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The role of epigenetic changes in chemoresistant breast cancer cells

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THE ROLE OF EPIGENETIC CHANGES IN CHEMORESISTANT BREAST CANCER CELLS

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Abstract

Cytotoxic chemotherapy is extremely important in adjuvant treatment of breast cancer. Yet, tumours frequently acquire chemoresistance that correlates with increased aggressiveness and poor prognosis. Three theories exist describing how the resistance develops: genetic, epigenetic and karyotypic theory. The epigenetic theory is the least explored. Here we analyzed the role of the epigenetic phenomena in the acquisition of drug resistance. To do so, we employed genome wide screens of microRNA and gene expression, DNA methylation and complete genome hybridization. We identified three novel microRNA interactions involved in the chemoresistant phenotype. These three microRNAs displayed depressed expression in the resistant cell lines and we were able to re-establish some level of drug sensitivity through ectopic expression of these under expressed microRNAs. In addition, we described the role of DNA methylation in impacting expression of a wide range of genes, thus, contributing to the phenotype of chemoresistance. Furthermore, we revealed a distorted global DNA methylation pattern that coincides with massive instability of the resistant genome. Finally, our results present a striking similarity between gene expression, epigenetic profiles and chromosomal aberrations in two different drug resistant cell lines. Taken together, this project suggests that the acquisition of chemoresistant phenotype is epigenetic in nature and may arise with a predictable pattern. Elucidating the specifics of this pattern may in the future prove useful in developing treatment and prognostic chemoresistance biomarkers.

Phor Phace and Phuzz

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[3H]dCTP - tritium labeled cytosine triphosphate
Akt - v-akt murine thymoma viral oncogene
Apaf 1 - apoptosis activating factor
ATM - ataxia
Bad - Bcl2-associated death promoter
Bak - Bcl-2 cell homologous antagonist/killer
Bax - Bcl2-associated X protein
Bcl-2 - B cell lymphoma 2
BCL-xl - B-cell lymphoma extra-large
Bcr-abl - break point cluster region-abelson murine leukemia oncogene
bp - base pair
BSA - bovine serum albumin
CDC 25 - cell division cycle 25
CDK2 - cyclin dependent kinase 2
CGH - complete genome hybridization
CIS – cisplatin
CNV - copy number variant
CO2 - carbon dioxide
CpG - cytosine in the 5' adjacent position to a guanine in a DNA strand
Cy 3 - cyanine 3
Cy 5 - cyanine 5
DAPI - 40,6-diamidino-2-phenylindole
dATP - deoxy adenosine triphosphate
dCTP - deoxy cytosine triphosphate
dGTP - deoxy guanine triphosphate
DHD - dihydropyrimidine dehydrogenase
DNA - deoxynucleic acid
DNMT - DNA methyltransferase
dNTP - deoxy nucleotide triphosphates
DOX – doxorubicin
dsDNA - double stranded DNA
EDTA - 2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetra-acetic acid
EGFR - epidermal growth factor
EMT - epithelial to mesenchymal transition
ERCC1 - Excision repair cross-complimenting repair deficiency 1
FAM - fluorescin amidite
FANCF - Fanconi anemia group F
FEN1 - flap endonulcease 1
G1 - gap1 growth phase
G2 - gap 2 growth phase
GC - guanine/cytosine
GO - gene ontology

GSH – glutathione
GST - glutathione-S-transferase
H – histone
h – hours
H2O – water
HM27 - Illumina human methylation27 bead array
HR - homologous recombination
http - deoxy thymine triphosphate
IC50 - inhibitory concentration to produce 50% cell death
MBD - methyl binding domain
MCF7 - Michigan cancer foundataion7 (cell line)
MCF7/CDDP - MCF7 cells resistant to cusplatin
MCF7/DOX - MCF7 cells resistant to doxorubicin
Mcl-1 - myeloid cell leukemia 1
MDA-MB-231 -
MDR1 - multi-drug resistant1
MeCP2 - methyl-CpG binding protein 2
MGMT - O-6-methylguanine DNA methyltransferase
miRNA – microRNA
MLH1 - MutL homolog1
MMR - mismatch repair
MRP - multi-drug related protein 2
MRP1 - multi-drug related protien1
MSH2 - MutS homolog2
MTX – methotrexate
NCBI - national center for biotechnology information
NEB - New England biolabs
NFkB - nuclear factor kappa-light-chain enhancer of activated B cells
NHEJ - non-homologous end join
NIH - National Institute of Health
nT – nucleotide
nT – nucleotide
N-terminal - the amino end of an amino cid polypeptide strand
PAGE - poly acrylamide gel electrophoresis
PBS - phosphate buffered saline
PCNA - proliferating ell nuclear antigen
PCR - polymerase chain reaction
P-gp - P glycoprotein
pol – polymerase
PP2 - 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine
PTGS - post transcriptional gene silencing
qRT-PCR - quantitative reverse transcription- polymerase chain reaction
Ras - Rat sarcoma
Rb – retinoblastoma
RISC - RNA silencing complex

RNA - ribonucleic acid
SAM - S-adenomethionine
ser/thr - serine/threonine
SIP1 - survival of motor neuron protein-interacting protein 1
SNP - single nucleotide polymorphism
SOCS1 - suppressor of cytokine signaling 1
S-phase - synthesis (DNA) phase
STAT - signal transducer and activator of transcription
TF – Transcription Factor
TGS - transcriptional gene silencing
TS - thymidine synthase
TX – Texas
UTR - untranslated region
V – volts
VA – Virginia
Wrnip - Werner's helicase interacting protein 1
WT - wild type
XP - Xeroderma pigmentosum A
ZEB1 - zinc finger E-box binding homeobox 1

1. General Introduction

1.1 Epidemiology

At 28% of all new cancers, breast cancer is the most common and the second deadliest (15%). With the exception of lung cancer, it is the only cancer type that has increased in incidence over the last 35 years[2]. Furthermore, it is expensive to treat, with total treatment expenditures nearing $14 billion in 2006—the most expensive of any single cancer (NIH Cancer Trends Report, 2010). Unfortunately, breast treatment does not pose a onetime challenge as ~30% of remised patients will endure recurrences which are predominantly metastatic and resistant to treatment and, thus, present a dismal outcome.

1.2 Cancer Treatment

Cancerous tumours are treated by surgical removal (resection), radiation exposure and chemical treatment (chemotherapy) or a combination of the three as dictated by cancer type, tumour size and location (NIH, 2010). Since tumour masses originate from a single cell, it is important to eradicate all cells of an existing tumour to prevent recurrence. This goal is severely hindered in resistant tumours as typically a reduced response is seen following both chemotherapeutic and radiation regimes. Although the exact mechanisms are not yet understood, the newly acquired resistance (both chemical/drug and radio-resistant) appears to coincide with increased invasiveness[3,4].

1.2.1 Chemotherapy

The use of chemotherapy as a strategy to treat cancer emerged in the 1940's. The first example of its use in clinical practice involved the treatment of a malignant lymphoma with nitrogen mustard; achieving a regression in the disease. However, a second dose

elicited a lesser affect and a third led to no response at all[5]. So, for as long as chemotherapy has been employed, acquired drug resistance has followed.

The goal of chemotherapeutic agents is cytotoxicity. Usually this is achieved through interfering with key life processes including DNA replication and repair or mitosis. However, in the last few decades several drugs that facilitate the cytotoxic effects by repressing growth signals or promoting apoptosis have been designed and licensed (NIH, 2010). At current, chemotherapeutic agents fall into six categories: i) direct DNA damaging agents; ii) anti-metabolites that prevent viable DNA/RNA production (e.g. nucleoside analogs); iii) DNA intercalators that interfere with replication machinery; iv) topoisomerase inhibitors that prevent the proper management of replication forks; v) anti mitotic agents that prevent cytokinesis, and; vi) targeted therapies that are engineered for specific targets in specific tumour types or towards the tumour microenvironment (NIH, 2010). Instances of resistance have been described for drugs in each category[6].

1.2.1.1 Cisplatin and Doxorubicin

Cisplatin and doxorubicin belong to the DNA damaging and DNA intercalating categories; respectively, as such they are considered genotoxic agents. Both of these drugs function at the level of replication. Cisplatin, an alkylating agent, belongs to the platinum-based drug class and was developed over 30 years ago. It is a neutral, planar molecule that requires activation via two aquation reactions upon entry into the cell. In its active form, cisplatin causes bulky, intrastrand, cross linked adducts between adjacent purine residues[7]. The slightly older (developed ~50 years ago) anthracycline, doxorubicin—a large planar molecule with several aromatic rings, intercalate into DNA

strands by positioning itself between base pairs and the minor groove of the DNA backbone. When in position, it is capable of impeding transcriptional and replication machinery[8]. These adducts, if left unrepaired, stall the machinery preventing proper replication and ultimately invoke an apoptotic signal through the DNA damage signalling pathway[7,8]. Both drugs are amongst the widest prescribed and most effective, however, acquired resistance is frequently observed in treated patients. For example, ovarian cancer initially responds favourably about 70% of the time to cisplatin, however, 5-year survival rates are ~15-20% as resistance is acquired to a broad range of drugs[7]. Although doxorubicin is among the most active agents in breast cancer treatment, many women will experience a relapse. Furthermore, approximately half of women with metastatic breast cancer will fail to respond to doxorubicin, and the majority of those showing initial benefit will subsequently deomstrate acquired clinical resistance, as demonstrated by tumor growth despite ongoing antracycline treatment[9].

1.3 Molecular Mechanism of Drug Resistance

The main goal of chemotherapy is to impart a cytotoxic effect on tumour cells. In short, this relies on three critical points: i) drug uptake; ii) interaction with target, and; iii) induction of apoptosis. Although the preceding sentence greatly oversimplifies the complexities of each step, it demonstrates that there are a finite number of opportunities by which a cancer cell may evade the effects of a drug. From a molecular perspective, this evasion is achieved through: i) reduced intra-cellular concentrations (impaired uptake; increased efflux); ii) increased or altered targets, drug inactivation or drug compartmentalization, and; iii) evasion of apoptosis via altered checkpoints or repairing DNA damage[1,6] (Figure 1.1)

1.3.1 Drug Uptake

The first important hurdle in achieving an effective drug response, is delivery to the cell. Congruently, the first mechanism of resistance is achieved by hindering transport into or accumulation in the cell. For example, resistance to methotrexate (MTX), an anti-metabolite drug whose toxic effects are achieved through preventing nucleoside synthesis, is observed in leukemia patients. MTX enters the cells through a solute carrier membrane protein. Reduced expression of this transport protein is associated with increased MTX resistance in cell lines and tumours and poor patient prognosis[10-12]. Besides import proteins, reduced accumulation can be the result of increased efflux. Drug efflux pumps are large transmembrane proteins that actively transport molecules across the membrane[13]. Although these transporters serve important physiological functions such as moving xenobiotics in a unidirectional fashion, protecting cells from toxic species and concentrating metabolic products; over expression and activity is

frequently observed in resistant phenotypes of solid tumours and leukemia[6]. Furthermore, the broad specificity of substrates for these pumps can account for the multi-drug resistant phenotype. The efflux pump most studied in a wide variety of cancers is aptly named *multi-drug resistance-1* (MDR1). It can transport a wide range of neutral and positively charged hydrophobic drug species, including doxorubicin[14,15]. Unfortunately, large scale cloning studies, demonstrated that many multi-drug resistant tumours did not express MDR1, however, they did express another member of the efflux family known as multi-drug resistance associated protein-1 (MRP1). Although MRP1 functions in the same manner as MDR1, it has a preference for negatively charged drugs including conjugated-cisplatin[1] and MRP1 is known to play a role in cisplatin resistance[7,16,17].

1.3.2 Interaction with target

1.3.2.1 Drug Inactivation

Drug metabolism circumvents the cytotoxic nature of a drug typically by reducing availability of free drug to interact with its target. 5-fluorouracil, a nucleoside analog that is frequently prescribed for solid tumours, is catabolised and inactivated by the cytosolic enzyme dihydropyrimidine dehydrogenase (DHD). Over expression of DHD both *in vitro* and *in vivo* is linked to 5-fluorouracil resistance[18,19]. Although this represents an outright destruction, more subtle modifications can still manage to impact the efficacy of a drug. Cisplatin is known to be modified by the cytosolic, scavenging, antioxidant glutathione (GSH). In the presence of glutathione-S-transferase (GST), cisplatin is covalently linked to GSH. This conjugated form is a better substrate for the MRP1 pump than cisplatin alone and, thus, is shuttled out of the cell. High levels of GST and GSH

expression are linked to cisplatin resistance[20,21]. Furthermore, repression of GST has reversed cisplatin resistance in breast cancer cells[22].

1.3.2.2 Altered Targets

Mutated drug targets or their over expression can impact the efficacy of a drug. For example, the expression levels of thymidine synthase (TS), the target of 5-fluorouracil, regulates chemosensitivity. Patients exhibiting an activating polymorphism in the promoter region of thymidine synthase over express TS and demonstrate resistance to the drug[23]. In another instance, decreased topoisomerase II activity due to reduced protein or mutations in the gene, confers resistance to doxorubicin[24,25]. Finally, resistance to taxanes, drugs that act on the microtubule dynamics, has been observed in patients that display altered microtubule mass or express different tubule isotypes[26,27].

1.3.3 Induction of apoptosis

Apoptosis is mediated through several interconnecting pathways and a multitude of proteins including, those employed in the DNA damage response, cell cycle, intrinsic apoptotic and proliferative pathway. For ease of presentation, these pathways are addressed below independently, however, it is impossible to expect changes in one of the pathways to not heavily influence the activity of the other three.

1.3.3.1 Repairing DNA damage

Many chemotherapeutic regimes attempt to induce massive DNA damage, either directly (e.g. alkylating agent-cisplatin) or indirectly (e.g. stalling replication/translational machinery- doxorubicin). If plentiful enough, this damage should induce apoptosis. However, in some instances, the over expression of DNA repair genes can efficiently

reverse any acquired damage. For example, cisplatin causes intra-strand adducts between adjacent purines that are readily reversed by the nucleotide excision repair pathway. Although this is an intricate pathway that involves in a multitude of different genes, over expression of the few rate-limiting players is sufficient to induce cisplatin resistance. ERCC1 and XPA over expression, both involved in the excision of the damaged strand, correlate well with cisplatin resistance in clinical samples of numerous tumour types[28,29] .

Interestingly, cases of resistance are reported in repair deficient cells as well. Abrogated mismatch repair proteins MSH2 and MLH1 have been implicated in the acquired resistance of ovarian tumours and cells to both cisplatin and doxorubicin[30-32]. This loss of MMR was linked to DNA methylation and microsatellite instability (two epigenetic features; explained elsewhere)[33,34]. Furthermore, the loss of MMR coincided with increased translesion synthesis, suggesting a plausible mechanism which allows these resistant cells to evade death[35].

1.3.3.2 Altered Checkpoints

Cells employ an elaborate system of checks and balances, termed cell cycle checkpoints, to ensure that genetic integrity is maintained between generations. In the event that it is not, a normal cell will induce apoptosis over proliferation. Circumventing any of these checkpoints can shift this balance. The master switch between DNA damage detection, cell cycle arrest and apoptosis is the p53 protein. Indeed, its importance is illustrated by the observation that p53 is mutated in up to 50% of cancers. However, it appears that p53 gene mutations do not correlate with expression in 30-40% of cases[6].

Conflicting reports also exist about its role in drug resistance. For example, opposing outcomes (sensitising and desensitizing) have been reported on the effect of p53 mutations in cisplatin treated cells (*in vitro*)[36,37]. From a molecular perspective it is also difficult to deduce the effects of p53 on resistance. On one hand a lack of p53 may prevent a cell from inducing apoptosis while on the other hand, WT or increased expression may increase the amount of time for DNA repair during cell cycle arrest: either way both result in a resistant phenotype. Furthermore, some studies indicate that it is unclear whether p53 status indicates poor prognosis due to platinum-base drug resistance proper or rather due to the increased aggressiveness seen in several resistant tumour types[38]. These conflicting results are observed in other genome damaging drugs as well such as 5-fluorouracil. Interestingly, doxorubicin seems to have a more predictable outcome: its sensitivity is dependent upon a WT p53 function with mutated and null p53 leading to resistance[39].

1.3.3.3 Apoptotic Pathway

Apoptosis is the death of a cell through a purposeful, mechanistic dismantling of the cellular machinery. It is induced through a number of signalling molecules with the most proximal being the Bcl-2 family of proteins. This family includes both pro-apoptotic (Bad, Bak and Bax) and anti-apoptotic members (Bcl-2, Bcl-xl and Mcl-1). Not surprisingly, there is good correlation between the expression levels of the Bcl-2 family of proteins and response to a wide range of chemotherapeutic agents. Specifically, down regulation of the anti-apoptotic members Bcl-2 and Bcl-xl (*in vitro*) increases sensitivity to a platinum drug, while loss of pro-apoptotic Bax decreases sensitivity[40]. Likewise, over expression of Bax in breast cancer cells increased sensitivity to cisplatin and

radiation, thus, providing some explanation of the cross resistance mechanism[41,42]. Once again, the status of Bcl-2 family members does not appear to be universal as others have reported a relationship between high Bcl-2 expression and increased sensitivity. In this instance, it has been suggested that the grade and aggressiveness may affect the death potential of the apoptotic proteins, with lower grade and less aggressive tumours responding to treatment regardless of Bcl-2 status[43].

1.3.3.4 Proliferative Signals

As mentioned above, the proliferative pathway interacts directly with the apoptotic, cell cycle, and DNA damage pathways. Built into proliferative pathways are proteins capable of inhibiting apoptotic signals. One such protein is Akt, an intracellular ser/thr kinase involved in the EGF pathway. Akt gains its anti-proliferative properties by inhibiting the pro-apoptotic proteins Bad and Casp9 and promoting NFκB. NFκB in turn promotes the pro-apoptotic Bcl2/Bcl-xl and 'inhibitor of apoptosis proteins'. Inhibiting the activity of AKT or NFκB was shown to increase sensitivity to several drugs and radiation therapy[44-47].

1.4 Theories of drug resistance

Although the molecular and cellular characteristics of drug resistance are frequently observed and explored, there is less information on how cells acquire these molecular changes in the first place. Several theories have been described. They will be detailed below.

1.4.1 Development of Resistance

As mentioned before, numerous cellular pathways have been inlcated in drug reistance. Yet, why and how are these pathways affected? As an added difficulty, tumours often demonstrate cross-resistance to an array of drugs that may be chemically different or even to other cytotoxic treatments i.e. radiation therapy. Boehm and Hahn attempted to evaluate the minimal and necessary mechanism that could elicit a drug resistant phenotype. They found that by introducing a number of transgenic mutations in a stepwise fashion they could create a tumorgenic cell. These mutations involved maintaining telomere length (increased telomerase activity), diminishing cell cycle control (p53 and RB inactivation) and promoting aberrant signalling (PP2 inactivation; constitutive Ras activity) and were performed in several primary epithelial cells including mammary gland, prostate, ovary, trachea and bronchia. Interestingly, with the exception of the post-telomerase mutation, treatment of these transformed cells following each step with doxorubicin demonstrated a drug-resistant phenotype[48]. This model revealed that the acquisition of drug resistance likely occurs through a mechanism similar to that of malignant transformation. However, it should be noted that not all naturally occurring

tumours exhibit the aforementioned marks and, thus, just as tumorgenesis involves a multi-faceted mechanism, so does the acquisition of drug resistance[49].

Through rapid Darwinian evolution and clonal expansion, resistant cells experience positive selection and, ultimately, will increase their frequency in the tumour cell population. When combined with the high proliferative rate observed in tumours, the acquisition of a resistant phenotype for the tumour as a whole becomes a relatively quick process. With the heterogenic nature of tumours, the degree of resistance can vary within a single tumour[50]. Iwasa *et al.* have managed to mathematically model the acquisition of resistance and not surprisingly they noted that probability of acquiring resistance increases with higher proliferative rates and increased genomic instability[51].

The clinical endpoint referred to as drug resistance is usually a multi-faceted characteristic resulting from a several different events within a single cell. Delineating the underlying mechanism and attempting to circumvent it is a daunting, yet necessary, task since drug resistance diminishes treatment efficacy in 90% of cancer cases and nearly all recurring cancers are resistant and metastatic[6]. The phenomenon of chemotherapeutic drug resistance has been observed since the first use of chemotherapy as an anti-cancer agent[5]. Several models attempt to explain the acquisition of drug resistance. These include the genetic, epigenetic and karyotypic theories. The genetic theory attempts to explain resistance through acquisition of mutations that impart an added fitness to the cells. This fitness is qualified through increased resistance to the chemotherapeutic regime. Conversely, epigenetics implicates non-mutational alterations that impact gene function (DNA methylation, histone changes and/or small, non-coding

RNA regulation) as the driving force behind the resistant phenotype. Finally, the karyotypic theory aims to illustrate the role of massive chromosomal aberrations and/or aneuploidy in the generation of the novel drug resistant phenotype[52]. Although evidence supporting each of the three theories exists, it is unlikely that any one of the three can solely explain the phenomenon: they likely all play some role but to varying extents.

1.4.1.1 Genetic Theory

Genetic mutation is thought to be a hallmark of the carcinogenesis process. The relationship between mutations and tumorgenesis describes a scenario where an initial mutation causes a phenotype that promotes and permits subsequent mutation events: over time this process culminates in cancer as, together, these mutations impact a number of cellular functions including apoptosis, cell cycle control, DNA repair, proliferation, invasiveness and transcription; for example. Large scale studies have: a) confirmed a high mutation rate in tumour cells, and; b) demonstrated a lack of similarity in mutation profiles between various tumour-types or within cells of the same tumour[53]. Therefore, it seems reasonable to expect that some of these mutations may impact the sensitivity of the cell. This premise is further bolstered by the observation that the acquisition of drug resistance can occur concurrently with carcinogenesis and it is only following initial rounds of treatment, which removes sensitive cells, that the resistant cells flourish giving rise to a new, resistant tumour mass[5].

