Surviving Stress with the Tumour
Microenvironment: Understanding Complex
Cell - Cell Interactions

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Abstract

Each year more than 18 million people are diagnosed with cancer. Stress survival plays a key role in tumour resistance which is a leading cause of cancer deaths. Understanding how cells respond to stress is key to understanding and treating cancer. Within a tumour, cells interact with many other cell types in the surrounding tumour microenvironment. The tumour microenvironment acts to both assist and resist tumorigenesis and metastasis depending on these cellular interactions.

Rho Zero cells are cells that contain no mitochondrial DNA and therefore lack a functional electron transport chain and rely on glycolysis for energy metabolism, these cells require supplementation to survive in cell culture. When deprived of essential supplementation rho zero cells exhibit severe metabolic stress. Yet when implanted into mice they form tumours after a 2-3 week delay. After this they contain new mitochondrial DNA acquired from the tumour microenvironment.

This thesis explored how cells under stress interact with a model tumour microenvironment and exploit normal cellular processes for continued survival. Using confocal microscopy, IncuCyte microscopic videography, and MinION sequencing techniques we have established that rho zero cells under metabolic stress in a co-culture environment uptake mitochondrial DNA from the surrounding microenvironment and that this cell survival is contact associated. Nars1 was identified as a potential signal from the rho zero cells to the tumour microenvironment when placed under metabolic stress. This identified Nars1 as potential part of a novel stress response mechanism for severe metabolic stress. I then investigated if these findings could be replicated in cisplatin and doxorubicin treated wild type cells. While contact dependant survival was implied in the doxorubicin treated cells no Nars1 signal was found. However, an upregulation of Cxcl5 and Cxcl1 was identified as a commonly upregulated transcript between the rho zero and both chemotherapy treatments in 4T1 breast cancer implicating a conserved chemokine signalling response to stress. Together these results illustrate the importance of chemokine signalling in complex cellular interactions as part of a cellular survival stress response.

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Contents

Abstract	2
Acknowledgements.....	3
List of Tables:	10
List of Figures:	12
1 Introduction.....	14
1.1 <i>Cancer</i>	14
1.1.1 The Hallmarks of Cancer	16
1.2 <i>Cancer Cell Types Used in this Thesis</i>	26
1.2.1 Breast Cancer	26
1.2.2 Melanoma	27
1.2.3 Glioblastoma	28
1.3 <i>Tumour Microenvironment</i>	29
1.3.1 Cancer associated Fibroblasts (CAFs).....	29
1.3.2 Mesenchymal Stromal Cells (MSC)	30
1.3.3 Mononuclear cells.....	32
1.3.4 Pericytes.....	34
1.3.5 Endothelial Cells.....	35
1.4 <i>Mitochondria</i>	35
1.4.1 Mitochondrial Matrix: mtDNA	35
1.4.2 Mitochondrial Matrix: TCA cycle	36
1.4.3 Mitochondrial Cristae: Cellular Respiration	37
1.4.4 Mitochondrial Matrix and Cristae: ROS signalling.....	37
1.4.5 Mitochondrial Matrix: Biosynthesis pathways.....	38
1.4.6 Mitochondria and cancer	38
1.4.7 Mitochondrial Networks	39
1.5 <i>Cellular stressors</i>	39
1.5.1 Rho Zero (ρ^0) cells.....	39
1.5.2 Strengths and weaknesses of ρ^0 lines.....	43
1.6 <i>Chemotherapy as Cellular Stress</i>	44
1.6.1 Doxorubicin	44
1.6.2 Cisplatin.....	45

1.6.3	Chemotherapy resistance	46
1.7	<i>Cellular stress responses and survival</i>	47
1.7.1	Endoplasmic Reticulum (ER) stress response.....	47
1.7.2	Inflammatory Stress Response.....	48
1.7.3	Mitochondrial Transfer	48
1.8	<i>MinION cDNA sequencing for Differential expression analysis:</i>	54
1.8.1	MinION long read vs short read transcriptomics	55
1.9	<i>Aims:</i>	56
2	Materials and Methods:	57
2.1	<i>Materials:</i>	57
2.1.1	Reagents:	58
2.1.2	Kits	59
2.1.3	Chemicals	59
2.1.4	Equipment.....	60
2.2	<i>Cell culture:</i>	61
2.2.1	Cell Lines:	61
2.2.2	Cell culture Media:	61
2.2.3	Culture method:.....	62
2.2.4	Mycoplasma Testing:	63
2.2.5	Cryopreservation:	63
2.2.6	Transfection of cell lines:	63
	<i>Plasmid generation</i>	63
2.2.7	Deprivation of ρ° s:	65
2.2.8	Chemotherapeutic Treatment:	65
2.2.9	Ex-vivo/primary culture and generation of Compact Bone Derived Cells:.....	65
2.3	<i>Microscopy:</i>	67
2.3.1	Staining:	67
2.3.2	Image Generation:	68
2.3.3	Co-Culture experiments with CBDC:	69
2.3.4	Image Analysis:.....	69
2.4	<i>Flow Cytometry:</i>	70
2.4.1	Preparing and staining cells:	70
2.4.2	Flow Cytometry on LSR and Fortessa:.....	71
2.4.3	Fluorescence Activated Cell Sorting (FACS):	71
2.5	<i>Molecular biology:</i>	71

2.5.1	DNA extraction.....	71
2.5.2	RNA extraction.....	72
2.5.3	Endpoint PCR.....	72
2.5.4	Quantitative Real Time PCR	73
2.6	<i>Sequencing</i>	74
2.6.1	MinION:.....	74
2.6.2	Sanger:	76
2.7	<i>Analysis:</i>	76
2.7.1	Bioinformatics:.....	76
3	Proof of Concept: Stress-Induced Transfer of Mitochondria to ρ° Cells.....	78
3.1	<i>Mitochondrial recipients</i>	78
3.1.1	Metabolic Stress in Recipient cells.....	78
3.1.2	Cell lines used.....	79
3.2	<i>Mitochondrial Donors</i>	79
3.3	<i>Mitochondrial Crosstalk</i>	80
3.4	<i>Results</i>	81
3.4.1	Characterising of Mitochondrial Recipients.....	81
3.4.2	Characterisation of the Cellular Aspect of the Supporting Microenvironment	86
3.4.3	Assessment of Mitochondrial Transfer Between Donor/Recipient Populations	93
3.5	<i>Discussion</i>	103
3.5.1	Co-Cultures.....	103
3.5.2	<i>In vitro</i> vs <i>ex vitro</i> / <i>in vivo</i> work:	106
4	Contact Dependent Survival Advantage of ρ° Cells in Co-culture with CBDC.....	108
4.1	<i>Contact Dependant Survival of ρ° Cell Lines in Co-Culture with CBDC</i>	108
4.1.1	Proliferation of ρ° Without Pyruvate and Uridine in Co-Culture with CBDC	110
4.1.2	Survival of ρ° with and without Co-Culture and Contact with CBDC	112
4.1.3	Percent Contact of ρ° with CBDC.....	115
4.2	<i>Differential Transcriptomics of ρ° Cell Lines under Metabolic Stress</i>	117
4.2.1	DESeq2 of Astrocyte ρ°	119
4.2.2	DESeq2 of 4T1 ρ°	122
4.2.3	DESeq2 of B16 ρ°	124
4.2.4	Comparison of differential transcripts for all 3 ρ° lines	126
4.2.5	Comparison of differential transcripts for 4T1 ρ° and Astrocyte ρ° cell lines.....	129

4.2.6	Comparison of Differential Transcripts for Cancer ρ° Lines.....	132
4.3	<i>Gene Set Enrichment Analysis of ρ° Cell Lines in Metabolic Stress.</i>	136
4.3.1	GSEA of Astrocyte ρ°	137
4.3.2	GSEA of 4T1 ρ°	138
4.3.3	GSEA of B16 ρ°	139
4.3.4	Comparison of GSEA results.....	140
4.4	<i>Discussion</i>	146
4.4.1	Contact Associated ρ° Survival of Metabolic Stress.....	146
4.4.2	Contact Associated Survival and Nars1 Mediated Signalling.	147
5	Survival of Chemotherapy Treated Cells in Co-Culture with Compact Bone Derived Cells.	151
5.1	<i>Chemotherapy treatment</i>	151
5.1.1	Doxorubicin.....	151
5.1.2	Cisplatin.....	152
5.2	<i>Contact Dependent Survival of Doxorubicin Treated Cancer and Untreated Cell Lines in Co-Culture with CBDC.</i>	153
5.2.1	Proliferation of Cancer cell lines under Doxorubicin Treatment and Co-culture with CBDC.	155
5.2.2	Contact Dependant Survival of Cancer Cells under Doxorubicin Treatment in Co-culture with CBDC.	156
5.2.3	Percent Contact of Cancer Cells under Doxorubicin Treatment in Co-Culture with CBDC.	158
5.3	<i>Contact Dependent Survival of Doxorubicin Treated Cancer and Untreated Cell Lines in Co-Culture with CBDC.</i>	160
5.3.1	Proliferation of Cancer Cell Lines under Cisplatin Treatment and Co-Culture with CBDC.	161
5.3.2	Contact Dependant Survival of Cancer Cells under Cisplatin Treatment in Co-culture with CBDC.	163
5.3.3	Percent Contact of Cancer Cells Under Cisplatin Treatment in Co-Culture with CBDC.....	164
5.4	<i>Discussion</i>	166
5.4.1	CBDC and Treated Cell Lines	167
6	Similarities and Differences Between Stressed Cancer Cell Lines.	169
6.1	<i>Transcriptomic Analysis of Chemotherapy treated Cancer Cell Lines</i>	170
6.2	<i>Results</i>	171
6.2.1	Differential Gene Expression in 4T1 Treated with Doxorubicin.	172
6.2.2	4T1 Treated with Cisplatin Differential Expression.	175
6.2.3	B16 Treated with Doxorubicin Differential Expression.	177
6.2.4	B16 Treated with Cisplatin Differential Expression.....	179

6.2.5	Comparisons	181
6.2.6	Chemotherapy Specific Response in Different Cell Lines.....	182
6.2.7	Cell Line Specific Response to Different Chemotherapies.	184
6.2.8	Cell Line Specific Response to Different Stressors.	186
6.3	GSEA.....	191
6.3.1	GSEA of 4T1 Treated with Doxorubicin and Cisplatin.	192
6.3.2	GSEA of B16 Treated with Doxorubicin and Cisplatin.	195
6.3.3	Chemotherapy Specific Response in Different Cell Lines.....	198
6.3.4	Cell Line Specific Response to Different Chemotherapies.	202
6.3.5	Cell Line Specific Response to Different Stressors.	207
6.4	Discussion	212
6.4.1	Differential Sequencing and GSEA	213
6.4.2	Differential sequencing and GSEA.....	214
7	Discussion:	216
7.1	Summary.....	216
7.1.1	Proof of Concept: Stress-Induced Transfer of Mitochondria to ρ° Cells.....	217
7.1.2	Contact Dependant Survival Advantage of ρ° Cells in Co-culture with CBDC.....	218
7.1.3	Survival of Chemotherapy Treated Cells in Co-Culture with CBDC.	221
7.1.4	Similarities and Differences Between Stressed Cancer Lines.....	222
7.2	Moving Forward:	224
7.2.1	Chemokine Signalling	225
7.2.2	Single Cell Transcriptomics	226
7.2.3	CBDC and ρ° Co-culture Experiments	226
7.2.4	Treated cell lines and CBDC	228
8	Final Conclusions:.....	229
	References	230
	Appendix 1: Attempted mUNG creation of ρ°	261
	Appendix 2: DESeq2 results.....	269

List of Tables:

Table 2-1: Cell Lines Used in this Research.....	61
Table 2-2: Plasmids used in this Thesis.....	63
Table 2-3: Conjugated Antibodies Used in this Project	70
Table 4-1: Total mapped reads for barcodes on each flow cell.....	118
Table 4-2: Upregulated genes with significant p-values common among all 3 ρ° cell lines.	128
Table 4-3: Upregulated genes with significant p-values common between 4T1 ρ° and Astrocyte ρ° cell lines.	130
Table 4-4: Down regulated genes with significant p-values common between 4T1 ρ° and Astrocyte ρ° cell lines.	131
Table 4-5: Upregulated genes with significant p-values common among cancer ρ° cell lines.	133
Table 4-6: Downregulated genes with significant p-values common among cancer ρ° cell lines. significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.	133
Table 4-7: qPCR results for sequencing confirmation.	134
Table 4-8: Positively enriched gene sets with significant p-values common among ρ° cell lines.	144
Table 4-9: Negatively enriched gene sets with significant p-values common among ρ° cell lines.	144
Table 4-10:Positively enriched gene sets with significant p-values common among 4T1 ρ° and Astrocyte ρ° cell lines.	144
Table 4-11: Negatively enriched gene sets with significant p-values common among 4T1 ρ° and Astrocyte ρ° cell lines.....	144
Table 4-12: Positively enriched gene sets with significant p-values common among cancer ρ° cell lines.....	145
Table 4-13: Negatively enriched gene sets with significant p-values common among cancer ρ° cell lines.	145

Table 6-1: Table of total mapped reads for treated sequencing	171
Table 6-2: Up regulated genes common among 4T1 treated with doxorubicin and cisplatin and ρ° with p-values less than 0.05.	189
Table 6-3: Down regulated genes common among 4T1 treated with doxorubicin and cisplatin and ρ° with p-values less than 0.05.	189
Table 6-4: Down regulated genes common among B16 treated with doxorubicin and cisplatin and ρ° with p-values less than 0.05.	190
Table 6-5: Positively enriched gene sets with significant p-values common among doxorubicin treated cell lines.	200
Table 6-6: Negatively enriched gene sets with significant p-values common among doxorubicin treated cell lines.	200
Table 6-7: Positively enriched gene sets with significant p-values common among cisplatin treated cell lines.	201
Table 6-8: Negatively enriched gene sets with significant p-values common among cisplatin treated cell lines.	201
Table 6-9: Positively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line.....	204
Table 6-10: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line.....	205
Table 6-11: Positively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated B16 cell line.	206
Table 6-12: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated B16 cell line.	206
Table 6-13: Positively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line and ρ° deprived 4T1.	210
Table 6-14: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line and ρ° deprived 4T1.	210
Table 6-15: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated B16 cell line and ρ° deprived B16.....	211

List of Figures:

Figure 1-1: Mitochondrial metabolism and glycolysis.	42
Figure 3-1: Astrocyte ρ° response to pyruvate and uridine deprivation:.....	82
Figure 3-2: 4T1 ρ° response to pyruvate and uridine deprivation.	84
Figure 3-3: B16 ρ° response to pyruvate and uridine deprivation.	85
Figure 3-4: Phenotyping and classification of Compact Bone Derived Cells:	89
Figure 3-5: Effect of pyruvate and uridine deprivation on CBDC.	91
Figure 3-6: Astrocyte ρ° + CBDC:.....	97
Figure 3-7: 4T1 ρ° + CBDC:	101
Figure 4-1: Proliferation of ρ° without pyruvate and uridine in co-culture with CBDC.	110
Figure 4-2: Contact dependant survival of ρ° cell lines in co-culture with CBDC.	112
Figure 4-3: Percent contact between CBDC and ρ° cells.	115
Figure 4-4: DESeq2 analysis of Astrocyte ρ° cell line treated deprived of pyruvate and uridine for 24 hours.	119
Figure 4-5: DESeq2 analysis of 4T1 ρ° cell line treated deprived of pyruvate and uridine for 24 hours.....	122
Figure 4-6: DESeq2 analysis of B16 ρ° cell line treated deprived of pyruvate and uridine for 24 hours.....	124
Figure 4-7: Comparison between ρ° lines and their differentially expressed gene lists.	127
Figure 4-8 Comparison between 4T1 ρ° and Astrocyte ρ° lines and their differentially expressed gene lists.	129
Figure 4-9: Comparison between cancer derived ρ° lines.	132
Figure 4-10: Gene Set Enrichment Analysis of Astrocyte ρ°	137
Figure 4-11: Gene Set Enrichment Analysis of 4T1 ρ°	138
Figure 4-12: Gene Set Enrichment Analysis of B16 ρ°	139
Figure 4-13: Comparison between ρ° lines and their up and down enrichment lists.	141
Figure 4-14: Comparison between 4T1 ρ° and Astrocyte ρ° lines and their up and down enrichment lists..	143

Figure 5-1: IncuCyte analysis of cell lines treated with doxorubicin in co-culture with CBDC.	155
Figure 5-2: Survival of cell lines after treatment with doxorubicin in co-culture with CBDC.	157
Figure 5-3: Percent contact of doxorubicin treated cells with CBDC while in co-culture over 5 days.	158
Figure 5-4: IncuCyte proliferation analysis of cell lines treated with cisplatin in co-culture with CBDC.	161
Figure 5-5: Survival of cell lines after treatment with cisplatin in co-culture with CBDC.	163
Figure 5-6: Percent contact of cisplatin treated cells with CBDC while in co-culture over 5 days.	165
Figure 6-1: Differential gene expression induced by doxorubicin in 4T1 cells.	173
Figure 6-2: Differential gene expression induced by cisplatin in 4T1 cells.	175
Figure 6-3: Differential gene expression induced by doxorubicin in B16 cells.	177
Figure 6-4: Differential gene expression induced by cisplatin in B16 cells.	179
Figure 6-5: Comparison between individual chemotherapies and their differentially expressed gene lists of 4T1 and B16 cell lines.	182
Figure 6-6: Comparison of gene expression changes under chemotherapy treatment within a cell line.	184
Figure 6-7: Comparison of gene expression changes under ρ^0 zero deprivation and chemotherapy treatment within the 4T1 cell line.	186
Figure 6-8: Gene Set Enrichment Analysis of 4T1 cells treated with 500ng/ml doxorubicin for 24 hours.	192
Figure 6-9: Gene Set Enrichment Analysis of 4T1 cells treated with 500ng/ml cisplatin for 24 hours	193
Figure 6-10: Gene Set Enrichment Analysis of B16 cells treated with 2000ng/ml doxorubicin for 24 hours.	195
Figure 6-11: Gene Set Enrichment Analysis of B16 cells treated with 2000ng/ml cisplatin for 24 hours.	196
Figure 6-12: Chemotherapy specific comparison between 4T1 and B16 cell lines GSEA.	198
Figure 6-13: Cell line response comparison within 4T1 and B16 cell lines GSEA.	202
Figure 6-14: Comparison within 4T1 and B16 cell lines GSEA.	207

1 Introduction

This thesis explores the interactions between cancer cells under stress and cells present in the tumour microenvironment. Cell models from different tissues were used to explore these interactions *in vitro* and extract potential similarities in cell responses. Co-cultures, proliferation and survival analysis, cell to cell contact, cell fate analysis and differential transcriptomics were all used to examine the role different stressors play in the interactions cells have with their surrounding microenvironment.

1.1 Cancer

Cancer is a global health issue with 18 million cases of cancer in 2018, 9.5 million men and 8.5 women were diagnosed ^{1,2}. The cancers with the highest incidence are lung, breast and colorectal cancer cumulatively accounting for 35.2% of all cancers worldwide ^{1,2}. In New Zealand 24,453 people were diagnosed with cancer in the most recent year available 2017 ³. The overall rate of cancer diagnosis was higher in Māori than in non-Māori, with an average incidence rate between 2008 and 2017 per 100,000 of 430.3 to 320.8 respectively ³. Between 2008 and 2017 more non-Māori men were diagnosed with cancer than non-Māori women (an average incidence of 354.9 and 291.0 per 100,000 respectively) whereas the incidence in Māori men was lower than Māori women (average of 422.9 and 437.9 per 100,000 respectively) ³.

The different rates of diagnosis and different treatment outcomes for different ethnic and gender groups reflect the socioeconomic stratification of the New Zealand society ⁴. These disparities underscore the importance of repurposing known low-cost treatments to address inequalities in patient outcomes. The development of cancer cell treatment resistance is a key limiter to these 'old-fashioned' treatments effectiveness. Understanding how cancer cells interact differently with their microenvironment when they are exposed to these treatments may allow us to find ways to overcome drug resistance and thus improve

patient outcomes. Cancer mortality rates have dropped significantly in the last hundred years, particularly for the treatment of primary cancers which have not spread beyond their original site. However, we have been less successful in treating secondary cancers, with 90% of cancer deaths caused by metastatic spread ⁵.

Cancer is a set of diseases where cells no longer respond to the body's anti-proliferation or death signals; that is to say cancer is a condition where a group of cells continues to survive and proliferate despite the body's signals to stop. One way to explain the process of tumorigenesis and the resulting differences between cancer cells and 'normal' cells is by looking at the hallmarks of cancer which describe salient features of neoplastic disease ⁶. Six hallmarks of cancer were first set out in 2000 by Hanahan and Weinberg⁷ and were updated in 2011; the update included the six primary hallmarks, two emerging hallmarks and two enabling characteristics. The primary hallmarks are sustaining proliferative signalling, evading growth suppressors, enabling replicative immortality, resisting cell death, deregulating cellular energetics, inducing angiogenesis, and activating invasion and metastasis. The enabling characteristics are genome instability and mutation, and tumour promoting inflammation. The emerging hallmarks are reprogramming energy metabolism and evading immune destruction ⁸.

The hallmarks are caused by somatic mutations or epigenetics changes that give those altered cells an advantage over the surrounding tissue ⁹. These changes give cells the ability to ignore external or internal regulation of growth and cellular death. This dysregulation results in continuous division of a single cell into a 'clone' which rapidly evolves and forms a tumour in solid tissue or outcompetes 'normal cell populations' in liquid tissues ¹⁰. It is the stepwise acquisition of a number of mutations that drive and result in carcinogenesis that lends itself to the idea that cancer is a Darwinian disease ⁹. Some of these mutations are inherited from parents and some mutations are caused by viruses, exposure to UV and ionising radiation or chemical mutagens. Most mutations, however, occur during the cells normal replicative cycle and thus cannot be prevented ¹⁰. The mutations that contribute to the cancer phenotype disrupt the cell's normal metabolic and reproductive processes through protein, regulatory and epigenetic changes ¹¹.

1.1.1 The Hallmarks of Cancer

Sustaining Proliferative Signalling

In normal somatic tissue the growth and expansion of cells is tightly controlled; this is not the case in cancerous tissue. Tissues control cell growth and proliferation by producing and releasing growth promoting signals, maintaining homeostasis of cell number, tissue architecture and function ⁶. For example, upregulation of growth signals and receptors, such as insulin like growth factor (IGF-1) signalling, are crucial for tumour transformation and survival of malignant cells. Conversely IGF-1 signalling is not highly involved in non-tumour cell growth ¹². When the TP53/mouse double minute 2(MDM2)/IGF-1 receptor (R) network is disturbed, this causes IGF-1R upregulation which leads to a growth advantage in cancer cells ¹². Another example is the strong upregulation of estrogen receptor expression, in particular in estrogen receptor positive breast cancer, this is upregulated at the transcript level, the gene level, with increased stabilisation of the protein and a decrease in control of protein levels ¹³. Patients with estrogen receptor positive breast cancer have a better prognosis as the estrogen receptor is easily inhibited by removing its ligand, estrogen from the patient through hormone therapy ¹³.

A key difference between normal cells and cancer cells is that normal cells cease to divide once proliferation signals disappear whilst cancer cells continue to divide ¹⁴. Tumour cells do not halt proliferation when subjected to growth inhibitory signals or in the absence of growth stimulatory signals ¹⁴. For instance, PTEN is a tumour suppressor gene whose normal function includes maintaining genomic stability, cell survival, migration, proliferation and metabolism ¹⁵. A decrease in abundance or a loss of function of PTEN amplifies phosphoinositide 3-kinase (PI3-K)/ protein kinase B (AKT)/ mechanistic target of rapamycin (mTOR) signalling which is involved in control of cellular metabolism, growth and proliferation ^{8,15}. An excess of signalling can also cause senescence, for example the MAPK signalling pathway is upregulated in senescent tumour cells. Senescent tumour cells while not dividing themselves secrete exosomes that promote nearby tumour growth ^{16,17}. Another example of the effect of proliferation signals on tumour growth is that an

upregulation of Urokinase Receptor (uPAR) results in invasion and metastasis, whereas downregulation of uPAR induces tumour dormancy^{18,19}.

Evading Growth Suppressors

In order to prevent uncontrolled growth and proliferation, cells have internal checks they must pass before entering the next stage of the cell cycle; many of these cell cycle checkpoints are dysregulated or suppressed in cancer cells. There are three checkpoints one at the transition from G0 to G1 which is regulated by proteins such as cyclin D1 and cdk 4/6, the G1 to S checkpoint which is regulated by TP53, p21 and MDM2 and the G2 to M checkpoint which is regulated by the ATM serine/threonine kinase, checkpoint kinase 2 (CHK2) and TP53²⁰.

The inactivation of tumour suppressors such as TP53, through mutation and epigenetic silencing, results in tumours that are resistant to DNA damage associated apoptosis²¹. TP53 responds to DNA damage by stopping cell cycle progression from G1 to S phase and initiating DNA repair or apoptosis; and is mutated in more than 50% of cancers. Absence of functional TP53 allows persistent cell proliferation invasion and metastasis^{21,22}.

Contact inhibition is another mechanism used to control cellular growth in normal tissues. In non-cancerous tissue, growth halts when cells come into physical contact with each other. Evading contact inhibition and continuing to grow when in contact with other cells is an important feature of many cancers. Loss of E-cadherin is associated with loss of contact inhibition and increased motility, which is associated with advanced cancer stages²³. When cells get close to one another, E-cadherin binding between cells initiates a signalling cascade which halts proliferation²³. Upregulation of transforming growth factor beta (TGF- β) results in the downregulation of E-cadherin and thus the loss of contact inhibition²⁴.

When TGF- β is upregulated in tumour associated macrophages (TAMs) it induces genome wide changes in DNA methylation²⁵, which lead to the epithelial to mesenchymal transition (EMT) in cancer cells²⁶. The EMT facilitates invasion and migration of cancer cells. This process cannot occur in the presence of contact inhibition²⁶.

Avoiding Immune Destruction

One of the emerging hallmarks of cancer is avoiding immune destruction. Part of this hallmark is based on the idea that surveillance of all stromal cells by the immune system is constant and that potential malignant cells are eradicated almost all of the time but some slip through. These cells that evade immune destruction are the cells that can generate tumours²⁷. Conventional dendritic cell activity is dampened during low surveillance and normal in high surveillance²⁸. Poorly metastatic melanomas inhibit their own metastasis by secreting exosomes that stimulate an innate immune response²⁹. However, some exosomes are also immune suppressive, altering cytokine secretion levels of CD8+ T cells, blocking cell cycle progression and inducing apoptosis of CD8+ T cells³⁰.

There is an increase in cancer incidence in immunocompromised individuals, however most of these cancers are virus induced^{31–33}. Most of the immune surveillance for tumour cells must then be the elimination of viral infected cells and reduction of viral burden⁸. Tumours also arise more frequently and grow more rapidly in immune compromised mice³⁰. In immune competent mice CD8+ T cells, CD4+ Th1 helper cells, and NK cells all seem to be partially responsible for the removal of carcinogenic cells^{30,34}. In support of this, tumours derived from immunocompromised mice are often highly immunogenic and they fail to proliferate in immune competent models^{35,36}. Patients with tumours that exhibit high levels of immune infiltration have a better prognosis than patients with tumours that exhibit low levels of immune infiltration^{35,36}. Cancer cells can also secrete immune suppressing factors such as TGF β to reduce their 'immunogenicity' assisting to evade the immune systems surveillance^{37,38}.

Tumour Promoting Inflammation

Some tumours are densely infiltrated by cells of the innate and adaptive immune systems, this mirrors the immune infiltration of inflammatory conditions in non-cancerous tissues³⁹. However, immune infiltration is both beneficial and destructive for tumours with both evasion of immune destruction and antitumoral immune responses being associated with infiltration^{39,40}. The innate immune system and inflammation can have tumour promoting effects⁴¹. These immune cells can contribute to tumorigenicity by supplying bioactive molecules to the tumour microenvironment (TME)⁴². These bioactive molecules include

growth factors that sustain proliferative signalling, survival factors, proangiogenic factors and signals that lead to activation of the EMT ⁴². Infiltrating immune cells are also known to secrete reactive oxygen species (ROS) that are mutagenic for nearby cancer cells further promoting tumorigenesis ⁴¹.

Enabling Replicative Immortality,

Each time that cells divide, they lose a part of their telomeres, the highly repetitive sequences at the end of their chromosomes. Over an organisms life span, there is a progressive shortening of telomeres ⁴³. Telomere dysfunction or shortening results in senescence which usually results in the halting of cell division. If cell division is allowed to continue, extreme telomere dysfunction results in cellular crisis ⁴⁴. Telomerase (TERT), the enzyme that restores telomere length is silenced in adult somatic cells but highly upregulated in cancer and immortalised cell lines ⁴⁵. This allows tumour cells to continue dividing indefinitely by avoiding senescence and crisis. The TERT promoter is therefore a promising target for cancer therapies ⁴⁵. Other mechanisms for telomere lengthening are seen in tumours without TERT mutations. Interestingly, *p53* mutations may also allow for telomere shortening without crisis, repairing the damage induced by replication and enabling replicative immortality ⁴⁶. Alternative telomere lengthening (ALT) is another mechanism by which 4-11% of cancers obtain replicative immortality ^{47,48}. ALT is telomerase independent, recombination dependant telomerase maintenance; where extrachromosomal telomere DNA recombines with chromosomal telomeres lengthening them ⁴⁹. ALT is present in cancers of mesenchymal origin and associated with poor patient outcome ⁴⁸.

Genome Instability

Genome instability is an enabling characteristic of cancer. Cancer is a genetic disease where genetic mutations give cell populations a selective advantage, to survive and proliferate within the body's tissues ⁵⁰. Spontaneous mutation rates are low in normal tissues but high in tumours, increasing the likelihood of cells containing mutations with selective advantages occurring in the tumour ⁵¹. Darwinian selection plays a considerable role in tumorigenesis and resistance to chemotherapeutics and other treatments ^{50,51}. Increased replication itself

is a driver of genome instability and tumorigenesis and defects in genome maintenance and repair are instrumental for tumour progression ^{6,52}.

There are 'caretakers of the genome' that detect and repair DNA damage and inactivate or intercept mutagenic molecules before DNA damage occurs ^{53,54}. These caretakers act as tumour suppressors whose normal function is inherently anti-tumorigenic and thus the inactivation of these caretakers promotes tumorigenesis. *p53* is a tumour suppressor, overseeing progression through the G1 to S cell cycle check point and is considered the 'guardian of the genome' ⁴⁶. *p53* copy number and DNA proofreading is increased in large animals, this is believed to be due to the large number of cell divisions from the zygote required to reach adulthood and thus the increased chance of accumulating hazardous mutations ⁵⁵. The loss of telomeres also results in genomic, specifically karyotypic, instability ^{46,56}. Epigenetic modification of tumours results in increased genome instability. This is shown by the fact that histone deacetylase SIRT1 knockouts display increased tumorigenesis ^{57,58}.

Resisting Cell Death

There are many mechanisms of cell death, including programmed, partially programmed and otherwise. Apoptosis is a type of programmed cell death and is characterised by cell membrane blebbing, cell shrinkage and the formation of apoptotic bodies ⁵⁹. There are three main apoptosis pathways, intrinsic, extrinsic and mitochondrial mediated, all of which involve proteases called caspases ^{59,60}. Caspases are inactive until cleaved which occurs after receipt of apoptosis signal, and this initiates the caspase cascade that is characteristic of apoptosis ⁶¹. The intrinsic pathway is activated after DNA damage and stress and involves mitochondrial release of cytochrome C ⁵⁹. In cancer the loss of *p53* leads to increased DNA damage survival which mitigates the intrinsic apoptosis pathway ⁵⁵. The apoptosis pathways themselves are also mutated in majority of cancers ⁵⁵. BCL-2-associated X protein (BAX) and BCL2 antagonist (BAK) are tumour suppressors, members of BCL-2 family ⁶². BAX and BAK act to permeabilise the mitochondrial membrane in mitochondrial mediated apoptosis, mutation of either of them results in resistance to mitochondrial mediated apoptosis ^{62,63}. The extrinsic pathway is activated after specific death receptors are activated by ligand binding, triggering the caspase cascade ⁵⁹. The endoplasmic reticulum stress (ER stress)

induced pathway is triggered by ER stress and the unfolded protein response involves the JNK- and p38 MAPK signalling cascade⁵⁹.

Necrotic cell death is an inflammatory form of cell death and involves the rupture of the cell membrane and release of lysosomal enzymes in the cell direct environment ⁵⁹. Necrosis can be triggered by more severe forms of stress such as shock, can be electrical, chemical and poison induced, high pressure and aerobiosis or by ATP depletion ⁵⁹. Necrosis is characterised by a rapid increase in the permeability of the mitochondrial membrane ⁵⁹. In contrast to the suppression of apoptosis necrosis and the pro inflammatory signals it releases are beneficial in metastasis and invasive tumours⁸.

Methuosis, necroptosis, NETosis, pyronecrosis and pyroptosis share mechanisms and similarities with necrosis but are ultimately different forms of cell death ⁵⁹.

Methuosis is characterised by displacement of the cytoplasm by large fluid filled vacuoles derived from macropinosomes. It is similar to necrosis by the loss of metabolic capacity and plasma membrane integrity, while the lack of cell shrinkage and no nuclear fragmentation is unlike apoptosis ⁶⁴. Methuosis is often found in cancer cells that are resistant to apoptosis causing drugs ⁶⁴.

Necroptosis is a form of regulated necrosis activated by ligands of death receptors under apoptotic deficient conditions, it requires kinase activity of receptor interacting protein 1 (RIP1) which activates receptor interacting protein 3 (RIP3) and mixed lineage kinase domain like protein (MLKL) ⁶⁵. Necroptosis is implicated in TNF mediated hypothermia, systemic inflammation, ischemic reperfusion injury, neurodegeneration, progressive atherosclerotic lesions and more ⁶⁵. NETosis is a form of neutrophil specific cell death mediated by the neutrophil extracellular traps (NET) in response to ROS and chromatin unfolding ⁶⁶. NETosis is insensitive to both caspase inhibition and necrostatin and thus is neither apoptosis nor necrosis ⁶⁶.

Pyronecrosis is a nucleotide-binding oligomerisation domain (NLRP) dependant proinflammatory cell death, a subtype of regulated necrosis, it is caspase independent but cathepsin dependant, and morphologically similar to necrosis ⁶⁷. Genetic inducement by

mutations in the NLRP3 gene and microbial pathogens such as shigella flexneri lead to pyronecrosis ⁶⁷.

Pyroptosis is a proinflammatory form of regulated cell death, it is caspase dependant but involves cell lysis. Early permeabilization of cell membrane distinguishes it from apoptosis ⁶⁸. However, it is similar to apoptosis in that it involves DNA fragmentation, chromatin condensation and caspase 3 and 7 maturation, as well as the cleavage of PARP1⁶⁸.

Autosis is autophagy mediated non-apoptotic cell death ⁵⁹. It is characterised by being positively sodium and potassium regulated and, inversely ATPase regulated ⁶⁹. It can be induced by autophagy inducing peptides, starvation and hypoxia ischemia ⁷⁰ and was first characterised in 2013 ⁶⁹. Morphologically it is characterised by the disappearance of endoplasmic reticulum and swelling of perinuclear space ⁷⁰.

Other cellular death mechanisms include entosis, ferroptosis, lysosome dependant cell death and parthanosis ⁵⁹. Entosis is engulfment mediated cell death where the entotic cells assist in their own engulfment. Other engulfing cell death mechanisms also includes phagoptosis and suicidal emeriopolesis ⁷¹. Entosis is triggered in culture by the detachment of adherent cells from the extracellular matrix. Epithelial adherent junctions, e cadherin and α -catenin, play a role in the entotic cells uptake via RhoA-GTPase activity ⁷¹. This leads to the formation of cabalistic cell in cell structures ⁷¹. Ferroptosis is characterised by smaller than normal mitochondria, reduced or vanished mitochondrial cristae, condensed mitochondrial membrane density's and outer mitochondrial membrane rupture ⁷². Ferroptosis is caused by the accumulation of lipid peridoxidation products and lethal reactive oxygen species (ROS) derived from Iron metabolism and can be inhibited by iron chelators ⁷². Lysosome dependant cell death, is characterised by lysosomal membrane permeabilization, and results in apoptosis ⁷³. Parthanosis is a PARP dependant, caspase independent, form of cell death. It is dependent on the nuclear translocation of mitochondrial associated apoptosis inducing factor (AIF) and is found in terminally differentiated cells such as neurons ⁷⁴.

Deregulating Cellular Energetics

Hanahan and Weinberg included 'deregulating cellular energetics' as an emerging hallmark in 2011. This hallmark pertains to the changes in energy metabolism that enable dysregulated cellular proliferation. Cancer cells rely on glucose for metabolism and many but not all cancer cells, rely on glycolysis to oxidise glucose, even in the presence of oxygen (aerobic glycolysis). Glycolysis has an 18-fold lower efficiency for releasing energy from glucose compared with mitochondrial oxidative phosphorylation ⁷⁵. Glycolytic metabolism in the presence of oxygen is called the Warburg effect, and was first noticed by Otto Warburg in 1926 ^{75,76}. His idea was that cancer was caused by irreversible mitochondria damage and this led to an increased dependence on glycolysis ⁷⁵ and now altered energy metabolism is regarded as one of the hallmarks of cancer ^{6,77,78}. In order to fuel themselves, cancer cells upregulate glucose transporters such as GLUT2, which transport glucose across plasma membrane into the cell, and glycolytic enzymes such as hexokinase II ⁷⁹. In highly glycolytic cancers, pyruvate is primarily reduced to lactate through lactate dehydrogenase, rather than being further oxidised by the TCA cycle in the mitochondria ⁸⁰. Lactic acid is secreted by highly glycolytic cells leading to microenvironmental acidosis. This is an advantage for cancer cells that are generally resistant to acidic environments ⁹.

In conjunction with the switch to glycolysis, glutamine transportation and metabolism increases. Glutamine is used in the TCA cycle via α ketoglutarate which allows the TCA cycle to run without pyruvate being transformed into acetalCoA. This allows pyruvate to be primarily used for lactate production and NAD⁺ recycling ⁸¹. Some tumour cells become glutamine addicted and glutamine is required for rapid cell growth as it provides nitrogen and nucleic acid precursors ⁸¹. This glutamine metabolism is upregulated by MTORC1 ⁸¹.

Solid tumours contain hypoxic regions which can be very transient due to the unreliable and compromised blood supply of tumours. The hypoxic regions of the tumour upregulate the hypoxia response, glucose transport and glycolytic rates ⁸² and promotes the epithelial to mesenchymal transition (EMT) which facilitates metastasis ⁸³. The hypoxia response is mediated by hypoxia inducible factors (HIF), which mediate immune responses and induce angiogenesis, this results in upregulation of GLUT 1 ^{84,85}. The HIFs are transcription factors that mediate the tissues response to hypoxia and upregulate angiogenesis ⁸⁶.

As well as remodelling of the TME, increased glycolytic rates along with an increase in glutamine metabolism allow for the diversion of TCA cycle intermediaries into biosynthetic pathways including the generation of nucleosides and amino acids ^{87,88}. These intermediaries form the macromolecules required for continued cancer cell proliferation in the same way that, embryos with rapidly dividing cells use mainly aerobic glycolysis to generate energy ^{89,90}.

While many tumour cells are glucose dependant and release lactate, other cells are lactate dependant and use lactate as their main fuel. Lactate is oxidised to pyruvate in the mitochondria by mitochondrial LDH and the resulting NADH is re-oxidised in the mitochondrial electron transport chain, in the presence of oxygen ^{91–93}. Altered fatty acid metabolism also occurs in cancer cells with an upregulation of mTOR signalling and increased fatty acid synthesis by cancer cells, this too contributes to the remodelling of the TME ⁹⁴.

Inducing Angiogenesis

Angiogenesis is the formation of new vasculature; in the tumour this vascularisation results in better metabolite acquisition and waste removal. Angiogenesis is only transiently turned on in adult stroma but is constantly upregulated in the tumour ⁹⁵. Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis and is upregulated by hypoxia ⁹⁵. Upregulation of VEGF results in angiogenesis, increased blood supply to the tumour allows tumours to reach increased size ⁹⁵. Hypoxia occurs when tumour growth outstrips angiogenesis. The hypoxia inducible factor HIF1 α mediates the cell's hypoxic response because its expression and stability are dependent on oxygen levels ⁸⁶. Under normoxia HIF1 α is expressed at low levels and the protein is unstable ⁸⁶. Conversely HIF1 α is stable and has increased expression in hypoxic conditions ⁸⁶. Thus HIF1 α it is a key mediator of the hypoxic switch from mitochondrial to glycolytic energy production ⁸⁶ and is a key target for cancer therapies ⁹⁶.

In contrast to normal tissue angiogenesis, tumour angiogenesis is not orderly and appears 'pathological' in phenotype ⁹⁵. The tumour vasculature is also 'leaky', with the new vessels missing a basement membrane and fewer tight junctions between epithelial cells; this leakiness results in suboptimal blood flow and transient hypoxia and thus more VEGF is produced ⁹⁵. This angiogenic switch, to abnormal vasculature, is induced early in tumour

models before tumours reach a couple of mm in size. Pericytes, the cells that surround blood vessels and are involved in controlling blood flow; are decreased in number and activity in the tumour or have been turned into cancer associated fibroblasts (CAFs) by exosomes from cancer cells ⁹⁷. Bone marrow derived cells such as macrophages, neutrophils, mast cells and myeloid cells contribute to angiogenesis in the tumour and play a role in resistance to VEGF targeted therapies ⁹⁸.

Activating Invasion and Metastasis

Invasion and metastasis occur by local invasion, introvasation, transit, extravasation and colonisation. Tumours arising from epithelial tissue tend to be more malignant and invasive ⁹⁹. In invasion and metastasis, adhesion molecules normally associated with migration in embryogenesis are upregulated ¹⁰⁰. E-cadherin binds cells to one another and the inactivation of E-cadherin occurs before metastasis in most tumours, but ductal breast carcinomas require E-cadherin for metastasis ^{101,102}. In ductal breast cancers the loss of E-cadherin increases invasion but reduces proliferation and survival, circulating tumour cell numbers, seeding of cancer cells in new environments and metastasis outgrowth ¹⁰¹.

Epithelial cells acquire the abilities necessary to invade by going through the EMT process which is induced by TGF- β ^{103,104}. In the process of EMT cells lose epithelial characteristics such as polarity and specialised cell-cell contact and become migratory as they move into surrounding tissue ¹⁰³[Click or tap here to enter text..](#) EMT occurs at the borders of tumours and suppression of the EMT increases cell proliferation and sensitivity to chemotherapies ^{105,106}.

The crosstalk between cancer cells and the associated neoplastic stroma is highly involved in metastasis and invasion ¹⁰⁷. Cancer cells secrete and receive paracrine factors such as hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), C-X-C motif chemokine 12 (CXCL12) and Interleukin-6 (IL-6); all are active in progression and metastasis ¹⁰⁸. Mesenchymal stromal cells also secrete chemokine ligand five (CCL5) in response to signals from cancer cells, to recruit immune cells, this can induce chemoresistance ¹⁰⁹.

Tumour associated macrophages (TAMS) stimulate key tumour progression steps ¹¹⁰. TAMS secrete cytokines, growth factors and proteases that help remodel the tumour microenvironment into a supportive microenvironment ¹¹¹. IL-4 activated macrophages promote growth and metastasis ¹¹². TAMS supply EGF to the tumour cells which reciprocate by producing colony stimulating factor-1 (CSF-1) ¹¹³. Cancer cells undergo the EMT and travel to a new site, then revert via the mesenchymal to epithelial transition (MET), after invasion to generate the metastatic colony ^{103,114}. Some micro-metastasis remain dormant until changes in TME occur that allow their outgrowth unto tumours ¹¹⁴.

1.2 Cancer Cell Types Used in this Thesis

In order to study cancer *in vitro* model cancer cell lines were used in this research, in particular murine breast cancer and melanoma, and an immortalised murine Astrocyte cell line that represents a precursor cell for astrocytoma, a brain cancer.

