis was previously linked to cisplatin resistance - SFN. The entire lists of commonly up- and down-regulated genes were then examined in order to determine if genes which had not been represented in the pathway analysis

were associated with cisplatin resistance. This identified one further gene (Table 4.32).

Table 4.32. Commonly Dysregulated Genes in the A2780 and A2780cis 'Hypoxic Only' Response to Cisplatin which have Been Directly Linked to Cisplatin Resistance.

Gene	Annotation	Reference	Alteration in Expression
SFN	Stratifin	[686]	Up
E2F5	E2F transcription factor 5, p130-binding	[678]	Down

4.3.9 Discussion: The Effect of Hypoxia on Response to Cisplatin in A2780 and A2780cis

In Chapter 3, drug-treating A2780 cells in hypoxia induced nearly 10-fold resistance to cisplatin compared to A2780 cells which were treated in normoxia. Gene expression data generated by microarrays was explored in order to identify genes or pathways which may contribute to the increased resistance.

The top up-regulated pathways in A2780 treated with cisplatin in normoxia included p53 signalling and lysosome. These pathways were not surprising as cisplatin treatment has been shown to induce apoptosis. Indeed, mutation in the p53 gene leading to loss of function has been shown to cause resistance to cisplatin [695,696], and adenovirus-mediated transfer of exogenous p53 to a p53-null lung cancer cell line induced apoptosis and increased the cells' sensitivity to cisplatin [697]. The microarray analysis identified a number of molecules related to p53 signalling which were up-regulated. Fas is a member of the TNF receptor superfamily which induces cell death on stimulation by a ligand [698]. Activation of Fas results in the formation of a multimolecular protein complex known as the death-inducing signalling complex (DISC) and involves the proteins FADD (Fas-associated death domain), and caspase 8 [698]. Previous studies have shown up-regulation of Fas on cells treated with cisplatin [699,700], although cisplatin can induce apoptosis independent of Fas activation [701]. Ovarian tumours resistant to chemotherapy generally express less Fas than chemosensitive tumours [702].

CDKN1A (p21) is a cyclin-dependent kinase inhibitor and is induced by cisplatin as part of the apoptotic response [703,704]. Underexpression of CDKN1A has been linked to cisplatin sensitivity [705], whereas CDKN1A overexpression was shown to increase cisplatin resistance [706]. In ovarian cancer cell lines, cytoplasmic CDKN1A expression was associated with cisplatin resistance, while siRNA-mediated CDKN1A knock down sensitized the cells [707]. Similarly, in prostate carcinoma, siRNA directed against CDKN1A in testicular cancer also sensitized the cells to cisplatin treatment [708]. SU5416, a VEGF receptor-selective inhibitor has been shown to

ameliorate the effects of cisplatin through down-regulation of CDKN1A, along with other cell cycle-related proteins [308], thus demonstrating its potential as a therapeutic target.

Lysosomes are cellular organelles which contain a number of enzymes associated with cellular degradation. Cathepsins are protein-degrading enzymes located within the lysosome [709]. Cathepsin L has been implicated in a number of different carcinogenic processes including apoptosis, proliferation, angiogenesis and invasion [709]. Expression of cathepsin L has been correlated to tumour grade, recurrence and poorer survival in urothelial carcinoma [710]. Cathepsin L has been explored as a potential diagnostic biomarker for ovarian cancer – serum levels were significantly higher in patients with malignant disease compared with benign or healthy controls – however, its sensitivity and specificity is low [711,712]. In addition, a further study found that supplementation with an anti-oxidant, Vitamin B, following chemotherapy resulted in reduced cathepsin activity [713]. A previous study in melanoma has also identified cathepsin L as up-regulated in hypoxia [714]. Its potential usefulness as a therapeutic target was demonstrated in glioma cells. Transfection of cisplatin-resistant glioma cells with the oncolytic virus parvovirus resulted in significant cell death with activation of cathepsins B and L [715]. The lysosomal-associated membrane proteins 2 and 3 (LAMP2 and LAMP3) were also up-regulated following cisplatin treatment in normoxia in A2780. *In vitro* studies have identified LAMP2 as a marker of cisplatin sensitivity. Reduced LAMP2 expression was shown to be associated with cisplatin resistance in an ovarian cancer cell line [716], however over-expression of LAMP2 has also been shown to confer resistance to cisplatin in colon and breast carcinoma cell lines [717]. LAMP3 has not yet been associated with response to cisplatin, however, LAMP3 over-expression has been associated with metastasis in cervical cancer [718].

In A2780 cells treated in hypoxia, some markers of sensitivity to cisplatin were also up-regulated following cisplatin treatment. PMAIP1 (phorbol-12-myristate-13-acetate-induced protein 1/NOXA) is a pro-apoptotic protein which is activated by

p53 [719]. PMAIP1 has been shown to be induced by HIF-1α, and to induce cell death in association with release of cytochrome c from the mitochondria and caspase activation [720]. It has been shown to be induced by proteasome inhibitors such as bortezomib, after which it induces apoptotic cell death in melanoma [721]. Similarly, it is induced by diallyl trisulfide, an organosulfur molecule which modulates drug metabolism systems, and facilitates cell death in prostate carcinoma [722]. PMAIP1 has previously been shown to be inducible in response to cisplatin treatment in gastric cancer [723], breast cancer [724] and cervical cancer [725] and has been shown to be involved in the synergistic effects induced by the Bcl inhibitor ABT-737 when combined with cisplatin treatment in head and neck squamous cell carcinoma cells [726]. A study by Difeo *et al.* identified a therapeutic potential for PMAIP1 inhibition – they showed that targeting the tumour suppressor KLF6-SV1 in a mouse model of ovarian cancer resulted in increased survival times due to increased apoptosis mediated through PMAIP1 [32].

In A2780 and A2780cis treated in normoxia and hypoxia with cisplatin, there was down-regulation of many genes related to DNA replication and the cell cycle. There was wide-spread down-regulation of DNA polymerase enzymes. Cisplatin treatment has previously been shown to inhibit the action of DNA polymerases [253,727]. Indeed, increased DNA polymerase beta activity has been observed in a cisplatin-resistant leukaemia cell line [728]. In addition, increased DNA polymerase alpha activity has been shown in a cisplatin resistant ovarian cancer cell line, A2780CP [729] and cisplatin resistant colon carcinoma cell line, HCT8 [730]. siRNA-mediated down-regulation of DNA polymerase beta has been shown to sensitize cells to the cytotoxic activity of cisplatin [731]. Primase 1 and 2 (PRIM1 and PRIM2) enzymes were also down-regulated in response to cisplatin. Primase enzymes work in conjunction with DNA polymerase to synthesise new DNA strands and a functioning primase is required for DNA synthesis and repair to occur following DNA damage [732]. Thus, reduced levels of primase, as observed in this study, limit the repair of DNA following genotoxic stress and lead to triggering of apoptosis. Indeed, several

studies in a number of different cancers have shown that inhibition of primase leads to apoptosis [733-735].

Down-regulation of cell cycle components was also observed in cells treated with cisplatin in both hypoxia and normoxia. Cell cycle progression is positively regulated by cyclin dependent kinases (CDKs) which are activated by cyclins [22]. There was down-regulation of both CDKs and cyclins in response to cisplatin in normoxia and hypoxia in both cell lines. CDK1 is responsible for cell cycle progression through G_2 to M phase, while CDK2 is responsible for cell cycle progression through G_1 to S phase [22]. In both A2780 and A2780cis, CDK1, 2 and cyclins A2, B2, E1 and E2 were all down-regulated in response to cisplatin, while CDK1, 2 and cyclins A2, B1 and B2 were down-regulated in the hypoxic response. Cisplatin has previously been shown to induce cell cycle arrest in a number of cell lines. A study of bladder carcinoma showed that cisplatin and another cytotoxic drug gemcitabine, were able to induce cell cycle arrest at G_1 and G_2 phases, and in addition, they showed that cisplatin induced apoptosis only occurred in p53-wild type cells [736]. A study of cervical carcinoma also showed cell cycle arrest following treatment with cisplatin [737]. Overexpression of CCNA2 has been linked to chemoresistance and poor prognosis in endometrial carcinoma [738]. Similarly, overexpression of CCNE1 promotes cell survival in ovarian cancer cell lines [739].

The 'hypoxic only' response to cisplatin – i.e. the genes differentially expressed by the response to cisplatin in hypoxia but not normoxia were examined in order to identify genes which may be associated with resistance, rather than just the normal response to cisplatin-induced DNA damage as discussed above.

Notch signalling is a highly conserved pathway with functions in embryogenesis and tissue self-renewal [740]. NOTCH1 is a receptor for the ligands Jagged 1 and 2 and Delta-like 1, 3 and 4 [740]. It was up-regulated in the A2780 'hypoxic only' response to cisplatin. In cervical cancer Notch1 expression has been observed both in tumour samples and cell lines [685]. It was shown to interact with NF-KB pathway and

antagonize cisplatin-mediated cytotoxicity [685]. Another study in head and neck cancer associated NOTCH1 expression with resistance to cisplatin [741]. siRNA-mediated targeting of NOTCH1 in ovarian cancer cell lines was shown to inhibit cell proliferation [742]. Similarly, inhibitors of gamma-secretase, the enzyme that cleaves the NOTCH1 intracellular domain to initiate signalling, were also shown to inhibit cell proliferation and stimulate apoptosis [743]. Treatment of ovarian cancer cells with the novel therapeutic agents xanthohumol and withaferin A resulted in reduced NOTCH1 mRNA and protein expression, reduced cell proliferation, cell cycle arrest and apoptosis [744,745]. Thus NOTCH1 is a potentially useful therapeutic target for the treatment of ovarian cancer. A study of ovarian cancer found that NOTCH1 expression was higher in cancer tissue compared to normal tissue, and that its expression was correlated with differentiation and tumour stage – poorly differentiated tumours expressed higher levels of NOTCH [746].

BCl2l1 (BCl2 like 1) is an anti-apoptotic protein which was up-regulated in the A2780cis 'hypoxic only' response to cisplatin. Expression of BCl2l1 has been linked to the pathophysiology of many different cancers through its effects on apoptosis including Hodgkin's Disease [747], breast cancer [748] and ovarian cancer [749]. In ovarian cancer BCl2l1 expression has been widely studied. BCl2l1 expression is higher in malignant ovarian tissue compared to benign, and correlated with progesterone receptor levels [749]. Lower BCl2l1 in ovarian tumours compared to normal ovarian tissue has also been observed, indicating a potential alternative role apart from anti-apoptosis [750]. In addition, no difference in BCL2l1 expression has been observed between serous and mucinous or clear cell ovarian tumours [751,752]. It has also been linked to pathology associated with stem cells – it is up-regulated by the stem cell marker Piwil2 [753]. Previous microarray analysis of serous ovarian cancer identified BCL2l1 as over-expressed [754], and its expression has been linked with a shorter progression free survival [755,756]. BCl2l1 expression has been associated with cisplatin resistance in ovarian cancer cell lines [757], and is expressed in protein sampled from ascetic fluid [758]. Lack of down-

regulation of BCL2l1 in response to cisplatin, as observed in this study, was shown to correlate with cisplatin resistance [759]. In addition, hypoxia has been shown to increase BCL2L1 expression [760]. BCl2l1 has demonstrated potential usefulness as a therapeutic target. Inhibition of BCl2l1 with siRNA in an ovarian cancer cell line has been shown to induce anoikis – apoptosis induced from anchorage-independent growth – and reduce tumourgenicity in cells transplanted to *in vivo* models [761]. It has also been implicated in the action of novel therapeutics such as curcumin [762] and the green tea extract, epigallocatechin-3-gallate [763].

MAD2L1 is a highly conserved spindle checkpoint protein whose main function is to ensure faithful chromosome segregation during mitosis in conjunction with other proteins such as BUB1 [764]. It was down-regulated in the A2780 'hypoxic only' response to cisplatin. MAD2L1 expression has been associated with resistance to cisplatin in several cancer types. In nasopharyngeal carcinoma cell lines, low MAD2L1 expression was associated with cisplatin, and could be overcome with MAD2L1 transfection [765]. Similarly, in gastric carcinoma, silencing of MAD2L1 induced cisplatin resistance [766]. MAD2L1 expression has been shown to sensitize cancer cells to cisplatin through activation of the MEK pathway [767] and through interference with the DNA repair pathway [768]. Prencipe *et al*. examined MAD2L1 expression in ovarian cancer cells exposed to hypoxia, and found that MAD2L1 was down-regulated – although this was independent of promoter methylation – and validation of this work in ovarian tissue samples confirmed a reciprocal relationship between MAD2L1 protein expression and expression of CA9, a surrogate marker for hypoxia [769].

E2F1 was down-regulated in the 'hypoxic only' response in A2780 and A2780cis cell lines. E2F1 is a transcription factor which has a role in activating genes involved in cell cycle progression, apoptosis and senescence, and is negatively regulated by the retinoblastoma protein, pRb [770]. Its expression has long been shown to induce apoptosis mediated by p53 [771]. DNA damaging agents and other stresses such as hypoxia [772] have been shown to induce expression of E2F1, and this expression

has been shown to be mediated through the ATM/ATR kinase pathway [773]. Its expression in hypoxia is regulated by another member of the E2F family, E2F6 [774]. In addition, E2F1 depletion has been associated with increased tumour angiogenesis through VEGF activation [775]. Over-expression of E2F1 has been directly linked to cisplatin sensitivity [776]. In addition, E2F1 has been shown to play an important role in cisplatin-mediated nephrotoxicity in *in vitro* and *in vivo* studies [777]. However, studies of E2F1 expression in ovarian carcinoma have shown E2F1 to be up-regulated in serous carcinomas compared to borderline tumours [778], however previous studies correlated E2F1 expression with tumour stage and grade, thus highlighting its potential as a prognostic indicator for ovarian cancer [779,780].

4.3.10 Hypoxic Biomarker Discovery

This chapter has provided a comprehensive analysis of the transcriptional changes induced by exposure of ovarian cancer cells to hypoxia. We have explored the unique genetic changes which occur in cells receiving drug treatment in hypoxia, as well as identifying common genetic alterations between cisplatin-induced and hypoxia-induced chemoresistance. There is a paucity of literature utilizing microarray data to glean new information regarding the role of hypoxia in ovarian cancer chemoresistance. For the first time, we have used a paired platinum resistance cell line model to investigate the transcriptional alterations invoked by hypoxic exposure. We have identified many novel biomarkers, including markers not previously associated with ovarian cancer, hypoxia, and/or chemoresistance. As mentioned previously, there are relatively few surrogate markers of tumour hypoxia, and HIF-1α itself has certain problems due to difficulties in isolation of its protein and conflicting evidence in the literature regarding its significance in terms of survival. Therefore, as hypoxia is proving to be such an important mediator of chemoresistance, it is imperative to discover consistent markers of its presence. This study has identified many markers influenced by hypoxia, and linked them to platinum resistance in ovarian cancer and others. In Chapter 5, we provide an initial evaluation of their expression in a cohort of ovarian tumour samples.

4.3.11 Summary

The experiments examining the effect of hypoxia on the response of A2780 and A2780cis to cisplatin revealed a number of findings:

1. Similar pathways and a large number of genes are commonly dysregulated both in normoxia and hypoxia and in both cell lines.
2. Many gene changes were as would be expected in a cell line faced with the genotoxic stressors of cisplatin and hypoxia.
3. A number of changes in hypoxic cells were identified which did not entirely fit with a normal response to cisplatin – these genes were often markers of resistance to cisplatin, and hold potential prognostic/therapeutic value.

