

Building an *ex vivo* Model of
Breast Cancer Lung
Metastasis

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Declaration

I confirm that all the work in this thesis is my own and I take full responsibility for it. All collaborative efforts have been recognised in the text or in the acknowledgements.

Abstract

Cell cultures are traditionally studied as 2D monolayers with use of 3D culture systems gradually becoming more prevalent. The advantages of 3D culture over traditional methods are numerous as they: do not force apical-basal cell polarity, allow the formation of 3D cell structure and allow the study of complex microenvironments, amongst others. The study of biological processes can be hindered by the limitations of monolayer culture. One such process is the metastasis of malignant cells, which requires understanding of different chronological biological processes for efficacious secondary tumour formation. This thesis shows how the many biological events occurring during metastasis can be broken down and subsequently scrutinised by traditional methods, to gain insight into the strategies employed by different breast cancer sub types. Understanding each individual process alone has led to significant gains in our understating of metastasis, bringing all components together is now possible when integrating cells into a bioreactor 3D culture system. Findings here have shown the metastatic strategies of 'luminal A' breast cancer is delamination whilst triple negative 'basal B' breast cancers used a nomadic, single-cell strategy. It was found that the single-cell strategy induced large changes in phenotype when cells were cultured in suspension, including an increased resistance to paclitaxel, mitochondrial health and resistance to anoikis, and a 'suspended animation' phenotype. Importantly, these behaviours were seen to revert to a migratory, proliferative phenotype upon re-adherence to a substrate. These findings were then tested in 3D culture systems. Embryonic lung fibroblast organoids were first tested as a 3D substrate before decellularised rat lung matrices were selected as the most representative. Rat decellularised lung matrix was tested as a substrate for mimicking metastasis in the context of bioreactor system which allowed the transfer of cells from one matrix to another via the flow of media. This thesis shows that reconstituting models of metastasis is possible outside of animal models.

Publications

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Abbreviations

ABL1/2	Tyrosine-protein kinase ABL1/2
ACTB	Actin, cytoplasmic 1 (β -actin)
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADP	Adenosine diphosphate
AKT	RAC- α serine/threonine-protein kinase
AMPK	Adenosine monophosphate kinase
APAF1	Apoptosis protease-activating factor 1
APC	Adenomatous polyposis coli protein
ATM	Serine-protein kinase ATM
ATP	Adenosine triphosphate
ATR	Serine/threonine-protein kinase ATR
AURKB	Aurora kinase B
BAG1	BAG family molecular chaperone regulator 1
BAK	BCL-2 homologous antagonist/killer
BAX	Apoptosis regulator BAX
BCL-X(L)	BCL-2-like protein long isoform
BCL2	Apoptosis regulator BCL-2
BDNF	Brain derived neurotrophic factor
BID	BH3-interacting domain death agonist
BIR	Baculoviral IAP repeat
BIRC1	Baculoviral IAP repeat-containing protein 1
BIRC2	Baculoviral IAP repeat-containing protein 2
BIRC3	Baculoviral IAP repeat-containing protein 3
BIRC5	Baculoviral IAP repeat-containing protein 5
BIRC6	Baculoviral IAP repeat-containing protein 6
BIRC7	Baculoviral IAP repeat-containing protein 7
BIRC8	Baculoviral IAP repeat-containing protein 8

BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRUCE	BIR repeat-containing ubiquitin-conjugating enzyme
c-FOS	Proto-oncogene c-Fos
c-JUN	Transcription factor c-Jun
CASP3	Caspase 3
CASP7	Caspase 7
CASP8	Caspase 8
CCNB1	G2/mitotic-specific cyclin-B1
CD4	Cluster of differentiation protein 4
CD4	Cluster of differentiation protein 8
CD68	Macrosialin
CDC42	Cell division control protein 42
CDCA8	Borealin
CDK2	Cyclin-dependent kinase 2
CDKN1A	Cyclin-dependent kinase inhibitor 1
CFLAR	CASP8 and FADD-like apoptosis regulator
CHK1	Serine/threonine-protein kinase CHK1
cIAP1	Cellular inhibitor of apoptosis 1
cIAP2	Cellular inhibitor of apoptosis 2
CKII	Casein kinase II
CLOCK	Circadian locomotor output cycles protein kaput
CoA	Coenzyme A
CPC	Chromosomal passenger complex
CTCs	Circulating tumour cells
CTSL2	CTD small phosphatase-like protein 2
CTTN	Cortactin
CYC1	Cytochrome c

DISC	Death inducing signalling complex
DIABLO	Diablo homolog, mitochondrial
DNA	Deoxyribonucleic acid
E2F	Transcription factor E2F
EAA1	Excitatory amino acid transporter 1
ECM	Extracellular matrix
EEF1A1	Elongation factor 1-alpha 1, EF-1-alpha-1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHS	Engelbroth-Holm-Swarm
EMI	Emilin homology domain
ER	Oestrogen receptor
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
ERE	Oestrogen response element
ERK1	Extracellular signal-related kinase 1
ESR1	Oestrogen receptor 1
ESR2	Oestrogen receptor 2
FACIT	Fibril-associated collagen with interrupted helices
FAD	Flavin adenine dinucleotide (oxidised)
FADH₂	Flavin adenine dinucleotide (reduced)
FADD	FAS-associated death domain protein
FAK	Focal adhesion kinase
FAS	Tumour necrosis factor receptor superfamily member 6
FASL	Tumour necrosis factor ligand superfamily member 6
G₀	Gap 0 phase
G₁	Gap 1 phase
G₂	Gap 2 phase

GAG	glycosaminoglycan
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDI	Guanosine nucleotide-dissociation inhibitor
GEF	Guanosine nucleotide exchange factor
GRB2	Growth factor receptor-bound protein 2
GRB7	Growth factor receptor-bound protein 7
GSK3β	Glucose synthase kinase 3 β
GSTM1	Glutathione S-transferase Mu 1
GTP	Guanosine triphosphate
GUS	β -glucuronidase
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-pipirazineethanesulfonic acid
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HR	Hormone receptor
IAP	Inhibitor of apoptosis
IGF	Insulin-like growth factor
INCENP	Inner centromere protein
Ki67	Proliferation marker protein Ki67
KPNB1	Importin subunit beta-1
MACIT	Membrane associated collagen with interrupted helices
MAPK	Mitogen activated protein kinase
MCL1	Induced myeloid leukaemia cell differentiation protein MCL-1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase

MMP11	Matrix metalloproteinase 11
MOMP	Mitochondrial outer membrane permeabilisation
MUC4	Mucin-4
MYBL2	Myb-related protein B
NAD⁺	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NCOA1/2	Nuclear coactivator 1/2
NF-κB	Nuclear factor κB
NOXA	Protein Noxa
NTRK2	BDNF/NT-3 growth factors receptor
PARP	Poly [ADP-ribose] polymerase
PCR	Polymerase chain reaction
PDX	Patient-derived xenograft
PEA15	Astrocytic phosphoprotein PEA-15
PGR	Progesterone receptor
P_i	Phosphate (inorganic)
PI3K	Phosphatidylinositol 3-kinase
PI3KC2B	Phosphatidylinositol 4-phosphate 3-kinase C2 domain containing subunit β
PIK3R	Phosphatidylinositol 3-kinase receptor
PKC	Protein kinase C
PLK1	Polo-like kinase 1
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
PPP	Pentose Phosphate Pathway
PR	Progesterone receptor
PTGS2	Prostaglandin G/H synthase 2
PTM	Post-translational modification
PUMA	p53 up-regulated modulator of apoptosis

qPCR	Quantitative real-time polymerase chain reaction
RB	Retinoblastoma protein
RAB10	Ras-related protein Rab-10
RAC	Ras related C3 botulinum toxin substrate
RAF	RAF proto-oncogene serine/threonine-protein kinase
RAN	GTP-binding nuclear protein Ran
RAS	GTPase Ras
RASA1	Ras GTPase-activating protein 1
rDLM	Rat decellularised lung matrix
RGD	Arginine glycine aspartic acid (motif)
RHO	Transforming protein p
RIPK	Receptor-interacting serine/threonine protein kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPLP0	60S acidic ribosomal protein 1
SCUBE2	Signal peptide, CUB and EGF-like domain containing protein
SHC1	SHC-transforming protein 1
SP1	Transcription factor Sp1
SP3	Transcription factor Sp3
SRC	Proto-oncogene protein tyrosine kinase Src
SYK	Tyrosine-protein kinase SYK
TBP	TATA-box-binding protein
TCA	Tricarboxylic acid
TFRC	Transferrin receptor protein 1
TNFα	Tumour necrosis factor α
TP53	Cellular tumour antigen p53
TRAIL	TNF-related apoptosis inducing ligand
TRAILR1/2	TRAIL receptor 1/2

TUBB	Tubulin β chain
VDAC	Voltage-dependent anion-selective channel protein 1
VWFA	von Willebrand factor domain A
WNT	Protein WNT
XAF1	XIAP-associated factor 1
XIAP	X-linked inhibitor of apoptosis
YY1	Transcriptional repressor protein YY1

Chapter 1

Literature Review

Metastatic breast cancer is a complex disease, causing greatly increased mortality by comparison with less severe pathologies of breast tumour (Weigelt, Peterse & Veer, 2005; Siegel, Miller & Jemal, 2015). The biology of breast cancer is related to the metamorphosis events of pubertal breast development (Javed & Lteif, 2013). As such, oestrogen, progesterone and other growth factor receptors, such as *human endothelial growth factor receptor* (HER), are tightly associated with breast tumourigenesis (Naccarato *et al.*, 2000; Jackson-Fisher *et al.*, 2004).

Metastasis comprises sequential events, which have association with other stages of tumourigenesis. Hanahan and Weinberg (2011) defined six biological changes which collectively must occur within a population of cells, not necessarily a single cell, to confer formation of a malignant tumour. These events are potentiated by genome instability and prolonged localised inflammation. To introduce metastasis properly, it is necessary to understand some of the facets of the other hallmarks of cancer: replicative immortality, evasion of cell death, sustained proliferative signalling, evasion of growth suppression, induction of angiogenesis, evasion of the immune system and dysregulated cellular energetics.

The common biological markers associated with breast cancer are closely associated with the cell cycle, sustained replicative signalling and replicative immortality. The signalling in these systems is associated with evasion of cell death and the mitochondria, which also ties in metabolism and dysregulated cellular energetics. These events together with increased migratory potential lead to metastatic populations which then traverse the extracellular matrix in response to nutrient/oxygen diffusion gradients to come into interface with the circulatory system and traverse to a secondary site.

1.1 Breast Cancer Incidence

Breast cancer is the most common form of cancer worldwide, accounting for approximately 25% of new cases and 14% of deaths in 2012, with a notable disparity in mortality rates between developed and developing countries, summarised in Table 1.1 (Cancer Research UK, 2012; Torre *et al.*, 2015). The disparity between developed and developing countries is proposed to be due to multiple factors, including: treatment type and availability, mammography programmes, age of menarche, lifetime exposure to oestrogen, age at first pregnancy and/or menopause, and the incidence of post-menopausal obesity (Pike *et al.*, 1983; Hsieh *et al.*, 1990; Althuis *et al.*, 2005; Kohler *et al.*, 2015; Torre *et al.*, 2015).

Table 1.1 – Worldwide incidence and mortality rates for breast cancer. New cases and mortality are displayed as a percentage of total cases / deaths from all cancers. All data are to 2 significant figures [Adapted from: Torre *et al.*, 2015].

	New Cases (%)	Mortality (%)	Mortality as a percentage of new cases (%)
Worldwide	25	14	31
Developed Countries	28	15	25
Developing Countries	23	14	37

There has been a continuous increase in breast cancer survival since 1988 due to improvement in early detection practices, improved treatment regimens and awareness (ACBCS, 2006). Despite this, there is a drastically reduced 5 year survival rate with disease which progresses to form secondary tumours distant from the primary site (Siegal, Miller & Jemal, 2015). As a result, time of diagnosis is of particular importance in breast cancer prevention. Mammography screening in the UK for those between 50 and 70 years of age has decreased the mortality rate by 35% since its introduction in 1988 (ACBCS, 2006). This is because late stage metastatic cancers correlate with at least 60% lower 5 year survival rate by comparison with tumours which are localised or regional in pathology, as shown in Figure 1.1 (Weigelt, Peterse & Veer, 2005; Siegel, Miller & Jemal, 2015).

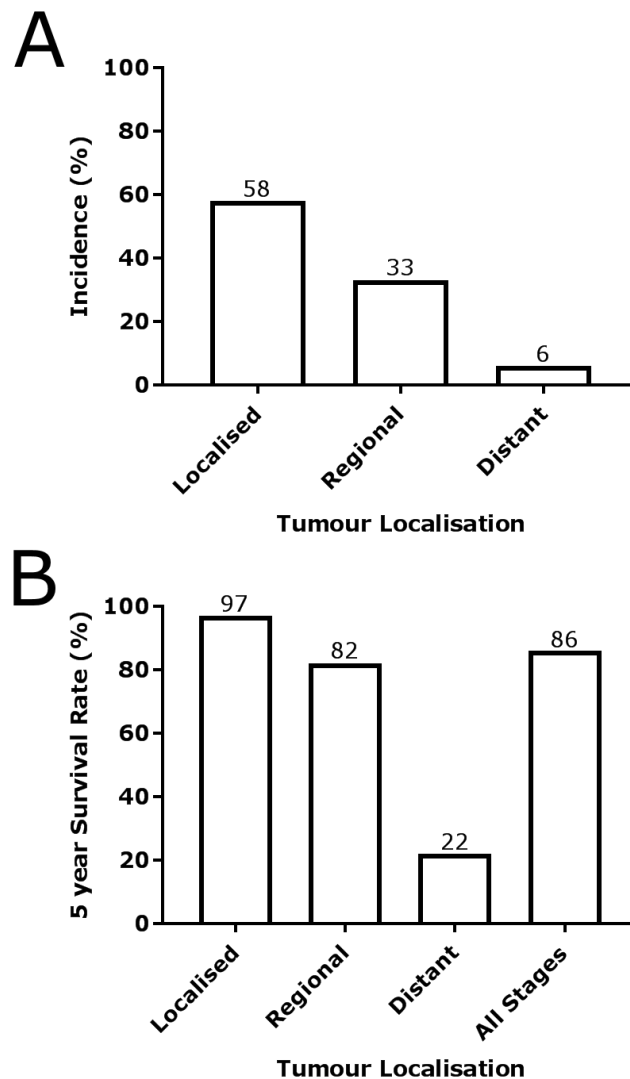


Figure 1.1 – Breast cancer incidence by localisation and the associated 5 year survival rates. **A**, percentage incidence of tumours at different loci. **B**, 5 year survival rate of tumours at said loci. Percentage incidence does not add up to 100% due to missing values. A localised pathology is where malignancy is restricted to a single tissue. A regional pathology is where a malignancy is present in tissue and its adjacent tissues. A distant pathology includes a metastatic population [Adapted from: Siegel, Miller & Jemal, 2015]

1.2 Breast Cancer Biology

To understand the pathology with which breast cancer is associated, it is necessary to understand the biological markers currently used in diagnostics. Breast cancer has common biomarkers: HER2 encoded by *ERBB2* and the nuclear receptors for oestrogen and progesterone, encoded by *ESR1/ESR2* and *PGR* respectively (Gibson & Saunders, 2012).

Recent analysis of breast cancer incidence in the United States has added to the well-established relevance of these biomarkers in terms of histological grade and receptor expression. Hormone receptors positive (HR⁺) tumours with no HER2 expression (HER2⁻) were found to be the most well differentiated, or lowest grade, and triple negative (HR⁻/HER2⁻) tumours were the most poorly differentiated, or highest grade (Kohler *et al.*, 2015). The receptor status of breast cancer is beginning to become subdivided by emerging characteristics, as highlighted in Table 1.2. Furthermore, over 40 discrete types of tumour mutation profiles have been catalogued in a recent publication by Nik-Zainal *et al.* (2016).

Table 1.2 – Breast tumour classification. Luminal A/B tumours are endocrine responsive owing to hormone receptor expression and similarly HER2, and luminal B tumours are responsive to trastuzumab (Herceptin), a clinical anti-HER2 antibody. Ki67: Antigen Ki67, EGFR: Epidermal growth factor receptor, KRT5/6: cytokeratin-5/6. [Adapted from: Kao *et al.*, 2009; Holliday & Speirs, 2011].

Classification	Receptor Status	Emerging characteristics
Luminal A	ER ⁺ , PR ^{+/-} , HER2 ⁻	Ki67 low, chemotherapy responsive
Luminal B	ER ⁺ , PR ^{+/-} , HER2 ⁺	Ki67 high, variable chemotherapeutic response
Basal A	ER ⁻ , PR ⁻ , HER2 ⁻	Ki67 high, chemotherapy responsive, EGFR ⁺ , KRT5/6 ⁺
Basal B (Claudin-Low)	ER ⁻ , PR ⁻ , HER2 ⁻	Ki67 positive, intermediate chemotherapeutic response
HER2	ER ⁻ , PR ⁻ , HER2 ⁺	Ki67 high, chemotherapy responsive

The emerging characteristics, in terms of response to chemotherapy and Ki67 expression, exemplify the advancement of understanding in tumour heterogeneity and malignant cell behaviour has guided the diagnosis and treatment selection.

Oncotype DX[®] is a quantitative real-time polymerase chain reaction (qPCR) assay, which analyses the expression of 16 target genes with 5 reference genes. The resulting data help to guide treatment by subdividing patients into treatment categories according to their tumour biology (see Table 1.3) (Paik *et al.*, 2004). The commercialisation of Oncotype DX exemplifies the significance of understanding breast cancer biology and the related prognosis.

Table 1.3 – Target genes for Oncotype DX. The genes were selected from a pool of 250 candidates by qPCR involving 447 patients. The chosen genes were selected based on recurrence and probe performance across three discrete studies [Adapted from: Paik *et al.* 2004].

Category	Gene
Proliferation	<i>Ki67, STK15, BIRC5</i> (Survivin), <i>CCNB1</i> (cyclin B1), <i>MYBL2</i>
Invasion	<i>MMP11</i> (stromolysin 3), <i>CTSL2</i> (cathepsin L2)
HER2	<i>GRB7, HER2</i>
Hormones	<i>ESR1, PGR, BCL2, SCUBE2</i>
Miscellaneous	<i>GSTM1, CD68, BAG1</i>
Reference	<i>ACTB</i> (β -actin), <i>GAPDH, RPLP0, GUS, TFRC</i>

Oncotype DX is limited to a relatively discrete group, those with oestrogen receptor positive and lymph node negative breast cancer. The increased understanding of breast cancer biology should lead to more treatment programmes being developed (Onitilo *et al.*, 2009; Feero & Guttmacher, 2011; Harris *et al.* 2015).

1.2.1 **Steroid Hormone Receptors**

Hormones can be either peptides or steroids with peptide hormones, such as insulin, requiring active transport mechanisms to cross the cell membrane. Steroid hormones derive from cholesterol, which makes them membrane permeable and thus allows them to diffuse freely through the cell membrane and interact with their respective cytoplasmic receptors. With respect to mammary development, the most important hormones produced by the ovaries are the steroid hormones progesterone and oestrogen. The tissue plasticity instigated by these two hormones can also be deployed to modify tissues during tumourigenesis.

1.2.1.1 **Oestrogen Receptors**

ESR1 and *ESR2* encode the oestrogen receptors α and β (ER α and ER β) respectively. Both proteins are transcription factors that can homo- or heterodimerise before binding to oestrogen response elements (ERE; Pettersson *et al.*, 1997). ER activation and conformational change occurs upon ligand binding, freeing it from cytoplasmic chaperones and allowing DNA binding of the ERE (Smith & Toft, 1993; Gruber *et al.*, 2004). Binding of this site often occurs

in tandem with a plethora of co-activators such as *nuclear co-activator 1/2* (NCOA1/2) and as a result binding at discrete loci can be affected by multiple variables such as: chromatin state, co-activator presence and homo- or heterodimeric state. ER α does not only act alone, it can also heterodimerise with *proto-oncogene c-Fos* (c-FOS) and *transcription factor c-JUN* (c-JUN) to allow ERE independent signalling (Saville *et al.*, 2000; Stoner *et al.*, 2000; Teyssier *et al.*, 2001; Gruber *et al.*, 2004). This becomes particularly relevant in transformed cells where *ESR1* and the ER binding partners are commonly upregulated (Holst *et al.*, 2007).

1.2.1.2 Progesterone Receptor

PGR encodes the progesterone receptor (PR), which is structurally and functionally similar to ER, has hormone-dependent and hormone-independent activities. In the presence of progesterone, PR is highly phosphorylated at many serine residues, conferring transcription factor binding and downstream epigenetic activities (Zhang *et al.*, 1997; Knotts *et al.*, 2001; Pierson-mullany & Lange, 2004; Faivre *et al.*, 2008).

To some extent the plasticity of the breast during prenatal and pubertal development influences carcinogenesis, as migratory behaviours and morphological changes are necessary for normal breast development (Javed & Lteif, 2013). Tissue plasticity can be linked to the activity of ER and PR in progenitor cell lineages, whilst mammary stem cells do not express many of the hormone receptors (Shackleton *et al.*, 2006; Asselin-Labat *et al.*, 2010; Joshi *et al.*, 2010). The breast tissue develops by complex paracrine signalling between progenitors and stem cells, exemplified by the ductal elongation and branching elicited by oestrogen and progesterone respectively (Briskin *et al.*, 1998). Interestingly BCL2, an inhibitor of apoptosis, and HER2 have also been implicated in the development of ductal morphology and branching (Naccarato *et*

al., 2000; Jackson-Fisher *et al.*, 2004). Significantly, both proteins are among those used as diagnostic markers in Oncotype DX.

1.2.2 HER2

HER2 has activity as both a tyrosine kinase and transcriptional co-activator (Madson *et al.*, 2006). HER2 activity in receptor complexes is dependent on its homo- or heterodimerisation with the other HER family proteins: HER1, HER3 and HER4, notably the kinase in HER3 is inactive and HER2 has a native conformation as if permanently ligand-bound. As a result, HER2 acts to potentiate HER signalling, whilst HER3 can mitigate it (Rubin & Yarden 2001; Lazaro, 2015). The dimerisation state of the HER proteins directs downstream pathways that are activated by altering the intracellular kinase interface (Alroy & Yarden, 1997; Tzahar *et al.*, 1998). In the context of non-malignant cells HER2 has very low expression. As such, overexpression of HER2, and the increase in HER2 heterodimers cause hypersensitivity to growth factors and external stimuli thus significantly contributing to transformation. In this case HER2 can also dimerise with itself, causing downstream signalling in the absence of any ligand. To this end, ligand variability is also implicated in constitutive signalling, (Tzahar *et al.*, 1998). HER2 is an indicator of poor prognosis and has been successfully targeted for treatment by immunological therapy with the antibody trastuzumab (Herceptin) (Rubin & Yarden 2001; Hudis, 2007).

Commonly targeted proteins by HER2/HERX dimers are PIK3R, SHC1, SYK, ABL1/2 and RASA1; collectively associated with PIP₂/IP₃, non-receptor tyrosine kinase and *mitogen associated protein kinase* (MAPK) signalling (Jones *et al.*, 2006). Upon ligand binding of the receptor domain, HER proteins autophosphorylate in trans. The phosphorylated residue defines which of a variety of downstream effectors are targeted. (Thelemann *et al.*, 2005; Fuller, Sivarajah & Sugden, 2008; Kozloski, Carothers-Carraway & Carraway, 2010). A key downstream target of signalling through HER2 is the PI3K complex with

subsequent signalling via the AKT signalling axis (Jones *et al.*, 2006). Another important action of HER2 is its ability to work in transcriptional transactivation, implicated in the *CDKN1A* locus which is commonly dysregulated in cancer (Yu *et al.*, 1998; Giri *et al.*, 2005; Thelemann *et al.*, 2005; Madson *et al.*, 2006; Ansari, Rundhaug & Fischer, 2008; Wang & Dubois, 2010; Li *et al.*, 2011).

1.2.3 Genetic Risk Factors

Aside from the protein expression studied for prognostic and diagnostic purposes, there are many genetic risk factors that predispose carriers to breast cancer. *Breast cancer type 1* and *type 2 susceptibility proteins* (BRCA1 and BRCA2), are the most commonly mutated proteins in hereditary breast cancers (Rebbeck *et al.*, 2018). BRCA1 linked cancers, specifically in women, are typically associated with basal A biology, are diagnosed as high grade, commonly have lymphocytic infiltration and are more aggressively metastatic (van 't Veer *et al.*, 2002; Kao *et al.*, 2009).

Both BRCA1 and BRCA2 are heavily involved in homologous recombination, a DNA repair pathway that responds to damage by ultraviolet light, ionising radiation and various chemical agents which cause double strand breaks. In brief, homologous DNA is melted and recombined with the damaged molecule. The homologous DNA is then used as a template to bypass the damaged DNA through the formation of a Holliday junction and subsequent excision of damaged DNA (Li & Heyer, 2008). Homologous recombination is reliant on the correct function of many proteins including BRCA1 and BRCA2 amongst others. Mutant forms of these proteins lead to reduced fidelity in DNA repair, resulting in an increased risk of mutant clone expansion (Silvestri *et al.*, 2016; Rebbeck *et al.*, 2018). The transformation process is accelerated by this mechanism. As such, many BRCA1 hereditary cancers have an early age of onset (Somasundaram *et al.*, 1997).

BRCA1 is phosphorylated by *ataxia telangiectasia mutated* and *ataxia telangiectasia and Rad3 related* proteins (ATM and ATR) in response to DNA damage. Phosphorylated BRCA1 interacts with *cellular tumour antigen p53* (TP53) leading to p21^{Cip1} activity and the subsequent attenuation of cell cycle progression through G₂ (Somasundaram *et al.*, 1997). BRCA1/2 involvement in both DNA repair and cell cycle progression earmarks them as gatekeepers of genomic stability, disruption of which increases the rate at which malignancies develop (Taneja *et al.*, 2010)

1.3 Cell Cycle and Replicative Immortality

Amongst the hallmarks of cancer, replicative immortality is necessary for the development of both malignant and non-malignant tumours. Akin to all other tumourigenic processes, replicative immortality is necessary for correct development and tissue renewal throughout the life of multicellular organisms. In normal circumstances, cell cycle advancement through S-phase and mitosis is prevented if any of a multitude of signalling circuits are not behaving correctly. In the context of tumours, the cell cycle and its failsafe checkpoints are hijacked leading to replicative immortality. This combined with decreased fidelity of DNA repair/response is sufficient to begin the process of tumourigenesis (Taneja *et al.*, 2010, Lim & Kaldis, 2013). In terms of metastasis, replicative immortality is necessary for the formation of secondary tumours, whether seeded by a single stem-like cell or group of cells, containing a sub-population capable of sustained proliferation. The molecular mechanisms behind the control of the cell cycle, and how they are dysregulated in malignant populations, are well understood with regards to *cyclin* and *cyclin-dependent kinase* (CDK) activities. These are the key effectors for passage through each cell cycle stage (Vermeulen *et al.*, 2003).

In normal, fully developed tissues, most cells are in gap 0 (G₀) phase of interphase and are not able to progress through the cell cycle. The end of gap 1 (G₁) phase of interphase is the committing step for entry into the cell cycle and

is also known as the 'restriction point'. In normal tissues, very few cells can employ the genetic and epigenetic programmes to overcome the restriction point. Any cells that can enter G₁ must be either totipotent, pluripotent or multipotent, that is, a stem cell or transiting amplifying cell rather than a senescent, fully differentiated cell (Lim & Kaldis, 2013).

To advance from G₁ to synthesis (S) phase, a cell must relieve repression of E2F family transcription factors. In interphase cells not committed to the cell cycle, E2F transcription factors are bound by *retinoblastoma protein* (RB1) which represses E2F-family activity. This family of transcription factors initiates S phase by upregulating transcription of cyclin E. Cyclin E expression creates a strong positive feedback loop when in complex with CDK2 (Hydbring, Malumbres. & Sicinski, 2016; Figure 1.2). This creates an irreversible commitment to the cell cycle, engaging the transcriptional circuitry necessary for genome replication, leading into gap 2 (G₂) phase. Genome replication is an energetically demanding process; explaining the stringent epigenetic control at the restriction point.

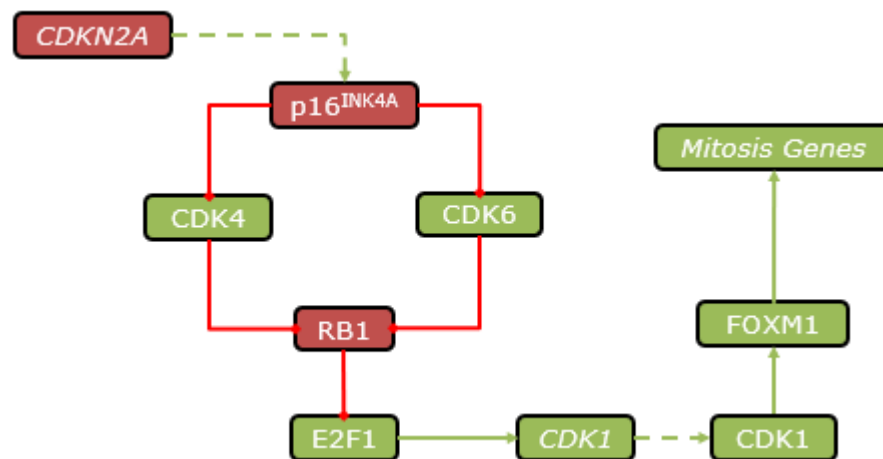


Figure 1.2 – Regulation of the restriction point by RB1 phosphorylation. Transcription of the CDKN2A locus leads to synthesis of p16^{INK4A} which inhibits CDK4 and CDK6. RB1 binding of E2F1 inhibits E2F1 transcription of CDK1 leading to a cascade of transcriptional events leading to advancement in the cell cycle. RB1 inhibition of E2F1 is alleviated by hyperphosphorylation by CDK4 and CDK6. Red lines represent inhibitory action, green solid arrows represent direct induction of activity, dotted green arrows represent transcription and italicised labels indicate transcription or genes respectively. Red proteins/genes are inhibitors of cell cycle advance, green proteins/genes are activators.

G₂ phase has a key DNA damage checkpoint that prevents cells with damaged DNA from advancing into mitosis until the damage is repaired. ATM and ATR proteins are vital for DNA damage to be detected correctly, and as such are commonly dysregulated or mutated in cancer (Li & Martin, 2016). Similarly, downstream of ATM and ATR is tumour suppressor TP53 which is the most commonly mutated gene in cancer, with the nickname: '*guardian of the genome*'. A recent study by Nik-Zainal *et al* (2016) found that amongst 560 breast cancer tumours, TP53 was a driver mutation in ~20% of ER⁺ and 80% of ER⁻ tumours. Once a cell has passed the DNA damage checkpoint mitosis can begin.

1.3.1 Mitosis

The intracellular signalling axis responsible for the mitotic response to successful DNA replication and G₂ operate through AKT1, CLOCK, AURKB and finally PLK1. The orchestration of this signalling cascade is intricate and requires precise chronological phosphorylation and acetylation events for mitosis to occur correctly (Vong *et al.*, 2005; Colnaghi & Wheatley, 2010; Akle *et al.*, 2017; Luciano *et al.*, 2018).

Mitosis can be divided into five key morphological checkpoints: prophase, prometaphase, metaphase, anaphase, and telophase; summarised in Figure 1.3. Prophase sees the beginning of DNA condensation into chromosomes, and disintegration of the nuclear envelope (Sivakumar & Gorbsky, 2015). At the beginning of metaphase, termed prometaphase, microtubules generate a spindle that connects to the kinetochores of each chromosome, which are enriched at the centromeres with the chromosomal passenger complex (borealin, INCENP and survivin), and the chromosomes fully congress during metaphase (Carmena *et al.*, 2012; Landino, *et al.*, 2017). At anaphase, one set of 46 chromosomes translocate to one spindle pole, whilst the second set of chromosomes translocate to the opposite pole. Leading to two sets of aligned chromosomes at

each pole of the cell (Sivakumar & Gorbsky, 2015). The final phase of mitosis is telophase, when chromosomes decondense and are re-encapsulated to form two daughter nuclei. As this phase reaches its end, cytokinesis, the process of dividing the cytoplasm, cell membrane and other organelles into two daughter cells occurs (Pohl, C. & Jentsch, S. 2008). This process is achieved by an actomyosin contractile ring (Sivakumar & Gorbsky, 2015).

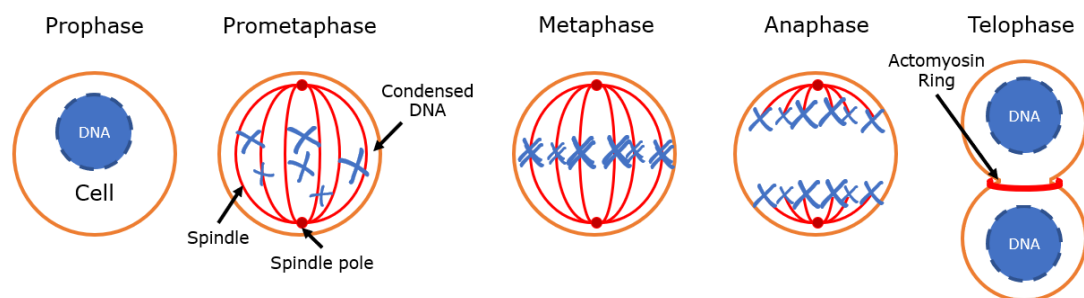


Figure 1.3 – Morphological mitosis checkpoints. **Prophase:** the nuclear envelope begins to break down, and DNA begins to condense. **Prometaphase:** the spindle poles fully develop, and chromosomes (fully condensed DNA) begins to align. **Metaphase:** chromosomes align on the metaphase plate and become associated with specific spindles. **Anaphase:** Chromosomes are translocated along the spindles to each spindle pole. **Telophase:** DNA begins to de-condense and be enveloped by new nuclear envelopes, an actomyosin ring begins to divide the cells, this process terminates in cytokinesis.

Traditionally, the inhibition of mitosis has been a proposed method for chemotherapeutics. Many treatments have been developed to prevent chromosome congression by targeting the microtubule cytoskeleton. The formation of a bipolar spindle requires the kinesin-like motor protein EG5. Inhibition of this protein leads to monopolar spindle formation and stalling of mitosis at pro-metaphase (Colnaghi & Wheatley, 2010). The EG5 inhibitor, dimethylenastron, was initially developed as a chemotherapeutic agent but is more well known for its use in stalling cells in mitosis for biological and biochemical study. One of the oldest treatments for breast cancer is a drug that makes microtubules hyper-stable: paclitaxel®, or taxol, stabilises spindles to the point where they cannot be depolymerised and therefore cannot be detached from the kinetochores of mitotic chromosomes (Kreger *et al.*, 2016).

An interesting family of proteins involved in mitosis are the *baculoviral inhibitor of apoptosis (IAP) repeat (BIR)* family. These multifunctional proteins are needed for correct execution of mitosis and to prevent apoptosis. All but 2 members of the BIR family, NAIP and survivin, are E3 ligases which self-regulate by autoubiquitination (Varfolomeev *et al.*, 2007). The transcriptional regulation of BIR family proteins has been linked to multiple pathways including the NF- κ B signalling system and DNA damage response pathways (Stehlik *et al.*, 1998a; You *et al.*, 1997; Stehlik *et al.*, 1998b; Gu *et al.*, 2009; Nestal de Moraes *et al.*, 2015; Saldivar *et al.*, 2018). BIR-family protein function, binding partners and their regulation are summarised in Table 1.4.

cIAP1 and survivin are essential for correct mitotic execution with knockout models of either being lethal *in utero* of murine models. Due to the similar structures of cIAP1, cIAP2 and XIAP there is some functional redundancy in this family (Mahoney *et al.*, 2008; Vasilikos *et al.*, 2017). BRUCE (*BIRC6*) has been shown to be necessary for mid-body breakdown after abscission (Pohl & Jentsch, 2008).

Transcriptional regulation of the BIR family of proteins varies with data suggesting cIAP1, cIAP2 and XIAP are more widely expressed at all cell-cycle stages and mainly self-regulated (Varfolomeev *et al.*, 2007). Whereas survivin is tightly regulated so that temporal expression coincides precisely with signals eliciting the transition from G₂ to mitosis (Nestal de Moraes *et al.*, 2015; Saldivar *et al.*, 2018). Tight regulation of survivin is likely why dysregulated expression leads to so many different cellular changes in interphase malignant cells. Tightly regulated proteins, by nature, have transient interactions which are dependent on their temporal expression. In the case of survivin, the interactome should be that of a G₂ or mitotic cell. Persistent expression of survivin means it can interact with proteins not normally co-expressed, potentially leading to mitochondrial

localisation, interactions with SRC and liaisons with autophagic machinery (Wang *et al.*, 2011; Wang *et al.*, 2014; Dunajová *et al.*, 2016).

Table 1.4 – Summary of BIR family protein targets and inhibitors. Adapted from uniprot entries: Q13075, Q13490, Q13489, P98170, O15392, Q9NR09, Q96CA5, Q96P09.

Protein	Gene	E3-Ligase	Binding Partners	Inhibitors
NAIP	<i>BIRC1</i>	No	CASP3, 7 & 9 APAF1	
cIAP1	<i>BIRC2</i>	Yes	RIPK1, 2, 3 & 4 CASP3, 7 & 8 MAP3K5 & 14 TRAF2, NEMO and MXD1	Itself, DIABLO
cIAP2	<i>BIRC3</i>	Yes	RIPK1, 2, 3 & 4 CASP3, 7 & 8 MAP3K14 TRAF1, NEMO and BCL10	Itself, DIABLO
XIAP	<i>BIRC4</i>	Yes	RIPK1 CASP3, 7, 8 & 9 MAP3K2 AIFM1, CCS and survivin	Itself, DIABLO
Survivin	<i>BIRC5</i>	No	CASP3, 7 & 9	DIABLO
BRUCE	<i>BIRC6</i>	Yes	CASP3, 7 & 9 DIABLO	Itself
Livin	<i>BIRC7</i>	Yes	CASP3, 7 & 9 DIABLO and MAPK8 & 9	Itself
ILP2	<i>BIRC8</i>	Yes	BAX	

1.3.2 Senescence

Every time the genome is duplicated the telomeres at the terminus of each chromatid decrease in length slightly. Successive mitotic events lead to further telomere shortening and reaching of the so-called Hayflick limit, the ultimate number of divisions a cell can perform before being unable to divide again, termed “senescence” (Dou *et al.*, 2015). In stem cell populations senescence is circumvented by the activity of telomerase, a ribozyme which extends telomeres with GC rich DNA (Zhao *et al.*, 2009).

1.4 Evasion of Cell Death

Another hallmark of cancer is the evasion of cell death which enables malignancies to survive a host of biochemical stresses and chemotherapeutic assaults (Hanahan & Weinberg, 2011). Cell death can be induced by several ligands as well as from increased intracellular stress and can occur in a controlled or uncontrolled manner, the latter being referred to as “necrosis”. Controlled cell death was formerly referred to as “apoptosis” but more recently, several other controlled forms of cell death have been described: necroptosis

(elicited through the RIPK family), ferroptosis and pyroptosis, However, these processes are extremely context specific (Pasparakis & Vandenabeele, 2015).

1.4.1 Apoptosis

Apoptosis is a controlled process by which a cell kills itself. It can be triggered by numerous stimuli and can be executed in a variety of manners. Uncontrolled cell death, necrosis, is associated with an increased local inflammatory response (Pasparakis & Vandenabeele, 2015). The best studied cell death processes are extrinsic and intrinsic apoptosis, which differ in the source of pro-death signalling. Extrinsic apoptosis is elicited by signalling factors outside of the cell whereas intrinsic apoptosis is triggered by internal stress such as extensive DNA damage and reactive oxygen species (ROS; Pasparakis & Vandenabeele, 2015).

1.4.1.1 Extrinsic apoptosis

Extrinsic apoptosis is induced by a transmembrane receptor complex, part of which, *death inducing signalling complex* (DISC), is on the intracellular side. Binding of a pro-apoptotic ligand, such as *tumour necrosis factor ligand superfamily member 6* (FASLG), to its receptor, *tumour necrosis factor receptor superfamily member 6* (FAS), induces a conformational change in *FAS-associated death domain protein* (FADD), which then recruits caspase 8 which activates by autocatalysis. Importantly, free cytoplasmic domains of β -integrins can recruit caspase 8, independent of FADD (Scaffidi *et al.*, 1999; Simpson Anyiwe & Schimmer, 2008; Figure 1.4).

Caspase 8 cleaves the executioner caspases 3 and 7, activating them resulting in the process of cells breaking themselves down, by targeting proteins with an "Asp-Xaa-Yaa-Asp" consensus, where Xaa is hydrophobic, and Yaa is hydrophilic (Simpson Anyiwe & Schimmer, 2008). Important caspase targets include BID and PARP. When cleaved, BID increases mitochondrial outer

membrane permeabilisation (MOMP). PARP is important in DNA repair, apoptotic cells halt DNA repair by PARP cleavage, perpetuating apoptotic signalling (Igney & Krammer, 2002; Yuan *et al.* 2010).

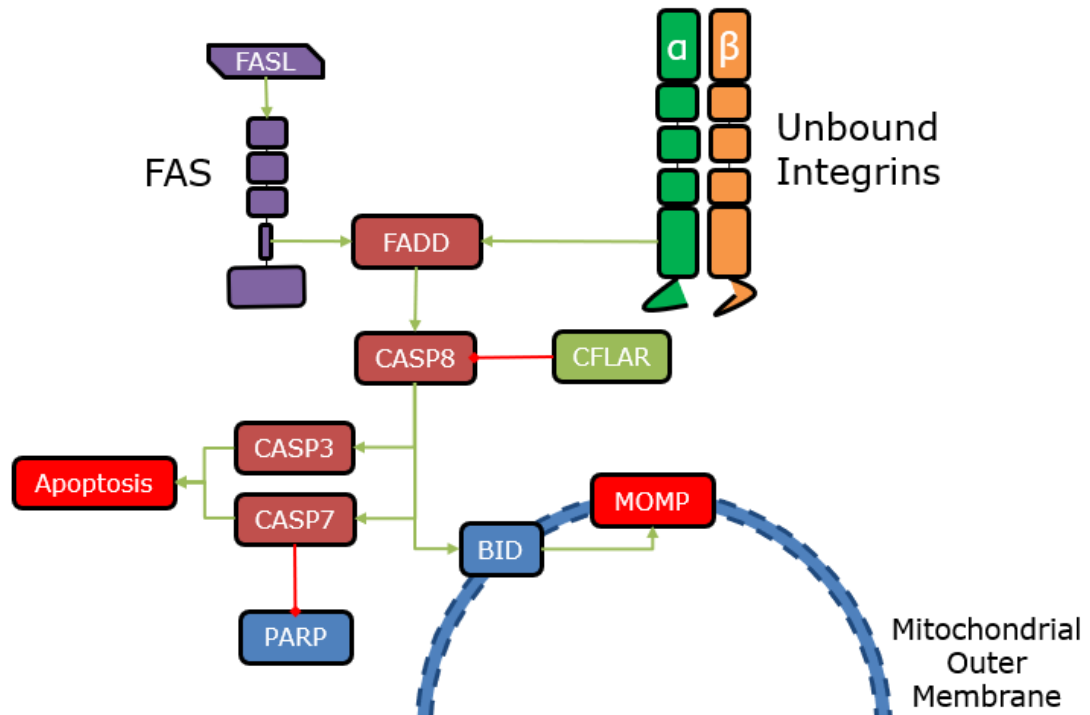


Figure 1.4 – Extrinsic apoptotic signalling cascade. Binding of death inducing ligands, or unbound integrins lead to FADD induction of caspase 8 (CASP8) autocatalysis. Active CASP8 is competitively inhibited by CFLAR. CASP8 cleaves caspase 3 and 7 (CASP3 and 7), and BID leading to apoptosis, PARP degradation and mitochondrial outer membrane permeabilisation (MOMP) respectively.

1.4.1.2 Intrinsic apoptosis

The net result of intrinsic apoptosis is the same as extrinsic apoptosis, but it is activated differently. A key step for intrinsic apoptosis is the release of cytochrome c from the inter-membrane space of the mitochondria. Cytochrome c is released by increased outer mitochondrial membrane permeability which can be caused by multiple processes. Cytochrome c release is necessary for intrinsic apoptosis, as it binds with APAF1 and caspase 9 to form the apoptosome, a protease complex which cleaves other executioner caspases, triggering apoptosis (see Figure 1.5; Owens *et al.*, 2010).

The best studied of these processes is through the t-BID, BAX, BAK pore complex. This complex is formed by a cascade of upstream events starting with

those that detect cellular stresses (Owens *et al.*, 2010). For example, extensive DNA damage leads to the recruitment of TP53 by ATM and ATR through CHK1. Activated TP53 leads to large modifications in gene expression, in general promoting expression of pro-apoptotic factors (Saldivar *et al.*, 2018). Active TP53, which has been exported from the nucleus, is commonly bound by BCL-X(L) in malignant cells, halting pro-apoptotic signalling. In normal cells, BCL-X(L) is not expressed sufficiently for TP53 to be fully sequestered. Alternatively, non-malignant cells can relieve BCL-X(L) inhibition of TP53 by competitive binding of PUMA, which relieves inhibition of TP53 allowing it to activate NOXA (Grossmann, 2002). NOXA activity has a positive feedback mechanism which perpetuates pro-apoptotic signalling, whereby it binds and inhibits anti-apoptotic BCL2-family proteins. This leads to increased TP53-NOXA interactions and releases BID, BAX and BAK from BCL2-family mediated inhibition; thus, allowing formation of outer mitochondrial membrane permeabilisation complexes (Mehlen & Puisieux, 2006). Furthermore, the inhibition of BCL-X(L) and other BCL2-family proteins leads to opening of the porin, VDAC, which confers cytochrome c release into the cytosol (Shoshan-Barmatz, Krelín & Shteinfein-Kuzmine, 2018).

Apoptotic cells are easily distinguished from healthy neighbours as their DNA is hyper-condensed, their plasma membrane blebs, and phosphatidylserine and phosphatidylethanolamine are flipped into the outer cell membrane. The membrane of a bleb eventually has increased curvature such that it buds off, with a fate of being engulfed and destroyed by macrophages (Emoto *et al.*, 1997; Vance & Tasseva, 2013).

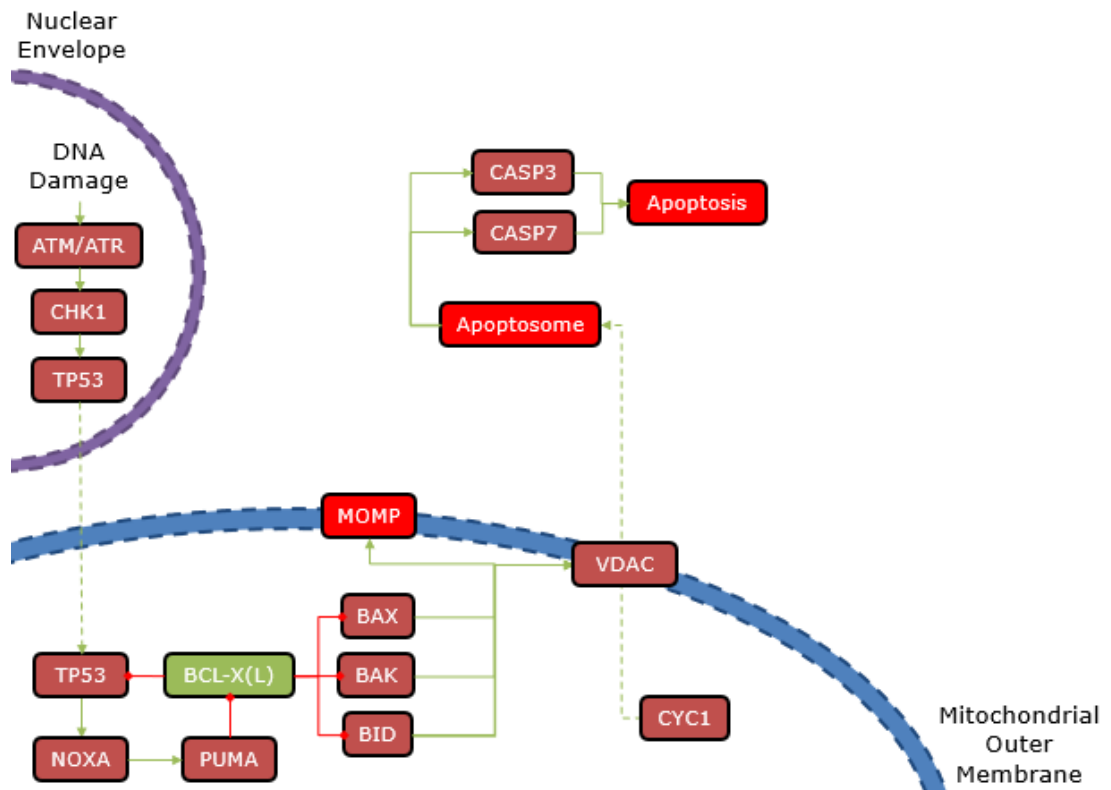


Figure 1.5 – Intrinsic apoptotic signalling cascade. DNA damage as an intrinsic apoptosis activator is detected by ATM/ATR which signal through CHK1 to confer TP53 translocation to the mitochondria. TP53 can be bound and inhibited by BCL2 family proteins such as BCL-X(L), but a critical mass leads to NOXA binding and subsequent PUMA inhibition of BCL2 family proteins. BCL2-family proteins normally prevent MOMP through BAX, BAK and BID, so normal cells with extensive DNA damage, eventually undergo MOMP allowing cytochrome c (CYC1) release and formation of the apoptosome. This complex activates CASP3 and CASP7 leading to apoptosis.

Cell viability involves a delicate balance between pro- and anti-apoptotic factors. The stoichiometry of factors such as BID, BAX, PUMA and NOXA versus BCL2, BCL-X(L), MCL1 and BIR-family proteins is tilted depending on the strength of signalling circuits affecting each family. For example, robust signalling through TP53 and TNF α tips the balance in favour of apoptosis; whereas constitutively active signalling through MAPKs will affect transcription in favour of anti-apoptotic factors (Mehlen & Puisieux, 2006; Simpson, Anyiwe & Schimmer, 2008; Owens *et al.*, 2010; Laetsch *et al.*, 2014).

1.4.2 BIR-Family Proteins as Inhibitors of Apoptosis

The apoptotic cascade can be prevented at multiple steps, many of which involve the BIR protein family. Many IAPs are tightly associated with mitosis

(section 1.3.2), but all members contain at least one BIR domain which bind and inhibit caspases (Chai *et al.*, 2001).

The most potent IAPs in the family are cIAP1, cIAP2 and XIAP, the latter of which is the most well studied of the BIR-family and is only known to act at the executioner caspase level (Suzuki, Nakabayashi & Takahashi, 2001). It is inhibited by DIABLO binding, which in turn is prevented by the binding of survivin to DIABLO (Pavlyukov *et al.*, 2011). Similarly, survivin binding to the BIR-domains of XIAP can prevent auto-degradation of XIAP (Nakamura *et al.*, 2010). Stabilisation of XIAP is a key factor in preventing apoptosis and occurs in a targeted fashion during development and in cancer (Liu *et al.*, 2007; Kang *et al.*, 2010). The stabilisation of XIAP by post-translational modifications or protein-protein interactions, can be attenuated by binding of XAF1. This promotes degradation of survivin and autoubiquitination of XIAP, making XAF1 a potent tumour suppressor (Arora *et al.*, 2007; Nakamura *et al.*, 2010).

cIAP1 and cIAP2 have been shown to have functional redundancy with XIAP in the context of mitosis. Both cIAP1 and cIAP2 are active further up the apoptotic cascade than XIAP and all are associated with inflammation and signalling through NF- κ B, TNF α and MAPKs (Varfolomeev *et al.*, 2007; Mahoney *et al.*, 2008; Takeda *et al.*, 2014). cIAP1/2 bind strongly with RIPK proteins, targeting them for proteasomal degradation. RIPK proteins are active at the cell surface at the point of pro-apoptotic ligand binding and activity of different combinations of the RIPK family can lead to alternative cell death processes such as necroptosis (Pasparakis & Vandenabeele, 2015).

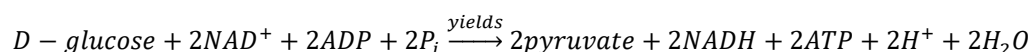
1.5 Metabolism and Dysregulated Cellular Energetics

Metabolic changes in malignancies are a hallmark of tumour progression with the first instance described by Warburg (1950) and named the “Warburg effect”. However, it is only recently that the true level of change it confers on the malignant phenotype has been appreciated (Hannahan & Weinberg 2011).

Tumourigenic cells are selected by multiple environmental stresses, a crucial one being hypoxia. Hypoxic environments exert selective pressures on cells that can survive in oxygen deprived regions of tumours, driving them to change their metabolic state. Therefore, malignant cells respiring anaerobically are selected for, as they are the only ones that can survive hypoxic conditions. These subpopulations are often the most metastatically aggressive and hardy, leading some to suggest hypoxia can raise the risk of metastatic disease (Devignes, Aslan & Provot, 2018; DiGiacomo & Gilkes, 2018; Klaus *et al.*, 2018).

1.5.1 Glycolysis

Glycolysis is the process of converting monosaccharides such as glucose and fructose, into pyruvate and ATP. This process requires ten enzymatic conversion steps, the net reaction is summarised below:



The most important point is that no oxygen or carbon dioxide is used or generated in this process. The terms “aerobic and anaerobic glycolysis” refer to the destination of the pyruvate, to avoid confusion the terms “aerobic and anaerobic respiration” will be used. Any pyruvate generated can be trafficked through the mitochondrial membranes or remain in the cytosol. Mitochondrial pyruvate is converted into acetyl-CoA by pyruvate dehydrogenase in the mitochondrial matrix. Acetyl-CoA is the fuel for the tri-carboxylic acid (TCA) cycle, the flux of which generates many products useful to the cell such as:

NADH, GTP and reduced quinone. Flux of the TCA cycle also generates carbon dioxide.

Cytosolic pyruvate can be converted into lactate by anaerobic respiration, a reaction catalysed by lactate dehydrogenase that only occurs in the presence of NADH. Lactate is responsible for increased acidification of hypoxic tissue as its export requires a proton as a cofactor (Chiche, Brahimi-Horn, & Pouysségur, 2010). It has recently been shown that the knockdown of both lactate dehydrogenase A and lactate dehydrogenase B is required to revert the Warburg effect phenotype to one of oxidative metabolism (Peter *et al.*, 2018).

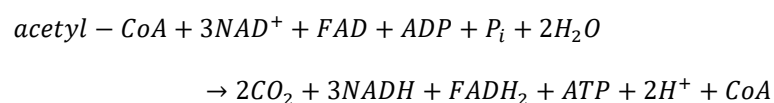
Despite anaerobic respiration leading to a net production of ATP 16 times smaller than aerobic respiration, most malignancies select for anaerobically respiring cells (Hanahan & Weinberg, 2011). This is due, in part, to the integration of glycolysis with the pentose phosphate pathway (PPP). Current research is focused on changing the destination of pyruvate, but the molecular mechanisms controlling this are complex (Pivovarova & MacGregor, 2018; Ždravlević, 2018).

1.5.2 Pentose Phosphate Pathway

The PPP is a catabolic process by which cells can convert glucose and fructose into ribose, a necessary component of DNA and RNA. Any cell lacking in ribose production will not be able to efficiently replicate its genome and as such will be out competed by faster replicating malignant sub-clones. The PPP is implicated in many different forms of malignant disease. and has been particularly tightly associated with the starved state of cells and AMPK activity (Fogarty & Hardie, 2010). As a result, targeting of the PPP could potentially be used to target cells that are able to survive prolonged nutrient deprivation, typically the hardest of malignant sub-clones, that increase the risk of metastatic disease (Guo *et al.*, 2018).

1.5.3 Tri-Carboxylic Acid Cycle

The TCA cycle was originally described as a circular series of enzymatically propagated chemical reactions, however the more that is learnt about metabolism the more it is apparent that each metabolite within the TCA cycle is also involved in many other linear and cyclical metabolic processes. This can be exploited by malignancies, such as survival with only glutamine as a carbon source (Desideri, Vegliante & Ciriolo, 2015). The net reaction of the TCA cycle is summarised below:



The NADH generated in the mitochondrial matrix has one common, energetically favourable fate, reduction into NAD^+ by NADH dehydrogenase, or complex I, of the oxidative phosphorylation cascade (OxPhos).

1.5.4 Oxidative Phosphorylation

OxPhos requires at least 4 of 5 transmembrane complexes which bridge the inner mitochondrial membrane; termed complexes I-V. NADH generated in the matrix is reduced by complex I leading to the passage of electrons and sequential reduction/oxidation of ubiquinone, complex III, cytochrome c, and complex IV; with oxygen being the final acceptor of electrons. reduction of quinone. Importantly, the conformational changes of complex I through complex IV, associated with the passage of electrons from NADH to quinone, confers the passage of protons from the mitochondrial matrix into the inter-membrane space (IMS; Figure 1.6). Active transport of protons into the IMS generates a large charge gradient across the inner mitochondrial membrane, termed "membrane potential" (Sazanov, 2015). A high proton concentration gradient leads to rapid diffusion of protons back into the mitochondrial matrix, via ATP synthase, complex V, leading to the generation of ATP from ADP.

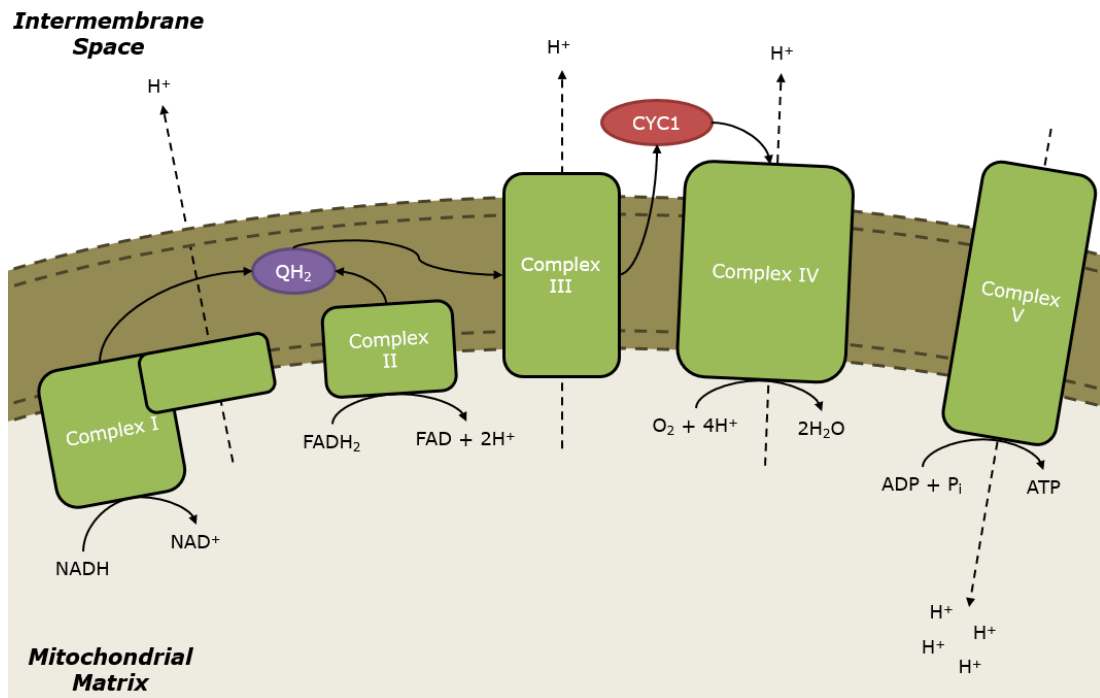


Figure 1.6 – Electron transport chain. The oxidation of NADH to NAD^+ or FADH_2 to FAD by complex I and complex II respectively confers the reduction of quinone (Q) to QH_2 . This cofactor is then oxidised back to its Q-form whilst reducing complex III which subsequently reduces CYC1 in the intermembrane space (IMS). Reduced CYC1 oxidises whilst reducing complex IV which finally reduces oxygen to water in the presence of protons in the mitochondrial matrix. Each time a complex is reduced, conformational changes induce proton transport into the IMS. Protons then diffuse through complex V down the concentration gradient, into the mitochondrial matrix. Diffusion of 4 protons through complex V provides the energy necessary for phosphorylation of ADP to form ATP.

