INVESTIGATION INTO MECHANISMS OF TREATMENT RESISTANCE AND HETEROGENEITY IN BREAST CANCER AND ACUTE PROMYELOCYTIC LEUKEMIA

by

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Although it has been 15 years since you were gone, this one is for you dad.

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ABSTRACT

Treatment failure remains a leading contributor to cancer related death despite considerable advances in detection and treatment approaches. Several factors contribute to treatment failure including the high degree of tumor heterogeneity across patient populations and within individual patient tumors. While this heterogeneity can present an obstacle to successful treatment, it can provide new possibilities for personalized approaches to target resistant cancers and achieve better outcomes. This can be achieved through predicting patient response to different drugs to determine which treatment would be most effective as well as determining resistance mechanisms so that treatment strategies can be tailored to avoid these mechanisms.

In this body of work, we utilized breast cancer and acute promyelocytic leukemia (APL) models to investigate resistance mechanisms associated with different therapies and the potential effect of using combination therapy to reduce resistance and enhance treatment outcome. Cancer stem cells (CSCs) are a source of tumor heterogeneity and facilitate tumor initiation and progression. To determine the role of different breast CSCs in the interplay with the immune system, we analyzed the differential expression of immune genes in the Aldefluor+ and CD44+CD24- breast CSCs and demonstrated that Aldefluor+ cells silence a key antigen presentation gene leading to their enhanced survival under immune pressure. Next, I applied a genome-wide shRNA screen to identify potential mediators of paclitaxel resistance in breast cancer, which is a common problem in the treatment of the cancer. This led to the identification of B cell lymphoma 6 (BCL6) as a contributor to paclitaxel resistance. I demonstrated enhanced tumor growth suppression in breast tumors treated with a BCL6 inhibitor and paclitaxel, suggesting a potential combination therapy that could be used overcome treatment resistance in breast cancer patients. Finally, I investigated the lasting benefits of combination treatment all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) treatment in APL cells. Only combination therapy was able to induce sustained demethylation and expression of target genes and in turn lasting changes in differentiation. Together, these studies highlight the importance of the studying heterogenous populations of tumors and mechanisms of resistance to inform strategies for combination treatments and improve patient outcomes.

List of Abbreviations and Symbols Used

µL	Microliter
µM	Micromolar
5mc	5-Methylcytosine
7-AAD	7-Aminoactinomycin D
AA	Antibiotic-Antimycotic
ABCB1	ATP Binding Cassette Subfamily B Member 1
ABCB5	ATP Binding Cassette Subfamily B Member 5
ABCG2	ATP-binding cassette super-family G member 2
ADA	adenosine deaminase
AKT	Protein kinase B
ALDE-	Aldefluor-
ALDE+	Aldefluor+
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
AML	Acute myeloid leukemia
APAF1	apoptotic peptidase activating factor 1
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
ARF1	ADP-ribosylation factor
ASB2	Ankyrin Repeat and SOCS Box Containing 2
ATCC	American Type Culture Collection
ATM	ATM Serine/Threonine Kinase
ATO	arsenic trioxide
ATP	Adenosine triphosphate
ATRA	all trans retinoic acid
B2M	Beta-2-Microglobulin
BAAA	BODIPY® – aminoacetaldehyde
BCL2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
BCL6i	BCL6 inhibitor

BRCA1/2	breast cancer type 1/2 susceptibility
C	Celsius
CCAC	Canadian Council on Animal Care
CCL2	C-C Motif Chemokine Ligand 2
CCND1	Cyclin D1
CCND1	Cyclin B1
CD	Cluster of differentiation
CD80/86	Cluster of differentiation (80/86)
CDK	cyclin-dependent kinase
CDK1	Cyclin Dependent Kinase 1
CDK4	Cyclin Dependent Kinase 4
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
ChIP	Chromatin Immunoprecipitation
CHKB	choline kinase beta
CLCN3	chloride voltage-gated channel 3
CLL	Chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CO2	Carbon dioxide
COPS4	COP9 signalosome subunit 4
CSCs	Cancer stem cells
CTBP2	C-terminal-binding protein 2
CXCL2	Chemokine ligand 2
CXCR4	C-X-C chemokine receptor type 4
DARS	aspartyl-tRNA synthetase
DCIS	ductal carcinoma in situ
DCs	dendritic cells
DEAB	diethylaminobenzaldehyde
Decitabine	5-aza-20-deoxycitidine
DIS3L	DIS3 like exosome 3'-5' exoribonuclease

DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNAJA4	DnaJ heat shock protein family (Hsp40) member A4
DNMT	DNA methyltransferase
DTT	Dithiothreitol
Dusp8	dual specificity phosphatase 8
EDTA	Ethylenediaminetetraacetic acid
EGAD	Enhanced Gene Analysis and Discovery
EGFR	epidermal growth factor receptor
EIF6	eukaryotic translation initiation factor 6
EMT	epithelial-mesenchymal transition
ER	Estrogen receptor
FBS	Fetal Bovine Serum
FCER1G	Fc fragment of IgE receptor Ig
FISH	fluorescent in situ hybridization
FL	Fluorescence
FOXS1	forkhead box S1
FSC	forward scatter
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GEO	Gene Expression Omnibus
HAS2	hyaluronan synthase 2
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HER2	human epidermal growth factor receptor 2
HIST1H2BA	histone cluster 1 H2B family member a
HIST1H2BK	Histone H2B type 1-K
HLA	Human leukocyte antigen
HMT	Histone methyltransferase

HRPT1	Hypoxanthine-guanine phosphoribosyl transferase
IER3	Immediate Early Response 3
IPO9	importin 9
KDM3B	Lysine Demethylase 3B
kg	Kilogram
LINE-1	Long interspersed element-1
m2	Squared meter
MDR-1	Multi drug resistance 1
MDS	Myelodysplastic syndromes
MDSCs	myeloid-derived suppressor cells
mg	Milligram
MHCI/II	major histocompatibility complex I/II
MICA/B	MHC Class I Polypeptide-Related Sequence A/B
ml	Milliliter
mM	Millimolar
MPO	Myeloperoxidase
MRTF-A	myocardin related transcription factor-A
MT1X	metallothionein 1X
MYL9	myosin regulatory light chain 9
N/A	Not applicable
NCL	Nucleolin
NCOR	nuclear co-repressor
NDUFB10	NADH: Ubiquinone Oxidoreductase Subunit B10
NK	Natural killer
nM	Nanomolar
NOD/SCID	non-obese diabetic severe combined immunodeficiency
nt	Nucleotide
NT	No treatment
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase
Pac	Paclitaxel
PAEP	progestagen-associated endometrial protein

PBS	Phosphate-buffered saline
PCAF	p300/CREB-binding protein-associated factor
PCR	Polymerase chain reaction
pCR	Pathological complete response
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDX	patient-derived xenografts
PI3K	phosphoinositide 3-kinase
PISD	phosphatidylserine decarboxylase
PLZF	promyelocytic leukemia zinc finger
PML	promyelocytic leukemia
PR	Progesterone receptor
PRC2	polycomp repressive complex 2
PRKCQ	protein kinase C theta
PRTN3	Proteinase 3
PUM1	Pumilio RNA Binding Family Member 1
QPCR	quantitative polymerase chain reaction
RAB33A	RAB33A, Member RAS Oncogene Family
RAB4B	RAB4B, member RAS oncogene family
RANBP17	RAN binding protein 17
RAREs	retinoic acid response elements
RARα	retinoic acid receptor α
RARβ	retinoic acid receptor beta
RHBDF2	rhomboid 5 homolog 2
RNAi	RNA interference
RNF144A	ring finger protein 144A
RPL7a	Ribosomal Protein L7a
RXR	retinoid X receptor
s	Second
shRNA	Short hairpin RNA
SLC6A7	solute carrier family 6 member 7

SMYD3	SET And MYND Domain Containing 3
SNPH	syntaphilin
SOCS1	suppressor of cytokine signaling 1
SSC	Side scatter
STAT3	signal transducer and activator of transcription 3
STAT5B	signal transducer and activator of transcription 5b
TAM	Tumor associated macrophage
TAP1/2	Transporter associated with antigen processing 1/2
TAPBP	TAP binding protein
TBP	TATA-binding protein
TGFβ1	transforming growth factor beta 1
TGM2	Transglutaminase 2
TICs	Tumor initiating cells
TNBC	Triple negative breast cancer
TNM	Tumor, Node, Metastasis
TOR1A	torsin family 1 member A
Tregs	T regulatory cells
U/ml	Unit per ml
UGP2	UDP-glucose pyrophosphorylase 2
XRN1	5'-3' exoribonuclease 1

* Names and abbreviations immune genes upregulated/downregulated in breast CSCs will appear in Appendix 1

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CHAPTER 1: INTRODUCTION

Copyright statement

Portions of this chapter have been previously published in the following manuscript. The corresponding text has been edited for length, consistency, and to include recent findings.

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1.1 Cancer

Cancer is a term used to describe a group of related diseases characterized by abnormal cellular division that can be associated with the ability to invade and spread to the surrounding tissue. Cancer can arise from most tissues in the body, resulting in an array of diseases with different characteristics and treatment approaches. Cancer was first described around 3000 B.C. in ancient Egypt in a book about trauma surgery known as Edwin Smith Papyrus. The term cancer was coined by the ancient Greek physician Hippocrates to describe tumors with crab-like projections and it is still used in our current days to describe tumors. Presently, one in two Canadians are expected to develop cancer during their lifetimes with a slightly higher probability in males. Despite medical advances, one in four Canadians are expected to die from cancer and mortality rates correlate positively with age of diagnoses. This can be observed in the increased numbers of cancer related death despite the decrease in the overall mortality rates due to the aging population (Smith et al. 2019). This highlights the continuous need to improve cancer treatment through investigating potential novel treatments and overcoming resistance to current therapies.

1.2 Cancer Development

As previously mentioned, the term cancer includes an array of diseases; thus, it is understandable that individual types cancer have different characteristics, depending on their origin and driving events. However, despite the wide spectrum of cancer characteristics, certain hallmarks have been proposed to define tumors and in turn shed light on the process of tumorigenesis (Hanahan and Weinberg 2000, 2011). The first set of cancer hallmarks were described in 2000 as six functional abilities

present in all cancers despite being acquired through different mechanisms and mutations. Cell growth in normal cells is controlled through several mechanisms including the availability of growth and anti-growth signals; however, cancer cells develop abilities to maintain continuous growth while being insensitive to mechanisms that would halt growth and division (Hanahan and Weinberg 2000). Many mechanisms have been proposed to explain these abilities, which led to identification of driver mutations in key oncogenes such as the RAS oncogene family (Pylayeva-Gupta, Grabocka, and Bar-Sagi 2011) and tumor suppressor genes, including TP53 (Fromentel and Soussi 1992). Cancer cells demonstrate sustained replicative abilities, due to loss of regular control of cell cycle progression observed in normal cells (Diaz-Moralli et al. 2013). In addition to abnormal growth and replication control, certain cancer cells within a tumor display the capacity for metastasis; to break away from the primary tumor and subsequently invade distant or adjacent organs to form secondary tumors through several complex mechanisms (Pantel and Brakenhoff 2004). Additionally, cancer cells acquire the ability to inhibit apoptosis through down regulation of key signalling pathways including caspases mediated apoptosis (Lowe and Lin 2000). Finally, tumorigenesis is also characterized by ability to maintain and promote sustained angiogenesis to provide oxygen and nutrients to tumor cells (Carmeliet and Jain 2000).

A decade later, four more hallmarks were added to address the emerging and novel discoveries made by numerous researchers to identify common and essential features seen across the spectrum of cancer diseases (Hanahan and Weinberg 2011). These hallmarks include genomic instability and mutations, tumor promoting inflammation and the ability to evade immune detection and destruction. These three

features were directly associated with the research questions I asked in my later chapters and will be discussed further in following sections. The fourth and final emerging hallmark of cancer is the ability to downregulate cellular energetics. Cancer cells restructure their metabolism to support their continues growth and proliferation. Unlike normal cells, cancer cells utilize anerobic glycolysis even in the presence of functional mitochondria and oxygen. This effect is known as the Warburg effect and leads to decreased adenosine triphosphate (ATP) production in cancer cells and in turn deregulated cellular energetics (Liberti and Locasale 2016).

1.3 Tumor Heterogeneity

Despite medical advances, enhancing treatment efficacy remains a major goal for cancer research due the high degree of heterogeneity between different patient tumors (intertumoral heterogeneity) and within an individual tumor (intratumoral heterogeneity) (Fig. 1.1). While each type of heterogeneity can present a different challenge in designing treatment protocols, they also provide an opportunity to utilize individual treatment plans that are tailored to each patient or group to achieve the best possible outcome.

The personalized medicine approach utilizes molecular diagnostic tools to determine treatment options based on the specific characteristics of a patient or their tumor, rather than the traditional generalized treatment for the disease. Intertumoral heterogeneity results in differential response to treatment depending on the overall tumor resistance capacities, which varies from tumor to tumor. Thus, it is imperative that different patients of certain type of cancer be divided based on their tumoral

characteristics and treated accordingly with treatment tailored to successfully target their tumors (Fig. 1.1A)

Intratumoral heterogeneity describe the diversity of cancer cells and other components within an individual tumor (Fig.1.1B). It arises from genetic and epigenetic alterations in tumor cells and can be shaped by the tumor microenvironment as well as the selective pressures of the immune system or different therapies (Fig.1.1B). The heterogeneity is observed between tumor cells as well as the surrounding microenvironment to ensure tumor growth and survival.

1.4 Genomic Alterations Drive Intratumoral Heterogeneity

During our lifetime, cells acquire somatic mutations (Martincorena and Campbell 2015). Most of these mutations have no notable outcome and do not affect cellular function and thus are called passenger mutations (Stratton, Campbell, and Futreal 2009). However, some mutations can affect key cellular functions and promote tumorigenesis (Greenblatt et al. 1994). These mutations are called driver mutations and have been identified in different types of cancer including lung (Kris et al. 2011), breast (Nik-Zainal et al. 2012), liver (Fujimoto et al. 2012), colorectal (Starr et al. 2009), head and neck (Forastiere et al. 2001), melanoma (Hodis et al. 2012) and chronic lymphocytic leukemia (Landau et al. 2013). Identification of these different mutations sparked interest in studying tumor evolution dynamics and led to the construction of several models that explain the relationship between different mutations in an evolutionary manner. Among these models, branched evolution has been well supported and heavily studied. In the branched evolution model, different clones share a common ancestor but due to different intrinsic and extrinsic factors,

multiple new mutations emerge and the tumor evolves to achieve the best fitness (Davis, Gao, and Navin 2017). Mutations shared amongst all the cells in a tumor are called clonal mutations, indicating an early point in tumor evolution. In contrast, mutations present in only some cells are called sub-clonal mutations and are believed to be later events in tumor evolution (Mazor et al. 2016). Driver mutations are mostly considered to be clonal; however, there is accumulating evidence of sub-clonal driver mutations during tumor evolution, especially post treatment (Brastianos et al. 2015; McGranahan et al. 2016; Morrissy et al. 2016). The accumulation of these mutations in different cells within a tumor leads to intratumoral heterogeneity. Thus, to a large extent genomic instability begets intratumoral heterogeneity (McGranahan and Swanton 2015; Villamón et al. 2013). Genomic instability can occur at the single nucleotide level, at the copy number level or at the chromosomal level (McGranahan and Swanton 2015) and is a key contributor to intratumoral heterogeneity. Together, genomic alterations constitute the intrinsic driving forces of intratumoral heterogeneity.

1.5 Epigenomic Alterations and Intratumoral Heterogeneity

Epigenomic modifications can also promote intratumoral heterogeneity through DNA methylation (Quek et al. 2017) and histone modifications (Torres et al. 2016). The importance of epigenetic modification in tumor progression and treatment response have been reported in different solid and blood malignancies and will be further discussed in following chapters (Duenas-Gonzalez et al. 2008; Galm, Herman, and Baylin 2006).

1.5.1 DNA Methylation

DNA methylation is an important mechanism utilized by eukaryotic cells in development, cell replication and other cellular processes (Handy, Castro, and Loscalzo 2011). During cell replication, newly synthesized strands lack methylation initially allowing checkpoints and DNA repair mechanisms to maintain DNA integrity (Hare and Taylor 1985). Methylation patterns also affect gene expression. Hypermethylation in the promoter region of a gene hinder normal activity of the replication machinery and results decreased gene expression. Methylation occurs when a DNA methyltransferase (DNMT) adds a methyl group to the 5' position of a cytosine ring in a CpG dinucleotide (Jaenisch and Bird 2003; Moore, Le, and Fan 2013). In cancer, hypermethylation has been shown to downregulate the expression of key tumor suppressor genes (Das and Singal 2004). In contrast, global hypomethylation (mainly observed in the gene body) has been also been linked to tumorigenesis through promoting genomic instability and upregulating expression of genes associated with invasion and metastasis (Ehrlich 2009) . Thus, it is very important to consider the interplay between DNA methylation and different treatments in order to understand and overcome treatment resistance.

Several studies have demonstrated the role of methylation in promoting intratumoral heterogeneity in different types of cancer. High level of heterogeneity was observed in the methylation status of 48 spatial tumor regions isolated from 11 lung adenocarcinomas and their matched normal tissue (Quek et al. 2017). Similar finding were observed in esophageal squamous cell carcinoma (ESCC) when methylation analysis was preformed on different regions from three tumors and their

matched healthy tissue (Hao et al. 2016). In both studies, high correlation between genomic and epigenomic landscape heterogeneity was observed, despite the difference of the percentage of clonal events in each, which suggests that methylation alterations are later events in tumoral evolution.

These studies highlight the important role of methylation in providing tumor cells with enhanced fitness under different conditions during tumor evolution resulting in intratumoral heterogeneity. They also provide an example of both genomic and epigenomic alterations working together to shape tumor evolution.

1.5.2 Histone Modifications

In addition to DNA methylation, histone modifications serve as post-translational epigenetic modification that can alter gene expression in the DNA sequences interacting with the histones (Cedar and Bergman 2009; Esteller 2007). Chromosomal DNA is tightly packaged in repeated complexes called nucleosomes. Within each nucleosome negatively charged DNA get wrapped around a complex of 8 positively charged protein called histones. Each nucleosome has tow copies of histones 2A, 2B, 3 and 4 forming a histone octamer that get wrapped with 146 base pairs of DNA. Additionally, histone 1 bind an additional 20 base pairs. Each chromosome contains 1000s of nucleosomes linked together by short sequences of DNA (about 20 base pair) (Lowary and Widom 1997). Some modifications increase histones interaction with DNA, thus, decreasing the ability of the transcription machinery to access the DNA and in turn resulting in downregulation of gene expression. Enzymes facilitating post translation modifications can be classified as

eraser, writers and readers. Writers add modifications, erasers remove them and readers recognize them (Gillette and Hill 2015). Writers include histone methyltransferases (HMTs) and histone acetyltransferases (HATs). HMTs facilitate the addition of (1-3) methyl groups to lysine and arginine resides on a histone. Depending on the site of methylation, HMTs can lead to transcription repression or activation as chromatin becomes more packed or relaxed respectively (Simon and Lange 2008). HATs on the other hand add acetyl groups to lysine residues to promote gene expression.

In contrast, histone deacetylases (HDACs) can remove an acetyl group from lysine residues and are considered an eraser modification (Bolden, Peart, and Johnstone 2006). Finally, readers recognize, interpret and mediate the effect of post translational modifications (Biswas and Rao 2018). Thus it is not surprising that different readers are associated with distinct writers and erasers at specific sites and are investigated in various cancers (Hyun et al. 2017).

Understanding the role of epigenetic alterations in tumor progression and treatment response is essential for successful therapies. DNA demethylating agents and HDAC and HMT inhibitors have been and continue to be investigated as potential therapies that can be used to enhance treatment efficacy in both solid and hematological malignancies (Gallipoli and Huntly 2018; Liu, Gao, and Li 2019).

1.6 Intratumoral Heterogeneity on the Cellular Level

Intratumoral heterogeneity expands beyond tumor cells to include different cell populations that constitute the tumor microenvironment. Together, the extracellular matrix, fibroblasts, endothelial cells, and immune cells constitute the rest

of the tumor composition and contribute to tumor growth, behavior and treatment resistance. The role of cancer associated fibroblasts in tumor growth and treatment response have been long recognized and studied to account for its possible impact of disease progression and patient survival (Östman and Augsten 2009). Heterogeneity can also be observed among tumor associated fibroblasts as well, with different subsets with distinct function present in the diverse tumor types (Orimo and Weinberg 2007). Fibroblasts, stromal cells and other components of the extracellular matrix can also mediate the interaction between cancer cells and other cells in tumor microenvironment to mediate tumor growth (Okuda et al. 2012).

Intratumoral heterogeneity is also affected by other components of the tumor microenvironment including the type of infiltrating immune cells and the abundance of these cells (Fig. 1.1). This relationship between intratumoral heterogeneity and immune cells has had increased attention due to the increased interest in immunotherapy to target cancer cells. Among the immune fraction of tumor infiltrating cells, different innate and adaptive immune cells have varied roles in either tumor targeting and/or tumor promotion. These cells include: tumor associated macrophages (TAMs), neutrophils. dendritic cells, natural killer (NK) cells, B cells and T cells. Many immunotherapy approaches depend on activation of cytotoxic T cells that can recognize and destroy cancer cells. Naturally, this has led to an interest in teasing apart the relationship between intratumoral heterogeneity and response to immune targeting, given that different tumor cells posses different abilities to evade immune recognition and destruction.

1.7 Cancer Stem Cells

Intratumoral heterogeneity can also be observed on the tumor cellular level through the existence of different population of tumor cells with distinct tumorigenic and metastatic capacities (Marjanovic, Weinberg, and Chaffer 2013). Cancer stem cells (CSCs) [also commonly referred to as tumor-initiating cells (TICs), cancer-initiating cells or stem-like cancer cells] are a subpopulation of tumor cells that are believed to initiate cancer, mediate metastasis and contribute to therapeutic resistance and recurrence (Al-Hajj et al. 2003; Dalerba, Cho, and Clarke 2007; Ginestier et al. 2007). Their percentages differ between individual tumors from the same type of cancer, with higher levels of CSCs associating with more aggressive and resistant tumors (Cho and Clarke 2008; Marotta and Polyak 2009).

The CSCs population was first defined in the 1990s by Dr John Dick's laboratory, which isolated a population of acute myeloid leukemia (AML) cells with distinct cell surface marker expression (i.e. CD34+CD38−) that had significantly enhanced leukemogenesis properties in immunocompromised mice (Lapidot et al. 1994). Shortly after, the same group was able to identify CSCs, or leukemia-initiating cells, in three other forms of leukemia (Bonnet and Dick 1997). Investigation for the presence of cell populations with tumor-initiating and self-renewal properties led to the discovery of the first CSC population in solid tumors by Dr Michael Clark's group in breast cancer (Al-Hajj et al. 2003). They illustrated that breast cancer cells with CD44+CD24− surface marker expression had increased tumorigenicity and yielded tumors with heterogeneous cell surface marker expression. Subsequently, CSCs were isolated and described in other forms of solid tumors. Many of these CSC populations were identified by CD133+, such as in glioblastoma (Singh et al. 2004), liver (Ma et

al. 2007), colon (O'Brien et al. 2007) and pancreatic cancer (Hermann et al. 2007), or by CD44+CD24−, such as in pancreatic cancer (Li et al. 2007) and prostate cancer (C. Liu et al. 2011). Additional CSC cell surface markers have since been discovered, such as CD90 for glioma, liver and breast cancer; CD166 for colon and prostate cancer; CD117 for lung and ovarian cancer; CD166 for colon and prostate cancer; and CD20 and CD271 for melanoma (Bruttel and Wischhusen 2014; Medema 2013).

In addition to identification of CSCs by cell surface marker expression, the CSCs of many cancers can be identified by vital dye exclusion due to the increased expression of efflux pumps (Komuro et al. 2007). In fact, transporter ATP-binding cassette super-family G member 2 (ABCG2) identifies pancreatic CSCs, and transporters ATP Binding Cassette Subfamily B Member 5 and 1 (ABCB5 and ABCB1) are used to identify melanoma CSCs (Wouters et al. 2013). Additionally, increased aldehyde dehydrogenase (ALDH) activity as measured by the Aldefluor assay is used to identify the CSCs of a number of cancers including breast, colon, lung, melanoma, pancreatic and prostate cancer (Awad et al. 2010; Clay et al. 2010; Ginestier et al. 2007; Silva et al. 2011). It is important to note that the identification of cells that express these markers do not necessarily define them as CSCs, but rather identifies a population of tumor cells that are enriched for the cells with the CSC phenotype. The identification of tumor cells based on a combination of these markers (e.g. ALDH+CD44+CD24−) identifies tumor cells of greater tumorigenicity than those identified based on single markers (Ginestier et al. 2007). This suggests that the most robust CSC studies should use multiple markers and assays to confirm identification of the population.

1.8 A Role for Cancer Stem Cells in Treatment Resistance

In addition to increased tumorigenicity, further investigations on CSCs have revealed that these cells have increased resistance to conventional treatments, which could contribute to incomplete response to chemotherapy and radiation, and therefore cause relapse and decreased patient survival. Several mechanisms have been described that cause decreased sensitivity to these conventional treatments including increased expression of detoxification enzymes such as ALDHs (Sládek et al. 2002), quiescence (Liu et al. 2009), hyperactivation of the DNA damage response (Bao et al. 2006) and increased drug efflux pump activity (Loebinger et al. 2008).

1.9 Cancer Stem Cells and the Interplay with the Immune System

The role of the immune system in promoting and maintaining tumorigenesis has been largely attributed to innate immune cells [i.e. macrophages, myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs) and T regulatory (Treg) cells] (Coussens and Werb 2002). This phenomenon promoted interest in investigating the role of innate immune cells in regulating CSCs maintenance and tumorigenesis. Interestingly, several studies found TAMs to contribute to CSC maintenance, tumorigenesis and drug resistance by several mechanisms. These mechanisms include activation of signal transducer and activator of transcription 3 (STAT3) and the hedgehog signaling pathways in CSCs, which promote their drug resistance (M. Jinushi et al. 2011), production of transforming growth factor beta 1 (TGFβ1), to drive epithelial-mesenchymal transition (EMT) towards the CSC phenotype (Fan et al. 2014) and interacting directly with CSCs to activate NF-κB and Src signaling pathways to maintains the CSC state (Lu et al. 2014). Breast CSCs also interact with

TAMS via upregulation of hyaluronan synthase 2 (HAS2) and the production of pericellular hyaluronan. This interaction activates the stromal cells (e.g. fibroblasts) in the tumor microenvironment and enhances CSC self-renewal ability (Okuda et al. 2012).

CSCs also interact with other types of innate immune cells. MDSCs promote ovarian cancer cell stemness by inducing mir-101 expression in cancer cells to inhibits C-terminal-binding protein 2 (CTBP2) and increases expression of stemness genes and CSC-associated properties (Cui et al. 2013). Tumor microenvironment can also promote the interaction between CSCs and immune cells. The pancreatic cancer tumor microenvironment can induce monocyte transformation to MDSCs by activating STAT3 signaling, which increases EMT of the tumor cells and the frequency of ALDH+ pancreatic tumor cells (Panni et al. 2014). Other immune cells have been shown to interact with CSCs in different models as well. Follicular DCs interact with follicular lymphoma CSCs via the Chemokine ligand 2/C-X-C chemokine receptor type 4 (CXCL2/CXCR4) signaling axis to promote tumorigenesis (Lee et al. 2012). Regulatory T cells (Tregs) promote the expansion of colorectal CSCs by producing IL17 (Yang et al. 2011).

Additionally, CSCs promote the expansion of protumorigenic immune phenotypes. Several examples have been highlighted in different tumor models. Glioblastoma and melanoma CSCs can promote macrophage polarization toward the M2 immunosuppressive phenotype, away from the M1 proinflammatory and anti-tumorigenic phenotype (Jinushi 2014; Theocharides et al. 2012). Additionally, glioblastoma, head and neck squamous cell carcinoma and melanoma CSCs inhibit

cytotoxic T-cell proliferation and recruit Tregs to the tumor microenvironment (Chikamatsu, K, Takahashi G, Sakakura K, Ferrone S 2011).

While all previously mentioned interactions highlight CSCs ability to promote more pro-tumorigenic environment, CSCs can also employ several mechanisms to evade immune detection and destruction. Activated T cells can recognize and destroy abnormal cells. However, several signals are required to activate T cells including functional major histocompatibility complex I/II (MHC-I/II) to present the antigens to T cells, functional Transporter Associated With Antigen Processing 1/2 (TAP1/2)to process and load the antigen on MHC and costimulatory signals via CD80/86 and activating cytokines. CSCs can interfere with antigen presentation and costimulatory signals to prevent cytotoxic T cells from targeting them (Brahmer et al. 2012; Lee and Sunwoo 2014; Volonte et al. 2014). Examples of such interference have been reported in several models. Head and neck squamous cell carcinoma CSCs can downregulate MHC-I/II molecules to prevent antigen presentation to T cells and therefore limit their activation (Chikamatsu, K, Takahashi G, Sakakura K, Ferrone S 2011). CSCs from various cancers downregulate TAP1/2 resulting in a lack of antigen presentation to T cells, limiting their activation (Schatton et al. 2010; Volonte et al. 2014). Additionally, CSCs have been shown to downregulate costimulatory signalling such as CD80 and upregulate inhibitory Programmed death-ligand 1 (PDL1) to induce T-cell anergy (Lee and Sunwoo 2014; Di Tomaso et al. 2010).

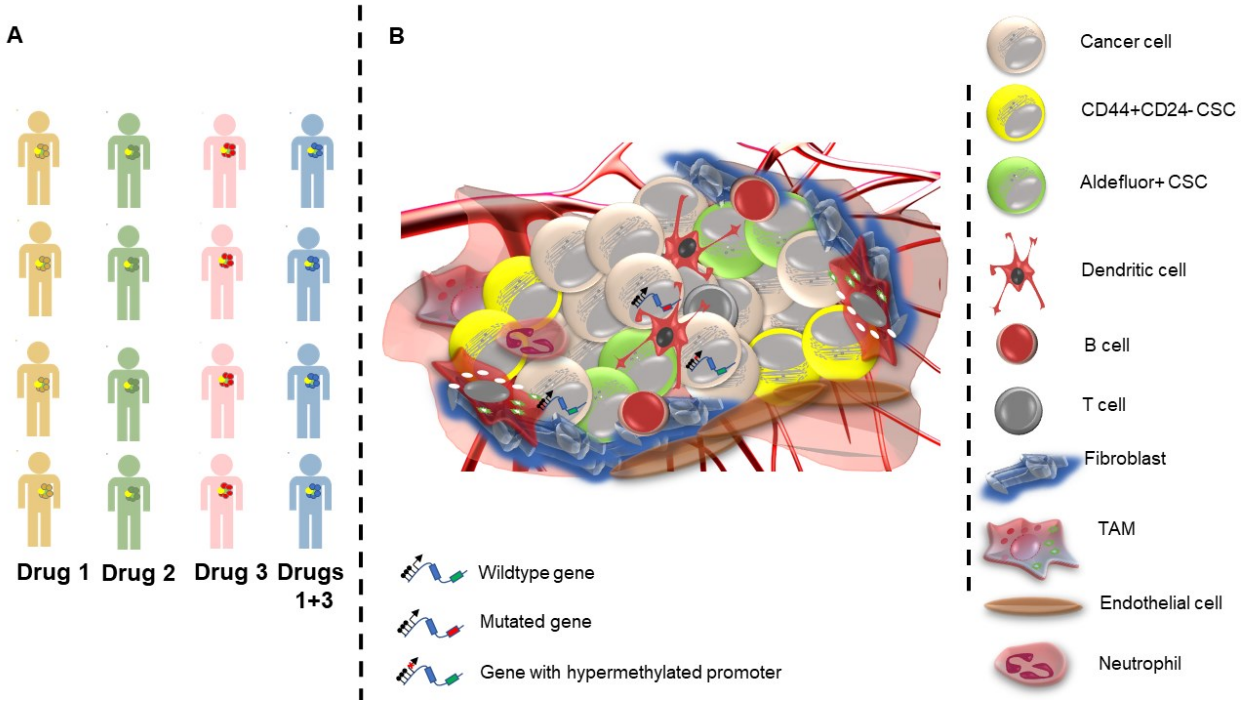


Figure 1. 1 Intertumoral and intratumoral heterogeneity. (A) Intertumoral heterogeneity requires different patients to be treated based on their tumor characteristics. While some patients benefit from individual treatments (drugs 1,2,3), other require combination therapy (drugs 1+3) to overcome resistant and achieve better response(B) Different tumor cells and tumor infiltrating cells contribute to intratumoral heterogeneity (example breast cancer).

1.10 CSCs and Epigenetic Changes

Given the importance of epigenetic regulations during development and cellular differentiation, they are hypothesized to play important role in CSCs differentiation and maintenance. This hypothesis was tested in several models, including breast and liver cancers where CSCs had lower levels of promoter regions DNA methylation and repressive modification in comparison to non-CSCs (Vincent and Van Seuningen 2012; Yasuda et al. 2010). Similar, findings were also observed in AML (Chavez-Gonzalez et al. 2017) and head and neck cancer (Zuo et al. 2009), suggesting an important role for epigenetic modification in altering gene expression once CSC differentiate and give rise to bulk tumor cells.

The role of epigenetic modifications in regulating CSC differentiation has been demonstrated for many markers used to identify these cells (Vincent and Van Seuningen 2012). Furthermore, several CSC markers have been demonstrated to have enzymatic activities that facilitate such modification (Vincent and Van Seuningen 2012). Thus, it is imperative to both understand and examine epigenetic mechanisms utilized by CSCs when investigating these cells resistance to therapy and potential tools to overcome it.

1.11 Breast Cancer

Breast cancer is the most common malignancy diagnosed in Canadian women accounting for 25% of the all newly diagnosed cases in 2019 (Smith et al. 2019). One in eight Canadian women are expected to be diagnosed with breast cancer during their lifetime with 40% of the cases between the age 30 and 59. Mortality rates associated with breast cancer have declined since the 1980s due to advancement in treatment and

early diagnosis. However, one in 33 women are expected to still die from breast cancer in Canada (Smith et al. 2019), which highlights the importance of developing new treatments and overcoming resistance to enhance patient survival.

Mammary malignancies encompass an array of types that are treated differently under the umbrella of breast cancer. Breast tumors are most often classified to be either ductal or lobular histologically depending on their originating site in the breast (Li et al. 2003). The majority of mammary malignancies arising from epithelial origins and thus are classified as adenocarcinomas. Among the ductal breast cancer subtypes, is ductal carcinoma in situ (DCIS), which is not considered invasive due to the containment of proliferation of the epithelial cells by the basement membrane. However, when DCIS is untreated, some patients will develop an invasive ductal carcinoma (Burstein et al. 2004).

Breast cancer is also classified based on the tumor grade and stage as well as using molecular diagnostic tools. Tumor grade is used to describe tumor cell differentiation in comparison to normal tissue. In breast cancer, tumors are classified from grade 1-3. Lower grade tumors have well differentiated cells and tend to have slower progression and better outcomes. In contrast, in high grade tumors cells are less differentiated or poorly differentiated. Higher grade tumors are typically more invasive with less favorable outcomes. Meanwhile, staging describes the size and the spread of the tumor. In breast cancer the most commonly used staging system is the Tumor, Node, Metastasis (TNM) system, which includes 5 stages (0-4) (Cserni et al. 2018). The higher stages correspond to bigger and more metastatic tumors (Table 1.1).

Breast tumors are also classified based on the presence or the absence of the estrogen (ER) and progesterone (PR) receptors and the human epidermal growth factor receptor 2 (HER2) as ER+/-, PR+/- and HER2+/- respectively (Fig.1.2). Additionally, tumors lacking all three receptors are classified as triple-negative breast cancer (TNBC) (Fig 1.2). TNBCs account for 12-17% of all breast cancers and cannot be targeted with hormonal therapy (Foulkes, Smith, and Reis-Filho 2010). Breast cancer tumors can also be molecularly classified based on gene expression into luminal A, luminal B, HER2+, basal like and claudin low subtypes (Cserni et al. 2018; Dias et al. 2017). Tumors in the luminal A and B subtypes express the ER receptor and can be phenotypically differentiated based on the presence or absence of the other two receptors and the levels of the protein KI6 (a proliferation marker) (luminal A (ER^{+}, $PR^{+/-}$ and $HER2^{-}$, $KI\text{-}67^{low}$) luminal B (ER^{+}, $PR^{+/-}$, $HER2^{+/-}$and $KI\text{-}67^{high}$)). ER positive tumors can be targeted with estrogen receptor targeting therapy as part of their treatment regimen (Ariazi et al. 2006). Tumors expressing the HER2 receptor and lacking the ER and PR receptors overlap with the HER2+ molecular subtype. HER2 targeting agents can be used as part of the treatment regimen for HER2+ tumors (Cheang et al. 2009; Piccart-Gebhart et al. 2005). Finally, the majority of the basal like and claudin tumors phenotypically overlap with TNBC subtype (Dias et al. 2017; Prat et al. 2015). However, it is important to note that the two methods of subtyping breast cancer do not completely overlap as can be observed in some basal like tumors expressing the ER and PR receptors (Bidard et al. 2007).

Using the different classification methods, different treatment approaches are implemented to achieve higher survival rates for individual patients. An investigation of published studies demonstrating breast cancer patient survival based on their tumor

subtype reveals the ER+ subtypes (luminal A and B) to have the highest survival. In contrast, HER2+ and TNBC tumors have the lowest survival, and thus should be the prime target to investigate new treatments and methods to overcome resistance (Table 1.2).

Stage	Tumor size	Node involvement	Distant metastasis
0	DCIS and Paget disease	None	No
1A	Tumor ≤ 20 mm	None	No
1B	No evidence of primary tumor	Micro metastases to axillary lymph nodes	No
	Tumor ≤ 20 mm		
2A	No evidence of primary tumor	Metastases to movable axillary lymph nodes	No
	Tumor ≤ 20 mm		
	Tumor > 20 mm but ≤ 50 mm	None	
2B	Tumor > 20 mm but ≤ 50 mm	Metastases to movable axillary lymph nodes	No
	Tumor > 50 mm	None	
3A	Tumor ≤ 20 mm	Metastases to fixed axillary lymph nodes or mammary lymph nodes in the absence of axillary lymph node	No
	Tumor > 20 mm but ≤ 50 mm		
	Tumor > 50 mm	Metastases to movable axillary lymph nodes	
	Tumor > 50 mm	Metastases to fixed axillary lymph nodes or mammary lymph nodes in the absence of axillary lymph node	
3B	Tumor reaching chest wall and skin	None	No
	Tumor reaching chest wall and skin	Metastases to movable axillary lymph nodes	
	Tumor reaching chest wall and skin	Metastases to fixed axillary lymph nodes or mammary lymph nodes in the absence of axillary lymph node	
3C	Any tumor size	Metastases to infraclavicular lymph nodes +/- axillary lymph nodes metases	No
4	Any tumor size	Absent or present	Yes

Table 1.1 TNM anatomical staging of breast cancer based on the American Joint Commission on Cancer- revisions of the eight edition (2017). (Giuliano et al. 2017)

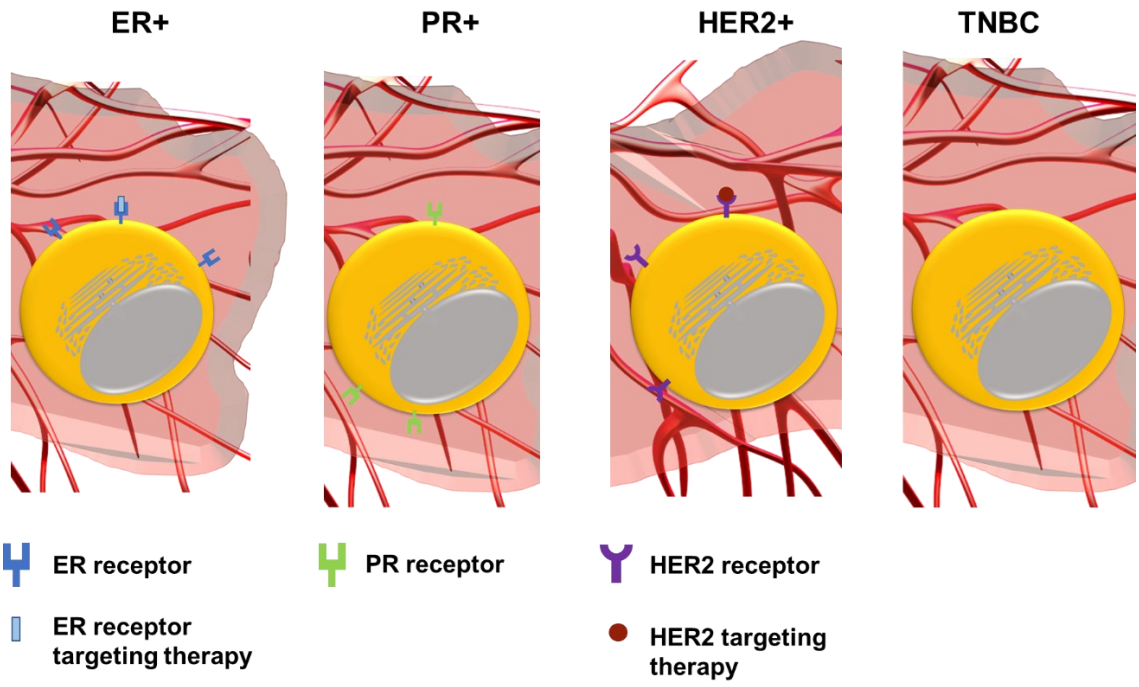


Figure 1.2. Breast cancer subtypes based on the expression of ER, PR and HER2 receptors. Breast cancer tumors can be classified in 4 subtypes based on the presence or lack of the hormone receptors and the HER2 receptor as ER+/-, PR+/-, HER2+/- and TNBC. The expression of these receptors allows the use of targeted therapies to treat these tumors. Tumors lacking the expression of the three receptors are classified as TNBC and thus, can not be targeted with these therapies.

Table 1.2. Breast cancer patient disease-free and over all survival based on their tumor subtype. Luminal A and B subtypes have higher survival than the TNBCs and HER2+ subtypes. * This study analyzed survival of patients with highly aggressive inflammatory breast cancer and thus overall survival rates were lower than the other studies where patients were at different stages. ** Metastasis-free survival.

Subtype	Disease-free survival	Overall survival	study
Luminal A	86.8%	90.3%	(Onitilo et al. 2009)
	88.6%	93.3%	(Xue et al. 2012)
	NR	69.7%	(Li et al. 2011)*
	93.2%**	80.8%	(Ihemelandu et al. 2008)
Luminal B	83.2%	88.7%	(Onitilo et al. 2009)
	73.1%	89.4%	(Xue et al. 2012)
	NR	73.5%	(Li et al. 2011)*
	94.4%**	83%	(Ihemelandu et al. 2008)
HER2+	66.0%	78.8%	(Onitilo et al. 2009)
	64.1%	77.5%	(Xue et al. 2012)
	NR	54.0%	(Li et al. 2011)*
	88.5%**	72.7%	(Ihemelandu et al. 2008)
TNBC	73.5%	79.0%	(Onitilo et al. 2009)
	74.1%	85.5%	(Xue et al. 2012)
	NR	42.7%	(Li et al. 2011)*
	80.25%	63.9%	(Ihemelandu et al. 2008)

1.12 Epigenetics in Breast Cancer

Epigenetic alterations have been heavily investigated in breast cancer to understand their role in promoting and maintaining breast cancer as well as for the identification of potential targets for therapy. Examples of these alteration includes hypermethylation in the promoter region of several key tumor suppressor genes and interference with normal histone chromatin dynamics. Several studies have highlighted the increased levels of methylation in the promoter region of the breast cancer type 1 susceptibility (BRCA1) resulting in decreased expression and impaired tumor suppressor activity (Dobrovic and Simpfendorfer 1997; Rice, Massey-Brown, and Futscher 1998). Moreover, methylation of the BRCA1 promotor has been shown to have similar effects to having a BRCA1 mutation in tumors (Wong et al. 2011). BRCA1 and BRCA2 have been reported to contribute DNA repair through transcriptional changes in response to DNA damage and mediating cell cycle arrest and apoptosis. Additionally, both proteins have been shown to contribute to genomic stability and mutations in these genes are associated with genomic instability (Yoshida and Miki 2004). Epigenetic silencing was also found to affect other tumor suppressor genes in breast cancer, including TP53 (Kang et al. 2001) and ATM Serine/Threonine Kinase (ATM) (Vo et al. 2004).

Chromatin modeling due to histone deacetylation is another epigenetic modification that is heavily indicated in breast cancer. HDAC1 is highly expressed in ER+ positive breast cancers, while HDAC2 and HDAC3 are highly expressed in HER2+ and hormone receptor negative tumors (Müller et al. 2013). HDACs have been also associated with breast cancer cell invasive and metastatic abilities, and targeting HDACs was shown to halt these abilities (S. Y. Park et al. 2011). Other

histone modifications have been also indicated in breast cancer. Different HMTs have been shown to have both antitumorigenic and protumorigenic activity in breast cancer. Inhibition of HMT NSD3L resulted in increased metastatic and invasive abilities of MDA MB 231 breast cancer cell line suggesting its potential role as a tumor suppressor gene (Zhou et al. 2010). In contrast, HMTs hSETD1A and SET And MYND Domain Containing 3 (SMYD3) were found to promote invasion and metastasis in breast cancer through the activation of matrix metalloproteinases and myocardin related transcription factor-A (MRTF-A) induced upregulation of myosin regulatory light chain 9 (MYL9), respectively (Luo et al. 2014; Salz et al. 2015). BRCA2, a tumor suppressor gene heavily investigated in breast cancer, has been shown to have histone acetyltransferase activity that is important for its function (Fuks, Milner, and Kouzarides 1998; Siddique et al. 1998). In contrast, histone acetyltransferase HBO1- a positive regulator of DNA replication - was found to be highly expressed in breast cancer cells among other tumor types (Iizuka et al. 2009).

1.13 Breast Cancer Treatment

Breast cancer treatment involves an array of local and systemic therapies. Local therapies include surgery, radiation and some novel targeted therapies. Surgery aims to remove primary tumors and some of the surrounding healthy tissue to minimize the chance of missing malignant cells within the healthy tissue margins (De La Cruz et al. 2016). Surgery could be preceded or followed by other type of treatments including chemotherapy and radiation. Radiation therapy can be applied following surgery to kill remaining cancer cells that were not removed during surgery and remained within the healthy tissue, thus, preventing recurrence. Systemic

therapies include hormonal therapies, chemotherapy and targeted therapies (Meattini et al. 2017).

Chemotherapy has been a key component in breast cancer treatment in the last few decades. Chemotherapy can be administered to breast cancer patients as neoadjuvant therapy prior to surgery to decrease tumor size or as adjuvant therapy following surgery to kill any remaining cancer cells and prevent recurrence (Mauri, Pavlidis, and Ioannidis 2005). Chemotherapy regimens for breast cancer typically involve the use of combination of several chemotherapeutic agents including paclitaxel, doxorubicin, 5-fluorouracil and cyclophosphamide (Rouzier et al. 2005). Hormonal therapy targets the ER and PR receptors that were previously mentioned. Several drugs targeting the ER receptor have been investigated with some modulating the receptors including tamoxifen and raloxifene and other belonging to the aromatase inhibitor family including letrozole. ER receptor modulators and aromatase inhibitors are currently used to treat patients with ER+ tumors (den Hollander, Savage, and Brown 2013). PR receptor targeting is not widely used as a treatment for breast cancer; however, studies have shown benefits for modulating PR receptor in inhibiting breast cancer cell proliferation (Giulianelli, Molinolo, and Lanari 2013; Lanari et al. 2012).

Targeted therapies are currently used to treat HER2+ breast cancer tumors including Herceptin (trastuzumab) and lapatinib. Trastuzumab is a monoclonal antibody targeting the HER2 receptor while lapatinib is an orally active drug with a dual kinase inhibitor activity targeting both HER2 and the epidermal growth factor receptor (EGFR). In TNBCs, several selective inhibitors are currently being investigated in clinical trials and preclinical models including poly (ADP-ribose)

polymerase inhibitors (Anders et al. 2010), retinoids and vitamin A derivatives (Merino et al. 2016), phosphoinositide 3-kinase (PI3K) pathway inhibitors, and cyclin-dependent kinase (CDK) inhibitors to inhibit cell cycle progression (McCann, Hurvitz, and McAndrew 2019).

Given the importance of epigenetic modifications in breast cancer progression, there was an increased interest in investigating them as potential therapeutic targets. Demethylation agents including azacytidine and decitabine have been investigated as potential therapeutics for targeting breast cancer. Promising effects were observed in preclinical models when demethylating agents were used alone or as part of combination therapies (Mirza et al. 2010; Thakur et al. 2012). However, clinical trial findings were less convincing with minor or no effects observed on patient survival and response to therapy (Connolly et al. 2017), suggesting more research is needed to translate the success in the preclinical models into the clinical settings.

HDAC inhibitors were heavily investigated in breast cancer as novel targeted therapies. Preclinical investigation highlighted the ability of some HDAC inhibitors to induce cell cycle arrest (Munster et al. 2001) and apoptosis (Hsieh et al. 2015) in breast cancer cells and enhance the antiproliferative activity of other drugs when used in combination therapy (Raha et al. 2015). Similar to demethylating agents, HDAC inhibitors did not show significant effects when investigated as a stand a lone therapy in clinical settings (Luu et al. 2008). In contrast, combining HDAC inhibitors with other drugs such as paclitaxel, tamoxifen and bevacizumab enhanced patient survival and decreased resistance to these drugs (Munster et al. 2011; Ramaswamy et al. 2012; Tu et al. 2014).

The increased interest in utilizing the immune system in targeting tumor cells was also evident in breast cancer. Ongoing immunotherapy clinical trials are examining the role of immunotherapy in breast cancer management to enhance the outcome of resistant patients that failed conventional therapy. Checkpoint inhibitors including anti-programmed cell death protein 1 (PD1) and anti-PD-L1 have shown promise in targeting highly metastatic TNBCs and are currently being investigated among other combinations in clinical trials (Emens 2018).

The extensive advancement in molecular diagnostics was also evident in breast cancer management. The oncotype DX is a prognostic test currently used in clinical practice to assess the likelihood of recurrence and whether adding chemotherapy would be beneficial for early stage ER+/HER2- patient. The test examines the expression of 21 genes and determine a recurrence score (0-100) (Sparano et al. 2018). A lower score is associated with decreased recurrence risk and less benefits of chemotherapy, while a higher score has the opposite indications. Other genomic test have developed to predict breast cancer recurrence risk including the Breast Cancer Index Test (Zhang et al. 2013)and EndoPredict test (Dubsky et al. 2013); however, they are not as widely used as the oncotype DX text.

As previously mentioned, treatment plans for breast cancer patients are affected by several factors (e.g. tumor grade, stage, hormone receptor expression, molecular subtype), which dictate what combination of treatment a patient will receive (Table 1.3,) (Figure 1.3). Notably, TNBCs are among the most aggressive types of breast cancer with the worst outcomes and limited treatment options (Prat et al. 2015); thus, it is important to continue to investigate potential avenues to enhance patient survival and treatment outcomes.

Stage	Surgery	Radiation therapy	Chemotherapy
0	**+ (main)**	**+/- (following surgery)**	**-**
1	**+ (main)**	**+/- (following surgery, primary tumor and lymph nodes)**	**Adjuvant (not common)**
2	**+ (standard)**	**+/- (following surgery, primary tumor and lymph nodes)**	**Adjuvant and neoadjuvant (larger tumors)**
3	**+**	**+/- (following surgery, all of the breast, chest muscles and lymph nodes)**	**(main) usually adjuvant. Neoadjuvant (stage 3A, prior to conserving surgery)**
4	**-**	**-**	**+ main**

Table 1.3. Treatment for breast cancer based on stage. Current treatment for breast cancer patients based on stage (data adopted from the Canadian cancer society website). Hormonal and targeted therapies are available for patients from different stages based on their receptors status and the expression of the designated targets of targeted therapies.

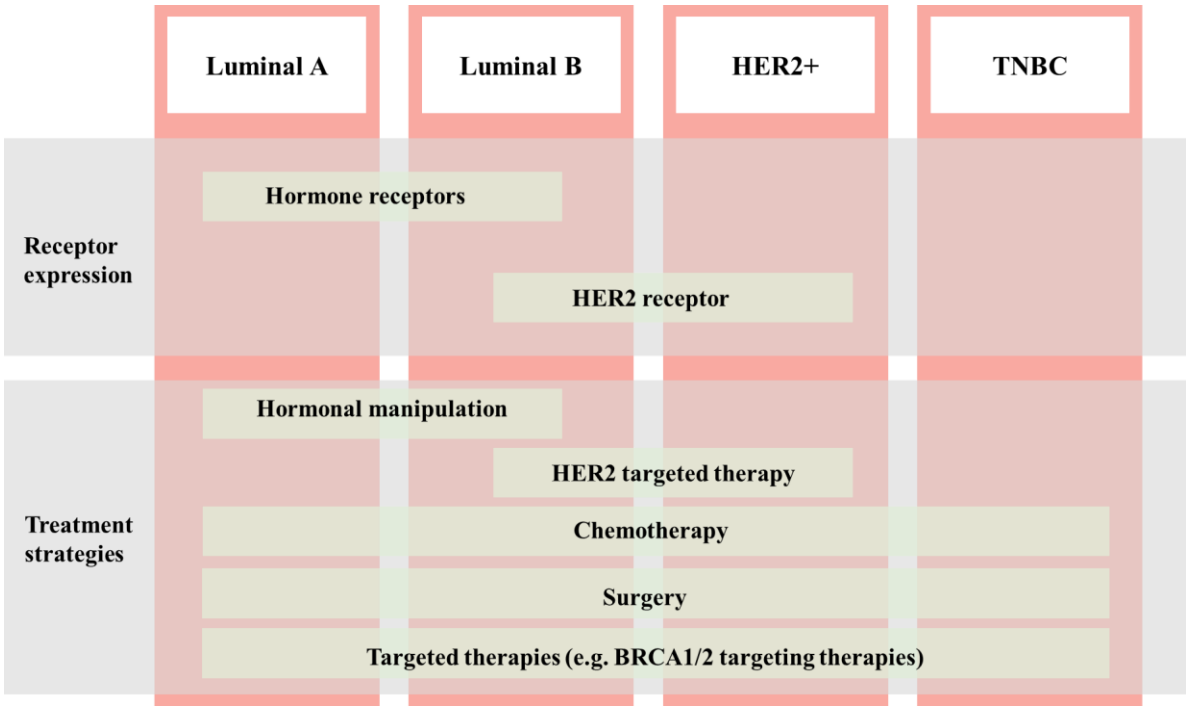


Figure 1.3. Treatment strategies for breast cancer based on molecular subtypes. Chemotherapy and surgery remain the most common approaches to treat breast among the four subtypes. Luminal A, Luminal B and HER2+ patients can be targeted with hormone manipulating drugs and HER2 targeting drugs are based on their receptor expression.