In a well documented example of resistance, dozens of mutations interfering with drug-target interactions have been characterized in BCR-ABL kinase: the cellular protein target of imatinib (a targeted chemotherapy regime). These mutations, however, were not

"acquired" following initiation of the treatment, but rather existed in a subset of tumour cells and were selected for during the treatment[54-56]. In another instance, patients with non-small cell lung cancer display resistance to the EGFR antagonists gefitinib and erlotinib through mutations in EGFR or k-Ras gene (an early member of the EGFR-induced signally pathway)[57,58]. However, the narrow scope of these drugs (one drug: one protein target) allows a cell to evade their cytotoxicity through mutations affecting a single gene. Therefore, it doesn't explain multi-drug or cross-resistance which is usually the case. Furthermore, an identified mutation in the coding sequence of a gene does not necessarily impact the structure, function or stability of the resulting protein and, thus, mutations identified in resistant tumours may apply little pressure on the drug-target interaction[59]. Finally, one may speculate that multi-drug resistance can be the result of mutations in genes that have a global impact on the cell. For example, mutations in p53 coincide with resistance to many cytotoxic drugs, and p53 status is important in determining a prognosis for responsiveness to platinum based chemotherapies[60-62]. However, these p53 mutations/status were innate properties of the tumour cells that existed prior to treatment and, thus, do not explain acquired drug resistance observed during the course of treatments or in secondary cancers[63].

Another blow to the genetic theory arises from the observation that cells gain resistance at rates higher than the mutational rate. Spontaneous resistant rates are approximately 10^4 to 10^{11} fold higher in tumour cells under selection pressure than the mutational rate (10^{-7} and 10^{-14}, for mono-allelic and bi-allelic genes per cell generation, respectively) and, therefore, at best mutation could only account for ~1 in every 10^4 resistant cells[52].

Thus, it is necessary to develop a theory that can account for a larger number and wider range of gene changes.

1.4.1.2 Epigenetic Theory

Because mutations are either: impotent at affecting a large enough change in protein structure, function or stability to allow for altered drug interactions; or, cause such specific changes to a narrow niche of the cell that many characteristics of the drug resistant phenotype remain unexplained, the genetic theory is insufficient in explaining drug resistance. Resistance is more likely the result of alterations in expression of multiple genes in varied pathways and a feasible model must address this scenario. The epigenetic theory works toward this ends[63]. Epigenetic modifications such as DNA methylation, histone modifications and small, non-coding RNA regulations can have a large impact of gene expression. Although epigenetics does not necessarily explain aberrant function of a particular gene product, it can explain dysregulated pathways and distorted expression patterns. Furthermore, it has the potential to impact a multitude of genes concurrently—a necessary characteristic in explaining drug resistance. Like genetic mutations, epigenetic alterations are stable, heritable changes that are propagated in clonal fashion and, thus, can explain subpopulations of resistant cells stemming from an initially resistant tumour. Perhaps the strongest suggestion that epigenetics may play a role in acquired drug resistance lies in the observation that cancer cells already demonstrate some degree of epigenetic dysregulation. They are known to be globally hypomethylated with localized hypermethylation at particular gene promoters and display aberrant silencing histone modifications. Moreover, it has been shown that the cellular profiles for small, non-coding RNAs, microRNAs, are also drastically different[64,65].

Therefore, it is plausible that epigenetic dysregulation may play a role in the acquisition of a drug resistant phenotype.

Examples of epigenetic influences of drug resistance include silencing of pro-apoptotic genes and silencing of DNA repair genes. Apaf1, an apoptosis activating factor, is heavily methylated in chemoresistant melanoma lines. Since the end goal of all chemotherapeutic agents is to induce death, loss-of-function in any necessary member of the apoptotic pathway will manifest as a resistant phenotype. The hypermethylation of the Apaf1 promoter silenced the activity of the gene and, thus, prevented apoptosis. Relief of Apaf1 repression and increasing sensitivity of the cells was observed following treatment with a demethylating agent, 5-azacytidine[66]. In addition, DNA repair capacities can impact drug-sensitivity. Many drugs, such as cisplatin, impart their effect by causing DNA damage that, if left unrepaired, is lethal[7]. In one such example, methylation of the DNA repair gene MGMT that reverses the damage induced by DNA alkylating agents has been shown to be a strong prognostic tool for determining the treatment response of gliomas, i.e. methylated MGMT conferred sensitivity to the drugs[67]. In another example, methylation of FANCF, a gene involved in regulating an S-phase/G2 arrest checkpoint, is responsible for cisplatin sensitivity in ovarian cells. Demethylation of the FANCF promoter coincided with the acquisition of cisplatin resistance[68]. Although, at current, no specific examples linking particular histone modifications to a drug resistant phenotype have been described, the fact that DNA methylation and histone modifications usually occur together, suggests they likely play a role in silencing or permitting expression of key genes[69].

It has been noted that altered miRNA expression in cancer cells coincides with altered drug metabolism in several human cells. Specifically, Blower *et al.*, found that of the 31 miRNAs that correlated with anti-cancer compound activity of 14 drugs, 10 were aberrantly over expressed in several cancer tumour types. One of these, miRNA21, was able to significantly impact the efficacy of six of the 14 drugs tested between 2 and 4-fold—it increased sensitivity in two cases and increased resistance in four[70]. Since a single miRNA has the potential to target hundreds of transcripts, it is rather unremarkable that miRNA 21 impacted the efficacy of six different drugs. However, it is not clear what targeted transcripts and the resulting reduction or increase in their translation imparted this effect.

Where the one mutation: one affected target of the genetic theory falls short in describing the polygenic nature of resistance, the wide reaching impact of miRNAs (and to a lesser extent DNA methylation) damage the epigenetic theory. That is, it is possible that the wide sweeping effects of 'epimutations' likely impact so many aspects of the cellular function, that is in only a few cases that cells acquire an advantageous mutation that results in drug resistance. For example, Luhzna *et al.* recently demonstrated that the cross resistance to radiation observed in a doxorubicin resistant breast cancer line could be sensitized in the presence of SAM, a methyl donor for the DNA methylation event. However, SAM treatment of the already sensitive parental line results in the acquisition of radio-resistance. Therefore, it appears that DNA methylation (and perhaps other the epigenetic regulators) needs to be precisely modulated to achieve a favourable effect[71]. It will be necessary to explore the epigenetic profiles of multiple resistant cells to determine whether a common profile exists.

1.4.1.3 Karyotypic Theory

The karyotypic theory implicates gross chromosomal alterations as the cause of acquired drug resistance. For example, just as trisomy 21 generates a new phenotype—Downs syndrome; aberrations in cancer cells can alter the stoichiometry and integrity of multigenic transcriptomes. The karyotypic theory has foundation in the fact that existing cancer cells already demonstrate some degree of aneuploidy and, thus, experience chromosomal instability. With the existing aneuploidy comes a misbalanced potential for synthesis, repair and mitotic segregation and the rate of chromosomal alterations is proportional to the degree of aneuploidy. Similar to the genetic theory, the role of the karyotype is not inherent to the phenomenon of acquired drug resistance, but rather is a extension of the pre-existing characteristics of cancer cells[52].

Duesberg *et al.* have presented several compelling arguments to support the role of the karyotype in acquired drug resistance. These include: i) karyotypic changes coincide with drug resistance; ii) the rate of the karyotypic changes is similar to the rate of spontaneous acquisition of drug resistance, and; iii) cells are usually multi-drug— and cross resistant and display other changes in quantifiable characteristics (e.g. morphology and invasiveness) necessitating an event capable of impacting numerous genes[52]. However, they do not speculate about the underlying mechanism of the pre-requisite aneuploidy. Epigenetics is a plausible culprit. Genome stability is maintained through the resistance and reversal of genetic changes including mutations, rearrangements and breaks and is mediated through the epigenetic mechanisms of DNA methylation and histone modifications (with a postulated role for small, non-coding RNAs). Therefore, it

is likely that the chromosomal aberrations observed by Duesberg *et al.*, (importantly) in the absence of any genotoxic agent, were dependent upon epigenetic mechanisms.

1.5 Epigenetic Regulation

Most cellular mechanisms depend heavily on upon gene expression and organization, as well as on the accessibility of DNA in DNA-protein interactions. These domains are governed by epigenetic processes—meiotically heritable and mitotically stable alterations in gene expression. Epigenetics includes DNA methylation and histone modifications with a recent addition of small, non-coding RNA regulation. It is important to note that these mechanisms are not mutually exclusive of each other and have an added responsibility of controlling genome stability[72].

1.5.1 DNA Methylation

Mammalian DNA methylation has only been described as a covalent addition of a methyl group at the 5-carbon position of the cytosine base and it has a well investigated role in controlling gene expression, genetic imprinting, and tissue- or temporal- specific gene expression. DNA methylation is a stable and heritable, yet reversible epigenetic trait of mammalian genomes[73,74]. There are three main contributing proteins involved in establishing and maintaining DNA methylation patterns within mammalian cells: DNA methyltransferase (DNMT) 1, DNMT3a, and DNMT3b[75,76]. DNMT3a and DNMT3b are responsible for *de novo* methylation of sequences and appear to hold an indispensable function, as mutant mice lacking either of these genes die within weeks of birth or are not viable through the embryonic stages; respectively. Not surprisingly, DNMT3a and DNMT3b are extremely active during development where cellular differentiation is achieved through high levels of *de novo* DNA methylation in promoter regions of pluripotency genes[77,78]. In contrast DNMT1 is responsible for restoring the methylation pattern on hemi-methylated DNA following replication. It is localized to the replication

fork, where it could directly modify nascent DNA immediately after replication [73,75,79]. Its function is also mandatory as DMNT1-/- mice are embryonic lethal[80].

A correlation between methylation status and expression of endogenous genes is apparent[81]. Cytosine methylation is observed at CpG dinucleotides that tend to cluster into islands containing >55% GC content over a 500 base region[73]. These CpG islands are observed within the promoters of about ~72% of human genes and methylation of CpG-rich promoters frequently coincides with reduced gene activity[82]. The reduced expression is achieved directly through disruption of the transcription factor and RNA polymerase binding as well as, indirectly, through the recruitment of methyl-CpG binding domain proteins as subsequent chromatin remodelling[83] (the implications of which are to be discussed in the following section).

Besides controlling gene expression, DNA methylation is complicit in suppressing parasitic DNA sequences such as transposonable elements and endogenous retroviruses[84]. It is postulated that up to 35% of the human genome is composed of parasitic sequences and that most of them are methylated in an attempt to quarantine these sequences[85]. Active transposable elements are highly mutagenic as they tend to insert within expressed genes disrupting its normal function and can cause illegitimate recombination events and genomic rearrangements[86]. Interestingly, global hypomethylation is a hallmark of all stages of tumour cells with a 20%-60% decrease in methylated cytosines. This decrease in methylated DNA coincides with the reactivation of transposable elements, mitotic recombination (leading to loss of heterozygosity) and aneuploidy[73,87,88]. Furthermore, cells lacking the activity of DNMT3b display high levels of chromosome aberrations[89].

Therefore, in a hypomethylated environment chromosomal instability increases and genome integrity is challenged.

1.5.2 Histone Modifications

In the nucleus, eukaryotic DNA closely interacts with histones[72,87]. Histones are structural proteins that provide scaffolding for DNA molecules to wrap around in a predictable manner forming many nucleosomes in a 'beads-on-a-string' manner. The nucleosome contains 4 core histones, H2A, H2B, H3 and H4 which exist in duplicate forming an octamer around which approximately 150 base pairs of DNA is wrapped[90]. The most obvious role of the histones is to provide scaffolding for the DNA molecule—mechanical support that protects the linear DNA molecule from becoming tangled with other molecules or breaking during the movement experienced throughout the cell cycle. In the last few decades, it has been discovered that histones are subject to numerous posttranslational modifications. These modifications can affect their interactions with DNA and other proximal non-histone proteins. These modifications have a profound impact on transcriptional expression, DNA repair and genome stability[91,92]. The combination of DNA wrapped around histones is referred to as chromatin and it generally falls into two categories: heterochromatin; a tightly wound, inaccessible state, or euchromatin, an open, accessible form.

Post-translational modifications to the N-terminal ends of histones include phosphorylation, acetylation, methylation, sumolation and ubiquitination events that define and/or change the chromatin state. Collectively, histone modifications are known as the histone code and constitute part of the epigenome of a given cell[91,93-95]. Chromatin

remodelling frequently occurs to modify the transcriptional activity of a gene. It is achieved through the above mentioned post-translational modifications. Unlike DNA methylation where a single chemical modification occurs at a single position on a single base, histone modifications are much more complex. The general school of thought is that these chemical modifications on histones alters the shape of the chromatin, thus, affecting the ease with which non-histone proteins may access and bind, in turn altering the transcriptional outcome. Histone modifications are achieved through the work of several different enzymes including histone methyltransferases, histone acetylases and histone deacetylases amongst others[91].

Histone modifications that are indicative of silencing commonly occur along with DNA methylation. Furthermore, it was recently shown that tumours undergo a massive shift in the profile of their histone code[96,97]. This loss occurs along with DNA hypomethylation and is linked to chromatin relaxation and aberrant expression. It was suggested to be a universal marker for malignant transformation and genome instability[96]. Although it has been stipulated that one event may beget the other, evidence supporting which is the antecedent action waivers between the two. Furthermore, it is not obligatory to have DNA methylation changes that coincide with silencing histone modifications or vice versa[98].

Besides providing flexibility with expression levels, histone modifications play an important role in genomic stability. Highly repetitive regions such as telomeres, centromeres and transposable elements can challenge genome integrity if the surrounding chromatin adopts a more relaxed state. For example, the nature of telomeres (i.e. they are

basically a dsDNA break) make them good candidate for errant NHEJ and HR—events that would lead to gross chromosomal aberrations, aneuploidy and/or gene duplication[99]. Likewise, histone modifications work in conjunction with DNA methylation to maintain transposonable elements in a heterochromatic state. In mouse models transposon re-activation was associated with chromosomal segregation defects[86].

1.5.3 Small, non-coding RNA regulation

Recently it has been discovered that small RNA molecules can act in a potent gene silencing mechanism that in mammalian cells involves the inhibition of translation. This mechanism—post-transctriptional gene silencing (PTGS), involves a class of short (21-25 nTs), non-coding RNA molecules. These molecules are generated through a two-step cleavage of the long primary RNA transcript that forms secondary hairpin structures. The first cleavage is performed in the nucleus by the RNase III protein Drosha and results in a shortened hairpin that is transported to the cytoplasm where a second RNase III protein, Dicer, performs the second cleavage. The Dicer-dependent cleavage results in short dsRNA molecules that are 19 to 23 base pairs in length. These short dsRNA molecules are loaded onto a RNA-induced silencing complex (RISC) where they are further processed and matched with their complimentary sequence within the 3' untranslated region (UTR) of mRNA transcripts. This mature, single stranded RNA molecule is termed a microRNA (miRNA). Imperfect base pairing between the miRNA and a cognate 3'UTR results in inhibition of the translational machinery and eventual sequestering and destruction of the messenger molecule[100]. PTGS is a widely conserved mechanism for controlling gene expression across several kingdoms. However, it is also employed in plants, fungi and some invertebrates as an effective mechanism for

achieving transcriptional gene silencing (TGS). During TGS, transcribed dsRNA are cleaved into small perfectly matched molecules capable of guiding the acquisition of DNA methylation patterns and/or silencing histone modifications at their complimentary sequences: the result being a localized change in the epigenetic profile of that cell. Although no such mechanism has been described in somatic mammalian cells, there has been some evidence that this is possible[101-104]. If true, this would incorporate small, non-coding RNA regulation as a *bona fide* epigenetic mechanism.

1.6 Preliminary Data

MCF-7 cells were exposed to increasing concentrations of the chemotherapeutic drugs cisplatin and doxorubicin; respectively. The concentrations of the drugs were increased gradually from 0.5 to 15 μg/mL over a six month period. The resulting cells LD_{50} was 12μM and 94μM for the MCF7 and MCF7/CDDP lines, respectively; and 1μM and 24μM for the MCF7 and MCF7/DOX lines, respectively). Besides increased tolerance for the drug, these cells demonstrated: increased resistance to radiation treatment, global hypomethylation and reduced background levels of apoptosis[21,71]. Furthermore, no reversal of the resistant phenotype was observed following cessation of the drug treatment.

Overall, from the literature and the preliminary data obtained by the Kovalchuk laboratory and our collaborators, we have learned that global, and some promoter-specific, DNA methylation changes and global microRNAome alterations are important in breast cancer drug resistance. However, the vast majority of studies have analyzed either global genome changes which lack vital locus-specific details, or use a reductionist-type 'pick-and-choose' approach to analyze genes and miRNAs. Thus, there is still much to be learned regarding the exact details of epigenetic DNA methylation and microRNAome changes in drug resistant cells and the exact contributions of known epigenetic mechanisms on the development of drug resistance. Breast cancer cell lines representing the extremes of drug resistance constitute an excellent model system for initial epigenome mapping.

Therefore, I set out to establish an important and clinically relevant cell-line based - ‘inventory’ of drug resistance-related epigenetic changes to form a solid basis for our future translational efforts.

1.7 Hypotheses

Rationale

The literature and our results suggest that epigenetic changes play important roles in breast cancer drug resistance. While the role of global DNA methylation in drug resistant breast cancer cells was shown, the exact nature of these epigenetic phenomena in cells resistant to cytotoxic DNA damaging agents and anti-estrogen agents need to be defined and compared. This is especially important, since it was recently proven that alterations in DNA methylation in cancer cells occur in defined regions, suggesting locus-specific and non-random global DNA dysregulation[98,105,106].

Furthermore, the phenomenon of drug resistance has not been fully explored in the microRNAome domain. The precise roles of differentially expressed miRNAs in cytotoxic and anti-estrogen drug resistance must be delineated and compared.

Such an approach will allow us to resolve the epigenetically affected loci and identify differentially expressed miRNAs that may serve as general and/or drug-specific markers of chemoresistance. Additionally, this research will enable us to define the resistance changes that are specific to cytotoxic chemotherapy.

The epigenome is a plastic characteristic of cells that responds acutely to stimuli within a cells environment. Epigenetic changes allow for flexible control over gene expression, thus, impacting a multitude of cellular processes. Unfortunately, besides maintaining a regulated homeostatic state, epigenetic modifications have been implicated in disease onset and progression including malignant transformation. Furthermore, epigenetic mechanisms have a large impact on genomic stability. Yet again, genomic instability is

known to play an integral role in disease. Therefore, it is important to explore the role of epigenetics in acquired drug resistance of cancerous cells; cells known to display altered epigenetic patterns and genomic instability.

I hypothesize that:

1. Drug resistant cells will exhibit a well-defined pattern of DNA methylation at a variety of loci. These changes may be linked to the expression of the given loci, thus contributing to the drug resistant phenotype.

2. MicroRNAome changes play a crucial etiological role in the generation and maintenance of drug resistance and that this dysregulation will be a common feature for resistance to DNA damage-inducing agents. Alterations of these miRNAs by means of their over expression or targeted inhibition may lead to changes in the resistant phenotype.

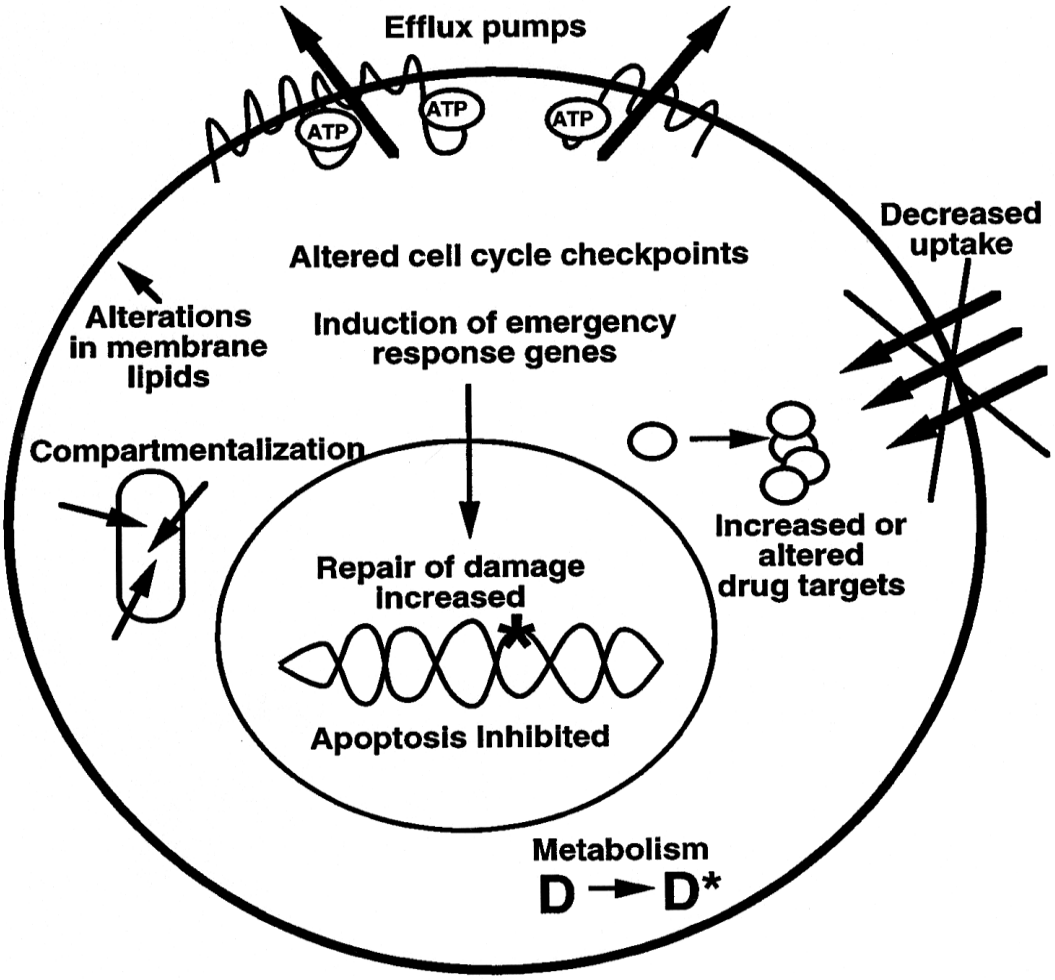

Figure 1.1 Molecular mechanisms of drug resistance[1].