Highly efficient OxPhos leads to a large membrane potential and commonly this is absent in malignancies. Inefficient OxPhos does not only change the membrane potential of the inner mitochondrial membrane, it is also associated with increased electron leakage and formation of reactive oxygen species, which can in turn lead to increased mutation in mitochondrial DNA and generally more 'unhealthy' mitochondria (Rivadeneira *et al.*, 2015).

1.6 Metastasis

Typically, the final stage of malignant tumourigenesis is the ability of cells in a primary tumour to disseminate to a secondary site, a process termed "metastasis". Successful metastatic tumours require significant changes in cell polarity and adhesive properties, they must be able to migrate or delaminate to intravasate, evade the immune system, evade anoikis and survive in the

circulatory system, then finally re-adhere, extravasate and colonise; summarised in Figure 1.7.

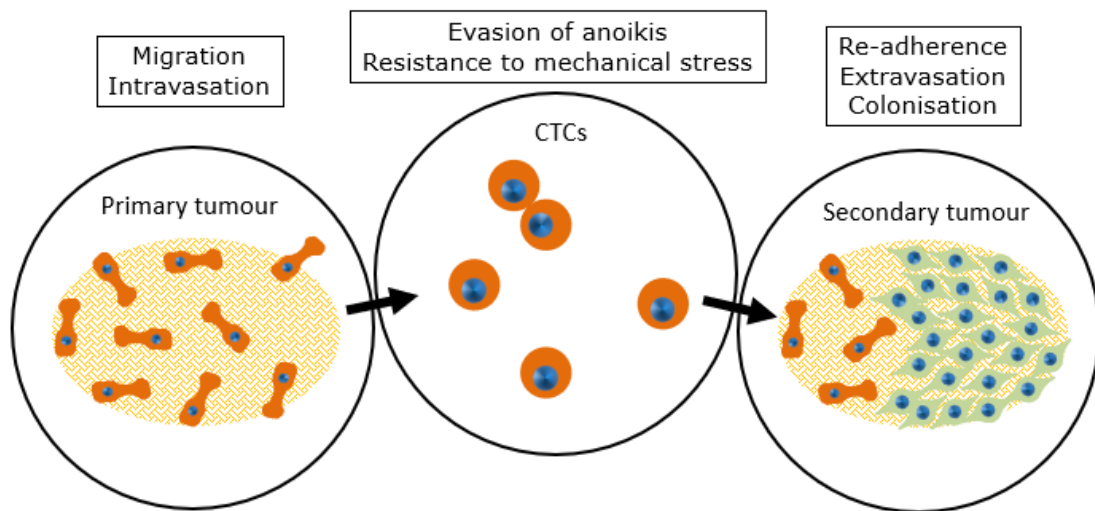


Figure 1.7 – The metastatic process. Cells can detach and intravasate by migration or delamination from the primary tumour, becoming circulating tumour cells (CTCs), before re-adhering and extravasating into a secondary site.

1.6.1 Cell Polarity and Adhesion

The polarity and associated adhesive properties of cells must be adaptable for a cell to metastasise. Cell polarity refers to the asymmetrical localisation of a plethora of biological molecules which define intercellular junction loci and matrix adhesion loci (Iden & Collard, 2008). Epithelial cells are highly polarised. They are attached via several junction types at distinct subcellular positions such as: tight junction, adherens junction, desmosomes, and hemi-desmosomes thus, are good models for the biochemical study of cell polarity (Etienne-Manneville & Hall, 2002; Suzuki & Ohno, 2006).

Cell junction formation involves a complex network of proteins including members of the *partitioning defective homolog* (PARD) family, *protein kinase C* (PKC) subtypes and small GTPases such as *cell division control protein 42 homolog* (CDC42), *Ras related C3 botulinum toxin substrate* (RAC) and *transforming protein p* (RHO) (Iden & Collard, 2008). The tight junction and adherens junction are stacked in epithelia, creating two concentric rings that

mutually exclude basolateral domain related proteins and are vital in maintaining apicobasal polarity.

The actin cytoskeleton is necessary for the maintenance and stability of the ring of adherens junctions. When active, GTPases regulate actin cytoskeletal dynamics, the cortical filaments of which are important in maintaining tissue and junctional structure (Iden & Collard, 2008). The small GTPase RHO regulates actomyosin contractility and RHO dysregulation is a common cause for dissolution of cell polarity and a migratory phenotype (Etienne-Manneville & Hall, 2002). Similarly, RAC is typically associated with dynamic cortical actin-based structures such as filopodia, invadopodia, podosomes and membrane ruffling; cellular structures which are highly plastic in aggressive malignancies (Webb, Eves & Mak, 2006; Figure 1.8).

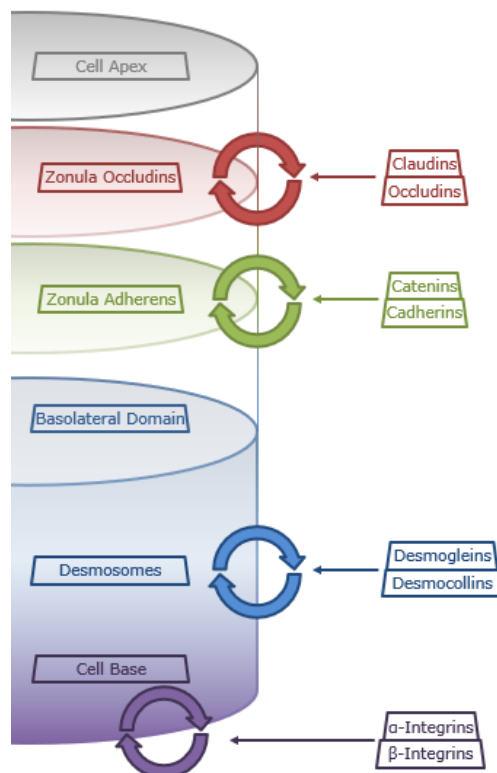


Figure 1.8 – Epithelial cell polarity schematic. Just below the cell apex is the tight junction which acts to occlude all but the smallest of molecules passing between cells in the epithelium. Basal to the tight junction is the adherens junction. In the basolateral domain desmosomes are found. The base of the cell is connected to the basal lamina by hemidesmosomes. All adhesion complexes have high levels of protein turnover and regulation [Adapted from: Angst, Marozzi & Magee, 2001; Etienne-Manneville & Hall, 2002; Assémat, *et al.*, 2008; Frisch, Schaller & Cieply, 2013].

The adherens junction is composed of cadherins and catenins, the former of which can homo- or heterodimerise intercellularly allowing juxtacrine interactions and signalling (Angst, Marcozzi & Magee, 2001). Desmogleins and desmocollins, proteins in the cadherin superfamily, are the main constituents of desmosomes, a basolateral intercellular protein complex also capable of juxtacrine signalling (Angst, Marcozzi & Magee, 2001; Assémat, *et al.*, 2008; Hutz *et al.*, 2017).

Focal adhesions, or hemidesmosomes, share the cytoskeleton-dependent characteristic of the adherens junction. In focal adhesions, the cadherins are functionally replaced by integrins, whilst catenins are functionally replaced by focal adhesion kinase (FAK), paxillin, vinculin and α -actinin. Signalling via these loci is not elicited via catenins but rather by non-receptor protein kinases recruited to the constructs, such as *proto-oncogene protein tyrosine kinase Src* (SRC) and downstream phosphorylation targets such as the MAPKs (Guo & Giancotti, 2004).

Transformed cells lack adherens junctions and tight junctions, with many cancers exhibiting severely depleted *CDH1* expression (encoding E-cadherin), alongside depletion of claudins specifically in the Basal B sub-type of breast cancer previously mentioned in section 1.2 (Neve *et al.*, 2009). Hemidesmosomes are significantly more plastic in their signalling properties as the alpha and beta subunits of the integrins together with the isoforms of many extracellular matrix (ECM) proteins allow differential intracellular signalling (Guo & Giancotti, 2004).

1.6.2 Migration, Invasion, and Intravasation

Focal adhesions are super-complexes comprising both core, and transient associated protein components. In tandem with proteinases and proteases, they are necessary for a cell to attach to the ECM, and for the motility required for metastasis (Guo & Giancotti, 2004). Through a combination of focal adhesion

turnover and matrix metalloproteinase (MMP) activity it is possible for mesenchymal, amoeboid, and collective cells to translocate across and through the ECM and between cells such as those in the vascular or lymphatic endothelium. Interestingly in the tumour microenvironment the aberrant angiogenic and lymphangiogenic signals cause 'leaky' vessels which can facilitate circulatory access.

The human genome encodes 21 MMP genes, thus the repertoire of MMPs available to a transformed cell is large. These may act in concert with another family of proteinases known as *a disintegrin and metalloproteinase with thrombospondin motifs* (ADAMTSs) of which there are 19 encoded in the human genome (Yamamoto, Murphy & Troeberg 2015). Together MMPs and ADAMTSs target many substrates, the catabolites of which can act as angiogenic and lymphangiogenic signals, reviewed by Cal & López-Otín (2015). Once they have remodelled the local ECM, malignant cells must survive a host of other stresses to metastasise successfully. These stresses are more apparent the closer migratory cells get to the vascular or lymphatic sub-systems of the circulatory system, and therefore the immune system.

1.6.3 Immune Evasion

During tumourigenesis and metastasis malignant cells must avoid assaults by many apoptotic stimuli from the immune system and local microenvironment. Host CD8⁺ and CD4⁺ T cells recognise peptides presented by major histocompatibility complex (MHC) class I and II respectively. MHC defects are common in malignant cells, preventing the presentation of antigens and thus evading identification by the immune system. Interestingly, the defects can be of two types, so-called "soft wired" or "hard wired". Soft wired defects are epigenetic and generally histone deacetylase (HDAC) associated, whereas hard wired defects are more substantial, including loss of heterozygosity, deletions, and other genetic damage (Igney & Krammer, 2002; Garrido, Cabrera &

Aptsiauri, 2010; van der Burg *et al.*, 2016). Abrogation of MHC expression is only one method of immune escape. Malignant cells may also be physically protected by surrounding stroma as well as being insensitive to the apoptotic cytokines associated with an innate immune response.

Apoptosis signalling by FASLG and *tumour necrosis factor* (TNF)-related *apoptosis-inducing ligand* (TRAIL) are well understood (Igney & Krammer, 2002). Both systems are associated with ligand binding of their specific receptors (FAS and TRAILR1/2 respectively) and subsequent downstream activity through FADD. The ligand and receptor components included here are at the top of the extrinsic apoptotic cascade (section 1.4.1.1). With respect to this ligand/receptor induction of cell death, early exposure of malignant cells to FAS and TRAIL may act as a selective pressure (Igney & Krammer, 2002). This may allow late stage malignancy to have the cellular components required to avoid apoptosis; whether from innate immune stimuli or due to detachment from the ECM, termed “anoikis”.

1.6.4 Evasion of Anoikis and Survival in Circulation

The ability for a cell to survive death due to detachment from the ECM and the mechanical stresses of transit from one locus to another is vital for successful metastasis. Metastasising cells breaking through the endothelium and becoming suspended in circulating blood or lymph is sufficient to induce anoikis.

1.6.4.1 Anoikis

Integrin signalling in adherent cells acts as a constitutive pro-survival stimulus that acts through *GTPase Ras* (RAS) – *GTPase Raf* (RAF) and the MAPK cascade. Unbound integrins do not elicit pro-survival stimuli and so the biochemical balance is tipped in favour of apoptosis (Simpson, Anyiwe, & Schimmer, 2008). In malignancies, this can be circumvented by disruption of downstream apoptotic effectors, such as DISC and caspase-8. *CASP8 and FADD-*

like apoptosis regulator (CFLAR) is a key inhibitor of this process as it allows the DISC to form partially but prevents the enzymatic cleavage of the caspase 8 zymogen (Condorelli *et al.*, 1999; Scaffidi *et al.*, 1999). Dysregulation of this process in favour of DISC inhibition confers resistance to anoikis. Equally, further downstream, disruption directly to executioner caspases by IAP-family proteins can have the same outcome (Simpson, Anyiwe, & Schimmer, 2008). The extracellular cleavage of cadherins with RGD (Arg-Gly-Asp) motifs in their extracellular domain can confer resistance to anoikis by binding of integrins and maintenance of pro-survival signalling (Bartolomé *et al.*, 2014; Bartolomé, *et al.*, 2015). Evasion of anoikis has been linked to activation of the PI3K γ -AKT signalling axis (Brazzatti *et al.*, 2012). Interestingly, when highly expressed, the *brain derived neurotrophic factor* (BDNF) receptor NTRK2 (also known as TRKB) significantly reduces anoikis, potentially acting upstream of the PI3K-AKT pathway (Kim, Lee & Jin, 2016). AKT activation leads to upregulation of many proteins, including the IAP survivin (Semba *et al.*, 2006). The role of specific IAPs in evasion of anoikis is yet to be determined.

1.6.4.2 Mechanotransduction and mechanical stress

Having evaded anoikis, one of the next survival challenges to metastasis cells is exposure to physical forces during transit, which can mechanically destroy cells. Increases in membrane tension due to mechanical stress allow Ca²⁺ to diffuse more readily into the cytoplasm, and this influx has been linked to increased activity of the metalloproteinase ADAM10 which cleaves extracellular domains of cadherins as a mechanism of relieving membrane tension (Ito *et al.*, 1999; Reiss *et al.*, 2005). However, in doing so cadherins may be released and this process may in fact contribute to metastasis through the binding of RGD-motif-containing cadherins to integrins (Bartolomé *et al.*, 2014; Bartolomé, *et al.*, 2015). Ca²⁺ influx can cause calmodulin and proteins containing calmodulin-like domains to elicit numerous intracellular changes

which can, in turn, tip the scales in favour of apoptosis, or survival (Ito *et al.*, 1999; Reiss *et al.*, 2005).

1.6.5 Re-adherence, Extravasation and Colonisation

The processes metastasising cells must evade to get to the point of successful re-adherence makes those that achieve it likely to form secondary tumours. By this point, malignant cells must have survived: DNA damage and/or chromatin dysregulation, hypoxia, the innate immune system, anoikis and mechano-destruction. Interestingly, the mechanical properties of blood itself give metastasising cells more time to re-adhere in distant capillaries. Blood, a shear-thinning non-Newtonian fluid, reduces in viscosity under increased shear stress. As blood flow decreases, the shear stress decreases adjacent to the endothelium, increasing viscosity and therefore increasing residency time of cells in vessels. This is vital for efficient gas exchange in the lungs but also allows metastasising cells longer to re-adhere and/or extravasate.

Of the many cells that intravasate from the primary locus, very few survive (Wang *et al.*, 2015). The major question, about re-adherence, extravasation and colonisation; is whether circulating cells only form secondary tumours in tissues which they are predisposed to survive by the described processes, or whether they can quickly remodel a secondary locus to suit them best. Given the specificity of cancers and tumourigenesis between different tissues of origin, it is likely to be a combination of the two.

1.7 The Extracellular Matrix (ECM)

There is an intimate relationship between the mechanical properties of the ECM and biochemistry of adherent cells. Most of the total anatomical ECM is made of collagens, but many of the biochemical properties derive from other ECM glycoproteins, proteoglycans and polysaccharides. The complexity of ECM ultrastructure is furthered by the incorporation of multiple isoforms of each protein component which is often overlooked. The mechanical and chemical properties of the ECM can influence proliferation, apoptosis, metabolism and migration; all facets related to metastasis and described in previous sections (Ricard-Blum, 2011; Mouw, Ou & Weaver, 2014).

1.7.1 Collagens

Collagens make up ~30% of human physiological protein mass, being key components in all solid organs and tissues (Ricard-Blum; 2011; Fratzl-Zelman *et al.*, 2016). The nomenclature of collagens can be generalised. For example, collagen I refers to a collagen I trimer made up of two collagen I $\alpha 1$ chains and one collagen I $\alpha 2$ chain; this is further complicated by the availability of different isoforms, making the level of ECM molecular variety very large in terms of only collagen I.

There are six families of collagen: fibrillar forming collagens, network forming collagens, fibril-associated collagen with interrupted helices (FACIT), membrane-associated collagens with interrupted triple helices (MACIT), and multiple triple helix domains with interruptions (multiplexins). The forms of collagen in each family are summarised in Table 1.5.

Table 1.5 – Families and forms of collagens. [Adapted from Ricard-Blum (2011)].

Family	Collagen Form
Fibrillar	I, II, III, V, XI, XXIV, XXVII
Network forming	IV, VIII, X
FACIT	IX, XII, XIV, XVI, XIX, XX, XXI, XXII
MACIT	XIII, XVII, XXIII, XXV
Multiplexin	XV, XVIII
'Other'	VI, VII, XXVI, XXVIII

1.7.1.1 Fibril Forming Collagens

Fibril forming collagens are most dense in ligaments, tendons, cartilage, and bone; but still comprise a substantial proportion of many other tissues. Collagen triple helical coiled coils, termed proto-fibrils, bundle by hydrogen bonding to form interdigitated fibrils; which are then tethered together by cross-linking, in large part by FACIT collagens such as collagen XII. Fibrils are further bundled to form striated tapes or fibres (Ushiki, 2002; Yamauchi & Sricholpech, 2012). A graphical representation of a proto-fibril is shown in Figure 1.9.

Of the fibrillar collagen family, collagen I, II, III, V and XI have been studied the most widely with collagens XXIV and XXVII being the more recently described members of this family with roles in organogenesis including in the generation and maturation of cartilage (Koch *et al.* 2003; Pace *et al.* 2003; Wang *et al.*, 2012). The importance of collagens in development may be key in elucidating their role in tumourigenesis. This is exemplified by the tight relationship between the oestrogen receptors, progesterone receptor and HER2 with mammary development and breast tumourigenesis; as previously described (section 1.2.1.2).

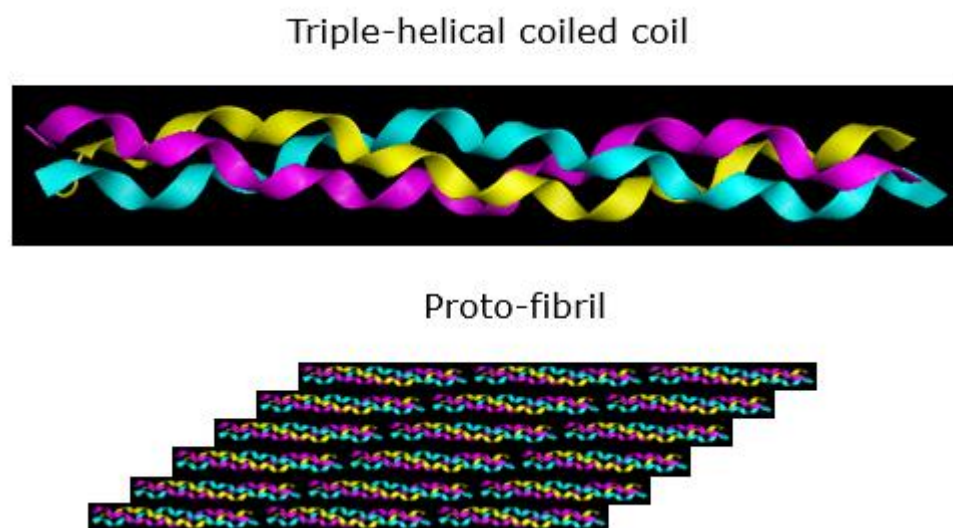


Figure 1.9 – Schematic representation of collagen I. 3D representation of a portion of ternary collagen I, comprised of two collagen I(α 1) and one (α 2) chain, and how these interdigitate to form a protofibril, PDB entry 1CAG.

1.7.1.2 Network Forming Collagens

Network forming collagens are non-fibrillar meshwork forming collagens commonly found in basal laminae and between discrete tissue types such as between the capillary bed and alveolar epithelium in the lung. Metastasising cells are very likely to encounter network forming collagens.

The first elucidated and most well studied of these are the collagen IV family, of which there are six forms. The special nature of collagen IV is furthered when considering the bioactive nature of its cleavage products, so called "matricryptins" (Davis, G.E. *et al.*, 2000). Collagen IV(α 1) chains contain the cleavage product arresten in the C-terminal domain. Arresten inhibits angiogenesis and tumour formation (Aikio *et al.*, 2012). Matricryptins are present in all other collagen IV chains (α 2-6) and known as canstatin, tumstatin, tetrastatins 1-3, pentastatin 1-3 and hexastatin 1-2 respectively. These bioactive molecules are also linked to anti-angiogenic and anti-tumourigenic behaviours (Kamphaus, G.D. *et al.*, 2000; Maeshima, Y., Colorado, P.C. & Kalluri, R., 2000; Ricard-Blum, 2011).

1.7.1.3 FACIT and MACIT Collagens

FACIT and MACIT collagens are anchors for fibrillar collagens. FACIT collagens tether cells to the fibrillar and network forming collagens indirectly by bridging between integrins on the cell surface and the collagen network. Collagen XII is an important component in development of tissue architecture and mechanical properties (Zhang, G. *et al.*, 2005; Ricard-Blum, 2011).

MACIT collagens are very similar in structure to FACIT collagens with many interrupted collagenous domains; the key difference being the transmembrane regions that allow direct tethering of cells to fibrillar collagens rather than the indirect tethering through hemi-desmosomes associated with FACITs (Ricard-Blum, 2011).

1.7.1.4 Collagen and Tissue Plasticity

Fibril forming collagens are enzymatically cross-linked to form fibrils from the multimeric proto-fibrils (Yamauchi & Sricholpech, 2012). This process in combination with protein-protein interactions with both network-forming collagens, FACIT collagens and elastin, regulate the flexibility of many tissues. This leads to the mechanical properties of cartilage, bone, ligaments and tendons; all of which are collagen rich but differ considerably in terms of their mechanical properties (Zhang, G. et al., 2005). Abundance of collagen fibrils or networks does not constitute a rigid tissue. The regulating factor in rigidity is the number of cross-links between parallel fibrils or networks (Fratzl-Zelman *et al.*, 2016). The regulation of the FACIT collagens and therefore cross-linking is associated with the genetic differentiation program as well as epigenetic changes brought on by mechanical stress. For example, over-stressed tendon tissue during post-natal development has been linked to shortening and stiffening of the tendon in mice (Zhang, G. et al., 2005). This kind of mechanotransduction could also be of importance in *de novo* vascularisation of tumours.

1.7.2 Laminins

Laminins are heterotrimers consisting of α , β and γ chains; they also constitute an important component of the ECM encountered by metastasising cells, and differ greatly in abundance between tissues and organs. Although there are 60 possible combinations of chains to form trimers, only 16 have been observed (Aumailley *et al.*, 2005). The trimers form a crucifix or 'T'-shaped molecule depending on the length of the central α -chain. Laminin nomenclature follows a simple rule with α -chain isoform numbers preceding β -chain isoform numbers and so on to form a three-digit number; laminin-511 is $\alpha 5$, $\beta 1$ and $\gamma 1$ for example (Aumailley *et al.*, 2005). Laminin-111 is the most widely studied as it is a core component in murine Engelbreth-Holm-Swarm tumour, the extract of which is commonly known as Matrigel® (Miller, 2017). However, it is becoming

clear as research moves forward that many properties of laminin-111 have very few properties that are comparable to other laminins, as such Matrigel is likely not the best 3D culture environment for most cell types. For a complete review of all laminins, see Domogatskaya, Rodin & Tryggvason (2012). Here the laminins found in lung tissues will be described; as these are anatomically relevant metastatic niche of triple negative breast cancer (Ignatov *et al.*, 2018).

Lungs are highly homogenous organs dominated by alveolar tissue. This tissue is highly enriched in basal lamina proteins such as laminins and collagen IV. This is because most of the cells within the lung are either pneumocytes, which comprise alveoli, or endothelial cells, and make up the capillary beds in interface with the alveoli. Between these two layers of cells lies the laminin rich basal lamina. Laminin-511 and laminin-332 are produced by small airway epithelial cells, inferring their enrichment in lung tissue (Carpenter *et al.*, 2017).

Laminin-332 has two forms of differing function. α -chain isoform 3A is truncated and forms a T-shaped molecule, which is missing the 12 N-terminal *EGF-like* domains and the *laminin IV type A* domain found in the longer isoform 3B. A study by Carpenter *et al.* (2012) showed a decrease in breast cancer cell motility in the absence of laminin-332, making laminin-332 a good candidate ECM component of tissues that breast cancer cells can easily invade. This is consistent with primary breast cancers developing a motile phenotype in a laminin-332 rich environment such as the mammary epithelium (Carpenter *et al.*, 2009).

There is a distinct lack of research into the use of specific, tailored 3D culture substrates for different cell types. Many research groups default to commercial products such as Matrigel which oversimplify the highly complex ECM.

1.8 Cell Culture Systems

So far, the effect of ECM components and niches of tumours and tissues on tumour behaviour and how the ECM of metastatic loci affects the efficacy of secondary tumour formation have been described (Chapter 1, section 7). Some of the experiments that have elucidated these processes, and associated phenotypic and genotypic changes, have been performed in conventional 2D culture systems (Carpenter *et al.*, 2017; Moilanen *et al.*, 2017). Alternatively, much of breast cancer research is performed in animal models. Both systems of research have significant drawbacks in terms of the biological relevance of these microenvironments, and ethics respectively. Here the use of biomaterials to replace animals in research whilst maintaining a contextually relevant biological microenvironment, lost in 2D culture systems; will be reviewed.

1.8.1 Benefits and Limitations of Monolayer Culture

The process of culturing cells on 2D substrates is a core component of cell biology, but limitations arise when the whole process is scrutinised. Many cell lines could be used as examples but for brevity breast cancer lines derived from pleural effusion will be used.

During their establishment, cell lines derived from pleural effusion have been transferred from suspension or aggregate culture and introduced in to a single two-dimensional substrate. In doing this once, cells able to attach to the substrate are selected. Successive passages start with washing in PBS, or similar, removing cells that have not attached and are viable in suspension above the substrate along with cells which have attached and following a biological cue, detached. These cells are removed from the culture and each round selects increasingly for cells with an adhesive profile. Alternatively, the opposite is true if a suspension line is being developed. The selectivity of this process will change cell line characteristics over time and to varying extents,

when the variability of seeding density, media and cryptic stress factors are considered (Baker & Chen, 2012).

The sole benefit in the use of cell lines is comparability despite the limitations. Cultures changing characteristics per passage number is insignificant when the differences in discrete tumours are considered. Similarly, the comparability between primary cells is considerably more limited unless there is a large sample and detailed background information to account for tumour heterogeneity. However, research is now moving toward a much more complex understanding of tumourigenesis and tumour heterogeneity. This combined with the ease of application and reduction in cost of omics-techniques means that using primary cells is easier than ever before, without the draw-back of gene/protein characterisation (Hanahan & Weinberg, 2011)

1.8.2 Organoid Culture

The use of organoid cultures allows the investigation of cell behaviour in a more realistic environment than in 2D, thus the use of organoids has led to an improved understanding of drug response and hypoxia (Chiche, Brahimi-Horn & Pouyssegur, 2010; DiGiacomo & Gilkes, 2018). The morphology of organoids can vary from spheroids, to more disordered cellular bodies. Spheroids are generally more commonly used as their behaviour is more regular. Some spheroid cultures, such as MCF10A, generate a lumen in the centre of the spheroid whilst other cell lines, such as MCF7, are denser and form a necrotic, hypoxic core. The culture methods used to generate organoids vary widely, with the common factor of no adherent material present other than the cells themselves (Gaiko-Shcherbak *et al.*, 2015; Froehlich, K. *et al.*, 2016). The presence and role of ECM proteins in organoids has not yet been fully understood (Gaiko-Shcherbak *et al.*, 2015).

1.8.3 Biomaterials

Biomaterials are becoming used more commonly in the study of cell biology and the study of cancer metastasis is no exception. The use of collagen, Matrigel® and more recently, decellularised matrices, has begun to improve the understanding of cell behaviour in a 3D culture setting (Dunne *et al.*, 2014). These models add an extra level of complexity by comparison with organoid cultures. Not only is there a third dimension that cells can exploit, but cellular cues from the extracellular matrix will also be encountered, making the system more physiologically relevant. Furthermore, the mechanical properties of ECM microenvironments are significantly different between common breast cancer metastatic loci, such as bone and lung.

1.8.3.1 Hydrogels and Matrigel®

Hydrogels are synthetic biocompatible matrices, typically derived from ECM proteins. The use of hydrogels allows the construction of complex niches from its component parts. Hydrogels can vary in mechanical and biochemical properties with various chemical constituents providing a large variety of tools from which to generate a synthetic 3D matrix. Cross-linking agents, such as pro-anthacyanadin and glutaraldehyde can be used to modify substrate stiffness, whilst remaining biologically compatible (Yang, Ritchie, & Everitt, 2017). The mechanical properties of a substrate are key if one is to accurately mimic a desired tissue, as outlined (section 1.7.1.6). In addition, the constituents of any hydrogel can be altered to change the biochemical properties, eg. growth factors and ECM proteins can be integrated to challenge cells for any desired purpose, see Sokol *et al.* (2016). Matrigel® is commonly used commercialised hydrogel comprising collagen, laminin, entactin and many solubilised growth factors which, importantly, differ in abundance between batches (Hughes, Postovit & Lajoie, 2010).

1.8.3.2 Decellularised Matrices

The benefit of using decellularised matrices over other matrices is the complexity of the ECM. The number of proteins, glycoproteins and glycosaminoglycans (GAGs) specific to any one tissue is vast and difficult to replicate artificially, making decellularised matrices the best substrate for studying cell behaviour *in vitro*. This is shown by Dunne *et al.* (2014), where MCF7 and BT474 cell behaviour in decellularised adipose closely resembled a breast tissue murine xenograft model.

1.8.4 Bioreactors and Microfluidics

Accurate mimesis of physiological conditions is not only performed by introduction of a physiologically mimetic substrate, but also by the flow of nutrients and fluid through/past cellular structures. Dynamic culture, such as those used in bioreactor systems can also be used to simulate the flow of serum past or through a tissue. If the configuration has a secondary chamber to which cells can migrate or communicate, the system can be used to study complex processes such as endocrine signalling or metastasis (Laronda *et al.*, 2013; Laronda *et al.* 2017). Considering current advancements in terms of both material science and machining technologies; bioreactor development is only restricted by creativity.

One example is rotating wall vessels, where a horizontally rotating barrel containing inoculated media is used to suspend cells in 'microgravity'. The organoids which form are recirculated such that they are never settled at the bottom of the vessel (Figure 1.10A). A second example is a perfusion bioreactor system, which can be designed to suit almost any experimental question in terms of biological processes. Correctly controlled fluid dynamics within perfusion bioreactors is vital for accurate replication of the respective biological context being mimicked. Schematics of both recirculating and single-pass perfusion bioreactors are shown in Figure 1.10B-C.

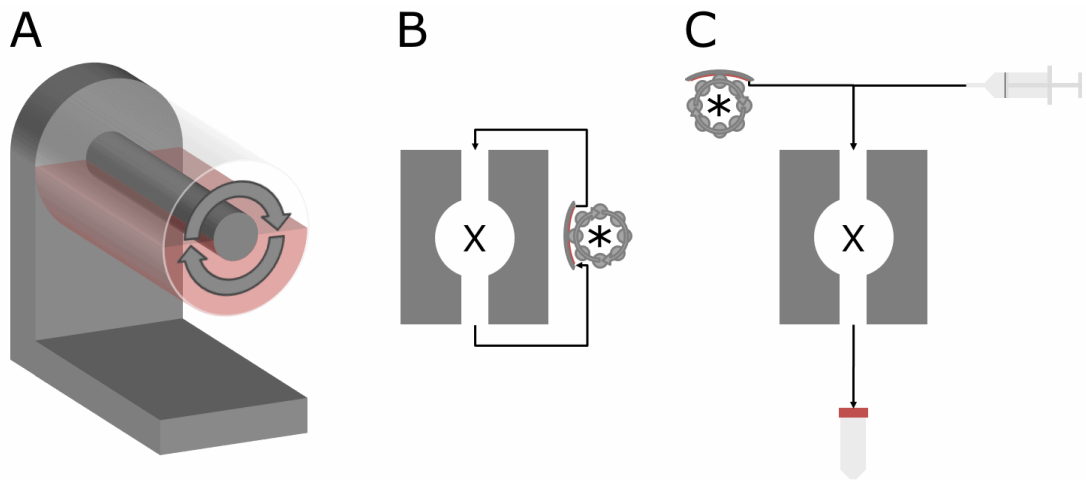


Figure 1.10 – Bioreactor schematic. **A**, rotating wall vessel which keeps cells suspended by generating 'microgravity'. **B**, recirculating perfusion bioreactor where medium passes through the chamber and continues to circulate via serial pump-chamber configuration. **C**, single-pass perfusion bioreactor where medium passes from a pump or syringe through the chamber into a collection reservoir. Bioreactor chamber (X), peristaltic pump (*), arrows represent flow of media.

1.9 Rationale

The main aim of this thesis is to gain a better understanding of the process of metastasis. The metastatic process can be broken down into seven key steps: Loss of cell polarity, invasion or delamination, intravasation, evasion of anoikis and survival in circulation, extravasation, re-adherence, and colonisation. The complete process can be studied holistically, as is common in patient-derived xenograft (PDX) mouse models (Byrne *et al.* 2017), or by studying one or two of the above processes in isolation, for example, migration, within a Boyden chamber trans-well assay (Perkel, 2012). Here, a biomimetic bioreactor system is developed to study the process of metastasis holistically, in tandem with the breakdown of individual processes into component parts.

1.10 Thesis Aims

The aims of the research here are to analyse basal characteristics of breast cancer cell lines, mimicking each challenge of metastasis (Figure 1.11):

- Invasion and intravasation
- Evasion of anoikis and survival in circulation
- (re)adherence and extravasation
- Colonisation of a secondary site.

Furthermore, the broken-down process will then be reconstructed in an in vitro setting by using biomaterials to represent different physiologically relevant niches to:

- Assess the efficacy and accuracy of lung-organoids as a model for breast cancer lung metastasis
- Assess the biocompatibility and accuracy of decellularised lung matrix as a model for secondary lung tumour formation, in 3D

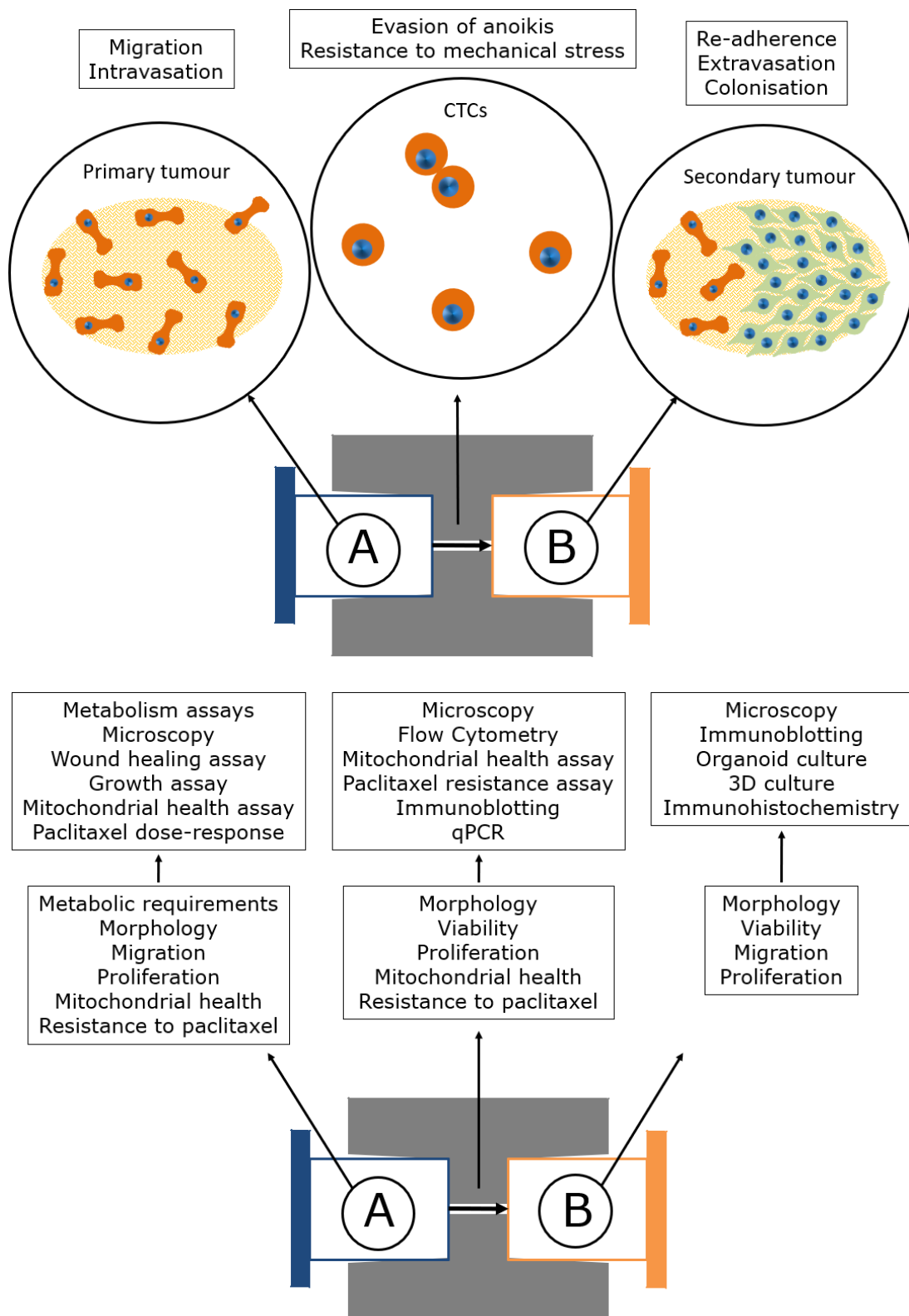


Figure 1.11 – Work-flow breakdown and relation to metastasis. With the intention of assessing metastasis in a 2-chamber bioreactor system, each location was assessed in an isolated fashion before being combined into one system, assessing migration, intravasation, evasion of anoikis and resistance to mechanical stress, re-adherence, extravasation and colonisation.

Chapter 2

Materials & Methods

2.1 Cell Culture

All cell-related experiments used cells of passage 20-30. Where possible all triplicate repeats were of consecutive passage number or as close to this as possible.

2.1.1 2D

MCF7^{CASP3} cells were sourced from Janicke *et al.* (1998, hereon referred to as MCF7); MDA231, MRC5^{GFP} (embryonic lung fibroblasts) and HeLa (cervical cancer) cell cultures were derived from ATCC lines (HTB-26, CCL-171 and CCL-2 respectively). Cells were cultured at 37°C, 5% CO₂ in a humidified atmosphere, hereon referred to as 'standard conditions'. Cells were passaged at ~90% confluency and harvested using trypsin-EDTA solution (Sigma, T4174) diluted in Dulbecco's phosphate buffered saline (DPBS; Sigma, D8537). All cells were cultured in Dulbecco's modified Eagle's medium (Sigma, D6429) with 10% (v/v) foetal bovine serum (Sigma, F7524), known from here on as complete media. 1 mmol·l⁻¹ G418 (ACROS organics) was used as a selective pressure on transfected cells.

2.1.2 Suspension Culture

MCF7 and MDA231 cells were seeded at a density of 2.5x10⁶ per 40 ml of media, in sealed 50 ml Falcon tubes (Sarstedt) at 37°C and agitated at 200 rpm in an orbital incubator (Sanyo) with no humidity or CO₂. Cells were cultured in complete media supplemented with 25 mmol·l⁻¹ HEPES (pH 7.5).

2.1.3 Re-adherent Colony Forming Assay

A 1 ml aliquot of suspension culture was taken and seeded onto a petri dish before culturing for 7 days in standard conditions. Plates were stained with methylene blue solution (1% methylene blue (w/v) in 30% ethanol) and viewed by bright field microscopy at 20x magnification (Nikon Eclipse, TE2000-S). Images (.tiff) were processed using Fiji software.

2.1.4 Decellularised Matrices

MCF7, MDA231 and MRC5^{GFP} cells were seeded on top of sterile, equilibrated matrices in a dropwise fashion at a density of 2.5×10^4 per 6 mm diameter matrix disc (see 2.3.1), before incubation in otherwise standard tissue culture conditions.

2.1.5 Bioreactor

The bioreactor comprised one 20x90x70 mm Acetal block (Direct Plastics, Sheffield, UK) with 12 identical 1/8 British standard pipe (BSP) diameter threaded holes on each face connected by a 1 mm channel connecting chambers 'A' and 'B' (Figure 2.1). Acetal was chosen due to its biocompatibility and ease of machining. Seeded 3D matrices (see 2.3.1) cultured in static conditions for 96 h were transferred into one of the chambers and secured by screwing a 1/8-27 NPT Luer-threaded fitting (Cole-Parmer UK, St Neots) into the bioreactor body. A peristaltic pump was then used to perfuse the chamber at a rate of $16 \mu\text{l} \cdot \text{min}^{-1}$ (section 2.1.5.1). Polyurethane Luer tubing (Cole-Parmer UK, St Neots) connected the chambers to 5/32" bore silicone tubing (Masterflex® L/S® Precision Peroxide-Cured Silicone Pump Tubing) via polypropylene 5/32" hose barb connectors (all fluid circuit parts supplied by Cole-Parmer UK, St Neots). Luer tubing was used to connect the pump outlet to the medium reservoir. As seen in Figure 2.1, the pump was used to draw medium through the bioreactor, so that static pressure within the chambers could be controlled by adjusting the vertical distance between the reservoir and the bioreactor. Medium was introduced at the base of the bioreactor to minimise trapped air within the culture chambers.

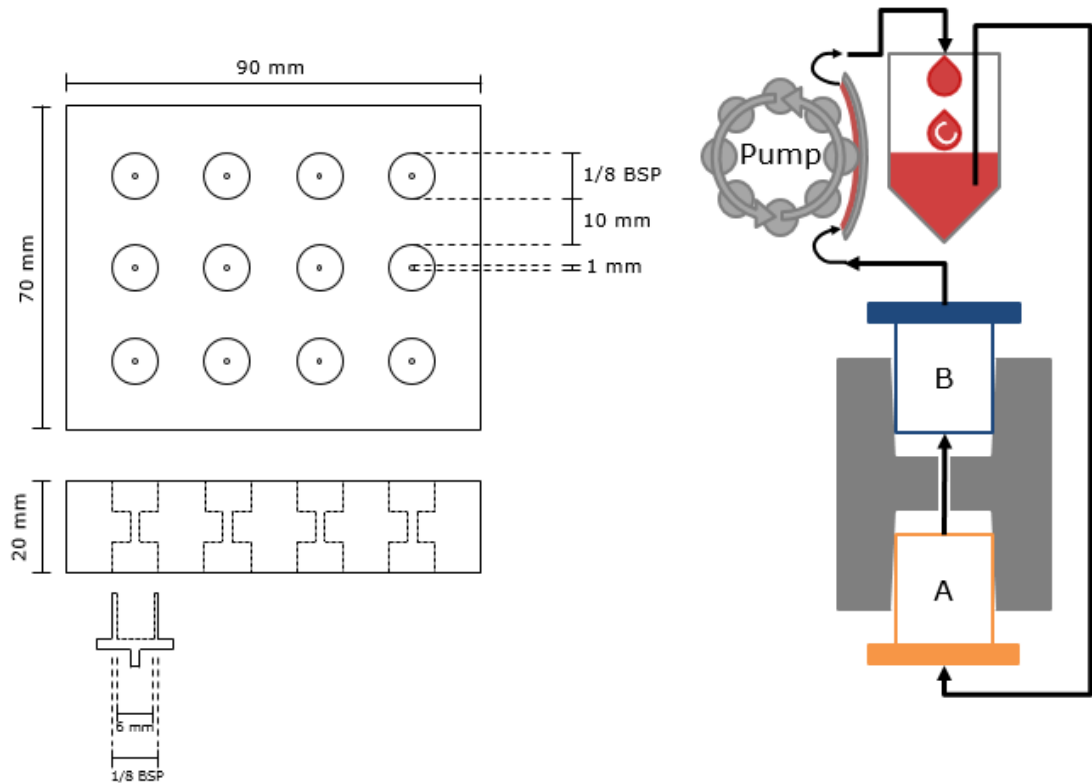


Figure 2.1 – Bioreactor system schematic. Chamber 'A' contains matrix representing a primary locus. Chamber 'B' contains matrix representing a secondary locus. Media was circulated via a peristaltic pump (black arrows).

2.1.5.1 Optimum Flow Rate

The flow rate within the bioreactor was set to achieve a Reynolds number similar to that reported for flow of blood within a tissue capillary of 3×10^{-3} by Li (2004). The fluid density and apparent viscosity of culture medium in this calculation was taken to be similar to those for water at 40°C (Howatson, Lund & Todd, 1972). From the definition of the Reynolds number:

$$Re = \frac{vD\rho}{\mu}$$

Where v is the fluid velocity, ρ is the density, D is the characteristic dimension (chamber diameter) and μ is the dynamic viscosity of the fluid. If the diameter of the chamber is $6 \times 10^{-3} \text{ m}$, and the porosity of the decellularised lung material is 30% (measured by histological analysis, see 2.6), the cross-sectional area through which culture medium can flow is given by: $A = \pi r^2 \times 0.3 = \pi \times 2.7 \times 10^{-6} \text{ m}^2$, thus to determine flow rate (Q):

$$v = \frac{Q}{A} \text{ and therefore } Re = \frac{Q \times D \times \rho}{\pi \times \mu \times 2.7 \times 10^{-6}} \text{ and } Q = \frac{Re \times \mu \times \pi \times 2.7 \times 10^{-6}}{D \times \rho}$$

Measurement of the pore diameter by optical microscopy gave a mean pore diameter within the tissue of $3 \times 10^{-4} \text{ m}$. The dynamic viscosity of the culture medium was taken as $0.651 \times 10^{-3} \text{ Ns} \cdot \text{m}^{-2}$ and the density of culture medium was taken as $0.992 \times 10^3 \text{ kg m}^{-3}$ to achieve a Reynolds number of 3×10^{-3} :

$$Q = \frac{(3 \times 10^{-3}) \times \pi \times (0.651 \times 10^{-3}) \times 2.7 \times 10^{-6}}{(3 \times 10^{-4}) \times (0.992 \times 10^3)} = \frac{\pi \times 5.27 \times 10^{-12}}{0.2976} = 5.56 \times 10^{-11} \text{ m}^3 \cdot \text{s}^{-1}$$

$$= 0.334 \mu\text{l} \cdot \text{min}^{-1}$$

The optimum flow rate was limited by the minimum rate of the peristaltic pump which was $16 \mu\text{l} \cdot \text{min}^{-1}$, with the consequence of higher shear stress applied to cultured cells.

2.1.6 Cell Proliferation Assay

5×10^3 MCF7 and MDA231 cells were seeded in quadruplicate for each condition in a 96-well plate. Cell number was calculated by resazurin assay and a standard curve.

2.1.6.1 Resazurin Assay

At specified time points, medium was aspirated and replaced with 1 volume of $40 \mu\text{mol} \cdot \text{l}^{-1}$ resazurin in complete media and cells incubated in standard culture conditions for 1 h. Fluorescence was measured with excitation set at 530 nm and emission at 590 nm on a spectrophotometer (BMG Labtech, FLUOstar Galaxy). This assay measures the conversion of resazurin (blue, non-fluorescent) to resorufin (purple, fluorescent when excited by 530-560 nm light). Resorufin can be further metabolised to dihydroresorufin (colourless, non-fluorescent) and it is recommended that metabolic assays employing this

reagent are performed over a short time (Grivennikova, Kareyeva & Vinogradov, 2018).

2.1.6.2 NADH Turnover Assay

5×10^3 MCF7 and MDA231 cells were seeded in triplicate in a glass-bottom black-walled 96-well plate (Eppendorf) and left to adhere for 3 h in standard culture conditions. Medium was then aspirated and replaced with 1 volume of fresh media before resazurin was diluted to $500 \mu\text{mol}\cdot\text{l}^{-1}$ in the first row of the plate, then diluted two-fold, serially, down to $15.625 \mu\text{mol}\cdot\text{l}^{-1}$. This left 6 concentrations (500, 250, 125, 62.5, 32.25 and $15.625 \mu\text{mol}\cdot\text{l}^{-1}$) of resazurin, in triplicate, alongside an identical resorufin dilution series, acting as standards. The amount of resorufin produced in 1 h was quantified by spectrophotometry as above (2.1.6.1). Readings were normalised by removal of equimolar blanks and a resorufin standard curve was used to convert sample fluorescence into remaining concentration of resazurin. NADH oxidation velocity was then calculated by dividing readings by the duration of the assay in minutes. These values were then plotted against relative resazurin substrate concentrations, as a Michaelis-Menten function. The resultant logarithmic line of best fit allowed the derivation of maximal NADH turnover (V_{max}), specific to each cell line and culture condition.

2.1.7 Wound Healing Assay

MCF7, MDA231 or MRC5^{GFP} cells were cultured to confluency on 10 mm x 0.1 mm circular coverslips in complete media, a scratch in the monolayer was made with a 200 μl pipette tip 24, 16, 8 or 0 h prior to fixing. Coverslips were washed in PBS, fixed in 4% formaldehyde solution (v/v) for 5 min, washed 3 times in PBS and permeabilised in 0.15% Triton X-100 (v/v) for 2 min all at room temperature. Coverslips were stained with $200 \text{ nmol}\cdot\text{l}^{-1}$ rhodamine conjugated phalloidin (Life Technologies) and $1 \mu\text{g}\cdot\text{ml}^{-1}$ 4',6-diamino-2-phenylindole (DAPI) and mounted in Mowiol (Aldrich) mounting media. Slide

scanner images were quantified using Zen 2 Lite software; or in Fiji (ImageJ) when imaging was performed on a Zeiss 200M microscope at 10x magnification.

2.1.8 Dose-Response Assay to Cell Cycle Inhibitors

Cells were seeded at a density of 2.5×10^4 per well in a 96-well plate (Corning) and left to adhere (typically 3 h). Cell cycle inhibitor drugs then underwent a tenfold serial dilution alongside the relevant vehicle (plate layout shown in Table 2.1). Cells were incubated for 96 h in standard conditions before a resazurin assay was performed (section 2.1.6.1). Samples, minus blank values, were then expressed as a percentage of vehicle only treated samples to give percentage viability for all treatments. Mimosine was dissolved in complete media, thymidine was dissolved in Opti-MEM (Sigma, 11058021) and paclitaxel was dissolved in dimethyl sulphoxide (DMSO; Sigma, 472301).

Table 2.1 – Cell cycle-inhibitor dose-response assay plate layout. Drugs were dissolved tenfold, serially; alongside their respective vehicle.

	Mimosine	Medium	Thymidine	Optimem	Paclitaxel	DMSO
Row/column	1-2	3-4	5-6	7-8	9-10	11-12
A	500 $\mu\text{mol}\cdot\text{l}^{-1}$	N/A	10 $\text{mmol}\cdot\text{l}^{-1}$	20%	10 $\mu\text{mol}\cdot\text{l}^{-1}$	0.1%
G	5 $\text{nmol}\cdot\text{l}^{-1}$	N/A	100 $\text{nmol}\cdot\text{l}^{-1}$	$2 \times 10^{-6}\%$	100 $\text{pmol}\cdot\text{l}^{-1}$	$1 \times 10^{-8}\%$
H	No Treatment					

2.2 Flow Cytometry

2.2.1 Count and Viability Assay

MCF7 and MDA231 cells grown in suspension were sampled immediately after media inoculation and every 24 h thereafter for 96 h. Samples were diluted tenfold in Muse® 'count & viability reagent' and counted using the Muse Cell Analyser (Merck Millipore) as per the manufacturer's instructions. 1000 events were measured, and detection thresholds set at 1 for debris and 0.75 (45°) for live/dead.

2.2.2 Cell Cycle Assay

2.2.2.1 Sample Collection

2.5x10⁶ MCF7 and MDA231 cells were seeded in 40 ml suspension cultures in duplicate alongside 1x10⁵ cells seeded in a 6-well plate (Corning). All cells grown in 2D and one suspension culture were treated with 1 $\mu\text{mol}\cdot\text{l}^{-1}$ of dimethylenastron (DMA, Enzo life sciences). 8 ml was sampled at each timepoint and centrifuged at 500 g for 5 min at 4°C. Pellets were washed in PBS then resuspended in 250 μl of PBS. From the 250 μl samples 50 μl was removed for use in a Count and Viability assay. The remaining 200 μl was then slowly mixed with 1 ml of ice cold 70% ethanol and stored at -20°C for at least 3 h; as per the manufacturers' instructions.

2.2.2.2 Assay

For analysis, 600 μl of samples were diluted 1:1 with PBS and pelleted by centrifugation (500 g for 5 min at 4°C). Cell pellets were then resuspended in Muse Cell Cycle reagent and incubated for 30 min at room temperature. Subsequent analysis was performed on the Muse Cell Analyser (Merck Millipore) as per the manufacturer's instructions. 5000 events were measured, detection thresholds set as recommended by the manufacturer and were kept constant throughout.

2.3 Biomaterials

2.3.1 Decellularisation

Decellularisation of organs was performed using the immersion method developed by Atala (2002). Dissected rat lungs were immersed in sterile distilled water (SDW) for 48 h with water replaced every 24 h or when it became turbid. Cell debris was then removed by immersion in decellularisation buffer (0.05% NH₃OH (v/v), 0.5% Triton X-100 (v/v) dissolved in SDW) for 24 h. Decellularisation buffer was washed out by immersion in SDW for 24 h, all steps were performed at 4°C with stirring. Decellularised organ was cut into 6 mm

diameter discs with a steel borer and placed into 24-well plate inserts with 3 μm pores (Greiner Bio One) before equilibration in 244 $\mu\text{mol}\cdot\text{l}^{-1}$ penicillin, 172 $\mu\text{mol}\cdot\text{l}^{-1}$ streptomycin in DPBS for at least 24 h in standard tissue culture conditions. DPBS was aspirated and replaced with complete media before incubation in standard tissue culture conditions for at least 24 h to confirm sterility and equilibrate the matrix with media. Matrices were routinely tested for cellular material by immunoblotting and DNA staining with haemalum.

2.3.1.1 3D Migration Assay

MDA231 cells were seeded as described in section 2.1.4 and incubated in standard tissue culture conditions for 7 days before fixing and histological processing (section 2.6). Serial 5 μm sections were taken through the entirety of each sample before all samples were stained with DAPI as described (section 2.6.3). A representative area of every section was then imaged using a Zeiss 200M widefield microscope. All images were then processed in a semi-automated fashion with Fiji software (Chapter 8, section 1.3.2); where the tissue in each image was outlined with the point tool then nuclei counted automatically. Once all images were processed Microsoft Excel was used to determine the distance of each nucleus from the nearest point on the tissue perimeter. These data were then collated across 3 independent repeats and the distance of each nucleus from the edge of the matrix was binned into 10 μm intervals. The frequency for each bin was then plotted in GraphPad Prism software.

2.4 Molecular Biology

2.4.1 Real-Time Quantitative PCR (qPCR)

At all steps during RNA and cDNA handling, filtered pipette tips were used (Sarstedt), and all buffers were treated with diethylpyrocarbonate (DEPC) or made up in DEPC treated dH₂O.

2.4.1.1 RNA Extract Preparation

Approximately 7×10^6 live cells at 90% confluency were scraped into 500 μ l of media and lysed by addition of 1 ml TRI reagent and incubation for 5 min at room temperature. 1-bromo-3-chloro-propane (BCP; 11.76% BCP (v/v) in TRI reagent and complete media) was then added and samples mixed by inversion before incubation for 3 min at room temperature, fractionating protein/lipids, DNA and RNA. Phase separation was performed by centrifugation at 10,000 g for 15 min at 4°C. The top phase was removed and incubated at -20°C overnight in acidified isopropanol solution (256 mmol·l⁻¹ sodium acetate pH 4, 36% isopropanol (v/v)). Extracted RNA was precipitated by centrifugation (17,000 g for 15 min at 4°C), before being washed in 70% ethanol and centrifuged further (17,000 g for 15 min at 4°C). Samples were then DNase treated with RNase-free DNase I (Qiagen, Cat No. 79254) according to the manufacturer's instructions. DNase I treated RNA was isolated by phenol-chloroform-isoamyl alcohol (50% phenol-chloroform-isoamyl alcohol (v/v) 125:24:1 in dH₂O) centrifuge phase separation at 11,000 g for 9 min at 12°C. The top phase was then removed and precipitated overnight in acidified ethanol solution (323 mmol·l⁻¹ sodium acetate pH 5.2, 65% ethanol (v/v)). Finally, samples were centrifuged (17,000 g for 15 min at 4°C); the pellet washed in 70% ethanol and centrifuged (17,000 g for 15 min at 4°C); the resulting pellet was then dissolved in dH₂O. Purity and yield was calculated by spectrophotometry (Nanodrop 2000, Thermo Scientific) and samples stored at -80°C.

2.4.1.2 RNA Integrity

RNA integrity was determined using denaturing agarose gel electrophoresis (20 mmol·l⁻¹ MoPS, 8 mmol·l⁻¹ sodium acetate, 1 mmol·l⁻¹ EDTA, 6.7% formaldehyde (w/v), 1.5% agarose (w/v), 0.02% SYBR safe (v/v); cast at ~50°C). Samples were prepared in denaturing loading buffer (400 mmol·l⁻¹ formamide, 20 mmol·l⁻¹ MoPS, 8 mmol·l⁻¹ sodium acetate, 1 mmol·l⁻¹ EDTA, 4.44% formaldehyde (w/v), 40 ng·µl⁻¹ RNA, 1x purple loading buffer (NEB)) then denatured at 65°C for 15 mins. Gels were pre-run with no samples at 55 V for 5 min, loaded and run at 55 V for 2 h before imaging (BIO-RAD, Gel Doc™ XR+).

2.4.1.3 cDNA Synthesis

1 µg of RNA was used as template for cDNA synthesis by reverse transcription using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. All reactions were run in duplicate, one reaction was the complete mixture (RT⁺) and the second was an enzyme negative control (RT⁻). cDNA was stored at -20°C.

2.4.1.4 Primer Optimisation

PCR for qPCR-primer annealing temperature (T_m) optimisation was performed with REDTaq® polymerase summarised in Table 2.2 and Figure 2.2. Primers were designed with an optimum T_m of 60°C and primers were tested at 58, 60, 62 and 64°C using a temperature gradient thermocycler (Bio-Rad, T100™ Thermal Cycler). All reactions originated from the same master mix without the template.