Chapter 5

Evaluation of Novel Hypoxic Biomarkers of Ovarian Cancer

5.1 Introduction

Based on the array analysis of Chapter 4, four potential biomarkers of hypoxia and ovarian cancer were selected for validation in a cohort of ovarian tumour samples. In the array analysis, a number of hypoxia-related biomarkers of ovarian cancer were identified that were altered in (i) both cisplatin resistance and hypoxia resistance, (ii) general response to hypoxia in one or both cell lines and (iii) response to cisplatin in hypoxia in one or both cell lines. Markers were chosen based on their potential to serve as markers of chemoresistance in ovarian cancer, due to their expression patterns in cells exposed to hypoxia/cisplatin/both, and based on information available in the literature on their connection to cisplatin resistance.

i) Angiopoietin-like 4 (ANGPTL4) – Up-regulated in A2780 cells exposed to hypoxia (8.65-fold); A2780cis cells exposed to hypoxia (3.48-fold)

ii) BDNF – Up-regulated in A2780 cells exposed to hypoxia (2.69-fold)

iii) HER3 – Up-regulated in A2780cis compared to A2780 (normoxia, 2.71-fold); A2780 cells exposed to hypoxia (3.44-fold); down-regulated in A2780 'hypoxic only' response (i.e. gene expression was altered when cells were treated with cisplatin in hypoxia, but not when treated in normoxia) to cisplatin (-3.2-fold)

iv) HIF-1α – although the expression of HIF-1α was not altered in these samples, its expression is widely studied in the literature in terms of hypoxia

A fifth biomarker, MAD2L1 was validated in the hypoxia matrix, however, its expression was not determined in the tissue sample cohort, as its expression in the same cohort was previously determined by one of our collaborating groups [781]. On the array analysis it was down-regulated in the A2780 response to cisplatin in normoxia (-2.01-fold) and the A2780 and A2780cis response to cisplatin in hypoxia (-3.41-fold and -4.91-fold respectively).

5.1.1 Angiopoietin-like 4

The ability of tumours to produce their own blood supply by stimulating the growth of blood vessels (angiogenesis) is one of the hallmarks of cancer [16]. Tumour angiogenesis is stimulated in a number of ways. Vascular endothelial growth factor (VEGF) is released by tumour cells leading to proliferation of endothelial cells, platelet derived growth factor (PDGF) released by activated platelets stimulates perivascular cells, and angiopoietins signal through the insulin-like growth factor pathway [41]. In 2000, angiopoietin-like 4 (ANGPTL4) was discovered and designated as hepatic fibrinogen/angiopoietin-related protein (HFARP) by Kim *et al.* [782]. In the same year, two other groups discovered the same protein and named it fasting induced adipose factor (FIAF) [783] and PGAR (PPAR gamma angiopoietin related) [784]. Studies in a variety of malignant and non-malignant cell types have shown ANGPTL4 to have a number of functions including prevention of apoptosis [782], regulation of fat and carbohydrate metabolism [785,786], induction of angiogenesis [787], inhibition of angiogenesis [788,789], inhibition of metastasis [790] and facilitation of metastasis [791]. Thus, ANGPTL4 is seen to have paradoxical functions.

In vitro experiments have shown ANGPTL4 to be methylated in gastric cancer [792] and breast cancer [793] cell lines and transfection of hepatocellular carcinoma cells with recombinant ANGPTL4 was shown to reduce cell proliferation and reduce tumour growth in mice [794]. The angiogenesis inhibitor ZD6474, which acts via the VEGF receptor as well as the epidermal growth factor receptor (EGFR), has been shown to up-regulate expression of ANGPTL4 while reducing tumour growth and metastasis in mice [795]. However, a study examining the gene expression profiles of breast tumour metastases found that ANGPTL4 was up-regulated in the tumour metastases [796], and high expression of ANGPTL4 has been associated with poor survival in oral tongue squamous cell carcinoma [797]. ANGPTL4 has been suggested as a diagnostic biomarker for renal cell carcinoma [798]. In addition, up-regulation of ANGPTL4 has been implicated in angiogenesis in Kaposi's sarcoma

[799] and invasion in gastric carcinoma [800]. In oesophageal carcinoma, ANGPTL4 expression correlates with invasion and poorer overall survival [801], while in endometrial cancer, its expression correlates with invasion and better overall survival [802]. Thus there is conflicting evidence in the literature on the function of ANGPTL4 and its effects in different cancer types. Currently, there is no evidence in the literature on ANGPTL4 expression and its relationship with cisplatin resistance, and there is very limited information on ANGPTL4 expression in ovarian cancer. One study has shown ANGPTL4 to be expressed in the ovarian cancer cell line SKOV3, and in xenograft tumours derived from it [803].

5.1.2 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a growth factor which binds the TrkB receptor resulting in enhanced excitement of neurons and protection against cellular stresses [804]. BDNF over-expression has been observed in many cancer types including hepatocellular carcinoma [805]. A study of colorectal carcinoma found BDNF to be produced in the cellular response to stress, and suppressed apoptosis [806]. Hypoxia has been shown to induce BDNF expression *in vitro* and *in vivo* [807,808]. In breast cancer, BDNF was found to be over-expressed when compared to normal tissue, and was associated with a number of features of tumour aggressiveness such as lymph node metastasis and reduced progression-free and overall survival [809]. In a study of cervical cancer, BDNF was up-regulated in cancer tissue and was associated with low FIGO (International Federation of Obstetricians and Gynaecologists) stage and reduced features of invasion [810]. Indeed, over-expression of BDNF in a normal endothelial cell line stimulated many tumorigenic properties such as increased cell proliferation, survival, invasion, and angiogenesis [811]. In fact, BDNF has been shown to stimulate production of VEGF in a HIF-1α-dependent manner [812]. BDNF has recently been shown to play a role in cisplatin resistance in head and neck squamous cell carcinoma through affecting the ratio of pro- and anti-apoptotic proteins, and through up-regulation of enzymes such as glutathione-s-transferase and the multidrug resistance protein, MDR-1 as well as

activation of the Akt and MAPK signalling pathways [516]. In ovarian cancer, BDNF has been shown to increase cell proliferation and migration through activation of the Akt pathway [813]. A study of BDNF expression in ovarian cancer tissues found no difference in mRNA expression between malignant and non-tumour tissue, however high levels of its receptor, TrkB were noted and correlated with poor survival [814]. Inhibition of BDNF has been shown to induce apoptosis in hepatocellular carcinoma and breast cancer, thus demonstrating its therapeutic potential [805,815]. Similarly, the Akt inhibitor perifosine has been shown to exert its effects, at least partially, through down-regulation of BDNF [816].

5.1.3 HER3

HER3 (ERBB3) is a member of the epidermal growth factor receptor (EGFR) family. Activation of HER3 through ligand binding stimulates its dimerization with another member of the ERBB family – HER3 has no intrinsic enzymatic (tyrosine kinase) activity of its own [817]. Following activation, members of the EGFR family stimulate several signalling pathways within the cell including Ras/MAPK, PI3K/Akt, Src/NFκB, PAK-1/Rac and catenin/cytoskeleton [818]. HER3 is frequently co-expressed with HER2 in breast cancer [819]. Knockdown of HER3 with siRNA has been shown to decrease metastatic potential [820]. In colon carcinoma, elevated HER3 is associated with reduced progression free survival in tumour samples, and knockdown of HER3 in cell lines resulted in reduced proliferation, migration and invasion of cells [821]. Signalling through HER3 has been linked to resistance to a number of therapeutic agents including gefitinib in lung cancer [822] and paclitaxel in breast cancer [823]. A study of glioblastoma has identified HER3 expression as prominent in a putative cancer stem cell population [824]. *In vivo* studies in ovarian cancer have identified that HER3 is involved in stimulating cell proliferation, and that targeting HER3 with siRNA reduces cell growth and increases progression-free and overall survival in mice [21]. In breast cancer, the HER2/HER3 heterodimer is associated with decreased survival [825], and oncogenic signalling through HER3 can attenuate the effects of HER2-mediated therapies [826]. Four alternative splice

variants of HER3 have been identified in ovarian cancer cell lines and normal tissues [827]. Previous cDNA microarray studies have identified over-expression of HER3 in serous ovarian adenocarcinoma tissue [828]. Other studies of EGFR expression in ovarian cancers have shown that EGFR is expressed in only a small subset of tumours [829,830], however its expression has been associated with poorer survival [830]. Interestingly, a study in ovarian cancer cell lines showed that treatment with heregulin, a ligand for HER3, and EGF significantly decreased HER3 expression in some cell lines, while HER3 expression was reduced to a lesser amount in others [831]. This was associated with activation of the MAPK pathway, and was also linked to sensitivity to the monoclonal α-HER2 antibody, pertuzumab, which prevents dimerization of HER2 molecules.

5.1.4 MAD2L1

MAD2L1 is a member of the spindle checkpoint assembly of proteins [832] whose function is to ensure faithful chromosome segregation during mitosis [764]. Loss of one MAD2L1 allele results in a defective spindle checkpoint and inappropriate chromosome segregation [833]. Over-expression of MAD2L1 has been observed in gastric cancer and colon cancer, and is associated with metastasis [834,835]. A previous microarray study of endometrial carcinoma identified MAD2L1 as over-expressed in cases with lymph node positivity [836], while in thyroid cancer, over-expression of MAD2L1 was observed in anaplastic carcinoma, a particularly aggressive subtype [837]. Expression of MAD2L1 in hepatocellular carcinoma is associated with tumour grade and reduced overall survival [838]. Reduced expression of MAD2L1 in ovarian cancer cell lines is associated with a loss of checkpoint control [839] and low MAD2L1 expression has been observed in the chromophobe subtype of renal carcinoma – which is associated with chromosomal monosomy [840], and in a large proportion of marginal zone B-cell lymphomas due to hypermethylation [841]. Knockdown of MAD2L1 in cervical cancer cells was shown to result in chromosome loss in mitosis and reduced viability [842]. However, in gastric cancer, inhibition of MAD2L1 with siRNA was shown to reduce

apoptosis following treatment with the cytotoxic drugs vincristine and cisplatin [766]. Similarly, a study of MAD2L1 in testicular carcinoma correlated high MAD2L1 expression with sensitivity to cisplatin mediated through the ERK signalling pathway [767]. This may be mediated, at least in part, by interference of MAD2L1 with DNA repair proteins [768]. An *in vitro* study by Sudo *et al.* showed that over-expression of MAD2L1 in an ovarian cancer cell line which is low in endogenous MAD2L1, OVCA432, increased sensitivity to paclitaxel [843]. Indeed, high MAD2L1 expression induces cellular senescence, and paclitaxel resistance [844]. A study by our collaborators in University College Dublin showed that low MAD2L1 expression is associated with resistance to paclitaxel in ovarian cancer cell lines, and low MAD2L1 expression in ovarian tumours is associated with reduced progression free survival [781].

5.1.5 HIF-1α

Hypoxia-inducible factor 1α (HIF-1α) is a transcriptional activator which is stabilized in hypoxic conditions [845]. There have been a large number of articles which investigate its potential utility as a prognostic or therapeutic biomarker in cancer. *In vivo* studies have linked HIF-1α expression to tumourigenesis, and knock-down of HIF-1α has been shown to reduce tumour growth and metastasis [846]. The potential usefulness of HIF-1α as a monitor for response to therapy was demonstrated in a study of liver cancer, which showed reduced HIF-1α levels in the sera of patients who had undergone transcatheter arterial chemoembolization (TACE) to restrict tumour blood supply [847]. Similarly, HIF-1α has also been shown to have potential as a prognostic biomarker – its expression predicts progression free survival in rectal cancer [848] and in pancreatic adenocarcinoma it is associated with reduced overall survival [849]. In addition, polymorphism analysis of HIF-1α in non-small cell lung cancer correlated certain polymorphisms of HIF-1α with prognosis [850].

HIF-1α and many of its target genes have been identified as up-regulated in clear cell ovarian carcinoma [851], and in serous ovarian carcinoma its expression was linked with reduced progression free survival and overall survival [200]. It has also been identified as up-regulated in platinum-resistant ovarian tumours [852]. HIF-1α expression is higher in poorly differentiated ovarian tumours where its expression correlates with vascular endothelial growth factor (VEGF) [853]. Interestingly, high HIF-1α expression was associated with increased progression-free survival in suboptimally debulked ovarian cancer patients who subsequently underwent platinum/taxane based chemotherapy [201].

The expression of surrogate markers of HIF-1α such as carbonic anhydrase 9 (CA9) and glucose transporter 1 (GLUT-1) has also been examined in ovarian tumours. Expression of CA9 is present in all types of ovarian neoplasm, however it is higher in mucinous tumours [854]. Another study found that expression of CA9 was higher in mucinous and endometrioid ovarian tumours and linked it to shorter overall survival [855]. GLUT-1 is over-expressed in invasive tumours compared to borderline [856,857] and has been linked to tumour grade [858]. In addition it has been shown to be over-expressed in poorly differentiated tumours and associated with shorter progression free survival [859]. Previous work by our group examined expression of vascular endothelial growth factor (VEGF) in ovarian carcinomas, and found that low expression of VEGF conferred a survival advantage [860].

5.1.6 Aim

The aim of this chapter was two-fold:

i) To validate the results of the hypoxia matrix, and to evaluate the effect of differing lengths of hypoxic exposure on gene expression

ii) To evaluate some of the biomarkers identified in Chapter 5 in a cohort of ovarian tumour samples

5.2 Methods

5.2.1 Samples – Cell Lines

The quality of gene expression data from arrays can be variable depending on the platform used and thus real-time polymerase chain reaction (PCR) is often employed to validate microarray results. Markers which were selected from the microarray analysis for further investigation were validated using real-time Reverse Transcription-PCR (RT-PCR). In addition, as only a very small subset of the hypoxia matrix had been interrogated on the microarrays, the expression of these genes was determined in alternative matrix conditions. Expression of four genes (ANGPTL4, BDNF, HER3 and MAD2L1) was validated using Taqman RT-PCR on the samples interrogated on the Affymetrix arrays as well as some other points of the hypoxia matrix, summarized in Table 5.1. Initial experiments revealed some inconsistency in C_T values between some biological replicates within sample groups, therefore all samples were 'cleaned up' by washing in ice-cold isopropanol followed by reconstitution in RNase-free water, in order to remove any residual contaminating salts following RNA extraction. Following clean-up, samples of the same type all displayed expression levels within one C_T of each other. All experiments were carried out for n=3. 18S, GAPDH (glyceraldehydes) and β-actin were evaluated as endogenous controls in a range of samples, and β-actin was chosen due to its low variation in expression.

5.2.2 Samples – Tumour Tissue

Expression of four genes (ANGPTL4, BDNF, HER3 and HIF-1α) was examined in a cohort of 35 serous papillary ovarian tumour samples. The patient characteristics are summarized in Table 5.2. Tumour regions were macrodissected from formalin fixed paraffin embedded (FFPE) sections and RNA was extracted using the RNeasy FFPE kit (Qiagen, UK). Sectioning and RNA isolation was carried out by one of our group's collaborators in University College, Dublin. RNA was converted to cDNA and Taqman PCR was carried out as described in Chapter 2. A list of the probes is

displayed in Table 5.3. GAPDH was used as an endogenous control for these samples, as β-actin displayed an unacceptable degree of variation between samples.

Table 5.1. List of Samples Used for Validation Based on the Hypoxia Matrix. Expression in these samples was determined for both A2780 and A2780cis.