Data adopted from (Adam Sharp 2014)

1.14 Taxanes

Taxanes are a family of chemotherapeutics widely used to treat variety of cancers including breast, ovarian, lung, and head and neck cancers. Taxanes are microtubules interfering agents that disrupt cell cycle progression in a rapidly dividing cell. The name taxane is derived from the pacific yew tree (Taxus brevifolia) from which the first taxane "paclitaxel" was derived from in 1971. Paclitaxel remains highly used today, along with docetaxel – a semi synthetic second-generation drug associated with less hypersensitivity than paclitaxel.

Taxanes bind with high affinity to the beta-tubulin subunit of microtubules and inhibit their depolymerization. In a rapidly dividing cells like cancer cells this will inhibit the segregation of sister chromatids between metaphase and anaphase during the M phase of the cell cycle (McGrogan et al. 2008). This cell cycle arrest can lead to several outcomes (Fig. 1.4). Some cells will directly undergo apoptosis through activation of the apoptotic cascades. Other cells can undergo mitotic slippage where they override checkpoint inhibition and continue to divide without segregating the sister chromatids. Cells that underwent mitotic slippage will either continue to divide as tetraploids or undergo apoptosis (Blagosklonny 2007). Paclitaxel has also been shown to induce mitotic catastrophe leading to cell death without activating apoptotic pathways (Morse et al. 2005). Additionally, several reports have shown paclitaxel to induce a G1 cell cycle arrest when administrated at low doses leading directly to apoptosis (Giannakakou et al. n.d.; Shu et al. 1997).

Taxane side effects include peripheral neuropathy, suppression of bone marrow cells differentiation resulting in decreased red and white cells and platelets, joint and muscle pain, and skin reactions (Cella et al. 2003). Paclitaxel is

administrated in a solution of chromophore oil as a solvent due to its hydrophobic nature. This solution is what is thought to cause the hypersensitivity skin reaction and thus it is recommended to treat patients with steroids prior to paclitaxel administration to limit this reaction. However, steroid treatment can further compromise patient immunity leaving them to be more susceptible to sicknesses and comorbidities. In contrast, docetaxel solution is associated with less hypersensitivity and can be used as an alternative in patients that display these reactions (McGrogan et al. 2008).

Several mechanisms have been identified to cause resistance to paclitaxel including downregulation of apoptotic machinery, alteration to beta tubulin to inhibit binding and overexpression of the multi-drug resistant gene MDR-1 (Yusuf et al. 2005). Several other resistance mechanisms where identified in breast cancer models. Transcriptional changes such as Twist upregulation of Protein kinase B (AKT) (Cheng et al. 2007) and miR125-b inhibition of B-cell lymphoma 2 (BCL2) have been shown to promote paclitaxel resistance in breast cancer. Similarly, breast cancer cell lines were shown to downregulate key apoptotic pathways and upregulate autophagy pathways causing resistance to paclitaxel (Ajabnoor, Crook, and Coley 2012).

Given the widespread use of taxanes in cancer treatment in general and in breast cancer specifically, it is important to investigate potential mechanisms to overcome resistance enhance patient outcomes. Identification of a novel resistance mechanism that can be targeted with combination therapy to decrease paclitaxel resistance could be particularly beneficial for TNBC patients who are treated mainly with combination of chemotherapies that contain paclitaxel. Additionally, such combinations could be beneficial for other types of cancers that get treated with taxanes.

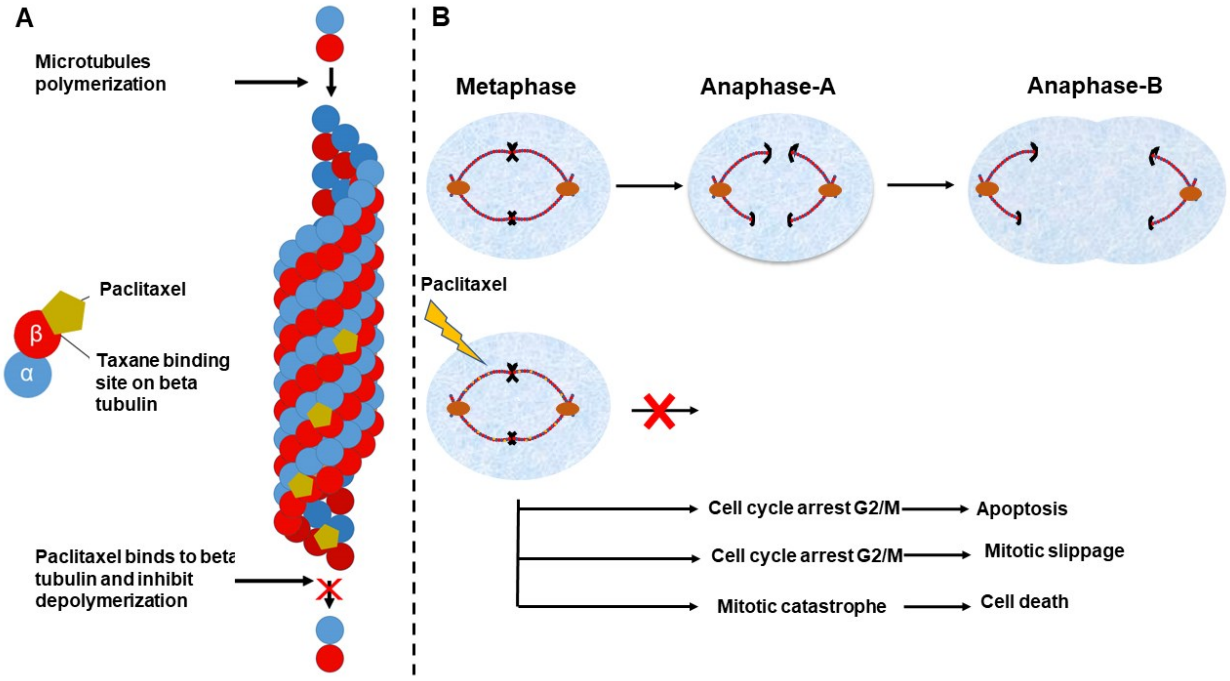


Figure 1.4 Paclitaxel a microtubule interfering agent that disrupt cell cycle progression in a rapidly dividing cell. (A) Paclitaxel binds to the taxane binding site on beta tubulin and inhibit microtubules depolymerization (**B)** thus, inhibiting sister chromatid segregation and leading to a cell cycle arrest between the metaphase and the anaphase during the M phase of cell cycle.

1.15 Breast Cancer in vitro Models

Human and mouse breast cancer cell lines are widely used for *in vitro* modeling of breast cancer. These cell lines are easily accessible with verified growth information and can provide low-cost models to complete a diverse array of assays investigating different stages of the disease progression and the effect of therapy (Neve et al. 2006). The available cell lines provide tools to study different subtypes of breast cancer and develop targeted therapies utilizing their unique characteristics (Chavez, Garimella, and Lipkowitz 2010).

The use of breast cancer cells *in vitro* provided a platform for extensive investigation of many drugs effects, response and mechanisms in breast cancer (Imamura et al. 2015). However, there are several limitations that arise when using these models that drives the need to further investigate and validate findings in *in vivo* models (Burdall et al. 2003; Imamura et al. 2015). Such limitations stem from the environment in which the cells are grown in. Most cell lines are grown in serum rich media under certain temperature and gaseous conditions. This provide less heterogeneity in the cell population (Burdall et al. 2003) unlike when a tumor develops in a patients where different portions of the tumor have different access to oxygen and nutrients (Hao et al. 2016; Jiang et al. 2012).

Cell lines are also characterized for mutations and genetic characteristics, which could provide an advantage when studying a specific model with certain characteristics. However*, in vitro* manipulation renders cell line cultures susceptible to genetic drifts, thus, giving rise to false positive observations and providing inaccurate picture of tumor behavior and during development and response under different treatment conditions (Burdall et al. 2003). Finally, numerous publications have

highlighted the importance of the tumor microenvironment in shaping tumor behaviors and responses to treatment (Ciriello, M. L. L. Gatza, et al. 2015; Fan et al. 2014; Zhang et al. 2016). The lack of tumor microenvironment *in vitro* hinders the complete understanding of tumorigenesis and investigation of potential therapies. Several solutions were proposed to overcome the limitations of *in vitro* cultures including using hypoxia champers (R. Wang, Jin, and Zhong 2014), complex co-culture assays (Saad et al. 2000) and the use of three dimensional mammosphere cultures (Weigelt, Ghajar, and Bissell 2014). While these assays could be beneficial when successful, they are associated with more limitations in term of failure of cell growth. Thus, it is imperative that when *in vitro* models are used for primary discovery that validation occurs in concordance with *in vivo* models to obtain a more comprehensive understanding of the research question.

1.16 Breast Cancer in vivo Models

In vivo models provide a platform to further investigate breast cancer and treatments in addition to *in vitro* experiments. Most *in vivo* experiments involving human breast cancer cell lines involve xenografting the cells in immunocompromised mouse models (Clarke 1996). These models include non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice (Milsom et al. 2013), recombination activating gene (Rag) deficient mice and athymic nude mice (Zhang et al. 2012). As their names indicate, these mice lack a functional immune system due to impairment in their adaptive immunity which allows human cells to form a tumor in a mouse without a graft versus host immune response.

In vivo breast cancer models include the use of patient-derived xenografts (PDXs). These xenografts are developed through implanting tumor pieces or isolated cancer cells from a patient into an immunocompromised mouse. Once successfully implanted, PDXs are then passaged to allow changes to the tumors microenvironment that favor growth in mice (Whittle et al. 2015). Interestingly, more aggressive tumors such as basal-like TNBCs have the highest success take rate when trying to establish a PDX. This can be attributed to downregulation of key immune modulators facilitating interaction with the immune system (Moon et al. 2015). PDXs provide a closer resemblance to patient tumors and more preserved tumor heterogeneity which allows for more accurate findings and observations. Together, cell lines xenografts and PDXs provide a valuable tool to investigate treatments and biology of breast cancer. These models provide important preclinical data to study breast cancer.

However, it is important to highlight that not all human breast cancer cell lines can be successfully xenografted in immunocompromised mice (Visonneau et al. 1998). Several factors could contribute to these limitations including hormonal requirement and other microenvironment differences that could be essential for the human cell line but are lacking in a mouse model (Sflomos et al. 2016). Additionally, metastasis patterns differ in mice (Kuperwasser et al. 2005), which could be attributed to the impaired immune system given the key role played by immune cells in facilitating invasion and metastasis (Coffelt et al. 2015; DeNardo, Johansson, and Coussens 2008).

In contrast, immunocompetent *in vivo* models can solve some of the limitations associated with immunocompromised models, while also having limitations of their own. Among the most common used models to study breast cancer

in an immune competent setting are syngeneic models that arose from spontaneous murine mammary tumors (Park, Lee, and Lee 2018). Cell lines derived from these tumors have been immortalized and used for *in vitro* and *in vivo* investigations. Among these models are the 4T1 murine breast cancer cell line that was initially derived from a spontaneous tumor in a BALB/C mouse. This model can now be used to develop highly metastatic and aggressive tumors in BALB/C mice and provide a tool to answer scientific questions in the presence of the immune system (Ghochikyan et al. 2014). Other syngeneic models were developed from different spontaneous tumors arising in different immune backgrounds including C57BL/6J mice (Davie et al. 2007).

Transgenic mice models serve as another important *in vivo* platform to investigate breast cancer (Li, Hively, and Varmus 2000). These mice are genetically altered to expresses or lack the expression of important genes and can help answer questions directed at the target gene and its pathways. Thousands of models have been generated targeting tumor suppressor genes (Blackburn and Jerry 2002), oncogenes (Reilly et al. 2000) and immune modulators (Knutson et al. 2006) resulting in numerous advancements in understanding and targeting breast cancer. Genetic alterations could be permanent such as generating over expression or knockout strains (Blackburn and Jerry 2002) or it could be inducible using systems such as the tetracycline induction (J. Liu et al. 2011). Furthermore, these transgenic models could be designed in immunocompetent and immunocompromised models providing more tools and options to investigate using human and mouse derived models.

While these models provide an additional tool to study the effect of the immune system on a specific treatment or pathway in breast cancer, they also have

their limitations. In additions to decreased intratumoral heterogeneity, intra-species differences are more pronounced when trying to draw conclusions for a research question in human tumors (Day, Merlino, and Van Dyke 2015). Such differences could be in the genetic components of non-conserved pathways leading to difference in investigated markers or tumor composition and microenvironment between human and murine cell lines (Klein et al. 2007).

In recent years, there has been effort to establish dual xenograft models in which mice are xenografted with a human cancer cell line and a functional human immune system. These models involve the abolition of the mouse immune using radiation and replacing it with human immune cells (De La Rochere et al. 2018). Several approaches have been taken to establish such models including transplanting human stem cells which successfully can differentiate in an immunocompromised mouse to establish a functional immune system without the need to transplant human thymic tissue into the mouse (Capasso et al. 2017). However, it is important to highlight that these models are still in development and not widely used in breast cancer research yet.

In addition to mice, other *in vivo* models have been used to study breast cancer. These models include the use of rats (Kumar et al. 2017) or zebrafish (Bentley et al. 2015; De Boeck et al. 2016) as an alternative to mice. However, these models are not as widely used in breast cancer research due different limitations including reduced genomic similarities between zebrafish and humans (Howe et al. 2013) and the limited/absence of metastases in rat models (Simmons et al. 2015).

It is essential to realize the advantages and limitation of each model and utilize them appropriately to answer specific questions. This includes the use of *in vitro* and

in vivo models as well as utilizing immunocompromised and immunocompetent models when available.

1.17 Leukemia

Leukemia is a malignancy of hematopoietic stem cells accounting for about 25% of hematological malignancies in Canada (Hodgson et al., 2016). Under normal conditions hematopoietic stem cells differentiate in response to different growth signals to give rise to different blood cells. Hematopoietic stem cells differentiate into lymphoid and myeloid lineages and then each lineage will further differentiate to establish different populations of blood cells (Jagannathan-Bogdan and Zon 2013) (Fig. 1.5).

Leukemia occurs when normal differentiation of hematopoietic stem cells is halted resulting in increased numbers of highly proliferative leukemic cells. The leukemic cells continue to divide overcrowding the bone marrow and overtaking normal hematopoietic stem cells and in turn mature blood cells (Lowenberg, Downing, and Burnett 1999). The lack of functioning blood cells in turn interferes with all functions carried out by blood cells in normal human physiology including oxygen delivery tissue, immune response and coagulation cascades.

Leukemia can occur in both the lymphoid and myeloid lineages and thus is classified based on the lineage and the cells its affecting as lymphocytic (Pui, Relling, and Downing 2004) or myelogenous (Lowenberg et al. 1999). Leukemia is also classified based on the onset duration of the disease as chronic or acute (Lee et al. 2007). Together these two classification criteria help differentiate between the different types of leukemia. Therefore, leukemias are considered a spectrum of

different disease with different prevalence (Figure 1.6), characteristics and treatment approaches (Hamieh and Sadelain 2018; Kruth et al. 2017; Talpaz et al. 2018; Tamamyan et al. 2017). Treatment approaches include the use of chemotherapeutics, targeted therapies, immunotherapies as well as hematopoietic stem cell transplants (Kantarjian et al. 2017; Landau et al. 2017; Lichtenegger et al. 2017; Pratz et al. 2019).

While leukemias and hematological malignancies differ from solid tumors in some aspects of tumorigenesis and interactions with the tumor microenvironment, they share key aspects that allows researchers to investigate similar targeting strategies with therapeutics that could be beneficial for both types.

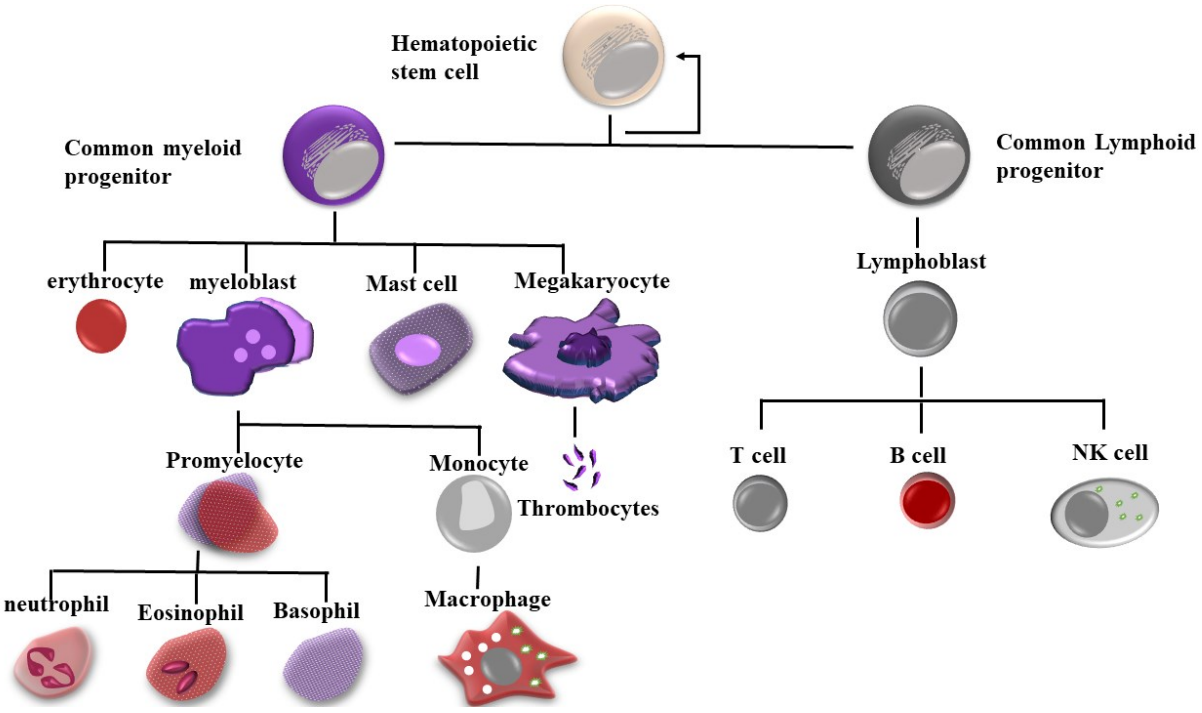


Figure 1.5 Hematopoiesis in healthy individuals. Hematopoietic stem cells give rise to common myeloid and lymphoid progenitor cells which in turn give rise to different populations of blood cells. A mutation in the promyelocytic cells leads to acute promyelocytic leukemia (APL), which in turn interferes with normal hematopoiesis leading to the manifestation of the disease symptoms due to a decrease in the number of healthy blood cells.

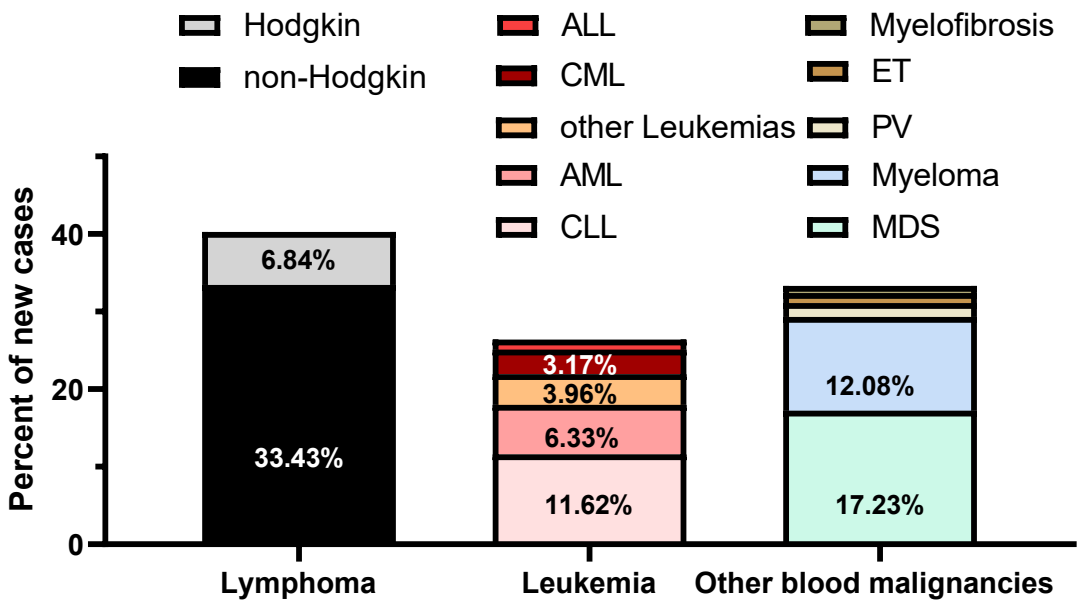


Figure 1.6 Percent of newly diagnosed blood malignancies in Canada in 2016. Lymphomas are the most common blood malignancies diagnosed in Canada accounting for about 40% of the cases. Leukemias are the second common type of blood malignancies (26%) followed by myelodysplastic syndrome) then Myelomas (12.08%). Among the leukemias, AML accounts for 6.33% of the newly diagnosed cases with (10-15%) of these cases belonging to the APL subset. Other blood malignancies including polycythemia vera, essential thrombocytosis and myelofibrosis, which account for the remaining 4%.

1.18 Acute Promyelocytic Leukemia (APL)

As the name indicates, APL is a type of leukemia characterized by interrupted normal hematopoietic cell differentiation at the promyelocytic stage. APL occurs in the myeloid lineage of hematopoiesis and thus, it is considered a subset of acute myeloid leukemia (AML), accounting for 10-15% of newly diagnosed AML cases in adults (Wang and Chen 2008).

Similar to other leukemias, malignant APL cells overtake the bone marrow and out crowd the healthy blood cells inducing disease symptoms including fever, infections, fatigue, anemia and hemorrhagic bleeding (Choudhry and DeLoughery 2012; Girmenia et al. 2003; Kantarjian et al. 1986).

Histologically, APL cells are hyper-granular due to excessive levels of primary granules. Genetically, APL is characterized with a single chromosomal translocation between chromosomes 15 and 17 resulting in the formation of a pathogenic fusion protein between promyelocytic leukemia (PML) and the retinoic acid receptor α (RARα). The resulting PML-RARα protein leads to a loss of normal function associated with the individual proteins (Wang and Chen 2008; Warrell et al. 1993). In normal non-malignant cells PML is a tumor suppressor gene that plays a key role in regulating cell cycle, DNA replication and transcription, and apoptosis. PML carries its effects through forming PML nuclear bodies which in turn help regulate transcription, interact with transcription factors and other tumor suppressor genes and induce apoptosis (Borden 2002; Dellaire and Bazett-Jones 2004; De Stanchina et al. 2004). On the other hand, RARα binds to retinoic acid and activate signalling pathways associated with hundreds of genes (Chambon 1996). Unliganded RARα can act as transcription repressor through formation of a heterodimer with the

retinoid X receptor (RXR) and binding to retinoic acid response elements (RAREs) in the genome (Dawson and Xia 2012).

The RARα/RXR heterodimer promote chromatin repression through interaction with HDACs (Bastien and Rochette-Egly 2004). This activity is amplified in the fusion protein, as PML- RARα can bind more readily to RXR and in turn the heterodimer binds more readily to RAREs and HDACs leading abnormal gene silencing (Kamashev, Vitoux, and De Thé 2004; Liquori et al. 2020; di Martino and Welch 2019). Additionally, the fusion protein interferes with the normal physiological role RARα plays in granulocytes differentiation and self renewal leading to the arrested malignant phenotype (Vitaliano-Prunier et al. 2014). Finally, the formation of the fusion protein interferes with PML's normal function leading to loss of its tumor suppressor activity and its role in DNA repair and apoptosis, and in turn promoting uncontrolled proliferation (Eskiw and Bazett-Jones 2002). In contrast, several reports have reported an oncogenic role for PML in TNBC (Arreal et al. 2020; Martín-Martín et al. 2016; Ponente et al. 2017). High levels of PML were associated with enhanced growth TNBC cell line MDA MB 468 (Arreal et al. 2020). PML was also shown to promote metastasis in TNBC but not other breast cancer subtypes through regulating the expression of several HIF1A target genes (Ponente et al. 2017). Interestingly, inhibiting PML in TNBC cell lines and tumor models was shown to induce growth arrest and inhibited metastasis through promoting senescence (Arreal et al. 2020; Ponente et al. 2017).

The formation of the PML- RARα fusion protein is a used as clinical marker to detect the vast majority of APL cases using molecular diagnostic tools including fluorescent *in situ* hybridization (FISH) and reverse transcriptase polymerase chain

reaction (RT-PCR) (Fujita et al. 2003; Gallagher et al. 2003). While PML- RARα is a characteristic of about 98% of APL cases, RARα can also form a fusion protein with different proteins including signal transducer and activator of transcription 5b (STAT5B) (H. Chen et al. 2012), promyelocytic leukemia zinc finger (PLZF) (Sainty et al. 2000) and BCL6 corepressor (BCOR)(Yamamoto et al. 2010). These mutations serve as biomarkers and predictors for treatment response and, thus, can be used to guide treatment approach similar to what is seen with breast cancer subtyping.

1.19 All Trans Retinoic Acid and Arsenic Trioxide in APL Treatment

Once a deadly disease, APL now is considered a malignancy with favorable outcome due to the use of targeted therapies to induce granulocyte differentiation. Treatment of APL involves the use of targeted therapies directed at the PML-RARα fusion protein including all trans retinoic acid (ATRA) (Huang 1988), arsenic trioxide (ATO) (Ghavamzadeh et al. 2006) and chemotherapeutics agents such as anthracyclines (Montesinos et al. 2009). The informed use of these targeted therapies have significantly increased survival rate in APL; however, recent reports have shown increased rate of resistance and relapse following treatments with single agents and combination therapy with chemotherapy (Gallagher 2002; Hattori et al. 2018; Jimenez et al. 2020; Noguera et al. 2019) . Thus, it is imperative to determine therapy resistance mechanisms and potential approaches to overcome it and enhance patient survival.

Targeting APL cells with ATRA induces the degradation PML-RARα, restoring granulocytic differentiation and inducing apoptosis in APL cells (Idres et al.

2001; Yoshida et al. 1996). However, ATRA as a stand alone therapy was not effective at achieving long term disease free status and many patients relapsed and required additional therapy (Takeuchi et al. 1998). Thus, ATRA was combined with anthracycline, where ATRA serves as induction therapy to promote differentiation followed by anthracycline treatment to target the malignant cells (Fenaux et al. 1999). This approach was successful in increasing treatment efficacy and reducing relapse incidents. However, patients who failed this therapy acquired a mutation in PML-RARα, that rendered them unsensitive to ATRA treatment (Marasca et al. 1999). Moreover, the side effects from anthracycline treatment remained a negative impacting factor on patient overall survival.

It was not until ATO was used to treat APL patients (Shen et al. 1997; Zhang et al. 2001), that the idea of using non-chemotherapeutic based regimen to induce long lasting effects on patients survival in APL was realized. Similar to ATRA, ATO induced transcriptional changes to restore granulocytic differentiation through the degradation of PML-RARα (Lallemand-Breitenbach et al. 2012). ATO restored the formation of PML-nuclear bodies, allowing SUMO- mediated degradation of PML and PML-RARα (Lallemand-Breitenbach et al. 2008). Moreover, ATO treatment had significant effects on APL associated epigenetic alterations including promoting demethylation of key genes that promote cell cycle arrest as well as decreasing overall methylation status through inhibition of HMT activity (Hassani et al. 2018).

The promising effect of each individual therapy prompted the investigation of ATRA and ATO combination therapy on APL. Several studies have shown ATO and ATRA combination therapy to have high efficacy in promoting disease free survival and overall survival (Lo-Coco et al. 2012). Combination therapy survival rates were

higher than those achieved by either drug alone and were comparable to those achieved by ATRA and anthracycline combination therapy (Mayor 2013). Thus, using this chemotherapy-free combination prevented the unwanted side effect of chemotherapies while maintaining good outcomes for APL patients. This effect was largely attributed to synergistic effects of each individual drug in promoting PML-RARα and targeting different genetic and epigenetic abnormalities in APL (Nitto and Sawaki 2014). However, it remains unclear what modifications are responsible for the long-lasting effect of combination therapy in comparison to treatment with either individual drug.

1.20 Epigenetic Alterations in APL

Similar to breast cancer, epigenetic modifications play an essential role in APL progression and treatment response. Alteration in methylation profiles and chromatin modification in APL are associated with transcriptional repression of important genes involved in granulocyte differentiation and tumor suppression (Cheung and So 2011). These effects are in large due to interactions between the PML-RARα fusion protein with DNMTs and histone modification enzymes including HDACs and HMTs (Arteaga et al. 2015).

Different DNMTs have been reported to be overexpressed in APL including the DNMT3a (a de novo methyltransferase) that binds to PML-RARα (Cole et al. 2016). High levels of DNMTs are associated with gene specific hypermethylation of several granulocytic differentiation genes associated with PML-RARα including retinoic acid receptor beta (RARβ) and transglutaminase 2 (TGM2) (Benedetti et al. 1996; Csomós et al. 2010). However, other reports indicated that methylation can

occur in APL in a PML-RARα independent matter as well (Schoofs et al. 2013); thus, using demethylating agents in APL could be beneficial in treating the leukemia.

In addition to gene specification methylation, malignant APL cells display elevated levels of genome-wide methylation in comparison to healthy progenitor cells (Chim, Wong, and Kwong 2003). Moreover, DNA methylation pattern observed in APL samples differ from those observed in other leukemia subtypes (Figueroa et al. 2010) which further highlight the importance of understanding and potentially targeting DNA methylation in APL.

APL fusion proteins interacts and recruit histone modifying enzymes to promote APL progression. These interactions can be seen with HDACs, HATs and HMTs. Both PML-RARα and PLZF-RARα can recruit nuclear co-repressor (NCOR) HDAC complex, which in turn plays an important role PML progression and response to treatment (Grignani et al. 1998). Other reports have demonstrated that the use of an HDAC inhibitor can enhance ATRA treatment efficacy and prevent mice death following NB4 APL cell line injection (Kosugi et al. 2001). Similarly, the pan HDAC inhibitor panobinostat was able to induce cell cycle arrest and apoptosis in NB4 cells as stand alone therapy and had synergistic effects when combined with ATO (Mosleh, Safaroghli-Azar, and Bashash 2020). HMTs were also reported to play a key role in APL pathogenesis and could possibly be targeted to enhance treatment efficacy. The polycomp repressive complex 2 (PRC2) methyltransferase activity has been reported to play a role in promoting methylation of PML-RARα target genes and mediating APL progression. Moreover, targeting the SUZ12 subunit of PRC2 reverted these effects and promoted normal differentiation of APL cells (Villa et al. 2007). In contrast histone demethylases, such as Lysine Demethylase 3B (KDM3B), were

found to inhibit APL progression by restricting chromatin access, which in turn decreased transcription of key genes involved in APL progression. Furthermore, KDM3B was also shown to play an important role in degradation of the PML-RARα fusion protein (Wang et al. 2019). Likewise, HATs have also been associated with APL response to retinoic acid treatment. HAT p300/CREB-binding protein-associated factor (PCAF) were shown to play an essential role in ATRA induced differentiation of APL cells. Therefore, targeting PCAF and its signalling pathway might provide increased ATRA treatment efficacy (Sunami et al. 2017).

Thus, it is imperative to understand and consider epigenetic alterations during APL progression and treatment. These alterations can provide additional treatment possibilities for patients that fail conventional therapy and require more treatment.

1.21 APL in vitro Models

The few APL cell lines available are widely used for *in vitro* modeling of the disease progression and treatment investigation. These cell lines are well characterized tools for investigating different aspect of APL and its respond to therapy. The most widely used cell line to study APL is the NB4 cell line (Lanotte et al. 1991). This cell line provides a platform to understand the role of the PML-RARα fusion protein and its effect on therapy. In contrast another APL cell line, HL-60, lacks the fusion protein and, thus, cannot be compared to NB4 in term of progression and response to treatment (Collins 1987). This limitation has prompted artificial creation of resistant clones of NB4, such as the NB4-MR2 cell line, which display

resistance to ATRA therapy (S. Chen et al. 2012). These clones allow the investigation of the role of combination therapy in overcoming ATRA resistance and achieving more favorable outcomes.

1.22 Research Rational and Objectives

In order to enhance treatment efficacy and patient survival it is important to understand the role of tumor heterogeneity in treatment response and promoting resistance. Moreover, genetic and epigenetic heterogeneity could provide new targets to enhance cancer treatment and increase patient survival and overcome resistance. The collective aim of this body of work is to investigate different mechanisms affecting breast cancer and APL response to therapies such as chemotherapy and immune modulating therapy. These mechanisms include tumor heterogeneity at the tumor cell level (i.e. CSCs), epigenetic modifications and the expression of therapy resistance mediators. Furthermore, I hypothesized that investigating these mechanisms could identify potential candidates for targeted combination therapies and enhance treatment efficacy in breast cancer and APL. This goal was achieved through the following objectives:

- Investigating breast CSC gene expression and epigenetic differences in the context of immune interactions. (Chapter 3)
- Determining the role and mechanism of Aldefluor+ breast CSCs in evading immune evasion and destruction. (Chapter 3)
- Identifying the underlining mechanism for this effect and possible mechanisms to overcome it. (Chapter 3)

- Identifying genes important in paclitaxel response in breast cancer. (Chapter 4)
- Investigating combination therapy by targeting paclitaxel resistance mediators to enhance treatment efficacy. (Chapter 4)
- Investigating the effect of ATRA and ATO combination therapy on NB4 APL cells and the ATRA-resistant NB4-MR2 APL cells. (Chapter 5)
- Determining key gene expression induced by the combination therapy of APL NB4 cells and NB4-MR2 cells which correspond to treatment outcomes. (Chapter 5)
- Investigating the role of epigenetic modifications in promoting the response combination therapy in NB4 and NB4-MR2 cells (Chapter 5)

Taken together, findings from the following chapters provide examples of the importance of investigating tumor heterogeneity to identify novel therapeutic targets to enhance treatment outcome.

CHAPTER 2: METHODS

Copyright statement

Portions of this chapter have been previously published in the following manuscripts. The corresponding text has been edited for length, consistency, and to include recent findings.

Mohammad Sultan, Dejan Vidovic, Simmone A Paine, Thomas T Huynh, Krysta M Coyle, Margaret Lois Thomas, Brianne M Cruickshank, Cheryl A Dean, Derek R Clements, Youra Kim, Kirsten Lee, Shashi Gujar, Ian CG Weaver, Paola Marcato. Epigenetic Silencing of TAP1 in Aldefluor+ Breast Cancer Stem Cells Contributes to Their Enhanced Immune Evasion. Stem Cells, Volume 36, Issue 5, May 2018, Pages 641–654.

Thomas T. Huynh, Mohammad Sultan, Dejan Vidovic, Cheryl A. Dean, Brianne M. Cruickshank, Kristen Lee, Chao-Yu Loung, Ryan W. Holloway, David W. Hoskin, David M. Waisman, Ian C. G. Weaver, Paola Marcato. Retinoic acid and arsenic trioxide induce lasting differentiation and demethylation of target genes in APL cells. *Sci Rep* **9,** 9414 (2019).

Contribution statement

I prepared the first manuscript and its figures for publication with the guidance of my supervisor Dr. Paola Marcato. Dejan Vidovic, Simmone Paine, Thomas Huynh, Krysta Coyle, Margaret Thomas, Brianne Cruickshank, Cheryl Dean, Derek Clements, Youra Kim, Kristen Lee, Dr. Shashi Gujar and Dr Ian Weaver supported the writing, editing and the revision of the manuscript. Kirsten Lee and Dr. Ian C. G. Weaver performed the DNA bisulfite pyrosequencing.

Thomas Huynh and my self equally contributed to the preparation of the second manuscript and its figures for publication with the guidance of our supervisor Dr. Paola Marcato. Dejan Vidovic, Cheryl Dean, Brianne Cruickshank, Kristen Lee, Chao-Yu Loung, Ryan Holloway, Dr. David W. Hoskin, Dr. David M. Waisman and Dr. Ian C. G. Weaver contributed to data generation, and supported the writing, editing and the revision of the manuscript. Kirsten Lee and Dr. Ian C. G. Weaver performed the DNA bisulfite pyrosequencing.

2.1 Cell Lines and Vectors

Different cell lines used in this body of work were either obtained from the American Type Culture Collection (ATCC), DSMZ-German Collection of Microorganisms and Cell Cultures GmbH or kindly provided from other labs (Table 2.1). Cell lines were cultured in recommended growth media (Table 2.1) at 37°C in a 5% CO_2 humidified incubator. shRNA knockdown clones were generated using the lentiviral vectors with either the shRNA scramble sequence or shRNA sequences specific to the target gene (Tables 2.2) transfected along with packaging plasmids into 293T cells following procedure highlighted in Figure 2.1. shRNA containing lentivirus particles were applied to the parental cell line and the selection of stable transfectants commenced two days post with the addition of cell line specific selection dose of puromycin (Sigma-Aldrich) (Table 2.2, Fig.2.2). Clones were then maintained in their appropriate growth medium supplemented with specific maintenance dose of puromycin. Knockdown efficiencies of target genes were evaluated by quantitative polymerase chain reaction (QPCR) using gene-specific primers (Tables 2.8 and 2.9).

2.2 In vitro Treatment Protocols

Different cell lines used in this body of work were treated with different therapeutic agents (Table 2.3) using a cell line-drug specific treatment schedule to investigate different mechanisms associated with treatment resistance. Different reagents were obtained and stored per manufacture recommendations. Decitabine (Sigma Aldrich) was obtained and suspended in dimethyl sulfoxide (DMSO, Millipore Sigma) in a 100µM aliquots and stored at -80^{o}C for no longer than 1 month.

Paclitaxel 6 mg/ml suspensions in Cremophor EL oil (Biolyse) were obtained and stored in 50µL aliquots at room temperature for no longer than 3 months. BCL6 inhibitor (BCL6i) (79-6 – Calbiochem, Millipore Sigma) was obtained and suspended in DMSO in 50µL (50 mg/ml) aliquots and stored at -20^{o}C for no longer than 3 months.

ATRA (Sigma-Aldrich) was dissolved in DMSO as a 100mM stock solution and ATO (Sigma-Aldrich) was dissolved in NaOH as a 100mM stock solution and stored in aliquots at -80^{o}C. Prior to use, they were serially diluted in media to working concentrations.

Cell line	Growth media	Chapter	Source
MDA MB 231	Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1X Antibiotic-Antimycotic (AA)	4	ATCC
MDA MB 468	DMEM with 10%FBS and 1XAA	4	ATCC
HEK293T	DMEM with 10%FBS and 1XAA*	3-4	ATCC
4T1	RPMI-1640 Medium with 10%FBS and 1XAA	3	ATCC
NB4	RPMI-1640 Medium with 10%FBS and 1XAA	5	DSMZ
NB4-MR2	RPMI-1640 Medium with 10%FBS and 1XAA	5	Dr. Wilson Miller Jr., McGill University, Montreal, QC

Table 2.1. Cell lines used in results chapters. Growth conditions and sources of different cell lines used in this body of work. DMEM, FBS and AA (ThermoFisher). Puromycin (Sigma Aldrich). * HEK293T cells were maintained in without the addition of AA in the media during lentivirus preparation.

Target gene	Cell line	shRNA	Vector	Chapter	Puromycin selection dose	Puromycin maintenance dose
TAP1	4T1	shRNA-1: TRCN0000066352	PLKO.1	3	5µg/ml	1.5µg/ml
TAP1	4T1	shRNA-2: TRCN0000066349	PLKO.1	3	5µg/ml	1.5µg/ml
BCL6	MDA MB 231	shRNA-1: V2HS_271606	GIPZ	4	1.5µg/ml	0.25µg/ml
BCL6	MDA MB 231	shRNA-2: V3LHS_404721	GIPZ	4	1.5µg/ml	0.25µg/ml

Table 2.2. Lentiviral vectors used to create gene specific knockdown clones in the results chapters. PLKO.1 vectors were obtained from (Dharmacon) and GIPZ vectors were obtained from Enhanced Gene Analysis and Discovery (EGAD) Core Facility at Dalhousie University.

Cell line/clone	# cells seeded	Treatment	Treatment schedule	Chapter
4T1	1*10^4	Decitabine	1 μM starting 24 hours post seeding and lasting for 72 hours (treatment media replaced every 24 hours)	3
4T1 TAP1 knockdown	1*10^4	N/A	No treatment- proliferation assay	3
MDA MB 231 (unaltered, scrambled control, BCL6 knockdown)	2*10^4	Paclitaxel*	7.5 nM starting 24 hours post seeding and lasting for 24 hours	4
MDA MB 468	2*10^4	Paclitaxel*	3.75 nM starting 24 hours post seeding and lasting for 24 hours	4
MDA MB 231, MDA MB 468	2*10^4	BCL6i *	50 μM starting 24 hours post seeding and lasting for 72 hours (treatment media replaced after paclitaxel treatment termination in combination therapy wells)	4
NB4 and NB4 MR2	5*10^5 cell/ml	ATRA**	1 μM for 72 hours or 168 hours	5
NB4 and NB4 MR2	5*10^5 cell/ml	ATO**	0.5 or 1.5 μM for 72 hours or 168 hours	5

Table 2.3. Treatments outlines for different *in vitro* experiments in the results chapters. All experiments contained a no treatment vehicle condition in addition to treatment conditions highlighted above. * Paclitaxel and BCL6i combination therapy used the individual concentration for each drug. ** ATRA and ATO combination therapy used the individual concentration for each drug.

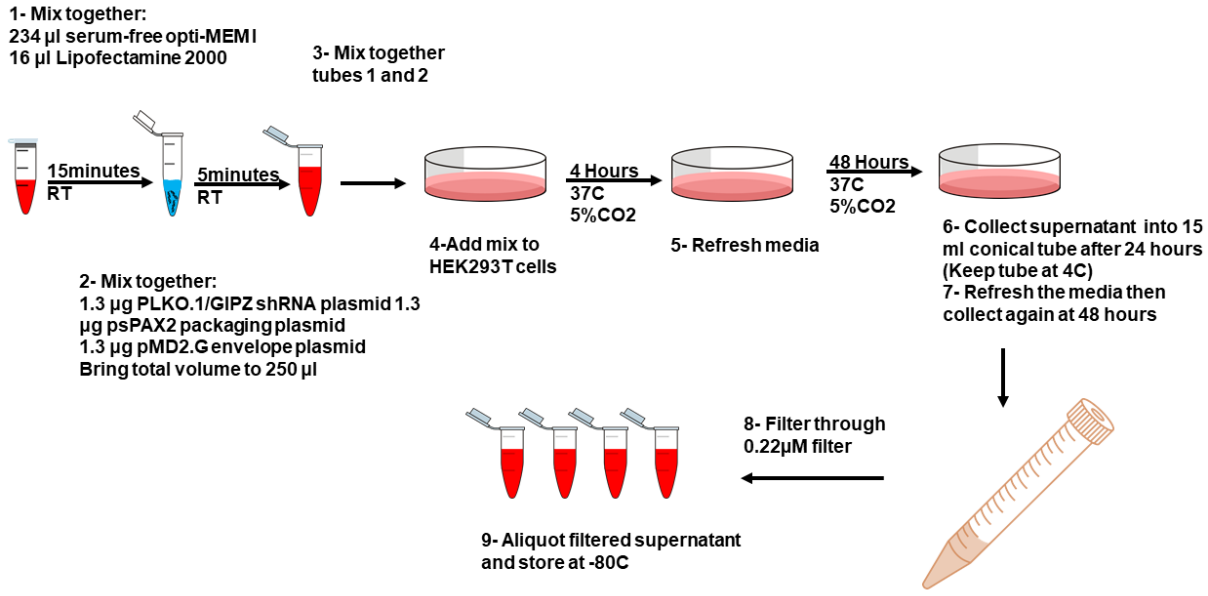


Figure 2.1. Lentiviral vectors preparation protocol for knockdown studies. shRNA vectors are acquired from different suppliers (Table 2.2). Packaging plasmids psPAX2 and pMD2.G are acquired from Dharmacon.

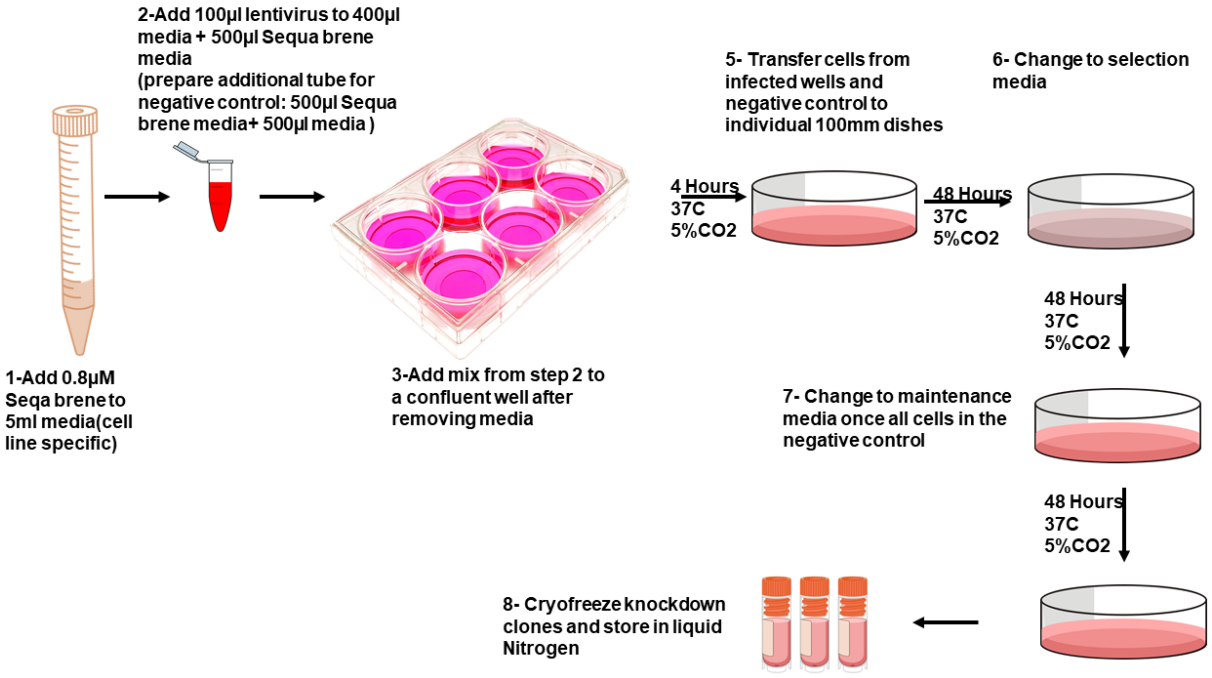


Figure 2.2. Knockdown clone preparation using lentiviral vectors. Sequa brene was obtained from Sigma-Aldrich.

2.3 Tumor Models

All animal studies detailed in this body of work (Table 2.4) have been conducted in accordance with the ethical standards set by the Declaration of Helsinki and the Canadian Council on Animal Care (CCAC) standards and a protocol approved by Dalhousie University Committee on Laboratory Animals.

Tumor volumes were calculated for the duration of the experiments (Table 2.5) using calipers to measure the dimensions of the tumors and the volume formula (tumor volume = length*width* height/2). Once tumor/humane end points were reached in at least one mouse, experiments were terminated, and the tumor tissue harvested and weighed.

Different tumor models were either investigated in tumorigenicity studies or treated with different therapeutic agents (Table 2.5) using a tumor-drug specific treatment schedule to investigate different mechanisms associated with treatment resistance. Paclitaxel 6 mg/ml suspensions in Cremophor EL oil (Biolyse) were obtained and stored in 350µL aliquots at room temperature for no longer than 3 months. The BCL6i was suspended in DMSO in 350µL (50 mg/ml) aliquots and stored at -20^{o}C for no longer than 3 months.

Tumor origin	Chapter	Injected /Implanted	In vivo model	Study type
Spontaneous mammary tumor in a NOD/SCID mouse	3	Injected	NOD/SCID	Tumorigenicity: 5 000 or 50 000 Aldefluor+ or Aldefluor - cells
Spontaneous mammary tumor in a NOD/SCID mouse	3	Implanted	NOD/SCID BALB/C	Equal 2 mm^3 pieces sterilely implanted. Resulting tumors were sorted for characterization and detection infiltrating cells
4T1	3	Injected	BALB/C	Tumorigenicity: Aldefluor+ and – (100, 1 000 or 10 000 tumor cells)
4T1 (TAP1 knockdown and scrambled control)	3	Injected	BALB/C	Study the effect of TAP1 knockdown on tumor growth kinetics (3000 cells)
Genome-wide shRNA screen	4	Injected	NOD/SCID	Identify novel effectors in paclitaxel response
MDA MB 231 (BCL6 knockdown and scrambled control) and (BCL6i studies)	4	Injected	NOD/SCID	Study the effect of inhibiting BCL6 on paclitaxel induced tumor regression (2*10^6 cells)

Table 2.4. *In vivo* study parameters described in the results chapters. Cells and tumor pieces were either injected (admixed 1:1 with Matrigel (BD Bioscience)) or implanted into mammary fat pad number four of the mouse model used in the experiment. Numbers highlighted above represent cell numbers injected per mouse.

Study	Number of mice	Treatment protocol/Tumor measurement schedule	Experiment ended
Spontaneous mammary tumor in a NOD/SCID mouse tumorigenicity study	3 mice per group	N/A	Day 21 post cell injection*
Spontaneous mammary tumor in a NOD/SCID and BALB/C mice. Tumor composition and infiltration study	4 mice per group	N/A	Day 14 post tumor implantation*
4T1 tumorigenicity study	4 mice per group	N/A	Day 21 post cell injection*
4T1 TAP1 knockdown studies	8 mice per group	Tumor volumes recorded every 3 days starting at day 7	Day 24 post cell injection*
Genome wide shRNA screen in MDA MB 231 cells	6 mice per group	Treatment started day 24 post cell injections** (8 days daily injections) Paclitaxel: 10 mg/Kg	Day 32 post cell injection
MDA MB 231 paclitaxel studies	12 mice per treatment group	Treatment started day 21 post cell injections** (7 days daily then 14 days every second day) Paclitaxel: 7.5 mg/Kg BCL6i: 50 mg/Kg Tumor volumes recorded every 5 days starting at day 21	Day 42 post cell injections

Table 2.5. Treatment protocols for *in vivo* studies described in results chapters. * experiment ended when at least one mouse reached humane end point. ** No treatment group received Phosphate-buffered saline (PBS) injection of equal volumes on the same treatment schedule.

2.4 Aldefluor Sorting of a Spontaneous Murine Mammary Tumor and 4T1 Cells (Chapter 3)

A spontaneous mammary tumor originating from a female NOD/SCID mouse was harvested and sorted for tumorigenicity studies based on Aldefluor activity (Aldefluor assay kit, Stemcell Technologies, Vancouver, BC, Canada). Prior to sorting with a FACSAria (BD Pharmingen), the tumor was minced and digested with a 225U/mL of collagenase III (Bioshop) for one hour at 37ºC with rotation. After straining with 70µM filter for the removal of undigested tissue, the red blood cells were lysed with red cell lysis buffer and remaining cells washed with phosphate buffered saline before resuspension in the Aldefluor buffer, and staining BODIPY® – aminoacetaldehyde (BAAA) substrate and allophycocyanin (APC) conjugated mouse specific lineage antibodies (anti-CD45.2, anti-CD31, anti-CD140b, Biolegend). Cells were stained with viability dye 7-Aminoactinomycin D (7-AAD) (Biolegend) to discard dead cells. Side scatter (SSC) and forward scatter (FSC) were used to eliminate debris. The inclusion of a control sample with the addition of ALDH inhibitor diethylaminobenzaldehyde (DEAB) was added to verify that an Aldefluor+ population of cells had been identified, as per the manufacturer's instructions (Stemcell Technologies). Similarly, harvested single cell suspensions of 4T1 were sorted based on Aldefluor activity; however, the mouse specific lineage antibodies were not included.

2.5 Flow Cytometry Tumor Composition Studies in NOD/SCID Versus BALB/C Mice

After mice were euthanized, tumors were harvested, weighed and processed for analysis with a FACSCalibur (BD Pharmingen), by mincing and straining. Cell suspensions were lysed of red blood cells, washed and stained for percentage live cells by 7-AAD, percentage of cells of leukocyte, myeloid, and endothelial origin (lineage markers) with APC conjugated anti-CD45.2, anti-CD31, anti-CD140b, and percentage of Aldefluor+ cells as described above.

2.6 Patient Data Analysis

2.6.1 Gene Expression Analysis of Microarray Data

Genome wide expression raw data files were obtained from the Gene Expression Omnibus (GEO) for studies highlighted in Table 2.6. Raw data was normalized using Affymetrix Expression Console and then processed using Affymetrix Transcriptome Analysis Console. Dataset GSE54326 was analyzed using the GEO2R platform. Fold changes in gene expression between groups were calculated and reported. P values were calculated using repeated measure one-way ANOVA paired test or unpaired t-test.

2.6.2 cBioportal Data Analysis (Chapter 3)

Data was extracted from TCGA, Cell 2015 dataset (Ciriello, M. L. Gatza, et al. 2015) to determine the correlation between TAP1 expression and methylation at site cg16890093.