Table 2.2 –qPCR primer optimisation summary. A, reaction stoichiometry. **B**, PCR programme. **C**, primer sequences for reference genes and genes of interest (GOI).**A**

Constituent	Volume (µl)	Final Concentration
10X REDTaq® Buffer	2	1X
Forward Primer	0.5	250 nmol·l ⁻¹
Reverse Primer	0.5	250 nmol·l ⁻¹
dNTPs	0.5	250 µmol·l ⁻¹ (62.5 µmol·l ⁻¹ ATP, CTP, GTP and TTP)
Template	1	500 pg·µl ⁻¹
REDTaq® Polymerase	1	0.05 U·µl ⁻¹
DEPC treated ddH ₂ O	14.5	N/A
Final Volume	20	

B

Step	1	2	3	4	5	6	7
Temperature (°C)	95	95	T _m	72	Return to 2	72	12
Time (s)	180	30	30	60	X39	300	∞

C

Gene	NCBI accession number		Length (bp)	Sequence (5`-3`)
Reference				
<i>ACTB</i>	NM_001101.3	F R	23 20	GCAGAAAACAAGATGAGATTGGC TGTGAACTTTGGGGGATGCT
<i>EEF1A1</i>	NM_001402.5	F R	20 20	GGACACGTAGATTCGGGCAA CACGTTACGCTCAGCTTTC
<i>GAPDH</i>	NM_001289746.1	F R	22 22	CAACAGCCTCAAGATCATCAGC TGGCATGGACTGTGGTCATGAG
<i>TBP</i>	NM_001172085.1	F R	20 20	GTGGGGAGCTGTGATGTGAA ACGTCGTCTTCCTGAATCCC
<i>TUBB</i>	NM_178014.3	F R	20 21	ACTTTGTATTTGGTCAGTCT TTGCTGATAAGGAGAGTGCCC
Mitosis GOI				
<i>AURKB</i>	NM_004217	F R	20 20	CGATCATGGAGGAGTTGGCA TGCACCACAGATCCACCTTC
<i>CDCA8</i>	NM_001256875	F R	20 20	GACACCCCTGAAATCTGCCA ACAGTGTTAGCACGGCTTGA
Anoikis GOI				
<i>BID</i>	NM_197966.2	F R	20 18	CTGGGAAACTGTTGAGTGGC GTGGCGACAGAATCCGCA
<i>CFLAR</i>	NM_003879	F R	20 23	TGCTCTACAGAGTGAGGCGA AGGTCCAAGAACTCTTCTCCTT
<i>FADD</i>	NM_003824.3	F R	22 21	CTAGACCTCTTCTCCATGCTGC GAGCCAGCCTTCTCCAATCTT
<i>PMAIP1</i>	NM_021127.2	F R	20 21	ACTGTTTCGTGTTTCAGCTCGC GAGTAGCACACTCGACTTCCA
BIR family GOI				
<i>BIRC2</i>	NM_001166.4	F R	19 19	ATCGTGCGTCAGAGTGAGC CGCCGACAAGGAGATACGG
<i>BIRC3</i>	NM_001165.4	F R	20 22	CTCTGGGCAGCAGGTTTACA AACTACCTCCCGAGATTAGACT
<i>BIRC4</i>	NM_001167.3	F R	23 22	GTGTCTGGTAAGAACTACTGAGA CCTTGAAACTGAACCCCATTCG
<i>BIRC5</i>	NM_001168.2	F R	20 19	AGGTCCCCGCTTTCTTTGGA CCGAGATGACCTCCAGAGG
<i>BIRC5-1</i>	NM_001168.2	F R	20 21	AGGACCACCGCATCTCTACA TATGTTCTCTATGGGGTCGT
<i>BIRC5-2</i>	NM_001012271.1	F R	21 21	GAGCCAGATGACGACCCCAT TTCCTCTCTCGTGATCCGCCC
<i>BIRC5-3</i>	NM_001012270.1	F R	20 20	GGACCACCGCATCTCTACAT TTGGTTTCCTTTGCATGGGG
Other GOI				
<i>CTTN</i>	NM_138565.2	F R	20 22	GTGGTTTTGGCGGCAAGTAT ACTTTGTCCTTGTCGATACCGT
<i>YY1</i>	NM_003403.4	F R	20 19	GAAGATGATGCTCCAAGAAC AGCGTTTCCACAGCCTTC

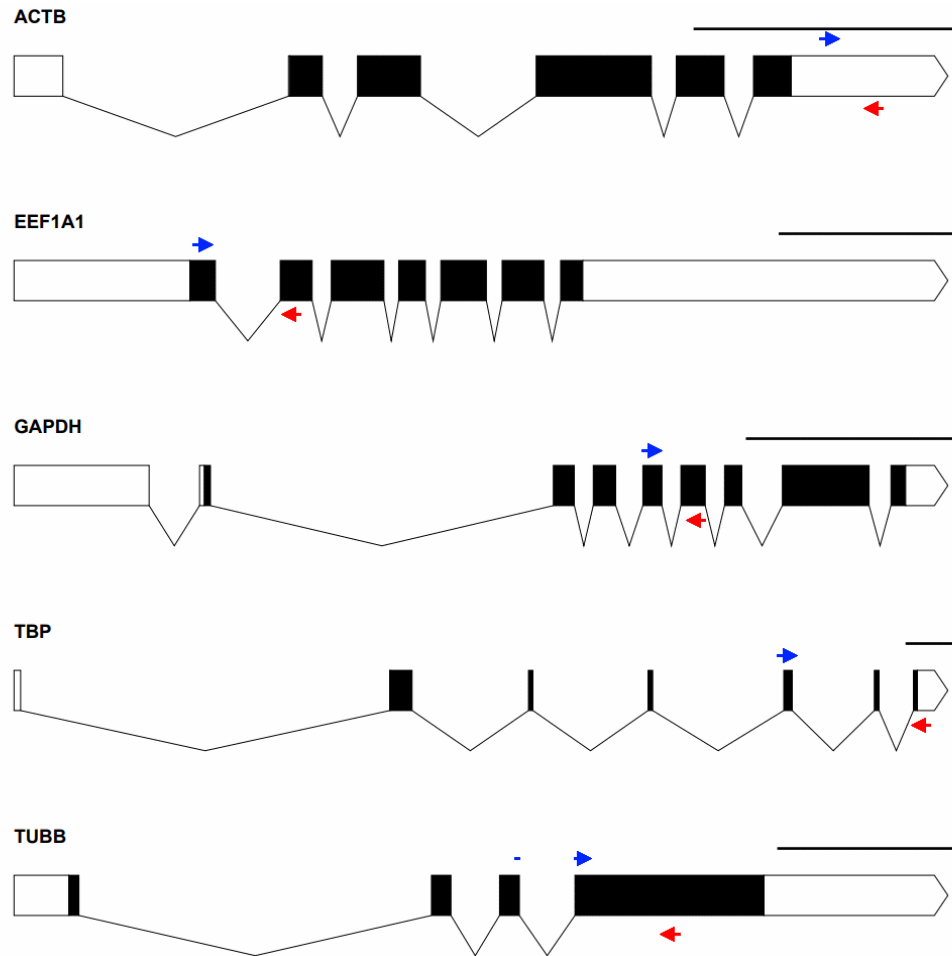


Figure 2.2 – Reference gene structures and PCR amplicons. Genes are represented in a 5`-3` orientation with white regions indicating untranslated regions (UTRs), black regions representing exons and lines representing introns. **Blue** arrows represent forward primers, **red** arrows represent reverse primers. 1000 bp scale bar. Reference genes are used as an example, all other primer amplicons can be found in the appendix (section 8.1.1).

2.4.1.5 Primer Efficiency

qPCR efficiency was calculated by quantitative analysis of the threshold breaking cycle (C_T) for six, threefold serial dilutions of template, with the same primers, alongside blank and RT⁻ controls and run in duplicate on a qPCR thermocycler (7500 Fast Real-Time PCR, Applied Biosciences). The final concentration of each reaction, the PCR programme and plate layout are shown in Table 2.3 respectively. The gradient of the standard curve generated was used to calculate reaction efficiency in accordance with the MIQE guidelines (Bustin *et al.*, 2009) with the following equation, where m is the gradient:

$$qPCR\ efficiency = 10^{\frac{-1}{m}} - 1$$

If low concentrations of cDNA led to no amplification or C_T values of $38 \leq$, the starting concentration of the dilution series was doubled to $2500 \text{ pg} \cdot \mu\text{l}^{-1}$.

Table 2.3 – qPCR primer efficiency summary. A, reaction stoichiometry. **B,** thermocycler programme. **C,** plate layout; RT^- is the reverse transcriptase negative control (no cDNA), H_2O is the reagent contamination control (no added RNA or cDNA).

A

Constituent	Volume (μl)	Final Concentration
2X iTaq™ Universal SYBR® Green Supermix	10	1X
Forward Primer	0.5	$250 \text{ nmol} \cdot \text{l}^{-1}$
Reverse Primer	0.5	$250 \text{ nmol} \cdot \text{l}^{-1}$
Template	1	-
DEPC treated ddH ₂ O	7.5	N/A
Final Volume	20	

B

Step	1	2	3	3	4	6
Temperature ($^{\circ}\text{C}$)	50	95	95	60	Return to 3	Default melt curve programme
Time (s)	120	60	15	60	X39	

C

Column Row	GOI1 1-2 ($\text{pg} \cdot \mu\text{l}^{-1}$ cDNA)	GOI2 3-4 ($\text{pg} \cdot \mu\text{l}^{-1}$ cDNA)	...GOI6 ...11-12 ($\text{pg} \cdot \mu\text{l}^{-1}$ cDNA)
A	1250.00	1250.00	1250.00
B	416.67	416.67	416.67
C	138.89	138.89	138.89
D	46.30	46.30	46.30
E	15.43	15.43	15.43
F	5.14	5.14	5.14
G	RT^-	RT^-	RT^-
H	H_2O	H_2O	H_2O

2.4.1.6 qPCR

qPCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) as per the manufacturer's instructions on a qPCR thermocycler (7500 Fast Real-Time PCR, Applied Biosciences). cDNA final concentration was optimised to each gene but was typically $166.66 \text{ pg} \cdot \mu\text{l}^{-1}$ and raised to $312.5 \text{ pg} \cdot \mu\text{l}^{-1}$ relative to mRNA of low abundance or $625 \text{ pg} \cdot \mu\text{l}^{-1}$ in extreme cases. Data were analysed, and reference genes verified by the Pfaffl method (2011) and using REST 2009 software (QIAGEN, bootstrap: 10000).

2.4.1.7 Agarose Gel Electrophoresis

Agarose was dissolved in tris-acetate-EDTA (TAE) buffer ($40 \text{ mmol} \cdot \text{l}^{-1}$ Trizma, 5.7% acetic acid (v/v), $1 \text{ mmol} \cdot \text{l}^{-1}$ EDTA) at a concentration of 1% (w/v) by microwaving before being cooled to $\sim 50^\circ\text{C}$ and SYBR® safe (Invitrogen) added to a final concentration of 0.01% (v/v). Gels were run at 100 V in TAE buffer for 45 min.

2.5 Protein Analysis

2.5.1 Sample Preparation

2.5.1.1 Monolayer WCE

Samples for whole cell extracts (WCE) from adherent cultures were harvested by scraping in ice cold PBS. Samples were then centrifuged at 500 g for 5 min at 4°C , resuspended in PBS and centrifuged again, then cells lysed by resuspension in Tris-Triton buffer ($100 \text{ mmol} \cdot \text{l}^{-1}$ NaCl, $10 \text{ mmol} \cdot \text{l}^{-1}$ Trizma, $1 \text{ mmol} \cdot \text{l}^{-1}$ EDTA, $1 \text{ mmol} \cdot \text{l}^{-1}$ EGTA, 10% Glycerol (w/v), 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v)) with an inhibitor cocktail ($2 \text{ mmol} \cdot \text{l}^{-1}$ β -glycerophosphate, $1 \mu\text{g} \cdot \text{ml}^{-1}$ 1:1:1:1 chymostatin : leupeptin : antipain : pepstatin A (CLAP), $100 \mu\text{mol} \cdot \text{l}^{-1}$ phenylmethylsulphonyl fluoride (PMSF), $20 \text{ mmol} \cdot \text{l}^{-1}$ Na_3VO_4), vortexed briefly for 30 min on ice with vigorous agitation. Protein samples were then sonicated at 50 Hz for 5 s and protein concentration was determined by Bradford assay. Typically extracts were

$\sim 5 \mu\text{g} \cdot \mu\text{l}^{-1}$. An aliquot of extract was reduced in sample buffer ($100 \text{ mmol} \cdot \text{l}^{-1}$ β -mercaptoethanol, $50 \text{ mmol} \cdot \text{l}^{-1}$ Trizma pH 6.8 with HCl, 10% glycerol (w/v), 2% SDS (w/v), 0.01% bromophenol blue (w/v)) for 10 min at 95°C , in preparation for SDS PAGE and immunoblotting.

2.5.1.2 Dynamic Suspension Culture WCE

WCEs from suspension culture were harvested by centrifugation at 500 g for 5 min at 4°C before resuspension in PBS and lysis as described for adherent whole cell extract.

2.5.1.3 Decellularised Matrix WCE

WCEs from decellularised matrices were prepared by washing the matrix 3 times in PBS for 5 mins at 4°C before lysis in Tris-Triton buffer as described. Samples were then subsequently sonicated at 20 Hz for 10 s before being incubated on ice for 3 mins. This process was repeated 3 times before samples were centrifuged (500g for 5 min at 4°C) and the pellet discarded along with excess matrix. Monolayer and empty-matrix samples harvested in parallel were treated in the same way.

2.5.2 Bradford Assay

1 μl of sample protein was diluted in Bradford reagent (20% v/v) and Bradford assay performed as per the manufacturer's instructions against BSA standards of $1\text{-}10 \mu\text{g} \cdot \text{ml}^{-1}$.

2.5.2.1 Proliferation in Suspension

MDA231 cells were grown in suspension as described with samples being taken at each timepoint and pelleted by centrifugation (500 g for 5 min at 4°C). Pellets were washed by resuspension in PBS and repeat centrifugation (500 g for 5 min at 4°C), supernatant aspirated and pellet stored at -20°C until all samples in triplicate were collected. Pellets were lysed in 50 μl of SDW ($2 \text{ mmol} \cdot \text{l}^{-1}$ β -glycerophosphate, $1 \mu\text{g} \cdot \text{ml}^{-1}$ CLAP, $100 \mu\text{mol} \cdot \text{l}^{-1}$ PMSF, $20 \text{ mmol} \cdot \text{l}^{-1}$ Na_3VO_4),

and left on ice for 30 min with vigorous agitation. 2 μ l of each sample was then subjected to a Bradford assay in triplicate (chapter 2, section 5.2).

2.5.3 SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) for the detection of proteins below $\sim$ 130 kDa was performed by standard methods on 15% polyacrylamide gels (390 mmol $\cdot$ l $^{-1}$ Trizma pH 8.8 with HCl, 12-15% acrylamide:bisacrylamide (37.5:1) (v/v), 3.5 mmol $\cdot$ l $^{-1}$ SDS, 3.4 mmol $\cdot$ l $^{-1}$ TEMED, 0.42 mmol $\cdot$ l $^{-1}$ ammonium persulphate). Gels were loaded and run at 200 V in running buffer (192 mmol $\cdot$ l $^{-1}$ glycine, 25 mmol $\cdot$ l $^{-1}$ Trizma, 3.5 mmol $\cdot$ l $^{-1}$ SDS), typically 20 μ g of sample was loaded per well. To detect proteins above $\sim$ 130 kDa, 8% polyacrylamide gels without SDS in tris-acetate resolving gel buffer (192 mmol $\cdot$ l $^{-1}$ Trizma pH 7 with glacial acetic acid, 8.6 mmol $\cdot$ l $^{-1}$ TEMED, 6-10% acrylamide:bisacrylamide (37.5:1) (v/v), 0.44 mmol $\cdot$ l $^{-1}$ ammonium persulphate) were used. Gels were run at 50 mA in L-running buffer (50 mmol $\cdot$ l $^{-1}$ tricine, 50 mmol $\cdot$ l $^{-1}$ Trizma, 3.5 mmol $\cdot$ l $^{-1}$ SDS, 2.4 mmol $\cdot$ l $^{-1}$ sodium bisulphite). Actin was always used as a loading control, as stable expression had been confirmed by qPCR (see appendix 8.2.2).

2.5.4 Immunoblotting

Immunoblotting was performed by standard protocols. In brief, Tris-HCl gels were transferred onto nitrocellulose (Amersham Biosciences) at 350 mA for 1.5 h in transfer buffer (192 mmol $\cdot$ l $^{-1}$ glycine, 25 mmol $\cdot$ l $^{-1}$ Trizma, 20% methanol (v/v)), whilst Tris-acetate gels were transferred at 70 V for 2 h in transfer buffer containing 3.5 mmol $\cdot$ l $^{-1}$ SDS and 5% methanol. Membranes were blocked in 5% non-fat dry milk in tris-buffered saline with Tween-20 (TBST; 150 mmol $\cdot$ l $^{-1}$ NaCl, 50 mmol $\cdot$ l $^{-1}$ Trizma, 5% non-fat dried milk (w/v) 0.1% Tween-20 (v/v) pH 7.4 with HCl) before incubation in primary antibody at 4°C over-night. Membranes were washed three times in TBST (5 min at room temperature), then incubated in the relevant HRP-conjugated secondary antibody at a 1:2000

(75 $\mu\text{g}\cdot\text{ml}^{-1}$) dilution (Dako; 45 min at room temperature) before being washed three times in TBST. ECL reagents (Amersham Biosciences) were mixed 1:1 and left at RT for 2 min then membranes were incubated for 2 min in ECL reagent and finally exposed to photographic film in the dark for 1, 5 and 30 min. Table 2.4 summarises all antibodies used and the diluent.

Table 2.4 – Summary of antibodies and buffers used for immunoblotting. Gt secondary antibody is anti-sheep diluted 1:1000, Ms and Rb secondary antibodies diluted 1:2000.

Antigen	Vendor	1° Buffer	1° Dilution	2° Buffer
β -Actin	Sigma	TBST-5% milk	1:1000	TBST-5% milk
AURKB	Sigma	TBST-5% milk	1:1000	TBST-5% milk
Borealin	Sigma	TBST-5% milk	1:1000	TBST-5% milk
Cadherin (E, N, P, R)	CST	TBST-5% BSA	1:1000	TBST-5% BSA
E-Cadherin	Abcam ab76055	TBST-3% BSA	1:500	TBST-3% BSA
cIAP1	CST	TBST-5% BSA	1:1000	TBST-5% BSA
Collagen I	Abcam ab88147	TBST-5% milk	1:500	TBST-5% milk
Cortactin	CST #3502	TBST-5% milk	1:1000	TBST-5% milk
FAK	CST	TBST-5% milk	1:1000	TBST-5% milk
GFP	In-house	TBST-5% BSA	1:500	TBST-5% BSA
Histone H3 (phospho S10)	Sigma	TBST-5% BSA	1:1000	TBST-5% BSA
INCENP	Sigma	TBST-5% BSA	1:1000	TBST-5% milk
Ki67	Bio-Rad AbD02531	TBST-1% BSA	1:1000	TBST-3% milk
Laminin (Total)	Abcam ab11575	TBST-5% BSA	1:500	TBST-5% milk
OPA1	Abcam	TBST-1% milk	1:1000	TBST-1% milk
Survivin	Novus NB500-201	TBST-5% milk	1:1000	PBST only
Talin	Santa Cruz	PBST-5% milk	1:1000	TBST-5% milk
XIAP	BD biosciences	TBST-5% milk	1:1000	TBST-5% milk

2.5.4.1 Semi-quantitative Analysis

Immunoblots were quantified using Fiji software. Band intensity values were measured, and the values were then pooled into sample groups, with all untreated/treated replicates in single pools. The intensity values for each sample and protein within each pool were then expressed as a percentage of the average of the loading control for that pool. Calculated values were then expressed as a percentage of the average of the same protein in the control pool. The final value was then achieved by expressing percentages as a decimal and transforming values as a function of a base 2 logarithm ($\log_2$). This process is summarised below.

$$\text{normalised abundance} = \frac{\text{sample intensity}}{\text{sample pool loading control average intensity}}$$

$$\text{normalised relative abundance} = \frac{\text{normalised abundance}}{\text{control sample pool average normalised abundance}}$$

$$\text{fold change} = \log_2(\text{normalised relative abundance})$$

2.6 Histology

2.6.1 Embedding

Acellular lung was fixed in 4% formaldehyde (Thermo Scientific) in PBS for 15 min, then washed in PBS twice for 20 min and dH₂O twice for 20 min. Samples were dehydrated by successive 20 min washes in 70%, 95% and 100% ethanol with three subsequent 20 min washes in 100% xylene. All washes were performed at room temperature. Samples were then immersed in 50% paraffin in xylene (v/v) at 64°C for 20 min before being immersed in 100% paraffin at 64°C for 20 min twice and set in a cassette at room temperature overnight.

2.6.2 Sectioning

Unless otherwise stated, paraffin embedded samples were sectioned at 5 μm using a Leica DSC1 microtome. Prepared slides were dried overnight at 42°C on a hot-plate (Thermo Scientific), then stored at room temperature.

2.6.3 Immunohistochemistry

Tissue sections were deparaffinised and hydrated by successive 5 min washes in xylene (x2), 100% ethanol, 95% ethanol 70% ethanol and two 5min washes in dH₂O (all at room temperature). Antigen unmasking was achieved by a microwaving (ProLine, 750W) with either citrate or EDTA buffers, summarised in Table 2.5. Samples were washed 3 times in TBST for 5 min before being blocked (10% goat serum, 5% BSA in TBST) for 1 h at room temperature. Samples were washed three times in TBST and incubated in primary antibody, diluted in blocking buffer, overnight at 4°C. Antibody information is shown in Table 2.5. Three further washes preceded incubation in secondary antibody solution (1:200 in blocking buffer) for 1 h at room temperature before samples were counterstained in 1 $\mu\text{g}\cdot\text{ml}^{-1}$ DAPI for 10 min and washed 3 times in TBST, then mounted with Mowiol (Aldrich) mounting media. Stained samples were typically stored overnight at 4°C before imaging.

Table 2.5 – Antibodies used for immunofluorescent detection in 5 μm sections of paraffin embedded samples. Citrate buffer: 10 $\text{mmol}\cdot\text{l}^{-1}$ tri-sodium citrate dihydrate pH 6 with HCl. EDTA buffer: 10 $\text{mmol}\cdot\text{l}^{-1}$ Trizma, 1 $\text{mmol}\cdot\text{l}^{-1}$ EDTA pH 9 with HCl.

Antibody	Vendor	Concentration		Unmasking buffer
		Dilution	Mass	
Collagen I	Abcam ab88147	1:100	6.8 $\mu\text{g}\cdot\text{ml}^{-1}$	Citrate
Pan-laminin	Abcam ab11575	1:100	5 $\mu\text{g}\cdot\text{ml}^{-1}$	
Survivin	Novus NB500-201	1:100		
Ki67	Bio-Rad AbD02531	1:100		
Laminin-332	DHSB	2:5	9.6 $\mu\text{g}\cdot\text{ml}^{-1}$	
Collagen IV	DHSB	2:5	20 $\mu\text{g}\cdot\text{ml}^{-1}$	
Collagen XII	DHSB	2:5	13.2 $\mu\text{g}\cdot\text{ml}^{-1}$	
E-cadherin	Abcam ab76055	1:100	5 $\mu\text{g}\cdot\text{ml}^{-1}$	EDTA
Cortactin	CST #3502	1:100	5 $\mu\text{g}\cdot\text{ml}^{-1}$	

For chemical staining (periodic acid, Schiff-diastase; PAS-D), deparaffinised samples were treated with 0.5% (w/v) α -amylase (Sigma) for 30 min at 37 °C, 0.5% periodic acid for 5 min, followed by Schiff's reagent (Sigma) for 20 min, both at room temperature. Samples were counterstained with neat haemalum (Thermo Scientific, LAMB-170-D) nuclear stain for 3 min at room temperature followed by a series of dehydration washes in increasing concentrations of ethanol (70, 95 and 100%) and two washes in xylene, all for 5 min at room temperature. Finally, samples were mounted with distyrene plasticiser xylene (Sigma) mounting media (Grey *et al.*, 2018).

2.7 Microscopy

2.7.1 Live Imaging

2.7.1.1 Suspension Culture Aggregation

100 μ l 0.01% (w/v) poly-lysine was added to each well of an 8-well live imaging chamber (Ibidi) then left to air dry for 1 h at room temperature. The live imaging chamber was then sterilised by 10 J·cm² 245 nm UVC irradiation. Cells were cultured in DMEM without phenol red (D1145) with supplementary 25 mmol·l⁻¹ HEPES (p, 4 mmol·l⁻¹ L-glutamine and 1 mmol·l⁻¹ sodium pyruvate. 15 min prior to imaging, 1 drop NucBlue (Life Technologies) was added per ml to sampled suspension cultures.

Mitochondrial Health

A single well of a live imaging chamber (Ibidi) was poly-L-lysine treated as previously before 1x10⁵ MDA231 cells were seeded into a single untreated well of the live imaging chamber, ~48 h before imaging. Immediately before imaging, 1 ml of suspension culture was transferred to a microfuge tube before 1 drop of NucBlue (Life Technologies) was added alongside 500 nmol·l⁻¹ MitoTracker Green (Life Technologies) and 200 nmol·l⁻¹ tetramethylrhodamine ethyl ester perchlorate (TMRE, AAT Bioquest). The medium of 2D cultured cells was replaced in parallel with identical media containing identical live imaging stains; the cells were left for 15 min in fresh media at 37°C with no CO₂ or

humidity in the sealed live imaging chamber before imaging. 2-3 random fields of view were acquired at 20x magnification before 10 random fields of view were acquired at 60x magnification for each sample. Exposure was set to suit the highest level of fluorescence in each channel.

2.7.1.3 Image processing

Images of cells stained with NucBlue, MitoTracker Green and TMRE were deconvolved using an iterative refinement method built into the microscope software; and quantified in Fiji by a fully automated macro with no user guidance (appendix 8.1.3.1). The macro was programmed to threshold each channel of each image identically before saving a colour composite image and a greyscale image of each channel alone. The programme then counted the number of nuclei in the image and measured the minimum, maximum and mean signal intensity of MitoTracker Green and TMRE. These values were then collated in Microsoft Excel and transferred to GraphPad Prism software.

2.7.2 Fixed Sample Imaging

Fixed samples were imaged in the following manners:

Table 2.6 – Summary of imaging systems used.

Experiment	Microscope	Magnification	Excitation and Emission
Wound Healing Assay	Zeiss 200M	5x and 10x	DAPI: 390/435 GFP: 475/523 Rhodamine-Phalloidin: 542/594
Immunohisto-fluorescence	Deltavision Elite	20x	DAPI: 390/435 Fluorescein: 475/523 Texas Red: 542/594
PAS-D	Zeiss Axioplan	5x and 20x	N/A
Wholemount immune-fluorescence	Zeiss Z.1 Light Sheet	20x	DAPI: 390/435 Fluorescein: 475/523 Rhodamine Phalloidin: 542/594
3D Migration	Zeiss 200M	10x	DAPI: 390/435

Images (.dv or .tif) were processed in Fiji software with the use of fully, or semi-automated macros. Macros are summarised in section 8.1.3. Resultant data were processed in Excel before being summarised and displayed in GraphPad Prism software.

2.8 Bioinformatics

2.8.1 Primer Design

2.8.1.1 qPCR

Primers for qPCR were designed using primer3 (Ye, 2012), BLAST and OligoAnalyzer 3.1 online resources for the relevant genes (Table 2.2). Amplicons and extensive primer information are shown in chapter 8 section 1, in accordance with the MIQE guidelines (Bustin *et al.*, 2009). Primer3 default settings were modified in the following way: PCR product size set to 100-250, primers must span an exon-exon junction or primers must include an intron (altered to accommodate genes with few introns and those with CG or AT base pair bias). Predicted RefSeq transcripts and uncultured/environmental sample sequences were excluded. For primers with exceptionally consistent, low dimer energies (ΔG lower than -8 kcal.mol^{-1}) the "Max Self" and "Max Pair" complementarity settings were altered. BLAST default settings were modified in the following way: the database was altered to RefSeq RNA, Models (XM/XP) were excluded and the search was for "somewhat similar sequences (blastn)". OligoAnalyzer 3.1 default settings were modified by raising the Mg^{2+} concentration from 0 mmol.l^{-1} to 2 mmol.l^{-1} only.

2.8.2 Phylogenetics

Phylogenetic analysis of protein sequences was performed by the MAFFT (Multiple Alignment using Fast Fourier Transform) E-INS-i iterative refinement algorithm, scored by BLOSUM62 (Blocks Substitution Matrix) (1.53 gap opening penalty, 0 offset value); and visualised using Archaeopteryx software (Katho *et al.*, 2002; Han & Zmasek, 2009). Protein sequences were downloaded from uniprot with the search criteria: "COL1A1", "COL1A2" "COL28A1". All sequences were classified as "reviewed".

For laminins, protein sequences were downloaded from uniprot with the search criteria "LAMA#", "LAMB#" or "LAMC#". All sequences were classified as "reviewed".

2.8.3 The Transcription Element Listening System (TELIS)

Down-regulated qPCR genes were searched using TRANSFAC TELIS (Quandt *et al.*, 1995; Cole *et al.*, 2005). Microarray was set to "All Human Genes", the gene list was as follows: *BIRC2*, *BIRC3*, *BIRC4*, *BIRC5*, *AIM1*, *FADD*, *PMAIP1*, *BID*; promoter size was set as "-1000 to +200", stringency was set to "High (.90)". The algorithm did not recognise the aliases for *BIRC4* and *AIM1* which are also known as *XIAP* and *AURKB* respectively. The algorithm did not recognise *CDCA8* or the previous name for this gene: *PESCRG3*, as such it was not included in analysis.

2.8.4 Online Resources

Many online tools were used to understand the functional regions and facets, of genes and proteins of interest. Their use is summarised in Table 2.7.

Table 2.7 – Summary of online databases and resources used.

Tool	Use	Website
Phyre2	Protein modelling	http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index
COTH	Protein binary interaction modelling	https://zhanglab.ccmb.med.umich.edu/COTH/
SymmDock	Protein homo-dimerisation modelling	http://bioinfo3d.cs.tau.ac.il/SymmDock/
Phobius	Secretion signal consensus search	http://phobius.sbc.su.se
Peptide Cutter	Protease consensus site search	https://web.expasy.org/peptide_cutter/
Translate Tool	DNA to protein translation	https://web.expasy.org/translate/
Prot Param	pI, half-life and residue number summary	https://web.expasy.org/protparam/
Reverse Complement Tool	Reverse complementing for primer sequence generation	http://www.bioinformatics.org/sms/rev_comp.html
IARC TP53 Search	Cell line TP53 status	http://p53.iarc.fr/CellLines.aspx
COSMIC	Catalogue of somatic mutations in cancer	https://cancer.sanger.ac.uk/cell_lines/
PROSITE	Protein conserved domain search	https://prosite.expasy.org/
Clustal Omega	Multiple sequence alignment for HHblits and Ali2D	https://toolkit.tuebingen.mpg.de/#/tools/clustalo
HHblits	Protein homology search	https://toolkit.tuebingen.mpg.de/#/tools/hhblits
Ali2D	Protein secondary structure search	https://toolkit.tuebingen.mpg.de/#/tools/ali2d
uniprot	Protein database	https://www.uniprot.org/
Gene Cards	Protein/gene database	https://www.genecards.org/
Phospho Motif	Phospho-motif search	http://www.hprd.org/PhosphoMotif_finder
WormWeb	Exon/Intron maps of genes	http://wormweb.org/exonintron
Protein String	Interaction mapping	https://string-db.org/
EVEX	Protein/gene literature search engine	http://evexdb.org
Pubmed	Protein/gene literature search engine	https://www.ncbi.nlm.nih.gov/pubmed/

2.9 Statistical Analyses

Data were assessed for pairing by interrogation of the experimental design. Normality was assessed by Shapiro-Wilks normality test (H_0 = The data do not significantly deviate from a normal distribution). Variance was assessed by *F*-test of equality of variance (H_0 = The two populations do not have significantly different variance) (Table 2.8)

More complex experimental strategies employing repeated measures, or a nested design were analysed by 2-way ANOVA with *post hoc* two-stage linear step up procedure by Benjamini, Krieger & Yekutieli (2006) commonly employed for multiple comparisons. Two-stage linear step-up was used when intercomparing all conditions and samples and acted to adjust for false discovery of significant *P*-values ($\alpha_P=0.05$ $\alpha_Q=0.05$).

Table 2.8 – Summary of statistical tests employed. ANOVA, analysis of variance.

Sample Number	Sample pairing	Normality	Variance	Test
2	Paired	Normal	N/A	Paired samples t-test
		Not normal	N/A	Wilcoxon t-test
	Independent	Normal	Equal	Independent samples t-test
			Unequal	Welch's t-test
		Not-normal	N/A	Two sample Wilcoxon-test
>2	Independent samples	Normal	Equal	1-way ANOVA
			Unequal	Kruskal-Wallis test
		Not normal	N/A	
	Repeated measures	N/A	N/A	2-way ANOVA
	Nested			

Linear and non-linear regression analysis was employed to determine differences between trends in data. Where possible, linear regression was compared to an appropriate non-linear regression curve by extra sum of squares *F*-test (H_0 = Linear regression provides the best fit to the data). Alternatively, in the case of exponential non-linear regression, the extra sum of squares *F*-test was used to test significant differences between trends in dependent variables within a nested design (H_0 = One line of best fit accurately represents all data).

Correlation analysis was performed using combined, Pearson's and Spearman's rank. Pearson's rank informed on the linearity of any correlation and if the data were monotonic; a rank of 1 represents a change in one variable is matched perfectly by a linear proportional change in the other. Spearman's rank informed on the non-linear relationship between two variables; a rank of 1 represents a change in one variable is proportional to the change in the other, this can take the form of any non-linear change.

Chapter 3

Breast Cancer Cell Characteristics In Dissected Metastatic Contexts

3.1 Overview

The process of metastasis is complex, with several sequential steps required for development of a secondary tumour. In primary tumours of high grade and with tissue infiltrates cells have: 1. become immortal, 2. successfully broken down the basal lamina and, 3. induced local angiogenesis or co-opted vessels, putting malignant cells in interface with the circulatory system. Cells actively migrating away from solid tissues into the liquid tissues of the circulatory system are presented with another set of challenges that must be overcome for secondary tumour formation to occur. Biochemical and mechanical stresses act in tandem to kill cells not designed for attachment-independent survival. Successful metastases comprise only of cells that can intravasate and survive in suspension, but do not completely nullify their adherent potential.

3.1.1 **Survival in Circulation**

Non-transformed cells that detach from the extracellular matrix enter a default programme of apoptosis, termed 'anoikis'. Integrin signalling through RGD motifs confers sustained anti-apoptotic messages within the cell (Bartolomé *et al.*, 2014; Bartolomé, *et al.*, 2015). Transformed cells must sustain this process to become circulating tumour cells; this challenge represents the first step in surviving in transit from primary to secondary sites. Cell aspiration into vessels of increasing diameter, flow and pressure, provides the next obstacle for metastasising cells in the form of mechanical stress. In this context circulating cancer cells must survive the conditions that blood, and lymphoid cells are adapted for. Increased tension across the cell membrane due to shear stress increases membrane permeability (Reiss *et al.*, 2005) and an influx of Ca^{2+} causes many cellular changes including, but not limited to, increased metalloproteinase and calmodulin activity (Umasankar *et al.*, 2012; Reichow *et al.*, 2013). Understanding how the circulating cancer cells avoid anoikis, is key, as is how these cells can be 'primed' for successful secondary tumour formation. This may be achieved by hijacking mechano-transduction pathways described; or alternatively, by anti-anoikis biochemistry in proliferating cells or cells of liquid tissues. Anoikis can signal through both the intrinsic and extrinsic apoptosis pathways, see (section 1.6.4).

Pro-survival signals to protect cells from anoikis can come in many forms, but the strongest is via MAPK signalling through KRAS (GTPase KRas) and BRAF (Serine/threonine protein-kinase B-raf), which are commonly mutated in cancer. If this pro-survival signalling is absent, anoikis can still be circumvented by IAP activity. IAPs are active at multiple points in the apoptosis signalling cascades:

1. They block apoptosis by ubiquitin-mediated degradation of their pro-apoptotic targets;
2. They are competitive inhibitors of caspases via the familial BIR-domain;
3. cIAP1 and cIAP2 can inhibit apoptosis at the cell surface by

interaction with RIP kinases, upstream of DISC formation;

4. XIAP is the best studied IAP and inhibits executioner caspases. This can be abrogated by DIABLO binding of XIAP. However, survivin, another IAP, can directly bind DIABLO preventing any interaction with XIAP. The expression of IAPs and how this confers survival of circulating cancer cells was of particular interest here (Arora *et al.*, 2007).

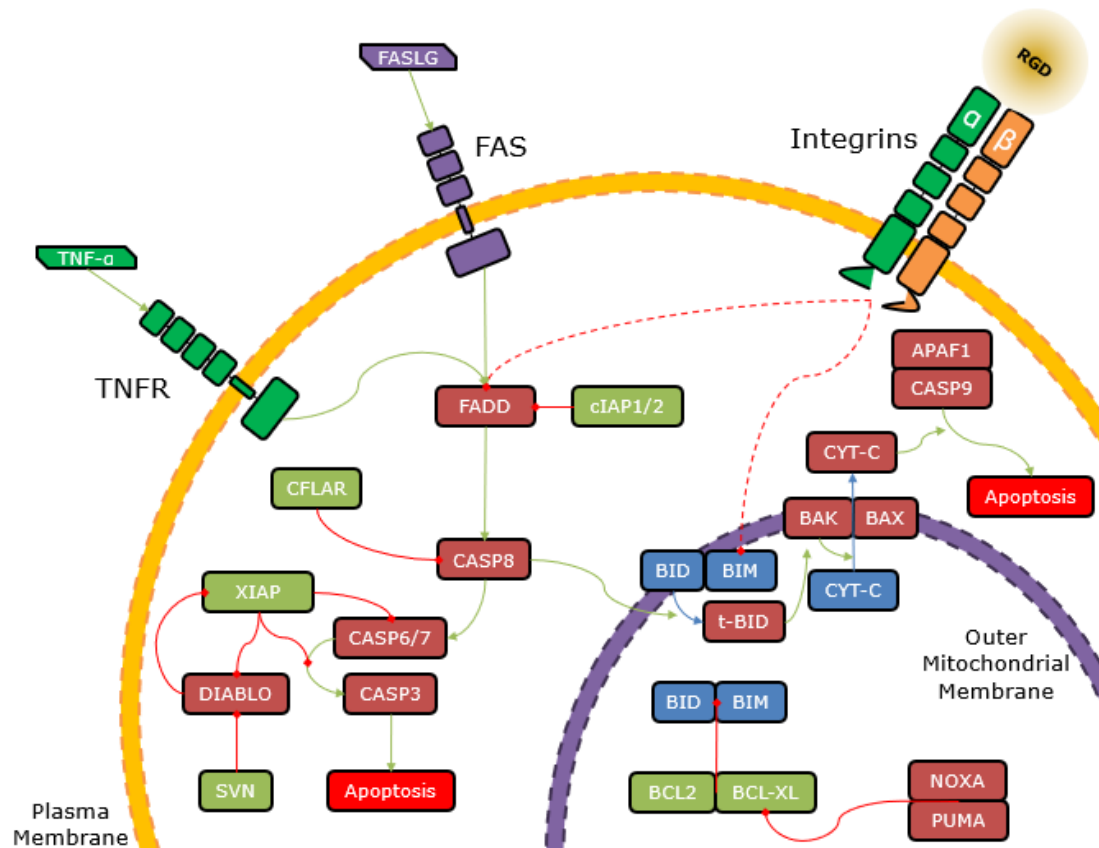


Figure 3.1 – Extrinsic and intrinsic apoptotic cascades. Extrinsic apoptosis is related to the cytosolic proteins, downstream of TNFR, FAS and integrins, whereas intrinsic apoptosis is downstream of pro-death factors such as NOXA and PUMA. Pro-apoptotic factors are in red, antiapoptotic factors are in green, neutral factors are in blue [Adapted from: Mehlen & Puisieux, 2006; Simpson, Anyiwe & Schimmer, 2008; Owens *et al.*, 2010; Laetsch *et al.*, 2014].

Specifically, this chapter assesses phenotypic changes on the level targeted by treatments: mitotic potential, vulnerability to extrinsic apoptotic factors and changes in IAP biochemistry. IAP biochemistry in this respect is particularly relevant as many of the BIR family IAPs have essential mitotic behaviours paired to activity at various points throughout both intrinsic and extrinsic apoptosis pathways (Chai *et al.*, 2001; Suzuki, Nakabayashi & Takahashi, 2001; Arora *et al.*, 2007; Nakamura *et al.*, 2010).

3.1.2 Rationale

The aim of this chapter was to understand the behaviours of breast cancer cells during their metastatic journey. This was done stage-by-stage to mimic the sequential steps required for metastasis to occur:

1. Invasion
2. Evasion of anoikis
3. Re-adherence
4. Colonisation

This was performed so these processes could be understood in the context of the cells used, providing a clearer picture for the suspected behaviour in 3D culture, with the aim of analysing metastatic behaviours holistically.

3.1.3 Cell Lines

MCF7 and MDA-MB-231 (MDA231) cells were chosen due to the different phenotypes and behaviours in culture. Both cell lines derive from pleural effusions, express wild type BRCA1, do not express HER2, and have a *CDKN2A* locus deletion. MCF7 and MDA231 cell line characteristics are summarised in Table 3.1. MCF7 cells are of the luminal cluster of breast cancers, derive from an invasive ductal carcinoma and have an epithelial phenotype. Increased lipid kinase activity of PIK3CA acts as a driver mutation and causes MDM2 and AKT1 hyperactivity. MCF7 cells are normally caspase 3 negative, meaning their apoptotic responses are weak; by using an engineered sub-line of MCF7, with caspase 3, this problem is circumvented (Janicke *et al.*, 1998).

MDA231 cells are triple negative basal B cluster of breast cancers, derived from an adenocarcinoma and are so-called claudin-low, so they are unable to form tight junctions. This, combined with E-cadherin promoter repression by hypermethylation, confers a mesenchymal phenotype with minimal intercellular junctions. In addition, constitutively active KRAS and BRAF, and mutant TP53 (no DNA binding activity) act as driver mutations.

Table 3.1 – MCF7 and MDA231 cell line characteristics. Genetic characteristics of cell lines are specific to: hormone receptor and cadherin expression, MAPK signalling mutations and, caspase 3 and TP53 status. -, not expressed; -^η, promoter hypermethylation; ++, overexpression; Δ, deletion; M, mutant; WT, wild type [Compiled from Olivier *et al.*, 2002, Neve *et al.* 2006; Hollestelle *et al.* 2010].

Protein	Cell Line	
	MCF7	MDA231
ER	WT	-
PR	WT	-
HER2	-	-
E-cadherin	WT	- ^η
N-cadherin	-	-
P-cadherin	WT	-
TP53	WT	++M ^{R280K}
MDM2	++	WT
CDKN2A locus	Δ	Δ
CDKN2B locus	-	Δ
BRCA1	WT	WT
KRAS	WT	M ^{G13D}
BRAF	WT	M ^{G464V}
AKT1	++	WT
PIK3CA	M ^{E545K}	WT
Caspase 3	Δ	WT
Driver Mutations	5	5
Ki-67 score	33-66% positive	>66% positive

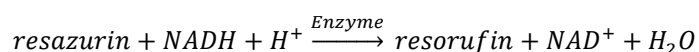
3.2 Results

3.2.1 Characteristics of Breast Cancer Cells in Monolayer (2D) Culture

To gain an understanding of the characteristics of MCF7 and MDA231 cells in regular monolayer culture, their growth, migration and protein expression were analysed.

3.2.1.1 MCF7 and MDA231 Proliferation and Glucose Dependency

To understand the metabolic requirements of MCF7 and MDA231 cells; a resazurin based assay was employed to measure the rate of growth for each cell line in 4 different media: high glucose serum⁺, high glucose serum⁻, low glucose serum⁺ and low glucose serum⁻. High glucose was 25 mmol·l⁻¹ whereas low glucose was 4 mmol·l⁻¹. Resazurin is an organic molecule that is converted to resorufin in the presence of NADH-dependent enzymes. Resorufin is fluorescent in 530nm light, making the amount of resazurin converted in a set time quantifiable. The reaction is shown below:



For both cell lines tested, high glucose serum⁺ medium conferred the highest proliferative rate (Figure 3.2A-B). There was no statically significant difference in doubling time of MCF7 cells in any of the conditions, but low glucose serum⁺ slowed growth rate the most, with cells taking ~20 h more to double. MDA231 showed similar growth regardless of glucose concentration. The most remarkable difference was in doubling of MDA231 in high glucose serum free media. MDA231 cells were unable to grow beyond ~48 h leading to no net growth over 96 h and thus doubling time could not be accurately calculated (Figure 3.2, see section 8.3 for statistical tables).

By comparing the rate of cell proliferation within each media type, it was clear that these two cell lines have distinct metabolic profiles (Figure 3.2B). Both

cell lines grow at similar rates in the most stringent media: low glucose serum free, whereas growth in low glucose serum⁺ media was markedly different between MCF7 (67.85 h) and MDA231 (33 h). MCF7 growth rate in high glucose serum⁺ media was ~50% slower than MDA231 cells, 36.34 h by comparison with 23.22 h respectively.

Doubling time was calculated using an exponential line of best fit, as a result, low growth rates where the gradient of the line tends to 0 caused doubling time to tend to infinity. This alters the efficacy of statistical analysis and as a result, accurate analysis could only be performed on the other growth conditions if high glucose serum⁻ data were excluded. In this case there was a significant difference in proliferation between cell lines grown in low glucose serum⁺ medium, and a significant difference between MCF7 proliferation in low and high glucose serum⁺ medium (Figure 3.2C-D).

Table 3.2 – MCF7 and MDA231 doubling time in different media.

Media Cell Line	Mean Doubling Time (h)			
	Low Glucose		High Glucose	
	Serum ⁻	Serum ⁺	Serum ⁻	Serum ⁺
MCF7	47.7	67.85	60.04	36.34
MDA231	43.99	33	-17.92	23.22

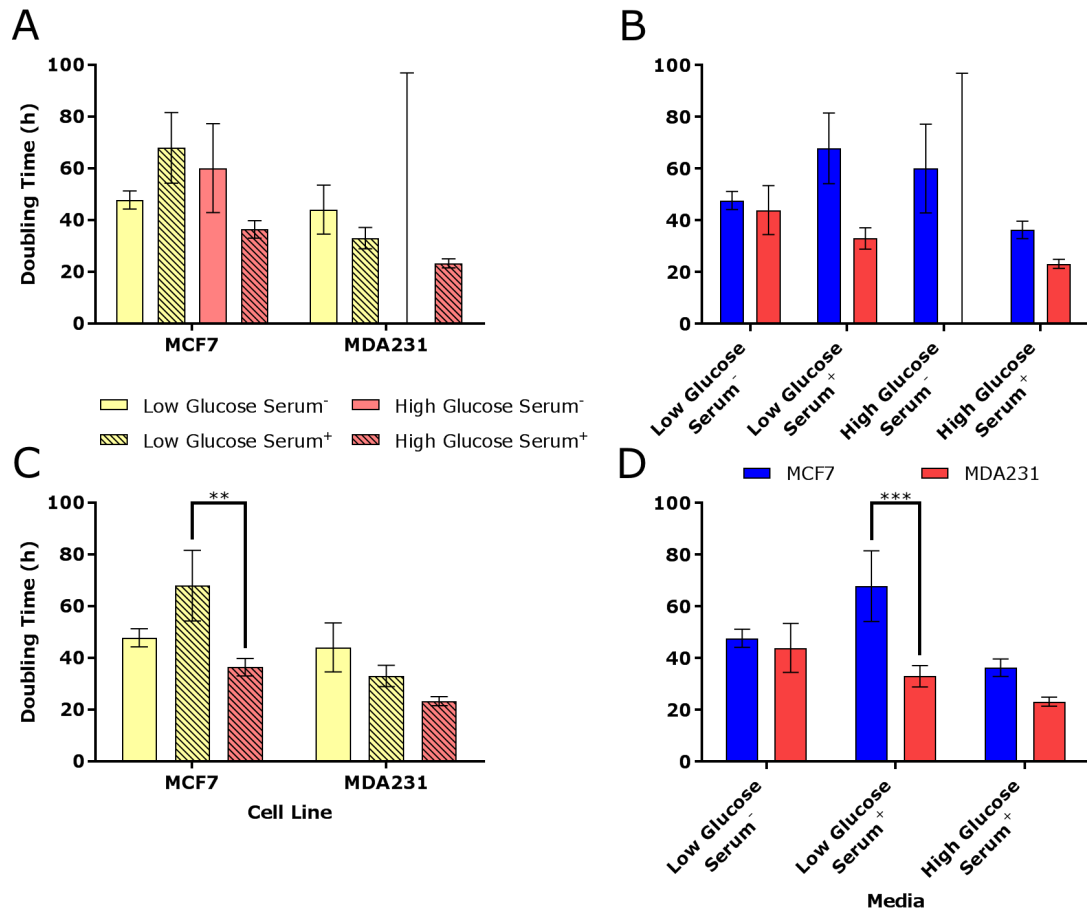


Figure 3.2 – Growth rate of MCF7 and MDA231 cells and dependence on glucose and serum. **A**, comparison of doubling time analysed by 2-way ANOVA with *post hoc* two-stage linear step-up. **B**, comparison of doubling times between each cell line, grouped within media conditions. **C**, 'A' with high glucose serum⁻ excluded. **D**, 'B' with high glucose serum⁻ excluded; analysed by 2-way ANOVA with *post hoc* two-stage linear step-up. Growth rate is measured as time (h) taken for cells to double in number (**, $P=0.01$, $Q=0.021$; ***, $P=0.0055$, $Q=0.0115$; $n=3$; all data were normally distributed).

3.2.1.2 NADH Turnover is Higher in MCF7 Than in MDA231

As growth rates of MCF7 and MDA231 differed, it was hypothesised that MCF7 cells would have a higher rate of NADH turnover than MDA231 cells in high glucose media due to increased TCA cycle and OxPhos activity.

To test this an assay was developed using a twofold serial dilution of resazurin from $500 \mu\text{mol}\cdot\text{l}^{-1}$ to $15.625 \mu\text{mol}\cdot\text{l}^{-1}$; alongside an identical serial dilution of resorufin (the product of the reaction between NADH and resazurin) as a standard. Raw spectrophotometry readings for wells containing cells and resazurin represent the amount of resorufin product produced in 1 h at 37°C . From this, the equimolar resorufin standards can be used to calculate the

amount of resazurin remaining and thus the turnover rate of resazurin. These data plotted as a Michaelis-Menten function were used to calculate the maximum turnover rate ($V_{\max}$) of within different cell lines and conditions (Figure 3.3).

As shown in Figure 3.3A, the $V_{\max}$ for MCF7 cells was higher than that of MDA231 cells regardless of the media type. There was no significant difference in $V_{\max}$ between media or in independent variable interaction (Figure 3.3A). There was however, a significant difference in $V_{\max}$ between MCF7 and MDA231 cells in serum⁺ media, with none in serum⁻ media (Figure 3.3B).

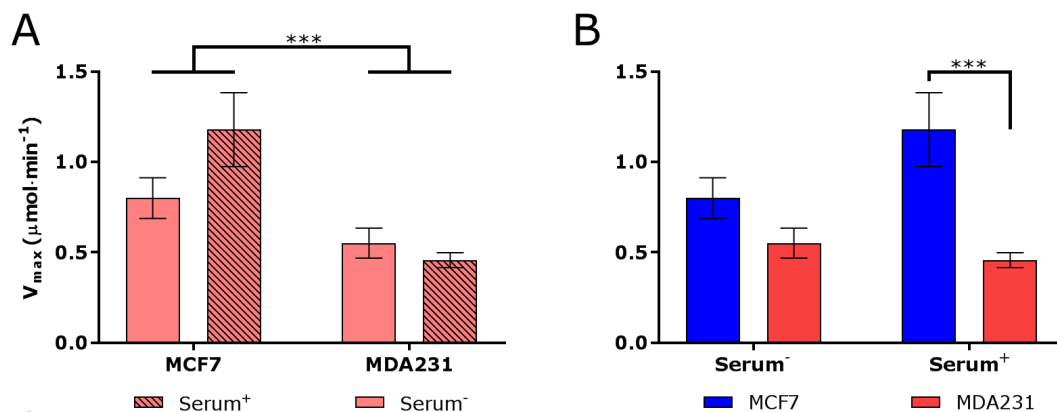


Figure 3.3 –NADH metabolism of MCF7 and MDA231 cells in high glucose. Maximal NADH turnover ($V_{\max}$) in MCF7 and MDA231 cells. Analysed by 2way ANOVA with *post hoc* two-stage linear step-up (ns, not significant; **A**: **, $P=0.0014$; **B**: ***, $P=0.0009$ $Q=0.0038$; $n=5$). A sum-of-squares F-test was employed to compare the fit of the Michaelis-Menten model with the allosteric sigmoidal model of kinetics, and in each case the Michaelis-Menten model fit data most accurately ($\alpha=0.05$).

3.2.1.3 MCF7 Cells Do Not Migrate and MDA231 Cells Are Highly Motile

To characterise the migratory behaviour of each cell line in 2D monolayer, a wound healing assay was performed, with cell and nuclear diameter of cells on the edge, or within the wound area measured with Zen Blue software. These acted as a measure of cell elongation and flattening. 0, 16 and 24 h after wounding. MCF7 cells did not migrate across the wound individually, but instead advanced as a front, notably growth was not arrested in this assay. By contrast MDA231 cells migrated to fully cover the wound in ≤ 16 h (Figure 3.4). MDA231 cells in the wound area were seen to be elongated and firmly attached, with MCF7 cells remaining more uniform in shape, and anchored to each other. There

were no significant changes in MCF7 nuclear diameter between 0 and 16 h post-wounding (Figure 3.5A-B); this contrasted with significant changes in MDA231 nuclear diameter, indicating cell spreading. MCF7 cell diameter, also remained unchanged over the course of the assay with MDA231 cells having significantly increased cell diameter (Figure 3.5C-D). Cell volume, or cell diameter: nuclear diameter ratio was significantly different between cell lines but, most significantly was unchanged between 0 and 16 h post-wounding in MDA231 cells (Figure 3.5E-F).

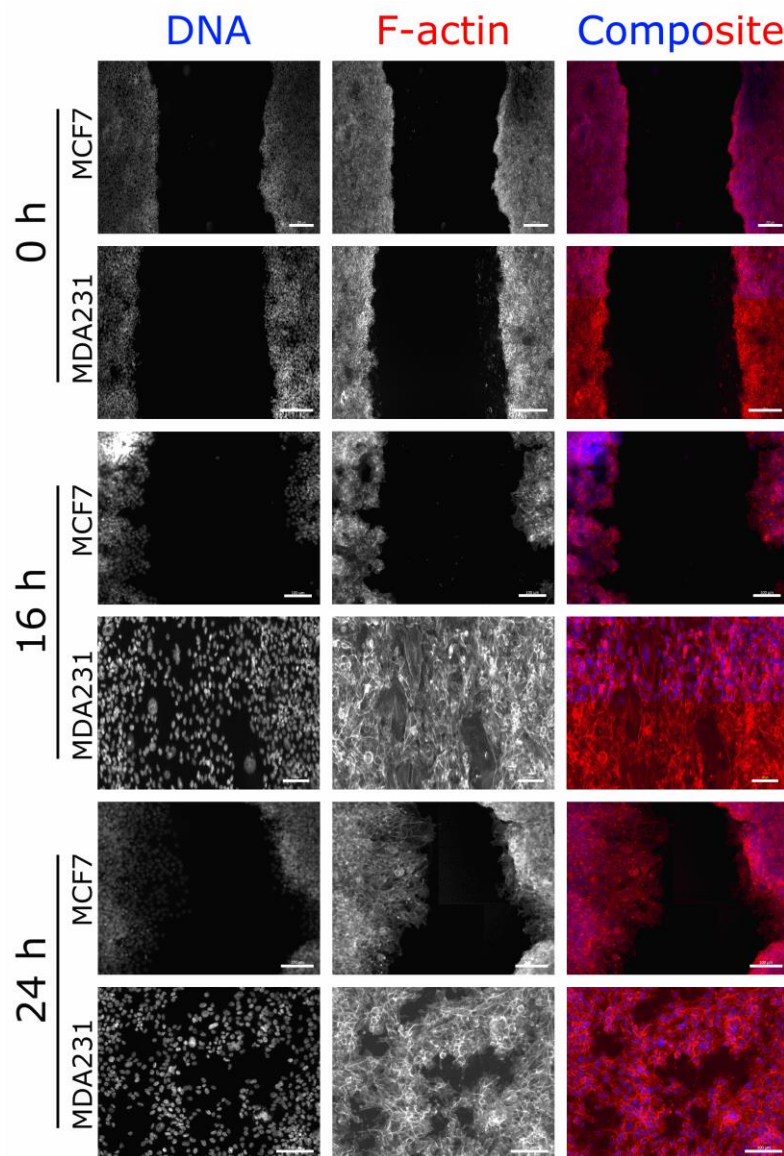


Figure 3.4 - Wound healing assays of MCF7 and MDA231 cells, 0 h, 16 h and 24 h after scratching. Scale bar 100 μ m.

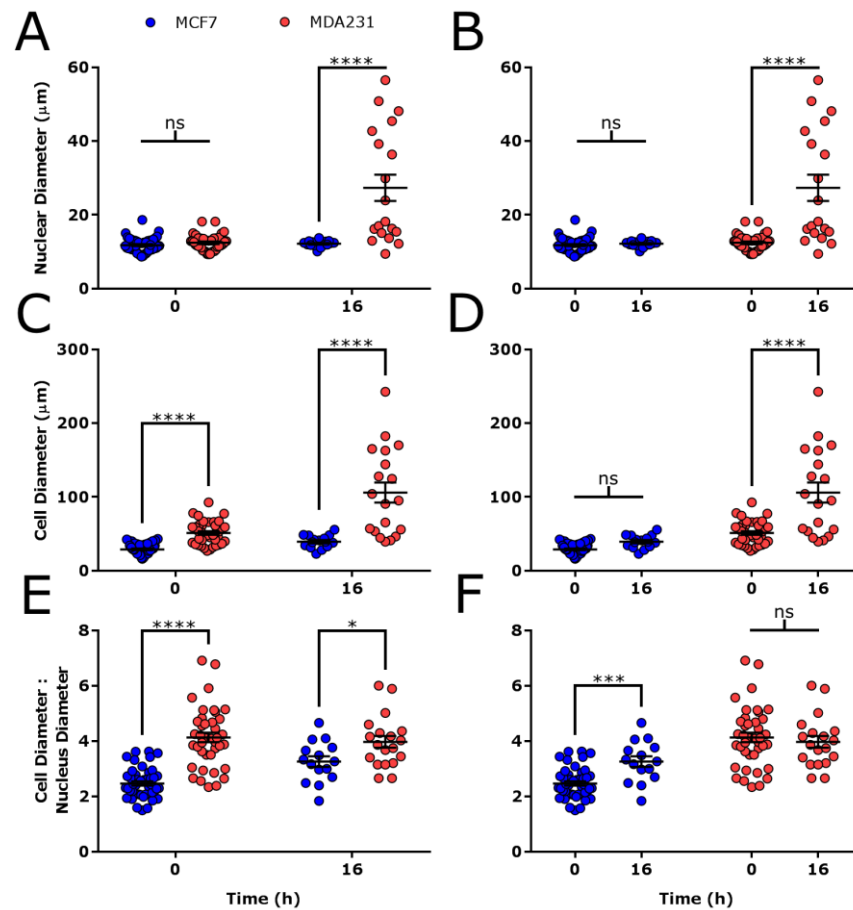


Figure 3.5 – Cell morphology of migratory MCF7 and MDA231 cells. **A-B**, nuclear diameter as an indicator of cell elongation and spreading of MCF7 and MDA231 cells at 0 and 16 h post-wounding. **C-D**, cell diameter as an indicator of cell elongation of MCF7 and MDA231 cells at 0 and 16 h post-wounding. **E-F**, cell diameter:nuclear diameter ratio as an indication of cell spreading of MCF7 and MDA231 cells at 0 and 16 h post-wounding (ns, not significant; *, $P=0.0150$ $Q=0.0032$; ***, $P=0.0018$ $Q=0.0005$; ****, $P<0.0001$ $Q<0.0001$).

3.2.2 Characteristics of Breast Cancer Cells in Dynamic Suspension Culture

Given the link between cell-cell contacts and inhibition of proliferation (Theveneau & Mayor, 2012), it was hypothesised that the mitotic profiles of each cell line would differ in suspension with MDA231 cells maintaining a highly proliferative phenotype due to lower cadherin and claudin expression. It was also hypothesised that the MCF7 aggregated cells would have greater contact inhibition and thus proliferate less when aggregated. To assess this, immunoblotting, qPCR and flow cytometry were employed to assess breast cancer cells in suspension.

3.2.2.1 Dynamic Suspension Culture Conditions

When cultured in dynamic suspension the culture was buffered against media acidification with 25 mmol·l⁻¹ HEPES (pH 7.5) and there was an air gap at the surface that allowed gas exchange. Orbital shaking generated a rigid-body vortex within the culture vessel. There are two types of vortices: rigid-body and irrotational; a rigid-body vortex has higher angular velocity proportional to the distance from the centre, in which case particles will traverse the vortex in the same time, regardless of their position relative to the vortex centre (Figure 3.6A). In contrast, an irrotational vortex is one where the angular velocity of the liquid is greater at the centre than the edge, such as that caused by a magnetic stirrer centrally placed in the vessel (Figure 3.6B).

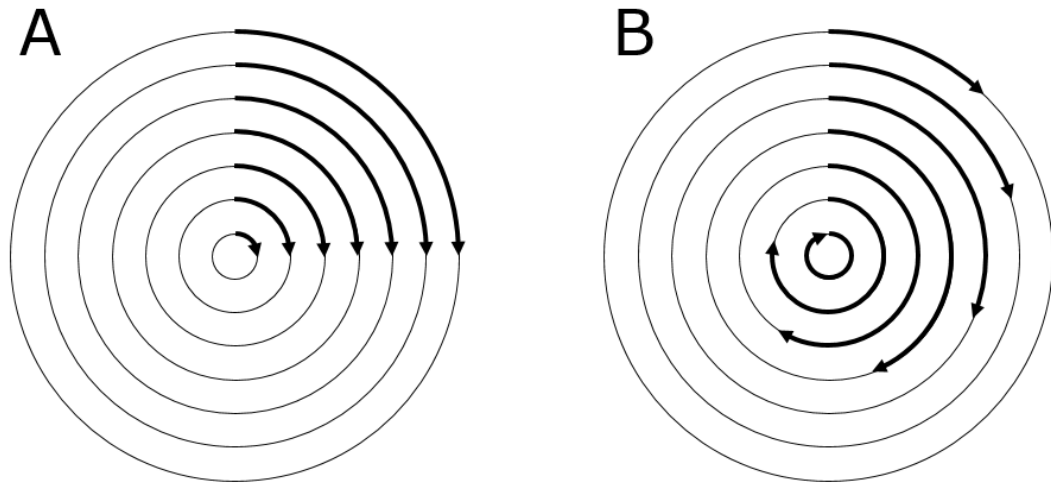


Figure 3.6 – Vortices schematic. **A**, rigid-body vortex, particles traverse the vessel in the same time. **B**, irrotational vortex particles traverse the vessel faster the closer they are to the centre.