1.2.1 Breast Cancer

Breast cancer is the most common cancer in women worldwide ¹¹⁵. It is curable in 70-80% of patients when the disease is recognised early and is pre-metastatic ¹¹⁵. Advanced breast cancer with metastasis is incurable with current therapies ¹¹⁵. The main features that distinguish different types of breast cancer are the expression of human epidermal growth factor (HER2+), oestrogen (ER+) and progesterone receptors (PR+) and/or BRCA 1 or 2 mutations ¹¹⁵. Estrogen receptor negative disease represents 20-30% of cases with a higher proportion in younger women and women of African ancestry ¹¹⁶. Triple negative breast cancer (HER2-, ER- and PR-) is more aggressive, with a worse prognosis, largely because there are fewer treatment options ¹¹⁷. The mortality for breast cancer in NZ is 16.9 per 100,000 people age standardised to the world health organisation (WHO) standard population ³.

The tumour microenvironment of different subtypes of breast cancer differs widely, in saying that there are some common TME prognostic features. High tumour associated macrophage content is associated with poor outcome in breast cancer ¹¹⁸. Tumour associated neutrophils are associated with poor outcome in triple negative breast cancer ¹¹⁸.

Higher circulating levels of plasmacytoid dendritic cells are indicative of good prognosis as they indicated less tumour infiltration ¹¹⁸.

4T1 Breast Tumour Model

The 4T1 murine breast cancer model has been in use since 1992 and was created from a spontaneous tumour in MMTV* BALB/c mouse ^{119–121}. The tumour line grows *in vivo* in BALB/c mice and *in vitro* cell culture ³. The 4T1 cell line is 6-thioguanine resistant which allows for easy isolation of the tumour cells from the surrounding tissue ^{121,122}. 4T1 tumours are highly tumorigenic, invasive and spontaneously metastasise to lymph nodes, liver, blood, lung, brain and bone ^{121,122}.

1.2.2 Melanoma

Melanoma is the most aggressive and deadly form of skin cancer ¹²³. The main risk factor for developing melanomas is UVA from sun exposure in childhood and adulthood ¹²⁴. Therefore, it is unsurprising that the highest rates of melanoma occur in New Zealand and Australia with incidences of 35.8 and 34.9 per 100,000 person years respectively ¹²⁵. Worldwide, there are 55,500 deaths annually for cutaneous melanomas, and diagnoses account for 1.7% of all malignant cancers diagnosed each year ¹²⁵. Metastasis of melanoma is indicative of very poor prognosis ¹²⁵. Melanoma's resistance to therapy is common and problematic ¹²³. BRAF inhibitors and mitogen-activated protein kinase inhibitors have had some improvement on survival, but prognosis remains poor for metastatic disease ¹²⁵. Immunotherapies such as Pembrolizumab have shown marked success in treating melanoma in recent years ¹²⁶. This is due to melanoma tumours being highly immunogenic, they have high mutational load leading to many neoantigens that in turn elicit immune responses ¹²⁷. The mortality for melanoma in NZ is 3.7 per 100,000 people, age standardised to the WHO standard population ³.

B16 Melanoma Model

The B16 murine melanoma model was established in the 1970's ¹²⁸. It was derived from a spontaneous C57BL/6- derived melanoma B16 melanoma cells ^{128,129}. B16 tumours are highly tumorigenic, invasive and form spontaneous metastasis in the lung ¹²⁸.

1.2.3 Glioblastoma

Glioblastoma is the most common, most aggressive and deadly primary adult brain tumour^{130–133}. It is a stage 4 glioma and can arise spontaneously or progress from a lower grade glioma¹³⁰. Glioblastoma has a high burden of disease with an incidence rate of 3.2 per 100,000 in the United States^{132–134}. Prognosis for patients is very poor and this is primarily due to the resistance against radiation and chemotherapy and the difficulty in getting drugs and immunotherapies across the blood brain barrier. The danger and risk of serious complications following conservative resection of tumours is also well established^{131,133,135}. Many targeted therapies show promise in preclinical models, but all have failed in clinical trials. Recent advances in prognostic factors have also not resulted in advances in treatment or patient outcomes since 2005^{132,133}. The current best treatment plan for glioblastoma patients is maximal safe resection followed by temozolomide and radiotherapy concurrently, first reported in 2005 by Stupp et al¹³². Glioblastoma have a highly immune suppressive TME¹³⁶. This is exemplified by the significant dampening of anti-tumour T-cell responses in glioblastoma¹³⁶.

GL261

GL261 is a tumour cell line that is the current standard for murine models of glioma and glioblastoma. It is representative of a carcinogen induced glioma model and similar to lower grade gliomas but not necessarily glioblastoma¹³⁷. GL261 cells carry mutations in K-ras and p53, they form intracranial and subcutaneous tumours and are radiosensitive¹³⁸. GL261 is the only available murine ‘glioblastoma’ line, however the experiments in this thesis were carried out on ρ^0 cells and GL261 did not survive this transformation, (see Appendix 1). Instead, a non-cancer C57 eGFP Astrocyte line was used as a ‘representative’ cell line.

C57 eGFP Astrocytes

Astrocytes are the most numerous cell type in the brain, they are star shaped glial cells or neuroglia^{139,140}. They are heavily involved in both the normal physiology and pathology of the brain^{139,140}.

The cell line henceforth referred to as the ‘Astrocytes’ is an Astrocyte cell line created and immortalised by Dr Remy Schneider in our laboratory¹⁴¹. It was derived from the neonatal

brain tissue of an eGFP mouse. eGFP expression in these mice is cytoplasmic and under the control of a chicken beta actin promoter and cytomegalovirus enhancer ¹⁴².

1.3 Tumour Microenvironment

Cancer cells do not exist in monoculture within the body, they are surrounded by and are a part of the tumour microenvironment (TME). Therefore, to understand cell-cell interactions it is important to evaluate the TME. The TME consists of all of the cells in the area in between and around tumour cells as well as the physical environment. This includes structural cells such as epithelial cells and infiltrating immune cells such as tumour associated macrophages (TAMs). Depending on the cancer type, tumour location and host-related factors, the TME differs greatly between different cancers, and even between cancers of the same type. Differences in the microenvironment can be caused by different cell types, their metabolic products, nutrient and oxygen levels as well as the extracellular matrix ¹⁴³. Most of the non-cancer cells present in the TME are also present in the normal tissue but the proportions of certain cell types change in relation to each other as a tumour develops ¹⁴⁴. Infiltrating immune cells make up a significant part of the TME. This change in TME is particularly obvious in brain tumours where immune cells are not often found before the tumour has compromised the blood brain barrier (BBB), and many are found after ¹⁴⁴. The TME plays a significant role in disease progression or suppression¹⁴⁵. Interactions between cancer cell and cell of the TME may explain why tumours with similar histology behave differently under treatment and disease progression and thus prognosis differs between patients ¹⁴⁵.

1.3.1 Cancer associated Fibroblasts (CAFs)

CAFs are highly abundant in many tumours and originate from a mesenchymal lineage ^{146,147}. They are present from tumour inception to end stage metastatic spread ¹⁴⁶. CAFs have a diverse secretome, they express signalling receptors and are involved in the epigenetic reprogramming of cancer cells^{146,148}. CAFs secrete extracellular matrix proteins tenascin C (TN-C), fibronectin (FN), periostin (POSTN), collagens, matrix metalloproteinases (MMPs), lysyl oxidase (LOX), tissue inhibitor of metalloproteinases (TIMPs), TGF- β , VEGFs,

platelet derived growth factor (PDGFs), EGF, FGFs, prostaglandin E₂ (PGE₂), connective tissue growth factor (CTGF), stromal cell-derived factor 1 (SDF-1) and secreted Wnt ligands¹⁴⁶. CAFs express platelet derived growth factor receptor α and β (PDGFR α/β), fibroblast activation protein (FAP), vascular cell adhesion protein1 (VCAM1), discoidin domain-containing receptor 2 (DDR2), podoplanin, α -smooth muscle actin (α SMA), Fibroblast specific protein 1 (FSP1/S100A4), vimentin, desmin, caveolin1 (CAV1^{low}), TGF β RI/II, Bone morphogenetic protein receptors 1 and 2 (BMPRI/II), EGFR, FGFRs¹⁴⁶. CAFs are sourced from bone marrow, EMT, pericytes and resident quiescent fibroblasts¹⁴⁶. CAFs provide physical support for tumour cells and can either promote or retard tumour growth under different conditions¹⁴⁹. They have antitumour roles at the start of tumour progression but change to pro tumour roles in progressed tumour where they promote metastasis^{146,148}. In some tumours such as pancreatic and breast cancers they occupy 80% of cancer volumes and 40-50% of cell population/number¹⁵⁰. As our understanding of CAFs is still limited they represent an important avenue for further exploration and a potential target for immunotherapy and novel chemotherapies¹⁵¹.

1.3.2 Mesenchymal Stromal Cells (MSC)

Mesenchymal stromal or stem cells (MSC) can also be called multipotent stromal cells. This abbreviation has previously been used to refer to solely as mesenchymal stem cells, but as this term is restrictive in its use, I will be using MSC to describe mesenchymal stromal cells which encompasses the stem cells along with all the stromal cells that are derived from this lineage¹⁵². They are found in tissue types that are derived from the mesoderm including umbilical cord blood, endometrial polyps, bone marrow, compact bone and adipose tissue^{153–155}. They are a heterogeneous subset and can differentiate into anything with a mesodermal origin including adult bone marrow, osteoblasts, chondrocytes, monocytes, marrow stromal cells, tendon ligament fibroblasts, adipocytes and skeletal tissue^{154,156,157}. MSC with stem cell ability derived from different tissues are not limited to differentiation into those tissues, for example bone marrow derived MSC can differentiate into osteoblasts, chondrocytes, adipocytes and neural cells¹⁵⁸. These cells are only studied after *in vitro* culture and proliferation, at which point they are considered MSC if they are negative for the markers CD45, CD34, CD14 and positive for CD73 CD90 and CD105¹⁵⁴. Variable levels of

other markers, such as CD44, CD71, ganglioside GD2IN and CD271 have also been observed in MSC demonstrating the heterogeneous nature of this cell subset ¹⁵⁴.

MSC are highly involved in tissue repair by their differentiation capacity and contribute to tissue repair *in vivo* ¹⁵⁹. MSC transplanted into animals rapidly engraft and differentiate ¹⁶⁰. Their final tissue specific contribution is determined by initial engraftment and proliferative capacity of the ensuing tissue specific cells that the MSC differentiate into ¹⁶⁰. There is some debate as to if they are an intrinsically reparative cell or a stem cell ¹⁵⁶. They were originally thought to be multipotent stem cells, however, bone marrow derived MSC (BM-MSC) are rare and heterogeneous, and the grouping contains cells with different levels of commitment to the mesodermal lineage, hence the stromal over stem distinction ¹⁶¹. Because of this multipotency and residency in adult tissue they are currently being investigated as potential stem cell therapeutic agents ¹⁵⁷. Both extrinsic (microenvironment) factors and intrinsic (genetic) potential cause MSC to differentiate ¹⁶².

MSC are strongly involved in immunomodulation by the secretion of cytokines and growth factors; they also have paracrine and autocrine activity ¹⁵⁶. These secreted bioactive factors suppress the local immune system and inhibit fibrosis and apoptosis, they enhance angiogenesis and stimulate mitosis and the differentiation of tissue ¹⁵⁶. These trophic effects are different and distinct from the differentiation of MSC into cells that repair tissue. The effects of mesenchymal stem cell secreted bioactive molecules can be direct or indirect ¹⁵⁶. They are known to interact with the innate and adaptive immune cells and have modulator effector function ¹⁵⁴. MSC are currently used as a treatment in infarction (heart injury), stroke and meniscus regeneration, among others ¹⁵⁶. After *in vivo* administration, MSC migrate to injured tissue and release proinflammatory cytokines to promote survival of damaged cells ¹⁵⁴. Allogenic MSC transplantation alleviates the severity of disease such as idiopathic pneumonia syndrome, through significant contribution of their immunomodulatory capacity ¹⁶³.

MSC are not only associated with repair and migration to sites of injury, they also migrate to the tumour microenvironment ¹⁶⁴¹⁶². The presence of MSC in the TME increases the stemness of tumour cells, mediates migration, promotes angiogenesis, suppresses the

immune response and induces drug resistance ¹⁶⁴. MSC are also known to differentiate into other pro tumorigenic stromal components, enhance the EMT and increase cancer cell survival ¹⁶⁵. However, MSC also have antitumour functions, which include enhancement of immune response, inhibition of angiogenesis, regulation of cellular signalling and induction of tumour cell apoptosis ¹⁶⁵. There is less evidence for this tumour suppressing role, and it appears to be dependent on the ratio of MSC to tumour cells, the higher the ratio of MSC to cancer cells, the more suppressive the phenotype¹⁶⁵. In addition, MSC are known to induce therapy resistance in acute myeloid leukemia (AML) cancer cells that lack the mutations required to form resistance themselves ¹⁶⁶.

One of the mechanisms that MSC lead to tissue and cellular repair is through the transfer of mitochondria/ mitochondrial activity. It has been well documented through the use of dyes, rho zero (ρ^0) cells and genetic methods that MSC can donate mitochondria to injured cells ^{167,168}. MSCs can move many different cellular components to other cells and have been known to outsource mitophagy, shuttle microRNAs and other activities through extracellular vesicles ¹⁶⁹. mtDNA and protein transfer has been recorded from MSC in many different tissue types as well, such as cornea and airway epithelial cells, and they have been shown to protect or repair mitochondrial damage in many situations including Friederichs ataxia ^{170,171}. MSC have also been shown to donate mitochondria to monocytes and enhance phagocytosis by these monocytes ^{172,173}. MSC derived from compact bone generated using a method established by Dr Schneider in our lab, have been shown to donate mitochondria to ρ^0 cells ¹⁴¹.

1.3.3 Mononuclear cells

The mononuclear phagocyte system contains monocytes, dendritic cells and macrophages ¹⁷⁴.

Monocytes

Monocytes are white blood cells produced in the bone marrow from myeloid progenitor cells. They circulate in blood, bone marrow and spleen, and do not proliferate in steady state ¹⁷⁵. Macrophages and dendritic cells can be derived from monocytes ¹⁷⁵. These

immune effector cells have chemokine receptors and adhesion receptors that mediate migration from blood to tissues during infection ¹⁷⁵.

Dendritic Cells (DCs):

Specialised antigen presenting and processing cells, they are highly phagocytic when immature and generate high levels of cytokines as mature cells ^{175,176}. DCs are highly migratory, they move from tissues to lymph nodes and to T and B cells for antigen presentation ¹⁷⁷. They are involved in regulation of T cell responses, are short lived and replaced often ¹⁷⁷.

There are many DC subsets, however all DC subsets can be found in the tumour and in general their density correlates with positive prognosis ¹⁷⁸. Conventional DCs recruit and activate T-cells against the tumour cells and so play an important role in the anti-tumour response ¹⁷⁸. Plasmacytoid DCs however, correlate with poor prognosis¹⁷⁸.

Macrophages:

Macrophages, like DCs, can originate from monocytes and are the resident phagocytic cells in lymphoid and non-lymphoid tissue involved in tissue homeostasis and the clearance of apoptotic cells ^{175,179}. Most macrophages originate from embryonic progenitors ¹⁷⁴. Macrophages have broad pathogen recognition receptors, and this makes them efficient at phagocytosis, they also induce production of inflammatory cytokines ¹⁷⁵. In inflammation they are involved in antigen presentation, phagocytosis and immunomodulation through cytokine and growth factor production ^{175,180}. Macrophages are recruited to endothelial cells in response to stress in the inflammatory response ¹⁸¹. They mediate the body's immune response and form a crucial part of the mononuclear phagocyte system: they circulate as monocytes and then infiltrate tissues in inflammation before becoming macrophages ^{175,179}. Monocyte function also alters following major injury ¹⁸². Macrophage development and activation is dependent on cytokines such as interferon gamma, granulocyte-monocyte colony stimulating factor and TNF- α ^{175,180}. Deactivation of macrophages occurs when they are exposed to IL-10 and TGF- β ^{180,182}.

Tumour Associated Macrophages (TAMs)

Key drivers of tumour promoting inflammation, these immune cells promote genetic instability, nurture cancer stem cells, support metastasis and inhibit protective immunity¹⁸³. TAMs express molecular triggers of checkpoint proteins that regulate T-cell activation and are targets of checkpoint blockade immunotherapies¹⁸³. Another target of TAMs is the inhibition of TAM recruitment and survival in tumours¹⁸³. There are two types of TAMs: M1 and M2. M1 TAMs are fully polarised, exhibit antitumour activity and express IL-12 and TNF- α ^{183,184}. In contrast M2 TAMs are alternatively activated and promote cancer progression they express IL-10, IL-1 receptor agonist (IL-1ra) and type II IL-1 decoy receptor^{184,185}. Phenotype switching from M2 TAMs to M1 TAMs is a good therapeutic target as a high M1/M2 ratio is associated with good prognosis while high M2/M1 ratios are associated with poor prognosis¹⁸⁵.

1.3.4 Pericytes

The normal function of pericytes is to regulate blood vessel wall integrity¹⁸⁶. They are the cells that surround the blood vessel, they are perivascular and make up an important part of the blood brain barrier¹⁸⁶. In this position pericytes play a key role in vascular homeostasis, rheology and the regulation of blood flow¹⁸⁷. The origin of pericytes is still uncertain however they are often thought to be very similar to MSC^{186,188}. Many researchers think that pericytes and MSC are just subsets of the same cell type, or cells that share a common progenitor, that have different primary functions but retain the ability to do each other's functions¹⁸⁸. They are generally thought to be CD146+, CD34-, CD45- and CD56-¹⁸⁸. Pericytes are multipotent for osteogenic, chondrogenic, adipogenic and myogenic lineages and all known MSC lineages *in vitro*. However, there is some debate over this ability *in vivo*^{188,189}. Like MSC they are a heterogeneous population of cells and the functions of both MSC and pericytes are thought to be different in developing tissues than in adult tissue^{186,188}. Like MSC they secrete lots of bioactive molecules that have immunomodulatory effects¹⁸⁸. Both MSC and pericytes migrate to damaged tissue¹⁸⁸.

In the TME pericytes provide paracrine support signals to normally quiescent endothelium⁶. They are known to be key signalling cell types in the TME¹⁹⁰. Pericytes are recruited to

tumours by platelet-derived growth factor- β ¹⁹⁰. Pericytes that are Nestin + / neuron glial antigen (NG2)+ are type 2 pericytes, they promote endothelial cell survival by secreting VEGFA, ANGPT1 and ECM components ¹⁹⁰. These cells also recruit other pericytes which in turn promotes vascular maturation ¹⁹⁰. Pericytes are involved in cancer stem cell maintenance, metastasis and the immune microenvironment ¹⁹⁰. TME pericytes upregulate PD-L1, CD90, PDGFR β , CD248 and RGS5 which inhibit CD4+ and CD8+ cytotoxic T-cell activity supporting immunosuppression and immune evasion of tumour cells ¹⁹¹.

1.3.5 Endothelial Cells

The inner lining of blood vessels, endothelial cells regulate perfusion, fluid and solute exchange, homeostasis, coagulation, inflammatory responses, vasculogenesis and angiogenesis ¹⁹². They play a key role in maintaining the blood brain barrier and in angiogenesis ^{186,193}. Tumour associated epithelium of tumour endothelial cells are thought to support tumour cells in the TME ¹⁹⁴.

1.4 Mitochondria

One distinct type of interaction between tumour cells and cells in their environment in the exchange of mitochondria through a process called mitochondrial transfer. Mitochondria are organelles with a double membrane and are responsible for aerobic respiration in a cell ¹⁹⁵. The inner membrane forms finger like protrusions called cristae that enlarge the surface area and where upon cellular respiration takes place ¹⁹⁶. Inside of the inner mitochondrial membrane is the mitochondrial matrix contains the mitochondrial DNA (mtDNA), the TCA cycle and numerous biosynthetic pathways ¹⁹⁷. Mitochondria are the organelles primarily responsible for cellular respiration and ATP production ¹⁹⁸, but are also involved in signalling, apoptosis, calcium homeostasis and biosynthesis of many macromolecules ¹⁹⁹.

1.4.1 Mitochondrial Matrix: mtDNA

The mitochondrion is an intracellular organelle descended from an ancestral free-living protoeukaryote 2 billion years ago ¹⁹⁷. Mitochondria have their own circular genome, which divides independently of the cell's nuclear DNA/genome. Both genomes contribute to a

cell's DNA ²⁰⁰. Mitochondrial DNA (mtDNA) is a 16.5 kb circular molecule encoding 13 subunits of the 86 required for four of the five respiratory subunits, 22 tRNAs and 2 rRNAs ²⁰¹. The nuclear genome encodes a vast number of proteins, 1500 of these are imported into the mitochondria ²⁰⁰. These mito-nuclear proteins form the remaining subunits of the respiratory complexes and are involved in mitochondrial DNA, RNA and protein synthesis, as well as mitochondrial function and survival ²⁰¹. Also encoded on the mtDNA within the rRNA genes are small open reading frames (ORFs) that encode small peptides. Humanin, MotC and others enable communication between the mitochondria and the nucleus ²⁰². These peptides are associated with ageing and stress response ²⁰³, humanin has been shown to increase *C. elegans* life span when overexpressed ²⁰².

The number of mitochondria varies significantly, depending mainly on how much energy is required. The number of mtDNA copies per mitochondrion has been found to be 1-10 ^{204,205}. Mitochondrial copy number can vary from under 10 copies per cell in sperm cells up to hundreds of thousands of copies in oocytes ²⁰⁴. Different stages in cell cycle, cell type and function can all affect mtDNA copy number ²⁰⁴.

1.4.2 Mitochondrial Matrix: TCA cycle

The TCA cycle (or citric acid cycle) which occurs within the mitochondrial matrix is an important metabolic process that produces NADH and FADH, this NADH is then used by the electron transport chain to produce ATP ²⁰⁶. In this process pyruvate, Oxaloacetate (OAA) and Acetyl CoA form Citrate which is converted to iso-citrate, which in turn is converted to 2-oxoglutarate with the production of 1 CO₂, 2-oxoglutarate is converted to succinate with another CO₂ produced, then succinate is converted to fumarate and then to malate and back to OAA ²⁰⁷. The pyruvate which allows this process to happen is supplied by glycolysis, which oxidises glucose to pyruvate, which is shuttled into the mitochondria via pyruvate translocase to be converted to acetyl CoA ²⁰⁷.

1.4.3 Mitochondrial Cristae: Cellular Respiration

Cellular respiration, which generates ATP is the reason for the nickname “The Powerhouse of the cell”, given to mitochondria. NADH and FADH₂ that are generated during oxidation of glucose and other carbon energy sources during glycolysis and the TCA are recycled by the mitochondria to enable continued glycolysis and TCA cycle activity ²⁰⁸. High energy electrons from NADH and FADH₂ are passed through four respiratory complexes in the mitochondrial electron transport chain ²⁰⁸. Three of these respiratory complexes act also as proton pumps which translocate protons from the mitochondrial matrix to the intermembrane space ²⁰⁹. To generate an electrochemical gradient ATP synthase is used to generate ATP from ADP and inorganic phosphate (Pi) through a process called oxidative phosphorylation (OXPHOS) ²⁰⁹. Possibly the second most well-known function of the mitochondria is apoptosis, which is regulated and initiated by the release of cytochrome C into the cytosol ²¹⁰. Much of the intra-cellular signalling in a cell is due to mitochondrial metabolites, released during electron transport, ATP synthesis and citric acid cycle activity ¹⁹⁹.

1.4.4 Mitochondrial Matrix and Cristae: ROS signalling

Reactive Oxygen Species (ROS) are often considered a by-product of cellular metabolism and aerobic respiration, they are mainly produced in the mitochondria when oxygen is not fully reduced to water but to superoxide ²¹¹. Superoxide can be transformed to hydrogen peroxide or, in the presence of iron and copper, form the highly aggressive hydroxyl radicals ²¹². These ROS are unstable and highly reactive and can damage the cell's macro molecules through oxidation ²¹¹. Cells have a strong antioxidant defence system, consisting of antioxidant enzymes, such as superoxide dismutase, catalase and a number of peroxidases, and non-enzymatic oxidants such as vitamins A, C and E and polyphenols. If the amount of ROS overwhelms the antioxidant defence system, the cell will suffer oxidative damage leading to mtDNA, nuclear DNA mutations and possibly cell death ^{213,214}.

ROS also play an important role in cellular signalling and regulation ²¹³. Low doses of ROS such as hydrogen peroxide and super oxide are involved in basal cell signalling and in stress

response signalling ^{168,215}. ROS are important in the inflammatory response and injury repair due to their pivotal role in cell growth, adaption and cellular death ²¹⁴.

1.4.5 Mitochondrial Matrix: Biosynthesis pathways

The mitochondria are involved in nuclear communication, protein degradation/production, and signalling. However, they can also be considered to be metabolic hubs. Biosynthetic pathways in the mitochondrial matrix produce pyrimidines (uridine, cytosine and thymine) ²¹⁶, CO₂, CoA (Co-enzyme A), Heme A, the respiratory complexes (COX – cytochrome C oxidase, ATP synthase). They facilitate fatty acid and cholesterol synthesis, and the first steps of urea synthesis as well as several other factors involved in anabolic metabolism ^{209,217–220}. Disruption of any of these pathways can cause serious disease in humans such as mitochondrial neuromuscular disorders, mtDNA depletion syndrome (MDS), neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP) and maternally inherited Leigh's syndrome (MILS) ²¹⁸.

1.4.6 Mitochondria and cancer

Altered energy metabolism such as a switch from mitochondrial oxidative phosphorylation to predominantly glycolysis is now regarded as one of the hallmarks of cancer, although most cancer cells retain the ability to use oxidative phosphorylation ^{8,77,78}. Apoptosis is dependent on mitochondrial function, and it is a fundamental feature of cancer cells to evade it. Mutations in the mtDNA can lead to cancer, and cancer cells use less aerobic respiration and more glycolysis than non-cancer cells, although there is incomplete glycolysis of glucose in some cancer cells ^{8,77,78,221}. Many cancer types have complete function of their mitochondria, but they choose instead to utilise glycolysis as their sole source of energy ⁷⁸. In brain cancers inhibiting glycolysis induces higher rates of oxidative phosphorylation, which could deprive cancer cells from their ability to respire and may prevent the formation of tumours ^{222,223}.

1.4.7 Mitochondrial Networks

Mitochondria are often not singular discrete objects, but can form multibranched networks within a cell, and their structure can tell us much about the health of a cell ²²⁴. These networks act to dampen biochemical fluctuations, support active metabolism and aid calcium transport ^{224,225}. Full mitochondrial networks are associated with increased energy production, protection against apoptosis and increased cellular proliferation ^{224,226}. Mitochondrial networks are formed by mitochondrial fusion processes and degraded and maintained with mitochondrial fission processes ^{168,227}. Punctate and dispersed mitochondrial networks are associated with apoptosis and cellular stress ²²⁸. In ρ^0 cells there is no mitochondrial network, the mitochondria themselves are swollen /enlarged and without cristae ²²⁹.

Mitochondrial cybrids are cells where the mitochondria are removed and replaced with mitochondria from a different nuclear background. These cybrids are used to evaluate mitochondrial to nuclear crosstalk in tumour formation and other tumour properties ²³⁰. In experiments where mitochondria from a non-cancerous cell were supplanted into a cancerous cell, crosstalk from the non-cancerous mitochondria suppressed oncogenic pathways and inhibited metastasis ²³⁰. Non-cancerous mitochondria also increased the level of mitochondrial dependant energy production in cancerous cells ²³⁰.

1.5 Cellular stressors

1.5.1 Rho Zero (ρ^0) cells

First generated in 1989 by King and Attardi these human cells lack mitochondrial DNA and rely on pyruvate and uridine supplementation in order to survive ²³¹. They have no respiratory chain and are primarily generated by low dose ethidium bromide exposure ²³¹. Mammalian ρ^0 cells were first created in the 1980s ²³¹ many years after the equivalent *Saccharomyces cerevisiae* petite cells with missing or mutated mtDNA were created in 1968 ²³².

When mtDNA is completely removed from a cell, the cell is referred to as ρ^0 and these cells develop swollen defective mitochondria and only carry out glycolysis as the mitochondrial

electron transport chain and oxidative phosphorylation are absent ²³¹. The ρ^0 model is the most extreme form of mtDNA damage a cell can sustain. These cells still carry out glycolysis which occurs in the cytoplasm and maintain biosynthetic pathways²²⁹.

Glycolysis in ρ^0 cells will only continue in the presence of supplemented pyruvate because all the pyruvate generated during glycolysis (Figure 1.1) must be reduced to lactate to recycle the NADH, generated during glycolysis, into NAD⁺ and ensure continued glycolysis ²³³. This does not leave any pyruvate to enter the citric acid cycle to generate intermediaries for the many biosynthetic pathways that occur in the mitochondrial matrix ⁹.

Uridine is a precursor to the pyrimidines (cytosine, thymine and uridine) necessary for producing DNA/RNA and needs to be supplemented to circumvent the inability of ρ^0 to synthesise pyrimidine. Pyrimidine synthesis requires the activity of the enzyme dihydroorotate dehydrogenase (DHODH), located at the outer surface of the inner mitochondrial membrane, which catalyses the fourth step in pyrimidine biosynthesis ²³⁴. DHODH mediates the oxidation of dihydroorotate to orotate, electrons from this are transported to complex 3 using co-enzyme Q (CoQ) ^{143,231}. DHODH thus requires a continued supply of coenzyme Q, ²²⁹. The inability to re-oxidise coenzyme Q because of the lack of electron transport, makes ρ^0 cells incapable of synthesising pyrimidine precursors ²¹⁶.

In the absence of oxidative phosphorylation ρ^0 cells can process glutamine through a modified reversed TCA cycle that results in the production of acetyl CoA and intermediaries for biosynthesis ²³⁵. Anomalies in a ρ^0 cells processing ability are prime candidates for stress response signals that cause the uptake of mitochondria.

1.1.1.1 Production of ρ^0 cell lines

The two main methods of ρ^0 production are those that use ethidium bromide (EtBr) and those that use endonucleases targeted to the mitochondrial genome.

Ethidium Bromide (EtBr)

Many people in the scientific community regard EtBr as a hazardous substance due to its ability to intercalate strongly with double stranded DNA ²³⁶. It has been used primarily for gel electrophoresis and also in plants as a mutagen to cause genetic mutations ²³⁷. However, it is also used for anti-trypanosomal treatment for African cattle by inhibition of nuclear replication²³⁸.

Ethidium bromide has been used in the production of ρ° and petite cells since they were first produced in 1968 ^{239,240}. Ethidium bromide is a positively charged molecule and thus is attracted to both the mitochondrial membrane and negatively charged DNA. When it comes into contact with mtDNA it interferes with replication and therefore mtDNA is lost after a series of replications ^{241,242}. It is believed that ρ° cells generated via low dose EtBr over 4-6 weeks have also accumulated a selection of nuclear mutations throughout treatment ²⁴³. This is because EtBr also interferes with nuclear DNA replication, albeit at higher concentrations than used to generate ρ° lines. Therefore, nuclear mutations caused by EtBr cannot be ruled out ²⁴¹.

Figure 1-1: Mitochondrial metabolism and glycolysis in cells with (A) and without mitochondrial DNA (B). Q depicts coenzyme Q, I, II, III and IV denote the mitochondrial respiration complexes 1 through 4. A) Wild type metabolism. B) ρ^0 metabolism. See text for further explanation of metabolic processes in both cell types.

Endonucleases

The other method of ρ° production is the use of a mitochondrially targeted endonucleases. Endonucleases encoded for on the mutant uracil-N-glycosylase (mUNG) plasmid have been used in recent years to create ρ° lines²⁴⁴. The mUNG plasmid encodes a Y147A mutant uracil-N-glycosylase which has been shown to decrease mitochondrial copy number to undetectable (ρ°) levels utilising a mitochondrial matrix targeting sequence²⁴⁴. Mitochondrial nucleoids begin to disappear 48 hours post transfection and therefore this method allows for the generation of ρ° lines within a matter of weeks²⁴³. mUNG causes single stranded DNA breaks and a-basic sites which are not repaired by mtDNA repair mechanisms²⁴⁵. Due to their specificity this method should be less mutagenic than traditional EtBr ρ° production.

Restriction endonucleases such as mitochondrially targeted ECOR1 can be used to create ρ° lines but with less efficacy than mUNG²⁴⁴. The modified ECOR1 is targeted to the mitochondrial DNA and cleaves it causing sufficient damage to allow for total degradation of the mitochondrial DNA by the cells own mitochondrial endonucleases²⁴¹.

There are other methods of depleting mitochondrial DNA such as dideoxycytidine (DDC) treatment DDC is a cytidine analogue which also blocks mtDNA replication, however no ρ° has yet been produced using DDC alone²⁰⁵.

This PhD project has used a murine cancer ρ° model. Murine cancer cell lines (4T1 or B16) depleted of mtDNA by a low sustained dose of EtBr, grow slower and require supplemental pyruvate and uridine to survive²²⁹. Removal of uridine and pyruvate results in cell death over 3-5 days¹⁴¹.

1.5.2 Strengths and weaknesses of ρ° lines

The main function of ρ° cells other than in mitochondrial transfer projects has been to assess normal and abnormal mitochondrial functions that are dependent on mtDNA²⁴¹. Because ρ° cells lack mtDNA, detecting foreign mtDNA is relatively easy. One of the main weaknesses of ρ° models is that they grow at about half the speed of the parental wildtype

cells ²⁴⁶. Another major limitation is that the p^o model is artificial and cannot be directly translated to clinical situations as there are no known physiological conditions where mtDNA number is depleted to zero.

1.6 Chemotherapy as Cellular Stress

At the beginning of the 20th century Paul Ehrlich coined the term chemotherapy, as the use of chemicals to treat disease ²⁴⁷. Cytotoxic chemotherapies against cancer work best on rapidly dividing cells; however, tumours consist of many different phenotypes because of spatial and other environmental constraints and not all cells will be dividing rapidly.

1.6.1 Doxorubicin

Doxorubicin is used to treat a wide range of malignancies such as breast, ovarian, sarcomas, lymphomas and acute leukemias, and works in several different ways²⁴⁸. Doxorubicin is a type of anthracycline which are antibiotics that interfere with the DNA replication machinery ²⁴⁹. Doxorubicin is metabolised to doxorubicinol in the liver ²⁴⁹. Doxorubicin intercalates double stranded DNA²⁵⁰. The peak saturation concentration of doxorubicin is 340 μ M, which indicates that 1 molecule is intercalated at every 5th base pair of DNA^{249,251}. Doxorubicin binds to and inhibits topoisomerase enzymes 1 and 2 which classifies doxorubicin as a topoisomerase inhibitor ²⁵². Topoisomerase is responsible for the separation of the two strands of DNA during replication ²⁵³.

Doxorubicin-DNA-adducts induce apoptosis which activates the BCL-2/BAK caspase cascade ²⁵⁴. Doxorubicin-DNA-adducts form when doxorubicin is co-administered with non-toxic levels of formaldehyde; these adducts are a more cytotoxic topoisomerase poison than doxorubicin on its own ²⁵⁴. However, doxorubicin lesions can be repaired by PARP-1 thus doxorubicin hyperactivates PARP-1 which leads to autophagy and can lead to a necrosis type cell death while inhibition of PARP-1 inhibition results in apoptosis ²⁴⁹.

Doxorubicin causes the formation of free radicals, which in turn cause more DNA damage which cannot be repaired due to the topoisomerase inhibition action of doxorubicin ²⁵⁵.

Doxorubicin binds to plasma membrane proteins, which causes hydroxyl free radicals which

are responsible for most of the drug's toxic side effects ²⁴⁹. Inhibition of DNA polymerase is also known to be caused by doxorubicin ²⁵⁵.

1.6.2 Cisplatin

Cisplatin was first derived in 1845 and identified as an anticancer agent in the 1960's²⁵⁶. It reacts readily with proteins, RNA, membrane phospholipids, cytoskeletal filaments and thio-containing molecules such as glutathione, cystine or methionine ²⁵⁷. Cisplatin acts primarily on DNA and causes DNA damage. However, less than 1% of the bioactive cisplatin available to the cell interacts with DNA and causes DNA damage ²⁵⁸. The cisplatin that does interact with DNA binds to purines and forms intra- and inter-strand crosslinks ^{258,259}. These crosslinks/adducts trigger responses such as replication arrest, transcription inhibition, cell cycle arrest, DNA repair and apoptosis ^{258,260}. These DNA interactions play a crucial component of cisplatin's antiproliferative activity.

Cancer cells administered with lethal doses of cisplatin have altered transcription only after 48 hours and double stranded DNA breaks are detected after one day ²⁶¹. DNA replication continues for some time after cell replication halts ²⁶². For the cells that can survive, and repair high levels of DNA damage cisplatin is still effective, these cells arrest in G2 at the G2/M transition and undergo apoptosis ^{258,261}. Cisplatin induces both apoptosis and necrosis in the same cultures, apoptosis at low concentrations (<100μM) and necrosis at high concentrations (>100μM)²⁵⁷.

Cisplatin affects the plasma membrane, by interacting with the negatively charged phospholipids and plasmalemmal proteins, reducing the activity of some ion channels and transporters ²⁶³. This causes destabilisation of the membrane and increased fluidity ²⁶⁴. Then apoptosis is induced by plasma membrane disruption triggering the Fas death receptor pathway ²⁶⁴.

Interactions with the phospholipid membrane is the cause of most cisplatin related toxicity ²⁶³. Cisplatin toxicity mainly affects inner ear and renal tubules ²⁶⁵. It stresses different

organelles such as mitochondria, lysosomes, endoplasmic reticulum, nucleus, cell membrane, cytoskeleton and is found in cytosol ²⁶⁵.

The cancer cell metabolic switch from oxidative phosphorylation to glycolysis is facilitated by the upregulation of pyruvate dehydrogenase kinase (PDK), this inhibits pyruvate dehydrogenase which is responsible for the movement of pyruvate into the mitochondria to be oxidised to acetyl CoA. With no pyruvate entering the mitochondria the result is sole reliance on glycolysis, increased lactic acid production and high glucose and glutamine demands ²⁵⁸. Oxidative phosphorylation is needed to activate many cellular death mechanisms ⁶². Drugs that block PDK activity force cells into oxidative phosphorylation and result in cell death via P53 ²⁶⁶. This is due to a reversal of mitochondrial oxidative phosphorylation suppression, resulting in reduced cancer cell viability, lactate production and increased apoptosis. Cisplatin blocks PDK activity ²⁶⁷. This forced shift from glycolysis to mitochondrially generated energy production in cancer cells results in apoptosis ²⁶⁶.

1.6.3 Chemotherapy resistance

There are many mechanisms by which cells become resistant to chemotherapy. The tumour microenvironment can confer resistance by inhibiting drug penetration ¹⁹⁰. The TME also aids in the regeneration of a tumour following treatment ¹⁹⁰. The abnormal vasculature of the tumour causes low drug penetration due to areas of hypoxia ¹⁹⁰. As most drugs enter the cell via diffusion a frequent method of chemoresistance is drug efflux where they are pumped out again ²⁶⁸. p-glycoprotein is one of the best characterised drug transporters associated with chemoresistance ²⁶⁹. P-glycoprotein is part of the ATP binding cassette (ABC) family and aids to reduce the intra cell concentrations of many drugs to sub therapeutic levels ²⁶⁹. Most if not all chemoresistance is caused by mutations and tumorigenic mechanisms such as genome instability are key to this.

Doxorubicin Resistance

Resistance to doxorubicin can arise, via cell and extracellular matrix interactions that play a role in acquisition of doxorubicin resistance ²⁷⁰. Autophagy plays a role in doxorubicin resistance and some autophagy targeting treatments have some desensitising effect ²⁷¹.

Drug resistance autophagy is different from starvation autophagy, and suppressors of starvation induced autophagy Beclin1 and Atg 7 had no effect on drug resistance ²⁷². Activation of NRF pathways correlated with acquisition of doxorubicin resistance ²⁷³. The loss of PTEN is also associated with resistance to doxorubicin ²⁷⁴.

Cisplatin resistance

Colorectal, lung, and prostate cancers are intrinsically resistant to cisplatin and ovarian cancers often acquire resistance after exposure over time ²⁷⁵. Cisplatin resistant cells have lowered uptake of 2-deoxyglucose compared to cisplatin sensitive cells, they metabolise less glucose and thus will be less affected by the transition to oxidative phosphorylation ²⁶⁷. Decreased expression of *Glut1* and overexpression of *Sirt1* in cancer cells is correlated with cisplatin resistance use ²⁶⁷. SIRT1 shifts energy metabolism back to oxidative phosphorylation resulting in reduced glucose use ²⁶⁷. SIRT 1 is a histone deacetylase and its upregulation plays a role in cancer cell growth and chemoresistance ²⁷⁶.

Upregulation of *Bcl-2* is associated with cisplatin resistance via inhibition of BAX ²⁷⁷. Cisplatin induced apoptosis is dependent on BCL-2 and BAX/BAK induction and thus resistance is dependent on their repression ⁶². BAX and BAK are crucial in apoptosis and are also activated in response to endoplasmic reticulum stress ^{62,278}.

Mitochondrial DNA mutations are involved in cisplatin resistance ²⁷⁵. Crosstalk between the mitochondria and nucleus and rewiring of cellular metabolism allow cells to escape from cisplatin and induced metabolic stress ²⁷⁵. Induction of the ER stress response and activation of p38 protect cancer cells from cisplatin and doxorubicin induced apoptosis ²⁷⁹.

1.7 Cellular stress responses and survival

1.7.1 Endoplasmic Reticulum (ER) stress response

The ER is a membrane bound organelle responsible for the folding and modification of proteins. Under pathological conditions, the ER lacks sufficient resources to perform its role. Aggregation of unfolded and misfolded proteins in the ER lumen leads to the unfolded protein response (UPR) ²⁸⁰. ER stress and the UPR induce apoptosis ²⁸⁰. In malignant

tumours this response can also induce autophagy²⁸⁰. ROS are also produced by ER stress, this leads to the degradation of cellular organelles²⁸⁰. Sustained ER stress can trigger an inflammatory response²⁸⁰. MAPK signalling is activated by ER stress and MAPK is involved in resistance to cisplatin and doxorubicin²⁷⁹. Cisplatin and doxorubicin induce the ER stress response and ER induction activates p38²⁷⁹. Inhibition of p38 removes effects of ER stress induced resistance to apoptosis from cisplatin and doxorubicin treatment²⁷⁹.

1.7.2 Inflammatory Stress Response

Well established in normal tissues, inflammation is also essential in tumour development. ER stress and the UPR can both induce inflammation²⁸¹. Inflammation and infection can also contribute to tumorigenesis²⁸². This inflammation then changes the TME which contributes to tumour maintenance.

Non-cancerous inflammation was first described millennia ago as redness, warmth, pain, swelling and loss of function²⁸³. Pathogen associated molecular patterns (PAMPS) and Danger associated molecular patterns (DAMPs) are recognised via pattern recognition receptors (PRRs) on antigen presenting cells causing TNF- α and IL-1 β to be secreted which leads to activation of macrophages and neutrophils^{283–285}. Metformin (a blood glucose reduction agent used to treat diabetes mellitus type II) inhibits NF- κ B and results in a weaker inflammatory signal, which decreases tumour growth in initial tumour formation^{286,287}. NF- κ B signalling is a pivotal mediator of inflammatory responses, it induces the expression of cytokines and chemokines and is key in inflammasome regulation²⁸⁸. NF- κ B plays a key role in the survival, activation and differentiation of inflammatory T cells and innate immune cells^{288,289}.

1.7.3 Mitochondrial Transfer

Transfer of respiratory competent mitochondria into cells with respiratory deficient mitochondria has been associated with cellular repair and restoration of mitochondrial function¹⁶⁷. Both *in vitro* and *in vivo* studies have identified mitochondrial transfer between cell types in the last 15 years. The first studies *in vitro* between two different cell types

showing the transfer of organelles via tunnelling nanotubules were conducted by Rustom *et al.* in 2004 ²⁹⁰. The first studies of mitochondrial transfer, between cancer cells and ρ° cells, were published by Spees *et al.* in 2006. These studies initiated the notion that either whole mitochondria or mtDNA could be transferred between cells, with restoration of respiration in injured cells a possible mechanism of cellular repair ¹⁶⁷. These *in vitro* models allow for easy manipulation of a system where many variables can be controlled.