2.6.3 Survival Analysis (Chapter 4)

Survival data was extracted and analyzed using the KMPlotter breast cancer platform by comparing the survival based on individual gene expression. Patients in the high and low expression group are classified based top vs bottom thirds in expression values for a given gene respectively. Patients that underwent systematic chemotherapy treatment were selected in the analysis cohort. Patients that underwent endocrine therapy were excluded from the analysis cohort.

Study	Groups compared	Origin and umber of replicates	Chapter
GSE7513	CD44+CD24- versus CD44-CD24+	cells isolated from 14 breast cancer patients	3
GSE52327	Aldefluor+ versus Aldefluor- cells	Cells isolated from 8 breast cancer patients	3
GSE54326	Epirubicin sensitive and resistant breast cancer cell lines	3 replicates of each group in MDA MB 231, MCF7 and SKBR3 cell lines	4
GSE90062	AML cells versus healthy donors' bone marrow cells	Cells isolated from 3 AML patients and 3healthy donors bone marrow	5
GSE73157	NB4 cells cultured alone or cocultured with bone marrow cells	3 replicates	5
GSE115812	ATO sensitive and resistance NB4	1 replicate of ATO sensitive NB4 3 replicate of ATO resistant NB4	5
GSE68844	Hematopoietic progenitor cells isolated from wild type and DNMT3A -/- mice	6 replicates per group	5

Table 2.6. Geo datasets analyzed in results chapters.

2.7 Genome-Wide shRNA Screen (Chapter 4)

MDA MB 231 cells were previously transduced with a pool of concentrated lentiviral particles containing 20,862 shRNAs targeting genes with well-characterized biological functions (Dharmacon) by my supervisor Dr. Paola Marcato. Transduction efficiency was confirmed using Thermo Scientific Decode RNAi Pooled Lentiviral shRNA Screening Libraries protocol (ThermoFisher). The genome-wide RNAi transduced MDA MB 231 cells were then injected in NOD/SCID mice as detailed in table 2.4 and treated with paclitaxel (Table 2.5). Following treatment termination, tumor tissue was harvested and genomic DNA (gDNA) was isolated from control and treatment tumors. The gDNA was then amplified using polymerase chain reaction (PRC) to amplify the molecular barcodes of the screen shRNAs. The purified DNA was then labeled with fluorescent dye-labeling Cy3 and Cy5 for control and treatment group respectively. Next we purified the labelled DNA to remove unbound dye and asses labeling efficiencies. Purified labeled DNA from control tumors and treated tumors were then hybridized and processed on six Custom Decode Agilent 2x105K microarrays (Agilent's Technologies). Data was processed through Agilent's Feature Extraction software version 11.5.1.1 using the protocol Neg_Sel_2009 and the grid file 020719_D_F20080627_clean and normalized gene expression values is supplied for each sample. Samples were normalized using the percentile shift algorithm. No baseline transformation was performed.

2.8 Quantitative PCR

RNA was extracted from isolated samples highlighted in Table 2.7, using TRIzol (Invitrogen) combined with the Purelink RNA kit (Invitrogen) and reverse transcribed with iScript™ cDNA Synthesis Kit (Bio-Rad) as per manufacturer's recommended protocols. QPCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) and gene-specific primers (Tables 2.8 and 2.9) as per manufacturer's recommended protocol using a 96CFX and a 384CFX Touch Real-Time PCR Detection System (Bio-Rad). Standard curves were generated for each primer set, and primer efficiencies were incorporated into the CFX Manager software (Bio-Rad). mRNA expression of all samples was calculated relative to reference genes (Table 2.7) and normalized to respective controls.

Samples	Reference gene(s)	Chapter
Isolated Aldefluor+ and – sorted samples	glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	3
Decitabine treated samples	GAPDH	3
4T1 TAP1 knockdowns and scrambled control	GAPDH	3
MDA MB 231 BCL6 Knockdown and scrambled control (Paclitaxel experiments: 24 hours post treatment and 72 hours post treatment termination)	Pumilio RNA Binding Family Member 1 (PUM1) and ADP-ribosylation factor (ARF1)	4
MDA MB 231 and MDA MB 468 (Paclitaxel and BCL6i experiment: 24 hours post treatment and 72 hours post treatment termination)	PUM1 and ARF1	4
NB4 and NB4 MR2 (ATRA and ATO experiments: 72 hours post treatment and 96 hours post treatment termination)	Hypoxanthine-guanine phosphoribosyl transferase (HRPT1) and TATA-binding protein (TBP)	5

Table 2.7. Samples investigated by QPCR in the different results chapters.

Primer	Direction	Sequence	Chapter
GAPDH	Forward	GGAGTCAACGGATTTGGTCGTA	3
	Reverse	TTCTCCATGGTGGTGAAGAC	3
TAP1	Forward	CCCAGTGGTCTGTTGACTCC	3
	Reverse	AGCACTAAGACATCTGGGCG	3
TAP2	Forward	TCTCAGGGAGACAGTCAGGG	3
	Reverse	CTCTGCAGGGGGTTGTGAAA	3
TAPBP	Forward	ACAGCGTAGGCCTTTTCCTG	3
	Reverse	CTTGCAGGTGGACAGGTAGAC	3
B2M	Forward	AGGCTATCCAGCGTACTCCA	3
	Reverse	CGGATGGATGAAACCCAGACA	3
CD80	Forward	GCAGGGAACATCACCATCCA	3
	Reverse	TCACGTGGATAACACCTGAACA	3
CD86	Forward	ACACGGTTACCCAGAACCTAAG	3
	Reverse	ACGTCGTACAGTTCTGTGACAT	3
STAT3	Forward	GCCAGCAAAGAGTCACATGC	3
	Reverse	GACTCTTGCAGGAATCGGCT	3
CXCR4	Forward	GCCCTCCTGCTGACTATTCC	3
	Reverse	CATTGGGGTAGAAGCGGTCA	3
HAS2	Forward	TTTGCCTTTTTGGAGCACCG	3
	Reverse	GATAGGCAGCGATGCAAAGG	3
MICB	Forward	TGCTTCAGAGTCAACGGACAG	3
	Reverse	TCTTGCAACAAGGGACACAGA	3
BCL6	Forward	GCCTCCTCGTGAAGAGTTCC	4
	Reverse	TTGTTCTCCACCACCTCACG	4
CDKN1A (P21)	Forward	GCGACTGTGATGCGCTAATG	4
	Reverse	GAAGGTAGAGCTTGGGCAGG	4
ARF1	Forward	GTGTTCGCCAACAAGCAGG	4
	Reverse	CAGTTCCTGTGGCGTAGTGA	4
PUM1	Forward	GGCGTTAGCATGGTGGAGTA	4
	Reverse	CATCCCTTGGGCCAAATCCT	4
CDK4	Forward	GGAAACTCTGAAGCCGACCA	4
	Reverse	GCAGGGATACATCTCGAGGC	4
CCND1	Forward	TGAGGAGCCCCAACAACTTC	4
	Reverse	CCGGGTCACACTTGATCACT	4
CDK1	Forward	GGAAGGGGTTCCTAGTACTGC	4
	Reverse	TCCTGCATAAGCACATCCTGA	4
CCNB1	Forward	AGGCGAAGATCAACATGGCA	4
	Reverse	AGCTGTTCTTGGCCTCAGTC	4

Primer	Direction	Sequence	Chapter
CDKN1B	Forward	CCCCTAGAGGGCAAGTACGA	4
	Reverse	GCGGGGGTCTGTAGTAGAAC	4
CDKN2A	Forward	CAGAGGCAGTAACCATGCCC	4
	Reverse	CTCAGAGCCTCTCTGGTTCTTTC	4
TP53	Forward	CAGATCCGTGGGCGTGAG	4
	Reverse	CTGGGCATCCTTGAGTTCCAA	4
TGM2	Forward	GAGCGAAGGGACGTACTGC	5
	Reverse	GACAAAGGGCGCATCGTACTT	5
RARβ	Forward	TTCTCAGACGGCCTTACCCT	5
	Reverse	GCTGGTTGGCAAAGGTGAAC	5
CCL2	Forward	GAAAGTCTCTGCCGCCCTT	5
	Reverse	GGGGCATTGATTGCATCTGG	5
ASB2	Forward	GAGCCGGACATCTCCAACAA	5
	Reverse	CACCAGAATCTTCACGGCCT	5
IER3	Forward	CAGAGGACGCCCCTAACG	5
	Reverse	TGTTGCTGGAGGAAAGTGCT	5
PRTN3	Forward	TGCCGGCCACATAACATTTG	5
	Reverse	CCCCAGATCACGAAGGAGTC	5
RPL7a	Forward	CAAAAGAGACCTCACCCGCT	5
	Reverse	CAAAAGAGACCTCACCCGCT	5
RARα	Forward	GTGTCACCGGGACAAGAACT	5
	Reverse	CGTCAGCGTGTAGCTCTCAG	5
RAB33A	Forward	GCTGGTTGGCAAAGGTGAAC	5
	Reverse	GCTGGTTGGCAAAGGTGAAC	5
NDUFB10	Forward	CTACTACCACCGGCAGTACC	5
	Reverse	TCTTCCACTGCATTTCGGCT	5
NCL	Forward	GCTGGTTGGCAAAGGTGAAC	5
	Reverse	GCTGGTTGGCAAAGGTGAAC	5
MPO	Forward	CGCCAACGTCTTCACCAATG	5
	Reverse	CATGGGCTGGTACCGATTGT	5
HIST1H2BK	Forward	ACCTCCAGGGAGATCCAGAC	5
	Reverse	TGTACTTGGTGACGGCCTTG	5
TBP	Forward	GGCAACCACTCCACTGTATCC	5
	Reverse	GCTGCGGTACAAATCCCAGAA	5
HPRT1	Forward	GACCAGTCAACAGGGGACAT	5
	Reverse	CCTGACCAAGGAAAGCAAAG	5

Table 2.8 Primer sequences used to investigate human genes expression in the results chapters.

Gene	Primer direction	Sequence	Chapter
GAPDH	Forward	GCGAGACCCCACTAACATCA	3
	Reverse	GGCGGAGATGATGACCCTTT	3
TAP1	Forward	CTGTTCAGGTCCTGCTCTCC	3
	Reverse	CCACAAGGCCTTTCATGTTT	3
TAP2	Forward	GCTGTGGGGACTGCTAAAAG	3
	Reverse	GCAGAAGCCACTCGGACTAC	3
TAPBP	Forward	CTGGGAATGGGACCTTCTGG	3
	Reverse	GTAGGGCAGGTGTACCGTAG	3
B2M	Forward	ATGGGAAGCCGAACATACTG	3
	Reverse	CAGTCTCAGTGGGGGTGAAT	3
CD80	Forward	CAAAGCCTCGCTTCTCTTGG	3
	Reverse	AGGATCCTGGGAAATTGTCGT	3
CD86	Forward	ATGGACCCCAGATGCACCA	3
	Reverse	ACAGCATCTGAGATCAGCAAGAC	3
H2D	Forward	GAGTGAGCCTGAGGAACCTG	3
	Reverse	AGCCAGACATCTGCTGGAGT	3
STAT3	Forward	GAGCTGCACCTGATCACCTT	3
	Reverse	GGAGATCACCACAACTGGCA	3
CXCR4	Forward	ATGGAACCGATCAGTGTGAGT	3
	Reverse	AAGCAGGGTTCCTTGTTGGA	3
HAS2	Forward	CTTCCTCAGCAGCGTGAGAT	3
	Reverse	CAGAGGACCGCTTATGCACT	3
MICB	Forward	GCCCTTGGGATGCTGGATTA	3
	Reverse	TCCCCCTGAGTTTCTCTTCCA	3

Table 2.9 Primer sequences used to investigate mouse genes expression in Chapter 3.

2.9 Chromatin Immunoprecipitation (ChIP)/re-ChIP (Chapter 3)

Assays were performed following the ChIP assay kit protocol [49] (Cat#06-599, Upstate Biotechnology). Mouse 4T1 cells (n = 3 samples/treatment) were fixed with 4% paraformaldehyde and then the resultant cross-linked protein–DNA complexes were sonicated followed by immunoprecipitation using antibodies against 5mC (Catalog number BI-MECY-0500, AnaSpec, Inc.), NF-kB (Catalog C15310256, Diagenode) as well as the control normal rabbit IgG (Catalog number sc-2027, Santa Cruz Biotechnology). After dissociating the DNA-protein complexes, pulled-down DNA along with the input DNA (devoid of antibody) were subjected to QPCR analysis using the SsoFast™ EvaGreen® Supermix and primers to interrogate the TAP1 promoter region NF-κB response element (GenBank: D14566.1; 1844-1981) using gene -specific primers (Table 2.10). The specificity of the amplified PCR products was assessed by melt curve analysis. Results are expressed as the amount of DNA detected in immunoprecipitated fraction minus the amount of DNA detected in the non-immune IgG (negative control) fraction normalized to the input DNA. For sequential ChIP (ChIP–reChIP) experiments (with both 5mC and NF-kB antibodies), the protein bound to the beads with the first antibody was incubated (30 min, 37 ºC) twice with Dithiothreitol (DTT) (20 mM) and the combined elutes were suspended in ChIP dilution buffer, which was then immunoprecipitated (14 h, 4 ºC) with the second antibody. Only DNA sequences that bind both proteins concurrently are detected by this assay.

2.10 Chromatin Immunoprecipitation-Quantitative PCR (ChIP-qPCR) (Chapter 5)

At 72h and 96h post treatment termination timepoints, treated NB4 cells were fixed with 1% formaldehyde and the resulting crosslinked protein–DNA complexes were sonicated using a Q800R2 sonicator (QSonica) into 150-250 bp length fragments confirmed using a QIAxcel Advanced System Bioanalyzer. Immunoprecipitation was then performed using rabbit IgG (Diagenode, C15410206) and ChIP-grade rabbit polyclonal IgG antibodies from Diagenode against human H3K9/14ac, H3K9me3 and H3K27me3 (C15410200-10, C15410193-10, C15410195-10). Post immunoprecipitation, the precipitated DNA-protein complexes were dissociated from the protein A conjugated Dynabeads (Invitrogen, Thermo Fisher) and isolated using a Purelink PCR purification kit (Invitrogen, Thermo Fisher) along with their respective total input controls (pre-immunoprecipitation DNA). QPCR analysis was then performed using primers targeting the promoter regions of RARβ and TGM2 (Table 2.10) with equipment and reagents described above. Results are expressed as the fold change of the enrichment of the DNA detected under the treatment conditions against the DNA detected under the no treatment conditions. This was determined by dividing the signals obtained from the ChIP by the signals obtained from the total input control sample and normalizing for the DNA detected by the non-immune IgG (negative control).

Gene	Primer direction	Sequence
TAP1	Forward	GCGCTGCACAGGAGTCT
	Reverse	GTCGGCTTTCGGTTTCTTCTTC
TGM2	Forward	CTTCACCGAGCCTCAGTTTC
	Reverse	GATAAGCCCCAGAGGTCACA
RARβ	Forward	GGGAGAGAAGTTGGTGCTCAA
	Reverse	CACAAGCCGGCGTTTTCTTT

Table 2.10 Primers sequences used in the chromatin immunoprecipitation assays.

2.11 Analysis of DNA Methylation by Pyrosequencing

Using a Purelink gDNA mini kit (Invitrogen Thermo Fisher) and following the manufacturer's instructions, genomic DNA was extracted from Aldefluor sorted spontaneous tumor cells and 4T1 cells (chapter 3), decitabine treated 4T1 cells (chapter 3) and treated NB4 cells at 72h and 96h post treatment termination timepoints (chapter 5). DNA-methylation was analysed by sodium bisulfite pyrosequencing on a PyroMark Q24 Advanced pyrosequencer using the DNA EpiTect Fast DNA Bisulfite Kit and PyroMark PCR Kit (Qiagen N.V, Venlo, Netherlands) according to the manufacturer's instructions beginning with 500 ng template DNA. Bisultfie treatment converts unmethylated cytosine groups to uracil while leaving 5-methyl cytosine residue unaffected. These changes in the DNA sequence of interest can then be detected using PCR. To this end, five custom assays covering the TAP1/LMP2 (GenBank: D14566.1; 1881-2200), the TAP2 (GenBank: AF307513.1; 2914-3077; exon1 underlined), LINE-1, RARβ and TGM2 promoters were designed using PyroMark Assay Design software (v2.0; Qiagen N.V, Venlo, Netherlands) and validated to amplify single PCR products (TAP1 = 319 nt; TAP2 = 163 nt LINE-1 = 400nt, RARβ = 400nt, TGM2 = 428nt) using primer sequences listed in Table 2.11. PCR conditions for both assays: 95°C, 15 min; (94°C,30s; 56°C, 30s; 72°C, 30s) x 50 cycles; and 72°C, 10 min.

2.12 Cell Proliferation Assay

Different cell lines were seeded and treated as indicated in Table 2.3. Cells were then counted at different time points (Table 2.12) using Trypan Blue exclusion cell viability staining (ThermoFisher).

Gene/ Chapter	Primer direction	Sequence	Sequencing primer
TAP1/ Chapter 3	Forward	(Biotin)GTGGGGAAGGAAGAAGGG	ATCTACCCAAAAAC AAATAAC
	Reverse	ACAAACCTAAACAAAACAAATCTACC	
TAP2/ Chapter 3	Forward	TTGTTTTGTTTTGGTTTTTTTTGGTTTTGT	AGTATTTTAGATTTT TAAGAGTTT
	Reverse	(Biotin) AATTCTATCCAAAAAAACTCACC	
TGM2/ Chapter 5	Forward	TTGGTGTTTTTTTTTTT TGTTGATGAG	TTGTTGATGAGGTGG
	Reverse	(Biotin)TCTCCTCCTCCCTAAACAAAAT	
RARβ/ Chapter 5	Forward	GTTAAAGGGGGGATTAGAATTT	GGGGGATTAGAATTTT TTTAT
	Reverse	(Biotin)AACTCTACCCCTTTTTTAACA	
LINE1/ Chapter 3	Forward	AGGGAGAGTTAGATAGTG	GGAGAGTTAGATA GTGG
	Reverse	(Biotin)AACTATAATAAACTCCACCC	

Table 2.11 Primers sequences for bisulfite pyrosequencing in Chapters 3 and 5.

Cell line/clone	Treatment	Counting schedule	Chapter
4T1 TAP1 knockdown	N/A	24, 48 and 72 hours post seeding	3
MDA MB 231 (unaltered, scrambled control, BCL6 knockdown)	Paclitaxel*	24 hours post treatment (48 hours post seeding) 72 hours post treatment termination (120 hours post seeding)	4
MDA MB 231, MDA MB 468	Paclitaxel +/- BCL6i *	24 hours post treatment (48 hours post seeding) 72 hours post treatment termination (120 hours post seeding)	4

Table 2.12 Cell proliferation assay schedules used in Chapters 3 and 4. Individual and combination therapies used the individual concentration for each drug highlighted in Table 2.3.

2.13 Cell Cycle Analysis (Chapter 4)

MDA MB 231 cells with control vector and BCL6 targeting shRNA were treated with paclitaxel as previously described. MDA MB 231, MDA MB 468 cells were treated with paclitaxel and BCL6i as previously mentioned. Cells from treatment and no treatment wells were collected at 24 hours post treatment as well as 3 days post treatment termination using 0.05% trypsin (GIBCO) and washed with PBS (ThermoFisher). The cells were suspended in 50 µl PBS, mixed with 450 ul 70% ethanol and fixed at -20 C for 48 hours. The samples were then washed with PBS (ThermoFisher) and stained with 1% propidium iodide stain. Samples were processed using the BD FACS Canto II analyzer and then analyzed using ModFit analysis software (Verity software house).

2.14 Flow Cytometry Apoptosis Analysis (Chapter 4)

Scrambled control and BCL6 shRNA knockdown MDA MB 231 clones were treated with either paclitaxel as previously mentioned and collected at the 24 hours post treatment and 72 hours post treatment termination timepoints for flow cytometry apoptosis analysis. Cells were collected using 0.05% trypsin (GIBCO) and washed with PBS (ThermoFisher). Samples were then suspended in blocking buffer consisting of PBS supplemented with 1% FBS and 1% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) and incubated with 3 µL Alexafluor 647 conjugated antibody (Invitrogen, ThermoFisher) in 100 µL annexin binding buffer for 30 min at 4 °C. The staining was then removed, and the cells were resuspended in annexin binding buffer with 7-AAD diluted to 1/50. Samples were processed using the BD FACS Canto II analyzer to detect the percentage of live cells (annexin V- and 7-

AAD-) apoptotic cells (annexin V+) apoptotic and necrotic cells (annexin V+ and 7-AAD+) and dead cells (7-AAD+ only). The flow cytometry data was analyzed using FCS Express 4 Research Edition software (De Novo Software).

2.15 Flow Cytometry Analysis of APL Cells (Chapter 4)

Treated NB4 cells were collected at 3 timepoints for flow cytometry analysis (72h, 168h and after 96h post treatment for 72h). Cells were collected by centrifugation (500xg) and each sample was then washed in PBS and suspended in blocking buffer consisting of PBS supplemented with 1 % FBS and 1% EDTA and incubated with 3µL Alexafluor 488 conjugated anti-human CD11b monoclonal mouse antibody (clone M1/70.15, Invitrogen, ThermoFisher) in 100µL of PBS containing 1% FBS, 1% EDTA for 30 min at 4°C. Afterwards, the cells were centrifuged at 500xg and resuspended in PBS containing 1% FBS, 1% EDTA and 7-AAD diluted to 1/50. A fluorescence activated cell sorter (FACS) Calibur (BD Pharmingen) was then used to detect the percentage of differentiated cells (CD11b+ only or CD11b+ and 7-AAD+) and dead undifferentiated cells (7-AAD only). The flow cytometry data was analyzed using FCS Express 4 Research Edition software

2.16 Statistical Analysis (Chapters 3,4 and 5)

All statistical analyses were performed with GraphPad Prism Version 7. ANOVA (one-way analysis of variance or repeated measures) was performed followed by post-tests Dunnett or Bonferroni (specified in the figure legends) when multiple comparisons were made. Significant p values are represented as follows: * <0.05, ** <0.01 and *** <0.001.

CHAPTER 3

Copyright statement

This chapter have been previously published in the following manuscript. The corresponding text has been edited for length, consistency, and to include recent findings.

Mohammad Sultan, Dejan Vidovic, Simmone A Paine, Thomas T Huynh, Krysta M Coyle, Margaret Lois Thomas, Brianne M Cruickshank, Cheryl A Dean, Derek R Clements, Youra Kim, Kirsten Lee, Shashi Gujar, Ian CG Weaver, Paola Marcato. Epigenetic Silencing of TAP1 in Aldefluor+ Breast Cancer Stem Cells Contributes to Their Enhanced Immune Evasion. Stem Cells, Volume 36, Issue 5, May 2018, Pages 641–654.

Contribution statement

I prepared this manuscript and its figures for publication with the guidance of my supervisor Dr. Paola Marcato. Dejan Vidovic, Simmone Paine, Thomas Huynh, Krysta Coyle, Margaret Thomas, Brianne Cruickshank, Cheryl Dean, Derek Clements, Youra Kim, Kristen Lee, Dr. Shashi Gujar and Dr Ian Weaver supported the writing, editing and the revision of the manuscript. Kristen Lee, and Dr Ian Weaver performed the pyrosequencing and methylation analyses.

3.1 Abstract

Avoiding detection and destruction by immune cells is key for tumor initiation and progression. The important role of CSCs in tumor initiation has been well established, yet their ability to evade immune detection and targeting is only partly understood. To investigate the ability of breast CSCs to evade immune detection, we identified a highly tumorigenic population in a spontaneous murine mammary tumor based on increased aldehyde dehydrogenase activity. We performed tumor growth studies in immunocompetent and immunocompromised mice. In immunocompetent mice, growth of the spontaneous mammary tumor was restricted; however, the Alde+ population was expanded, suggesting inherent resistance mechanisms. Gene expression analysis of the sorted tumor cells revealed that the Aldefluor+ tumor cells has decreased expression of TAP genes and co-stimulatory molecule CD80, which would decrease susceptibility to T cells. Similarly, the Alde+ population of patient tumors and 4T1 murine mammary cells had decreased expression of TAP and co-stimulatory molecule genes. In contrast, breast CSCs identified by CD44+CD24- do not have decreased expression of these genes but do have increased expression of CXCR4. Decitabine treatment and bisulfite pyrosequencing suggests that DNA hypermethylation contributes to decreased TAP gene expression in Alde+ CSCs. TAP1 knockdown resulted in increased tumor growth of 4T1 cells in immuno-competent mice. Together, this suggests immune evasion mechanisms in breast CSCs are marker specific and epigenetic silencing of TAP1 in Aldefluor+ breast CSCs contributes to their enhanced survival under immune pressure.

3.2 Introduction

Self-renewing CSCs are highly tumorigenic cells that are able to give rise to new tumors with high efficiency com-pared to differentiated non-CSC tumor cells (Lapidot et al. 1994; Ma et al. 2007; O'Brien et al. 2007; Singh et al. 2004). The CSC populations of many solid tumors are identified by the expression of specific cell surface markers, such as CD44+CD24- in breast cancer (Al-Hajj et al. 2003). Additionally, increased ALDH activity as measured by the Aldefluor assay is used to identify the CSCs of many tumors, including breast(Ginestier et al. 2007; da Silveira et al. 2017), colon (Huang et al. 2009), liver (S. Ma et al. 2008), lung (Jiang et al. 2009), prostate (van den Hoogen et al. 2010), glioblastoma (Rasper et al. 2010), melanoma (Luo et al. 2012), and head and neck (Clay et al. 2010). The resistance of CSCs to conventional treatments suggests they may mediate the incomplete response to chemotherapy and radiation, and contribute to recurrence (Liu et al. 2006; S Ma et al. 2008; Silva et al. 2011). Thus, with the increased interest in the clinical impact of immunotherapies on different types of tumors (Kantoff et al. 2010; Rosenberg, Yang, and Restifo 2004), it is important to understand the interactions between CSCs and the immune system and whether CSCs have enhanced mechanisms for immune evasion (Sultan et al. 2016). Mechanisms of tumor immune evasion include reduced antigen processing and presentation, limiting T cell detection (Roemer et al. 2016); inhibition of T cell activation and proliferation (Kusmartsev et al. 2004; Woo et al. 2002); and the induction of an immunosuppressive tumor microenvironment via the recruitment of Tregs (Curiel 2008), myeloid derived suppressor cells (Ostrand-Rosenberg and Sinha 2009), and M2 macrophages (Sica et al. 2006). There is accumulating evidence that CSCs exhibit enhanced tumor immune evasion, which may be of potential concern in the successful implementation of immunotherapies

(Chikamatsu, K, Takahashi G, Sakakura K, Ferrone S 2011; Inoda et al. 2011; Sultan et al. 2016; Volonte et al. 2014). The CSCs of various cancers have increased capacity to inhibit the activity of effector immune cells (Lee and Sunwoo 2014), and can promote their maintenance via interactions with innate immune cells (Jinushi 2014; Wu et al. 2010). For breast CSCs specifically, the evidence is limited and has predominately focused on CSC interactions with innate immune cells which increase CSC maintenance/renewal as well as CSC susceptibility to NK cell killing (Ames et al. 2015; B. Wang et al. 2014). Murine mammary CSCs identified by CD90/Thy1 expression interact directly TAMs to maintain their CSC state (Lu et al. 2014). Similarly, CD44+CD24- CSCs identified in human breast cancer cell lines upregulate HAS2, leading to interaction with TAMs in nude mice and increased CSC renewal (Okuda et al. 2012). Furthermore, contrasting reports have found that breast CSCs are both highly susceptible to NK cells (Ames et al. 2015) and resistant to NK cell targeting (B. Wang et al. 2014). Thus, the interaction between breast CSCs and the immune system remains under-investigated and has been limited to studies where these cells are identified based on cell surface markers. Ideally, for the characterization of breast CSC immune cell interactions in murine models to accurately reflect patient tumor CSC interactions with the immune system, the same markers to identify CSCs should be used. Notably, although CD44+CD24- is commonly used to identify breast CSCs in humans, these markers are not commonly used to identify murine mammary CSC populations, which are typically defined by Sca1+, Thy1+CD24+, or CD24+CD29+(Cheng et al. 2010; Grange et al. 2008). In contrast, Aldefluor activity has been reported to define CSCs in the murine 4T1 mammary tumor model (Choi et al. 2012; Kim et al. 2013; Nguyen et al. 2015; S.-J. Park, Kim, and Nam 2011; Zhuang et al. 2012). To increase our understanding of how immune

pressure affects breast CSCs, we performed comparative tumor growth and cell population analyses on a spontaneous murine mammary tumor grown under immunocompetent versus immuno-compromised backgrounds. We hypothesized that Aldefluor+ cells will have a growth advantage under immune pressure due to their ability to manipulate the immune environment by altering the expression of key immune markers. Growth of the tumor in the immunocompetent background resulted in immune cell infiltration, tumor growth restriction, and enriched Aldefluor+ cells. The increased survival of the Aldefluor+ cells under immune pressure suggests they may have enhanced immune evasion mechanisms leading to decreased CSC detection and destruction. Consistent with this possibility, the Aldefluor+ cells of patient tumors, the spontaneous murine mammary tumor and 4T1 murine mammary cancer cells had decreased expression of transporter associated with TAP genes and T-cell co-stimulation genes. In contrast, breast cancer patient CSCs identified by CD44+CD24- did not have decreased expression of the antigen processing and presentation and T cell co-stimulation genes but did have increased expression of CXCR4. Methylation analysis by bisulfite pyrosequencing suggest that DNA hypermethylation contributes to the decreased expression of TAP1 and TAP2 in Aldefluor+ breast CSCs. Finally, knockdown of TAP1 in 4T1 cells did not alter their in vitro growth rates but did increase tumor growth in BALB/C mice suggesting a functional role for the anti-gen processing gene in tumor growth. Together, this data provides evidence that mechanisms for avoiding immune cell detection and destruction in breast CSCs is marker specific and that Aldefluor+ breast CSCs have enhanced immune evasion mechanisms related to epigenetic silencing and decreased expression of genes involved in the processing of antigens.

3.3 Result

3.3.1 Aldefluor+ CSCs of a Spontaneous Mammary Tumor Are Enriched in an Immunocompetent Host

A spontaneous mammary tumor which developed in a 7-month-old NOD/SCID female mouse provided an opportunity to investigate the behavior of the tumor-initiating population in an immunocompromised versus immunocompetent background. First, we characterized the cells of the spontaneous tumor using the Aldefluor assay. Once 7-AAD-positive (dead) cells and lineage-negative cells (of non-cancer origin) were eliminated from the analysis, two distinct populations of low and high Aldefluor activity were identified, termed ALDE- and ALDE+, respectively (Fig. 3.1A). The addition of the ALDH inhibitor, N,N-diethylaminobenzaldehyde (DEAB), effectively eliminated the Aldefluor+ + population, confirming that we had successfully identified two distinct tumor cell populations based on ALDH activity. Next, we injected 5,000 or 50,000 of the Aldefluor+ or Aldefluor- in NOD/SCID mice, and of the mice injected with the lower number of cells (5,000 cells), only Aldefluor+ sorted cells formed tumors in the mice (Fig. 3.1B). This confirmed that the spontaneous mammary tumor contained a population of cells with increased tumorigenic capacity, identifiable based on the CSC marker of high Aldefluor activity. Having identified a population with increased tumor initiating potential, we next examined how tumor growth, viability, cell infiltration, and tumor cell composition would be affected by the presence of a fully functional immune system (Fig. 3.2). BALB/C mice have the same H2-Kd haplotype (Caudrillier et al. 2015; Markees et al. 1999) as NOD/SCID mice, preventing rejection of tumor cells due to MHC I mismatch .The resulting tumors in BALB/C mice were significantly smaller (Fig. 3.2A), with fewer

live cells (Fig. 3.2B). The proportion of infiltrating cells (identified by APC cocktail, including CD45.2+ leukocytes, CD140b+ fibroblast cells, and CD31+ endothelial cells) was significantly increased in the tumors grown in BALB/C mice (Fig. 3.2C). The smaller tumors, demonstrated decreased tumor cell viability and increased proportion of infiltrating cells is consistent with an active immune surveillance system in the immunocompetent mouse which is restricting tumor growth and viability. Next, we analyzed the percentage of live tumor cells that were Aldefluor+. Tumors grown in the immunocompetent BALB/C mice had an increased percentage of Aldefluor+ cells compared to the immunocompromised NOD/SCID mice (Fig. 3.2D). In comparison to the immunocompetent BALB/C counterpart, NOD/SCID mice lack functional T and B cells and have hampered NK cell activity (Bosma, Custer, and Bosma 1983). The higher proportion of Aldefluor+ tumor cells in the immunocompetent BALB/C background suggests these cells may have enhanced mechanisms for avoiding detection and eradication by lymphocytes.

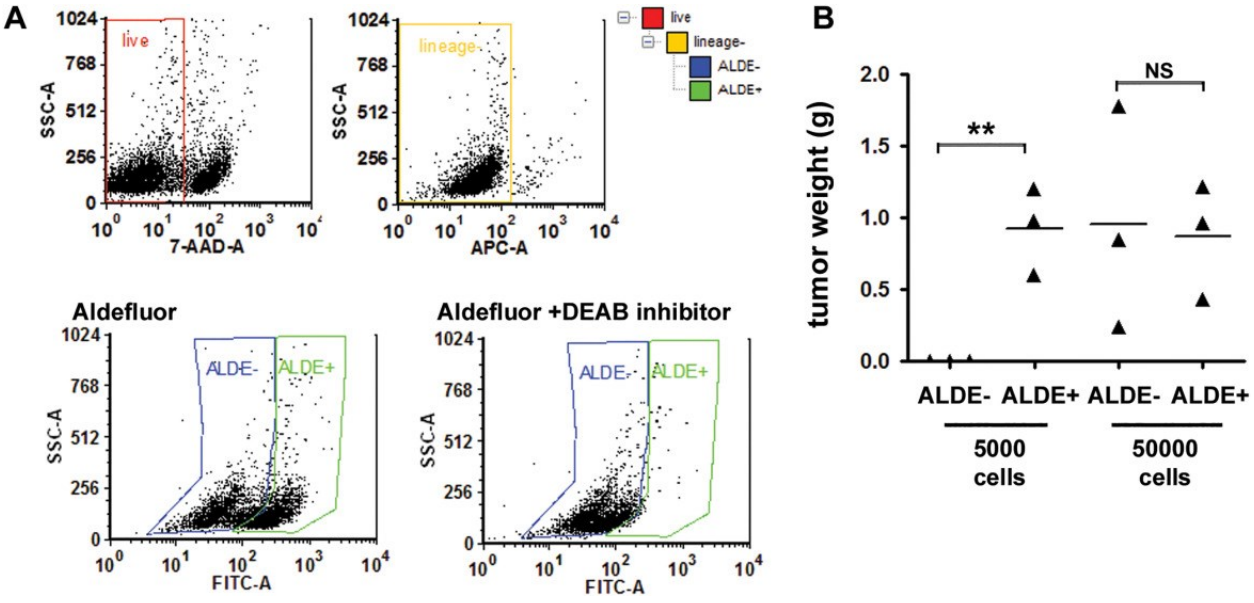
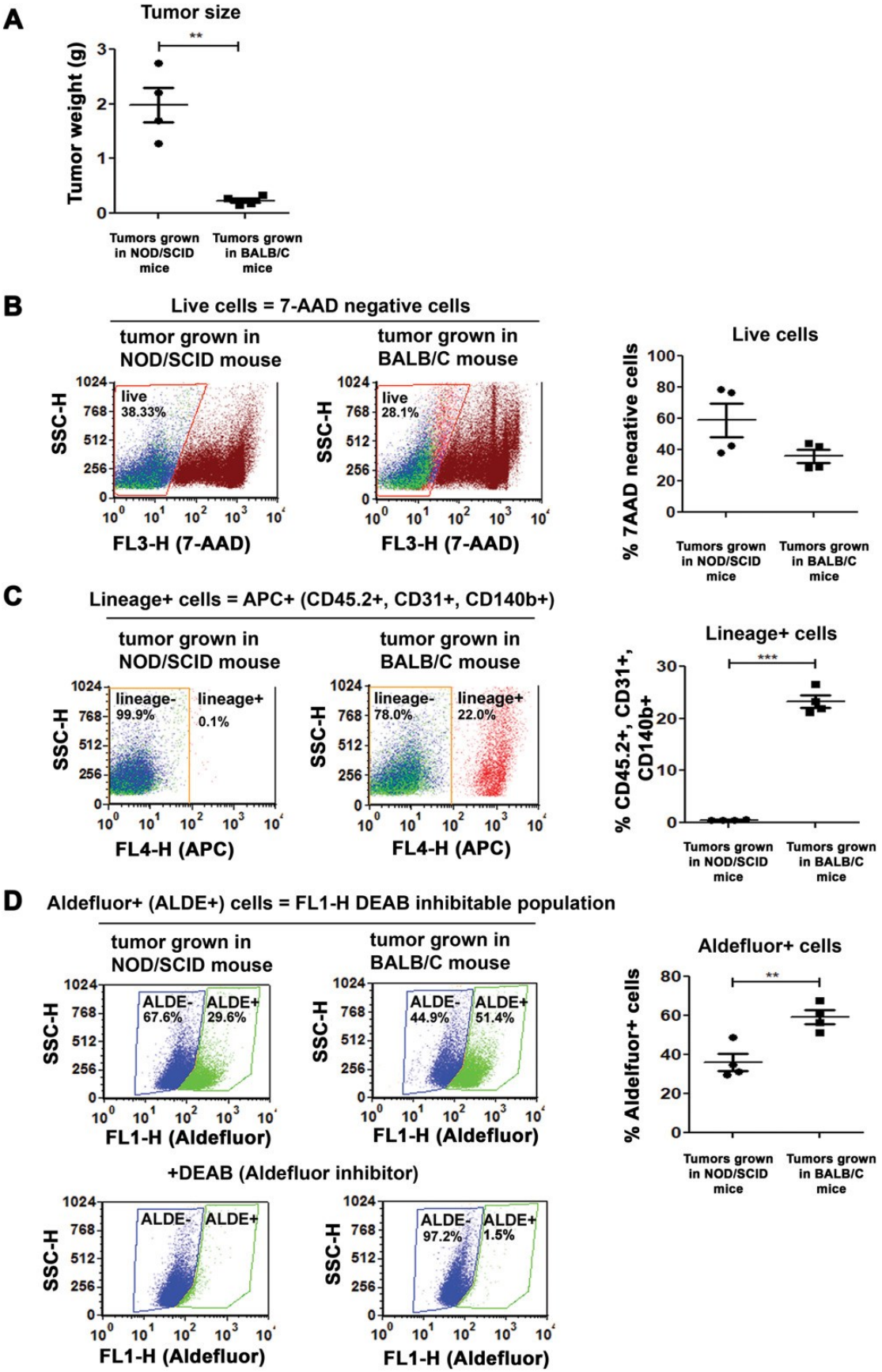


Figure 3.1 Aldefluor+ cells isolated from a spontaneous murine mammary tumor have increased tumorigenicity. (A): Aldefluor+ (ALDE+) and Aldefluor− (ALDE−) cells were isolated from a spontaneous mammary tumor that developed in a 7-month-old NOD/SCID female mouse using the Aldefluor assay and dead cells (7-AAD staining) and lineage cells (anti-CD45.2, anti-CD31, anti-CD140b staining) removed. (B): Resulting weights of tumors that developed in NOD/SCID female mice (n = 3) from either 5,000 or 50,000 sorted Aldefluor+ and Aldefluor− cells injected in the mammary fat pads (triangles represent the weights of individual tumors harvested from mice, significance determined by unpaired t test, **, $p < .01$.

Figure 3.2 The Aldefluor+ tumor cells of the spontaneous mammary murine tumor are enriched under immune pressure. The spontaneous mammary murine tumor was implanted in NOD/SCID and BALB/C mice and resulting tumors (n = 4) assessed by tumor weights (A) and flow cytometry (B–D). (B): The percentages of live cells were determined by negative 7-AAD staining of single cell suspensions (n = 4). (C): Of the live cells, the percentage of infiltrating lineage cells was determined by a combination of anti-CD45.2, anti-CD31, anti-CD140b staining. (D): Of the live and lineage- cells, the percentage of Aldefluor+ (ALDE+) and Aldefluor− (ALDE−) was determined. Significance was determined by unpaired t test, **, $p < .01$; ***, $p < .001$.


A
Tumor size
**
Tumor weight (g)
3
2
1
0
Tumors grown in NOD/SCID mice
Tumors grown in BALB/C mice
B
Live cells = 7-AAD negative cells
tumor grown in NOD/SCID mouse
tumor grown in BALB/C mouse
live 38.33%
live 28.1%
SSC-H
1024
768
512
256
0
FL3-H (7-AAD)
Live cells
% 7AAD negative cells
100
80
60
40
20
0
Tumors grown in NOD/SCID mice
Tumors grown in BALB/C mice
C
Lineage+ cells = APC+ (CD45.2+, CD31+, CD140b+)
tumor grown in NOD/SCID mouse
tumor grown in BALB/C mouse
lineage- 99.9%
lineage+ 0.1%
lineage- 78.0%
lineage+ 22.0%
FL4-H (APC)
Lineage+ cells
***
% CD45.2+, CD31+, CD140b+
30
20
10
0
Tumors grown in NOD/SCID mice
Tumors grown in BALB/C mice
D
Aldefluor+ (ALDE+) cells = FL1-H DEAB inhibitable population
tumor grown in NOD/SCID mouse
tumor grown in BALB/C mouse
ALDE- 67.6%
ALDE+ 29.6%
ALDE- 44.9%
ALDE+ 51.4%
FL1-H (Aldefluor)
+DEAB (Aldefluor inhibitor)
ALDE-
ALDE+
ALDE- 97.2%
ALDE+ 1.5%
Aldefluor+ cells
**
% Aldelfuor+ cells
80
60
40
20
0
Tumors grown in NOD/SCID mice
Tumors grown in BALB/C mice

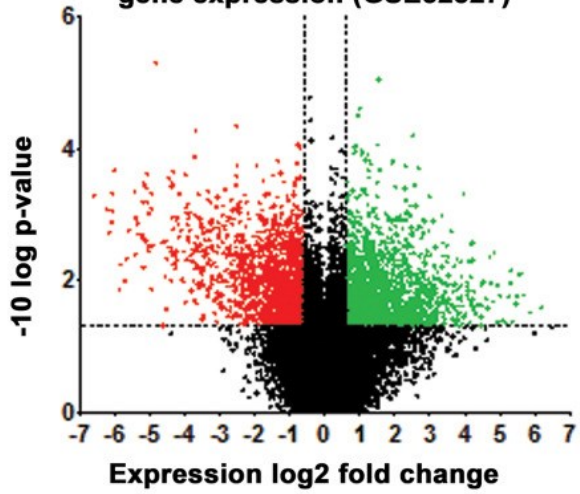
3.3.2 Aldefluor+ Sorted Breast Tumor Cells Downregulate Antigen Processing and Presentation Genes and Co-Stimulatory Molecule Genes

Potentially, changes in expression of genes related to various immune functions contribute to the observed Aldefluor+ + CSC enrichment under immune pressure (Fig. 3.2). To identify potential clinically relevant gene expression changes contributing to Aldefluor+ breast CSCs interactions with the immune system, we analyzed microarray gene expression data of Aldefluor sorted breast tumor cells from patient samples (GSE52327) (S. Liu et al. 2014). Based on uncorrected p value of <.05 and fold change greater than 1.6, 1,099 genes were downregulated and 1,286 genes upregulated significantly in Aldefluor+ cells (Fig. 3.3A) Over 700 genes are identified by nanoString pan cancer immune panel to play a role in immune function in the tumor microenvironment; 109 of which are predominately expressed by immune cells, while 621 are expressed by many types of cells including cancer cells . Of the Aldefluor+ upregulated and downregulated genes, 28 and 129 genes, respectively, are genes related to immune functions (Fig. 3.3B, Tables 3.1 and 3.2). Of note, genes key for T-cell recognition and targeting, including TAP1, TAP2, TAPBP, MHCI (human leukocyte antigen [HLA]), and co-stimulatory CD80 and CD86 molecules are downregulated (Fig. 3.3C). Downregulation of TAP1, TAP2, TAPBP, HLA, CD80, and CD86 by Aldefluor+ cells could render these cells resistant to detection and killing by cytotoxic T cells. Also among the downregulated genes in Aldefluor+ patient tumor cells were MHC-I Polypeptide-Related Sequence B (MICB, Fig. 3.3C). Downregulation of MICB may lead to decreased susceptibility of breast CSCs to NK cell killing and is consistent with the findings of Wang et al. who reported that CSCs isolated from breast cancer patient tumors

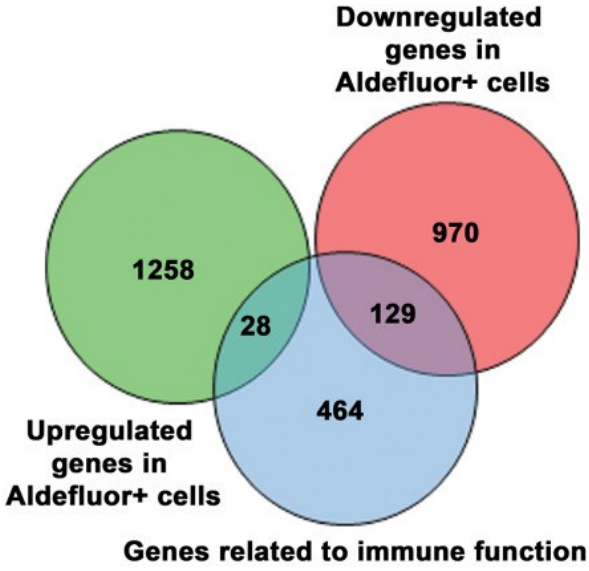
had low levels of MICA/B and were resistant to lysis by NK cells (B. Wang et al. 2014). We also noted the expression of CXCR4 and STAT3, which are important in establishing pro-tumorigenic microenvironment via interactions with immune cells (Hira et al. 2015; Lee et al. 2012; Panni et al. 2014; Wan et al. 2014; Zhu et al. 2014), are significantly downregulated in Aldefluor+ patient tumor cells (Fig. 3.3C). If these genes were involved in Aldefluor+ CSC enrichment under immune pressure, we would have expected that CXCR4 andSTAT3 would be upregulated as reported in CSC tumor models using other markers (Hira et al. 2015; Panni et al. 2014; Wan et al. 2014; Zhu et al. 2014). Finally, among the upregulated immune function genes, we noted HAS2 (Fig. 3.3C), which is consistent with a previous study where HAS2 was upregulated in CSCs leading to interactions with TAMs and enhanced self-renewal (Okuda et al. 2012).

Figure 3.3 Expression of immune function genes in Aldefluor+ versus Aldefluor− tumor cells isolated from breast cancer patient tumors. (A): The change in gene expression in Aldefluor+ versus Aldefluor− tumor cells isolated from eight patient tumors (GSE52327). The Log2 fold change in expression is plotted versus the −Log10 ANOVA p value of over 54,600 probes covering 22,479 Refseq (Entrez) genes. Probes with a 1.6-fold or greater change in expression ($Log2 \geq 0.678$ or ≤ -0.678) and a p value of less than .05 are indicated as green or red dots in the volcano plot. (B): Genes corresponding to probes with a 1.6-fold or greater change in expression and p value of less than .05 are indicated, with the immune function genes overlapping in the Venn diagram. (C): The fold change in expression of noted genes and corresponding probes.

A



B



C

Transcript Cluster ID	Fold Change (linear) (ALDE+ vs. ALDE-)	ANOVA *p*-value	Gene Symbol
Antigen presentation and processing			
202307_s_at	-1.76	.000171	TAP1
204770_at	-1.9	.012405	TAP2
225973_at	-2.08	.016616	TAP2
208829_at	-1.62	.005511	TAPBP
200905_x	-1.71	.005148	HLA-E
206247_at	-3.68	.020521	MICB
Costimulatry molecules			
1554519_at	-2.06	.002207	CD80
1555689_at	-2.83	.026815	CD80
210895_s_at	-2.35	.016279	CD86
Tumor microenviroment immune modulation			
209201_x_at	-13.22	.00103	CXCR4
211919_s_at	-11.88	.001427	CXCR4
217028_at	-3.98	.002932	CXCR4
243213_at	-3.02	.006056	STAT3
230372_at	4.01	.020116	HAS2
206432_at	5.34	.023155	HAS2

ZAP70	CASP8	IL15	ITGA4	TNFRSF1B	LCP1
IL12RB1	TAP1	CD79B	IRF4	ETS1	CCR5
CXCR4	LAG3	NFATC3	CD37	ISG20	IRF8
NCF4	CD53	ITK	PECAM1	CD247	KIR3DL3
AMICA1	TAPBP	KIR3DL1	CCR6	CD48	ST6GAL1
BTLA	HLA-DQB1	ITGB2	CD244	CYLD	TCF7
CD4	CD58	CD3D	IL2RA	SH2D1B	LY9
IRF1	GZMK	PIK3CD	LYN	IRF2	NOD2
CD1D	ABCB1	EOMES	CD80	SLAMF7	ITGAE
SH2D1A	TNFRSF4	TNFRSF13C	NLRC5	INPP5D	CYFIP2
NLRP3	CTSS	MAP3K1	PIK3CG	CD5	IL2RB
RUNX3	TNF	MR1	TAP2	IL7R	PAX5
CARD11	LCK	CD86	TYK2	IL16	NOTCH1
SELL	TIGIT	ICAM2	CD79A	IL10RA	ITGAL
TNFRSF9	HLA-DPA1	POU2F2	CXCR3	ICAM3	KLRB1
HLA-E	IL1A	TNFAIP3	IL1R2	CD96	CD1C
POU2AF1	LTB	TXNIP	SELPLG	IL4R	PSMB9
NFKB2	CD7	HLA-DQA1	HLA-DPB1	CCL5	MEF2C
HLA-DRA	CD27	IL18RAP	ULBP2	HLA-DMA	IL2RG
CTSW	CD207	SLAMF6	CD74	IL32	
TRAF3	STAT3	CCR7	MICB	JAK3	
SLAMF1	CASP1	PTPRC	TNFSF8	JAK1	

Table 3.1 Genes related to immune function that are downregulated genes in Aldefluor+ cells. Overlapping genes downregulated in Aldefluor+ cells with the nanoString pan cancer immune panel identified 129 genes related to immune functions that are downregulated in ALDE+ CSCs.

C1R	SERPING1	IFI27	CCL11	CLU	SPA17
NRP1	LTBR	THY1	C1S	CXCL12	LAMP2
LAMP1	EGR1	COLEC12	CD59	CD63	MASP1
CSF1	FN1	COL3A1	LRP1	C2	IL1R1
CFD	PDGFC	APP	C6		

Table 3.2 Upregulated genes related to immune function in Aldefluor+ cells.

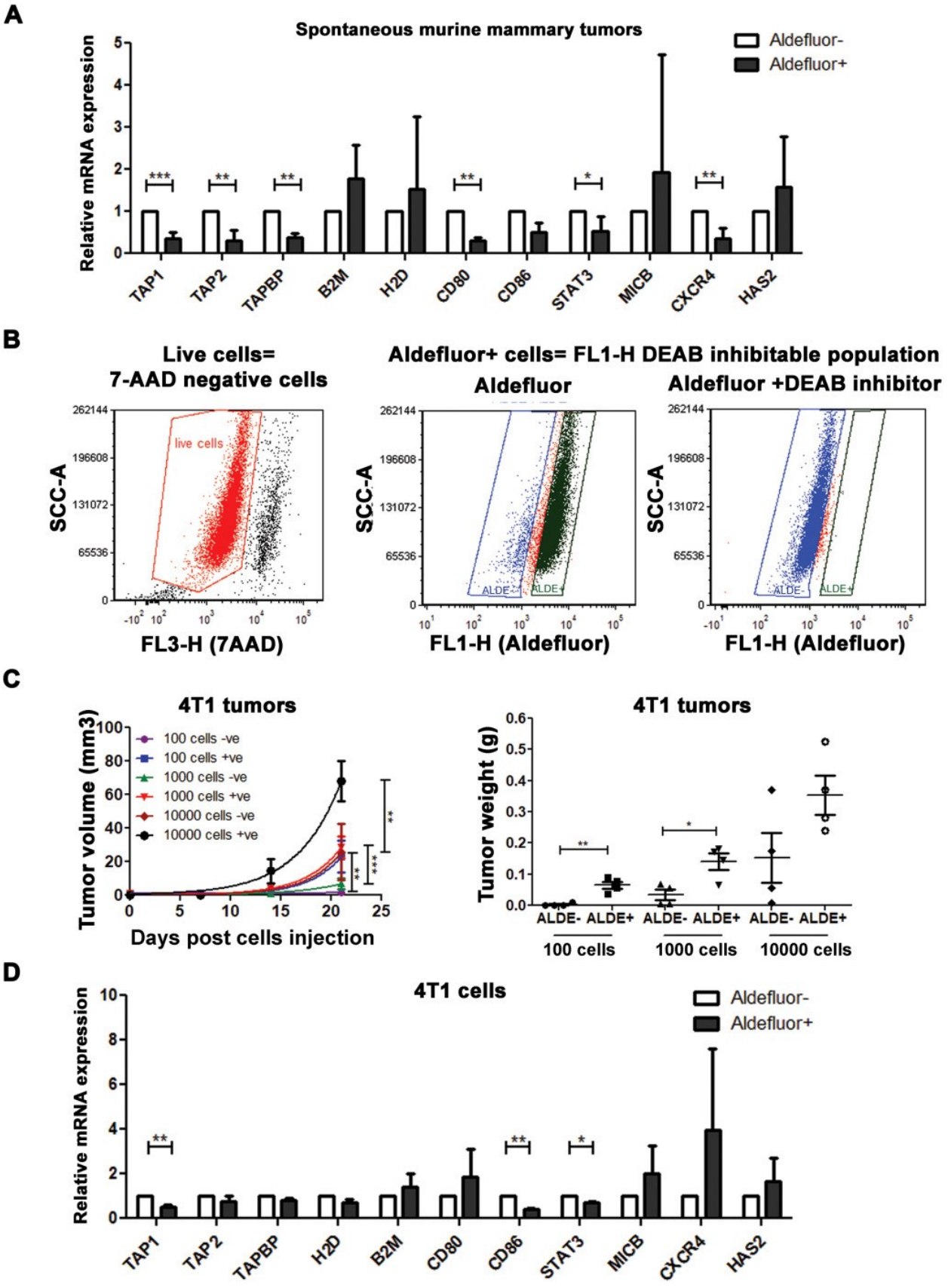
Overlapping genes downregulated in Aldefluor+ cells with the nanoString pan cancer immune panel identified 28 genes related to immune functions that are upregulated in Aldefluor+ CSCs.