3.2.2.2 MCF7 and MDA231 Cells Are Viable in Dynamic Suspension Culture

As both MCF7 and MDA231 cells are derived from a sub-population which had successfully metastasised into the pleural cavity it was proposed that they must be able to evade anoikis and survive in suspension. To test this, cells were grown in an orbital incubator for 96 h. Samples were taken at 24 h intervals to test cell viability by flow cytometry. Both cell lines remained viable in suspension for the duration of the assay with MCF7 at ~95% viable for the duration; and MDA231 increasing significantly from ~92.5% to ~97% at 48 h, ~98% at 72 h and ~95% at 96 h (Figure 3.7). MCF7 cells readily aggregated in suspension in contrast to MDA231 cells which were rarely seen in clusters of more than ~10 cells.

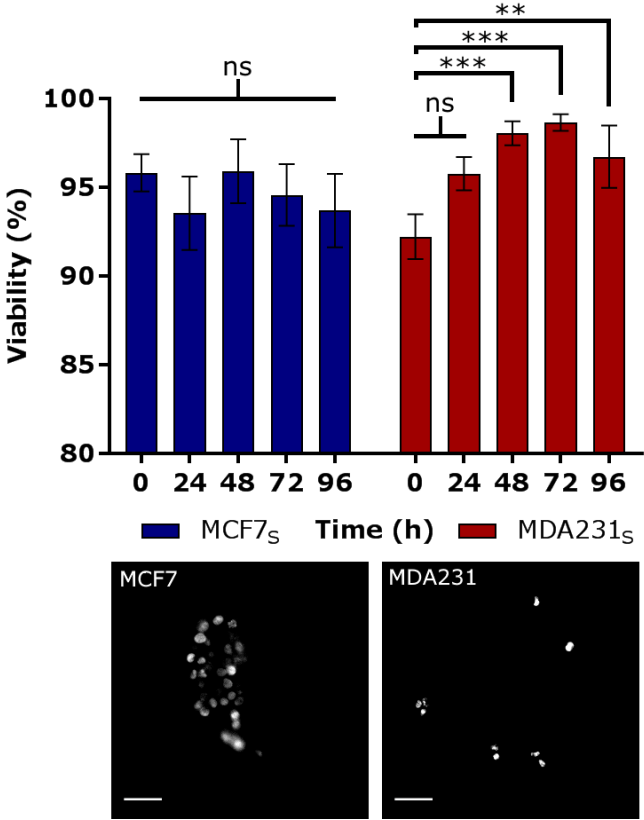


Figure 3.7 –Cell viability of MCF7 and MDA231 suspension cultures. Viability profile of MCF7 and MDA231 suspension cultures (ns, not significant; **, P=0.0089 Q=0.0218; 48: ***, P=0.0009 Q=0.0033; 72: ***, P=0.0003 Q=0.0021). Scale bar 50 μm.

3.2.2.3 MCF7 and MDA231 Cells Downregulate Mitotic Effectors in Suspension

To determine the mitotic state of MDA231 and MCF7 cells in suspension three independent protein extracts were made from MCF7 and MDA231 cells grown in suspension and compared to cultures grown in 2D monolayer and analysed by immunoblotting. Ki67 was used as a marker for cells between G₁ and M-phase. INCENP (inner centromere protein), AURKB (aurora kinase B) and borealin are components of the CPC (chromosomal passenger complex), essential for mitosis to occur. pH3 is a marker of mitotic chromosomes.

As shown in Figure 3.8A the proliferation markers (Ki67, INCENP, AURKB, borealin and phosphor-histone 3^{S20}) were less abundant in suspension culture samples of both cell lines by comparison with 2D. The most marked differences were exhibited by MDA231 cells, which showed a complete loss of Ki67, INCENP and borealin in MDA231_s samples. Low levels of the mitotic marker, pH3, in MDA231_{2D} samples are notable due to similar growth rates of MCF7 and MDA231 cells in standard 2D culture conditions (section 3.2.1.1, Figure 3.2). Blots were quantified using Fiji software (Figure 3.8B), with protein abundance normalised to β -actin and expressed as relative to 2D culture; this was then expressed as log₂ fold change to be consistent with qPCR data. Upon quantitation, of n=3, changes in INCENP, AURKB and borealin were not significant in MCF7 samples; however, Ki67 and pH3 were significantly downregulated. In contrast, when comparing MDA231_s to the control (MDA231_{2D}); all proteins assessed were significantly down-regulated. The AURKB and borealin (*CDCA8*) results were corroborated by qPCR of MDA231 cells in 2D and suspension. This acted to assess changes on the transcriptional level of AURKB, the catalytic component of the CPC along with borealin and survivin, all of which are essential components of the CPC. *BIRC5*, encoding survivin, was assessed alongside other BIR-family genes of interest (section 3.2.2.4).

ACTB (encoding β -actin) and *GAPDH* acted as reference genes for qPCR experiments, they were selected from a panel of genes (section 8.2.2); also establishing β -actin as an appropriate protein loading control. *TUBB* was originally chosen as a reference gene but showed a small but significant down-regulation in suspension culture samples (Figure 3.8C). *AURKB* and *CDCA8*, encoding borealin, were ~ 5 fold and ~ 6 fold downregulated respectively, showing that changes seen at the protein level were due to changes in transcription.

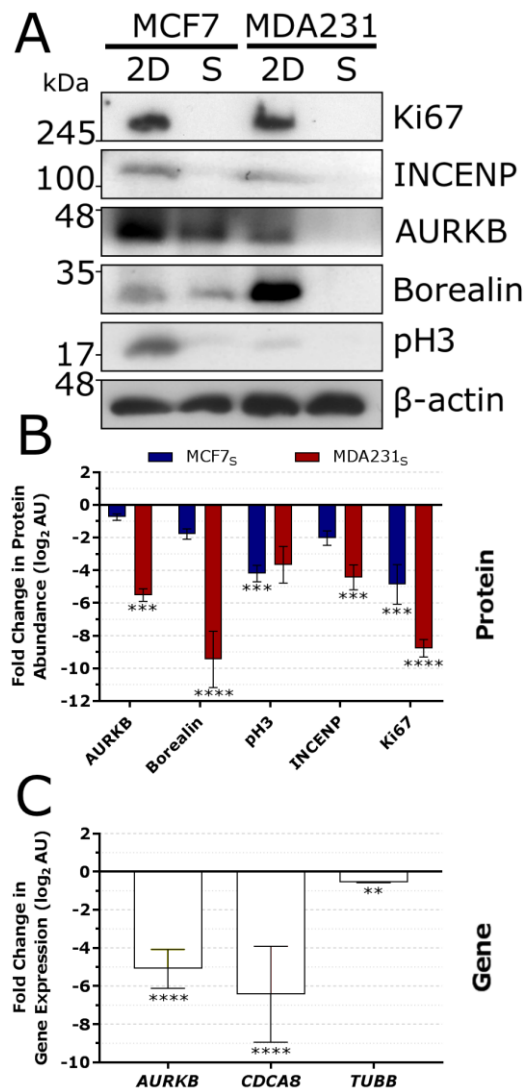


Figure 3.8 – Comparison of mitotic protein abundance in MCF7 and MDA231 cells grown in monolayer (2D) and in suspension (S). **A**, immunoblot of Ki67, INCENP, AURKB, borealin and histone 3^{S20~P}. **B**, quantification of immunoblots (MDA231_S relative to 2D) represented in **A**, analysed by 2-way ANOVA and *post hoc* two-stage linear step-up (AURKB: $P=0.0009$ $Q=0.0018$; borealin: $P<0.0001$ $Q<0.0001$; pH3: $P=0.0003$ $Q=0.0024$; INCENP: $P=0.0028$ $Q=0.0039$; $n=3$). **C**, qPCR analysis of *AURKB*, *CDCA8*, and *TUBB* in MDA231_S (**, $P\leq 0.01$; ****, $P\leq 0.0001$; $n=3$). All data plotted with SEM.

3.2.2.4 BIR-Family Proteins Are Downregulated in MCF7 and MDA231 Suspension Cultures

The BIR-family of proteins are multifunctional and have activities in both mitosis and apoptosis, with many of the family exhibiting E3-ligase activity. Given the downregulation of mitotic protein and gene expression (section 3.2.2.3) it was hypothesised that *BIRC* family proteins not necessary for mitosis (*BIRC3* and *XIAP*) would be unchanged or upregulated to enhance apoptotic inhibition in suspension culture samples. Conversely it was hypothesised that *BIRC2* (cIAP1) and *BIRC5* (survivin) would be down-regulated due to their necessity in mitosis and the lack of survivin protein in the suspension culture samples. Analysis of protein extracts from both MCF7 and MDA231 cells revealed changes in survivin expression but no change in XIAP (Figure 3.9A-B). No cIAP1/2 (encoded by *BIRC2/3*) was detected; however, a non-specific secondary band ~5 kDa larger than XIAP was not present in MDA231s samples. This has been shown to be an unspecific band (Hou, Allan & Clarke, 2017). Bioinformatic analysis of the antibody epitope suggested this band to be either cIAP1 or 2 (data not shown) but this could not be verified. Protein data were corroborated by qPCR analysis of MDA231s cells and revealed that all *BIRC* family genes were down-regulated (Figure 3.9C): *BIRC2* and *BIRC3* decrease by 3.36 fold and 3.05 fold respectively, and *XIAP* was 0.95 fold downregulated. In addition, the canonical isoform of *BIRC5* was downregulated 5.34 fold, along with its mitotically-incompetent isoform *BIRC5-2* and *BIRC5-3*, which were downregulated 4.82 fold and 8.76 fold respectively. Total *BIRC5* transcript (*BIRC5*^{3'UTR}) was also shown to be significantly down-regulated 5.78 fold.

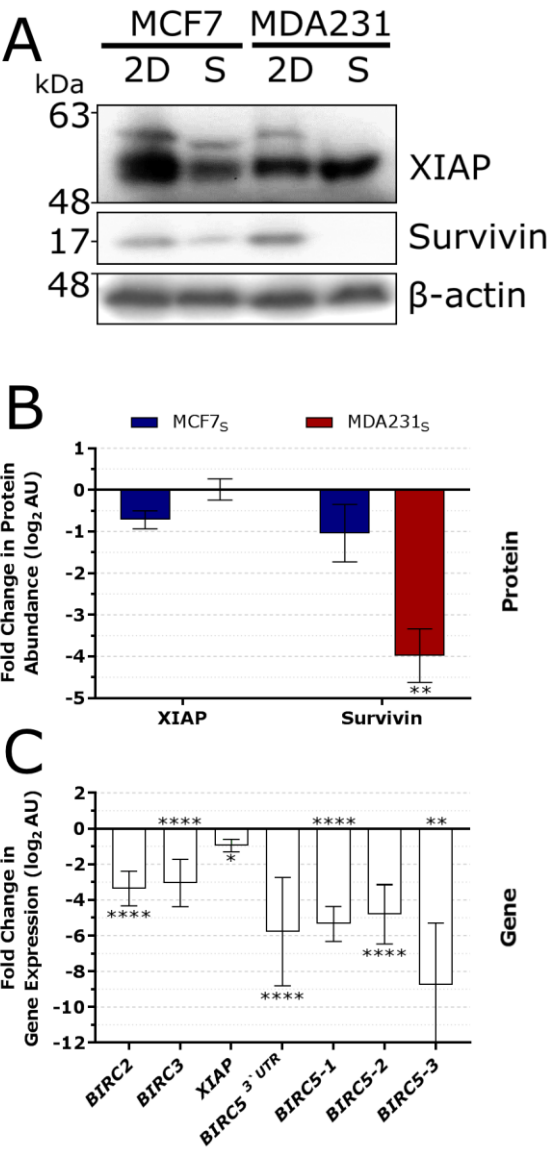


Figure 3.9 – Comparison of BIR-family protein abundance in MCF7 and MDA231 cells grown in standard conditions (2D) and in suspension. A, immunoblot of XIAP and survivin. **B**, quantification of immunoblots represented in **A**, analysed by 2-way ANOVA and *post hoc* two-stage linear step-up (survivin: $P=0.0044$ $Q=0.0046$; $n=3$). **C**, qPCR analysis of *BIRC2*, *BIRC3*, *XIAP* and *BIRC5* (*, $P<0.05$; **, $P<0.01$; ****, $P<0.0001$; $n=3$). All data plotted with SEM.

3.2.3 **MDA231 Cells in Suspension Do Not Progress Through the Cell Cycle**

Imaging of MCF7 and MDA231 cells in suspension suggested a lack of mitotic cells in MDA231 suspension cultures (Figure 3.10), which is consistent with expression data of mitosis-related protein abundance with gene expression (3.2.2). As a result; the stage at which suspended MDA231 cells were in the cell cycle was assessed. MDA231s cultures were sampled at 24 h intervals for 96 h and cell cycle analysis of DNA content was carried out using a MUSE cell cycle kit. Viability was assessed in parallel. Throughout the 96 h time course viability did not drop below 80% (Figure 3.10A). Analysis of DNA content by flow cytometry with the same periodicity showed no change in 2n, S, 4n or >4n cells as a proportion of their population throughout the assay.

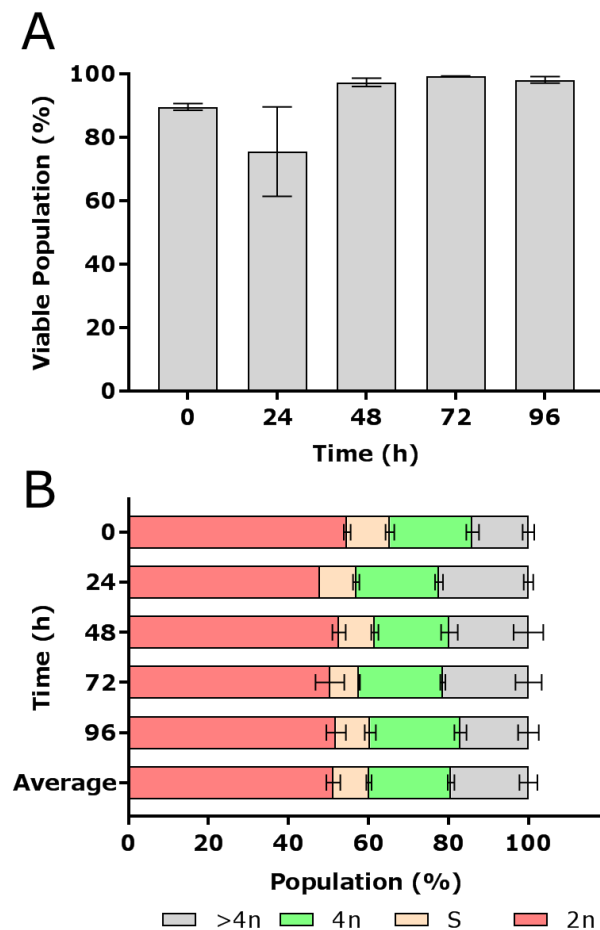


Figure 3.10 – Cell cycle distribution of MDA231 cells at 24 h intervals over 96 h. **A**, viability. **B**, DNA content profile. n=3 for all times but 96 h, n=2. All data plotted with SEM.

3.2.3.1 MDA231 Cells Arrest Growth After 24 Hours in Suspension Independent of Cell Cycle Stage

As the population of MDA231 cells in suspension appeared static, cell cycle and viability experiments were repeated in the presence of dimethylenastron (DMA), which causes monopolar spindle formation by inhibiting activity of the kinesin-like motor protein, KIF11. This prevents chromosome segregation, and therefore stalling in prometaphase. It was hypothesised that MDA231 cells cultured as a monolayer would arrest in mitosis and their viability would fall, whilst MDA231_s cells would be unaffected by DMA treatment.

After only 24 h both DMA treated MDA231_s and MDA231_{2D} cell populations changed in demographic from primarily 2n, to primarily 4n (Figure 3.11A-C). No such change was observed in untreated MDA231_s. Surprisingly, the viability of DMA treated MDA231_s cells did not follow the same trend as DMA treated MDA231_{2D} cells (Figure 3.11D-E). DMA treated MDA231_{2D} cells had a highly significant drop in viability from ~90% to ~65% over the time course, but there was no significant change in either treated, or untreated MDA231_s cells (Figure 3.11D). Similarly, at each time sampled, there was a significant difference between treated MDA231_{2D} and both treated and untreated MDA231_s at 24, 48, 72 and 96 h (Figure 3.11E). Overall this shows that the DMA treatment works to arrest cells in M-phase for 2D monolayer and suspension cultures, but the key observation is that suspension culture cells are less severely affected, in terms of viability, and significantly fewer cells get arrested in M-phase after 24 h.

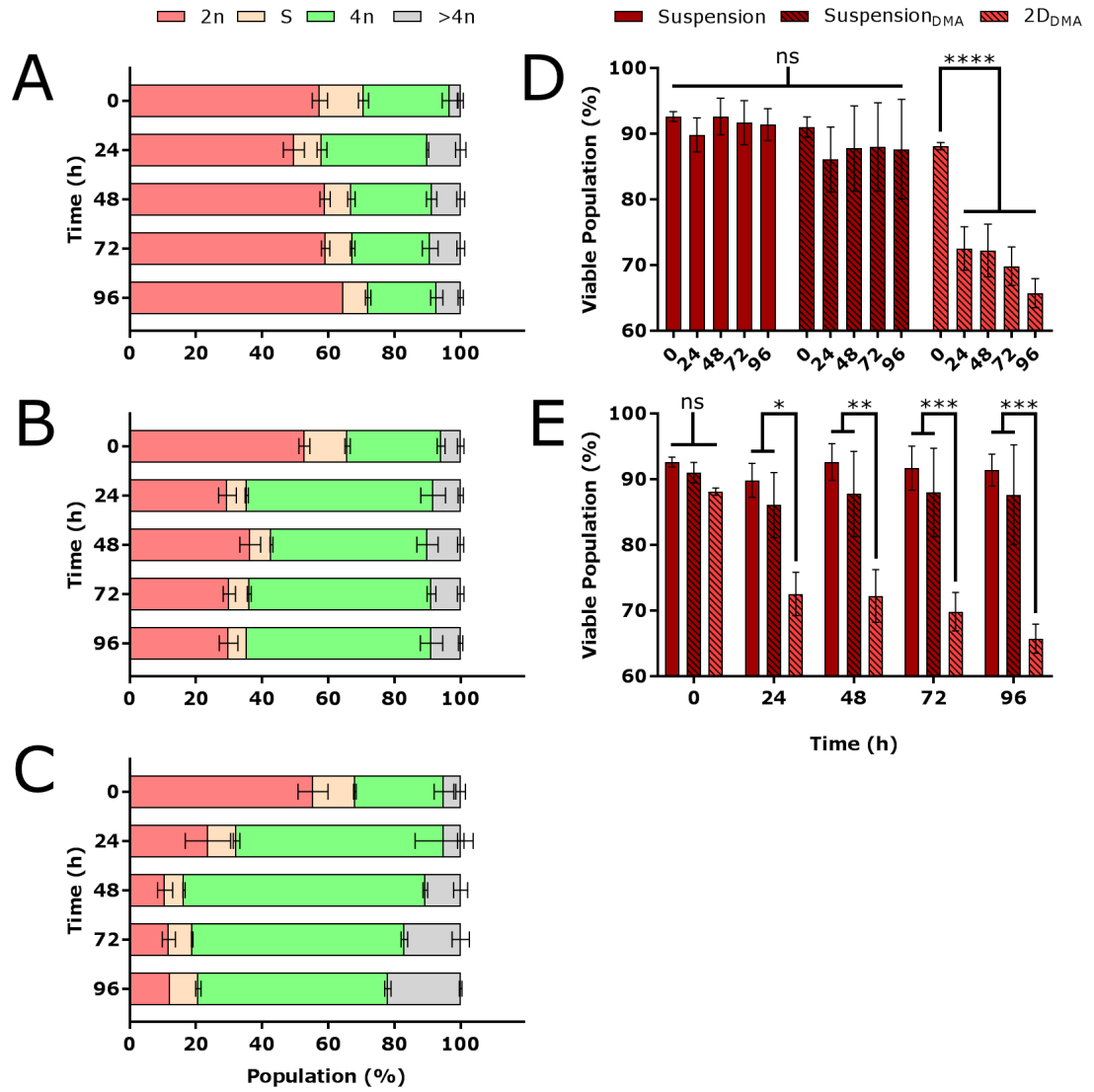


Figure 3.11 – Cell cycle distribution of DMA-treated MDA231 in suspension and 2D. **A-C**, cell cycle distributions of MDA231 suspension culture (**A**), MDA231 suspension culture with DMA treatment (**B**), and MDA231 monolayer culture with DMA treatment (**C**). **D-E**, viability profiles of each condition and time. Data were analysed by 2-way ANOVA with *post hoc* two-stage linear step-up, constrained across treatments (**D**), or constrained through time (**E**) (ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; n=3).

Data shown in Figure 3.11A-C were replotted, allowing comparison of all subpopulations of all samples in terms of DNA content (Figure 3.12). The 2n population of untreated MDA231_s cells stayed constant with no significant changes over the time course. However, both treated samples, regardless of culture method, showed significant drops in their respective 2n populations over the first 24 h. This trend continued through to 48 h for DMA-treated 2D cultured cells, whereas DMA-treated suspended cells showed no changes from 24 h through to 96 h (Figure 3.12A-B), with no change in S-phase cells throughout (data not shown). A similar set of changes was observed in 4n and >4n cells, such that as the 2n population dropped, the 4n population increased proportionally up to 48 h (Figure 3.12C-F). At this point 2n, 4n and >4n populations of DMA-treated MDA231_s cells, remained constant. Conversely, DMA-treated MDA231_{2D} cells continued to try to divide such that when the 4n population appeared stay static, the >4n population continued to increase significantly through from 24 h to 96 h, as exemplified by combining the 4n and >4n populations, see Figure 3.12G-H.

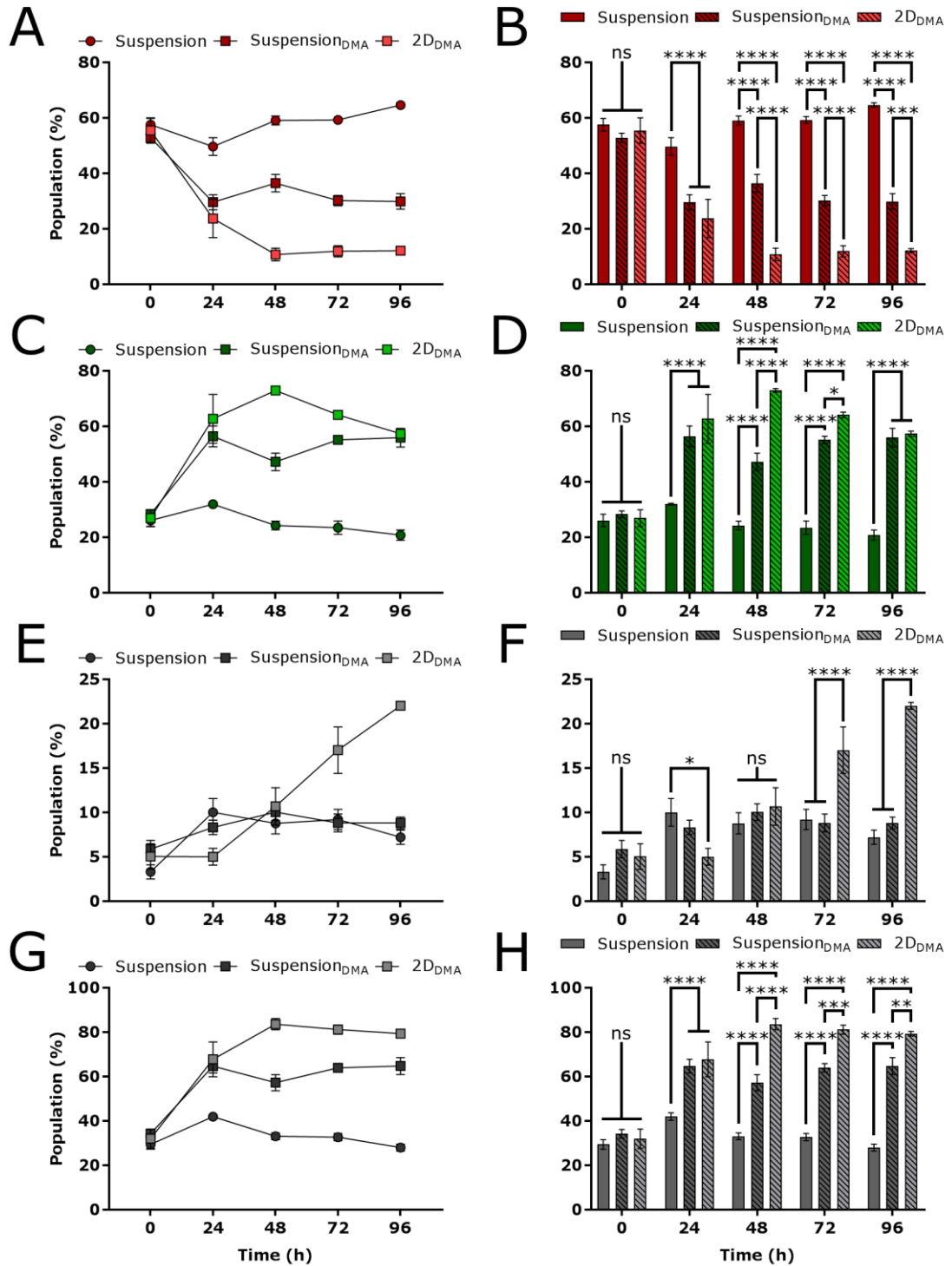


Figure 3.12 – DNA content profiles of DMA-treated MDA231 cells cultured in suspension and 2D. Changes in 2n (A-B), 4n (C-D), >4n (E-F) and ≥4n (G-H) DNA content of MDA231 cells over 96 h in 2D and suspension, with and without DMA treatment. The same data are plotted in two different ways to show the profile over time (A, C, E, G) and the statistical significance of any changes by comparison with other treatment groups (B, D, F, H). Data were analysed by 2-way ANOVA with *post hoc* two-stage linear step-up, (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$).

3.2.3.2 MDA231_s Cells Do Not Accumulate in S-phase

At 48 h MDA231_s cells were not progressing into mitosis. There was also no evidence for a build-up in S-phase but due to sub-100% viability it was proposed that the 5% of cells in S-phase were the same 5% that were no longer viable. One possible explanation for this could be that the cell cycle was still slowly progressing, but cells in S-phase, unable to finish duplicating their genome, underwent apoptosis. To determine whether this was the case, MDA231 cells were therefore submitted to a thymidine block to arrest cells in S-phase. This treatment upsets the delicate balance between adenosine, cytidine, guanosine and thymidine required for genome replication in S-phase. Viability and cell cycle profiles monitored over 96 h. As shown in Figure 3.13, there were no significant changes in cell viability over the time course; however, at 48 h there was a significant rise in the S-phase population in thymidine-treated 2D monolayer samples (Figure 3.13). This change was not seen in treated suspension samples or control samples. By 96 h post-treatment, the S-phase population had almost returned to normal in 2D_T samples with a significant difference observed only between 2D_T and S samples.

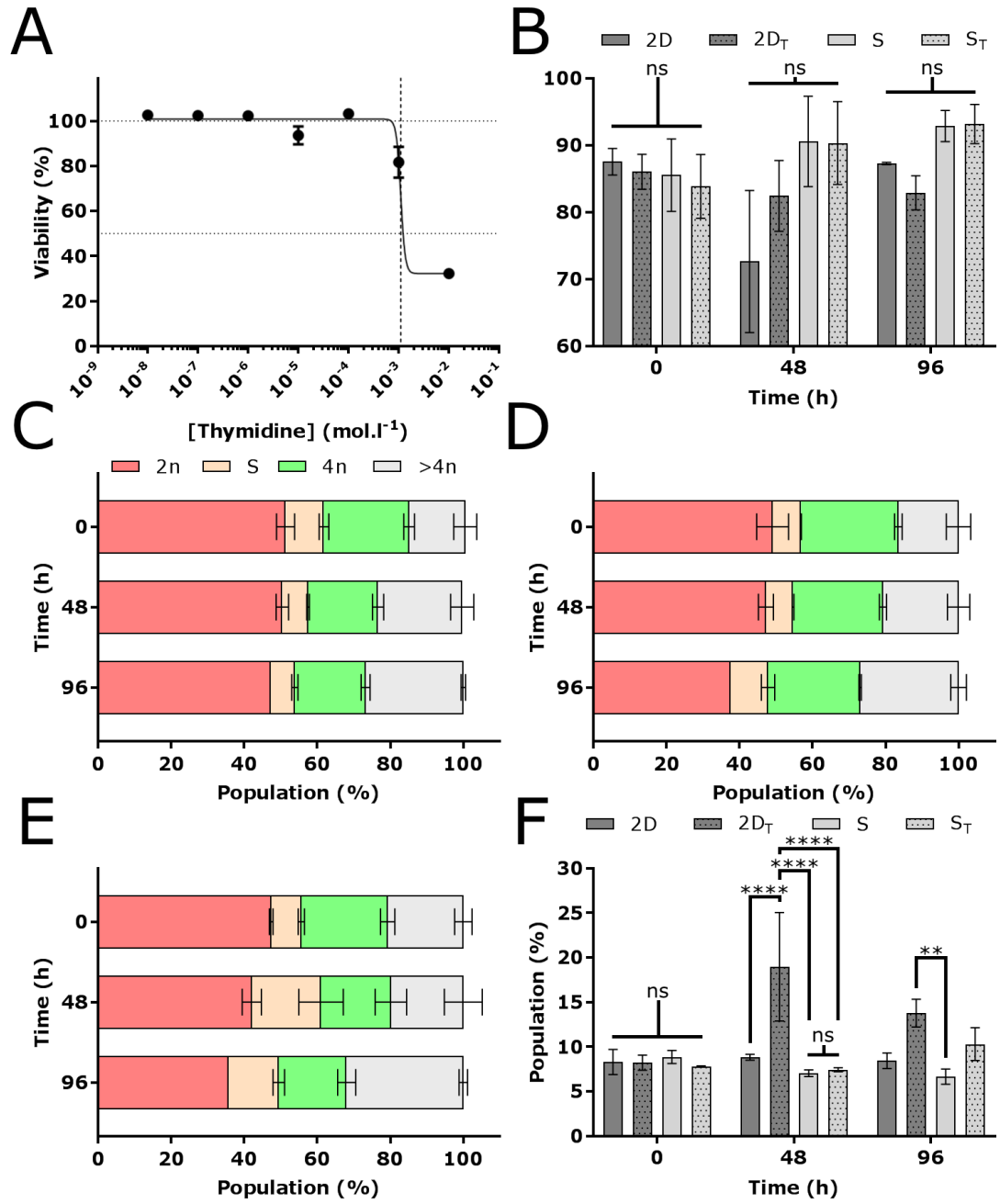
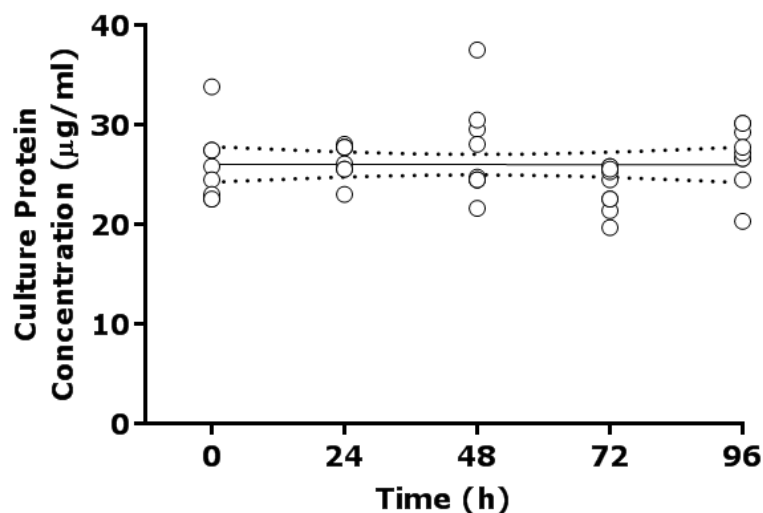


Figure 3.13 - Viability and cell cycle profiles for thymidine treated MDA231 cells in suspension and 2D. **A**, drug-response curve of MDA231 monolayer culture to thymidine treatment. **B**, viability profile of thymidine (T)-treated MDA231 cells (ns, not significant). **C**, untreated suspension culture. **D**, T-treated suspension culture. **E**, T-treated monolayer MDA231 culture. **F**, S-phase population of T-treated (**, $P=0.0035$ $Q=0.0303$; ****, $P<0.0001$ $Q<0.0001$).

3.2.3.3 Biomass of MDA231_s Cultures Remains Constant

Finally, to determine any change in suspension culture cell number, a protein-concentration of whole cell extracts was determined by Bradford assay, which showed no correlation between time and culture biomass, indicating a static growth of the culture (Figure 3.14).



	<i>r</i>	R ²	P-value
Pearson's Rank	-0.0107	0.0001144	0.9864
Spearman's Rank	0.2	-	0.7833

Figure 3.14 – Protein concentration of MDA231 suspension cultures over time. Pearson's and Spearman's rank correlation tests showed no significant correlation between time and culture protein concentration over 96 h. Linear line of best fit is represented by the solid line with 95% confidence interval represented by the dotted line.

3.2.3.4 Cell Cycle Arrest and Chemo-Resistance in MDA231_s Cells

Broad-spectrum chemotherapeutic agents target highly proliferative cells; as these cells are a key component in tumourigenesis. Considering the observations made in MDA231_s cells, the efficacy of paclitaxel, a chemotherapeutic agent which targets rapidly proliferating cells, was tested against MDA231_s cells. To do this, a paclitaxel dose-response curve was generated using monolayer MDA231 cells. From this, the IC₅₀ concentration for monolayer MDA231 cells was calculated, and this was used to treat cells in suspension culture. Cell viability and cell cycle profiles were then assessed by flow cytometry. Paclitaxel is a microtubule hyper-stabilising agent which prevents microtubule depolymerisation and turnover. This can cause adverse effects in both mitotic and non-mitotic cells.

No changes were observed in viability, 4n, >4n or ≥4n populations of cells over 48 h (Figure 3.15B-H). However, 96 h post-treatment, a significant decrease in 2D_{PTX} cell viability was observed by comparison with S and S_{PTX} samples (Figure 3.15B), which was concurrent with an increase in the >4n population of 2D_{PTX} cells relative to untreated 2D samples (Figure 3.15C-E,G).

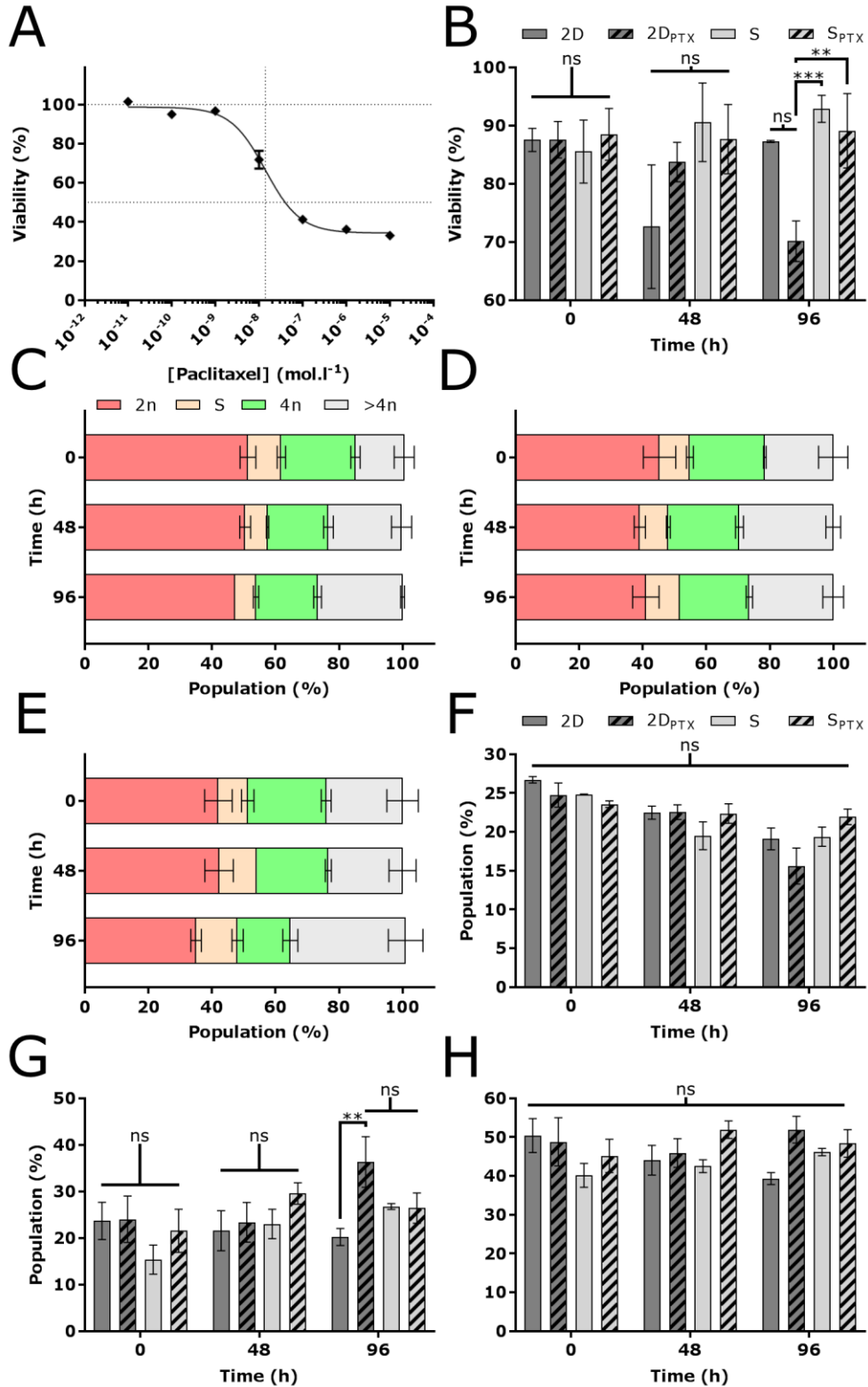


Figure 3.15 – Viability and cell cycle profiles of paclitaxel treated MDA231 cells in suspension and 2D. **A**, response curve of MDA231 monolayer culture to paclitaxel. **B**, viability profile of paclitaxel (PTX)-treated MDA231 cells (ns, not significant; **, P=0.0047 Q=0.0299; ***, P=0.0006 Q=0.0073). **C**, untreated suspension culture. **D**, PTX-treated suspension culture. **E**, PTX-treated monolayer MDA231 culture. **F**, 4n population of PTX-treated MDA231 cells. **G**, >4n population of PTX-treated MDA231 cells (**, P=0.0016 Q=0.0461). **H**, ≥4n population of PTX-treated MDA231 cells. n=3.

3.2.4 Evasion of Anoikis Through Changes in Apoptotic Responses in MDA231 Cells

Having confirmed the cause for growth inhibition and the associated effects in MDA231s cells, the next characteristic of interest was the ability for cells to survive in transition from adherent to non-adherent culture conditions, that is, to evade anoikis. To understand the anti-anoikis machinery active in MDA231 cells, qPCR was used to test for changes in gene expression related to anoikis. Candidate genes related to anoikis were selected based on signalling nodes, such as at the DISC, and at the most upstream points in the apoptotic cascade (Figure 3.16). Relative to the cell membrane transduction bottle-neck, the pro-apoptotic gene *FADD* was 1.61 fold downregulated and the anti-anoikis gene *CFLAR* was upregulated 1.12 fold. At the mitochondrial level of the apoptotic machinery, *PMAIP1* (encoding NOXA) was significantly downregulated 3.86fold ($P \leq 0.0001$); and *BID* was 2.7 fold downregulated.

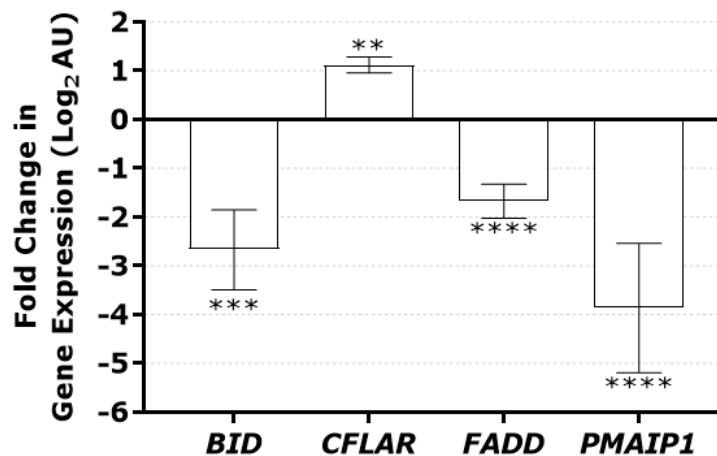


Figure 3.16 – Changes in anoikis-related gene expression between MDA231 2D and suspension culture systems. Plotted with SEM (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n=3$).

3.2.4.1 MDA231 Mitochondrial Health is Improved in Dynamic Suspension Culture

To determine the mitochondrial health of MDA231 cells in suspension they were imaged live with MitoTracker Green, a cell permeable mitochondrial dye that enters the mitochondria and accumulates thus highlighting all mitochondria; and TMRE, which enters mitochondria with a large membrane potential more readily but does not accumulate, thus highlighting highly active mitochondria. The combination of the two allows definition of inner mitochondrial membrane polarisation. Due to the downregulation of *BID* shown by qPCR, it was hypothesised that cells in suspension would have an increased IMM potential and thus stronger TMRE signal. To determine the mean average, minimum and, maximum fluorescence intensity for MitoTracker Green and TMRE in each culture condition, 30 random fields were acquired at 60x magnification and processed in Fiji. Figure 3.17A shows an example image comparing MDA231 cells in suspension and 2D, and their associated quantification of IMM polarisation in Figure 3.17B. Cells in suspension were found to have more polarised IMM when measured by mean signal ratio. However, by analysing minimum and maximum signal ratios, it was apparent that this was due to a subset of cells which had considerably more polarised IMM, with cells showing no significant difference in minimum signal ratio. This indicates that there is a subset of cells in suspension culture which are able to restore IMM polarisation, this requires decreased OMM permeability which is associated with a decrease in pro-apoptotic factors, such as BID. This is indicative of healthy mitochondria.

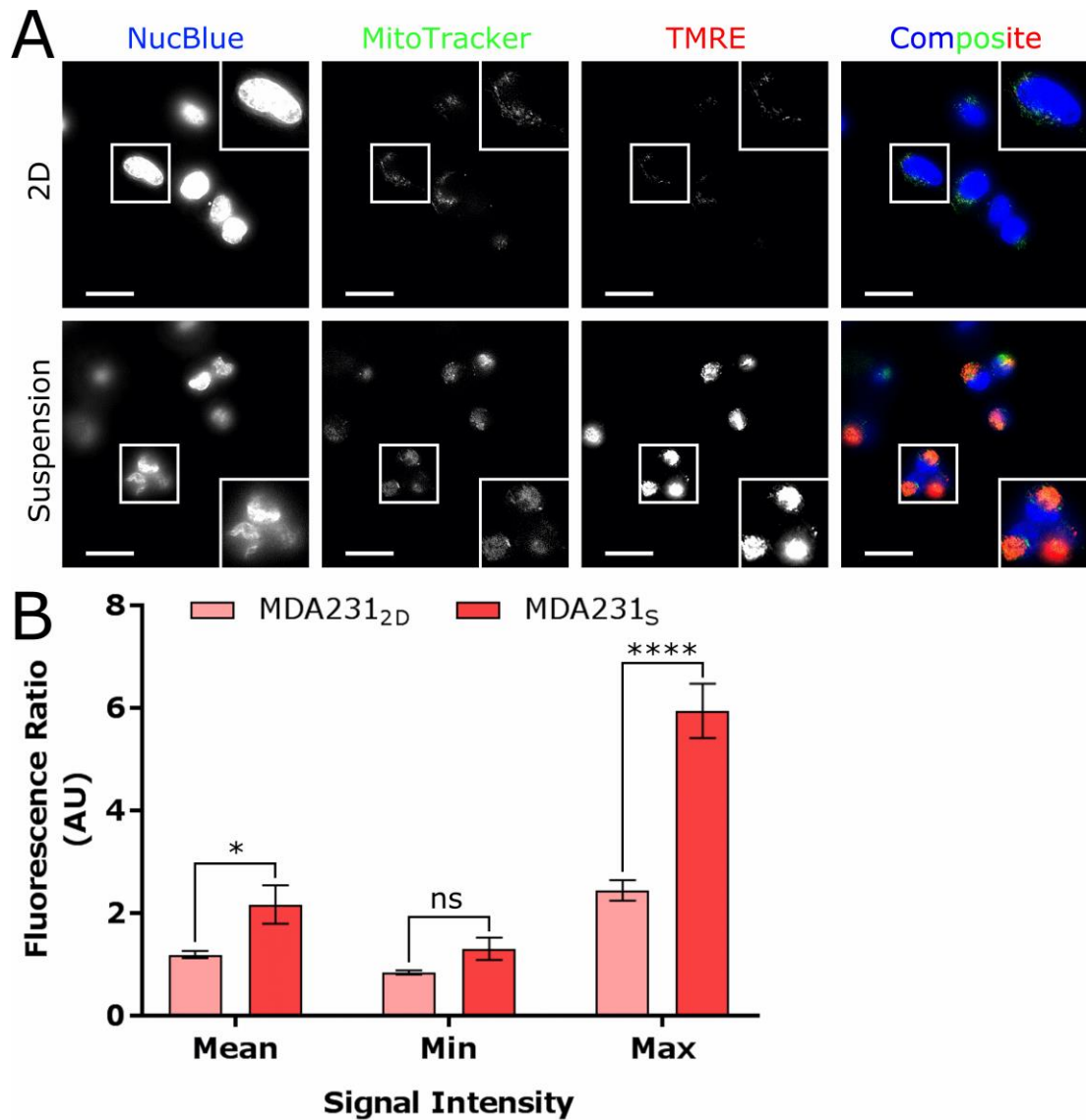


Figure 3.17 – Mitochondrial polarisation in MDA231 suspension culture vs. 2D monolayer. **A**, MDA231 cells in 2D and suspension stained with NucBlue, MitoTracker Green and TMRE, scale bar 20 μ m. **B**, quantified fluorescence intensity from 30 random fields of view for MDA231 cells cultured in 2D and suspension (ns, not significant, $P=0.2659$, $Q=0.0931$; *, $P=0.0197$, $Q=0.0104$; ****, $P<0.0001$, $Q<0.0001$; $n=3$; $N=200$).

3.2.5 **Changes in MDA231s Gene Regulation are Linked to Transcriptional Master-Regulators.**

Considering the downregulation of all the *BIRC* family genes in combination with *AURKB*, *TUBB*, *CDCA8*, *BID*, *FADD* and *PMAIP1*, it was hypothesised that these genes were co-regulated. To deduce which transcription factors could identify promoters of the genes mentioned, and which of these could identify, multiple genes, the transcription element listening system (TELIS) bioinformatic tool on the TRANSFAC database was employed. The results were then filtered for transcription factor consensus sequences found in all eight of the genes of interest. This tool revealed no significant hits above the false discovery rate (FDR) for promoter elements in all genes of interest. However, when genes were limited to the *BIRC* family and mitosis-related genes, FOXD3 (H2H) was identified as the top hit with a frequency ratio of 4.4, meaning FOXD3 can identify 4.4 times more consensus sequences in these promoters compared to the genome-wide average (Table 3.3A). MYB was identified the top hit for the downregulated anoikis related genes searched with a frequency ratio of 4.2 (Table 3.3B).

Table 3.3 – TELIS top hits identifying genes of interest. TELIS analysis of all mitosis-related genes of interest including *BIRC* family genes (**A**) and all downregulated anoikis genes of interest (**B**).

A

Transcription Factor	Frequency (z-test)
FOXD3 (H2H)	$z=3.66$ $P=0.0002$ Sample mean=2.000 Population mean=0.453 Ratio=4.4

B

Transcription Factor	Frequency (z-test)
MYB	$z=2.88$ $P=0.0040$ Sample mean=1.333 Population mean=0.317 Ratio=4.2

3.2.6 Characteristics of Re-Adherent Breast Cancer Cells

Once a circulating tumour cell has survived suspension, it must be able to adhere at a new location to form a secondary tumour. To test if this was possible with MCF7 and MDA231 lines, cells grown in suspension for 96 h were seeded at low density on petri dishes and colony formation was assessed 7 days later.

MCF7 cells formed successful colonies (Figure 3.18), demonstrating that they maintained adherent potential in suspension. Interestingly, MCF7 cell aggregates that formed in suspension did not readily disperse once adhered but remained in a 3D colony and exhibiting little migration (Figure 3.18). In contrast, MDA231 cells were able to migrate once adhered; forming both large and small colonies, not dissimilar from cells handled in normal tissue culture conditions (Figure 3.18).

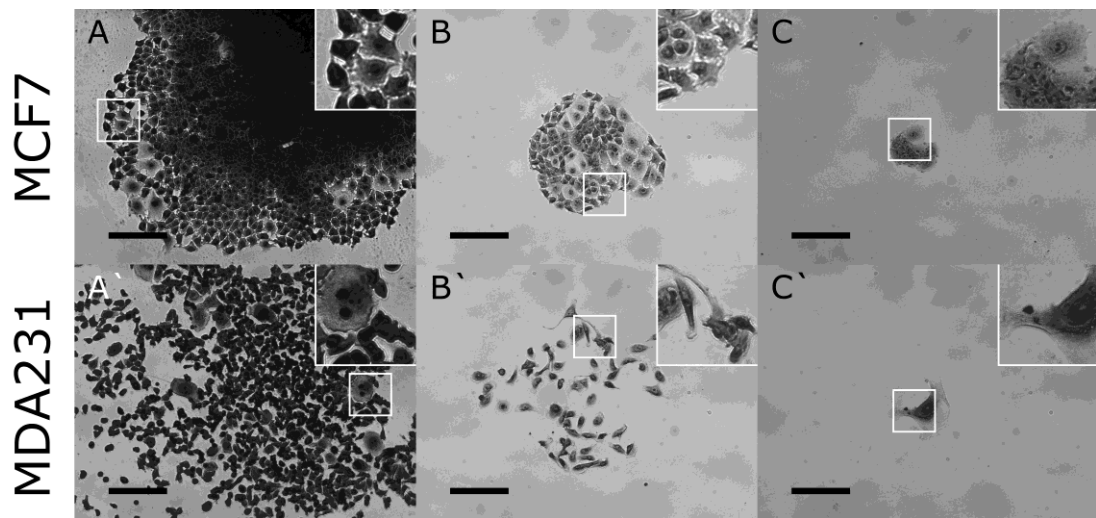


Figure 3.18 – Colony formation “re-adhesion assay”. Density of colonies decreases from left to right. Scale bar: 150µm; n=3.

3.2.6.1 MDA231 Cells Reactivate the Cell Cycle Within 24 h of Re-Adhesion

To determine the proliferative potential of cells that had re-adhered, protein extracts were made from 96 h suspension cultures and from 96 h suspension cultures that had been transferred to 2D monolayer for 24 h. Samples were processed for immunoblotting as previously and extracts from 2D cultured cells were used as a control. It was hypothesised that MCF7 cells would revert their proliferative potential to the rate prior to suspension culture; as the cell cycle was not arrested when in suspension. In contrast, it was hypothesised, that MDA231 suspension cultures would restart mitosis once adhered to a substrate as MDA231 cells were observed to have a static cell cycle in suspension but are highly metastatic.

Surprisingly, across all samples MCF7 in standard culture conditions had the highest level of survivin expression, with expression decreasing in suspension and remained so in re-adherent culture (Figure 3.19). Similarly, MDA231 cells in 2D had the highest level of survivin expression across MDA231 samples; however, expression in suspension samples of MDA231 was not detected and once re-adhered there was survivin expression recovered, albeit incompletely. Furthermore, XIAP expression appeared to be raised in MCF7 suspension and re-adherent samples by comparison with 2D. In contrast, XIAP levels were lowest in re-adherent MDA231 samples by comparison with 2D and suspension where XIAP was consistent, indicating a change in anti-apoptosis biochemistry. The non-specific band above XIAP changed in accordance with survivin in both MCF7 and MDA231 samples. No changes in cadherins detected across MCF7 samples and none was detected in any MDA231 samples correlating with the phenotype of re-adherent colony phenotype.

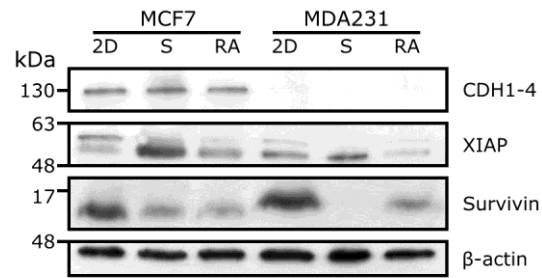


Figure 3.19 – Cadherin, XIAP and survivin immunoblot of MCF7 and MDA231 in 2D, suspension (S) and re-adherent (RA) cultures. CDH1-4 are E-, N-, P- and R-cadherin respectively. XIAP appeared as a double band, the lower band was XIAP and the upper band was a non-specific protein likely to be cIAP1 or cIAP2.

3.3 Discussion

3.3.1 Dependence of Cancer Cells on Glucose and Serum

The dysregulation of cellular energetics and metabolism was termed an 'emerging hallmark' of cancer by Hanahan and Weinberg (2011). Since then it is becoming clearer how altering of the cellular energetics in cancer is intrinsically linked to many other malignant activities, such as: changes in mitochondrial dynamics, and the link between hypoxic tumours with induction of a highly metastatic phenotype (Rivadeneira *et al.*, 2015; Devignes, Aslan & Provot, 2018; DiGiacomo & Gikes, 2018). Data presented here reiterate the metabolic differences between genotypic sub-types of breast cancer cell lines in a traditional culture context; with a new resazurin based-assay designed to measure metabolic changes due to metabolic stresses in a short time-frame. Additionally, in the context of dynamic suspension culture, marked changes in metabolism were observed in MDA231 cells by comparison with monolayer culture. This is especially relevant in terms of the origin of metastatic clones, and how efficiently they can form secondary tumours as a result of previous metabolic stress.

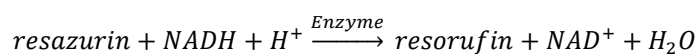
3.3.2 Resazurin-Based Metabolic Assays

It is important to note that traditional resazurin (Alamar/Presto Blue) based assays can have altered readout depending on the cellular metabolic state. In the standard resazurin-based growth curve assay it was shown that MCF7 cells were more reliant on high concentrations of glucose in the presence of serum by comparison with MDA231 cells. This was consistent with significantly faster NADH turnover in MCF7 cells.

3.3.2.1 Differences in Metabolic Load Between Different Breast Cancer Sub-Types

This assay relied on the reduction of resazurin to resorufin concomitant with NADH oxidation into NAD⁺, a reaction catalysed by many cellular metabolic

enzymes including complex I of the electron transport chain, NADH dehydrogenase (Zhang, Du & Zhang, 2004; Abu-Amero & Bosley, 2005).



As the concentration of resazurin is increased, the reaction is altered so that NADH concentration becomes the limiting factor of the described reaction, resazurin is therefore in excess. The concentration at which no more resazurin processed per unit time was calculated by the Michaelis-Menten.

The $V_{\max}$ and Michaelis constants (K_M) calculated by this assay revealed some interesting characteristics. Calculated $V_{\max}$ values showed an increased rate of NADH turnover in MCF7 by comparison with MDA231. Significantly the media type did not change the rate of NADH turnover in either cell line implying a hard-wired metabolic profile. This explains the death of MDA231 cells in serum free media as they are unable to adapt to the lack of scavangeable protein and signalling molecules contained within serum. Furthermore, this also implies that within 48 h, before cells began to die during growth curve experiments, MDA231 cells were unable to synthesise the necessary *de novo* growth factors *in situ*. Reduced NADH turnover in MDA231 combined with stagnant growth in serum free conditions could also imply heavy reliance on glycolysis and lactate metabolism in line with the Warburg effect.

Finally, in the medium most commonly used in the laboratory, DMEM with 10% FCS; MCF7 cells had a significantly higher rate of NADH turnover at $1.2 \mu\text{mol}\cdot\text{min}^{-1}$ by comparison with $0.5 \mu\text{mol}\cdot\text{min}^{-1}$ in MDA231 cells. This means that although the doubling time of MDA231 cells in this medium is comparable to MCF7 cells, MDA231 NADH turnover is almost 1/3 of that seen in MCF7 cells. This raises many questions which warrant further investigation. Are MCF7 cells less metabolically efficient? Could this be due to a harsher selective pressure in the tumour from which MDA231 cells derive, such as increased hypoxia?

3.3.3 Triple Negative Breast Cancer Cells Have Increased Mitochondrial Health in Suspension

Data here show that there is a marked change in metabolic behaviours between MDA231_{2D} and MDA231_s cells. A significant sub-population of MDA231_s cells have highly polarised mitochondria. This requires vast changes in protein stoichiometry of metabolic, apoptotic and mitochondrial dynamics pathways. Future work should be focused on resolving the changes which occur, but more how they are regulated. Stopping metabolic plasticity in malignancies may provide a novel angle for new treatments. These data would be complemented by future 2-photon autofluorescence which can be used to determine the intracellular NADH concentrations in the cytoplasm and mitochondrion of live cells (Yu & Heikal, 2009).

3.3.4 Hard and Soft-Wired Cell Cycle Dysregulation

It has been shown here that expression of the mitotic proteins/genes *AURKB*, *BIRC2/3*, borealin, *INCENP*, survivin (*BIRC5-1/2/3*), *TUBB* and *XIAP* are significantly downregulated when cells are grown in suspension. *AURKB*, *BIRC5-3* (survivin Δ Ex3), *CDCA8* (borealin) and *XIAP* were difficult to detect in MDA231_s samples suggesting expression of these genes is extremely low. The hard-wired dysregulation of the cell cycle in MDA231 cells is shown in Figure 3.20.

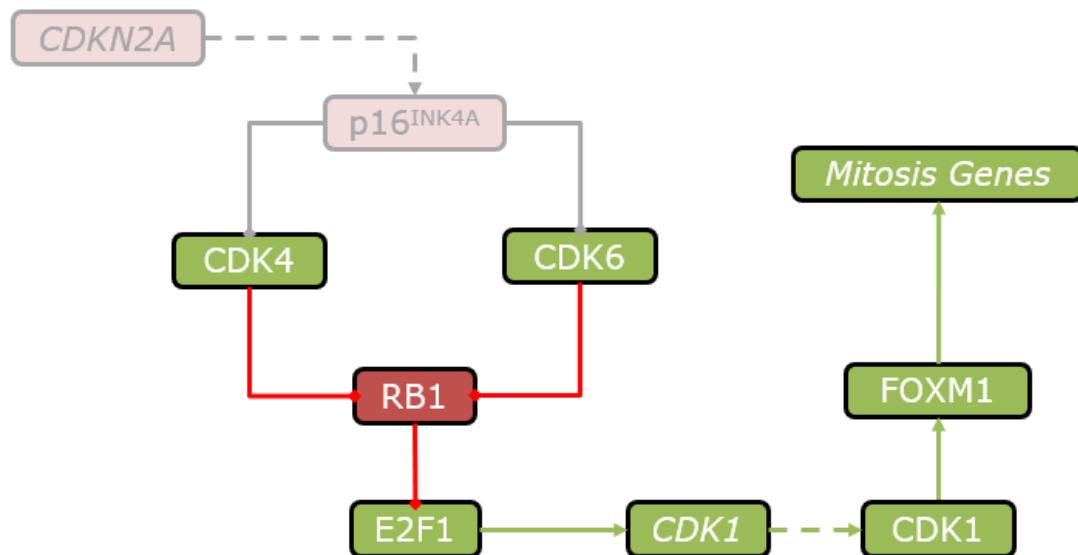


Figure 3.20 – Cell cycle signalling dysregulation in MDA231_{2D} and MDA231_S cells. MDA231 cells have no *CDKN2A* locus preventing expression of p16^{INK4A} which normally strongly regulates CDK4/6 by inhibition. CDK4/6 hyperphosphorylate RB1 preventing its inhibition of E2F1 and the subsequent cascade of pro mitotic signalling. Green boxes represent pro-mitotic proteins, arrows represent pro-mitotic interactions; red boxes and arrows represent the opposite. Greyed-out boxes/arrows are not present in MDA231 cells.