In vivo models have begun to show how this phenomenon could be clinically relevant. Many of the initial papers that showed mitochondrial transfer between mammalian cells *in vivo* were mostly under pathophysiological conditions, such as mitochondrial transfer that historically occurred (tested via deep sequencing) in a canine venereal tumour originating 10000 years ago ^{291,292}. Research has also been published that demonstrates mitochondrial transfer with lung injury ²⁹¹. These studies all contribute to the growing evidence that this is a process that is likely to occur across all mammals including humans.

Our group was the first to demonstrate mitochondrial transfer *in vivo* by ρ° tumour implantation into mice, which showed tumour formation by both B16 ρ° melanoma and 4T1 ρ° breast cancer only after a lag phase and the acquisition of mtDNA correlated with restoration of mitochondrial respiration ²⁰¹. The Berridge group in conjunction with the McConnell group have since explored the phenomenon of mitochondrial transfer further, investigating mitochondrial transfer in neural cells and establishing robust, quantitative methods of transfer detection in non ρ° cells ^{141,293}. Mitochondrial transfer has been detected in stressed and non-stressed ρ° lines but has only been shown to be sustained under metabolic stress ^{141,293}.

The primary candidates for mitochondrial donor cell types are MSC, pericytes, endothelial cells and monocytes, and have all been implicated as potential universal donors ¹⁶⁸. MSC are effective mitochondrial donors in models of human disease ²⁹⁴. Given that MSC from different tissues can differentiate into cells from other tissues, it is interesting that MSC from different origins have different mitochondrial transfer potential. Studies have been published using mitochondrial labelling dyes and cell tracking stains, that assessed

mitochondrial transfer via flow cytometry and fluorescence imaging. Production of mitochondrial ROS and oxygen consumption levels were also assessed ²⁹⁴. These studies concluded that MSC from adipose tissue and bone marrow had higher rates of mitochondrial transfer than dental pulp and Wharton's jelly MSC ²⁹⁴. However dental pulp and Wharton's jelly generated MSC suppressed mtROS levels in stressed recipient cells²⁹⁴. This points to an inverse correlation between transfer efficiency and recovery of mitochondrial respiration ²⁹⁴. Bone marrow MSC are known to be capable of repairing wounded epithelial cells in the lung through cytoplasmic and mitochondrial donation; the mechanism of this is still unknown ²⁹⁵. MSC donate mitochondria to epithelial cells of the airway, this rescues mitochondrial respiration in models of lung injury ²⁹⁶. Mitochondrial transfer from MSC to corneal epithelial cells is enhanced by rotenone induced oxidative stress ¹⁷⁰. Physical separation between cell types appears to stop mitochondrial transfer¹⁷⁰. Mitochondrial transfer provides protection from oxidative stress induced mitochondrial damage in several models ¹⁷⁰.

Data from the McConnell and Berridge groups show transfer of mitochondria *in vitro* from pericytes ¹⁸⁹ but no studies as of yet have shown them as an *in vivo* donor, potentially due to their lowered stem abilities *in vivo* compared to *in vitro* ¹⁸⁹. Endothelial cells have also been shown *in vitro* to transfer mitochondria and confer chemoresistance to recipient cells via tunnelling nanotubes (TNTs) ¹⁹³.

These studies provide evidence that mitochondrial transfer occurs in response to stress and that this transfer occurs with physical proximity and not via free floating mitochondria. There is also evidence that chemotherapy treated cells receive mitochondria from endothelial cells and that this reduces apoptosis ²⁹⁷. This means that there could be much wider implications for mitochondrial transfer than currently understood.

There are several mechanisms by which mitochondrial transfer can occur, tunnelling nanotubes are the most prominent theory, although other methods such as cellular associated particles and cellular fusion have also been proposed ^{291,298}. Transfer of plastids in plants has been known to happen for decades ²⁹⁹. This, combined with the mammalian

cell evidence described above, indicates a potential evolutionarily conserved process that must perform some vital function for it to have persisted for so long ²⁹⁹.

1.7.3.1 Mitochondrial Transfer mechanisms:

Tunnelling nanotubes

First described in 2004, tunnelling nanotubes (TNT) are tubes or channels that connect the cytoplasm of two or more cells ^{290,300}. TNTs are diverse transport mechanisms that can transfer proteins, lipids, ions, cytosolic molecules, plasma membrane components, mRNA, miRNA organelles and viruses ^{193,300,301}. There are two main types of TNTs; Type 1 TNTs are larger and longer with a large clear cytosolic tunnel, these contain microtubules and microfilaments ³⁰⁰. Type 2 TNTs are smaller, shorter, contain F-actin and are tubulin deficient ³⁰⁰.

Forming after large membrane adhesions between cells TNTs play an important role in intercellular communication ^{193,302}. They have been observed in many cell types *in vivo* and *in vitro* and have been observed in developing embryos ^{302,303}.

TNTs do not have contact with the substrate of the culture but can 'hover' in the medium and they can vary widely in diameter ^{290,303}. TNTs can enable the propagation of depolarising signals for up to 70µm ³⁰⁴.

With regards to mitochondrial transfer mechanisms, the strongest evidence is for transfer via TNT. TNTs have been shown to rescue UV treated apoptotic pheochromocytoma (PC) cells by mitochondrial donation from healthy PC cells at 24 hours ²⁹⁸. TNTs are present in co-culture situations as early as 45 minutes after mixing between bone marrow MSC and lung tissue MSC or bronchiolar lavage MSC ²⁹⁵. Mitochondrial transfer itself is associated with an increase in TNT formation between MSC and corneal epithelial cells ¹⁷⁰. TNTs are a known method of removing aggregated α synuclein compounds in Parkinson's disease ³⁰⁵. They have been shown to mediate transfer of mitochondria towards cancer cells associated with a more invasive tumour phenotype *in vitro* and *in vivo* and TNT mediated transfer has been shown to modulate chemoresistance in doxorubicin treated cells ^{193,300}. Importantly these are not just associations - anti-microtubule compounds such as cytochalasin and β alkaloids

can inhibit and/or decrease mitochondrial transfer implying that type 1 TNTs play a role in mitochondrial transfer ³⁰⁰. F-actin and α -tubulin containing TNTs transport mitochondria and other genetic material between laryngeal cell carcinoma cells ^{306,307}. Additionally, knockdown of *Miro1* which is involved in microtubule formation is associated with decreased TNT formation and decreased mitochondrial transfer, conversely an upregulation of *Miro1* increased transfer ³⁰⁰.

Extracellular vesicles; microvesicles, macrovesicles and exosomes

Extracellular vesicles have been emerging as an important cellular communication tool ³⁰⁸. Vesicles are a heterogeneous group of cell derived membranous structures comprising exosomes and microvesicles ³⁰⁹. Exosomes originate from the endosomal system and microvesicles are shed from the plasma membrane ³⁰⁹. They are often associated with apoptosis, where cells release membrane bound sacs into the microenvironment ³¹⁰.

Vesicles are present outside the cell and are of varying size and produced by different mechanisms. Exosomes are small (less than 150nm), while extracellular vesicles are larger 200-500 nm and up to 1-10 μ m in cancer cells ^{300 311}. All membrane vesicles are of endosomal or plasma membrane origin ³¹¹. These vesicles are released by cells into the microenvironment where they come into contact with other cells ³¹¹. This is very important for cellular communication and as a vehicle for transfer of cytosolic proteins, lipids, RNA and extra chromosomal DNA between cells ^{311,312}. The mRNA found in the vesicles can be translated into protein by recipient cells ^{311,313,314}. Mitochondria and other organelles have also been found in, and transferred by, the vesicles ³¹³. Extracellular vesicles are likely to be involved in the regulation of immune responses, as most immune cell types release extracellular vesicles of some form ³¹². Vesicles are found in plasma and other bodily fluids such as breast milk, semen, saliva, urine and sputum ³¹². They appear to be conserved evolutionarily as both eukaryotes and prokaryotes produce some sort of vesicle³¹⁵.

Cellular and cytoplasmic fusion

Cellular fusion is mitochondrial transfer in the way that demolishing a house is knocking down a wall or two, that is to say yes mitochondria are 'transferred' but so is everything else in the cell of which mitochondria are only a small part. Fusion is one of the lesser

documented mechanisms by which mitochondrial transfer has been reported to occur. However, it is important to talk about it because of the volume of mitochondria and other material that can be 'transferred' ^{297,316}. Possibly the best-known version of cellular fusion is when a spermatozoal nucleus and oocyte meet and fuse forming a zygote ³¹⁷. Aside from sexual reproduction, fusion is a normal part of the life of some cell types such as myocytes and neurons, which fuse partially or wholly as part of their normal cellular processes ^{318,319}. Cytoplasmic fusion has also been shown to be a mechanism of protein transfer in donor/host transplant situations ³²⁰. In myocytes the process of forming sarcomas by fusion is regulated by a host of genes and proteins including myogenin, JAM-B and JAM-C, and c-MET ^{318,321,322}. Neural cells are shown to fuse in culture frequently; these cells that fuse initially have 2 nuclei but after extended culture only 1 nucleus remains ³²³. In pathological tissues such as cancer, fusion is an important process with multinucleate giant cells (MGC) associated with poorer prognosis for patients ^{324–327}. These MGC are thought to be products of the fusions of macrophages with cancer cells and are also associated with immunological and non-immunological stress ^{327,328}.

Gap Junctions:

Gap Junctions are specialised transmembrane regions that contain collections of transmembrane channels which link adjacent cells ³²⁹. Gap Junctions allow for the direct cell-cell transfer of ions, small molecules and metabolites ^{329,330}. Connexins form channels between neighbouring cells and are very important in cancer cell communication ^{329–332}. Gap junctions are highly associated with mitochondrial transfer. They appear to play an important role in this process, but it is unlikely that mitochondria actually pass through them due to their size. However, the inhibition of gap junctions has been shown to decrease mitochondrial transfer ^{295,333}. This could be because of the introduction of calcium which inhibits binding of MIRO1 to KIF5 kinesin motor ³⁰⁰. Mitochondrial transfer from bone marrow MSC to leukemia cells appears to be dependent on calcium exchange through the generation of connexin 43 containing gap junctions ³⁰⁰. Retinoid acid (a gap junction potentiator) also increased the quantity of mitochondria transferred from bone marrow derived MSC to motor neurons in a rat spinal injury model ³³³. Whether gap junctions are a primary method of transfer or simply facilitate another form of contact dependant transfer

i.e., TNTs³⁰⁶ or cellular fusion remains to be confirmed, however they look to play some sort of role in the transfer of mitochondria from one cell to another.

1.1.1.2 Signals of transfer and survival

We hypothesised that if mitochondrial transfer is a stress response, then there must be some sort of signal that the stressed cells emit in order for transfer to occur. Many other stress responses have a signalling component, in particular ROS signalling is important in stress responses and are generated via abnormal mitochondrial functioning¹⁶⁸. There have been a few proposed mechanisms that could be signals of mitochondrial transfer, for example the overexpression of *Miro1* in MSC caused increase of mitochondria transferred via TNT in stressed epithelial cells³³⁴. Humanin, which is mitochondrially encoded and a key regulator of mitochondrial – nuclear crosstalk, has been associated with a wide range of diseases and tumour progression^{335,336}. Humanin has also been implicated as a potential mitochondrial transfer signal³³⁷. However, there may be many mechanisms by which mitochondrial transfer occurs and thus many initiating signals.

1.8 MinION cDNA sequencing for Differential expression analysis:

This theses used MinION sequencing to answer questions about interactions occurring between cell types. MinION is a third-generation sequencing technology that uses biological pores to directly sequence DNA or RNA strands, it was first released commercially in 2014³³⁸. cDNA (complimentary DNA) is DNA produced from reverse transcribing mRNA, it is more stable than pure RNA and is often used in transcriptome analyses instead of unstable native RNA which still includes rRNA transcripts and other non-polyadenylated RNAs. Differential transcriptomics is a new area for long read sequencing but has particular advantages for identifying differentially spliced transcripts as the read length is long enough for full transcripts^{2 70,271}. MinION technology is highly valuable in the identification of onco-fusion genes and transcripts however is unsuitable for SNP detection³³⁹. Previous studies on the MinION have found that MinION reads are an order of magnitude longer than Illumina reads³⁴¹.

1.8.1 MinION long read vs short read transcriptomics

With long read technology emerging as a cheaper and effective alternative to short read sequencing more and more people are opting to use it for their genomics and transcriptomics projects. New advances in long read technology are overcoming early limitations in accuracy and throughput and new analysis technologies are constantly being developed to better take advantage of this ³⁴². Long read sequencing has access to high GC content regions granting resolution to complex regions that short read sequencing cannot penetrate ³⁴³. Full length transcriptomics of mRNA can look at isoforms and alternative splicing ³⁴³. Some benefits for long read sequencing is that it improves de novo assembly of genomes ³⁴² and it can be used directly for long range characterisation of methylation patterns ³⁴³. However, short read is cost effective, accurate and has lots of analytical tools ³⁴². Most analysis software and pipelines designed for long read data are designed for base calling, error correction and polishing, detecting structural variation, detecting base modifications and in particular alternative splicing and isoform detection ³⁴². Long read transcriptomic analysis still uses tools designed for short read sequencing such as limma, edgeR and DESeq2 ³⁴². Short read transcriptomics have 20-50M reads per sample with long read sequencing averaging between 4-10M ³⁴⁴.

1.9 Aims:

I hypothesised that when under stress ρ° cells would signal other cells in the microenvironment that would enable cellular survival and that these signals could be conserved across different stresses. I wanted to understand if induction of stress in ρ° cells under pyruvate and uridine deprivation initiated mitochondrial transfer and whether this was a model that that was more broadly applicable.

To do this I set out to:

- Establish a TME and ρ° co-culture setting to assess survival of cells under stress.
- Observe and understand the interactions between ρ° cancer cells and cells of the TME under stress.
- Determine any signals the ρ° cells elicit under stress that may encourage interactions with the TME.
- Assess the differences and similarities in how cancer cells that are either stressed ρ° cells and wild type cells treated with chemotherapies interact with the TME.
- Assess the differences and similarities in the signal's these stressed ρ° and treated cancer cells elicit.

2 Materials and Methods:

2.1 Materials:

Name	Source
Nunc TM EasYFlask TM 75cm ² Nunclon TM Delta Surface	Thermo Scientific
Falcon 15ml High-Clarity Polypropylene Conical Tube	Falcon
Nunc TM EasYFlask TM 25cm ² Nunclon TM Delta Surface	Thermo Scientific
Corning R 175cm ² Cell Culture Flask	Corning
Disposable Scalpel	Kai Medical
Pyrex Cloning Cylinders	Corning Inc
BD 10ml Syringe	BD
Cover Slips 25mm round	Menzel-Glaser
Microscope Cover Glasses 22x60 mm	Marienfeld
50ml Polypropylene Conical Tube	Falcon
MicroAmp Optical 96-Well Reaction Plate	Applied Biosystems
0.2ml THIN WALL, CLEAR, PCR Tubes	Axygen INC
Qubit assay tubes	Invitrogen
MicroAmp Optical 8-Tube Strip (0.2mL)	Applied Biosystems
MicroAmp Optical 8-Cap Strip (0.2mL)	Applied Biosystems
0.2 mL SnapStrip II PCR Tubes, Natural	SSIbio
Microtubes 1.7mL	Axygen INC
Microtubes 2mL	Axygen INC
12cm Tissue Culture Dish	Falcon
1ml Syringe	BD
15ml High-Clarity Polypropylene Conical Tube	Falcon
Fisherbrand Superfrost Microscope slides	Fisher Scientific
Acrodisc Syringe Filters	Pall Corporation
Tissue culture plate 96 well Flat bottom with Low evaporation Lid	Falcon
Tissue culture plate 12 well Flat bottom with Low evaporation Lid	Falcon
Tissue culture plate 6 well Flat bottom with Low evaporation Lid	Falcon
Tissue culture plate 24 well Flat bottom with Low evaporation Lid	Falcon
MicroAmp Optical Adhesive Film	Applied Biosystems

2.1.1 Reagents:

Name	Source
Nuc Blue	Invitrogen
Penicillian Streptomycin	Gibco
Collagenase type II	Gibco
Dulbecco's Modified Eagle Medium +Glucose + Pyruvate - L-Glutamine	Gibco
Dulbecco's Phosphate Buffered Saline	Gibco
GlutaMAX -L (100X)	Gibco
Trypsin EDTA 0.05%	Gibco
Viafect	Promega
Propidium Iodide	
LongAmp taq 2X master Mix	New England Bio Labs
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen
Deoxynucleotide(dNTP) Solution Mix	New England Bio Labs
10mM dNTP Mix	Invitrogen
0.1M DTT	Invitrogen
5X RT Buffer	Thermo Scientific
SuperScript Reverse Transcriptase	Invitrogen
Exonuclease 1	New England Bio Labs
Quick-Load 2 Log DNA Ladder	New England Bio Labs
Trackit 100bp DNA Ladder	Invitrogen
10x Blue Juice Loading Buffer	Invitrogen
Trackit 1Kb DNA Ladder	Invitrogen
Trackit Cyan/orange Loading Buffer	Invitrogen
PowerUp SYBR Green Master Mix	Applied Biosystems
Quantiflour ONE dsDNA System	Promega
Dulbecco's Modified Eagle Medium +Glucose - Pyruvate - L-Glutamine	Gibco
Dulbecco's Phosphate Buffered Saline	Gibco
Kapa HiFi Hotstart ReadyMix	Roche
Fetal Bovine Serum	Gibco

2.1.2 Kits

Name	Source
Quick-DNA Miniprep Kit	Zymo Research
Dneasy Blood and Tissue Kit	Quiagen
DNA Clean and Concentrator	Zymo Research
Quick-DNA Microprep Kit	Zymo Research
RNeasy Mini Kit	Quiagen
RNeasy Plus Micro Kit	Quiagen
PureLink Hi Pure Plasmid Maxiprep kit	ThermoFisher, USA
PureLink Hi Pure Precipitator Module	ThermoFisher, USA
Qubit dsDNA BR Kit	ThermoFisher, USA
MycoAlert Mycoplasma Detection Kit	Lonza

2.1.3 Chemicals

Name	Source
Cisplatin	Cayman Chemical, USA
Ethidium Bromide	Sigma Life Sciences
Doxorubicin Ebewe 1 vial 200mg in 100mL	Sandoz
Sodium Chloride	Sigma Life Sciences
(3-Aminopropyl)triethoxysilane	Aldrich
Acetone, analytical grade	Scharlau
Ethyl alcohol, Pure	Sigma Aldrich
Ethanol absolute anhydrous	Carlo Erba
Methanol, analytical grade	Scharlau
Sodium Pyruvate (100mM)	Gibco
Geneticin Selective antibiotic (G418 Sulfate)	Gibco
Nuclease-Free Water (not DEPC treated)	Ambion
DEPC- Treated Water	Ambion
DBL Cisplatin Injection 100mg in 100mL	Onco-Tain
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
2-Mercaptoethanol	Gibco
6-Thioguanine	Sigma Life Sciences

2.1.4 Equipment

Name	Source
IncuCyte® S3 Live- Cell Analysis System	Essen Bioscience, USA
FLUOVIEW FV3000 Laser Scanning Microscope	Olympus
Eppendorf® Research® plus pipette variable volume 0.2-2, 2-20 10-100, 20-200, 100-1000 uL	Eppendorff
Eppendorf® Research® pipette variable volume 0.2-2, 2-20 10-100, 20-200, 100-1000 uL	Eppendorff
Thermomixer compact	Eppendorff
Centrifuge 5424	Eppendorff
C1000 Touch TM Thermo Cycler	Bio-Rad
Veriti 96 Well Thermo Cycler	Applied Biosystems
Grant SUB Aqua 12 Plus	ThermoFisher Scientific
HERAcell 150i CO2 incubator	Thermo Scientific
HERAcellCO2 incubator	Heraeus
Megafuge 2.0R	Heraeus Instruments
Liquid Nitrogen	
QuantStudio 7 Flex	Applied Biosystems
Minion Sequencer	Oxford Nanopore
IX51 Microscope	Olympus
U-RFL-T Lamp	Olympus
TH4 – 200 mercury lamp	Olympus
LSR II Benchtop Analyser	BD
LSR Fortessa	BD
Influx Cell Sorter	BD
NanoDrop One	Thermo Scientific
Quantus Fluorometer	Promega
CK2 Microscope	Olympus
FV1200 Confocal Microscope	Olympus
Heat Block	Thermolyne
Coverslip Cell Chamber	Aireka Scientific Company, China
Qubit™ Fluorometer	ThermoFisher Scientific

2.2 Cell culture:

2.2.1 Cell Lines:

Table 2-1: Cell Lines Used in this Research

Line Name	Origin	Strain	Status	Tissue Type
4T1	Murine	Balb	Immortalised	Breast Cancer
B16	Murine	C57BL/6	Immortalised	Melanoma
GI261	Murine	C57BL/6	Immortalised	Glioma
eGFP- Astrocyte	Murine	C57BL/6	Immortalised	Astrocyte
Compact Bone Derived Cells	Murine	C57BL/6 DsRed x Balb F1	Primary	Stromal/osteo
4T1p°	Murine	Balb	Immortalised	Breast Cancer
B16p°	Murine	C57BL/6	Immortalised	Melanoma
eGFP- Astrocytep°	Murine	C57BL/6	Immortalised	Astrocyte

2.2.2 Cell culture Media:

Complete p° Culture Medium

DMEM

10% FBS

1% Glutamax

1% Pyruvate

0.4% Uridine

Deprivation Media (minus Pyruvate and Uridine)

DMEM

10% FBS

1% Glutamax

Primary Culture Medium

DMEM

10% FBS

1% Glutamax at 100X

1% Pyruvate at 100mM

1% of 10,000U/mL Penicillin 10,000ug/mL Streptomycin

Freezing Medium:

Complete Medium for specific cell line

10% DMSO

20% FBS

Penicillin and Streptomycin (PenStrep):

The antibiotic mix PenStrep was not used in cell line culture unless that cell line was co-cultured with a primary culture. When Pen Strep was used it was made up 1% of the final volume of media used. The stock concentration of this was 10,000 Units per mL Penicillin and 10,000 µg/mL Streptomycin and working concentration was at 100 Units per mL Penicillin and 100 µg/mL Streptomycin.

2.2.3 Culture method:

All cell lines used in this project were maintained as adherent cultures. All cells were maintained at 37°C in an humidified incubator with an atmosphere of 5% CO₂. Cells were passaged at 70-80% confluence and re-plated at a suitable confluency level to allow for 3-4 days between passages.

Passaging Adherent cells:

Adherent cell lines were passaged regularly to maintain exponential growth. Once cells had reached 80% confluency media was removed from the culture flask. The cells were washed with DPBS to the same volume as the discarded media. 0.05% Trypsin EDTA was added in sufficient volume to cover the base of the flask, and this was incubated at 37 degrees until cells no longer adhered to the plate/flask. To stop the trypsin reaction complete media (containing FBS) was added. The media/cells/trypsin mix was then removed from the flask/plate and centrifuged for 5 minutes at 1000g. The supernatant was then discarded and cell pellet re-suspend in completed media before cells were split as follows to allow for 3-4

days growth before becoming 80% confluent; Astrocyte ρ° 1/5, Astrocyte wt 1/10, 4T1 ρ° 1/10 4T1 wt 1/40, B16 ρ° 1/10 and B16 wt 1/40.

2.2.4 Mycoplasma Testing:

Approximately every three months a mycoplasma test was performed using the Lonza MycoAlert Mycoplasma Detection kit. Two millilitres of cell suspension taken at passage, were spun in a microfuge for 2 minutes to pellet cells in an Eppendorf tube, 100 μ L of supernatant was added to a fresh Eppendorf tube. Then 100 μ L of reconstituted MycoAlert reagent was added to the sample, incubated at room temp for 5 minutes before luminescence was read. Following this 100 μ L of MycoAlert substrate was added to the sample followed by a 10 min room temp incubation and a second luminescence reading. If the second reading was greater than the first there was mycoplasma contamination.

2.2.5 Cryopreservation:

Cells were centrifuged for 5 minutes at 1000 x g and then resuspended to 1×10^6 cells/mL in freezing medium. 1 mL was then aliquoted into cryovials. Vials were cooled to -80°C using a controlled rate cooler. Once cells had reached -80°C they were transferred to vapour phase liquid nitrogen for long term storage.

2.2.6 Transfection of cell lines:

Plasmids

Table 2-2: Plasmids used in this Thesis

Name	Origin	Inserts	Source
pMA3790,	Alexeyev Lab	EGFP,hUNG Y147A,G418 resistance	Addgene
GFP	Backbone	Ampicillin resistance ,Neomycin resistance	Addgene

Plasmid generation:

Plasmids were prepared from transformed *E.coli* in agar stabs or frozen glycerol stocks in the McConnel laboratory using standard lab protocol as follows. Transformed *E.coli* were cultured overnight on Luria Bertani (LB) agar plates containing 100 $\mu\text{g/mL}$ Ampicillin.

Colonies were picked using a sterile p1000 pipet tip and then inoculated into 2mL sterile liquid LB/Ampicillin in a 15mL tube, which was grown at 37°C for 16hrs under constant shaking at 200 rpm. From this culture 500µL was added to 400mL liquid LB/Ampicillin in a 1L conical flask and incubated at 37°C for 16hrs at 200rpm shaking. Centrifugation at 5000 x g for 10 minutes was used to pellet the bacteria. The PureLink™ HiPure Plasmid Maxiprep Kit and PureLink™ HiPure Precipitator module were used as directed to extract plasmid DNA from the bacterial cell pellets. Plasmid DNA was quantified by Qubit™ Fluorometer using the Qubit™ dsDNA BR kit, according to the manufactures protocol.

Transfection:

All transfections were performed using Viafect™ liposomal reagent according to the manufactures protocol. Cells were seeded in the appropriated culture vessel and allowed to reach approximately 70% confluence overnight. For each well of a 6 well plate, plasmid DNA was diluted to 3µg/300µL serum free media. To this 6µL, 9µL, 12µL and 18µL of room temperature Viafect™ was added to create ratios of 2:1, 3:1, 4:1, 6:1 Viafect™ reagent to plasmid DNA. This complex was incubated for 20 minutes at room temperature to allow for complex formation. All 300µL of the Viafect™ DNA serum free media complex was added to 1.7mL of complete media (total volume 2mL) and added to one well of 6 well plate to incubate for 24 hours to allowing for maximum transfection. Transfection rate was assessed at this point via EGFP fluorescence using an Olympus IX51 microscope with Olympus U-RFL-T Lamp and Olympus TH4 – 200 mercury lamp.

For 75cm flasks all transfections were performed at 1:4 rations of DNA to Viafect™. The Viafect™ DNA complex was made using 23.4µg DNA in 2.34mL serum free media and 93.76 µL Viafect™, this whole 2.43mL mix was added to a flask containing 18.75mL complete media and seeded cells this was allowed to incubate for 24 hours before all cells were lifted using Trypsin ready for clonal selection and bulk sorting.

Clonal selection and bulk sorting:

Cells were sorted into 96 well and 6 well plates with 1 cell/well and 5 cell/well in the 96 well plates and 10,000 (or as many cells as possible) in the 6 well plates using the FACS protocol below.

2.2.7 Deprivation of ρ° s:

ρ° cells were deprived of Pyruvate and Uridine to induce extreme metabolic stress. Cells were plated at an appropriate density either in Nunc culture flasks or in corning culture plates containing AT treated coverslips. After 24 hours complete culture media was removed, and cells were washed with multiple volumes of PBS until PBS was clear and colourless. Deprivation media without pyruvate and uridine was then added to the cells.

2.2.8 Chemotherapeutic Treatment:

Doxorubicin stock: 2mg/mL

Cisplatin stock: 1mg/mL

Both doxorubicin and cisplatin were obtained from the Wellington Hospital pharmacy and were dissolved in physiological saline, they were diluted with complete media to the appropriate concentrations. PBS was diluted as a vehicle control.

Cells were plated 24 hours before treatment and allowed to reach 70% confluency. Cells were washed with 37°C PBS before complete media with appropriate concentration of treatment was added.

4T1 cells were cultured in 6-thioguanine at 20 μ M for 2 weeks to remove non 4T1 cells.

2.2.9 Ex-vivo/primary culture and generation of Compact Bone Derived Cells:

1.1.1.3 Mouse strains:

All mouse lines were acquired from the Biomedical Research Unit at the Malaghan Institute of Medical Research and taken under ethics approval number 26793. This research used three strains: C57B/6J, BALB/cByJ and an F1 cross between the DsRed C57B/6J and the BALB/cByJ. The DsRed C57B/6J are a transgenic mouse with cytosolic expression of DsRed from the constitutively active promoter (DS red 2004). Male DsRed C57B/6J were crossed

with female BALB/cByJ, to generate a mouse with cytosolic DsRed expression and a BALB/cByJ mitochondrial genotype.

1.1.1.4 Preparation of BDC

Sterile PBS was mixed with 10% FBS and 1% EDTA, this is referred to as PBSFE. Mice were euthanised and the hind legs removed by cutting the skin off around the leg and by cutting up the groin towards the hip excising the muscle. The hip was dislocated by pulling firmly on the leg above the knee while holding the rest of the mouse. Once the hip was dislocated the leg was removed and femur kept intact where possible. Skin and feet were removed, and dismembered legs were placed in a small aliquot of PBSFE in a 50 mL flacon tube.

Using tweezers, the legs were picked up and as much tissue as possible was removed. The fibula was discarded but the tibia and the femur were kept and cleaned. The leg bones were scraped with a gloved fingernail or scalpel to remove any residual flesh from the shaft of the bone, tissue still present attached to the epiphyses of the bones was removed later. As they were cleaned bones were placed into another dish containing more PBSFE. Once all bones were cleaned a scalpel was used to remove the epiphyses, shafts were placed in another culture dish. Once all epiphyses had been removed the scalpel was used to break the bones into smaller pieces, care was taken to break the bones slowly as this avoided flying chip of bone which desiccated rapidly in the tissue culture hood.

After all the bones were broken into 2- 5mm chips they were washed two times with the PBSFE. The chips were then removed from the culture dish and placed in 1 well of a 6 well culture dish at approximate density of 6 legs (3 mice) to each well. One leg (2 bones) yielded approximately 30,000 cells after 3 days culture. Ten mL of type 2 collagenase was prepared at 1 mg/mL in complete media and 5mL of collagenase media mix was added to each well containing chips. This was then incubated at 37°C in a shaking incubator at 200 rpm for 3-4 hours or until the chips no longer clumped together. Chips were then washed with the PBSFE two more times before 5 mls of culture media was added and incubated in a cell culture incubator for 3 days, after which cells were visible migrating out of compact bone chips.

After 3 days the chips were removed from the 6 well plate and any larger pieces were cut to the desired 2-5mm size. The chips were distributed into piles of approximately equal size and placed in desired final culture vessel. For confocal microscopy (3-Aminopropyl) triethoxysilane treated coverslips were added to the bottom of 6 well plates with the chips on top. After chips were plated, media was added, and cells were incubated for a further 3 days.

After 3 further days of culture media was removed from the wells and 4 mL PBS was added to each well. To remove the chips from the culture, the end of a 1 mL pipet tip was removed to generate a wide bore. The culture dish was tilted and swirled to dissociate the attached chips from the base of the dish, 500uL of the PBS/bone chip mix was rapidly pipetted up trying to get as close to the chips as possible helped this, this often took 2 or 3 tries and was repeated until all the chips had been removed from the well. Sometimes extra PBS was added to help this process. Once chips had been removed all remaining PBS was also removed and primary culture media was added to the wells. The compact bone derived cells (CBDC) were then ready for downstream applications such as staining for microscopy and flow cytometry or trypsinisation.

2.3 Microscopy:

2.3.1 Staining:

Ethidium Bromide

The mitochondrial networks were stained with a solution of 5mg/mL ethidium bromide in complete media for 20 minutes, which was followed by two washes with PBS to remove excess ethidium bromide. Following this complete media was added for live cell imaging.

CFSE and Cell Trace Violet staining:

CFSE and Cell Trace Violet were made up to a stock of 5mM with DMSO. Cells were stained in a 50mL falcon tube by placing 5µL of stock solution on the lid of the Falcon tube containing the cells suspended in 5mL warmed PBS, after securing the lid the tube was inverted 2-3 times creating a final staining concentration of 5µM. This was then incubated

for 20 minutes at 37 °C, followed by the addition of 25mL of complete media and incubated for 5 minutes at 37 °C. Cells were then pelleted by spinning at 1000g for 5 minutes and resuspended in complete media ready for plating.

NucBlue staining:

To visualise the nuclei of cells in live culture NucBlue was added at a concentration of 1 drop per mL live culture media 15 minutes prior to imaging.

Imaging medium:

Cells were imaged in their complete medium.

Preparation and (3-Aminopropyl) Triethoxysilane Treatment of Coverslips:

All coverslips used in culture were prepared for adherent cells by treatment with (3-Aminopropyl) triethoxysilane. Coverslips of desired size and thickness were added to a 100mL glass bottle. One millilitre of (3-Aminopropyl) triethoxysilane was added to 50mL acetone in the glass bottle containing the coverslips. The coverslips were then subjected to constant agitation for 5 minutes, washed twice with 100% ethanol and stored in 70% ethanol until use.

Live cell imaging chambers:

Cells were cultured on treated coverslips in 6 well plates until ready for use, staining was performed in the well and then coverslips were transferred to the base of a live cell imaging chamber (LCIC). The top of the LCIC was then screwed into place with care to not exert excessive pressure onto the coverslip, to avoid caused warping of the glass and difficulties imaging. Complete media was then added to the LCIC and imaging commenced.

2.3.2 Image Generation:

IncuCyte:

For 6 well plates 49 fields of view (FOV) were selected for each well, and images recorded every 3-6 hours over 5 days. For 96 well plates 4 images per well were selected and the

same time points were performed. The images were then analysed according to the instructions in 2.3.4: Image Analysis.

Confocal:

Single stained controls were used to set exposure and gain and to assess autofluorescence and leakage of fluorophores into other channels. Images were taken as Z stacks for the whole depth of the adhered cells. Slices were between 0.5-2 μm apart, exposure and other parameters were adjusted for optimal image generation. Images were 800x800 to 1024x1024 pixels in size.

2.3.3 Co-Culture experiments with CBDC:

To assess if mitochondrial transfer had taken place between cell lines and Compact Bone Derived cells (CBDC), CBDC were plated into empty or coverslip ((3-Aminopropyl) triethoxysilane treated) containing 6 well flat-bottomed plates. After removal of bone chips cell lines were plated with the CBDC on the coverslips.

For ρ° cells the CBDC media was replaced with ρ° medium supplemented with 1% Penicillin Streptomycin. For confocal experiments only, ρ° cells were stained with CFSE if not GFP positive and were plated at determined appropriate density. After 3 days of co-culture the complete medium was removed, cells were rinsed with PBS, and the co-cultures replenished with deprivation media supplemented with Penicillin and Streptomycin. After cells had been deprived for the appropriate time, they were stained with EtBr and NucBlue and imaged on the confocal microscope.

4T1 cells were co-cultured with 6TG for 14 further days to remove any remaining CBDC and allow for assessment of viability without CBDC.

2.3.4 Image Analysis:

Confocal Image Analysis:

Confocal image analysis of Z stacks was performed using FIJI (Image J).

IncuCyte Image Analysis of CBDC and Cell Line Cultures:

Inbuilt IncuCyte analysis software was used to identify confluency of cells under phase and fluorescence microscopy. As only one cell type in each experiment was fluorescent, the confluency of the other cell type was determined by subtracting the fluorescent confluency from the phase confluency, confluency determined by the IncuCyte, $\text{conf Phase} - \text{conf Fluor}$. Standard error was calculated by $\sqrt{((\text{SE Phase})^2) + ((\text{SE RFP})^2)}$.

Contact Analysis:

Videos were created for each individual FOV. Each well was analysed by recording the outcome of the cells from the cell line in the culture. Survival and contact with CBDC was recorded for the cells that began the video in frame and did not leave frame until either they had died or the video ended. Each cell was coded for whether it survived or died and whether it came into contact with CBDC during that time.

2.4 Flow Cytometry:

Table 2-3: Conjugated Antibodies Used in this Project

Name	Clone	Source	Conjugate	Amount added to 50 μ L stain
CD105	REA 794	Miltenyi Biotech, Germany	APC-VIO770	150ng
CD73	TY/11.8	Miltenyi Biotech, Germany	FITC	150ng
CD90.2	30-H12	Miltenyi Biotech, Germany	VioBlue	150ng
CD45	30-F11	BD Biosciences	BV510	100ng
CD11b	M1/70	BD Biosciences	APC-Cy7	100ng

2.4.1 Preparing and staining cells:

Cells were harvested by trypsinisation and washed twice with DPBS , and then in FACS buffer (DPBS with 10%FBS and 1% EDTA) and spun at 1000g for 5 minutes to remove any remaining media and trypsin. A Fc block used (Mouse BD Fc Block) 250 μ g was incubated with the cells for 30minutes at 37°C, to block non-specific interactions between cells and the conserved Fc domain of the antibodies.

Fluorochrome conjugated antibodies were used to label cell surface epitopes. As described in the table 100,000- 1,000,000 cells were stained in 50µL on ice for 30 minutes protected from light. Cells were then washed in FACS buffer and centrifuged at 1000 x g for 5 minutes twice before being resuspended in 500µL FACS buffer. Finally DAPI was used to stain cell nuclei 1µL of 5mg/mL stock in 5 mL was used to create a working stock concentration of 1ng/mL and 50ul of this was added to the cells and they were incubated on ice for 20 minutes before being analysed.

Single stains were prepared on beads to determine spectral overlap and 'fluorescence minus one' FMO controls were prepared on untreated/unused sample cells.

2.4.2 Flow Cytometry on LSR and Fortessa:

Single stained and FMO controls were used to set appropriate fluorescence and laser levels on the flow cytometers. Between 10,000 -100,000 cells were analysed for controls and 100,000 + cells were collected for samples. Flow cytometry data was analysed using FlowJo™.

2.4.3 Fluorescence Activated Cell Sorting (FACS):

To separate cells for clonal isolation FACS was carried out on a BD Influx™ Cell sorter fitted with a 200 µm nozzle. The cytometer was operated by staff members of the Hugh Green Cytometry Centre based at the Malaghan Institute for Medical Research. Cells were prepared as above for Flow Cytometry but with the addition of filtration of the cells through a 100µm filter prior to the cells being stained with DAPI and incubated on ice.

2.5 Molecular biology:

2.5.1 DNA extraction

DNA was extracted for PCR using either Zymo Quick DNA Miniprep Kit or Qiagen DNeasy Blood and Tissue Kit. Kits were used followed according to instructions but with the addition

of one additional wash step and an additional 2-minute centrifugation at 10,000 x g to dry the membrane prior to elution. This was done to avoid contamination of the eluted DNA.

2.5.2 RNA extraction

RNA was extracted using the Qiagen RNeasy Miniprep and RNeasy Microprep Kits. Kits were followed according to instructions with the addition of a 10,000 x g spin prior to elution of RNA to dry the membrane.

2.5.3 Endpoint PCR

For Confirmation of ρ° Status:

For confirmation of ρ° status two concurrent reactions were used one for mitochondrial DNA and one for nuclear DNA. This was performed both every three months and every time a result was observed that could be attributed to mitochondrial DNA presence.

For each sample two 10 μ L reaction were performed, one with primers to the nuclear genome and one with primers specific to the mitochondrial genome. Each 10 μ L reaction contained 5 μ L Long Amp taq 2X, 0.5 μ L of forward primer, 0.5 μ L of reverse primer (primer stock concentration 10mM) and 20ng of DNA diluted in 4 μ L.

Each reaction was then run on a 2% Agarose gel at 70mV for 1 hour. A sample was considered ρ° if it returned a positive PCR product at 580bp for nuclear DNA, no band for mitochondrial DNA, both the no template controls were negative, and both the positive nuclear control and the positive mitochondrial control were of the appropriate molecular weight. Cells were considered positive for mitochondrial DNA if they returned all the products above and a positive mitochondrial band in the sample lane at 2145bp.

For mitochondrial DNA, primers used:

mitoFWD 5' TCA GTA CTT CTA GCA TCA GGT GT 3'

mitoREV2 5' GCT AGG CAG AAT AGG AGT GAT G 3'

For nuclear DNA, primers used:

CD45FWD 5' CGC AGA CTC TCA CAT TAG TTC 3',

CD45REV 5' ATT TTG TTC ACG TCC CAG ATC 3'

Cycling for both of these reactions was as follows one cycle at 95°C for 3 minutes, then 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute 50 seconds. This was followed by a final extension at 72°C for 5 minutes and 4°C hold.

2.5.4 Quantitative Real Time PCR

For quantitative real time PCR detection of mitochondrial copy number. Mitochondrial DNA was compared to nuclear DNA the following reactions and analysis were performed.

Reactions were carried out in 20µL with 10µL PowerUp SYBR Green Master Mix 2x, 20ng of DNA in 8µL and 5µM of each primer diluted in 2µL. For each primer set a standard curve was performed, with 0.02 ng, 0.2 ng, 2 ng and 20 ng of genomic DNA. There were triplicate reactions for each reaction and each sample was run with both primer sets (Mitochondrial and Nuclear). The MicroAmp Optical 96 well plate was sealed with MicroAmp Optical Adhesive film and centrifuged at 2000 x g for 2 minutes to remove any bubbles and then run on the Quantstudio at the following conditions:

1. 50°C for 2 minutes
 2. 95°C for 2 minutes
- Followed by 40 cycles at
3. 95°C for 1 second
 4. 60°C for 30 seconds.

Analysis was performed by using the mean Ct from the triplicate wells, calculate $2^{-(Ct)}$ for both Mitochondrial and Nuclear. Then relative mtDNA levels were calculated using $2^{-(NUC Ct)} - 2^{-(MITO Ct)}$.

Mitochondrial primers

QPCR MITOFWD ND4 5' GCC TAG CAA ACT CCA ACT ACG 3'

QPCR MITOREV ND4 5' ACC ATT TGA AGT CCT CGG GC 3'

Nuclear Primers

QPCR NUC FWD 5' CAC GTG GGC TCC AGC ATT 3'

QPCR NUC REV 5' TCA CCA GTC ATT TCT GCC TTT G 3'

2.6 Sequencing

2.6.1 MinION:

PCR-CDNA barcoding kit (SQK-PCB109): The sample protocol found on the Oxford Nanopore site and associated with this kit was used, this protocol was last adapted on 10/10/2019.

Previous updates including the transition from two kits (The cDNA PCR kit and PCR barcoding Kit) to one combined kit did not change the protocol. Any subsequent changes in protocol have not been used in the data presented in this thesis.

For each sample of RNA, 50ng total RNA (volume x) was added to 1 μ L VN Primer (at 2 μ M), 1 μ L 10mM dNTPs, and 9-x water to create a total volume of 12 μ L this was then incubated at 65°C for 5 min before snap cooling on ice. Next 4 μ L 5x RT buffer, 1 μ L RNaseOUT and 1 μ L Strand Switching Primer (at 10 μ M) was mixed and added to annealed RNA and then incubated at 42°C for 2 minutes. One microliter Maxima H Minus Reverse Transcriptase was added and mixed by tube flicking and spinning down briefly, this was then then incubated at 42°C for 90 minutes for reverse transcription and strand switching, and 85°C for 5minutes for heat inactivation before being held at 4°C.