3.3.3 Aldefluor+ Cells Isolated from a Spontaneous Murine Mammary Tumor and 4T1 Cells Downregulate Antigen Processing and Co-Stimulatory Signal Genes

Having identified a short list of genes with altered expression in Aldefluor+ patient tumor cells that would give the cells advantage under immune pressure (Fig. 3.3C), we next investigated expression of these genes in the sorted spontaneous murine mammary tumor cells. Consistent with Aldefluor+ sorted patient tumor cells, Aldefluor+ tumor cells isolated from spontaneous tumors had decreased expression of TAP1, TAP2, TAPBP, CD80, STAT3, and CXCR4, but did not have significantly altered expression of MHC I molecule genes, CD86, MICB, or HAS2 (Fig. 3.4A). The decreased expression of TAP1, TAP2, TAPBP, and CD80 is consistent with Aldefluor+ being less susceptible to T cell detection and targeting, resulting in enrichment of Aldefluor+ murine mammary tumor cells under immune pressure (Fig.3. 2D). Since MICB was not decreased in expression as was seen in the patient tumors (Fig. 3.3C), it suggests that resistance to NK cell killing is a less likely contributor. The lack of change of HAS2 suggests that hyaluronan interactions with TAMs, as reported in CD44+CD24- CSCs of human breast cancer cell lines in nude mice (Okuda et al. 2012), is not a likely contributor to the increased percentages of Aldefluor+ murine mammary tumor cells observed in the BALB/C mice(Fig. 3.2).To further determine which genes are important in the immune cell interactions of breast CSCs, we investigated another murine mammary tumor model, 4T1 cells, which also harbour a Aldefluor+ CSC population (Choi et al. 2012; Kim et al. 2013; Nguyen et al. 2015; S.-J. Park et al. 2011; Zhuang et al. 2012). Notably, the Aldefluor+ fraction represented the majority of the 4T1 cells (Fig. 3.4B). We isolated the Aldefluor+ and Aldefluor- cells and injected 100, 1,000, or 10,000 of the cells into the

mammary fat pads of BALB/C mice. The tumors that formed from the Aldefluor+ sorted cells were significantly larger than the tumors that formed from the Aldefluor- sorted cells (Fig. 3.4C). This confirmed increased tumorigenic capacity of 4T1 cells identified based on the CSC marker of high Aldefluor activity. Next, we investigated whether the same immune function genes interrogated in the spontaneous tumor have altered expression in sorted 4T1 cells. Consistent with the spontaneous tumor, expression of TAP1 and STAT3 was significantly decreased in Aldefluor+ cells. Notably, CD86 was significantly decreased, which would have the same functional effect as decreased CD80 in the spontaneous tumor (Fig. 3.4D). We were also curious if we would see similar changes of expression in cultured human cell lines and for this purpose we used Aldefluor-sorted MDA MB 231 cells and performed QPCR analysis on the same panel of genes (Fig 3.5). We did not observe reduction in TAP1, TAP2, CD80 or CD86, and on the contrary, HAS2 and CXCR4 were downregulated in MDA MB 231 Aldefluor+ cells. Therefore, the observed changes in expression of antigen processing and presentation genes and other immune genes in patient tumors, mouse tumors, and 4T1 cells could be tumor type specific, a consequence of the tumor microenvironment, and may not be translatable to long-term cultured human cell lines.

Fig 3.4 Aldefluor+ cells isolated from murine mammary tumors and 4T1 murine breast cancer cells have decreased expression of genes involved in antigen processing and T cell activation. (A): The fold change in TAP1, TAP2, TAPBP, MICB, CD80, CD86, CXCR4, STAT3, and HAS2 gene expression of Aldefluor+ versus Aldefluor- tumor cells was determined by QPCR using RNA isolated the sorted cells of the spontaneous murine mammary tumor. Error bars represent standard deviation and significance determined by paired t test. (B): Aldefluor+ (ALDE+) and Aldefluor− (ALDE−) cells were isolated from 4T1 cells using the Aldefluor assay and dead cells removed (7-AAD staining). (C): Resulting tumors that developed in BALB/c female mice (n = 4) from either 100, 1,000, or 10,000 sorted Aldefluor+ and Aldefluor− cells injected in the mammary fat pads. Error bars represent standard error and significance determined by unpaired t test. Tumor volume was modeled using a nonlinear (exponential) regression and compared by extra-sum-of-squares F test. (D): QPCR was used to assess fold change in the expression of TAP1, TAP2, TAPBP, MICB, CD80, CD86, CXCR4, STAT3, and HAS2 gene expression of Aldefluor+ versus Aldefluor− 4T1 cells. Error bars represent standard deviation and significance determined by paired t test, *, $p < .05$; **, $p < .01$.


A
Spontaneous murine mammary tumors
Aldefluor-
Aldefluor+
Relative mRNA expression
TAP1
TAP2
TAPBP
B2M
H2D
CD80
CD86
STAT3
MICB
CXCR4
HAS2
B
Live cells=
7-AAD negative cells
live cells
SCC-A
FL3-H (7AAD)
Aldefluor+ cells= FL1-H DEAB inhibitable population
Aldefluor
Aldefluor +DEAB inhibitor
ALDE-
ALDE+
FL1-H (Aldefluor)
C
4T1 tumors
100 cells -ve
100 cells +ve
1000 cells -ve
1000 cells +ve
10000 cells -ve
10000 cells +ve
Tumor volume (mm3)
Days post cells injection
4T1 tumors
Tumor weight (g)
ALDE- ALDE+
100 cells
1000 cells
10000 cells
D
4T1 cells
Aldefluor-
Aldefluor+
Relative mRNA expression
TAP1
TAP2
TAPBP
H2D
B2M
CD80
CD86
STAT3
MICB
CXCR4
HAS2

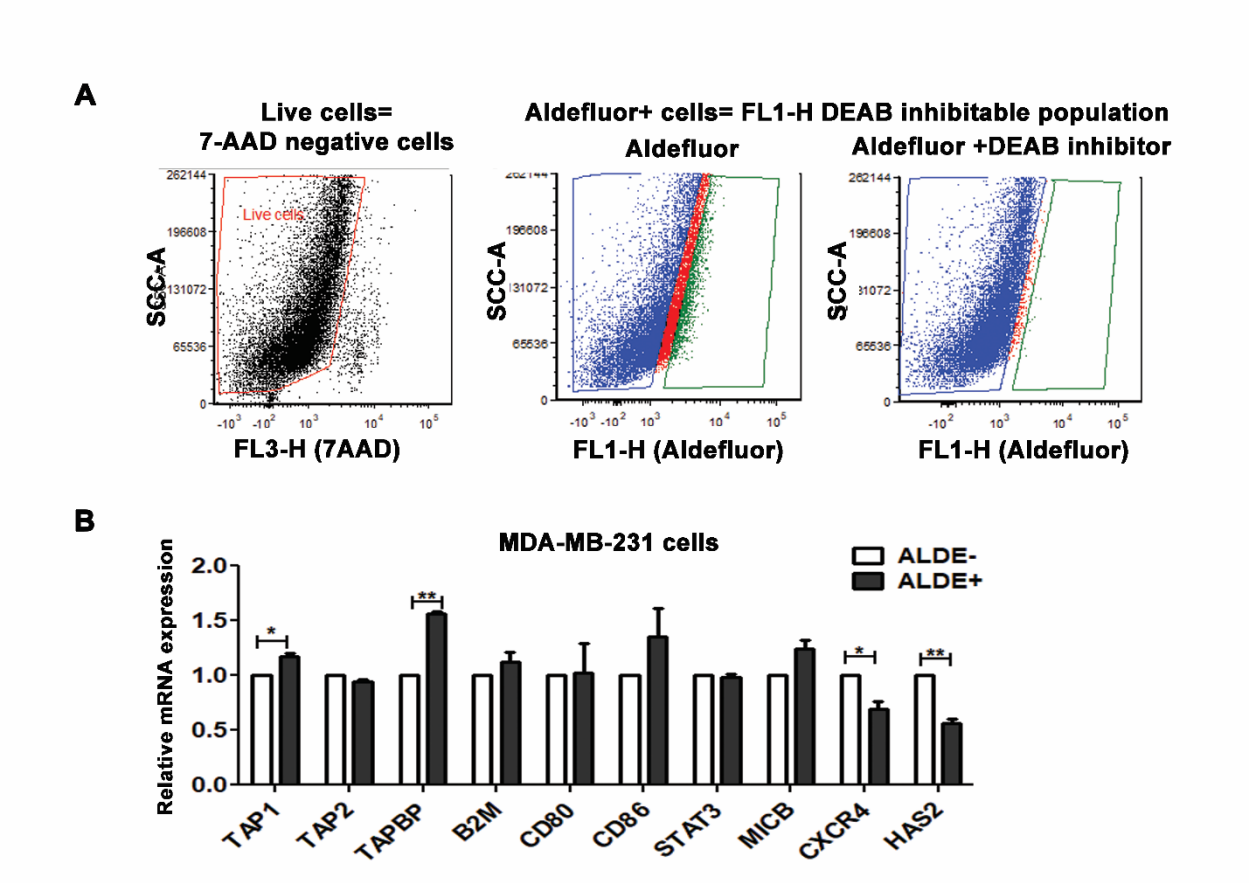
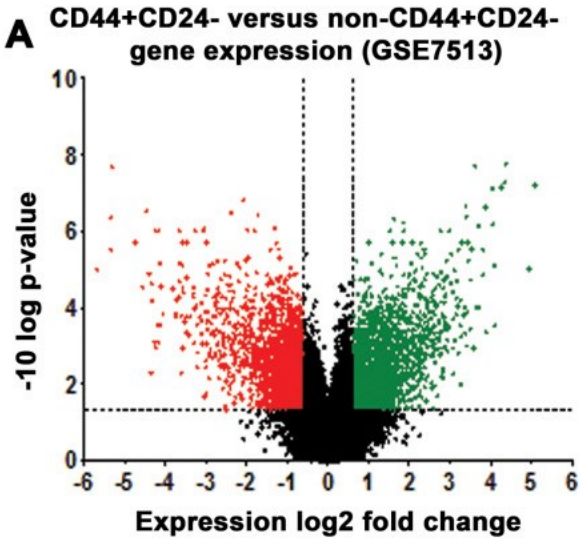


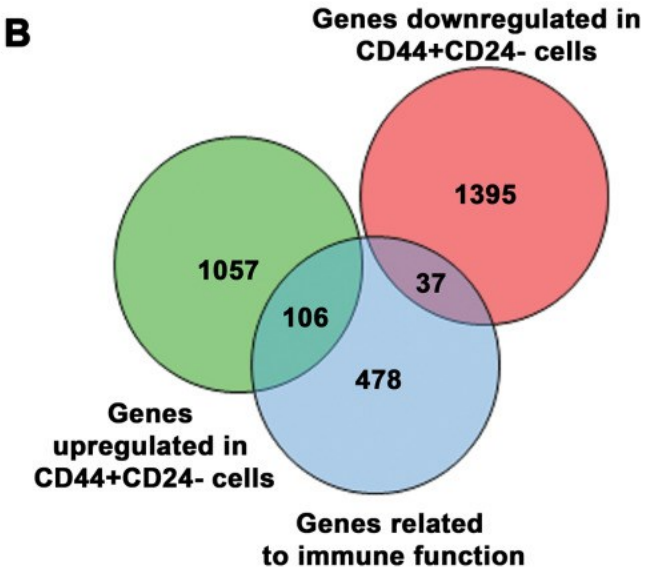
Figure 3.5 Aldefluor+ cells isolated from MDA MB 231 human breast cancer cells have increased expression of TAP1 and TAPBP and decreased expression of CXCR4 and HAS2. (A) Aldefluor+ (ALDE+) and Aldefluor- (ALDE-) cells were isolated from MDA-MB-231 cells using the Aldefluor assay and dead cells (7-AAD staining) were excluded. (B) QPCR was used to assess the fold change in the expression of TAP1, TAP2, TAPBP, MICB, CD80, CD86, CXCR4, STAT3, and HAS2 gene expression of Aldefluor+ versus Aldefluor- MDA-MB-231 cells. Error bars represent standard deviation and significance determined by paired t-test, p value <0.05 =*, < 0.01 =**.

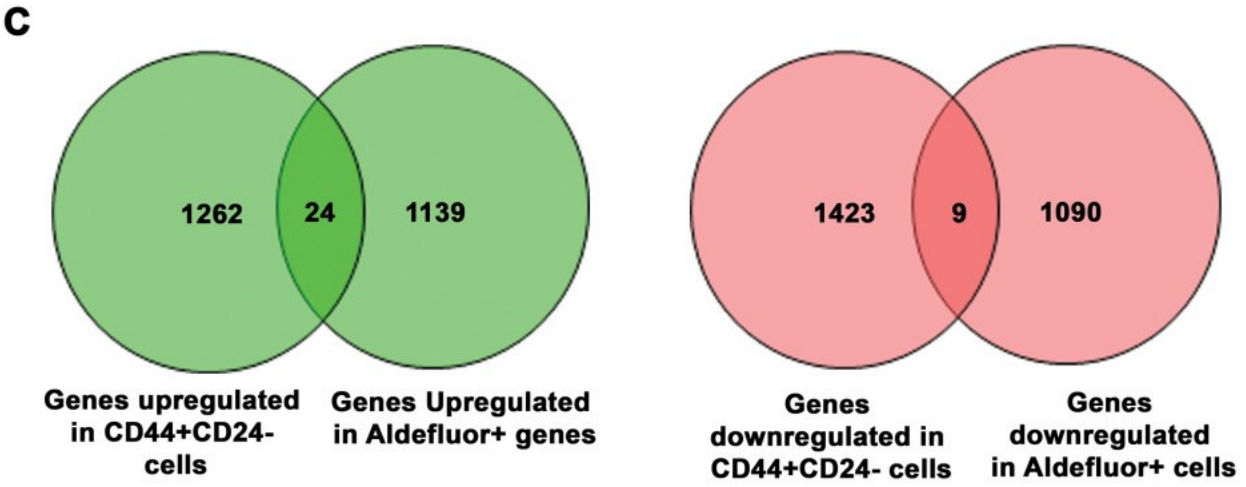
3.3.4 CD44+CD24- Breast Cancer Patient Tumor Cells Do Not Have Decreased Expression of the Antigen Processing and Presentation Genes

Having identified decreased expression of the key genes involved in antigen processing, presentation and T cell co-stimulation in the Aldefluor+ cells of patient tumors and murine tumor models (Figs. 3.3 and 3.4), we next wondered if breast CSCs defined by CD44+CD24- exhibited a similar altered gene expression of immune function genes. We analyzed microarray gene expression data of CD44+CD24- and CD44-CD24+ sorted breast tumor cells from patient samples (GSE7513) to identify differently expressed genes in CD44+CD24- CSC population (Fig. 3.6A). Based on the same cut offs (p value of <.05 and greater than 1.6 fold change), we identified 1,432 downregulated genes and 1,163 upregulated genes (Fig. 3.6B). Different immune function genes were upregulated (Table 3.3) and downregulated (Table 3.4) in CD44+CD24- sorted breast tumor cells compared to the Aldefluor+ sorted breast tumor cells (Fig. 3.6B). Notably, absent among the down-regulated genes were the genes related to antigen processing, presentation and T cell co-stimulation. This is not surprising considering the minor overlap in upregulated and downregulated genes among the CD44+CD24- and Aldefluor+ sorted breast tumor cells (Fig. 3.6C, Table 3.5). In further contrast to the Aldefluor+ cells, CXCR4 was among the upregulated genes in CD44+CD24- patient tumor cells (Fig. 3.6D), which is reported to play a role in immune induced maintenance/self-renewal of CSCs as observed in the CSCs of other tumor types (Hira et al. 2015; Lee et al. 2012). In summary, these findings suggest that depending on the markers used to identify breast CSCs, expression of different genes may be altered to give the cells a survival advantage under immune pressure.

Figure 3.6 Expression of immune function genes in CD44+CD24− versus non-CD44+CD24− tumor cells isolated from breast cancer patient tumors. (A): The change in gene expression in CD44+CD24− versus non-CD44+CD24− tumor cells isolated from 14 patient tumors (GSE7513). The Log2 fold change in expression is plotted versus the −Log10 ANOVA p-value of over 54,600 probes covering 22,479 Refseq (Entrez) genes. Probes with a 1.6-fold or greater change in expression ($Log2 \geq 0.678$ or ≤ -0.678) and a p value of less than .05 are indicated as green or red dots in the volcano plot. (B): Genes corresponding to probes with a 1.6-fold or greater change in expression and p value of less than .05 are indicated, with the immune function genes overlapping in the Venn diagram. (C): Genes corresponding to probes with a 1.6-fold or greater change in expression and p value of less than .05 are indicated, with overlapping upregulated and downregulated genes in Aldefluor+ cells (from Fig. 3) and CD44+CD24− cells illustrated in the Venn diagrams. (D): The fold change in expression of CXCR4 and corresponding probes.







D

Transcript Cluster ID	Fold Change (linear) (CD44+CD24- vs. non-CD44+CD24-)	ANOVA p-value	Gene Symbol
Tumor microenviroment immune modulation			
209201_x_at	3.67	0.005475	CXCR4
211919_s_at	3.46	0.006067	CXCR4
217028_at	3.02	0.00206	CXCR4

CD44	ABCB1	TNFRSF13C	CXCR3	REL	IL2RG
IL12RB1	BTK	SERPINB2	CREB1	ENTPD1	HLA-DQB1
CXCR4	IFI16	ICAM2	SYK	HLA-DMA	CD58
BTLA	CD86	POU2F2	TLR7	HLA-B	NFATC3
HLA-DRB4	CTSS	TXNIP	SELPLG	JAK3	ITK
NLRP3	TNF	HLA-DPA1	HLA-DPB1	CD84	PIK3CG
RUNX3	ADA	HLA-DQA1	CD74	JAK1	CD79A
FCGR2B	FYN	IL18RAP	ETS1	CD22	IL4R
CARD11	FCER1A	CCR7	SOCS1	TFEB	STAT2
SELL	LTB	PTPRC	TNFRSF14	IRF8	KLRB1
TNFRSF9	TANK	CXCR5	CD48	NFKB1	MEF2C
HLA-E	CD37	ITGA4	CYLD	ST6GAL1	IL1RL1
POU2AF1	CD27	IRF4	ITGAX	LY9	CD79B
HLA-DRA	PTGS2	BLNK	SLAMF7	IL18R1	LILRB1
TRAF3	NT5E	HLA-DMB	INPP5D	HAVCR2	TNFRSF13B
CD40	CD200	CCR6	IL7R	CYFIP2	CSF2RB
SLAMF1	CD83	LYN	IL16	PAX5	
CD53	CCL4	MAPK1	IL10RA	LY96	

Table 3.3 Upregulate genes related to immune function in CD44+CD24- cells.

Overlapping genes upregulated in CD44+CD24- cells with the nanoString pan cancer immune panel identified 106 genes related to immune functions that are upregulated in CD44+CD24- CSCs.

TGFB2	LIF	IL17RB	TNFRSF12A	CX3CL1	F12
PLAU	LTBR	CD9	EPCAM	LTF	LCN2
CEACAM6	EGR1	HRAS	CD59	ARG2	CEACAM1
F2RL1	RIPK2	CD46	CD24	LAMP2	BCL10
MUC1	VEGFC	ITGA2	CDH1	CFB	TTK
TNFSF15	TNFRSF1A	S100A8	CLU	MIF	APP
GPI					

Table 3.4 Downregulated genes related to immune function in CD44+CD24- cells.

Overlapping genes downregulated in CD44+CD24- cells with the nanoString pan cancer immune panel identified 37 genes related to immune functions that are upregulated in CD44+CD24- CSCs.

Downregulated genes					
AP1S2	TNFRSF10A	OBFC1	RDH13	CXorf40A	ERO1A
NEDD9	HNRNPLL	GCNT1			
Upregulated genes					
VWA5A	FLCN	APH1B	STXBP5	KANSL1L	WDR41
SLC35A5	SYNE1	HEXA	SLC44A1	TRIM13	MEG3
LXN	PDE4DIP	PKD2	CPM	SNRPN	PCSK5
PLEKHM3	SSPN	PLA2G4A	CCPG1	SLC41A2	TRAPPC6B

Table 3.5 Genes with altered expression in Aldefluor+ and CD44+CD24- cells. 9 genes were downregulated in both Aldefluor+ + and CD44+CD24- cells. In contrast 24 genes were upregulated in both populations.

3.3.5 TAP Gene DNA Hypermethylation in Aldefluor+ Cells

Notably, TAP1 was consistently downregulated across three Aldefluor+ breast tumor models (Figs. 3.3 and 3.4); this observation suggests the potential functional importance of the gene and is of interest for further study. Therefore, we investigated the cause for decreased TAP1 gene expression in Aldefluor+ breast tumor models. Given the important role of DNA methylation in regulating gene expression (Jaenisch and Bird 2003) and promoting cancer progression (Baylin 2005), as well as its association with decreased expression of antigen processing and presentation genes in tumors (Hasim et al. 2012; Poage et al. 2012; Qifeng et al. 2011; C. Wang et al. 2014), it suggests a likely mechanism for the decreased expression of TAP1 in the Aldefluor+ cells. Inline with the role of DNA methylation in regulating gene expression of TAP1, expression and methylation of the gene at site cg16890093 is significantly negatively correlated in breast cancer patient tumors in the TCGA, Cell 2015 dataset (Ciriello, M. L. Gatza, et al. 2015) (Fig. 3.7A). To investigate this further, we treated 4T1murine breast cancer cells with the DNA demethylating agent,5-aza-20-deoxycitidine (decitabine), and evaluated its effect on TAP1 gene expression. Decitabine treatment significantly increased expression of TAP1 consistent with a gene that is regulated by promoter methylation (Fig. 3.7B). We next interrogated the region upstream of the transcription start site previously functionally confirmed as the promoter region for TAP1 (Brucet et al., 2004; Wright et al., 1995) (Fig. 3.7C) by Chip/re-Chip and bisulfite pyrosequencing (Fig. 3.7D, 3.7E, respectively). Decitabine increased the binding of NF-jB (a transcription factor that promotes TAP1 expression (Wright et al., 1995) to the promoter region of TAP1 (Fig. 3.7D). At the same time, decitabine decreased 5 mC levels in the promoter region (Fig. 3.7D). The sequential

ChIP (ChIP–reChIP) experiments (with both 5 mC and NF-jB antibodies), revealed decreased enrichment of the promoter sequence. This is consistent with NF-jB binding poorly to the promoter region when it is hypermethylated, since only DNA sequences that bind both antibodies concurrently are detected in the ChIP–reChIP assay. Bisulfite pyrosequencing confirmed that decitabine correspondingly reduced the methylation of the 18 CpG sites in the TAP1 promoter region (Fig. 3.7E). Together, this confirms epigenetic regulation of this gene and defines the CpG sites associated with altered TAP1 expression depending on DNA methylation levels. Having identified CpG sites in the promoter region of TAP1, whereby higher methylation corresponds to decreased expression, we next interrogated the methylation of these 18 CpG sites in the Aldefluor sorted pooled samples of the spontaneous murine mammary tumor. Bisulfite pyrosequencing confirmed that in the TAP1 promoter region CpG sites are hypermethylated in Aldefluor+ cells (Fig.3.7F), consistent with the decreased expression of TAP1 in the cells (Fig. 3.4A). We similarly interrogated the CpG methylation of the TAP2 promoter and found evidence of hyper methylation in the Aldefluor+ sorted cells, consistent with the decreased expression of the gene in the cells (Fig. 3.8). Together, this data provides evidence of epigenetic silencing by DNA hypermethylation as a mechanism of decreased TAP gene expression in the Aldefluor+ CSCs.

Fig 3.7 TAP1 gene promoter is hypermethylated in Aldefluor+ cells of a spontaneous murine mammary tumor. (A): TAP1 mRNA expression is plotted against level of DNA methylation in breast cancer patient tumor samples from the TCGA Cell 2015 data set and shows a significant negative correlation. (B): The fold change in TAP1 gene expression in 4T1 cells post decitabine treatment was determined by QPCR (n = 6). (C): The location of the 18 CpG sites interrogated by pyrosequencing relative to the location of the primers, the TAP1 transcription start site (underlined) and a NF-κB binding site and the region of DNA that was interrogated in the ChIP assays. (D): ChIP and re-ChIP assays with antibodies against 5-mC and NF-κB were performed on 4T1 cells with or without decitabine treatment. (E, F): The methylation status of individual CpG sites and total C-methylation of the promoter regions of TAP1 in 4T1 cells post decitabine treatment (E) or in Aldefluor+ versus Aldefluor− sorted five pooled samples of the spontaneous mouse tumors (F). Error bars represent standard deviation and significance determined by paired t test, *, $p < .05$; **, $p < .01$.

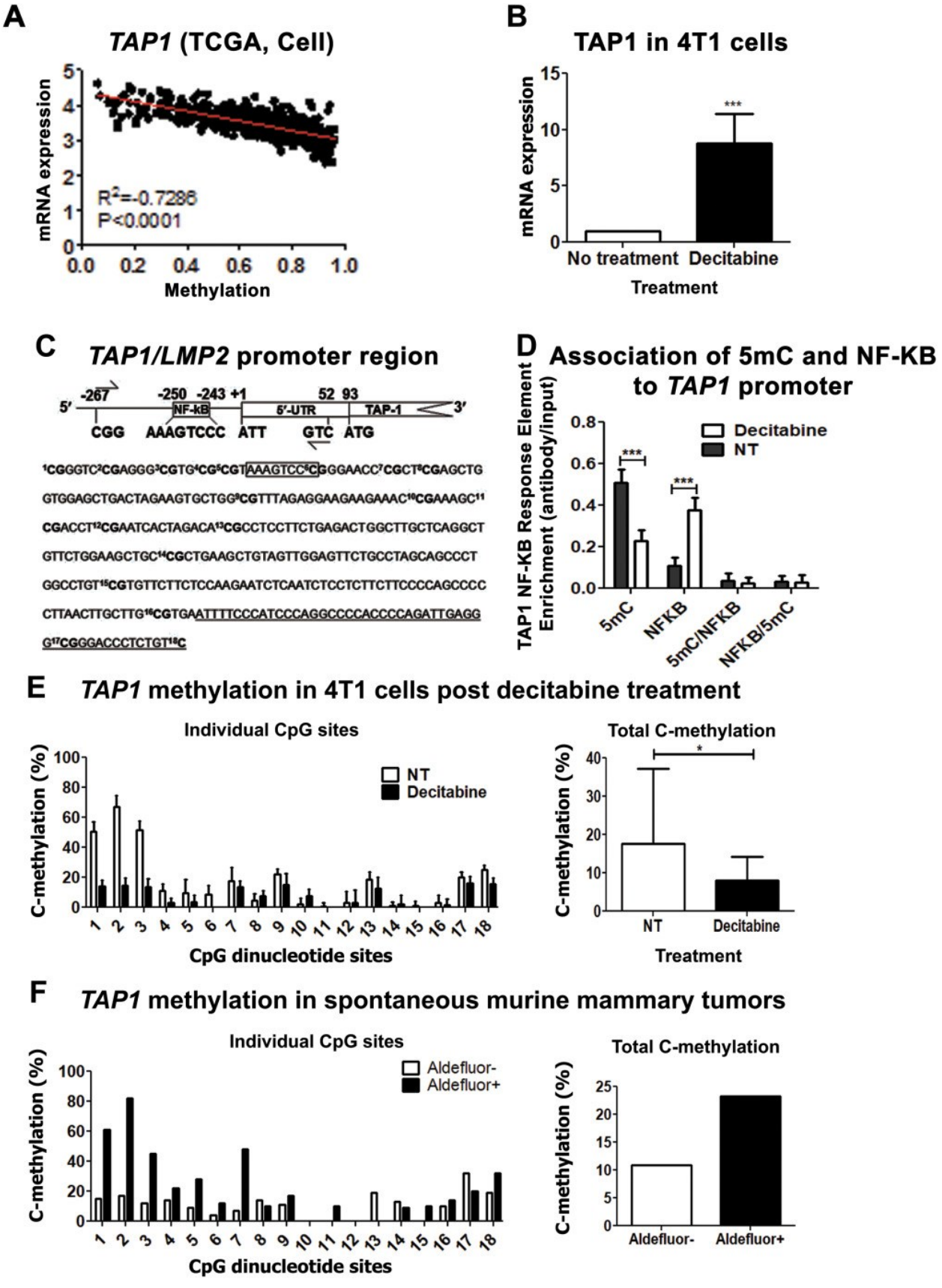
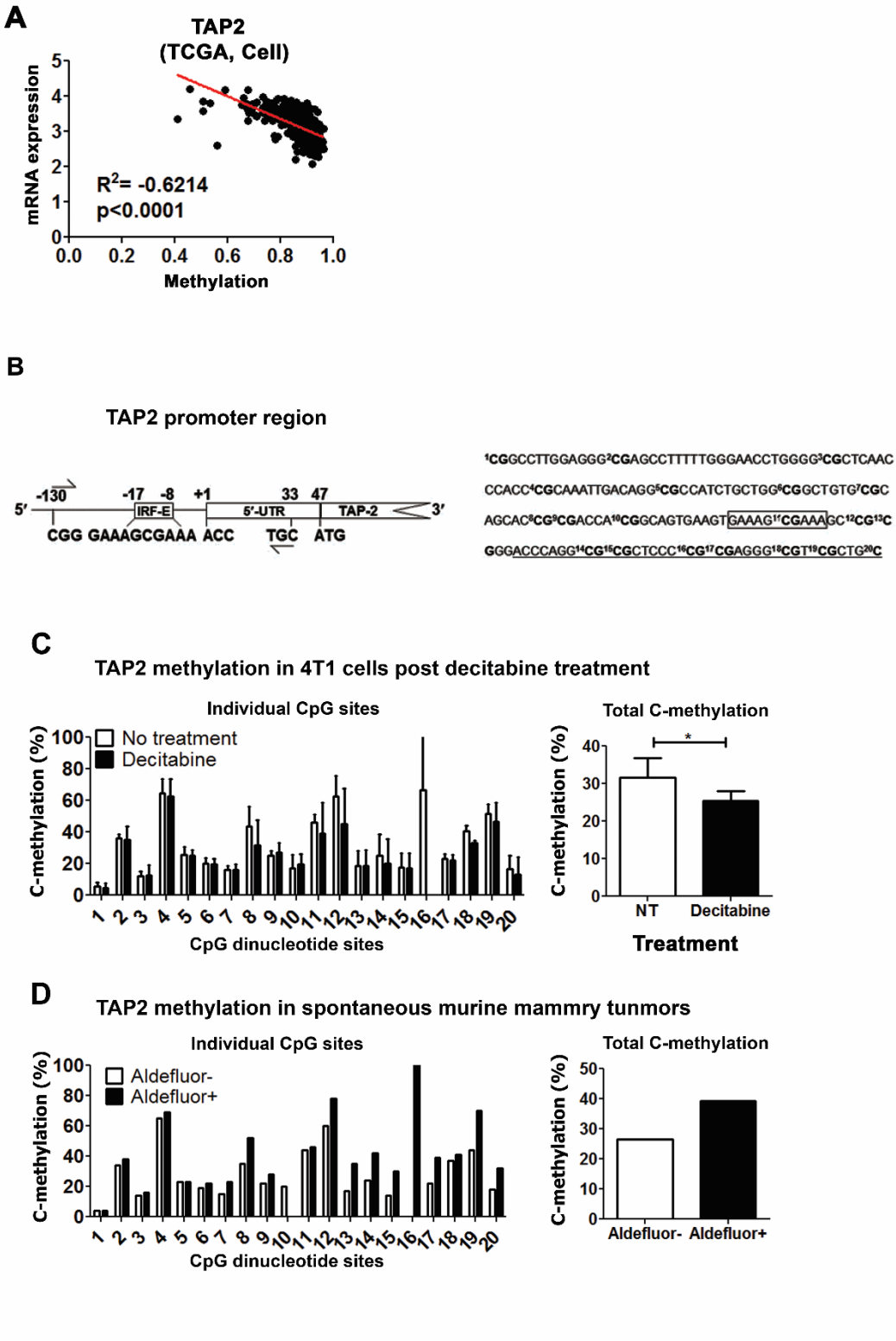

A
TAP1 (TCGA, Cell)
mRNA expression
R²=-0.7286
P<0.0001
Methylation
B
TAP1 in 4T1 cells
mRNA expression
***
No treatment
Decitabine
Treatment
C
TAP1/LMP2 promoter region
-267
-250
-243
+1
52
93
5'
3'
NF-kB
5'-UTR
TAP-1
CGG
AAAGTCCC
ATT
GTC
ATG
D
Association of 5mC and NF-KB to TAP1 promoter
TAP1 NF-KB Response Element Enrichment (antibody/input)
Decitabine
NT
***
***
5mC
NFKB
5mC/NFKB
NFKB/5mC
E
TAP1 methylation in 4T1 cells post decitabine treatment
Individual CpG sites
C-methylation (%)
NT
Decitabine
CpG dinucleotide sites
Total C-methylation
*
NT
Decitabine
Treatment
F
TAP1 methylation in spontaneous murine mammary tumors
Individual CpG sites
C-methylation (%)
Aldefluor-
Aldefluor+
CpG dinucleotide sites
Total C-methylation
Aldefluor-
Aldefluor+

Figure 3.8 TAP2 gene promoter is hypermethylated in Aldefluor$^+$ cells of a spontaneous murine mammary tumor. (A) TAP2 mRNA expression is plotted against level of DNA methylation in breast cancer patient tumor samples from the TCGA Cell 2015 data set and shows a significant negative correlation. **(B)** The location of the 20 CpG sites interrogated by pyrosequecing relative to the location of the primers, the TAP2 transcription start site (underlined) and a IRF-E binding site. **(C and D)** The methylation status of individual CpG sites and total C-methylation of the promoter regions of TAP2 in 4T1 cells post decitabine treatment **(C)** or in Aldefluor+ versus Aldefluor- sorted five pooled samples of the spontaneous mouse tumors **(D).** Error bars represent standard deviation and significance determined by paired t-test, p value >0.05 =*, > 0.01 =**.


A
TAP2
(TCGA, Cell)
mRNA expression
R2= -0.6214
p<0.0001
Methylation
B
TAP2 promoter region
-130
-17
-8
+1
33
47
IRF-E
5'-UTR
TAP-2
CGG GAAAGCGAAA ACC
TGC
ATG
C
TAP2 methylation in 4T1 cells post decitabine treatment
Individual CpG sites
No treatment
Decitabine
C-methylation (%)
CpG dinucleotide sites
Total C-methylation
NT
Decitabine
Treatment
D
TAP2 methylation in spontaneous murine mammry tunmors
Individual CpG sites
Aldefluor-
Aldefluor+
Total C-methylation
Aldefluor-
Aldefluor+

3.3.6 TAP1 Knockdown in 4T1 Cells Increased Tumor Growth.

Having identified that TAP1 is downregulated in Aldefluor+ cells isolated from patient tumor s, mouse tumors and 4T1 cells, and given the important role that TAP1 plays in the induction of T cell anti-tumor response, we investigated whether decreased TAP1 gives 4T1 cells a growth advantage when implanted into BALB/C mice. We generated stable knock down clones of TAP1 in 4T1 cells using two different shRNAs and confirmed the clones had decreased TAP1 expression compared to a scramble control shRNA clone (Fig. 3.9A). Next, we tested if the decreased TAP1expression in 4T1 cells alters their growth kinetics and found that TAP1 knockdown did not alter proliferation of the cells. (Fig. 3.9B). However, when 3,000 cells were implanted in the mammary fat pads into groups of BALB/C mice, TAP1 knockdown resulted in increased tumor growth (Fig. 3.9C, shRNA-1; Fig. 3.9D, shRNA-2). This suggests that tumor cells with lower TAP1 expression have a growth advantage *in vivo*.

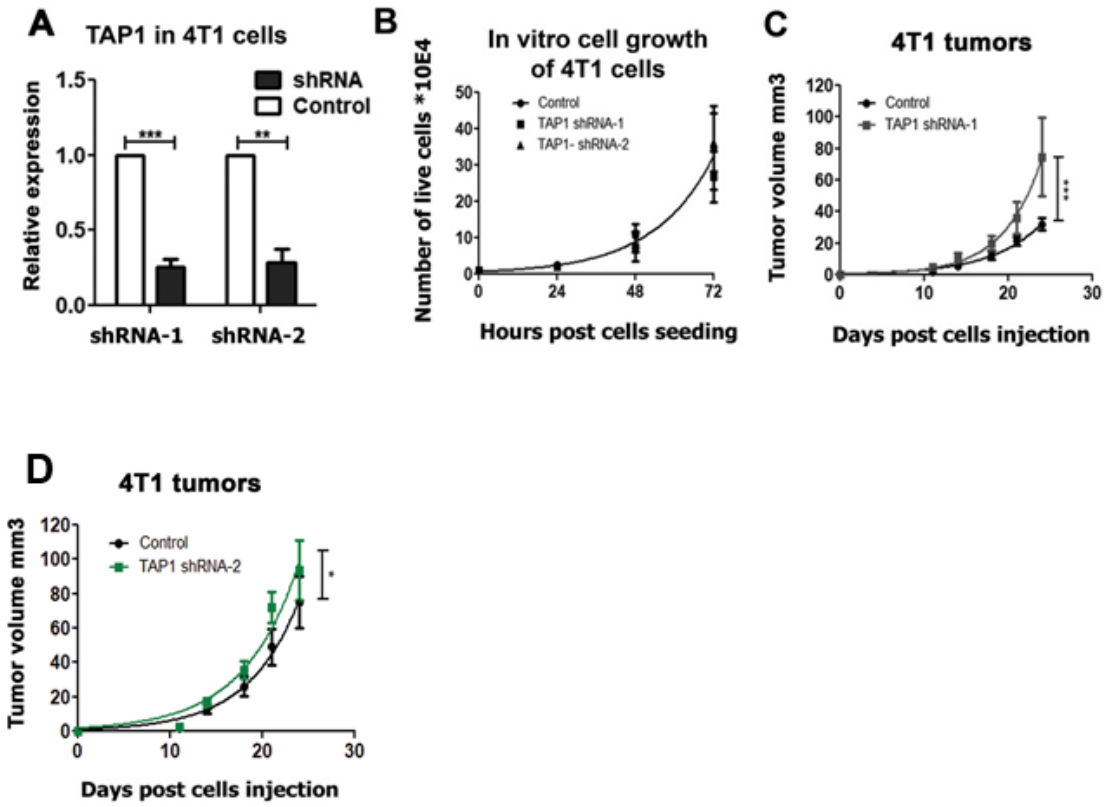


Fig. 3.9 TAP1 Knockdown enhances 4T1 tumor growth in BALB/C mice. (A): shRNA knockdown clones of TAP1 in 4T1 cells were verified by QPCR, and compared to scramble shRNA cloned by repeated measure ANOVA with Bonferroni post test. (B): The effect of TAP1 knockdown on in vitro cell proliferation compared with the scramble shRNA using a nonlinear (exponential) regression and compared by extra-sum-of-squares F test. (C and D): Effect of TAP1 knockdown on tumor growth was quantified in 4T1 cells implanted into BALB/C mice female mice. Tumor volume was modeled using a nonlinear (exponential) regression and compared by extra-sum-of-squares F test. For all statistical comparisons, *, $p < .05$; **, $p < .01$; ***, $p < .001$.

3.4 Discussion

The role of the immune system in carcinogenesis has been heavily investigated since the early 1950s (Thomas 1959). Many discoveries were made to establish both the anti-tumorigenic role (Dunn, Old, and Schreiber 2004) and the pro-tumorigenic role (Coussens and Werb 2002) of different immune cells in tumorigenesis. In fact, it is only when a cancer cell acquires mechanisms to avoid immune detection and destruction that tumors develop (Dong et al. 2002; Drake, Jaffee, and Pardoll 2006). This sparked an interest in harnessing the anti-tumorigenic effect of immune cells while hampering their pro-tumorigenic mechanisms to target cancer cells with immunotherapy (Brahmer et al. 2012; Leon et al. 2010). Immunotherapies encompass a range of drugs which stimulate various elements of the immune system to target cancer cells (Rosenberg et al. 2004). These include monoclonal antibodies which act as checkpoint inhibitors and prevent downregulation of cytotoxic T cells by blocking PD1/PDL1 or CTLA-4 signaling (Pardoll 2012), nonspecific immuno-therapies such as interleukins (Colombo and Trinchieri 2002) and interferons (Kane and Yang 2010) that stimulate the immune system toward targeting the cancer cells, chimeric antigen receptor T-cell therapy (Ritchie et al. 2013), NK cell therapy (Cheng et al. 2013), cancer vaccines against tumor-specific (Coulie et al. 2001) and tumor-associated antigens (Okada et al. 2011), and immune-boosting oncolytic viral therapies (Gujar et al. 2010). Arguably, the long-term effectiveness of immunotherapies also requires effective targeting of CSCs. As such, characterizing immune evasion characteristics of CSCs populations in tumors may reveal potential limitations in certain immunotherapies that could be circumvented by combi-nation therapies which target CSCs. The tumor initiating properties of CSCs have led to the hypothesis that CSCs have enhanced mechanisms for avoiding immune detection.

Reports in recent years have provided evidence that supports this hypothesis, illustrating CSC-mediated expansion of pro-tumorigenic immune cells, inhibition of anti-tumorigenic immune cells, and in some cases downregulation of antigen presentation (Lu et al. 2014; Okuda et al. 2012; Sultan et al. 2016; Wu et al. 2010). Yet in many cases, the underlining mechanisms for this immune privilege is unclear. Furthermore, the range of markers used to interrogate CSCs of certain tumor types makes it unclear if the immune system interactions observed are marker-specific. This is likely, at least in part, due to the fact that CSC markers only identify tumor cell populations enriched for CSCs and different markers identify different populations of cells within a tumor (Y. Liu et al. 2014). This was first evidenced in breast cancer by the increasing tumorigenicity of CSCs defined by multiple markers (i.e., the fraction of cells that were both Aldefluor+ and CD44+CD24-) .Herein, the differences between breast cancer cells identified by Aldefluor+ and CD44+CD24- are illustrated by their largely disparate gene expression profiles, including differential regulation of genes involved in immune cell interactions (e.g., antigen presentation genes, co-stimulatory molecules vs. induction of innate immune response). This reinforces the importance of markers when defining CSC characteristics, which often are marker-specific (Y. Liu et al. 2014). Furthermore, in immune cell interaction studies, ideally the markers that are used to define CSCs in murine model studies are also applicable in patient tumors, resulting in a greater likelihood that the results will have clinical relevance. This has been a limitation of the CD44+CD24-, but not Aldefluor+, breast CSC markers (Choi et al. 2012; Kim et al. 2013; Nguyen et al. 2015; S.-J. Park et al. 2011; Zhuang et al. 2012).

3.5 Conclusion

In this study, we primarily investigated the effect of immune pressure on breast CSCs defined by Aldefluor activity, which is a well-recognized marker of CSCs across cancer types (Clay et al. 2010; Ginestier et al. 2007; van den Hoogen et al. 2010; Huang et al. 2009; Jiang et al. 2009; Luo et al. 2012; S. Ma et al. 2008; Rasper et al. 2010).We observed an enrichment of Aldefluor+ murine mammary tumor cells when implanted in immunocompetent hosts, suggesting these cells possess mechanisms for enhanced immune privilege. This is consistent with the decreased expression of antigen processing and co-stimulatory genes, also observed in Aldefluor+ cells of patient tumors, but not CD44+CD24- patient tumors. At least in the context of the TAP genes, we found evidence of increased DNA methylation in the Aldefluor+ cells. Epigenetic silencing via DNA methylation of antigen processing and presentations genes (including TAP1and 2) is a common mechanism of immune cell evasion in tumors and is a predictor of recurrence and survival (Hasim et al. 2012; Poage et al. 2012; Qifeng et al. 2011; C. Wang et al. 2014). Therefore, it is not surprising that this mechanism of downregulating expression of genes key in antigen processing is found further enhanced in the tumor initiating population of mammary tumors. Furthermore, we demonstrated that down-regulating TAP1 in 4T1 cells gives them a growth advantage during tumor formation under immune pressure. Taken together, our data suggest that markers are a defining parameter when investigating immune evasion mechanisms of CSCs, and that Aldefluor+ breast CSCs may be resistant to immune therapies which harness T cell activity due to their ability to use epigenetic mechanisms to silence the expression of the TAP genes. Demethylating agents can be used to increase antigen presentation and sensitize the tumors to T cell or check point inhibitor immunotherapy. Moreover, the ability of

demethylating agents to upregulated antigen presentation could result in the discovery of new tumor specific antigen than can be used to develop anti-tumor vaccines. Thus, combining therapies that include drugs that override downregulation of antigen processing, such as the DNA demethylating agent decitabine, maybe an effective strategy.

CHAPTER 4

Copyright statement

This chapter have not been previously published.

This chapter will be submitted in whole or as part of a manuscript with these contributing authors: Mohammad Sultan, Jacob T Nearing, Justin M Brown, Wasundara Fernando, Marie-Clair D Wasson, Brianne M Cruickshank, Margaret Lois Thomas, Cheryl A Dean, Dejan Vidovic, Emily Lamoureux, Morgan Gi Langille and Paola Marcato. A Genome-wide *in vivo* RNAi screen identifies BCL6 as a novel mediator of paclitaxel resistance in breast cancer.

Contribution statement

I designed the experiment, collected the data and prepared this manuscript and its figures for publication with the guidance of my supervisor Dr. Paola Marcato. Jacob Nearing, Justin Brown, Wasundara Fernando, Marie-Clair Wasson, Brianne Cruickshank, Margaret Thomas, Cheryl Dean, Dejan Vidovic and Dr. Morgan Gi Langille supported the data collection and analysis for this manuscript.

4.1 Abstract

Treatment for breast cancer often involves the use of paclitaxel; however, some patients do not respond to treatment and would be better treated with an alternative drug. Thus, being able to identify the genes which when expressed in a tumor predict paclitaxel response prior to administration would improve treatment efficacy and patient survival. A genome-wide RNAi screen was performed with MDA-MB-231 tumor xenografts in female NOD/SCID mice. This allowed the identification of enriched and depleted shRNA sequences that theoretically target paclitaxel sensitivity and resistance genes, respectively. The screen identified several potential novel paclitaxel response genes in breast cancer, including B Cell Lymphoma 6 (BCL6). To test if BCL6 is an important effector of paclitaxel response in breast cancer, we generated individual knockdowns of BCL6 in MDA-MB-231 cells and orthotopically implanted the knockdowns or control clones into mammary fat pads of NOD/SCID female mice. The tumor bearing mice were treated systemically with either paclitaxel or phosphate-buffered saline (PBS). Paclitaxel induced regression of the MDA-MB-231 control clone tumors and BCL6 knockdown caused more regression in paclitaxel treated mice thus validating the role of BCL6 as a novel mediator of paclitaxel resistance in breast cancer. *In vitro* mechanism studies revealed knocking down BCL6 to affect paclitaxel treatment outcome through altering cell cycle progression and inducing apoptosis. Similarly, inhibiting BCL6 using a small molecule inhibitor enhanced paclitaxel treatment efficacy both *in vitro* and *in vivo* in breast cancer models. Together, this data suggests that the genome-wide

shRNA knockdown screen has identified BCL6 as a novel resistance mediator of paclitaxel in breast cancer.

4.2 Introduction

Breast cancer remains one of the leading causes of death in woman worldwide despite medical advances and increased awareness (DeSantis et al. 2019). Treatment for breast cancer patient is determined based on several factors including, tumor stage and grade, metastasis and whether the tumors express the estrogen receptor (ER+), progesterone receptor (PR+) and the human epidermal growth factor receptor 2 (HER2) receptor (Waks and Winer 2019). Based on the presence or absence of these three receptors, breast cancer tumors can be classified as luminal A or B, HER2+ or triple negative tumors (Waks and Winer 2019). Tumors in different groups have different gene expression profiles and in turn different treatment approaches (Cejalvo et al. 2017). Patients with ER+ positive tumors can often benefit from targeting the ER receptor (Shanle and Xu 2010), while patients with HER2+ are treated with HER2 targeted therapy (Lewis Phillips et al. 2008). Triple-negative breast cancer (TNBC) patients lack the expression of these receptors and are targeted with conventional therapy approaches (Isakoff 2010).

Treatment for TNBC patients and patients of other subtypes with advanced stage disease often includes the use of chemotherapies (Waks and Winer 2019). Patients who achieve pathological complete response (pCR) to chemotherapeutic treatment have excellent outcomes with lower risk of recurrence, while those who experience residual disease (non-pCR) have poorer outcomes (Cortazar et al. 2014). Identification of genes which either cause resistance or sensitivity to chemotherapies,

or those genes that when expressed in tumors are predictive of patient response to certain chemotherapies, would lead to improved treatment efficacy for patients (Cardoso et al. 2016). Additionally, molecular profiling of tumors and the application of proven prognostic gene signatures can prevent under- and over-treatment (e.g. Oncotype DX) and in turn minimize unwanted side effects of treatment (Cardoso et al. 2016; Carlson and Roth 2013).

Taxanes including paclitaxel and docetaxel are among the most widely used chemotherapeutic drugs and often used to treat breast cancer patients with locally advanced and metastatic disease (McGrogan et al. 2008), as well as ovarian (Chan et al. 2016), lung (Govindan et al. 2017) and pancreatic cancer (Von Hoff et al. 2013). Taxanes inhibit microtubule depolymerization resulting in cell cycle arrest and apoptosis of cancer cells (McGrogan et al. 2008). Despite their widespread use, many patients are resistant for generally unknown reasons. In recent years, the availability of large datasets of tumor gene expression profiles combined with patient outcomes has allowed progress into generation of predictive gene signatures for taxane response. However, the genes identified by these methods often do not have functional relevance in chemoresistance or sensitivity, limiting their translational application into novel drug discovery for chemoresistance sensitization approaches.

An alternative to gene expression analysis is functional-based screening approaches, which make use of RNAi or CRISPR technologies to identify chemoresistance and sensitivity genes and novel drug targets. For example, an *in vitro* screen targeting all known kinases identified a set of genes which contribute to sensitivity or resistance of paclitaxel, which was later shown to have predictive power

as a gene signature for paclitaxel response (Juul et al. 2010; Swanton et al. 2007). Similarly, an *in vitro* RNAi screen targeting 428 genes in HeLa cells identified novel druggable targets for paclitaxel chemoresistance in breast cancer (Bauer, Chakravarthy, et al. 2010). These studies illustrate the potential of RNAi screening technologies to both identify novel drug targets and to generate gene signatures for prognostic purposes. However, these studies included less than 5% of the protein-coding genes in the genome and were performed *in vitro*, making *in vivo* application possibly more difficult.

To address these potential weaknesses, identify novel genes required for paclitaxel sensitivity and resistance and construct a gene signature with more predictive power and better clinical application we employed a genome-wide shRNA library that has been used successfully to characterize the components of many pathways (Schlabach et al. 2008; Silva et al. 2008).

Among the top resistance genes identified in the screen was oncogenic B cell lymphoma 6 (BCL6), a transcriptional repressor, which is being investigated as a therapeutic target for B cell lymphoma and breast cancer (Leeman-Neill and Bhagat 2018). BCL6 dysregulation promotes the development of B cell lymphomas. In breast cancer, BCL6 is commonly overexpressed in high grade and high stage breast cancers (Logarajah et al. 2003), associated with poor survival (Ang et al. 2017), and is highly expressed in some breast cancer cell lines (e.g. MDA-MB-231 cells) promoting their invasiveness and migration (Chen et al. 2013; Yu et al. 2015). BCL6 inhibitors (e.g. small molecule 79-6) have demonstrated anti-lymphoma and breast cancer activity in preclinical models (Cerchietti et al. 2010). Herein we demonstrate

an important role played by screen hit BCL6 in paclitaxel resistance in breast cancer as well as the enhanced response to treatment when BCL6 is inhibited either by gene silencing or using a BCL6 inhibitor.

4.3 Results

4.3.1 Genome-Wide shRNA Screen Identifies Potential Paclitaxel Response Mediators

Given paclitaxel importance in breast cancer treatment we hypothesized that identifying novel mediators of paclitaxel response will enhance treatment efficacy and enhance patient survival. To this end we performed an unbiased *in vivo* genome-wide RNAi screen using the well characterized TNBC cell line MDA MB 231 to identify novel effectors of paclitaxel response (Fig. 4.1A). Given shRNAs effects on its target gene expression, we hypothesized that down regulated shRNAs targets resistance genes and gives cells growth disadvantage under paclitaxel treatment. In contrast, up regulated shRNAs targets sensitivity genes and gives cells growth advantage under paclitaxel treatment. Average fold change was calculated for each individual shRNA barcode was calculated relative to the untreated control and was plotted against it is significance to determine the top enriched and depleted hits (Fig. 4.1B). We identified the top 15 resistance genes (Fig. 4.1C) and top 15 sensitivity genes (Fig. 4.1D) that were significantly changed more than 2-fold with p value < 0.01. We further assessed the top genes for their potential role in resistance and sensitivity We determined if expression of the genes correlated with patient survival in a cohort of breast cancer patients treated with systemic chemotherapy that have not received hormonal therapy using the online portal KMPlotter (Győrffy et al. 2010a) (Fig. 4.2A and B). Additionally, we investigated the effect of these genes in a sub cohort of

patients with basal-like tumors to determine if the screen identified genes are specific to basal-like/TNBC. Among the screen identified potential top resistance genes, BCL6 had the most significant correlation with worse patient survival under the above described parameters, as patients with high levels of BCL6 had decreased survival in comparison to those with low expression (Figure 4.2A). We observed similar correlations with hazard ratio for high levels of BCL6 expression in gastric and ovarian cancer; gastric cancer patients with high levels of BCL6 expression in their tumors have decreased survival when compared to patients with low BCL6 expression (Fig 4.2C and D).