Sample Type
Normoxia, Untreated
Hypoxia, Untreated
Normoxia, Cisplatin Treated
Hypoxia, Cisplatin Treated
Acute hypoxia (4 hours), Untreated
Chronic hypoxia (5 days). Untreated

Table 5.2. List of Patient/Tumour Characteristics

Histology	Stage	Grade	Number
Serous Adenocarcinoma	2	3	2
Serous Adenocarcinoma	3	1/2	3
Serous Adenocarcinoma	3	2	7
Serous Adenocarcinoma	3	2/3	2
Serous Adenocarcinoma	3	3	15
Serous Adenocarcinoma	4	1/2	1
Serous Adenocarcinoma	4	2/3	1
Serous Adenocarcinoma	4	3	4

Table 5.3. List of Probes for Taqman® PCR. All probes were obtained from Applied Biosystems, USA.

Probe	Assay Code
ANGPTL4	Hs01101127_m1
BDNF	Hs00380947_m1
HER3	Hs00176538_m1
HIF-1α	Hs00153153_m1
MAD2L1	Hs03063324_g1
ACTB	4333762F
GAPDH	4326317E

5.2.3 Sample Grouping and Relative Quantitation

Patient samples were grouped as outlined in Table 5.4 according to their response to platinum/taxane chemotherapy obtained from follow-up data. Progression-free survival (PFS) and overall survival (OS) were determined as the number of months following completion of platinum/taxane chemotherapy until recurrence or death respectively.

Relative gene quantitation for both cell line and tissue samples was determined using the $2^{-\Delta\Delta Ct}$ method, as described in Chapter 2. For cell line samples, the calibrators (reference samples) used were either A2780 (normoxia, untreated) or A2780cis (normoxia untreated). For all patient samples, the calibrator was the group of patients deemed to have responded to chemotherapy (Table 5.4).

Once fold-changes had been determined, statistical significance was determined by a one-sided Student's t-test of the hypothesis that the fold change = 1, with $p<0.05$ determined as significant. Data was graphed and statistical analysis carried out using GraphPad Prism Software.

Table 5.4. Classification of Tumour Samples Based on Response to Chemotherapy.

Class	Response	Number of Samples
Responders	Recurrence >12 months following completion of chemotherapy	17
Partial Responders	Recurrence between 6 – 12 months following completion of chemotherapy	11
Non-Responders	Recurrence <6 months following completion of chemotherapy	7

5.2.4 Kaplan-Meier Survival Analysis

Relationships between gene expression and progression free survival (PFS) and overall survival (OS) were analysed using Kaplan-Meier survival curves on Prism software. This method allows for analysis of data where some data might be censored – the end-point has not yet occurred (e.g. death). This analysis is based on several assumptions outlined by Bland and Altman [861]. Firstly, it is assumed that the censored patients have the same survival prospects as those who are followed – in this study, patient data is censored if the patient has not yet progressed or died. Secondly, it is assumed that survival probabilities are the same for patients recruited early and late in the study – this is not relevant for this study as all patients were 'recruited' at the same time. Finally, it is assumed that the event occurred at the time specified. This is true for certain events e.g. date of death is relatively unambiguous. However, time of progression is more complex, as it is not always clear from patient records exactly when progression occurred. In the case of ovarian cancer management, Ca125 levels are checked regularly, at 6-month intervals. A rising Ca125 can be indicative of recurrent disease, however, not all clinicians conclude that a patient has recurred until they are symptomatic – thus it can be unclear exactly when a recurrence has taken place. We determined time of

recurrence as when it was identified on radiology and logged in the patient's notes by their consultant. Curves are generated using a product limit formula and compared using the log rank test, a non-parametric test which can be used to compare groups containing censored data. It tests the null hypothesis that there is no difference between the groups in the probability of an event at any given time point, and is based on the same assumptions as those used to generate a survival curve [862]. If an individual's data is censored, it is considered to be at risk of an event at the time of censoring, but not in subsequent time points [862].

Follow-up data for PFS and OS with Taqman data was available for 27 of the 35 patients. Graph Pad Prism software was used for calculating Kaplan-Meier curves based on the follow-up data. For each gene, the samples were split into two groups, high expressers and low expressers, and the PFS and OS data was entered (Table 5.5). If the event had occurred at the most recent follow-up point available, i.e. the patient had recurred or died, the data was given the binary code 1. If the event had not yet occurred, i.e. the patient was still healthy/alive at the most recent follow-up point, the data was given the binary code 0 to facilitate censoring of data. To separate the samples into high and low expressers, the median value for expression for each gene (ΔC_T) was determined for all samples. A higher C_T value on Taqman® analysis for a gene, indicates that a lower amount of target is present, therefore samples with a higher ΔC_T value were classed as low expressers while samples with a lower ΔC_T value were classed as high expressers.

Table 5.5. Breakdown of High And Low Gene Expression.

Gene	Number of High Expressers	Number of Low Expressers
ANGPTL4	14	13
HER3	16	13
HIF-1α	15	12

5.3 Results

5.3.1 Array Validation

A summary of array validation is displayed in Figures 5.1 and 5.2 and Table 5.6.

5.3.1.1 ANGPTL4

ANGPTL4 was up-regulated in response to hypoxia in A2780 and A2780cis on arrays and was confirmed with RT-PCR (Figures 5.1B and 5.1C). In addition, RT-PCR identified expression of ANGPTL4 as up-regulated in A2780cis when compared to A2780 (Figure 5.1A). ANGPTL4 was up-regulated in the response to cisplatin in normoxia in A2780 and also in the hypoxic response to cisplatin in A2780cis (Figure 5.2D).

5.3.1.2 BDNF

BDNF was identified as up-regulated on the array analysis in A2780 cells in response to hypoxia (Figure 5.1B) and was confirmed using RT-PCR. In addition, RT-PCR picked up expression of BDNF in the response to hypoxia in A2780cis (Figure 5.1C). BDNF was up-regulated in response to cisplatin in normoxia in A2780cis and down-regulated in response to cisplatin in hypoxia in both A2780 and A2780cis on PCR (Figure 5.2).

5.3.1.3 HER3

On arrays, HER3 was up-regulated in A2780cis compared to A2780 (Figure 5.1A) and up-regulated in the response to hypoxia in A2780 (Figure 5.1B). It was down-regulated in the hypoxic response to cisplatin in A2780 (Figure 5.2B). The same changes were observed by Taqman analysis (Figure 5.1B). In addition, HER3 expression was found to be up-regulated in response to hypoxia in A2780cis, in the A2780cis response to cisplatin in normoxia, and down-regulated in response cisplatin in hypoxia in both cell lines (Figure 5.2).

5.3.1.4 MAD2L1

On arrays, MAD2L1 was down-regulated in response to cisplatin in normoxia and hypoxia in A2780 and in hypoxia in A2780cis (Figure 5.2). Taqman PCR revealed MAD2L1 to be up-regulated in A2780cis compared to A2780. It was also up-regulated in response to hypoxia (Figure 5.1B and 5.1C) and in the response to cisplatin in normoxia in both cell lines (Figure 5.2A and 5.2C), however it was found to be down-regulated in the hypoxic response to cisplatin in both cell lines (Figure 5.2B and 5.2D).

Table 5.6. Comparison of Fold-Changes in Validation Genes on Array and Taqman® (TM). Taqman® findings which were contrary to the array findings are highlighted in red. Novel findings on Taqman® not identified in the array analysis are highlighted in blue.

Gene	A2780 v A2780cis (Normoxia)		A2780 Normoxia v Hypoxia		A2780cis Normoxia v Hypoxia		A2780 Untreated v Cisplatin (Normoxia)		A2780 Untreated v Cisplatin (Hypoxia)		A2780cis Untreated v Cisplatin (Normoxia)		A2780cis Untreated v Cisplatin (Hypoxia)	
	Array	**TM**	**Array**	**TM**	**Array**	**TM**	**Array**	**TM**	**Array**	**TM**	**Array**	**TM**	**Array**	**TM**
BDNF	-	1.10	2.69	4.79	-	28.64	-	-1.20	-	-2.5	-	4.52	-	-6.25
ANGPTL4	-	4.86	8.65	22.20	3.48	9.59	-	2.48	-	1.28	-	1.96	-	3.45
HER3	2.71	11.86	3.44	7.11	-	7.78	-	2.66	-3.20	-5.00	-	7.99	-	-3.45
MAD2L1	-	27.10	-	7.43	-	4.14	-2.01	2.16	-3.91	-4.70	-	9.24	-4.91	-20.00

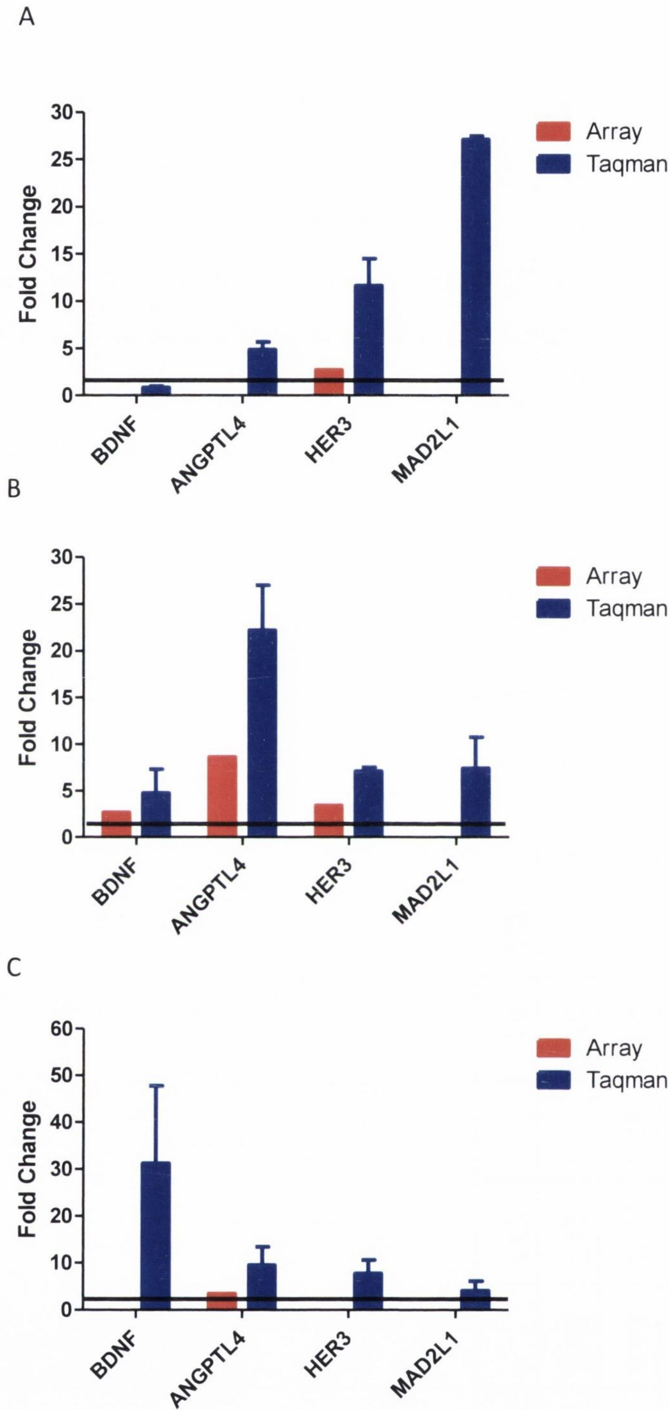


Figure 5.1. Comparison of Taqman® Analysis and Array Data. Taqman® and Array fold-changes are compared for A2780 v A2780cis (A); A2780 normoxia v hypoxia (B) and A2780cis normoxia v hypoxia (C). Threshold of 2-fold change is indicated by the black bar. n=3

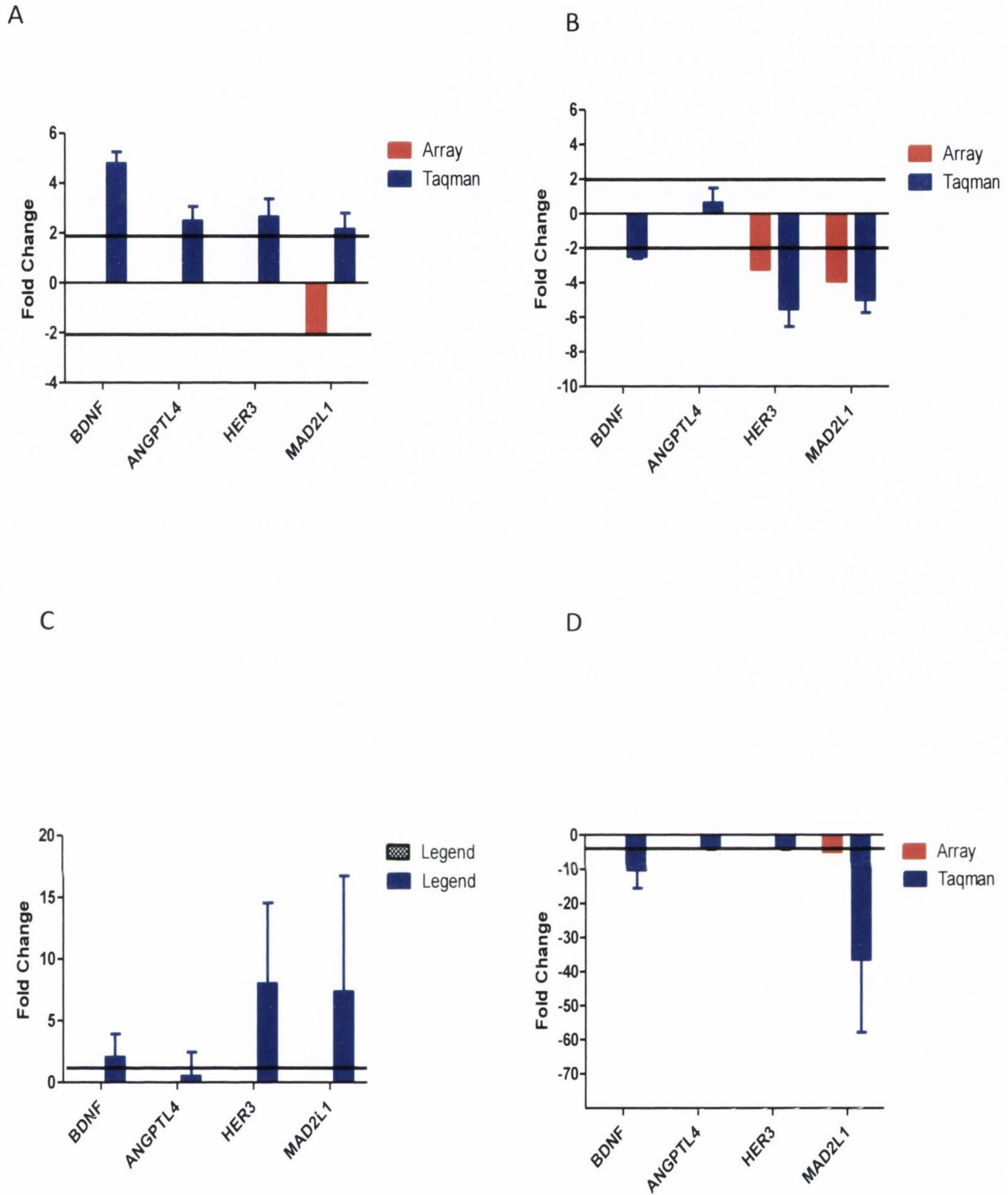


Figure 5.2. Comparison of Array and Taqman® fold changes for cisplatin response. Fold-changes identified on arrays and Taqman® for (A) A2780 Untreated v Cisplatin (normoxia); (B) A2780 Untreated v Cisplatin (hypoxia); (C) A2780cis Untreated v Cisplatin (normoxia); (D) A2780cis Untreated v Cisplatin (hypoxia). Threshold of 2-fold change is indicated by the black bar. n=3

5.3.2 Further Evaluation of the Hypoxia Matrix

Expression of the identified genes was analysed in other parts of the hypoxia matrix in order to determine if other types of hypoxic exposure affected their expression. The hypoxia matrix had explored the effect of acute (4 hours) and chronic (5 days) exposure to hypoxia prior to drug treatment, as well as the effect of hypoxia concomitant with drug treatment without previous exposure. The array data was based on samples which had been exposed to hypoxia for the treatment duration (72 hours), so in this experiment we evaluated the effect of the other hypoxia durations on gene expression.