2. Involvement of microRNA-451 in resistance of the MCF7 breast cancer cells to the chemotherapeutic drug doxorubicin

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2.1 Abstract

Many chemotherapy regiments are successfully used to treat breast cancer; however, often breast cancer cells develop drug resistance that usually leads to a relapse and worsening of prognosis. We have shown recently that epigenetic changes such as DNA methylation and histone modifications play an important role in breast cancer cell resistance to chemotherapeutic agents. Another mechanism of gene expression control is mediated via the function of small regulatory RNA, particularly microRNA; its role in cancer cell drug resistance still remains unexplored. In the present study, we investigated the role of miRNA in the resistance of human MCF-7 breast adenocarcinoma cells to doxorubicin (DOX). Here, we for the first time show that DOX-resistant MCF-7 cells (MCF-7/DOX) exhibit a considerable dysregulation of the miRNAome profile and altered expression of miRNA processing enzymes Dicer and Argonaute 2. The mechanistic link of miRNAome deregulation and the multidrug resistant phenotype of MCF-7/DOX cells was illustrated by a remarkable correlation between specific miRNA expression and corresponding changes in protein levels of their targets, specifically those ones that have a documented role in cancer drug resistance. Furthermore, we show that microRNA-451 regulates the expression of multidrug resistance 1 gene. More importantly, transfection of the MCF-7/DOX-resistant cells with microRNA-451 resulted in the increased sensitivity of cells to DOX, indicating that correction of altered expression of miRNA may have significant implications for therapeutic strategies aiming to overcome cancer cell resistance.

2.2 Introduction

Resistance of cancer cells to chemotherapy continues to be a major clinical obstacle to the successful treatment of cancer, including breast cancer[14,107,108]. Causes of cancer-specific drug resistance are currently believed to be linked to the random drug-induced mutational events (genetic hypothesis), to the drug-induced non-mutational alterations of gene function (epigenetic hypothesis), and, recently, to the drug-induced karyotypic changes (karyotypic hypothesis[49,51,52,63,109]). The absence of convincing evidence that genetic changes have a role in acquired clinical resistance following anticancer therapy undermines the genetic hypothesis[63]. In contrast, conclusive data show that increased resistance of cancer cells to chemotherapeutic agents is associated with epigenetic alterations that include changes in DNA methylation and histone modifications[49,63,109]. The karyotypic hypothesis[52] is closely related to the epigenetic one in view of the well known fact that epigenetic changes are a necessary prerequisite to karyotypic changes[110]. In this regard, karyotypic changes may be considered as a consequence of the epigenetic alterations progression and may serve as indirect evidence of the importance of epigenetic dysregulation in the acquisition of cancer drug resistance.

Currently, extensive studies have indicated the existence and importance of another mechanism of nonmutational regulation of gene function mediated by means of short noncoding RNA[111-113]. Aberrant levels of microRNA (miRNA) have been reported in a variety of human cancers[65,114], including breast cancer[115,116]. They have been shown to have both diagnostic and prognostic significance and to constitute a novel target for cancer treatment[117,118]. Considering the critical role of miRNA in cancer, we

hypothesized that the acquisition of drug resistance by cancer cells may also be modulated via the changes in miRNA levels. A recent study by Climent *et al.*[119] suggests that the increased sensitivity of breast cancer patients to anthracycline-based chemotherapy may be related to the deletion of chromosome 11q, a region containing miR-125b gene. This finding was the first evidence to indicate a possible link between miRNA dysregulation and cancer drug resistance; however, the role of miRNA in the acquisition of drug resistance by cancer cells still remains elusive.

Our present study for the first time shows that breast cancer cells resistant to doxorubicin (DOX) exhibit a pronounced deregulation of miRNA expression and the altered expression of miRNA processing enzymes. Moreover, we show that microRNA-451 (miR-451) regulates the expression of the multidrug resistance 1 (mdr1) gene, a crucial factor in drug resistance, and this interaction may have an important functional consequence in the formation of cancer cell resistance to chemotherapeutic agents.

2.3 Materials and Methods

2.3.1 Cell Lines and Cell Culture

The human breast adenocarcinoma MCF-7 cell line and MCF-7/DOX were cultured using Iscove's modified Dulbecco medium (Sigma) containing 10% newborn calf serum (HyClone) and 40 μg/mL gentamicin at 37°C in a 5% CO_2 atmosphere. The MCF-7/DOX drug-resistant variant of the MCF-7 cell line was established by stepwise selection after prolonged (>6 months) treatment of MCF-7 cells to increasing concentrations of DOX at a range of 0.5 to 25 μmol/L in the medium[21]. After 6 months of culturing in the presence of DOX, the IC50 (inhibitory concentration to produce 50% cell death) values were 24 and 1 μmol/L DOX for the MCF-7/DOX and parental MCF-7 cells, respectively. Cells were seeded at a density of 0.5 X 10^6 viable cells per 100 mm plate, and the medium was changed every other day for 6 days. Trypsinized cells were washed in PBS and immediately frozen at -80°C for subsequent analyses. The experiments were independently reproduced twice, and each cell line was tested in triplicate.

2.3.2 Immunocytochemistry and Immunofluorescence

Expression of P-glycoprotein (P-gp), a product of the mdr1 gene, in the MCF-7 and MCF-7/DOX cells was detected by immunocytochemistry as described by Chekhun *et al.*[21] and by immunofluorescence. Cells were cultured on glass coverslips for 24 h and fixed in PBS containing 0.4% paraformaldehyde. The fixed cells were then rinsed with PBS and incubated with the primary mouse anti-human P-gp monoclonal (clone C494) antibodies (DAKO) diluted 1:100 at room temperature for 60 min. Horseradish peroxidase–coupled secondary antibodies and DAKO EnVision System were used for

visualization. For immunofluorescence, the fixed and permeabilized cells were incubated with the primary anti- P-gp antibodies (1:100; Abcam). After washing, the cells were incubated with Alexa Fluor secondary antibodies and counterstained with 4',6-diamidino-2-phenylindole.

2.3.3 miRNA Microarray Expression Analysis

Total RNA was extracted from MCF-7 and MCF-7/DOX cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The miRNA microarray analysis was done by LC Sciences. Total RNA (10 μg) was size fractionated (<200 nucleotides) by using a mirVana kit (Ambion) and labelled with Cy3 and Cy5 fluorescent dyes. Dye switching was done to eliminate the dye bias. Pairs of labelled samples were hybridized to dual-channel microarrays. Microarray assays were done on a μParaFlo microfluidics chip with each of the detection probes containing a nucleotide sequence of coding segment complementary to a specific miRNA sequence and a long non-nucleotide molecule spacer that extended the detection probe away from the substrate.

A miRNA detection signal threshold was defined as twice the maximum background signal. The maximum signal level of background probes was 180. Normalization was done using a cyclic LOWESS (locally weighted regression) method to remove the system-related variations[120]. Data adjustments included data filtering, log2 transformation, and gene centering and normalization. The t test analysis was conducted between MCF-7 and MCF-7/DOX samples, and miRNA with p values < 0.05 were

selected for cluster analysis. The clustering analysis was done using a hierarchical method and average linkage and Euclidean distance metrics[121].

2.3.4 Quantitative Real-time PCR Analysis for miRNA Expression

The quantitative real-time PCR (qRT-PCR) was done by using SuperTaq Polymerase (Ambion) and a mirVana qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR Primer Sets (Ambion) specific for human miR-127, miR-200a, miR-200c, miR-34a, miR-15a, miR-16, miR27b, let-7, miR-21, miR-28, miR-106a, miR-206, and miR-345. Human 5S rRNA served as a positive control. qRT-PCR was done on a SmartCycler (Cepheid). The level of each miRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method [122]. The results are presented as fold change of each miRNA in the MCF-7/DOX cells relative to the parental MCF-7 cells.

2.3.5 Western Immunoblotting

Total cellular extracts were prepared by homogenization of $3X10^6$ to $5X10^6$ cells in 500 μL lysis buffer [50 mmol/L Tris-HCl (pH 7.4); 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; 1 μg/mL each of aprotinin, leupeptin, and pepstatin; 1 mmol/L Na_3VO4; and 1 mmol/L NaF], sonication, and incubation at 4°C for 30 min followed by centrifugation at 10,000X g at 4°C for 20 min. Small aliquots (10 μl) of extracts were reserved for protein determination using protein assay reagents from Bio-Rad. Equal amounts of proteins (20 μg) were separated by SDS-PAGE in slab gels of 8% or 12% polyacrylamide, made in duplicate,

and transferred to polyvinylidene difluoride membranes (GE Healthcare Biosciences). The membranes were incubated with antibodies against Dicer (1:500; Santa Cruz Biotechnology), AGO2 (1:500; Santa Cruz Biotechnology), RB1 (1:750; Labvision Neomarkers), PTEN (1:1000; Cell Signalling Technology), K-RAS (1:500; Santa Cruz Biotechnology), CYP1B1 (1:500; Santa Cruz Biotechnology), ERa (1:500; Cell Signalling Technology), P-gp (1:200; Abcam), BCL6 (1:200; Santa Cruz Biotechnology), BRCA1 (1:200; Santa Cruz Biotechnology), and NOTCH1 (1:250; Abgent). Antibody binding was revealed by incubation with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and an ECL Plus immunoblotting detection system (GE Healthcare Biosciences). Chemiluminescence was detected by GE ECL Hyperfilm (GE Healthcare Biosciences). The unaltered polyvinylidene difluoride membranes were stained with Coomassie blue (Bio-Rad), and the intensity of the Mr 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software.

2.3.6 Luciferase Reporter Assay for Targeting the mdr1 3’-Untranslated Region

For the luciferase reporter experiments, a 3’-untranslated region (UTR) segment of mdr1 gene corresponding to a region of 610 bp (4,262-4,872 nucleotides of the total transcript) for mdr1 (accession no. NM_000927) was amplified by PCR from human genomic DNA using primers that included a XbaI and EcoRI tails on the 5’ and 3’ strands, respectively. PCR products were restricted with both XbaI and EcoRI restriction endonucleases and then gel purified. The amplified 3’-UTR of mdr1 contains a XbaI restriction site; therefore, mdr1-3’-UTR was ligated into the pGL3-control vectors (Promega) by using

the XbaI site located immediately downstream of the stop codon of luciferase. The HEK293 cells were transfected with the firefly luciferase UTR-report vector, control Renilla luciferase pRL-TK vector (Promega), and precursor miR-451 for the mdr1-3'-UTR construct using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). Twenty-four hours after transfection, cells were lysed with a 1X passive lysis buffer and the activity of both Renilla and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.3.7 Cell Survival Analysis

The MDR-7/DOX cells were seeded in six-well plates at density of $1X10^5$/mL and transfected with scrambled RNA oligonucleotide (control) or 100 nmol/L miR-451 (Ambion) in three independent replicates LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). Seventy-two hours after transfection, cells were reseeded in 96-well plates in the presence of miR-451 at density of $1X10^4$ per well and treated with DOX at a range of concentration of 2.5 to 200 μmol/L in the medium for 72 h. Cell survival was analyzed by using the CellTiter-Blue Cell Viability Assay (Promega).

2.4 Results

2.4.1 Expression of miRNA in MCF-7 and MCF/DOX Breast Cancer Cells

miRNA microarrays were used to analyze the miRNA expression profiles in the human breast adenocarcinoma MCF-7 cell line and its drug-resistant MCF-7/DOX variant. The cluster analysis revealed that the MCF-7 breast cancer cells with acquired resistance to DOX were characterized by significant changes in miRNA expression. We identified 137 miRNA genes (63 up-regulated and 75 down-regulated) that were differentially expressed ($P < 0.05$) in the MCF-7/DOX cells compared with the parental MCF-7 cells. Furthermore, 84 of these miRNA genes were differentially expressed at a level of $P < 0.01$ (Fig. 2.1A). The results obtained by miRNA microarray analysis were independently confirmed by the qRT-PCR. We analyzed the status of differentially expressed miR-127, miR-200a, miR-200c, miR-34a, miR-15a, miR-16, miR27b, let-7, miR-21, miR-28, miR-106a, miR-206, and miR-345 genes in MCF-7 and MCF-7/DOX cells. The qRT-PCR confirmed the data obtained by microarray analysis (Fig. 2.1).

2.4.2 Expression of Dicer and Argonaute 2 Proteins in MCF-7 and MCF/DOX Breast Cancer Cells

Having revealed the profound alterations in the miRNA profile in the MCF-7/DOX drug-resistant cells, we decided to analyze the protein levels of the main miRNA processing enzyme, Dicer. Several studies pointed toward the putative role of Dicer in tumorigenesis[116,123,124]; however, its involvement in cancer drug resistance has not been addressed yet. Figure 2.1C shows a very strong down-regulation of Dicer levels in the MCF-7/DOX cells. Additionally, we have detected a pronounced down-regulation of the

Argonaute 2 protein, a member of Argonaute protein family that has an important role in RNA silencing[125] in theMCF-7/DOX cells compared with MCF-7 cells. The significantly decreased levels of the Dicer and Argonaute 2 proteins may, in part, explain the profoundly dysregulated miRNAome profile in the MCF-7/DOX cells.

2.4.3 Association between miRNA Expression and Levels of miRNA Target Proteins

To establish the significance of miRNA expression dysregulation with respect to the acquired cancer cell drug resistance, we determined the protein levels of the experimentally confirmed targets of these differentially expressed miRNA. We found a strong negative correlation between expression of particular miRNA and levels of confirmed target proteins associated with cancer cell drug resistant phenotype. For instance, Western blot analysis of the MCF-7/DOX cells showed the increased levels of anti-apoptotic proteins BCL6, NOTCH1, and K-RAS and up-regulation of CYP1B1 protein leading to the increased metabolism of DOX. The up-regulation of these proteins was associated with down-regulation of the corresponding miRNA: miR-127, miR-34a, miR-27b, and let-7[126-130], respectively (Fig. 2.1D). In contrast, up-regulation of the miR-206, miR-106a, miR-21, and miR-214 miRNA (Fig. 2.1A and B; Supplementary Table S1) in MCF-7/DOX cells was associated with the decreased level of their targets ERa, RB1, and PTEN proteins[131-135], respectively, resulting in estrogen insensitivity and increased survival of MCF-7/DOX resistant cells. Additionally, MCF-7/DOX cells were characterized by aberrant expression of several miRNA, such as miR-10, miR-21, miR-155, and miR-200c, associated with increased cell invasion and metastasis[135-137].

2.4.4 miR-451Regulates Expression of mdr1

One of the major mechanisms involved in cancer cell resistance to chemotherapeutic agents is an increased energy-dependent efflux of drugs from cancer cells mediated by the ATP-binding cassette transporter P-gp, which is encoded by the mdr1 gene[14,15]. Several studies have shown a critical role of this protein in the intrinsic or acquired drug resistance[14,138]. The importance of epigenetic mechanisms in the regulation of the mdr1 gene has also been well documented[21,139,140]. Specifically, the increased resistance of cancer cells, including breast cancer cells, to chemotherapy is associated with pronounced hypomethylation and altered histone modifications at the mdr1 promoter region and up-regulation of the mdr1 gene expression[21,139,140]; however, the role of miRNA in the mdr1 regulation has not been addressed. Figure 2.2 shows that the MCF-7/DOX cells exhibit very high levels of P-gp, a product of mdr1 gene, compared with the parental MCF-7 cells as detected by immunocytochemistry and immunofluorescence.

Computational analysis of the 3'-UTR of mdr1 revealed a putative binding site for a single miRNA, miR-451, mature sequence UUGAGUCAUUACCAUUGCCAAA, at 4,742 to 4,763 nucleotides (Fig. 2.3A). This prediction was further confirmed by the miRGen software[141]. Therefore, we determined the role of miR-451 in the regulation of mdr1expression. The cellular level of this miRNA in the MCF-7/DOX cells was below the detection limit of the microarray. To examine whether mdr1 is indeed functionally targeted by miR-451, the segment of mdr1-3'-UTR containing the miR-451 complementary site was cloned into the 3'-UTR of a luciferase reporter system. The resulting reporter vector was transfected into the HEK293 cells together with transfection

controls and miR-451, anti-miR-451, or miRNA that do not have binding sites within the 3'-UTR of mdr1.

Figure 2.3B shows that miR-451 inhibited the luciferase activity from the construct with the mdr1-3'-UTR segment in a concentration-dependent manner (Fig. 2.3B). There was no change in the luciferase reporter activity when the cells were cotransfected with the negative control (scrambled oligonucleotides) or unrelated miRNA such as miR-7, miR-127, or miR-345 (data not shown). No luciferase expression changes were observed when the cells were transfected with the plasmid lacking the mdr1-3'-UTR fragment (data not shown).

To further confirm that miR-451 indeed affects the protein levels of P-gp in the MCF7/DOX cells, these cells were transfected with either the miR-451 or miR-451 and anti-miR-451, and the level of P-gp was determined by Western blotting 48 h after transfection. Figure 2.3C shows that transfection of MCF-7/DOX cells with miR-451 resulted in a decrease of P-gp levels, whereas the simultaneous transfection of miR-451 and anti-miR-451abolished the inhibitory effect of miR-451 efficiently.

2.4.5 Inhibition of mdr1 Expression Results in the Increased Sensitivity of theMCF-7/DOX Cells to DOX

The finding that the miR-451 targets mdr1 suggested that down-regulation of expression of this miRNA contributes to the cancer drug resistance; therefore, the restoration of a miR-451 level in the resistant MCF-7/DOX cells may increase their sensitivity to DOX.

To address this issue, we transfected MCF-7/DOX with miR-451 and determined the sensitivity of cells to DOX treatment. Figure 3.3D shows that transfection of MCF-7/DOX cells with miR-451 resulted in the increased sensitivity of the resistant MCF-7/DOX cells to DOX. The IC50 of MCF-7/DOX cells transfected with miR-451 was 2.5 times lower ($P < 0.05$) compared with the MCF-7/DOX cells transfected with scrambled oligonucleotide. In contrast, transfection of MCF-7 cells resistant to cis-dichlorodiammine platinum with miR-451 did not change sensitivity of the MCF-7/cis-dichlorodiammine platinum–resistant cells to cis-dichlorodiammine platinum treatment (data not shown).

2.5 Discussion

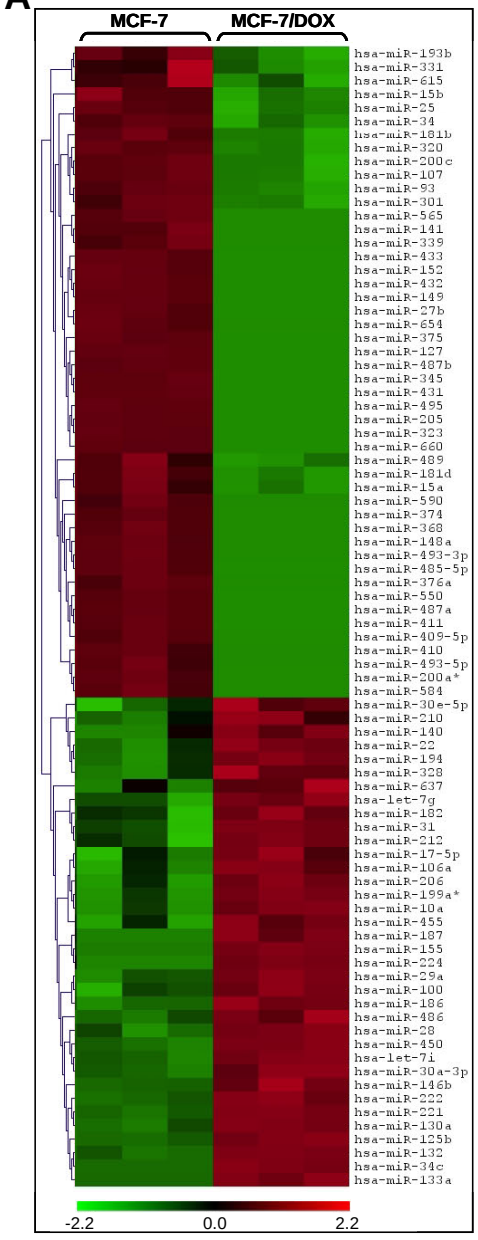
Recent findings have confirmed a critical role of miRNA as powerful diagnostic and prognostic indicators of human breast cancer[115,116,136,137,142], resulting in the development of novel approaches to breast cancer management[142]. Despite the well-established role of miRNA in cancer[65,115] and the dedication of research and resources to the elucidation of the molecular mechanisms involved in the development of resistant cancer cells to chemotherapy, the role of miRNA in cancer drug resistance remains largely unexplored. In this report, we provide data indicating the importance of miRNA dysregulation in the acquisition of cancer cell resistance to chemotherapeutic drugs. This was evidenced by the pronounced alteration in expression of miRNA genes in the MCF-7/DOX-resistant cells compared with parental MCF-7 cells. The mechanistic connection of miRNAome dysregulation with the establishment of a multidrug-resistant phenotype in MCF-7/DOX cells was evidenced by the correlation between expression of specific miRNA and corresponding changes in the protein levels of their targets, specifically those targets that have a documented importance in the development of cancer cell drug resistance. At present, the cancer drug resistance is considered as a multifactorial phenomenon involving several major mechanisms, such as decreased uptake of water-soluble drugs, increased repair of DNA damage, reduced apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cell[107,109]. The pattern of miRNA expression in the MCF-7/DOX cells affecting multiple genes simultaneously provided support for this multifactorial polygenic drug resistance hypothesis. This was evidenced by (a) down regulation of miR-27b and consequent up-regulation of CYP1B1 leading to the increased

metabolism of DOX; (b)down-regulation of miR-127 and miR-34a resulting in a reduced apoptotic program via inhibition of p53 network; (c) down-regulation of miR-200c resulting in up-regulation of TCF8[137] and consequent decreased expression of E-cadherin inducing cell invasiveness and metastasis, (d)up-regulation of miR-21 and miR-214 targeting PTEN[133,134] leading to increased cell survival; (e) up-regulation of miR-28 leading to loss of BRCA1 expression, which is associated with increased resistance to DOX treatment[143]; and (f) up-regulation of miR-206 leading to loss of the ERa-mediated signal transduction. Furthermore, our results of miR-125 down regulation in the MCF-7DOX-resistant cells support suggestion by Climent *et al.* [119] regarding the link between miRNA dysregulation and breast cancer drug resistance.