Figure 4.1. *In vivo* genome-wide RNAi screen identifies novel mediators of paclitaxel response in breast cancer. (A) MDA MB 231 cells bearing an RNAi library was injected into 12 NOD/SCID mice. The mice were divided into treatment and control groups. Following treatment termination tumor tissues were harvested and genomic DNA was extracted, equalized and labeled with fluorescent dyes. The labeled DNA was hybridized to microarrays to determine fold changes in shRNA representation. Depleted shRNAs would theoretically be present in cells that they impart a growth disadvantage under paclitaxel treatment and thus their targets are potential resistance genes. In contrast, enriched shRNAs would theoretically be present in cells that they impart a growth advantage under paclitaxel treatment and thus their targets are potential sensitivity genes. (B) Average fold change was reported between the treatment and no treatment group (n=6) for 23,506 shRNA targeting genes with known biological functions and was plotted against the -log10 of their p values. The 15 top (C) resistant and (D) sensitivity genes with p value < 0.01 were prioritized for further validation. Significance was determined using unpaired T Test as per microarray manufacturer recommendation.

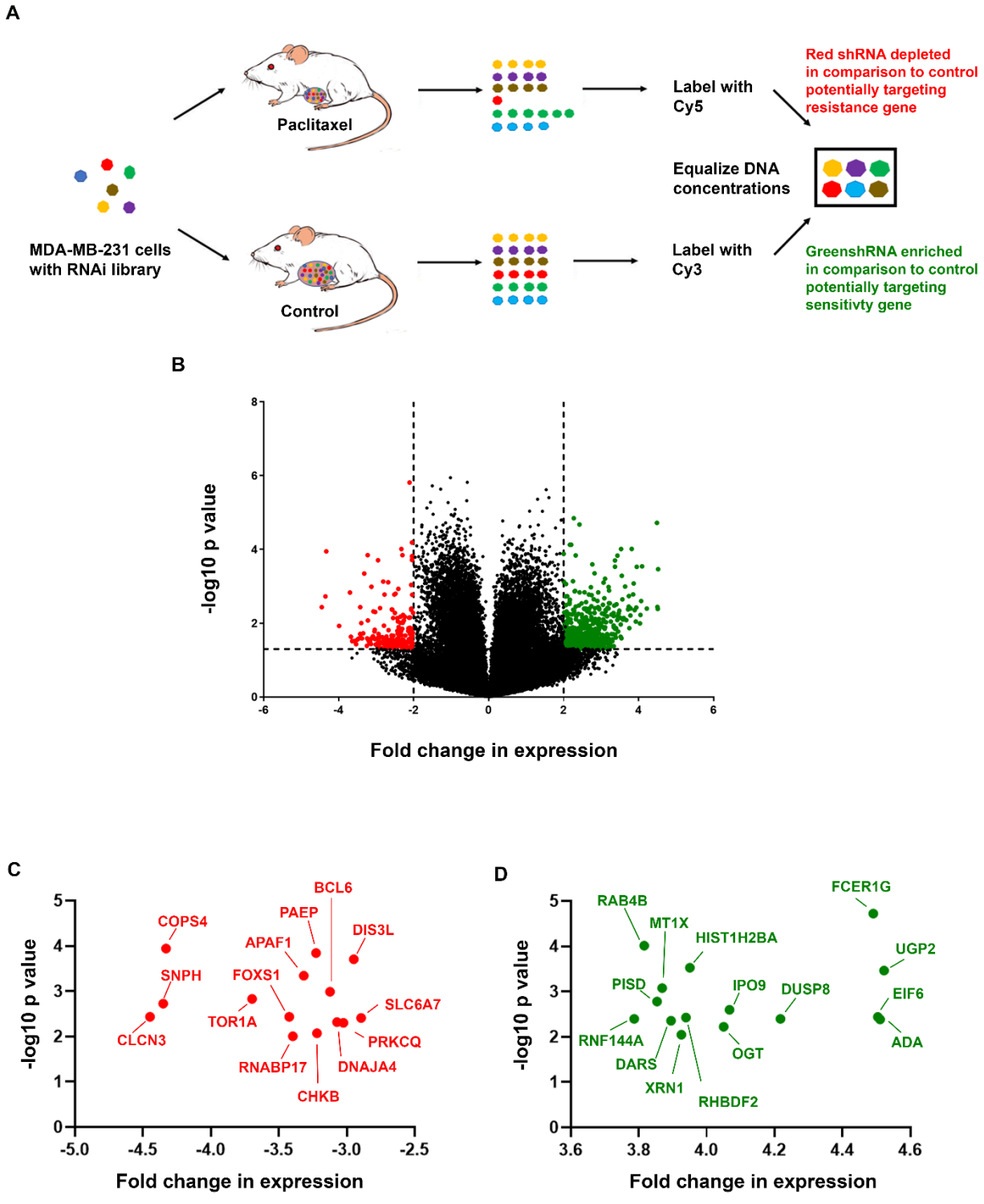

A
Paclitaxel
Label with Cy5
Red shRNA depleted in comparison to control potentially targeting resistance gene
Equalize DNA concentrations
MDA-MB-231 cells with RNAi library
Control
Label with Cy3
GreenshRNA enriched in comparison to control potentially targeting sensitivty gene
B
-log10 p value
Fold change in expression
C
-log10 p value
COPS4
PAEP
BCL6
DIS3L
APAF1
SNPH
FOXS1
SLC6A7
TOR1A
CLCN3
PRKCQ
RNABP17
DNAJA4
CHKB
Fold change in expression
D
-log10 p value
FCER1G
RAB4B
MT1X
HIST1H2BA
UGP2
PISD
IPO9
DUSP8
EIF6
RNF144A
OGT
ADA
DARS
XRN1
RHBDF2
Fold change in expression

Figure 4.2. High levels of BCL6 are associated with decreased survival in patients treated with chemotherapies in different types of cancer. Using Kmplotter (Győrffy et al. 2010b) we investigated the expression correlations of each of the top 15 screen identified (A) resistance and (B) sensitivity genes on breast cancer patient survival in the context of chemotherapy treatment and report their HR and the significance of the difference between patient with high vs low expression of each gene. The patient cohort consisted of 602 patients, expression for each gene was investigated using the specified probe in the high (upper third) vs low (bottom third) groups of patients. Lower HR (less than 1) indicates that high levels of a specific gene is associated with a better outcome for patient survival. In contrast, higher HR (more than 1) indicates that high levels of a specific gene associate with worse outcome for patient survival. Among the top 15 resistance genes, BCL6 had on of the highest HR and the lowest p value, in the context of chemotherapy treated patients, suggesting that BCL6 could be a prime candidate for further investigation as potential chemotherapy resistance mediator in breast cancer. Similarly, we investigate the effect of BCL6 expression on patients using same probe a group separation in (C) a cohort of 76 gastric cancer patients treated with adjuvant chemotherapy and (D) a cohort of 614 ovarian cancer patients treated with chemotherapy.

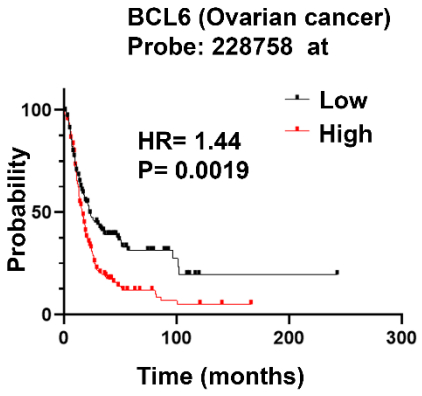
A

Gene	Screen fold change	probe	HR	P value	HR-Basal like	P value- basal like
CLCN3	-4.45	201733_at	1.28	0.19	1.68	0.094
SNPH	-4.35	205104_at	1.25	0.21	1.29	0.036
COPS4	-4.33	218042_at	0.59	0.008	0.72	0.31
TOR1A	-3.70	202349_at	0.85	0.39	1.50	0.17
FOXS1	-3.42	229731_at	3.95	0.000019	1.99	0.094
RANBP17	-3.40	219661_at	1.19	0.37	0.61	0.19
APAF1	-3.32	204859_s_at	1.38	0.09	2.56	0.0054
PAEP	-3.23	206865_s_at	0.95	0.80	1.26	0.46
CHKB	-3.22	204193_at	1.10	0.63	1.23	0.50
BCL6	-3.12	228758_at	2.86	0.0019	2.48	0.036
DNAJA4	-3.07	225061_at	0.90	0.81	1.20	0.67
DIS3L	-2.95	235005_at	1.10	0.76	1.63	0.25
SOCS1	-3.99	210000_s_at	0.68	0.046	0.58	0.074
PRKCQ	-3.02	210039_s-at	0.72	0.072	0.52	0.034
SLC6A7	-2.89	208494_at	0.60	0.0052	0.72	0.33

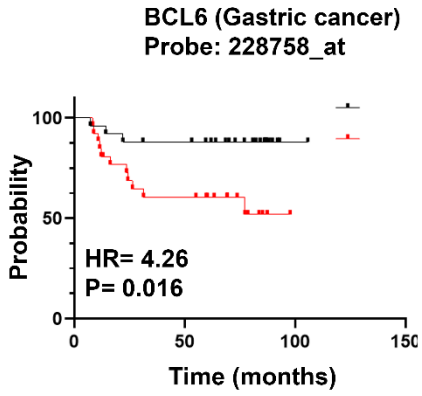
B

Gene	Screen fold change	probe	HR	P value	HR-Basal like	P value- basal like
UGP2	4.52	205480_s_at	0.94	0.74	0.66	0.094
ADA	4.51	216705_s_at	0.66	0.026	0.42	0.036
EIF6	4.5	210213_s_at	1.1	0.63	1.17	0.31
FCER1G	4.49	204232_at	0.66	0.16	0.54	0.17
Dusp8	4.22	206374_at	1.68	0.094	1.73	0.094
IPO9	4.07	217885_AT	1.5	0.03	1.45	0.19
OGT	4.05	220594_at	0.78	0.21	0.72	0.0054
HIST1H2BA	3.95	241519_at	1.07	0.81	0.72	0.46
RHBDF2	3.94	219202_at	1.2	0.35	0.83	0.50
XRN1	3.93	1570394_at	0.78	0.38	0.47	0.036
DARS	3.89	201623_s_at	0.91	0.61	0.48	0.67
MT1X	3.87	204326_x_at	1.48	0.036	1.34	0.25
PISD	3.85	214402_s_at	1.35	0.11	1.19	0.074
RAB4B	3.82	219807_x_at	0.73	0.099	0.92	0.034
RNF144A	3.79	204040_at	1.42	0.069	1.15	0.33

C



D



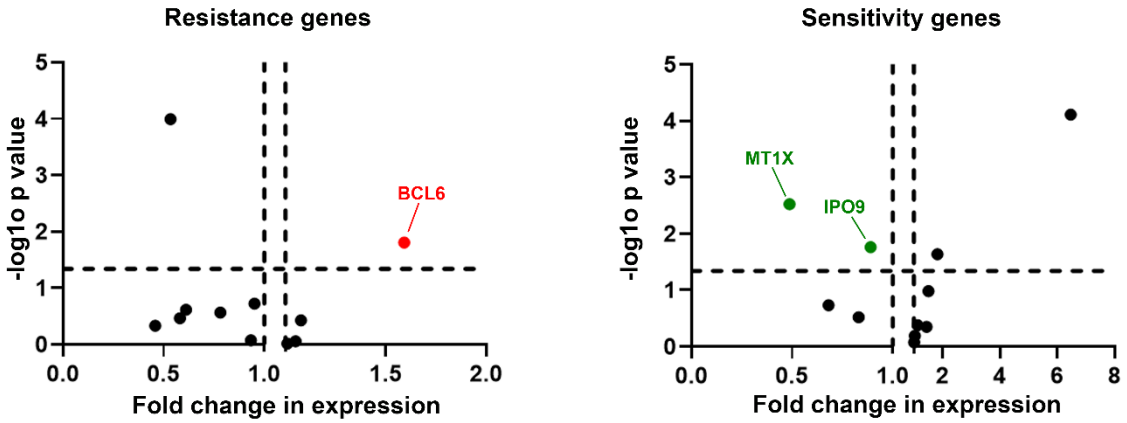
4.3.2 BCL6 Expression is Associated with Anthracyclines Resistance in Breast Cancer.

Taxanes and anthracyclines (e.g. epirubicin) are commonly used in combination to treat breast cancer patients, thus, it was important to determine if any of the screen identified paclitaxel sensitivity and resistance genes also associate with anthracyclines response. To achieve this goal, I utilized a previously published dataset GSE54326 with gene expression from three breast cancer cell lines from different subtypes and their epirubicin resistant counterparts. Using the GEO2R analysis online platform, I investigated the expression of the top 15 resistance and sensitivity genes identified in the screen (Fig. 4.2 A and B) to determine if any of these genes were differentially expressed in the resistant cell lines. Among the resistance genes, only BCL6 was significantly upregulated in the epirubicin resistance MDA MB 231 (Fig 4.3A), MCF7 (Fig 4.3 B) and SKBR3 cell lines (Fig 4.3C). This finding is consistent with the potential importance of BCL6 in predicting breast cancer patient's response and prompted further investigation of BCL6 inhibition in combination with paclitaxel. In contrast, we expected the expression of the sensitivity genes to be downregulated in the resistant cell lines if they were implicated in epirubicin response. Interestingly, none of the screen identified genes were significantly downregulated across all the three cell lines (Fig. 4.3); therefore in isolation, these genes are perhaps less relevant hits for predicting response to chemotherapeutic treatment of breast cancer in general.

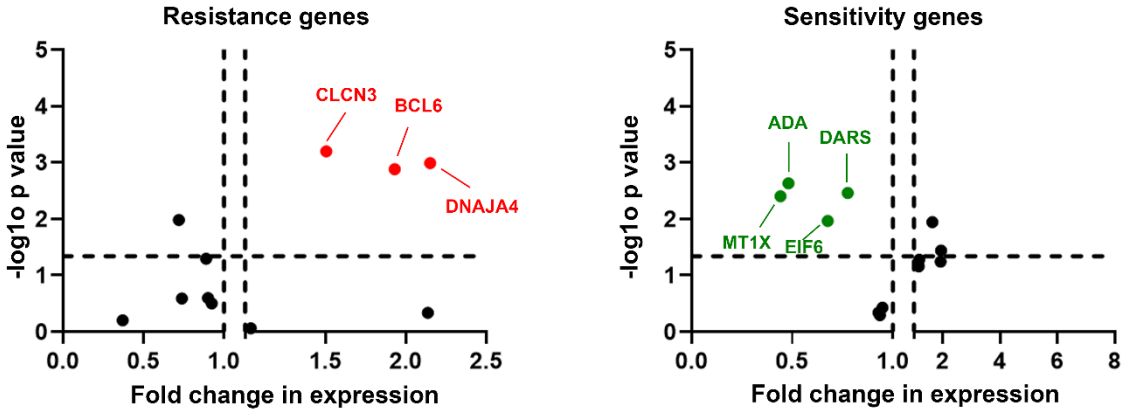
Together, these findings (Figs. 4.1, 4.2, and 4.3) along with the availability of a small molecule inhibitor to target BCL6 (Cerchietti et al. 2010) promoted us to individually investigate the effect of inhibiting/silencing BCL6 in the context of paclitaxel treatment in breast cancer.

Figure 4.3. Screen identified paclitaxel response mediator are differentially expressed in epirubicin resistant breast cancer cell lines. The change in gene expression of screen identified top hits in parental versus epirubicin resistant cell lines (GSE54326) in (A) MDA MB 231, (B) MCF7 and (C) SKBR3. Error bars represent standard deviation (n=3) and significance was determined using unpaired test between parental and resistant cell line for each gene.

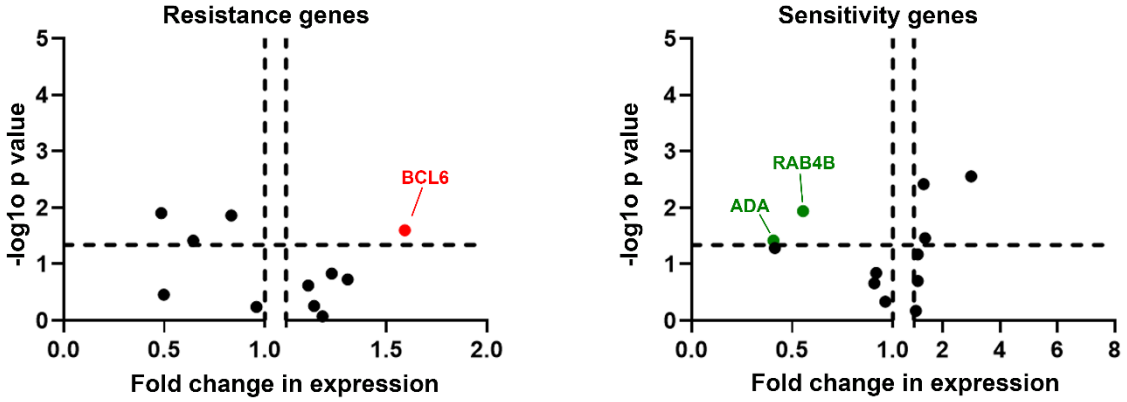
A. Relative expression levels of top screen hits in epirubicin resistant MDA MB 231 cells.



B. Relative expression levels of top screen hits in epirubicin resistant MCF7 cells.



C. Relative expression levels of top screen hits in epirubicin resistant SKBR3 cells.

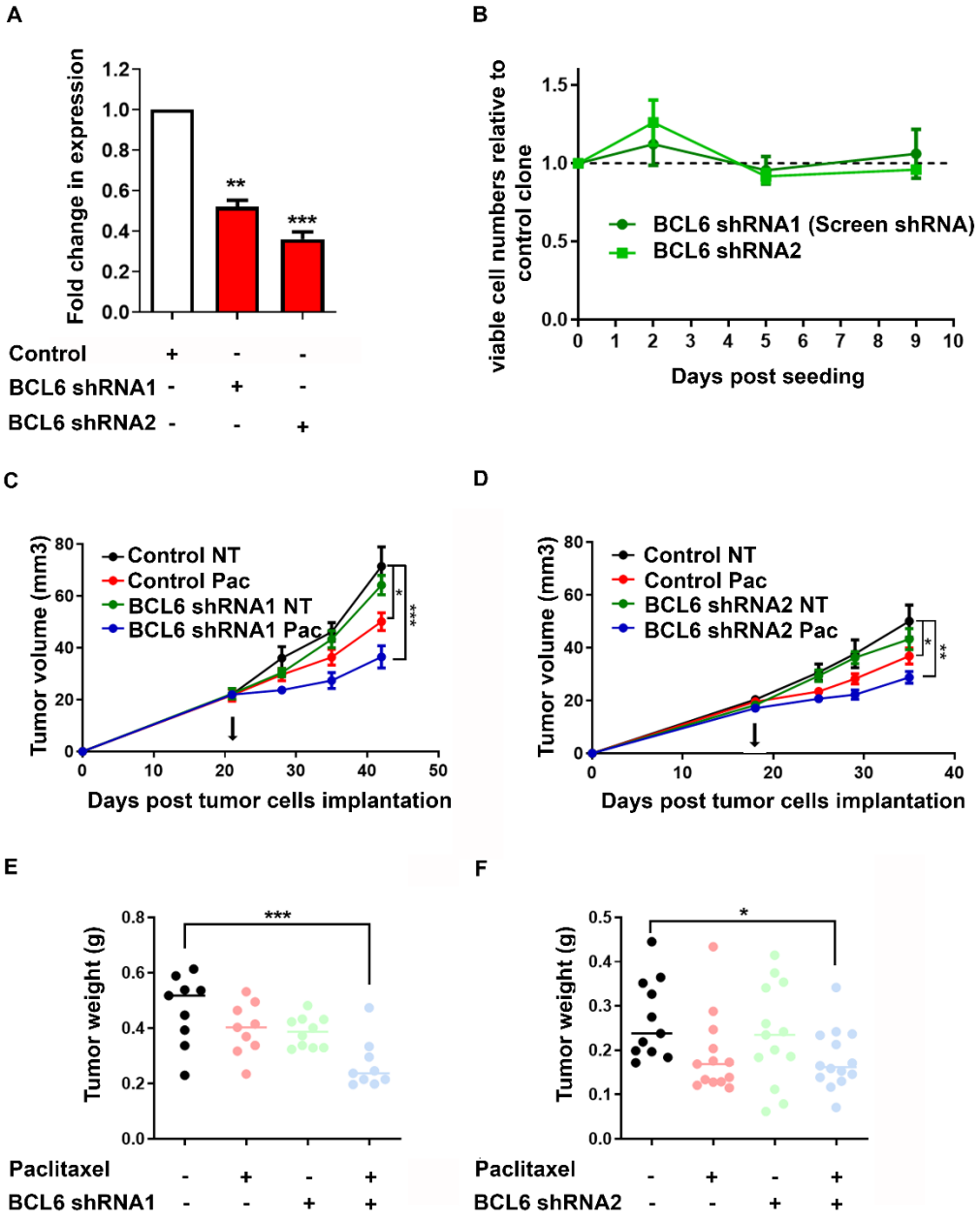


4.3.3 BCL6 Knockdown Increases Paclitaxel Treatment Efficacy in MDA MB 231 Tumors

Having identified BCL6 in the shRNA genome-wide screen as a top paclitaxel-resistance hit, with significant hazard ratio correlations in patients treated with chemotherapy and upregulation in epirubicin resistant cell lines, we prioritized BCL6 for further study and validation. We investigated whether decreased BCL6 expression leads to increased paclitaxel treatment efficacy in MDA MB 231 tumors implanted in NOD/SCID mice. We generated stable knockdown clones of BCL6 in MDA MB 231 cells using two different shRNAs (including the clone identified in the screen) and confirmed that knockdown clones resulted in decreased BCL6 expression compared to a scramble control shRNA clone (Fig. 4.4A). Next, we tested if the decreased BCL6 expression in MDA MB 231 cells alters the growth kinetics of the cells and found that BCL6 knockdown alone did not alter proliferation. (Fig. 4.4B). When assessed *in vivo* in tumors bearing BCL6 targeting shRNA, we similarly noted no significant difference in tumor volume when compared to tumors bearing the scrambled control shRNA (Fig. 4.4C and Fig 4.4D). Notably when the tumor weights were assessed at the endpoint tumors with BCL6 knockdown that received no treatment were smaller than the untreated scrambled control tumor (Fig. 4.4E and Fig 4.4F); however, the change was not significant. Paclitaxel treatment caused a significant decrease in the treated control shRNA tumor volumes when compared to their untreated counterparts (Fig 4.4C and D) and notable decrease in tumor weights (Fig 4.4E and Fig 4.4F). BCL6 knockdown with either shRNA caused an enhanced response to paclitaxel treatment observed in further decrease in tumor volumes and weights (Fig 4.4 C, D,E and F) relative to the untreated control and when compared to

paclitaxel treatment alone. This confirmed the proposed role for BCL6 as a paclitaxel resistance mediator and promoted further investigation of the mechanism by which targeting BCL6 enhance paclitaxel effect in TNBC.

Figure 4.4 BCL6 knockdown enhances paclitaxel induced regression of MDA MB 231 tumors. (A) BCL6 knockdown with two different shRNAs was confirmed using qPCR (shRNA1= screen shRNA). **(B)** The effect of BCL6 knockdown on *in vitro* cultured cell growth was made relative to a scrambled control. The BCL6 role as a potential resistance mediator was assessed in NOD/SCID mice by comparing tumor volumes **(C and D)** and tumor weights **(E and F)** (n=12). Error bars represent standard deviation for panels A and B and standard error of the mean for the panels C, D, E and F. Significance was determined using repeated measures one way anova followed by Tukey test, p value >0.05 =*, > 0.01 =**, > 0.001 =***.


A
Fold change in expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
Control + - -
BCL6 shRNA1 - + -
BCL6 shRNA2 - - +
B
viable cell numbers relative to control clone
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10
Days post seeding
BCL6 shRNA1 (Screen shRNA)
BCL6 shRNA2
C
Tumor volume (mm3)
80
60
40
20
0
0 10 20 30 40 50
Days post tumor cells implantation
Control NT
Control Pac
BCL6 shRNA1 NT
BCL6 shRNA1 Pac
*
***
D
Tumor volume (mm3)
80
60
40
20
0
0 10 20 30 40
Days post tumor cells implantation
Control NT
Control Pac
BCL6 shRNA2 NT
BCL6 shRNA2 Pac
*
**
E
Tumor weight (g)
0.8
0.6
0.4
0.2
0.0
***
Paclitaxel - + - +
BCL6 shRNA1 - - + +
F
Tumor weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
*
Paclitaxel - + - +
BCL6 shRNA2 - - + +

4.3.4 BCL6 Knockdown Enhances Paclitaxel Effect in MDA MB 231 Cells in vitro by Inducing G1/S Phase Cell Cycle Arrest and Promoting Apoptosis

Using the stable knockdown clones of BCL6 generated by the previously mentioned two shRNAs along with the scramble control clone, we investigated the effect of BCL6 expression in MDA MB 231 *in vitro* proliferation under paclitaxel treatment. Following 24 hours of paclitaxel treatment neither the scrambled control nor the cells bearing the BCL6 shRNAs induced a significant change on the number of viable cells (i.e. cells lacking trypan blue stain) when compared to the untreated control (Fig. 4.5A). We preformed apoptosis and cell cycle analyses on control and knockdown cells under treatment and normal conditions from both time points. Apoptosis analysis revealed no changes in the level of apoptotic cells in any of the treatment or untreated groups at the 24 hours post treatment time point (Fig 4.5B), which was expected as we did not observe a change in the number of viable cells assessed by the proliferation assay (Fig. 4.5A). Unlike the proliferation and apoptosis assays, the cell cycle analysis revealed significant changes in the cell cycle progression of cells bearing BCL6 knockdown following 24 hours of paclitaxel treatment (Fig. 4.5C). As previously reported (Schmidt 2014) paclitaxel induced a G2/M cell cycle arrest in the control samples; however, when treated with paclitaxel, cells harboring BCL6 targeting shRNA sequences experienced a G1/S cell cycle arrest.

To determine if the this change in cell cycle progression at 24 hours, would translate to later effects on proliferation and have enduring lasting effects, we repeated the experiments outline above on samples collected 72 hours post treatment

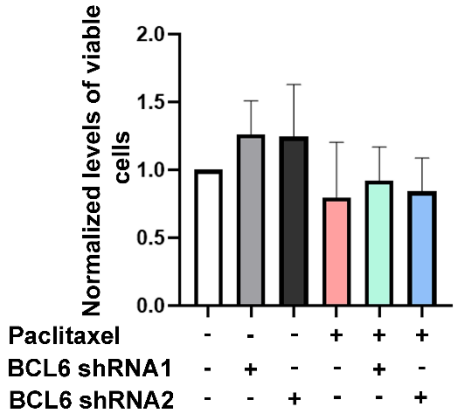
termination. Interestingly, when the level of viable cells was assessed at this time point the treated scrambled control had a significant decrease in the level of viable cells and this effect was further enhanced in cells with both shRNAs targeting BCL6 (Fig 4.6A).

Apoptosis analysis revealed similar findings to the cell viability assay. The treated control samples had a decrease in the level of live cells accompanied with an increase in the level of early apoptotic cells.. Importantly, this effect was enhanced in cells with BCL6 knockdown as they showed a further decrease in level of live cells and an increase in early apoptotic cells to a greater extent than observed in the control treated cells (Fig. 4.6B).

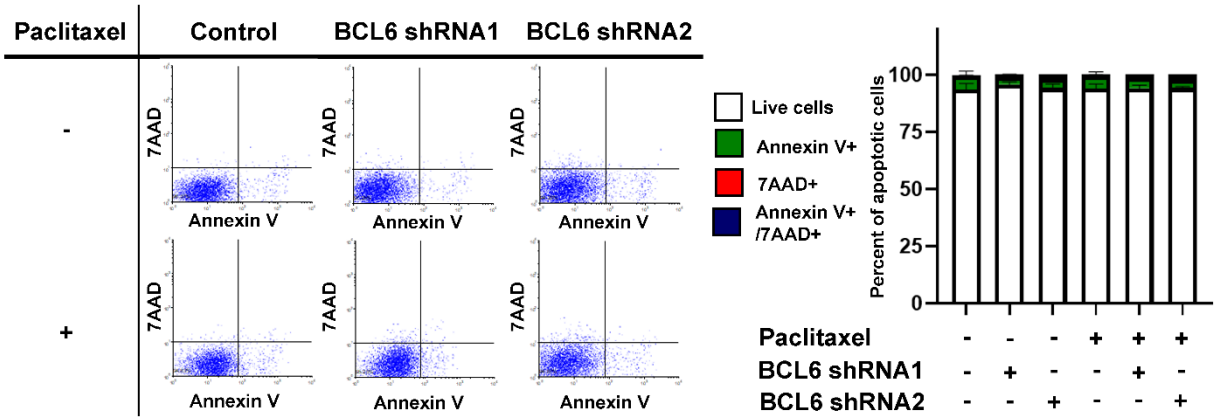
Cell cycle analysis of samples collected 72 hours post treatment termination showed the control samples treated with paclitaxel remain in a G2/M cell cycle arrest (Fig. 4.6C) to a lesser extent than observed at treatment termination (Fig.4.5C). In contrast, cells harboring BCL6 targeting shRNAs were completely arrested in G1/S phases with almost no cells in the G2 phase (Fig. 4.6C). These results suggests that changes in the cell cycle progression at 24 hours post treatment induced by BCL6 knockdown in the context of paclitaxel treatment, lead to the later observed effects on reduced cell numbers and increased apoptosis (Fig 4.6A and B). Together these findings are consistent with BCL6 enhancing paclitaxel activity in MDA MB 231 cells by increasing paclitaxel induced apoptosis and promoting G1/S phase cell cycle arrest.

Figure 4.5 BCL6 knockdown alters paclitaxel induced cell cycle arrest in MDA MB 231 cells after 24 hours of treatment. (A) Trypan Blue cell viability assay was preformed on control and BCL6 knockdown cells following 24 hours of paclitaxel treatment and the level of viable cells was reported relative to untreated control (n=3). **(B)** Flow cytometry apoptosis analysis was preformed on all samples collected at this time point using Annexin V and 7AAD antibodies to determine change in level of live, early apoptotic and late apoptotic cells (n=4). **(C)** Flow cytometry cell cycle analysis was performed on all samples to determine the effect of BCL6 knockdown on paclitaxel induced cell cycle arrest (n=4). Significance was determined using repeated measures one-way anova followed by Tukey's test, p value >0.05 =*, > 0.01 =**, > 0.001 =***.

A. Cell viability of MDA MB 231 cells treated with paclitaxel



B Apoptosis analysis in MDA MB 231 cells treated with paclitaxel



C. Cell cycle analysis in MDA MB 231 cells treated with paclitaxel

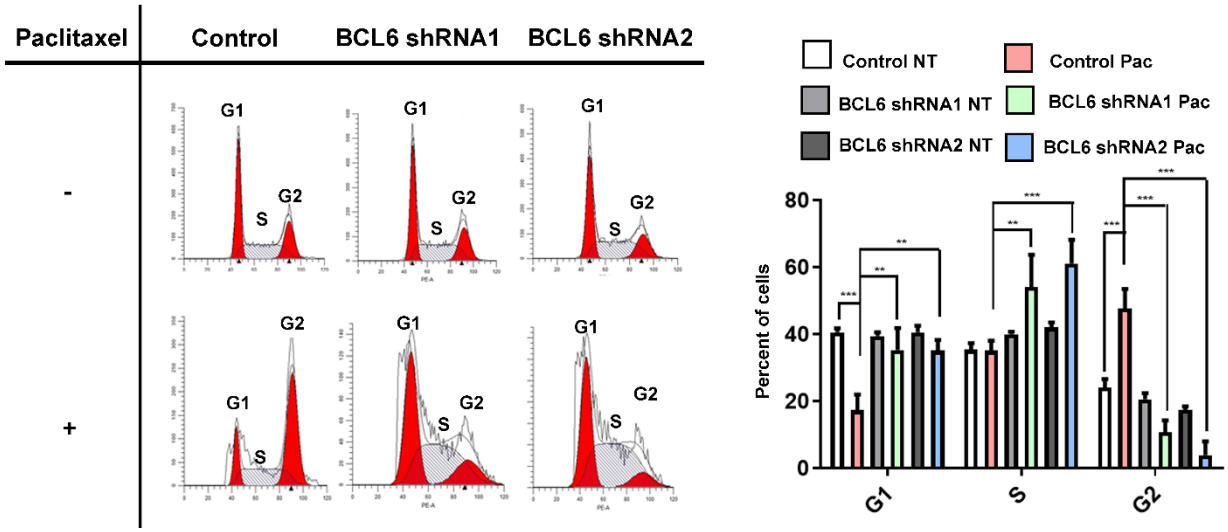
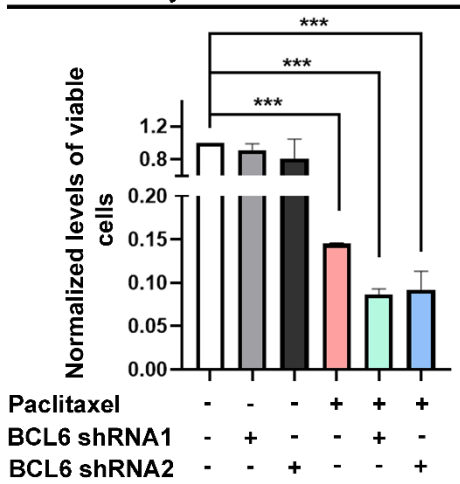
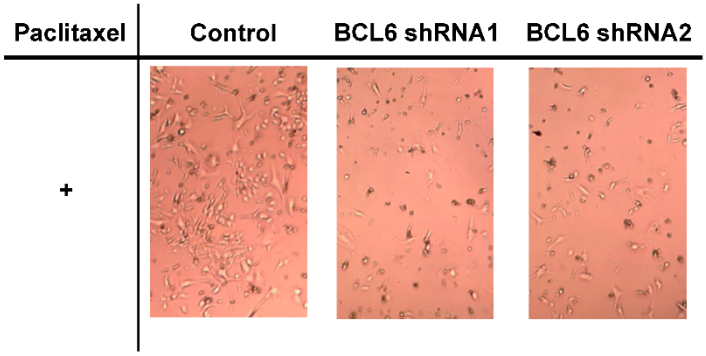


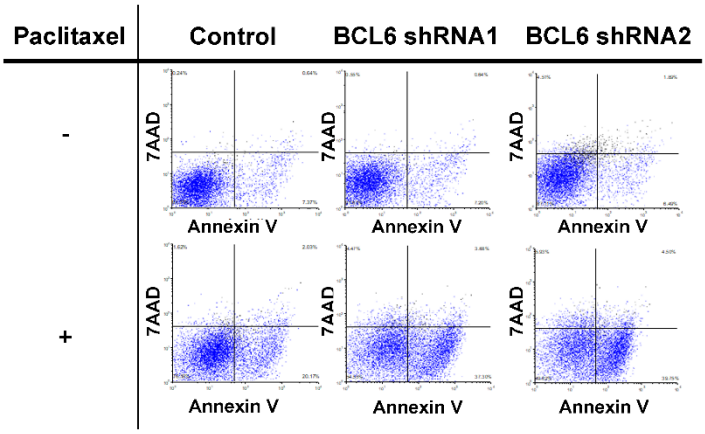
Figure 4.6. BCL6 knockdown enhances paclitaxel induced cell death and reduces number of viable cells in MDA MB 231 cells 72 hours post treatment termination. **(A)** Trypan Blue cell viability assay was preformed on control and BCL6 knockdown cells collected at 72 hours post treatment termination and the level of viable cells was reported relative to untreated control (n=3). **(B)** Flow cytometry apoptosis analysis was preformed on all samples collected at this time point using Annexin V and 7AAD antibodies to determine change in level of live, early apoptotic and late apoptotic cells (n=4). **(C)** Flowcytometry cell cycle analysis was performed on all samples to determine the effect of BCL6 knockdown on paclitaxel induce cell cycle arrest (n=4). Significance was determined using repeated measures one-way anova followed by Tukey's test, p value >0.05 =*, > 0.01 =**, > 0.001 =***.

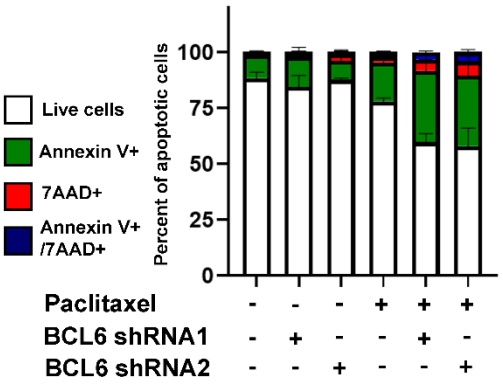
A. Cell viability of MDA MB 231 cells treated with paclitaxel (72 hours post treatment termination)



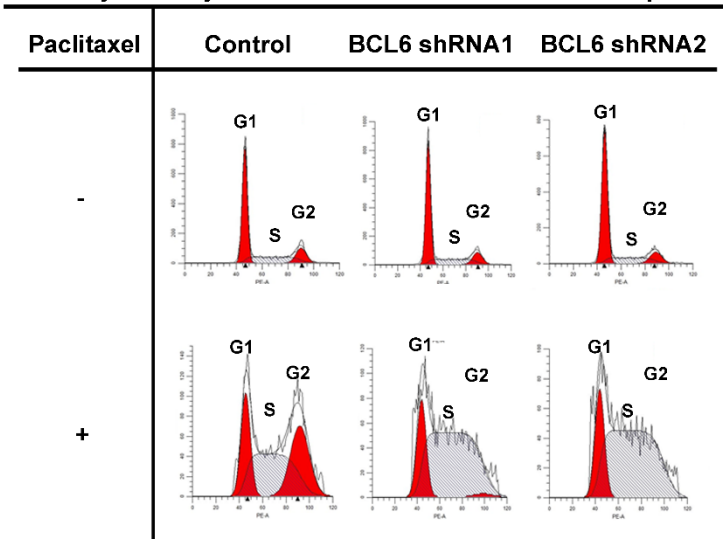


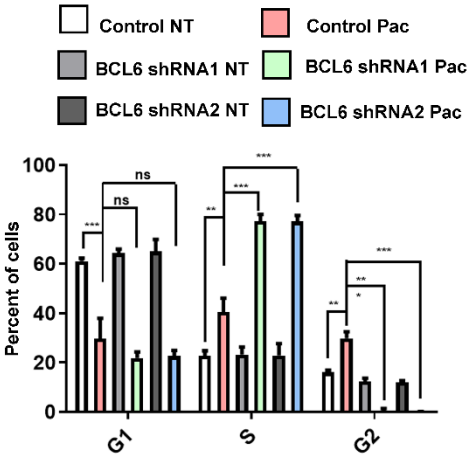
B Apoptosis analysis in MDA MB 231 cells treated with paclitaxel (72 hours post treatment termination)





C. Cell cycle analysis in MDA MB 231 cells treated with paclitaxel (72 hours post treatment termination)





4.3.5 BCL6 Inhibitor Increases Paclitaxel Treatment Efficacy in MDA MB 231 in vivo and in vitro Models

To determine if BCL6 can be used as a therapeutic target in combination with paclitaxel, we investigated the effect of combining BCL6 inhibitor 79-6 (BCL6i) with paclitaxel on MDA MB 231 tumor growth. Group of mice receiving individual treatments with either BCL6i or paclitaxel alone showed a decrease in tumor volume when compared to the no treatment control; however, the changes were not significant (Fig. 4.7A). In contrast, combination therapy with BCL6i and paclitaxel caused a significant reduction in tumor volume in comparison to the no treatment group (Fig. 4.7A). Changes in tumors weights with individual and combination therapies reflected the tumor volume changes; however, none of the changes were significant (Fig. 4.7B). Findings from this experiment suggests that BCL6i could be beneficial in enhancing paclitaxel induced tumor regression; however, further investigation is needed to assess the best dosage of BCL6i and timing of treatment to achieve this goal. Next, we tested if inhibiting BCL6 in MDA MB 231 cells alters cell proliferation under paclitaxel treatment *in vitro*. Following 24 hours of paclitaxel treatment, both individual treatments caused a non-significant decrease in the level of viable cells when compared to the no treatment control (Fig. 4.7C). In contrast, combination therapy with BCL6i and paclitaxel caused a significant reduction in the level of viable cells when compared to the negative control (Fig. 4.7C).When we assessed the level of viable cells 72 hours post treatment termination, we observed paclitaxel treatment alone to have a significant decrease in the level of viable cells and this effect to be further enhanced in cells when paclitaxel is combined with BCL6i (Fig 4.7D).

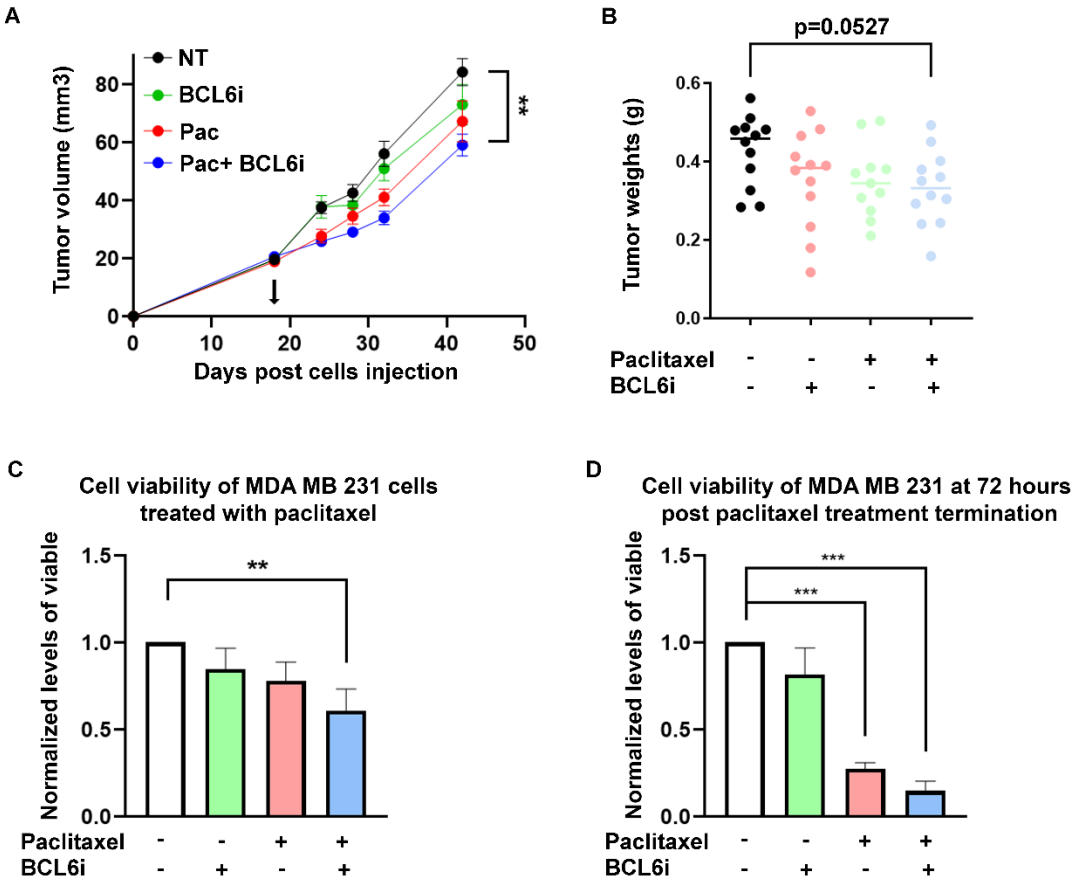
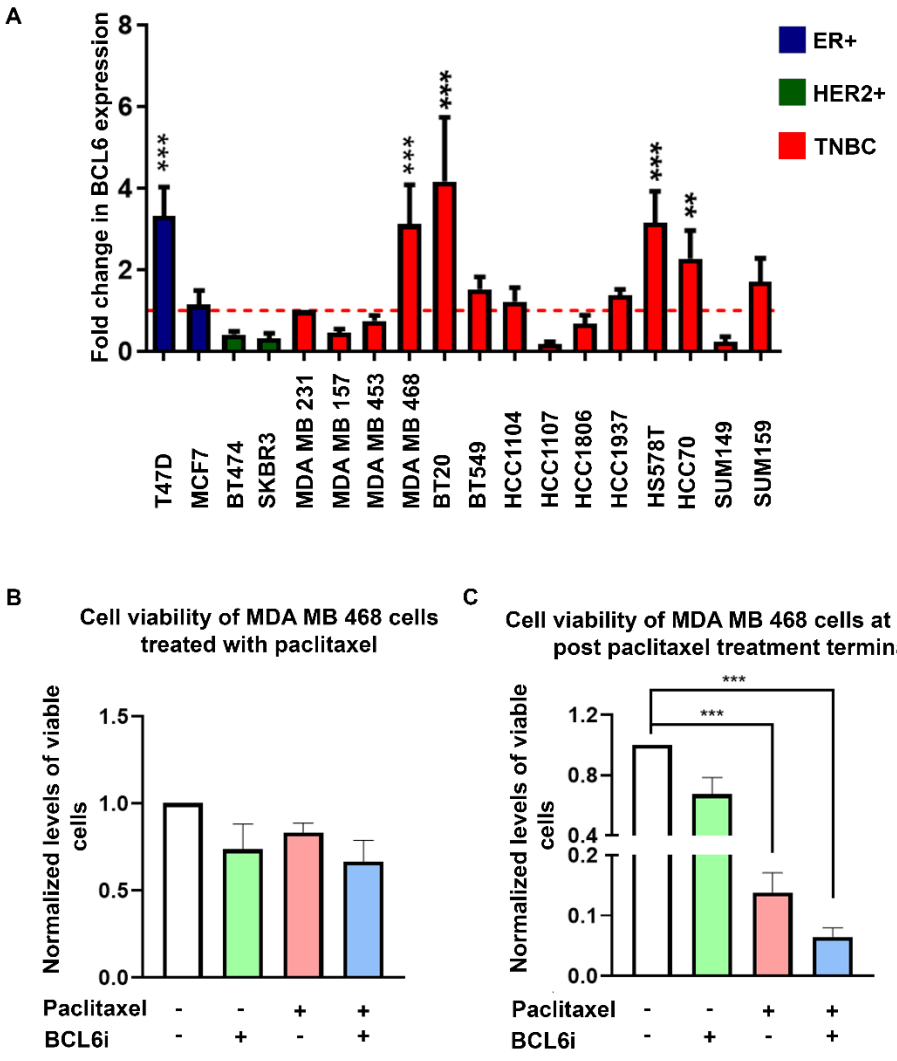


Figure 4.7. BCL6i enhances paclitaxel induced MDA MB 231 tumor regression and reduction in cell viability. The potential use of BCL6i in combination with paclitaxel to enhance treatment effect was investigated *in vivo* (n=12) by comparing tumor **(A)** volumes and **(B)** weights**.** Cell viability assay was used to investigate the effect of combination therapy *in vitro* (n=4) after **(C)** 24 hours treatment and **(D)** 72 hours post treatment termination. Error bars represent standard error of the mean for panels A and B and Standard deviation for panels C and D. Significance was determined using Brown–Forsythe test anova for panel A and B and repeated measures anova for panels C and D. Both tests were followed by Dunnett test, p value >0.05 =*, >0.01 =**, >0.001 =***.

4.3.6 BCL6 Inhibitor Enhances Paclitaxel Effect in MDA MB 468 Cells

Next, we investigated BCL6 expression in a panel of breast cancer cell line to identify another model to further investigate the effect of combining BCL6i with paclitaxel to enhance its effect. The panel included 18 cell lines, including 14 TNBCs, 2 HER2+ and 2 ER+ cell lines. Notably, BCL6 expression was high in several TNBCs cell lines (e.g. MDA MB 468 cells) as well as ER+ T47D cells (Fig. 4.8A). We decided to further investigate the effect of inhibiting BCL6 on paclitaxel induced cell regression in MDA MB 468 cells. Using the same treatment protocol followed in MDA MB 231 cells with paclitaxel and BCL6i, we assessed the level of viable MDA MB 468 cells at 24 hours post treatment and 72 hours post treatment. Following 24 hours of treatment, combination therapy induced a greater reduction in the number of viable cells than either individual treatment (Fig. 4.8B); however, the changes were not significant. Similar to MDA MB 231, we assessed the level of viable cells at 72 hours post treatment termination (Fig. 4.8C). While paclitaxel treatment alone resulted in a significant decrease in the level of viable cells, this effect was further enhanced in cells when paclitaxel is combined with BCL6i (Fig. 4.8C). Together, this data further validates BCL6 proposed role in paclitaxel treatment response in TNBC; however, further investigation is required to determine if these effects would be evident in other breast cancer cell lines.

Figure 4.8. BCL6i enhances paclitaxel induced reduction of viable cells in MDA MB 468 cells post treatment termination. (A) BCL6 expression in a panel of 18 breast cancer cell lines is assessed by qPCR. (B)Normalized level of viable cells following individual and combination treatment termination for 24 hours in MDA MB 468. (C) Normalized level of viable cells at 72 hours post treatment termination in MDA MB 468 cells. Error bars represent standard deviation (n=4) and significance was determined using repeated measures anova followed by Dunnett test, p value >0.05 =*, >0.01 =**, >0.001 =***.


A
Fold change in BCL6 expression
0
2
4
6
8
ER+
HER2+
TNBC
***
***
***
***
**
T47D
MCF7
BT474
SKBR3
MDA MB 231
MDA MB 157
MDA MB 453
MDA MB 468
BT20
BT549
HCC1104
HCC1107
HCC1806
HCC1937
HS578T
HCC70
SUM149
SUM159

B
Cell viability of MDA MB 468 cells treated with paclitaxel
Normalized levels of viable cells
0.0
0.5
1.0
1.5
Paclitaxel - - + +
BCL6i - + - +

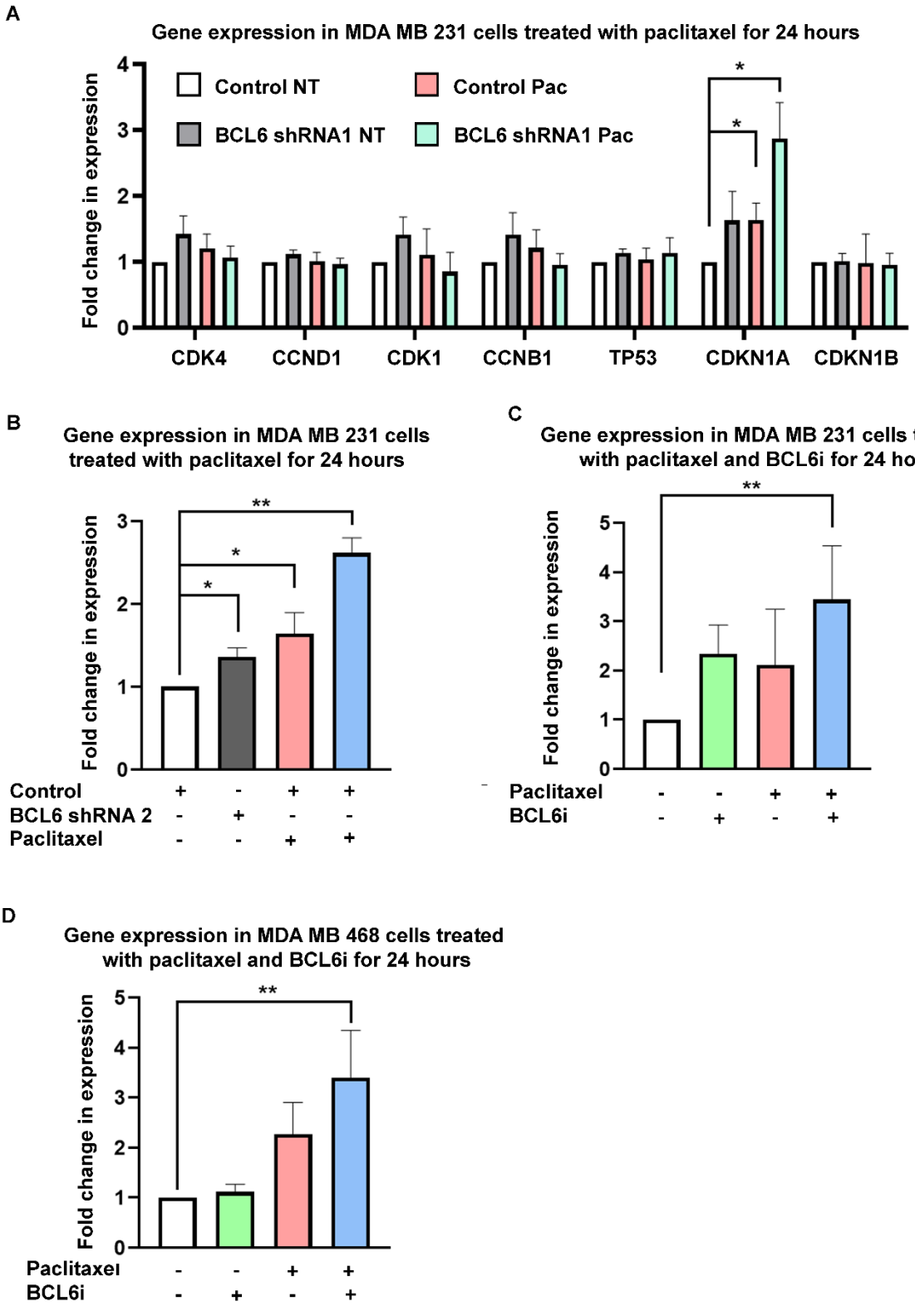
C
Cell viability of MDA MB 468 cells at 72 hours post paclitaxel treatment termination
Normalized levels of viable cells
0.0
0.1
0.2
0.4
0.8
1.2
***
***
Paclitaxel - - + +
BCL6i - + - +

4.3.7 BCL6 Inhibition Increases Cyclin Dependent Kinase Inhibitor 1A Expression under Paclitaxel Treatment in Breast Cancer Cells

Previous reports have demonstrated the important role of BCL6 as a transcription regulator involved in silencing many important genes involved in cell cycle progression and apoptotic pathways (Ci et al. 2009; Phan et al. 2005). Thus, we hypothesized that the effect we observed with BCL6 silencing on cell cycle progression and apoptosis in the context of paclitaxel treatment (Figs. 4.5C and 4.6C) is associated with altered transcriptional profile of some of the key regulators of cell cycle. While several check points exist in the mammalian cell cycle, we focused on the regulators of cell cycle progression from G1 to the S phase and from the G2 to the M phase. The qPCR analyses included genes coding for cyclins, cyclin dependent kinases as well as major regulators of cell cycle regulation pathways such as TP53 and cyclin dependent kinase inhibitor 1A and 1B (CDKN1A) and (CDKN2A).While the expression of many of these cell cycle regulators was unaffected by BCL6 inhibition in MDA MB 231 cells with the screen shRNA (BCL6 shRNA1) in combination with paclitaxel treatment, we observed a significant increase in CDKN1A levels in samples collected at 24 hours post treatment. (Fig. 4.9A). Similarly, CDKN1A levels were upregulated in samples treated with paclitaxel for 24 hours when BCL6 expression was downregulated using shRNA2 in MD MB 231 (Fig 4.9B). Additionally, BCL6i treatment resulted in an increase in CDKN1A mRNA levels in (Fig. 4.9C) MDA MB 231 cells and (Fig. 4.9D) MDA MB 468 in the context of paclitaxel treatment. This result was in accordance with a previous study that demonstrates that BCL6 inhibits CDKN1A and cell cycle arrest in B cell lymphoma

(Phan et al. 2005). The elevated levels of CDKN1A (encodes P21) could explain the additive effect we observed following BCL6 inhibition on paclitaxel induced apoptosis and the G1/S cell cycle arrest. Together theses findings demonstrate that additional benefit of inhibiting BCL6 in combination with paclitaxel in breast cancer could be attributed to its regulation of CDKN1A a known regulator of apoptosis and cell cycle progression.