5 μ L of the reverse transcription reaction (the other 15 μ L was used to repeat or in separate reactions to be pooled later) was added to 1.5 μ L barcode primer, 18.5 μ L Nuclease free water and 25 μ L 2x LongAmp Taq Master Mix and then amplified in a thermocycler under the following conditions:

- 1) 95°C for 30sec
- 2) 95°C for 15 sec
- 3) 62°C for 15sec
- 4) 65°C for 50sec per kb (approximately 3kb therefore 2.5min)
- 5) 65°C for 6minutes
- 6) 4°C hold

After amplification 20 units of NEB Exonuclease 1 (1 μ L) was added to each tube followed by an incubation at 37°C for 15 minutes and at 80°C for a further 15 minutes. Reactions containing the same barcode were pooled (usually 2) and 0.8x equivalent resuspended AMPure XP beads were added to bind the DNA and allow washing and incubated on a Eppendorf Thermomixer Compact at 200 RPM at room temperature to thoroughly mix the samples. Samples were spun down briefly and then pelleted on a magnet, supernatant was discarded, and pellets were washed 2x with 200 μ L of freshly prepared 70% ethanol, ethanol wash was then discarded. Beads were spun down and pelleted on the magnet and any residual ethanol was pipetted off before the tube was removed from the magnet and beads were resuspended in 12 μ L of MinION Elution Buffer. This was then incubated for 10 minutes on the Eppendorf Thermomixer Compact before being spun down and pelleted on the magnet. The eluate containing the cDNA was pipetted off and retained and the beads were discarded. Eluate was analysed for size quantity and quality using the Quantet and the nanodrop and then run on a 1.5% agarose gel. A total of 100-200 fmol each barcoded cDNA was pooled and made up to a final volume of 12 μ L. If the samples had a low concentration that caused the total volume with all samples having 100fmol to be over 12 μ L the lowest concentrations were discarded until there was less than 12 μ L, all samples were then increased to the maximum volume while still having between 100-200fmol. If there was 2 or more samples from each condition at this stage 1 μ L Rapid adapter was added and incubated at room temperature for 15 minutes to create the DNA library.

MinION Flow cells were prepared according to Oxford Nanopore instructions. Approximately 20-30 μ L of flow cell buffer was removed from the flow cell via the priming port, 800 μ L of priming mix (30 μ L of Flush Tether in 1 vial of Flush Buffer) was added via priming port and then left for 5 minutes.

The library was prepared by combining, 37.5 μ L Sequencing Buffer, 25.5 μ L freshly mixed Loading beads and 12 μ L DNA library. 200 μ L of the priming mix was added to the priming port with the sample port open and then 75 μ L of the sample was added dropwise to the

sample port (priming port still open). Both ports were closed, and the flow cell was run for 48 hours.

2.6.2 Sanger:

Sanger sequencing was used to confirm mitochondrial genotypes of cell lines. A nested PCR was performed using mitochondrial genome-specific primers (mitoFWD and mitoREV2) to create a mitochondrial specific product.

A 1047bp portion of this product was sequenced using the mitoFWD forward primer and an internal sequencing primer mitoREV1 as follows. After PCR, the products were cleaned using the Zymo Clean and Concentrate kit. For each primer 50 ng of cleaned PCR product and 0.4µL of 10µM primer were made up to 20µL and sent for sequencing at Massey Genome service.

mitoFWD 5' TCA GTA CTT CTA GCA TCA GGT GT 3'

mitoREV1 5' TGG GGA TTG GTA TGG AGC TT 3'

mitoREV2 5' GCT AGG CAG AAT AGG AGT GAT G 3'

2.7 Analysis:

2.7.1 Bioinformatics:

Sequencing analysis was performed by me unless otherwise stated with assistance from Dr David Eccles for problem solving R code and pipeline design.

Mapping and alignment:

Dr David Eccles performed clean-up, mapping and alignment of reads on all data generated by MinION Sequencing, detailed pipeline for this can be found at protocols.io <https://www.protocols.io/view/nanopore-data-analysis-8azhsf6>³⁴⁵.

MinION software MinKNOW and GUPPY were used for base calling and LAST was used to demultiplex the reads. Reads were then stranded mapped to the GCF 000001635.20

Murine Genome before being made into a CSV table of transcripts and read counts for each sample and sent on to me for further analysis.

Differential sequencing analysis – DESeq2:

For differential sequencing analysis DESeq2 was used. The CSV file denoting transcripts and read counts was filtered to remove transcripts with less than 5 reads, and those transcripts mapping in the reverse direction³⁴⁶. The DESeq2 estimates size factors, dispersion and then calculates the negative binomial GLM fit and WALD statistic. Once a DESeq2 result list was generated common transcripts were found from comparisons of the relevant result lists using Euler plots.

Gene Set Enrichment Analysis – FGSEA

GSEA analysis was used on all DESeq2 results using FGSEA. All transcripts that were differentially expressed were ranked by the 'stat' generated during the DESeq2 protocol. The 'stat' is a function of the Wald statistic³⁴⁷. These differentially expressed transcript IDs and their corresponding Stats were compared to a list of gene sets from retrieved from the MSigDB of the broad institute at <http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp>.

Other packages used to facilitate the formation, data manipulation and visualisation of results includes ggplot2, tidyverse, dplyr, stringr, lubridate, ggfortify, Glimma, pheatmap, purrr, magrittr, EnrichmentBrowser and EnhancedVolcano

3 Proof of Concept: Stress-Induced

Transfer of Mitochondria to ρ° Cells

The overall aim of this research was to identify signals that enhance survival of stressed cells. This chapter focuses on the donor/recipient interplay that results in mitochondrial transfer to cells under extreme metabolic stress.

Experiments in this chapter included the assessment and characterisation of the 4T1, B16 and Astrocyte cell lines under metabolic stress and the creation, assessment and characterisation of a donor population that modelled aspects of the tumour microenvironment. Finally, the assessment of mitochondrial transfer between donor and recipient cell populations was examined over short and medium term.

3.1 Mitochondrial recipients

A mitochondrial recipient is a cell that can receive mitochondria from a mitochondrial donor. One of the main limitations with mitochondrial transfer studies is the ability to detect whether a cell has received mitochondria or not. This is why ρ° lines which lack mitochondrial DNA are used, the presence of acquired mitochondria and mitochondrial DNA is very easy to demonstrate. The mitochondrial recipients used in this chapter were ρ° lines that have been produced from 4T1 and B16 tumour lines and a non-tumour Astrocyte line using long term exposure to ethidium bromide³⁴⁸.

3.1.1 Metabolic Stress in Recipient cells

ρ° cells lack mitochondrial DNA and thus a functional electron transport chain (ETC) ²³¹. These cells are therefore constitutively glycolytic and require supplementation with pyruvate and uridine to produce NAD⁺ and pyrimidines (see page no 40) ²²⁹, when pyruvate

and uridine are removed, the ρ^0 cells enter severe metabolic stress as they are unable to survive.

3.1.2 Cell lines used

The lines used in this work are B16, 4T1 and Astrocyte lines. Both B16 and 4T1 cell lines have been instrumental in shaping the current understanding of how tumours and their metastases and chemoresistance form¹²⁸. Both B16 and 4T1 ρ^0 cells have been shown to accept mitochondria from unknown mitochondrial donors *in vivo*²⁰¹. Astrocyte ρ^0 have previously been shown to accept mitochondria *in vitro*¹⁴¹.

3.2 Mitochondrial Donors

As mitochondrial transfer happens *in vivo* we can expect the mitochondrial 'donor' to be a cell that is present in the microenvironment^{167,201,291}. There are four cell types that have been shown to donate mitochondria in some capacity: these are mesenchymal stem cells (MSC), monocytes, endothelial cells and pericytes.

There is a large body of evidence that supports MSC as a mitochondrial donor in most tissues and tumour types^{294,295,349}. There is some evidence that monocytes and endothelial cells are mitochondrial donors^{193,350} and previous work from our group²⁹³, and others have shown pericytes with their high degree of similarity to MSC are also a likely candidate^{188,293}. Due to the many types and varied nature of the microenvironment it is probable that different cell types play a role in mitochondrial transfer of some form.

Prior work in our group showed that adherent cells isolated from compact bone are able to donate mitochondria to ρ^0 cells¹⁴¹. The work in this chapter attempts to understand the interplay and timing between these compact bone derived cells (CBDC) and metabolically stressed ρ^0 cells.

There are three primary proposed mechanisms for mitochondrial transfer referred to in more detail on pages 54-56. Donor cells are thought to transfer mitochondria via

mechanisms such as tunnelling nanotubules (TNTs), extracellular vesicles and/or cellular fusion, all potentially with assistance from gap junctions. There is the most evidence for TNTs but Gap Junctions and EVs are possibly involved ^{295,300,351}.

3.3 Mitochondrial Crosstalk

Communication between mitochondria and other organelles within a cell is crucial to normal cellular functioning ¹⁶⁸. Crosstalk is primarily involved in the assembly of nuclear and mitochondrially encoded subunits for the electron transport chain which is tightly controlled by retrograde signalling from the mitochondria ¹⁶⁸. Communication between mitochondria and the nucleus is thought to play an important role in tumorigenesis and a lack of communication is thought to be important in mitochondrial transfer ^{168,201,352}. Metabolic stress responses rely on the crosstalk between the mitochondria and nucleus, and pathways such as the nuclear factor erythroid 2 (NRF2)/ antioxidant response element (ARE) signalling pathway are involved in cellular response to oxidative stress ^{353–355}.

The following work attempted to elucidate the donor/ recipient interplay that results in mitochondrial transfer when cells are under extreme metabolic stress. This included the assessment and characterisation of the ρ^0 lines B16, 4T1 and Astrocyte under severe metabolic stress. After the characterisation of the ρ^0 cell lines, a 'donor population was needed to assess transfer. This entailed the creation of, assessment and characterisation of a donor population/ microenvironment. Finally, the assessment of any mitochondrial transfer between these two cell populations was assessed over both the short and medium terms.

3.4 Results

3.4.1 Characterising of Mitochondrial Recipients

In order to confirm the model of stress and stress response, I needed to first establish what stress looks like in the recipient cell population. To characterise the recipient cells and their response to stress, established ρ^0 cell lines were deprived of the essential supplements pyruvate and uridine and then observed using flow cytometry, IncuCyte microscopy and confocal microscopy.

Figure 3-1: Astrocyte ρ° response to pyruvate and uridine deprivation:

ρ° Astrocyte cells were plated in complete ρ° media and after 24 hours complete media was exchanged for ρ° deprivation media. A) Proliferation of deprived cells. Cells were placed on the IncuCyte for 5 days and images were recorded every 6 hours. Error bars represent the standard error of the mean of 3 replicates of 49 fields of view each. B) Dead cells were distinguished by PI staining and the percentage of live and dead cells was measured using flow cytometry over 4 days, C) Confocal imaging of cells at 0, 24 and 48 hours without pyruvate and uridine, CFSE was used as a cytoplasmic stain (blue) and NucBlue for nuclei (yellow). Scale bar, 50 μ m. All data for B and C are from a single replicate.

Without pyruvate and uridine supplementation Astrocyte ρ° cells failed to proliferate over five days (Figure 3-1a), and their viability decreased (Figure 3-1b). The morphology of the remaining cells changed with the cells becoming rounder with increased blebbing, reminiscent of the blebbing seen as characteristic of apoptosis.

This loss of proliferation and viability was also observed when pyruvate and uridine was removed from the 4T1 ρ° and B16 ρ° cells, although over a longer timeframe in the B16 ρ° and 4T1 ρ° (Figures 3-2 and 3-3). The B16 ρ° were slower to respond and the effect on cell morphology was less pronounced, but increased blebbing was still observed. The 4T1 ρ° grew in a different manner than the Astrocyte ρ° and B16 ρ° . They grew in clusters and these clusters got smaller as deprivation continued.

This shows that all the ρ° cell lines were dependant on pyruvate and uridine, and that deprivation led to cellular death, as expected ^{229,231}.

Figure 3-2: 4T1 ρ° response to pyruvate and uridine deprivation.

ρ° 4T1 Cells were plated in complete rho media and after 24 hours, complete media was exchanged for DMEM, not containing either pyruvate or uridine A) Proliferation of deprived cells. Cells were placed on the IncuCyte for 5 days and images were recorded every 6 hours. Error bars represent the standard error of the mean of 3 replicates of 49 fields of view each. B) Dead cells were distinguished by PI staining and the percentage of live and dead cells was measured using flow cytometry over 4 days, R1, R2 and R3 were replicate experiments. C) Confocal imaging of cells at 0, 24 and 48 hours without pyruvate and uridine, CFSE was used as a cytoplasmic stain (blue) and NucBlue for nuclei (yellow). Scale bar, 50 μ m. All data for C are from a single replicate.

Figure 3-3: B16 ρ^0 response to pyruvate and uridine deprivation.

ρ^0 B16 cells were plated in complete rho media and after 24 hours, complete media was exchanged for DMEM, not containing either pyruvate or uridine A) Proliferation of deprived cells. Cells were placed on the IncuCyte for 5 days and images were recorded every 6 hours. Error bars represent the standard error of the mean of 3 replicates of 49 fields of view each. B) Dead cells were distinguished by PI staining and the percentage of live and dead cells was measured using flow cytometry over 4 days, C) Confocal imaging of cells at 0, 24 and 48 hours without pyruvate and uridine, CFSE was used as a cytoplasmic stain (blue) and NucBlue for nuclei (yellow). Scale bar, 50 μm . All data for B and C are from a single replicate.

3.4.2 Characterisation of the Cellular Aspect of the Supporting Microenvironment

This proof of principle model is based on mitochondrial transfer as the mechanism of support, where the stressed cell receives mitochondria from a supporting cell. In the literature the most common mitochondrial donor is the MSC mesenchymal stem cell, or stromal cells. Previous work in our group has demonstrated that adherent mesenchymal-like stromal cells could be isolated from compact bone, and that these cells were competent mitochondrial donors ¹⁴¹.

Compact bone derived cells (CBDC) were isolated from the hind leg of a maternal Balb/C x paternal C57 F1 cross. The bone was digested using type 2 collagenase and cells were allowed to migrate out of the bone over a period of 6 days. The cells that emerged were then phenotyped using a flow cytometry panel (Figure 3-4).

A

a

b

B

Figure 3-4: Phenotyping and classification of Compact Bone Derived Cells:

Sections A and B on previous pages. In section A) the bottom row is the unstained control, and the top row is the stained sample. In a) DAPI was used to assess viability, DAPI negative cells analysed. b) CD45 positive cells were examined for CD11b expression. B) CD45 negative cells were examined for CD73, CD105 and CD90.2 expression. C) Co-expression of CD105, CD90, CD73 in CD45 negative cells.

First the CD45 positive hematopoietic cell component of the culture and the CD45 negative non-hematopoietic cells, of stromal origin were identified. The viable cells were gated on size and exclusion of DAPI. Almost 15% of cells were CD45 negative while 85% of were CD45

positive. CD45 positive cells were almost 100% CD11b positive, which indicated a macrophage-like myeloid population³⁵⁶. The single cell gate for the stained sample has lower singlets than the unstained control. As they fell outside the gate this should not have affected the results.

Next, mesenchymal stromal cells markers were examined, CD73, CD90.2 and CD105. All of these markers have varying levels of expression in MSC¹⁵⁴. The CD45 negative cells were 66.1% CD73 positive, 32.8% CD105 positive and 19.4% CD90.2 positive. However, there was little correlation between all three markers. Only 18.7% of CD45- cells were both CD105 and Cd73 positive, 12.6% were CD73 and Cd90.2 positive and only 5.9% of CD45- cells are both CD105 and Cd90.2 positive. The classic mesenchymal stem cell is often defined by expression of all three of these markers however, only 1% of the CD73 positive cells were positive for both CD90.2 and CD105, indicative very few classic mesenchymal stem cells are present in this population.

Once we knew what types of cells we were working with, we wanted to know if they would be affected by the pyruvate and uridine depletion that placed ρ° cells under stress. To do this CBDC were placed in the IncuCyte, and growth with and without pyruvate and uridine was assessed.

A Compact Bone Derived Cell Survival in Pyruvate and Uridine negative conditions

B

Figure 3-5: Effect of pyruvate and uridine deprivation on CBDC.

Using the IncuCyte microscope system images were taken every 3 hours, A) shows the proliferation of CBDC using confluency determined using the IncuCyte with and without pyruvate and uridine. Error bars represent the standard error of the mean of 3 replicates of 49 fields of view each. B) shows a representative image of CBDC in each culture condition from the IncuCyte.

CBDC were unaffected by the loss of pyruvate and uridine (Figure 3-5). This was expected as wild type cells can produce their own NAD⁺ and pyrimidines through their functional electron transport chain. There are two cell types seen in the representative images, cells with large and small cell surface area. The large cells are very spread out and have irregular morphology, the small cells have concentrated cellular bodies and are more regular morphologically.

3.4.3 Assessment of Mitochondrial Transfer Between Donor/Recipient Populations

To demonstrate that metabolic stress in ρ^0 cells induced by pyruvate and uridine loss led to support from CBDC, a co-culture system was utilised. Primary cultures of CBDC were grown with ρ^0 cell for 3 days before pyruvate and uridine supplementation were removed. Cells were then imaged on a confocal microscope at multiple timepoints during the subsequent culture.

Support of ρ^0 cells in the absence of pyruvate and uridine had previously been shown for the ρ^0 Astrocytes, via the transfer of mitochondria ¹⁴¹. To determine how conserved this process of mitochondrial transfer was, and any signals driving it, I developed these co-culture models for two ρ^0 cell lines: Astrocytes and 4T1.

When Astrocyte ρ^0 cells were placed in co-culture with CBDC in complete ρ^0 medium, ρ^0 cells continued to proliferate and there was no sign of mitochondrial transfer.

When pyruvate and uridine supplementation were removed at 3 days in co-culture, cellular fusion occurred between CBDC and Astrocytes, as shown in Figure 3-6 A by the cell with two different nuclei in one cell body (arrow). Mitochondrial networks contributed by the CBDC were visible in the ρ^0 cells at 24 and 48 hours (white arrows), but only in the minus pyruvate and uridine condition. By contrast no networks were seen in ρ^0 cells in the plus pyruvate uridine condition even after 10 days.

A

B

C

Figure 3-6: Astrocyte ρ^0 + CBDC:

Sections A, B and C on previous pages. ρ^0 Astrocytes (eGFP - blue) were placed in co-culture with CBDC (DsRed - red) at a 1:2 ratio of Astrocytes to CBDC in complete ρ^0 media. Mitochondrial networks were stained with ethidium bromide (red) and nucleus stained with NucBlue (yellow). Images are in pseudo-coloured using Image J. After 3 days of co-culture medium was changed to either ρ^0 deprivation medium without pyruvate and uridine (-PU) or complete ρ^0 medium containing pyruvate and uridine (+PU). A) confocal microscopy at 24 hours deprivation. B) Confocal microscopy at 48 hours deprivation. C) Confocal microscopy at 7 days deprivation. D) Long term PU deprivation at 52 and 82 days deprivation and wild type Astrocyte. Scale bar = 50 μ m. Experiments performed in biological triplicate, all representative images are from one experiment.

Interestingly, these mitochondrial networks were not visible in the ρ^0 cells minus pyruvate and uridine at day 7, despite the fact that the ρ^0 cells continued to survive the loss of pyruvate and uridine. However, at 52 days the ρ^0 cells in the minus pyruvate and uridine condition did show visible perinuclear mitochondrial networks, which were maintained as long as 82 days in culture without pyruvate and uridine.

To examine whether stress was required for cell fusion and restoration of mitochondria by the CBDC cells, the pyruvate and uridine were removed from the plus pyruvate and uridine co-culture after 2 weeks. This was approximately the same amount of time pure CBDC cultures took to become senescent. At this point almost no CBDC were visible and the remaining ρ^0 cells did not survive the pyruvate and uridine deprivation at this later time point, despite the prior two weeks in co-culture with CBDC. This suggests that the mitochondrial transfer occurs only under metabolic stress in Astrocyte ρ^0 cells when the mitochondrial donors are healthy and had not occurred during the co-culture without stress.

A similar experiment (Figure 3-7) was carried out with the breast cancer cell line 4T1 ρ^0 , stained with CFSE (shown in blue) to distinguish the 4T1 ρ^0 from the CBDC. After staining the 4T1 ρ^0 were plated onto DS-red CBDC, allowed to culture in + pyruvate and uridine medium for 3 days before the medium was exchanged for pyruvate and uridine depleted medium. Cells were then imaged using a confocal microscope at 24 and 48 hours. After 6-thioguanine treatment, cells were imaged again at 35 days post pyruvate and uridine removal.

A

3

Figure 3-7: 4T1p° + CBDC:

Sections A and B on previous pages. 4T1p°(blue) cells were placed in co-culture with CBDC (red) at a 1:4 ratio of 4T1 to CBDC in complete p° media with penicillium and streptomycin. After 3 days of co-culture media was changed to either p° deprivation media not containing pyruvate and uridine or complete p° media containing pyruvate and uridine. Cells were kept in a 37°C incubator at 5% CO₂ for the remainder of the experiment. A) Confocal microscopy at 24 hours deprivation. B) Confocal microscopy at 48 hours deprivation. 4T1 stained with CFSE (blue) C) Long term deprivation and wild type confocal imagery. At 2 weeks deprivation all samples were treated with 6TG to remove remaining CBDC. Those that were initially cultured with pyruvate and uridine did not survive deprivation of pyruvate and uridine at this point. The surviving cultures were placed on the confocal at 35 days deprivation (C). Cells were stained with ethidium bromide at 5mg/mL for 6 minutes, 4T1 were initially stained with CFSE (no longer present in C) and CBDC are ds-red positive. NucBlue was used as the nuclear stain. Scale is 50 μm. Experiments performed in biological triplicate; all representative images are from one experiment.

Similar to the results seen in the Astrocyte experiment, the 4T1 ρ° cells survived the loss of pyruvate and uridine in co-culture, but there was no visible signs of cellular fusion or mitochondrial transfer in either the plus or minus pyruvate and uridine condition, at either 24 or 48 hours. At 7 days I was unable to distinguish the 4T1 ρ° cells from the CBDC as the CFSE had diluted out due to continued cellular proliferation, in both the presence and absence of pyruvate and uridine. However, 4T1 cells are resistant to the chemical 6-thio-guanine (6TG) ³⁵⁷, so at 14 days the co-cultures were subjected to treatment with 6TG, and pyruvate and uridine were removed from the remaining plus pyruvate and uridine cultures. Removal of the remaining CBDC by 6TG allowed for the analysis of mitochondrial networks in the remaining cells. At 35 days after the initial removal of pyruvate and uridine, established mitochondrial networks were seen in the minus pyruvate and uridine ρ° cells.

As seen in the Astrocyte experiment, simply being in co-culture with CBDC was not enough to induce the transfer of mitochondria to the ρ° cells. The ρ° cells not subjected to the initial metabolic stress did not survive the loss of pyruvate and uridine during the 6TG treatment. This demonstrates again that the transfer of mitochondria in this setup occurs without pyruvate and uridine from healthy CBDC to stressed ρ° cells and that this enables the ρ° cells to survive that would normally prove lethal to them.

3.5 Discussion

ρ^0 cells that are lacking mitochondrial DNA are a very extreme example of DNA damage, the removal of pyruvate and uridine simulates a very extreme model of metabolic stress.

Therefore, any experiments using ρ^0 cells have limited physiological relevance.

The main limitation of the CBDC is that they are not representative of any actual tumour or non-tumour microenvironment. One of the reasons for using this method was to ensure as little artificial modification as possible in the system. Potentially it would be better for understanding how these interactions work *in vitro* if we could isolate the mesenchymal type cells and monocytes separately or get a better representation of the tumour microenvironment of melanoma, breast cancer and brain cancers.

One of the main limitations of the co-culture experiments was the inability to observe what was happening in the first 24 hours of deprivation and the inability to follow the cells of interest over an extended period of time due to the CFSE stain being lost after a short period of time (5-7 days). Because of this we potentially missed some of the events of interest in the Astrocytes and any significant events of interest in the 4T1 lines.

The co-culture experiment was also set up with the B16 ρ^0 , again staining with CFSE to distinguish from ds red CBDC. However, the B16 ρ^0 cells proliferated faster than the 4T1 ρ^0 and during the 3-day incubation step before pyruvate and uridine deprivation the CFSE signal was lost and the B16 ρ^0 cells could not be identified positively from the CBDC. In addition, unlike the 4T1 ρ^0 cells there was no simple way to separate the B16 ρ^0 cells from the CBDC and so this experiment was never completed.

3.5.1 Co-Cultures

In co culture Astrocyte ρ^0 cells survived deprived of pyruvate and uridine, and at 24 hours without pyruvate and uridine I saw fused cells with characteristics from both halves of the co-culture. Interestingly this cell fusion was reduced at 48 hours and was not visible at all at 7 days.

This was not just something that occurred whenever these two cell types were in co-culture together as this was never visible in the non-deprived (+pyruvate and uridine) samples. The ρ^0 cells that were not deprived in the initial experiment did not survive the removal of pyruvate and uridine after the first two weeks of co-culture. Cells that were only exposed to pyruvate and uridine for the first 3 days of co-culture did survive. The 3-day supplemented condition showed no visible evidence of mitochondrial networks at 7 days but, went on to have perinuclear networks at 52 days deprived and full 'healthy' networks at 82 days deprived. As I saw whole cell fusion in these experiments, the cells would possibly have to establish single nuclear control and mitochondrial nuclear crosstalk before fully developing a mitochondrial network.

4T1 ρ^0 cells acted differently to Astrocytes in co-culture and did not show the same transfer of mitochondrial networks after 24 hours. However they did survive the loss of pyruvate and uridine with CBDC, similar to the Astrocyte ρ^0 cells. After removal of the CBDC with 6TG, the 4T1 ρ^0 also showed replete mitochondrial networks, indicating that transfer did occur although perhaps not by the same mechanism. Also consistent with the Astrocytes the 4T1 ρ^0 cells that were deprived of pyruvate and uridine at two weeks did not survive the removal of supplementation once CBDC were absent.

The CBDC that migrate out of the bone were a mixed culture that contained cells of a mesenchymal origin and macrophage like cells. There is clearly a mitochondrial donor in this population, which could be from either the CD45 – mesenchymal stromal cell, or the CD45+ CD11b- cells. The classic donor cells, MSC, were not found in the mixed culture in significant numbers. However, I aimed to set up a mitochondrial donor culture with the CBDC and it is entirely possible that the macrophage population was the donor in this situation.

Cell Fusion:

With the Astrocyte ρ^0 cells we see evidence of fusion early on at 24 hours, so it is likely that these events play a role in the resistance to deprivation and development of mitochondrial networks seen later with these cells. I cannot rule out that these fusion events can occur spontaneously in co-cultured cells, but it is worth noting that these events were only seen in the fully deprived condition. Astrocytes are cells that are 'prone to fusion' *in vitro* so it is not

unexpected that they would fuse with other cells in this setting³²³. These Astrocytes are often seen to be multinucleated under normal culture conditions, which adds further evidence that they have the potential, inclination and ability to fuse with each other without external influences³²³.

Interestingly, the Astrocytes that fused at 24 hours had two nuclei of different colours, and yet no cells were seen with multiple differently coloured nuclei at 48 hours or at 7 days. It appears that at some point one of the nuclei of the fused cells 'outcompeted' the other nucleus leaving one nucleus in control with all of the remaining cytoplasm of the fused cell.

Unlike the Astrocytes I did not see any obvious evidence of fused cells in the 4T1p^o cultures despite observing hundreds of cells, but as this was a time point analysis, I cannot rule out that I missed some fusion events that fell between timepoints. Due to the normal growth patterns of 4T1 p^o cells into small groups/colonies of small highly rounded cells, it is much more difficult than it is in the Astrocytes to detect minor amounts of mitochondrial stain. Therefore, it is possible that at 24 hours there was some amount of mitochondrial stain from a fusion event that occurred earlier.

Other Mechanisms for Mitochondrial Transfer: Extracellular Vesicles and Tunnelling Nanotubules:

There are other mechanisms by which mitochondrial transfer could have occurred in the 4T1 p^o cells in particular. Extracellular vesicles have been described to transfer mitochondria³¹³. However, no extracellular vesicles were seen during these analyses, although most EV sizes would be too small for visualisation due to the microscopy resolution I was using. Further, the images were disconnected timepoints, rather than a time-lapse, so it is possible that we missed any evidence of extracellular vesicles. It is possible that specks and dots present in the images could also be extracellular vesicles that were not resolved at 60X optical magnification.

Tunnelling nanotubes are the other commonly observed mechanism for mitochondrial transfer. No TNTs were specifically seen in the samples that showed fusion and transfer despite being an adequate resolution to observe these structures³⁰², but again as this was not a time course, I could have missed these events or because the cells that were

'obviously transferred' were the fused cells we missed the TNTs while looking at fusion. I also did not see any TNTs in the 4T1 ρ° samples that had evidence of transfer later, this could be because I missed the events due to the time points or because the 4T1 ρ° cells grow in clusters which make looking for small cytoplasmic protrusions difficult. Either way both extracellular vesicles and TNTs could be the events responsible for the transfer that has happened in the 4T1 ρ° , but neither was observed in the cultures.

3.5.2 *In vitro* vs *ex vitro*/ *in vivo* work:

Within a tumour's microenvironment many of the cellular components would be activated to encourage tumour growth such as TAMs. I have tried to ensure some degree of activation of the CBDC by co-culturing the cells for 3 days before depriving them of supplementation, but we did not test the CBDC to see if they displayed classical activation markers of tumour associated cells.

1.1.1.5 Lag phase

The publication that initiated this work Tan *et. al.* 2015 showed that 4T1 ρ° and B16 ρ° cell lines grew tumours in a murine model after a 2-3 week delay compared with the parental lines of 4T1 and B16 ³⁴⁸. After the tumours were removed they were found to have mitochondrial DNA that matched the murine host and not the parental line ³⁴⁸. The conclusion from this study was that mitochondrial DNA and mitochondria was essential to tumorigenesis ³⁴⁸.

One of the reasons for studying the 4T1 ρ° and B16 ρ° *in vitro* is to understand what happened in the cells prior to the tumours removal. This lag phase appears to correlate with the disappearance and reappearance of mitochondrial networks I see in this work. Here I hypothesize and expand upon Tan *et. al.* 's findings and say that not only is the mitochondrial genome necessary for tumour growth but a functional mitochondrial network is also necessary. Both Astrocyte ρ° and 4T1 ρ° lines displayed a delayed onset of full/complete networks. The 4T1 ρ° line is the same line that was used in the Tan *et. al.* findings and the 'lag' we see here between stress induction and network reestablishment is similar to the 'lag' seen before tumour formation *in vivo* ²⁰¹.

As mitochondrial networks are associated with oxidative phosphorylation and cellular health, we presume that in these cells, as has been previously documented, the appearance of networks coincides with a resumption of oxygen dependant respiration ²²⁴. This reestablishment of networks indicates a reversal of the aberrant metabolism exhibited by ρ^0 cells in a rescue event which indicates that mitochondrial transfer and mitochondrial network resumption play a role in cellular survival and stress response ^{228,229}.

The wild type 4T1 cell line and the ρ^0 4T1 cell line both have faster growth rates than their Astrocyte counterparts which indicates that many cellular processes may be faster in the cancer line. I hypothesise that the faster reestablishment of networks in the 4T1 ρ^0 is due to a general faster rate of cellular processes. This could also be due to cellular size and area, the Astrocyte lines are cells that cover a large basal surface area in irregular spread out shapes whereas the 4T1 cells are small and rounded, the difference in time taken to re-establish networks could be that the Astrocytes have a more complex network structure and therefore take more time to fully establish.

4 Contact Dependent Survival

Advantage of ρ° Cells in Co-culture with CBDC

Cells that have no mitochondrial DNA are described as ρ° cells, they are constitutively glycolytic and auxotrophic for pyruvate and uridine^{229,231}. When pyruvate and uridine are removed from a ρ° cell culture the cells enter a form of severe metabolic stress and generally die within 3-5 days. In the previous chapter I described the phenomenon of mitochondrial transfer from CBDC to cell lines 4T1 ρ° cells and Astrocyte ρ° cells under extreme metabolic stress while in co-culture. In this chapter I examined interactions between the CBDC and ρ° cells and determined that ρ° cellular survival was enhanced by contact with CBDC. Finally I assessed the RNA transcriptome of the pyruvate and uridine deprived ρ° cells to begin to identify a signal that could result in mitochondrial transfer or cellular survival.

4.1 Contact Dependant Survival of ρ° Cell Lines in Co-Culture with CBDC.

In order to evaluate the interactions between CBDC and ρ° cells co-cultures between the two were established. To differentiate the two cell groups CBDC were created from cytosolic Ds-RED fluorescent mice, and the Astrocyte ρ° cells express cytosolic eGFP. The ρ° cells were plated onto established CBDC for three days before pyruvate and uridine were removed.

At pyruvate and uridine removal the plates were placed in an IncuCyte where images were taken every 3-6 hours for five days. From these data, proliferation of both the CBDC and the ρ^0 cells were analysed by assessing the change in confluency generated by the IncuCyte. At the conclusion of the experiment time-lapse videos were created of the consecutive images, for field of view photographed on the IncuCyte. These videos allowed individual cells to be followed through pyruvate and uridine deprivation, and the fate of ρ^0 cells in the co-culture were tracked through the experiment. Their survival or demise was recorded along with their contact with CBDC, where this occurred. Cells that migrated out of the field of view during the course of the time lapse were discounted, as their fate could not be determined.

The proportion of ρ^0 cells that came into contact with the CBDC was also recorded allowing the determination of whether increased or decreased cellular contact resulted in increased or decreased cellular survival.

Figure 4-1 shows the proliferation of the different ρ^0 cell lines in the absence of pyruvate and uridine in the presence and absence of CBDC. The Astrocyte ρ^0 cells (Figure 4-1 A) proliferated more when in co-culture with CBDC, than in culture without CBDC. The Astrocyte ρ^0 cells made up approximately 1.5% of the surface area of the co-culture when pyruvate and uridine was removed. The -CBDC conditions confluency decreased to approximately zero while the +CBDC condition decreased a little to approximately 0.5% confluency, before increasing back up to 1% confluency. The initial decrease of the 'with CBDC' condition also appeared to be less extreme than that of the '- CBDC' condition.

4.1.1 Proliferation of ρ^0 Without Pyruvate and Uridine in Co-Culture with CBDC

Figure 4-1: Proliferation of ρ^0 cells without pyruvate and uridine in co-culture with CBDC. -PU means minus pyruvate and uridine, Hours is hours elapsed since deprivation. Error bars are standard error. Statistical significance is calculated using the area under the curve and a paired T-test. Experiments were repeated in triplicate. * is a p-value of <0.0001. Rho = ρ^0 . A) Astrocyte ρ^0 cell proliferation with and without CBDC. Y-axis is from 0%-3% confluency. B) Proliferation of 4T1 ρ^0 cells with and without CBDC. y-axis is 0%-30% confluency. C) B16 ρ^0 cells proliferation with and without CBDC. y-axis is 0% -30% confluency.

Similarly, to the Astrocyte ρ^0 cells the 4T1 ρ^0 cells (Figure 4-1 B) proliferate more when in co-culture with CBDC than in culture without CBDC. The 4T1 ρ^0 cells with and without CBDC started at approximately 2% area of the co-culture with the negative CBDC condition decreasing or stable confluency at approximately 2% while the co-cultures with CBDC increased to approximately 10% confluency.

In addition to the 4T1 ρ^0 cells and Astrocyte ρ^0 cells, the B16 ρ^0 cells (Figure 4-1 C) cells also proliferated more when in co-culture with CBDC than in culture without CBDC. The B16 ρ^0 cells with and without CBDC started at approximately 2% of the co-culture with the negative CBDC condition stable or barely increased in confluency from 2-3% while the cells in culture with CBDC increased up to 10% confluency. This was consistent with the increased resilience of the B16 ρ^0 cells to pyruvate and uridine deprivation described in chapter 3.

Taken together, these results show that all ρ^0 cells proliferated more under pyruvate and uridine deprivation when in co-culture with CBDC than without CBDC co-culture.

4.1.2 Survival of ρ° with and without Co-Culture and Contact with CBDC

Figure 4-2: Contact dependant survival of ρ° cell lines in co-culture with CBDC. Experiments performed in triplicate of 49 videos per replicate, error bars are range and statistical significance calculated via paired t-test. Rho = ρ° . A) Astrocyte ρ° cell contact with CBDC and Astrocyte ρ° cell survival over 5 days, the p-value of -PU contact vs no contact is 0.0002, p-value of +PU contact vs -PU contact is <0.0001. B) 4T1 ρ° cell contact with CBDC and Astrocyte survival over 5 days. C) B16 ρ° cell contact with CBDC and Astrocyte ρ° cell survival over 5 day the p-value of -PU contact vs no contact is 0.0011, p-value of +PU contact vs -PU contact is 0.0290.

The survival of individual cells in co-culture with CBDC is shown in figure 4-2, this shows both co-culture with and without the removal of pyruvate and uridine.

The Astrocyte ρ^0 cells (Figure 4-2 A) in positive pyruvate and uridine conditions had almost 100% survival over 5 days and contact with CBDC made little difference to their survival. In the minus pyruvate and uridine with CBDC condition however there was a significant difference in survival between those cells that had come into contact with the CBDC and those that had not. Those that came into contact had approximately 60% survival while those without contact had approximately 10% survival, this contact associated survival was significant with a p-value calculated via t-test of 0.0002.

This impact of co-culture was less clear in the 4T1 ρ^0 cells (Figure 4-2 B). As expected, the cell in the positive pyruvate and uridine conditions regardless of contact state had almost 100% survival over 5 days. In the pyruvate and uridine negative with CBDC condition there appears to be a much smaller difference (not significant) in survival between those cells that had come into contact with the CBDC and those that had not. With those cells having contact with the CBDC having an approximately 90% survival and those without contact have approximately 70% survival. This less clear advantage was likely due to the 4T1 ρ^0 cell growth patterns in which 4T1 ρ^0 cells grow in clumps of cells as shown in chapter 3. This means that CBDC contact with individual cells is difficult to determine as the cells are so close together.

Similarly, to the Astrocyte ρ^0 cells, the B16 ρ^0 cells (Figure 4-2 C) in the positive pyruvate and uridine conditions had almost 100% survival over 5 days and that their contact with CBDC made no difference to their survival. Conversely in the pyruvate and uridine negative CBDC positive conditions there was a significant difference in survival between cells that had come into contact with the CBDC and those that had not. Those cells that had CBDC contact had a roughly 78% survival while those without contact had roughly a 20% survival, this contact associated survival was significant with a p-value calculated via t-test of 0.0011.

Together these results show that CBDC contact had no effect on cell survival in pyruvate and uridine positive conditions, but conferred significant survival in two of the three ρ° cell lines without pyruvate and uridine.

4.1.3 Percent Contact of ρ^o with CBDC

Figure 4-3: Percent contact between CBDC and ρ^o cells.

Experiments performed in triplicate, error bars are range and statistical significance calculated by paired t-test. Rho = ρ^o . A) The total percent contact between Astrocyte ρ^o cells and CBDC in both pyruvate uridine conditions. B) The total percent contact between 4T1 ρ^o cells and CBDC in both pyruvate uridine conditions. C) The total percent contact between B16 ρ^o cells and CBDC in both pyruvate uridine conditions p-value is 0.0083

Finally, the total percentage of ρ° cells that made contact with CBDC was calculated from all the cells that remained in the field of view until the end of the experiment or their death. For the Astrocyte ρ° cells with and without pyruvate and uridine (Figure 4-3 A) there was no significant difference in the percentage of cells that came into contact with the CBDC in either pyruvate and uridine condition. The 4T1 ρ° cells however (Figure 4-3 B) shows there was potentially an increase of 7% in the percentage of cells that come into contact with the CBDC in the minus pyruvate and uridine condition with a p-value nearing significance of 0.0619 . The B16 ρ° cells (Figure 4-3 C) were similar to the 4T1 ρ° cells showing a significant increase of approximately 20% in the percentage of cells that come into contact with the CBDC in the minus pyruvate and uridine condition with a p-value calculated via T-test of 0.0080.

This indicates that a stress-induced increase in contact between ρ° cells and CBDC may be cell specific as it was not consistent across all three cell lines.

4.2 Differential Transcriptomics of ρ^0 Cell Lines under Metabolic Stress.

The presence of CBDC increased survival of the ρ^0 cells under severe metabolic stress. To identify signals that may be produced by ρ^0 cells to encourage contact between ρ^0 and CBDC and subsequent survival I took ρ^0 cell lines 4T1 ρ^0 , B16 ρ^0 and Astrocyte ρ^0 and deprived them of pyruvate and uridine. RNA was collected at a timepoint of 24 hours. This was chosen for several reasons. Firstly, cellular fusion was observed in the Astrocyte ρ^0 cells within the first 24 hours indicating that the signalling event occurred rapidly. Secondly, this timepoint was designed to detect immediate changes in gene expression. Finally, 24 hours was sufficient time to induce severe metabolic stress, but with as little cell death as possible to allow isolation of RNA of sufficient quality for analysis. The RNA was then extracted, converted to cDNA and underwent long read sequencing on the MinION sequencer (Oxford Nanopore Technologies). Independent triplicate samples were barcoded, and the deprived and non-deprived samples were then run on the same flow cell to minimise batch variation and allow for direct comparisons between the two.

In order to identify genes where expression was altered by pyruvate and uridine loss, the minus pyruvate and uridine samples were compared to the positive pyruvate and uridine controls and differential expression analysis was performed using DESeq2.

These changes, in the first 24 hours of deprivation, could be the signal to initiate cellular contact, survival and/or mitochondrial transfer.

Table 4-1: Total mapped reads for barcodes on each flow cell

	Total mapped reads	Treatment	Cell Line
RD003.2 BC02	1030503	UT	4T1 ρ°
RD003.2 BC03	958921	UT	4T1 ρ°
RD003.2 BC04	1506762	T	4T1 ρ°
RD003.2 BC05	1698553	T	4T1 ρ°
RD003.3 BC01	271634	UT	Astro ρ°
RD003.3 BC02	990983	UT	Astro ρ°
RD003.3 BC03	584564	UT	Astro ρ°
RD003.3 BC04	1287698	T	Astro ρ°
RD003.3 BC06	814985	T	Astro ρ°
RD003.6 BC01	1016542	T	B16 ρ°
RD003.6 BC02	1964566	T	B16 ρ°
RD003.6 BC04	1298306	UT	B16 ρ°
RD003.6 BC05	2709250	UT	B16 ρ°
RD003.6 BC06	2074089	UT	B16 ρ°

Table 4-1 shows the total number of mapped reads for each barcode used in this chapter, Treatment shown as T is 24 hours without pyruvate and uridine and UT is with pyruvate and uridine. The range in reads is from 271,634 to 2,074,089 with most barcodes having more than 1,000,000 reads.

4.2.1 DESeq2 of Astrocyte ρ^0

A

B

C

Figure 4-4: DESeq2 analysis of Astrocyte ρ^0 cell line treated deprived of pyruvate and uridine for 24 hours. cDNA generated by PCR and sequenced using a MinION long read sequencer, transcripts were removed if they had less than 5 reads or mapped in the reverse direction. A) Volcano Plot of the DESeq2 coverage showing Log 2-Fold Change on the X axis and Log₁₀P to demonstrate statistical significance on the Y axis, green dots are log 2-Fold change of greater than 1, blue dots denote significant p-values and red denotes both. B) PCA plot showing the amount of variance explained by the variables untreated (+pyruvate and uridine) and treated (-pyruvate and uridine). C) Unsupervised heatmap of the individual samples and their differential expression.

After differential sequencing the data was visualised in three separate ways (Figure 4-4). First the fold change value for every transcript was plotted against the negative log 10 of its own p-value (Figure 4-4 A) in a volcano plot. From this the colour co-ordination allows us to see both the volume of significantly changed transcripts and the volume of transcripts with significant p-values. The volume and scale of the differential expression i.e., the number of transcripts which are both significantly changed (Log 2-fold > 1) and have significant p-values (<0.05) can thus be evaluated. For the Astrocyte ρ^0 there was a strong differential signature between pyruvate and uridine deprived Astrocyte ρ^0 and non-deprived Astrocyte ρ^0 .

The Astrocyte ρ^0 cells deprived of pyruvate and uridine had more genes upregulated than downregulated that had significant p-values and had a log 2-fold change of greater than one which is a doubling of gene expression. This resulted in a volcano plot with a high and narrow shape. This means that of the transcripts that have significant p-values and significant log 2-fold changes, most of the log 2-fold changes were low, in this case most are less than 2.5.