Because the increased energy-dependent efflux of chemotherapeutic drugs is a major mechanism associated with resistance of cancer cells to DOX, we have focused on a potential role of miRNA as regulators of the mdr1expression. Our reporter assay experiments show significant reduction of MDR1 expression, clearly indicating that miR-451 is a regulator of MDR1. More importantly, transfection of the MCF-7/DOX cells with miR-451 resulted in the increased sensitivity of resistant cells to DOX. This finding indicates that correction of altered expression of miRNA may have significant implications for therapeutic strategies aiming to overcome cancer cell resistance. Indeed, recent reports on the positive results in the use of small interfering RNA [144,145] or miRNA [146] to suppress resistance of cancer cells to chemotherapeutic drugs support this suggestion. In our previous study using MCF-7/DOX cells, we have found profound alterations of cellular epigenetic landscape[21] (20), including hypomethylation of the mdr1

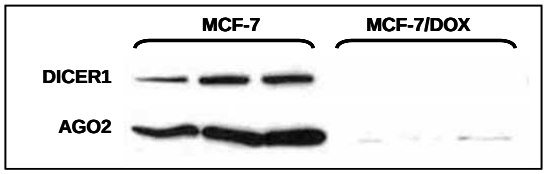
gene. Those epigenetic abnormalities in MCF-7/DOX cells, specifically loss of cytosine methylation, may be partially related to the increased expression of miR-22, miR-29a, miR-194, and miR-132 miRNA that target DNA methyltransferases 3A and 3B and methyl CpG binding protein 2[147,148].

In conclusion, we have shown that the development of multidrug resistance is associated with the pronounced deregulation of miRNA expression. Additionally, we have shown that expression of miR-451 is inversely correlated with mdr1 expression in breast cancer drug-resistant cells. Furthermore, the enforced increase of miR-451 levels in the MCF-7/DOX cells down-regulates expression of mdr1 and increases sensitivity of the MCF-7-resistant cancer cells to DOX. These results provide a strong rationale for the development of miRNA-based therapeutic strategies aiming to overcome cancer cell resistance. However, these alterations are not necessarily indicative of the causative role of miRNA deregulation in the cancer drug resistance development and the ultimate goal of future studies is to address this question.



B

Differentially expressed miRNAs in MCF-7/DOX cells		
MicroRNA	**Fold change**	**Protein targets**
Down-regulated		
hsa-miR-127	-17.4	BCL6
hsa-miR-200c	-32.1	TCF8 (ZEB1)
hsa-miR-34	-7.1	E2F3, Notch1
hsa-miR-27b	-2.2	CYP1B1, Notch1
hsa-miR-21	-4.4	PTEN
hsa-let-7	-1.3	K-RAS
Up-regulated		
hsa-miR-28	4.6	none
hsa-miR-22	6.5	none
hsa-miR-106	2.0	RB1
hsa-miR-206	4.8	ERα



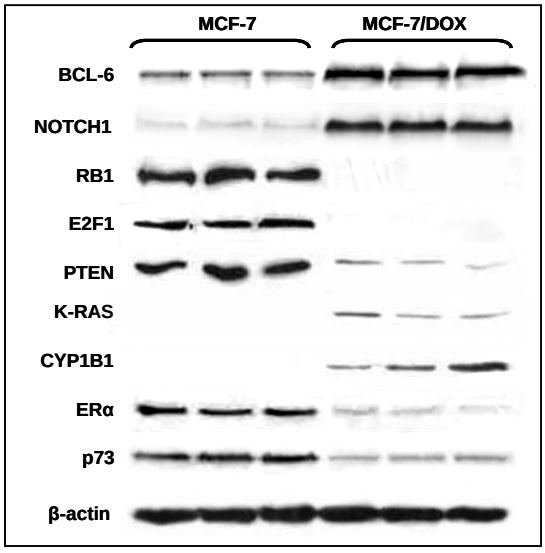


Figure 2.1. MicroRNAome changes in the drug resistant breast cancer cells.
A) Expression of miRNAs in MCF7 and MCF7/DOX breast cancer cells. B) qRT-PCR confirmation of miRNA microarray analysis, data presented as fold change compared to control ($p<0.05$). C) Levels of dicer and Ago2 protiens in mCF7 and MCF7/DOX cells. D) Changes in the expression of selected confirmed miRNA targets.

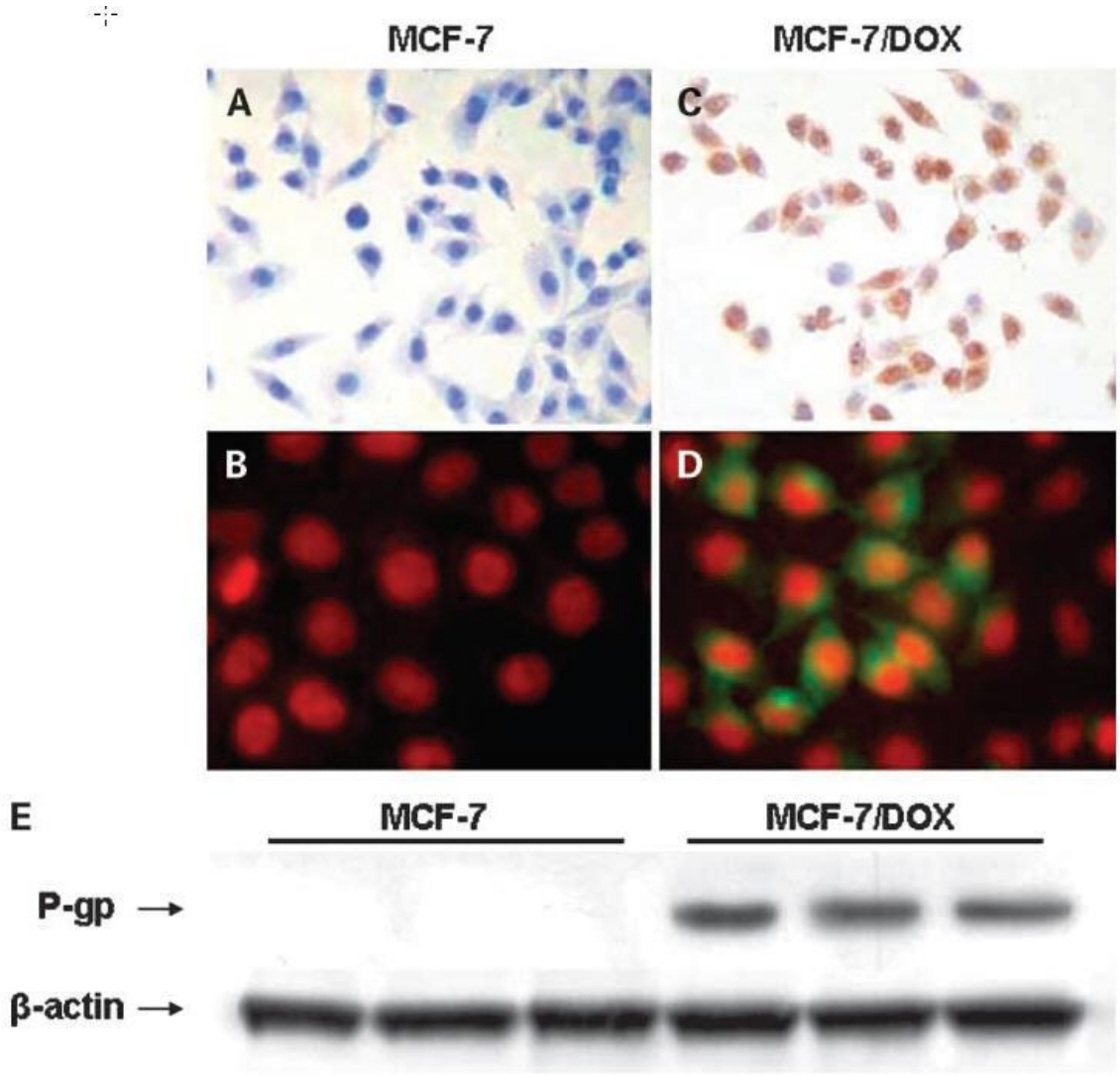


Figure 2.2 . The resistant cells display different levels of expression of P-gp.

Expression in the MCF7 and MCF7/DOX human breast adenocarcinoma cells as detected by immunocytochemistry (A and C), immunofluorescence (B and D) and immunoblotting (E).

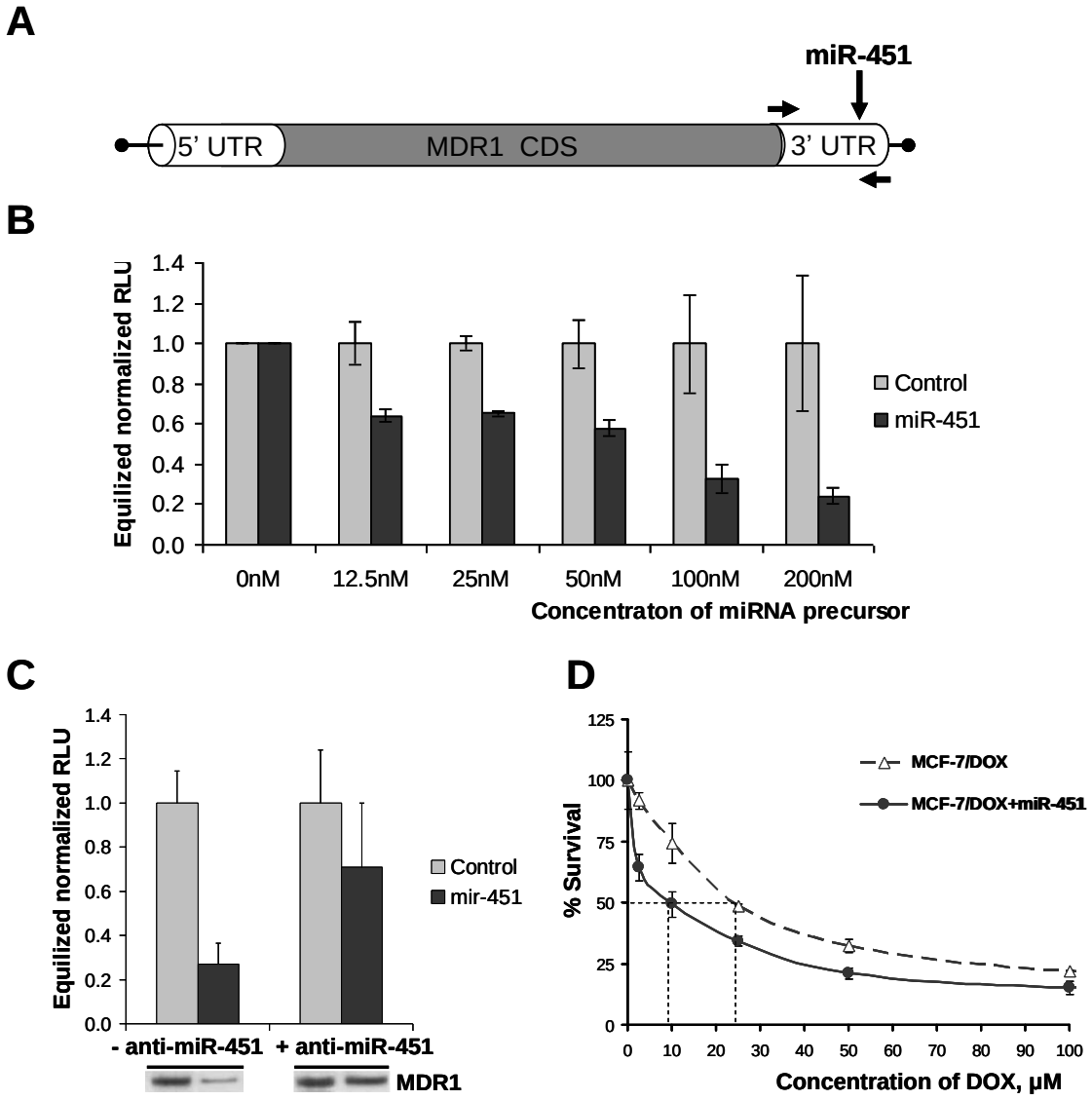


Figure 2.3. miR-451 regulates expression of *mdr1*.

(A) A putative miR-451 binding site at the 3'UTR of *mdr1, as identified* using MiRanda software. (B) miR-451 inhibits luciferase activity in a concentration-dependent manner from the construct with the *mdr1*-3'UTR segment. (C) Transfection of MCF-7/DOX cells with mir-451 results in a decrease of Pgp (MDR1 product) levels, while the simultaneous transfection of miR-451 and anti-miR-451 efficiently abolishes the inhibitory effect of miR-451. (D) Suppression of MDR1 expression by miR-451 results in increase sensitivity of MCF-7/DOX cells to DOX**.**

3. Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin

Chapter 3 accepted for publication in its entirety:
Pogribny, IP., Filkowski, JN., Tryndyak VP., Golubov, A., Shpyleva, SI., and Kovalchuk O. Alterations of microRNAs and their targets are associated with acquired resistance MCF7 breast cancer cells to cisplatin. Int. J. Cancer 2010, 127: 1785-1794.

3.1 Abstract

Cancer cells that develop resistance to chemotherapeutic agents are a major clinical obstacle in the successful treatment of breast cancer. Acquired cancer chemoresistance is a multifactorial phenomenon, involving various mechanisms and processes. Recent studies suggest that chemoresistance may be linked to drug-induced dysregulation of microRNA function. Furthermore, mounting evidence indicates the existence of similarities between drug-resistant and metastatic cancer cells in terms of resistance to apoptosis and enhanced invasiveness. We studied the role of miRNA alterations in the acquisition of cisplatin-resistant phenotype in MCF-7 human breast adenocarcinoma cells. We identified a total of 103 miRNAs that were over expressed or under expressed (46 up regulated and 57 down regulated) in MCF-7 cells resistant to cisplatin. These differentially expressed miRNAs are involved in the control of cell signalling, cell survival, DNA methylation and invasiveness. The most significantly dysregulated miRNAs were miR-146a, miR-10a, miR-221/222, miR-345, miR-200b and miR-200c. Furthermore, we demonstrated that miR-345 and miR-7 target the human multidrug resistance-associated protein 1. These results suggest that dysregulated miRNA expression may underlie the abnormal functioning of critical cellular processes associated with the cisplatin-resistant phenotype.

3.2 Introduction

Breast cancer is the most common malignancy in women. In the United States, the incidence of invasive breast cancer, the most serious form of breast cancer, was estimated as 182,460 new cases and 40,480 deaths in 2008[2]. Despite advances in understanding the molecular mechanisms of breast cancer biology, as well as advances in early detection and treatment, in 50% of cases cancer cells will either rapidly acquire resistance against numerous cytotoxic drugs or are intrinsically resistant[149]. Furthermore, ~30% of all patients with early-stage breast cancer will have recurrent disease, which becomes predominantly metastatic and resistant to treatment[6,150]. It is believed that resistance to chemotherapy or administration of ineffective chemotherapeutic agents causes treatment failure in 90% of patients with metastatic cancer[6]. Currently, acquired drug resistance and metastasis are major obstacles in the successful treatment of breast cancer[151,152]. Thus, increasing tumour cell sensitivity to chemotherapeutic agents and predicting chemotherapeutic agent effectiveness without developing drug resistance in individual patients are attractive goals for improving the clinical management of cancer. Recent evidence suggests that drug-induced dysregulation of microRNA (miRNA) function may modulate the sensitivity of cancer cells to chemotherapeutic agents and may be involved in the acquisition of cancer cell resistance to chemotherapy[49,70], including breast cancer drug resistance[153-155], and breast cancer metastasis[136,156]. In addition, there is increasing evidence that there are great similarities between drug-resistant and metastatic cancer cells; particularly, in terms of profound resistance to apoptosis and enhanced invasiveness[157]. With this in mind, the aims of this study were as follows: (i) to determine whether or not the acquired drug-resistant phenotype of breast cancer cells to cisplatin

(cis- dichlorodiammine platinum (II) (CDDP)), a major chemotherapeutic agent used to treat a range of human malignancies[158], is associated with secondary miRNA alterations and (ii) to determine whether or not these miRNA abnormalities are associated with specific known mechanisms of cisplatin based resistance.

3.3 Materials and Methods

3.3.1 Cell lines and cell culture

The MCF-7 and MDA-MB-231 human breast cancer lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained according to ATCC's recommendations. Cisplatin (CDDP) is a chemotherapeutic agent used to treat a range of cancers including ovarian and breast cancer[159,160]. The MCF-7 drug-resistant variant to CDDP (MCF-7/CDDP) cell line was established by stepwise selection after prolonged (>6 months) treatment of MCF-7 cells to increasing concentrations of CDDP (Sigma) at a range of 0.5–25 μM in the medium[21]. After 6 months of culturing in the presence of CDDP, the IC50 (inhibitory concentration to produce 50% cell death) values were 94 and 12 μM of CDDP for the MCF-7/CDDP and parental MCF-7 cells, respectively. Cells were seeded at a density of 0.5 $X10^6$ viable cells per 100 mm plate, and the medium was changed every other day for 6 days. Trypsinized cells were washed in phosphate-buffered saline and immediately frozen at -80°C for subsequent analyses. The experiments were independently reproduced twice, and each cell line was tested in triplicate.

3.3.2 miRNA microarray expression analysis

Total RNA was extracted from MCF-7 and MCF-7/CDDP cells using TRIzol reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. The miRNA microarray analysis was performed by LC Sciences (Houston, TX). Ten micrograms of total RNA was size-fractionated (<200 nucleotides) by using a mirVana kit (Ambion, Austin, TX) and labelled with Cy3 and Cy5 fluorescent dyes. Dye switching was

performed to eliminate dye bias. Pairs of labelled samples were hybridized to dual-channel microarrays.

Microarray assays were performed on a lParaFlo microfluidics chip with each of the detection probes containing a nucleotide sequence of coding segment complementary to a specific miRNA sequence and a long non-nucleotide molecule spacer that extended the detection probe away from the substrate. A miRNA detection signal threshold was defined as twice the maximum background signal. The maximum signal level of background probes was 180. Normalization was performed using a cyclic LOWESS (locally weighted regression) method to remove system-related variations[120,154]. Data adjustments included data filtering, log2 transformation, gene centering and normalization. T-test analysis was conducted between MCF-7 and MCF-7/CDDP samples, and miRNAs with p-values < 0.05 were selected for cluster analysis. The clustering analysis was performed using a hierarchical method and average linkage and Euclidean distance metrics[121,154].

3.3.3 Quantitative real-time PCR analysis for miRNA expression

The qRT-PCRs were performed by using SuperTaq Polymerase (Ambion, Austin, TX) and a mirVana qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR primer sets (Ambion) specific for human miR-127, miR-126, miR-200c, miR-29a, miR-29b, miR-206 and miR-345. Human 5S rRNA served as an internal control. qRT-PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA), and each cell line was run in triplicate. The level

of each miRNA expression was measured using the $2^{\Delta\Delta Ct}$ method[122,154]. The results are presented as fold change of each miRNA in the MCF-7/CDDP cells relative to the parental MCF-7 cells. Indicated changes are significant at 95% confidence level ($p < 0.05$).

3.3.4 Western blot analysis of protein expression

The protein levels of DNA methyltransferase 3A (DNMT3A), methyl-CpG-binding protein 2 (MeCP2), ZEB1, E-cadherin, MRP1 and b-actin in the MCF-7, MCF-7/CDDP cells were determined by Western blotting using protocols described previously[21,154,161].

3.3.5 Immunofluorescence

Expression of ZEB1 and E-cadherin in the MCF-7 and MCF-7/CDDP cells was detected by immunofluorescence. Cells were cultured on glass coverslips for 24 hr and fixed in PBS containing 0.4% paraformaldehyde. Fixed and permeabilized cells were incubated with the primary ZEB1 (1:50, Santa Cruz Biotechnology) and E-cadherin (1:50, Cell Signalling). After washing, the cells were incubated with Alexa Fluor secondary antibodies and counterstained with 40,6-diamidino-2-phenylindole (DAPI).

3.3.6 Cytosine DNA methylation analysis

The extent of global DNA methylation was evaluated with a cytosine extension assay as described previously[21,162].

3.3.7 Analysis of the invasiveness of cisplatin-resistant MCF-7 cells

MCF-7 and MCF-7/CDDP cells were plated in the upper chamber of BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) and grown as recommended by the manufacturer. After 48 hr, cells on the bottom side of membrane were stained and mounted onto glass slides, and the mean number of cells in 4 replicates was determined. Representative images (magnification 125X) of stained membranes are shown.

3.3.8 Luciferase reporter assay for targeting MRP1-3'-UTR

Cloning of the UTR was based on transcript NM_004996 for the MRP1(Abcc1) gene. UTR was defined as the sequence between 4772 and 6564 bps. For the luciferase reporter experiments, a 30-UTR segment of the MRP1 gene (nucleotides 4742–6564) that contains putative binding regions for hsa-miR-345 (nucleotides 4819–4838, NM_004996) and hsamiR-7 (nucleotides 5346–5368) was amplified by PCR from human genomic DNA using primers that included an XbaI and EcoRI tails on the 50 and 30 strand, respectively. PCR products were restricted with both XbaI and EcoRI restriction endonucleases and then gel purified. The amplified 3'-UTR of MRP1 contains an XbaI restriction site; therefore, MRP1-3'-UTR was ligated into the pGL3-control vectors (Promega, Madison, WI) by using the XbaI site located immediately downstream of the luciferase stop codon. In parallel, we mutated the miR-7 (nucleotides 5361–5368) and miR-345 (4830–4837) seed sequence-binding regions in the 3'-UTR segment of the MRP1 gene to 50-AGAGGAAG-30 and 50-AAGAGAGA-30, respectively. HEK293 cells were transfected with firefly luciferase MRP1-3'-UTR construct or mutated MRP1-

3'-UTR construct, a control Renilla luciferase pRL-TK vector (Promega) and synthetic precursors of miR-345 and miR-7 (Ambion) using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Scrambled oligonucleotides or unrelated miRNA that is not predicted to target MRP1 (miR-127) (Ambion) served as controls. Twenty-four hours after transfection, cells were lysed with a 1X passive lysis buffer, and the activity of both Renilla and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions[154].