3.3.4.1 Hard-Wired Defects Can Influence a Soft-Wired Phenotype

A plethora of transcriptional, soft-wired, changes to mitotic gene expression have been shown here, which can now be integrated into the context of inherent, 'hard-wired', mutations in MDA231 cells.

MDA231 cells have no *CDKN2A/B* loci and mutant *TP53*^{839G>A} (encoding p53^{R280K} a DNA binding null mutant, Olivier *et al.*, 2002). These mutations, in combination, confer the ability for all cells to proceed through both cell cycle arrest check points unhindered despite DNA damage. Ordinarily, loss of *CDKN2A/B* alleviates RB inhibition of E2F1 and thus confers E2F1 its full repertoire of transcriptional activities (Okamoto *et al.*, 1994; Bockstaele *et al.*, 2006). In G₀ cells, E2F1 is bound and repressed by RB protein until it is hyperphosphorylated by the CDK2-cyclin E or CDK4/6-cyclin D complexes (Hydbring, Malumbres. & Sicinski, 2016). Downstream of this, CDK1 activation leads to the transcription of FOXM1, a transcription factor which regulates *BIRC5* expression. Therefore, FOXM1 activity is indirectly negatively regulated by p14^{INK4A}, encoded at the *CDKN2A* locus. If survivin is not expressed and cells are

not in mitosis, it is being correctly regulated. If it is being correctly regulated, FOXM1 cannot be expressed and therefore CDK1 cannot be present; equally E2F1, CDK4 and CDK6 cannot be active and RB1 must be hypophosphorylated (Figure 3.20). Therefore, MDA231_s cells must be in a G₀ state when in suspension; this is fully supported by flow cytometry data, showing no metaphase arrest for MDA231_s cells treated with DMA, along with no decrease in viability associated with an extensive mitotic stalling (Hou, Allan & Clarke, 2017).

In MDA231 cells, KRAS and BRAF are both mutated leading to sustained proliferative signalling through the MAPK cascade (Davies *et al.*, 2002). Combining this with the observations seen and other signalling circuits described; substantial, alternative genetic changes must be in play for the phenotype observed to exist. To avoid extensive single target approaches such as immunoblotting and qPCR; future studies should focus on the use of RNAseq to provide novel insight into how cell cycle arrest is achieved.

A noteworthy observation relating to *BIRC5* expression is its robust action as a marker of mitosis. Survivin is not overexpressed in MDA231 cells and the mitotic arrest phenotype heavily implies that it is correctly regulated and not constitutively expressed. As a result, the expression of survivin indicates the mitotic population, as any MDA231 cells expressing survivin will be going through cell division. This also correlates with changes in *TUBB* expression, which has been previously shown to be cyclically linked to the cell cycle. *TUBB* expression slightly increases toward the end of G₂ and through mitosis, meaning that a mitotically inactive population will exhibit slightly reduced *TUBB* expression (Schedl *et al.*, 1984). Surprisingly, data here infer that, contrary to popular belief, expression of *TUBB* and β -tubulin is not constant during the cell cycle; in agreement with previous research (Schedl *et al.*, 1984).

3.3.4.2 MYB and FOXD3 Are Master Regulators for a Suspended Animation Phenotype

After establishing concomitant downregulation of so many mitosis-related, and BIR-family genes; a bioinformatic approach was taken to try to evaluate whether an overarching regulatory system was modifying the behaviour of all the genes. Lack of inhibition through CDK4 and CDK6 can indirectly confer FOXM1 transcriptional activity; however, FOXM1 has not been shown to influence the transcription of all the genes shown to be downregulated in MDA231_s cells. This was also combined with the anoikis-related genes of interest to determine whether it was possible that suspended animation was caused by combined inhibition of anoikis and arrest of the cell cycle.

TELIS analysis identified FOXD3 as a potential regulator of the suspended animation phenotype. FOXD3 is of the same family as FOXM1; this suggests that the forkhead-box family of transcription factors could be key in understanding the suspended animation phenotype. FOXD3, specifically is necessary for pluripotent stem cells in the pre-implantation and peri-implantation stages of embryogenesis (Guo *et al.*, 2002). Paradoxically, this suggests that MDA231 cells in suspension are not of a pluripotent stem cell phenotype. This combined with NANOG, OCT4 and SOX2 not being detected on the mRNA level leads to a surprising suggestion (data not shown). Metastasising MDA231 cells may not be or contain a typical stem cell sub-population, and as such, may not fit the current models of single-cell secondary tumour seeding (REF).

Using TELIS to assess the changes in anoikis-related genes (*BID*, *FADD* and *PMAIP1*) revealed MYB as potential regulator. MYB is a general transcription factor associated with breast cancer tumourigenesis (Liu *et al.*, 2018). More specifically the induction of MYB has been recently shown to reduce lung metastases of breast cancer by preventing cell seeding (Knopfová *et al.*, 2018). This is suggested to occur through a dampened inflammatory response.

Concordant with data here, an inflammatory response in tumours typically leads to large responses to TNF α , interleukins and interferons all of which can interact with cells to induce extrinsic apoptosis. Cells, in an exponentially inflamed microenvironment, are selected for based on their ability to resist these signals. Considering the down-regulation of *BID*, *FADD* and *PMAIP1* in MDA231s cells here suggests that these cells can modulate the extrinsic apoptosis pathway based on increased signalling through this axis (Figure 3.21). Potentially, this can occur regardless of whether pro-apoptotic signalling is based on anoikis, or the increased cytotoxic paracrine signalling associated with inflammation. Significantly, the qPCR assay commercialised as Oncotype DX includes *MYB* as a readout gene for treatment stratification in HR⁺ and lymph node negative breast cancers (Paik *et al.*, 2004). The role of MYB in a suspended animation phenotype is therefore of clinical importance.

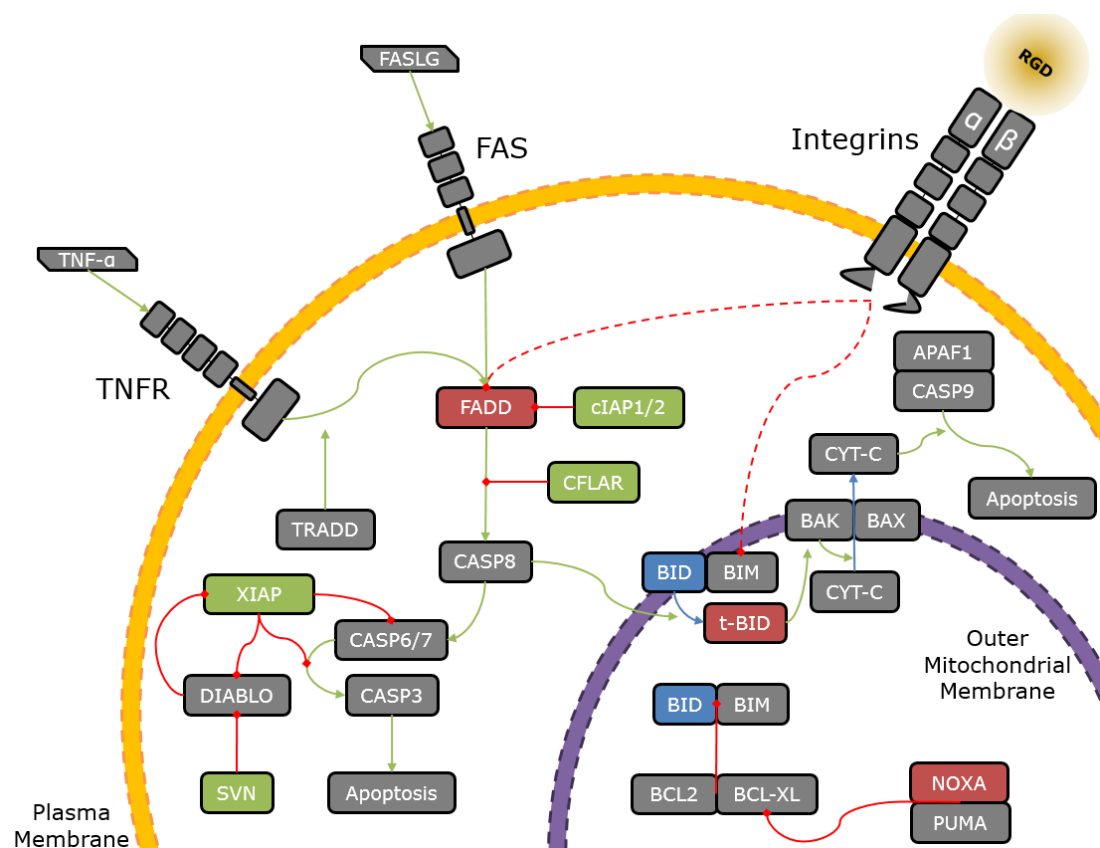


Figure 3.21 – Intrinsic and extrinsic anoikis pathway genes of interest. Proteins in colour were those selected for qPCR analysis.

3.3.5 Improved Mitochondrial Health and Evasion of Cell Death Are Associated with A Suspended Animation Phenotype

Due to the presence of constitutively active KRAS and BRAF, IAPs are not required for the evasion of anoikis in MDA231 cells (Hollestelle *et al.*, 2010). However, changes in mitotic and mitochondrial dynamics suggest extensive changes in MOMP; alongside changes in IAP biochemistry.

3.3.5.1 Mitochondrial Health is Greatly Increased in Suspended Triple Negative Breast Cancer Cells

BID is a key component of apoptosis that acts as a bottleneck for pro-apoptotic signals downstream of both intrinsic and extrinsic signalling axes. Caspase mediated cleavage of BID allows the formation of a BID^{p15}, also known as t-BID (Figure 3.21). This product can form a heterodimer with BAX which allows cytochrome c release from the mitochondria and subsequent formation of the apoptosome. BID cleavage is prevented by BCL2-family proteins which sterically hinder caspase cleavage by direct BID interaction through BH2-BH3 domain interfaces (Mehlen & Puisieux, 2006).

Upstream of BCL2, NOXA (encoded by *PMAIP1*), alongside PUMA and others acts to prevent BCL2-family proteins interacting with BID (Grossmann, 2002). qPCR data showed that both *PMAIP1* and *BID* were significantly downregulated 3.8fold and 2.7fold respectively. This, in combination, confers a decreased ability for cells to inhibit BCL2-family competition with caspases for BID binding, leading to a decreased potential for mitochondrial outer membrane permeabilisation (MOMP). Similarly, decreases in *BID* expression will prevent MOMP by altered BID^{p15}-BAX stoichiometry. Together, these data are permissive to decreased MOMP and increase IMM potential. The intrinsic link between MOMP, mitochondrial metabolic load and circumvention of pro-apoptotic signals provides a strong selective advantage to these cancer cells.

Analysis of mitochondrial health showed a vast increase in the maximum polarisation possible in MDA231_s by comparison with MDA231_{2D}; with a small increase in the mean polarisation state. However, the minimum signal remained the same suggesting there is a sub-population of cells which is not responding to detachment in the same way. Taken together, this suggests that a small sub-population which is 'diluted' out in the RNA samples used for qPCR analysis may have different BID/anti-apoptosis biochemistry. It would be interesting to isolate these cells by integration of TMRE staining with FACS sorting for qPCR analysis.

3.3.5.2 IAP Expression is Altered in Suspension

Inhibitor of apoptosis (IAP) family proteins are a small, multifunctional family of proteins, all containing at least 1 baculoviral inhibitor of apoptosis domain. NAIP and survivin are the only family members not to have RING-domain mediated E3-ligase activity. This family of 8 proteins is associated with proliferation, evasion of cell death, inflammation, metabolic reprogramming, and metastasis (Chai *et al.*, 2001; Varfolomeev *et al.*, 2007; Mahoney *et al.*, 2008; Takeda *et al.*, 2014; Nestal de Moraes *et al.*, 2015; Saldivar *et al.*, 2018). All IAPs assessed were down-regulated in suspension culture samples, which is interesting due to the activity of IAPs as anti-apoptotic proteins, as well as mitotic proteins.

Direct interactions of survivin with borealin and aurora kinases is essential for competent mitosis (Guvenç *et al.*, 2013). In terms of apoptosis, the sequestration of DIABLO by survivin in the cytoplasm of challenged cells prevents DIABLO inhibition of XIAP, allowing XIAP E3-ligase activity targeted at activator and executioner caspases. Direct interactions of cIAP1, cIAP2 and XIAP have been associated with NF- κ B signalling and the downstream inflammatory responses (Varfolomeev *et al.*, 2007). cIAP1, cIAP2 and XIAP were all found to be significantly downregulated in MDA231_s samples, this is interesting because the regulation of inflammation and evasion of anoikis appear mutually exclusive.

This could occur such that upregulation of cIAP1/2 and XIAP confers reduced inflammatory signalling by negative regulation of NF κ B, paired with increased resistance to anoikis by negative regulation of signal transduction through CASP8 or via CASP3/7 inhibition. This marks a crucial step of tumourigenesis elicited by detachment, as upon reattachment cells can rapidly reengage an inflammatory programme by re-engaging cIAP1, cIAP2 and XIAP expression.

In many clinical examples elevated survivin in circulating tumour cells is a prognostic indicator for high levels of metastasis (Ning *et al.*, 2015; Adams *et al.*, 2016). At first this appears inconsistent with results shown here. However, in the clinical environment circulating tumour cells (CTC) could have been in circulation for any amount of time, having recently shed from the primary tumour or having been in circulation for an extended period. Considering this, any sampled CTC population is going to be contaminated with recently shed cells which potentially have high *BIRC5* expression. Experiments *in vitro*, have therefore inferred an interesting component of long-lived CTCs. MDA231 cells in suspension have significantly down-regulated *BIRC5* expression. This implies that the most dangerous of CTCs are those which have the transcriptional plasticity to favour cell cycle arrest in suspension; leading to severely reduced vulnerability to apoptotic factors targeting highly proliferative cells. In this way, treating high-grade tumours with radiation and Paclitaxel only goes to further select the most dangerous cells, in terms of metastatic potential. In the specific case of triple-negative cancers (ER^{-/-}, PR^{-/-}, HER2^{-/-}) it is therefore extremely important to identify the mechanism by which adhesion (re)activates mitotic potential. Keeping cells dividing when in suspension presents as counterintuitive, but it would lead to sensitisation of the highly metastatic population to treatments. In the context of this *in vitro* study the next step is proteomic and transcriptomic analysis to fully understand the full range of changes present in the long-lived CTC population of which MDA231 cells are so capable of forming.

3.3.5.3 Paclitaxel Resistance in Suspension Cell Cultures

Paclitaxel is a beta-tubulin binding agent which hyper-stabilises microtubules and prevents their disassembly. In use at chemotherapeutic concentrations, this prevents chromosome congression in pro-metaphase. In highly-proliferative sub-populations of tumours, paclitaxel acts to block mitosis, leading to mitotic cell death. However, there are off-target effects of paclitaxel, some of which have not been studied. One such effect is microtubule-kinetochore slippage which stops efficacious microtubule attachment to kinetochores during prometaphase (Kreger *et al.*, 2016). Regardless of the off-target effects, MDA231 cells grown in suspension are unaffected by it at the IC50 concentrations of monolayer MDA231 cells. This is particularly relevant as ~90% of cancer fatalities are caused by secondary tumour formation. It is therefore vital that more chemotherapeutic agents are tested on cells grown in non-canonical culture systems.

3.3.6 Breast Cancer Organoids Partially Retain Structure After Re-adherence

MCF7 cells were shown to have equivalent survivin expression in dynamic suspension as in re-adherent culture. This suggests that re-adherence of organoids formed in suspension does not change the mitotic behaviour gained by culturing in suspension. In connection with this, canonical cadherin (CDH1-4) expression was not altered between culture conditions suggesting equivalent cell-cell ligation in each context. These findings are concordant with the repressive effect of an increase in number of adherens junctions between cells (Okamoto *et al.*, 1994). Importantly the change in phenotype and protein expression between suspension samples and re-adherent samples is minimal, this implies that MCF7 cells in suspension have reverted to a more 'natural' phenotype in suspension and so maintain this phenotype when re-adherent. These findings also suggest that MCF7 cells in 2D culture, prevented from getting over-confluent and forming 3D colonies, are not truly representative of

cell behaviour. This may have implications for other luminal A breast cancer cells and requires further investigation.

3.3.7 Triple Negative Breast Cancer with a Suspended Animation Phenotype Can Proliferate and Migrate upon Re-adherence

In opposition to the observations in MCF7 samples, the protein expression profiles of MDA231 cells across 2D, suspension and re-adherent culture showed marked and un-sustained changes in survivin and XIAP (nonspecific band, 3.2.2.4). MDA231 cells stopped expressing survivin in suspension before expressing survivin within the first 24 h of re-adherence. This suggests that the suspended animation phenotype of MDA231 cells in suspension is purely transcriptional. Thus, the suspension culture conditions are not selecting for an MDA231 sub-population which is senescent and able to circumvent anoikis. The phenotype observed here, consistent with a complete lack of canonical cadherin expression, is one of a highly aggressive, drug resistant tumour which readily metastasises and forms secondary tumours.

Consistent with the literature, in monolayer migration assays, MCF7 cells were non-migratory whereas MDA231 had a highly migratory phenotype (Fox & Kandpal, 2004). This behaviour was maintained in re-adherent cultures in which MCF7 colonies simply attached as organoids with no lone cells. Large MDA231 colonies were dispersed with many cells alone and few in direct interface. This data supports the conclusion that dynamic suspension culturing of MCF7 and MDA231 cells does not inherently change their migratory behaviour. This is important to consider when assessing the metastatic strategy of luminal A and basal B breast cancers respectively.

3.3.8 Summary

The data presented here have shown an altered dependence of MCF7 and MDA231 cells on glucose and serum, via resazurin based, growth curve assays. Furthermore, a more detailed assessment of MCF7 and MDA231 cell metabolism showed increased NADH turnover in MCF7 cells with a kinetics-based approach. Previously observed migratory phenotypes of both MCF7 and MDA231 cells were confirmed in the context of a wound healing assay; marking differences in the metastatic potential of these breast cancer sub-types (luminal A and basal B respectively).

The next phase of metastasis, survival in suspension, was assessed showing differences in MCF7 and MDA231 aggregation and markedly reduced mitotic phenotype in MDA231 cells specifically. It was then shown that MDA231 cells ceased to progress through the cell cycle, seemingly regardless of at which position the cells were, 2n, S-phase or 4n. These observations were paired with marked changes in MDA231 mitochondrial phenotype; suggesting a large increase in oxidative phosphorylation of suspended MDA231 cells by comparison with those grown in monolayer. Furthermore, this phenotype was also paired with an increased resistance to paclitaxel, a very important observation when considering the ability of these cells to remain in suspension for long periods of time. This means they have the potential, as dormant cells, to seed secondary tumours immediately after chemotherapy, providing a new, dangerous phenotypic character.

In terms of (re)adherent properties of both MCF7 and MDA231 cell lines, grown in suspension: both cell lines maintained their migratory behaviour with MDA231 immediately migrating and making colony-forming assays impossible. MCF7 cells retained their aggregate phenotype and migrated as a moving front, consistent with wound healing assays, and the metastatic method associated

with luminal A cancers: migration as an aggregate getting lodged in an adjacent tissue, typically the axillary lymph nodes (under the arm).

Working with common cell lines such as MCF7 and MDA231 raised many questions about the current state of cancer behaviour methodology. Given the incidence of cancer across the world, it is inexplicable that cell lines originating from the 1970s are still used. Robust characterisation procedures of a new cell line, generated from a recent biopsy of the relevant cancer, before the desired experiments are performed should be more than sufficient to make more salient insights into cancer biology. Furthermore, old methods which continue to persist are, in the view of this thesis, inhibiting progress particularly in the form of high glucose-content media, and the lack of media flow. New innovations to make the removal of these from widespread research, with minimal disruption to current working procedures in tissue culture facilities, is essential to continuing to move forward with more accurate models and understating of cancer behaviour.

Chapter 4

Breast Cancer Cell Behaviour In Biomimetic Environments

4.1 Overview

The previous chapter described the behaviour of MCF7 and MDA231 cells in conventional suspension and re-adherent culture conditions. The present chapter brings the contribution of the ECM into the equation. This important facet of *in vivo* metastasis is assessed in the context of organoid co-culture, and employment of decellularised matrices as culturing substrates.

4.1.1 The Extracellular Matrix

The ECM is a complex network of proteins and its ultrastructure is influenced by many cell types, with fibroblasts generating most ECM proteins; the key structural constituents of which are collagens and laminins (Aumailley *et al.*, 2005; Ricard-Blum; 2011).

Cells with strong inter-cellular connections can form organoids when grown in non-adherent conditions. The integrity of organoids is influenced by the ECM, but it does not necessarily contain all the ECM components of a mature organ. Integrating metastatic lines into organoids generated from non-malignant cell lines may provide a more physiologically relevant platform for assessing cell invasion than traditional 2D-methods.

An alternative to organoid culture is the use of decellularised matrices that can provide a biomimetic ECM. Decellularised matrices are essentially all the extracellular biological material of an organ remaining after removal of cellular material. The harsh treatments used can also remove biologically relevant components such as GAGs and solubilised growth factors. It is important to assess the efficacy of cell debris removal with the chosen decellularisation method as decellularisation buffers have varying effects on different tissues and organs and remaining cellular material will affect subsequent use of the matrix. This is typically performed by assessment of matrix DNA content and cellular markers (Crapo, Gilbert & Badylak, 2011).

4.1.2 Organoid Culture

Organoid systems are commonly used to generate 3D cellular structures in static conditions. Organoids from MCF7 cells can generate a lumen; whilst stem cell organoids can be directed to simulate early stages of organ development upon exposure to growth factor treatments (Shackleton *et al.*, 2006). In the previous chapter it was found that MDA231 cells do not form aggregates or organoids in dynamic suspension culture. MDA231 cells derive from a pleural effusion, thus they derive from a location which has an interface with the lung. It was therefore hypothesised that the generation of embryonic lung fibroblast (MRC5) organoids could provide a biomimetic destination for MDA231 to integrate into and colonise. The interaction of these two cell lines was tested in 2D and suspension conditions and their ability to form MRC5-MDA231 co-culture organoids was assessed. For comparison, the growth rate and migration of MDA231 cells in the presence and absence of MRC5 cells was determined in monolayer culture. Protein expression, histological and microscopy techniques were also employed to assess the efficacy of MDA231 integration into MRC5 organoids.

4.1.3 Decellularised Matrices

To investigate the potential contribution of the ECM or 'soil' on the behaviour of metastatic breast cancer, decellularised matrices were generated and used as a 3D substrate. Decellularised matrices have typically been employed for use in regenerative medicine as scaffolds for tissue and organ regeneration; or in stem cell research where decellularised organs are repopulated with the aim of generating live functional tissues (Abolbashari *et al.*, 2016). Similarly, it has been previously shown that decellularised lung matrices are biocompatible with breast cancer cells with respect to proliferation and viability, but the invasive potential was not assessed (Xiong *et al.*, 2015). Here, cell behaviour in the context of a decellularised matrix was assessed with

particular attention given to intercellular adhesive properties, proliferative potential, invasive potential and matrix remodelling by *de novo* ECM synthesis. These facets of secondary tumour formation were tested by immunoblotting and immunohistochemistry, in both static and conventional culture, and using a perfusion bioreactor.

4.2 Results

4.2.1 Fibroblast – Breast Cancer Cell Co-culture

To add further complexity to the systems described, the embryonic lung fibroblast cell line, MRC5, engineered to express GFP, was used as a lung milieu-mimetic. In co-culture conditions, changes in proliferation and migration were of interest. The ability of MRC5 cells to form an organoid-based substrate for MDA231 cells with which to integrate, was also investigated.

4.2.1.1 MDA231 Growth Arrest is Relieved by Fibroblast Co-culture

MDA231 cells rely on serum during culturing (section 3.2.1.1). To determine whether the presence of MRC5 cells and any associated paracrine signalling by them could rescue this dependency. MDA231 cells were seeded into a 96 well plate and MRC5 cells added. The co-culture conditions were always seeded with the same total number of cells with either 25%, 50% or 75% MDA231, with the remainder being MRC5 cells. MRC5 and MDA231 seeded alone acted as controls. Growth was assessed over 96 h using a resazurin assay, growth curves generated, and doubling time calculated.

As shown previously, MDA231 cells experienced a significantly reduced rate of growth in serum free conditions. If serum was present however, glucose concentration did not have a significant effect on doubling time (Figure 4.1A). When grown in 2D co-culture, there was no difference in doubling time across all media conditions with any ratio of MDA231:MRC5 cells, a finding that is mirrored by MRC5 response alone. Interestingly, co-culturing of MDA231 cells with MRC5

cells rescued the serum starved response of MDA231 (Figure 4.1B). In serum⁻ conditions there was a significant difference between MDA231 cells alone and all co-culture samples, regardless of glucose concentration. It was also apparent that the phenotype of MRC5 serum starved cells was less severe than the MDA231 response in terms of an increase in doubling time. In addition, the inclusion of 25% MDA231 cells in the presence of MRC5 cells significantly reduced the doubling time in low glucose and serum⁻ conditions, but not high glucose without serum.

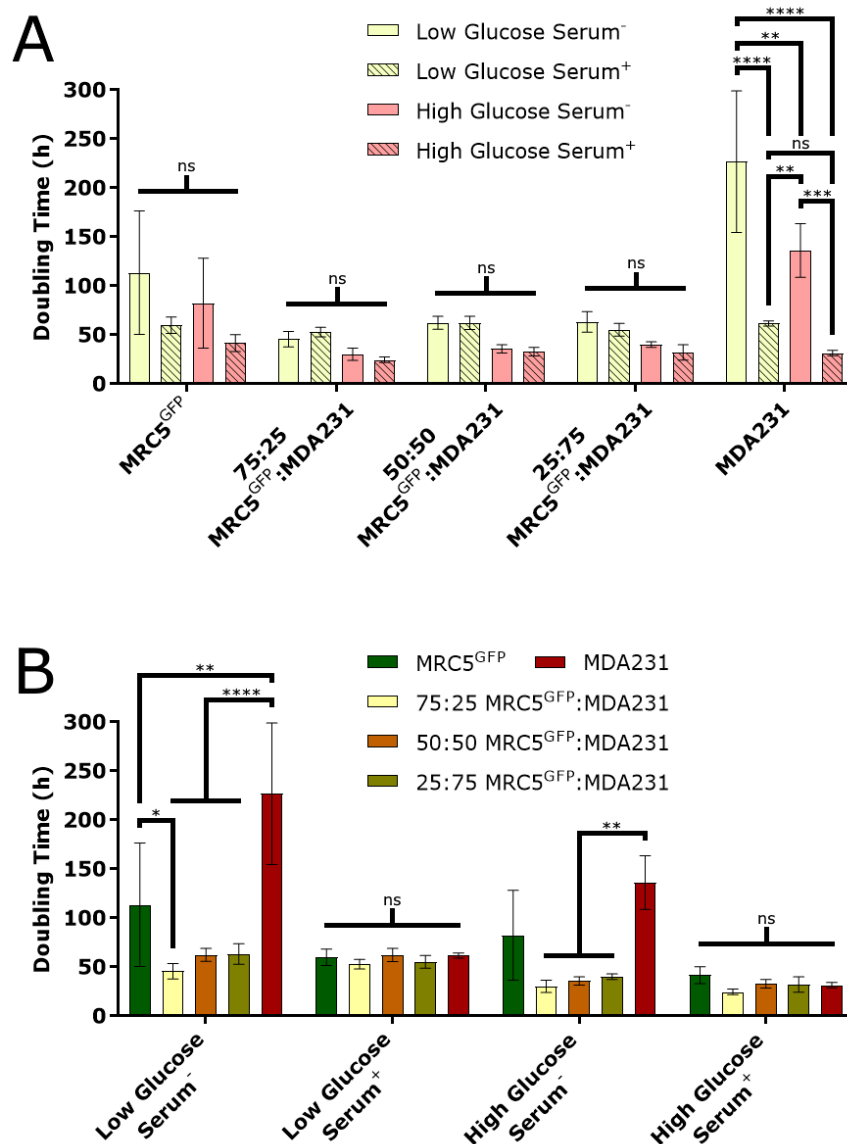


Figure 4.1 – MDA231 and MRC5^{GFP} doubling time in different co-culture conditions. **A**, MDA231 and MRC5^{GFP} growth rates in different co-culture ratios and media conditions. **B**, data from 'A' replotted to highlight changes between media conditions. Data analysed by 2-way ANOVA with *post hoc* two-stage linear step-up procedure (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $N = 58$, $n = 3$). Plotted with SEM.

4.2.1.2 MRC5 Cells Inhibit MDA231 Cell Migration and Causes Delamination

To assess the level of MDA231 cell migration in monolayer conditions, in the presence and absence of MRC5 cells, a wound healing assay was performed. Previous work with MDA231 cells in this context showed complete wound coverage within 16 h. As a result, the assay here was adapted to assess migration at 0, 8 and 16 h, post-wounding.

As previously (section 3.2.1.3), in the absence of MRC5 cells, MDA231 cells migrated to completely cover the wound area within 16 h. When in the presence of MRC5 cells however, there was a different phenotype, with migration inhibited and delamination induced (Figure 4.2). In co-culture the wound area was not migrated across within 16 h. In the case of MCR5 cells alone and in 2 of the 3 samples tested for co-culture, the cell monolayer delaminated from the substrate in response to wounding.

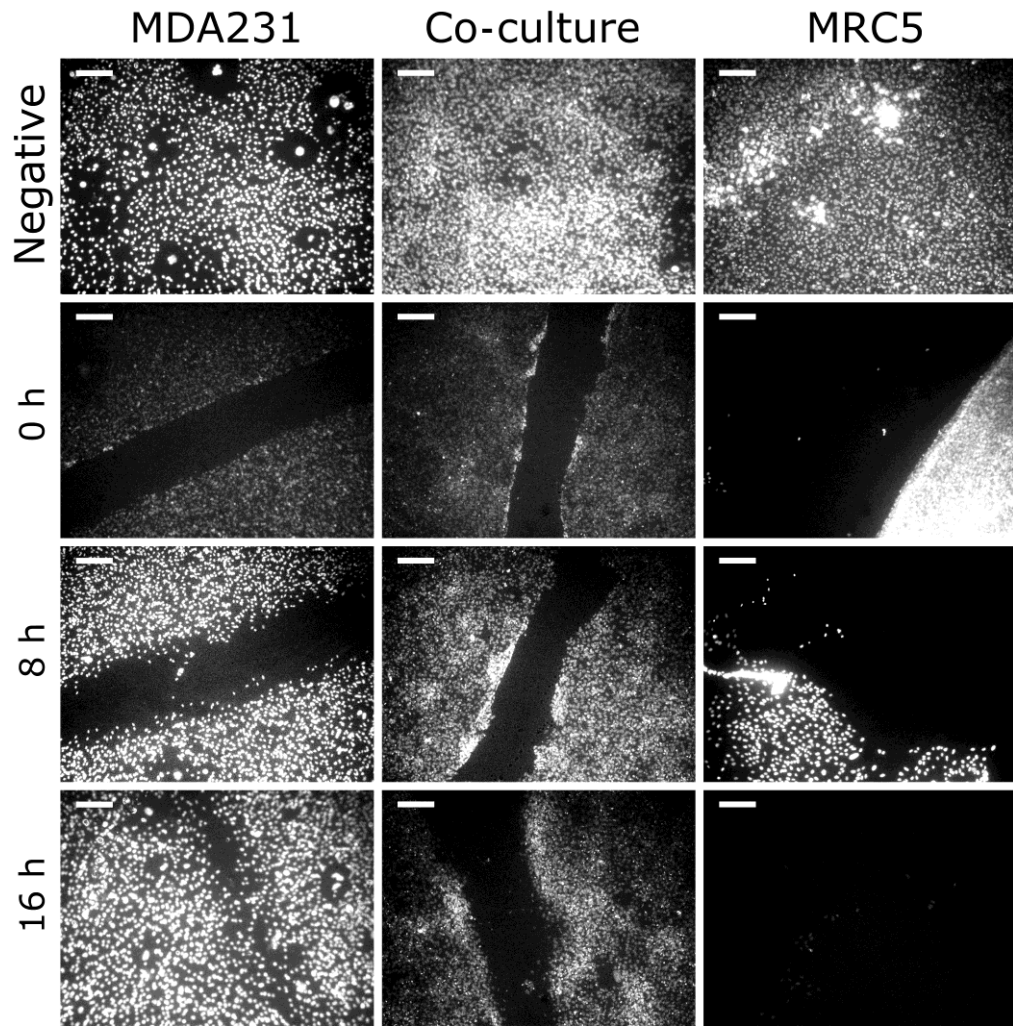


Figure 4.2 – MDA231 cell migration in the presence and absence of MRC5 cells. **Left**, MDA231 cell migration in the absence of MRC5 cells. **Middle**, MDA231-MRC5 co-culture migration. **Right**, MRC5 cell migration in the absence of MDA231 cells. Stained with DAPI. 200 μm scale bar.

4.2.1.3 MRC5 Cells Form Organoids in Dynamic Suspension

To understand the distribution of cells in the MRC5 organoids, samples were fixed, permeabilised, then stained to visualise nuclei and the actin cytoskeleton, before mounting for light sheet microscopy. MRC5^{GFP} organoids formed ~250x500 μm oblate spheroids with many apoptotic and mitotic cells within the organoid, see (Figure 4.3).

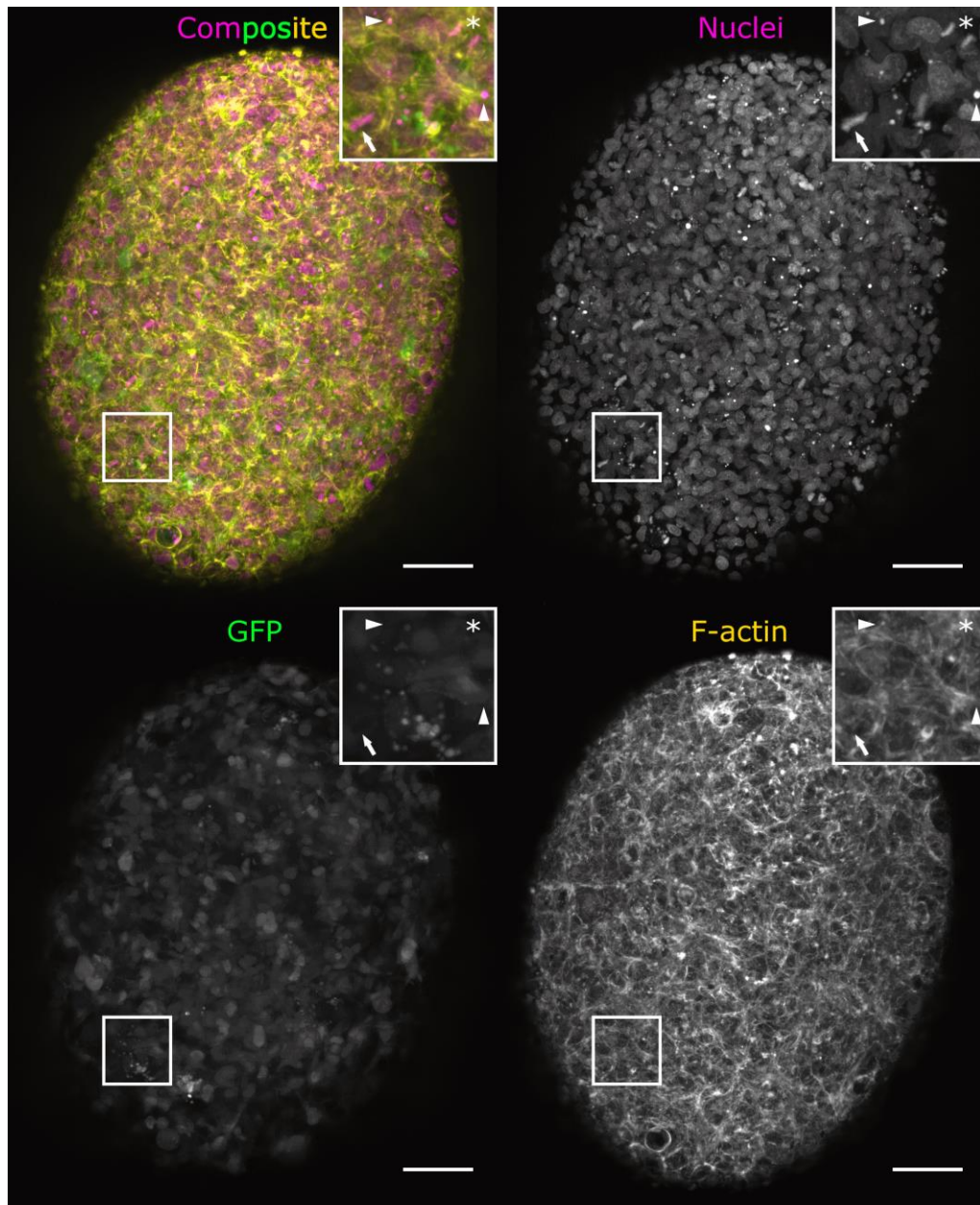


Figure 4.3 – MRC5^{GFP} organoid wholemount stained for nuclei and F-actin. MRC5^{GFP} organoid imaged by light-sheet microscopy (20x). Apoptotic cells (arrow-heads), anaphase/telophase chromosomes (*) and metaphase chromosomes (arrow). Scale bar 100 μm .

4.2.1.4 Survivin expression Changes in Lung Fibroblast Organoids

MRC5 cells readily aggregate during handling in tissue culture so it was hypothesised they would form organoids in dynamic suspension culture conditions. This was the case, therefore, the next question was whether MDA231 cells could integrate within them to mimic a metastatic lung tumour microenvironment.

To test this MRC5 and MDA231 cells were cultured in 2D and suspension alone and in co-culture for 96 h, then protein extracts were prepared and immunoblotted. GFP acted as a loading control for the MRC5 subpopulation of the co-culture and β -actin acted as a general loading control.

As described in, survivin abundance dropped in MDA231 suspension cultures (Figure 4.4). In contrast, MRC5^{GFP} showed a small increase in survivin abundance between monolayer and suspension, however, when co-cultured, no change in survivin abundance was observed between 2D to suspension (Figure 4.4). Quantification of three independent immunoblots yielded no significant differences between 2D and suspension for any cell line or co-culture (Figure 4.4B). Similarly, the sub-population of MRC5^{GFP} in co-culture was indeterminate due to an apparent rise in GFP expression in suspension samples compared to 2D.

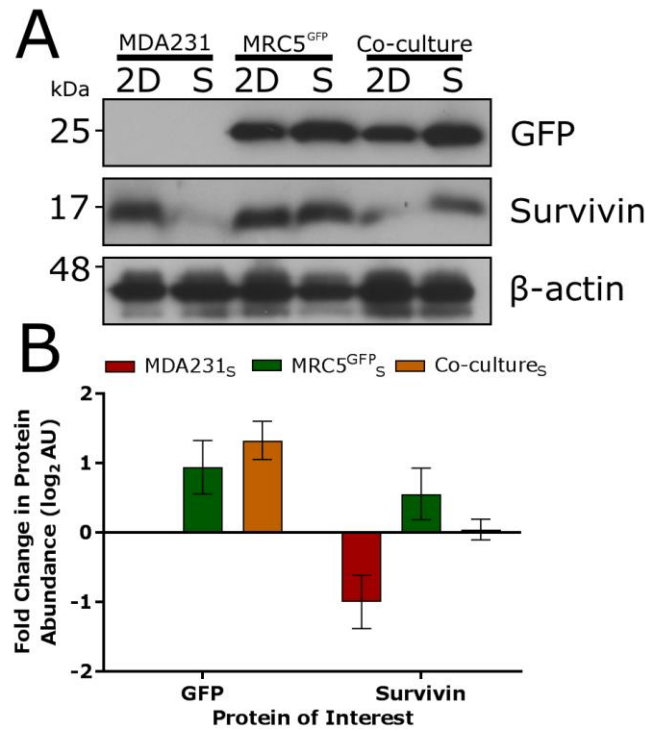


Figure 4.4 – Comparison of survivin expression between 2D and suspension cultures. **A**, immunoblot for survivin with GFP and β-actin loading controls. **B**, quantification of triplicate of **A**, no results were significant, plotted with SEM (2-way ANOVA with post hoc two-stage linear step-up procedure; n=3).

4.2.1.5 MRC5 and Co-culture Organoids Maintain Proliferative Potential

As survivin was detected in both MRC5^{GFP} and co-culture suspension samples; MRC5^{GFP} and MDA231 cells were cultured in suspension as previously described then processing for immunohistological staining for survivin. Survivin was seen as enriched in mitotic cells with low levels present in interphase nuclei (Figure 4.5A). Quantification of colocalised puncta and expression as a percentage of total nuclei displayed no significant difference in mitotic cell number between MRC5 only and co-culture organoids, indicating no difference in the growth rate of cells in either context (Figure 4.5B).

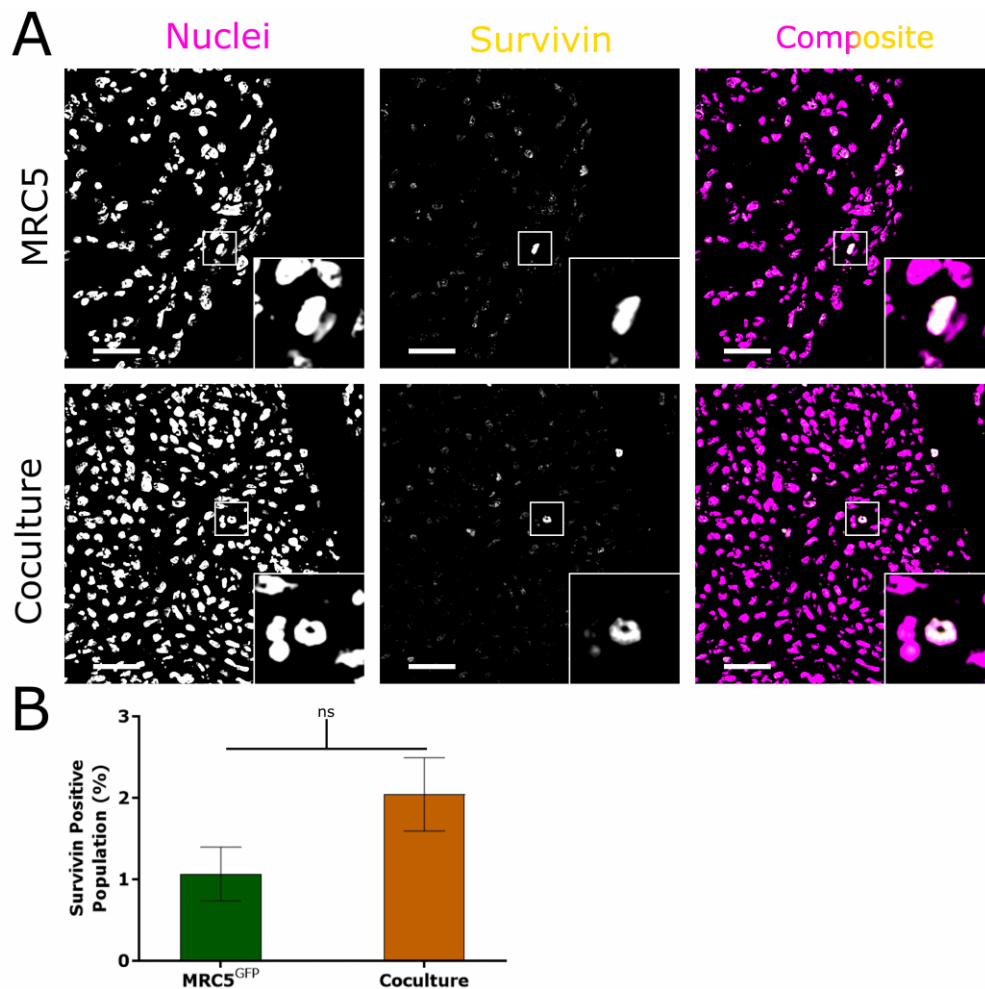


Figure 4.5 – Survivin staining of MRC5 organoids in the absence and presence of MDA231 cells. **A**, survivin colocalisation with mitotic chromosomes in MRC5 (metaphase) and co-culture (prometaphase) organoids. **B**, quantification of colocalised puncta (Welch's t-test; ns, not significant, $P=0.1078$). Scale bar 50 μm . $n=3$.

4.2.1.6 MDA231 Cells Disrupt MRC5 Organoid Structure

Upon staining for cells in mitosis it was noted that the co-culture organoids were smaller and more disordered than those without MDA231 cells. To test whether this was a cause of changes in ECM protein expression, samples were stained for collagen I and laminin. GFP signal was quenched during processing allowing the use of 488nm fluorescent secondary antibodies.

In MRC5 organoids, a distinct outer layer of cells was found to be rich in collagen I but to have very low levels of laminins (Figure 4.6A). The central region of the organoid was devoid of collagen I and laminins. Interestingly the presence of MDA231 cells in suspension alongside MRC5 cells reduced the size and caused a more irregular organoid shape. Within co-culture organoids cells were arranged in swirls, which were rich in collagen I and low in laminin (Figure 4.6A). Area fraction, the percentage coverage of the stain as a proportion of the image, and fluorescence intensity for both collagen I and laminin were quantified. This revealed a smaller but more concentrated distribution of collagen I within MRC5 organoids by comparison with co-culture organoids (Figure 4.6B-C), however there was no significant difference when tested by 2-way ANOVA.

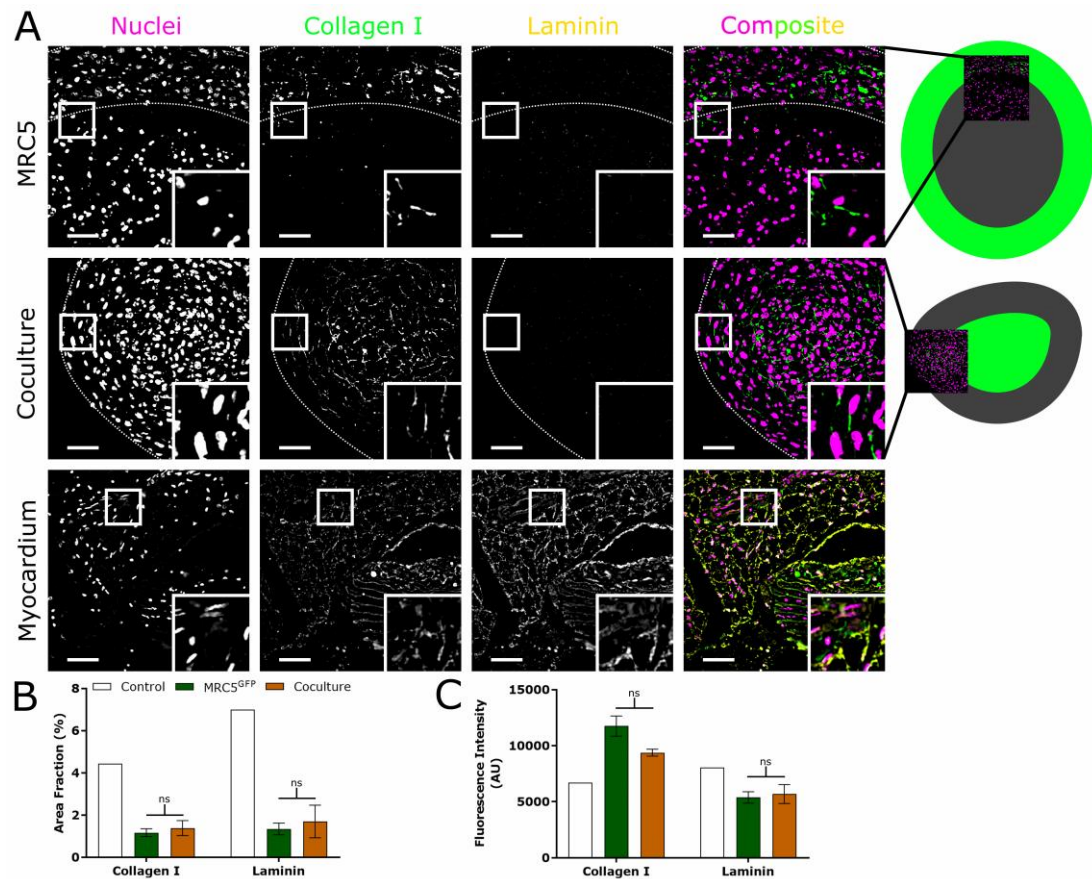


Figure 4.6 – Collagen I and laminin staining of MRC5 and MDA231 co-culture organoids. **A**, collagen I and total laminin distribution in MRC5 and co-culture organoids. Dotted line represents the outer layer of organoids. **B**, area fraction of collagen I and laminin. **C**, Fluorescence intensity of collagen I and laminin in organoid samples (2-way ANOVA with *post hoc* two-stage linear step-up; ns, not significant). Rat myocardium was used as a positive control. Scale bar 50 μm . $n=3$.

It was then hypothesised that the decreased size and disorder may be due to the absence of network-forming and FACIT collagens, ECM components that are key for the maintenance of tissue ultrastructure. It was also speculated that induced expression of ECM proteins associated with MRC5 cell scattering may also contribute to this phenotype.

Surprisingly both MRC5 and co-culture organoids were devoid of collagen IV, collagen XII and laminin-332 (Figure 4.7A-C).

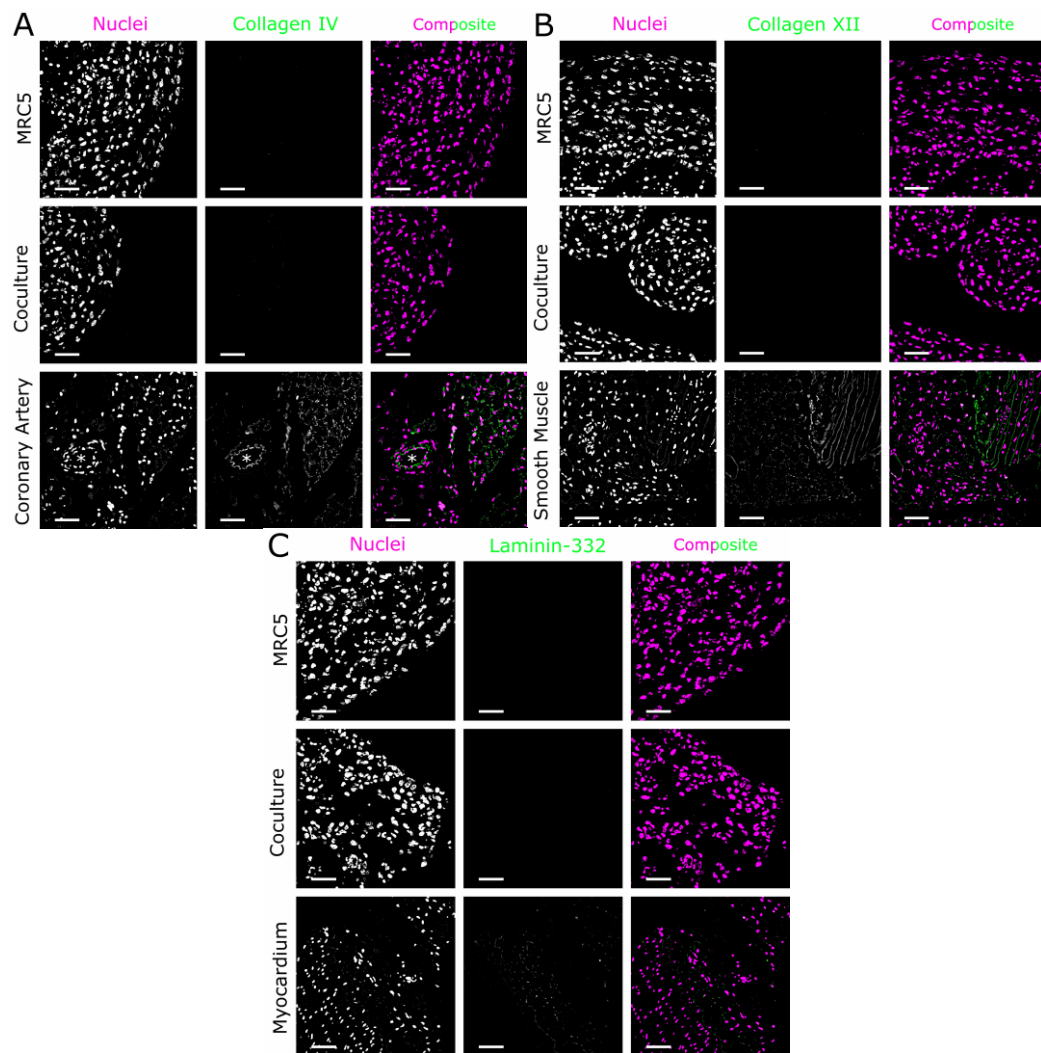


Figure 4.7 – Collagen IV and XII, and laminin-332 staining of MRC5 organoids in the absence and presence of MDA231 cells. A, collagen IV staining of organoids, rat coronary artery was a positive control (*). **B,** collagen XII staining of organoids, vena cava smooth muscle was a positive control. **C,** laminin-332 staining of organoids, myocardium was a positive control. Scale bar 50 μ m. n=3.

4.2.1.7 MRC5 Organoids Have Altered Adhesive Properties

Co-culture organoids had a more inconsistent phenotype, in terms of size and shape, by comparison with MRC5 organoids. This did not correlate with changes in ECM protein expression. To determine if the morphological changes were due to altered intercellular adhesion, organoids were stained for cortactin and E-cadherin.

Surprisingly, MRC5^{GFP} cells did not express much E-cadherin in normal or co-culture conditions, those that did, expressed a similar amount in both samples (Figure 4.8A-C). In contrast, cortactin was much more widely expressed in co-culture samples than in MRC5 organoids (Figure 4.8B), with similar bright nuclear puncta present in both sample sets (Figure 4.8C).

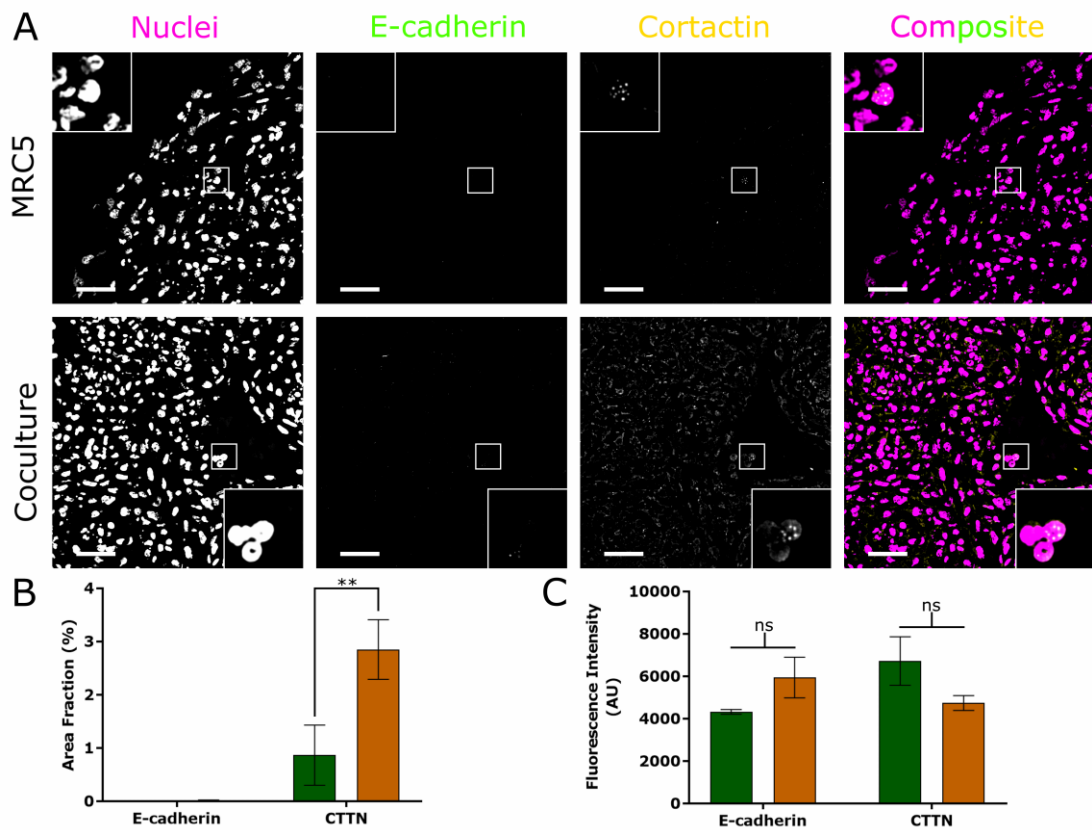


Figure 4.8 – E-cadherin and cortactin staining of MRC5 organoids in the absence and presence of MDA231 cells. **A**, E-cadherin and cortactin staining of MRC5 and co-culture organoids. **B**, Quantification of E-cadherin and cortactin area fraction. **C**, quantification of E-cadherin and cortactin mean fluorescence intensity. Scale bar 50 μ m. n=3.

4.2.2 Decellularised Matrix as a Biomimetic Environment for Secondary Tumour Formation

Integration of MDA231 cells into MRC5 organoids was trialled with the intention of providing a 3D structure more closely related to lung tissue than that in monolayer culture, for MDA231 cells to colonise and invade. As shown, many key ECM proteins are absent in the organoids, changes in MRC5^{GFP} expression occurred in suspension, and histological quenching of the GFP signal combined to make it difficult to analyse the behaviour of MDA231 cells in this context.

To overcome these problems and to more accurately represent the ECM microenvironment breast cancer lung metastases would encounter in the body, a different biomaterials approach was taken. Decellularisation allows the whole ECM structure of an organ to be retained whilst removing cellular material. Decellularisation of lung tissue would theoretically provide a biomimetic substrate for colonisation by malignant cells such as MCF7 and MDA231.

4.2.2.1 Phylogenetic Analysis of Extracellular Matrix Proteins

In order to select an appropriate species from which to source lungs for decellularisation, a phylogenetic approach was employed. First, it was determined which ECM protein would be a good candidate for comparisons with other species. Second, the ECM homology of candidate species was assessed. The two largest families of ECM proteins are collagens and laminins, comprising 45 and 12 members respectively. These two families were assessed for a suitable protein with which to perform cross-species phylogenetic analysis.

FASTA sequences of collagen were identified on the uniprot database by searching for “reviewed” human sequences with a “COL#A#” consensus; where ‘#’ stands for any number, then downloaded and formatted for multiple sequence alignment using MAFFT E-INS-I; with a BLOSUM62 scoring matrix. The results were then exported to Archaeopteryx software for visualisation (Han & Zmasek, 2009). The resultant phylogenetic tree (Figure 4.9) showed a wide

spread of relationships with various clusters for single collagen families. Fibrillar collagens could be found in 3 discrete groups, network forming collagens in 5 groups, FACITs in 5 groups, MACITs in 3 groups, multiplexins in 2 and others in 1 group. Due to the complex evolutionary history of collagens, it was difficult to determine what candidate collagen would be best for cross-species phylogenetic analysis; thus, the laminin family was selected as the next best candidate.