The same data were analysed using a principal component analysis (PCA) (Figure 4-4 B) to identify how similar or different the deprived of pyruvate and uridine or not deprived samples were from one another. PCA separates the variation in an experiment by a series of components that each account for a proportion of the experimental variation. In a transcriptomic experiment each principal component is a gene or set of genes, the differential expression of which explains the variation of the samples. A PCA plot is the first two principal components plotted against one another, each sample is plotted within this based on the expression of the gene or genes in the principal component. The first principal component (PC1) for the Astrocyte ρ^0 cells separated the non-deprived from the deprived samples and explained 60% of the variance in the sample. The second principal component (PC2) identified some variation in the 3 non-deprived samples, with two clustering together and one separated. However, this was a smaller effect, and the two sample types were easily separated using the first PC

Finally, in this analysis the heatmap (Figure 4-4 C) allows the scale of the differential expression to be seen. The top 50 transcripts were selected and put through an unsupervised clustering analysis. Where there were multiple different transcripts for a single gene, a representative transcript was chosen. The 'between condition' and 'within condition' variation that is represented in the PCA plot (Figure 4-4 B) was confirmed. There was a clear divide between the pyruvate and uridine conditions that have clustered within condition. The heatmap also showed the range of log 2-fold changes for the top 50 transcripts with all falling between 1.5 and -1.5.

4.2.2DESeq2 of 4T1 ρ^0

A

B

C

Figure 4-5: DESeq2 analysis of 4T1 ρ^0 cell line treated deprived of pyruvate and uridine for 24 hours. cDNA generated by PCR and sequenced using a MinION long read sequencer, transcripts were removed if they had less than 5 reads or mapped in the reverse direction. A) Volcano Plot of the DESeq2 coverage showing Log 2-Fold Change on the X axis and Log₁₀P to demonstrate statistical significance on the Y axis, green dots are log 2-Fold change of greater than 1, blue dots denote significant p-values and red denotes both. B) is a PCA plot showing the amount of variance explained by the variables untreated(+pyruvate and uridine) and treated (-pyruvate and uridine) . C) Shows an unsupervised heatmap of the individual samples and their differential expression.

The 4T1 ρ° cells differential sequencing (Figure 4-5) was visualised in three ways in the same manner as the previous results for the Astrocyte ρ° cells, by volcano plot, PCA plot and heatmap. In contrast to the Astrocyte ρ° cells, in the volcano plot (Figure 4-5 A) for the 4T1 ρ° cells there were more downregulated differentially expressed transcripts than upregulated transcripts with significant p-values. The 4T1 ρ° cells had both more highly differentially expressed transcripts and more significant transcripts than the Astrocyte ρ° giving the volcano plot a wider and more filled appearance. Many of the transcripts had a bigger change than the Astrocyte ρ° cells, between 1 and 5 log 2-fold change. This resulted in a stronger differential signature of 4T1 ρ° cells deprived of pyruvate and uridine compared than the Astrocyte ρ° cells at the same timepoint.

The PCA plot for 4T1 ρ° cells (Figure 4-5 B) shows that the PC1 separated the with pyruvate and uridine samples from the without pyruvate and uridine samples, this between condition variation accounted for 90% of the variation in the samples. The PC2 separates the within condition variance and this accounts for 8% of the variance in the experiment. Therefore, there is much more between condition variation than within condition variation in this experiment.

The unsupervised clustering of the 4T1 ρ° cells DESeq2 data using the top 50 most differentially expressed transcripts (Figure 4-5 C) demonstrated the same pattern as the PCA plot, driven by dependence on pyruvate and uridine condition. This confirms the PCA result that the between condition variation is greater than the within condition variation. The range for the log 2-fold changes of these transcripts fell between 2 and -2.

4.2.3 DESeq2 of B16 ρ^0

Figure 4-6: DESeq2 analysis of B16 ρ^0 cell line treated deprived of pyruvate and uridine for 24 hours. cDNA generated by PCR and sequenced using a MinION long read sequencer, transcripts were removed if they had less than 5 reads or mapped in the reverse direction. A) Volcano Plot of the DESeq2 coverage showing Log 2-Fold Change on the X axis and Log10P to demonstrate statistical significance on the Y axis, green dots are log 2-Fold change of greater than 1, blue dots denote significant p-values and red denotes both. B) is a PCA plot showing the amount of variance explained by the variables +PU (+pyruvate and uridine) and -PU (-pyruvate and uridine). C) Shows an unsupervised heatmap of the individual samples and their differential expression.

In contrast to the effect of pyruvate and uridine deprivation at 24 hours on the 4T1 ρ^0 cells and Astrocyte ρ^0 cells there was a smaller effect on the B16 ρ^0 cells. This is evidenced by the volcano plot (Figure 4-6 A) which unlike the 4T1 ρ^0 cells and Astrocyte ρ^0 cells has no transcripts with p values < 0.05 and few transcripts with log 2-fold change of greater than one. This leads to a visually low narrow volcano plot.

Similar to the PCA plots of the 4T1 ρ^0 cells and Astrocyte ρ^0 cells the PC1 of the B16 ρ^0 (Figure 4-6 B) separated the pyruvate and uridine controls from the without pyruvate and uridine samples, however this separation is less than the previous examples with the PC1 accounting for 49% of the total sample variation. The PC2 is also similar to the other ρ^0 cell samples in that it identified variation within the 'with pyruvate and uridine' samples, again like the B16 ρ^0 cells PC1 to a lesser degree than the others. This PC2 accounts for 37% of the sample's total variation. This indicates that there is less within sample variation than between sample variation. Unlike the 4T1 ρ^0 cells and Astrocyte ρ^0 cells the PC2 of the B16 ρ^0 accounted for a much higher proportion of the variation indicating that there is less variation between conditions, more variation within conditions compared to the other ρ^0 cells or a combination of both.

The lower amount of variation between conditions seen in the PCA (Figure 4-6 B) is further evidenced in the heatmap (Figure 4-6 C) where one of the with pyruvate and uridine samples clustered not within its condition but with the pyruvate and uridine deprived samples. Despite this mostly successful unsupervised clustering, as a log 2 fold of zero is yellow it is apparent that most of the top 50 transcripts have a very small transcriptional change, as seen in the volcano plot (Figure 4-6 A). Those transcripts that do change range between 1 and -1.5 log 2 fold change. This all results in a differential sequencing analysis for the B16 ρ^0 cells that was less informative than it was for the Astrocyte ρ^0 cells and 4T1 ρ^0 cells. This was consistent due to the lesser effect that pyruvate and uridine deprivation had on the B16 ρ^0 cells throughout.

4.2.4 Comparison of differential transcripts for all 3 ρ^0 lines

Next, I wanted to know what transcripts were differentially expressed in common across ρ^0 cells that had been deprived of pyruvate and uridine.

Because the B16 ρ^0 cells in particular had a low number of transcripts that were significant using the adjusted p-value of <0.05 , a list of differentially expressed genes was generated using a non-adjusted p-value of <0.05 . This analysis was also performed without the B16 ρ^0 due to the low number of differentially expressed transcripts.

To do this I produced a series of Euler plots from the genes that had p-values of less than 0.05. The adjusted p-values were then used later to exclude genes in the results that had no significant adjusted p-values.

The B16 ρ^0 cell results in particular had a low number of genes with significant adjusted p-values and this method was used in order to compare the gene sets and use the B16 ρ^0 cells dataset.

Figure 4-7: Comparison between p^0 lines and their differentially expressed gene lists.

Lists of transcripts with p-value of <0.05 when deprived of pyruvate and uridine for 24 hours. $p0 = p^0$ A) Shows a Euler plot comparing upregulated genes common between 4T1 p^0 cells, B16 p^0 cells and Astrocyte p^0 cell. B) Shows a Euler plot comparing downregulated genes common between 4T1 p^0 cells, B16 p^0 cells and Astrocyte p^0 cells.

In order to evaluate the similarities between the cell lines the differentially expressed transcript lists were separated into upregulated and downregulated transcripts. First the up regulated transcripts were compared to each other (Figure 4-7 A), where the 4T1 p^0 cells had the highest number of differentially expressed transcripts with p-values <0.05 , followed by the Astrocyte p^0 cells and finally the B16 p^0 cells. The overlap between the different sets showed that both the 4T1 p^0 cells and B16 p^0 cells lists had more in common with the Astrocyte p^0 cells list than each other, and that the Astrocyte p^0 cells list was more similar to the B16 p^0 cells list. There are four transcripts common among all 3 p^0 cell line transcript lists. Proportionally the overlap represented, 0.23% of the 4T1 p^0 cells, 1.45% of the B16 p^0 cells and 0.74% of the Astrocyte p^0 cells.

Next the downregulated transcripts were analysed (Figure 4-7 B). Unlike the upregulated transcripts there were no commonly downregulated genes between all three p^0 lines. However, 1.2% of the 4T1 p^0 cells set was represented in the B16 p^0 cells set and 1.8% was represented in the Astrocyte p^0 cells set. There was 2.5% of the B16 p^0 cells set represented in the 4T1 p^0 cells set and 10.8% represented in the Astrocyte p^0 cells set. The Astrocyte p^0

cells had 8.5% in common with the B16 ρ° cells and 2.9% with the 4T1 ρ° cells. Consistent with the upregulated transcripts this showed that in the downregulated transcripts the 4T1 ρ° cells and the B16 ρ° cells had slightly more in common with the Astrocyte ρ° cells than with each other and the Astrocyte ρ° cells had more in common with the B16 ρ° cells rather than the 4T1 ρ° cells. This indicates that the B16 ρ° cells and Astrocyte ρ° cells may respond to stress in more similar ways than the 4T1 ρ° cells.

Table 4-2: Upregulated genes with significant p-values common among all 3 ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Transcript	Astro log 2-Fold Change	Astro padj	4T1 log 2-Fold Change	4T1 padj	B16 log 2-Fold Change	B16 padj
<i>Nars.5</i>	1.6196	0.0003	1.3195	0.0038	1.0461	1.0000
<i>Nars</i>	1.5092	0.0000	0.7068	0.0671	0.6128	1.0000
<i>Nars.4</i>	1.4133	0.0082	1.0186	0.0479	0.9726	1.0000
<i>Nars.1</i>	1.3457	0.0007	0.5916	0.1885	0.5672	1.0000

The overlap from Figure 4-7 A is shown above. The 4 transcripts are all of the *Nars1* gene and were upregulated in all 3 ρ° cell datasets. The adjusted p-values were significant in both the 4T1 ρ° cells and Astrocytes ρ° cells for 2 of the transcripts. In the B16 ρ° cells none of the changes in expression were significant.

Nars1 (Asparaginyl-tRNA synthetase1) is a protein coding gene expressed in the cytoplasm^{358,359}. The normal functioning of *Nars1* is to load amino acids onto their tRNA for protein production^{358,360}. However, the Aminoacyl tRNA synthetases (*Ars*) to which *Nars1* belongs, are known to play a role in neurodevelopmental disorders and chemokine signalling³⁶¹.

4.2.5 Comparison of differential transcripts for 4T1 ρ^0 and Astrocyte ρ^0 cell lines

As the B16 ρ^0 cells dataset was not significant the same analysis as 4.2.4 was run using only the 4T1 ρ^0 cells and Astrocyte ρ^0 cells datasets.

Figure 4-8 Comparison between 4T1 ρ^0 cells and Astrocyte ρ^0 cells lines and their differentially expressed gene lists.

Lists of transcripts with p-value of <0.05 when deprived of pyruvate and uridine for 24 hours. $p0 = \rho^0$

A) Shows a Euler plot comparing upregulated genes common between 4T1 ρ^0 cells and Astrocyte ρ^0 cells. B) Shows a Euler plot comparing downregulated genes common between 4T1 ρ^0 cells and Astrocyte ρ^0 cells.

This analysis shown in figure 4-8 A produced 30 commonly upregulated transcripts and 20 commonly downregulated transcripts. This accounted for 5.6% of the Astrocyte ρ^0 list and 1.7% of the 4T1 ρ^0 cells list. This was as expected from what was seen in 4.2.4. In 4-8 B there was 20 overlapping transcripts which accounts for 1.8% of the 4T1 ρ^0 cells list and 2.8% of the Astrocyte ρ^0 list.

Tables of the upregulated and downregulate transcripts were generated to see if any further insight from 4.2.4 could be gleaned.

Table 4-3: Upregulated genes with significant p-values common between 4T1 ρ^0 and Astrocyte ρ^0 cell lines.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Transcript	Astro log 2-Fold Change	Astro padj	4T1 log 2-Fold Change	4T1 padj
<i>Cdc6.2</i>	2.4877	0.4855	4.3594	NA
<i>Cdc6</i>	2.3905	0.4024	4.7918	NA
<i>Cdc6.1</i>	2.2575	0.6399	4.5270	NA
<i>Fen1</i>	1.6302	0.1290	1.3320	0.1708
<i>Fen1.1</i>	1.6220	0.1316	1.4525	0.1276
<i>Nars.5</i>	1.6196	0.0003	1.3195	0.0038
<i>Fen1.2</i>	1.5525	0.2148	1.3108	0.2082
<i>Nars</i>	1.5092	0.0000	0.7068	0.0671
<i>Fen1.3</i>	1.4268	0.4927	1.4829	0.2159
<i>Nars.4</i>	1.4133	0.0082	1.0186	0.0479
<i>Nars.1</i>	1.3457	0.0007	0.5916	0.1885
<i>Hsph1</i>	1.1077	0.6905	1.1647	0.1257
<i>Hnrnp.3</i>	1.0978	0.7816	0.8183	0.1946
<i>Amd1.3</i>	1.0449	0.6343	0.8574	0.1393
<i>Hnrnp.2</i>	1.0415	0.8681	0.9504	0.0863
<i>Dhfr</i>	1.0352	0.7997	2.1365	0.0605
<i>Hnrnpm</i>	0.9984	0.6928	1.0887	0.0202
<i>Prim1</i>	0.9960	0.9546	1.8017	0.1304
<i>Hsph1.1</i>	0.9796	0.9991	1.0234	0.2455
<i>Amd1.1</i>	0.9516	0.5667	0.6628	0.2697
<i>Amd1.2</i>	0.9516	0.5667	0.6628	0.2697
<i>Tcof1.1</i>	0.9444	0.8164	0.9278	0.1308
<i>Amd2</i>	0.9441	0.6501	0.7089	0.1806
<i>Tcof1</i>	0.9280	0.8615	0.9590	0.1117
<i>Bc1</i>	0.8862	0.5330	0.9529	0.0128
<i>Mcm2.1</i>	0.8732	0.9991	1.1108	0.2157
<i>Hnrnp.1</i>	0.8560	0.9991	1.1023	0.0205
<i>Pcna</i>	0.6635	0.9991	1.2789	0.0048
<i>Pa2g4</i>	0.5396	0.9991	0.7134	0.0805
<i>Hsp90aa1</i>	0.5238	0.9991	0.5590	0.1373

Table 4-4: Down regulated genes with significant p-values common between 4T1 ρ° cell and Astrocyte ρ° cell lines.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Transcript	Astro log2FoldChange	Astro padj	4T1 log2FoldChange	4T1 padj
<i>Ip6k2.6</i>	-4.5824	0.9991	-3.5307	NA
<i>Ip6k2.7</i>	-4.5824	0.9991	-3.5307	NA
<i>Scx</i>	-2.7077	0.0424	-1.1978	0.1160
<i>Wincrl</i>	-2.0451	0.0034	-4.8007	NA
<i>Senp6.3</i>	-1.6745	0.8907	-1.9094	0.1677
<i>Wincrl.1</i>	-1.6637	0.1103	-3.5037	NA
<i>Senp6.1</i>	-1.4013	0.9991	-1.5183	0.2039
<i>Eif5.4</i>	-1.1760	0.5426	-0.8624	0.1076
<i>Rpl24.2</i>	-1.1117	0.1698	-0.6983	0.0986
<i>Eif5.3</i>	-1.0839	0.5823	-0.8952	0.0652
<i>Eif5.5</i>	-1.0322	0.9991	-1.1120	0.0726
<i>Rpl24.1</i>	-1.0306	0.0852	-0.5220	0.2434
<i>Zfp467</i>	-0.9857	0.9991	-0.7136	0.1691
<i>Zfp467.1</i>	-0.9857	0.9991	-0.7136	0.1691
<i>Zfp467.2</i>	-0.9857	0.9991	-0.7136	0.1691
<i>Eif5</i>	-0.8855	0.4866	-0.7716	0.0781
<i>Gm10263</i>	-0.8673	0.9991	-0.8812	0.1344
<i>Eif5.1</i>	-0.8484	0.7482	-0.8403	0.0385
<i>Eif5.2</i>	-0.7967	0.9991	-0.8438	0.0419
<i>Ptgs2</i>	-0.6524	0.9991	-2.0190	0.0000

These tables 4-3 and 4-4 show that no new transcripts are identified as significant to both transcript sets past the two *Nars1* transcripts. These tables however identify many further transcripts that are significant to one cell line and present but not significant in the other.

Next, as the Astrocyte ρ° cell line was not cancer derived, the potential for a cancer specific signal for ρ° was evaluated.

4.2.6 Comparison of Differential Transcripts for Cancer ρ^0 Lines

Figure 4-9: Comparison between cancer derived ρ^0 lines.

Differentially expressed gene lists with p-value of <0.05 when deprived of pyruvate and uridine for 24 hours, $p_0 = \rho^0$. A) Shows a Euler plot comparing upregulated genes common between cancer lines 4T1 ρ^0 cells, B16 ρ^0 cells . B) Shows a Euler plot comparing downregulated genes common between cancer lines 4T1 ρ^0 cells, B16 ρ^0 cells .

As with the ρ^0 cell lines the upregulated and downregulated transcripts were separated. It had already been established that there are few genes in common between the ρ^0 cancer lines. However this overlap, small though it is may represent a common mechanism for survival of stress in cancer. The upregulated transcripts (Figure 4-8 A) show there were 11 commonly upregulated genes between both the B16 ρ^0 cells and 4T1 ρ^0 cells, this represented only 0.6% of the 4T1 ρ^0 cells set but 4% of the B16 ρ^0 cells set. In the downregulated transcripts (Figure 4-8 B) this overlap was 13 genes that represented 1.2% of the 4T1 ρ^0 cells set and 2.5% of the B16 ρ^0 cells set.

This reiterated that at the transcript level, there was very little in common between the two cancer lines.

Table 4-5: Upregulated genes with significant p-values common among cancer ρ° cell lines. significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Gene	4T1 log 2-Fold Change	4T1 padj	B16 log 2-Fold Change	B16 padj
<i>Psmc3.4</i>	2.3206	0.0369	1.2770	1.0000
<i>Psmc3.5</i>	2.2446	0.0622	1.3546	1.0000
<i>Psmc3.3</i>	1.9394	0.0415	1.1474	1.0000
<i>Ran.2</i>	1.8359	0.2713	1.1333	1.0000
<i>Set.4</i>	1.3882	0.1001	1.2177	1.0000
<i>Set.3</i>	1.3411	0.0908	1.2247	1.0000
<i>Rny3</i>	1.3300	0.0010	1.2193	1.0000
<i>Nars.5</i>	1.3195	0.0038	1.0461	1.0000
<i>Nars.4</i>	1.0186	0.0479	0.9726	1.0000
<i>Nars</i>	0.7068	0.0671	0.6128	1.0000
<i>Nars.1</i>	0.5916	0.1885	0.5672	1.0000

Table 4-6: Downregulated genes with significant p-values common among cancer ρ° cell lines. significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Transcript	4T1 log 2-Fold Change	4T1 padj	B16 log 2-Fold Change	B16 padj
<i>Gsta1</i>	-4.0285	0.0000	-0.8646	1.0000
<i>Gm3776</i>	-4.0047	0.0000	-0.8303	1.0000
<i>Cryab.1</i>	-2.0209	0.0635	-0.9043	1.0000
<i>Commd9</i>	-2.0064	1.0000	-0.9198	1.0000
<i>Cryab.2</i>	-1.9029	0.1944	-0.7481	1.0000
<i>Cryab</i>	-1.7556	0.0836	-0.7973	1.0000
<i>1810058I24Rik</i>	-1.3115	0.0040	-0.6035	1.0000
<i>Mt1</i>	-0.9905	0.0020	-0.8301	1.0000
<i>Ctsb</i>	-0.9187	0.0239	-0.6372	1.0000
<i>Ssr3</i>	-0.9023	0.2402	-0.7528	1.0000
<i>Gm49701</i>	-0.8903	0.0665	-0.5725	1.0000
<i>Fkbp9</i>	-0.7596	0.0615	-0.7150	1.0000
<i>Cst3</i>	-0.6487	0.1942	-0.8299	1.0000

The common transcripts from Figure 4-8 are shown in the tables above (Tables 4-2 and 4-3). These tables show that there are only significantly upregulated or downregulated genes in the 4T1 ρ° cells dataset. The upregulated genes common to both B16 ρ° cells and 4T1

ρ° cells , and significant to 4T1 ρ° cells are two *Psmc3* transcripts, *Rny3* and the 2 *Nars1* transcripts. The downregulated genes are *Gsta1*, *Gm3776*, *1810058/Rik*, *Mt1* and *Ctsb*.

The RNA-sequencing experiments were multiplexed in batches to reduce any possibility for inter flow cell transcriptional differences with the untreated/ non deprived samples being spread across multiple flow cells. This allowed us to normalise between flow cells. A quantitative PCR experiment was designed to assess the coverage and to confirm differential expression. Transcripts with both low expression (*Ccl20* and *Irf7*) and high expression (*Nars1* and *Crip1*) were identified from the RNA seq data, as well as unchanged 'housekeeper' genes *Hprt* and *Ubb*. The aim of this experiment was to examine how effective the coverage of the full RNA seq was and also to determine if this had an effect of differential expression observed in the results.

Table 4-7: qPCR results for sequencing confirmation.

4T1p0			
Gene	read count range	deseq2(UT -PU)	ddCT(qPCR)
UBB	219-384		
HPRT	12-24		
CCL20	No reads		0.02590471
IRF7	No reads		0.344394
NARS	206-568	0.4-1.3	-0.0645999
CRIP	98-705	1.7-1.8	-1.3722974
4T1dox			
UBB	No reads		
HPRT	No reads		
CCL20	12-45	2.4-2.7	-1.0671338
IRF7	0-19	4.6-5.4	0.41517682
NARS	0-236		
CRIP	176-353		

As shown in table 4-7 genes with low or no transcription via the MinION sequencing results still amplify via qPCR, this indicated that we have low sequencing coverage and were potentially missing from the sequencing results where there could have been differentially

expressed interesting results. *Nars1* was expected to show a positive log 2-fold change from the DESeq2 results for the 4T1 ρ^0 cells. However, we see that there is a slight negative log 2-fold change in these results. This is likely due to the DESeq2 being performed at transcript level as opposed to the qPCR being performed at gene level. With only 2 of the 14 *Nars1* transcripts/potential transcripts (ENSMUST00000236392 and ENSMUST00000236186) being significantly differentially expressed it is possible that the results are diluted by the non-differentially expressed transcripts. Thus, the differences seen between the qPCR results and the DESeq2 results such as the *Cc/20* result could be due to differences in differential expression at the transcript level or due to low sequencing coverage in the experiment due to multiple barcoded samples per flow cell.

4.3 Gene Set Enrichment Analysis of ρ^0 Cell Lines in Metabolic Stress.

Gene set enrichment analyses (GSEA) allow extrapolation of gene/transcript sets into pathways and biological functions. This is a more broad analysis than looking at specific gene and transcript function and determining relationships individually. The GSEA were performed using fGSEA, a Bioconductor package for R ³⁴⁷. The following is a description of how GSEA are conducted using DESeq2 data.

The normalised expression data (DESeq2), is ranked by the Wald statistic generated in the DESeq2 analysis. This ranked list (L) is then compared to a set of genes associated with a phenotype (S) e.g. angiogenesis. For each gene in S its presence or absence in L is recorded, the associated rank score and the gene's correlation to the phenotype ³⁴⁷. Depending on the presence, rank and correlation these genes are classified as hits or misses. The enrichment score (ES) is calculated from hits – misses. If the L was randomly distributed the ES would be roughly zero and therefore not enriched. The normalised enrichment score (NES) is calculated from the ES and is normalised for the size of the gene set, and using the Benjamini-Hochberg procedure is corrected against multiple testing³⁴⁷.

The gene sets used in this analysis were the 'hallmarks' gene sets from MSigDB, they are a collection of gene sets for specific and defined biological processes ³⁶². They are updated frequently and include many processes fundamental to the hallmarks of cancer ³⁶². This was used to hopefully allow a better understanding of the processes occurring in ρ^0 cells deprived of pyruvate and uridine and potentially how these processes could initiate cell-cell contact, mitochondrial transfer and cellular survival.

By using the GSEA it was hoped that even with the low numbers of transcripts in the B16 ρ^0 cells there would be enrichment of the same gene sets across all three cell lines.

4.3.1 GSEA of Astrocyte ρ° .

Figure 4-10: Gene Set Enrichment Analysis of Astrocyte ρ° cells.

Astrocyte ρ° cells without pyruvate and uridine for 24 hours using the hallmarks of cancer gene sets from the Broad institute and the fGSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the Astrocyte ρ° cells differential gene list is enriched or not in a given gene set (listed on the y axis). Size of the dots denotes how many of the genes in the gene set were found in the Astrocyte ρ° cells sample, the colour of the dots denotes the strength of the padj, which is the adjusted p-value, this corrects for multiple testing. The differentially expressed gene set used in this analysis was generated using DESeq2 as shown previously.

4.3.2 GSEA of 4T1 ρ^0 .

Figure 4-11: Gene Set Enrichment Analysis of 4T1 ρ^0 cells.

4T1 ρ^0 cells without pyruvate and uridine for 24 hours using the hallmarks of cancer gene sets from the Broad institute and the fgSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the 4T1 ρ^0 cells differential gene list is enriched or not in a given gene set (listed on the y axis). Size of the dots denotes how many of the genes in the gene set were found in the 4T1 ρ^0 cells sample, the colour of the dots denotes the strength of the padj, which is the adjusted p-value, this corrects for multiple testing. The differentially expressed gene set used in this analysis was generated using DESeq2 as shown previously.

4.3.3 GSEA of B16 ρ^0

Figure 4-12: Gene Set Enrichment Analysis of B16 ρ^0 cells.

B16 ρ^0 cells without pyruvate and uridine for 24 hours using the hallmarks of cancer gene sets from the Broad institute and the fGSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the B16 ρ^0 cells differential gene list is enriched or not in a given gene set (listed on the y axis). Size of the dots denotes how many of the genes in the gene set were found in the B16 ρ^0 cells sample, the colour of the dots denotes the strength of the padj, which is the adjusted p-value, this corrects for multiple testing. The differentially expressed gene set used in this analysis was generated using DESeq2 as shown previously.

Figure 4-9 displays the GSEA of the Astrocyte ρ° cells using the corresponding DESeq2 dataset (Figure 4-9), this had a roughly equal distribution of positively and negatively enriched gene sets. Slightly more significantly enriched sets had a positive NES compared to gene sets with a negative NES.

In contrast to the Astrocyte ρ° cells GSEA the 4T1 ρ° cells GSEA (Figure 4-10) generated using the corresponding DESeq2 dataset had more positively enriched gene sets than negatively enriched with a large number of sets with significant adjusted p-values.

Differently from both the 4T1 ρ° cells and Astrocyte ρ° cells GSEAs there were no enriched gene sets for B16 ρ° cells GSEA (figure 4-11) that have an adjusted p-value < 0.05 . More of the gene sets were negatively enriched than positively enriched.

4.3.4 Comparison of GSEA results

As with DESeq2 data the lists of enriched gene sets from the GSEA were separated into positively and negatively enriched gene sets. Again because of the less informative B16 ρ° cells results the lists were filtered on p-values < 0.05 . The adjusted p-values and NES were then used to evaluate the importance of the gene sets.

Figure 4-13: Comparison between ρ° cells lines and their up and down enrichment lists. Enriched gene sets with p-value of <0.05 when deprived of pyruvate and uridine for 24 hours. A) Shows a Euler plot comparing up-enriched gene sets common between 4T1 ρ° cells, B16 ρ° cells and Astrocyte ρ° cells. B) Shows a Euler plot comparing down-enriched gene sets common between 4T1 ρ° cells, B16 ρ° cells and Astrocyte ρ° cells. C) Shows a Euler plot comparing up-enriched gene sets common between 4T1 ρ° cells and B16 ρ° cells. D) Shows a Euler plot comparing down-enriched gene sets common between 4T1 ρ° cells and B16 ρ° cells.

From the commonly enriched hallmark gene sets (Figure 4-12) there were few gene sets with p-values < 0.05 and less still that were commonly enriched among all three ρ° cell lines, with just one positively enriched set and one negatively enriched sets in total.

The one positively enriched gene set that is common among all three cell lines represents 3.3% of the 4T1 ρ° cells, 100% of the B16 ρ° cells and 4.8% of the Astrocyte ρ° cells (figure 4-12 A and C)

There is one commonly negatively enriched gene set all three ρ° cell lines, this represents 20% of the 4T1 ρ° cells, 4.6% of the B16 ρ° cells and 5.3% of the Astrocyte ρ° cells (Figure 4-12 B).

The Astrocyte ρ° cells enriched gene sets shared 4.76% in common with the B16 ρ° cells and 47.62 % in common with the 4T1 ρ° cells. The Astrocyte negatively enriched gene sets share 52.63 % in common with the B16 ρ° cells and 10.53% in common with the 4T1 ρ° cells.

The 2 common negatively enriched gene sets common among both cancer cell lines sets, represent 40% of the 4T1 ρ° cells sets and 9.09% of the B16 ρ° cells sets (Figure 4-12 D).

As expected compared with the DESeq2 individual transcript data there was proportionally more in common between all of the cell lines, meaning the conclusions drawn from the GSEA analyses are more broad.

As the transcripts of the B16 ρ° cells list were not significant, this analysis was repeated with just the Astrocyte ρ° cells and 4T1 ρ° cells.

Figure 4-14: Comparison between 4T1 ρ° cells and Astrocyte ρ° cells lines and their up and down enrichment lists. Enriched gene sets with p-value of <0.05 when deprived of pyruvate and uridine for 24 hours. A) Shows a Euler plot comparing up-enriched gene sets common between 4T1 ρ° cells and Astrocyte ρ° cells. B) Shows a Euler plot comparing down-enriched gene sets common between 4T1 ρ° cells and Astrocyte ρ° cells.

As shown in figure 4-14 there was 10 positively enriched gene sets common among both cell lines representing 33.3% of the 4T1 ρ° cells, and 47.6% of the Astrocyte ρ° cells (figure 4-14 A).

There are two commonly negatively enriched gene sets for both ρ° cell lines, representing 40% of the 4T1 ρ° cells and 10.5% of the Astrocyte ρ° cells (Figure 4-14 B).

Table 4-8: Positively enriched gene sets with significant p-values common among ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	Astro padj	Astro NES	4T1 padj	4T1 NES	B16 padj	B16 NES
HALLMARK TNFA SIGNALING VIA NFκB	0.0461	1.9167	0.4672	1.1428	0.8070	1.1429

Table 4-9: Negatively enriched gene sets with significant p-values common among ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	Astro padj	Astro NES	4T1 padj	4T1 NES	B16 padj	B16 NES
HALLMARK TGF β SIGNALING	0.1748	-1.5793	0.4672	-1.1233	0.7111	-1.3004

Table 4-10: Positively enriched gene sets with significant p-values common among 4T1 ρ° and Astrocyte ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	Astro padj	Astro NES	4T1 padj	4T1 NES
HALLMARK APOPTOSIS	0.0229	2.5695	0.0174	2.0383
HALLMARK MTORC1 SIGNALING	0.0229	2.4456	0.0112	2.6528
HALLMARK TNFA SIGNALING VIA NFKB	0.0461	1.9167	0.4672	1.1428
HALLMARK PI3K AKT MTOR SIGNALING	0.0461	1.8345	0.3551	1.2743
HALLMARK OXIDATIVE PHOSPHORYLATION	0.1124	1.7369	0.4672	1.1418
HALLMARK MYC TARGETS V1	0.3746	1.2977	0.0220	1.9580
HALLMARK DNA REPAIR	0.3746	1.2879	0.1543	1.5385
HALLMARK APICAL JUNCTION	0.3746	1.2773	0.2333	1.3918
HALLMARK KRAS SIGNALING UP	0.4012	1.1986	0.0218	1.9519
HALLMARK CHOLESTEROL HOMEOSTASIS	0.5306	1.0075	0.0112	2.8029

Table 4-11: Negatively enriched gene sets with significant p-values common among 4T1 ρ° and Astrocyte ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	Astro padj	Astro NES	4T1 padj	4T1 NES
HALLMARK TGF BETA SIGNALING	0.1748	-1.5793	0.4672	-1.1233
HALLMARK HEME METABOLISM	0.1225	-1.6326	0.2154	-1.4136

Table 4-12: Positively enriched gene sets with significant p-values common among cancer ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 padj	4T1 NES	B16 padj	B16 NES
HALLMARK TNFA SIGNALING VIA NF κ B	0.4672	1.1428	0.8070	1.1429

Table 4-13: Negatively enriched gene sets with significant p-values common among cancer ρ° cell lines. Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 padj	4T1 NES	B16 padj	B16 NES
HALLMARK TGF β SIGNALING	0.4672	-1.1233	0.7111	-1.3004
HALLMARK HYPOXIA	0.2315	-1.3621	0.8436	-1.0275

Tables 4-7,8,11 & 12 show fewer significantly positively or negatively enriched gene sets after pyruvate and uridine derivation compared to the basic differential gene analysis in Tables 4-1,2,&3 . However this is likely due to the less informative results from the B16 ρ° cells resulting in fewer significant sets to compare overall. When only 4T1 ρ° cells and Astrocyte ρ° cells were compared in tables 4-9&10 there were more positively enriched gene sets including Apoptosis and MTORC1 signalling which were significant to both gene sets and not found in the B16 ρ° cells. While Heme metabolism was additionally present in the negatively enriched gene sets, it was not significant.

Interesting TNF α signalling via NF κ B was significantly positively enriched in the Astrocyte ρ° dataset but not in the 4T1 ρ° cells or B16 ρ° cells and TGF beta was negatively enriched in all three ρ° cells but not significantly so in any one.

4.4 Discussion

ρ° cells are not clinically relevant, there is currently no naturally occurring ρ° like phenotype, they represent an extreme model of metabolic stress and any results gathered from them may have limited clinical relevance. This is why in the following chapters I have used the techniques and processes set out in this chapter to evaluate chemotherapy treated parental lines.

4.4.1 Contact Associated ρ° Survival of Metabolic Stress

The results generated in this chapter shows that there was a proliferation advantage for ρ° cells under severe metabolic stress (deprived of pyruvate and uridine) in the presence of CBDC and this was consistent across all three cell lines. Enhanced contact associated survival may have been caused by mitochondrial transfer, metabolite transfer, a combination of both or neither of these mechanisms. The experiments conducted in this chapter did not explain how the advantage was achieved, only that it occurred. This is due to the nature of these experiments where the cells were cultured undisturbed for as long as possible in the IncuCyte to gather as much interaction data as possible. This meant that the cells 'overgrew' resulting in cultures unable to be used for confocal mitochondrial network analysis. The IncuCyte has limitations on the colours that can be used with it and it does not have the resolution required to see mitochondrial trafficking. The timing of many of the interactions I saw in the experiments on the Astrocyte ρ° CBDC co-cultures were after the first 24 hours, from chapter 3 it is known that mitochondrial transfer occurs in an Astrocyte ρ° CBDC co-culture within the first 24 hours of pyruvate and uridine deprivation.

There was an increase in contact with the CBDC in the minus pyruvate and uridine condition with the B16 ρ° cells, this could indicate chemotaxis of either CBDC or B16 ρ° cells. In order to confirm this hypothesis, a chemotaxis assays or similar could be performed.

4.4.2 Contact Associated Survival and Nars1 Mediated Signalling.

The aim of the RNA-sequencing was to identify potential signals produced by the ρ^0 cell deprived of pyruvate and uridine for 24 hours. These signals could contribute to the interactions between the ρ^0 cells deprived of pyruvate and uridine and CBDC and potentially lead to the contact associated survival advantage observed. Each of the ρ^0 cell lines had a clear survival advantage in the presence of CBDC, but the transcriptomic analyses found that there were few genes and pathways in common in severely stressed ρ^0 cells. The removal of pyruvate and uridine from ρ^0 cell cultures puts those cells under a lot of acute metabolic stress. I chose the 24 hour timepoint for RNA-sequencing to avoid as many death signals and signatures as possible. It was also the timepoint at which the Astrocyte ρ^0 cells showed cellular fusion in the chapter 3 and so I hypothesised that any signal to initiate contact must have occurred by 24 hours. I hypothesised that a signal would require a transcriptional change and that it would be visible at 24 hours.

With the low number of differentially expressed genes from the B16 ρ^0 cells it might have been more effective to deprive the B16 ρ^0 cells for a timepoint where a comparable amount of stress was observed and then compare gene expression changes to the 4T1 ρ^0 cells and Astrocyte ρ^0 cells. Metabolic stress could be measured or a timepoint used where there was a similar amount of cell death in the B16 ρ^0 cells, compared to the 4T1 ρ^0 cells and Astrocyte ρ^0 cells at 24 hours. This was a major contributor to the low statistical significance in the comparison work. With the B16 ρ^0 cells under more stress they would exhibit a stronger stress response that would induce a stronger differential transcription signature. With a stronger signature I believe I may have seen more common genes between the three cell lines. However, there would also have been more indirect effects present as well.

One of the other contributors to the low statistical significance is likely due to the multiplexing of samples using the MinION. On average 4-6 samples were multiplexed on each flow cell, this meant controls were run on the same flow cell as experimental samples, to ensure comparisons between controls and deprived samples were as precise as could be.

However it reduced the amount of reads per sample, which in turn reduced the statistical power of the experiment. MinION long read sequencing is a constantly evolving technology and over the 1.5 years in which all the sequencing was performed the ability to call reads has improved dramatically increasing the mapping performance which in turn with re-analysis has improved these results. A low read count due to multiplexing would also potentially exclude some transcripts from sequencing, if transcripts were low abundance they may have been missed due to multiplexing which could result in lower differential sequenced transcript results. Promising candidate genes were analysed by quantitative RT-PCR in each of the cell lines (table 4-7). These reagents were ordered, but due to substantial global manufacturing and shipping delays induced by the Covid19 pandemic these experiments were unable to be optimised for this thesis, the initial results are shown.

One gene, *Nars1* was consistently up regulated in all ρ^0 cells within 24 hours of pyruvate and uridine deprivation. *Nars1* is a highly conserved protein and is present in all branches of life ³⁶³. NARS1 functions in the cytoplasm while the closely related NARS2 functions in the mitochondria ³⁵⁸. The family of proteins to which NARS1 belongs is the ARS family. Mutations in ARS genes result in neurodevelopmental disorders including Charcot-Marie-Tooth neuropathies and hypomyelination of the brain stem and spinal cord³⁵⁹.

ARS are known to cause migration of CD4+ and CD8+ lymphocytes, interleukin-2- activated monocytes and immature dendritic cells in a CCR-5 dependant manner ³⁶⁴. ARS are associated with P43, P43 induces TNF production, p43 associated TNF leads to activation of NF κ B, therefore ARS leads to NF κ B activation ³⁶¹. ARS molecules released during inflammation and apoptosis are chemoattractant for lymphocytes, macrophages and antigen presenting cells ³⁶⁵. MHC class 1 and 2 presentation of ARS antigen invoke ARS specific autoimmune response ³⁶⁵.

NARS1 has also been associated with autoimmune diseases such as inflammatory myopathy interstitial lung disease for which autoantibodies have been found ^{364,366}. *Nars1* mutations are associated with problems in pathways such as cell cycle progression, DNA repair and replication (G2/M checkpoint), homology directed repair and telomere synthesis ³⁵⁹.

NARS1 interreacts with chemokine receptor 3 (CCR3)³⁶⁴ and has been shown to induce migration of lymphocytes, activated monocytes and immature dendritic cells³⁶⁷. The CBDC culture contains monocytes for which migration could be induced by NARS1.

Chemokine receptor 3 (CCR3) is expressed in eosinophils and when activated initiates eosinophil migration. It is also involved in metastasis and tumour invasion³⁶⁸. CCL7 interacts with CCR3, resulting in enhanced cellular proliferation invasion and migration³⁶⁸. CCR3 is upregulated in human renal cell carcinoma and presence correlated with grade of tumour³⁶⁹.

If the upregulation of *Nars1* transcripts ENSMUST00000236392 and ENSMUST00000236186 in these samples is confirmed, it could mean that a secretion of NARS1 or a release of NARS1 during apoptosis could be initiating CBDC monocyte migration via CCR3 activation and initiating cellular contact in the co-culture samples. A migration of these cells would bring more into contact with the ρ^0 cells possibly allowing for metabolite exchange and/or mitochondrial transfer and thus survival of stress. Currently Helena Abolins-Thompson a masters student in the McConnell lab is working on this and she has had some success in observing differential expression of *Nars1* transcripts via qPCR.

Gene set enrichment analysis of all three cell lines showed nothing significant in the cancer ρ^0 cell lines but a significant positive enrichment of TNF α signalling via NF κ B in the Astrocyte ρ^0 cells. As mentioned before, ARS are known to activate NF κ B³⁶¹. TNF α is a cytokine that is associated with inflammation, cell survival, proliferation and differentiation and NF κ B is a transcription factor highly linked to immune regulation³⁷⁰. TNF α signalling via NF κ B is known to promote invasion and metastasis of tumours by promoting an inflammatory tumour microenvironment³⁷¹.

It is therefore possible that this signalling mechanism outlined above is playing a role in the phenomenon of contact associated survival observed in the ρ^0 cell lines. This supports the *Nars1* findings as there are ARS genes involved in TNF- α signalling via NF- κ B. More evidence

would be needed to draw any conclusions to a role of NARS1, TNF-A and NFκB in the survival of p^o cells in co-culture environments.

5 Survival of Chemotherapy Treated

Cells in Co-Culture with Compact

Bone Derived Cells.

Throughout life our body's cells are exposed to a variety of stressors, either as a normal bi-product of living or intentionally during therapy. The previous chapter described the contact dependant survival of ρ^0 cell lines under metabolic stress, by co-culture with CBDC. The ρ^0 system is an extreme system of metabolic stress and is useful for determining what a complete lack of mitochondrial ATP and shutdown of the Citric acid cycle induces in cells. However there are no known treatments or conditions that cause such a severe phenotype. In the previous chapter I proposed mechanisms that could be responsible for this survival, based on cDNA sequencing.

In this chapter I will describe the effect of CBDC co-culture on cancer cells that have been treated with chemotherapeutics. The aim of this chapter is to understand if treated cells exhibit the same contact dependant survival seen in ρ^0 cells deprived of pyruvate and uridine. I also aim to understand if different chemotherapies exhibit the same or different interactions than those seen in the previous chapter.

5.1 Chemotherapy treatment

5.1.1 Doxorubicin

Doxorubicin is an anthracycline style drug³⁷². It is one of the most commonly used chemotherapeutic agents today and is used in the treatment of solid tumours, lymphomas and leukemias²⁵⁴. It intercalates with dsDNA and acts as a topoisomerase antagonist, these DNA-Doxorubicin adducts lead to double stranded DNA breaks and apoptosis^{250,254}. These

double strand breaks initiate apoptosis in cells through the BCL2/BAK caspase cascade^{249,254}. Resistance to Doxorubicin is common and problematic, it is particularly associated with loss of function of PTEN activating the mTOR/TP53 inhibition of apoptosis^{373,374}.

5.1.2 Cisplatin

Cisplatin has been used as a chemotherapeutic agent in humans since it was approved for use in cancer in 1979^{375,376}. It is one of the most widely used chemotherapeutic agents and is used in many solid malignant tumours such as testicular, ovarian, head and neck, colorectal, bladder and lung cancers^{376,377}.

Cisplatin generates DNA lesions activating the DNA repair response by intercalating with double stranded DNA³⁷⁷. It is also involved in switching cells from glycolytic metabolism to oxidative phosphorylation allowing TP53 mediated apoptosis²⁶⁶. Cisplatin therefore induces mitochondrially mediated apoptosis and is implicated in ferroptosis³⁷⁷. Use of cisplatin often results in chemoresistance and therapeutic failure^{375,377}.