3.3.9 Analysis of the effect of miR-7 and miR-345 on cellular levels of MRP1

MCF-7/CDDP cells were transfected with miR-7, miR-345 or scrambled RNA oligonucleotides (100 nM). Twenty-four hours after transfection, the cellular levels of MRP1 were detected by Western immunoblotting using anti-MRP1 antibodies (Abcam) according to the manufacturer's instructions.

3.3.7 Statistical analysis

Statistical analysis was performed using MS Excel 2007 and JMP5 software packages.

3.4 Results and Discussion

Microarrays were used to analyze the expression of miRNAs in the MCF-7 human breast adenocarcinoma cell line and its cisplatin-resistant variant MCF-7/CDDP. Cluster analysis revealed that the MCF-7/CDDP cells were characterized by significant changes in miRNA expression. We identified 103 miRNA genes (46 up regulated and 57 down regulated) that were differentially expressed ($p < 0.01$) in the MCF-7/CDDP cells compared to the parental MCF-7 cells (Fig. 3.1). Microarray data were confirmed by qRT-PCR. Table 1 lists a number of miRNAs that exhibited pronounced changes in expression in the MCF-7/CDDP cells when compared with the parental MCF-7 breast cancer cells. Specifically, the MCF-7/CDDP cells were characterized by the greatest alterations in miRNAs involved in the control of several indispensable cellular processes and pathways, including cell signalling, cell survival and apoptosis, invasiveness and DNA methylation (Table 1).

The most up regulated miRNAs in the MCF-7/CCDP cells were miR-146a, miR-10a and miR-221/222 (Table 3.1). These miRNAs regulate the cellular levels of breast cancer-associated BCRA1 protein, homeobox family HOXD10, tumour suppressor p27 and estrogen receptor A[136,155,163-166]. The role of these proteins in the pathogenesis of breast cancer, cancer drug resistance and metastasis is well established[136,155,163-166]. Additionally, several miRNAs (miR-29a, miR-29b, miR-132 and miR-194), up regulated in MCF-7/CDDP cells, are known to target DNA methyltransferases (DNMTs) and MeCP2 and therefore affect DNA methylation patterns[147,148]. Specifically, we found a significant up regulation of miR-29a and miR-29b (14.6 and 52.4 times, respectively), which target *de*

novo DNMT3A and -3B[147]. DNMT3A and -3B are *de novo* methyltransferases responsible for setting up DNA methylation patterns in the cells. Recently, Fabbri *et al.* have shown that the cellular levels of miR-29a and miR-29b are inversely correlated to DNMT3A and -3B in cancer tissues, and that miR-29a and miR-29b directly target both DNMT3A and -3B. Over expression of these miRNAs led to significant decreases in the DNMT3A and -3B levels[147]. In addition, MCF-7/CDDP cells were characterized by a significant (56.9 times) increase in the levels of miR-132 that targets MeCP2[148]. MeCP2 is a transcriptional repressor that belongs to a family of methyl-CpG-binding domain proteins (MBD). MeCP2 selectively recognizes methylated DNA and plays a central role in chromatin remodelling. Recently, Klein *et al.* reported that MeCP2 translation is directly regulated by miR-132. Furthermore, blocking miR132-mediated repression effectively increased cellular MeCP2 levels[148].

In light of this, we analyzed whether or not the up regulation of miR-29a, miR-29b and miR-132 in the drug-resistant MCF-7/CDDP cells resulted in altered cellular levels of DNMT3A and MeCP2. As predicted, we found a significant decrease in the cellular levels of DNMT3A and MeCP2 in the MCF-7/CDDP drug-resistant cells (Fig. 3.2a). As dysregulation of DNA methyltransferases and methyl binding proteins is often associated with altered levels of DNA methylation, we next studied the status of global DNA methylation in parental MCF-7 cells and its MCF-7/CDDP drug-resistant variant. A well-established cytosine extension assay confirmed that decreased cellular levels of DNMT3A and MeCP2 were accompanied by a significant decrease in global DNA methylation in the MCF-7/CDDP cells (Fig. 3.2b). It has been suggested that one of the

features of cancer drug-resistant cells is enhanced invasiveness[157]. Indeed, we detected altered expressions of several miRNAs, including miR-10a, miR-10b, miR126, members of miR-200 family (miR-200b, miR-200c, miR-141 and miR-429) and miR-205, whose aberrant expression has been linked to increased metastatic properties of breast cancer cells[136,156,167]. Amongst these miRNAs, the most pronounced changes were specific for miR-200c and miR-200b, whose levels in MCF-7/CDDP cells were 497 and 1,000 times lower, respectively, when compared with parental MCF-7 cells (Table 1). These miRNAs play a major role in defining cellular epithelial phenotype by suppressing the expression of ZEB1/deltaEF1 and SIP1/ZEB2[167]. Both ZEB1 and SIP1 are transcriptional repressors of E-cadherin and have been implicated in the epithelial to mesenchymal transition (EMT). Manipulation of the miR-200 family can induce EMT[168]. EMT has been implicated in tumour progression and metastasis, and a major step in this process is the down regulation of E-cadherin[169,170]. A recent study by Gregory *et al.* provided evidence that ZEB1 and SIP1 expression is controlled by the miR-200 family and that of miR-200 is an essential early step in tumour metastasis[167]. In the MCF-7/CDDP cells, the decreased levels of the miR-200 family (Table 1) were associated with increased levels of ZEB1 protein and consequently with the decreased expression of E-cadherin (Fig.3.3a). Although ZEB1 is a nuclear protein, we noticed that immunofluorescence for ZEB1 still indicated some weak cytoplasmic staining for MCF-7 cells. In contrast, MCF-7/CDDP cells exhibited strong nuclear staining and some relatively strong cytoplasmic staining. Some weak cytoplasmic staining is sometimes observed in certain tumour cells[171-173]. This may be indicative of some basal levels of translation. Indeed, some basal low levels of ZEB1are also seen on the ZEB1 Western blot (Fig. 3.3b). Furthermore, the MCF-

7/CDDP cells exhibited increased invasiveness when compared with the MCF-7 cells (Fig. 3.3b). Interestingly, the extent of the invasiveness of the MCF-7/CDDP cells was similar to the highly invasive MDA-MB-231 breast cancer cells (data not shown). More importantly, the IC50 (inhibitory concentration to produce 50% cell death) values for the MDA-MB-231 cells to CDDP (100 μM) were similar to that observed in the MCF-7/CDDP cells. Regulation of drug efflux is yet another key mechanism involved in drug resistance[174]. Specifically, one of the mechanisms of resistance to platinum-based chemotherapeutic drugs in cancer cells is the formation of platinum and glutathione intracellular complexes[7,16,175]; however, these complexes are themselves toxic. It has been suggested that MRP1and MRP2 may mediate the resistance of cancer cells to cisplatin via enhancing the transport of cisplatin-glutathione-S conjugate out of cancer cells[7,16]. Indeed, the results of Negoro *et al.* recent study demonstrated the important role of MRP1 and MRP2 up regulation in the establishment of a cisplatin-resistant cell line[17]. Using Western immunoblotting, we determined that cisplatin-resistant MCF-7/CDDP cells were characterized by a significant up regulation of MRP1 when compared with the sensitive MCF-7 cells (Fig. 3.4). Therefore, as a next step, we analyzed the profile of miRNA expression in MCF-7 and MCF-7/CDDP lines to identify any novel miRNAs that may potentially target efflux pumps. The results of miRNA microarray and qRT-PCR analyses demonstrate that one of the miRNAs with the greatest difference in MCF-7/CDDP expression was miR-345, whose expression was 17.0 times lower when compared with parental MCF-7 cells. While examining potential targets of miR-345 using several *in silico* methods for target gene predictions, such as Sanger miRNA database[176] and miRGen miRNA database[141], we noted that this miRNA may target the 30-UTR of the

human MRP1 (Abcc1) gene. Interestingly, the MRP1 gene was also predicted to be targeted by miR-7. MiR-7 levels are 2.3 times down regulated in MCF-7/CDDP cells when compared with MCF-7 cells. Detailed *in silico* analysis revealed that the 3'-UTR of MRP1 contains putative regions (nucleotides 4819–4838, NM_004996 and nucleotides 5346–5368, NM_004996) that match the sequences of hsa-miR-345 and hsa-miR-7, respectively (Fig. 3.5a).

To validate these predicted miRNA-target interactions, the segment of MRP1-3'-UTR (nucleotides 4742–6564) containing the miR-345 and miR-7 complementary sites was cloned into the 3'-UTR of a luciferase reporter system. Additionally, we have mutated the miR-7 and miR-345 seed-interacting sequences in the MRP1-3'-UTR. The resulting reporter vectors were transfected into HEK293 cells together with miRNA that do not have binding sites within the 3'-UTR of the MRP1-luciferase UTR-report vector and either with miR-345 or miR-7 alone or with both miR-345 and miR-7. No change in the luciferase reporter activity was observed in HEK293 cells that were cotransfected with negative control (scrambled oligonucleotides) or unrelated miRNAs such as miR-127. In contrast, transfection of the HEK293 cells with either miR-345 or miR-7, or both miRNAs together, significantly inhibited luciferase activity from the construct with the MRP1-30-UTR segment. No luciferase inhibition was noted in the constructs containing mutated MRP1-3'-UTR segments (Fig. 3.5b). Most importantly, transfection of the MCF-7/CDDP cells with miR-345 and miR-7 resulted in a significant decrease in the cellular levels of MRP1 24 hr after transfection (Fig. 3.5c) and an increased sensitivity of MCF-7/CDDP to CDDP, as evidenced by a decrease in IC50 values from 94 μM in MCF-7/CDDP cells to 43 μM in MCF-7/CDDP cells transfected with miR-345 and miR-7 (Fig.

3.5d). Furthermore, one of the predicted targets of miR-489, down regulated in MCF-7/CDDP cells, is MRP2 (Table 1). Thus, we concluded that miRNAs may be important regulators of the cellular levels of efflux pump proteins such as MRP1 and MRP2. Furthermore, this data further substantiate the previously reported role of miR-451 in the regulation of another efflux pump—MDR1, in another cancer drug-resistant cell line model—breast adenocarcinoma cells resistant to doxorubicin[154]. Overall, acquired drug resistance is a multifactorial phenomenon, involving multiple mechanisms and processes that include decreased uptake of drugs, alterations in cell cycle and signal transduction pathways, increased repair of DNA damage, reduced apoptosis, increased efflux of hydrophobic drugs, DNA damage tolerance and altered DNA methylation and chromatin structure. The results of our study demonstrate that dysregulation of miRNA expression is associated with abnormal functioning of some of the critical cellular processes associated with the drug-resistant phenotype in MCF-7/CDDP cells. Specifically, we identified miRNA changes associated with increased efflux of drugs, changed DNA methylation and altered DNA repair. We also found miRNAs that may contribute to increased EMT and the invasiveness of CDDP cells. However, these miRNA alterations are not necessarily indicative of the causative role of miRNA dysregulation in cancer drug resistance development. The ultimate goal of future studies is to address and identify these roles during the stepwise acquisition of molecular changes during development of drug resistance, among other questions. Our study thus serves as a roadmap for the future analysis of the roles of miRNAs in drug resistance.

Table 3.1 miRNA expression profile in MCF-7 and MCF/CDDP breast cancer cells.

Up-regulated						Down-regulated				
miRNA	Log2 CDD P/MCF-7, array	Fold change, array	Fold change, qRT-PCR	Targets	Consequences	miRNA	Log2 CDD P/MCF-7, array	Fold change, array	Fold change, qRT-PCR	Targets	Consequences
hsa-miR-146a	7.2	144.5		BRCA1, TRAF6	Cell survival	**hsa-miR-345**	-4.1	-17.0	**-17.8**	MRP1	Drug resistance
hsa-miR-10a	7.1	141.4		HOXD10	Invasion/metastasis	**hsa-miR-200c**	-10.1	-1008.0	**-40.6**	ZEB1	Invasion/metastasis
hsa-miR-221	6.4	83.8		p27,p57, ERα	Cell survival, Estrogen unresponsiveness	hsa-miR-200b	-9.3	-497.1		ZEB1	Invasion/metastasis
hsa-miR-132	5.8	56.9		MECP2	DNA methylation	**hsa-miR-127**	-5.1	-34.2	**-7.0**	BCL6	Cell survival
hsa-miR-29b	5.7	52.4	**46.6**	DNMT3A	DNA methylation	hsa-miR-429	-6.5	-855.1		ZEB1	Invasion/metastasis
hsa-miR-222	5.2	36.7		p27,p57, ERα	Cell survival, Estrogen unresponsiveness	hsa-miR-141	-5.7	-107.3		ZEB1	Invasion/metastasis
hsa-miR-29a	3.9	14.6	**9.1**	DNMT3A	DNA methylation	hsa-miR-196	-5.2	-56.9		ANXA1	Invasion/metastasis
hsa-miR-214	2.8	7.1		PTEN	Cell survival	hsa-miR-205	-4.0	-19.4		ZEB1	Invasion/metastasis
hsa-miR-206	2.8	6.7	**3.2**	ERα	Estrogen unresponsiveness	**hsa-miR-126**	-3.7	-12.7	**-3.4**	CRK	Invasion/metastasis
hsa-miR-10b	2.5	5.5		HOXD10	Invasion/metastasis	hsa-miR-342	-3.2	-10.1		JMJD*	Histone demethylation
hsa-miR-194	2.3	4.9		DNMT3A, MECP2	DNA methylation	hsa-miR-489	-2.9	-10.8		MRP2*, ITGB8*	Drug resistance Invasion/metastasis
						hsa-miR-7	-1.2[†]	-2.3[†]		MRP1	Drug resistance

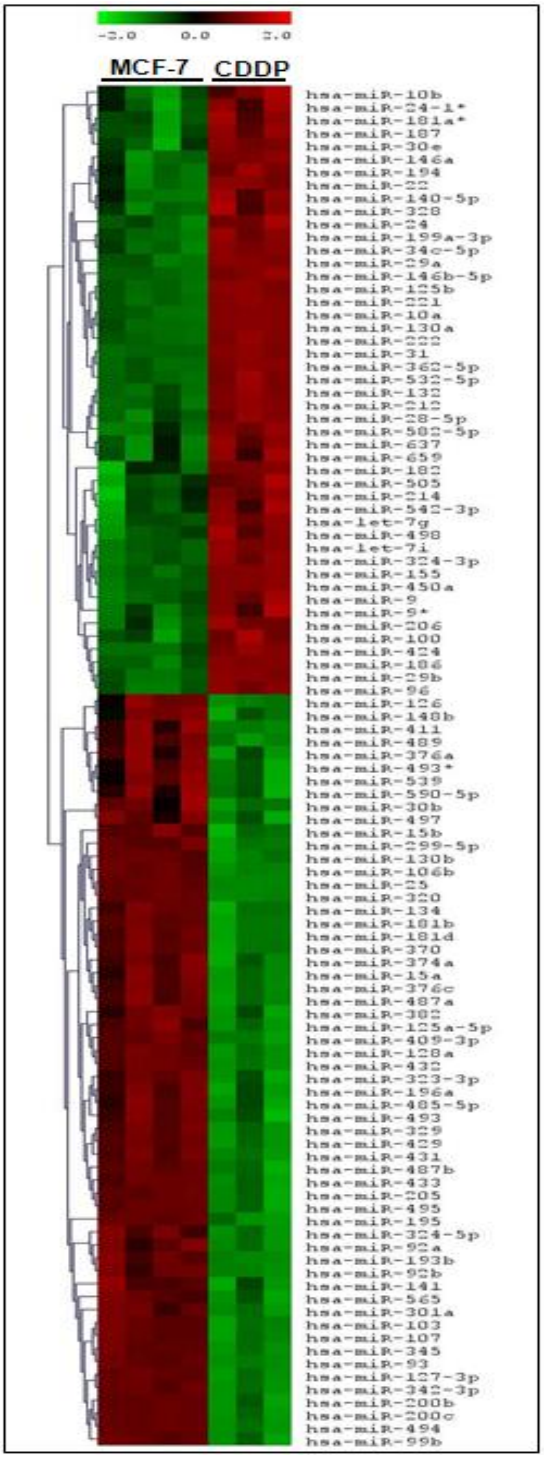


Figure 3.1. Hierarchial clustering of the differentially expressed miRNA genes (as determined by ANOVA) in the MCF7 and MCF7/CDDP cells. For each, red color denotes high expression levels whereas green means low expression levels Each listed miRNA is significantly different ($p<0.01$) between the resistant and parental cell line.

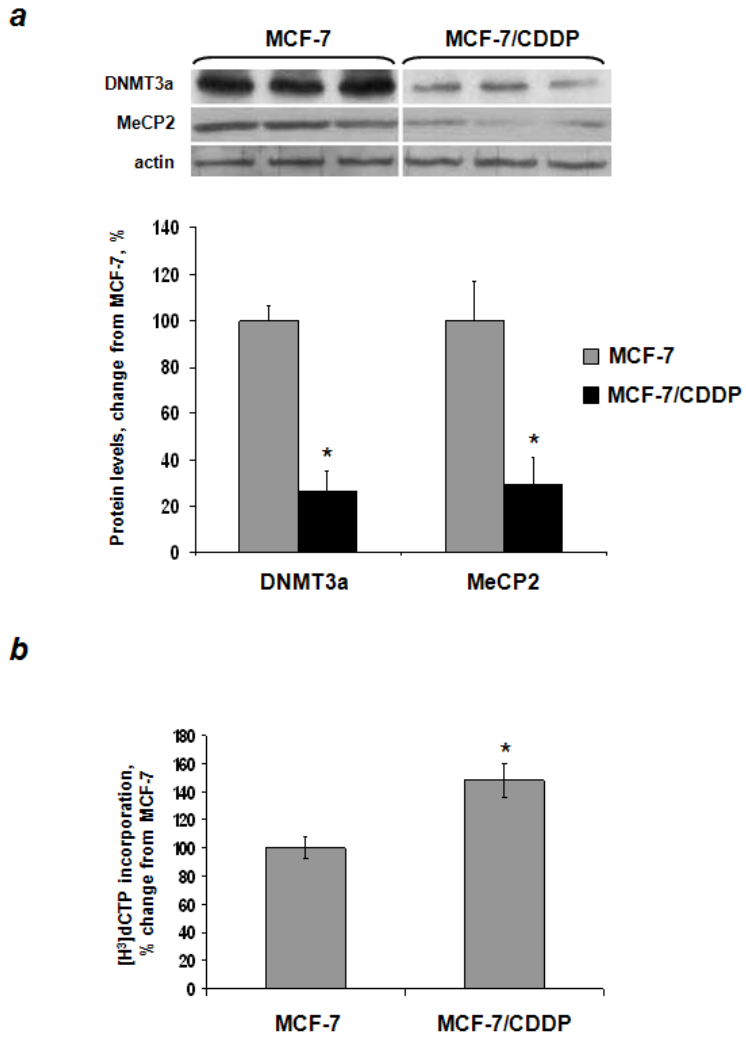


Figure 3.2. Association between DNMT3a and MeCP2 expression and aberrant DNA methylation in cisplatin-resistant MCF-7 cells.

a. Decreased protein levels of DNMT3A and MeCP2 in MCF-7/CDDP cells, as detected by Western immunoblotting. Graph represents a quantitative evaluation of the DNMT3A and MeCP2 protein levels in the MCF-7/CDDP cells relative to those in the MCF-7 cells.

b. Global DNA hypomethylation in the MCF-7/CDDP cells as determined by [3H]dCTP extension assay after digestion of genomic DNA with methylation sensitive restriction endonuclease HpaII.

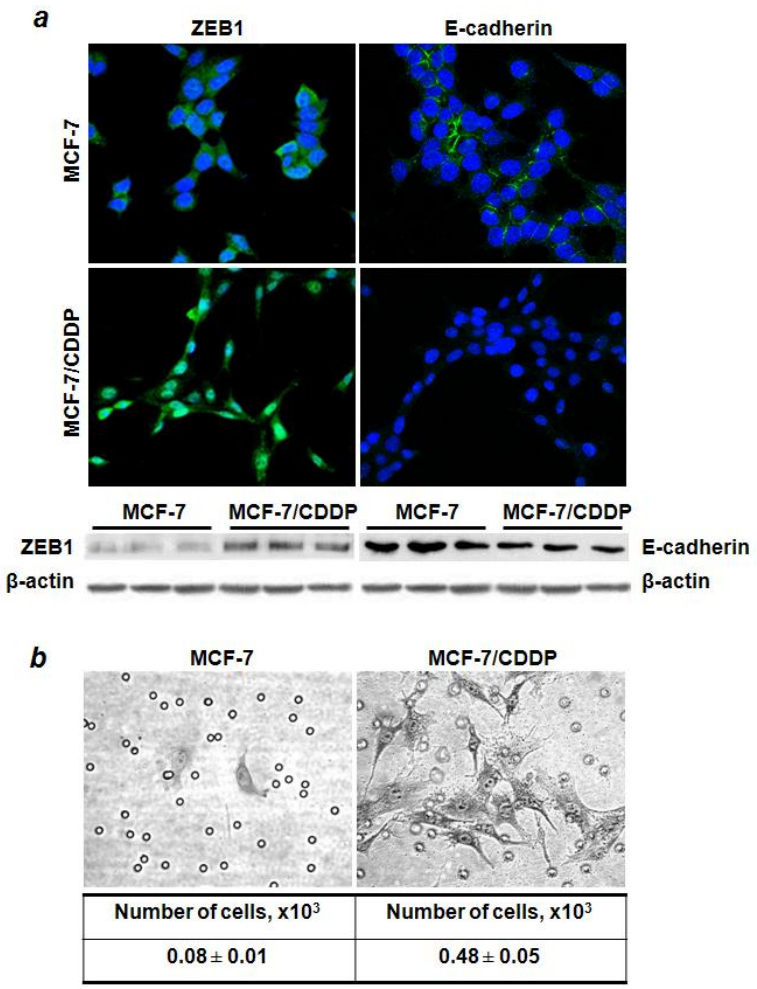


Figure 3.3. Altered levels of ZEB1 and E-cadherin, and invasive phenotype of cisplatin-resistant MCF-7 cells.
A) Increased levels of ZEB1 and decreased levels of E-cadherin in the MCF-7/CDDP cells as detected by immunofluorescence using primary antibodies against ZEB1 (green) and E-cadherin (green) and by western immunoblotting.
B) Increased invasiveness of the cisplatin-resistant MCF-7 cells. MCF-7 and MCF-7/CDDP cells were plated in invasion chambers. After 48 hours, cells on the bottom side of membrane were stained and mounted onto glass slides and mean number of cells in four replicates was determined.