5.3.2.1 ANGPTL4

When A2780 (normoxia) was used as a calibrator, ANGPTL4 was found to be significantly increased following 72 hours of hypoxia (Figure 5.3A). It was also increased following acute and chronic hypoxia, although this did not reach significance. When A2780cis (normoxia) was used as a calibrator (Figure 5.4A), there was a trend for increased expression in 72 hours hypoxia, however expression was not significantly different from normal in acute or chronic hypoxia.

5.3.2.2 BDNF

When A2780 (normoxia) was used as a calibrator, BNDF was found to be significantly decreased following acute hypoxia (Figure 5.3B). There was a trend for it to be increased following 72 hours of hypoxia, and chronic hypoxia although this was not significant. When A2780cis (normoxia) was used as a calibrator (Figure 5.4B), there was a trend of increased expression in 72 hours hypoxia, however expression was not significantly different from normal in acute or chronic hypoxia.

5.3.2.3 HER3

When A2780 (normoxia) was used as a calibrator (Figure 5.3C), HER3 was significantly up-regulated following 72 hours hypoxia. HER3 expression was not significantly different from normal oxygen in A2780 following acute hypoxia.

There was a trend for increased expression following chronic hypoxia, although this was not significant. When A2780cis (normoxia) was used as a calibrator (Figure 5.4C), HER3 was reduced in acute and chronic hypoxia although this was not significant.

5.3.2.4 MAD2L1

When A2780 (normoxia) was used as a calibrator (Figure 5.3D), MAD2L1 was increased following acute hypoxia, but its expression decreased with increasing time in hypoxia. When A2780cis (normoxia) was used as a calibrator (Figure 5.4D), MAD2L1 expression showed a trend to increase following 72 hours and chronic hypoxia, although this was not significant due to the high amount of variation between groups.

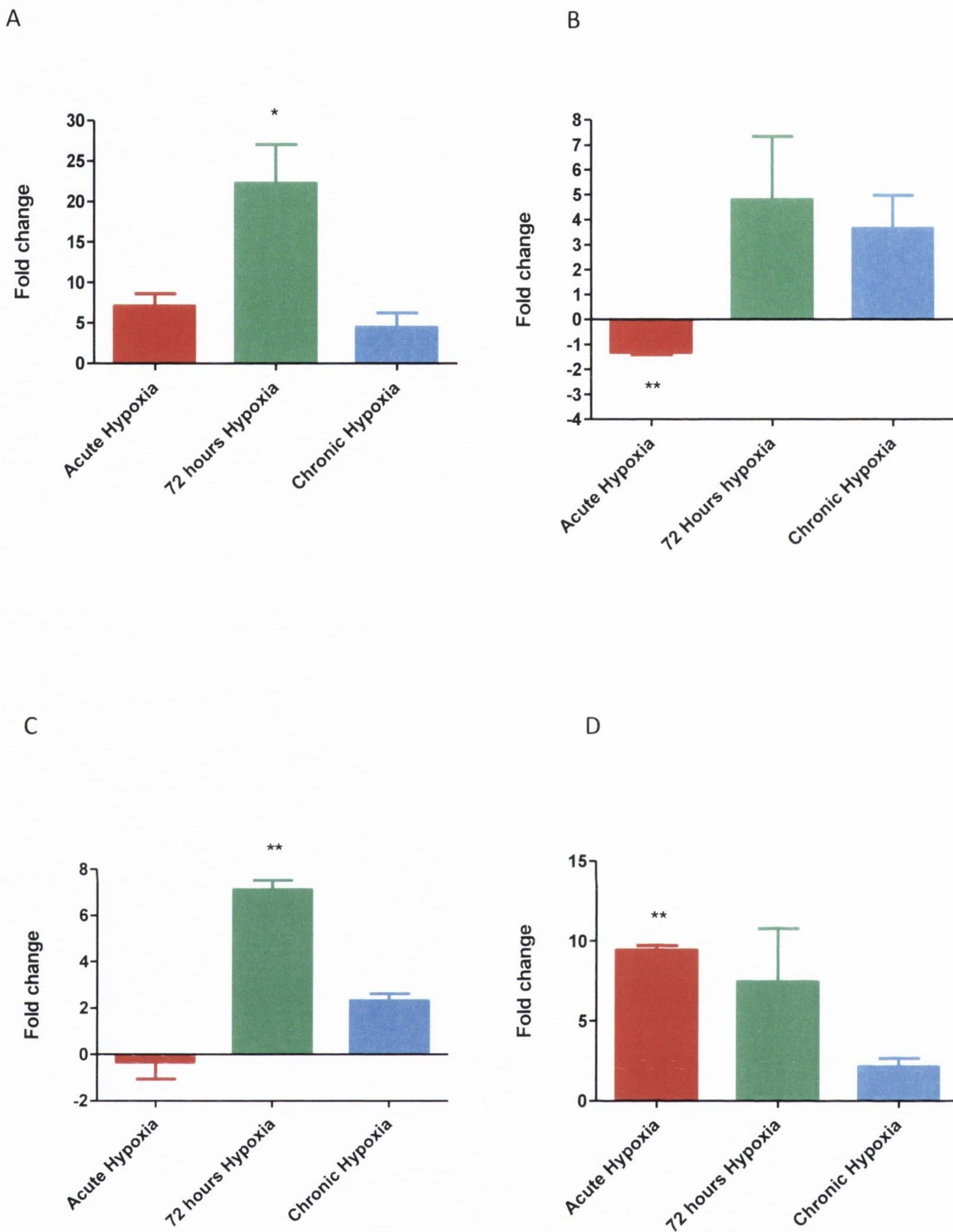


Figure 5.3. Changes in Gene Expression in A2780 in the Hypoxia matrix. Changes in gene expression of ANGPTL4 (A), BDNF (B), HER3 (C) and MAD2L1 (D) as measured by Taqman RT-PCR. A2780 (normoxia) was used as a calibrator. BDNF expression was reduced in acute hypoxia while increased following longer hypoxic exposures (A); ANGPTL4 was increased in hypoxia (B); HER3 was increased following 72 hours hypoxia (C); MAD2L1 was increased following acute hypoxia, but this reduced with longer exposure to hypoxia (D) n=3 *p<0.05 **p<0.01

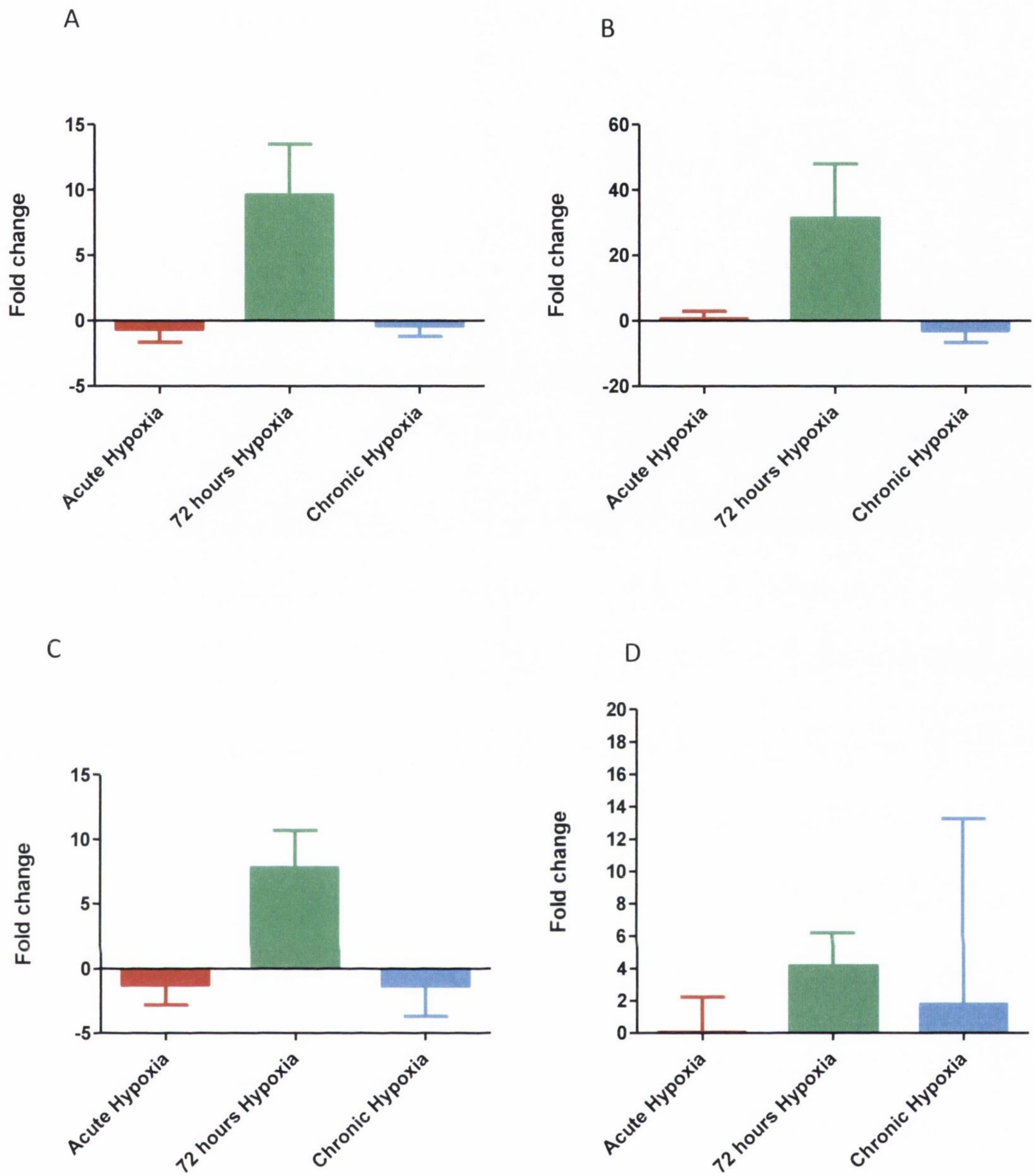


Figure 5.4. Changes in Gene Expression in A2780cis in the Hypoxia Matrix. Changes in gene expression of ANGPTL4 (A), BDNF (B), HER3 (C) and MAD2L1 (D) as measured by Taqman RT-PCR. A2780cis (normoxia) was used as a calibrator. BDNF, ANGPTL4, HER3 and MAD2L1 were all increased following 72 hours of hypoxia compared to normoxia. n=3

5.3.3 Expression of ANGPTL4, BDNF, HER3 and HIF-1α in Tumour Sample Cohort

Following the validation of ANGPTL4, BDNF HER3 and MAD2L1 in the cell line work, their expression was determined in a cohort of ovarian tumour samples. As mentioned previously, MAD2L1 expression has previously been determined in this set of samples by our collaborators, therefore the expression of HIF-1α, a marker of tumour hypoxia was determined instead.

5.3.3.1 ANGPTL4

ANGPTL4 expression was significantly up-regulated in partial responders and significantly down-regulated in non-responders to chemotherapy (Figure 5.5A) compared to responders to chemotherapy. Kaplan-Meier survival analysis showed a trend towards shorter PFS and OS for samples highly expressing ANGPTL4, however this was non-significant (Figure 5.5B $p = 0.18$ and 5.5C $p = 0.96$, Table 5.7).

5.3.3.2 BDNF

BDNF expression was observed in only 6 of the 35 samples assayed, thus a thorough analysis of its significance was not possible in this study. It could be studied as part of a larger cohort of samples, and perhaps in different types of samples e.g. recurrent vs. primary.

5.3.3.3 HER3

HER3 expression was significantly down-regulated in partial- and non-responders (Figure 5.6A) compared to responders to chemotherapy. Kaplan-Meier survival analysis showed a trend towards longer PFS and shorter OS for samples highly expressing HER3, however this was non-significant (Figure 5.6B $p = 0.11$ and 5.6C $p = 0.7$, Table 5.7).

5.3.3.4 HIF-1α

HIF-1α expression was significantly up-regulated in partial- and down-regulated in non-responders (Figure 5.7A) compared to responders to chemotherapy. Kaplan-Meier survival analysis showed a trend towards longer PFS and slightly shorter OS for samples which over-expressed HIF-1α, however this was non-significant (Figure 5.7B $p = 0.3$ and 5.7C $p = 0.9$, Table 5.7).

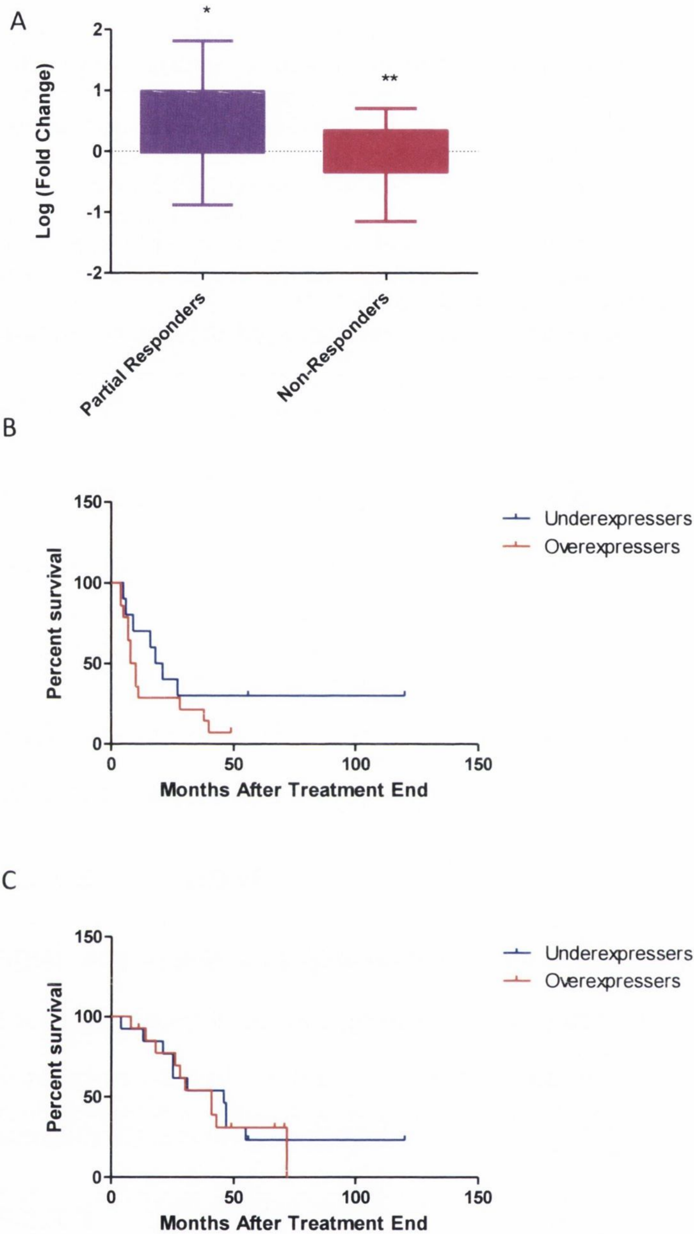


Figure 5.5. Expression of ANGPTL4 in Cohort of Tumour Samples. ANGPTL4 was significantly up-regulated in patients with a partial response (n = 11), while it was significantly down-regulated in non-responders (n = 7) compared to responders (n = 17) to chemotherapy (A). Kaplan-Meier curves indicated a trend towards reduced progression free survival (B) and overall survival (C) in samples which over-expressed ANGPTL4 (n = 14) compared to under-expressers (n = 13), although this was non-significant. *p<0.05 **p<0.01