5.2 Contact Dependent Survival of Doxorubicin Treated Cancer and Untreated Cell Lines in Co-Culture with CBDC.

For this section of study, the primary aim was to assess if cells that are treated with chemotherapy respond to co-culture in the same way their stressed ρ^0 counterparts do. To do this I evaluated Doxorubicin treated Astrocytes, 4T1 and B16 cell lines, with and without CBDC. Cells were treated for 24 hours and then lifted from the plate using trypsin, stained with CFSE and replated onto ds-Red expressing CBDC. The proliferation and cell-cell contact of the cancer cells was observed in the IncuCyte for 5 days in complete media with images taken every 3-6 hours.

In order to understand if any proliferation advantages also conferred a survival advantage, I conducted a visual analysis of survival. As the survival was contact mediated in the ρ^0 cells contact with CBDC was also observed in these treated cells. This visual analysis of survival and contact was performed by observing cells within the field of view and determining survival and CBDC contact condition. Each cancer cell within the field of view at the beginning of the experiment was coded and then that cell's survival and its contact with CBDC were recorded throughout the time frame. Cells were excluded if they left the field of view. Finally, I wished to understand if cells were coming into contact with CBDC more or less in the treated cultures and so percentage contact between treated cells and CBDC was also assessed. This was to identify if cellular migration played a role in the phenomena observed.

A proliferation based MTT assay was used to determine initial concentrations of chemotherapies to be used. The cells were plated for 24 hours before being treated for 24 hours at a range of concentrations. After 24 hours media containing drugs was removed and the cells were lifted and replated into 96 well plates and left to grow for 5 days. After 5 days MTT was added and cells with minimal staining were selected as minimal proliferation while still having an effect. Initially for doxorubicin I selected 135ng/ml for 4T1, 2000ng/ml for B16 and 30ng/ml for Astrocytes. Initially for cisplatin I selected 300ng/ml for 4T1, 3000ng/ml for B16 and 100ng/ml for Astrocytes. These concentrations were selected to kill some but

not all of the cancer cells, while still leaving cells alive at initiation of co-culture after lifting and replating cells.

These initial concentrations were optimised for use in the IncuCyte this resulted in concentrations as follows: for doxorubicin 500ng/ml for 4T1, 2000ng/ml for B16 and 30ng/ml for Astrocytes. For cisplatin 500ng/ml for 4T1, 2000ng/ml for B16 and 55ng/ml for Astrocytes were the final concentrations.

5.2.1 Proliferation of Cancer cell lines under Doxorubicin Treatment and Co-culture with CBDC.

Figure 5-1: IncuCyte analysis of cell lines treated with doxorubicin in co-culture with CBDC. UT denotes Untreated cells, error bars represent standard error and experiments were carried out in triplicate. A) Astrocyte line treated with 30ng/ml doxorubicin. B) B16 line treated with 2000ng/ml doxorubicin. C) 4T1 line treated with 500ng/ml doxorubicin.

Figure 5-1 showed the proliferation of 4T1, B16 and Astrocyte cell lines under treatment with doxorubicin with and without co-culture with CBDC. The treatment concentrations of doxorubicin had an effect on 4T1, B16 and Astrocyte proliferation, the CBDC had no effect on untreated cells and when treated cells were in co-culture with CBDC there was increased proliferation in all three cell types. The Astrocytes had a proliferation advantage from 19% without CBDC to 25% with CBDC the B16 and 4T1 from almost 0% to approximately 5%.

5.2.2 Contact Dependant Survival of Cancer Cells under Doxorubicin Treatment in Co-culture with CBDC.

Next, cells within the field of view were followed over five days. Individual treated cells were observed, and their survival was documented as well as their contact with CBDC. Cells that left the field of view were discounted as their fate could not be recorded. Survival of cells was calculated by number of surviving cells/ total cells that did not leave the field of view.

Figure 5-2 shows survival of treated cancer cells with and without contact with CBDC. This showed that in untreated cells there was no significant difference in survival between cells that came into contact with CBDC or not. This also shows that in Astrocyte and 4T1 there was an increase in survival in those cells that came into contact with CBDC compared with cells cultured without CBDC and those cultured with CBDC that did not come into contact with them. This however was not statistically significant. In the B16 however we see no difference in survival between cells that did or did not come into contact with CBDC.

Figure 5-2: Survival of cell lines after treatment with doxorubicin in co-culture with CBDC. Co-culture condition denoted by + and –, + CBDC is with and – CBDC is without. + Contact are the cells that came into contact with CBDC and – Contact are the cells that did not. Percent survival is the number of cells that were still living after 5 days for Astrocyte and B16 and 2.5 days for 4T1. Error bars are range calculated from biological triplicates of 49 fields of view each. A) Astrocyte line treated with 30ng/ml doxorubicin. B) B16 line treated with 2000ng/ml doxorubicin. C) 4T1 line treated with 500ng/ml doxorubicin.

5.2.3 Percent Contact of Cancer Cells under Doxorubicin Treatment in Co-Culture with CBDC.

Cells were finally assessed for percent contact with CBDC in treated and untreated conditions. This was calculated by cells that came into contact with CBDC/ cells in treatment condition.

Figure 5-3: Percent contact of doxorubicin treated cells with CBDC while in co-culture over 5 days. Calculated by taking the number of cells that came into contact with CBDC and did not exit the field of view divided by the total number of cells observed that did not exit the field of view. Error bars are range calculated from biological triplicates of 49 fields of view each. A. Astrocyte line treated with 30ng/ml doxorubicin. B. B16 line treated with 2000ng/ml doxorubicin. C. 4T1 line treated with 500ng/ml doxorubicin.

Figure 5-3 showed the percentage contact between cancer cells treated with doxorubicin and CBDC. There is no significant difference between doxorubicin treated Astrocyte and 4T1 cell lines and their untreated counterparts. In contrast to this the B16 doxorubicin treated cells had increased contact with CBDC compared to their untreated counterparts and that this is significant calculated via T-test with a p value of 0.0017.

5.3 Contact Dependent Survival of Doxorubicin Treated Cancer and Untreated Cell Lines in Co-Culture with CBDC.

I wished to understand if a chemotherapy with a different primary method of action would give the same pattern of enhanced proliferation, increased survival and increased contact with the CBDC in some of the cell lines and not the others comparing to the doxorubicin and the ρ^0 response from chapter 4, so I looked at cisplatin treatment in the three cell lines. I followed the same experimental plan and data analysis as the doxorubicin and ρ^0 cells. Similarly, to the doxorubicin, a proliferation-based assay was used to determine concentrations of cisplatin, and 500ng/ml for 4T1, 2000ng/ml for B16 and 55ng/ml for Astrocytes were chosen. These concentrations were picked to cause stress in the cancer cells while leaving some cells alive at initiation of co-culture.

5.3.1 Proliferation of Cancer Cell Lines under Cisplatin Treatment and Co-Culture with CBDC.

Figure 5-4: IncuCyte proliferation analysis of cell lines treated with cisplatin in co-culture with CBDC. UT denotes Untreated cells, error bars represent standard error and experiments were carried out in triplicate. A) Astrocyte line treated with 55ng/ml cisplatin. B) B16 line treated with 2000ng/ml cisplatin. C) 4T1 line treated with 500ng/ml cisplatin.

Figure 5-4 showed that all the concentrations of cisplatin decreased cell line proliferation. While the decrease in proliferation is similar to the doxorubicin the B16 and 4T1 cisplatin treatment was less dramatic than the doxorubicin treatment. In contrast to the doxorubicin treated lines there is little to no difference in proliferation between the 4T1 and Astrocyte lines with and without co-culture with CBDC where in the doxorubicin treated cells there was a marked increase in proliferation in co-culture. The B16 appears to show an increase in survival in the co-cultured sample however the co-cultured line runs in parallel to the line without co-culture. Thus, no conclusions can be drawn from these data.

5.3.2 Contact Dependant Survival of Cancer Cells under Cisplatin Treatment in Co-culture with CBDC.

Figure 5-5: Survival of cell lines after treatment with cisplatin in co-culture with CBDC.

+ and – CBDC denotes co-culture condition, + is with and – is without. Percent survival is the number of cells that were still living after 5 days, divided by number of cells counted total that did not leave the field of view. Contact with CBDC during this time denotes + and – contact condition. Error bars are range calculated from biological triplicates of 49 fields of view each. A) Astrocyte line treated with 55ng/ml cisplatin. B) B16 line treated with 2000ng/ml cisplatin. C) 4T1 line treated with 500ng/ml cisplatin.

Similarly, to the proliferation data on the doxorubicin treated cells figure 5-5 shows that for the B16 and Astrocyte lines there is no difference between survival of untreated cells that had contact with CBDC and cells that did not. In the 4T1 however there appears to be a slight survival advantage of those cells that came into contact with CBDC over those that did not although this is not statistically significant. In contrast to the doxorubicin treated data figure 5-5 also shows that for B16, 4T1 and Astrocyte cells treated with cisplatin cells in co-culture that came into contact with CBDC had a survival increase over cells not in co-culture, and this was statistically significant in the B16 data with statistical significance calculated via t-test giving a p-value of 0.0022. This increase in survival was also apparent within the 4T1 and Astrocyte co-cultured treated samples, cells that came into contact with CBDC have increased survival over those that didn't come into contact with CBDC, however this was not statistically significant.

5.3.3 Percent Contact of Cancer Cells Under Cisplatin Treatment in Co-Culture with CBDC.

Figure 5-6 shows that there is no difference in the amount of contact between untreated 4T1 cells with CBDC compared with cisplatin treated 4T1 cells. This is in contrast to the B16 and Astrocyte results which show an increased amount of contact between cisplatin treated cells and CBDC compared to untreated cells, this is significant in the B16 cells calculated via t-test with a p-value of 0.0049. This is different to the doxorubicin treated cells where only the doxorubicin treated B16 showed an increase in percent contact compared to untreated.

Figure 5-6: Percent contact of cisplatin treated cells with CBDC while in co-culture over 5 days. Calculated by taking the number of cells that came into contact with CBDC and did not exit the field of view divided by the total number of cells observed that did not exit the field of view. Error bars are range calculated from biological triplicates of 49 fields of view each. A) Astrocyte line treated with 55ng/ml cisplatin. B) B16 line treated with 2000ng/ml cisplatin. C) 4T1 line treated with 500ng/ml cisplatin.

5.4 Discussion

The biggest limitation with this work is the lack of statistical significance between aspects of treated and untreated cells. This is due primarily to two things: treatment concentrations and the need for more analytical replicates. These experiments were also impacted by limitations on laboratory time brought on by the SARS CoV2 coronavirus pandemic.

In terms of treatment concentrations, we can see there are differences in survival of cells in co-culture between cells in contact with CBDC and those that are not. With more experiments the concentrations of these drugs could be fine-tuned to provide a larger effect on survival response from the co-culture which could result in statistical significance of these differences. Longer term treatments of cisplatin in particular could lead to more cellular stress and a greater survival advantage for those in CBDC contact.

For the doxorubicin treatment concentration and duration, we see in the B16 and 4T1 cell lines that there is nearly no proliferation when treated alone, but proliferation in co-culture CBDC with treatment.

The need for more analytical replicates is because the cell tracking, and cell fate work was largely analysed manually and required many hours of image analysis time. An attempt was made to analyse these data via an image analysis pipeline with help from Olympus, but as of December 2020 they were unable to analyse all three parameters simultaneously, these parameters are cellular tracking in flat cells, cellular survival and cellular contact. While there are limitations with the current manual analysis method it was the only method available at the time.

The way that the chemotherapies were used in these experiments did not completely model the clinical use of these drugs. A single one-off dose of chemotherapy was given, which was then removed after 24 hours, and cells lifted and replated before co-culture was initiated. This is not representative of the way the tumour, and the tumour surrounding microenvironment would be exposed to drugs *in vivo*. Both tumour and surrounding

microenvironment would be treated, and treatment would continue for a more sustained length of time.

5.4.1 CBDC and Treated Cell Lines

The aim of this work was to assess if the same contact dependent survival could be seen in DNA damaging chemotherapy treated cell lines as ρ^0 cell lines under pyruvate and uridine deprivation.

One of the findings from this work is that when looking at proliferation of cell lines under doxorubicin chemotherapy with and without CBDC, treated cells had a proliferation advantage in co-culture compared to their monocultured treated counterparts. Interestingly the proliferation advantage in figure 5-1 did not correlate to a significant survival advantage in figure 5-2 and thus the advantage seen needs to be further investigated.

The B16 in particular in figure 5-2 shows no decrease in the survival of doxorubicin treated cells between co-culture and not co-cultured, CBDC contact condition irrespective. This is interesting as they showed a clear proliferation advantage in figure 5-3. This is possibly a result of cell cycle arrest or senescence occurring in the treated monoculture, where the cells do not die but they also do not proliferate.

The same however cannot be said for cisplatin. While the cisplatin concentrations definitely affected proliferation there was no distinct proliferation advantage to those grown in co-culture. Strangely this does not correlate with the survival advantage data as one of the few statistically significant findings in this chapter is that B16 cisplatin treated cells that have contact with a CBDC survive more than those that are not in co-culture. This is also strange given the results that B16 and Astrocyte cells increased contact with the CBDC when treated with cisplatin as this implies potential migration and chemotaxis of one or both cell types.

Therefore, there are some similarities between treatment of cell lines with chemotherapies - doxorubicin and cisplatin and ρ^0 deprivation of pyruvate and uridine. However, the evidence is not conclusive. As for similarities between the two treatments it appears that

they differ somewhat as there is a proliferation advantage seen for doxorubicin and not cisplatin. Largely I found that the interactions and response to stress of chemotherapy was different for both treatments used and both of these were different to the ρ^0 interactions and response. As it stands these are interesting preliminary results that need to be investigated further to fully understand the interactions at play between chemotherapy treated cells and CBDC and indeed the tumour microenvironment.

6 Similarities and Differences Between Stressed Cancer Cell Lines.

The previous chapters described how CBDC enhanced the survival of ρ° cells stressed by the loss of pyruvate and uridine. Also described was the response of treated cell lines in co-culture with CBDC. The ρ° model is an extreme model, however cancer cells are subjected to substantial stress when undergoing treatment. In this chapter I analysed the transcriptomics of the cancer lines B16 and 4T1 after treatment with doxorubicin and cisplatin, and compared them to their stressed ρ° counterparts. To do this I used the same cDNA sequencing approach and differential analysis utilised in chapter 4 to look at similarities in expression profiles between cell lines and treatments. The treatments and concentrations used were the same as used in the previous chapter with both cisplatin and doxorubicin demonstrating substantial decreases in proliferation.

6.1 Transcriptomic Analysis of Chemotherapy treated Cancer Cell Lines

In order to (i) understand the relationship between treated cells and CBDC and to (ii) assess signals that may initiate contact between the two, B16 and 4T1 cell were treated with doxorubicin and cisplatin for 24 hours and then RNA was harvested and converted to cDNA using MinION cDNA PCR synthesis before being barcoded. Samples were multiplexed before being loaded onto a MinION flow cell and run for 48 hours. The resulting sequences were mapped to individual transcripts on a murine reference genome and the read counts determined³⁴⁵. The DESeq2 was run on the samples to find the differential transcriptome results. The untreated cells were the same between both gene sets for each cell line and were sequenced across 2 MinION flow cells, with the doxorubicin treated samples on one flow cell with two untreated samples and the cisplatin treated samples on another flow cell with the other two untreated samples. The two flow cells were then sequenced at the same time.

6.2 Results

Table 6-1: Table of total mapped reads for treated sequencing

	Total mapped reads	Treatment	Duration	Cell Line
RD005.8 Dec19.BC01	949710	Dox	24hr	4T1wt
RD005.8 Dec19.BC02	753889	Dox	24hr	4T1wt
RD005.8 Dec19.BC03	980532	Dox	24hr	4T1wt
RD005.8 Dec19.BC04	995829	Dox	24hr	4T1wt
RD005.8 Dec19.BC05	768504	UT	24hr	4T1wt
RD005.8 Dec19.BC06	802667	UT	24hr	4T1wt
RD005.8 Dec19.BC07	969460	UT	24hr	4T1wt
RD005.8 Dec19.BC08	873357	UT	24hr	4T1wt
RD005.8 Dec19.BC09	1036082	Cisp	24hr	4T1wt
RD005.8 Dec19.BC10	849946	Cisp	24hr	4T1wt
RD005.8 Dec19.BC11	1042476	Cisp	24hr	4T1wt
RD005.8 Dec19.BC12	562120	Cisp	24hr	4T1wt
RD005.10 Jun20.BC01	2262372	Cisp	24hr	B16wt
RD005.10 Jun20.BC02	384495	Cisp	24hr	B16wt
RD005.10 Jun20.BC03	1808207	Cisp	24hr	B16wt
RD005.10 Jun20.BC04	2192919	UT	24hr	B16wt
RD005.10 Jun20.BC05	730009	UT	24hr	B16wt
RD005.10 Jun20.BC07	789571	Dox	24hr	B16wt
RD005.10 Jun20.BC09	828443	Dox	24hr	B16wt

Table 6-1 shows the total number of mapped reads for each barcode used in this chapter, Treatment shown as T is 24 hours without pyruvate and uridine and UT is with pyruvate and uridine. The range in reads is from 384,495 to 2,262,372 with most barcodes having more than 700,000 mapped reads.

6.2.1 Differential Gene Expression in 4T1 Treated with Doxorubicin.

Initially, the fold change value for every transcript was plotted against its own p-value, indicating significance of the change thus identifying significantly differentially expressed genes (figure 6-1 A). There was a strong differential signature between 4T1 that were treated with 500ng/ml doxorubicin and those that were not. A differential signature is a global description of how different treated samples are from untreated paired samples, a weak signature indicates few changes to the transcriptome and a strong signature indicated lots of changes to the transcriptome. More genes were upregulated than downregulated, as defined by significant (<0.05) adjusted p-values and a log 2-fold change of greater than one. The volcano plot was high, with a number of significant genes but not particularly wide - with those significant genes having log 2-fold changes of mostly 1-2 (2 – 4-fold increase).

Next the same data were analysed using a principal component analysis to identify how similar or different the treated and untreated samples were from each other. Figure 6-1 B showed a clear separation of treated and untreated samples, with 74% of the variance explained by the set of transcripts that comprised the first principal component. The second principal component transcripts showed the variation within the treated samples, but not between treated and untreated samples. In total 81% of the variance in the sample is explained by the first two principal components.

A heatmap (Figure 6-1 C) was used to visualise the scale of the differential expression. The 50 most differentially expressed transcripts were selected and put through an unsupervised clustering analysis. When there were multiple differentially expressed transcripts for one gene a representative transcript was chosen.

The treated and untreated samples clustered together, showing consistency within sample groups as well as differences between them. As seen from the PCA analysis, both the within condition variation and between condition variation was confirmed by the heatmap. The colour scale also shows differential expression of the top 40 transcripts lay between -1.5 and 1.5 log 2-fold change differences.

6.2.2 4T1 Treated with Cisplatin Differential Expression.

Figure 6-2: Differential gene expression induced by cisplatin in 4T1 cells.

The 4T1 cell line was treated with 500ng/ml cisplatin, cDNA sequenced using a MinION log read sequencer, transcripts were removed if they had less than 5 reads or mapped in the reverse direction . A) Volcano Plot of the DESeq2 coverage showing Log 2 fold change on the X axis and Log10P (statistical significance) to demonstrate significance on the Y axis. Green dots are genes with a log 2-Fold change of greater than 1, blue dots denote significant p-values and red denotes both genes with significant p-value and log 2 fold change. B) is a PCA plot showing the amount of variance explained by the variables in untreated and treated samples. C) Shows an unsupervised heatmap of the individual samples and their differential expression.

In comparison to the effect of doxorubicin on 4T1, the effect of cisplatin on the gene expression of 4T1 cells was much less pronounced. The same differential expression analysis was carried out comparing cells treated with 500ng/ml cisplatin to untreated cells. This indicated that within the first 24 hours there was not a highly significant change in transcriptional signature, the volcano plot shows (figure 6-2 A) that there were few genes that had significant p-values and a log 2-fold change of greater than one. The reduced height of the volcano plot compared to the doxorubicin data indicates that the p-values were less significant, and the width showed that those genes that were significant had smaller log 2-fold changes around 1.

Despite the small scale of the changes in expression, more than 50% of the variance that was in the cisplatin-treated cells was explained by the first two principal components (figure 6-2 B). The first principal component separated the variance within the cisplatin treated sample while the second separated the variance between the cisplatin and untreated samples. The variance explained for the cisplatin treated samples along the first principal component also included two of the untreated samples, thus there is less separation in this PCA than in the doxorubicin treated one for the same cell line.

When these data were visualised via heatmap (figure 6-2 C) the variability across the replicates was apparent; surprisingly, the unsupervised clustering has grouped untreated and treated samples separately. This was surprising because most of the top 50 transcripts shown are yellow across most of the samples which corresponds to a log 2 fold change of close to 0. There is very little range in the log 2 fold changes presenting the top 50 transcripts as they range from 1 to negative 1.

6.2.3 B16 Treated with Doxorubicin Differential Expression.

Figure 6-3: Differential gene expression induced by doxorubicin in B16 cells.

The B16 cell line was treated with 2000ng/ml doxorubicin, cDNA sequenced using a MinION log read sequencer, transcripts were removed if they had less than 5 reads or mapped in the reverse direction. A) Volcano Plot of the DESeq2 coverage showing Log 2 fold change on the X axis and Log₁₀P (statistical significance) to demonstrate significance on the Y axis. Green dots are genes with a log 2-Fold change of greater than 1, blue dots denote significant p-values and red denotes both genes with significant p-value and log 2 fold change. B) is a PCA plot showing the amount of variance explained by the variables in untreated and treated samples. C) Shows an unsupervised heatmap of the individual samples and their differential expression.

Similar to the 4T1 cells treated with doxorubicin the B16 cells treated with doxorubicin show a strong differential signature between those B16 cells treated with 2000ng/ml and those not treated. The volcano plot (figure 6-3 A) shows there are a high number of differentially expressed transcripts and there are more transcripts downregulated than upregulated. In contrast to the 4T1 treated with doxorubicin the B16 treated with doxorubicin has differentially expressed transcripts with high Log 2-fold changes and comparatively lower p-values to those found in the 4T1 which had lower log 2-fold changes and higher p-values. This gave the appearance in the 4T1 of a high and narrow volcano plot whereas the B16 has a wide and lower volcano plot.

Again, with similarity to the 4T1 doxorubicin treated set, the B16 doxorubicin set PCA plot (Figure 6-3 B) shows that the first principal component accounted for most of the variation in the sample (94%) and separated the treated from the untreated samples. The second PCA also separates the variation only within the treated samples. This shows that the sample variation is represented primarily by the differences between treated and untreated samples than within those groups.

This variation is represented well in the heatmap (Figure 6-3 C). The unsupervised analysis tells us that treated and untreated samples cluster with their fellows.

With this the considerable range of Log 2-fold change results is apparent, with a range from -3.5 and 3.5 log 2-fold change the differential effect of doxorubicin on transcription in the B16 cells was greater than in their 4T1 counterparts.

6.2.4 B16 Treated with Cisplatin Differential Expression.

Figure 6-4: Differential gene expression induced by cisplatin in B16 cells.

The B16 cell line was treated with 2000ng/ml cisplatin, cDNA sequenced using a MinION log read sequencer, transcripts were removed if they had less than 5 reads or mapped in the reverse direction. A) Volcano Plot of the DESeq2 coverage showing Log 2-fold change on the X axis and Log₁₀P (statistical significance) to demonstrate significance on the Y axis. Green dots are genes with a log 2-fold change of greater than 1, blue dots denote significant p-values and red denotes both genes with significant p-value and log 2-fold change. B) is a PCA plot showing the amount of variance explained by the variables in untreated and treated samples. C) Shows an unsupervised heatmap of the individual samples and their differential expression.

In contrast to both doxorubicin treated sets yet in similarity to the cisplatin treated 4T1 set when B16 cells were treated with 2000ng/mL cisplatin for 24 hours there was a weak if any differential signature. The volcano plot (Figure 6-4 A) shows that very few genes have both a significant p-values and a log 2-fold change of greater than one. This makes the volcano plot short with very few significant genes with those significant genes having log fold changes around 1 (2-fold increase).

Similarly, to the cisplatin treated 4T1 the PCA plot (Figure 6-4 B) for the cisplatin treated B16 shows us that none of the variance in the sample was explained by the first two principal components. This implies that the variation within the conditions is greater than the variation between the conditions. This makes it difficult to group the treated and untreated into conditions using the PCA.

Very surprisingly using an unsupervised clustering the heatmap (Figure 6-4 C) clustered the samples into treatment condition. This demonstrates there was enough variation between the two conditions to cluster in this way. However, with a log 2-fold change of zero represented by yellow we can see that almost all the top 50 transcripts are barely differentially expressed across all five samples. The three transcripts identified in the volcano plot (Figure 6-4 A) *Ccng1*, *Cdkn1a* and *Snord13* all appear in the heatmap (Figure 6-4 C) and show differential transcription across the 5 samples corresponding with condition. It appears that the differential transcription of these three transcripts was enough for the unsupervised clustering to associate the treated and untreated samples with themselves. The heatmap also shows that the top 50 transcripts have between just under -1. and 1 log 2-fold change differences.

Overall, these data show that doxorubicin treatment of 4T1 and B16 cells resulted in significant transcriptional changes at 24 hours, whereas cisplatin treatment of these cell types had a much smaller effect on transcription at 24 hours. While whole transcriptomic changes in gene expression with cisplatin may not have occurred, there were individual transcripts that were altered.

6.2.5 Comparisons

After looking at all the transcript lists generated from the DESeq2 analysis I wanted to know what the common methods of cellular response to stress were between the two cell lines. The same analysis as in section 4.2.4 and 4.2.5 was performed. The list of transcripts was initially filtered to select differentially expressed transcripts with p-values of less than 0.05. These new lists were then compared with each other. Overlapping transcripts were then made into a table with their corresponding log 2 fold change and adjusted p-value. The adjusted p-value for DESeq2 was calculated by fGSEA using the Benjamini Hochberg method to mitigate false positives generated through multiple testing³⁴⁷. The adjusted p-values and log 2 Fold changes were used to evaluate the importance of the common transcripts. As noted above, cisplatin treatment in particular generated a low number of genes differentially expressed and these comparisons were used to help tease a signal out of the noise. The following Euler plots illustrate the number of differentially expressed genes that appear in each list. In order to see common methods of cell response to stress, stressors were compared within each cell line.

6.2.6 Chemotherapy Specific Response in Different Cell Lines.

Figure 6-5: Comparison between individual chemotherapies and their differentially expressed gene lists of 4T1 and B16 cell lines.

A) Euler plot comparing upregulated genes common between 4T1 and B16 cell lines treated with doxorubicin. B) Euler plot comparing downregulated genes common between 4T1 and B16 cell lines treated with doxorubicin. C) Euler plot comparing upregulated genes common between 4T1 and B16 cell lines treated with cisplatin. D) Euler plot comparing downregulated genes common between 4T1 and B16 cell lines treated with cisplatin.

These Euler plots (Figure 6-5) demonstrate how similar or dissimilar the B16 and 4T1 cell lines response is to the same stress. When comparing the upregulated transcripts of B16 and 4T1 under treatment with doxorubicin (Figure 6-5 A) we find 150 transcripts upregulated in both gene sets, this is 12.2% of the B16 dataset and 6.7% of the 4T1 set. The downregulated transcripts of doxorubicin treated 4T1 and B16 (Figure 6-5 B) share 131 transcripts this is 7% of the B16 and 8.3% of the 4T1 set. This is a small amount of the total transcripts that are differentially expressed and demonstrates a low level of similarity to how these cell lines deal with doxorubicin treatment.

When comparing cisplatin treated upregulated transcripts (Figure 6-5 C) there are only 5 transcripts differentially expressed in both B16 and 4T1 sets, this is 4.8% of the B16 dataset and 2.0% of the 4T1 set. In the downregulated transcripts (Figure 6-5 D) there are again 5 commonly differentially expressed transcripts this is 4.63% of the B16 set and 1.47% of the 4T1 set is. This shows that there is much less in common between the cisplatin sets compared to the doxorubicin sets. Therefore it is probable that these cell lines have very different responses to the same cisplatin treatment .

Cumulatively there are many more genes in the doxorubicin sets than in the cisplatin sets and these doxorubicin sets have slightly more differentially expressed transcripts in common than the cisplatin sets.

Full lists of the gene sets common to each of the above conditions are available in the appendix 2.

Due to the low levels of similarity between the cell lines responses to the same stress we then looked at the cell line specific response to treatment by comparing the datasets within the cell line that was treated.

6.2.7 Cell Line Specific Response to Different Chemotherapies.

Figure 6-6: Comparison of gene expression changes under chemotherapy treatment within a cell line. A) Euler plot of upregulated genes in the 4T1 cell line under chemotherapy treatment with doxorubicin and cisplatin. B) Euler plot of downregulated genes in the 4T1 cell line under chemotherapy treatment with doxorubicin and cisplatin C) Euler plot of upregulated genes in the B16 cell line under chemotherapy treatment with doxorubicin and cisplatin. D) Euler plot of downregulated genes in the B16 cell line under chemotherapy treatment with doxorubicin and cisplatin.

These Euler plots (Figure 6-6) illustrate the transcripts common to both doxorubicin and cisplatin treatment within each cell line. In the 4T1 cell line for transcripts upregulated by the chemotherapy treatment (Figure 6-6 A) there were 72 commonly differentially expressed transcripts this is 3.19% of the doxorubicin and 29.34% of the cisplatin set. In the downregulated transcripts (Figure 6-6 B) there were 96 commonly downregulated transcripts this was 6.05% of the doxorubicin transcripts and 28.15% of the cisplatin transcripts. Therefore much more of the smaller cisplatin set is found in the doxorubicin set than doxorubicin in the cisplatin set where very little overlap occurred.

In the B16 cell treated with chemotherapy the same observation was made (Figure 6-6). Of the upregulated transcripts (Figure 6-6 C) there were 28 common transcripts, this was 2.27% of the doxorubicin set and 26.67% of the cisplatin set. In the downregulated B16 transcripts (Figure 6-6 D) there were 40 common transcripts, this was 2.13% of the doxorubicin set and 37.04% of the cisplatin set. Compared to the 4T1, the same proportion of the cisplatin set was present in the doxorubicin set and an even smaller percentage of transcripts of the doxorubicin set was represented in the cisplatin set.

In both the 4T1 and B16 cells, a large proportion(25-37%) of the genes regulated by cisplatin were found in the doxorubicin transcript set. This showed that of the few transcripts differentially expressed in response to cisplatin, approximately 30% were found to be regulated by doxorubicin indicating that there may be some common mechanism in each cell line in response to chemotherapy stress.

Full lists of the gene sets common to each of the above conditions are available in the appendix 2.

One of the aims of this thesis was to understand if there was a cellular response mechanism that allowed cells to survive both ρ^0 deprivation stress and chemotherapy treatment stress. Therefore, we compared up and downregulated genes of both treated datasets for each cell line with the ρ^0 datasets from chapter five.

6.2.8 Cell Line Specific Response to Different Stressors.

Figure 6-7: Comparison of gene expression changes under ρ^0 zero deprivation and chemotherapy treatment within the 4T1 cell line.

dox = doxorubicin, cisp = cisplatin, rho = ρ^0 A) Euler plot of upregulated genes in the 4T1 cell line under chemotherapy treatment with doxorubicin and cisplatin and 4T1 ρ^0 cells under pyruvate and uridine removal stress. B) Euler plot of downregulated genes in the 4T1 cell line under chemotherapy treatment with doxorubicin and cisplatin and 4T1 ρ^0 cells under pyruvate and uridine removal stress. C) Euler plot of upregulated genes in the B16 cell line under chemotherapy treatment with doxorubicin and cisplatin and B16 ρ^0 cells under pyruvate and uridine removal stress. D) Euler plot of downregulated genes in the B16 cell line under chemotherapy treatment with doxorubicin and cisplatin and B16 ρ^0 cells under pyruvate and uridine removal stress.

These Euler plots (Figure 6-7) show us that there are very few genes/transcripts that are differentially expressed in the same direction across all three conditions, ρ° deprivation of pyruvate and uridine, doxorubicin and cisplatin treatment of wild type cells. I asked several questions of these data; was the relationship between ρ° and cisplatin the same or different from the doxorubicin? And what did the commonly expressed transcripts represent from the whole transcript sets,

Between cisplatin and ρ° in the upregulated 4T1 cells there were 21 transcripts present in both the cisplatin and the ρ° , this was 1.23% of the ρ° and 8.57% of the cisplatin. In the downregulated 4T1 cells there were 20 transcripts; this was 1.78% of the ρ° and 5.87 % of the cisplatin. In the B16 cells there was almost no overlap between the ρ° and cisplatin sets with no commonly upregulated transcripts and only three downregulated transcripts which represented was 1.37% of the ρ° and 6.48% of the cisplatin set.

There were many more differential expressed genes in the doxorubicin set compared to the cisplatin set and between the doxorubicin and ρ° sets. In the upregulated 4T1 cells there were 365 commonly upregulated transcripts, this represented 21.30% of the ρ° set and 16.19% of the doxorubicin set. In the downregulated 4T1 cells there were 136 commonly downregulated transcripts representing 12% of the ρ° and 8.6% of the doxorubicin sets. Between doxorubicin and ρ° in the upregulated B16 cells there were 12 upregulated transcripts which accounted for 4.4% of the ρ° and 0.97% of the doxorubicin sets. In the downregulated B16 cells there were 91 downregulated transcripts between ρ° and doxorubicin; this accounted for 20.7% of the ρ° and 5.5% of the doxorubicin sets.

The upregulated 4T1 cells had 9 transcripts (Figure 6-7 A) commonly upregulated, this was 0.53% of the ρ° , 3.67% of the cisplatin set and 0.4% of the doxorubicin set. Downregulated 4T1 cells (Figure 6-7 B) had 5 common transcripts for all three stressors; this was 0.44% of the ρ° , 1.47% of the cisplatin and 0.32% of the doxorubicin sets.

A very different relationship was seen in the B16 cells. In the upregulated B16 transcripts there was nothing in common. The down regulated transcripts shared 1 transcript, this represented 0.3% of the ρ° set, 0.9% of the cisplatin set and 0.05% of the doxorubicin set.

This shows there are more transcripts in common between all three sets in the 4T1 cell line than in the B16 cell line. This indicates a possible common mechanism for stress response amongst all 3 stressors in the 4T1 cell line. To determine what the mechanism might be, the identify and function of the conserved genes was investigated.

Table 6-2: Up regulated genes common among 4T1 treated with doxorubicin and cisplatin and ρ° with p-values less than 0.05.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Gene	4T1 ρ° log 2-Fold Change	4T1 ρ° padj	4T1 cisplatin log 2-Fold Change	4T1 cisplatin padj	4T1 doxorubicin log 2-Fold Change	4T1 doxorubicin padj
Cxcl1	3.5944	0.0005	0.4535	0.6831	1.0410	0.0000
Cxcl5.1	2.3762	0.0016	0.8075	0.0000	1.5215	0.0000
Cxcl5	2.3540	0.0015	0.8068	0.0000	1.5154	0.0000
Crip1	1.7422	0.0000	0.4647	0.9998	0.5103	0.0509
Lgals9	1.6803	0.1728	0.6998	0.9998	1.3114	0.0014
Tpm1.9	1.4525	0.0160	0.5444	0.9998	1.6218	0.0000
Klk10	1.2568	0.0038	1.1363	0.6658	1.7120	0.0000
Sparc	0.8814	0.0513	0.3239	0.9998	0.6022	0.0053
Tmsb4x	0.8537	0.0389	0.4531	0.9998	1.0505	0.0026

Table 6-3:Down regulated genes common among 4T1 treated with doxorubicin and cisplatin and ρ° with p-values less than 0.05.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Gene	4T1 ρ° log 2-Fold Change	4T1 ρ° padj	4T1 cisplatin log 2-Fold Change	4T1 cisplatin padj	4T1 dox log 2-Fold Change	4T1 doxorubicin padj
Trib3	-2.1757	0.0000	-0.9975	0.5665	-3.2725	0.0000
Eif1.3	-1.6462	0.0992	-1.1316	1.0000	-1.2900	0.1420
Chac1	-1.5674	0.0006	-0.8712	0.9998	-2.0726	0.0000
Nupr1	-1.4874	0.0021	-0.4516	0.9998	-2.1380	0.0000
Nupr1.1	-1.0712	0.1273	-0.8181	0.9998	-1.7185	0.0001

There was a significant upregulation in both treated 4T1 sets and in the ρ° set of two *Cxcl5* transcripts. There was also a significant upregulation in 4T1 treated with doxorubicin and 4T1 ρ° sets of *Cxcl1*, *Tpm1*, *Klk10* and *Tmsb4x*. In the same datasets there was a downregulation of *Trb3*, *Chac1*, *Nupr1* in the 4T1 treated with doxorubicin and 4T1 ρ° sets. With respect to the B16 stress response the only gene common among all three B16 sets was *Manf* and this was only significant in the doxorubicin set.

Table 6-4: Down regulated genes common among B16 treated with doxorubicin and cisplatin and ρ° with p-values less than 0.05.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Gene	B16p0 log 2-Fold Change	B16p0 padj	B16 cisplatin log 2-Fold Change	B16 cisplatin padj	B16 doxorubicin log 2-Fold Change	B16 doxorubicin padj
Manf	-1.5055	1.0000	-0.6065	0.9999	-0.7909	0.0336

6.3 GSEA

To look beyond individual genes and transcripts, gene set enrichment analyses (GSEA) were performed, to extrapolate what pathways could be impacted by the chemotherapy treatment. Enrichment analyses were performed using FGSEA the same as in Chapter 4. A GSEA is performed as follows:

Normalised expression data i.e. from DESeq2 is converted from transcript to gene level and then genes are ranked by the Wald statistic calculated during the DESeq2 analysis. This creates a ranked list L. A set of genes associated with a phenotype (S) is then compared to L. For every gene in S its presence and absence in L is recorded along with the rank. The enrichment score (ES) is calculated from this, taking into account the correlation of each gene to the phenotype of the S. ES is calculated by the number of Hits in the gene set – the number of misses, if the L was randomly distributed this would be roughly zero. The normalised enrichment score (NES) is the ES normalised for the size of the gene set and is corrected for multiple testing using the Benjamini-Hochberg procedure ³⁴⁷.

This analysis was performed in order to look at enriched gene sets within the differentially expressed dataset. The ‘hallmarks’ gene sets from MSigDB collection was chosen, a collection of 50 gene sets that represent specific and well defined biological states or processes. These gene sets are updates frequently and include many processes key to cancer survival and tumorigenesis ³⁶². This was used to allow us to understand the processes that were occurring in the treated samples and compare them to the p° results.

6.3.1 GSEA of 4T1 Treated with Doxorubicin and Cisplatin.

Figure 6-8: Gene Set Enrichment Analysis of 4T1 cells treated with 500ng/ml doxorubicin for 24 hours. Using the hallmarks of cancer gene sets from the Broad institute and the fgSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the differential gene list is enriched or unenriched in a given gene set (listed on the y axis). Size denoted how many of the genes in the gene set were found in the sample, padj is the adjusted p-value which corrects for multiple testing. Gene set used in this analysis was generated using Dseq2 as shown previously.

Figure 6-9: Gene Set Enrichment Analysis of 4T1 cells treated with 500ng/ml cisplatin for 24 hours Using the hallmarks of cancer gene sets from the Broad institute and the fgSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the differential gene list is enriched or unenriched in a given gene set (listed on the y axis). Size denoted how many of the genes in the gene set were found in the sample, padj is the adjusted p-value which corrects for multiple testing. Gene set used in this analysis was generated using Dseq2 as shown previously.

The 4T1 doxorubicin treated desq2 dataset (Figure 6-8) had a roughly equal distribution of positively and negatively enriched gene sets with slightly more significant negatively enriched sets.

Similar to the doxorubicin treated 4T1 the cisplatin treated 4T1 dataset (Figure 6-9) had a roughly equal distribution of positively and negatively enriched gene sets with slightly more significant positively enriched sets. There were fewer enriched sets with significant adjusted p-values than in the doxorubicin sets.

6.3.2 GSEA of B16 Treated with Doxorubicin and Cisplatin.

Figure 6-10: Gene Set Enrichment Analysis of B16 cells treated with 2000ng/ml doxorubicin for 24 hours. Using the hallmarks of cancer gene sets from the Broad institute and the fgSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the differential gene list is enriched or unenriched in a given gene set (listed on the y axis). Size denoted how many of the genes in the gene set were found in the sample, padj is the adjusted p-value which corrects for multiple testing. Gene set used in this analysis was generated using Dseq2 as shown previously.

Figure 6-11: Gene Set Enrichment Analysis of B16 cells treated with 2000ng/ml cisplatin for 24 hours. Using the hallmarks of cancer gene sets from the Broad institute and the fGSEA package in R. The NES is the normalised enrichment score and a measure of how enriched the differential gene list is enriched or unenriched in a given gene set (listed on the y axis). Size denoted how many of the genes in the gene set were found in the sample, padj is the adjusted p-value which corrects for multiple testing . Gene set used in this analysis was generated using Dseq2 as shown previously.

In contrast to the 4T1 doxorubicin treated dataset, the B16 treated dataset GSEA (Figure 6-10) showed more negatively enriched gene sets. However, both the 4T1 and B16 doxorubicin treated sets had more significantly positive or negatively enriched gene sets than the 4T1 and B16 cisplatin sets.

The B16 cisplatin GSEA (Figure 6-11) showed no gene sets with adjusted p values of <0.05 . While both the 4T1 and B16 cisplatin sets had fewer significant gene sets than their doxorubicin counterparts both the B16 sets had more negatively enriched gene sets than positively enriched gene sets.

6.3.3 Chemotherapy Specific Response in Different Cell Lines.

Figure 6-12: Chemotherapy specific comparison between 4T1 and B16 cell lines GSEA.

GSEA of B16 and 4T1 when treated with doxorubicin and cisplatin for 24 hours. Dox = doxorubicin, cisp = cisplatin A) Shows a Euler plot comparing up-enriched gene sets common between 4T1 and B16 treated with doxorubicin. B) Shows a Euler plot comparing down-enriched gene sets common between 4T1 and B16 treated with doxorubicin. C) Shows a Euler plot comparing up-enriched gene sets common between 4T1 and B16 treated with cisplatin. D) Shows a Euler plot comparing down-enriched gene sets common between 4T1 and B16 treated with cisplatin.

Similarly to the DESeq2 Euler plots these GSEA Euler plots show the similarities between the GSEA results for both treatments. Because there are fewer gene sets used in this analysis than transcripts the number of comparisons is much lower.

The positively enriched gene sets (Figure 6-12 A) of B16 treated with doxorubicin shared 28.57% with the 4T1 treated with doxorubicin sets and the 4T1 treated with doxorubicin shared 61.54% with the B16 set. The negatively enriched gene sets (Figure 6-12 B) for B16 treated with doxorubicin shared 44.44% with the 4T1 set and the 4T1 sets shared 42.11% with the B16 sets. The pathways enriched in both gene sets are shown in tables 6-4 and 6-5.

The positively enriched gene sets of cisplatin treated (Figure 6-12 C) B16 had 80% in common to the 4T1 gene sets, and the 4T1 had 30% in common to the B16 sets. In the negatively enriched gene sets (Figure 6-12 D) B16 had 72.73% in common to the 4T1 gene sets, and the 4T1 had 34.78% in common with the B16 sets. The pathways enriched in both gene sets are shown in tables 6-6 and 6-7.

This is different from the DESeq2 analysis of the same sets where there was a much lower proportion of similarly expressed transcripts. This indicates that some of the different transcripts that were not common between the two cell lines belong to the same gene sets.