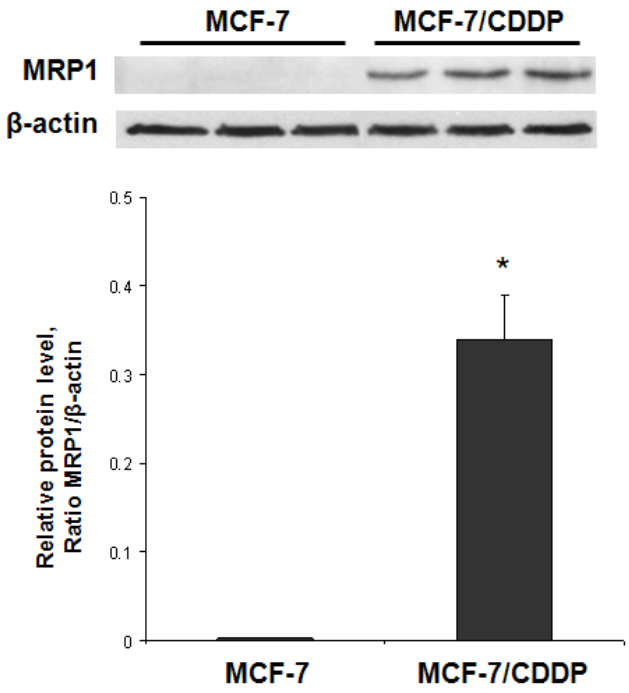


Figure 3.4. Levels of MRP1 in MCF-7 cells and MCF-7/CDDP cells. MCF-7 and MCF-7/CDDP cells were grown in triplicate. Each line represents a protein extract from an independent flask. Top, representative western immunoblot; bottom, a quantitative evaluation of the MRP1 protein levels in the MCF-7/CDDP cells relative to those in the MCF-7 cells. (*denotes $p<0.05$).

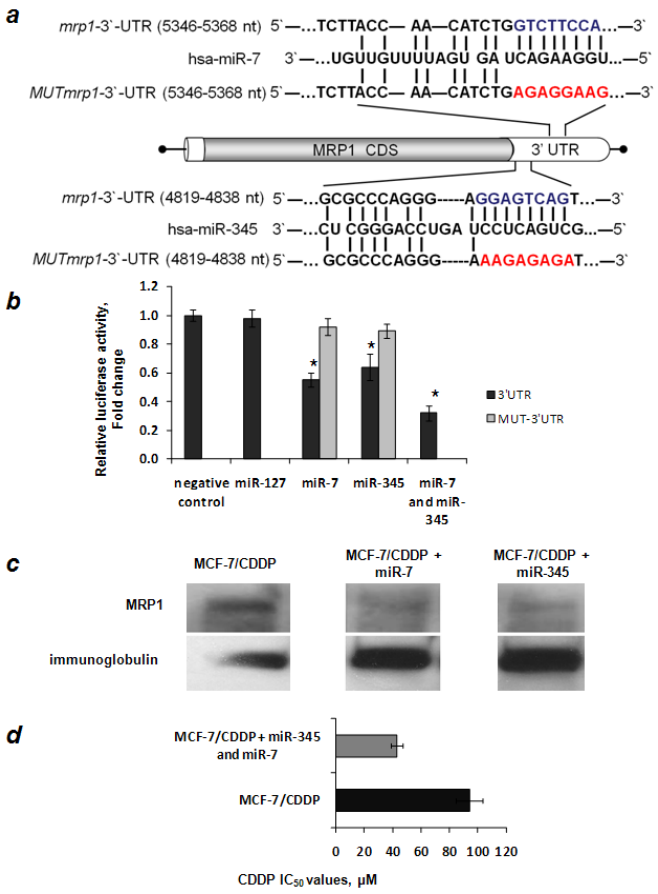


Figure 3.5. miR-345 and miR-7 target multidrug resistance protein 1 (MRP1).
A) Alignment of the 3'UTR of MRP1 by miR-345 and miR-7. The original cloning of the UTR was based on transcript NM_004996 for the MRP1 gene and the binding regions for miR-7 and miR-345 bound at 5346-5368 nTs and 4819-4838 nTs, respectively. Seed regions have been defined as at least 5 Watson-Crick base pairs within the 2-7 nucleotides of the 5' region of the miRNA. Mutant vectors abolished the corresponding seed sequeneces at the 3' end of the miRNA binding site within the vector. Nucleotides depicted in blue represent wild-type seed-interacting sequences. Red nucleotides depict mutated regions.
B) Targeting of the 3'UTR of MRP1 by miR-345 and miR-7. For the luciferase reporter experiments, a vector containing the 3'-UTR segment of the *MRP1 (Abcc1)gene*, hsa-miR-7 and hsa-miR-345 were co-transfected. Scrambled oligonucleotides and un-related miRNA (miR-127) served as controls. Activity was assayed after 24hours.
C) Decreased levels of MRP1 24 hours after transfection of MCF-7/CDDP cells with miR-7 and miR-345. The MCF-7/CDDP cells were transfected with miRNAs as described above and the levels of MRP1 were measured by western blotting.
D) Increased sensitivity of MCF-7/CDDP to CDDP after transfection with miR-7 and miR-345.

4. Doxorubicin and cisplatin resistant MCF-7 cell lines display strikingly similar DNA methylation profiles, copy number variation and gene expression patterns

4.1 Abstract

Many chemotherpauetic agents achieve their effect by eliciting extreme genotoxic stress. This includes two commonly prescribed chemotherapy drugs cisplatin and doxorubicin. Doxorubicin is widely used in curative-intent adjuvant breast cancer therapy. It is an intercalator which interferes with topoisomerase function, blocks replication and leads to induction of apoptosis. Cisplatin is an alkylating agent that causes crosslinks and therefore interferes with cell division. It is used to treat a range of cancers including ovarian and breast cancer[4,5].

We have previously generated two separate MCF7 cell lines resistant to either cisplatin or doxorubicin. The resistant lines demonstrated ~3-fold faster growth rates, increased invasiveness and cross resistance to radiation. In an attempt to evaluate genome wide locus-specific DNA methylation changes and the concomitant gene expression changes, we use the Illumina DirectHyb gene expression and Infinium Human Methylation27 bead chip assay. We noted a surprisingly congruent global DNA methylation profile between the MCF-7/DOX and MCF-7/CDDP lines. Our results show that >50% of the DNA methylation and gene expression changes are inversely correlated. Such a strong correlation between gene expression and DNA methylation strongly suggests that epigenetic control is important for acquired chemoresistant phenotype. Furthermore, comparative genome hybrization also revealed a pronounced degree of genome instability and highly similar patterns of genome rearrangements in MCF-7/DOX and MCF-7/CDDP cells.

We suggest that the increased instability of the resistant lines may be a consequence of the epigenetic dysregulation. Finally, our data support the notion that acquisition of drug

resistance is an epigenetic-regulated process and that certain commonalities exist between resistant cells acquired under different selection pressure, thus, explaining phenomenon of cross resistance.

4.2 Introduction

Nearly 90% of all recurring breast cancers exhibit multi-drug resistance, increased invasiveness, cross resistance to radiation and present a poor prognostic outcome[6]. This drug resistance is the result of several molecular mechanisms including reduced intracellular concentrations, altered targets, efficient repair of DNA repair and evasion of apoptosis[1,6]. The acquisition of the resistance has roots in genetic, epigenetic and karyotypic changes to the genome. Although genetic changes likely contribute in some instances, the 'one mutation:one altered protein' theory is too limiting to explain the multi-factorial phenotype of drug resistance and especially cross-resistance. The epigenetic and karyotypic theories allow for the robust, wide spread changes that are necessary to elicit the broad phenotype and, thus, are likely the major contributing chemoresistance mechanism[52]. The epigenetic theory hypothesizes that drug resistance is the result of DNA methylation, histone modifications and/or small, noncoding regulatory RNAs while the karyotypic implicates gross chromosomal aberrations as the driving force[52,63]. These theories are not mutually exclusive of each other as the genomic instability required by the karyotypic theory requires epigenetic alterations. The role of epigenetics in the acquisition of drug resistance has been described previously[63].

We have further explored the role of epigenetics through the investigation of two breast adenocarcinoma (MCF7) cell lines resistant to cisplatin or doxorubicin. We uncovered the role that small, non-coding RNAs play in the drug resistant phenotype by demonstrating their influence on over-expression of drug efflux pumps. Furthermore, we have demonstrated a global hypomethylation of in these drug resistant cell lines[21]. Here

we attempt to increase the understanding the role of DNA methylation in the process by increasing the resolution of our assay. We hypothesize that drug-resistant cells will exhibit a well-defined pattern of DNA methylation at a variety of loci. These changes may be linked to the expression of the given loci, thus contributing to the drug resistant phenotype. Furthermore, we believe the hypomethylated genome is responsible for diminishing genome stability.

To test our hypothesis we conducted a genome-wide analysis of the locus- specific DNA methylation profiles, gene expression and copy number variations in the MCF-7 cells and their variants resistant to doxorubicin and cisplatinum. Here we show that patterns of the three measured attributes were strikingly similar in MCF-7/DOX and MCF-7/CDDP cells. This prompted us to speculate that epigenetic and/or karyotypic profiles may not be unique to particular instances of resistance but rather provide plausible cross-resistance markers. Furthermore, we used our data to describe some of the phenotypic features of drug resistance including increased growth rate, diminished cell cycle control and reduced apoptosis.

4.3 Materials and Methods

4.3.1 Cell culture

The MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained according to ATCC's recommendations. The MCF-7 drug-resistant variants were established by stepwise selection after prolonged (>6 months) treatment of MCF-7 cells to increasing concentrations of CDDP (Sigma) or DOX at a range of 0.5–25 μM in the medium[21]. After 6 months of culturing in the presence of CDDP or DOX, the IC50 (inhibitory concentration to produce 50% cell death) values were 94 and 12 μM of CDDP for the MCF-7/CDDP and parental MCF-7 cells, respectively; and 24 and 1 μmol/L DOX for the MCF-7/DOX and parental MCF-7 cells, respectively. Cells were seeded at a density of 0.5 $X10^6$ viable cells per 100 mm plate, and the medium was changed every other day for 6 days. Trypsinized cells were washed in phosphate-buffered saline and immediately frozen at -80°C for subsequent analyses. The experiments were independently reproduced three times, and each cell line was tested in triplicate.

Cell growth rates were determined by seeding $2x10^5$ cells in a 10cm culture plate. Plates were photographed every 24 hours for the next 72 hours and cells counts performed on four field of views (n=4).

4.3.2 DNA Preparation and microarray based methylation and CGH analysis

DNA was collected from cells using the DNA Easy Blood and Tissue Extraction kit (Qiagen) according to manufactures protocols. Briefly, cells were lysed and the liberated

DNA was treated with RNAase (Sigma) and adsorbed to silica membranes. The membrane bound DNA was washed with the prescribed buffers and eluted with water.

The collected DNA (500ng) was bisulfite converted using the DNA EZ methylation kit from Zymo Research according to manufactures protocol. This process converts cytosine residues on single stranded DNA to thymine residues in the presence of quinine and sodium bisulfite. Methylated cytosines are not a proper substrate for this reaction and, therefore, are left unconverted. Methylation was determined using the Infinium assay on the Illumina platform. Data was collected from the >27, 000 probes represented on the HM27 microarray. These probes contain CpG dinucleotides from selected loci throughout the genome. All steps were carried out according to the manufacture's specifications and with Illumina supplied reagents. In short, bisulfite converted samples were amplified overnight, fragmented and purified. The resuspended sample was hybridized overnight to the microarray which harboured millions of bead-bound 50-mer oligos. Each interrogated loci is represented by two bead types: a methylated type, 'C' remains a 'C'; and an unmethylated type, 'C' becomes a 'T'. Hybridized chips were washed to remove unbound or non-specific DNA fragments. The resulting oligo-sample hybrid is then extended with a biotin linked dedeoxy cytosine and stained with streptividin. The relative intensity of the unmethylated to methylated bead for each allele provides a measure of relative methylation level.

Complete genome hybridization was also performed using the Infinium assay on the Illumina platform according to the manufactures protocol. The chemistry for this assay is

identical to what is described above with the exception that beads harbour oligos that represent each of the possible base SNP variations for the interrogated locus. Also, the extension of these loci will create a two color signal (red and green). The relative intensities of these colors indicate what base combination is present in the SNP. Copy number variation (CNV) was determined by using contiguous SNP data in an Illumina algorithm and comparison to a reference genome[177,178].

4.3.3 RNA preparation and expression microarrays

Total RNA was extracted from cells using Trizol reagent and according to the manufacturers protocols (Invitrogen). Extracted RNA was further purified with the RNA Easy kit from Qiagen. This purification step was necessary to remove all traces of phenol. The quality of the resulting RNA was assayed using a bioanalyzer 2100 (Agilent).

Gene expression analysis was achieved through the whole genome hybrization assay on the Illumina platform. Each line was assayed in quadruplicate. All practices were carried out according to Illumina protocol and using manufacturer supplied reagents. First, a library of cRNA was prepared using the Ambion TotalPrep RNA amplification Kit (Invitrogen) and 500ng of input total RNA. This involved an RT-PCR step followed by second strand synthesis. cDNA was then purified on a silica column and *in vitro* transcription was performed and the resulting cRNA purified again. For the bead arrays, 750ng of cRNA was hybridized to the human whole genome bead array chip HT-12v4. This array contained >47,000 probes that represent genes included in NCBI ReqSeq data

base. Following overnight hybrization, the microarrays were washed several times. This included a 10 minute, static high stringency (high temperature and salts) wash, five minute room temperature wash on an orbital shaker, ten minute wash in 100% ethanol on an orbital shaker and a second room temperature wash. Arrays were blocked for 10 minutes and stained with a streptividin-Cy3 tag for detection purposes. Next, arrays were washed a final time at room temperature, briefly centrifuged and dried. The bead arrays were scanned using an iScan (Illumina). Signal data was interpreted with the aid of the genome studio (Illumina) software.

4.3.4 Polymerase Fidelity Assay

A polymerase fidelity assay was performed to observe the relative levels of polymerase fidelity between the samples. This involved placing total protein extracts from the cells in a reaction mixture with dNTPs and a fluorescently labelled, primer-template hybrid of known sequence. Samples were run post-reaction on a 20% denaturing polyacrylamide gel and visualized using a phosphoimager. The specifics of the assay are as follows.

The primer/template complex for the assay was prepared by annealing the fluorescein amidite (FAM)-labelled 15 bp primer (5'-6-FAM-TCCCAGTCACGACGT-3', PAGE-purified) to the 30 bp template (5'-TCATCGAGCATGATCACGTCGTGACTGGGA-3', PAGE-purified). All components were mixed by pipetting in the following order (on ice): Tris-HCl (1M, pH 8.0) = 10 µl, β-Mercaptoethanol (14.3M) = 0.5 µl, BSA (10 mg/ml, NEB) = 2 µl, Primer (100µM) = 3 µl, Template (100µM) = 3 µl, and H2O = 183.5 µl for a total volume of 200µL. The reaction was incubated for 5 min in boiling water and then

allowed to cool to room temperature (20-25°C). The complex was prepared in advance and stored at -20°C.

Freshly harvested cells were sonicated in 1X PBS for 10, five second intervals. Samples were placed on ice between intervals to prevent heating. Following sonication, samples were spun down at 13,000Xg for 10 minutes. Protein levels were determined using a Bradford Assay. 70 µg of protein from the crude extract was used immediately in the polymerase reaction. The crude extract was mixed with 2.5 µl of the primer/template complex, 2.5uL of 2mM dNTPs, 2.5µl reaction buffer (10x Y+/Tango, Fermentas) and water for a total volume of 25µl. The reaction was carried out at 37 ºC for 15 min in a PCR machine, quenched with 50 µl of loading buffer (95% formamide, 50 mM EDTA, and 0.05% bromphenol blue), heated to 95°C for 3 min and cooled on ice for 2 min. A reaction including Klenow enzyme (NEB buffer 2) in place of a protein extract was used as a positive control (ladder).

After quenching, 15 µl of the sample/loading buffer mix was loaded on a 20% polyacrylamide gel (20x20x0.075 cm) containing 8 M Urea. Also, 0.5 µl of the 15 bp 6-FAM-labelled primer was mixed with 2.5 µl of loading buffer and loaded on the polyacrylamide gel to serve as a molecular weight marker. Electrophoresis was carried out in 1x TBE buffer for ~5 hours at 500 V. Gels were scanned using Typhoon 9410 at an excitation wavelength of 88 nm using a 520 BP 40 emission filter, the PMT voltage of 685 V, at a resolution of 100 µm. Images of scanned gels were analyzed using

ImageQuant 5.2 software (Molecular Dynamics). For the adjustment of total values of the object outline, the Local Median background correction method was applied

4.3.5 Western blot analysis of protein expression

The protein levels of Polymerase beta (polβ), epsilon (Polε) and iota (Polι) were determined by Western blotting using protocols described previously[21,154,161]. Polβ antibodies (Abcam) were used at a dilution of 1:1000 and probed with anti-rabbit secondary at 1:500 dilution (Santa Cruz Biotechnologies). Polε and Polι antibodies were procured from Santa Cruz Biotechnologies and employed at a dilution of 1:500. Polε necessitated an anti-mouse secondary and Polι an anti-goat, both were used at 1:500 dilution (Santa Cruz Biotechnology).

4.4 Results

4.4.1 Growth rate of resistant cells

The resistant cells grew ~3-fold faster than the sensitive parental MCF7 line (Fig. 4.1). All cells were initially seeded at a density of $2.7x10^3$ cells/cm^2 and counted every 24 hours over a 72 hour period, the p-values were calculated for the each resistant line relative to the MCF7 parental values. At the 24 hour time point, cell numbers were: $5.9x10^3$ (S.E.±126), $1.7x10^4$ (S.E.±238; $p<0.001$) and $1.6x10^4$ cells (S.E.±593, $p<0.01$) for the MCF7, MCF7/CDDP and MCF7/DOX, respectively. At 48 hours the cells count was: $1.6x10^4$ (S.E.±188cells), $3.4x10^4$ (S.E.±405; $p<0.001$) and $4.8x10^4$ cells/cm^2 S.E.±1442; $p<0.01$) for the MCF7, MCF7/CDDP and MCF7/DOX, respectively. Finally at 72 hours, cells counts were: $3.3x10^4$ (S.E.±342cells), $1.0x10^5$ (S.E.±1458; $p<0.001$) and $1.2x10^5$ cells/cm^2 (S.E.±1227; $p<0.001$) for the MCF7, MCF7/CDDP and MCF7/DOX, respectively. Furthermore, cell morphology and cell-cell interactions deviated visibly in both resistant lines relative to the sensitive parent. In the MCF7/CDDP cells display spindly out crops while the MCF/DOX cells seem to prefer a equidistant spacing with minimal physical contacts between adjacent cells. In contrast the MCF7 cells display a relatively round cell shape and prefer tight intercellular spacing. These differences in morphology have been linked to invasiveness and their implications have been discussed in previous chapters.

4.4.2 Gene Expression and methylation in the MCF7, MCF7/CDDP and MCF7/DOX cell lines

Microarray analysis using the Illumina platform revealed a total of 1329 and 1344 statistically significant ($p<0.05$; $0.5>$fold change>2) expression changes in the MCF7/CDDP and MCF7/DOX lines respectively (Fig. 4.2A). Interestingly, 1219 (424 down— and 795 up regulated) of these changes were common with similar direction and amplitudes of changes between the two lines. The net signal of these genes was ~3-fold higher in the resistant lines, thus, indicating a higher level of transcription.

Genome-wide methylation profiles were assayed using the Illumina based Infinium microarray. Again results between the two lines were similar. Of the 2252 differentially methylated loci in the MCF7/CDDP and 2227 loci in the MCF7/DOX, 2025 were common in amplitude and direction between the lines ($p,0.05$; $0.05>$fold change>2) (Fig. 4.2B). Of the common loci, 596 were hypomethylated and 1427 hypermethylated relative to the parental line. When comparing total signal values, the MCF7/CDDP and MCF7/DOX displayed ~1.3-fold more signal than the MCF7 lines.

There were 123 genes that demonstrated significant changes in both gene expression and methylation patterns (Fig. 4.3A). These genes were grouped into several ontology categories including in descending order with respect to the number of genes affected: metabolic processes (GO:0008152), nucleic acid metabolism (GO:0006139), transcription (GO:0006350), apoptosis (GO:0008219), proliferation (GO:0008283), cell cycle regulation (GO:0007049) and cell growth (GO:0016049). Most genes were binned

into metabolic processes and nucleic acid metabolism, 68% and 62%, respectively (Fig. 4.3B, Table4.1).

Of the 123 genes common between both lines with significant changes in gene expression and methylation, 58% had inversely proportionate changes. These genes obey the 'classical theory' of increased methylation leading to repressed expression and *vice versa* (represented in quadrant #1 and #3 in Fig.4.3). 37% exhibited an increase in both expression and methylation (quadrant #2 of Fig.4.3) while only 4% a decrease in both (quadrant#4 of Fig.4.3)—depicting a 'non-classical' model. In an attempt to explain the 'non-classical' genes we interrogated the entire genome for copy number variations (CNV). Again our analyses presented a strikingly similar profile between the two resistant lines relative to the MCF7 parental line. Specifically, 20 regions were commonly deleted between the two lines, however, none of the 'non-classical' probes could be mapped to these regions. These deleted regions spanned in size from ~$2x10^5$ to $2.5x10^6$, however, this was minuscule in comparison to the ~600 regions displaying amplification relative to the MCF7 parental line (Fig. 4.4A). Interestingly, when we over layed the CNV data for only the 123 genes showing changes in gene expression and methylation, the resistant lines finally demonstrated some individuality. Of the 123 genes, 26% and 35% of the genes did not deviate from the parental copy number in the both MCF7/CDDP and MCF7/DOX cells, respectively. However, ~45% and 25% were increased in MCF7/CDDP and MCF7/DOX cells, respectively. The opposite was trend was true for depleted regions where the MCF7/CDDP had fewer (30%) deletions and the MCF7/DOX more (~40%).