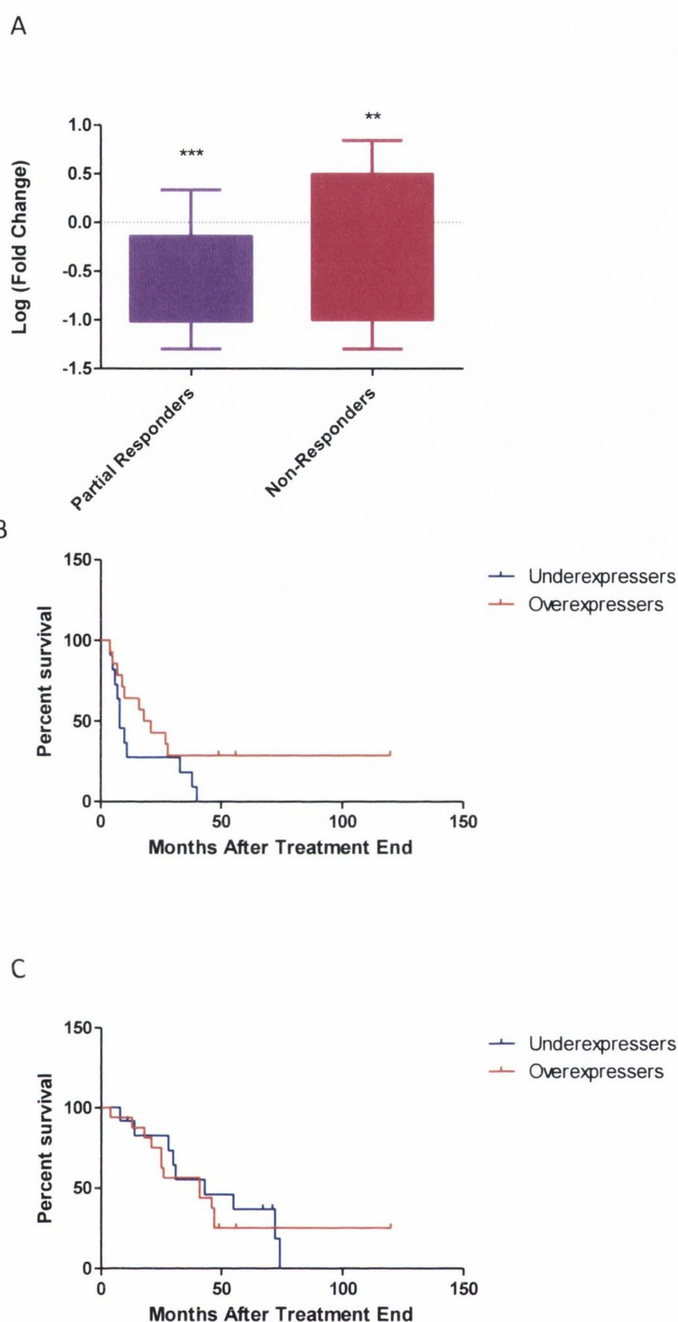
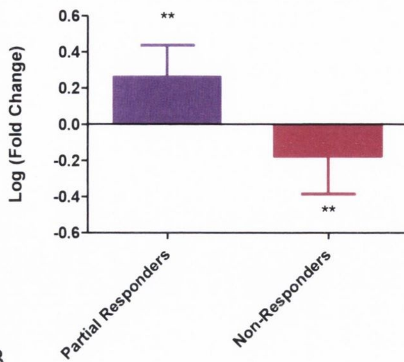
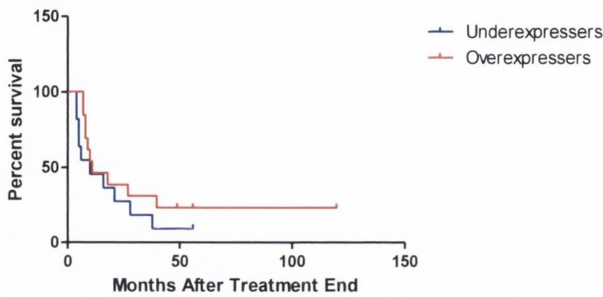


Figure 5.6. Expression of HER3 in Cohort of Tumour Samples. HER3 expression was significantly down-regulated in both partial (n = 11) and non-responders (n = 7) to chemotherapy compared to responders (n = 17) (A). Kaplan Meier survival analysis indicated a longer progression free survival (B) and shorter overall survival (C) for samples which over-expressed HER3 (n = 16) compared to those which under-expressed HER3 (n = 13) however this was non-significant. **p<0.01 ***p<0.001

A



B



C

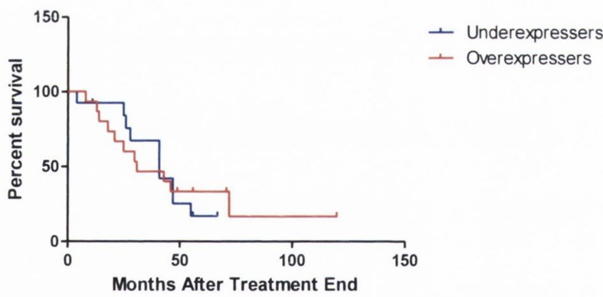


Figure 5.7. Expression of HIF-1α in Cohort of Tumour Samples. HIF-1α expression was significantly up-regulated in partial (n = 11) and down-regulated in non-responders (n = 7) to chemotherapy compared to responders (n = 17) (A). Kaplan-Meier analysis revealed a trend towards increased progression-free survival (B) and reduced overall survival (C) in samples which over-expressed HIF-1α (n = 15) compared to those which under-expressed HIF-1α (n = 12), however this was non-significant. $^{**}p<0.01$

Table 5.7. Summary of Kaplan-Meier Analysis. Median values for PFS and OS (months) in over- and under-expressers of ANGPTL4, HER3 and HIF-1α. Curves were not statistically significant as determined by the log rank test.

Gene	Median PFS		Median OS	
	Under-expressers	Over-expressers	Under-expressers	Over-expressers
ANGPTL4	19.5	9.0	46.0	41.0
HER3	8.0	19.5	43.0	41.0
HIF-1α	10.0	11.0	41.0	31.0

5.4 Discussion

The differential gene expression of ANGPTL4, BDNF, HER3 and MAD2L1 as determined by array analysis was validated using Taqman PCR before moving to examine their expression in ovarian tumour samples. Due to the potential for introduction of artefacts within array experiments, the results are generally validated by a variety of methods such as comprehensive literature search of the data previously published in the same system, RT-PCR analysis of the same samples, and protein validation [863]. Artefacts may be introduced at any stage during the experiment. For example, a sufficient number of replicates must be carried out in order to reduce any 'noise' in the data, and the software used for image acquisition and data normalization may affect the final gene list obtained. In addition, if arrays are carried out in 'batches', there can be differences between batches independent of biological significance [864]. In this study, the results of genes to be further analysed were first validated using RT-PCR on the same samples as the arrays.

5.4.1 Biomarker Selection

Selection of potential biomarkers to evaluate in the tumour sample cohort was based on a combination of analysis of patterns of gene expression on microarray, and thorough literature search in order to identify candidate genes which displayed strong potential for further development. As there was a limited time period allocated to the final experiments, we wished to select biomarkers which had previously displayed promise as biomarker candidates, yet retained novelty – in that they perhaps had shown promise in cancer types other than ovarian, or that they had been linked to chemotherapy resistance, but not necessarily platinum resistance. ANGPTL4 was chosen as it was up-regulated in both cell lines in response to hypoxia, and was particularly novel. There was previous evidence that expression of ANGPTL4 was a negative influence on survival in a number of cancers [797,801], however, there was relatively little information on its expression in ovarian cancer and no strong link between its expression and platinum resistance. BDNF was up-regulated in the parent A2780 cell line in

response to hypoxia. Previous studies on BDNF had shown that its expression was associated with a number of aggressive tumour properties such as invasion and angiogenesis [811], and it had been previously linked to cisplatin resistance in head and neck squamous cell carcinoma [516]. However, there was limited information available on BDNF expression, or its significance in relation to survival in ovarian cancer. In addition, BDNF had shown promise as a potential therapeutic target in ovarian cancer and others [805,865], and thus was of interest in our study population. HER3 displayed an unusual pattern of expression in the hypoxia matrix, and was up-regulated in response to hypoxia, yet down-regulated in the response to cisplatin in hypoxia. HER3 had previously been shown to be a potential therapeutic target in ovarian cancer [866], and had been linked to sensitivity to monoclonal antibody therapy with gefitinib [867] and pertuzumab [831], but the relationship between HER3 expression and cisplatin resistance was unclear. Although not a novel marker of hypoxia, or cisplatin resistance, HIF-1α was chosen to evaluate in the tumour sample population as it is a universal marker of hypoxia. In addition, there is some conflicting evidence in the literature regarding the association of HIF-1α expression with survival in ovarian cancer, with high expression of HIF-1α having been linked to both reduced and increased PFS and OS in ovarian cancer by two separate groups [200,201], therefore it was of interest to further examine the relationship between survival and HIF-1α expression in an ovarian cohort.

Thus, the biomarkers were chosen based on both novelty and previous knowledge on their role(s) in cancer. There were many alternative biomarker candidates which could have been chosen, and will be explored in future projects.

5.4.2 Cell Lines

Angiopoietin-like 4 (ANGPTL4) expression on RT-PCR correlated with the results observed on array analysis. In addition, RT-PCR identified ANGPTL4 as up-regulated in other samples in which the array analysis did not identify any change. This may be explained in two ways. Firstly, the analysis was constricted

by stringent statistical limits – only samples which passed a threshold of 2-fold change in expression with an FDR<0.05 were considered as truly 'changed'. It is possible that with less stringent limits i.e. relying on a p-value <0.05, the changes in ANGPTL4 expression could have been identified in the array analysis. In addition, RT-PCR is a more sensitive tool than the microarray for identifying differences in gene expression – on arrays, the ability to evaluate multiple targets simultaneously limits accurate quantification of low levels of gene expression [868].

Similarly, BDNF expression on RT-PCR concurred with the results on arrays and in addition it was identified as >2-fold up-regulated in hypoxic A2780cis cells, and was >2-fold down-regulated in the response to cisplatin in hypoxia for both cell lines.

The expression change for HER3 in A2780cis cells compared to A2780, and in A2780 cells exposed to hypoxia compared to A2780 concurred on both array and Taqman. Also, in A2780 cells treated with cisplatin in hypoxia, it was down-regulated both on array and Taqman. In addition, it was shown to be down-regulated in A2780cis cells treated with cisplatin in hypoxia on Taqman, although no change was observed on array analysis. Also, Taqman identified HER3 as up-regulated in response to hypoxia in A2780cis.

MAD2L1 results on Taqman validated for most of the array time-points. Both array and Taqman identified MAD2L1 as down-regulated in response to cisplatin in hypoxia. In addition, MAD2L1 was found to be up-regulated in the A2780cis cell line compared to A2780 cells. While Taqman analysis displayed MAD2L1 as down-regulated in response to cisplatin in normoxia in A2780, Taqman demonstrated MAD2L1 as up-regulated. There are several reasons for non-concordance between array and Taqman data. As mentioned earlier, Taqman is more sensitive than array analysis, and low fold-changes identified on arrays may not always be robust. Indeed it has been shown previously that genes with less than 4-fold changes in gene expression are not always consistently validated [869].

The expression of the four genes were then analysed on samples which were exposed to acute and chronic hypoxia in order to determine if the length of hypoxic exposure affected the expression levels. In A2780 cells BDNF was found to be significantly reduced following acute hypoxia, yet was up-regulated following 72 hours and 5 days, although these were not statistically significant. Most articles investigating BDNF expression following hypoxia examine acute intermittent hypoxia i.e. cycles of periods of hypoxia followed by reoxygenation. Some groups show that BDNF expression is increased *in vivo* [870,871], while others demonstrate reduced BDNF expression [872]. One study of BDNF expression in mice following continuous hypoxia for eight days found that BDNF was significantly increased, although this depended on the breed of mouse [873]. However, there are inherent limitations when comparing *in vivo* studies to *in vitro* studies such as this. In addition, most *in vitro* studies of BDNF are carried out on neuronal cell cultures. Two other studies are available in the literature examining the relationship between hypoxia on BDNF expression in cancer cells. Both studies were published by the same group and showed that in normal cerebellar granule neurons and in neuroblastoma cells, incubation with exogenous BDNF is shown to regulate expression of VEGF in a HIF-1α-dependent manner thus placing it up-stream of the HIF-1α signalling pathway and presenting one mechanism in which HIF-1α signalling may be mediated in non-hypoxic conditions [812,874]. Our study observed a dual-response to hypoxia in terms of BDNF production. There was an initial drop in BDNF expression which may be explained by an early response to hypoxic insult within the cells. For example, acute hypoxia is known to mediate a transient DNA damage response which initially results in phosphorylation of Chk1 and cell cycle arrest, however after chronic periods of hypoxia, Chk1 is no longer phosphorylated, thus removing its block on cell cycle progression [573]. A similar type of response may be occurring in the BDNF response to hypoxia, although there is nothing as yet in the literature to confirm this. The following increase in BDNF expression following longer periods of hypoxia most likely indicates the adaptive response to hypoxia, and the utilization of the protective effect of BDNF on cell survival [875,876]. In A2780cis, the BDNF response to hypoxia was different. While

BDNF was not induced by acute hypoxia it was up-regulated following 72 hours of hypoxia. However, this response was dampened by 5 days of hypoxia, when the response was again not different from normoxia. While A2780cis did display increased resistance to cisplatin following chronic hypoxia in Chapter 3, the fold-changes were not nearly as large as those observed in A2780, or as those observed following cisplatin treatment in hypoxia for 72 hours. It may be that the reduced expression of BDNF following chronic hypoxia plays a part in the reduced levels of resistance observed following chronic hypoxia in A2780cis. This may also be explained by a differential gene expression pattern which can be observed following different periods of hypoxic exposure [573].

ANGPTL4 expression was increased following all lengths of exposure to hypoxia in A2780, while the expression pattern was similar to that of BDNF in A2780cis. Previous microarray analysis has shown ANGPTL4 to be up-regulated from 24 hours exposure to hypoxia in adipocytes [877]. In oesophageal carcinoma, ANGPTL4 was shown to be up-regulated from as early as 30mins in hypoxia in both single cells and 'bulk cells' [878], while in renal cell carcinoma, ANGPTL4 was found to be raised to six times the basal level following 2 hours hypoxia, reaching a maximum of 8 times basal level at 20 hours [787]. This indicates that the amount of ANGPTL4 produced may be rate-limited, and does not increase continuously. In our study, it was found in both cell lines that ANGPTL4 was at its highest following 72 hours of hypoxia, and this declined following chronic hypoxia, which fits in with the previous data. In addition, it was found that while ANGPTL4 was still raised in A2780 cells following chronic hypoxia, although not statistically significant, in A2780cis, ANGPTL4 levels had returned to normoxic levels (<1-fold difference). This may be due to biological differences between the two cell lines, and indeed, ANGPTL4 expression has been found to vary between different subtypes of cancer e.g. renal cancer [787]. In addition, Taqman had identified ANGPTL4 as over-expressed in A2780cis compared to A2780, thus although the levels may have reduced following chronic hypoxia, it is likely that they are still at a high level of expression. ANGPTL4 expression is mediated through HIF-1α [554], and in colorectal cancer, ANGPTL4 has been

shown to be induced by hypoxia through prostaglandin E2, a potent prostaglandin produced by COX-2, and has been shown to promote cell proliferation both *in vitro* and *in vivo* [879].