Table 6-5: Positively enriched gene sets with significant p-values common among doxorubicin treated cell lines.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 doxorubicin padj	4T1 doxorubicin NES	B16 doxorubicin padj	B16 doxorubicin NES
HALLMARK INTERFERON GAMMA RESPONSE	0.0221	2.0355	0.0948	1.6142
HALLMARK INTERFERON ALPHA RESPONSE	0.0221	1.9867	0.0169	2.0384
HALLMARK ANGIOGENESIS	0.0654	1.8154	0.0291	1.8162
HALLMARK OXIDATIVE PHOSPHORYLATION	0.0585	1.7637	0.0107	3.0591
HALLMARK COAGULATION	0.2048	1.4308	0.0107	2.7017
HALLMARK ALLOGRAFT REJECTION	0.2356	1.3990	0.1819	1.4337
HALLMARK EPITHELIAL MESENCHYMAL TRANSITION	0.4693	1.1404	0.0107	2.2133
HALLMARK COMPLEMENT	0.5459	1.0582	0.0819	1.5962

Table 6-6: Negatively enriched gene sets with significant p-values common among doxorubicin treated cell lines.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 doxorubicin padj	4T1 doxorubicin NES	B16 doxorubicin padj	B16 doxorubicin NES
HALLMARK MYC TARGETS V2	0.3350	-1.2233	0.0808	-1.6334
HALLMARK NOTCH SIGNALING	0.1877	-1.4483	0.4198	-1.1627
HALLMARK PI3K AKT MTOR SIGNALING	0.0275	-2.3915	0.4986	-1.0742
HALLMARK E2F TARGETS	0.0221	-2.4198	0.0107	-2.3198
HALLMARK MYC TARGETS V1	0.0221	-2.5381	0.0107	-2.6844
HALLMARK G2M CHECKPOINT	0.0221	-2.7805	0.0107	-2.1746
HALLMARK UNFOLDED PROTEIN RESPONSE	0.0221	-3.2583	0.0852	-1.5348
HALLMARK MTORC1 SIGNALING	0.0221	-3.5903	0.0852	-1.4692

Tables 6-5 and 6-6 show a positive enrichment in both doxorubicin treated 4T1 and B16 gene sets for Interferon alpha response, and negative enrichments in both doxorubicin treated sets for MYC target, E2F targets and G2M Checkpoint gene sets.

Table 6-7: Positively enriched gene sets with significant p-values common among cisplatin treated cell lines.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 cisplatin padj	4T1 cisplatin NES	B16 cisplatin padj	B16 cisplatin NES
HALLMARK INFLAMMATORY RESPONSE	0.0581	1.9688	0.2227	1.5326
HALLMARK TNFA SIGNALING VIA NFkB	0.2888	1.6117	0.4850	1.2498
HALLMARK INTERFERON GAMMA RESPONSE	0.2888	1.6007	0.1100	1.8700
HALLMARK DNA REPAIR	0.2888	1.5859	0.7575	1.0491
HALLMARK COAGULATION	0.2888	1.4496	0.6584	1.1061
HALLMARK INTERFERON ALPHA RESPONSE	0.2888	1.3906	0.1100	1.8700
HALLMARK IL2 STAT5 SIGNALING	0.5288	1.1138	0.4850	1.2008
HALLMARK ESTROGEN RESPONSE LATE	0.5401	1.0529	0.2227	1.5701

Table 6-8: Negatively enriched gene sets with significant p-values common among cisplatin treated cell lines.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 cisplatin padj	4T1 cisplatin NES	B16 cisplatin padj	B16 cisplatin NES
HALLMARK MYC TARGETS V1	0.4752	-1.1602	0.1715	-1.8375
HALLMARK HEME METABOLISM	0.2888	-1.3885	0.4341	-1.2449
HALLMARK MYC TARGETS V2	0.2888	-1.5786	0.2186	-1.3300
HALLMARK UNFOLDED PROTEIN RESPONSE	0.0422	-2.3854	0.5384	-1.2155

Tables 6-7 and 6-8 show that none of the positively enriched gene sets common to both 4T1 and B16 treated with doxorubicin were statistically significant. Similarly, to the doxorubicin sets interferon alpha, interferon gamma responses and coagulation were present in the positively enriched sets. In the negatively enriched gene sets common to both 4T1 and B16 treated with cisplatin unfolded protein response was only significant in the 4T1 set although it was also present in the doxorubicin sets, MYC targets were also present in both the doxorubicin and cisplatin comparisons but were not significant for cisplatin treatments.

6.3.4 Cell Line Specific Response to Different Chemotherapies.

Figure 6-13: Cell line response comparison within 4T1 and B16 cell lines GSEA.

GSEA of 4T1 and B16 when treated with doxorubicin and cisplatin for 24 hours. Dox = doxorubicin, cisp = cisplatin A) Shows a Euler plot comparing up-enriched gene sets common between 4T1 cells treated with doxorubicin and cisplatin. B) Shows a Euler plot comparing down-enriched gene sets common between 4T1 cells treated with doxorubicin and cisplatin. C) Shows a Euler plot comparing up-enriched gene sets common between B16 cells treated with doxorubicin and cisplatin. D) Shows a Euler plot comparing down-enriched gene sets common between B16 cells treated with doxorubicin and cisplatin.

These Euler plots (Figure 6-13) make evident how comparable a cell line's response is to two chemotherapies with a different method of action. In the positively enriched gene sets for the 4T1 (Figure 6-13 A) 57.89% of the cisplatin gene sets were common to the doxorubicin gene sets, and 84.62% of the doxorubicin gene sets are common to the cisplatin gene sets. In the negatively enriched gene sets (Figure 6-13 B) 84.61% of the cisplatin gene sets were common to the doxorubicin gene sets, and 47.83% of the doxorubicin gene sets were common to the cisplatin gene sets. This is a much higher proportion than the individual treatments had in common.

In the positively enriched gene sets of the B16 (Figure 6-13 C) 55.56% of the cisplatin gene sets were common to the doxorubicin gene sets, and 35.71% of the doxorubicin gene sets were common to the cisplatin gene sets. In the negatively enriched gene sets of the B16 (Figure 6-14 D) 60% of the cisplatin gene sets were common to the doxorubicin gene sets, and 27.27% of the doxorubicin gene sets were common to the cisplatin gene sets.

In contrast to the chemotherapy specific comparisons the Euler plots in Figure 6-13 show that there was more in common within a cell line and its response to chemotherapy than there was between cell lines for the same chemotherapy.

Table 6-9: Positively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 doxorubicin padj	4T1 doxorubicin NES	4T1 cisplatin padj	4T1 cisplatin NES
HALLMARK INTERFERON GAMMA RESPONSE	0.0221	2.0355	0.2888	1.6007
HALLMARK INFLAMMATORY RESPONSE	0.0221	2.0183	0.0581	1.9688
HALLMARK INTERFERON ALPHA RESPONSE	0.0221	1.9867	0.2888	1.3906
HALLMARK ANGIOGENESIS	0.0654	1.8154	0.0422	1.3214
HALLMARK OXIDATIVE PHOSPHORYLATION	0.0585	1.7637	0.2888	1.4603
HALLMARK DNA REPAIR	0.1614	1.5186	0.2888	1.5859
HALLMARK COAGULATION	0.2048	1.4308	0.2888	1.4496
HALLMARK ALLOGRAFT REJECTION	0.2356	1.3990	0.4353	1.2236
HALLMARK EPITHELIAL MESENCHYMAL TRANSITION	0.4693	1.1404	0.3586	1.3527
HALLMARK TNFA SIGNALING VIA NFKB	0.4693	1.1250	0.2888	1.6117
HALLMARK COMPLEMENT	0.5459	1.0582	0.1270	1.8527

Table 6-10: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 doxorubicin padj	4T1 doxorubicin NES	4T1 cisplatin padj	4T1 cisplatin NES
HALLMARK MYC TARGETS V2	0.3350	-1.2233	0.2888	-1.5786
HALLMARK ANDROGEN RESPONSE	0.1810	-1.5553	0.5288	-1.0844
HALLMARK UV RESPONSE UP	0.1210	-1.6046	0.2888	-1.5175
HALLMARK P53 PATHWAY	0.0986	-1.6174	0.4619	-1.1881
HALLMARK APOPTOSIS	0.0789	-1.6832	0.5401	-1.0582
HALLMARK HEME METABOLISM	0.1205	-1.7259	0.2888	-1.3885
HALLMARK PI3K AKT MTOR SIGNALING	0.0275	-2.3915	0.2888	-1.2781
HALLMARK E2F TARGETS	0.0221	-2.4198	0.3868	-1.2931
HALLMARK MYC TARGETS V1	0.0221	-2.5381	0.4752	-1.1602
HALLMARK UNFOLDED PROTEIN RESPONSE	0.0221	-3.2583	0.0422	-2.3854
HALLMARK MTORC1 SIGNALING	0.0221	-3.5903	0.2888	-1.4228

Of the positively enriched gene sets (Table 6-9) both angiogenesis and inflammatory responses were approaching significance in both doxorubicin and cisplatin treated 4T1 fGSEA analysis. The unfolded protein response was significantly negatively enriched in both doxorubicin and cisplatin treated 4T1 fGSEA analysis (Table 6-10).

Table 6-11: Positively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated B16 cell line.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	B16 doxorubicin padj	B16 doxorubicin NES	B16 cisplatin padj	B16 cisplatin NES
HALLMARK COAGULATION	0.0107	2.7017	0.6584	1.1061
HALLMARK FATTY ACID METABOLISM	0.0107	2.2518	0.7703	1.0089
HALLMARK INTERFERON ALPHA RESPONSE	0.0169	2.0384	0.1100	1.8700
HALLMARK HYPOXIA	0.0107	2.0320	0.2186	1.3076
HALLMARK XENOBIOTIC METABOLISM	0.0107	1.9310	0.4850	1.2740
HALLMARK P53 PATHWAY	0.0271	1.9288	0.0778	2.3503
HALLMARK ESTROGEN RESPONSE LATE	0.0504	1.8833	0.2227	1.5701
HALLMARK INTERFERON GAMMA RESPONSE	0.0948	1.6142	0.1100	1.8700
HALLMARK CHOLESTEROL HOMEOSTASIS	0.3458	1.2191	0.3624	1.4428
HALLMARK IL2 STAT5 SIGNALING	0.3556	1.1767	0.4850	1.2008

Table 6-12: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated B16 cell line.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	B16 doxorubicin padj	B16 doxorubicin NES	B16 cisplatin padj	B16 cisplatin NES
HALLMARK UNFOLDED PROTEIN RESPONSE	0.0852	-1.5348	0.5384	-1.2155
HALLMARK MYC TARGETS V2	0.0808	-1.6334	0.2186	-1.3300
HALLMARK MYC TARGETS V1	0.0107	-2.6844	0.1715	-1.8375

None of the common positively or negatively enriched gene sets for B16 treated with doxorubicin and cisplatin were significant for both sets (Table 6-11 &12). However, Myc targets and unfolded protein response were present in negatively enriched B16 and 4T1 treated gene sets. Coagulation, interferon alpha response and interferon gamma response were present in positively enriched B16 and 4T1 treated sets.

6.3.5 Cell Line Specific Response to Different Stressors.

Figure 6-14: Comparison within 4T1 and B16 cell lines GSEA.

Positive and negative GSEA for 4T1 and B16 when treated with doxorubicin and cisplatin for 24 hours and ρ^0 cells deprived of pyruvate and uridine. Dox = doxorubicin, cisp = cisplatin rho denotes ρ^0 . A) Shows a Euler plot comparing up-enriched gene sets common between 4T1 cells treated with doxorubicin and cisplatin and 4T1 ρ^0 cells deprived of pyruvate and uridine for 24 hours. B) Shows a Euler plot comparing down-enriched gene sets common between 4T1 cells treated with doxorubicin and cisplatin and 4T1 ρ^0 cells deprived of pyruvate and uridine for 24 hours. C) Shows a Euler plot comparing up-enriched gene sets common between B16 cells treated with doxorubicin and cisplatin and B16 ρ^0 cells deprived of pyruvate and uridine for 24 hours. D) Shows a Euler plot comparing down-enriched gene sets common between B16 cells treated with doxorubicin and cisplatin and B16 ρ^0 cells deprived of pyruvate and uridine for 24 hours.

Comparisons were made between all three datasets for each cell line, doxorubicin treatment, cisplatin treatment, and ρ° deprivation, to aid in identification of common mechanisms of cellular response to stress.

In the 4T1 positively enriched gene sets (Figure 6-14 A) there were 8 gene sets common across all three 4T1 treated conditions. These 8 gene sets composed 26.7% of the ρ° gene sets, 42.1% of the cisplatin sets and 61.5% of the doxorubicin sets. In the negatively enriched 4T1 sets (Figure 6-14 B) there were two common negatively enriched gene sets, this represented 40% of the ρ° , 15.4% of the cisplatin and 8.7% of the doxorubicin. The positively enriched B16 sets (Figure 6-14 C) had no commonly enriched gene sets. The negatively enriched B16 sets however had (Figure 6-14 D) two common negatively enriched gene sets, this accounted for 9.1% of the ρ° , 40% of the cisplatin and 18.3% of the doxorubicin.

When comparing ρ° to cisplatin in the positively enriched 4T1 sets there were 13 gene sets in common representing 43.3% of the ρ° and 68.4% of the cisplatin. In the unenriched 4T1 gene sets there were 2 commonly unenriched sets, this accounted for 40% of the ρ° , and 15.4% of the cisplatin. In the positively enriched B16 there was 1 common gene set, this represented 100% of the ρ° , and 5.6% of the cisplatin sets. In the negatively enriched B16 sets 3 common gene sets were found accounting for 13.6% of the ρ° and 60% of the cisplatin sets.

When comparing the ρ° to doxorubicin 8 gene sets were found in the positively enriched 4T1 accounting for 26.6% of the ρ° and 61.5% of the doxorubicin sets. 3 gene sets were found in the negatively enriched 4T1, this accounted for 60% of the ρ° and 13.4% of the doxorubicin. There were no gene sets common between the ρ° and doxorubicin in the negatively enriched B16 sets yet 6 in the positively enriched B16 sets, making up 27.3% of the ρ° and 54.6% of the doxorubicin sets.

The common gene sets for both positively and negatively enriched gene sets are described in table 6-13 and 6-14. Overall, this shows there is more in common between the 4T1 gene

sets than the B16 ones. The less informative results of the B16 ρ° and B16 cisplatin results due to smaller biological effect of treatment is probably a significant contributor to this. In the 4T1 the upregulated ρ° were more related to the cisplatin but the downregulated ρ° had more in common with the doxorubicin.

Table 6-13: Positively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line and ρ° deprived 4T1.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 doxorubici n padj	4T1 doxorubici n NES	4T1 cisplatin padj	4T1 cisplatin NES	4T1 ρ° padj	4T1 ρ° NES
HALLMARK INTERFERON GAMMA RESPONSE	0.0221	2.0355	0.2888	1.6007	0.5166	1.1052
HALLMARK INFLAMMATORY RESPONSE	0.0221	2.0183	0.0581	1.9688	0.1710	1.4892
HALLMARK ANGIOGENESIS	0.0654	1.8154	0.0422	1.3214	0.0218	1.8879
HALLMARK OXIDATIVE PHOSPHORYLATION	0.0585	1.7637	0.2888	1.4603	0.4672	1.1418
HALLMARK DNA REPAIR	0.1614	1.5186	0.2888	1.5859	0.1543	1.5385
HALLMARK COAGULATION	0.2048	1.4308	0.2888	1.4496	0.0919	1.6945
HALLMARK EPITHELIAL MESENCHYMAL TRANSITION	0.4693	1.1404	0.3586	1.3527	0.1379	1.5516
HALLMARK TNFA SIGNALING VIA NFKB	0.4693	1.1250	0.2888	1.6117	0.4672	1.1428

Table 6-14: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated 4T1 cell line and ρ° deprived 4T1.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	4T1 doxorubici n padj	4T1 doxorubici n NES	4T1 cisplatin padj	4T1 cisplatin NES	4T1 ρ° padj	4T1 ρ° NES
HALLMARK P53 PATHWAY	0.0986	-1.6174	0.4619	-1.1881	0.1089	-1.5576
HALLMARK HEME METABOLISM	0.1205	-1.7259	0.2888	-1.3885	0.2154	-1.4136

Angiogenesis was significantly positively enriched or approaching significance across 4T1 treated with doxorubicin and cisplatin and the 4T1 ρ° (Table 6-13).

TNF α signalling via NF κ B appeared common among the 4T1 treated and 4T1 ρ° pyruvate and uridine deprived datasets and was common among all 3 ρ° line in the previous chapter although not significant here.

Table 6-15: Negatively enriched gene sets with significant p-values common among doxorubicin and cisplatin treated B16 cell line and ρ° deprived B16.

Significant adjusted p-values have been highlighted in blue with intensity of blue marking genes with more significant datasets.

Pathway	B16 doxorubicin p adj	B16 doxorubicin NES	B16 cisplatin p adj	B16 cisplatin NES	B16 ρ° p adj	B16 ρ° NES
HALLMARK UNFOLDED PROTEIN RESPONSE	0.0852	-1.5348	0.5384	-1.2155	0.8070	-1.1117
HALLMARK MYC TARGETS V1	0.0107	-2.6844	0.1715	-1.8375	0.7774	-1.2410

MYC targets were significantly negatively enriched only in the B16 doxorubicin dataset (Table 6-15), no other dataset had a gene set that was significant.

6.4 Discussion

In this chapter I compared ρ° deprivation to chemotherapy treatment. Deprivation of pyruvate and uridine in ρ° is an extreme model of metabolic stress. This is probably why the ρ° showed more in common between the three cell lines than observed in this chapter between both treatments of wild type cells and all conditions of each other.

One of the drawbacks of ρ° cells is they have been treated to long periods of ethidium bromide exposure, possibly resulting in substantial nuclear damage. This makes comparing them to the parental wild-type cell potentially difficult, as there may have been many sequential or regulatory differences in gene expression between parental and ρ° that were not due to the changes sustained in response to the stressor. This could have increased the noise when comparing between the ρ° cell and treated cells and decreased the power of the analyses.

There was little to no differential signature from the cisplatin. As cisplatin is known to affect transcription primarily from 48 hours this is due to the duration of treatment^{258,261}. 24 hours treatment was chosen based on the ρ° data for which a response in the Astrocyte ρ° was elicited in the first 24 hours in chapter 3. Cisplatin causes cellular death via necrosis due to blocking of apoptotic pathways²⁵⁸. This causes a massive NAD⁺ depletion^{258,261} and inability to produce ATP much like what we expect to cause cellular death in the ρ° cells when deprived of pyruvate.

Consistency in timing was prioritised as the most important variable to control for in the comparisons. Upon reflection it would have given a more consistent transcriptional signature to evaluate both the B16 ρ° and the B16 treated with cisplatin at 48 hours. As the mechanism of stress each condition would inflict upon the cells would be different, the 'level' of stress would not be controllable.

The doxorubicin concentration may have been too high in the 4T1. This could reduce the signals I would see overlapping with the B16 as potentially different levels of stress initiate different stress responses.

As mentioned previously multiplexing samples for sequencing reduced the batch variability but probably reduced the statistical power of these sequencing results. This could be fixed by sequencing single samples on single flow cells run concurrently on a GridION or a PromethION.

6.4.1 Differential Sequencing and GSEA

This chapter evaluated from the transcriptional analyses to answer three questions relating to the differential expression and gene set enrichment analysis of 4T1 and B16 cell lines treated with chemotherapies doxorubicin and cisplatin as well as the ρ^0 counterparts of these cells under 24 hours metabolic stress (pyruvate and uridine deprivation). These three questions were.

- 1) is there a common chemotherapy specific response between the two cell lines?
- 2) Is there a common cell line specific response to chemotherapy?
- 3) Is there a common cell line specific response to stress?

The results concluded that there was little in the way of a common chemotherapy specific response between cell lines. However, there was a cell line specific response to chemotherapy.

I found that approximately 30% of every cisplatin set was found in the corresponding doxorubicin set. This looks like a cell line specific response to both treatments in both cell lines. This implies that while most of a cell's response is different depending on the treatment, there are some conserved stress responses.

In looking for a cell line specific response to stress for each cell line I found for 4T1 via differential sequencing analysis that there was a significant upregulation in two *Cxcl5* transcripts across 4T1 cells treated with doxorubicin and cisplatin and 4T1 ρ^0 cells deprived of pyruvate and uridine for 24 hours and *Cxcl1* is upregulated in doxorubicin and ρ^0 samples. From the GSEA I also found an upregulation of angiogenesis in the 4T1 ρ^0 and cisplatin sets which was approaching significance in the doxorubicin sets.

In looking for a B16 specific response to stress only *Manf* was present, this was downregulated in doxorubicin and cisplatin treated samples and in the B16p^o dataset, however, was only significant in the doxorubicin set. This is probably due to the low significance of differences between the cisplatin and B16 p^o gene sets.

These data imply that for these treatments there are few common stress response mechanisms for each treatment. This is in contrast to the p^o work which showed a potential common mechanism of stress response across all three cell lines. This may be because the stress inflicted by chemotherapy is less than the wholesale destruction of a cell's ability to generate mitochondrial ATP and to synthesis, amino acids, fatty acids and pyrimidines and to replicate DNA.

6.4.2 Differential sequencing and GSEA

The upregulation of *Cxcl1* and *Cxcl5* in all stressed 4T1 cells is an intriguing finding. These results are yet to be confirmed via qPCR as the reagents to complete this important experiment were unexpectedly and substantially delayed due to the ongoing impact of the SARS-CoV2 pandemic on global manufacturing and shipping. Based on prior experience in our laboratory confirming minion sequencing differential expression studies via qPCR I expect to confirm the up regulation of these transcripts ³⁷⁸. When confirmed, these are excellent candidates for stress signalling molecules.

CXCL1 and CXCL5 are chemokines which are secreted chemo attractants with immune modulating properties. *Cxcl5* is overexpressed in tumour tissue, associated with advanced tumours and poor prognosis ³⁷⁹. It is expressed in cytoplasm and cellular membranes, overexpression is associated with migration and invasion of colorectal tumour cells ^{379,380}. Extracellular/secreted CXCL5 is a possible inducer of metastasis in prostate cancer ^{381,382}. Tumour formation may rely on CXCL5 ³⁸⁰ and yet in some tumours over expression of CXCL5 correlates with reduced metastatic potential ³⁸³ so this may be tumour type specific. CXCL5 is positively associated with TGF- β and is possible a TGF β target gene ³⁸⁰. CXCL5 recruits neutrophils and is found in advanced melanomas ³⁸³. CXCL5 is an angiogenic factor that promotes cell metastasis through CDC and this is enhanced via NF- κ B pathway ³⁸⁴.

CXCL1 and 5 are excreted by mesenchymal stromal cells encouraging migration in tumour metastasis ³⁸⁵. CXCL1 and 2 overexpression are primed for metastatic survival, they attract CD11b+ cells which then produce chemokines that aid cancer cell survival ³⁸⁶.

Chemotherapies often cause production of TNF α by endothelial and stromal cells ³⁸⁶. TNF α via NF- κ B heightens CXCL1/2 expression in cancer cells amplifying the loop and causing chemoresistance and was also found in my GSEA results for the all the 4T1 stressors ³⁸⁶.

Manf was decreased in all three B16 gene/transcript sets. MANF (Mesencephalic Astrocyte Derived Neurotrophic Factor) is a secreted protein that is also localised in the endoplasmic reticulum ^{387,388}. Decreased expression of *Manf* is associated with endoplasmic reticulum associated cellular death and with proliferation ³⁸⁸. It protects and restores damaged cells in rodent Parkinson's models ³⁸⁸. Endoplasmic reticulum stress can stimulate MANF expression and secretion and MANF also plays a role in ER homeostasis ^{387,389}. It is expressed in immune cells and has an autocrine immunomodulatory function, promoting anti-inflammatory activation ³⁹⁰. MANF is induced in response to several stress signals ^{389,390}. Once *Manf* is also confirmed via qPCR this is another great candidate for a stress response protein.

Angiogenesis is the formation of new blood vessels and capillaries ³⁹¹. It is upregulated in cancer and is essential in solid tumour formation ³⁹¹. Angiogenesis is also induced by CXCL5 via the AKT/NF κ B pathway ³⁸⁴.

Cxcl5, *Cxcl1* and *Manf* have the potential to be interesting genes for exploration into what the interactions between the tumour cells and microenvironment are, what cells initiate the interactions, when in tumour formation and chemoresistance development these interactions are the most important, why they occur and how they contribute to chemoresistance and tumour development.

7 Discussion:

This project's inception came from the highly intriguing, published data from the Berridge group²⁰¹. In these experiments ρ° B16 and 4T1 cell lines were injected subcutaneously into mice and, after a 2–3-week delay compared to the parental mitochondrial containing tumour cell lines, the ρ° cell lines formed tumours with mitochondrial DNA genotypes that matched the host and not the original cell line²⁰¹. This proved that mitochondrial transfer occurred *in vivo*, which was backed up by a growing body of evidence that suggested mitochondrial transfer could be a cellular rescue mechanism²⁰¹. This idea of one cell type rescuing another lead me to question what the behaviours of each cell type were, and what signals were exchanged between the ρ° cells and cells in the TME.

7.1 Summary

This thesis set out to investigate interactions between cells under extreme metabolic stress in a model tumour microenvironment, and to determine whether chemotherapy stress of the parental cell lines would have a similar response. Complex cell-cell interactions between cancer cells and cells in the microenvironment can be through physical contact, surface receptor-ligand interaction, cellular junctions or secreted stimuli from neighbouring cells or distant organs. Current work on the tumour microenvironment falls primarily into two camps 1.) the identification of the components of and differences between the TME of different tumour types, metastasis and chemoresistance, and 2.) the identification of how the tumour modulates and interacts with the TME and vice versa. This thesis research falls into the second camp - a complex cellular interaction involving mitochondrial transfer, that occurs within the tumour and in response to stress.

7.1.1 Proof of Concept: Stress-Induced Transfer of Mitochondria to ρ^0 Cells

My first objective was to establish a model of the tumour microenvironment using cells derived from compact bone. These CBDC were composed of a substantial monocyte population and a mesenchymal component. The CBDC were used with ρ^0 cells in a co-culture setting, to induce and assess stress survival.

The ρ^0 cells deprived of pyruvate and uridine failed to proliferate and died over a period of 5 days. In co-culture ρ^0 Astrocyte and 4T1 cell lines were rescued from severe metabolic stress by CBDC. The ρ^0 Astrocytes displayed visual evidence of mitochondrial transfer as mitochondrial networks at 24 hours and by 52 days were perinuclear. After 82 days they had visually fully replete mitochondrial networks. The 4T1 ρ^0 showed no immediate signs of mitochondrial transfer until 35 days, other than surviving in media that did not contain pyruvate and uridine. At 35 days the 4T1 ρ^0 also visually had fully replete mitochondrial networks, similar to the parental line.

At the onset of this work, Dr Remy Schneider, a previous PhD student in the McConnel and Berridge Labs had found that Astrocyte ρ^0 would form mitochondrial networks after co-culture with a CBDC-like culture ¹⁴¹. The 4T1 ρ^0 and B16 ρ^0 were known to form tumours *in vivo* after a 2-3 week delay, after which those tumours contained mitochondrial DNA matching the host and not the parental line ³⁴⁸. The timeline of the reestablishment of mitochondrial networks in both 4T1 ρ^0 and Astrocyte ρ^0 was not known and this timeline of reestablishment of mitochondrial networks corresponds with the onset of tumour formation in the 4T1 ρ^0 . This timeline of reestablishment of mitochondrial networks supports the idea that fully functional mitochondrial respiration and biosynthesis are required for tumour formation ²¹⁶, and suggests that a fully functional mitochondrial network with mitochondrial to nuclear crosstalk is also required ^{202,224}. The delay in tumour respiration may be caused by the time taken for the epigenetic regulation, transcriptional and proteomic response to allow network reformation in the ρ^0 cells after mitochondrial transfer.

This all led to several questions, was there an initial proliferation advantage of this co-culture or did this take time to develop? Was co-culture with the CBDC sufficient for ρ° survival? And was physical contact between the two cell types also necessary for survival?

7.1.2 Contact Dependant Survival Advantage of ρ° Cells in Co-culture with CBDC

My aims were, to understand the interactions between ρ° cancer cells and cells of the CBDC under stress and to determine any signals the ρ° cells give off under the same stress that results in interactions with the CBDC and mitochondrial transfer. I found that co-culture with CBDC not only allowed ρ° cells to survive pyruvate and uridine deprivation but gave a proliferation advantage, evident within the first 5 days of deprivation. I also discovered that the ρ° cells had a contact mediated survival advantage when co-cultured and that in the B16 this correlated with an increased amount of contact with CBDC when cultured without pyruvate and uridine.

When the ρ° cells were sequenced after 24 hours without pyruvate and uridine supplementation I found an upregulation of *Nars1*, an asparaginyl tRNA synthetase. Aside from helping load amino acids onto t-RNA, NARS1 is also involved with chemokine signalling initiating migration of monocytes and dendritic cells ^{364,367}, making NARS1 a good candidate for signalling in ρ° survival.

ρ° Cell Lines

Traditionally created ρ° are exposed to low levels of ethidium bromide over a period of weeks, which is taken preferentially into mitochondria, where it binds mitochondrial DNA and prevents replication until there is no detectable mt DNA left. However, there is also ethidium bromide exposure to nuclear DNA so it can be inferred that there is at least some nuclear damage in cell lines created in this manner ²³⁶. This nuclear damage could cause differences in cellular processes and responses which could cause issues when comparing ρ° cells to parental cells. As different cell lines have different chromatin structure, the location and abundance of non histone bound DNA will also differ ³⁹². Ethidium bromide

preferentially binds non-histone bound DNA and therefore I can presume the nuclear DNA damage caused by ethidium bromide differed for each ρ^0 cell line I used.

Mitochondrial specific endonuclease plasmids have been developed for generation of ρ^0 without nuclear damage. However, these methods require the stable transfection of the plasmid in order to completely remove the mtDNA. I attempted to use these methods in conjunction with clonal expansion, but the transfection could not be maintained for long enough to fully remove the mtDNA and either mitochondrial copy number rebounded, or cells did not survive clonal expansion, as shown in appendix 1. Therefore, I used traditionally created ρ^0 lines in this thesis. To limit the ethidium bromide damage specific signals comparisons between all three cell lines were made, as DNA damage is likely to be different in each cell line due to different chromatin structures.

The *ARS* genes and their corresponding proteins are tRNAase synthetases, responsible for charging amino acids to their respective tRNA molecules, the essential first step in protein translation ³⁹³. While first indicated in cellular migration 20 years ago, ³⁶⁷ it is only recently that *ARS* have been identified in neurodegenerative diseases ³⁹⁴. They have been shown to be involved in Charcot-Marie-Tooth neuropathy, neurodevelopmental epileptic encephalopathy and other neurodevelopmental and nervous system disorders ^{395–397}. In 2017 and 2018, their cell migration role was investigated, and *ARS* were found to play a role as global cell signalling mediators ³⁹⁸. *ARS* signalling pathways were also found to be important for cell viability ³⁹⁹.

Nars1 was first cloned in 1998 ³⁶³. In the same year *NARS1* was detected in the cytosol of cells, implicated in autoimmune reactions and identified as a human autoantigen ⁴⁰⁰. In 2002 *NARS1* was found to induce migration in lymphocytes, activated monocytes, IDCs and CCR3 transfected HEK-293 cells ³⁶⁷. This role was not published on again until December 2018 when the N terminal domain of *NARS1* was shown to elicit CCR3-mediated chemokine activity ³⁶⁴. *NARS1* activity was shown to be reduced by CCR3- targeting siRNA and the loop 3 region of the *NARS1* was shown to be the part of the N terminal domain that interacted with CCR3 ³⁶⁴. Recently reductions in *Nars1* mRNA and enzyme levels in fibroblasts and neural progenitor cells have been found in families with a novel unnamed neurodevelopmental disorder with epilepsy and neurodevelopmental delay ³⁵⁹. The authors

of this publication believe de novo and biallelic mutations in *Nars1* are significant cause of neurodevelopmental disease ³⁵⁹. Another publication recently indicates that a loss of *Nars1* leads to a decrease of proliferation in brain organoids ³⁵⁸.

CCR3 is highly expressed primarily on cerebral micro vascular cells, eosinophils, basophils, TH2 lymphocytes and fibrocytes ⁴⁰¹. It is however also expressed on 16.9% of non-classical CD14+ and CD16+ monocytes and 1.2% of classical CD14 + CD16- and 4.1% in HLA-DR high CD14+ monocytes ⁴⁰². The CBDC used for co-cultures contain monocytes and activated macrophages are one of the cell types for which migration is induced by NARS1.

At the start of this work, it was known that ρ^0 cells could not survive pyruvate and uridine deprivation unless implanted as a tumour or in co-culture ^{141,348}. This work found that ρ^0 survival of metabolite deprivation was enhanced in co-culture and that this survival was associated with contact to the cells in co-culture (CBDC). This led to the finding that *Nars1* was upregulated under ρ^0 metabolic deprivation. This adds to our understanding of cellular stress responses and mitochondrial transfer and adds to the body of evidence that NARS1 has a role as a signalling pathway with neurodevelopmental importance. This work indicates that NARS1 may also play a role in stress response to metabolic catastrophe, which has not been previously documented.

While I cannot tell what processes are occurring in my co-cultures that cause ρ^0 survival there are some interesting possibilities. For example, *Nars2* is the mitochondrially targeted paralogue of *Nars1* ³⁵⁸. *Nars2* mutations are associated with decreased oxygen consumption rates and impaired ETC activity ⁴⁰³. ρ^0 cells have no functional electron transport chain but the need for this is removed when supplemented with pyruvate and uridine. Both *Nars1* and *Nars2* mutations cause similar phenotypes of epilepsy and neurodevelopmental disease, exemplifying their similar roles ^{359,403,404}.

What if, in response to pyruvate and uridine depletion ρ^0 cells upregulate *Nars2* and as this cannot repair the ETC this causes a positive feedback loop. *Nars1* and *Nars2* as paralogues could be upregulated by the same transcription factors, like the HOX paralogues which are activated by different combinations of the same transcription factors ⁴⁰⁵. NARS2 is predicted

to not be secreted ⁴⁰⁶, unlike NARS1 which is secreted. Therefore, when *Nars1* is upregulated and secreted, this could initiate cellular migration, of CBDC, leading to cell-cell contact and mitochondrial transfer, resulting in survival of extreme metabolic stress and restoring ETC function. While this is an interesting possibility that needs to be investigated, it does not explain why we do not see an upregulation of *Nars2*, unless *Nars2* is upregulated in all ρ^0 cells, epigenetically silenced or not detected in our sequencing data. This is, of course, all very speculative at this stage.

Recent work in our group has indicated that ρ^0 4T1 cells upregulate immune pathways such as *Ccl5* and *Ccl2* when compared with the parental line ³⁷⁸. Both CCL5 and CCL2 are involved in monocyte migration ³⁷⁸. My results expand upon this and lead to a potential signalling pathway induced under severe stress.

7.1.3 Survival of Chemotherapy Treated Cells in Co-Culture with CBDC.

Thirdly I aimed to assess any difference and/or similarities in how cancer cells interact with the TME between the stressed ρ^0 cells and wild type cancer cells treated with chemotherapies. I conducted the same experiments as with the ρ^0 lines with cisplatin and doxorubicin treated parental cell lines and found similar results, limited primarily to the doxorubicin treatment. Treatment with cisplatin increased contact with CBDC in the B16 and not in the 4T1 and Astrocyte lines.

Choice of chemotherapies

Doxorubicin and cisplatin were chosen because both are used widely to treat many different primarily solid cancers, including breast cancer and melanoma ^{407–410}. They also have different modes of action, as doxorubicin is a topoisomerase inhibitor with DNA damaging activity ²⁵⁴, while cisplatin affects metabolic reprogramming by forcing cells to utilise oxidative phosphorylation. Both have some overlap in method of action in that they both elicit DNA damaging activity ^{266,267}.

From the previous chapter we knew that ρ^0 cells had a contact associated survival advantage when in co-culture with CBDC. These experiments found similar interactions to those observed in ρ^0 between treated cells and CBDC.

7.1.4 Similarities and Differences Between Stressed Cancer Lines

Finally, I aimed to assess any difference and/or similarities in the signals cancer cells emit by comparing the stressed ρ^0 cells to wild type cancer cells treated with chemotherapeutics. After differential sequencing analysis via DESeq2 we found that in the 4T1 doxorubicin and cisplatin treated and 4T1 ρ^0 cells there was an upregulation of *Cxcl5* and *Cxcl2*. CXCL5 is a chemokine that recruits neutrophils ³⁸³. The B16 treated and ρ^0 lines all had weak transcriptional signatures but showed a downregulation of *Manf*. MANF plays a role in the endoplasmic reticulum and is associated with stress signalling ^{389,390}.

To understand how my results fitted into the wider context of doxorubicin studies, I compared my doxorubicin treated GSEA results to published doxorubicin GSEA results. Some similarities between my work and published works include similar negatively enriched responses for cell cycle / G2/M checkpoint, MYC targets and hypoxia suppressed genes/ oxidative phosphorylation, which are all similar to my results despite some different naming systems ⁴¹¹. Conversely Maillet et al reported a positive enrichment of oxidative phosphorylation/ ETC related sets in which I did not find ⁴¹². However, both studies were conducted in cardiomyocytes and so many differences can be attributed to difference between cell types ^{411 412}.

Other studies of cisplatin-treated differential expression and gene set enrichment have been reported as well. Cisplatin treated HeLa cells showed similar hallmark enrichment to both the 4T1 and B16 samples. In this study cells were treated with 2 μ g/ml for 72 hours. This was a similar concentration of drug, although for a much longer period of time ⁴¹³. We both found TNF α , DNA repair and MYC targets V1 upregulated ⁴¹³. We found differences in our results in coagulation which was something that did not come up in their cisplatin dataset but was present in both B16 and 4T1 treated samples in my data ⁴¹³.

The differences between my data and other published work can be explained by different cell types and treatment durations. As shown in chapter 6 there were significant differences in transcriptome for the same treatment in different cell lines, so this would not be unexpected.

A similar response was seen in the treated cell lines to the p° cell lines when in co-culture with CBDC. However, drug-treated cells did not show an upregulation of Nars1 across the different treatments. Instead, I saw an upregulation of CXCL1 and CXCL5 in the 4T1 cells and a downregulation of Manf in the B16, although the latter was not statistically significant.

CXCL1 and CXCL5 are secreted chemoattractants that induce angiogenesis and expression is increased by $TNF\alpha$ ³⁸⁶. CXCL5 also recruits neutrophils ³⁸³. The most recent work on CXCL5 looks at the role cancer cell migration and neutrophil activation ^{414,415}. The underlying mechanism by which CXCL5 acts still remains unclear but it is shown to induce migration in both tumour and immune cells within the TME ^{415,416}.

This work plausibly adds to the cell types and conditions that CXCL5 is induced under, indicating that it may be induced under stress for immune recruitment or rapid migration of tumour cells in order to remove them from stressful environments, or towards supportive environments.

While less is known about MANF, it is also a signalling protein that is decreased in response to stress signals. The change in *Manf* in treated, stressed B16 cells is encouraging and is consistent with identification of stress-induced signalling pathways ^{389,390}.

Both NARS1 and CXCL5 are involved in chemokine signalling and immune infiltration, suggesting that these stressed cells recruit immune cells to aid their survival. Even MANF is involved in stress responses. While these three stressors – pyruvate and uridine deprivation, doxorubicin and cisplatin treatment- did not induce the same signals they all induced the same type of response, chemokine signalling. This indicates that signalling for help from other cells may be conserved and widely utilised by cells, leaving a target for researchers to exploit in our continued search for better and more effective treatment options for cancer.

Limits of Co-Culture experiments

As with any artificial system there are limitations to the capacity of the model tumour microenvironment used here to yield answers. While it allowed us to observe cell – cell interactions it under-represents the diversity of the TME. It also fails to recreate the ECM, hypoxic regions and complete endocrine signalling that is also present in the TME.

The CBDC come from an immune competent and cancer free mouse, and thus the cells in the microenvironment have not been activated by previous exposure to cancer cells. In the ρ^0 lines, the initial co-culture for 3 days before removal of pyruvate and uridine potentially allowed for some cross signalling between the ρ^0 and the CBDC. This could have resulted in tumour activation of some cellular components, and neither survival of the ρ^0 nor mitochondrial transfer occurred without co-culture prior to deprivation. In the chemotherapeutically treated samples, in an effort to not damage the CBDC, I treated the tumour cells before adding them to the CBDC and thus removed some opportunity for activation. Given that normal tissues are subjected to chemotherapies along with cancer cells, investigations where the whole co-culture was treated would yield results that are even more physiologically relevant.

The transcriptomic sequencing in this project was undertaken over 2 years 2019 and 2020. In that time the base calling, mapping and alignment tools improved. More reads were called and mapped from our first analysis in mid 2019 compared with our final analyses in February 2020 (runs performed after this were analysed using the same technology as in February). This indicated that as the technology improves it will become easier and more effective to use long read technology for transcriptomics, although perhaps not with quite so many barcoded samples.

7.2 Moving Forward:

One of the major contributions of this thesis is the co-culture approach to understanding the tumour microenvironment and cancer cell interplay. With this system it becomes easier to elucidate interactions without the need for *in vivo* mouse work. It is my hope that work such as this can contribute to the establishment of complex *in vitro* systems that mitigate our reliance on *in vivo* murine models to understand tumorigenesis and chemotherapy

resistance. This is in accordance with the New Zealand government's commitment to the 3 R's; the replacement of animals with non-animal alternatives, the reduction of animal use and the refinement of experimental procedure to reduce the pain or suffering of the animals used ⁴¹⁷.

7.2.1 Chemokine Signalling

With the *Nars1*, *Cxcl5* and *Manf* findings further investigation needs to be done.

The *Nars1* upregulation and CCR3 signalling has not been investigated in this TME model system but should be after verification of increased expression via qPCR. Chemokine and cytokine presence in the culture media would need to be assessed, via mass spectrometry, cytometric bead assay, ELISA or western blot. Migration assays would also add to this understanding. Trans-well plates and 'scratch' assays where different cells are plated on either side of a removable plug, would tell us if either cell type was migrating and which cell type was migrating towards or away from the other. These assay results could then be modified combined with the chemokine assay information to determine the role the chemokines play in this. There are commercially available CCR3 blockers that could be used to investigate a NARS1/CCR3 interplay within the CBDC ρ^0 co-culture setting. Cell lines not under stress could have *Nars1* expression increased via transient transfection.

Knock-downs/ knock-ins and siRNA silencing would allow us to look at what impact NARS1 has on the interactions we see. Conclusions could only be drawn after extensive genetic pathway analysis and experimental work. In conjunction with this, the assessment of the media for chemokine signals would allow us to fine tune the timing and determine the extracellular concentrations of the signalling factors required for CBDC- stressed cell interactions. The chemokines could also be used to 'stimulate' the CBDC and then their responses could be evaluated using transcriptomics, migration assays, motility assays and proteomics of both the culture media and the cells themselves.

CXCL5 and CXCL1 could also be investigated in this manner, to identify what cells initiate the interactions, when in tumour formation and chemoresistance development these interactions are the most important, why they occur and how they contribute to chemoresistance and tumour development.

7.2.2 Single Cell Transcriptomics

Single cell transcriptomics of the whole ρ° and treated co-cultures taken at time points is I believe one of the best next options for this work. It would allow for comprehensive analysis of all the cell types present in the co-culture, how they change over time and how co-culture affects them, for example are all ρ° cells aided by co-culture with CBDC or only a small subset? Do all 4T1 doxorubicin treated cells upregulate *Cxcl5*? Do these proportions increase over time?

These time points for the ρ° cells could be; before co-culture, during co-culture, on each of the initial 3 days before deprivation/treatment and after deprivation/treatment at 12, 24 and 48 hours. This would tell us how the transcriptome of the cells changes in response to the different conditions. If we could detect which cell had come into contact with the CBDC and vice versa via more advanced microscopy analysis combined with spatial transcriptomics to assess their basic transcriptome relative to the other ρ° / treated cells in culture that had no contact, we would be able to get a clearer picture of how cells respond to stress.

This should also be undertaken with tumour samples from patients to better understand the TME components of various cancers present in various tissues. This would allow us to better replicate the TME in vitro and thus give more accurate insights into how cancer cells survive stress.

7.2.3 CBDC and ρ° Co-culture Experiments

These experiments lead to many questions that should be investigated further, to begin by evaluating the mechanism of cellular death of each of the ρ° cell lines. This would be achieved by staining for different cellular death mechanisms and then using flow cytometry and confocal microscopy to assess what mechanisms were present with increased frequency in the stressed conditions ⁴¹⁸.