Figure 4.9. Silencing or inhibiting BCL6 in the context of paclitaxel treatment is associated with increased expression of CDKN1A in TNBC cell lines. (A) QPCR analysis of relative mRNA levels of several key cell cycle genes in MDA MB 231 cells harboring scrambled control and BCL6 shRNA1 following 24 hours paclitaxel treatment. QPCR analysis to detect CDKN1A mRNA levels in the context of paclitaxel treatment in (B) MDA MB 231 cells harboring scrambled control and BCL6 shRNA2 and in (C) MDA MB 231 and (D) MDA MB 468 cells treated with 50uM BCL6i. Error bars represent Standard deviation (n=4) and significance was determined using one way anova followed by Dunnett test, p value >0.05 =*, >0.01 =**.


A
Gene expression in MDA MB 231 cells treated with paclitaxel for 24 hours
Control NT
Control Pac
BCL6 shRNA1 NT
BCL6 shRNA1 Pac
Fold change in expression
CDK4
CCND1
CDK1
CCNB1
TP53
CDKN1A
CDKN1B
B
Gene expression in MDA MB 231 cells treated with paclitaxel for 24 hours
Control
BCL6 shRNA 2
Paclitaxel
C
Gene expression in MDA MB 231 cells treated with paclitaxel and BCL6i for 24 hours
Paclitaxel
BCL6i
D
Gene expression in MDA MB 468 cells treated with paclitaxel and BCL6i for 24 hours
Paclitaxel
BCL6i

4.4 Discussion

The wide use of taxanes in cancer treatment in general and breast cancer specifically (McGrogan et al. 2008) have sparked a continuous interest in further investigation of resistance mechanisms and methods to overcome this resistance to enhance patient's survival. Several studies have focused on the difference by which a cancer cell can become resistance to treatment including upregulating the multidrug resistance pumps (MDR) (Yusuf et al. 2005) and β tubulin overexpression (Nicoletti et al. 2001). Other studies have focused on the downregulation of apoptotic pathways (Ferlini et al. 2003) and the role of non-coding RNAs and epigenetic mechanisms in mediating resistance (Kojima et al. 2010). While many of these studies focused on specific mechanisms, several studies employed genome-wide screening technologies to investigate mediators of paclitaxel response on a larger scale (Bauer, Ye, et al. 2010; Swanton et al. 2007). However, most of these studies were preformed *in vitro* and thus, could be missing important factors associated with tumor development *in vivo*. Furthermore, many microenvironmental factors present *in vivo* can affect and modify resistance mechanisms identified *in vitro* such as hypoxia and inflammation (Diakos et al. 2014; McKeown 2014). This sparked our interest in preforming a genome-wide shRNA screen *in vivo* to identify genes with that play a role in paclitaxel response. Interestingly, genes identified in our screen were different from those identified in previous *in vitro* screens, highlighting how preforming such studies in an *in vivo* setting my identify factors that could be missed if screening is performed *in vitro*. Additionally, preforming gene set enrichment analyses for the top sensitivity and resistance genes did not identify any specific function clusters in either set.

Among the top resistance genes identified screen we focused our investigation on BCL6, a known mediator of tumor progression in B cell lymphoma (Polo et al. 2004) that has been previously investigated in breast cancer (Tran et al. 2010; Walker et al. 2015) but not in the context of taxane sensitivity. Our investigation revealed high levels of BCL6 is associated with decreased survival in breast, ovarian and gastric cancers treated with chemotherapy. Additionally, we investigated the expression of the top identified sensitivity and resistance genes and their association with epirubicin resistant in three different breast cancer cell line models. While several sensitivity genes where downregulated in at least one of the resistant cell lines, BCL6 was upregulated in all three resistant cell lines in comparison to the parental cell lines. This indicate that BCL6 could also be associated with epirubicin resistance and should be investigated as a potential target for enhancing anthracyclines effect in breast cancer.

Given BCL6 importance in B cell lymphoma, several inhibitors were developed to target and inhibit its activity (Cerchietti et al. 2010), making it an ideal candidate to investigate for combination therapy investigation with paclitaxel. To determine BCL6 importance in paclitaxel response, we generated two stable knockdowns of BCL6 in the MDA MB 231 cell line and investigated the effect of silencing BCL6 on paclitaxel treatment. Knocking down BCL6 enhanced paclitaxel effects *in vivo* (Fig. 4.4) and *in vitro* (Fig 4.6A), consistent with its potential role as novel paclitaxel resistance mediator. Given paclitaxel's reported mechanism as a microtubule stabilizer that induces cell cycle arrest and apoptosis, we next investigated the effect of BCL6 knockdown on cell cycle arrest and apoptosis in the

context of paclitaxel treatment. While knocking down BCL6 was associated with an additive effect on paclitaxel induced apoptosis (Fig 4.6B), it resulted in a shift in cell cycle arrest from the G2/M phases to G1/S (Figs 4.5C and 4.6C). This was associated with an increase in CDKN1A expression (Fig. 4.9), which was unsurprising given previous reports indicating BCL6 role as a transcription repressor of CDKN1A (Phan et al. 2005). While the increase in CDKN1A expression can explain the shift in cell cycle arrest that was observed following paclitaxel treatment, it also prompts further investigation given CDKN1A important role in promoting and maintaining senescence (Passos et al. 2010).

Using a small molecule inhibitor that targets BCL6 we were able to mimic the additive effect we observed in the knockdown studies on paclitaxel induced growth regression of MDA MB 231 cells *in vitro* and *in vivo* (Fig. 4.7). Inhibiting BCL6 was also associated with an enhanced reduction in the number of viable MDA MB 468 cells under paclitaxel treatment (Fig 4.8). In both TNBC cell lines MDA MB 231 and MDA MB 468 combination therapy with paclitaxel and the BCL6i resulted in an increase in CDKN1A levels (Fig 4.9C and D)

The success of the oncotype DX as a predictive tool for treatment response in ER+ positive breast cancer patients (Sparano et al. 2018), sparked an interest in developing predictive genes signature for taxanes response in breast cancer. Several taxane associated gene signatures were developed by analyzing gene expression from patients' tumors prior to treatment, leading to the identification of gene signatures which predicts response (Dorman et al. 2016; He et al. 2014). To this end, we are working on developing predictive gene signature by utilizing the top genes identified

in the genome-wide RNAi screen. To achieve this goal, we are employing machine learning random forest models to build the gene signature and test it in several published datasets. Preliminary data from this work is providing a promising outlook on the screen-derived signature accuracy and specificity in predicting patients' outcomes; however, further investigation is required to optimize its performance and compare it to existing taxane signatures.

CHAPTER 5

Copyright statement

This chapter have been previously published in the following manuscript. The corresponding text has been edited for length, consistency, and to include recent findings.

Thomas T. Huynh, Mohammad Sultan, Dejan Vidovic, Cheryl A. Dean, Brianne M. Cruickshank, Kristen Lee, Chao-Yu Loung, Ryan W. Holloway, David W. Hoskin, David M. Waisman, Ian C. G. Weaver, Paola Marcato. Retinoic acid and arsenic trioxide induce lasting differentiation and demethylation of target genes in APL cells. *Sci Rep* **9,** 9414 (2019).

Contribution statement

Thomas Huynh and my self contributed equally to the preparation of the above manuscript and its figures for publication with the guidance of our supervisor Dr. Paola Marcato. Dejan Vidovic, Cheryl Dean, Brianne Cruickshank, Kristen Lee, Chao-Yu Loung, Ryan Holloway, Dr. David W. Hoskin, Dr. David M. Waisman and Dr. Ian C. G. Weaver supported the writing, editing and the revision of the manuscript. Kristen Lee, and Dr Ian Weaver performed the pyrosequencing and methylation analyses.

5.1 Abstract

APL is characterized by arrested differentiation of promyelocytes. Patients treated with ATRA alone experience relapse, while patients treated with ATRA and ATO are often relapse-free. This suggests sustained changes have been elicited by the combination therapy. To understand the lasting effects of the combination therapy, we compared the effects of ATRA and ATO on NB4 and ATRA-resistant NB4-MR2 APL cells during treatment versus post treatment termination. After treatment termination, NB4 cells treated with ATRA or ATO reverted to non-differentiated cells, while combination-treated cells remained terminally differentiated. This effect was diminished in NB4-MR2 cells. This suggests combination treatment induced more permanent changes. Combination treatment induced higher expression of target genes (e.g., transglutaminase 2 and retinoic acid receptor beta), which in NB4 cells was sustained post treatment termination. To determine whether sustained epigenetic changes were responsible, we quantified the enrichment of histone modifications by chromatin immunoprecipitation, and CpG methylation by bisulfite-pyrosequencing. While ATRA and combination treatment induced similar histone acetylation enrichment, combination treatment induced greater demethylation of target genes, which was sustained. Therefore, sustained demethylation of target genes by ATRA and ATO combination treatment is associated with lasting differentiation and gene expression changes.

5.2 Introduction

APL accounts for approximately 10–15% of adult acute myeloid leukemias and until recently was associated with high mortality and poor patient outcomes(Zhou et al. 2007). The malignancy is characterized by arrested promyelocyte differentiation in the myeloid lineage of hematopoietic stem cells, resulting in the absence of mature granulocytes and an over accumulation of promyelocyte precursors (Warrell et al. 1993). The deficiency of this population of cells manifests in patients as severe coagulation defects that can culminate in fatal disseminated intravascular coagulation and systemic hemorrhaging(McCraw 2008). Uniquely, the cause in 98% of APL cases is attributed to a single chromosomal translocation event between chromosome 15 and chromosome 17(Adams and Nassiri 2015; Kakizuka et al. 1991; Mueller et al. 2006) This results in aberrant fusion between the PML and RARα genes located on the respective chromosomes. The resultant PML-RARα chimeric protein behaves as an altered RAR nuclear receptor, which changes the DNA binding specificity, and represses the transcriptional programs normally controlled by RAR-retinoid-X-receptor heterodimers, through enhanced interactions with corepressors (Ablain and De Thé 2014; Grignani et al. 1996; Zhou et al. 2006). Consequently, differentiation is abrogated and immortalization of the promyelocytes is promoted.

Similar to other malignancies, APL cells are characterized by aberrant epigenetic changes to DNA methylation and histone modifications which interfere with normal transcriptional programs and contribute to blocked granulocyte differentiation and disease progression (Cheung and So 2011). Specifically, PML-RARα binding to target gene promoters (e.g., retinoic acid receptor beta, RARβ; transglutaminase 2, TGM2) is

associated with decreased activating histone marks (e.g., acetylation of histone 3 lysine 9 and 14, H3K9/14ac), increased repressive histone marks (e.g., tri-methylation of histone 3 lysine, H3K9me3) and DNA hypermethylation, leading to repressed transcription and heterochromatin formation (Arteaga et al. 2013, 2015; Chim et al. 2003; Martens et al. 2010; Schoofs et al. 2013). PML-RARα binding to DNA is sufficient to induce histone modification changes, while DNA methylation changes can occur independent of PML-RARα and are believed to be a late event in leukemogenesis and associated with loss of transcription factor binding (Schoofs et al. 2013).

In the context of treatment, the oncogenic fusion gene provides an ideal target for therapeutic intervention, since supraphysiological levels of the RAR ligand, ATRA, induces degradation of the PML-RARα in APL cells and restores normal RAR transcriptional (Ablain and De Thé 2014; Arteaga et al. 2015; Yoshida et al. 1996). ATRA-induced target genes such as TGM2, mediate the differentiation of leukemic promyelocytes into mature granulocytes, restoring normal coagulation dynamics (Benedetti et al. 1996; Csomós et al. 2010). In terms of its effects on the aberrant epigenome of APL cells, ATRA induces genome-wide activating histone acetylation (e.g., H3K9ac and H3K9/14ac) of target genes, but it has negligible effects on histone methylation (e.g., H3K9me3 and H3K27me3) and DNA methylation (Arteaga et al. 2013, 2015; Chim et al. 2003; Martens et al. 2010; Schoofs et al. 2013). Since the first introduction of ATRA therapy for APL in 1985, it has had a dramatic impact in the survival outcomes of this once deadly disease (Z-Y Wang 2008). Used alone, ATRA induces a short-term remission in approximately 80% of patients . Its subsequent combination with anthracycline-based chemotherapies significantly reduced relapse and

increased response rates. Recent trials have demonstrated that the combination of ATRA with ATO treatment is superior to the combination of ATRA and anthracyclines; as a result, treatment recommendations are moving towards this newer combination (Abaza et al. 2017; Au et al. 2011; Coombs, Tavakkoli, and Tallman 2015; Estey et al. 2006; Huang 2016; Shen et al. 2004; Zeidan and Gore 2014).

ATO induces degradation of PML-RARα, modest differentiation and apoptosis of APL cells, and is synergistic with ATRA (Coombs et al. 2015; Giannì 1998; Shen et al. 1997; Zheng et al. 2005). The underlying mechanism behind the reduced relapse rates of the combination treatment is only partly understood and the effects of the combination treatment on epigenetic modifications have not been explored. In this study, we compare the short-term, long-term and the post treatment termination effects of ATRA and ATO on NB4 and ATRA-resistant NB4-MR2 APL cells, and characterized the epigenetic modifications of canonical target and differentiation genes RARβ and TGM2. The enhanced effectiveness of ATRA and ATO combination treatment in inducing terminal differentiation and expression of target genes was most evident 96 h after treatment was terminated. This effect was significantly diminished in ATRA-resistant NB4-MR2 cells. ATRA and ATO combination treatment reduced CpG island methylation of target gene promoters, which was sustained even after treatment was terminated. Together, this data provides new evidence of the benefits of ATRA and ATO post treatment termination in inducing long lasting effects of differentiation and apoptosis in APL cells and highlight possible underlying epigenetic mechanisms for the reduced relapse associated with the combination treatment.

5.3 Results

5.3.1 ATRA and ATO Combination Treatment Sustains Differentiation and Results in Cell Death of NB4 Cells Post Treatment Termination

While ATRA alone induces short-term remission in APL patients (Coombs et al. 2015), the complete remission induced by daily dosage of 45 mg/m^2 ATRA and 0.15 mg/kg ATO combination treatment(Abaza et al. 2017; Au et al. 2011; Estey et al. 2006), suggests the combination treatment is better at inducing long-term lasting effects, which are sustained post treatment termination. Pharmacokinetic studies in patients suggests that these doses translate to peak plasma concentrations of approximately 1 μM for ATRA (Lefebvre 1991; Muindi 1992), while ATO has been reported as peaking at 6.85 μM before troughing to less than 1 μM, to ranging from 0.08 to 0.40 μM (Cui et al. 2018), or from 0.11 to 0.37 μM (Fox et al. 2008). Therefore, to study the effect of these drugs alone or in combination for maintaining differentiation or inducing cell death post treatment termination, we treated the APL cell line NB4 or the MR2 ATRA-resistant clone with 1 μM ATRA and 0.5 μM ATO (Fig. 5.1) or 1.5 μM ATO (Fig. 5.2), alone or in combination, to capture the range of potential ATO doses in patient circulation. We treated the cells continuously for 72 h or 168 h (Fig. 5.1A). Alternatively, after 72 h, treatment was terminated by washing the cells and subsequently culturing the cells for an additional 96 h in treatment-free medium (Fig. 5.1B). We determined the percentage of differentiated cells (surface expression of myeloid marker CD11b) and dead cells (7-AAD staining) under these various treatment conditions by flow cytometry. The combination of 1 μM ATRA and 0.5 μM ATO induced a larger population of differentiated CD11b positive cells in comparison to single ATRA and ATO treated cells

after 72 h (Fig. 5.1C). As described previously, the higher dose of ATO induced greater cell death (Fig 5.2) (Chen 1996, 1997; Jing et al. 2001; Shen et al. 2004). The population of differentiated cells (and differentiated cells that had died) became significantly more pronounced at 168 h (CD11b/7-AAD positive cells, Fig. 1D). However, the potential synergistic benefits of ATRA and ATO combination treatments became most evident after treatment was terminated for 96 h (Fig. 51B,E, and Fig 5.2). Ninety-six hours post treatment termination, most of the cells treated with the single agents now lacked staining for the differentiation and death markers (Fig. 5.1E). We noted that the background cell death had increased 96 h post treatment termination. This was possibly due to overcrowding of the proliferating cells or stress that was induced by washing the cells.

Most strikingly and in sharp contrast to cells treated with single agents, the combination 1 μM ATRA + 0.5 μM ATO treated cells were still mostly differentiated and/or dead at 96 h post treatment termination (CD11b/7-ADD positive cells, Fig. 1D). This data mimics the clinical findings(Abaza et al. 2017; Coombs et al. 2015; Wang and Chen 2008; Zeidan and Gore 2014), whereby ATRA and ATO combination therapy result in sustained effects, which persist after the termination of therapy.

We applied the same treatment regimen to the ATRA-resistant NB4-MR2 cells (Fig. 5.3A,B). As expected, ATRA alone did not induce differentiation of the cells; however, the cells were partly differentiated by the ATRA and 0.5 μM ATO combination treatment, and predominately killed by 1.5 μM ATO (Fig. 5.3C). This is consistent with previously published findings, where the ATRA-resistant NB4-MR2 cells remain sensitive to ATO treatment (Chen 1996, 1997; Jing et al. 2001).

Fig 5.1 Combination 1 μM ATRA and 0.5 μM ATO treatment sustains differentiation and death of NB4 cells 96 h post treatment termination. (A) Schematic of treatment timeline and the timepoints NB4 cells samples were analyzed in (C and D). (B) Schematic of treatment timeline and the timepoint NB4 cells samples were analyzed in (E). (C–E) Representative flow cytometry dot plots of CD11b+, 7-AAD+, and CD11b+ /7-AAD+ NB4 cells under no treatment, 0.5 μM ATO, 1 μM ATRA, 0.5 μM ATO + 1 μM ATRA treatment after 72 h of continuous treatment (C), or 168 h of continuous treatment (D), or after 72 h treatment and subsequent 96 h post treatment termination. (C–E) The stacked bar graphs summarize the results of dot plots (n = 4, error bars represent standard deviation).

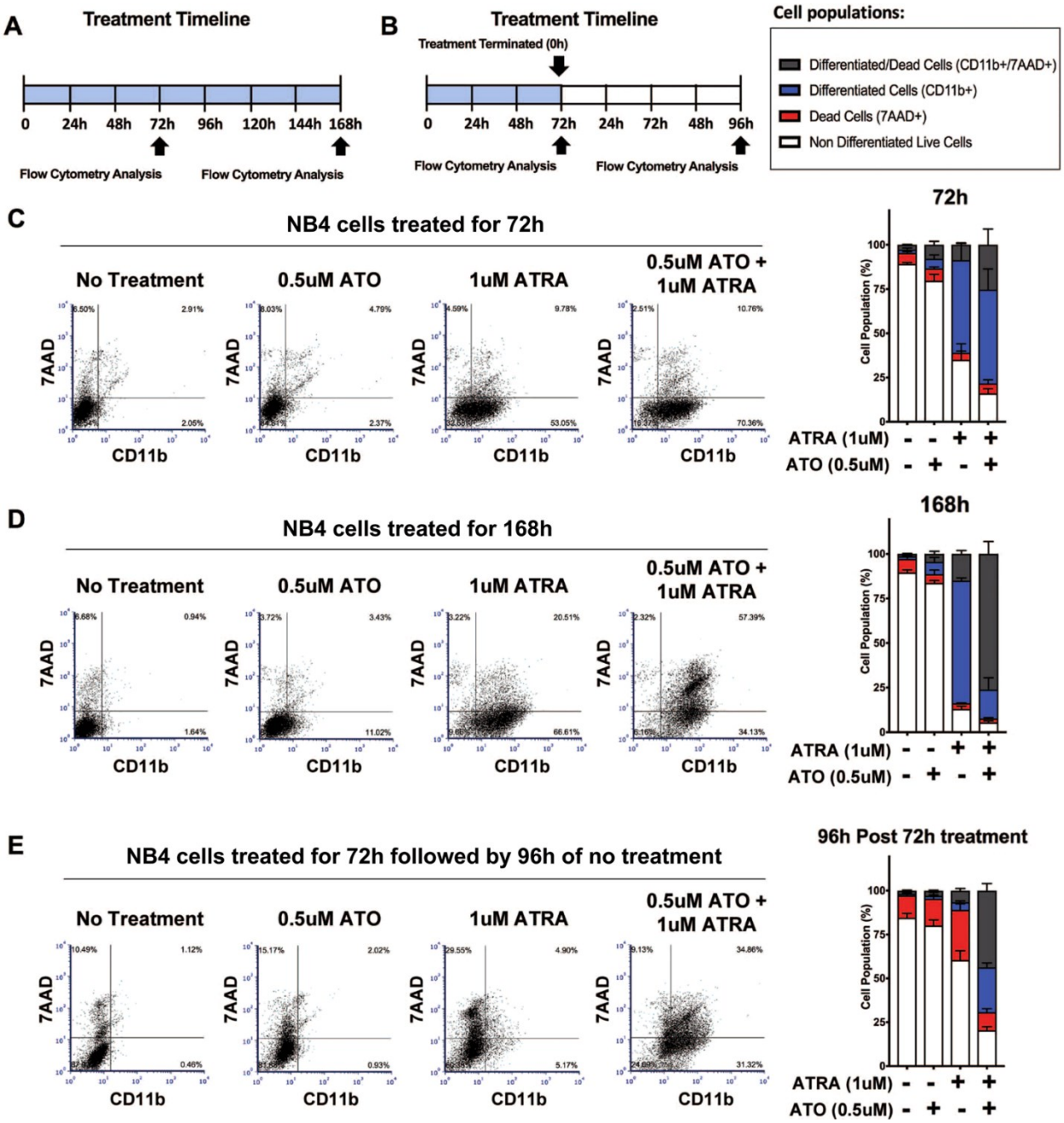

A
Treatment Timeline
0
24h
48h
72h
96h
120h
144h
168h
Flow Cytometry Analysis
Flow Cytometry Analysis
B
Treatment Timeline
Treatment Terminated (0h)
0
24h
48h
72h
24h
72h
48h
96h
Flow Cytometry Analysis
Flow Cytometry Analysis
Cell populations:
Differentiated/Dead Cells (CD11b+/7AAD+)
Differentiated Cells (CD11b+)
Dead Cells (7AAD+)
Non Differentiated Live Cells
C
NB4 cells treated for 72h
No Treatment
0.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
7AAD
CD11b
6.50%
2.91%
2.05%
8.03%
4.79%
2.37%
4.59%
9.78%
53.05%
2.51%
10.76%
70.36%
72h
Cell Population (%)
ATRA (1uM) - - + +
ATO (0.5uM) - + - +
D
NB4 cells treated for 168h
No Treatment
0.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
7AAD
CD11b
8.68%
0.94%
1.64%
3.72%
3.43%
11.02%
3.22%
20.51%
66.61%
2.32%
57.39%
34.13%
168h
Cell Population (%)
ATRA (1uM) - - + +
ATO (0.5uM) - + - +
E
NB4 cells treated for 72h followed by 96h of no treatment
No Treatment
0.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
7AAD
CD11b
10.49%
1.12%
0.46%
15.17%
2.02%
0.93%
29.55%
4.90%
5.17%
9.13%
34.86%
31.32%
96h Post 72h treatment
Cell Population (%)
ATRA (1uM) - - + +
ATO (0.5uM) - + - +

Figure 5.2. 1.5μM ATO treatment induces predominately cell death of NB4 cells, which is mostly sustained 96h post treatment termination and amplified when combined with 1μM ATRA. (A) Schematic of treatment timeline and the timepoints NB4 cells samples were analyzed in C and D. (B) Schematic of treatment timeline and the timepoint NB4 cells samples were analyzed in E. (C, D and E) Representative flow cytometry dot plots of CD11b+, 7-AAD+, and CD11b+/7-AAD+ NB4 cells under no treatment, 1.5μM ATO, 1.5μM ATO + 1μM ATRA treatment after 72h of continuous treatment (C), or 168h of continuous treatment (D), or after 72h treatment and subsequent 96h post treatment termination. (C, D and E) The stacked bar graphs summarize the results of dot plots (n=4, error bars represent standard deviation)

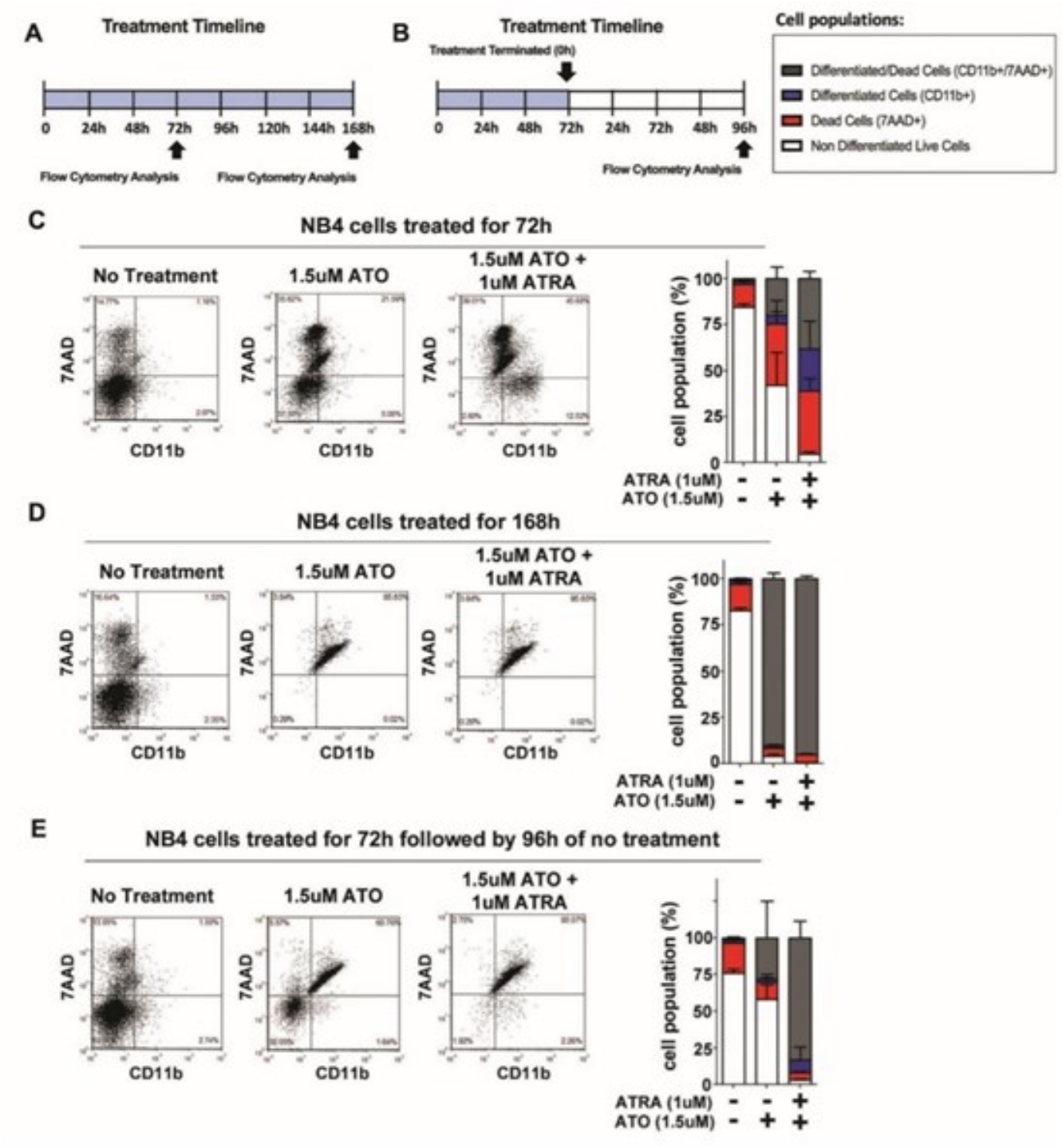
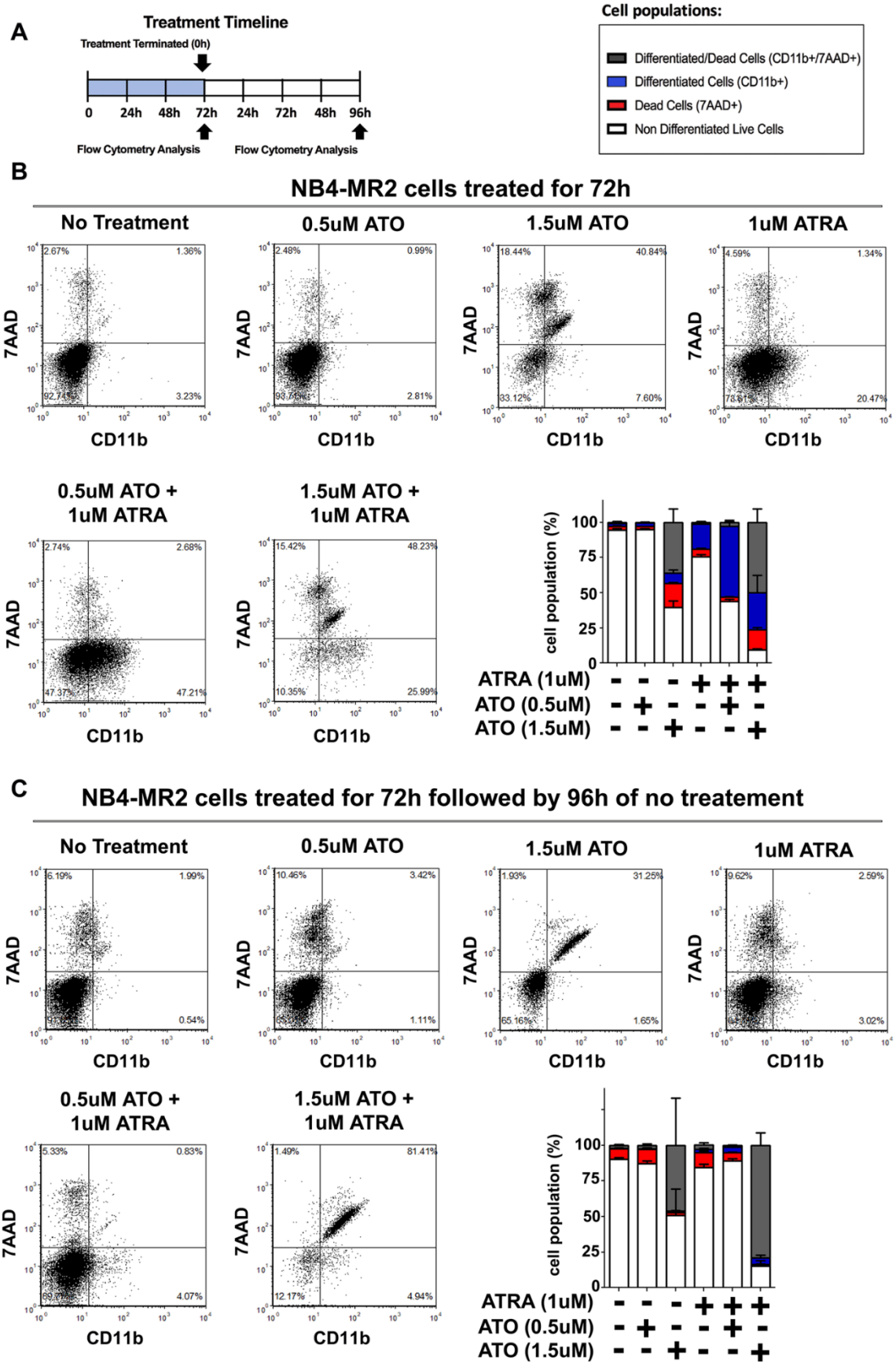

A
Treatment Timeline
0
24h
48h
72h
96h
120h
144h
168h
Flow Cytometry Analysis
Flow Cytometry Analysis
B
Treatment Timeline
Treatment Terminated (0h)
0
24h
48h
72h
24h
72h
48h
96h
Flow Cytometry Analysis
Cell populations:
Differentiated/Dead Cells (CD11b+/7AAD+)
Differentiated Cells (CD11b+)
Dead Cells (7AAD+)
Non Differentiated Live Cells
C
NB4 cells treated for 72h
No Treatment
1.5uM ATO
1.5uM ATO + 1uM ATRA
7AAD
CD11b
cell population (%)
100
75
50
25
0
ATRA (1uM) - - +
ATO (1.5uM) - + +
D
NB4 cells treated for 168h
No Treatment
1.5uM ATO
1.5uM ATO + 1uM ATRA
7AAD
CD11b
cell population (%)
100
75
50
25
0
ATRA (1uM) - - +
ATO (1.5uM) - + +
E
NB4 cells treated for 72h followed by 96h of no treatment
No Treatment
1.5uM ATO
1.5uM ATO + 1uM ATRA
7AAD
CD11b
cell population (%)
100
75
50
25
0
ATRA (1uM) - - +
ATO (1.5uM) - + +

Figure 5.3. ATRA and ATO treatment have a reduced effect on inducing and sustaining differentiation and cell death in NB4-MR2 cells. (A) Schematic of treatment timeline and the timepoints NB4-MR2 cell samples were analyzed in (B and C). (B and C) Representative flow cytometry dot plots of CD11b+, 7-AAD+, and CD11b+/7-AAD+ NB4-MR2 cells under no treatment, 0.5uM ATO, 1.5 μM ATO, 1 μM ATRA, 0.5 μM ATO+ 1 μM ATRA, or 1.5 μM ATO+ 1 μM ATRA treatment after 72 h of continuous treatment (B) and after treatment has been terminated for 96 h (C). The stacked bar graphs summarize the results of dot plots (n = 4, error bars represent standard deviation).

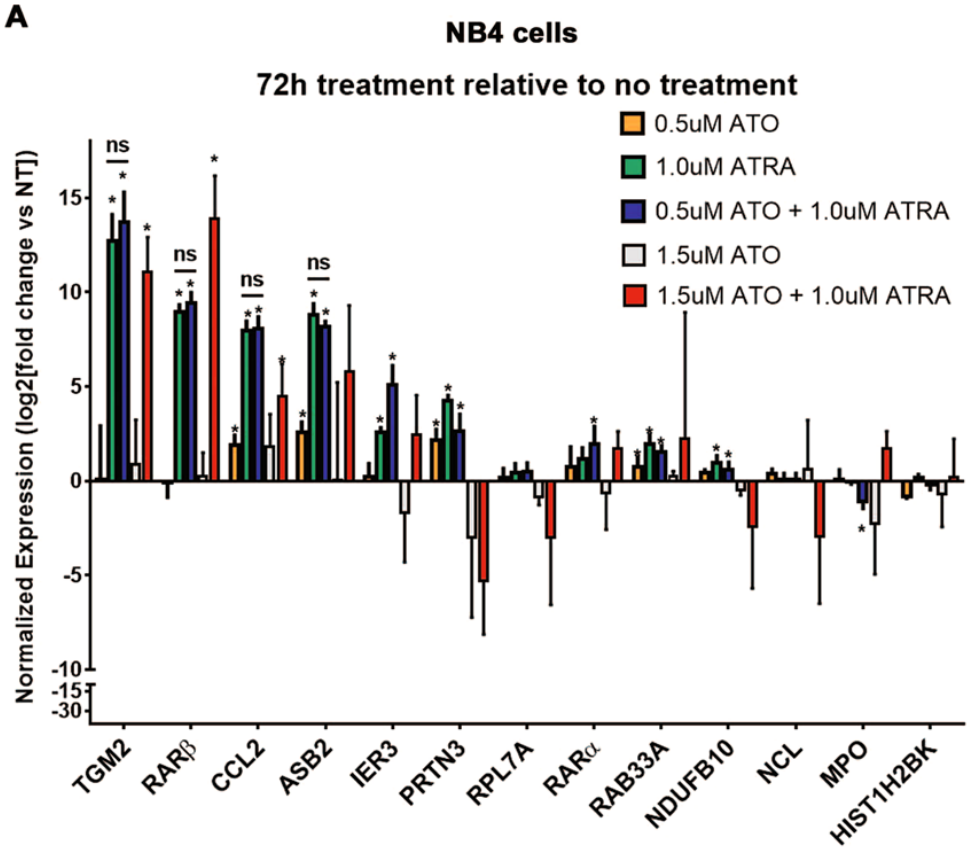

A
Treatment Timeline
Treatment Terminated (0h)
0
24h
48h
72h
24h
72h
48h
96h
Flow Cytometry Analysis
Flow Cytometry Analysis
Cell populations:
Differentiated/Dead Cells (CD11b+/7AAD+)
Differentiated Cells (CD11b+)
Dead Cells (7AAD+)
Non Differentiated Live Cells
B
NB4-MR2 cells treated for 72h
No Treatment
0.5uM ATO
1.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
1.5uM ATO + 1uM ATRA
7AAD
CD11b
2.67%
1.36%
92.74%
3.23%
2.48%
0.99%
93.76%
2.81%
18.44%
40.84%
33.12%
7.60%
4.59%
1.34%
78.61%
20.47%
2.74%
2.68%
47.37%
47.21%
15.42%
48.23%
10.35%
25.99%
cell population (%)
100
75
50
25
0
ATRA (1uM) - - - + + +
ATO (0.5uM) - + - - + -
ATO (1.5uM) - - + - - +
C
NB4-MR2 cells treated for 72h followed by 96h of no treatement
No Treatment
0.5uM ATO
1.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
1.5uM ATO + 1uM ATRA
6.19%
1.99%
0.54%
10.46%
3.42%
1.11%
1.93%
31.25%
65.16%
1.65%
9.62%
2.59%
3.02%
5.33%
0.83%
4.07%
1.49%
81.41%
12.17%
4.94%
cell population (%)
ATRA (1uM) - - - + + +
ATO (0.5uM) - + - - + -
ATO (1.5uM) - - + - - +

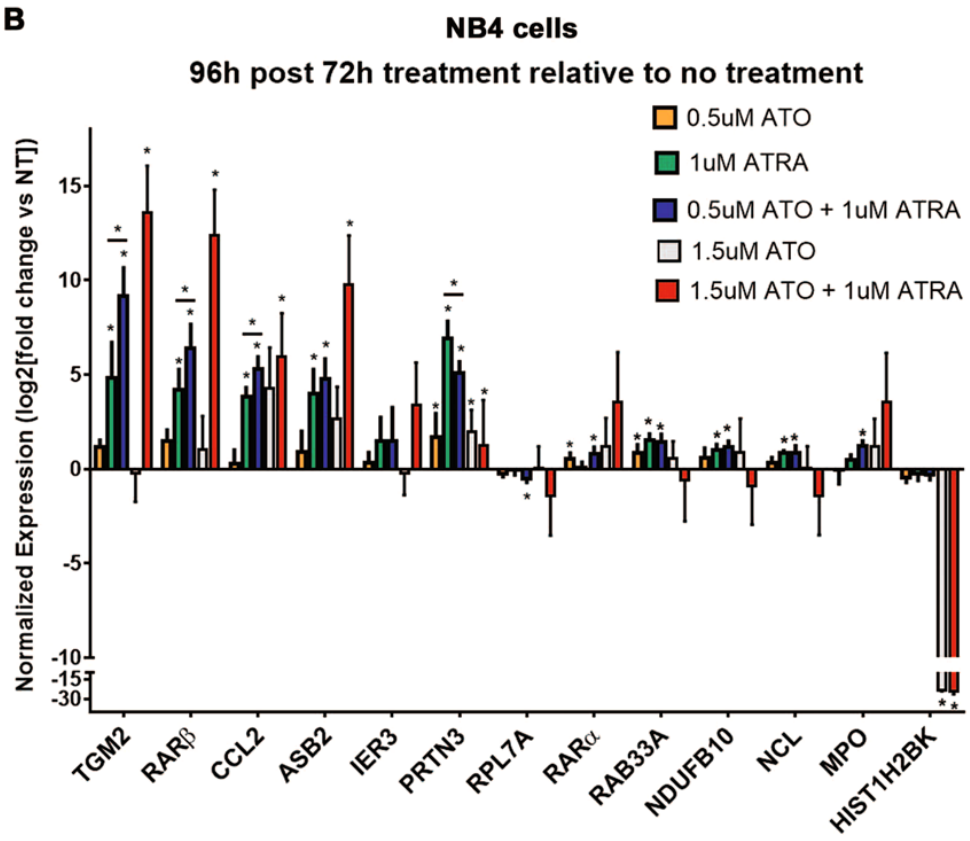
5.3.2 Combination Treatment of ATRA and ATO is More Effective at Maintaining High Transcript Levels of TGM2, RARβ, CCL2 and ASB2 in NB4 cells Post Treatment Termination

Having observed that ATRA and ATO combination treatment maintain the majority of NB4 cells in a state of terminal differentiation after treatment had ended (Figs. 5.1E and 5.2)), we next wondered if gene expression changes were similarly more persistent. Gene expression changes are a key component of the interfered differentiation and symptoms of APL disease (Lee et al. 2002)l. Supraphysiological levels of ATRA can restore the expression of epigenetically silenced target genes in APL cells. Using QPCR, we determined the effect of ATRA and ATO individually or in combination at 72 h and following 96 h post treatment termination, on the mRNA levels of several genes involved in APL processes such as leukemic differentiation (TGM2, RARβ), granulocyte function (MPO, PRTN3) and other ATRA-regulated targets (e.g., CCL2) in comparison to single agent treatment (Lee et al. 2002; Martens et al. 2010). ATRA alone and combination treatment resulted in significantly higher transcript levels of several genes (TGM2, RARβ, CCL2, ASB2, RPL7A, RARα, RAB33A, NDUFB10, NCL and HIST1H2BK) after 72 h of treatment (Fig. 5.4A). ATO treatment alone had a comparatively minor effect on expression of the genes. Notably, for most of the genes, there was no significant difference in the transcript levels induced by ATRA alone and combination treated cells (Fig. 5.4A). One notable exception was RARβ, which was induced to higher levels when NB4 cells were treated with combination treatment ATRA with higher dose 1.5 μM ATO. Interestingly, the sustained effects of the combination treatments over ATRA alone became much more apparent 96 h after termination of treatment, where the higher

transcript levels of TGM2, RARβ, CCL2 and ASB2 were still present (Fig. 5.4B). Therefore, the combination treatments maintained greater transcript levels of these genes once treatment had been terminated. The sustained expression of certain target genes may explain why terminal granulocytic differentiation is sustained in combination treated cells in comparison to single agent treated cells, especially considering the key role that TGM2 has in differentiation of NB4 cells (Csomós et al. 2010).

Figure 5.4. Combination treatment of ATRA and ATO is more effective at maintaining high levels of TGM2, RARβ, CCL2 and ASB2 mRNA 96 h post treatment termination. (A and B) QPCR analysis detects relative levels of mRNA of target genes in NB4 cells 72 h after treatment (A) and subsequent 96 h post treatment termination (B) with 0.5 μM ATO, 1.5 μM ATO, 1 μM ATRA, or combination treatments. The mRNA levels of target genes are log2 transformed and relative to the no treatment sample and reference genes (n = 4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *). Notably, the 1.5 μM ATO, 1.5 μM ATO + 1 μM ATRA combination treatment samples were completed at a later time and compared to their own no treatment control.

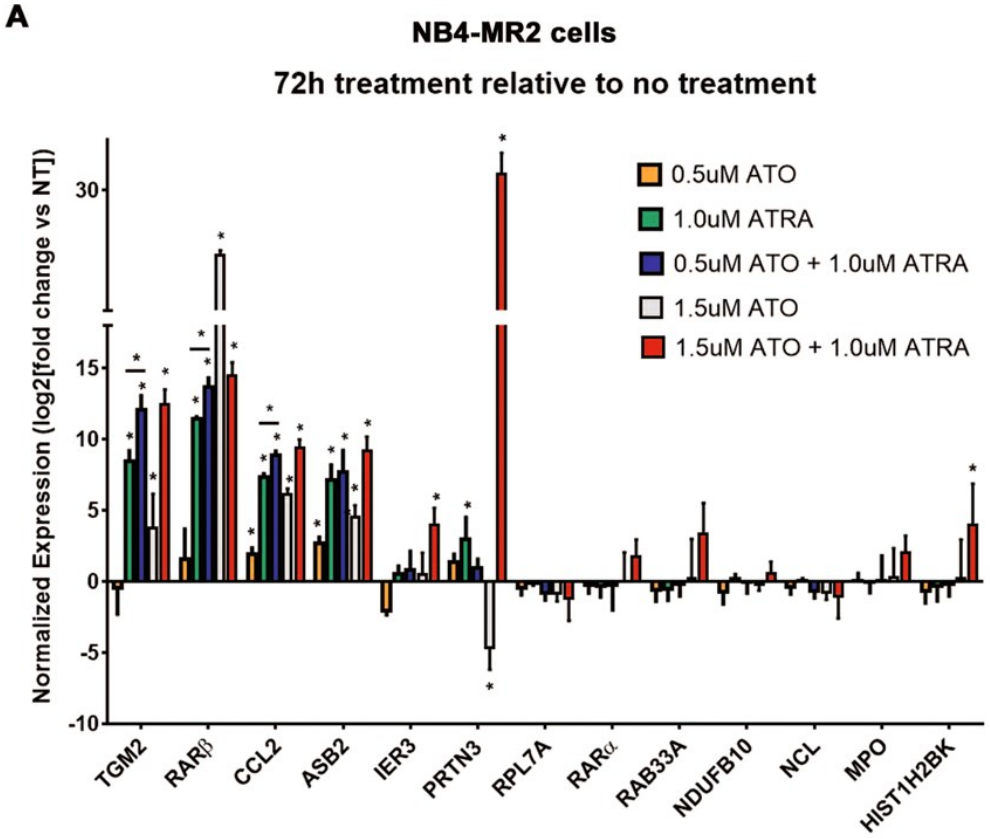

A
NB4 cells
72h treatment relative to no treatment
0.5uM ATO
1.0uM ATRA
0.5uM ATO + 1.0uM ATRA
1.5uM ATO
1.5uM ATO + 1.0uM ATRA
Normalized Expression (log2[fold change vs NT])
ns
TGM2
RARβ
CCL2
ASB2
IER3
PRTN3
RPL7A
RARα
RAB33A
NDUFB10
NCL
MPO
HIST1H2BK

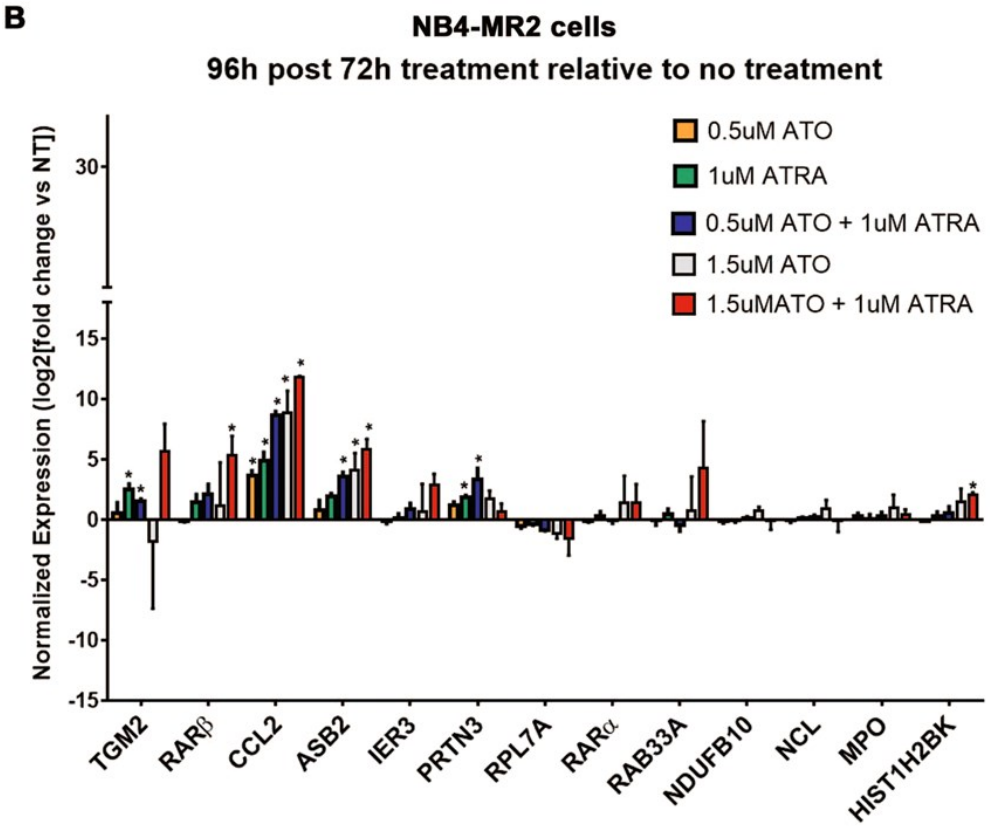

B
NB4 cells
96h post 72h treatment relative to no treatment
0.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
1.5uM ATO
1.5uM ATO + 1uM ATRA
Normalized Expression (log2[fold change vs NT])
TGM2
RARβ
CCL2
ASB2
IER3
PRTN3
RPL7A
RARα
RAB33A
NDUFB10
NCL
MPO
HIST1H2BK

5.3.3 Combination Treatment of ATRA and ATO Fails to Maintain High Transcript Levels of Target Genes in NB4-MR2 Cells Post Treatment Termination

We compared the expression of these genes in NB4-MR2 cells under the same treatment conditions. Notably the ATRA-resistant clone has demonstrated altered ligand binding of PML/RAR-alpha and retinoid-induced gene expression (Rosenauer 1996). We also found a somewhat different gene expression profile induced by the treatments at 72 h in NB4-MR2 cells, with generally more pronounced gene expression changes induced in the combination treatments (Fig. 5.5). A notable exception was that much greater levels of RARβ was induced in the cells treated with higher dose 1.5 μM ATO alone (Fig. 5.5A). However, once treatment was terminated for 96 h, the gene expression changes that had been induced in the NB4-MR2 cells were only weakly sustained by the combination treatment ATRA with higher dose ATO (Fig. 5.5B). This is in sharp contrast to the NB4 cells, in which the combination treatment ATRA with even the lower dose ATO resulted in generally well sustained expression of genes (e.g. TGM2 and RARβ) at 96 h post treatment termination (Fig. 5.4B).

Figure 5.5. ATRA and ATO induce gene expression changes in NB4-MR2 cells, which are weakly sustained post treatment termination. (A and B) QPCR analysis detects relative levels of mRNA of target genes in NB4-MR2 cells 72 h after treatment (A) and subsequent 96 h post treatment termination (B) with 0.5 μM ATO, 1.5 μM ATO, 1 μM ATRA, or combination treatments. The mRNA levels of target genes are log2 transformed and relative to the no treatment sample and reference genes (n = 4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by*).


A
NB4-MR2 cells
72h treatment relative to no treatment
Normalized Expression (log2[fold change vs NT])
0.5uM ATO
1.0uM ATRA
0.5uM ATO + 1.0uM ATRA
1.5uM ATO
1.5uM ATO + 1.0uM ATRA
TGM2
RARβ
CCL2
ASB2
IER3
PRTN3
RPL7A
RARα
RAB33A
NDUFB10
NCL
MPO
HIST1H2BK


B
NB4-MR2 cells
96h post 72h treatment relative to no treatment
Normalized Expression (log2[fold change vs NT])
0.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
1.5uM ATO
1.5uMATO + 1uM ATRA
TGM2
RARβ
CCL2
ASB2
IER3
PRTN3
RPL7A
RARα
RAB33A
NDUFB10
NCL
MPO
HIST1H2BK

5.3.4 ATRA Induces Sustained Enrichment of H3K9/14ac at the TGM2 and RARβ Promoters in NB4 Cells, Which is not Augmented by Combination Treatment

Decreased H3K9/14ac at target genes is key in their decreased expression in APL cells and ATRA-induced gene expression is associated with H3K9/14ac enrichment(Martens et al. 2010). We therefore wondered if the greater sustained expression post treatment termination of some target genes induced by combination treatment (Fig. 5.4B) was due to greater enrichment of H3K9/14ac. Since TGM2 and RARβ were most induced, with the greatest sustained expression by the combination treatment once treatment was terminated, we focused on these two genes for H3K9/14ac analysis by ChIP-qPCR. In agreement with previous reports, ATRA induced H3K9/14ac enrichment at TGM2 and RARβ promoters (Fig. 5.6A). The combination treatment of ATRA + 0.5 μM ATO did not further augment the H3K9/14ac enrichment at both 72 h and after 96 h post treatment termination. The lack of significant difference between the combination treatment versus the ATRA treatment alone (Fig. 5.6A) suggests that H3K9/14ac enrichment may not play a critical role in the greater sustained expression of the genes induced by combination treatment (Fig. 5.4B).