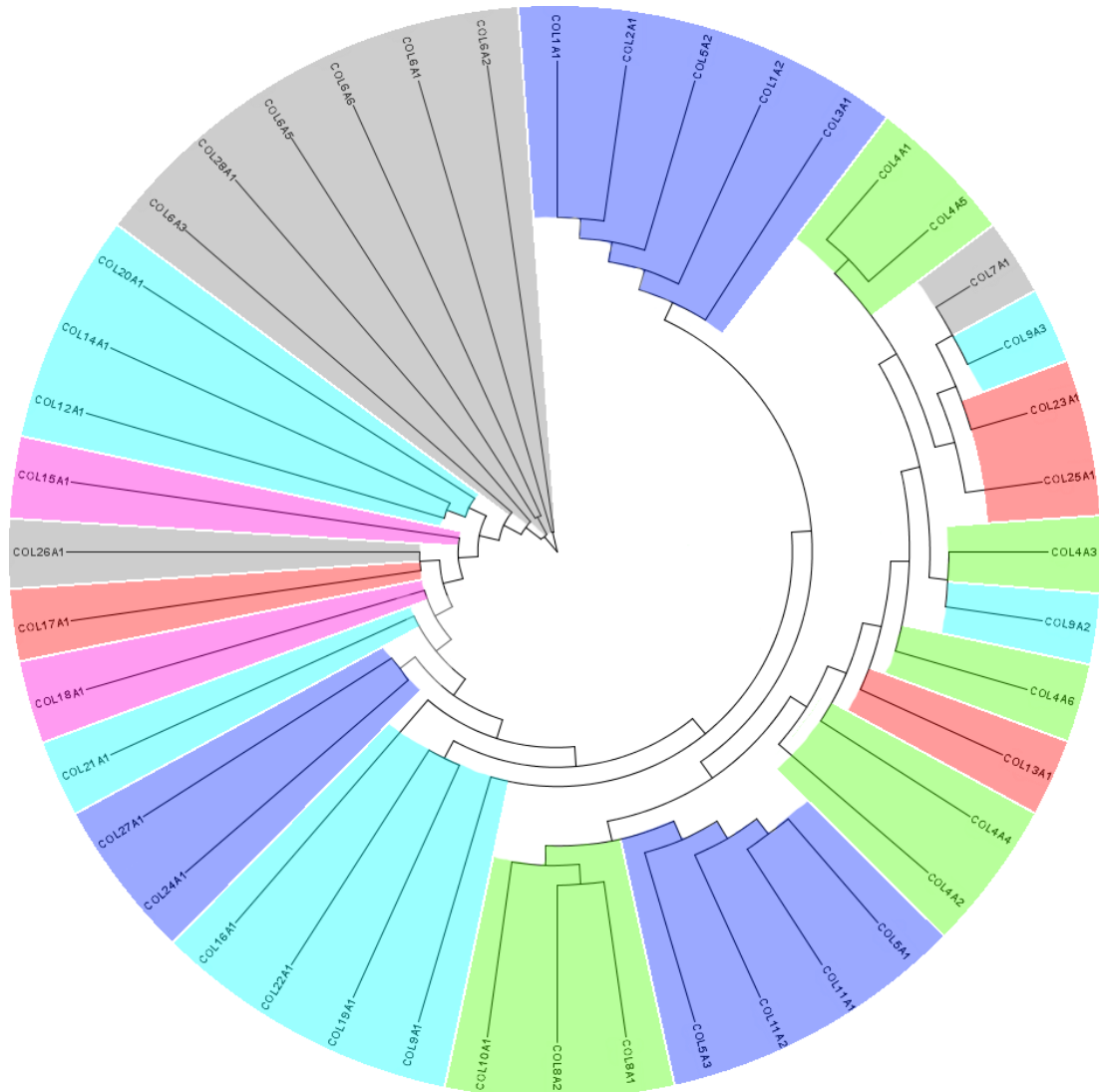


Figure 4.9 – Phylogenetic analysis of human collagens. FACIT collagens are highlighted in **cyan**, MACIT collagens in **red**, multiplexins in **magenta**, network forming collagens in **green**, fibril forming collagens in **blue** and others in **grey**.

The laminin family was examined in the same manner as described above, with protein FASTA sequences found using consensus for "LAMA#", "LAMB#" and "LAMC#". As expected the beta (LAMB) and gamma (LAMC) chain proteins formed groups; but surprisingly the LAMA3 and LAMA5 proteins were found to be diverged from a common ancestor with LAMC proteins before divergence from the other LAMA proteins (Figure 4.10). The analysis of laminin protein sequences also provided a clearly divergent candidate for cross-species phylogenetic analysis in LAMA4. Therefore, LAMA4 was taken forward for cross-species phylogenetic analysis.

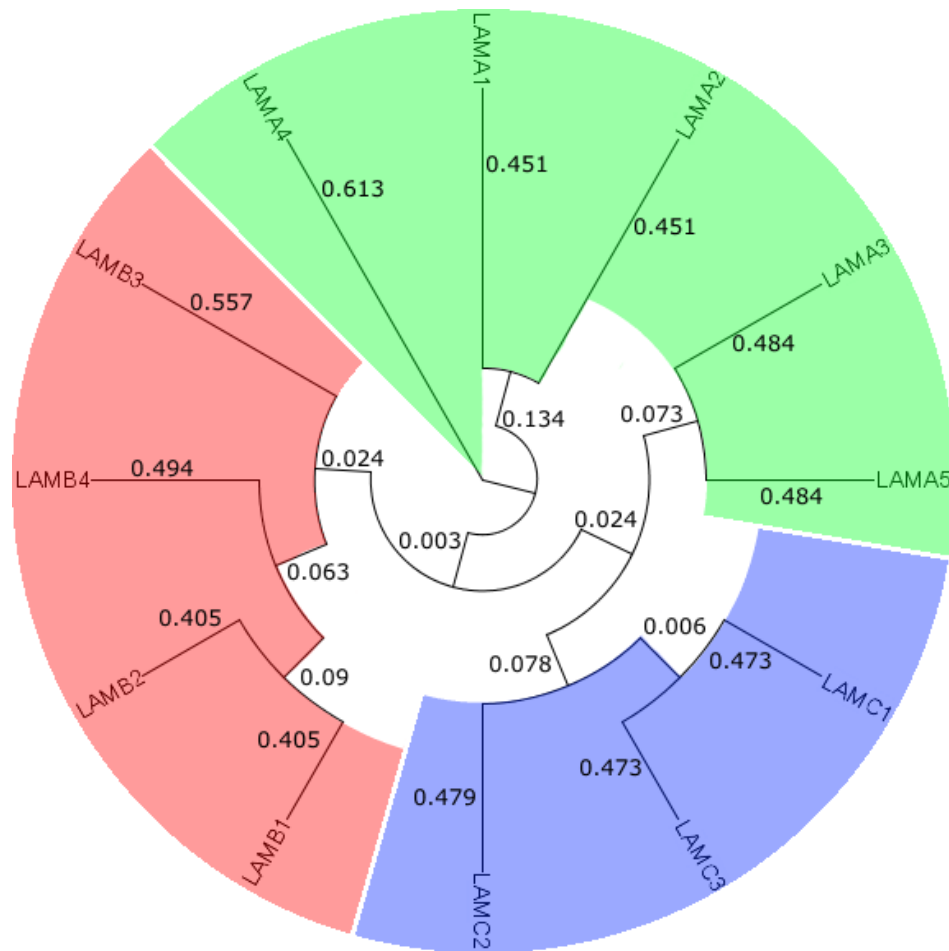


Figure 4.10 – Phylogenetic analysis of human laminins. α -chain laminins are highlighted in green, β -chain laminins in red and γ -chain laminins in blue. Values indicate branch length an arbitrary number for relative similarity.

For the cross-species analysis, sequences were no longer restricted to “reviewed” and “human” sequences. Proteins FASTAs under the gene name “LAMA4” were downloaded for all available species; in the case of duplicates amongst a species, the longest was always selected. The analysis here showed LAMA4 from all mammals, except for the monotreme: *Ornithorynchus anatinus* (duck-billed platypus); were grouped tightly together (Figure 4.11). Analysis was further constrained to common laboratory animals (rat, mouse, rabbit) and livestock (horse, pig, sheep, cow). Livestock Lama4 showed the closest relationships to human LAMA4 by comparison with laboratory animals (Figure 4.12). Of the livestock, bovine lung tissue was the most readily available with laboratory rat and mouse tissue being equally easily obtainable. Given the relation of human LAMA4 with mouse and rat Lama4 was equivalent, rat tissue was selected as their lungs are larger and thus provide a greater mass of matrix.

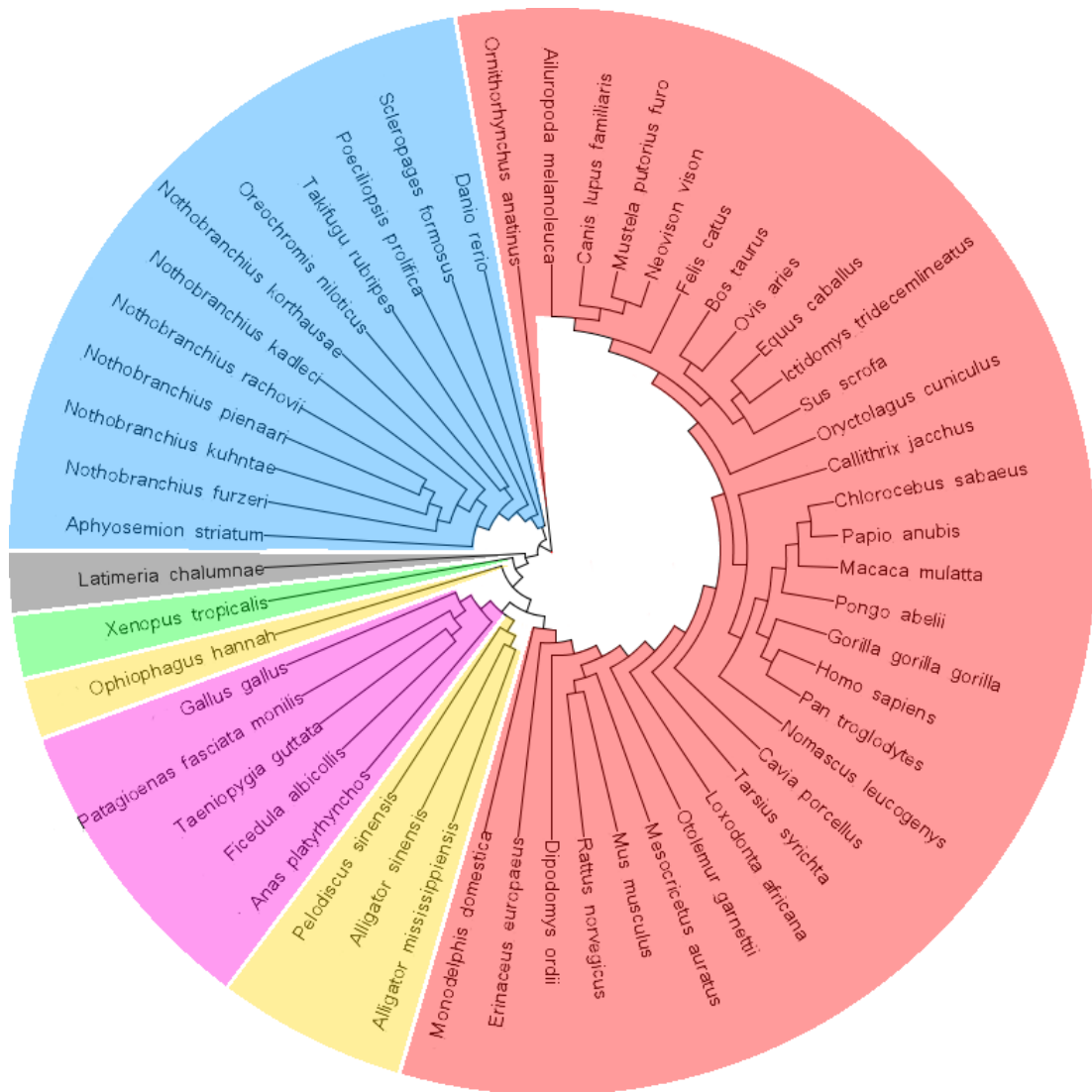


Figure 4.11 – Phylogenetic analysis of LAMA4. Mammalia highlighted in red, reptilia in yellow, aves in magenta, amphibia in green, sactopterygii in grey and actinopterygii in blue.

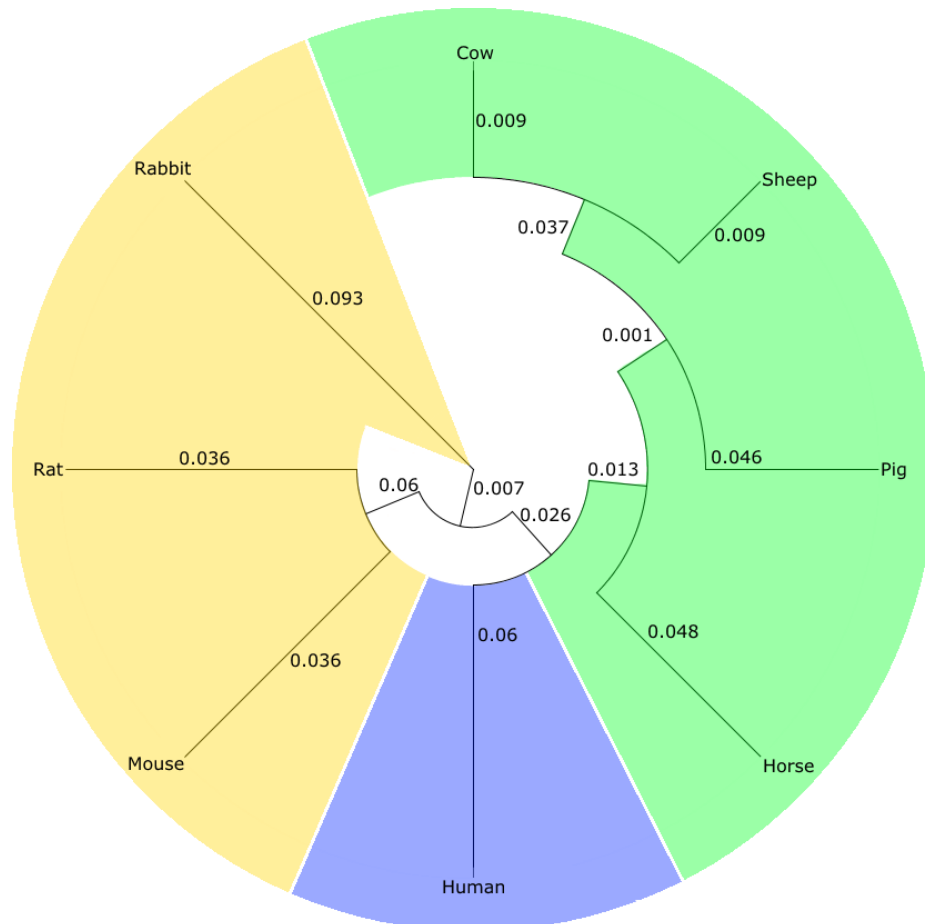


Figure 4.12 – Phylogenetic analysis of LAMA4 in decellularisation target species. Livestock are highlighted in **green**, human in **blue** and laboratory animals in **yellow**. Values indicate branch length and thus relative relationship.

To confirm LAMA4 was a good indicator of ECM protein similarities between human, rat and cow; a further phylogenetic analysis was used to compare all the laminin family members within each species (Figure 4.13). Notably, rats do not encode LAMB4. In most cases it was seen that the bovine protein was more closely related to the human except for in the cases of LAMB1 and LAMC3. Importantly, the distribution of rat and bovine laminin proteins very closely resembled that of the human with LAMA3 and LAMA5 branching from a common ancestor with LAMB proteins as opposed to other LAMA proteins.

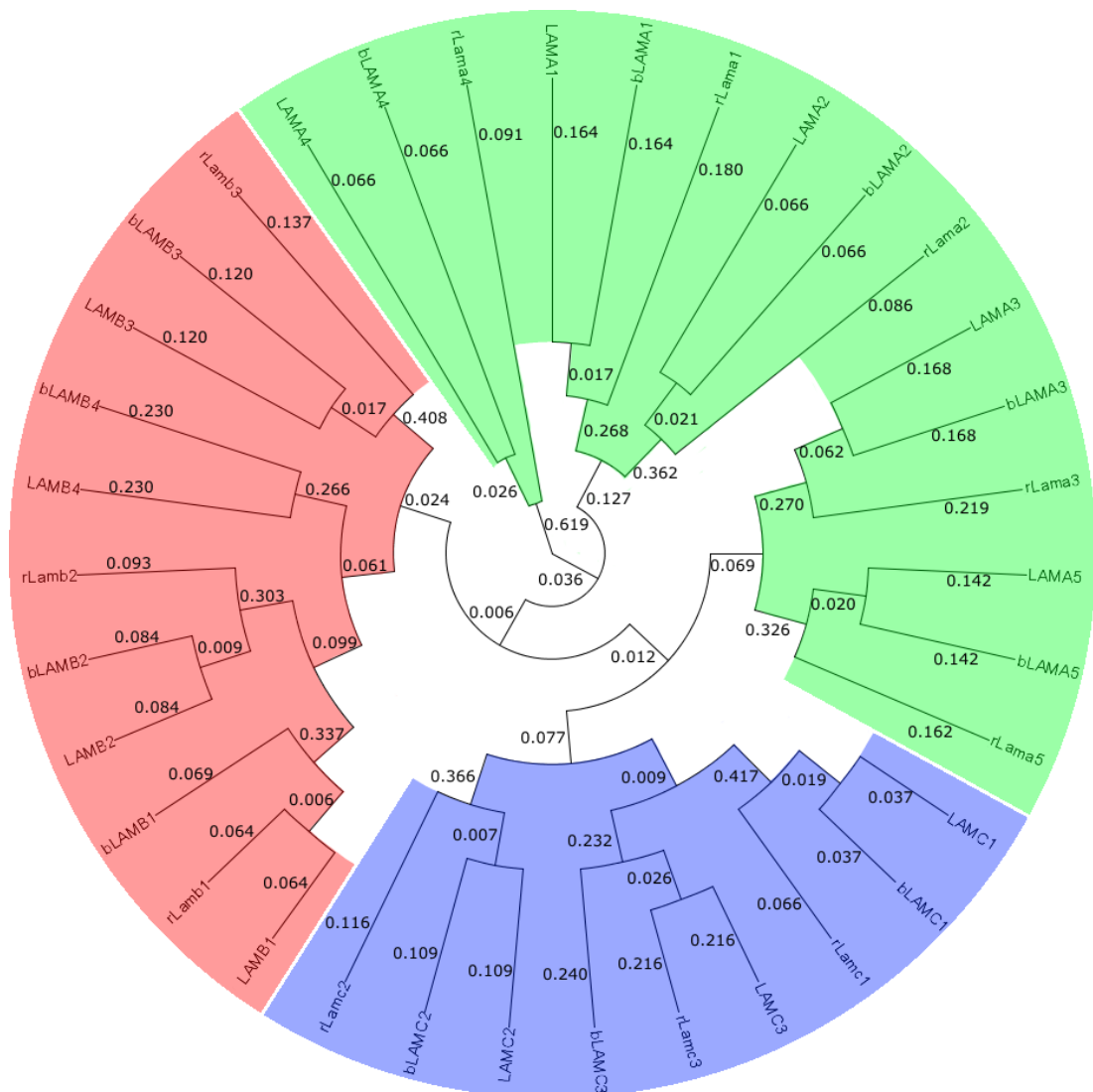


Figure 4.13 – Phylogenetic analysis of bovine, human and rat laminin chains. α -chain laminins are highlighted in green, β -chain laminins in red and γ -chain laminins in blue. Values indicate branch length and thus relative relationship. Bovine proteins are labelled 'b', rat proteins are labelled 'r'.

4.2.3 Decellularised Lung Matrix Preparation

4.2.3.1 Decellularised Bovine Lung Matrix (bDLM)

Considering the similarity by phylogenetic analysis, and the difference in tissue mass between rat and bovine lungs. Bovine tissue was procured for decellularised using the Atala immersion decellularisation protocol (2001), This entailed sequential washes of osmotic lysis in sterile distilled water, clearing with alkaline non-ionic detergents and equilibration in PBS. However, the produced matrix was prone to yeast infection.

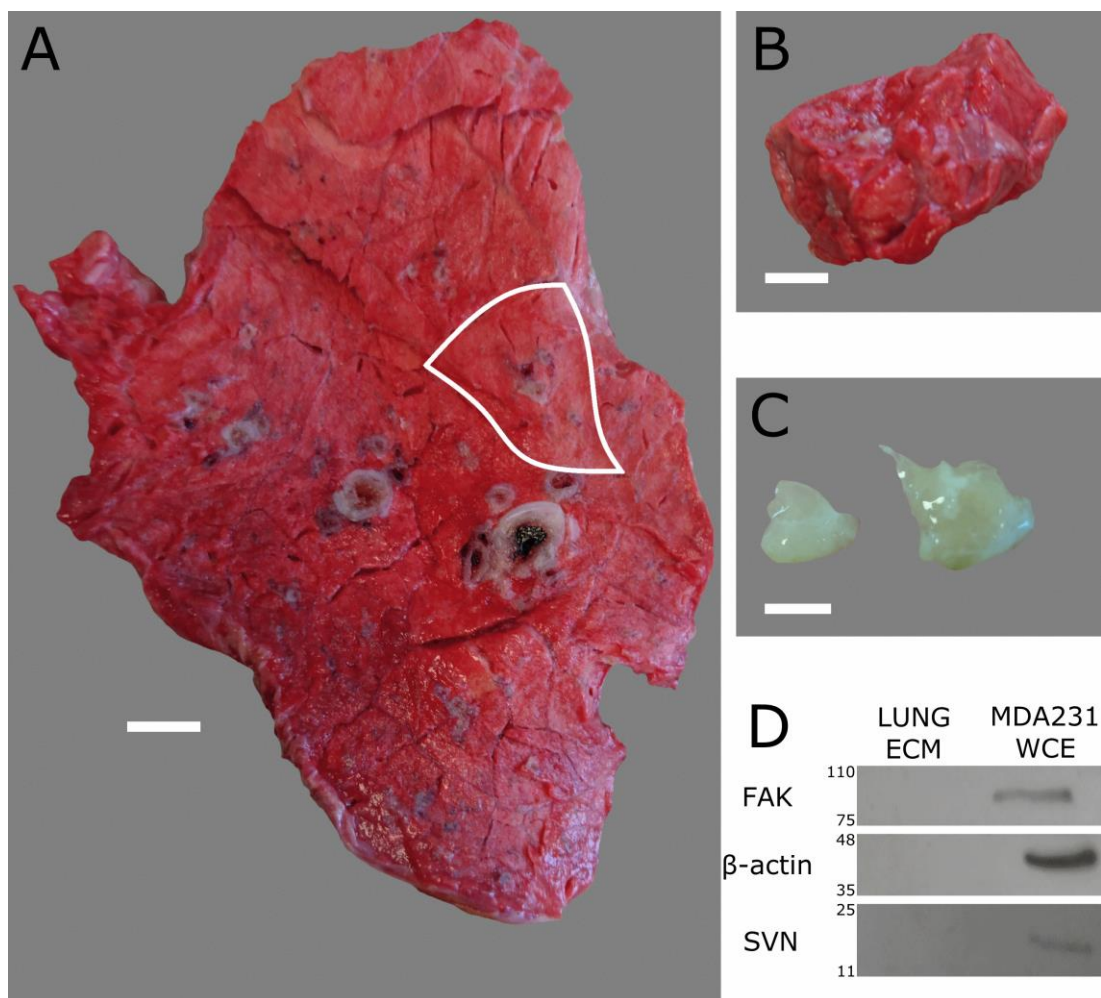


Figure 4.14 – Decellularisation of bovine lung. **A**, bovine lung cross section. **B**, resection of highlighted portion in **A**. **C**, acellular halves of **B**. **D**, immunoblot for cellular markers from protein extracts of **C**. Scale bar 10 mm.

4.2.3.2 Rat Decellularised Lung Matrix (rDLM)

At this point rat lung provided the next best alternative. Rat organs could be easily obtained from discarded animal house carcasses without the need for ethical approval or licensing as the animal's sacrifice was already permitted for another use, and so discarded material was considered 'waste'.

The size of the lungs made decellularisation much more efficient and did not require the resection of lobes, so entire sets of lungs could be decellularised (Figure 4.15A), yielding an amorphous, white matrix. The dry weight of the lung before and after decellularisation revealed that the ECM constituted approximately 10% of the weight of the organ (Figure 4.15A). To prepare the lung matrix for culturing, 6mm discs were bored from whole lung (Figure 4.15B-C) and equilibrated in PBS whilst suspended in a 24-well plate insert (Figure 4.15D). Prior to seeding the PBS was replaced with media and incubated in standard tissue culture conditions for at least 24 h, this also guaranteed the matrix was sterile.

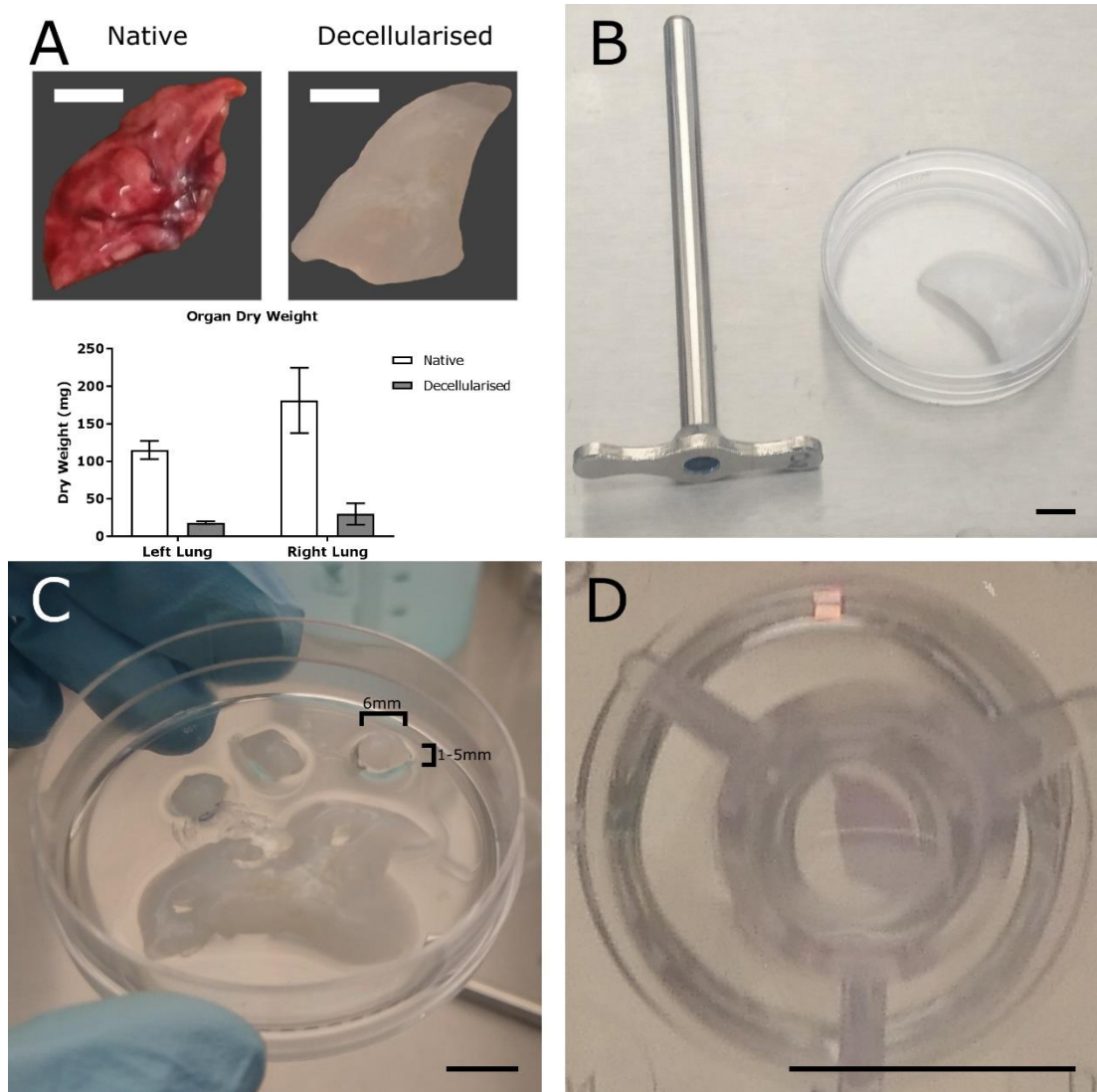


Figure 4.15 –Rat lung decellularisation. **A**, rat lung before decellularisation and after decellularisation with dry weight. **B**, 6 mm gauge boring apparatus. **C**, 6 mm diameter discs bored out of the rat decellularised lung matrix (rDLM). **D**, 6 mm disc equilibration in a 24-well plate insert. Scale bar 10 mm.

4.2.3.3 Decellularisation Efficacy

After successful decellularisation, the structural and biochemical integrity of rDLM was scrutinised. The periodic acid Schiff diastase (PAS-D) tissue stain is normally employed to show the presence of non-amino polysaccharides. This assay can be repurposed to show remaining sugars of a tissue that are not broken down by α -amylase, such as the glycosaminoglycans (GAGs) which are common post translational additions to ECM protein. Stain intensity is reduced when enzymatically treated with a diastase, in this case α -amylase. A pink stained matrix after treatment indicates the presence of amino sugars, with white matrix indicating no sugars. Stained sections of decellularised and native lung showed slightly reduced colour intensity indicating that the decellularisation process was not sufficiently harsh to remove all GAGs (Figure 4.16). When this stain was combined with a haemalum nuclear stain (a haematoxylin analogue), one can also prove that nuclear material has been removed.

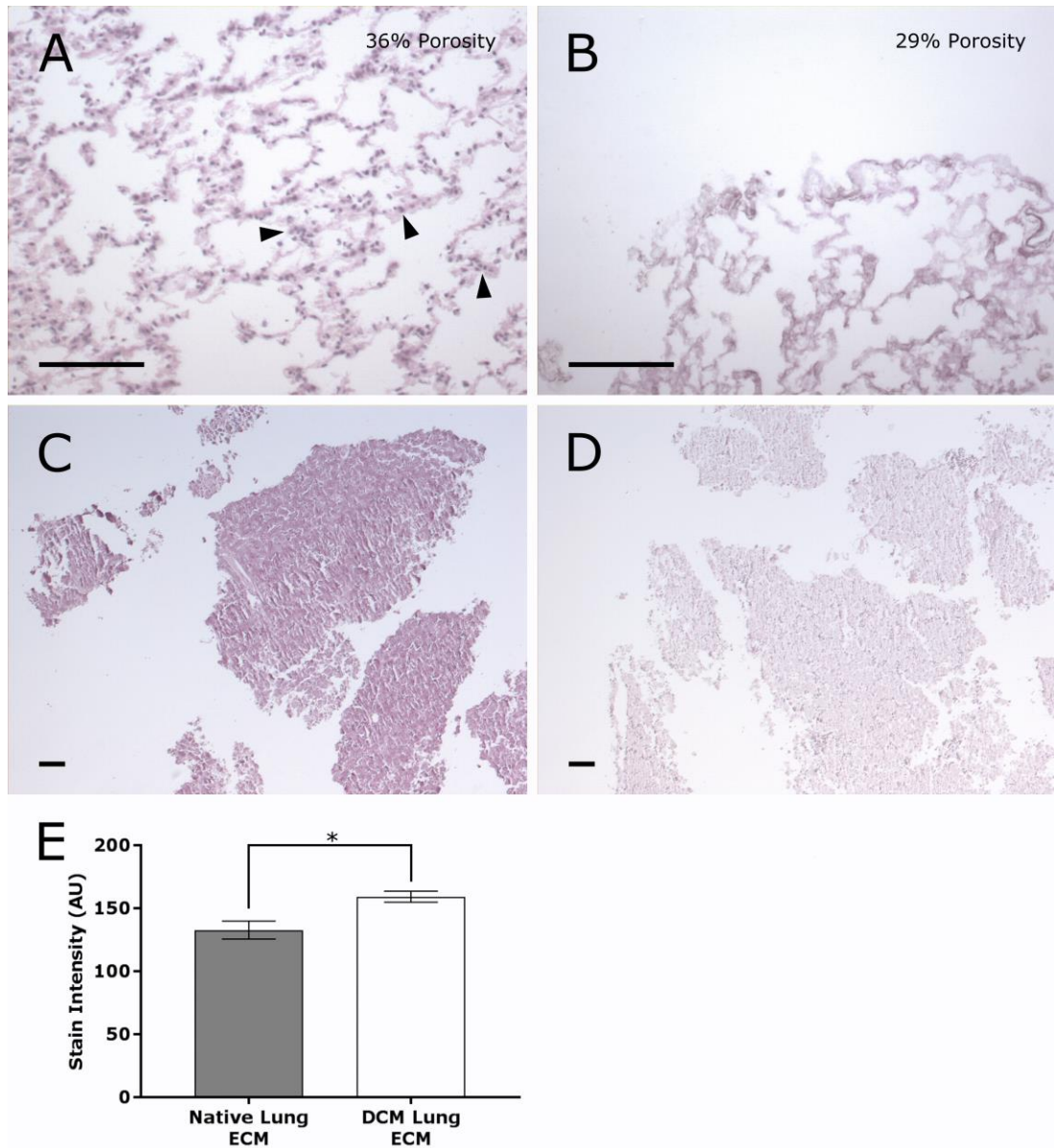


Figure 4.16 – PAS-D tissue stain of control samples. **A**, α-amylase treated rat lung, arrowheads indicate nuclei. **B**, α-amylase treated rDLM. **C**, un-treated rat liver. **D** α-amylase treated. **E**, quantification of stain intensity in **A** and **B** (Welch's t-test; *, $P=0.0451$, $n=3$). Nuclei are stained black, non-amino polysaccharides stain pink, amino sugars stain pale pink. Scale bar 50μm.

4.2.3.4 rDLM Biocompatibility

To determine if the matrix was biocompatible with cell lines. MDA231 cells were seeded and cultured on rDLM for 7 days, then harvested and cell lysates prepared by sonication and immunoblotted. β -actin acted as a cellular marker and total laminin as an ECM marker. There was significantly less β -actin detected in empty matrix (EM) samples by comparison with both 2D and 3D samples, indicating successful removal of cellular material (Figure 4.17). Contrastingly, there was comparable levels of β -actin in 2D samples and 7-day rDLM cultures (3D). pH3 was used as a mitotic marker and was not present in 3D samples. Importantly it must be noted that rat β -actin is detected by the antibody employed and would be visible as an identically sized band in immunoblots.

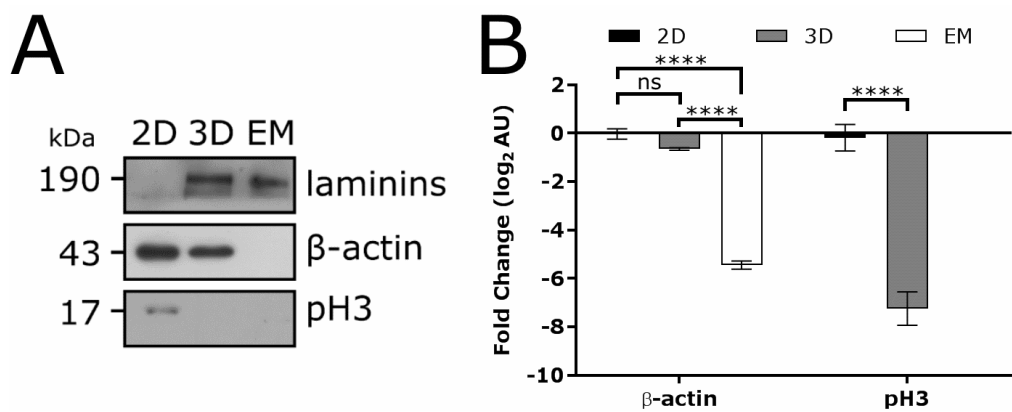


Figure 4.17 - Immunoblot of MDA231 in monolayer and rDLM culture. **A**, laminin, β -actin and histone H3 (phospho S20; pH3) immunoblot of MDA231 cells grown in 2D and rDLM with an empty matrix control. **B**, quantification of blot in 'A'.

4.2.4 MCF7 and MDA231 Cell Behaviour in rDLM

To test if the rDLM would allow growth, invasion and ECM remodelling, 1×10^6 MCF7 and MDA231 cells were seeded onto matrices and cultured in standard tissue culture conditions for 2 weeks refreshing media when necessary. Samples were then fixed, paraffin embedded, sectioned, and stained for proteins of interest. Changes in the distribution of collagen I and laminin-332 were used as markers for potential ECM remodelling. Laminin-332, cortactin and E-cadherin expression and distributions were used as markers for an invasive phenotype. Invasive cells are typically enriched in cortactin and colocalise with laminin-332 whilst repressing E-cadherin. Ki67 and survivin acted as markers non-quiescent and mitotic cells respectively.

4.2.4.1 MCF7 and MDA231 Cells Colonise rDLM

MCF7 and MDA231 cells both colonised the matrix; however, MCF7 cells aggregated in crevices, whilst MDA231 cells were more invasive and nomadic becoming more dispersed throughout the matrix (Figure 4.18). Interestingly, MCF7 cell aggregates stained positive for amino-sugars. It was not possible to determine if MDA231 cells stained positively for GAGs due to their distribution. Both MCF7 and MDA231 cells were compatible with the laminin-rich matrix (Figure 4.19)

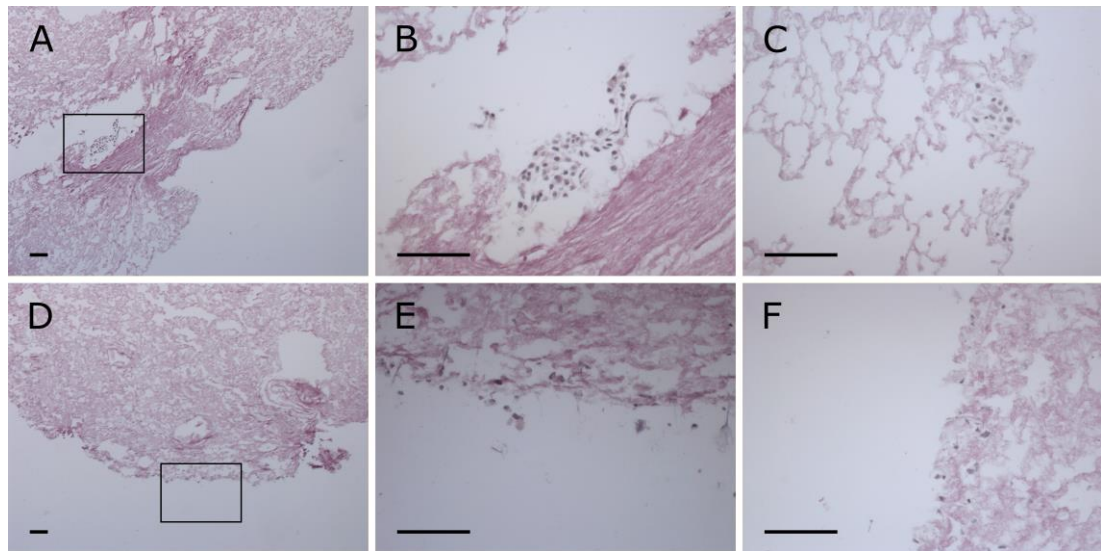


Figure 4.18 - Periodic acid Schiff diastase (PAS-D) tissue stain of MCF7 and MDA231 cells grown in static culture. A, large MCF7 aggregate. B, region shown in A. C, small MCF7^{casp3} aggregate. D, region shown in C. E, MDA231 matrix infiltration. F, representative region from E. Scale bar 50µm.

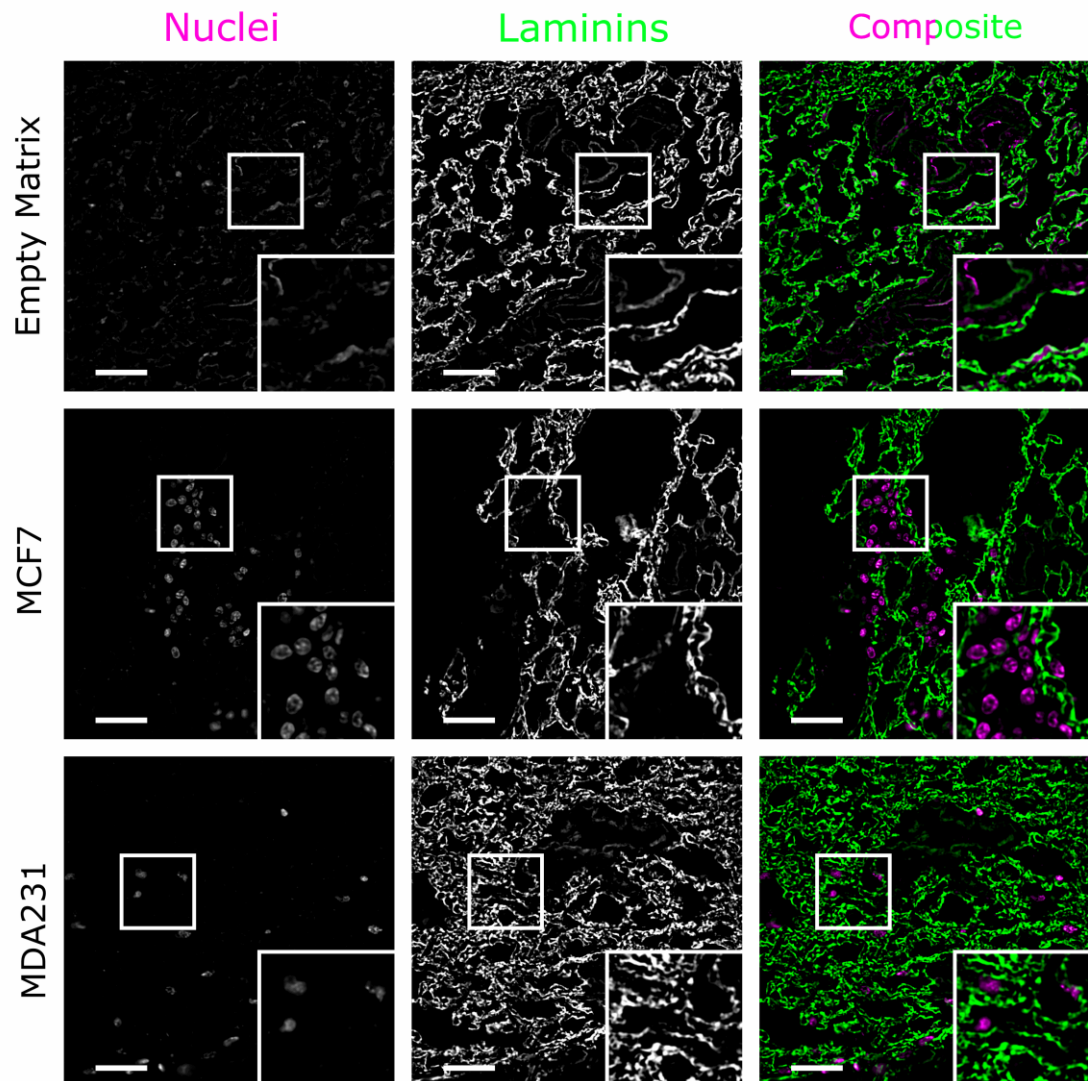


Figure 4.19 - Total laminin stain of MCF7 and MDA231 cells grown on acellular rat lung in standard culture conditions. Scale bar 50µm. n=3.

4.2.4.2 Neither MCF7 Nor MDA231 Cells Remodel rDLM with Collagen I or Laminin-332.

The rDLM generated contained essential GAGs and was found to be laminin rich (Figures 4.18 and 4.19). The possibility of matrix remodelling was now assessed by staining for the fibroblast-associated protein: collagen I. This protein makes up the majority of bone tissues, a common metastatic locus for breast cancer. As such, it was hypothesised that in the absence of collagen rich 'soil' breast cancer cells may synthesise collagen I *de novo*. Staining for collagen I revealed no *de novo* synthesis of collagen I in alveolar rDLM. The only positive stain observed was in decellularised blood vessels (Figure 4.20), and vascular smooth muscle which was used as a positive control.

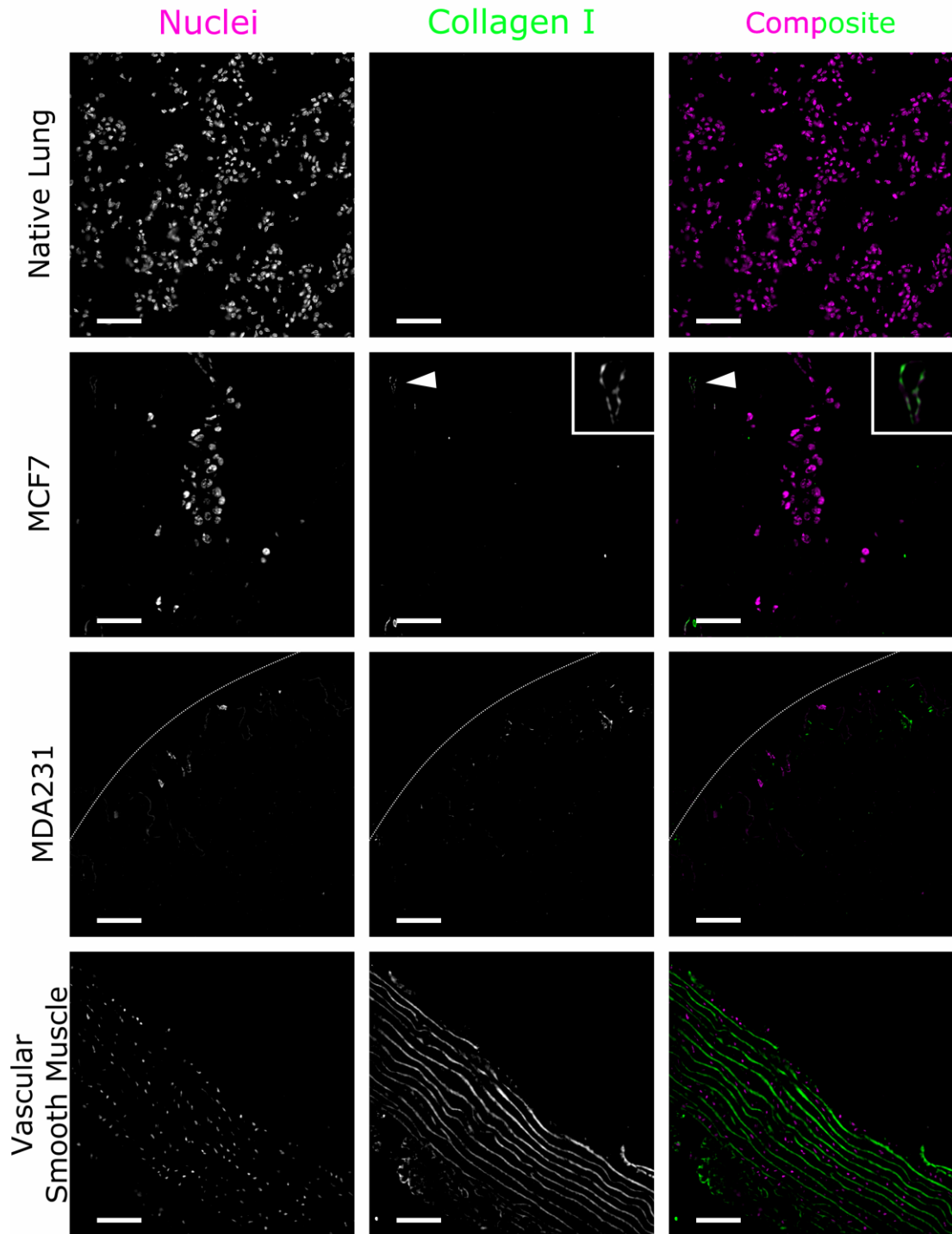


Figure 4.20 – Collagen I stain of MCF7 and MDA231 cells grown on rDLM in standard culture conditions. Collagen I was only observed in a decellularised blood vessel and in the positive control: vascular smooth muscle. Scale bar 50 μ m. n=3.

Carpenter et al (2009) have shown showed that laminin-332 is deposited by MCF7 cells in 2D monolayer and can induce migration. Therefore, immunohistological staining was undertaken to determine the presence of any *de novo* laminin-332 synthesis. Laminin-332 was absent in empty matrix controls, in MCF7 samples and MDA231 samples, with the only positive staining observed in the coronary artery endothelium control (Figure 4.21).

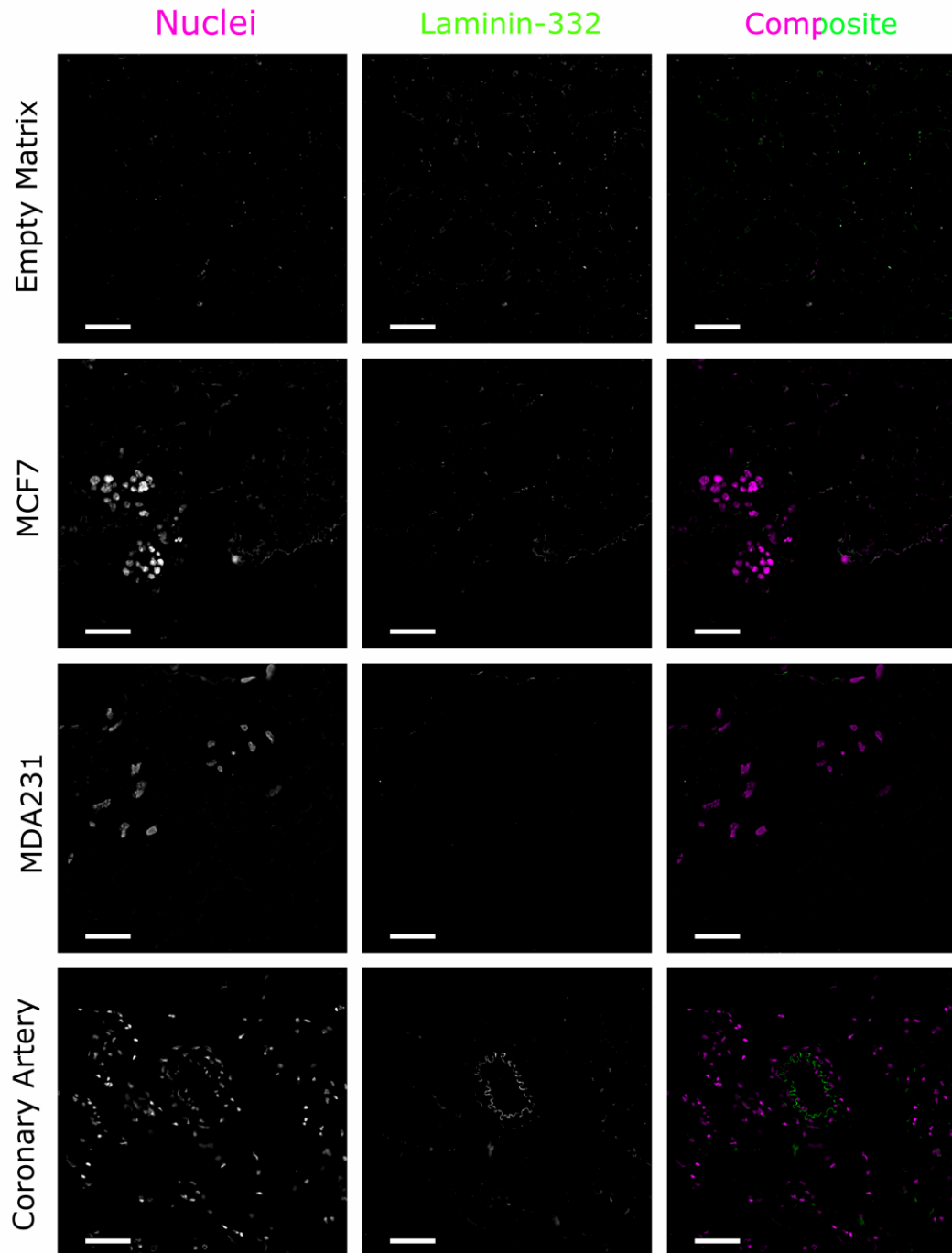


Figure 4.21 – Laminin-332 stain of MCF7 and MDA231 cells grown on rDLM in standard culture conditions. Scale bar 50µm. n=3.

4.2.4.3 MCF7 and MDA231 Cells Can Invade in rDLM

Having established that MCF7 and MDA231 cells do not remodel rDLM with collagen I or laminin-332, it was hypothesised that the mitotic and invasive potential of the cells would be reduced in rDLM. Colocalisation of cortactin and E-cadherin was used to mark static cells, with cortactin alone acting as an invasion marker. MCF7 cells expressed elevated levels of cortactin and low levels of E-cadherin (Figure 4.22). Whereas, MDA231 cells remained E-cadherin negative and surprisingly, cortactin was not detected in MDA231 cells despite the highly invasive phenotype in monolayer culture.

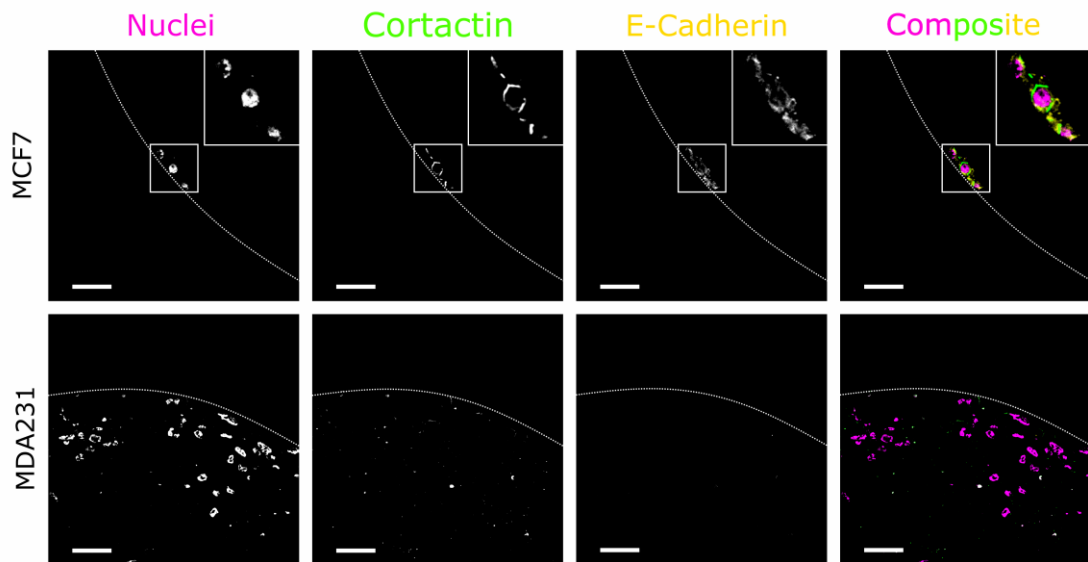


Figure 4.22 – Cortactin and E-cadherin stain of MCF7 and MDA231 cells grown on rDLM in standard culture conditions. Dotted line marks matrix edge. Scale bar 50µm. n=3.

4.2.4.4 rDLM Cultures of MCF7 and MDA231 Cells Maintain Proliferative Potential

Having assessed the expression of invasion markers in MCF7 and MDA231 cells, the next behavioural trait assessed was mitotic potential. Ki67-positive staining highlights cells that are not in G_0 , and survivin-positive staining highlights mitotic cells. Staining for Ki67-positive cells yielded multiple positive cells for both cell lines; with MCF7 cells having many nucleolar-localised Ki67 puncta (Figure 4.23). In contrast, MDA231 cells showed colocalisation of Ki67 with DNA but not nucleoli, which indicates few cells in G_1 - G_2 and many cells in M-phase, assuming this protein is localising correctly. Staining for survivin revealed a high prevalence of survivin in the cytoplasm of MCF7 cells, whereas in MDA231 cells survivin localised to the nuclei (Figure 4.24). These results were of particular interest because survivin is a protein associated with high grade metastatic tumours (Ning *et al.*, 2015; Adams *et al.*, 2016).

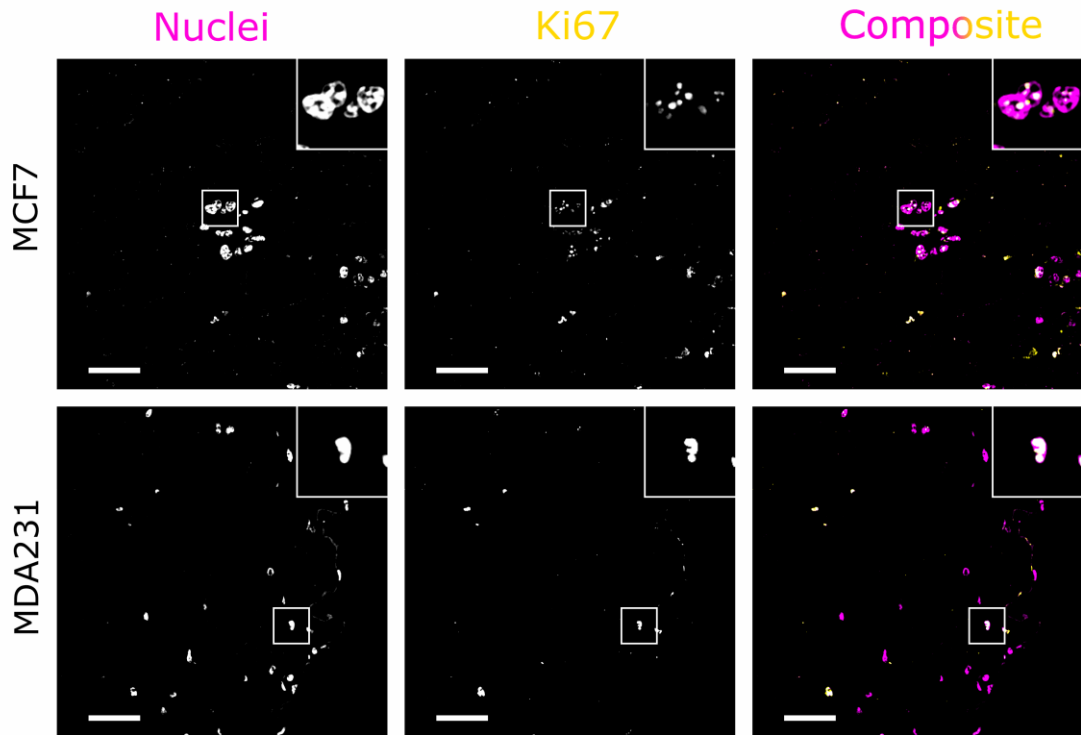


Figure 4.23 – Ki67 stain of MCF7 and MDA231 cells grown on rDLM in standard culture conditions. White portions in the composite image represent colocalisation. Scale bar 50 μ m. n=3.

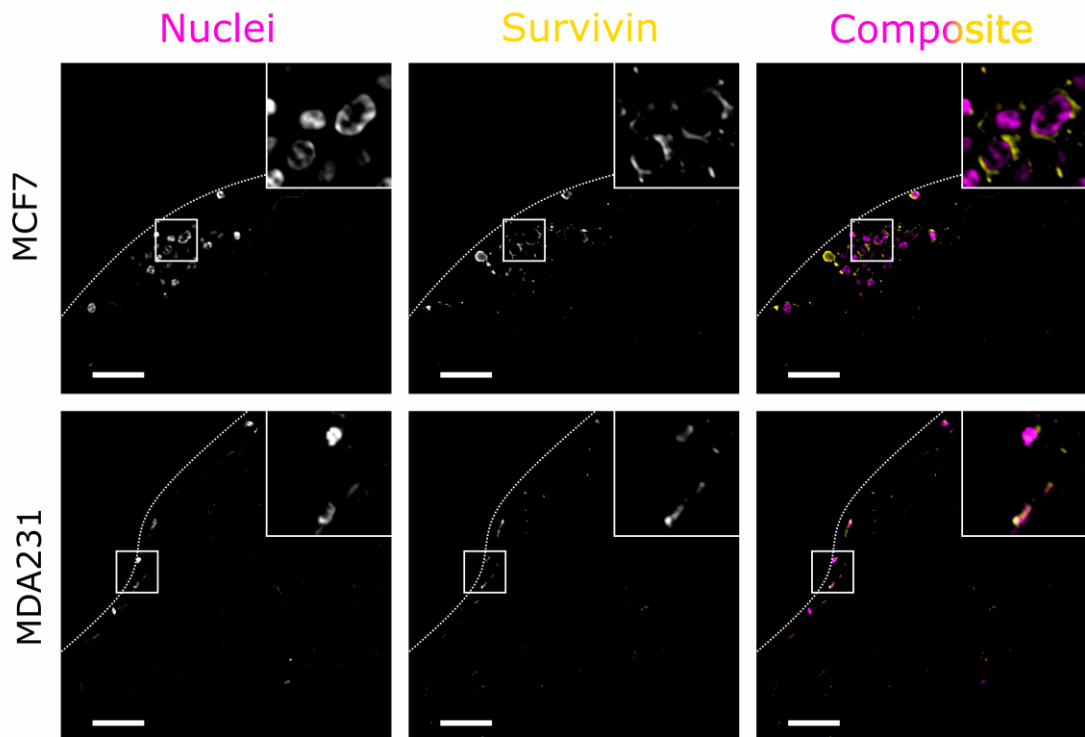


Figure 4.24 – Survivin stain of MCF7 and MDA231 cells grown on rDLM in standard culture conditions. White portions in the composite image represent colocalisation. Dotted line marks matrix edge. Scale bar 50 μ m. n=3.