If each potential mitochondrial donor from the CBDC was isolated, you could look at whether or not the same transfer and cellular interactions were witnessed with each donor type and therefore draw more conclusions about what cells can donate and to the mechanisms of each type. This would however be a completely artificial system. To counter this, you could form more realistic microenvironments by sampling the microenvironment in the 'normal' niche of the cell lines and constructing this as close as possible *in vitro*.

I would examine the transfer mechanisms at play in the 4T1 ρ° cell and Astrocyte ρ° cell co-cultures, we currently cannot rule out (or rule in) any other transfer mechanism other than cytoplasmic fusion. By staining for the other known mechanisms, (TNTs, gap junctions and EVs) we could see if the other mechanisms play a role in these cells' survival. In particular it would be interesting to see if fusion was the only mechanism of mitochondrial transfer in the Astrocyte ρ° survival or if the other mechanisms were used as well. It would also be interesting to see what mechanisms were at play in the 4T1 ρ° cells and B16 ρ° cells although some other more persistent method of staining would be needed for the B16 ρ° cells, possibly stable transfection.

Something that I think would benefit the work on mitochondrial transfer greatly is an accurate depiction of what is happening at the very early stages of stress induction. I would like to figure out what is happening in the first 24 hours. To do this I would take 1, 2, 6, 12 and 24 hr time points and use a process called rolling circle amplification (RCA) which allows us to look at the transfer of individual mitochondrial genomes²⁹³. This can be used in conjunction with immunohistochemistry staining for TNTs, EVs, Gap Junctions as well as CD45 staining. This would answer which of the two main cell types present in the CBDC was interacting with the ρ° cells, when they were interacting with the ρ° cells and if mitochondrial transfer was occurring in these interactions with the ρ° cells and via what processes. In answering these questions, we would have a much better picture of what is happening to these ρ° cells and this would allow us to start asking the same questions of chemotherapy treated cells in co-culture and ultimately in clinical samples.

Unfortunately, I do not believe there is currently the technology to answer this question fully *in vivo*, fluorescence producing tumour cell lines have been implanted into a mouse and removed at various timepoints⁴¹⁹. These could be examined to look for cell types and transfer mechanisms, but this would miss the early timepoints when transfer occurs, such as in the Astrocyte ρ^0 cells in chapter 3.

Inferring what cell type/s in the CBDC culture were responsible for the contact dependant survival would be a fascinating area to study, particularly the comparison with the cell type responsible for the mitochondrial transfer. As for mitochondrial transfer, individual cell types co-cultured with ρ^0 could determine if it is a single cell type or a mixture of many cell types that are responsible for the contact dependant survival advantage.

7.2.4 Treated cell lines and CBDC

A way to make *in vivo* observations of these interactions would be to take tumour sections and assess cell proximity in fixed cells, this however would limit the types of interactions seen and only whole tumour survival could be inferred.

I would also consider other common cancer treatments and chemotherapies to gain a better scope of the cell line dependant stress responses, these would include ionising radiation, immune therapies, alkylating agents other than and including cisplatin, antimicrotubular agents, topoisomerase inhibitors and other cytotoxic antibiotics such as bleomycin or mitomycin.

8 Final Conclusions:

Over the course of this PhD I have established, that mitochondrial transfer is a cellular response to stress in Astrocyte ρ^0 and 4T1 ρ^0 cell lines and established a timeline for this transfer. I have shown that the CBDC is a mixed culture of monocytes/ macrophages and mesenchymal stromal cells, this mixed culture contains a mitochondrial donor and helped ρ^0 cells survive under extreme metabolic stress. There is a proliferation advantage of ρ^0 in co-culture with CBDC when ρ^0 are under high levels of metabolic stress. The ρ^0 cells upregulate *Nars1* when deprived of pyruvate and uridine for 24 hours. That there is a proliferation advantage of doxorubicin treated wildtype Astrocyte, 4T1 and B16 and this correlated with a potential contact dependant survival advantage. Differential sequencing of cells treated with doxorubicin and cisplatin resulted in an upregulation of *Cxcl5* and *Cxcl1* in the 4T1 cells treated with doxorubicin and cisplatin as well as 4T1 ρ^0 cells deprived of pyruvate and uridine.

I conclude that cells survive stress better under co-culture and that different mechanisms of stress all induce an upregulation of chemokine related genes. I hypothesise that there are conserved cellular stress responses that result in the recruitment of immune cells from which a survival advantage is conferred through a contact mediated mechanism. The mechanism of survival whether it be conserved through different stresses or not is yet to be determined. However, the findings set out in this thesis should help guide future research that further develops our understanding as to how cancer cells survive stress with the help of the tumour microenvironment, with the hope that a better understanding can yield more effective and equitable treatment for those afflicted by cancer.

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Appendix 1: Attempted mUNG creation of

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Mutant Uracil N Glycosylase to Produce Rho Zero cells

The following work was conducted to attempt to create a rho zero line using GL261 to understand the role that mitochondrial transfer plays in glioblastoma and glioma. The mUNG plasmid was used to generate rho zero lines that contained less nuclear damage than those rho zero lines created using EtBr. One of the two protocols currently used to create rho zero cells by the mUNG plasmid results in retention of the plasmid and thus the perpetual removal of mtDNA²⁴⁴. The other protocol involved sorting and clonal selection which we modified in order to produce cells that would be able to produce mitochondrial acceptors for mitochondrial transfer experiments²⁴⁴.

Protocol for production of mUNG rho zero lines was as follows. Cell lines were transfected with mUNG plasmid for 24 hours before being sorted into 96 well plates at 1 or 5 cells per well based on GFP expression. The plates were then cultured until a significant volume of cells was available to test for mtDNA. Bulk samples of cells were taken for mtDNA copy number analysis at timepoints up until 8 days.

The plasmid used in the following work contains genes for enhanced green fluorescent protein (eGFP), ampicillin resistance, neomycin/G418 resistance and the mUNG construct. This allows for bacterial expansion of the plasmid using the Ampicillin resistance, detection of transfection efficiency using microscopy or FACS and the eGFP and the neomycin resistance allows for removal of non-transfected cells by drug treatment.

Initially cloning rings were attempted to isolate colonies from 6 well plates and 6 cm dishes that had been treated with G418 without success (data not shown), it was decided that a sorting strategy and clonal expansion from single sorted cells would provide more surety that the cells came from a single source. Cells were sorted into single and 5 cells per well of 96 well plates using the following FACS strategy. 5 cell wells and transfection with a GFP plasmid were used to determine if low survivability of clones was due to lack of intercellular communication or transfection with the mUNG plasmid itself.

Sorting the mUNG plasmid

A

B

The mUNG1 plasmid

This plasmid pMA3790 from Mikhail Alexeyev (Addgene plasmid # 70110 ;

<http://n2t.net/addgene:70110> ; RRIDNAAAddgene 70110) contains neomycin resistance, eGFP and a

mutant Y147A human uracil-N-glycosylase (mUNG1). A) Shows a complex schematic and B) shows a

simplified schematic of pMA3790 aka the mUNG1 plasmid. Source for A-B www.addgene.org, accessed

24 Aug 2020.

Sorting of mUNG transfected cell lines

Cells were transfected with a GFP plasmid and the mUNG plasmid and then run through the FACS sorter A) GL261 transfected with the GFP and mUNG plasmids, showing FSC[Perp] (perpendicular) vs FSC[Par] to gate for singlets, SSC vs FSC[Par] to gate for the cells of interest, SSC vs Dapi to gate for live cells and SSC vs GFP to sort for cells that are GFP positive. B) 4T1 transfected with the GFP and mUNG plasmids, showing FSC[Perp] (perpendicular) vs FSC[Par] to gate for singlets, SSC vs FSC[Par] to gate for the cells of interest, SSC vs Dapi to gate for live cells and SSC vs GFP to sort for cells that are GFP

positive. C) GL261 transfected with the GFP and mUNG plasmids, showing FSC[Perp] (perpendicular) vs FSC[Par] to gate for singlets, SSC vs FSC[Par] to gate for the cells of interest, SSC vs Dapi to gate for live cells and SSC vs GFP to sort for cells that are GFP positive. Sorting was performed by Sally Chappell of the Hugh Green Cytometry Core (HGCC) at the Malaghan Institute of Medical Research.

Cells were sorted on eGFP expression, under advice of the HGCC eGFP producing cells were selected for medium to high GFP expression, low expression levels were as we can see GFP expression was higher for all 3 cell lines with the mUNG plasmid.

1.1.1 GL261 mUNG transfection

GL261 Colonies Formed 1.5 weeks After Transfection With GFP or mUNG

GL261 colonies present in 96 well plate after transfection with either GFP plasmid or mUNG plasmid at 1.5 weeks post transfection. Cells were transfected at a ration of 4:1 Viafect: plasmid and cultured in DMEM supplemented with pyruvate, uridine and penicillin streptomycin after sorting.

We can see from “*GL261 Colonies Formed 1.5 weeks After Transfection With GFP or mUNG*” that mUNG transfection has lower colony formation/ survivability than GFP transfection. This indicates that transfection with the mUNG plasmid results in increased cellular death following sorting.

The bulk sorting of the cells was analysed via qPCR to detect changes in mitochondrial copy number as shown below.

GL261 Colonies and Copy Number after Transfection with mUNG Plasmid

mUNG plasmid was transfected at a ratio of 4:1 Viafect to DNA, cells were sorted for GFP positivity and cultured in complete rho DMEM with penicillin streptomycin for the first two weeks after which the pen strep was removed. Cells were either sorted into 96 well plates at either 1 cell per well or 5 cells per well or bulk sorted (up to 10,000 cells per well) into 6 well plates for culture and DNA extraction. A) Colony survival of mUNG transfected GL261 in 96 well plate over 6 weeks. B) mtDNA copy number detected via qPCR shows mtDNA change over 9 days post transfection with mUNG plasmid. R1 R2 and R3 denote replicates of the experiment.

mUNG colony number decreased until there were no viable colonies over the 6 weeks observed. No colonies were recovered successfully from this experiment and late stage (6 weeks) mtDNA presence was not observed for GL261. mtDNA copy number decreased in the 9 days observed showing the mUNG plasmids effectiveness at degrading mtDNA. This decrease in mtDNA in the short term coincides with a dramatic decrease in colony formation and survival in the long term. It appears that a decrease in mtDNA causes a decrease in viability in the GL261 cell line.

After the lack of success generating a rho zero GL261 line, I attempted to generate 4T1 and B16 rho zero lines with the MUNG plasmid in order to check that rho zeros could be generated using this method.

1.1.2 4T1 and B16 mUNG Transfection

4T1 and B16 mUNG Transfection

mUNG plasmid was transfected into cell at a ratio of 4:1 Viafect: DNA, cells were sorted for GFP positivity cultured in complete rho DMEM with penicillin streptomycin A) shows transfected 4T1 murine breast cancer cells transfected with mUNG plasmid and B) shows B16 murine melanoma cells transfected with mUNG plasmid C) PCR of mtDNA and nuclear DNA of both 4T1 and B16 punitive rho zero colonies that were randomly selected from those colonies that had grown out from the sort. Gel shows a positive control from wild type 4T1 with a mitochondrial band at 2145bp and a nuclear band at 580bp No template controls are marked NTC, Mt marks mitochondrial PCR and Nuc denotes nuclear PCR, Ctrl denotes the positive control. This is composite image from two halves of one gel, ladders have been kept bands consistent.

We can see from the figure above that mtDNA is decreased by exposure to the mUNG plasmid. The decrease is greatest at 2-4 days (48-96 hours) and begins to increase again following this.

All punitive rho zero colonies tested that grew enough to perform PCR had mitochondrial DNA remaining at 13 weeks post transfection. This table also shows that the percent of colonies decreased over time for 4T1 much like the GL261, but colonies did not substantially decrease for B16 transfected cells.

4T1 and B16 colony numbers after transfection with the mUNG plasmid

Table showing percent colony formation in 96 well plates following mUNG transfection. Weeks since transfection is the number of weeks since the cell lines were transfected with the mUNG plasmid and the number under the cell line is the number of cells seeded in a single well of a 96 well plate.

Weeks Since Transfection	4T1 5	4T1 1	B16 5	B16 1
1.5	45	14	94	61
3	24	4.5	95	62
5	14.5	3.5	95	58

Due to our inability to produce a GL261 rho zero line I elected to work with other established rho zero lines B16 and 4T1 as well as an in house generated Astrocyte line in order to understand how mitochondrial transfer and cellular interaction play a role in cell survival in cancer and non-cancer types.

Appendix 2: DESeq2 results

DESeq2 Chemotherapy Specific Response Upregulated Doxorubicin

Table of upregulated transcripts common among B16 and 4T1 cell lines treated with Doxorubicin

Transcript	B16 Dox log 2-Fold Change	B16 Dox padj	4T1 Dox log 2-Fold Change	4T1 Dox padj
Gm13056	5.27	-	0.78	0.10
Fkbp3.1	5.27	-	0.57	0.13
mt-Co1	5.27	0.00	0.79	0.01
Ccl2	5.26	-	1.88	0.00
n-R5s88	4.26	0.05	3.27	-
H2-K1	4.21	0.13	0.59	0.21
mt-Co2	4.19	0.00	1.10	0.00
Plk2	4.03	0.00	1.19	0.04
mt-Co3	3.89	0.00	0.75	0.02
Clu	3.76	0.01	0.82	0.04
Ubl5.4	3.60	0.08	1.01	0.02
mt-Rnr1	3.20	0.00	1.04	0.00
Dgat2	3.10	0.02	2.06	0.01
Uqcrh.2	3.03	0.08	0.48	0.15
Crip1	3.02	0.00	0.51	0.05
Ubl5.2	2.98	0.08	0.79	0.08
mt-Rnr2	2.82	0.00	0.81	0.00
Trappc6a.1	2.82	0.09	1.05	0.05
Emp3.1	2.75	0.00	0.59	0.07
B2m	2.67	0.00	0.40	0.20

Sparc.1	2.64	0.00	0.60	0.01
Hspb6	2.64	0.06	1.79	0.09
Sparc	2.59	0.00	0.60	0.01
Prkcsh	2.53	0.00	0.61	0.05
Akr1b3	2.52	0.00	2.17	0.00
Gstm2	2.49	0.00	0.89	0.02
Isyna1	2.41	0.00	0.81	0.06
Ndufa3.1	2.36	0.01	0.78	0.02
Ecm1	2.31	0.11	0.68	0.11
Ctsh	2.29	0.02	0.93	0.05
S100a4	2.24	0.00	0.38	0.20
Bst2	2.22	0.00	0.81	0.01
Fbxo2	2.21	0.03	0.91	0.01
mt-Nd2	2.20	0.00	0.44	0.19
Rnpep	2.18	0.09	1.89	0.00
Smardc1.1	2.16	0.01	1.60	0.03
Mrps18c.1	2.14	0.10	0.82	0.22
Pcolce	2.14	0.00	0.52	0.06
Snord104	2.09	0.00	1.73	0.00
Emb	2.08	0.00	0.81	0.06
Emp3.2	2.08	0.06	0.63	0.22
Atp5j2.1	2.07	0.06	0.49	0.15
Snord13	2.03	0.00	1.25	0.00
Ubl5	2.03	0.00	0.69	0.01
Gstm1	2.02	0.00	1.24	0.00
Nsf	2.02	0.13	2.40	0.00
mt-Nd4	2.02	0.00	0.84	0.00
Fuca1	2.01	0.00	1.35	0.08
H2-D1	2.01	0.00	0.49	0.04
Becn1	1.99	0.00	0.67	0.17
Atp6ap1	1.99	0.00	0.66	0.08

Trappc6a	1.98	0.00	1.01	0.02
mt-Nd5	1.98	0.00	0.58	0.13
Npc2	1.97	0.00	1.42	0.00
Ctsz	1.96	0.00	0.76	0.01
Ndufa1	1.96	0.00	0.55	0.14
Prkcsh.1	1.89	0.04	0.54	0.11
Ptgds	1.88	0.10	2.36	0.04
Anapc13	1.85	0.00	0.48	0.11
Iscu	1.84	0.00	0.98	0.00
Cox6b1	1.83	0.00	0.47	0.12
Bsg	1.79	0.00	0.37	0.28
Cox6c	1.79	0.00	0.34	0.26
Uqcr11	1.77	0.00	0.50	0.09
Gsn	1.76	0.07	0.87	0.09
Atox1	1.72	0.00	0.60	0.04
Atp5mpl.1	1.70	0.12	0.82	0.03
Plod1	1.70	0.00	1.10	0.17
Ptgds.1	1.70	0.00	2.14	-
Ptgds.3	1.70	0.00	2.01	-
Aif1l	1.69	0.02	1.21	0.07
Nedd8	1.66	0.00	0.49	0.05
Mrpl52.1	1.65	0.02	0.50	0.21
Ndufa3	1.64	0.00	0.53	0.08
Ifi27.1	1.64	0.03	1.92	0.00
Nme2.1	1.63	0.00	0.38	0.24
Tmem176a	1.63	0.00	0.65	0.11
Ost4	1.61	0.00	0.58	0.14
Gpx1	1.60	0.00	0.75	0.00
Psme1	1.57	0.00	0.88	0.00
Gxylt2	1.56	0.04	0.86	0.23
Prdx2	1.55	0.00	0.53	0.09

Atp5h	1.54	0.00	0.41	0.22
Uqcrcq.1	1.51	0.00	0.82	0.01
Sod2	1.50	0.00	0.61	0.11
Atp5k	1.50	0.00	0.47	0.09
Higd2a	1.48	0.00	0.65	0.20
Atp6v0c	1.47	0.00	0.72	0.01
Vps53	1.46	0.06	1.08	0.17
Cdc37	1.42	0.00	0.63	0.02
Gpi1	1.41	0.00	0.82	0.00
Rpl41	1.41	0.00	0.36	0.22
BC031181	1.41	0.00	0.89	0.16
Tomm7	1.41	0.00	0.40	0.26
Cox7a2	1.41	0.00	0.73	0.01
Atp5e	1.39	0.00	0.45	0.16
Tma7	1.38	0.00	0.58	0.03
Ndufa7.2	1.38	0.01	0.67	0.07
Rpl41.2	1.37	0.00	0.49	0.04
Cops9	1.36	0.00	0.38	0.21
Cox7b	1.36	0.00	0.57	0.05
Ndufc1	1.35	0.00	0.35	0.28
Tmx2	1.33	0.01	0.87	0.22
Atp5mpl	1.30	0.00	0.63	0.05
Bloc1s1	1.29	0.00	0.66	0.03
Pomp	1.28	0.00	0.63	0.01
Tmem176b.1	1.25	0.02	0.40	0.25
Lgals9	1.25	0.05	1.31	0.00
Mbp	1.22	0.03	1.50	0.14
Atp5md	1.22	0.00	0.76	0.01
Atp5j2	1.21	0.00	0.39	0.26
Ndufa12	1.21	0.01	0.48	0.07
S100a13	1.20	0.01	0.92	0.00

Rangap1	1.19	0.00	0.68	0.06
Mydgf	1.19	0.00	0.60	0.26
Ndufb1-ps	1.16	0.00	0.92	0.00
Alpk2	1.13	0.00	0.73	0.00
Romo1	1.12	0.00	0.59	0.02
Ndufa7	1.10	0.00	0.55	0.06
Usp37	1.03	0.09	0.58	0.09
Ndufa7.1	1.02	0.03	0.59	0.08
Cuedc2.1	1.01	0.10	0.57	0.21
Atp6v1f	1.01	0.00	0.52	0.09
Naa38	0.98	0.01	0.46	0.22
Uqcrh	0.97	0.00	0.47	0.09
Slc22a17	0.94	0.05	1.03	0.05
Atp5g1.1	0.94	0.01	0.47	0.12
Prdx2.1	0.94	0.02	0.54	0.10
Snf8	0.92	0.09	0.94	0.00
mt-Cytb	0.89	0.00	0.78	0.00
Ncbp3	0.87	0.04	0.81	0.02
Uqcr10.1	0.85	0.12	0.65	0.05
Lgals3	0.79	0.07	0.55	0.03
Atp6v0b	0.77	0.01	0.61	0.25
mt-Nd1	0.75	0.07	0.62	0.06
Tmbim4	0.75	0.07	0.73	0.09
Ddt	0.75	0.13	2.10	0.00
Uqcrcq	0.74	0.01	0.73	0.01
Mrps14	0.73	0.02	0.43	0.23
Tmem256.1	0.72	0.13	0.67	0.18
Rpl41.1	0.70	0.03	0.50	0.07
Ak2	0.68	0.11	0.51	0.20
Timm8b	0.64	0.07	0.90	0.02
Uqcr10	0.64	0.03	0.50	0.06

Psmb7	0.62	0.12	0.54	0.14
Ndufa11	0.62	0.06	0.67	0.08
Ndufb3	0.60	0.07	0.51	0.14
Phb	0.54	0.09	0.65	0.10
Ndufb9	0.53	0.08	0.51	0.06
Malat1	0.50	0.12	0.64	0.05

DESeq2 Chemotherapy Specific Response

Downregulated Doxorubicin

Table of downregulated transcripts common among B16 and 4T1 cell lines treated with Doxorubicin

Transcript	B16 Dox log 2-Fold Change	B16 Dox padj	4T1 Dox log 2-Fold Change	4T1 Dox padj
Rabggtb	-7.18	0.00	-1.00	0.23
H1f0	-7.05	0.00	-2.11	0.00
Trim35	-6.74	0.00	-0.57	0.21
Gas5.3	-6.47	0.00	-0.58	0.11
Kn11	-6.13	0.00	-0.93	0.02
2610002M06Rik	-5.95	0.00	-1.58	-
Hnrnpr.1	-5.71	0.01	-0.60	0.26
Ddx3x	-5.59	0.01	-0.59	0.07
Hist1h2ae	-5.43	0.00	-2.43	0.00
Rpp40	-5.42	0.01	-2.42	-
Chac1	-5.40	0.01	-2.07	0.00
Mitf	-5.34	0.00	-1.33	0.26
Spred1	-5.27	0.02	-1.24	0.03
Tsen2	-5.14	0.01	-3.86	-
Kras	-5.13	0.03	-0.76	0.21
Hist1h1e	-5.10	0.00	-2.99	0.00
Ftl1.1	-4.88	0.01	-0.38	0.16
Rsrc2	-4.86	0.05	-0.59	0.21
Prrc2c	-4.85	0.05	-0.56	0.16
Dnaja2	-4.80	0.00	-0.68	0.15
Cox6a2	-4.66	0.07	-2.41	-
Ubald2	-4.63	0.03	-1.05	0.01
Kmt2e	-4.59	0.08	-0.95	0.07
Thoc7.1	-4.52	0.08	-0.57	0.13

Cdkn1b	-4.46	0.10	-0.88	0.27
Caprin1.1	-4.46	0.10	-0.79	0.04
Tpx2.1	-4.39	0.10	-0.79	0.07
Rbm39.4	-4.36	0.11	-1.48	0.00
Tfg	-4.32	0.00	-1.11	0.04
Srsf3	-4.30	0.00	-0.50	0.09
Ube2d3	-4.30	0.00	-0.51	0.21
Snx4	-4.27	0.05	-0.85	0.16
Itsn2	-4.27	0.13	-0.69	0.17
Gnaq	-4.27	0.13	-1.63	0.05
Hmgb2	-4.25	0.00	-1.11	0.00
Plk1	-4.22	0.00	-1.53	0.00
Snrnp27	-4.18	0.00	-0.84	0.00
Calm2	-4.14	0.00	-0.35	0.22
Sub1	-4.06	0.00	-0.33	0.19
Cul1	-4.05	0.00	-0.77	0.26
Ckap5	-4.01	0.01	-0.66	0.23
Smc4	-4.01	0.01	-0.45	0.13
Hspa8	-3.89	0.00	-0.30	0.19
Usb1	-3.85	0.01	-3.25	-
Esf1	-3.77	0.08	-0.66	0.09
Reep4	-3.74	0.11	-0.68	0.11
Zrsr2	-3.74	0.02	-0.70	0.07
Cdc42se1	-3.71	0.00	-1.10	0.26
4921524J17Rik	-3.70	0.00	-0.97	0.17
Dnal4	-3.68	0.00	-2.19	-
Srxn1	-3.66	0.00	-1.09	0.00
Ube2e3	-3.65	0.02	-1.49	0.09
Dusp6	-3.64	0.03	-1.37	0.00
Ppp2r2a	-3.63	0.13	-0.57	0.21
Slc3a2.2	-3.60	0.13	-1.83	0.04

Nop53	-3.55	0.01	-0.80	0.01
Mt1	-3.53	0.00	-0.48	0.09
Mtap	-3.42	0.00	-0.84	0.00
Brd4	-3.36	0.05	-0.77	0.27
Spag7	-3.34	0.00	-0.57	0.15
Hnrnpa3.2	-3.27	0.06	-0.71	0.00
Dek	-3.19	0.00	-0.44	0.15
Prpf40a	-3.18	0.01	-0.41	0.25
Eif5	-3.15	0.00	-0.54	0.17
Pcbp1	-3.14	0.00	-1.42	0.00
Rad23b	-3.09	0.03	-0.49	0.21
Cryzl1	-3.06	0.00	-1.13	0.11
Rpl3.1	-3.05	0.01	-0.60	0.01
Mcrip1	-2.96	0.00	-1.08	0.02
Hint3	-2.86	0.01	-1.69	-
Eif2b4	-2.82	0.04	-0.87	0.14
Xbp1	-2.82	0.01	-2.38	-
Rrs1	-2.71	0.08	-0.63	0.26
Hnrnpa3.1	-2.69	0.08	-0.83	0.00
Csnk2a1	-2.66	0.01	-0.50	0.21
Rxylt1	-2.65	0.09	-1.22	0.04
Cbx3	-2.65	0.00	-0.58	0.05
Rpl22l1.2	-2.60	0.06	-1.25	0.00
Ywhah	-2.57	0.00	-0.56	0.10
Rad21	-2.47	0.07	-0.47	0.17
Eif4a1	-2.44	0.00	-0.36	0.19
Golgb1	-2.40	0.09	-0.92	0.10
A430005L14Rik	-2.39	0.00	-1.14	0.05
Eif3a	-2.38	0.00	-0.61	0.05
Fam3c	-2.32	0.05	-0.85	0.26
Nfe2l2	-2.29	0.01	-1.05	0.00

Hnrnpa0	-2.27	0.06	-0.89	0.00
Rbms1	-2.24	0.13	-0.80	0.03
Pfn1	-2.20	0.00	-0.87	0.00
Thrap3	-2.10	0.07	-0.60	0.25
Hnrnpa2b1	-2.08	0.00	-0.67	0.00
Dnm1l	-2.07	0.13	-0.73	0.17
Chordc1	-2.05	0.02	-0.55	0.22
Serbp1	-2.05	0.00	-0.82	0.00
Eif2s2	-2.03	0.00	-0.73	0.00
Sf3b1	-2.01	0.00	-0.58	0.18
Eif4b	-2.00	0.01	-0.87	0.06
Atf4	-1.98	0.00	-1.69	0.00
Serpinh1.1	-1.97	0.07	-1.20	0.00
Josd2.1	-1.96	0.04	-1.06	0.22
Rala	-1.96	0.04	-0.68	0.11
Ier3	-1.94	0.10	-0.63	0.02
Trabd	-1.84	0.13	-0.84	0.16
Rab2a	-1.83	0.01	-1.11	0.00
Pcif1	-1.83	0.13	-1.94	-
Dhx15	-1.82	0.09	-0.69	0.15
U2af1	-1.82	0.00	-0.60	0.26
Cltc	-1.80	0.02	-0.56	0.12
Ddx42	-1.77	0.12	-0.84	0.20
Serbp1.3	-1.76	0.11	-0.82	0.00
Manbal	-1.74	0.01	-1.12	0.05
Chmp2a	-1.65	0.00	-1.21	0.00
Sf3b4	-1.64	0.00	-0.72	0.13
Hmgb1	-1.62	0.00	-0.96	0.00
Pabpn1	-1.58	0.13	-0.47	0.23
Cdc20	-1.55	0.09	-1.00	0.08
Map2k3	-1.48	0.02	-0.80	0.12

Cln5	-1.47	0.14	-1.66	-
Serbp1.1	-1.47	0.01	-0.83	0.00
Hnrnpa2b1.2	-1.30	0.08	-0.52	0.04
Npm1	-1.28	0.00	-0.38	0.09
Hnrnpa3	-1.27	0.00	-0.83	0.00
H2afz	-1.22	0.00	-0.44	0.21
Thoc7	-1.21	0.01	-0.56	0.05
Utp3	-1.19	0.07	-0.95	0.00
Ralb	-1.18	0.12	-0.99	0.13
Sqstm1	-1.14	0.09	-0.90	0.00
Ptma	-1.10	0.00	-0.83	0.00
Hsp90b1	-0.86	0.02	-0.73	0.00
Sfr1	-0.76	0.05	-0.68	0.00
Dynlt1b	-0.69	0.09	-0.49	0.27

DESeq2 Chemotherapy Specific Response

Upregulated Cisplatin

Table of upregulated transcripts common among B16 and 4T1 cell lines treated with Cisplatin

Transcript	B16 cisp log 2-Fold Change	B16 cisp padj	4T1 cisp log 2-Fold Change	4T1 cisp padj
Nusap1	1.12	1.00	0.58	1.00
Crip1	1.10	1.00	0.46	1.00
Tubb6	0.92	1.00	0.48	1.00
Snora57	0.87	1.00	0.41	1.00
Ube2c	0.64	1.00	0.47	1.00

DESeq2 Chemotherapy Specific Response

Downregulated Cisplatin

Table of downregulated transcripts common among B16 and 4T1 cell lines treated with Cisplatin

Transcript	B16 cisp log 2-Fold Change	B16 cisp padj	4T1 cisp log 2-Fold Change	4T1 cisp padj
Hist1h2ae	-1.19	0.76	-1.41	0.00
Gm48610	-1.09	1.00	-0.37	1.00
Als2	-0.92	1.00	-0.45	1.00
Dlg1	-0.82	1.00	-0.40	1.00
Loxl2	-0.56	1.00	-0.28	1.00

DESeq2 Cell line Specific Response 4T1

Upregulated

Table of upregulated transcripts common among the 4T1 cell treated with both Doxorubicin and Cisplatin

Transcript	4T1 dox log 2-Fold Change	4T1 dox padj	4T1 cisp log 2-Fold Change	4T1 cisp padj
Irf7.1	5.40	0.00	2.89	-
Irf7.3	4.98	0.00	2.89	-
Ola1.4	3.83	-	3.54	-
Saa3.2	3.69	0.00	1.23	0.57
Saa3.1	3.51	0.00	1.56	0.00
Il18rap	3.49	-	3.00	-
Slc25a14.1	3.43	-	3.22	-
Fam13b	3.41	-	3.24	-
Parp11	3.30	-	3.04	-
Parp11.1	3.30	-	3.04	-
Gm33027	3.17	-	2.71	-
Zfp369.1	3.15	-	3.13	-
Bend4.2	3.02	-	2.67	-
Bend4	2.97	-	2.67	-
Bend4.1	2.97	-	2.67	-
Bend4.3	2.97	-	2.67	-
Bend4.4	2.97	-	2.67	-
Rab11fip1.1	2.78	-	2.91	-
Slc25a14.3	2.72	-	2.62	-
Lurap1l	2.65	-	2.59	-
Bloc1s1.1	2.64	-	2.35	-
Coro1a	2.63	-	2.81	-
Coro1a.1	2.63	-	2.81	-
Adar	2.57	-	2.32	-

lsg15	2.48	0.00	0.76	1.00
C3	2.38	0.00	0.66	1.00
Dnm2.5	2.31	-	2.28	-
lfitm6.1	2.26	-	2.30	-
Akr1b3	2.17	0.00	0.75	1.00
Fbxo21.1	2.06	-	1.81	-
Fbxo21	1.90	-	1.71	-
Dnm2.2	1.83	-	1.77	-
4931440P22Rik	1.82	-	1.78	-
Klk10	1.71	0.00	1.14	0.67
Tpm1.9	1.62	0.00	0.54	1.00
Cxcl5.1	1.52	0.00	0.81	0.00
Cxcl5	1.52	0.00	0.81	0.00
Pick1.2	1.44	0.04	1.07	-
Appt.2	1.40	0.13	1.67	-
Thra	1.39	0.19	1.43	-
Fuca1	1.35	0.08	1.09	-
Rnf19b.1	1.32	0.02	0.94	1.00
Lgals9	1.31	0.00	0.70	1.00
Rnf19b	1.25	0.03	0.84	1.00
H2-K2.1	1.23	0.16	1.32	-
Pigp	1.22	0.03	0.93	1.00
Tmsb4x	1.05	0.00	0.45	1.00
Cxcl1	1.04	0.00	0.45	0.68
H2-K2	1.04	0.22	1.17	1.00
Tbp	0.99	0.26	1.04	-
Mrpl50	0.99	0.23	1.01	1.00
Rad51.1	0.97	0.14	0.80	1.00
Prr13.1	0.97	0.01	0.75	1.00
Rad51	0.96	0.09	0.80	1.00

Prr13	0.95	0.00	0.56	1.00
Nipsnap1	0.89	0.14	0.75	1.00
Pspc1	0.87	0.25	1.00	1.00
mt-Nd4	0.84	0.00	0.29	1.00
Tiprl	0.84	0.19	0.80	1.00
Plscr1.1	0.73	0.24	0.99	0.93
Abcb1b.1	0.69	0.20	0.64	1.00
Arf3	0.69	0.17	0.60	1.00
Becn1	0.67	0.17	0.74	1.00
Malat1	0.64	0.05	0.42	0.90
Ssu72	0.62	0.25	0.64	1.00
Sparc	0.60	0.01	0.32	1.00
Emp3.1	0.59	0.07	0.52	1.00
H2-D1.1	0.54	0.02	0.36	1.00
Crip1	0.51	0.05	0.46	1.00
H2-D1	0.49	0.04	0.41	1.00
Nfkbia	0.48	0.08	0.63	0.57
B2m	0.40	0.20	0.48	1.00

DESeq2 Cell line Specific Response 4T1

Downregulated

Table of downregulated transcripts common among the 4T1 cell treated with both Doxorubicin and Cisplatin

Transcript	4T1 dox log 2-Fold Change	4T1 dox padj	4T1 cisp log 2-Fold Change	4T1 cisp padj
Col4a3bp.3	-3.63	-	-2.90	-
Prcp.2	-3.51	-	-3.46	-
Snora43	-3.36	-	-3.31	-
Trib3	-3.27	0.00	-1.00	0.57
Net1.1	-3.27	-	-3.20	-
Hist1h2ak	-3.17	-	-2.36	-
Gm2710	-3.08	-	-3.02	-
Asns.1	-2.99	0.00	-0.70	0.27
Hist1h1e	-2.99	0.00	-1.09	0.66
Asns	-2.95	0.00	-0.62	0.27
Hist1h2ae	-2.43	0.00	-1.41	0.00
Rere.2	-2.35	-	-2.27	-
Zfp667.2	-2.26	-	-2.21	-
Zfp667.1	-2.17	-	-2.12	-
Nupr1	-2.14	0.00	-0.45	1.00
H1f0	-2.11	0.00	-0.74	0.58
Hist1h2ap	-2.10	0.00	-0.88	0.00
Hmgb2.2	-2.10	0.00	-1.62	0.58
Gm44297	-2.09	-	-2.09	-
Gm44346	-2.09	-	-2.09	-
Chac1	-2.07	0.00	-0.87	1.00
Bag5.1	-2.07	-	-1.81	-
Stag1.5	-2.05	-	-1.58	-
Col4a3bp.2	-1.93	-	-2.39	-

Gm20633	-1.91	-	-1.89	-
Hmgb2.1	-1.87	0.00	-0.94	0.72
Hist1h2ao	-1.85	0.00	-1.10	0.00
Gm20632	-1.84	-	-1.60	-
Hist1h2ad	-1.82	0.03	-2.01	-
Ung.1	-1.79	0.02	-2.14	-
Ung	-1.77	0.02	-2.01	-
Atf4.1	-1.72	0.00	-0.93	0.10
Nupr1.1	-1.72	0.00	-0.82	1.00
Atf4	-1.69	0.00	-0.88	0.02
Hist1h2ag	-1.69	0.04	-2.11	-
Ung.2	-1.62	0.05	-2.08	-
Hist1h1b	-1.52	0.00	-0.88	0.58
Tars	-1.51	0.00	-0.49	1.00
Dbf4.4	-1.51	0.08	-1.26	-
Gm13167	-1.45	0.02	-1.16	0.92
Gm13167.1	-1.45	0.02	-1.16	0.92
Papss1.3	-1.44	0.11	-1.10	-
Gm13232	-1.43	0.02	-1.14	0.94
Cep83	-1.41	0.04	-1.15	1.00
Rbpj	-1.37	0.00	-0.42	1.00
Tars.1	-1.36	0.00	-0.50	1.00
Gm4739	-1.35	0.00	-0.85	0.44
Gm13237	-1.35	0.01	-1.09	0.58
Eif1.3	-1.29	0.14	-1.13	-
Gapdh.8	-1.28	0.15	-1.15	-
Tln1.2	-1.26	0.04	-0.86	1.00
Lmna.1	-1.25	0.00	-0.44	0.97
Lmna	-1.23	0.00	-0.41	1.00
Gapdh.6	-1.22	0.15	-0.96	-
Uck2.1	-1.22	0.14	-1.02	-

Gm33882	-1.21	0.00	-0.59	1.00
Sars	-1.19	0.00	-0.39	1.00
Iqca	-1.19	0.00	-0.50	1.00
Gapdh.7	-1.12	0.21	-1.01	-
Sars.2	-1.10	0.01	-0.60	1.00
Ahnak.1	-1.10	0.00	-0.40	1.00
Gm9800	-1.08	0.08	-0.75	1.00
Ptma.4	-1.02	0.09	-0.76	1.00
Zfp91	-1.01	0.01	-0.61	1.00
Sars.3	-0.99	0.09	-0.73	1.00
Gm4617	-0.99	0.03	-0.74	0.93
Ap2a2.1	-0.98	0.13	-0.74	1.00
Utp3	-0.95	0.00	-0.47	1.00
Apex1.1	-0.93	0.14	-1.33	0.58
Ptma.3	-0.93	0.12	-0.68	1.00
Ahnak.2	-0.90	0.01	-0.55	1.00
Snrnp27.1	-0.89	0.00	-0.54	1.00
Hnrnrm	-0.86	0.01	-0.50	1.00
Gm29257	-0.86	0.22	-0.90	1.00
Ptma.2	-0.86	0.04	-0.69	0.83
Hsp90ab1.1	-0.85	0.00	-0.47	1.00
Hnrnrm.1	-0.81	0.01	-0.46	1.00
Gps2.1	-0.80	0.21	-0.72	1.00
Gm5469	-0.75	0.11	-0.68	1.00
Me2	-0.74	0.25	-0.72	1.00
Cdv3.4	-0.72	0.14	-0.83	0.92
Gm5177	-0.71	0.05	-0.50	1.00
Cdv3-ps	-0.71	0.22	-0.83	1.00
Gapdh.5	-0.67	0.18	-0.77	1.00
Gm26917	-0.65	0.04	-0.69	0.54
Ipo4.6	-0.60	0.26	-0.58	1.00

Lars2.2	-0.58	0.03	-0.53	0.93
Gm43305	-0.58	0.00	-0.32	1.00
Tpm3	-0.55	0.05	-0.36	1.00
Tpm3.2	-0.51	0.10	-0.38	1.00
Actb.1	-0.47	0.06	-0.35	1.00
Mir6236	-0.47	0.08	-0.55	0.44
Mfsd11	-0.43	0.20	-0.46	0.83
Gm42031	-0.41	0.05	-0.31	1.00
Npm1.3	-0.40	0.16	-0.31	1.00
Pdap1	-0.30	0.26	-0.27	1.00

DESeq2 Cell line Specific Response B16

Upregulated

Table of upregulated transcripts common among the B16 cell treated with both Doxorubicin and Cisplatin

Transcript	B16 dox log 2-Fold Change	B16 dox padj	B16 Cisp log 2-Fold Change	B16 Cisp padj
Zranb2.1	6.41	0.01	4.39	1.00
A830082K12Rik	5.26	-	4.74	1.00
Plk2	4.03	0.00	1.98	1.00
Cbr2	3.85	0.00	2.13	1.00
mt-Rnr1	3.20	0.00	0.95	1.00
Crip1	3.02	0.00	1.10	1.00
mt-Rnr2	2.82	0.00	0.74	1.00
S100a11	2.55	0.00	0.82	1.00
Maf	2.41	0.02	1.54	1.00
Gm42031	2.35	0.00	0.90	1.00
Iqca	2.29	0.00	1.21	1.00
Bscl2	2.26	0.00	1.24	1.00
Ifitm3	2.21	0.00	0.90	1.00
Pqlc3	2.09	0.12	1.76	1.00
Snord13	2.03	0.00	1.52	0.00
AI506816	1.91	0.00	0.59	1.00
Dnaic1.2	1.86	0.00	0.85	1.00
Flii	1.84	0.05	1.12	1.00
Dcxr	1.83	0.02	1.57	1.00
Ephx1	1.79	0.00	1.02	1.00
Tmem14a	1.65	0.08	1.03	1.00
Cd9.1	1.65	0.00	0.54	1.00
Akr1b10	1.60	0.03	0.93	1.00
Abhd4	1.60	0.05	1.13	1.00

Csde1	1.54	0.09	1.40	1.00
Cald1	1.38	0.03	0.88	1.00
Mbp	1.22	0.03	0.67	1.00
Arl6ip1	0.97	0.00	0.96	0.12

DESeq2 Cell line Specific Response B16

Downregulated

Table of downregulated transcripts common among the B16 cell treated with both Doxorubicin and Cisplatin

Transcript	B16 dox log 2-Fold Change	B16 dox padj	B16 cisp log 2-Fold Change	B16 cisp padj
Timm10	-7.30	0.00	-0.64	1.00
Hist1h2ae	-5.43	0.00	-1.19	0.76
Cox6a2	-4.66	0.07	-6.12	0.12
Al413582	-4.53	0.03	-0.57	1.00
Srsf7.2	-4.12	0.06	-1.23	1.00
Gsr	-4.05	0.00	-1.06	1.00
Magohb	-3.90	0.00	-0.66	1.00
Polr2h	-3.77	0.00	-0.73	1.00
Srm	-3.71	0.00	-0.95	1.00
Ebna1bp2	-3.69	0.00	-0.52	1.00
Bri3	-3.68	0.00	-1.00	1.00
Srxn1	-3.66	0.00	-0.99	1.00
Tnfrsf12a	-3.20	0.00	-0.55	1.00
Nol12	-2.94	0.00	-0.73	1.00
Psmc2	-2.85	0.00	-0.63	1.00
Rpl28.1	-2.69	0.00	-1.18	1.00
Mcrip2	-2.67	0.00	-1.48	0.99
Gm47283	-2.57	0.00	-0.87	1.00
Gt(ROSA)26Sor	-2.56	0.01	-0.83	1.00
C1qbp	-2.55	0.00	-0.72	1.00
Alkbh3.1	-2.52	0.12	-1.55	1.00
Pfdn4	-2.52	0.00	-0.52	1.00
Tomm5	-2.51	0.00	-0.63	1.00
Hnrnpa0	-2.27	0.06	-1.07	1.00

Rpl27.1	-2.23	0.00	-0.69	1.00
Dqx1	-2.19	0.00	-0.90	1.00
Smim26	-2.11	0.00	-0.59	1.00
Orc6.1	-2.08	0.07	-0.95	1.00
Ube2i	-2.06	0.00	-0.78	1.00
Serbp1	-2.05	0.00	-0.76	1.00
Cnbp.2	-2.04	0.00	-0.54	1.00
Smyd5	-1.90	0.05	-0.98	1.00
U2af1	-1.82	0.00	-0.65	1.00
Stoml2	-1.75	0.00	-0.58	1.00
Brip1os	-1.48	0.04	-0.67	1.00
Serbp1.1	-1.47	0.01	-0.70	1.00
Fkbp11	-1.19	0.01	-0.66	1.00
Lyar	-0.98	0.10	-0.86	1.00
Manf	-0.79	0.03	-0.61	1.00
Lhfpl4	-0.73	0.12	-0.67	1.00