4.4.3 Polymerase Fidelity

Global polymerase activity was assayed via a polymerase fidelity assay that measures the efficiency and fidelity of single nucleotide incorporations on a known template. The results are presented in figure 4.6. As the nucleotides are incorporated, bands appear at sizes larger than the 15bp primer template substrate. Exonuclease activity is observed by the appearance of bands below the 15bp size. Relative to the MCF7 line, the resistant lines had higher activity, reduced fidelity and diminished exonuclease activity. In the presences of dATP, dCTP and dTTP, a 16 bp band is not expected as the template calls for a dGTP incorporation, and interestingly, none of the samples demonstrate any appreciable incorporation. Likewise, in the presence of dGTP, all the samples show a healthy 16 base pair band. However, when dGTP and dATP, are both supplied, the resistant cells show heavier bands of incorporation at 16 and 17 bp suggesting a higher rate of activity. Furthermore, the DOX cells appear to continuously incorporate the nucleotides beyond the two base pair demands of the template demonstrating a reduced fidelity (marked with red asterisks, Fig. 4.6). Interestingly, the resistant lines tend to display fewer and less intense bands at sizes below the initial 15 bp of the primer-template. These bands, indicative of exonuclease activity, suggest that the resistant lines do not display as much exonuclease activity.

Western blot analysis for specific polymerases (epsilon—ε, beta—β and iota—ι) revealed a striking difference between Polε protein expression—reduced to ~0.3 and 0.2-fold ($P<0.001$) in the MCF7/CDDP and MCF7/DOX lines, respectively. Polβ, an active member of the DNA repair protein contingency, was differentially expressed in the

MCF7/DOX resistant lines—1.15-fold in the MCF7/CDDP cells and 0.6-fold in the MCF7/DOX. However, with a P>0.1 for the MCF7/CDDP line, only the DOX cells were significantly altered ($p<0.01$). The translesion synthesis Polι was significantly increased in both lines albeit to a much higher extent in the DOX lines—1.7 ($p<0.0001$) and 3.94-fold ($p<0.001$) Fig. 4.5.

4.5 Discussion

The astonishing growth rates of the resistant cell lines prompted us to make several speculations. 1) These cells spend most of the time in S-phase replicating DNA and, thus, should have an increased polymerase capacity to achieve this feature of accelerated growth. 2) Any stalling for cell cycle checkpoints or damage repair is prevented. 3) Apoptosis is rare. 4) A massive level of genomic instability must exist.

A polymerase fidelity assay indicated that the resistant lines had a higher activity and, in the case of DOX cells, a lower fidelity of incorporation. This suggested that perhaps DNA polymerases responsible for bypassing damage or repairing damage would show altered levels of expression. Indeed, this was the case for Polι, a polymerase involved in translesion synthesis that localizes to stalled transcription machinery and quickly incorporates bases at highly distorted and uninformative templates[179,180]. DOX cells showed ~3-fold ($p<0.01$) increase in Polι protein expression while the MCF7/CDDP cells showed ~1.7-fold increase. On the other hand, the MCF7/DOX cells experienced a 0.6-fold change while MCF7/CDDP did not display a significant change. The differences in these expressions may be explained by the mechanism of action for each drug. Polβ is a high fidelity enzyme involved in DNA repair. It's over expression is linked to genomic stability and cisplatin resistance in ovarian and colorectal cancer cell lines[181,182]. Interestingly, Polβ was repressed in the MCF7/DOX lines suggesting that it plays less of a role in circumventing the effects of the drug. This may be due in part to the mechanism of action. Doxorubicin does not directly damage DNA, but rather acts as a physical interference to the transcriptional machinery—indicating a higher requirement for Polι.

Cisplatin exposure does result in direct DNA damage that would present as a suitable substrate Polβ activity. It is possible that due to selective pressure during the six months of drug exposure, each line sustained these beneficial alterations.

In addition, we assayed for the presence of Polε, one of the three major polymerases involved in replication. Polε possesses exonuclease activity and an added responsibility in DNA damage repair. Polε protein was down regulated ~0.2 and 0.3-fold in the MCF7/CDDP and MCF7/DOX cells, respectively. Interestingly, the resistant lines each displayed a considerably reduced exonuclease activity relative to the MCF7 parental lines. It is possible that with such low levels of Polε protein, global exonuclease activity was depressed. This would further indicate lower fidelity in the resistant lines as exonuclease activity is usually employed in reversing erroneous incorporation events. Combined with the high rate of incorporation (high growth rate), it is reasonable to expect that misincorporations are common and rarely reversed.

Since leading strand production relies heavily on Polε it is difficult to explain the extremely low protein levels. The rapid growth rate of these cells mandates expedited replication of the genome, however, one would expect a severe impairment of replication rates with such low Polε levels. Although mammalian Polε is not well investigated, studies into the budding yeast enzyme have revealed that the polymerase and exonuclease activity of Polε is nonessential as catalytically-incapable mutants are proficient at replication and repair[183]. Therefore, it is possible that the other polymerase members, for example the over expressed Polι, are capable of carrying the burden of rapid DNA

replication in the drug resistant cells. Finally, the Polε gene experienced ~2.5-fold hypermethylation in the resistant lines suggesting that the mechanism of repression may be epigenetic in nature.

Even in the absence of Polε, the remaining polymerases may increase their activity, thus, allowing the resistant cells to maintain an increased rate of activity. For example, Wrnip is an ATPase that is known to interact with polymerase delta increasing its activity up to 5-fold. It likely achieves this by acting as a modulator for initiating or restarting stalled Polδ[184]. Gene expression arrays demonstrated a ~2-fold increase in Wrnip expression while, methylation arrays demonstrated a 0.4-fold reduction of regulation.

Regulation of the cell cycle appears to be drastically impaired in the resistant cell lines as the cells are continuously replicating and dividing with virtually no stalling for important check points in the cell cycle. Key proteins involved in controlling the cell cycle are severely deregulated in the resistant lines. For example, CDK2, which is responsible for promoting the movement from G1 into (and then through) S-phase demonstrates a down regulation of 0.15-fold in expression with a concomitant 4.3-fold increase in methylation. This suggests that the deregulation of this gene is epigenetic in nature. If CDK2 –cyclinE and CDK2-cyclinA complexes are the driving force of S-phase, it would be expected that a reduction in the availability of CDK2 would result in a G1-S phase halt. Clearly this is not the case in the resistant lines. It is possible that another protein plays a redundant role for CDK2 in allowing progression through the cell cycle as CDK2 knockout mice are perfectly viable[185]. Interestingly though, these mice do display a slower entrance into S-

phase. We have postulated two possible reasons for the diminished CDK2 capacity. First, if one assumes that these cell lines have maintained some self-governing mechanism, it is possible that the large repression seen in the resistant lines is a futile attempt to slow and control S-phase. Second, we have previously shown (chapter 2) that Rb is practically non-existent in the resistant cell lines: Rb is a substrate for Cdk2 activity. With the loss of Rb, any requirement for CDK2 to drive the cell into S-phase via the relief of the oppressive nature of Rb is also lost. Therefore, the diminished CDK2 expression may have been relatively inconsequential relative to the loss of Rb. Additionally, the lack of CDK2 may partially explain the high radio-resistance observed previously in these lines[71]. Radiation induced damage is signalled through the ATM pathways to CDC25 which acts as repressor of CDK2 activity, thus, preventing S-phase progression. Perturbations in the CDK2-CDC25A pathway have been linked to a more radio-resistant DNA synthesis that allows cells to continue replication following ionizing radiation[186].

A similar ambiguity arises with the expression of the suppressor of cytokine signalling gene (SOCS1). SOCS1 appears to function exclusively as an anti-proliferative agent yet it seems impotent in the resistant lines. Again, this expression appears to be epigenetically controlled as the ~6-7-fold higher expression in the MCF7/CDDP and MCF7/DOX lines correlates with a reduction of <0.1-fold in methylation. Interestingly, aberrant SOCS1 hypermethylation is a common mark of cancer cells that imparts an increased proliferative capacity[187]. Similar to the CDK2 down regulation, the increased SOCS1 signalling seems counterintuitive to the observed phenotype. Although one could

speculate that it is another futile attempt to slow down the growth rate, this begs the question of why we do not see a 'purposeful' demethylation in normal tumours? Why should the more deviated cells employ such a level of regulation when the less deviated precursors do not? As is the case with CDK2, the downstream targets of SOCS1 (the STAT transcription factors) may be altered or non-existent themselves, thus, negating any effect that SOCS1 would normally impart.

Simply put, cell growth rate is a balance between proliferation and apoptosis. Besides the polymerase activity described above, our data have indicated several mechanisms that may contribute to the unchecked growth potential of the resistant cells including diminished apoptosis and altered DNA damage repair. For example, our expression/methylation screen revealed an increase in the expression (~5-fold expression, $p<0.001$; ~2.5 fold methylation, $p<0.01$) of Pim2, which acts as an anti- apoptotic protein. Through the phosphorylation of BAD, Pim2 is able to reverse BAD-induced cell death[188]. Pim2 represents an example of the 'non-classical' relationship between methylation and gene expression (i.e. both showing changes in the same direction). This scenario will be discussed below. Another example is provided by Fen1 an endonuclease responsible for the removal of the damaged strand during BER with reduced expression (~0.5 fold expression, $p<0.01$; 3.5-fold methylation, $p<0.01$)[189]. It does not appear as though the resistant cell lines recognize any deterrent to growth and, thus, likely do not attempt any repair. Therefore, reduced expression of a DNA repair protein such as Fen1 is a feasible scenario. Less feasible is the fact that Fen1 also has a role in Okazaki fragment processing which requires it to interact with PCNA and Polδ on the lagging

strand[190]. It is possible that due to the extra demand on Polδ the presence of Fen1 may be counterproductive to an expedient replication and, therefore, its reduced expression may favour the increased replicative rate of the resistant cells. However, this scenario demands an alternative processing mechanism for the high number of Okazaki fragments that must be produced in these cells[190,191]. Interestingly, Fen1 also has a direct role in promoting genomic instability. Microsatellite stability and telomere maintenance are both dependent on Fen1 activity[192,193]. Therefore, the reduced Fen1 profile is congruent with (and may even contribute to) the massive instability observed in these cells the observed phenotype of the cells.

As depicted in Figure 4.2, the correlation between methylation and gene expression does not always exhibit the 'classical model'. Of particular interest was the category with increased methylation and expression that account for nearly 40% of the changes of the altered in both (gene expression and methylation) pool. It is possible that probes in this group may arise from the amplified parts of the genome. The increased gene copy number may account for the increased expression despite the increased methylation. For example, figure4.4 illustrates that chromosome 6 is almost entirely, duplicated in the resistant lines. Of the approximately 1200 genes with altered expression levels, 73 are on chromosome six and 57 (of the 73) demonstrate increased expression. With respect to methylation, the resistant cell lines have 186 loci on chromosome six with a methylation pattern that deviates from the MCF7 parent. 120 of these loci are hypermethylated. This scenario allows for a plausible explanation of the nearly 40% 'non-classical' model of expression and methylation. Perhaps increased copy numbers lead to increased gene

expression, the increased amount of sequence may also be a target of methylation events, however, these events are insufficient at reducing the higher expression.

Qualitatively speaking, CNV gains account for the majority of the chromosomal aberrations. Although we have not officially investigated the physiological impact of each of these gains, we hypothesize that they may have a major impact on the observed phenotype. Furthermore, these changes provide support to the karyotypic hypothesis of acquisition of drug resistance and occur in conjunctions with massive epigenetic alterations.

Although methylation has a well described role in controlling gene expression, it has another responsibility in maintaining genomic stability. The comprehensive methylation profile we achieved through the use of microarray technology suggested that as a whole the genome of the resistant cells was hypermethylated—~1.3-fold higher signal in the resistant cell lines, however, our previous assay of global methylation via the cytosine extension assay revealed a hypomethylated genome. This discrepancy may lie in the biased focus of the microarray. Probes on the bead array are designed mainly towards known CpG islands in promoter regions, where as the cytosine extension assay presumably treats CpGs throughout the genome indiscriminately. Taken together, it is plausible that the hypomethylation seen in the extension assay results from a massive demethylation of non-transcribed or gene poor regions such as centromeres, telomeres and mobile elements; regions that are not well represented on the bead array. As such,

we would expect a large increase in chromosomal aberrations and anueploidy which was indeed the case (as described by the results of the CNV analysis).

This body of work has provided information into the epigenetic profile of two drug resistant lines and the possible consequences of such gross dysregulation. MCF7 cells resistant to either cisplatin or doxorubicin display growth rates ~3-fold larger than the parental line, increased invasiveness and cross resistance to radiation treatment. Investigation into the loci specific methylation patterns between the two resistant lines revealed a large deviation from the parental MCF7 cells. The altered methylation status was inversely proportional to gene expression greater than half the time, explaining, at least partially, the multifaceted phenotype frequently observed in drug resistant tumours. We also detailed a scenario where differential methylation patterns are related to chromosomal abnormalities. Data from our screens suggest that the resistant cells display altered ratios of polymerase, avoid cell cycle checkpoints, have reduced repair activity and evade apoptosis. Finally, and perhaps most importantly, we revealed that the molecular characteristics of the two different drug resistant lines is strikingly similar. Both changes in methylation, gene expression and chromosome aberrations were practically identical between the two lines suggesting that the mechanism leading to acquisition to drug resistance may have common, predictable characteristics regardless of the drug treatment. This provides promise for developing useful diagnostic and prognostic biomarkers. It will be important for future studies to elucidate the most proximal events in the acquisition of drug resistance.

		Fold Change	
Gene	Related Process	Gene Expression	Methylation
CDK2	*CELL CYCLE*	0.15	4.28
CEBPB	*TF*	7.80	5.43
CENPB	*MITOSIS*	0.65	0.97
CETN1	*MITOSIS*	3.66	0.38
DDX5	*TRANSCRIPTION*	0.15	6.70
EEF1A1	*TRANSLATION*	0.17	5.19
EEF1A1	*TRANSLATION*	3.44	5.19
FEN1	*DNA REPAIR*	0.46	3.58
GPR89A	*TF CONTROL*	0.62	7.29
HIST1H1A	*CHROMATIN*	3.12	4.29
HTRA2	*APOPTOSIS*	1.85	1.79
IHPK2	*APOPTOSIS*	2.64	0.73
JUNB	*TF*	2.28	4.17
LRDD	*APOPTOSIS*	2.42	0.52
MBD4	*DNA METHYLATION*	0.34	6.42
MBD6	*DNA METHYLATION*	4.18	0.39
NEIL1	*DNA REPAIR*	2.36	0.34
PCBP4	*APOPTOSIS*	1.29	0.34
PIM2	*APOPTOSIS*	1.93	2.47
RASSF5	*APOPTOSIS*	6.64	4.35
RNF7	*CELL CYCLE*	2.15	0.38
SMARCD2	*CHROMATIN*	0.21	6.59
SOCS1	*SIGNALLING*	6.84	0.48
SOCS4	*SIGNALLING*	2.89	11.38
UNG2	*DNA REPAIR*	3.64	4.45
WRNIP1	*REPLICATION*	2.17	0.39

Table 4.1. Selected genes demonstrating altered gene expression and DNA methylation profiles in the MCF7/CDDP and MCF7/DOX drug resistant lines relative to the sensitive, parental MCF7 line.

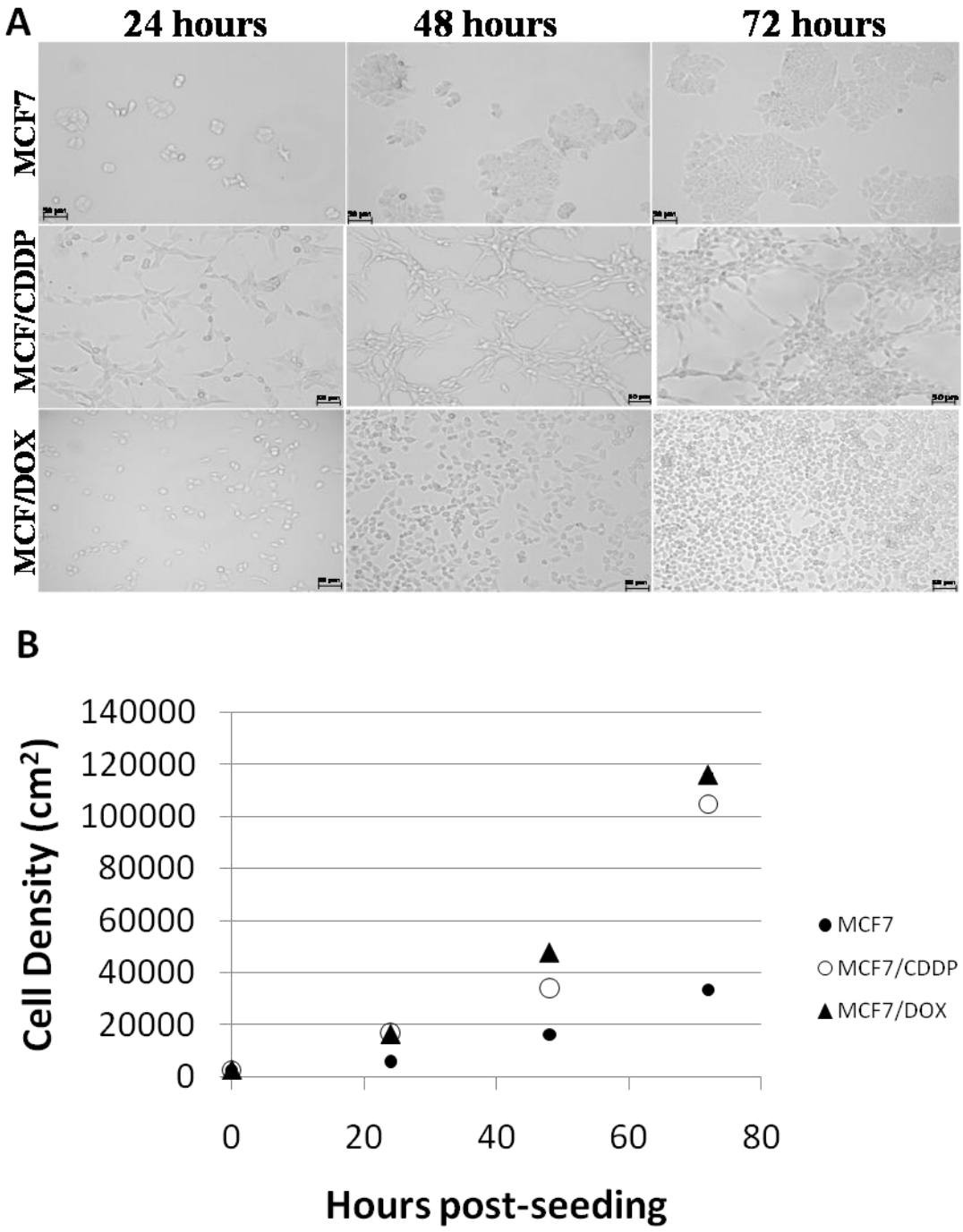


Figure 4.1. Drug resistant cell lines demonstrate a higher growth rate. A) MCF7, MCF7/CDDP and MCF7/DOX cells photographed 24, 48 and 72 hours post seeding. All plates were seeded with equal density (2x105 cells/ 10mm plate) B) Growth rate curve of the parental and resistant lines. The resistant cells grow nearly 3-fold faster than the MCF7 cells.

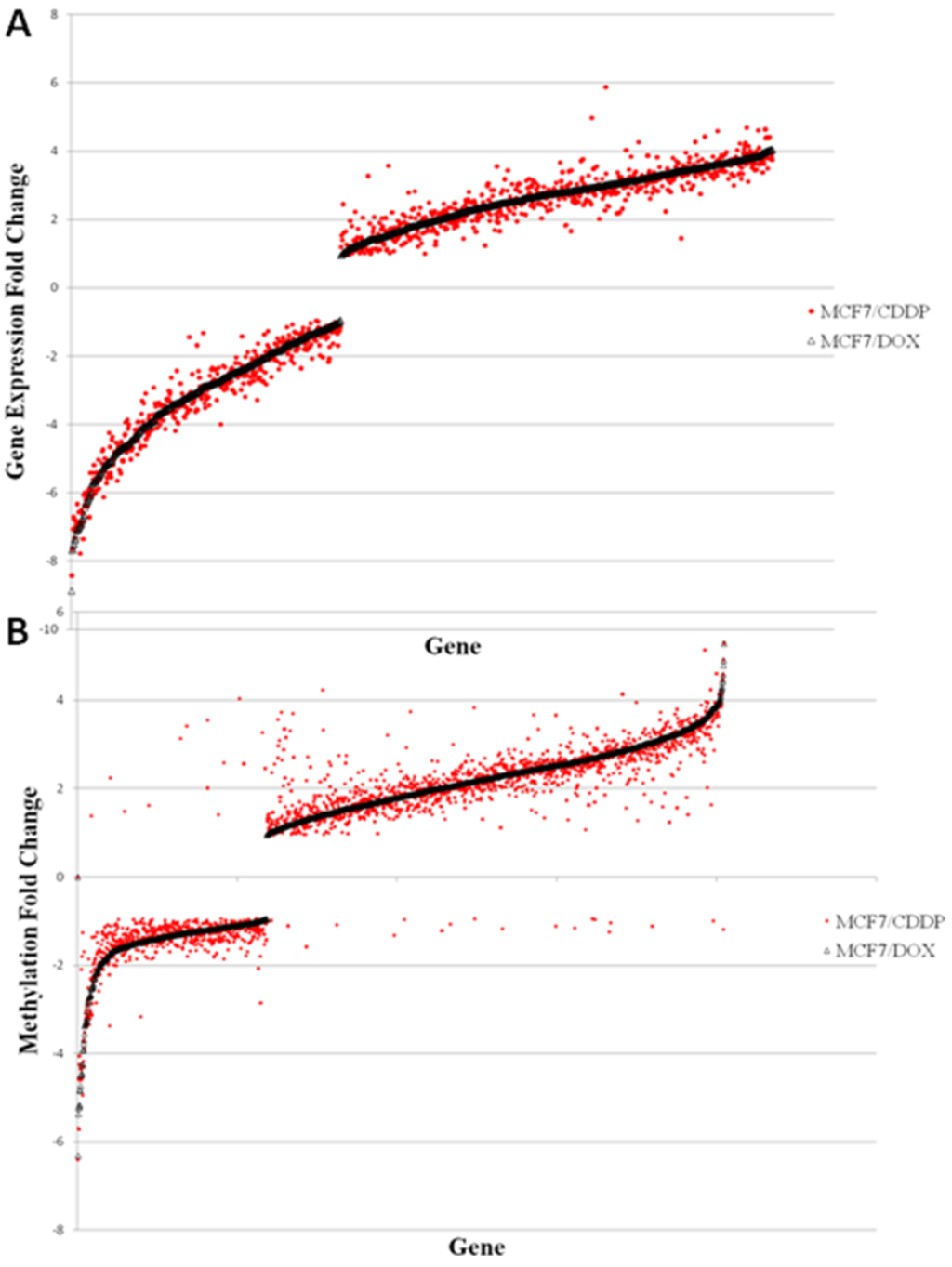


Figure 4.2. MCF7/CDDP and MCF7/DOX resistant lines demonstrate similar patterns of gene expression and differential methylation. A) 1219 genes displayed significant (fold change>2; $p<0.05$) in both the MCF7/CDDP and MCF7/DOX lines (785 up regulated and 424 down regulated). B) Genome wide locus specific methylation analysis revealed 2025 loci with methylation patterns significantly different from the MCF7 parental line (1427 increased methylation and 596 down methylated; fold change>2, $p<0.05$).