4.2.4.5 Invasion of MDA231 Cells Into rDLM

To investigate the invasive properties of MDA231 cells in the rDLM system, cells were cultured for 7 days. Samples were then embedded and sectioned in their entirety and stained to show the nuclei. A representative area of each section was then imaged, and images processed with a semi-automated nucleus counter and point reference system using Fiji software. This data set was then handled in Microsoft Excel to determine the minimum distance of each cell from the edge of the matrix. The section number determined the minimum distance through the z-axis, whilst the point reference system enabled determination of the minimum distance through the x- and y-axes. This process revealed that most cells growing on rDLM do not invade further than $\sim 150 \mu\text{m}$ (Figure 4.25). Approximately half this number invaded up to $300 \mu\text{m}$; with a very small sub-population ($\sim 2.5\%$) migrating the maximum distance of $450 \mu\text{m}$. The maximum distance migrated in 7 days was comparable to the linear distance migrated in 16 h in previous, conventional monolayer wound healing assays (section 3.2.1).

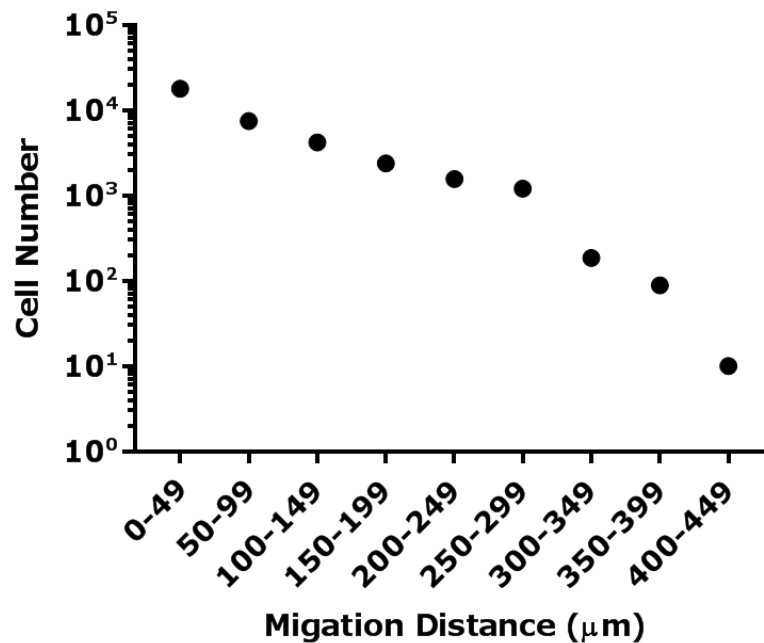


Figure 4.25 – Invasion of MDA231 cells into rDLM. MDA231 cell invasion distance was binned at $50 \mu\text{m}$ intervals. The number of cells in each bin roughly followed an exponentially decreasing trend. $N=34,986$, $n=3$.

4.3 Discussion

4.3.1 Lung Fibroblasts Influence Breast Cancer Cells

Data here shows that the influence of lung fibroblast cells (MRC5) on MDA231 is twofold: MDA231 cells are rescued in serum starved conditions by lung fibroblasts, and migration of MDA231 cells is inhibited by lung fibroblasts.

4.3.1.1 Lung Fibroblasts Rescue Serum Starved MDA231 Cells

Both MRC5 and MDA231 cells had increased growth rate (decreased doubling time) when in co-culture. This suggests paracrine and juxtacrine signalling play a role in MDA231 metabolic regulation. Robust androgen signalling is also a possible cause for increased growth rate and it has previously been shown that MDA231 cells are responsive to androgen. However, there is no data yet on whether MRC5 cells can produce androgen. (Cochrane *et al.*, 2014). The assessment of metabolic activity of MDA231 cells in the presence of MRC5 would be complemented by using TMRE as described (section 3.2.4.1) along with an NADH turnover assay. However, given the easy integration of these component into rDLM, starting in 3D would provide even more information on the response of MDA231 cells to apoptotic stimuli in the presence of fibroblasts and a biomimetic ECM.

4.3.1.2 Lung Fibroblasts Inhibit MDA231 Migration

Wound healing assays here revealed that MRC5 inhibited MDA231 migration. Conventionally, migration is assessed by wound healing or Boyden chamber trans-well assays, neither of which usually account for the presence of other cell types and the ECM in the tumour microenvironment. The migration of MDA231 cells into an empty space devoid of matrix or cells is not representative of tumour physiology. Migration of ~500 µm within 16 h in a real tumour, means cells would infiltrate surrounding tissues and distal sites within hours of a migratory population forming. Physiologically, most cancers, excluding childhood

cancers, develop over years with primary or pre-malignant tumours being more easily resected if they are detected.

Small airway epithelial cells have previously been shown to produce laminin-332 and laminin-511, both of which induced MCF7 cell migration (Carpenter *et al.*, 2012 & 2017). It was clear that the presence of MRC5 cells significantly perturbed MDA231 cell migration. This suggests that the location in which metastasising breast cancer arrive is significant in its ability to colonise.

4.3.2 Lung Fibroblast Organoid Structure is Influenced by MDA231 Cells

Organoid co-culture is an emerging area of research. The integration of multiple different cell lines into organoids has led to more informative data on drug response, metabolic changes and apoptotic response of *in vitro* malignancies (Chiche, Brahimi-Horn & Pouyssegur, 2010; DiGiacomo & Gilkes, 2018). It was shown here that morphological, mechanical and biochemical properties of MRC5 cells were disrupted by the presence of MDA231 cells.

4.3.2.1 Morphological and Mechanical Properties of Lung Fibroblast Organoids

Extracellular matrix proteins are responsible for a large proportion of the structural and mechanical properties of tissues and organs. Typically, collagens and laminins create the bulk of protein mass within any ECM with different forms. Isoforms and the ratio of collagen to laminin, defines the overall mechanical properties. Bones for example are highly enriched in highly cross-linked fibrillar collagens whereas lungs are considerably richer in laminins (Ricard-Blum, 2011; Xiong *et al.*, 2015). Lung fibroblast organoid cultures stained for collagens I, IV and XII revealed an enrichment of collagen I in the outer ring of cells with an absence of collagens IV and XII. The absence of network-forming collagen IV and FACIT collagen XII suggests the organoid structure is very fragile with intercellular adhesions contributing to organoid

stability. Staining for E-cadherin and cortactin, an adherens junction anchoring protein, revealed a distinct absence of both proteins throughout the organoid. Suggesting the involvement of protein junctions other than the adherens junction in the stability of the organoid.

MRC5 organoids that were generated in the presence of MDA231 cells were smaller in size and had similar ECM protein distribution. The difference in size plays a key role in cellular phenotype within the organoid, as large organoids tend to have necrotic cores if there is no lumen (Froehlich, K. *et al.*, 2016). The high concentration of collagen I in co-culture organoids suggests that these organoids are mechanically stable. In terms of intercellular adhesions, co-culture organoids had comparable levels of E-cadherin paired with a much more widely spread presence of cortactin, suggesting a change in inter-cellular adhesions by comparison with MRC5 organoids.

The distribution of cortactin in co-culture organoids not only varied but both samples had sporadic, highly concentrated cortactin puncta in some nuclei. There is very little known of any transcriptional role played by cortactin, however, the C-terminus of some cadherins can be cleaved by γ -secretase, before nuclear-trafficking and transcriptional activity, and cortactin binds indirectly to this region via catenins (Reiss *et al.*, 2005).

4.3.3 Organ Decellularisation Targets Directed by ECM Protein Phylogeny

Phylogenetic analysis of the collagen and laminin families revealed some interesting relationships. Surprisingly collagen proteins did not group according to their family suggesting multiple, unique, genetic events in the evolution of these proteins. This is exemplified by the separation of MACIT collagens which did share a common ancestor with other MACIT collagens. This strongly suggests that the transmembrane helix domain within each protein arose by two unique

evolutionary events. The complexity of this evolution made collagens a bad candidate for cross-species analysis.

Phylogenetic analysis of laminins revealed an interesting potential evolutionary pathway, whereby laminins $\alpha 3$ and $\alpha 5$ grouped with γ -chain laminins, suggesting that the common ancestor of all laminins was no larger than the size of short α -chain laminins or β - and γ -chain laminins. This is further supported by the two splice variants of laminin $\alpha 3$ being long (3,333aa, B-form) and short (1,724aa, A-form; Aumailley *et al.*, 2005). The tight grouping of laminin phylogeny when including bovine and rat protein sequences also suggests that the evolutionary history of laminin proteins is ancient.

Laminin $\alpha 4$ was selected as a candidate protein for cross-species analysis due to its ancient divergence from other laminin-family proteins. The subsequent multi-species analysis showed this to be an adequate method for selecting a candidate as multi-species analysis was close to the theory of evolution.

4.3.4 Decellularisation Efficiently Removed Rat Lung Cellular Material

Decellularisation can be performed in many ways, often with the use of osmotic lysis with subsequent flushing of cell debris utilising sodium dodecyl sulphate or Triton based buffers. The way these washes are applied also varies from simple submersion with agitation, to more complex perfusion systems (Crapo, Gilbert & Badylak, 2011; Guyette *et al.*, 2014; Kang *et al.*, 2016). Here, a basic Triton-X100 / ammonium hydroxide buffer was used to efficiently remove cellular material which was tested threefold: by staining with DAPI and haemalum, combined with immunoblotting for β -actin. Both stains were negative on histological sections, with β -actin being absent in immunoblots of empty matrix controls. Similarly, the retention of most GAGs and laminins, minimal changes in porosity and cellular viability on the matrix, provides substantial

evidence that the decellularisation process employed here is highly efficacious with minimal changes to the matrix ultrastructure.

The efficacious removal of cellular material in the fashion described is consistent with the literature, specifically relating to decellularisation of more porous organs and tissues (Crapo, Gilbert & Badylak, 2011). Also related to this methodology, the use of livestock-derived organs for decellularisation proved problematic due to persistent yeast infection. The process of decellularisation on bovine lung took longer than rat lung, likely due to the size of the resections. However, previous work on perfusion decellularisation has proved successful with whole bovine organs (Guyette *et al.*, 2014). Alternatively, antibiotics / antimycotics given to livestock may have led to the rise of a streptomycin-resistant strain of yeast in bovine tissues as this can be legally fed to cattle whereas ampicillin cannot (DEFRA, 2012).

4.3.5 rDLM Provides MCF7 and MDA231 Cells with Fertile 'Soil'

In the rDLM static culture system used here, MCF7 cells were seen to cluster and get 'trapped' in crevices within the matrix whilst MDA231 cells created a dispersed layer throughout the surface of the matrix with a small sub-population of cells invading deep into the matrix. These observations are consistent with a delamination strategy of metastasis adopted by MCF7 cells and an active, single cell, metastatic strategy in MDA231 cells. These behaviours also correlate to their tumour type clusters, luminal with respect to MCF7 and basal B with respect to MDA231 (Kao *et al.*, 2009; Holliday & Speirs, 2011). This shows that different breast cancers can employ different metastatic strategies, even if they are both epithelial in origin. MCF7 is associated with a primary invasive ductal carcinoma whilst MDA231 is associated with a primary adenocarcinoma (Neve *et al.*, 2006).

4.3.6 Proliferative Potential of MCF7 and MDA231 Cells in rDLM

Nuclear Ki67 staining identifies cells in G₂ or M-phase of the cell cycle, while nucleolar Ki67 is indicative of ribosome biogenesis and G₁ (MacCallum & Hall, 2000). As a result, a primarily nucleolar localisation of Ki67 in MCF7 samples suggests a more conservative proliferative-phenotype by comparison with MDA231 samples which had a primarily DNA colocalisation of Ki67. It would be interesting to determine the stem-cell like properties of these two cell lines when culture in rDLM, to determine if there is any further difference to their respective modes of metastasis.

4.3.7 Invasive Potential of MDA231 Cells in rDLM

MDA231 invasion in 3D was assessed by recapitulating cell locus in stacked histological sections of rDLM. This process, combined with a coordinated based system during quantitation, allowed the determination of the linear distance from each cell to the matrix surface. This led to the determination of a highly motile sub-population of cells which were able to migrate 450 µm in 7 days. This proves that MDA231 cells maintain their invasive potential when seeded on rDLM. Combined with the ease of generating rDLM, this shows that analysing cell migration in a more biomimetic environment is just as accessible as traditional methods.

4.3.8 Summary

The data presented here have further shown the dependence of MDA231 cells on serum and how this can be recovered by co-culture with normal lung fibroblasts. It has also been shown that lung fibroblasts inhibit MDA231 cell migration. However, MDA231 cells also influenced MRC5 cell organoid formation and structure, suggesting each cell line produces factors to which the other is responsive.

To expand on the relevance of ECM proteins in breast cancer cell behaviour, the use of both bovine and rat decellularised matrices was justified by protein homology. This led to validation of excess/discarded rat lung decellularised matrices in 3D cell culture, with subsequent examination of the metastatic potential of MCF7 and MDA231 cells revealing delamination and invasion behaviours respectively.

Chapter 5

Breast Cancer Metastasis In A Two-Chamber Bioreactor System

5.1 Overview

When biomaterials are employed in cell culture they can more accurately mimic many facets of a cells microenvironment. The ECM can be more accurately represented by decellularised matrices, Matrigel and other hydrogels. Similarly, the addition of other cellular cues such as growth factors can be easily added to media, with coculture providing another source for altered extracellular signalling molecules. A key component of the cellular microenvironment is forgotten however, the flow of blood, lymph or interstitial fluid past the cells of interest. Even extremely small flow rates can make a difference to cell phenotypes by altering shear stress across the cell surface (Reiss *et al.*, 2005).

5.1.1 Bioreactors

'Bioreactor' is a broad term, commonly describing systems for generating useful proteins, such as insulin, and carbon-based molecules, such as ethanol (Polez *et al.*, 2016; Rech *et al.*, 2018). Other bioreactor systems exist for a multitude of purposes including: decellularisation and cancer cell culture (Crapo, Gilbert & Badylak, 2011; Guyette *et al.*, 2014; Kang *et al.*, 2016). The bioreactor used here was designed to mimic cell transfer as in metastasis. Using a sequential chamber system with recirculated media allowing cells in a matrix to detach into the media and be carried by media flow to a second destination.

5.1.2 Microfluidics

A key difference in many bioreactors is the flow rate and the related flow turbulence, represented by a Reynold's number. In arterioles for example, Reynold's numbers are extremely low, meaning the flow is laminar (section 2.1.5.1). Completely laminar flow implies that all molecules in the liquid are moving in parallel layers in the same direction, with no turbulence. Microfluidic systems are exclusively laminar due to the low flow rates, measured in $\mu\text{l}\cdot\text{min}^{-1}$. Microfluidic systems can be in the form of chips (Campos Marín, Brunelli & Lacroix, 2018), as well as actively pumped (Calle, Petersen & Niklason, 2011).

Microfluidic culture chips are designed to emulate flow by osmotic pressure, whereas actively pumped microfluidic systems have accurately controlled flow rates which can be anastaltic or peristaltic. Anastaltic flow is consistent and peristaltic flow fluctuates between a higher and lower flow rate. Peristaltic flow is represented in the human circulatory system.

5.2 Results

5.2.1 MDA231 in Dual-Chamber Dynamic Culture

To build on the co-culture systems previously described (section 4.2.3.2), decellularised lung matrices were used in concert with a bioreactor system to mimic the full metastatic journey (Figure 5.1).

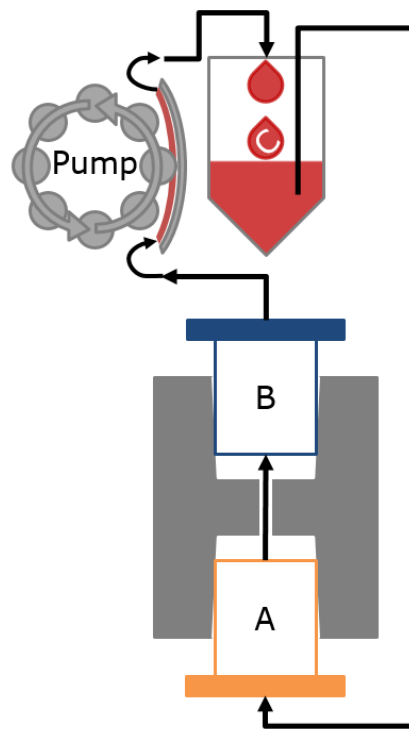


Figure 5.1 – Sequential chamber bioreactor schematic. Media is pulled through chamber A to B before being pushed into the media reservoir.

Samples were seeded and cultured in static conditions for 96 h to ensure sufficient adhesion, before being transferred into the bioreactor with the configurations shown in Table 5.1. Empty matrix, MDA231, MRC5^{GFP} and co-culture controls remained in static culture for the duration of the subsequent 96 h dynamic culture.

Table 5.1 – Bioreactor configurations.

Chamber	1	2	3
A	MDA231	Empty Matrix	MDA231
B	Empty Matrix	MRC5 ^{GFP}	MRC5 ^{GFP}

After dynamic culture, all samples had a small piece resected for light sheet imaging whilst the remainder was used for protein extract analysis. Immunoblot analysis revealed upregulation of FAK in MDA231 dynamic cultures, but only when empty matrix was in chamber B. Furthermore, FAK was only detectable in MRC5^{GFP} dynamic culture samples. MRC5^{GFP} cells also showed a high propensity for matrix remodelling by synthesising collagen I, in both static and dynamic culture conditions; but not when in co-culture (Figure 5.2). Significantly, β -actin was detected when empty matrix was a destination (chamber B) for MDA231 cells (chamber A). Very low levels of β -actin, only, were detected in all circulating cell samples.

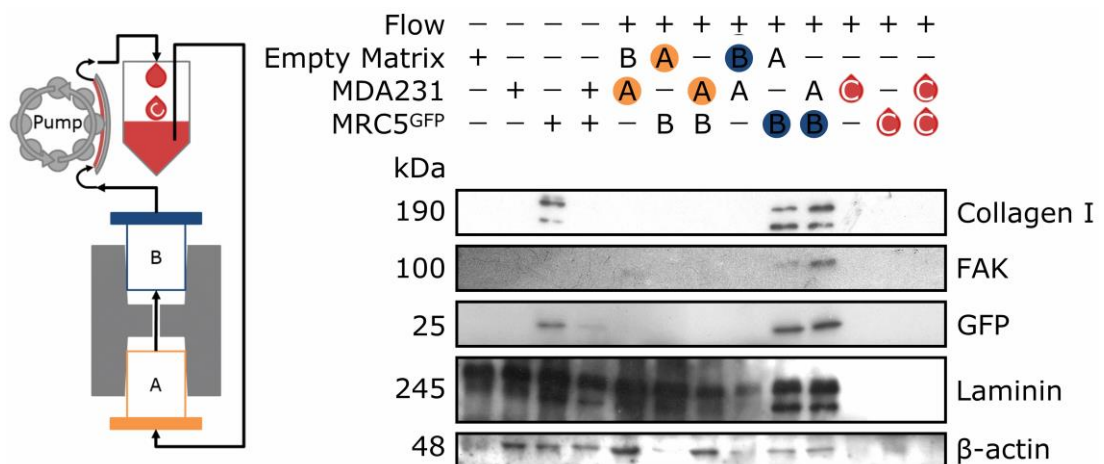


Figure 5.2 – MDA231 and MRC5^{GFP} in dual-chamber dynamic co-culture. 3 sets of samples were obtained for each bioreactor configuration; sample culture condition is highlighted by the orange (chamber A), blue (chamber B) and red (circulating cells) markers.

Light sheet imaging was performed on the same samples to visually detect any cell transfer; if present. Immunoblotting showed actin presence in the empty matrix (chamber B) downstream of MDA231 (chamber A). This was visually confirmed by microscopy (Figure 5.3), with a small number of cells present in the destination chamber. The number of cells present in co-culture dynamic conditions, where MRC5^{GFP} cells (chamber B) are downstream of

MDA231 (chamber A); is particularly surprising as the number of cells seeded at time 0 was identical to static co-culture samples.

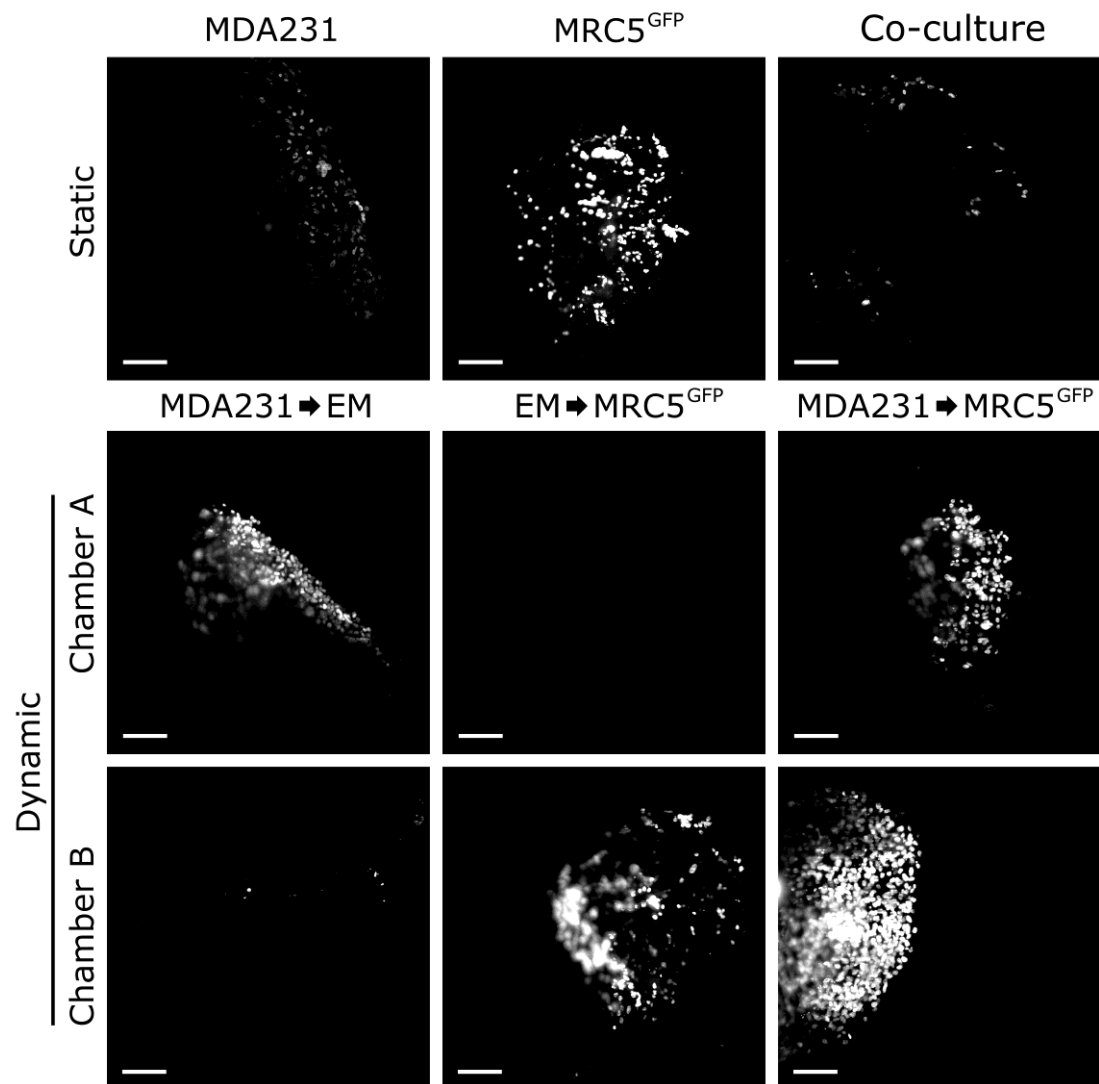


Figure 5.3 – Nuclear staining of bioreactor samples and controls. Chamber A samples are listed first with chamber B samples proceeding the arrow. EM, empty matrix. Scale bar 100 μm.

5.3 Discussion

5.3.1 Dynamic Bioreactor Culture Modifies Cell Behaviour

The alternative culture systems here include dynamic suspension culture and both static and dynamic decellularised matrix culture of breast cancer cell lines. These systems have put an alternative spin on alternative culture systems by introducing a dynamic slant to suspension culture. Furthermore, the integration and development of a two-chamber bioreactor system is beginning to build up the complexity for a representative *ex vivo* model of metastasis.

The dynamic culture system applied here integrated an element of stress to more accurately mimic the sheer stress applied across the surface of migrating cells. Altered flow rate has also been shown to modulate the rate of cell deposition onto 3D substrates (Campos Marín, Brunelli & Lacroix, 2018)

5.3.1.1 MDA231 and MRC5 Cells Upregulate FAK in Response to Media Flow

The bioreactor experiments described here have shown that FAK is upregulated in response to media flow. This suggests increased shear stress across the cell membrane is leading to increased anchorage. Shear stress across the cell membrane increases permeability to Ca^{2+} which binds calmodulin which subsequently modulates a host of intercellular activities. Previous work has shown how the presence of calcium can alleviate autoinhibition of PYK2, the second protein of the FAK family (Kohn *et al.*, 2008). In the context of FAK1, it would be interesting to determine the alterations mechanotransduction has; especially in the context of cells, which have constitutively active MAPK signalling via KRAS/BRAF mutation. These mutations are particularly common in colorectal cancer and melanoma, thus research into this area will assist multiple areas of oncology. Furthermore, current research has identified a high calcium diet to act synergistically with 5-fluorouracil treatment of colorectal cancers; leading to decreased tumour cell viability and increased necrosis through the destabilisation

of FAK (Jun *et al.*, 2018; Jeong *et al.*, 201; Park *et al.* 2017). The full biochemical understanding of this effect in the context of different cancers could be invaluable in understating the interplay between FAK activation, mechanical stress and metastatic tumour formation.

5.3.1.2 MRC5 Cells Remodel rDLM

The rise of FAK expression in samples exposed to MRC5 cells may be, in-part, due to the rise in collagen I detected in the matrix. Collagen I remodelling by MRC5 cells is consistent with a fibroblastic phenotype. Interestingly, collagen I was absent in static co-culture samples whereas MRC5 cells downstream of MDA231 in dynamic culture conditions remained expressing collagen I. This suggests that the physical presence of MDA231 cells is necessary for the repression of collagen I matrix remodelling; that is, juxtacrine signalling as opposed to paracrine or endocrine signalling. This seems unusual due to wide spread repression of cell junction forming molecules in MDA231 cells, and perhaps suggests the presence of an unknown signalling system regulating collagen I expression. Furthermore, the way that MDA231 cells integrate into MRC5 monolayer shown in the previous chapter (4.2.1.2), suggests that cell-cell junctions are still forming, presumably through non-canonical junction proteins.

5.3.2 Summary

From the data shown it can be concluded that the bioreactor system used, allows cell transfer via a liquid medium. It has also been shown that the flow of media induces changes in cell phenotype and behaviour related to matrix remodelling and adhesion.

Chapter 6

General Discussion

6.1 The Metastatic Process

In this thesis, the metastatic process described has been scrutinised in both a compartmentalised and holistic fashion. The breast cancer cells of interest, MCF7 and MDA231 cells, were assessed for their responses to nutrient deprivation; ability to migrate, behaviour in suspension, ability to re-adhere and proliferative potential upon re-adherence. Furthermore, invasion into a biologically representative substrate mimicking a secondary tumour site was assessed in both, using a decellularised matrix culture system.

The process of metastasis can be broken down into several stages that must be completed sequentially for formation of a secondary tumour. As a tumour develops uncontrolled proliferation leads to an increase in size raising the prevalence of nutrient-deprived cells and hypoxia. The cells that survive within the tumour, are those that evolve to manipulate and adapt to their changing, nutrient sparse, oxygen deficient environment (Devignes, Aslan & Provot, 2018; DiGiacomo & Gilkes, 2018; Klaus *et al.*, 2018). Some of the cells that are selected induce angiogenesis, thus enabling an increased flow of nutrients into the tumour, circumventing hypoxia and nutrient deprivation. In the absence of sufficient pro-angiogenic factors, hypoxic regions form, selecting for cells that can survive sustained nutrient deprivation and autophagic cell death, paired with metabolic reprogramming which contributes to survival (Peter *et al.*, 2018; Pivovarova & MacGregor, 2018; Ždralović, 2018). This process is linked to the Warburg effect whereby malignant cells adapt to their microenvironment by respiring anaerobically (Devignes, Aslan & Provot, 2018; DiGiacomo & Gilkes, 2018; Klaus *et al.*, 2018). This process is seen as a reduced dependence on oxidative phosphorylation and an increased rate of lactate metabolism, defined by the retention of pyruvate in the cytoplasm as opposed to its transport into the mitochondrial matrix (Chiche, Brahimi-Horn, & Pouyssegur, 2010; Peter *et al.*,

2018). Data here (3.2.1) have shown that different sub-types of breast cancer can have very different metabolic profiles.

In the context of the disease, the metabolic adaptations lead to formation of a pre-malignant tumour which either has many *de novo* blood vessels, or many cells which are resistant to pro-apoptotic environmental stresses. To focus on the latter, this process eventually causes selection for cells able to alleviate nutrient deprivation by inducing angiogenesis, or by selecting for cells which can migrate through the stroma and ECM, away from a nutrient deprived microenvironment (Hanahan & Weinberg, 2011). Either process enables cells to avoid nutrient deprivation and the associated apoptotic stress, and to interface with a more nutrient-rich location, closer to the circulatory system (Hanahan & Weinberg, 2011). Depending on their route to the circulatory system these cells can be motile or immotile. Typically, immotile cells enter the circulatory system by delamination, whereby shear-stress across a group of cells tears them away from the basal lamina, detaching as a cluster, or sheet. Alternatively, motile cells will simply migrate toward a more nutrient rich microenvironment, entering the blood, or lymph as a single cell, this mode of intravasation was observed in MDA231 cells (3.2.3).

Upon detachment, metastasising tumour cells have to circumvent anoikis, however, having evaded other pro-apoptotic assaults *en route* to the circulatory system they may already have developed such resistance. Alternatively, this stage may select for clones within the tumour, which is now malignant, to survive the stresses of the circulatory system, mechanical or biochemical. Data here have shown that MCF7 and MDA231 cells employ different strategies in this case, linked to their aggregation and nomadic suspension phenotypes respectively. MDA231 were particularly efficient in circumventing the stresses of circulation (3.2.4).

Regardless of the method for leaving the primary locus, circulating tumour cells must be able to re-adhere, maintain proliferative potential, and either remodel a new microenvironment to be more favourable, or adapt to a new microenvironment. Cells which metastasise by delamination can bring ECM “soil” with them, as it can be integrated into the small cluster of cells. The most important aspect of the process here is that there is no black and white between different methods of metastasis, the extremes are described: that is, single cell metastasis and delamination. It is possible for a range of tissue sizes, in terms of cell number, to metastasise, down to single cells actively migrating out of a primary tumour, into the circulatory system.

MCF7 and MDA231 cells were chosen in this case as they represent two distinct types of breast cancer, namely: luminal A and basal B, respectively. Luminal cancers are typically associated with a delamination mechanism of metastasis, are hormone receptor positive and HER2 null. Whereas, basal B cancers are typically triple negative cancers and are highly aggressive. As such, the literature describes MCF7 as non-invasive and MDA231 as highly invasive (Neve *et al.* 2009). Use of these two cell lines allowed clear resolution of behaviours between methods of metastasis.

6.2 Metabolic Stress as a Selective Pressure for Metastatic Clones

Chapter 3, section 2.1 outlined the growth characteristics of MCF7 and MDA231 cells in 2D monolayer culture when in high and low glucose, in combination with the absence and presence of serum. This informed on the metabolic flexibility of the non-invasive MCF7 cells and invasive MDA231 cells. MCF7 cells had a similar growth rate to MDA231 cells when they were cultured in the same media, with the most marked changes in both cell types observed in high glucose serum[−] medium. The data here suggest that MCF7 cells are metabolically flexible, able to survive low glucose conditions, regardless of the

presence of scavengeable protein or growth factors. This provides insight into the formation of luminal tumours as, in this case, their metabolic flexibility confers resistance to nutrient deprivation. Conversely, in glucose rich environments, MDA231 cells require an alternative source of scavengeable protein and growth factors to survive. Chapter 3, section 2.1 showed the death of MDA231 cells in the absence of serum in high glucose conditions, which was not matched by a similar phenotype in low glucose conditions. This suggests that a high concentration of glucose confers a phenotype in MDA231 cells which is incapable of prolonged survival. This is further supported by the data in chapter 4, section 2.1.1 which clearly show the rescuing of the serum starved MDA231 phenotype in the presence of MRC5 cells. This adds complexity in two forms: 1. MDA231 cells rely on paracrine, juxtacrine or endocrine signalling from cells in the metastatic niche to maintain proliferative potential when in conditions analogous to tissue culture serum starvation; 2. traditional assessment of cancer cells in culture as a single cell type can mis-inform. The reliance of MDA231 cells on paracrine signalling or serum is surprising due to its inherent sustained proliferative signalling by mutated, constitutively active KRAS and BRAF. These mutations typically confer unlimited proliferative potential through persistent MAPK signalling (Olivier *et al.*, 2002, Neve *et al.* 2006; Simpson, Anyiwe, & Schimmer, 2008; Hollestelle *et al.* 2010).

Assessment of cellular NADH turnover of MCF7 and MDA231 cells made it clear that the metabolic load of MCF7 cells was considerably higher than MDA231 cells in both media tested. This suggests that the metabolic programme employed by MCF7 is considerably different to that of MDA231 cells. Combining this with the observation that MDA231 cells die after ~72 h in high glucose, serum⁻ conditions suggests a heavy reliance on glucose in the absence of scavengeable protein. Perhaps reliance to such an extent, that these cells can exhaust the media of glucose in this time frame. For future work, assessing the

change in glucose consumption of MCF7 and MDA231 cells in physiologically representative media, and in the presence of stromal-mimetic cells for both primary and secondary tumour microenvironments is of relevance.

6.3 Migration as an Indicator for Metastatic Strategy

The migratory potential of cells has always been of particular interest in the study of metastatic cancers, as the most migratory cells in traditional culture systems are associated with aggressive metastatic cancers (Fox & Kandpal, 2004). Despite traditional wound healing and Boyden chamber migration assays not being physiologically representative, they have been successful in identifying which sub-types of breast cancer have the most aggressive phenotype (Kao *et al.*, 2009; Neve *et al.*, 2009; Holliday & Speirs, 2011). The previous results showing the non-migratory MCF7 phenotype and highly-migratory MDA231 phenotype have been replicated here (chapter 3.2.1 and 4.2.4). However, it is noteworthy that co-culturing with lung fibroblasts (MRC5), added another layer of complexity, inhibiting the migration of MDA231 cells. This suggests that in the formation of secondary tumours of the lung, breast cancers akin to MDA231 cells can have a different migratory phenotype, instructed by their microenvironment.

Further assessment of breast cancer cell migration in a biomimetic microenvironment was assessed using the rDLM model. With respect to both MCF7 and MDA231 cells, the migratory phenotype was similar to that in 2D monolayer wound healing assays. Culture of MCF7 and MDA231 cells on rDLM for 2 weeks showed MCF7 distribution to be one of cell-clusters, trapped in crevices on the surface of the matrix, with cells growing into the matrix, as opposed to actively invading. In contrast, and in accordance with the literature, MDA231 cells were sparsely distributed throughout the matrix with many cells remaining close to the matrix surface (Xiong *et al.*, 2015). This enforces the different methods of metastasis associated with MCF7 and MDA231 cells respectively, with

MCF7 cell metastasising by delamination and MDA231 cells metastasising by nomadic, active invasion. Further analysis led to the identification of a highly motile and invasive sub-set of MDA231 cells (~2.5%) migrating between 350-450 µm deep into the matrix within 7 days. These data reinforce the importance of preventing cells from getting to a secondary locus at all, as once there, dispersal throughout a new location can be rapid. The addition of more cell types into this system will greatly enhance the model, by making rDLM more physiologically representative. For example, as immune cells have been shown to have a much more important role in tumourigenesis than traditionally appreciated, it would be interesting to see the effects of macrophage and other white blood cell lines on breast cancer cell migration in rDLM (Hanahan & Weinberg, 2011).

6.4 Suspension Culture of Breast Cancer Cells Indicates Metastatic Potential

Breast cancer cells that generate secondary tumours must traverse the circulatory (lymphatic or vascular) system to be deposited in distal sites. Dysregulated biochemical machinery that confers resistance to apoptosis is essential for survival in circulation. Typically, when non-malignant cells detach from the ECM, unbound integrins recruit caspase 8 independent of FADD, leading to cell death in a detachment-dependent manner, termed "anoikis" (Scaffidi *et al.*, 1999; Simpson Anyiwe & Schimmer, 2008). Changes in downstream biochemistry or the expression of RGD-motif containing cadherins prevent the activation of caspase 8 and circumvent execution of apoptosis (Bartolomé *et al.*, 2014; Bartolomé, *et al.*, 2015).

This study has shown that the delamination mechanism of metastasis is employed by MCF7 cells which readily form aggregates in dynamic suspension, with these aggregates remaining intact on re-adherence to both traditional culture plates, and rDLM. In contrast, the behaviour of MDA231 cells in

suspension was seen to be nomadic, single cell strategy for metastasis, with minimal interactions with other cells in suspension.

Analysis of MCF7 cells showed they remained proliferative in suspension and formed organoid-like cell structures of varying sizes. This is in stark contrast to the behaviours of MDA231 cells in suspension, which reacted to detachment by ceasing expression of the mitotic proteins: borealin, survivin, INCENP and AURKB. As well as Ki67, suggesting cell cycle arrest. This finding was confirmed by cell cycle analysis by flow cytometry and qPCR analysis of *CDCA8*, *AURKB* and the *BIRC*-family. When combined with paclitaxel treatment it was also found that the suspended animation phenotype significantly reduced sensitivity to cell death induced by microtubule hyperstabilisation. Insensitivity to this widely used chemotherapeutic agent was further supported by decreased expression of *FADD*, *BID* and *PMAIP1*, increased expression of *CFLAR* and the increase in mitochondrial membrane potential. Collectively, these findings, indicate that transcriptional changes induced by detachment from a substrate, elicit changes in many different biochemical pathways leading to less-permeable outer mitochondrial membrane and insensitivity to treatments which target highly proliferative cells. These findings add to the importance of finding a treatment for late stage cancers which do not rely on killing highly proliferative cells and instead target the mechanisms behind the suspended animation phenotype. Candidates for the regulation of this phenotype were identified as *MYB* and *FOXD3*. *MYB* has been shown to behave as a master regulator of tumourigenesis (Liu *et al.*, 2018), with *FOXD3* associated more tightly with endodermal development (Guo *et al.*, 2002).

6.5 MCF7 and MDA231 Cells are Phenotypically Distinct at Secondary Sites

Phenotypically, circulating tumour cells change significantly, during transit to distal sites. To form a secondary tumour, the adaptations made by cells must

not impair adhesion and proliferation. The assessment of re-adhered MCF7 and MDA231 cells showed a distinct phenotype for each cell line, linked to their respective behaviours in suspension culture. Once adhered, MCF7 cells were seen to remain in an aggregate, with protein expression consistent with that seen in suspension cultures, maintaining proliferative potential, as indicated by expression of survivin. This reinforces the idea that the cells will establish a metastatic tumour wherever the aggregate gets trapped, and thus have time to adhere. This type of behaviour is consistent with axillary lymph node metastases, as most lymph from the breast drains through these nodes (Kao *et al.*, 2009; Neve *et al.*, 2009; Holliday & Speirs, 2011). In stark contrast, MDA231 cells maintained their nomadic, migratory phenotype upon re-adherence, exemplified by the lack of defined colony formation. Significantly, the expression of survivin was partially restored in re-adherent cells signifying the return of a proliferative subpopulation from suspension culture. The variation in expression of this mitotic protein is a good example of how flexible gene regulation in metastatic cancer is key in evading the host of stresses encountered during metastasis.

A key component for formation of secondary tumours, is the new microenvironment that is encountered, specifically the 'soil' or ECM. As a result, adherence of MCF7 MDA231 cells into biomimetic environments such as organoids and rDLM was assessed. The aspects of cell behaviour that were focused on in the context of 3D culture were: ECM remodelling, changes in cellular adhesive properties, proliferation and invasion. Metastasising cancers can adapt to a new microenvironment by altering behaviour and gene expression or altering the environment itself to enable the establishment of a viable population. To what extent specific cancers remodel and/or adapt to a new microenvironment is of interest as correlations of certain cancer subtypes with

specific 'predisposed' metastatic locations has the potential to greatly aid in the treatment of cancer, by being predictive.

MCF7 and MDA231 cells seeded into rDLM behaved similarly to re-adherent cultures with MCF7 cells remaining as aggregates and maintaining expression of survivin, Ki67 and E-cadherin. This was accompanied by with *de novo* synthesis of GAGs, suggesting matrix remodelling, which was not found to be related to collagen I, collagen IV, collagen XII or laminin-332. These data suggest that the sub-type of breast cancer that MCF7 cells represent, favours a remodelling mode of metastatic tumour formation as opposed to adaption to a new tumour microenvironment.

By contrast with MCF7 cell behaviour in rDLM, MDA231 cells, did not synthesise GAGs or ECM proteins, at least not those investigated. However, there was a high abundance of nuclear survivin and Ki67 detected. This paired with the highly dispersed cell distribution throughout the rDLM correlates with the phenotype of triple negative metastatic breast cancer and a poor prognosis. Importantly it appears that MDA231 cells do not alter the rDLM microenvironment, suggesting highly flexible genetic programming is the cause for easy adaptation to, and proliferation and/or invasion within rDLM. Defining the transcriptional regulation mechanisms, linked to *MYB* and *FOXD3*, as mentioned, is key to understanding and preventing a highly adaptive phenotype arising in metastasising triple negative breast cancers.

Conversely, MDA231-MRC5 organoid experiments showed the phenotype of MRC5 organoids was clearly influenced by the presence of MDA231 cells. It would be interesting to see if MDA231 cells can induce matrix remodelling indirectly by co-option of MRC5 cells. This also relates to the migration assays discussed, as there is a clear interplay between MRC5 and MDA231 cells in 2D monolayer.

6.6 The Holistic Study of Metastasis *ex vivo* in a Bioreactor System

Chapter 3 and 4 described how the process of metastasis can be broken down into several discrete events and how this can be integrated with 3D culture to produce a more physiologically relevant context to study metastatic breast cancer. This process required the use of many techniques and generations of multiple samples in the context of each stage of metastasis. Data in chapter 5 showed that all these characteristics can be integrated into a single system, from which many samples can be obtained, combining the individual steps of metastasis into a single system, with the added complexity of the flow of liquid, to complete physiological relevance.

The flexibility of this system is where the benefits are, media and matrices can be sampled at any time, and the order of matrices can be configured to map out human physiology, provided enough biomimetic matrices can be derived from the relevant tissues.

6.7 Summary

This thesis has shown two different phenotypes commonly associated with metastatic breast cancer, in MCF7 and MDA231 cells, representing a collective mode of metastasis and a single-cell mode. These behaviours were assessed in both a dissected form, by breaking each stage down, and assessing behaviours such as: nutrient dependence, migration, survival and behaviours in suspension, ability to (re)adhere, and cellular behaviours post-adherence.

Key findings in the dissected contexts were: the gross dependence of MDA231 cells on serum, the vastly different morphology and behaviours of MCF7 and MDA231 in suspension. Of particular importance was the discovery of MDA231 resistance to paclitaxel whilst in suspension, combined with a suspended animation phenotype. Furthermore, behaviours upon (re)adherence

reinforced the necessity for targeted therapies for breast cancers represented by MDA231 cells. In this context, MCF7 cells also showed an interesting phenotype where they retained semi-organoid morphology, suggesting typical monolayer cultures are insufficient to analyse breast cancers of the luminal A classification.

These observations acted as a good basis from which to build into 3D culture. Particularly important observations from these experiments was the ability for MRC5 organoids to generate a rudimentary ECM, and potentially act as a lung-metastasis model of breast cancer. However, this was disrupted by an undefined paracrine interaction between MRC5 and MDA231 cells. The ECM quality and efficacy of rat decellularised lung matrix was assessed and shown to be biocompatible with both MCF7 and MDA231 cells. This was combined with a retention of glycosaminoglycans and key ECM protein components such as laminin and collagen I (in vessels with the lung). The use of this matrix in the analysis of 3D cell migration was proven, along with the continuity of behaviours for non-migratory tumour types represented by MCF7 cells.

Finally, all the observations above were combined into a flexible bioreactor system which increased the physiological relevance of the 3D culture system further. The integration into the bioreactor showed cellular responses to media flow and proved it is possible to have cell migration from one matrix to another, via suspension in media.

Work in this thesis has built upon solid research in the use of biomaterials and engineering in the assessment of cell biology in a biologically relevant setting. Importantly this was performed, and had efficacy, outside of an animal model and in complete agreement with the tenets of NC3R: refine, reduce and replace.

Chapter 7

Bibliography

- Abolbashari, M. *et al.*, 2016. Repopulation of porcine kidney scaffold using porcine primary renal cells. *Acta Biomaterialia*, **29**, pp.52–61. doi: 10.1016/j.actbio.2015.11.026.
- Abu-Amero, K.K. & Bosley, T.M., 2005. Detection of mitochondrial respiratory dysfunction in circulating lymphocytes using resazurin. *Archives of Pathology and Laboratory Medicine*, **129**, pp.1295–8.
- Adams, D.L. *et al.*, 2016. Mitosis in circulating tumor cells stratifies highly aggressive breast carcinomas. *Breast cancer research : BCR*, **18**, pp. 44. 10.1186/s13058-016-0706-4
- Advisory Committee on Breast Cancer Screening, 2006. Screening for cancer in England: past and future. *Journal of Medical Screening*, **13**, pp.59–61.
- Aikio, M. *et al.*, 2012. Arresten, a Collagen-Derived Angiogenesis Inhibitor, Suppresses Invasion of Squamous Cell Carcinoma. *PLoS ONE*, **7**, pp.1–12.
- Akle, X. V. *et al.*, 2017. Circadian Kinetics of Cell Cycle Progression in Adult Neurogenic Niches of a Diurnal Vertebrate. *Journal of Neuroscience*, **37**, pp. 1900–9.
- Alroy, I. & Yarden, Y., 1997. The ErbB signaling network in embryogenesis and oncogenesis: Signal diversification through combinatorial ligand-receptor interactions. *FEBS Letters*, **410**, pp.83–6.
- Althuis, M.D. *et al.*, 2005. Global trends in breast cancer incidence and mortality 1973–1997. *International Journal of Epidemiology*, **34**, pp.405–12.
- Altieri, D.C., 2008. Survivin, cancer networks and pathway-directed drug discovery. *Nature reviews. Cancer*, **8**, pp.61–70.
- Amenta, P.S. *et al.*, 2005. Proteoglycan-Collagen XV in Human Tissues Is Seen Linking Banded Collagen Fibers Subjacent to the Basement Membrane. *Journal of Histochemistry & Cytochemistry*, **53**, pp.165–76.
- Angst, B.D., Marcozzi, C. & Magee, A.I., 2001. The cadherin superfamily: diversity in form and function. *Journal of Cell Science*, **114**, pp.629–41.
- Ansari, K.M., Rundhaug, J.E. & Fischer, S.M., 2008. Multiple signaling pathways are responsible for prostaglandin E2-induced murine keratinocyte proliferation. *Molecular Cancer Research*, **6**, pp.1003–16.
- Ao, Y. *et al.*, 2018. A role for the clock period circadian regulator 2 gene in regulating the clock gene network in human oral squamous cell carcinoma cells. *Oncology Letters*, **15**, pp.4185–92.
- Arnold, S.F. *et al.*, 1994. Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. *Molecular Endocrinology*, **8**, pp.1208–14
- Arnold, S.F. *et al.*, 1995. Phosphorylation of the human estrogen receptor on tyrosine 537 *in vivo* and by src family tyrosine kinases *in vitro*. *Molecular Endocrinology*, **9**, pp.24–33
- Arora, V. *et al.*, 2007. Degradation of survivin by the X-linked Inhibitor of Apoptosis (XIAP)-XAF1 complex. *Journal of Biological Chemistry*, **282**, pp.26202–9.

- Asselin-Labat, M.-L. *et al.*, 2010. Control of mammary stem cell function by steroid hormone signalling. *Nature*, **465**, pp.798–802.
- Assémat, E. *et al.*, 2008. Polarity complex proteins. *Biochimica et Biophysica Acta - Biomembranes*, **1778**, pp.614–30.
- Atala, A., 2002. Methods and Compositions for Organ Decellularisation. *United States Patent*, Patent No.: **6,376,244**.
- Aumailley, M. *et al.*, 2005. A simplified laminin nomenclature. *Matrix Biology*, **24**, pp.326–32.
- Baker, B.M. & Chen, C.S., 2012. Deconstructing the third dimension - how 3D culture microenvironments alter cellular cues. *Journal of Cell Science*, **125**, pp.3015–24.
- Barrett, R.M., Osborne, T.P. & Wheatley, S.P., 2009. Phosphorylation of survivin at threonine 34 inhibits its mitotic function and enhances its cytoprotective activity. *Cell Cycle*, **15**, pp.278–83.
- Bartolomé, R.A. *et al.*, 2014. An RGD motif present in cadherin 17 induces integrin activation and tumor growth. *Journal of Biological Chemistry*, **289**, pp.34801–14.
- Bartolomé, R.A. *et al.*, 2015. VE-cadherin RGD motifs promote metastasis and constitute a potential therapeutic target in melanoma and breast cancers. *Oncotarget*, **8**, pp.215–27.
- Benjaminin, Y., Krieger, A.M. & Yekutieli, D., 2006. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika*, **93**, pp.491–507.
- Bockstaele, L. *et al.*, 2006. Regulated Activating Thr172 Phosphorylation of Cyclin-Dependent Kinase 4(CDK4): Its Relationship with Cyclins and CDK "Inhibitors". *Molecular and Cellular Biology*, **26**, pp.5070–85.
- Brazzatti, J. A. *et al.*, 2012. Differential roles for the p101 and p84 regulatory subunits of PI3Ky in tumor growth and metastasis. *Oncogene*, **31**, pp.2350–61.
- Briskin, C. *et al.*, 1998. A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, pp.5076–81.
- Bustin, S.A. *et al.*, 2009. The MIQE guidelines: Minimum Information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, **55**, pp.611–622.
- Byrne, A. T. *et al.* 2017. Interrogating open issues in cancer precision medicine with patient-derived xenografts. *Nature Reviews Cancer*, **17**, pp.254–68.
- Calle, E.A., Petersen, T.H. & Niklason, L.E., 2011. Procedure for Lung Engineering. *Journal of Visualized Experiments*, **49**, doi: 10.3791/2651.
- Campos Marín, A., Brunelli, M. & Lacroix, D., 2018. Flow perfusion rate modulates cell deposition onto scaffold substrate during cell seeding. *Biomechanics and Modeling in Mechanobiology*, **17**, pp.675–87.

- Cancer Research UK, 2015. 'Breast Cancer Statistics' [ONLINE]. Available at: cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer [Last gained access to 11:07 28/10/15].
- Canty, E.G. *et al.*, 2004. Coalignment of plasma membrane channels and protrusions (fibropositors) specifies the parallelism of tendon. *Journal of Cell Biology*, **165**, pp.553–63.
- Cal, S. & López-Otín, C., 2015. ADAMTS proteases and cancer. *Matrix Biology*, **44-46**, pp.77–85. (*sic*)
- Caldas, H. *et al.*, 2005. Survivin splice variants regulate the balance between proliferation and cell death. *Oncogene*, **24**, pp.1994–2007.
- Carmena, M., *et al.*, 2012. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nature Reviews Molecular Cell Biology*, **13**, 789–803.
- Carpenter, P.M. *et al.*, 2009. Motility Induction in Breast Carcinoma by Mammary Epithelial Laminin 332 (Laminin 5). *Molecular Cancer Research*, **7**, pp.462–75.
- Carpenter, P.M. *et al.*, 2012. Migration of breast cancer cell lines in response to pulmonary laminin 332; implications for metastasis. *FASEB Journal*, **26**. doi: 10.1002/cam4.957
- Carpenter, P. M. *et al.*, 2017. Migration of breast cancer cell lines in response to pulmonary laminin 332. *Cancer Medicine*, **6**, pp. 220–34.
- Chai, J. *et al.*, 2001. Structural basis of caspase-7 inhibition by XIAP. *Cell*, **104**, pp.769–80.
- Chiche, J., Brahimi-Horn, M.C., & Pouyssegur, J., 2010. Tumour hypoxia induces a metabolic shift causing acidosis: A common feature in cancer. *Journal of Cellular and Molecular Medicine*, **14**, pp.771–94
- Chiquet, M. *et al.*, 2014. Collagen XII: Protecting bone and muscle integrity by organizing collagen fibrils. *International Journal of Biochemistry and Cell Biology*, **53**, pp.51–4.
- Clemm, D.L. *et al.*, 2000. Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. *Molecular endocrinology*, **14**, pp.52–65.
- Cochrane, D.R. *et al.*, 2014. Role of the androgen receptor in breast cancer and preclinical analysis of enzalutamide. *Breast cancer research : BCR*, **16**, p.R7. doi: 10.1186/bcr3599.
- Cole, S. *et al.*, 2005. Expression-based monitoring of transcription factor activity: The TELiS database. *Bioinformatics*, **21**, pp.803–810.
- Colnaghi, R. & Wheatley, S.P., 2010. Liaisons between survivin and Plk1 during cell division and cell death. *Journal of Biological Chemistry*, **285**, pp.22592–604.

- Condorelli, G. *et al.*, 1999. PED/PEA-15: an anti-apoptotic molecule that regulates FAS/TNFR1-induced apoptosis. *Oncogene*, **18**, pp.4409–15.
- Crapo, P.M., Gilbert, T.W. & Badylak, S.F., 2011. An overview of tissue and whole organ decellularization processes. *Biomaterials*, **32**, pp.3233–43.
- Davies, H. *et al.*, 2002. 6-Mutations of the BRAF gene in human cancer. *Nature*, **417**, pp.949–54
- Davis, G.E. *et al.*, 2000. Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. *American Journal of Pathology*, **156**, pp.1489–98.
- Department for Environment, Food and Rural Affairs (DEFRA), 2012. <https://www.gov.uk/guidance/cattle-health#hormonal-treatments-and-antibiotics-for-cattle>, last gained access to: 12:22, 08/10/2018.
- Desideri, E., Vegliante, R. & Ciriolo, M. R., 2015. Mitochondrial dysfunctions in cancer: Genetic defects and oncogenic signaling impinging on TCA cycle activity. *Cancer Letters*, **356**, pp.217–223.
- Deveraux, Q.L. & Reed, J.C., 1999. IAP family proteins - Suppressors of apoptosis. *Genes and Development*, **13**, pp.239–52.
- Devignes, C.-S., Aslan, Y., & Provot, S, 2018. Signaling Pathways Underlying Bone Metastasis: Hypoxia Signaling in Bone Metastasis and Beyond. *Current Molecular Biology Reports*, **4**, pp.69–79.
- DiGiacomo, J.W. & Gilkes, D.M., 2018. Tumor Hypoxia As an Enhancer of Inflammation-Mediated Metastasis: Emerging Therapeutic Strategies. *Targeted Oncology*, **13**, pp.157–73.
- Dohi, T., Xia, F. & Altieri, D.C., 2007. Compartmentalized Phosphorylation of IAP by Protein Kinase A Regulates Cytoprotection. *Molecular Cell*, **27**, pp.17–28.
- Domogatskaya, A., Rodin, S. & Tryggvason, K., 2012. Functional Diversity of Laminins. *Annual Review of Cell and Developmental Biology*, **28**, pp.523–53.
- Dou, Z. *et al.*, 2015. Autophagy mediates degradation of nuclear lamina. *Nature*, **527**, pp.1–17.
- Dunajová, L. *et al.*, 2016. The N-terminus of survivin is a mitochondrial-targeting sequence and Src regulator. *Journal of Cell Science*, **129**, pp.2707–12.
- Dunne, L.W. *et al.*, 2014. Biomaterials Human decellularized adipose tissue scaffold as a model for breast cancer cell growth and drug treatments. *Biomaterials*, **35**, pp.4940–9.
- Emoto, K. *et al.*, 1997. Exposure of phosphatidylethanolamine on the surface of apoptotic cells. *Experimental Cell Research*, **232**, pp.430–4.
- Etienne-Manneville, S. & Hall, A., 2002. Rho GTPases in cell biology. *Nature*, **420**, pp.629–35.

- Faivre, E.J. *et al.*, 2008. Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. *Molecular endocrinology*, **22**, pp.823–37.
- Feero, G.W. & Guttmacher, A.E., 2011. Genomics and the Continuum of Cancer Care. *The New England Journal of Medicine*, **364**, pp.340–50.
- Fratzl-Zelman, N. *et al.*, 2016. Bone matrix hypermineralization in prollyl-3 hydroxylase 1 deficient mice. *Bone*, **85**, pp.15–22.
- Fogarty, S. & Hardie, D. G., 2010. Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer', *Biochimica et Biophysica Acta - Proteins and Proteomics*, **1804**, pp.581–591
- Fox, B.P. & Kandpal, R.P., 2004. Invasiveness of breast carcinoma cells and transcript profile: Eph receptors and ephrin ligands as molecular markers of potential diagnostic and prognostic application. *Biochemical and Biophysical Research Communications*, **318**, pp 882–92.
- Froehlich, K. *et al.*, 2016. Generation of Multicellular Breast Cancer Tumor Spheroids: Comparison of Different Protocols', *Journal of Mammary Gland Biology and Neoplasia*. *Journal of Mammary Gland Biology and Neoplasia*, **21**, pp.89–98.
- Frisch, S. M., Schaller, M. & Cieply, B., 2013. Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. *Journal of cell science*, **126**, pp. 21–9.
- Fuller, S.J., Sivarajah, K. & Sugden, P.H., 2008. ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. *Journal of Molecular and Cellular Cardiology*, **44**, pp.831–54.
- Gaiko-Shcherbak, A. *et al.*, 2015. The Acinar Cage: Basement Membranes Determine Molecule Exchange and Mechanical Stability of Human Breast Cell Acini. *PloS one*, **10**, p.e0145174. doi: 10.1371/journal.pone.0145174.
- Garrido, F., Cabrera, T. & Aptsiauri, N., 2010. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: Implications for immunotherapy. *International Journal of Cancer*, **127**, pp.249–56.
- Gebauer, J.M. *et al.*, 2016. Structure, evolution and expression of collagen XXVIII: Lessons from the zebrafish. *Matrix Biology*, **49**, pp.106–19.
- Ghosh, J.C. *et al.*, 2006. Activated checkpoint kinase 2 provides a survival signal for tumor cells. *Cancer Research*, **66**, pp.11576–9.
- Gibson, D.A. & Saunders, P.T.K., 2012. Estrogen dependent signaling in reproductive tissues - A role for estrogen receptors and estrogen related receptors. *Molecular and Cellular Endocrinology*, **348**, pp.361–72.
- Giri, D.K. *et al.*, 2005. Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. *Molecular and cellular biology*, **25**, pp.11005–18.
- Grey, J.F.E. *et al.*, 2018 The use of decellularised animal tissue to study disseminating cancer cells. *Journal of Cell Science*, doi: 10.1242/jcs.219907.