HER3 expression was similar between both cell lines following all lengths of hypoxia – no change following acute hypoxia, increased HER3 following 72 hours hypoxia and no change in HER3 following chronic hypoxia. There is a paucity of literature describing HER3 expression in relation to hypoxia. Gui *et al.* isolated mesenchymal stem cells from rat bone marrow, subjected them to serum deprivation (SD) and anoxia (95%N_2, 5%CO_2) and found reduced levels of HER3 [880]. Heregulin, a ligand for HER3 was able to decrease the apoptosis observed following SD and anoxia. This is in contrast to the results observed in our study, however, there are a number of key differences between the two studies. Firstly, Gui *et al.* used a non-transformed primary cell culture of a normal cell type, while our study was using a malignant transformed cell line, which will have a large number of inherent differences to a non-malignant cell line. Secondly, the degree of hypoxia was different for both studies – while our study used 0.5% O_2, Gui *et al.* used virtually anoxic conditions, which would be more severe than those in our study, and in addition Gui *et al.* serum deprived their cells before subjecting them to hypoxia, which affects cell proliferation and reduces cell viability [881]. Thus it is not suitable to directly compare the two studies. Our study has provided novel information on the influence of hypoxia on HER3 expression in an ovarian cancer cell line model.

In A2780 cells, MAD2L1 expression was most highly expressed following acute hypoxia, and the expression reduced as the length of hypoxia increased, while in A2780cis, there was large variation observed in expression between replicates. The most consistent replicates were observed following 72 hours of hypoxia which showed an average of 4-fold increase in MAD2L1 expression compared to normoxia. Similarly to HER3, there is limited information on hypoxia in relation to MAD2L1 expression in the literature. Prencipe *et al.*, our collaborators in University College, Dublin, examined MAD2L1 expression in three ovarian cancer cell lines OVCA432, OVCA433 and UPN251 in 1% oxygen for 72 hours [769]. They

found decreased expression of MAD2L1 protein and mRNA following 24 hours of hypoxia. This is in contrast to our findings, however, there may be biologic differences between the cell lines to explain these differential responses. In addition, the level and length of hypoxia exposure in the study by Prencipe *et al.* was different than that used in our study, and it may be that the change in expression of MAD2L1 is dependent on the severity and duration of oxygen deprivation. MAD2L1 over-expression, as was observed in our study, has been linked to more aggressive tumour features such as liver metastasis in gastric cancer [834], reduced survival in colorectal cancer [835], lymph node metastasis in endometrial cancer [836] and increased tumour grade in hepatocellular carcinoma [838]. Interestingly, while MAD2L1 was found to be increased following expression to hypoxia, it was reduced following treatment with cisplatin in hypoxia, while it remained unchanged (A2780) or increased (A2780cis) following treatment with cisplatin in normoxia. Reduced MAD2L1 expression has been shown to reduce apoptosis induced by cisplatin in gastric cancer [766], and high cisplatin expression has been shown to sensitize cells to cisplatin in testicular carcinoma [767]. It could be postulated that the cells which survived cisplatin treatment in hypoxia and were subsequently assayed for gene expression changes did so because of reduced levels of MAD2L1. Expression of MAD2L1 was not assayed in the tumour population used in this study, as its expression had been determined in the same cohort by a collaborating group [781]. This study found that low MAD2L1 protein expression was associated with reduced progression-free survival following adjustment for stage, grade and debulking status.

5.4.3 Tumour Samples

ANGPTL4 was up-regulated in tumour samples from patients who displayed a partial response to platinum/taxane chemotherapy (6 – 12 months free from disease following chemotherapy) while it was down-regulated in tumour samples from patients who did not respond to chemotherapy (<6 months free from disease following chemotherapy) in comparison to patients determined to have had a response (>12 months free from disease following chemotherapy). This is

a novel finding, as, to my knowledge, there are currently no other studies in the literature which have examined its expression in serous ovarian cancer tumour samples. In breast cancer, high ANGPTL4 expression (as part of a panel of VEGF-associated genes) was associated with distant metastasis and reduced progression free survival [882], while in oral squamous cell carcinoma and oesophageal squamous cell carcinoma, high ANGPTL4 expression is associated with reduced overall survival [797,801]. A previous microarray study in endometrial cancer identified ANGPTL4 expression as part of a panel of markers to be associated with vascular invasion, increased histologic grade and necrosis [802]. The exact function of ANGPTL4 in carcinogenesis is slightly unclear, as some of its functions seem paradoxical. While certain studies have identified ANGPTL4 as a pro-angiogenic factor [787,799], others have noted that ANGPTL4 inhibits *in vivo* angiogenesis and suppresses tumour growth [788,794]. ANGPTL4 has been recognised as a down-regulated target for the tumour suppressor gene U94 in prostate carcinoma cell lines [883]. In addition, *in vivo* studies have demonstrated ANGPTL4 to prevent metastasis and invasion through reduction of vascular permeability and reduction of tumour cell motility [790]. Yet, in studies of breast tumours, ANGPTL4 expression has been linked to lung metastasis [791], and in hepatoma, it has been linked to resistance to anoikis [884]. In addition, ANGPTL4 has been shown to be methylated in a small percentage of breast tumours [793]. The observations in our study may represent a dual role for ANGPTL4 in the pathology of ovarian carcinoma. The exact role of ANGPTL4 may depend on the absolute quantity of transcript present, the cell type involved and its interplay with other genetic factors. The Kaplan-Meier analysis showed that patient samples which over-expressed ANGPTL4 tended to have shorter progression-free and overall survival than samples which under-expressed it, however this was non-significant. This is likely due to the limited number of samples which were examined in this study, and a larger cohort of samples should be assayed to confirm and expand on the results found here.

Average HER3 expression was significantly down-regulated in partial- and non-responders to chemotherapy compared to responders. Similarly, Kaplan-Meier

analysis demonstrated a trend for increased progression-free and reduced overall survival in patients who were high-expressers of HER3 compared to low-expressers, although this was non-significant. Previous studies of HER3 expression in ovarian cancer samples have observed higher levels of HER3 in cancer compared to benign disease [885] and correlated positive HER3 expression with decreased survival [830]. In endometrioid carcinoma of the ovary, HER3 expression was observed in half the tumours studied, and was associated with increased tumour grade [886]. However another study found that only a small proportion over-expressed HER3 and that over-expression was associated with well-differentiated tumours [456]. In breast cancer, high HER3 expression is associated with lymph node metastasis [453] and reduced progression free survival [887]. In oral squamous cell carcinoma, HER3 over-expression is associated with lymph node metastasis and poor survival [454], and in bladder carcinoma, HER3 expression in conjunction with HER2 is associated with reduced survival [888]. In colorectal cancer, HER3 over-expression is more common in early stage cancers [889] and negative HER3 expression has been associated with increased invasion and higher risk of disease recurrence [890]. The overwhelming evidence in the literature suggests HER3 over-expression to be associated with tumour aggressiveness and chemoresistance. HER3 over-expression has been linked to resistance to the HER2 inhibitor, trastuzumab (Herceptin®) [891], while up-regulation of HER3 has been linked to resistance to PI3K inhibitors in breast cancer cells [892]. HER3 has been shown to stimulate tumour cell migration [820]. Several therapies to target HER3 are being investigated. Monoclonal antibody therapy against HER3 has been shown to inhibit growth of breast cancer cells [893] and is in preclinical trials [894]. The multi-kinase inhibitor, Foretinib, has also been shown to inhibit phosphorylation of HER3 in gastric cancer cell lines [895]. In addition, expression of exogenous oestrogen receptor beta (ERβ) in human breast cancer cells was shown to reduce levels of HER3 and increase sensitivity to Tamoxifen therapy [896]. Our study found significant down-regulation of HER3 in patients who displayed a partial- or non-response to chemotherapy. Thus it is unlikely that in our study, low HER3 expression is linked to platinum chemoresistance in these patients. There are

several reasons which may explain this. Firstly, it may be that an as yet unknown molecule is negatively regulating HER3 in our sample population. In addition, it is recognized that tumour sampling is very important in terms of evaluation of molecular processes due to high degrees of intra-tumour heterogeneity – and the levels of biomarkers analysed in one study may not be representative of the whole tumour [897]. This has implications for all studies of potential biomarkers in cancer. It may be that the tumour regions which were analysed in this study were not representative of the whole tumour. It may be that subclones of cells are responsible for the effects of high HER3 expression. In addition, low HER3 expression has been previously identified in ovarian cancer [831], although its significance has not been adequately determined. Interestingly, low HER3 expression may identify patients who are suitable for alternate forms of chemotherapy. Alpha-TEA (alpha-tocopherol ether acetic acid analogue) is a Vitamin E analogue with anti-tumour properties through activation of Fas and JNK apoptotic pathways [898]. In addition, α-TEA down-regulates HER3 and thus down-regulates the Akt and survivin pathways [898]. Therefore tumours already low in HER3 may be more sensitive to α-TEA therapy, and identification of these may highlight patients who would benefit from this.

Average expression of HIF-1α was higher in patients who had a partial response, and lower in patients who had a non-response to chemotherapy compared to responders. Kaplan-Meier analysis indicated slightly longer progression-free and shorter overall survival in patients who expressed high levels of HIF-1α, although again this did not reach statistical significance. Previous studies have shown HIF-1α to be expressed in a large proportion of ovarian tumours [360], and HIF-1α expression has been correlated with VEGF expression, tumour grade and poor overall survival [199,899]. HIF-1α has also been shown to be highly expressed in clear cell ovarian carcinoma compared to other types – clear cell carcinoma is noted for its poor prognosis [900] and its expression is higher in serous than mucinous adenocarcinomas [204]. Expression of HIF-1α in serous ovarian carcinoma is associated with reduced overall survival in patients who have received carboplatin/paclitaxel chemotherapy [200]. Nuclear immunostaining,

but not cytoplasmic, of ovarian carcinoma with HIF-1α has been shown to predict poor prognosis [5]. Interestingly, one study linked HIF-1α expression with improved outcome following carboplatin/paclitaxel chemotherapy in ovarian tumours and particularly in those with suboptimal debulking [201]. In xenograft models, HIF-1α has been linked to microvascular density and inhibition of HIF-1α associated with reduced tumour growth when treated with cytotoxic drugs [901]. Oestrogen expression, a risk factor for ovarian cancer, has been shown to increase HIF-1α and VEGF expression and activate the PI3K pathway in ovarian carcinoma cell lines [902]. Certain types of cancer treatment have been shown to exert their effects through inhibition of HIF-1α. Fara-A (9-beta-D-Arabinofuranosyl-2-fluoroadenine), is a nucleotide analogue which is incorporated into DNA where it induces DNA damage and apoptosis in dividing cells [903]. A study of Fara-A in ovarian cancer cell lines found that it inhibited HIF-1α and activation of the Akt pathway [903]. Similarly, reservatrol, a natural plant product which has anticancer effects, was shown to reduce HIF-1α protein levels and inhibited Akt and MAPK signalling pathways [378]. Increased levels of HIF-1α in patients who received the antiangiogenic antibody therapy Bevacizumab were associated with increased progression-free survival, thus identifying it as a marker of patients who may benefit from this type of therapy [904]. Albendazole was initially used as an anti-parasitic in farm animals, however, it has also been evaluated as a potential anticancer agent, and was shown *in vitro* to inhibit accumulation of HIF-1α protein and *in vivo* to reduce tumour HIF-1α and VEGF expression [905]. A member of a new class of anticancer drugs, campothecins, has been shown to reduce accumulation of HIF-1α protein and to act synergistically with cisplatin [291]. HIF-1α itself has been shown to be a potentially useful direct target for therapeutic intervention. Treatment of ovarian cancer cells with HIF-1α antisense oligonucleotides attenuated the multi-drug resistance phenotype of the model [377]. Similarly a further study of ovarian cancer cells which inhibited HIF-1α with siRNA found reduced VEGF expression and angiogenic potential of the cells [164]. Thus, HIF-1α is not just a prognostic factor for ovarian cancer, but helps to identify patients who may benefit from a wide range of novel cancer therapies.

5.4.4 Summary

The aim of this chapter was two-fold:

1. To validate the results of the microarray experiments of Chapter 4
2. To evaluate a number of hypoxia-associated biomarkers identified on the array analysis in a cohort of ovarian tumour samples

There were a number of findings in this chapter.

1. BDNF expression is altered by hypoxia, and this is dependent both on the duration of hypoxia and the cell line – this is a novel finding
2. HER3 expression is altered by hypoxia, and is differentially regulated depending on the presence or absence of hypoxia during treatment with cisplatin – this is a novel finding
3. In an ovarian tumour cohort, ANGPTL4 was expressed in all samples, and the level of expression differed depending on the patient response to chemotherapy – this is a novel finding
4. In an ovarian tumour cohort, HER3 was under-expressed in partial- and non-responders to chemotherapy. This may identify patients who would benefit from alternative therapies such as α-TEA and pertuzumab
5. In an ovarian tumour cohort, HIF-1α was up-regulated in patients who had a partial and down-regulated in patients who had a non-response to chemotherapy

Chapter 6

General Discussion

6.1 Introduction

Ovarian cancer is a highly lethal malignancy, characterized by late diagnosis and the development of chemoresistance. Mechanisms of chemoresistance are multi-factorial, and the influence of the tumour microenvironment on chemoresistance is significant. Tumour hypoxia is the result of rapid tumour growth and is responsible for switching on many aggressive tumour features such as proliferation, invasion, metastasis, angiogenesis, and chemoresistance through expression of the master hypoxia regulator, HIF-1α. The aim of this study was to examine the effect of hypoxia on resistance to chemotherapeutic drugs in an ovarian cancer cell line model. Firstly, the direct effect of hypoxia on cell viability following drug treatment was analysed using MTT assays and a complex hypoxia matrix. Secondly, the global changes in gene expression in response to hypoxia and/or cisplatin were determined using Affymetrix Human Gene ST 1.0 arrays, and data was evaluated in the context of the literature to identify potential biomarkers of chemoresistance in relation to hypoxia. Finally, a selection of markers identified on array analysis was evaluated in a cohort of serous adenocarcinoma samples, in order to translate the cell line data to meaningful data in a 'real life' context.

6.2 Hypoxia and Ovarian Cancer – 2008

A Pubmed search for literature published in English using the search terms 'hypoxia' and 'ovarian cancer' up to the end of 2008 yields 171 articles, while searching for literature up to the current date yields an additional 100 articles in the space of three years, highlighting the fact that tumour hypoxia has become a relatively 'hot topic' in terms of cancer research. Up to 2008, a number of findings had been made in relation to hypoxia in terms of ovarian cancer.