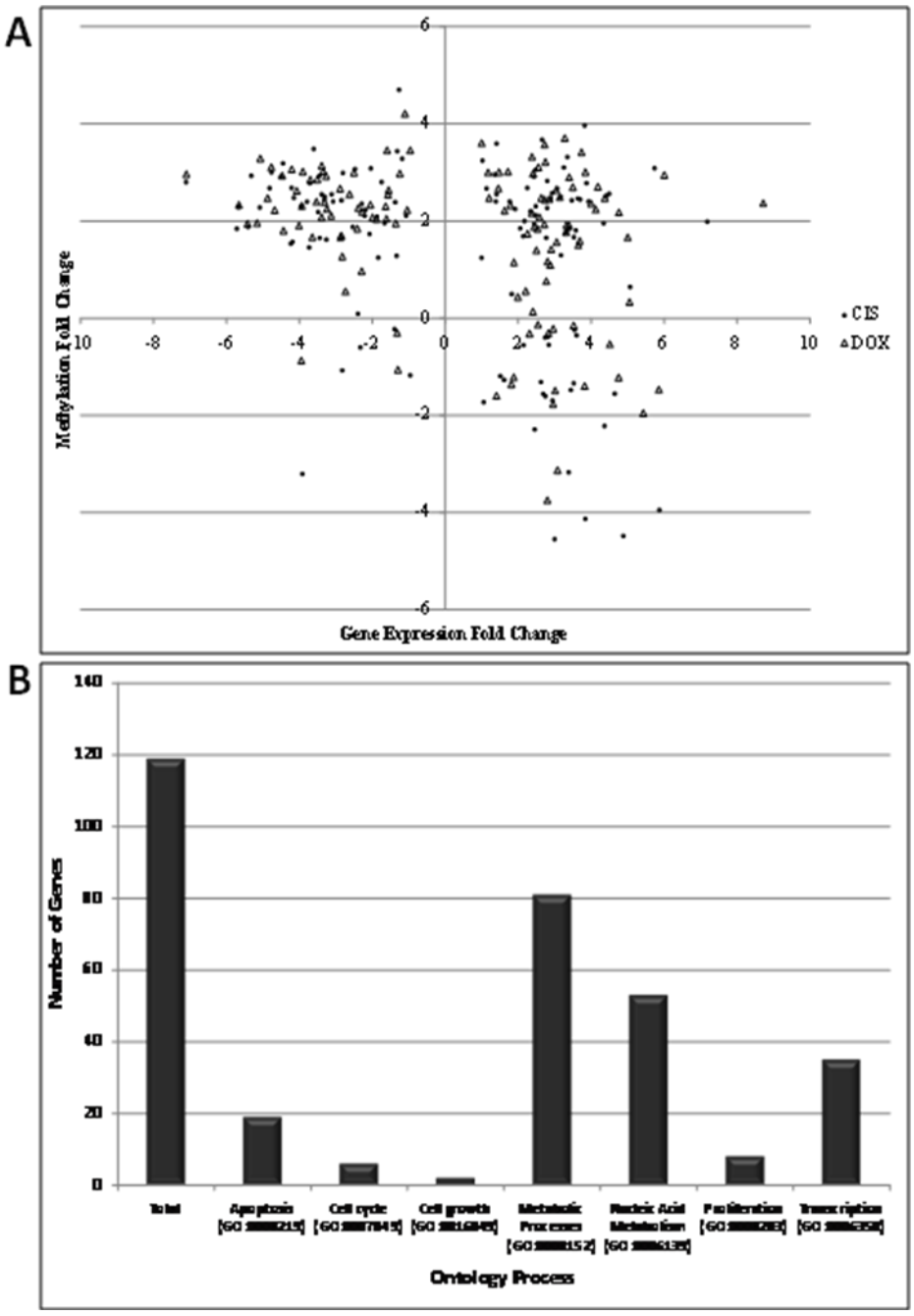


Figure 4.3. The majority of changes in gene expression that are opposite in direction of change in methylation status. A) 123 genes demonstrated significant change in both gene expression and methylation levels in both lines. Data points in quandrant #1 and #3 represent an expected relationship of inverse proportionality between the expression and methylation result ('classical model'). Points in quadrant #2 and 4 demonstrated directly proportional changes ('unclassical model'). B) The 123 genes group into several ontology processes.

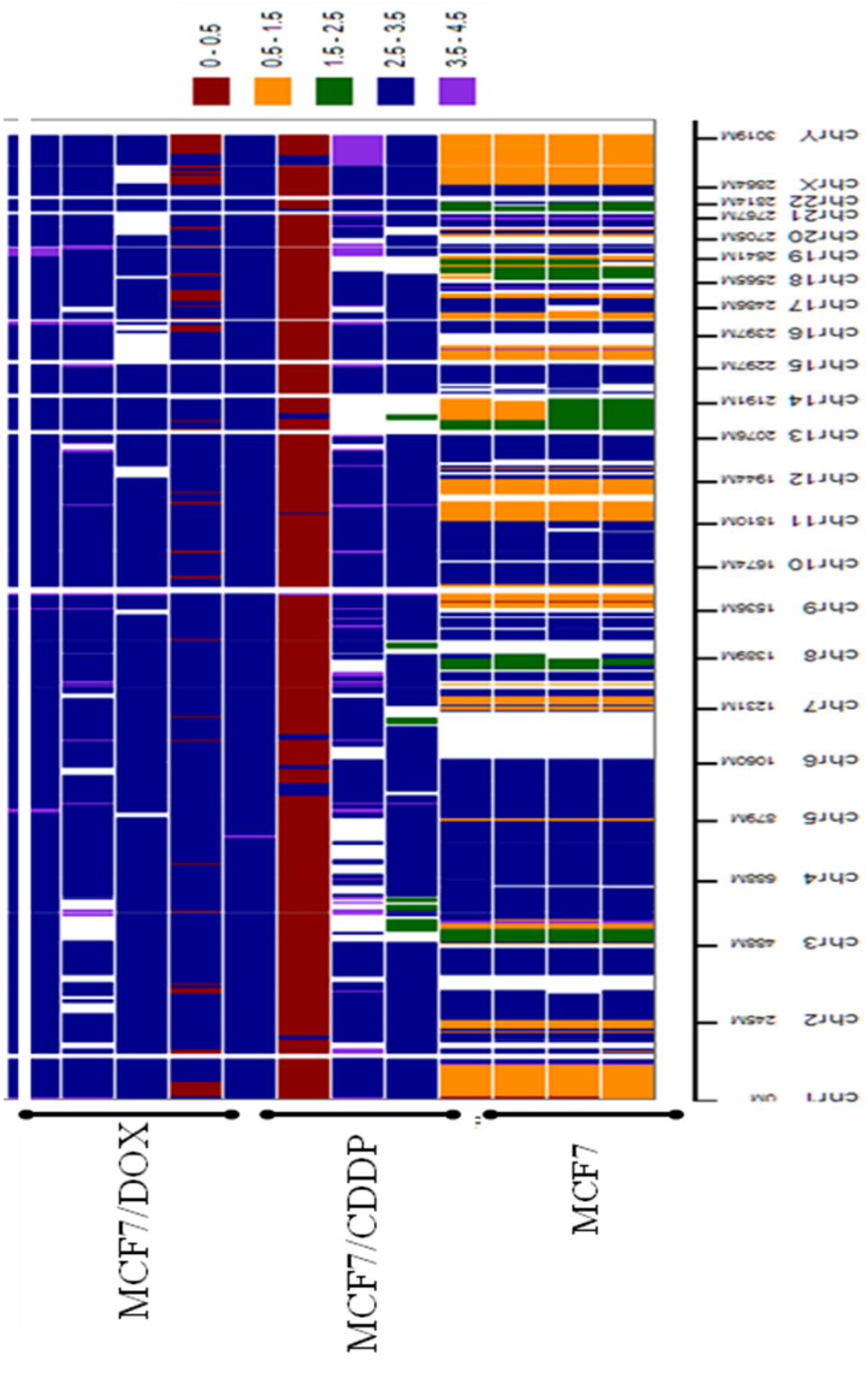


Figure 4.4. Resisatnt lines display many common CNV alterations, mostly gains. CNV in MCF7/CDDP and MCF7/DOX cell lines relative to the MCF7 parental line (continuous coordinates). Legend denots copy numbers of the loci.

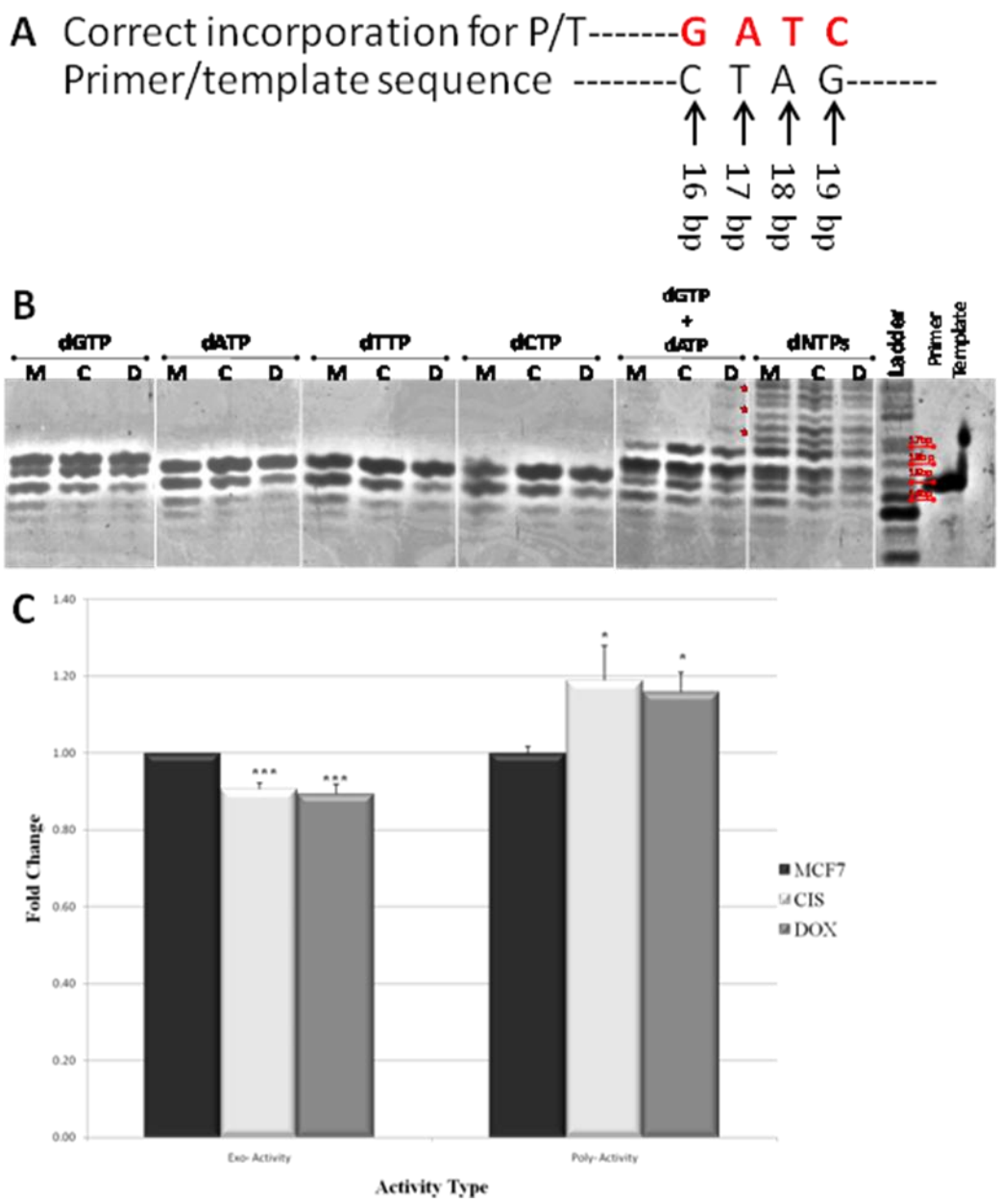


Figure 4.5. Drug resistant cell lines experience higher polymerase activity, lower fidelity and reduced exonuclease activity. A) The primer template construct used to measure endogenous polymerase activity. B) Endogenous polymaerase activity is observed by resolving reaction volume via 20% PAGE. All dNTPS represents the continuum of double starnded-template molecule produced in the presence of all dNTPS; ladder -template extension with Klenow fragment; primer/template – the fluorescently labelled 15 base pair (bp) substrate available for polymerase activity. Bands larger than 15 bp represent incorporations while bands beneath 15 bp are indicative of exonuclease activity. C) MCF7/CDDP and MCF7/DOX cells experience less exonuclease activity and higher levels of base incormporation (including mis-incorporated nucleotides).

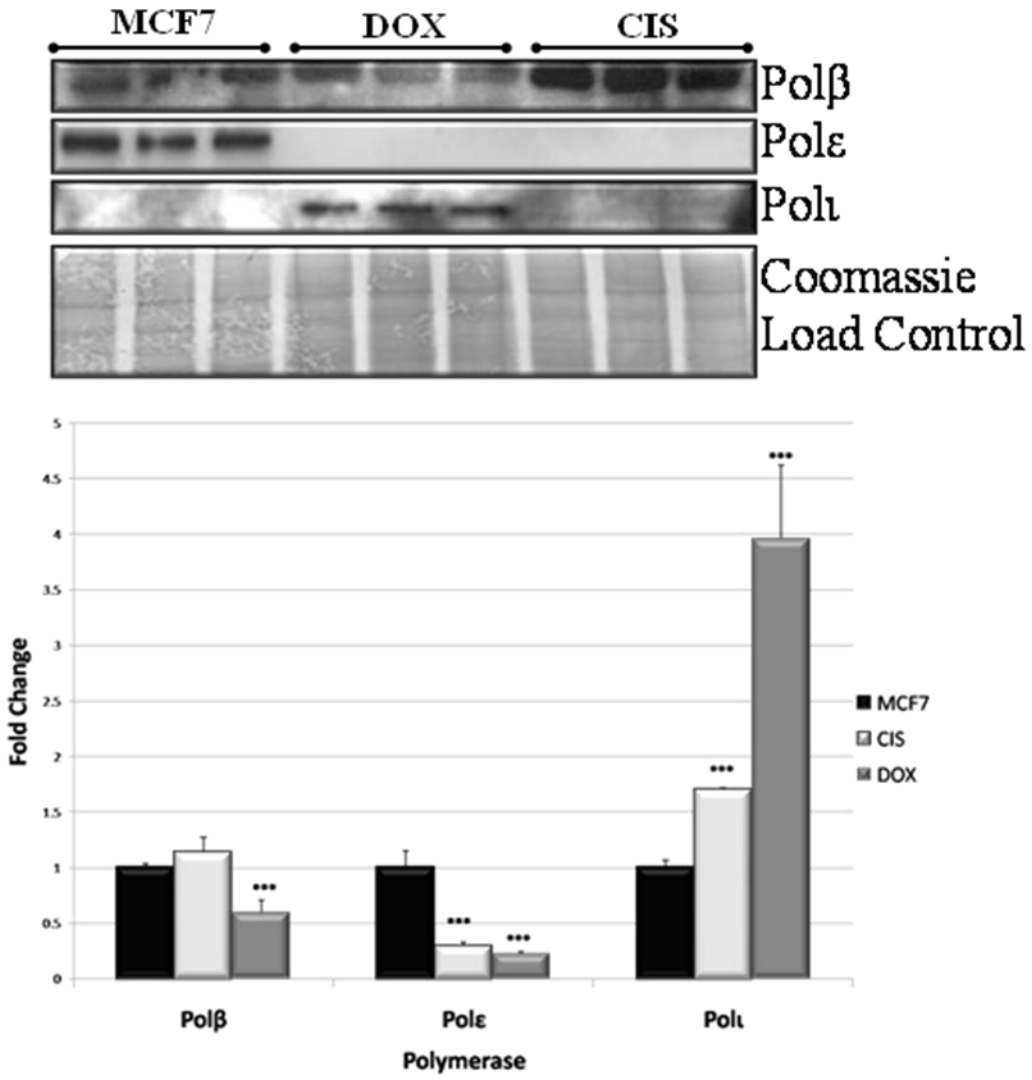


Figure 4.6. MCF7/CDDP and MCF7/resistant cell lines display distorted expression levels of DNA polymerases. Western blotting revealed a decrease in the DNA repair polymerase enzyme polymerase β in the MCF7/DOX line only while the translesion synthesis polymerase ι was significantly increased in both resistant lines. DNA polymeraseε, one of three polymerases involved in DNA replication, was severely depressed in both lines. (*** denotes $p<0.001$)

5. Conclusion and Future Directions

This work has attempted to evaluate what role epigenetic dysregulation plays in the drug resistant phenotype. The results of this work are several fold.

First, we have indicated that the microRNA profile of drug resistant cells is largely different from the parental line. Many of the dysregulated microRNAs have previously described roles in regulation proteins involved in cell cycle regulation, proliferation and apoptosis. We identified novel functions for three of the dysregulated microRNAs—hsa-miR-451, hsa-miR-7 and hsa-miR-365, in drug efflux. Drug efflux pump proteins MDR1 and MRP1 are greatly over expressed in the MCF7/CDDP and MCF7/DOX lines, respectively. We demonstrated that this over expression is at least in part to under expression of the above mentioned microRNAs. Through ectopic expression, we were able to partially recover sensitivity to the cisplatin or doxorubicin drugs—a result with therapeutic potential[70].

Second, we have illustrated a massive dysregulation in the methylation profile between the resistant and sensitive cell lines. In this, we describe a scenario of massive epigenetic dysregulation that >50% of the time impacts transcriptional outcome and, therefore, explains, at least in part, the phenotype of the MCF7/CDDP and MCF7/DOX resistant cell lines. The drug resistant phenotype typically displays multiple different characteristics. Explanation of this observation can prove difficult from a mutation-centric point of view[52]. The wide range of methylation and concomitant gene expression

on our results provides a good explanation of the underlying mechanism of drug resistance.

Third, the drug resistant phenotype coincides with a large level of genomic instability. Although the design of this experiment did not allow us to determine whether this is a cause or consequence of the resistant phenotype, it demonstrated that, similar to the epigenetic dysregulation, karyotypic alterations explain the wide phenotype[52].

Unfortunately, the work was designed around an end point that provided us with cells so drastically different from the parental line that it is arguable as to whether they still qualify as MCF7 cells. However, it did provide the striking demonstration that cells exposed to two different drugs with different modes of action can acquire drug resistance with an almost identical epigenetic and molecular profile. This alone suggests that the acquisition of drug resistance may involve predictable events or display particular characteristics that could act as biomarkers or prognostic tools[1,70].

In the future, it will be important to compare the results of this study to those seen in resistance to another class of drugs, for example taxanes or non-mutational, targeted therapies. Do the same epigenetic changes occur during acquisition of resistance to other types of drugs? It will also be important to further investigate the acquisition of resistance in the MCF7/CDDP and MCF7/DOX cells described here. Specifically, it would be interesting to perform the selection again and look at epigenetic profiles earlier on during the selection process as it is likely the majority of the characteristics observed here are

distal events. We would like to know what the most proximal events that we can observe are. How early are they observable? Recently, it was shown that methylation patterns are extremely plastic and that experiments performed with a single end point do not accurately demonstrate a level of persistence (Personal Communication, C. Klein). Thus, it is possible that our one-point-in-time measurement does not accurately convey the true frequency or magnitude in changes.

Finally, it is necessary to investigate the basic cytogenetics of the described lines. We were surprised at how different the lines behaved from their parental source, however, the experiment had been designed to gain molecular knowledge and, thus, the more historical experiments were not originally planned. Our molecular data, both the methylation and CNV analysis, suggest a plethora of massive chromosomal aberrations. This begs two questions: first, how do these cells survive with such a distorted genome, and; second, is it possible that the gross rearrangements of the chromosomes are not random but rather a specific, predictable combination that provides unparalleled survival capacity?

Just as only particular chromosomal trisomys allow for a viable embryo, it is possible that there is a narrow combination of karyotypes that would permit survival and even fewer that would allow for increased fitness at survival[52]. Thus, although the genomes of the resistant cell lines are severely distorted, perhaps they have achieved one of a few unique combinations of abberations that confer the added benefit of drug resistance[194]. This hypothesis allows one to explain the remarkable similarity in molecular changes (miR expression, DNA methylation patterns and gene expression) observed between the two

different lines created out of prolonged exposure to different drugs. It will be necessary to test this hypothesis by profiling drug resistant lines achieved through exposure to even more unrelated (non-genotoxic) drugs. If true, one would expect to see a similar level of congruency between drug resistant cells in general.

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