We next wondered if perhaps the combination treatment decreases repressive H3K9me3 and H3K27me3 marks associated with silencing of TGM2 and RARβ in APL cells13. Consistent with previous reports, ATRA had a minimal effect on H3K9me3 and H3K27me3 enrichment at the target genes (Fig. 5.6B and C). The combination treatment also had minimal effects on the enrichment of the two repressive histone marks (Fig. 5.6B and C). Overall, this indicates that the greater sustained TGM2 and RARβ mRNA levels

induced by combination treatment (Fig. 5.4B) is probably not due to changes in these histone modifications (Fig. 5.6).

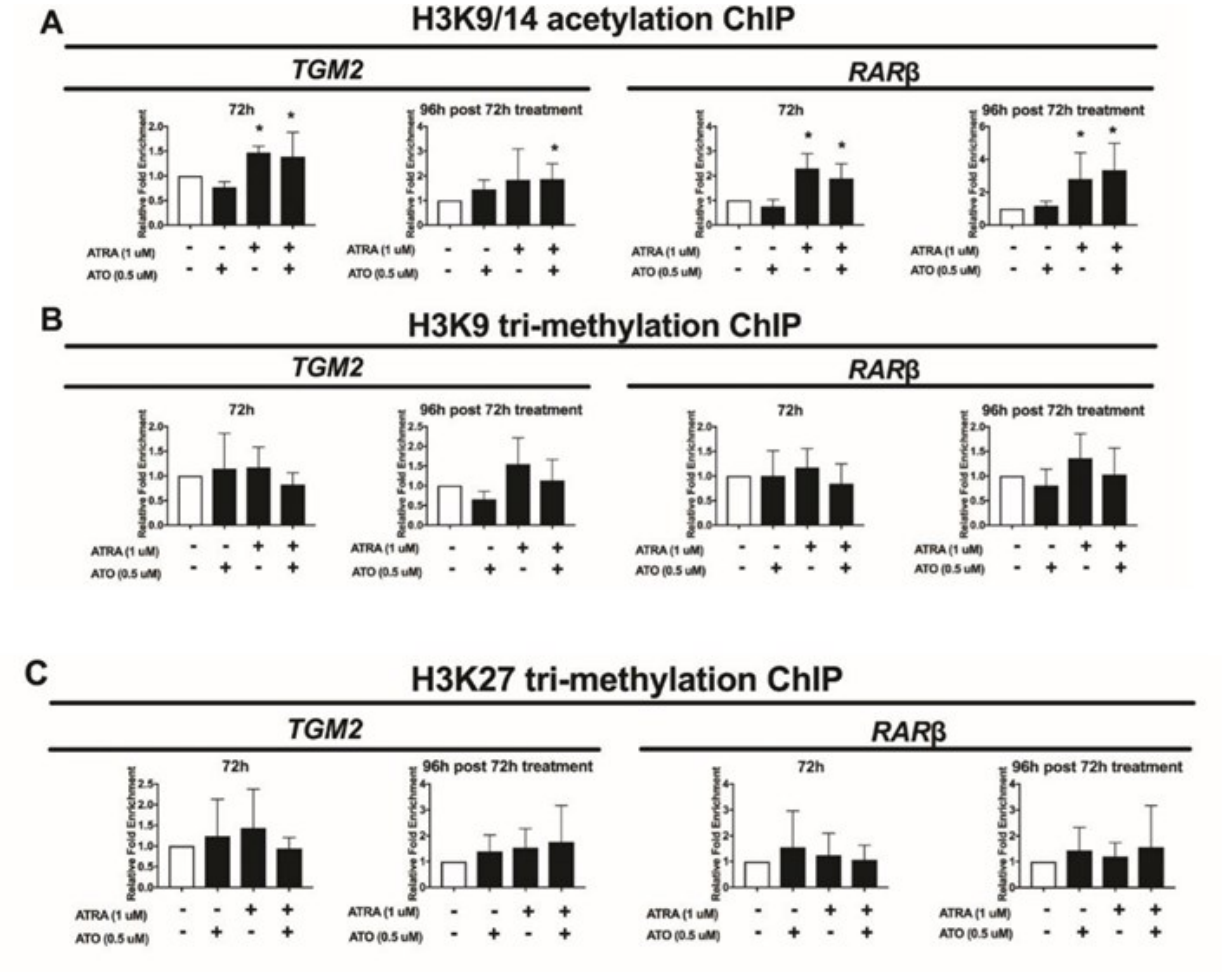


Figure 5.6. ATRA induces sustained enrichment of H3K9/14ac at TGM2 and RARβ promoters in NB4 cells. H3K9/14ac (A), H3K9me3 (B), and H3K27me3 (C) enrichment at TGM2 and RARβ promoters as measured by QPCR following ChIP with antibodies specific to the histone modification in NB4 cells following 72h of 0.5μM ATO, 1μM ATRA, or the combination treatment and subsequent 96h post treatment termination. Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *, n=4.

5.3.5 Combination Treatment Demethylates the CpG Sites in the Promoter Regions of TGM2 and RARβ in NB4 Cells, and Demethylation is Sustained Post Treatment Termination

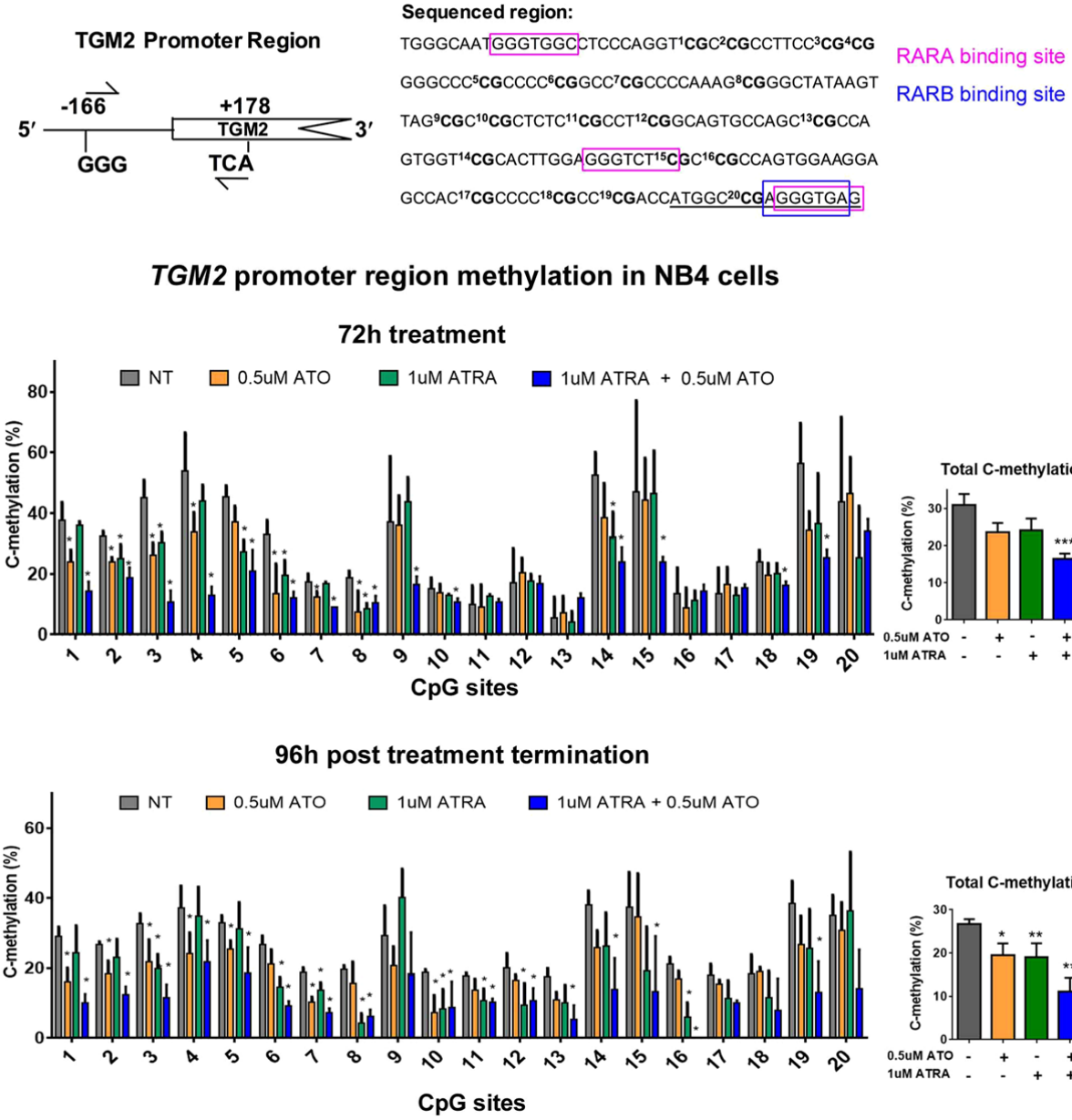
CpG island DNA hypermethylation silencing of genes contributes to the arrested differentiation of granulocytes in APL (Schoofs et al. 2013). Although ATRA is capable of inducing APL cells into a differentiated granulocytic phenotype, most evidence suggest that target genes, including differentiation-inducing gene TGM2 and canonical target gene RARβ, remain aberrantly hypermethylated upon ATRA treatment (Chim et al. 2003; Nouzova et al. 2004). However, we wondered if the combination treatment (or ATO) could be affecting the promoter methylation of the genes, contributing to the greater sustained transcript levels of the genes post treatment termination. Bisulfite pyrosequencing was used to interrogate the 20 CpGs in the CpG island in the TGM2 promoter (Fig. 5.7A). Combination treatment of ATRA with 0.5 μM ATO significantly reduced the total C-methylation of the TGM2 promoter region after 72 h treatment (Fig. 5.7B), and the demethylation was sustained 96 h after treatment had been terminated (Fig. 5.7C). Interestingly, the single agents did have some effect on methylation at individual sites and at 96 h after treatment had been terminated, ATRA and ATO alone did reduce overall methylation as well, albeit to lesser degree than the combination treatment. Notably, similar to the greater gene expression changes induced by combination ATRA treatment with the higher dose 1.5 μM ATO (Fig. 5.4), this combination also induced greater demethylation of the gene region (Fig. 5.8). We noted that RARα and RARβ binding sites are in the TGM2 promoter region (Fig. 5.7A), and CpG site 15 is located within a RARα binding site. CpG site 15 was among the more hypermethylated in the

TGM2 promoter region, and therefore its demethylation by the combination treatments (Fig. 5.7 and Fig. 5.8), may be particularly important for expression of TGM2 (Fig. 5.4).

We also interrogated the methylation of the 15 CpG sites in the CpG island neighboring the RARβ transcription start that contains RARα and RARβ binding sites by bisulfite pyrosequencing (Fig. 5.9A). Again, treatment with ATRA or 0.5 μM ATO alone did not significantly alter the overall CpG methylation of the region, but combination treatment significantly reduced the total C-methylation of the RARβ promoter region at 72 h (Fig. 5.9B). Importantly, this effect was sustained 96 h after treatment termination (Fig. 5.9C). These demethylation effects were augmented in the combination ATRA and higher dose 1.5 μM ATO treated cells (Fig 5.10). Of note as well is the overall shift in CpG methylation at some sites when comparing 72 h versus 96 h post treatment conditions, even in the no treatment condition samples (e.g. sites 11 and 14, Fig. 5.9). This is possibly reflective of the dynamic methylation status of some of the CpG sites in the region that may be susceptible to changes in cell culturing conditions (e.g. cell crowding, spent media, increased background cell death). Regardless, there is still a significant and sustained decrease in methylation when the cells are treated with the combination treatments. Together, the TGM2 and RARβ promoter analyses provides new evidence showing that the combination of ATRA and ATO reduces the aberrant methylation of key target genes, and similar to the transcript level analyses (Fig. 5.4), this effect was sustained after treatment had been terminated (Figs 5.7, 5.8, 5.9 and 5.10).

Figure 5.7 Combination 1 µM ATRA and 0.5 µM ATO treatment induces sustained demethylation of the CpG island in the promoter region of TGM2 in NB4 cells. (A) Schematic representation of the TGM2 promoter region and the specific 20 CpG sites located within the region that was bisulfite pyrosequenced. The binding sites for RARα and RARβ are indicated. (B and C) The methylation percentage of the individual 20 CpG sites and total C-methylation percentage of the region in NB4 cells following 72 h of treatment (B) and subsequent 96 h post treatment termination (C). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, n = 5.

A


TGM2 Promoter Region
-166
+178
TGM2
5′
3′
GGG
TCA
Sequenced region:
TGGGCAATGGGTGGCCTCCCAGGT1CGC2CGCCTTCC3CG4CG
GGGCCC5CGCCCC6CGGCC7CGCCCCAAAG8CGGGCTATAAGT
TAG9CGC10CGCTCTC11CGCCT12CGGCAGTGCCAGC13CGCCA
GTGGT14CGCACTTGGAGGGTCT15CGC16CGCCAGTGGAAGGA
GCCAC17CGCCCC18CGCC19CGACCATGGC20CGAGGGTGAG
RARA binding site
RARB binding site
TGM2 promoter region methylation in NB4 cells
72h treatment
NT
0.5uM ATO
1uM ATRA
1uM ATRA + 0.5uM ATO
C-methylation (%)
CpG sites
Total C-methylation
0.5uM ATO
1uM ATRA
96h post treatment termination
NT
0.5uM ATO
1uM ATRA
1uM ATRA + 0.5uM ATO
C-methylation (%)
CpG sites
Total C-methylation
0.5uM ATO
1uM ATRA

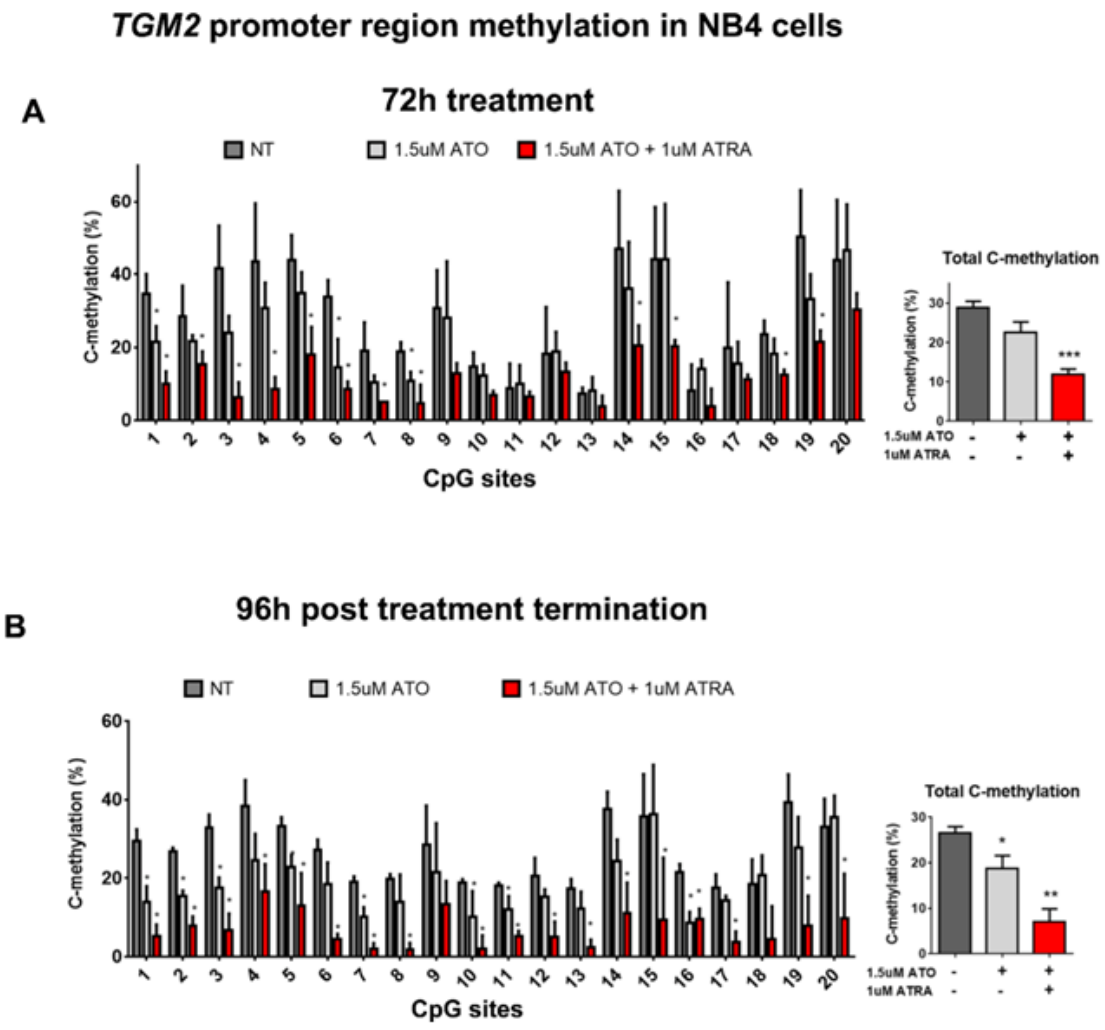
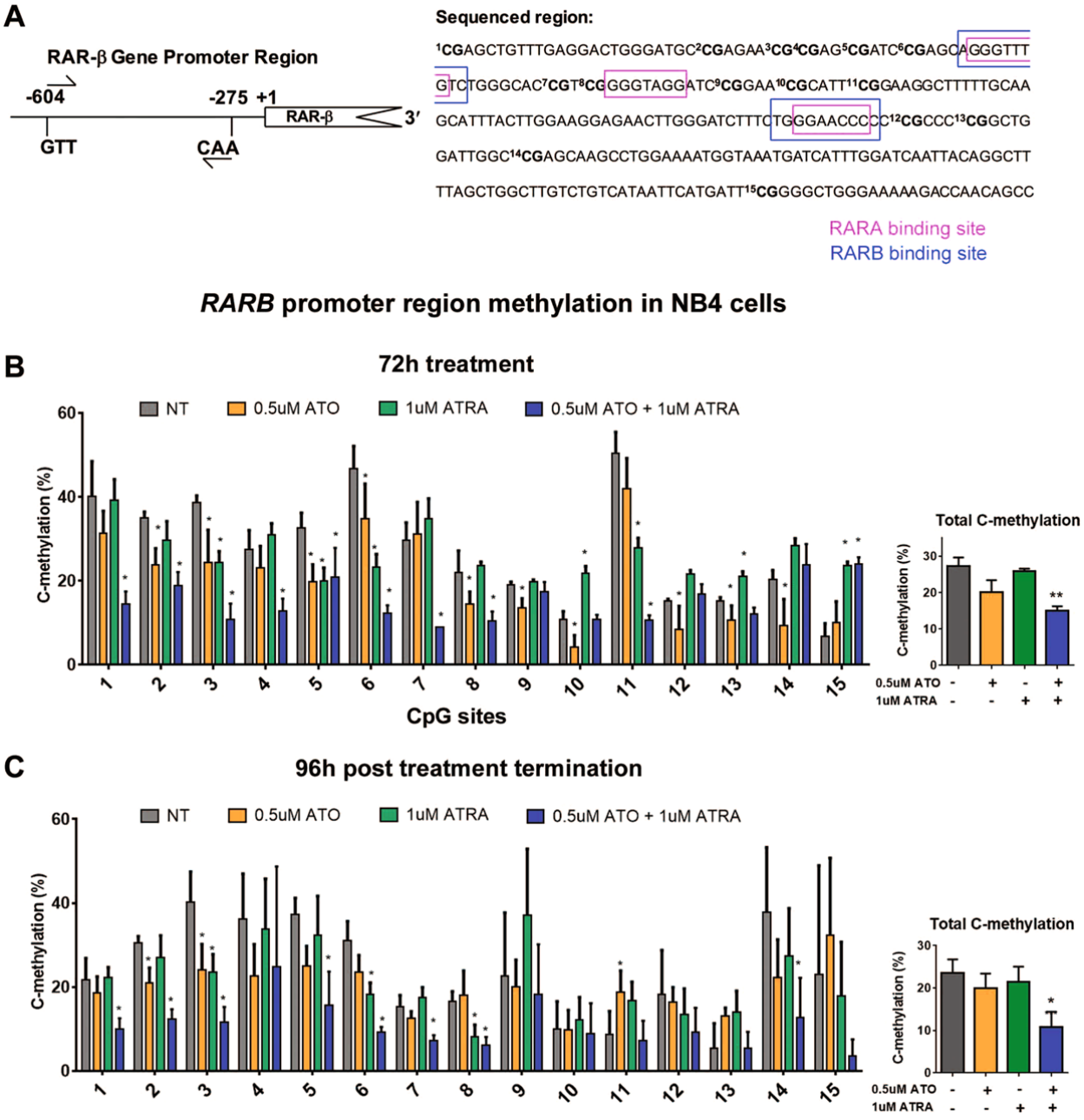


Figure 5.8. 1.5µM ATO reduces DNA methylation of the CpG island in the promoter region of TGM2 in NB4 cells to a greater degree when combined with 1µM ATRA. (A and B) The methylation percentage of the individual 20 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment (A) and subsequent 96h post treatment termination (B). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, n=4.

Figure 5.9 Combination 1 µM ATRA and 0.5 µM ATO treatment induces sustained demethylation of the CpG island in the promoter region of RARβ in NB4 cells. (A) Schematic representation of RARβ and the specific 15 CpG sites located within the region that were bisulfite pyrosequenced. The binding sites for RARα and RARβ are indicated. (B and C) The methylation percentage of the individual 15 CpG sites and total C-methylation percentage of the region in NB4 cells following 72 h of treatment (B) and subsequent 96 h post treatment termination (C). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, n = 5.


A
RAR-β Gene Promoter Region
-604
-275 +1
RAR-β
3′
GTT
CAA
Sequenced region:
RARA binding site
RARB binding site
RARB promoter region methylation in NB4 cells
B
72h treatment
NT
0.5uM ATO
1uM ATRA
0.5uM ATO + 1uM ATRA
C-methylation (%)
CpG sites
Total C-methylation
0.5uM ATO
1uM ATRA
C
96h post treatment termination

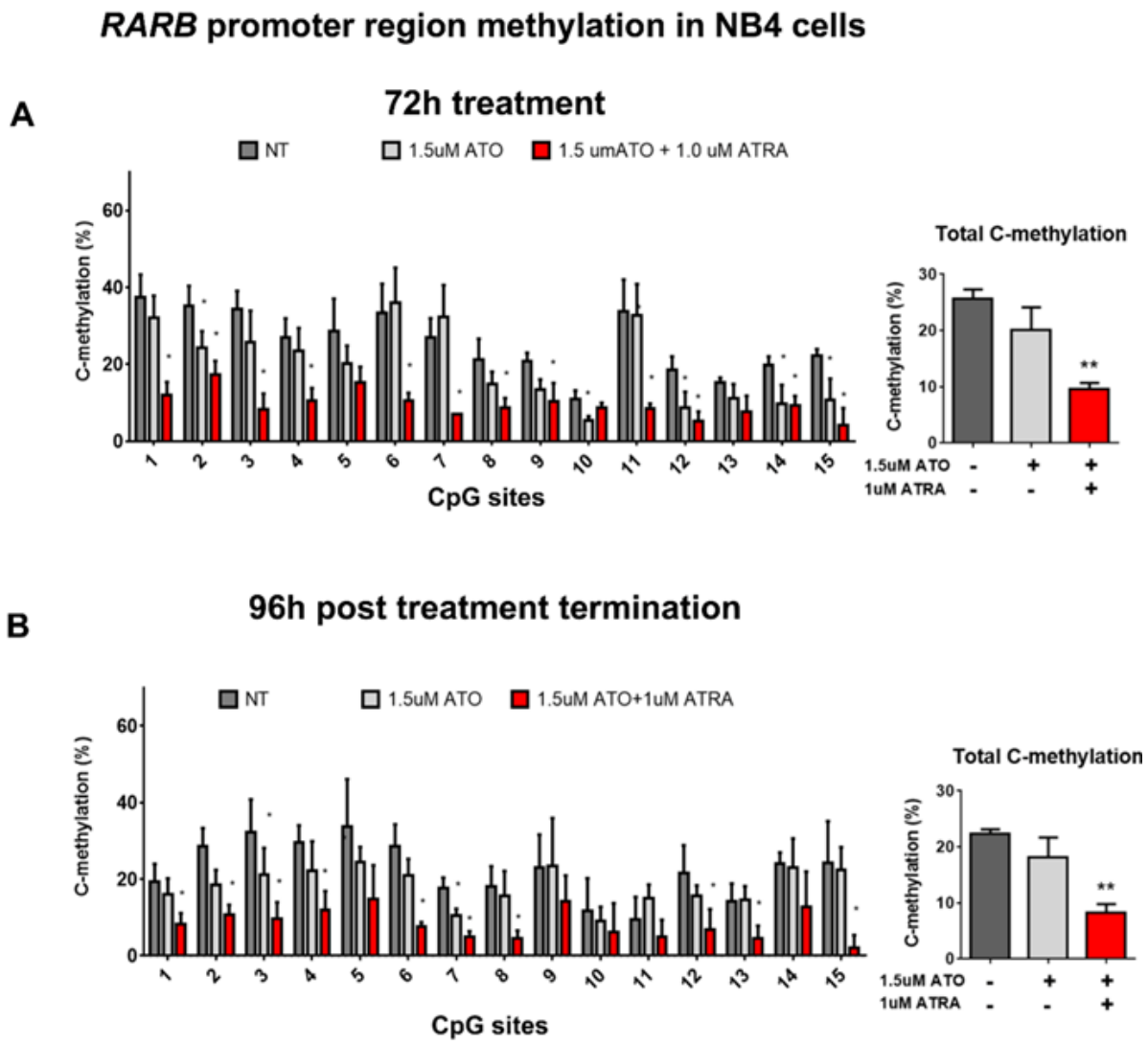


Figure 5.10. 1.5µM ATO reduces DNA methylation of the CpG island in the promoter region of RARβ in NB4 cells to a greater degree when combined with 1µM ATRA. (A and B) The methylation percentage of the individual 15 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment (A) and subsequent 96h post treatment termination (B). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, n=4.

5.3.6 Combination Treatment Demethylates of the CpG Sites in the Promoter Regions of TGM2 and RARβ in NB4-MR2 Cells, but Demethylation is not Sustained Post Treatment Termination

We next wondered how these treatment conditions would affect methylation of promoter regions of TGM2 and RARβ in NB4-MR2 cells, in which gene expression changes were induced, but unlike NB4 cells, the gene expression changes were not sustained post treatment termination (Fig. 5.5). Bisulfite pyrosequencing revealed that the combination treatments demethylated the promoter region of TGM2 in NB4-MR2 cells (Fig. 5.11A), but this effect was not sustained post treatment termination (Fig. 5.11B). These results mirrored the gene expression data for TGM2 in NB4-MR2 cells, which was also not sustained post treatment termination (Fig. 5.5). Intriguingly, for RARβ, higher dose 1.5 μM ATO treatment alone induced the greatest demethylation of the promoter region (Fig. 5.12A), which was again reflected in the gene expression changes (Fig. 5.5A). Importantly, these changes in RARβ methylation (like the changes in gene expression), were not sustained post treatment termination in NB4-MR2 cells (Fig. 5.5B). Together, this data strongly connects demethylation of the promoter regions of these genes with their increased expression.

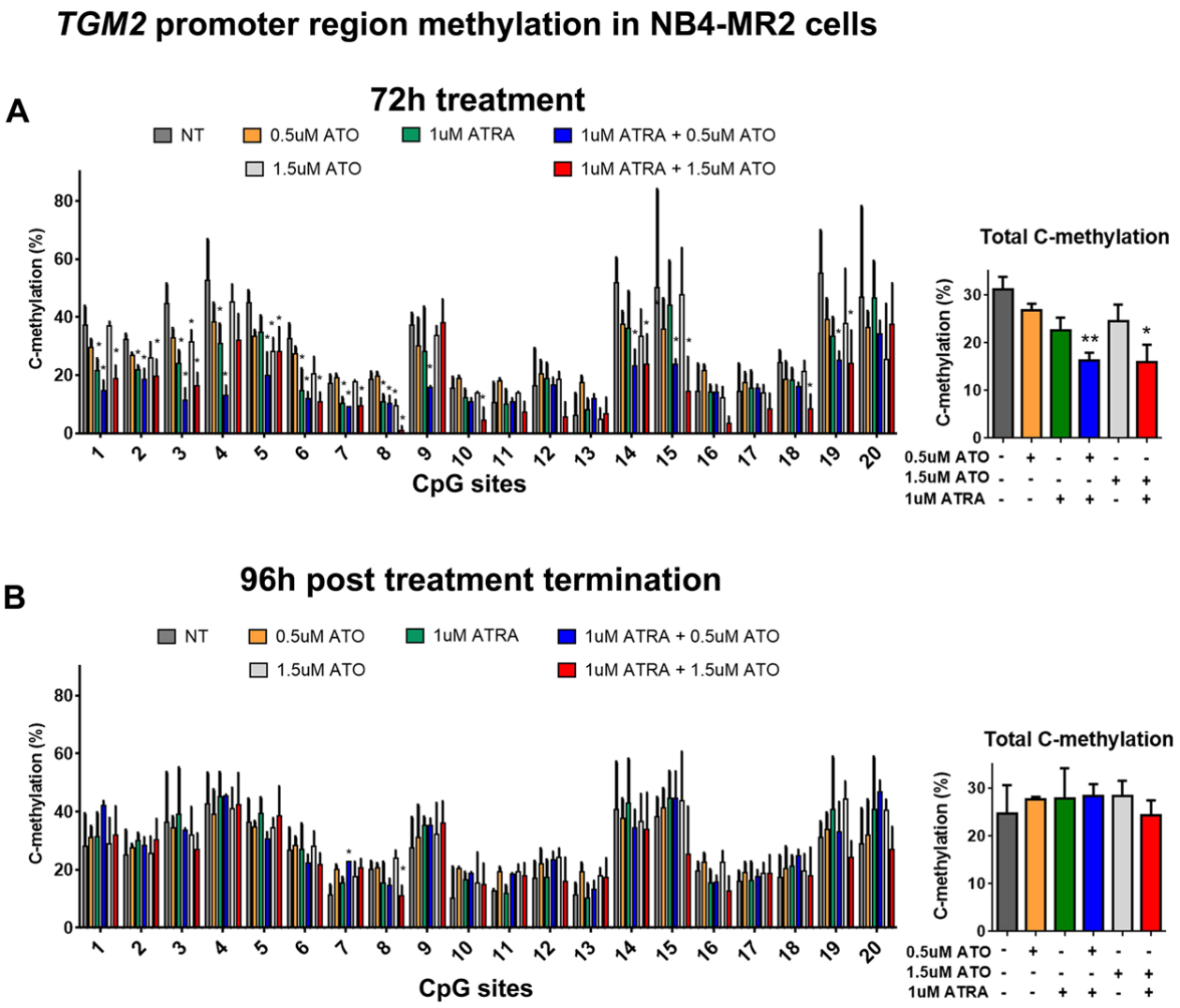


Figure 5.11ATRA and ATO combination treatment demethylate the CpG island in the promoter region of TGM2 in NB4-MR2 cells, but this is not sustained post treatment termination. (A and B) The methylation percentage of the individual 20 CpG sites and total C-methylation percentage of the region in NB4-MR2 cells following 72 h of treatment (A, n = 4) and subsequent 96 h post treatment termination (B, n = 3). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons.

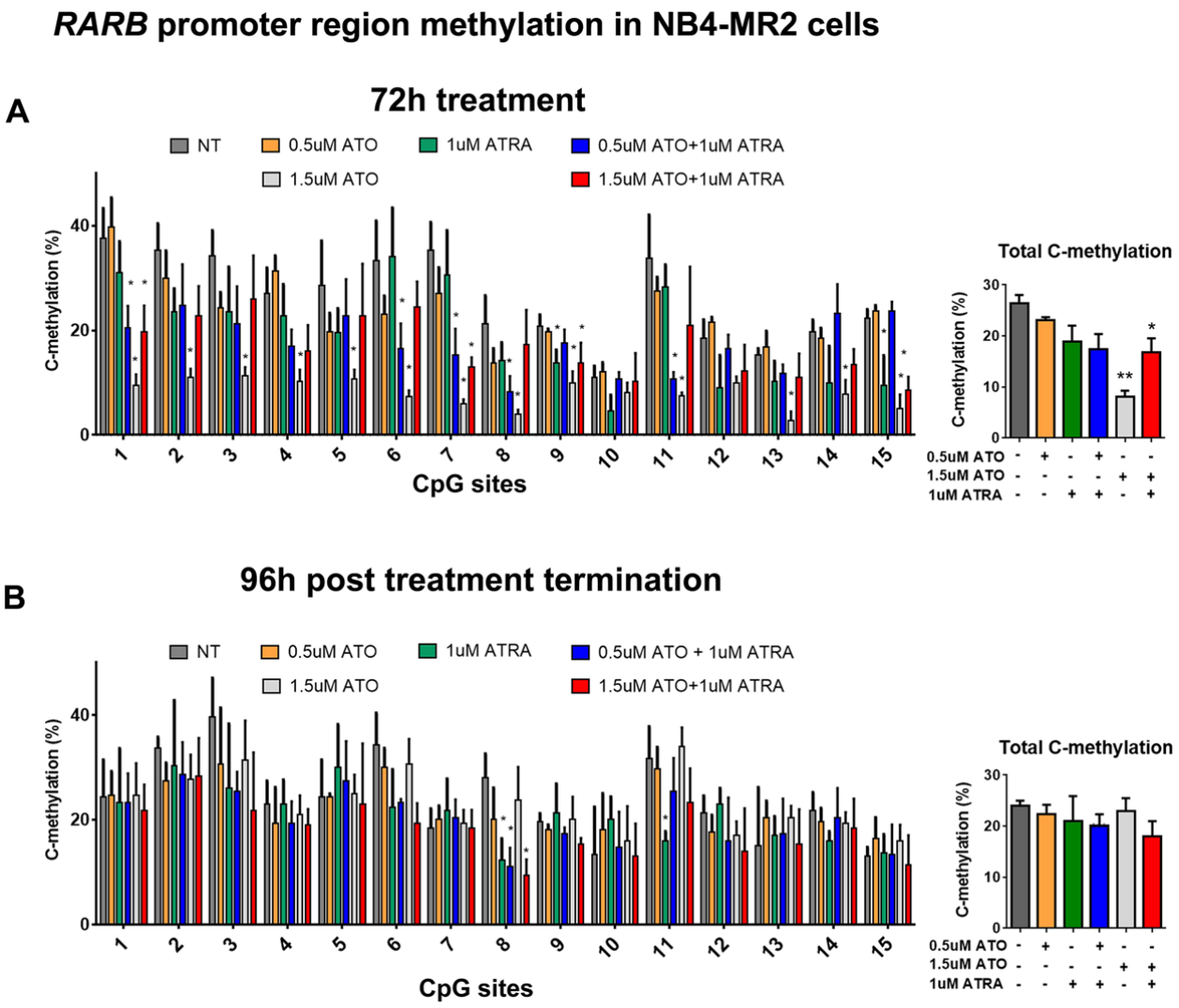
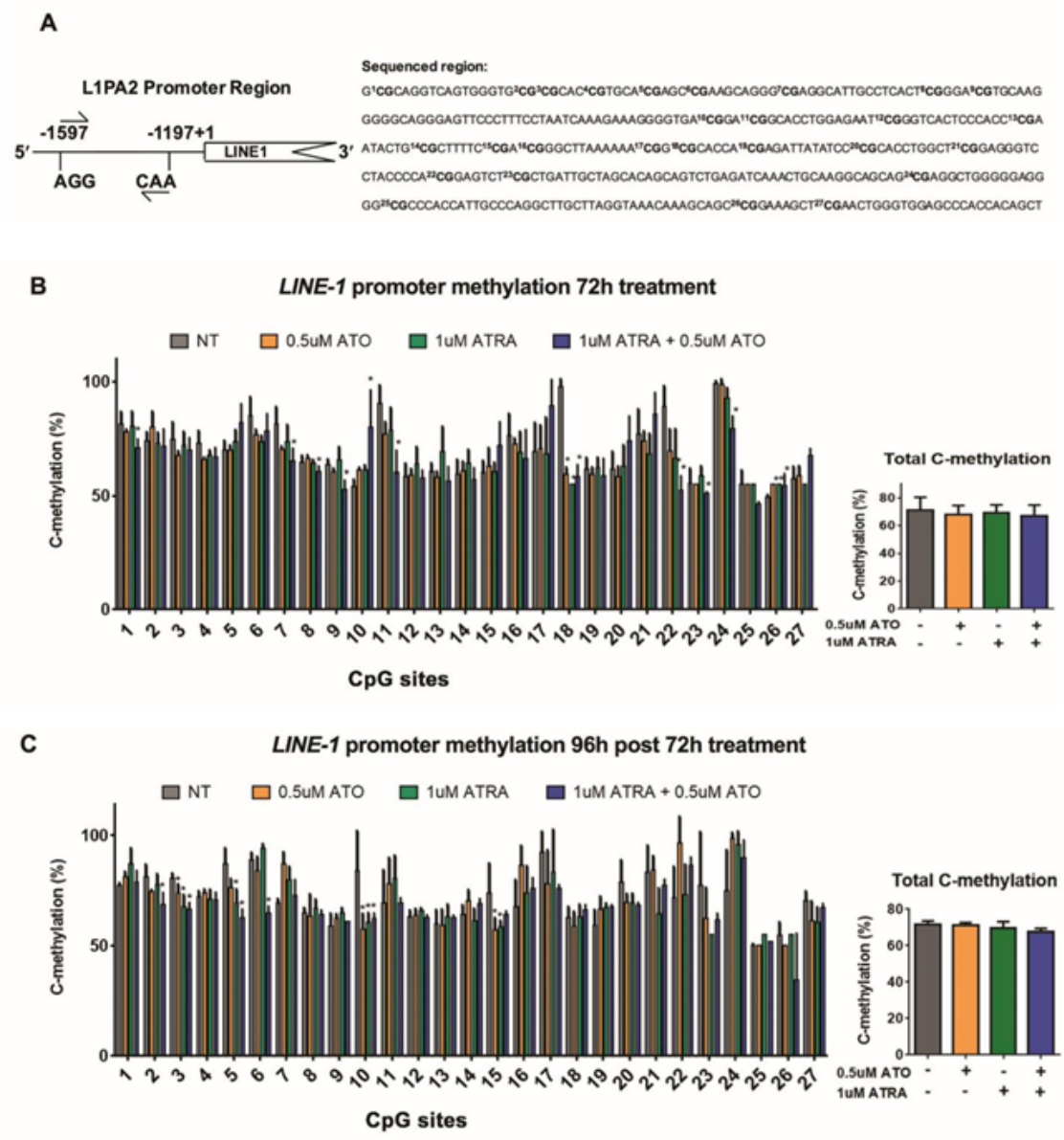


Figure 5.12. 1.5 µM ATO demethylates the CpG island in the promoter region of RARβ in NB4-MR2 cells, but this is not sustained post treatment termination. (A and B) The methylation percentage of the individual 15 CpG sites and total C-methylation percentage of the region in NB4-MR2 cells following 72 h of treatment (A, n = 4) and subsequent 96 h post treatment termination (B, n = 3). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons.

5.3.7 Global DNA Methylation Levels, Represented by Bisulfite Pyrosequencing of LINE-1, are Unchanged in ATRA, ATO or Combination Treated NB4 and NB4-MR2 Cells

We next wondered if the effect of combined ATRA and ATO treatment on CpG methylation extended beyond the target genes and was genome-wide. Long interspersed nucleotide element 1 (LINE-1) elements are transposable repetitive elements making up 17% of genomic DNA and are often heavily methylated, holding up to a third of the methylation in the genome. As such, assessing methylation of LINE-1 elements is an accepted surrogate for general global methylation levels of the genome (Cruickshanks and Tufarelli 2009; Ohka et al. 2011; Yang et al. 2004) Although this technique assesses relatively few CpG sites, LINE-1 bisulfite pyrosequencing has been shown to reflect global DNA methylation changes with high significance (Lisanti et al. 2013). We performed bisulfite pyrosequencing to assess methylation levels of 27 CpG sites within the promoter region of LINE-1 subfamily L1PA2, which also lacks RARα and RARβ binding sites (Fig 5.13A) (Cruickshanks and Tufarelli 2009; Mathews et al. 2003). Overall, LINE-1 methylation was unchanged by any of the treatment conditions at 72 h or 96 h after treatment was terminated in both NB4 cells (Figs 5.13 and 5.14) and NB4-MR2 cells (Fig. 5.15). This suggests that the reduced CpG methylation in response to treatment is associated with target genes (Figs 5.7, 5.9, 5.11 and 5.12), and is probably not global (Figs. 5.13, 5.14 and 5.15).

Figure 5.13. Supplemental Figure 5. Global DNA methylation levels, represented by bisulfite pyrosequencing of LINE-1, are unchanged in 1µM ATRA, 0.5µM ATO or combination treatment in NB4 cells. (A) Schematic representation of the LINE-1 subfamily PA2 and the 27 CpG sites located within the region that were bisulfite pyrosequenced. (B and C) The methylation percentage of the individual 27 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment (B) and subsequent 96h post treatment termination (C). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *, n=5.


A
L1PA2 Promoter Region
-1597
-1197+1
5′
LINE1
3′
AGG
CAA
Sequenced region:
B
LINE-1 promoter methylation 72h treatment
NT
0.5uM ATO
1uM ATRA
1uM ATRA + 0.5uM ATO
C-methylation (%)
CpG sites
Total C-methylation
0.5uM ATO
1uM ATRA
C
LINE-1 promoter methylation 96h post 72h treatment

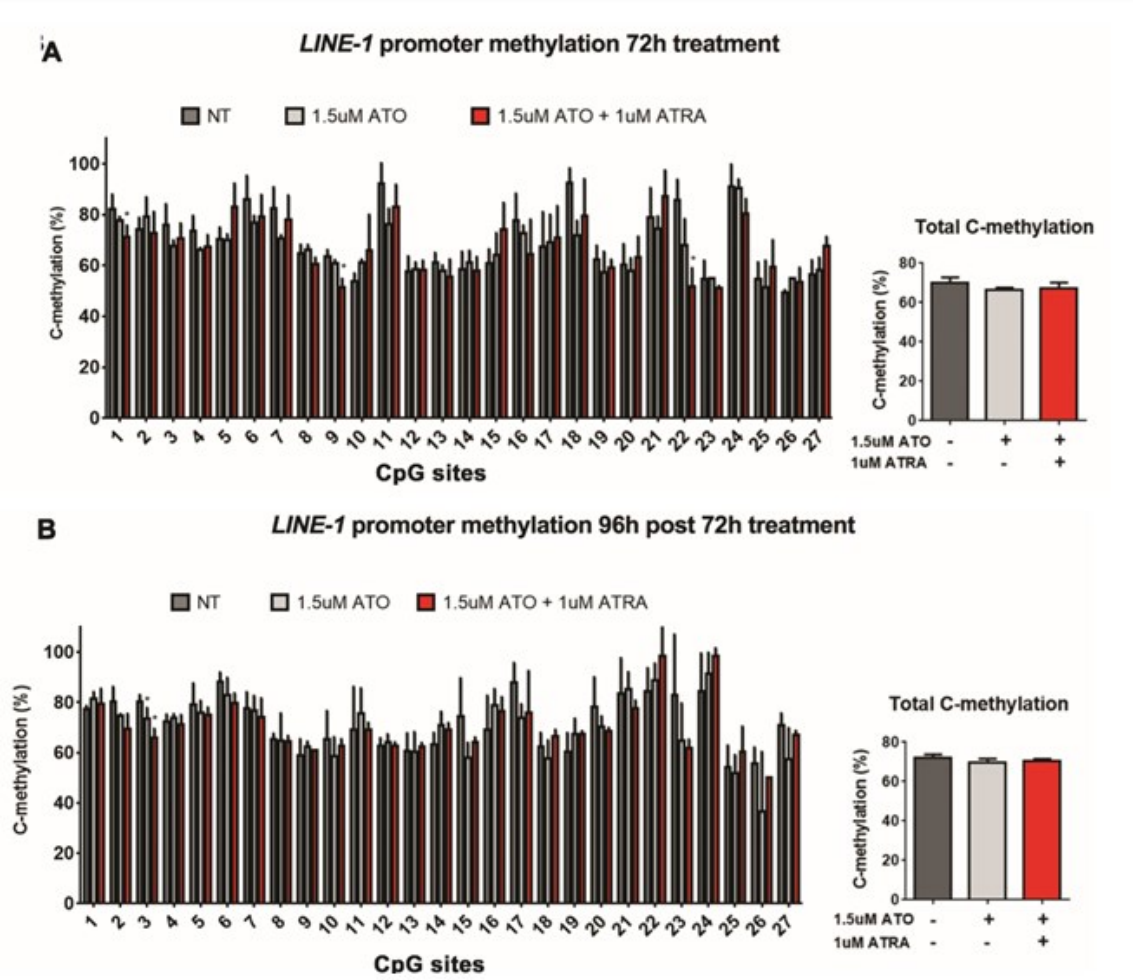


Figure 5.14. 1.5µM ATO (with or without ATRA) does not reduce global DNA methylation levels, represented by bisulfite pyrosequencing of LINE-1, in NB4 cells. (A and B) The methylation percentage of the individual 27 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment (A) and subsequent 96h post treatment termination (B). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, n=4.

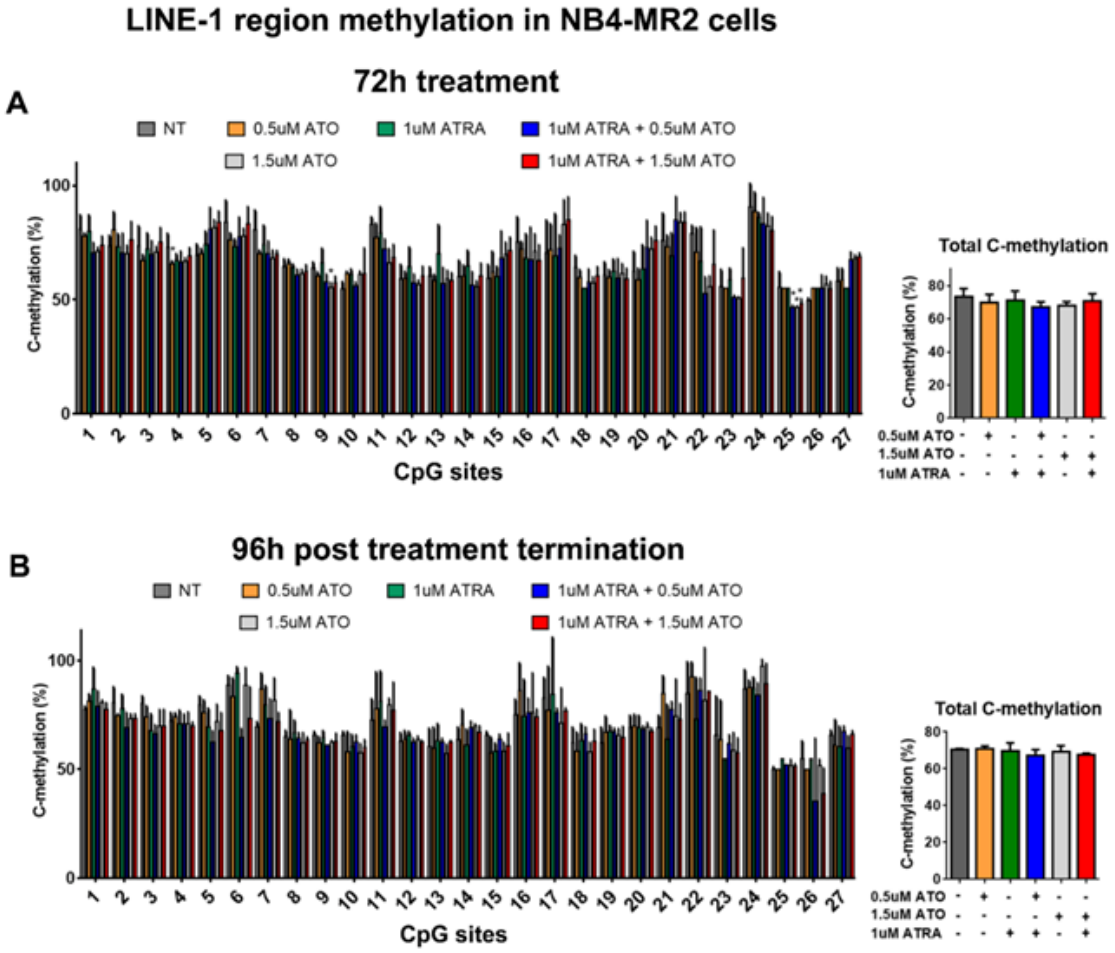


Figure 5.15. Global DNA methylation levels, represented by bisulfite pyrosequencing of LINE-1, are unchanged in ATRA, ATO or combination treated NB4-MR2 cells. (A and B) The methylation percentage of the individual 27 CpG sites and total C-methylation percentage of the region in NB4-MR2 cells following 72h of treatment (A, n=4) and subsequent 96h post treatment termination (B, n=3). Error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons.

5.3.8 BCL6 Expression is Associated with Increased ATO Resistance in APL

Having verified the role of BCL6 as a mediator of taxane resistance in breast cancer with a potential role in the treatment resistance of other solid tumors, we were interested in investigating its potential implications in APL and other blood malignancies.

Unlike, B cell lymphoma, no direct link has been established between AML or APL and BCL6 expression despite identifying BCL6 corepressor BCOR as a fusion protein partner for RARα in some APL cases (Yamamoto et al. 2010). We investigated BCL6 expression in AML patients in comparison to healthy donors using dataset GSE90062 and observed a significant increase in BCL6 expression in AML patients (Fig. 5.16A). However, we were unable to find any dataset to determine if BCL6 is also upregulated in APL patients.

Next, we wanted to investigate if BCL6 expression is associated with treatment resistance in APL cells. To achieve this goal, we analyzed BCL6 expression in two different datasets comparing genetic changes between ATO sensitive and resistant NB4 cells. In dataset GSE73157, BCL6 expression was upregulated in NB4 cell cocultured with bone marrow cells in comparison cells culture alone (Fig. 5.16B). Interestingly, cocultured NB4 cells, demonstrated an increased resistance to ATO treatment due to the protective effect of stromal cells (Ganesan et al. 2016). Similarly, when we performed expression analysis on an ATO resistant variant of NB4 cells from dataset GSE115812, we observed an increase in BCL6 expression in the resistant cell line in comparison to ATO sensitive NB4 cells (Fig. 5.16C).

Given the importance of DNA methylation in gene regulation during hematopoiesis (Hodges et al. 2011), we investigated BCL6 expression in common

myeloid and granulocyte-monocyte progenitor cells in the presence and absence of DNMT3A in dataset GSE68844. Interestingly, BCL6 expression was significantly increased in cells isolated from DNMT3A knockout mice in compassion to wild type mice (Fig 5.16D), suggesting that BCL6 expression may be negatively regulated through apparent methylation. Together, these findings suggest that BCL6 levels could be upregulated due to ATO's demethylating effects. However, additional investigation is required to determine if BCL6 upregulation in ATO resistant samples can be targeted to enhance treatment efficacy.

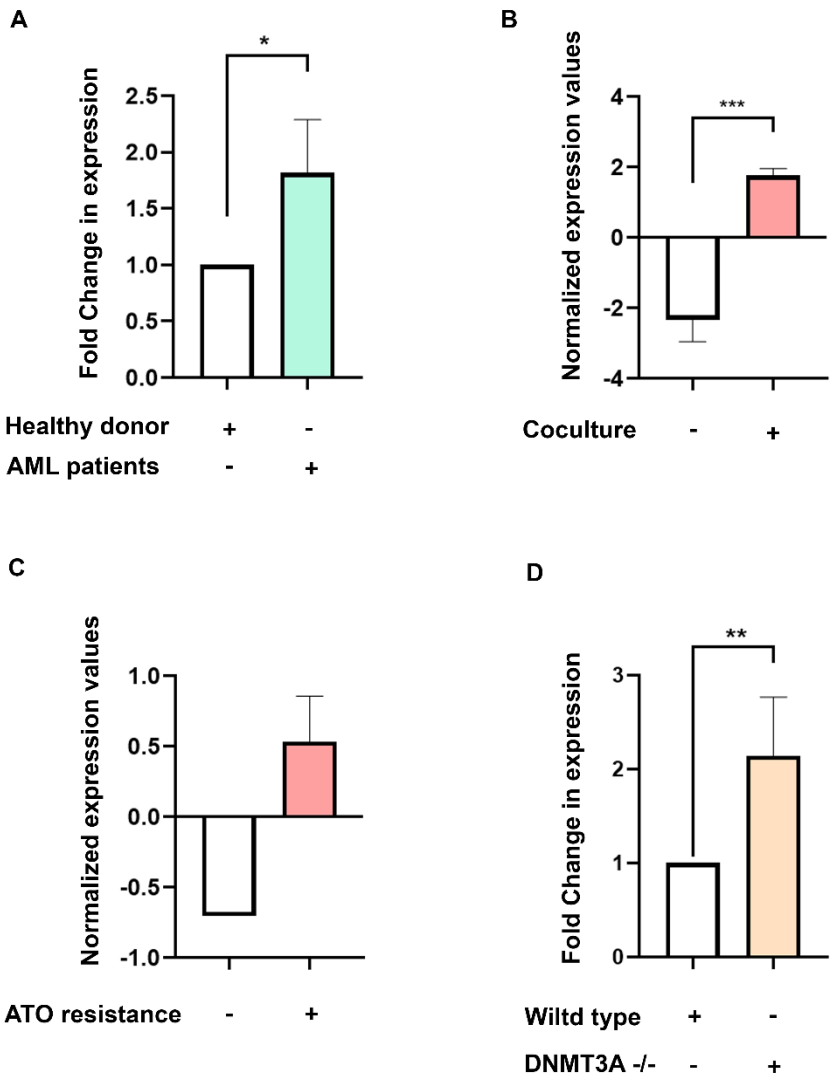


Figure 5.16 BCL6 expression is associated with increased ATO resistance in APL cells. Normalized BCL6 expression levels in (A) AML patients and healthy donors (GSE90062), in (B) NB4 cells culture individually or cocultured with bone marrow stromal cells, in (C) ATO sensitive and resistant NB4 cells and in (D) hematopoietic progenitor cells isolated from wild type and DNMT3A knockdown mice. Error bars represent standard deviation. Significance was determined using an unpaired t-test.