Tumour hypoxia had been shown to be associated with increased tumour volume and necrosis and correlated with reduced bioenergetic status due to increased metabolically active tumour cells [906,907]. In addition, glucose uptake into hypoxic regions was shown to be increased in melanoma and ovarian

tumours and immunohistochemical data linked this with increased GLUT-1 (glucose transporter 1) expression [908].

Early *in vitro* studies showed that exposure of ovarian cancer cells to anoxia for 24 hours did not affect cell viability, however reduced cell proliferation and induced cell cycle arrest at G_1 and G_2 associated with reduction in cyclin A and pRb expression [909]. Further studies of hypoxia and the cell cycle in ovarian cancer revealed that cell cycle arrest is associated with reduced CDK4 activity in relation to pRb, and reduction in Cyclins D1, D2 and increased p27 and Cyclin E [560]. Cell survival in hypoxia was shown to be mediated through integrin-linked kinase [910].

In addition, tumour hypoxia had been shown to abrogate the anti-tumour immune response through down-regulation of tumour necrosis factor alpha (TNFα)-induced monocyte chemoattractant protein 1 (MCP-1) [911]. Hypoxia was also shown to increase levels of the pro-inflammatory cytokine interleukin-8 (IL-8) through the NFKB pathway [912] and the PI3K pathway [913] as well as increasing chemokine receptors [914].

Hypoxia was associated with angiogenesis mediated through the adenosine receptor [915], vascular endothelial growth factor (VEGF) [916], prostaglandin E2 [917], chemokine stromal-derived factor CXCL12 [918], and activation of the PI3K signalling pathway [919,920]. Hypoxia-mediated dysregulation of PTEN and NDRG1 have been implicated in the carcinogenesis of endometrioid ovarian adenocarcinomas [921].

In addition, hypoxia was linked to ovarian tumour invasion and metastasis through reduced expression of E-cadherin and increased expression of SNAIL, a transcriptional repressor of E-cadherin [922]. Similarly, hypoxia has also been shown to mediate invasion through up-regulation of heparanase expression [923]. An additional link between hypoxia and metastasis was made by Kim *et al.* who showed that hypoxia increased ovarian cancer cell responsiveness to lysophosphatidic acid, a molecule which is expressed in ovarian cancer ascites and is involved in regulation of ovarian cancer biology [924]. Hypoxia was known

to confer protection against radiation in an *in vivo* setting [925,926], while *in vitro*, tumours composed of ovarian cancer cells were relatively more radioresistant than tumours containing lower hypoxic fractions [927].

A link was made between hypoxia and resistance to chemotherapy in ovarian tumours by Siemann and Alliet in 1987, who observed in *in vivo* studies that co-treatment of a particular type of sarcoma with misoindazole and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea (CCNU) resulted in increased cell kill in lung metastases, but this was reduced in ovarian metastases – postulated by the authors to be due to an increased fraction of hypoxic tumour cells in the ovarian metastases [928]. A subsequent study in Chinese hamster ovary (CHO) cells showed that resistance to a number of cytotoxic agents including etoposide, actinomycin D and vincristine was induced following exposure of the cells to anoxia for 24 hours [929]. In addition, hypoxia-related resistance to the antibiotic/cytotoxic drug mitomycin C, a drug which exhibits a cross resistance profile with cisplatin, was shown to be related to expression of detoxicating enzymes DT diaphorase and y-glutamylcystein synthase [930]. Studies of glucose-related stress, associated with the presence of tumour hypoxia, showed that induction of glucose-related stress resulted in increased resistance to a topoisomerase I inhibitor, campothecin, due to reduced drug accumulation and cell cycle arrest at G_1 [931]. In addition, hypoxia had been shown to induce expression of a number of chemoresistance associated proteins such as glutathione-s-transferase (GST), and glucose-related protein 78 (GRP78) [932]. HIF-1α activity and protein expression was shown to be reduced by cell treatment with cisplatin [309].

Hypoxia had been identified as a potential therapeutic target for treatment of ovarian cancer, and the concept of bioreductive hypoxia-selective drugs was being evaluated [933]. A hypoxia-activated drug, tirapazamine (TPZ), was shown to exert synergistic effects when ovarian cancer cells were co-incubated with activated TPZ and cisplatin, thus providing evidence for potential usefulness for hypoxia-activated drugs in the treatment of ovarian cancer [934]. Interestingly, ascorbate (vitamin C) was shown to markedly inhibit HIF-1α protein expression

and reduce expression of HIF-1α targets VEGF and GLUT-1 [935]. Other cytotoxic agents had been shown to mediate their effects at least partially through inhibition of HIF-1α [378,379,901,903,936,937]. Direct targeting of HIF-1α with siRNA was also shown to suppress resistance and enhance apoptosis [377,938]. Anti-angiogenic antibody bevacizumab was being evaluated in *in vivo* experiments [939,940].

Immunohistochemical studies of ovarian cancer observed positive HIF-1α expression in many cases [941], in particular the clear cell carcinoma [900,942] and serous carcinoma [204] subtypes, and co-expression of HIF-1α with p53 was shown to be associated with poor overall survival [360]. While HIF-1α expression alone was not determined to have prognostic significance by some [853], others have shown high HIF-1α expression to be predictive of poor overall survival [199,200]. In addition, HIF-1α tumour expression was associated with increased expression of VEGF and p27, and decreased expression of Cyclin D1 and pRb [943]. High HIF-1α and VEGF expression are associated with increased tumour grade [899] and stage [5]. One study linked high HIF-1α expression with better response to platinum/taxane based chemotherapy [201].

Thus, before this project started, there was already quite a large amount of knowledge on the role of hypoxia in terms of ovarian cancer biology, in terms of metabolism, proliferation, angiogenesis, invasion and metastasis. HIF-1α had also been recognised both as an important therapeutic and prognostic marker of ovarian cancer. However, while it was widely shown that hypoxia induced chemoresistance to a number of cytotoxic agents, there was little information on the genetics behind the resistance, and in particular in relation to resistance to cisplatin. An understanding of how hypoxia affects platinum resistance is extremely important in the context of ovarian cancer, as platinum-based treatment is the standard of care for first-line treatment, and platinum resistance in ovarian cancer has been associated with a poorer outcome for ovarian cancer patients.

6.3 Additions to Current Knowledge on Hypoxia and Ovarian Cancer Chemoresistance by this Project

The first aim of this project was to characterize the effect of various hypoxic challenges on the resistance profile of A2780 and its daughter A2780cis ovarian carcinoma cells. A hypoxia 'design matrix' was formulated in order to test various combinations of hypoxia and drug treatments. Cells were treated with the chemotherapeutic drugs cisplatin and paclitaxel in the presence or absence of hypoxia. In addition, the effect of acute and chronic exposure was examined by pre-exposing the cells to hypoxia before drug treatment. These experiments provided a number of novel findings in relation to drug resistance in ovarian cancer and hypoxia. Firstly, it was shown that in cisplatin-sensitive A2780 cells, resistance to cisplatin increases over time, while in the already cisplatin-resistant cell line A2780cis, although hypoxia does increase resistance to cisplatin, the magnitude of the increase is not as large in A2780, and the resistance diminishes over time, although remains significant. This indicates that a certain number of genes and pathways which are switched on in A2780 following chronic exposure to cisplatin may overlap with those which are switched on by hypoxia to induce resistance. This was a completely novel finding, as there had previously been no studies published in relation to absolute changes in resistance (IC_{50}) to cisplatin in relation to hypoxia and ovarian cancer. A recent study by Su *et al.* measured changes in cisplatin IC_{50} in the ovarian cancer cell line C13K following treatment with the hypoxia mimetic agent cobalt chloride, and found increasing resistance to cisplatin with increasing degree of hypoxia [306]. However, there are still no articles published in relation to the effect of pre-exposure of hypoxia on cisplatin resistance. Similarly, there are currently no articles published in A2780cis regarding the effect of hypoxia on cisplatin resistance.

Secondly, it was shown that there is a trend of increased resistance to paclitaxel in A2780 cells in response to hypoxia, although this is non-significant due to a number of potential confounding factors as discussed in Chapter 3. At the time of commencement of this project, there was no information published in relation to paclitaxel resistance in ovarian cancer associated with hypoxia. However, the

increased resistance we observed is in agreement with a study by Huang *et al.* in A2780 cells published in 2010 which observed increased paclitaxel resistance in cells treated for 24 hours in varying degrees of hypoxia [346]. Huang *et al.* demonstrated this response to be HIF-1α-induced, and related to cell cycle arrest in G_0/G_1. In addition, a study by Milane *et al.* in 2011 showed increased resistance to paclitaxel in SKOV3 and OVCAR5 ovarian cancer cell lines which were exposed to 0.5% O_2 during treatment [944]. However, there have been no articles published examining the effect of hypoxia pre-exposure on the response to paclitaxel, as was examined in our study. Interestingly, the resistance profile of A2780cis to paclitaxel was not altered in hypoxia. An inverse relationship between cisplatin and paclitaxel resistance has been noted in a number of cell lines [945], however this was not a feature of this cell line model. There is currently no information in the literature regarding paclitaxel resistance in hypoxia in A2780cis.

When we had established that hypoxia does cause chemoresistance in our ovarian cancer model, we aimed to identify genes and pathways causing this resistance by whole genome profiling. RNA was extracted from the point on the hypoxia matrix which gave the largest and most consistent changes in resistance to cisplatin in both cell lines – treatment with cisplatin in hypoxia with no pre-exposure to hypoxia. The changes in gene expression were determined using Affymetrix Human Gene ST 1.0 arrays and were analysed using Affymetrix Expression Console software (quality control), Bioconductor (normalization and statistics), and DAVID (pathway analysis).

In order to identify markers of chemoresistance, three main questions were focused on:

i) What are the common gene changes in A2780 cells chronically exposed to cisplatin (A2780cis) and A2780 cells exposed to hypoxia?

ii) What are the gene changes in A2780 and A2780cis cells in response to hypoxia?

iii) What is the 'hypoxic only' response to cisplatin in A2780 and A2780cis i.e. the gene changes which occur in response to cisplatin treatment in hypoxia but not normoxia

A plethora of genetic changes were identified in response to hypoxia and/or cisplatin treatment in both cell lines. This list was mined using pathway analysis and analysis of the individual genes through literature searches to identify any links to cisplatin resistance in ovarian cancer or others. When A2780cis cells were compared to A2780, a number of gene changes were identified which had previously been linked to cisplatin resistance in the literature including platelet-derived growth factor C (PDGFC) in head and neck squamous cell carcinoma [437], protein kinase C alpha (PRKCA) in ovarian cancer [946], Jak1 in osteosarcoma [446] and KIT ligand in ovarian cancer [8]. Thus, while we identified a number of genes which had previously been associated with cisplatin resistance in ovarian cancer as being altered in this cell line model, we also identified changes which have previously been identified in other cancer types as having potential involvement in cisplatin resistance in ovarian cancer.

In A2780 cells exposed to hypoxia, we identified a number of genes as dysregulated which had previously been observed in that cell line including FOS and telomerase RNA component [947]. However, the majority of genes identified have not been described as altered in A2780 in response to hypoxia previously, and no previous studies have analysed the gene changes in A2780 following hypoxic exposure on array. Similarly, there has been no previous work published on the effect of hypoxia on A2780cis cells. Thus, the vast majority of information gleaned from the arrays is novel. We identified a number of genes

associated with cisplatin resistance in both cell lines when exposed to hypoxia. These include genes which have been previously identified as associated with cisplatin resistance in ovarian cancer such as dual specificity phosphatase 1 (DUSP1) [613], FOS [948], collagen type VI alpha 3 (COL6A3) [949], insulin-like growth factor 1 receptor (IGF1R) [622], Rho-associated coiled-coil containing protein kinase 1 (ROCK1) [950], x-linked inhibitor of apoptosis protein (XIAP) [951], MET [952], PIK3CA [953] L1-CAM [556], and RBL2 [954]. In addition, cisplatin related genes which had not previously been linked to ovarian cancer were identified including BIRC2 (prostate cancer) [528], BIRC3 (lung cancer) [151], CXCR4 (lung cancer) [955], BDNF (head and neck cancer) [516], AKT3 (uterine cancer) [956], CD55 (oral squamous cell carcinoma) [628], SERPINE1 (glioblastoma) [957]. In addition, several of the markers had not previously been linked to hypoxia, including COL6A3, RBL2, and BIRC2. Thus the studies of changes in gene expression in hypoxia-exposed A2780 and A2780cis have identified novel markers of chemoresistance in ovarian cancer, as well as linking the expression of certain chemoresistance markers to hypoxia.

Gene profiling of A2780 and A2780cis cell lines following treatment with cisplatin in normoxia or hypoxia identified a wide range of genes which were dysregulated following drug treatment. A previous study by Varma *et al.* examined gene expression changes in A2780 cells following treatment with cisplatin using Affymetrix HG-U95 chips [693]. In their published article, they mainly focus on the effects of cisplatin on polyamine pathway genes. In our study, in general there was no change detected in the polyamine genes detected by Varma *et al.*, however this can be explained in a number of ways. Firstly, the methods used in the two studies were quite different. The drug treatment period was from 2 - 24 hours in Varma *et al.*, compared to 72 hours in this study. Also, Varma *et al.* incubated their cells in drug-free medium for 16 hours following cisplatin treatment before processing for array experiments, unlike our study, in which RNA was harvested directly following drug treatment. In addition, the statistical models used for analysing data by Varma *et al.* were different from those used in this study, and may affect the final gene lists generated. A subsequent article by

Brun *et al.* of the same group examined a wider range of genes in A2780 following cisplatin treatment [958]. Although some differences were noted between the differential gene expression from Brun *et al.* and our study, for many genes, the dysregulation followed the same pattern e.g. BTG2, CDKN1A, TP53I3 all up-regulated in both studies, and CENPA, MCM6 and PLK all down-regulated in both studies. There have been no studies published to date which have gene profiled A2780 or A2780 cell lines following cisplatin treatment in hypoxia. In fact, to my knowledge, there are no microarray studies published to date in any cancer type which examine the effect of cisplatin treatment in conjunction with hypoxia exposure. Our analysis of the 'hypoxic only' response to cisplatin treatment yielded a number of markers related to cisplatin resistance. While many of these had previously been linked with chemoresistance in ovarian cancer, e.g. BCL2L1 [959], PIK3R1 [670], RGS [688], PCNA [852], others had previously only been recognized as markers of chemoresistance in other cancer types e.g. E2F5 (lung) [678], RIPK1 (cervical) [669], SFN (colon) [686] and NOTCH1 (head and neck) [741]. Therefore, this microarray data has identified a number of novel chemoresistance markers of ovarian cancer.

Based on the questions asked of the array analysis, four genes were chosen for validation in a cohort of patient tumour samples:

i) HER3 – this was up-regulated in both A2780 cells exposed to chronic cisplatin (A2780cis) and A2780 cells exposed to hypoxia

ii) BDNF – this was up-regulated in A2780 cells exposed to hypoxia

iii) ANGPTL4 – this was up-regulated in both A2780 and A2780cis cells exposed to hypoxia

iv) MAD2L1 – this was down-regulated in both A2780 and A2780cis cells exposed to cisplatin in hypoxia

In addition, expression of HIF-1α was determined, as it is a universal marker of hypoxic change.

Expression was measured in a group of serous papillary carcinomas, which were categorized as responders (>12 months progression free survival, PFS), partial responders (6 – 12 months PFS) or non-responders (<6 months PFS). HER3 expression was significantly down-regulated in partial- and non-responders to platinum/taxane based chemotherapy. ANGPTL4 was up-regulated in partial-responders to chemotherapy, while it was down-regulated in non-responders. HIF-1α was up-regulated in both partial- and non-responders to chemotherapy, while BDNF expression was not detected in the majority of the samples tested. MAD2L1 expression was not evaluated in this study, as its expression in the same cohort had been previously evaluated by one of our collaborating groups in University College Dublin.