5.4 Discussion

Clinically, combined ATRA and ATO therapy is curative in APL patients, inducing long-lasting remission, whereas ATRA treatment alone is effective at eliciting short-term remission (Abaza et al. 2017; Au et al. 2011; Cicconi and Lo-Coco 2016; Coombs et al. 2015; Estey et al. 2006; Zeidan and Gore 2014). Previous studies of cultured APL cells describe the enhanced differentiation/cell death induction of the combination treatment over ATRA alone; however, these studies did not extend the analyses post treatment termination (Giannì 1998; Luesink et al. 2009; Nayak et al. 2010). This study compares, for the first time, the effects of these treatments four days after treatment termination. Unexpectedly, we observed that under these conditions the effects of the combination treatment were much more dramatic. ATRA induced granulocytic differentiation in the short-term (72 h) and was increased further after long-term continuous treatment (168 h); however, ATRA-induced effects were largely lost once treatment had been terminated (Fig. 5.1). This is in sharp contrast to the combination treatment, which resulted in the differentiation and/or death of most the of the NB4 cells. Our analyses also revealed the greater overall efficacy achieved by treating APL cells with higher doses of ATO in combination with ATRA; the benefits of which become most apparent in the ATRA-resistant variant clone NB4-MR2 cells (Fig. 5.3). These findings model the long-lasting effects induced by the combination therapy in patients, which ATRA treatment alone comparably fails to do. The results further illustrate the potential benefit of higher dose ATO in combination with ATRA. The data from most clinical studies illustrating the benefit of ATRA and ATO combination treatment employ 0.15 mg/kg/day ATO (Abaza et al. 2017; Au et al. 2011; Estey et al. 2006).

Pharmacokinetic studies report a range of plasma ATO levels based on this dose (Cui et al. 2018; Fox et al. 2008; Shen et al. 1997), although the 0.5 μM dose used in this study likely captures the lower reported plasma levels and the 1.5 μM may better represent the higher reported plasma levels. The results of a recent clinical trial utilizing 0.3 mg/kg/day ATO in combination with ATRA reported high rates of complete remission and overall survival (Ghavamzadeh et al. 2018). From the current available patient data, it is unclear if ATRA administered with 0.3 mg/kg/day ATO versus 0.15 mg/kg/day ATO results in significantly different outcomes for APL patients.

In our analyses, the sustained effects of the ATRA and ATO combination treatment post treatment termination was also detected at the transcript level of some target genes in NB4 cells (Fig. 5.4B) and encouraged us to evaluate the epigenetic modifications of these genes. Epigenetic modifications such as histone marks and DNA methylation regulate gene transcription and are aberrant in APL resulting in silencing of many target genes (Nouzova et al. 2004; Schoofs et al. 2013). Although the level of global methylation in APL NB4 cells remained unchanged by the combination treatments (Figs 5.13 and 5.14), the methylation of target genes was observed (Figs 5.7 and 5.9). An important contributing factor to the increased effectiveness of combination treatment may be the sustained demethylation of target genes that are aberrantly methylated in APL cells (Figs 5.7 and 5.9). The aberrant methylation and silencing of RARβ is particularly well described in APL (Chim et al. 2003; Di Croce et al. 2002; Martens et al. 2010; Nouzova et al. 2004).With the exception of one early study that used methylation-specific PCR to quantify RARβ methylation post ATRA treatment (Di Croce et al. 2002), other later studies using more quantitative techniques report that ATRA treatment alone fails to

revert the aberrant methylation of RARβ and other genes, including TGM (Castaigne 1990; Chim et al. 2003; Martens et al. 2010; Nouzova et al. 2004). Using the quantitative bisulfite pyrosequencing technique, our results show that ATRA alone does have some effects on the methylation of individual CpG sites of the target genes; however, the combination treatments are much more effective at demethylating the CpG islands of TGM2 and RARβ promoters (Figs 5.7 and 5.9) (Chim et al. 2003; Martens et al. 2010; Nouzova et al. 2004).

With respect to ATO alone, there are reports of its effects on DNA methylation in APL cells. A recent study showed that 2.0 μM ATO reduced DNA methylation and increased mRNA levels of cell cycle-related genes in NB4 cells (Hassani et al. 2018). ATO reduced transcript levels of DNA methyltransferases 1, 3 A and 3B in NB4 cells, which should have genome-wide demethylating effects on DNA (Hassani et al. 2018). This is consistent with another study on the cell line HL-60 (an APL-like cell line that lacks the PML-RARα fusion), in which 1 μM ATO modestly reduced global methylation (Peng et al. 2010). This suggests that more so than ATRA (Chim et al. 2003; Martens et al. 2010; Nouzova et al. 2004), 1-2 μM ATO has demethylating effects in APL cells (Hassani et al. 2018; Peng et al. 2010). In our study ATO did not reduce global methylation as measured by LINE-1 methylation, but the higher dose of 1.5 μM ATO did demethylate CpG sites in both NB4 and NB4-MR2 cells. Notably, in ATRA-resistant NB4-MR2 cells, 1.5 μM ATO alone induced the greatest demethylation of the CpG island in the RARβ promoter (Fig. 5.12A) and RARβ expression (Fig. 5.5A). However, these effects were not sustained post treatment termination (Figs 5.12B and 5.5B). Therefore, in general, our data is in agreement with those studies that concentrations of 1–2 μM ATO

has at least some demethylating effects (Hassani et al. 2018; Peng et al. 2010). In future studies, it would be interesting to determine if prolonged continuous treatment of ATRA/ATO (i.e. 168 h of treatment), or if the drugs are administered consecutively instead of in combination, affects differentiation/cell death and methylation of target genes.

Therefore, while ATRA and ATO do appear to have some effects on DNA methylation, it is their combination that induces sustained demethylation of key target genes TGM2 and RARβ post treatment termination (Figs 5.7 and 5.9). Restoration of TGM2 levels in NB4 cells is necessary in ATRA-induced granulocyte differentiation18. The sustained reversal of aberrant methylation of the TGM2 promoter (Fig. 5.7), and increased transcript levels (Fig. 5.4B) induced by the combination, could be a key event the sustained differentiation of NB4 cells post treatment termination (Fig. 5.1E). Notably, in the ATRA-resistant NB4-MR2 cells, TGM2 levels were increased and the gene demethylated by the combination, but these effects were not sustained post treatment termination. Therefore, the lack of sustained effects on DNA methylation could contribute to the resistance of NB4-MR2 cells to treatment.

The results from our gene-specific QPCR and bisulfite pyrosequencing studies highlight the need to perform transcriptome and genome-wide methylation analyses (e.g. whole genome bisulfite sequencing, or EPIC array which measures methylation of over 850,000 CpG sites (Pidsley et al. 2016; Yong, Hsu, and Chen 2016)) on combination ATRA and ATO treated NB4 cells and patient cells. This would reveal if the combination treatment induces sustained gene expression and methylation changes of other target genes with altered expression, while inducing limited genome-wide methylation changes

(as suggested by the surrogate LINE-1 methylation levels measured here). Thus far, genomic analyses have been primarily focused on the effects of ATRA only in APL cells (Martens et al. 2010; Schoofs et al. 2013); however, with the increasing utilization of ATRA and ATO in the clinic, the effects of the combination of ATRA and ATO on the epigenome and transcriptome needs to be understood. Differences in the effects of combination treatment may be crucial for APL patients who are resistant to therapy and experience disease relapse (J. Park et al. 2011). It is also possible that methylation changes of certain target genes (e.g. RARβ, TGM2), could be used as a predictor of complete response. Therefore, an increased understanding of the role of epigenetics in APL treatment response may help in the development of novel strategies to overcome treatment failure, and may also lead to strategies for the application ATRA-based therapies in other cancers (Coyle et al. 2018). Potential strategies include the use of demethylating agents such as decitabine or HDAC inhibitors such as vorinostat (CS Young 2017), which may improve on the demethylating effects demonstrated in our study.

Finally, we investigated the role of the novel taxane resistance mediator BCL6 in drug resistance in NB4 cells. Interestingly, BCL6 expression was upregulated in two different models of ATO-resistant NB4 cells (Fig. 5.16B and C). Additionally, we highlighted the potential role of DNMT3A in downregulating BCL6 expression in hematopoietic progenitor cells (Fig. 5.16D). Thus, additional investigations could determine the impact of ATRA and ATO treatments on BCL6 expression and the potential impact of inhibiting BCL6 on APL treatment efficacy.

CHAPTER 6: DISCUSSION

6.1 Preface

Treatment efficacy for different types cancers are governed and affected by different factors at the patient and tumor level. The high degree of intratumoral and intertumoral heterogeneity presents a challenge for treatment success and negatively impacts survival; however, it also provides different avenues that can be explored to reduce resistance through the implementation of precision medicine strategies.

At the tumor level, heterogeneity could be observed between tumor cells through the existence of different clones with different genetic and epigenetic alterations as a result of clonal evolution (Mazor et al. 2016). Several studies have shown different sections of an individual tumor to have different mutations (Boutros et al. 2015; Meyer et al. 2015), suggesting that targeted therapies against these mutations could result in selection for non-mutated clones resulting in treatment resistance. Additionally, heterogeneity manifests between different populations of tumor cells with different tumorigenic capacities (Marjanovic et al. 2013). Several studies have identified CSCs and demonstrated their increased abilities to initiate tumors and promote metastasis (Al-Hajj et al. 2003; Marcato et al. 2011; Singh et al. 2004). CSCs have been shown to have increased resistance to therapy which allow them to withstand treatment (Loebinger et al. 2008; Sládek et al. 2002). Similarly, several studies have demonstrated that CSCs have enhanced immune evasion capacities, allowing them to surpass immune surveillance (Chikamatsu, K, Takahashi G, Sakakura K, Ferrone S 2011) and utilize some immune cells to promote tumorigenesis and metastasis (Masahisa Jinushi et al. 2011). Heterogeneity can also be observed between different CSC populations in breast cancer.

Two different populations of CSCs have been identified; ALDE+ breast CSCs (Ginestier et al. 2007) and CD44+CD24- breast CSCs (Al-Hajj et al. 2003). A minimal overlap has been shown between the two populations, with each having different roles tumor progression and metastasis while maintaining their highly tumorigenic nature (S. Liu et al. 2014).

Intratumoral heterogeneity goes beyond tumor cells to include other infiltrating cell populations, constructing the tumor microenvironment. Several studies have demonstrated the importance of different cell populations within a tumor and its microenvironment in facilitating tumor progression and treatment response including fibroblasts and tumor infiltrating lymphocytes, macrophages, and neutrophils (Okuda et al. 2012; Östman and Augsten 2009). Furthermore, oxygen levels within different portions of the tumor resulting in hypoxic conditions that can affect treatment efficacy and outcomes (Widmer et al. 2013).

Additionally, several studies have highlighted the fluctuation in percentages of CSCs between different tumors from the same types of cancer and demonstrated that higher levels were associated with worse outcomes (Cho and Clarke 2008; Marotta and Polyak 2009). Similarly, levels of tumor infiltrating T cells have been indicated a prognostic factor that could predict treatment response and patients survival in different types of cancer (Hamanishi et al. 2007; Mahmoud, Macmillan, and Grainge 2011).

Heterogeneity defines the basis of personalized medicine that is designed to target specific patients and populations. This approach includes surveillance, screening, diagnostics and treatment of different patients using personalised and informed approach of their tumors (Liu, Dang, and Wang 2018).

The existence of certain mutations is used as screening tools for predicting the likelihood of developing cancer as well as predicting response to certain treatments. Inherit mutations in the BRCA1 or BRCA2 genes are associated with increased risk for developing breast and ovarian cancer (King, Marks, and Mandell 2003). Individuals with strong family history of these mutations undergo increased surveillance and different precautionary measures that allows for early detection of tumors and increases their chance of survival (Peto et al. 1999). Similarly, expression of different receptors (i.e. ER, PR and HER2) have been used to subtype breast cancer tumors. This allowed for treatment options targeted for individual subtypes and as result increased patient survival. Differential gene expression between different patients have also been used as predictive tool for treatment efficacy (DeSantis et al. 2019; Harris et al. 2006). This can be observed through the development and the use of gene signatures such as the Oncotype DX in breast cancer (Carlson and Roth 2013) and the detection and use of the PML-RARα fusion as a predictive marker for APL sensitivity to ATRA treatment (Testa and Lo-Coco 2016). Furthermore, different therapies have been shown to induce genetic and epigenetic changes in different tumor cells and other components of the tumor microenvironment to achieve the desired outcome (Okada et al. 2011; Peng et al. 2010; Yip et al. 2015). Thus, it is imperative to understand these changes and utilize this information to enhance treatment efficacy.

In this body of work, I explored several aspects of heterogeneity at the intratumoral and intertumoral patient level in cancer in the context of therapy and the interaction with immune system. I also explored differential gene expression both as predictive tool for treatment response in breast and cancer and as result of induction

treatment in APL. Additionally, I have identified several resistance mechanisms including epigenetic silencing of key genes, which can be further investigated as potential combination therapy approaches to enhance treatment efficacy.

6.2 Differential Expression of Immune Related Genes in Different Breast CSC Populations.

The ability of CSCs to give rise to tumors and mediate metastasis suggest a superior ability of these cells to evade immune detection and destruction. Several studies have shown CSCs can evade immune surveillance and utilize protumorigenic immune cells to promote tumor progression and maintenance (Chikamatsu, K, Takahashi G, Sakakura K, Ferrone S 2011; Masahisa Jinushi et al. 2011; Okuda et al. 2012). Several mechanisms have been highlighted by which CSCs from different cancer types evade immune destruction, including downregulation of antigen presentation machinery and costimulatory molecules (Brahmer et al. 2012; Volonte et al. 2014), and upregulation of T cell inhibitory signalling pathways such as PDL1 and CTLA4 (Di Tomaso et al. 2010). In contrast, several studies have shown the importance of different populations of innate immune cells in the maintenance and expansion of CSCs, including TAMs and neutrophils (Panni et al. 2014; Theocharides et al. 2012).

In the study detailed in Chapter 3, we were interested in investigating the gene expression of different immune markers in the CD44+/CD24- and ALDE+ breast CSCs population to determine the potential interaction of these populations with different immune cells. To achieve this goal, we utilized two previously published datasets where CSCs from either population were sorted along with their non-CSCs counterparts from two different sets of patient tumors. Using raw expression data, we re-analyzed both data

sets and determined differential gene expression (upregulated and downregulated genes) between the CSCs and their non-CSCs counterparts from each tumor. Similar to previous studies (Colacino et al. 2018; Shao et al. 2016), we observed a minimal overlap in the genes differentially expressed between the two CSCs populations (Fig 3.6C).

Next, we overlapped the differentially expressed genes in both populations with a panel of 621 genes related to immune function to determine different immune pathways altered in the two CSCs populations. Interestingly, the immune gene panel mainly overlapped with genes downregulated in the ALDE+ cells (Fig 3.3B) and genes upregulated in the CD44+/CD24- population (Fig 3.6B). Several of the immune genes downregulated in the ALDE+ population were genes associated antigen presentation and interaction with adaptive immune cells (Fig 3.3C) suggesting that ALDE+ have an enhanced immune evasion ability that allows them to escape detection and destruction by adaptive immune cells. In contrast, the immune gene panel overlapped with different genes upregulated in the CD44+/CD24- cells and several of these genes are one associated with interactions with innate immune cells and have been associated with protumorigenic cells. These findings indicate that the two breast CSC populations might play different roles in the interplay between tumor cells and the immune system. While the CD44+/CD24- cells could play a key role in promoting a protumorigenic immune environment, ALDE+ cells might play an essential role in evading immune detection and destruction.

6.3 ALDE+ CSCs Evade Immune Destruction and Downregulate TAP1 a Key Antigen Presentation Gene.

To determine the effect of immune pressure on tumor heterogeneity and breast CSCs we investigated tumor composition of a spontaneous mammary tumors in the presence and the absence of an immune system. As expected, tumors grown in the presence of the immune system were smaller and had an increase in infiltrating immune cells and in the level of apoptotic cells (Fig 3.2). Interestingly, despite the decrease in the level of viable cells under immune pressure we observed a significant increase in the percentage of ALDE+ cells (Fig 3.2) suggesting that these cells might have an enhanced ability to withstand immune targeting.

Having established that ALDE+ cells in the spontaneous mouse tumors had advantage in withstanding immune pressure, we aimed to determine the underlying mechanism of this advantage. Gene expression analysis of the antigen presentation that were downregulated in the patient sorted ALDE+ cells revealed a similar change in several of these genes including TAP1 (Fig. 3.4A). To further investigate this phenomenon, we isolated ALDE+ cells from the 4T1 murine cell line and confirmed their increased tumorigenic capacity in comparison to ALDE- cells (Fig 3.4B and 3.4C). Similarly, expression analysis of the antigen presentation genes in the sorted samples revealed TAP1 expression to be downregulated in the ALDE+ cells (Fig 3.4D).

The ability of ALDE+ cells isolated from three different models to downregulate TAP1 suggests a decrease in the antigen presentation capacity of these cells, which allows the cells to evade immune detection. It also indicates that these cells might be more resistance to immune therapy strategies that depends on T cell activation.

6.4 TAP1 Expression in ALDE+ Cells is Downregulated Through Epigenetic Silencing

Given the importance of DNA methylation in regulating key genes in CSCs, we investigated the effect of methylation on TAP1 expression in ALDE+ cells. We observed a negative correlation between TAP1 expression and its methylation in patient samples from the TCGA Cell 2015 dataset (Fig 3.7A) suggesting a potential role for DNA methylation in regulating TAP1 expression in breast cancer in general. These findings encouraged us to investigate the effect of hypermethylation on TAP1 expression in the Aldefluor sorted cells from the spontaneous mouse tumor. Pyro-sequencing analysis revealed increased level of total c-methylation and individual CpG site methylation of the TAP1 promoter in the ALDE+ cells in comparison to the ALDE- cells (Fig 3.7F). Therefore, we were able to identify epigenetic silencing as the underlying mechanism for TAP1 downregulation in ALDE+ CSCs, which can play an important role in their ability to avoid immune detection.

This encouraged us to investigate the effect of demethylating agents on the expression of TAP1 and the level of its promoter methylation. To achieve this goal, we used 4T1 murine cell line given the high proportion of ALDE+ population in these cells (Fig 3.4B) as well as previous reports indicating the poor immunogenic characteristics of this cell line. Importantly, treating 4T1 with demethylation agent decitabine resulted in a significant increase in TAP1 expression (Fig 3.7B) associated with a decrease in its promoter total c-methylation and the methylation of the individual CpG sites (Fig 3.7E).

The increase of the TAP1 expression following decitabine treatment provides a new prospect for investigating the use of demethylating agents in combination of T cell targeted immunotherapies to enhance antigen presentation in CSCs and in turn enhance

treatment efficacy. In fact, several clinical trials are currently investigating the effect of combining demethylating agents with T cell targeting immunotherapy in AML (NCT04277442), melanoma (NCT02608437) and prostate cancer (NCT03709550). Findings from these trials will determine the impact of these combinations on treatment outcomes and highlight any harmful effects associated with the broad-spectrum effect of demethylating agents of gene expression.

6.5 Chapter 3 Limitations

Investigation of immune gene expression in the two different breast CSC populations provided a starting point to begin to understand the potential role played by each population in the interplay between the tumor and the immune system. However, there are several limitations in this analysis that should be addressed in future investigations of breast CSC interactions with immune cells. First, given that the two different CSC populations were isolated in two separate experiments; it would be ideal to sort these population from the same patient tumors to understand their distinct roles in the context of similar microenvironments and eliminate differences that can be attributed to different experimental settings. Additionally, the level of immune infiltrating cells and percentage of CSCs was not reported for in the individual tumors, thus we are not able to confirm the effect of percentage directly on the immune interaction. Additionally, it is important to determine if cells displaying both phenotypes (ALDE+/CD44+/CD24-) are capable of altering the expression of the immune related genes in a similar fashion to the different CSCs population and thus have an advantage in interacting with both pro and anti tumorigenic immune cells.

While TAP1 was significantly downregulated in the ALDE+ cells isolated from the three models mentioned earlier, it was slightly upregulated in ALDE+ cells isolated from the MDA MB 231 cell line (Fig 3.5B). This could be due to the decreased levels of ALDE+ cells in MDA MB 231 cells (Fig 3.5A) as well as the prolonged *in vitro* expansion of these cells in the absence of immune pressure. This limitation could be overcome by isolating ALDE+ cells from cell line that has been cocultured with different immune cells to determine whether having the immune pressure will induce the downregulation of TAP1 in these cells. The cocultures also will provide a tool to investigate the ability to investigate the effect of TAP1 downregulation on the interaction between ALDE+ cells and the different immune cells through the performance of functional immune assays.

As previously mentioned, the use of demethylating agents provide a promising tool to enhance the expression of TAP1. However, it remains unclear whether the upregulation of TAP1 will be associated with increased antigen presentation and T cell activation. Analysis of levels of MHC I on cell surface following treatment could help clarify this point since higher levels of MHC I are expected due to increased antigen presentation. Another, limitation to consider with the use of demethylating agents is the undesired and unpredictable changes in gene expression due to the lack of target specificity when these drugs are delivered systematically. Intratumoral delivery of low dosages of demethylating agents might limit the unwanted wide-spectrum consequences of the demethylating agent. However, it remains unclear what effect delivering the demethylating agent will have on the different cell populations in the tumor.

6.6 Chapter 3 Future Directions

Several approaches could be taken to address limitations identified in the previous section and further investigate the role and potential targeting of TAP1 in immune targeting of ALDE+ CSCs. As previously mentioned, it is important to determine the differential gene expression of the immune genes in ALDE+ cells, CD44+/CD24- cells and the overlapping population under immune pressure to determine their immune characteristics and their role in the interplay between breast CSCs and immune cells. To achieve this goal we have screened several breast cancer PDXs and determined their HLA typing in preparation for a double engraftment of human immune cells and breast cancer PDXs in RAG-/- mice. This will establish models that allow for performance of functional immune assays in the presence of functional immune system. These models will be valuable tools to further validate our previous findings and test the effect of treatment of demethylating agents alone or in combination with immunotherapies on the CSCs and rest of the tumors.

Additionally, the next step would be to study the effect of low dose intratumoral injection of decitabine on 4T1 tumor growth and immune cell infiltration. Tumor growth should be assessed by comparing tumor volumes during treatment and tumor volumes and weights at end point. Additionally, immune cell infiltration and activation could be investigated by harvesting tumors, lungs, spleen and lymph nodes from different treatment groups and analyzing using flowcytometry.

Finally, proteomic analysis of different peptides presented in the MHC I molecules of the ALDE+ cells could give additional insights on development of targeted immunotherapies therapies directed against these cells. This analysis could be done in the

presence and absence of treatment with demethylating agents to further asses their potential in enhancing immune targeting of ALDE+ cells.

6.7 An in vivo Genome-Wide shRNA Screen Identifies Potential Novel Mediators of Paclitaxel Response in Breast Cancer.

The ability to determine patient response to treatment prior to its implementation helps in increasing treatment efficacy and increasing survival while decreasing unwanted side effects. Given paclitaxel's wide use in breast cancer treatment (McGrogan et al. 2008), we aimed to identify genes associated with paclitaxel sensitivity and resistance. Those genes can provide novel targets for combination therapies to increase sensitivity and overcome resistance. Furthermore, since paclitaxel is used to treat other types of cancer, the screen identified genes could also be investigated for their potential targeting in these cancers.

The screen identified hundreds of enriched and depleted shRNAs that potentially targeted paclitaxel sensitivity and resistance genes, respectively. Thus, we prioritized 10 potential sensitivity genes and 10 resistance genes to further analyze their effect on the survival of patients who received chemotherapy. Among the two sets of genes, high BCL6 expression was best associated with worse outcome in chemotherapy treated breast cancer patients, regardless of their tumor molecular subtype (Fig. 4.2). In contrast, when the same sets of genes were evaluated in a sub-cohort including the basal like patients, some genes displayed significant correlations with outcomes (Fig. 4.2). This may indicate

that many genes identified in the screen might have a role that is specific to basal-like while other markers, such as BCL6 might be more universal to include other subtypes.

6.8 BCL6 is a Novel Contributor of Paclitaxel Resistance in Breast Cancer

Having identified BCL6 as a possible mediator of paclitaxel resistance in breast cancer, we aimed to verify this role and investigate the potential use of BCL6 inhibitors to enhance paclitaxel response. Using several *in vitro* and *in vivo* models, BCL6 contributed to paclitaxel and the effect of inhibiting BCL6 on treatment efficacy. This was achieved by downregulating the expression of BCL6 using two different shRNAs and by inhibiting BCL6 protein activity by using the small molecule inhibitor BCL6i (Cerchietti et al. 2010). Both approaches were successful in enhancing the effect of paclitaxel on the TNBC cell line MDA MB 231 *in vivo* and *in vitro* (Figs 4.4, 4.5,4.6 and 4.7). Moreover, BCL6i (Fig. 4.8) combination enhanced paclitaxel effect in the TNBC breast cancer cell line MDA MB 468, further confirming BCL6 role as resistance mediator for taxane treatment.

The additive effect of BCL6 silencing on paclitaxel tumor growth inhibition prompted us to investigate the underlying mechanism by which this effect is achieved. Using different analyses we were able to demonstrate that BCL6 knockdown is associated with a shift in cell cycle arrest to the S phase in comparison to the typical G2/M arrest we see in the control (Figs. 4.5C and 4.6C). This shift in cell cycle arrest was also associated with an increase in the level of apoptotic cells and decrease in level of viable cells (Fig 4.6B).

To determine the mechanism by which inhibiting BCL6 promoted the shift in paclitaxel induced cell cycle arrest, we investigated changes in the expression of several key regulators of cell cycle progression and checkpoints. Interestingly, inhibiting BCL6 with both shRNAs and BCL6i was associated with a significant increase in CDKN1A levels in these cells (Fig. 4.9A,B and C). Similar effects were observed in MDA MB 468 cells (Fig. 4.9D). The increase in CDKN1A levels following BCL6 inhibition is in accordance with previous reports that indicate the role of BCL6 as negative transcription regulator of CDKN1A. Moreover, the change in CDKN1A levels could explain this shift in cell cycle arrest given its important in the progression of the G1 and S phases of cell cycle. This could be further validated by using CDKN1A knockdown/knockout models in the context of BCL6 inhibition and paclitaxel treatment to determine whether this effect will be lost. Additionally, a genome-wide transcriptome analysis could help build a complete picture of changes associated with paclitaxel treatment with/without BCL6 inhibition. However, it is interesting that inhibiting BCL6 alone was unable to induce similar effects on cell cycle progression (Figs 4.5C and Fig 4.6C) despite some increase in CDKN1A levels (Fig. 4.7A) and further investigation is required to fully understand this shift in cell cycle arrest.

6.9 BCL6 Association with Treatment Resistance for Other Drugs and Different Types of Cancer

Given the importance of BCL6 in progression and treatment of B cell lymphoma (Leeman-Neill and Bhagat 2018), we were intrigued to determine if BCL6 is associated with treatment response in other types of cancers. Similar to results observed in the breast

cancer patient cohorts, high levels of BCL6 were associated significantly with decreased survival in ovarian (Fig 4.2C) and gastric (Fig. 4.2D) cancers. Furthermore, analysis of different datasets of sensitive and resistance patients and cell lines in AML and APL revealed a significant increase of BCL6 level expression in the resistant samples (Fig 5.16). Together these findings indicate that BCL6 might have an important role in treatment resistance and predicting treatment outcome in different types of solid and blood malignancies and thus, it should be further investigated.

In addition to taxanes, chemotherapy protocols for breast cancer patients typically include anthracycline drug doxorubicin or epirubicin (Rouzier et al. 2005; Thomas et al. 2007). Anthracyclines are DNA intercalating agents and topoisomerase inhibitors (Hortobagyi 1997), thus, inducing cell death in cancer cells by different mechanisms than taxanes. However, we were interested to investigate the screen identified genes' expression in relation to anthracycline resistance, given that many of the patients in the previously mentioned breast cancer cohorts received combination therapy of taxanes and anthracyclines. To achieve this goal, we analyzed gene expression in three different breast cancer cell lines that are resistant to epirubicin in comparison to their sensitive parental cell lines. Among the screen identified resistant genes, BCL6 expression was upregulated in all three resistant cell lines (Fig. 4.3) while DNAJA4 and CLCN3 were upregulated in epirubicin resistant MCF7 (Fig. 4.3B). In contrast, different sensitivity mediators were downregulated in the different epirubicin cell lines (Fig 4.3). These findings further highlight a potential role for BCL6 in treatment resistance associated with other chemotherapies besides paclitaxel in breast cancer and inhibition of BCL6 would need to be tested in the context of epirubicin treatment.

6.10 Chapter 4 Limitations

Despite the advantages of preforming the genome-wide shRNA screen in an *in vivo* model in identifying markers associated with treatment response in patients, the model remains incomplete due to the absence of immune pressure in NOD/SCID mice. Several reports have indicated the importance of the immune system in providing response treatment outside the context of immunotherapies (Hamanishi et al. 2007; Mahmoud et al. 2011). Chemotherapy induced cell death of cancer cells has been associated with increase antigen availability for T cell activation (Liu et al. 2010) as well as induction of inflammatory response within the tumor (Mills et al. 2008). The induction of T cell responses might be beneficial in targeting cells with normal antigen presentation, but it can lead to selection for cells with downregulated antigen presentation (Schatton et al. 2010; Volonte et al. 2014). In contrast, induction of inflammatory response could have negative effects on patient survival as several report have demonstrated the role of protumorigenic inflammatory cell in promoting metastasis (Vyas, Laput, and Vyas 2014). Thus, it is important to realize this limitation as some important response mediators might not be detected in our model. Additionally, the increased, cost, time and efforts required to perform these screens and validate their findings *in vivo,* limits the ability to carry several screens by utilizing different cell lines. This approach can be more accessible if these screens were performed *in vitro* and can provide an advantage in identifying markers with generalized effects by prioritizing findings that common between the different cell lines.

Having identified BCL6 as a mediator of paclitaxel resistance in breast cancer, we inhibited its activity using two different methods to enhance paclitaxel response.

Although the two different shRNAs were successful in decreasing BCL6 expression, the knockdown efficiency was only (50-65%). Thus, more effective methods in inhibiting BCL6 expression including creating MDA MB 231 clones with BL6 knockout using Cas9 crisper technology might further enhance the additive effect on paclitaxel treatment. Similarly, the dose of BCL6i used in Fig 4.7 was determined based on a previous study that should no negative side effect of the drug in different organs harvest from mice post treatment (Cerchietti et al. 2010). However, it remains unclear if higher doses of BCL6i would further enhance paclitaxel activity and whether it will be associated any negative side effect.

The effect of inhibiting BCL6 on paclitaxel was measured on tumors treated following tumor establishment. This model provides an insight on the direct effect of the combination therapy on the primary tumor; however, it does not address the effect on the metastatic capacity of the tumor and whether it is affected by inhibiting BCL6. Further investigation is required to fully understand the interplay between BCL6 expression, metastasis, and treatment resistance.

The effect of inhibiting BCL6 on paclitaxel induced apoptosis and cell cycle arrest was only investigated in the knockdown studies but not in the inhibitor experiment. Thus, it remains unclear if the observed effect of the inhibitor is due to the same mechanisms despite the upregulation of CDKN1A in both sets of experiment. Additionally, it is important to investigate the effect of the combination therapy on senescence, given the important role played by CDKN1A in regulating and mediating this process in TP53 dependent/independent manner (Aliouat-Denis et al. 2005; Moh et al. 2008).

Similarly, as we continue to investigate additional screen identified hits, we might be able to identify other resistant genes that when inhibited that provide a synergistic effect to paclitaxel treatment and thus make potential targets for drug development and combination therapy. Additional analyses such Analytic Technique for Assessment of RNAi by Similarity (ATARiS) (Shao et al. 2013) could be used to further analyze the results of the shRNA screen and prioritize other screen hits for further validation.

6.11 Chapter 4 Future Directions

The promising results we observed with the BCL6i-combination therapy, have encouraged us to design an *in vivo* experiment to determine the effect of combination therapy breast cancer PDXs. This will help further verify the potential use of the inhibitor in the context of paclitaxel treatment for breast cancer. This model can also serve as starting point for a double xenograft experiment to assess the effects of immune pressure on this phenomenon. Additionally, an *in vivo* experiment has been designed to investigate the potential effect of BCL6i-paclitaxel combination on lung metastasis using breast cancer cell lines MDA MB 231 The experiment will be carried out for longer duration to be able to detect metastasis in different treatment groups.

Having collected RNA samples and formalin preserved tissue from all treatment groups in the different *in vivo* experiments outlined in Chapter 4, we can investigate the expression of CDKN1A, as well as markers of senescence following combination and individual therapies. Additionally, we can investigate apoptosis and morphological changes using immunohistology techniques. Observations from these studies along, with

cell cycle and apoptosis analyses on different cell lines treated with the inhibitor will help build a complete picture of the role of inhibiting BCL6 in enhancing paclitaxel response.

Given the different fates a cancer cell could face following paclitaxel treatment, it would be interesting to observe the effects of our treatments directly on the single cell level. This could be achieved through the use of microscopy to observe and record cell division of cells treated with different single and combination therapy to investigate their effect of paclitaxel induced cell cycle arrest.

Finally, the screen-derived gene signature that is currently being developed will be tested in the different subtypes of breast cancer in multiple datasets to determine the individual accuracies and specificities in comparison to the general datasets. This will provide further insight about the validity of the gene signature as a predictive tool for treatment response and identify which group of patients will most benefit from having the analysis performed on their tumors prior to treatment.

6.12 ATRA and ATO Combination Therapy Induce Lasting Effect on APL Post Treatment Termination.

While the effect of ATRA and ATO individually and in combination on APL cell differentiation and cell death have been previously reported (Elbahesh, Patel, and Tabbara 2014; Nitto and Sawaki 2014; Nouzova et al. 2004), the lasting effects post treatment termination for the individual and combination therapies remained unclear. Thus, we were interested in exploring the apoptosis and differentiation cellular changes and

corresponding changes in gene expression and epigenetic changes in APL cells post treatment termination.

Using flow cytometry analysis, we assessed the level of dead and differentiated cells following treatment with ATRA (1 μM) and two different doses of ATO (0.5 μM and 1.5 μM) alone or in combination at three different time points. Unsurprisingly, cells treated with the combination therapy had the most increase in the level of dead/differentiated cells following 72 hours treatment with either dose of ATO (Figs 5.1C and 5.2C). Similarly, combination treatment for 168 hours was the most effective in reducing the level undifferentiated viable APL cells. In contrast, individual treatment had a reduced effect, with ATRA treatment alone inducing cell differentiation and high dose ATO inducing cell death in APL cells (Figs 5.1D and 5.2D). Interestingly, when we investigated the effect of each treatment at 96 hours post 72 hours treatment termination, only cells treated with combination therapies had an increase in the level of dead/differentiated cells. In contrast, cells treated with individual therapies reverted back to the undifferentiated viable APL populations (Figs 5.1E and 5.2E). Together, these findings highlighted the long-lasting effectiveness of combination therapy without having to prolong treatment or increase the dose of ATO; thus, limiting the undesired toxicity associated with ATO.

The successful use of ATRA in APL treatment could be hampered by the increase in ATRA resistance APL cases. Therefore, it was important to our compare our findings in the NB4-MR2 ATRA-resistant APL cell line. Combination therapies with ATRA and either dose of ATO for 72 was still effective in reducing the level undifferentiated viable APL cells but to a lesser extent than in the ATRA sensitive NB4 cells (Fig 5.3B). In

contrast, the effect was mostly lost in all treatment conditions except ATRA and high dose ATO when the cell populations were investigated at 96 hours post treatment termination (Fig 5.3D). This further validates the importance of ATRA combination therapy in treating APL cells and overcoming ATRA resistance in ATO dose dependent manner.

6.13 ATRA and ATO Combination Therapy Restores the Expression of Several Key Genes Silenced in APL Through Induction of Transcription Permissive Epigenetic Changes

Given the important role of RARα in transcription regulation of hundreds of genes in the retinoic acid signalling pathways (Ablain and De Thé 2014), it was important to determine the effect of the combination therapy on the expression of previously identified genes that are silenced due to the PML-RARα fusion. While the individual and combination treatments have been previously shown to restore the expression of some of these genes to restore differentiation of APL cells (Ghavamzadeh et al. 2018; Nitto and Sawaki 2014), the lasting effects of these treatments on gene expression was largely unknown post treatment termination. Thus, we hypothesized that combination therapy can induce longer lasting effects post treatment termination, while these effects will be lost in cells treated with individual treatments which could explain the effects seen in on cell differentiation and death.

Interestingly, the expression of many RARα target genes was restored with both ATRA and the combination therapy after 72 hours of treatment with no significant

difference between the groups. In contrast, at 96 hours post treatment termination the expression of many of the genes induced by ATRA started decreasing, which can explain the loss of differentiation we observed in those groups. In contrast, the combination therapy successfully maintained the upregulation of these genes and in turn maintained the effectiveness of the therapy. Similar effects were observed in the resistant NB4-MR2 cell line following the 72 hours treatment; however, unlike with the NB4 cells, combination therapy effects on the expression of these genes were less evident and less significant.

Among the top changes in gene expression following treatment with ATRA and combination therapy was expression of TGM2 and RARβ; potential targets to further investigate the underlying mechanism by which combination therapy induced the last effects. Given the several reports on ATO ability to demethylate target genes in APL, we were intrigued to determine if this was the same mechanism utilized by the combination therapy to induce its effect. Interestingly, previous reports have highlighted that TGM2 and RARβ are hypermethylated in APL; however, ATRA treatment was unable to decrease their methylation status (Cheung and So 2011; Martens et al. 2010).

Pyrosequencing analysis for the promoter region of both genes in NB4 cells revealed a significant decrease in the level of total C-methylation and individual CpG site methylation both after treatment and at 96 hours post treatment termination (Figs. 5.7, 5.8,5.9 and 5.10). Similarly, combination therapy was able to decrease the methylation of the promoter regions of TGM2 and RARβ in NB4-MR2 following treatment (Figs 5.11A and 5.12A). However, these changes were lost in all treatment group at 96 hours post treatment termination (Figs. 5.11B and 5.12B). Together, these findings highlight the

important role of combination therapy in inducing long lasting effects on APL cell differentiation through the induction of epigenetic changes. Importantly, these changes were associated with a decreased in the methylation status of key regulators of promyelocytic cell differentiation that can largely be attributed to ATO demethylating effects (Peng et al. 2010).

Finally, we were interested in detecting changes in global methylation of these cells following different treatments. Interestingly, none of the treatment conditions outlined in Chapter 5 affected the global methylation status in either cell lines suggesting that effect seen in the TGM2 and RARβ are gene specific and possibly only applies to genes silenced in APL to inhibit normal differentiation of promyelocytic cells.

6.14 Chapter 5 Limitations

This study identified the underlying mechanism by which the combination therapy induces the long-lasting effect on gene expression changes and differentiation of APL cells in the NB4 cells and their ATRA-resistant derivative NB4-MR2. While the NB4 cells provide a good model to study APL and provide insight into treatment response, it lacks the heterogeneity present in patient samples. Similar to solid tumors, blood malignancies demonstrate a high degree of heterogeneity between the tumor cells which can associate with differential response to treatment. Additionally, the experiment performed on NB4 cells grown in culture do not account for environment mediated resistance to treatment. Previous studied have demonstrated increased resistance to ATO when NB4 cells were co-cultured with bone marrow cells (Ganesan et al. 2016). Thus, it

is important to determine the impact of the tumor environment on the effect observed with the combination therapy and its ability to upregulate gene express through targeting gene methylation.

Moreover, the genes interrogated following treatment were based on previous reports (Lee et al. 2002; Zheng et al. 2005) and, thus it could be an incomplete analysis and missing information on important mediators of treatment response. Therefore, it would be beneficial to further investigate the gene expression and epigenetic changes following treatment in both treatment sensitive and treatment resistant cell lines to identify genes that are differentially expressed depending upon treatment response.

6.15 Chapter 5 Future Directions

The findings from this study highlight the need to preform genome-wide studies on identify gene expression and epigenetic changes associated with different treatment conditions. Previously published studies have focused on the changes associated with either individual treatment but not the combination therapy. Thus, a combined study of genome wide RNA-seq and methylation microarray on samples treated with different treatment conditions will provide novel insights into APL treatment response and identify novel genes associated with the process.

The experience we gained preforming genome-wide RNAi studies in breast cancer can be applied to identify genes important for combination therapy response in APL. This will enable us to investigate potential mechanisms for overcoming resistance to enhance patient survival. Additionally, given the upregulation in BCL6 in different ATO resistant

sample, we could investigate the effect of combining BCL6i with ATO alone and ATO-ATRA combination therapy on the level of dead and differentiated NB4 cells. This can provide insights on the potential use of BCL6i treat resistant APL patients to enhance treatment outcomes.

To address the limitation associated with the lack of heterogeneity and environmental influence, the experiments outlined in Chapter 5 should be repeated using naïve and treated patient samples. Additionally, the use of transgenic APL mouse models (Pollock et al. 2001) could provide an additional platform to further validate out findings and determine the validity of our findings and their impact of treatment outcomes. This could be specifically applied to investigate the effect of combining demethylating agents such as decitabine in combination of ATO-ATRA therapy to further enhance the demethylating effects and promote differentiation.

6.16 Epilogue

My PhD journey unexpectedly led me to work on three distinctive projects with different hypotheses, techniques, and areas of focus. While initially I thought of these projects as separate entities, I realize now as they are coming to an end and I am writing my thesis that common mechanisms were present among the different chapters. This could be observed in our finding that highlighted the role DNA methylation in gene silencing of TAP1 in Aldefluor+ breast CSCs and TGM2 and RARβ in APL. Similarly, techniques I learned and employed in one study, were also employed in other chapters.

Such techniques include flow cytometry sorting, gene expression and methylation analysis, and patient dataset analyses.

Investigating resistance mechanisms associated with treatment failure can open new possibilities to enhance patient survival. Similarly, treatment strategies employed in one type of cancer can provide an exciting potential in targeting another type. The overarching theme of this work was understanding the role of intra- and intertumoral heterogeneity in the context of treatment response to identify resistance mechanisms that can be targeted through precision medicine.

The interplay between tumor cells and tumor microenvironment shape the progression of a tumor as well as its response to treatment. This was evident in the differential expression of immune related genes in the ALDE+ and CD44+/CD24- breast CSCs. While CD44+/CD24- cells altered the expression of genes associated with innate immunity, Aldefluor+ cells epigenetically silenced the expression of several key antigen presentation genes to evade immune destruction.

Heterogeneity among the patient population is a major contributor to treatment resistance that can be targeted through precision medicine. In this body of work, we have identified novel mediators of paclitaxel response in breast cancer that will be used to develop a gene signature to predict patient treatment outcomes. Moreover, we highlighted the impact of BCL6 expression on patient survival in different types of cancer and the potential of targeting it to enhance treatment response.

In this body of work, the evident benefits of combination therapy were most clearly observed in the ability of ATRA and ATO treatment to achieve a long-lasting effect on APL cells death and differentiation. While individual treatments were successful

in achieving the desired effect following treatment, these effects were mostly lost post treatment termination indicating a high risk of relapse associated with these treatments. In contrast, combination therapy effects remain prominent following treatment termination through induction of expression of genes that are epigenetically silenced in APL.

The different findings in these chapters provide encouraging grounds to further investigate the use of demethylating agents and BCL6i in combination therapy to enhance treatment efficacy in breast cancer and APL.

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APPENDIX 1: ABBREVIATIONS FOR IMMUNE GENES DIFFERENTIALLY EXPRESSED IN BREAST CANCER

ABCB1	ATP binding cassette subfamily B member 1
APP	amyloid beta precursor protein
ARG2	arginase 2
BCL10	B-cell CLL/lymphoma 10
BLNK	B-cell linker
BTK	Bruton tyrosine kinase
BTLA	B and T lymphocyte associated
C1R	complement C1r
C2	complement C2
CARD11	caspase recruitment domain family member 11
CASP1	caspase 1
CASP8	caspase 8
CCL4	C-C motif chemokine ligand 4
CCL5	C-C motif chemokine ligand 5
CCR5	C-C motif chemokine receptor 5 (gene/pseudogene)
CCR6	C-C motif chemokine receptor 6
CCR6	C-C motif chemokine receptor 6
CCR7	C-C motif chemokine receptor 7
CD1C	CD1c molecule
CD1D	CD1d molecule
CD37	CD37 molecule
CD3D	CD3d molecule
CD4	CD4 molecule
CD74	CD74 molecule
CD79B	CD79b molecule
CDH1	cadherin 1
CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1
CEACAM6	carcinoembryonic antigen related cell adhesion molecule 6

CFB	complement factor B
CFD	complement factor D
CLU	clusterin
COL3A1	collagen type III alpha 1 chain
COLEC12	collectin subfamily member 12
CSF1	colony stimulating factor 1
CSF2RB	colony stimulating factor 2 receptor beta common subunit
CTSS	cathepsin S
CTSW	cathepsin W
CX3CL1	C-X3-C motif chemokine ligand 1
CXCL12	C-X-C motif chemokine ligand 12
CXCR3	C-X-C motif chemokine receptor 3
CXCR5	C-X-C motif chemokine receptor 5
CYFIP2	cytoplasmic FMR1 interacting protein 2
CYLD	CYLD lysine 63 deubiquitinase
EGR1	early growth response 1
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1
EOMES	eomesodermin
EPCAM	epithelial cell adhesion molecule
ETS1	ETS proto-oncogene 1, transcription factor
F12	coagulation factor XII
F2RL1	F2R like trypsin receptor 1
FCER1A	Fc fragment of IgE receptor Ia
FCGR2B	Fc fragment of IgG receptor IIb
FN1	fibronectin 1
FYN	FYN proto-oncogene, Src family tyrosine kinase
GPI	glucose-6-phosphate isomerase
GZMK	granzyme K
HAVCR2	hepatitis A virus cellular receptor 2
HLA-B	major histocompatibility complex, class I, B
HLA-DMA	major histocompatibility complex, class II, DM alpha

HLA-DMB	major histocompatibility complex, class II, DM beta
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1
HLA-DPB1	major histocompatibility complex, class II, DP beta 1
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1
HLADQB1	major histocompatibility complex, class II, DQ beta 1
HLA-DRA	major histocompatibility complex, class II, DR alpha
HLA-DRB4	major histocompatibility complex, class II, DR beta 4
HLA-E	major histocompatibility complex, class I, E
HRAS	HRas proto-oncogene, GTPase
ICAM2	intercellular adhesion molecule 2
ICAM3	intercellular adhesion molecule 3
IFI16	interferon gamma inducible protein 16
IFI27	interferon alpha inducible protein 27
IL10RA	interleukin 10 receptor subunit alpha
IL12RB1	interleukin 12 receptor subunit beta 1
IL15	interleukin 15
IL16	interleukin 16
IL17RB	interleukin 17 receptor B
IL18R1	interleukin 18 receptor 1
IL18RAP	interleukin 18 receptor accessory protein
IL1A	interleukin 1 alpha
IL1R1	interleukin 1 receptor type 1
IL1R2	interleukin 1 receptor type 2
IL1RL1	interleukin 1 receptor like 1
IL2RA	interleukin 2 receptor subunit alpha
IL2RB	interleukin 2 receptor subunit beta
IL2RG	interleukin 2 receptor subunit gamma
IL32	interleukin 32
IL4R	interleukin 4 receptor
IL7R	interleukin 7 receptor
INPP5D	inositol polyphosphate-5-phosphatase D

IRF1	interferon regulatory factor 1
IRF2	interferon regulatory factor 2
IRF4	interferon regulatory factor 4
IRF8	interferon regulatory factor 8
ISG20	interferon stimulated exonuclease gene 20
ITGA2	integrin subunit alpha 2
ITGA4	integrin subunit alpha 4
ITGAE	integrin subunit alpha E
ITGAL	integrin subunit alpha L
ITGAX	integrin subunit alpha X
ITGB2	integrin subunit beta 2
ITK	IL2 inducible T-cell kinase
JAK1	Janus kinase 1
JAK3	Janus kinase 3
KIR3DL3	killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 3
KLRB1	killer cell lectin like receptor B1
KLRB1	killer cell lectin like receptor B1
LAG3	lymphocyte activating 3
LAMP1	lysosomal associated membrane protein 1
LAMP2	lysosomal associated membrane protein 2
LCK	LCK proto-oncogene, Src family tyrosine kinase
LCN2	lipocalin 2
LCP1	lymphocyte cytosolic protein 1
LIF	leukemia inhibitory factor
LILRB1	leukocyte immunoglobulin like receptor B1
LTB	lymphotoxin beta
LTBR	lymphotoxin beta receptor
LTF	lactotransferrin
LY9	lymphocyte antigen 9
LY96	lymphocyte antigen 96

LYN	LYN proto-oncogene, Src family tyrosine kinase
MAP3K1	mitogen-activated protein kinase kinase kinase 1
MAPK1	mitogen-activated protein kinase 1
MASP1	mannan binding lectin serine peptidase 1
MEF2C	myocyte enhancer factor 2C
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
MR1	major histocompatibility complex, class I-related
MUC1	mucin 1, cell surface associated
NCF4	neutrophil cytosolic factor 4
NFATC3	nuclear factor of activated T-cells 3
NFKB2	nuclear factor kappa B subunit 2
NLRC5	NLR family CARD domain containing 5
NLRP3	NLR family pyrin domain containing 3
NOD2	nucleotide binding oligomerization domain containing 2
NOTCH1	notch 1
NRP1	neuropilin 1
NT5E	5'-nucleotidase ecto
PAX5	paired box 5
PDGFC	platelet derived growth factor C
PECAM1	platelet and endothelial cell adhesion molecule 1
PIK3CD	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma
PLAU	plasminogen activator, urokinase
POU2AF1	POU class 2 associating factor 1
POU2F2	POU class 2 homeobox 2
PSMB9	proteasome subunit beta 9
PTGS2	prostaglandin-endoperoxide synthase 2
PTPRC	protein tyrosine phosphatase, receptor type C
REL	REL proto-oncogene, NF-kB subunit

RIPK2	receptor interacting serine/threonine kinase
RUNX3	runt related transcription factor 3
S100A8	S100 calcium binding protein A8
SELL	selectin L
SELPLG	selectin P ligand
SERPINB2	serpin family B member 2
SERPING1	serpin family G member 1
SH2D1A	SH2 domain containing 1A
SH2D1B	SH2 domain containing 1B
SLAMF1	signaling lymphocytic activation molecule family member 1
SLAMF6	SLAM family member 6
SLAMF7	SLAM family member 7
SPA17	sperm autoantigenic protein 17
ST6GAL1	ST6 beta-galactoside alpha-2,6-sialyltransferase 1
STAT2	signal transducer and activator of transcription 2
STAT3	signal transducer and activator of transcription 3
TANK	TRAF family member associated NFKB activator
TCF7	transcription factor 7 (T-cell specific, HMG-box)
TFEB	transcription factor EB
TGFB2	transforming growth factor beta 2
THY1	Thy-1 cell surface antigen
TIGIT	T-cell immunoreceptor with Ig and ITIM domains(TIGIT)
TNF	tumor necrosis factor
TNFAIP3	TNF alpha induced protein 3
TNFRSF12A	TNF receptor superfamily member 12A
TNFRSF13B	TNF receptor superfamily member 13B
TNFRSF13C	TNF receptor superfamily member 13C
TNFRSF14	TNF receptor superfamily member 14
TNFRSF1A	TNF receptor superfamily member 1A
TNFRSF1B	TNF receptor superfamily member 1B
TNFRSF4	TNF receptor superfamily member 4

TNFRSF9	TNF receptor superfamily member 9
TNFSF15	tumor necrosis factor superfamily member 15
TNFSF8	tumor necrosis factor superfamily member 8
TRAF3	TNF receptor associated factor 3
TRAF3	TNF receptor associated factor 3
TTK	TTK protein kinase
TXNIP	thioredoxin interacting protein
TYK2	tyrosine kinase 2
ULBP2	UL16 binding protein 2
VEGFC	vascular endothelial growth factor C
ZAP70	zeta chain of T cell receptor associated protein kinase 70

APPENDIX 2: PACLITAXEL GENOME-WIDE shRNA SCREEN FOLD CHANGES AND P-VALUES

This appendix will be uploaded as an online supplemental file as per Dalhousie Faculty of Graduate Studies guidelines. The file will include detailed fold changes and p values for all probes targeting functional genes from the *in vivo* paclitaxel genome-wide shRNA screen outlined in chapters 2 and 4.

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Mohammad Sultan, Krysta Mila Coyle, Dejan Vidovic, Margaret Lois Thomas, Shashi Gujar, Paola Marcato, Hide-and-seek: the interplay between cancer stem cells and the immune system, Carcinogenesis, Volume 38, Issue 2, 1 February 2017, Pages 107–118.

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