The tumour sample validation of biomarkers revealed a number of novel findings. While BDNF had been previously linked to cisplatin resistance, it was not linked to cisplatin resistance in ovarian cancer. Its expression, and expression of its receptor, had been previously demonstrated in normal ovarian tissue, where it plays a role in normal follicular development [960-962] and maturation of oocytes [963]. Since repeated ovulatory cycles are thought to be one reason for ovarian cancer development, there is potential for BDNF to play a role. Indeed, BDNF has previously been shown to induce ovarian cancer cell migration and proliferation [813], and expression of its receptor, TrkB, is associated with resistance to anoikis [964]. Expression of TrkB has been demonstrated in ovarian cancer tissues, and it is associated with poor outcome, while BDNF expression was not shown to be significantly different between normal and cancer tissues [814]. Unfortunately, in this study, the number of samples expressing BDNF was insufficient for thorough analysis – study in a larger cohort of samples will be necessary to fully evaluate it.

This is the first study to describe ANGPTL4 expression in serous ovarian cancer tissue. One study has identified ANGPTL4 as expressed in a xenograft model of

ovarian cancer [803], however there are no other studies demonstrating its expression in ovarian cancer tissues.

This study identified down-regulation of HER3, a marker whose up-regulation is commonly associated with chemoresistance – however, low expression of HER3 is associated with sensitivity to other therapeutics, and may identify patients who may benefit from alternative therapies. Tanner *et al.* found HER3 expression in approximately 50% of a cohort of 116 ovarian tumours, and associated HER3 over-expression with reduced overall survival [830]. This is in contrast to our study. As discussed in Chapter 5, others have also observed low levels of HER3 expression in ovarian cancer samples, therefore this was not a unique finding, and may be a feature of the particular sample cohort used in this study, a result of intra-tumour heterogeneity or due to HER3 promoter inhibition by an as yet undetermined molecule. Although we found HER3 over-expression to trend towards increased PFS, it also trended towards reduced OS, in agreement with the current literature. However, the cohort used in our study was very small, and all samples were positive for HER3 expression. It is likely that in a larger cohort, these results may change, however, as discussed, it may be that low HER3 expression may be an identifier for patients suitable for other therapies in addition to platinum/taxane.

We found HIF-1α to be over-expressed in patients with a partial-response and down-regulated in patients with a non-response to platinum/taxane based chemotherapy. HIF-1α over-expression has generally been associated with poor overall survival [199,200,360]. However other studies have shown HIF-1α expression to be not associated with survival [853], or associated with improved response [201]. Thus this study has added to the body of knowledge on HIF-1α as a prognostic indicator in ovarian cancer patient samples.

As MAD2L1 expression had previously been determined in the cohort of samples we used by Furlong *et al.* [781], we did not re-determine the expression in these tissues. Furlong *et al.* found that low MAD2L1 expression was associated with reduced progression free survival following carboplatin/paclitaxel-based

chemotherapy. Their group had previously shown MAD2L1 expression to be down-regulated by hypoxia and identified a reciprocal immunohistochemical staining pattern between MAD2L1 and CA9, a marker of hypoxia in ovarian tissues [769]. These are the only studies published in relation to MAD2L1 expression and ovarian cancer tissue to date, although MAD2L1 had previously been linked to platinum resistance in a number of other cancers including nasopharyngeal [765], gastric [766] and testicular cancer [768]. In addition, low MAD2L1 levels had also previously been linked to paclitaxel resistance in ovarian cancer cell lines [844,965].

6.4 Hypoxia and Ovarian Cancer - 2012; How this study compares

Since this study began, there have been several advances in the field of hypoxia and ovarian cancer.

Further studies were carried out on inhibition of the hypoxia-stimulated pathway mTOR as a therapeutic strategy for ovarian cancer [966]. In addition, new inhibitors of HIF-1α-related proteins were evaluated in cell lines and animal models such as ABT-510 [481], campothecin analogue NSC606985 [291], kaempferol [967], albendazole [905], BACPT [968], p70S6K1 [969] and sorafenib [970]. Further hypoxia-related prognostic markers of ovarian cancer were identified such as iNOS [202] and bone-morphogenetic protein 4 (BMP4) [971]. Importantly, the results of several phase II and phase III trials on the effectiveness of the anti-angiogenic, VEGF-targeting drug bevacizumab were published. In a phase II trial, Penson *et al.* evaluated the effects of combination bevacizumab with carboplatin/paclitaxel in ovarian cancer and found that combination therapy was associated with high remission and was well-tolerated [972]. A phase III trial by Perren *et al.* showed that addition of bevacizumab to standard chemotherapy significantly improved progression free survival, and in particular in patients at high risk of progression [549], thus demonstrating the clinical benefit of a drug targeted against an ovarian cancer/hypoxia biomarker.

Further insights into the biology of HIF-1α effects on ovarian tumourigenesis were discovered, such as the implication of hypoxia-induced REDD1 in Ras-mediated transformation [973]. In addition, glucose deprivation was shown to stimulate expression of angiogenic mediator VEGF [974] and follistatin, a tumourigenesis-associated protein [975]. The role of ovarian hormones oestrogen and progestin in mediating HIF-1α expression via the Akt pathway was discovered [205]. Some gene alterations in the adaptive response to hypoxia were identified including Cyclin D1 and V-src [148]. Hypoxic ovarian tumour cells were also shown to produce tissue factor-coagulation factor VII, a player in thrombosis observed in ovarian cancer patients [976], and truncated, inactive forms of the pro-apoptotic protein BNIP3 [977]. Akt and reactive oxygen species (ROS) were identified as important mediators of resistance to hypoxia-induced apoptosis [978,979]. NOTCH1 receptor ligand Delta-like 4 (Dll4) was identified as up-regulated by hypoxia and was shown to have potential as a therapeutic target [980]. S100A4 was shown to be up-regulated by hypoxia and associated with increased invasiveness [981].

Further insight into the mechanism of hypoxia-induced chemoresistance was demonstrated. Increased phosphorylation of STAT3 in hypoxia was shown to confer resistance to cisplatin and paclitaxel in A2780-derived xenograft tumours [189], and hyperbaric oxygen treatment was shown to reduce STAT3 levels and improve chemotherapy efficacy [982]. In addition, inhibition of the ROCK pathway was shown to increase cisplatin effectiveness in ovarian cancer cells [950].

Therefore, while there have been substantial advances in the understanding of the biology of hypoxia and ovarian cancer, there have been relatively few advances in the understanding of how hypoxia causes chemoresistance in ovarian cancer. Our study has significantly contributed to the body of knowledge of how hypoxia affects chemoresistance in terms of absolute fold-changes of resistance, genetic modifications in response to hypoxia, and the evaluation of novel biomarkers of chemoresistance.

6.5 Limitations of the Study

Although this study has provided some significant contributions to knowledge of the role of hypoxia in ovarian cancer chemoresistance, there are several limitations to the project.

The objective of the first results chapter, Chapter 3, was to observe the effects of hypoxia on chemoresistance in an ovarian cancer cell line model. We used a paired chemoresistance cell line model of A2780 and A2780cis. This is the only commercially available platinum resistance model of ovarian cancer. However, it would have been useful to repeat the experiments using other models. It was beyond the scope of this study to derive platinum resistant models from other ovarian cancer cell lines, however this is something that could be considered for future projects. Alternatively, it may be possible to source privately banked platinum resistant cell line models. In addition, while the response to cisplatin and paclitaxel in hypoxia was observed for the cell line model used, it would have been useful to observe some of the other effects of hypoxia on the cells. For example, the effect of hypoxia on cell cycle distribution through the use of flow cytometry could reveal whether hypoxia was inducing cell cycle arrest or progression, and indicate whether senescence, a tumour-preventative state consisting of irreversible cell cycle arrest [235], is being inhibited in order to enhance chemoresistance.

The sample size used in Chapter 5, in which the expression of a number of genes was evaluated in a cohort of ovarian tumour samples is perhaps the most significant limitation of the study. Due to time constraints, a small sample size of 35 was used to carry out an initial assessment of the expression levels of these genes in ovarian cancer tumour samples. The probability of obtaining a statistically significant result when performing a statistical test, or 'power' is essential when designing an experiment [983]. Power calculations are based upon the level of significance desired, α, the size of the biologically relevant difference, and the sample size, and power is generally greater with a larger sample size. Often, sample size calculations are based on a pilot study, a test

sample group which gives an indication of the significance level which should be chosen, and identifies any issues with the experimental process. In fact, the experiments carried out in this study could serve as a pilot study for a future large scale study of these biomarkers. It would provide information on expression levels in this population – for example, while ANGPTL4, HER3 and HIF-1α were expressed in all samples assayed, BDNF was only expressed in 6 of 35 samples, an important consideration when planning a larger scale study. Indeed, ANGPTL4 has been revealed as the most promising candidate based on the expression levels observed in this study, and will be of interest in future work.

6.6 Future Work

Future studies on this project could include:

i) Analysis of a hypoxia gradient
ii) Methylation analysis and hypoxia
iii) Evaluation of current biomarkers in a wider cohort, and at the protein level
iv) Evaluation of other biomarkers not evaluated in this study
v) Functional analysis of promising biomarkers

While in this study we analysed changes which occurred at levels of 0.5% oxygen, the oxygen levels within a tumour are not constant. There are gradients of hypoxia depending on how far cells are located from blood vessels [984]. Therefore, it would be of interest to determine the effects of varying levels of hypoxia on chemoresistance. A collaborating group in Dublin City University has developed a silicone based chip which is capable of generating gradients of oxygen. This will facilitate high-throughput testing of ovarian cancer cells in relation to varying concentrations of chemotherapeutic drugs at varying oxygen concentrations, and is one potential avenue for further research.

We observed concentrated regions of gene down-regulation in relation to cisplatin resistance and hypoxia. One reason for this may be gene hypermethylation, as hypoxia has previously been shown to induce gene

methylation in cancer [985,986]. Certain genes which were linked to cisplatin-resistance were down-regulated in response to hypoxia including DNA damage inducible transcript 3 (DDIT3) and tumour necrosis factor (TNF). Methylation of DDIT3 has previously been identified in chronic myeloid leukaemia [987] but not in ovarian cancer. Similarly, TNF has been identified as methylated in a large proportion of leukaemia cases [988] and demethylation of breast cancer samples has revealed a number of up-regulated TNF-related pathways [989]. Thus, it would be of interest to carry out methylation analysis of the genes which were down-regulated by hypoxia, and see if demethylation of the genes could improve chemosensitivity.

One limitation of this study was that the cohort of tumour samples used to validate the biomarkers was small. It would be necessary to validate the biomarkers in a much larger cohort of tumour samples in order to fully evaluate their potential. Importantly, it would be valuable to identify whether changes observed at the mRNA level were also present at the protein level. Proteins are the effectors of the genome i.e. it is the translated protein that carries out the function initiated by transcription of the gene. However, down-stream post-translational modification, protein folding and protein degradation can inhibit the desired effect of transcription [990]. Therefore, changes at the mRNA level do not necessarily represent changes at the protein level. Protein biomarkers which may be detected using simple assays e.g. immunohistochemistry, in which the protein is recognized by a specific antibody, and detected using enzymatic methods, are essential to become useful routine laboratory tests. Thus, the likely most valuable future work for this study would be to determine the protein status of the biomarkers identified.

In addition, we only examined expression in serous carcinoma cases categorized according to response. It would be of use to examine their expression in other histological subtypes of ovarian cancer, or perhaps compare expression in primary vs. metastatic/recurrent lesions in order to further understand their impact on tumour biology. Also, only a very small proportion of the potential biomarkers identified were validated in this study. Many other promising

chemoresistance biomarkers were identified and have potential to be validated in tumour samples. Functional analysis of promising biomarkers through over-expression or knock-down in hypoxia could be used to analyse their potential as therapeutic targets in ovarian cancer patients. VEGF is one hypoxia-related biomarker which has had success as a therapeutic target in ovarian cancer, thus the potential is there for success with other novel biomarkers. In our study, ANGPTL4 was the most promising biomarker candidate, and if future studies can confirm and expand the findings, it would be a promising marker to carry out functional work on.

6.7 Conclusion

Overall this study has had a number of findings in relation to hypoxia in terms of:

i) How duration and timing of hypoxia affect resistance

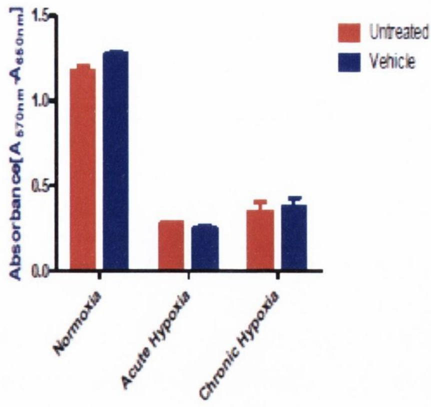
ii) Gene profiles of ovarian cancer cells in response to hypoxia and/or chemotherapy

iii) Validation of novel hypoxia-related biomarkers of chemoresistance

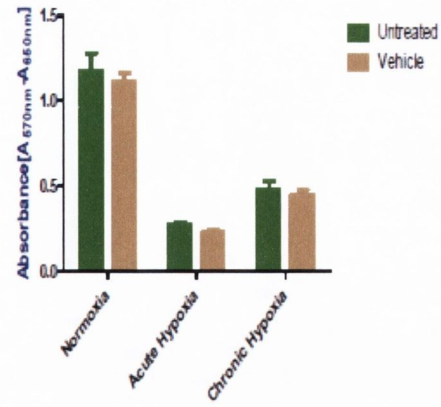
We have substantially added to the current body of knowledge in the literature in relation to genetic changes in response to hypoxia in an ovarian cancer, and provided initial information on the expression of potential biomarkers in tumour specimens.

Appendix

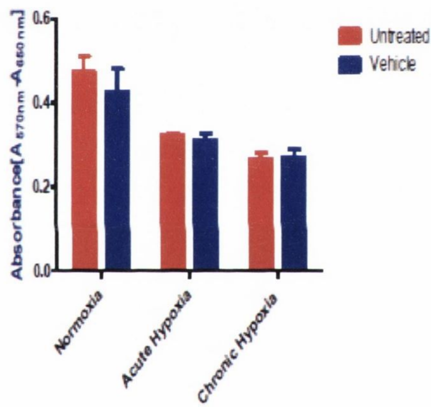
A



B



C



D

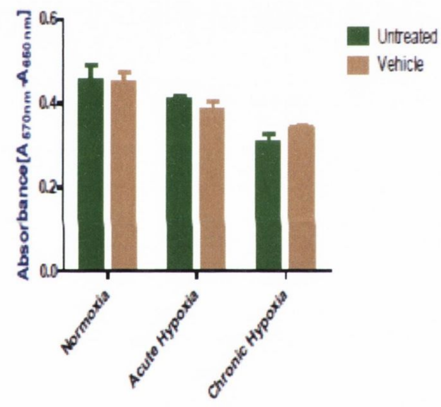


Figure A1. Comparison of Untreated Cells with Cells Treated with Vehicle Control Solutions. No difference in viability was observed between untreated A2780 cells and those treated with cisplatin vehicle (A) or paclitaxel vehicle (B). Similarly, no difference in viability was observed between untreated A2780cis cells and those treated with cisplatin vehicle (C) or paclitaxel vehicle (D). n = 3

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