- Grivennikova, V.G., Kareyeva, A.V. & Vinogradov, A.D., 2018. Oxygen-dependence of mitochondrial ROS production as detected by Amplex Red assay. *Redox Biology*, **17**, pp.192–199.
- Grossmann, J., 2002. Molecular mechanisms of “detachment-induced apoptosis--Anoikis”. *Apoptosis: an international journal on programmed cell death*, **7**, pp.247–60.
- Gruber, C.J. *et al.*, 2004. Anatomy of the estrogen response element. *Trends in Endocrinology and Metabolism*, **15**, pp.73–8.
- Gu, L. *et al.*, 2009. Article Regulation of XIAP Translation and Induction by MDM2 following Irradiation. *Cancer Cell*, **15**, pp.363–75.
- Guo, Y. *et al.*, 2002. The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. *Proceedings of the National Academy of Sciences*, **99**, pp.3663–7.
- Guo, W. & Giancotti, F.G., 2004. Integrin signalling during tumour progression. *Nature reviews. Molecular cell biology*, **5**, pp.816–26.
- Guo, H. *et al.*, 2018. Inhibiting 6-phosphogluconate dehydrogenase enhances chemotherapy efficacy in cervical cancer via AMPK-independent inhibition of RhoA and Rac1. *Clinical and Translational Oncology*, doi: 10.1007/s12094-018-1937-x.
- Guvenc, H. *et al.*, 2013. Impairment of glioma stem cell survival and growth by a novel inhibitor for survivin-ran protein complex. *Clinical Cancer Research*, **19**, pp.631–42.
- Guyette, J.P. *et al.*, 2014. Perfusion decellularization of whole organs. *Nature Protocols*, **9**, pp.1451–68.
- Han, M.V. & Zmasek, C.M., 2009. PhyloXML: XML for evolutionary biology and comparative genomics. *BMC Bioinformatics*, **10**, pp.1–6.
- Hanahan, D. & Weinberg, R.A., 2011. The Hallmarks of Cancer: The Next Generation. *Cell*, **144**, pp.646–674.
- Hanson, A J. *et al.*, 2012. XIAP Monoubiquitylates Groucho/TLE to Promote Canonical Wnt Signaling. *Molecular Cell*, **45**, pp.619–28.
- Harris, L. *et al.*, 2015. American Society of Clinical Oncology 2007 Update of Recommendations for the Use of Tumor Markers in Breast Cancer. *Journal of Clinical Oncology*, **25**, pp.5287–312.
- Heljasvaara, R. *et al.*, 2017. Collagen XVIII in tissue homeostasis and dysregulation - Lessons learned from model organisms and human patients. *Matrix Biology*, **57–58**, pp.55–75. (sic)
- Henikoff, S. & Henikoff, J.G., 1992. Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, pp.10915–9.
- Hill, R.C. *et al.*, 2015. Quantification of Extracellular Matrix Proteins from a Rat Lung Scaffold to Provide a Molecular Readout for Tissue Engineering. *Molecular & Cellular Proteomics*, **14**, pp.961–73.

- Holliday, D.L. & Speirs, V., 2011. Choosing the right cell line for breast cancer research. *Breast cancer research*, **13**, doi:10.1186/bcr2889.
- Hollestelle, A. *et al.*, 2010. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. *Breast Cancer Research and Treatment*, **121**, pp.53–64.
- Holst, F. *et al.*, 2007. Estrogen receptor alpha (ESR1) gene amplification is frequent in breast cancer. *Nature genetics*, **39**, pp.655–60.
- Hou, Y., Allan, L. A. & Clarke, P. R., 2017. Phosphorylation of XIAP by CDK1–cyclin-B1 controls mitotic cell death. *Journal of Cell Science*, **130**, pp.502–11.
- Howatson A.M., Lund P.G. & Todd J.D., 1972. Engineering Tables and Data. Chapman & Hall. London.
- Hsieh, C. *et al.*, 1990. Age at menarche, age at menopause, height and obesity as risk factors for breast cancer: associations and interactions in an international case-control study. *International Journal of Cancer*, **46**, pp.796–800
- Hubbard, S.R. & Till, J.H., 2000. Protein Tyrosine Kinase Structure and Function. *Annual Reviews in Biochemistry*, **69**, pp.373–98.
- Hudis, C.A., 2007. Trastuzumab – Mechanism of Action and Use in Clinical Practice. *New England Journal of Medicine*, **357**, pp.39–51.
- Hughes, C.S., Postovit, L.M. & Lajoie, G.A., 2010. Matrigel: a complex protein mixture required for optimal growth of cell culture. *Proteomics*, **10**, pp.1886–90.
- Hutz, K. *et al.*, 2017. Loss of desmoglein 2 promotes tumorigenic behaviour in pancreatic cancer cells. *Molecular Carcinogenesis*, **56**, pp.1884–95.
- Hydbring, P., Malumbres, M. & Sicinski, P. 2016. Non-canonical functions of cell cycle cyclins and cyclin-dependent kinases. *Nature Reviews Molecular Cell Biology*, **17**, pp.280–92.
- Iden, S. & Collard, J.G., 2008. Crosstalk between small GTPases and polarity proteins in cell polarization. *Nature reviews. Molecular cell biology*, **9**, pp.846–59.
- Ignatov, A. *et al.*, 2018. Patterns of breast cancer relapse in accordance to biological subtype. *Journal of Cancer Research and Clinical Oncology*, doi: 10.1007/s00432-018-2644-2.
- Igney, F.H. & Krammer, P.H., 2002. Immune escape of tumors: apoptosis resistance and tumor counterattack. *Journal of Leukocyte Biology*, **71**, pp.907–20.
- Ikeda, K. *et al.*, 2004. Protein phosphatase 5 is a negative regulator of estrogen receptor-mediated transcription. *Molecular endocrinology*, **18**, pp.1131–43.
- Ito, K. *et al.*, 1999. Calcium influx triggers the sequential proteolysis of extracellular and cytoplasmic domains of E-cadherin, leading to loss of beta-catenin from cell-cell contacts. *Oncogene*, **18**, pp.7080–90.

- Itoh, M. *et al.*, 2001. Junctional adhesion molecule (JAM) binds to PAR-3: A possible mechanism for the recruitment of PAR-3 to tight junctions. *Journal of Cell Biology*, **154**, pp.491–7.
- Jackson-Fisher, A.J. *et al.*, 2004. ErbB2 is required for ductal morphogenesis of the mammary gland. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, pp.17138–43.
- Janicke, R.U. *et al.*, 1998. Caspase-3 Is Required for DNA Fragmentation and Associated with Apoptosis. *The Journal of Biological Chemistry*, **273**, pp.9357–60.
- Javed, A. & Lteif, A., 2013. Development of the human breast. *Seminars in Plastic Surgery*, **27**, pp.5–12.
- Jeong, K.Y. *et al.*, 2017. Enhancing 5-Fluorouracil Efficacy in a Primary Colorectal Cancer by Long-lasting Calcium Supplementation. *Anticancer Research*, **37**, pp.2959–64.
- Jeyaprakash, A.A. *et al.*, 2007. Structure of a Survivin-Borealin-INCENP Core Complex Reveals How Chromosomal Passengers Travel Together. *Cell*, **131**, pp.271–85.
- Jiang, M. & Hart, G.W., 1997. A Subpopulation of Estrogen Receptors Are Modified by O⁶-Linked N⁶-Acetylglucosamine. *The Journal of biological chemistry*, **272**, pp.2421–8.
- Jones, R.B. *et al.*, 2006. A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature*, **439**, pp.168–74.
- Joshi, P. A *et al.*, 2010. Progesterone induces adult mammary stem cell expansion. *Nature*, **465**, pp.803–7.
- Jun, S.J., *et al.*, 2018. Investigation into Enhancing Capecitabine Efficacy in Colorectal Cancer by Inhibiting Focal Adhesion Kinase Signaling. *Anticancer Research*, **38**, pp.4667–76. 10.
- Kadler, K.E., Hill, A. & Canty-Laird, E.G., 2008. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. *Current Opinion in Cell Biology*, **20**, pp.495–501.
- Kamphaus, G.D. *et al.*, 2000. Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. *Journal of Biological Chemistry*, **275**, pp.1209–15.
- Kang, Y. J. *et al.*, 2010. Molecular interaction between HAX-1 and XIAP inhibits apoptosis. *Biochemical and Biophysical Research Communications*, **393**, pp.794–9.
- Kang, H.-W. *et al.*, 2016. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*, **34**, pp.312–9.
- Kao, J. *et al.*, 2009. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS ONE*, **4**, e6146.

- Katho, K. *et al.*, 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic. Acids Research*, **30**, 3059-66
- Kelley, L.A. *et al.*, 2015. The Phyre2 web portal for protein modelling, prediction, and analysis. *Nature Protocols*, **10**, pp.845-58.
- Kim, M.S., Lee, W.S. & Jin, W., 2016. TrkB Promotes Breast Cancer Metastasis via Suppression of Runx3 and Keap1 Expression. *Molecules and Cells*, **39**, pp.258-65.
- Klaus, A. *et al.*, (2018). Expression of Hypoxia-Associated Protein HIF-1 α in Follicular Thyroid Cancer is Associated with Distant Metastasis. *Pathology and Oncology Research*, **24**, pp.289-96.
- Knopfová, L. *et al.*, 2018. Transcription factor c-Myb inhibits breast cancer lung metastasis by suppression of tumor cell seeding. *Oncogene*, **37**, pp.1020-30.
- Knotts, T.A. *et al.*, 2001. Identification of a Phosphorylation Site in the Hinge Region of the Human Progesterone Receptor and Additional Amino-terminal Phosphorylation Sites. *Journal of Biological Chemistry*, **276**, pp.8475-83.
- Koch, M. *et al.*, 2003. Collagen XXIV, a Vertebrate Fibrillar Collagen with Structural Features of Invertebrate Collagens: Selective expression in developing cornea and bone. *Journal of Biological Chemistry*, **278**, pp.43236-44.
- Kohler, B.A. *et al.*, 2015. Annual Report to the Nation on the Status of Cancer, 1975-2011, Featuring Incidence of Breast Cancer Subtypes by Race/Ethnicity, Poverty, and State. *JNCI Journal of the National Cancer Institute*, **107**, pp.djv048-djv048 (*sic*)
- Kohno, T. *et al.*, 2008. Protein-tyrosine kinase CAK β /PYK2 is activated by binding Ca²⁺/calmodulin to FERM F2 α 2 helix and thus forming its dimer. *Biochemical Journal*, **410**, pp.513-23.
- Kozloski, G.A., Carothers-Carraway, C.A. & Carraway, K.L., 2010. Mechanistic and signaling analysis of Muc4-ErbB2 signaling module: New insights into the mechanism of ligand-independent ErbB2 activity. *Journal of Cellular Physiology*, **224**, pp.649-57.
- Kreger, B. T. *et al.*, 2016. The enrichment of survivin in exosomes from breast cancer cells treated with paclitaxel promotes cell survival and chemoresistance. *Cancers*, **8**. doi: 10.3390/cancers8120111.
- Kreuz, S. *et al.*, 2001. NF- κ B Inducers Upregulate cFLIP, a Cycloheximide-Sensitive Inhibitor of Death Receptor Signaling NF- κ B Inducers Upregulate cFLIP, a Cycloheximide-Sensitive Inhibitor of Death Receptor Signaling. *Molecular and Cellular Biology*, **21**, pp.3964-73.
- Laemmli, U.K., 1970. Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4. *Nature*, **227**, pp.680-5.
- Landino, J. *et al.*, 2017. Two mechanisms coordinate the recruitment of the chromosomal passenger complex to the plane of cell division. *Molecular Biology of the Cell*, **28**, pp.3634-46.

- Laronda, M.M. *et al.*, 2013. Recreating the female reproductive tract in vitro using iPSC technology in a linked microfluidics environment. *Stem cell research & therapy*, **4 (Suppl 1)**:S13. (sic)
- Laronda, M.M. *et al.*, 2017. A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nature Communications*, **8**. doi: 10.1038/ncomms15261
- Lazaro, G., 2015. Focal Adhesion Kinase (FAK) as a novel therapeutic target in HER2+ breast cancer. *PhD Thesis, Cardiff University*.
- Laetsch, T. W. *et al.*, 2014. Multiple components of the spliceosome regulate Mcl1 activity in neuroblastoma. *Cell death & disease*, **5**, p.e1072. doi: 10.1038/cddis.2014.40.
- Leehy, K.A. *et al.*, 2016. Modifications to Glucocorticoid and Progesterone Receptors Alter Cell Fate in Breast Cancer. *Journal of Molecular Endocrinology*. Available at: <http://jme.endocrinology-journals.org/content/early/2016/02/01/JME-15-0322.full.pdf+html>
- Li, L.Y. *et al.*, 2011. Nuclear ErbB2 enhances translation and cell growth by activating transcription of ribosomal RNA genes. *Cancer Research*, **71**, pp.4269–79.
- Li, S.K.H. & Martin, A., 2016. Mismatch Repair and Colon Cancer: Mechanisms and Therapies Explored. *Trends in Molecular Medicine*, **22**, pp.274–89.
- Li, X. & Heyer, W.D., 2008. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Research*, **18**, pp.99–113.
- Li, Y. *et al.*, 2014. Prognostic Significance of Survivin in Breast Cancer: Meta-analysis. *The Breast Journal*, **20**, pp.514–24.
- Lim, S. and Kaldis, P., 2013. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development*, **140**, pp.3079–93.
- Lin, Y. *et al.*, 2013. Targeting the XIAP / caspase-7 complex selectively kills caspase-3 – deficient malignancies. *The Journal of Clinical Investigation*, **123**, pp.3861–75.
- Lisignoli, G. *et al.*, 2017. Collagen type XV and the “osteogenic status.” *Journal of Cellular and Molecular Medicine*, XX(X), pp.1–9. doi: 10.1111/jcmm.13137
- Liu, W.-H. *et al.*, 2007. Notch inhibits apoptosis by direct interference with XIAP ubiquitination and degradation. *The EMBO journal*, **26**, pp.1660–9.
- Liu, T. *et al.*, 2017. CLOCK stabilizes CYCLE to initiate clock function in Drosophila. *Proceedings of the National Academy of Sciences*, **114**, pp.10972–7.
- Liu, X. *et al.*, 2018. Reassessing the potential of MYB-targeted anti-cancer therapy. *Journal of Cancer*, **9**, pp.1259–66.
- Luciano, A. K. *et al.*, 2018. CLOCK phosphorylation by AKT regulates its nuclear accumulation and circadian gene expression in peripheral tissues. *Journal of Biological Chemistry*. doi: 10.1074/jbc.RA117.000773.

- MacCallum, D.E. & Hall, P.A., 2000. The location of pKi67 in the outer dense fibrillary compartment of the nucleolus points to a role in ribosome biogenesis during the cell division cycle. *Journal of Pathology*, **190**, pp.537–44.
- Madson, J.G. *et al.*, 2006. Erbb2 regulates inflammation and proliferation in the skin after ultraviolet irradiation. *The American journal of pathology*, **169**, pp.1402–14.
- Maeshima, Y., Colorado, P.C. & Kalluri, R., 2000. Two RGD-independent $\alpha(v)\beta3$ integrin binding sites on tumstatin regulate distinct anti-tumor properties. *Journal of Biological Chemistry*, **275**, pp.23745–50.
- Mahoney, D. J. *et al.*, 2008. Both cIAP1 and cIAP2 regulate TNF -mediated NF- B activation. *Proceedings of the National Academy of Sciences*, **105**, pp.11778–83.
- Mahotka, C. *et al.*, 1999. Advances in Brief Survivin- DeltaEx3 and Survivin-2B : Two Novel Splice Variants of the Apoptosis Inhibitor Survivin with Different Antiapoptotic Properties. *Cancer Research*, **59**, pp.6097–102.
- Marinkovich, M.P., 2007. Tumour microenvironment: laminin 332 in squamous-cell carcinoma. *Nature reviews. Cancer*, **7**, pp.370–80.
- Marro, J. *et al.*, 2016. Collagen XII contributes to epicardial and connective tissues in the zebrafish heart during ontogenesis and regeneration. *PLoS ONE*, **11**, pp.1–23.
- Marusawa, H. *et al.*, 2003. HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO Journal*, **22**, pp.2729–40.
- McClements, L. *et al.* 2013. Targeting treatment-resistant breast cancer stem cells with FKBPL and Its peptide derivative, AD-01, via the CD44 pathway. *Clinical Cancer Research*, **19**, pp.3881–93.
- Mehlen, P. & Puisieux, A., 2006. Metastasis: a question of life or death. *Nature Reviews Cancer*, **6**, pp.449–58.
- Meyers, J., Craig, J. & Odde, D.J., 2006. Potential for control of signaling pathways via cell size and shape. *Current biology*, **16**, pp.1685–93.
- Miller, R.T., 2017. Mechanical properties of basement membrane in health and disease', *Matrix Biology*, **57–58**, pp.366–73.
- Miyauchi, A. *et al.*, 2006. $\alpha v\beta3$ Integrin ligands enhance volume-sensitive calcium influx in mechanically stretched osteocytes. *Journal of Bone and Mineral Metabolism*, **24**, pp.498–504.
- Moilanen, J.M. *et al.*, 2017. Significant Role of Collagen XVII And Integrin $\beta4$ in Migration and Invasion of The Less Aggressive Squamous Cell Carcinoma Cells. *Scientific Reports*, **7**, pp.1–11
- Mouw, J., Ou, G. & Weaver, V. 2014. Extracellular matrix assembly: A multiscale deconstruction. *Nature Review Molecular Cell Biology*, **15**, pp.771–785.

- Mseka, T., Bamburg, J.R. & Cramer, L.P., 2007. ADF/cofilin family proteins control formation of oriented actin-filament bundles in the cell body to trigger fibroblast polarization. *Journal of cell science*, **120**, pp.4332–44.
- Naccarato, A.G. *et al.*, 2000. Bio-morphological events in the development of the human female mammary gland from fetal age to puberty. *Virchows Archive*, **436**, pp.431–8.
- Nakamura, T. *et al.*, 2010. Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Molecular Cell*, **39**, pp.184–95.
- Narayanan, R., Edwards, D.P. & Weigel, N.L., 2005. Human progesterone receptor displays cell cycle-dependent changes in transcriptional activity. *Molecular and cellular biology*, **25**, pp.2885–98.
- Nde, C. B. M. *et al.*, 2018. Timeless Is a Novel Estrogen Receptor Co-activator Involved in Multiple Signaling Pathways in MCF-7 Cells. *Journal of Molecular Biology*, doi: 10.1016/j.jmb.2018.03.008.
- Nestal de Moraes, G. *et al.*, 2015. FOXM1 targets XIAP and Survivin to modulate breast cancer survival and chemoresistance. *Cellular Signalling*, **27**, pp.2496–2505.
- Neve, R.M. *et al.*, 2006. A collection of breast cancer cell lines for the study of functionally. *Cancer Cell*, **10**, pp.515–27.
- Nik-Zainal, S. *et al.*, 2016. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature*, doi:10.1038/nature17676.
- Ning, Y. *et al.*, 2015. Cytokeratin-20 and Survivin-Expressing Circulating Tumor Cells Predict Survival in Metastatic Colorectal Cancer Patients by a Combined Immunomagnetic qRT-PCR Approach. *Molecular Cancer Therapeutics*, **14**, pp.2401–8.
- O'Callaghan, E. K., Roig, M. N. B. & Mongrain, V., 2016. Cell Adhesion Molecules and Sleep. *Neuroscience Research*, doi: 10.1016/j.neures.2016.11.001.
- O'Connor, D.S. *et al.*, 2000. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, pp.13103–7.
- Okamoto, A. *et al.*, 1994. Mutations and altered expression of p16INK4 in human cancer. *Proceedings of the National Academy of Sciences*, **91**, pp.11045–9.
- Olivier, M. *et al.*, 2002. The IARC TP53 database: New online mutation analysis and recommendations to users. *Human Mutation*, **19**, pp.607–614.
- Onitilo, A.A. *et al.*, 2009. Breast Cancer Subtypes Based on ER / PR and Her2 Expression: Comparison of Clinicopathological Features and Survival. *Clinical Medicine & Research*, **7**, pp.4–13.
- Owens, T. W. *et al.*, 2010. Role for X-linked inhibitor of apoptosis protein upstream of mitochondrial permeabilization. *Journal of Biological Chemistry*, **285**, pp.1081–8.
- Pace, J.M. *et al.*, 2003. Identification, characterization and expression analysis of a new fibrillar collagen gene, COL27A1. *Matrix Biology*, **22**, pp.3–14.

- Paik, S. *et al.*, 2004. A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer. *The New England Journal of Medicine*, **351**, p.2817.
- Park, M. *et al.* 2017. Synergistically Anti-metastatic Effect of 5-Fluorouracil on Colorectal Cancer Cells via Calcium-mediated Focal Adhesion Kinase Proteolysis. *Anticancer Research*, **37**, pp.103-114.
- Pasparakis, M. & Vandenabeele, P., 2015. Necroptosis and its role in inflammation. *Nature*, **517**, pp.311–20.
- Pavlyukov, M.S. *et al.*, 2011. Survivin monomer plays an essential role in apoptosis regulation. *Journal of Biological Chemistry*, **286**, pp.23296–307.
- Perkel, J M. 2012. Go Cell, Go!. *BioTechniques*, **52**, pp.227–31.
- Peter, K. *et al.*, 2018. Double genetic disruption of lactate dehydrogenase A and B is required to ablate the “Warburg effect” restricting tumor growth to oxidative metabolism. *Journal of Biological Chemistry*, doi: 10.1074/jbc.RA118.004180.
- Petersen, O.W. *et al.*, 1992. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, pp.9064–8.
- Pettersson, K. *et al.*, 1997. Mouse Estrogen Receptor β Forms Estrogen Response Element- Binding Heterodimers with Estrogen Receptor α . *Molecular Endocrinology*, **11**, pp.1486–96.
- Pierson-mullany, L.K. & Lange, C.A., 2004. Phosphorylation of Progesterone Receptor Serine 400 Mediates Ligand-Independent Transcriptional Activity in Response to Activation of Cyclin-Dependent Protein Kinase 2. *Molecular Endocrinology*, **24**, pp.10542–57.
- Pike, M. *et al.*, 1983. “Hormonal” risk factors, “breast tissue age” and the age-incidence of breast cancer. *Nature*, **303**, pp.767–70.
- Pivovarova, A I., & MacGregor, G.G. (2018). Glucose-dependent growth arrest of leukemia cells by MCT1 inhibition: Feeding Warburg’s sweet tooth and blocking acid export as an anticancer strategy. *Biomedicine and Pharmacotherapy*, **98**, pp.173–9.
- Pohl, C. & Jentsch, S. 2008. Final Stages of Cytokinesis and Midbody Ring Formation Are Controlled by BRUCE. *Cell*, **132**, pp.832–845.
- Polez, S. *et al.*, 2016. A simplified and efficient process for insulin production in *Pichia pastoris*. *PLoS ONE*, **11**, pp. 1–15.
- Quandt, K. *et al.*, 1995. MatInd and MatInspector: New fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Research*, **23**, pp.4878–84.
- Rabajdova, M. *et al.*, 2016. The crucial role of emilin 1 gene expression during progression of tumor growth. *Journal of Cancer Research and Clinical Oncology*, **142**, pp.2397–402.

- Rebbeck, T.R. *et al.*, 2018. Mutational spectrum in a worldwide study of 29,700 families with BRCA1 or BRCA2 mutations. *Human mutation*, **39**, pp.593-620.
- Rech, F. R. *et al.*, 2018. Fermentation of hexoses and pentoses from sugarcane bagasse hydrolysates into ethanol by *Spathaspora hagerdaliae*. *Bioprocess and Biosystems Engineering*, doi: 10.1007/s00449-018-2016-y.
- Reche, P., 2008. *Sequence Identity And Similarity*. [ONLINE] Available at: <http://imed.med.ucm.es/Tools/sias.html>. [Gained Access To 8 June 2016]
- Reichow, S.L. *et al.*, 2013. Allosteric mechanism of water-channel gating by Ca²⁺--calmodulin. *Nature Structural & Molecular Biology*, **20**, pp.1085–92.
- Reiss, K. *et al.*, 2005. ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. *The EMBO journal*, **24**, pp.742–52.
- Ricard-Blum, S., 2011. The collagen family. *Cold Spring Harbor Perspectives in Biology*, **3**:a004978 (*sic*)
- Rivadeneira, D.B. *et al.*, 2015. Survivin promotes oxidative phosphorylation, subcellular mitochondrial repositioning, and tumor cell invasion. *Science Signaling*, **8**, pp.1–18. doi: 10.1126/scisignal.aab1624.
- Rubin, I. & Yarden, Y., 2001. The basic biology of HER2. *Annals of Oncology*, **12**, pp.3–8.
- Saldivar, J. C. *et al.*, 2018. An intrinsic S/G 2 checkpoint enforced by ATR. *Science*, **361**, pp. 806–10.
- Saville, B. *et al.*, 2000. Activation at GC-rich (Sp1) Promoter Elements *. *Journal of Biological Chemistry*, **275**, pp.5379–87.
- Sazanov, L. A., 2015. A giant molecular proton pump: structure and mechanism of respiratory complex I. *Nature Reviews Molecular Cell Biology*, **16**, pp.375–88.
- Scaffidi, C. *et al.*, 1999. The role of c-FLIP in modulation of CD95-induced apoptosis. *Journal of Biological Chemistry*, **274**, pp.1541–8.
- Schedl, T. *et al.*, 1984. Cell cycle regulation of tubulin RNA level, tubulin protein synthesis, and assembly of microtubules in *Physarum*. *Journal of Cell Biology*, **99**, pp.155–65.
- Scholzen, T. & Gerdes, J., 2000. The Ki-67 protein: From the known and the unknown. *Journal of Cellular Physiology*, **182**, pp.311–22.
- Semba, S. *et al.*, 2006. Fhit modulation of the Akt-survivin pathway in lung cancer cells: Fhit-tyrosine 114 (Y114) is essential. *Oncogene*, **25**, pp.2860–72.
- Shackleton, M. *et al.*, 2006. Generation of a functional mammary gland from a single stem cell. *Nature*, **439**, pp.84–8.
- Shoshan-Barmatz, V., Krelin, Y. & Shtein-Kuzmine, A., 2018. VDAC1 functions in Ca²⁺+homeostasis and cell life and death in health and disease. *Cell Calcium*, **69**, pp.81–100.

- Siegel, R.L., Miller, K.D. & Jemal, A., 2015. Cancer statistics, 2015. *CA: a cancer journal for clinicians.*, **65**, pp.5-29.
- Sievers, F. *et al.*, 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology*, **7**, pp.539.
- Silvestri, V. *et al.*, 2016. Male breast cancer in BRCA1 and BRCA2 mutation carriers: pathology data from the Consortium of Investigators of Modifiers of BRCA1/2. *Breast Cancer Research*, **18**, pp.15.
- Simon, H.U. & Friis, R., 2014. ATG5: A distinct role in the nucleus. *Autophagy*, **10**, pp.176–7.
- Simpson, C.D., Anyiwe, K. & Schimmer, A.D., 2008. Anoikis resistance and tumor metastasis. *Cancer Letters*, **272**, pp.177–85.
- Sivakumar, S. & Gorbsky, G. J., 2015. Spatiotemporal regulation of the anaphase-promoting complex in mitosis. *Nature Reviews Molecular Cell Biology*, **16**, pp. 82–94.
- Smith, D.F. and Toft, D.O., 1993. Steroid Receptors and their associated proteins. *Molecular Endocrinology*, **7**, pp.4-11
- Somasundaram, K. *et al.*, 1997. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/Cip1. *Nature*, **389**, pp.187–90.
- Span, P.N. *et al.*, 2006. Do the Survivin (*BIRC5*) Splice Variants Modulate or Add to the Prognostic Value of Total Survivin in Breast Cancer? *Clinical Chemistry*, **52**, pp.1693–700.
- St-Laurent, V. *et al.*, 2005. Selective hormone-dependent repression of estrogen receptor beta by a p38-activated ErbB2/ErbB3 pathway. *Journal of Steroid Biochemistry and Molecular Biology*, **94**, pp.23–37.
- Stauber, R.H., Mann, W. & Knauer, S.K., 2007. Nuclear and cytoplasmic survivin: Molecular mechanism, prognostic, and therapeutic potential. *Cancer Research*, **67**, pp.5999–6002.
- Stehlik, C. *et al.*, 1998a. Cytokine induced expression of porcine inhibitor of apoptosis protein (iap) family member is regulated by NF-kappa B. *Biochemical and biophysical research communications*, **243**, pp.827–32.
- Stehlik, C. *et al.*, 1998b. Nuclear Factor (NF)-kB-regulated X-chromosome-linked iap Gene Expression Protects Endothelial Cells from Tumor Necrosis Factor α-induced Apoptosis. *Journal of Experimental Medicine*, **188**, pp.211–6.
- Stoner, M. *et al.*, 2000. Inhibition of vascular endothelial growth factor expression in HEC1A endometrial cancer cells through interactions of estrogen receptor alpha and Sp3 proteins. *Journal of Biological Chemistry*, **275**, pp.22769–79.
- Suzuki, A *et al.*, 2000. Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. *Oncogene*, **19**, pp.1346–53.

- Suzuki, A. & Ohno, S., 2006. The PAR-aPKC system: lessons in polarity. *Journal of cell science*, **119**, pp.979–87.
- Suzuki, Y., Nakabayashi, Y. & Takahashi, R., 2001. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, pp.8662–7.
- Taneja, P. *et al.*, 2010. Classical and Novel Prognostic Markers for Breast Cancer and their Clinical Significance. *Clinical Medicine Insights. Oncology*, **4**, pp.15–34.
- Takeda, A.-N. *et al.*, 2014. Ubiquitin-dependent regulation of MEKK2/3-MEK5-ERK5 signaling module by XIAP and cIAP1. *The EMBO journal*, **33**, pp.1784–801.
- Teyssier, C. *et al.*, 2001. Characterization of the Physical Interaction between Estrogen Receptor α and JUN Proteins. *Journal of Biological Chemistry*, **276**, pp.36361–9.
- Thelemann, A. *et al.*, 2005. Phosphotyrosine Signaling Networks in Epidermal Growth Factor Receptor Overexpressing Squamous Carcinoma Cells. *Molecular & Cellular Proteomics*, **4**, pp.356–76.
- Theveneau, E. & Mayor, R., 2012. Neural crest delamination and migration: From epithelium-to-mesenchyme transition to collective cell migration. *Developmental Biology*, **366**, pp.34–54.
- Top, D. *et al.*, 2016. GSK-3 and CK2 Kinases Converge on Timeless to Regulate the Master Clock. *Cell Reports*, **16**, pp.357–67.
- Torre, L.A. *et al.*, 2015. Global Cancer Statistics, 2012. *CA: a cancer journal of clinicians*, **65**, pp.87–108.
- Tzahar, E. *et al.*, 1998. Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. *EMBO Journal*, **17**, pp.5948–63.
- Ubersax, J.A. & Ferrell Jr, J.E., 2007. Mechanisms of specificity in protein phosphorylation. *Nature Reviews Molecular Cell Biology*, **8**, pp.530–41.
- Umasankar, P.K. *et al.*, 2012. Distinct and separable activities of the endocytic clathrin-coat components Fcho1/2 and AP-2 in developmental patterning. *Nature Cell Biology*, **14**, pp.488–501.
- Ushiki, T., 2002. Collagen fibers, reticular fibers and elastic fibers. A comprehensive understanding from a morphological viewpoint. *Archives of Histology and Cytology*, **65**, pp.109–26.
- Valenzuela, M.M.A. *et al.*, 2015. Exosomes Secreted from Human Cancer Cell Lines Contain Inhibitors of Apoptosis (IAP). *Cancer microenvironment*, **8**, pp.65–73.
- van der Burg, S.H. *et al.*, 2016. Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat Rev Cancer*, **16**, pp.219–33.
- van 't Veer, L.J. *et al.*, 2002. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, **415**, pp.530–6.

- Vance, J. E. & Tasseva, G., 2013. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, **1831**, pp.543–54.
- Varfolomeev, E. *et al.*, 2007. IAP Antagonists Induce Autoubiquitination of c-IAPs, NF- κ B Activation, and TNF α -Dependent Apoptosis. *Cell*, **131**, pp.669–81.
- Vasilikos, L. *et al.*, 2017. Regulating the balance between necroptosis, apoptosis and inflammation by inhibitors of apoptosis proteins. *Immunology and Cell Biology*, **95**, pp. 160–5.
- Vermeulen, K., *et al.*, 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Proliferation*, **36**, pp. 131–49.
- von der Mark, K. *et al.*, 1977. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature*, **267**, pp.531–2.
- Vong, *et al.*, 2005. Chromosome alignment and segregation regulated by ubiquitination of survivin. *Science*, **310**, pp.1499–1504.
- Wang, D. & Dubois, R.N., 2010. Eicosanoids and cancer. *Nature reviews. Cancer*, **10**, pp.181–93.
- Wang, Q. *et al.*, 2011. Induction of autophagy-dependent apoptosis by the survivin suppressant YM155 in prostate cancer cells. *Cancer Letters*, **302**, pp.29–36.
- Wang, W. *et al.*, 2012. Collagen XXIV (Col24a1) promotes osteoblastic differentiation and mineralization through TGF- β /smads signaling pathway. *International Journal of Biological Sciences*, **8**, pp.1310–22. (*sic*)
- Wang, Y.F. *et al.*, 2014. Induction of autophagy-dependent cell death by the survivin suppressant YM155 in salivary adenoid cystic carcinoma. *Apoptosis*, **19**, pp.748–58.
- Wang, N. *et al.*, 2015. The frequency of osteolytic bone metastasis is determined by conditions of the soil, not the number of seeds; evidence from in vivo models of breast and prostate cancer. *Journal of Experimental and Clinical Cancer Research*, **34**, pp.1–12. doi: 10.1186/s13046-015-0240-8.
- Webb, B. a, Eves, R. & Mak, A. S., 2006. Cortactin regulates podosome formation: roles of the protein interaction domains. *Experimental cell research*, **312**, pp.760–9.
- Weigelt, B., Peterse, J.L. & van't Veer, L.J., 2005. Breast cancer metastasis: markers and models. *Nature Reviews Cancer*, **5**, pp.591–602.
- Xiong, G. *et al.*, 2015. Development of an ex vivo breast cancer lung colonization model utilizing a decellularized lung matrix. *Integrative Biology*, **7**, pp.1518–25.
- Yamamoto, K., Murphy, G. & Troeberg, L., 2015. Extracellular regulation of metalloproteinases. *Matrix Biology*, **44–46**, pp.255–63. (*sic*)
- Yang, Y., Ritchie, A.C. & Everitt, N.M., 2017. Comparison of glutaraldehyde and procyanidin cross-linked scaffolds for soft tissue engineering. *Materials Science and Engineering C*, **80**, pp.263–73.

- Ye, J. *et al.*, 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 13:134. (*sic*)
- You, M. *et al.*, 1997. ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. *Molecular and Cellular Biology*, **17**, pp.7328–41.
- Yu, D. *et al.*, 1998. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. *Molecular cell*, **2**, pp.581–91.
- Yu, Q. & Heikal, A.A., 2009. Two-photon autofluorescence dynamics imaging reveals sensitivity of intracellular NADH concentration and conformation to cell physiology at the single-cell level. *Journal of Photochemistry and Photobiology*, **95**, pp.46–57.
- Yuan, S. *et al.*, 2010. Structure of an apoptosome-procaspase-9 CARD complex. *Structure*, **18**, pp.571–83.
- Ždravlević, M. *et al.*, (2018). Disrupting the 'Warburg effect' re-routes cancer cells to OXPHOS offering a vulnerability point via 'ferroptosis'-induced cell death. *Advances in Biological Regulation*, **68**, pp.55–63.
- Zhang, Y. *et al.*, 1997. Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. *Molecular endocrinology*, **11**, pp.823–32.
- Zhang, H., Du, G. & Zhang, J., 2004. Assay of mitochondrial functions by resazurin in vitro. *Acta pharmacologica Sinica*, **25**, pp.385–9.
- Zhang, G. *et al.*, 2005. Development of tendon structure and function: Regulation of collagen fibrillogenesis. *Journal of Musculoskeletal Neuronal Interactions*, **5**, pp.5–21.
- Zhao, Y. *et al.*, 2009. Telomere extension occurs at most chromosome ends and is uncoupled from fill-in in human cancer cells. *Cell*, **138**, pp.463–75.

Chapter 8

Appendix

8.1 Materials & Methods

8.1.1 qPCR Amplicon Maps

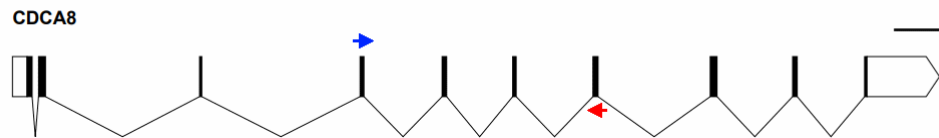
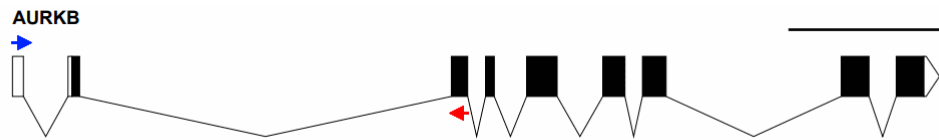


Figure 8.1 – Mitosis gene of interest amplicon maps. Blue arrows represent forward primers, red arrows represent reverse primers. Lines represent introns, black sections represent coding regions, white regions represent the 5' and 3' UTR. Scale bar 1000 bp.



Figure 8.2 – Anoikis gene of interest amplicon maps. Blue arrows represent forward primers, red arrows represent reverse primers. Lines represent introns, black sections represent coding regions, white regions represent the 5' and 3' UTR. Scale bar 1000 bp.

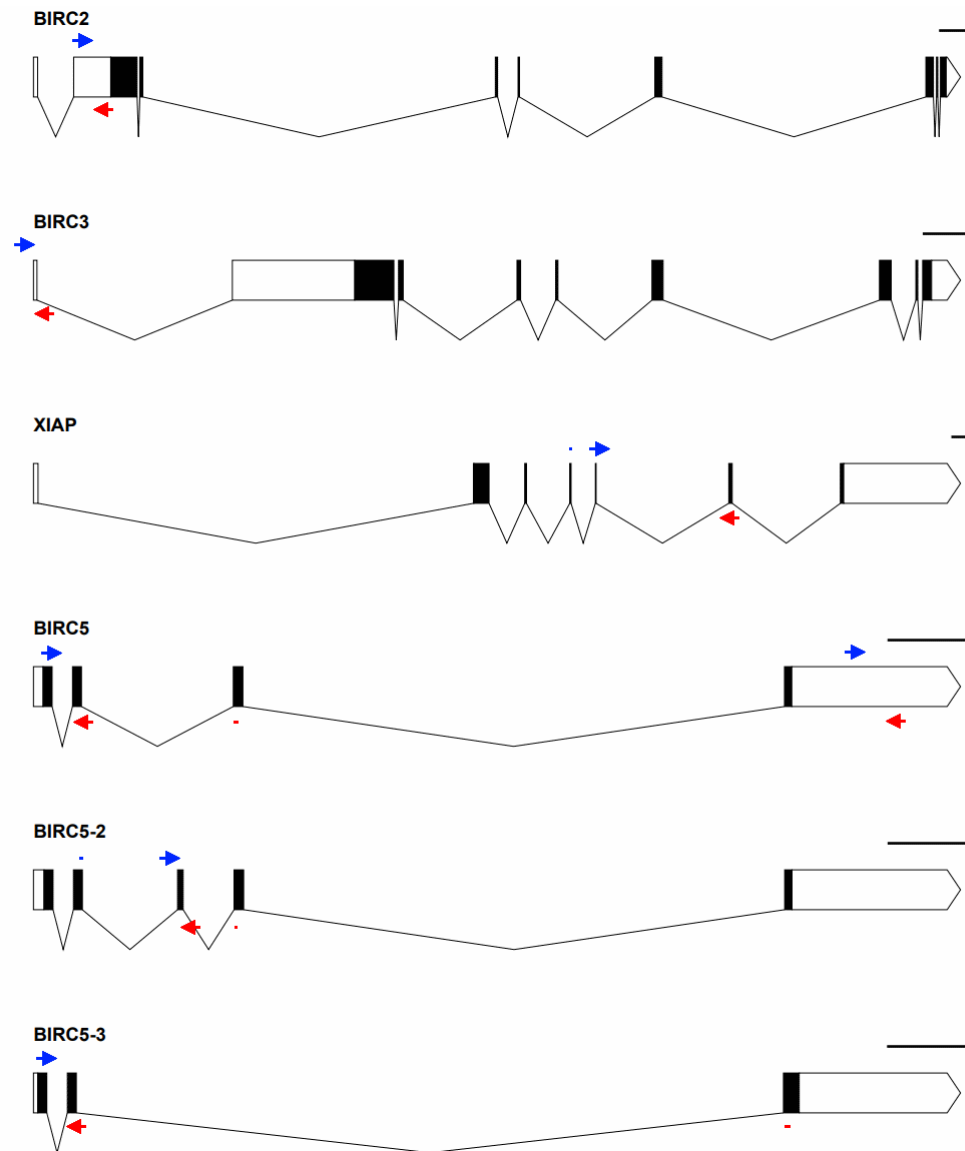


Figure 8.3 – BIRC gene of interest amplicon maps. Blue arrows represent forward primers, red arrows represent reverse primers. Lines represent introns, black sections represent coding regions, white regions represent the 5' and 3' UTR. Scale bar 1000 bp.

8.1.2 Primer Information

Table 8.1 – Primer dimerization and hairpin data.

Gene	Primer	T _m	Average T _m	Hairpin		Dimer Energy	
				ΔG	T _m	Homo-	Hetero-
<i>ACTB</i>	F	62.5	63.30	-0.74	32.8	-3.90	-5.09
	R	64.1		0.42	15.8	-3.14	
<i>GAPDH</i>	F	66.4	64.90	-2.49	47.8	-4.62	-6.21
	R	63.4		0.44	16.8	-8.53	
<i>TBP</i>	F	64.3	63.95	0.99	5.9	-6.34	-7.71
	R	63.6		0.60	10.1	-6.30	
<i>EEF1A1</i>	F	64.2	63.95	-1.14	38.2	-6.30	-6.30
	R	63.7		-1.37	43.7	-6.34	
<i>TUBB</i>	F	57.3	60.65	0.86	9.1	-2.94	-5.13
	R	64.0		-1.53	49.2	-3.14	
<i>Survivin UTR</i>	F	66.0	64.40	-0.30	30.2	-4.64	-7.58
	R	62.8		-2.16	49.8	-6.24	
<i>Survivin</i>	F	64.6	63.35	-0.39	30.6	-3.61	-6.24
	R	62.1		-0.92	37.5	-3.61	
<i>Survivin 2β</i>	F	66.0	66.55	0.32	19.0	-3.61	-6.53
	R	67.1		-0.58	35.4	-4.62	
<i>Survivin ΔEx3</i>	F	63.3	63.30	0.23	21.1	-3.61	-6.37
	R	63.3		0.03	24.6	-7.05	
<i>XIAP</i>	F	61.0	62.20	-0.66	30.8	-3.17	-5.02
	R	63.4		-0.70	33.1	-3.61	
<i>CFLAR</i>	F	64.6	64.05	-1.79	43.0	-4.77	-6.11
	R	63.5		-1.05	39.7	-5.12	
<i>PMAIP1</i>	F	65.0	64.20	-1.08	36.6	-6.34	-5.19
	R	63.4		-1.63	42	-6.76	
<i>BIRC2</i>	F	64.1	64.10	-0.07	26	-3.61	-6.75
	R	64.1		-1.98	46.3	-6.68	
<i>BIRC3</i>	F	64.3	63.15	-2.00	46.7	-3.55	-6.14
	R	62.0		-1.11	32.9	-3.61	
<i>FADD</i>	F	63.9	64.10	-0.63	33.9	-5.38	-6.69
	R	64.3		0.28	21.2	-3.17	
<i>BID</i>	F	63.2	63.95	-0.68	37.5	-3.29	-4.89
	R	64.7		-2.90	52.6	-6.75	
<i>CDCA8</i>	F	64.4	64.30	-0.35	31.1	-3.14	-6.21
	R	64.2		-1.41	40.6	-4.74	
<i>AURKB</i>	F	64.1	64.20	0.99	-0.3	-5.38	-6.60
	R	64.3		0.49	14.7	-7.05	

Generated by Oligoanalyzer 3.1, with 2 mmol·l⁻¹ Mg²⁺ and otherwise default settings.

8.1.3 Macros Used in ImageJ

8.1.3.1 Mitochondrial Health Assay

```
//Defines input and output directories
print("Hello World. Are you ready to generate some data?");
input = getDirectory("Select Input Folder");
output = getDirectory("Select Output Folder");
//Creates a shortcut for file list
list = getFileList(input);
//Starting batch mode
setBatchMode(true);
//Defines the measurements for results
run("Set Measurements...", "mean min display redirect=None decimal=3");
//Creates a work-flow loop for each file in the input folder
for (i=0; i<list.length; i++)
{
    showProgress(i+1, list.length);
    //Opens the first image
    open(input+list[i]);
    //Sets the file title as the original name
    origName=getTitle();
    selectWindow(origName);
    //Colour formats channel then asks for threshold
    //run("Channels Tool...");
    print(origName);
    Stack.setDisplayMode("composite");
    Stack.setChannel(1);
    run("Blue");
    setMinAndMax(200, 1000);
    Stack.setChannel(2);
    run("Green");
    setMinAndMax(500, 800);
    Stack.setChannel(3);
    run("Red");
    setMinAndMax(500, 2000);
    saveAs("PNG", output + origName + "composite");
    Stack.setDisplayMode("grayscale");
    Stack.setChannel(1);
    saveAs("PNG", output + origName + "DAPI");
    Stack.setChannel(2);
    saveAs("PNG", output + origName + "FITC");
    Stack.setChannel(3);
    saveAs("PNG", output + origName + "TRITC");
    newName=getTitle();
    run("Split Channels");
    selectWindow("C1-" + newName);
    //run("Threshold...");
    run("8-bit");
    run("Gaussian Blur...", "sigma=3");
    run("Auto Threshold", "method=RenyiEntropy white");
    run("Make Binary");
    run("Invert");
    setAutoThreshold("Default dark");
    run("Analyze Particles...", "size=20.00-1000.00 circularity=0.01-1.00
display exclude");
    close("C1-" + newName);
}
```

```
selectWindow("C2-" + newName);
//run("Threshold...");
setAutoThreshold("Default dark");
run("Create Selection");
run("Measure");
close("C2-" + newName);
selectWindow("C3-" + newName);
//run("Threshold...");
setAutoThreshold("Default dark");
run("Create Selection");
run("Measure");
close("C3-" + newName);
}
//Stops the batch mode
setBatchMode(false);
//Saves the results in the output folder as "TMRE.csv"
selectWindow("Results");
saveAs("Results", output + "TMRE.csv");
//Closes results
close("Results");
close("Color");
print("Finished");
```

8.1.3.2 Decellularised Matrix Serial Section Migration

```

//Defines input and output directories
input = getDirectory("Select Input Folder");
output = getDirectory("Select Output Folder");
//Creates a shortcut for file list
list = getFileList(input);
//Defines the measurments included in results
run("Set Measurements...", "area centroid display redirect=None decimal=0");
//Creates a workflow loop for each file in the input folder
for (i=0; i<list.length; i++)
{
    showProgress(i+1, list.length);
    //Opens the first image
    open(input+list[i]);
    //Sets the file title as the original name
    origName=getTitle();
    selectWindow(origName);
    //Creates point-list outline and adds their coordinates to the results
    setTool("multipoint");
    run("ROI Manager...");
    roiManager("Show All");
    waitForUser("select 80-100 points along outline, then click OK; selecting
no points will cause an error");
    roiManager("Add");
    roiManager("Select", 0);
    roiManager("Rename", "Points");
    roiManager("Measure");
    roiManager("select", 0);
    roiManager("Delete");
    close("roiManager");
    //Counts nuclei
    run("Color Balance...");
    run("Enhance Contrast", "saturated=0.35");
    run("Gaussian Blur...", "sigma=3");
    run("Auto Threshold", "method=RenyiEntropy white");
    run("Make Binary");
    run("Invert");
    setAutoThreshold("Default dark");
    run("Analyze Particles...", "size=10.00-1000.00 circularity=0.01-1.00
display exclude");
    //Closes the last image
    close(origName);
}
//Saves the results in the output folder as "Cell Number.csv"
selectWindow("Results");
saveAs("Results", output + "Cell Number.csv");
//Closes remaining windows
close("Results");
close("log");
close("color");

```

8.2 Breast Cancer Cell Characteristics in Dissected Metastatic Contexts

8.2.1 MCF7 and MDA231 Monolayer Growth Curves

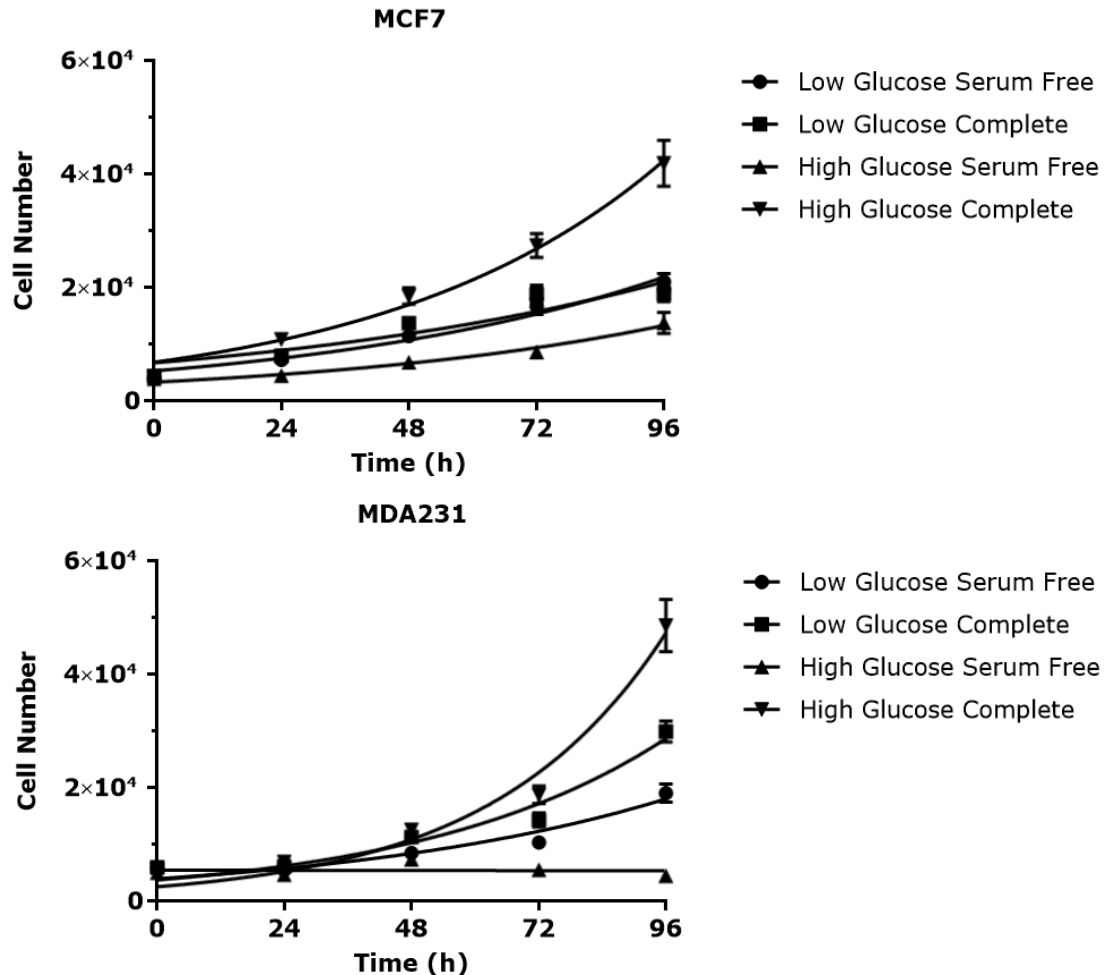
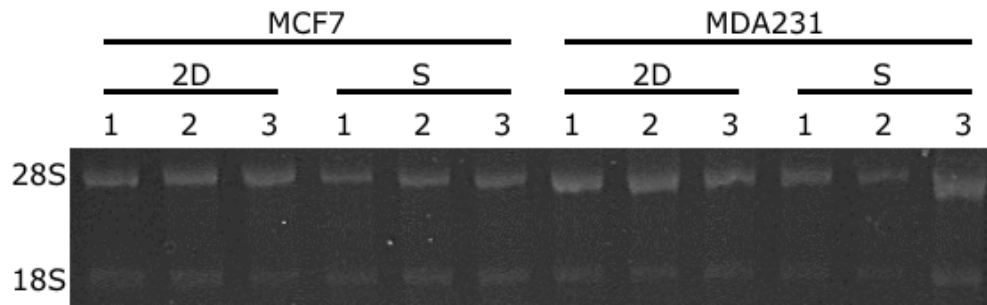


Figure 8.4 – Growth curves for MCF7 and MDA231 cells in monolayer culture. Note the downward sloping curve for MDA231 cells cultured in high glucose serum free media (n=3, N=12).

8.2.2 qPCR Optimisation

The protein abundance of many mitosis-related proteins was significantly reduced between 2D and suspension cultures of the cell lines analysed. To determine the cause of changes in protein abundance found by immunoblotting; RNA extracts were prepared with a view to performing quantitative real-time polymerase chain reaction of reverse transcribed RNA, hereon referred to as qPCR in accordance with MIQE guidelines (Bustin *et al.*, 2009). The flexibility of qPCR also allowed additional targets to be assessed in the context of mitosis and the BIRC family.

RNA was prepared from MDA231 and MCF7 cells grown in 2D and in suspension by a combination of Trizol/bromochloropropane phase separation and phenol-chloroform-isoamyl alcohol purification. Yield and purity was determined by spectrophotometry with RNA integrity assessed by denaturing formaldehyde agarose gel electrophoresis. RNA was seen to be intact and of adequate purity (Figure 8.5).



Cell Line	MCF7						MDA231					
Sample	2D			S			2D			S		
Repeat	1	2	3	1	2	3	1	2	3	1	2	3
260:280	1.90	1.95	1.94	1.89	1.88	1.84	1.97	1.96	1.92	1.89	1.86	1.84
260:230	2.19	2.25	2.26	2.35	2.43	2.41	2.23	2.28	2.32	2.37	2.41	2.38

Figure 8.5 – RNA integrity and purity of MCF7 and MDA231 samples. 1µg of RNA from each sample was run in reducing conditions with 28S and 18S rRNA being clearly seen as intact.

To test the RNA for genomic DNA contamination 500ng was reverse transcribed alongside a no-enzyme control, before 6.25ng of the resultant cDNA was used as a template for 40 cycle endpoint REDTaq PCR. Primers designed to target the 5' UTR of *ACTB* (encoding β -actin) were employed as the primers did not span an exon junction and there was no intron separating the hybridisation targets, allowing detection of both cDNA and genomic DNA. *ACTB* also has the benefit of being ubiquitously highly expressed and thus easily detected. PCR template deriving from enzyme positive reverse transcriptase reactions (RT⁺) all conferred amplification of the expected 150bp product; whereas template deriving from no-enzyme reverse transcriptase reactions (RT⁻) generated no product.

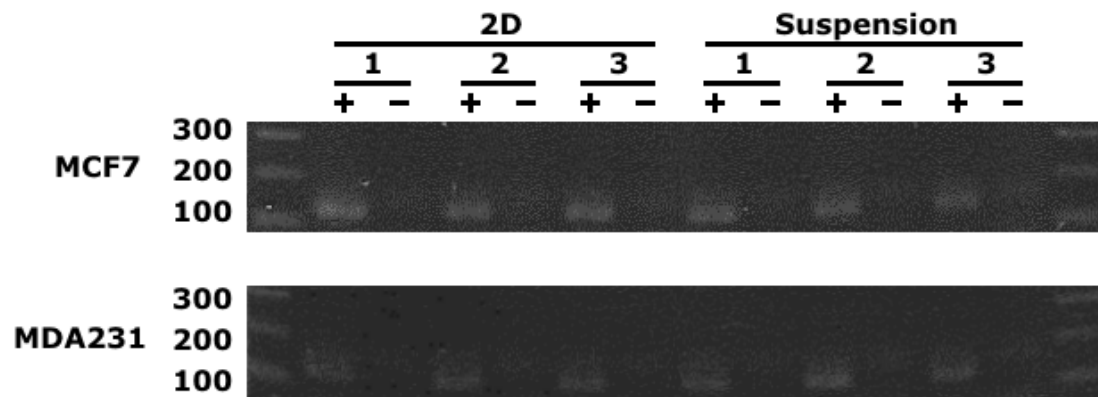


Figure 8.6 – cDNA genomic DNA contamination test of reverse transcribed MCF7 and MDA231 RNA samples. The 150bp product of ACTB 3'UTR specific primers were observed in all RT⁺ (+) samples and in no RT⁻ (-) samples.

At this point the RNA deriving from MCF7 and MDA231, 2D and suspension cultures had been shown to be intact, pure, and free of detectable genomic contaminants. To perform qPCR on multiple genes simultaneously it is necessary for primers to be close to maximum efficiency when hybridising to target cDNA at 60 °C; the polymerisation temperature of the polymerase used in qPCR, iTaq. Therefore, primers for reference genes and genes of interest were designed and their optimum melting temperatures verified as 60 °C using 40 cycle endpoint REDTaq PCR. REDTaq was used as it is not as efficient at making product as iTaq, and thus if any product was made by REDTaq it could be assumed the reactions would also be successful with iTaq. Primers which did not form sufficient product with a primer annealing temperature of 60 °C were redesigned and optimised before qPCR reaction efficiency could be calculated for each primer pair (Figure 8.7).

	T_m				
	58	60	62	64	
<i>ACTB</i>					150
<i>EEF1A1</i>					169
<i>GAPDH</i>					119
<i>TBP</i>					244
<i>TUBB</i>					196
<i>AURKB</i>					240
<i>CDCA8</i>					195
<i>BID</i>					203
<i>CFLAR</i>					229
<i>FADD</i>					223
<i>PMAIP1</i>					184
<i>BIRC2</i>					91
<i>BIRC3</i>					136
<i>XIAP</i>					113
<i>BIRC5</i>					145
<i>BIRC5-1</i>					189
<i>BIRC5-2</i>					96
<i>BIRC5-3</i>					189
<i>CTTN</i>					151
<i>YY1</i>					223

Figure 8.7 – qPCR primer T_m optimisation. All primers for all genes generated sufficient product with a T_m of 60 °C. Gene names are shown on the left with PCR product length on the right in base pairs. *BIRC5* refers to primers which detect all transcript variants of *BIRC5* via the 3' UTR. *BIRC5-1/-2/-3* refers to primers designed to only identify the canonical, 2 β and Δ Ex3 transcript variants of *BIRC5* respectively.

Once primers were confirmed to hybridise and prime polymerisation sufficiently at 60 °C, primer efficiency was calculated by iTaq SYBR green II qPCR with three-fold serial dilutions of template, in duplicate. The threshold breaking cycle (C_T) value for each reaction was used to generate a semi-log standard curve using GraphPad Prism; the gradient of each curve was then used to calculate the efficiency by the formula:

$$\text{reaction efficiency} = 10^{\frac{-1}{\text{standard curve gradient}}} - 1$$

Reaction efficiency between 0.9 and 1.1 was deemed acceptable (Table 8.2). Low efficiency is typically caused by low hybridisation efficiency whereas high reaction efficiency is due to primer-dimer formation.

Table 8.2 – qPCR efficiency.

Reference Genes	Gradient	R²	Efficiency
<i>ACTB</i>	-3.435	0.9993	0.9553
<i>EEF1A1</i>	-3.330	0.9990	0.9966
<i>GAPDH</i>	-3.216	0.9995	1.0466
<i>TBP</i>	-2.477	0.9080	1.5335
<i>TUBB</i>	-3.195	0.9967	1.0558
Mitosis GOI			
<i>AURKB</i>	-3.432	0.9360	0.9560
<i>CDCA8</i>	-3.476	0.9790	0.9395
Anoikis GOI			
<i>BID</i>			
<i>CFLAR</i>	-3.550	0.9638	0.9129
<i>FADD</i>	-3.579	0.9929	0.9029
<i>PMAIP1</i>	-3.515	0.9868	0.9253
BIRC Family GOI			
<i>BIRC2</i>	-3.584	0.9742	0.9012
<i>BIRC3</i>	-3.403	0.9729	0.9672
<i>XIAP</i>	-3.504	0.9878	0.9292
<i>BIRC5</i>	-2.963	0.9920	1.1752
<i>BIRC5-1</i>	-3.414	0.9975	0.9630
<i>BIRC5-2</i>	-3.400	0.9934	0.9688
<i>BIRC5-3</i>	-3.297	0.9983	1.0105

qPCR standard curve gradients, R² values and calculated efficiency for all reference genes and genes of interest (GOI).

Before analysing protein and mRNA abundance consistent stably expressed loading controls were established. 3 independent RNA extracts were made from MDA231 cells grown in 2D and suspension, and a panel of 4 reference genes were tested by comparative qPCR to determine which were stably expressed (Figure 8.8). Threshold breaking cycle (C_T) values were plotted against each other to visualise the consistency of gene expression between samples, with suspension culture sample 2 (S R2) showing slightly higher expression of all genes, especially *EEF1A1* (Figure 8.8A). REST software was then employed to determine which combination of reference genes served as the most consistent, and therefore stably expressed genes, with *ACTB* and *GAPDH* clearly fulfilling these criteria (Figure 8.8B-G). β -actin was therefore also used as a loading control in immunoblotting experiments.

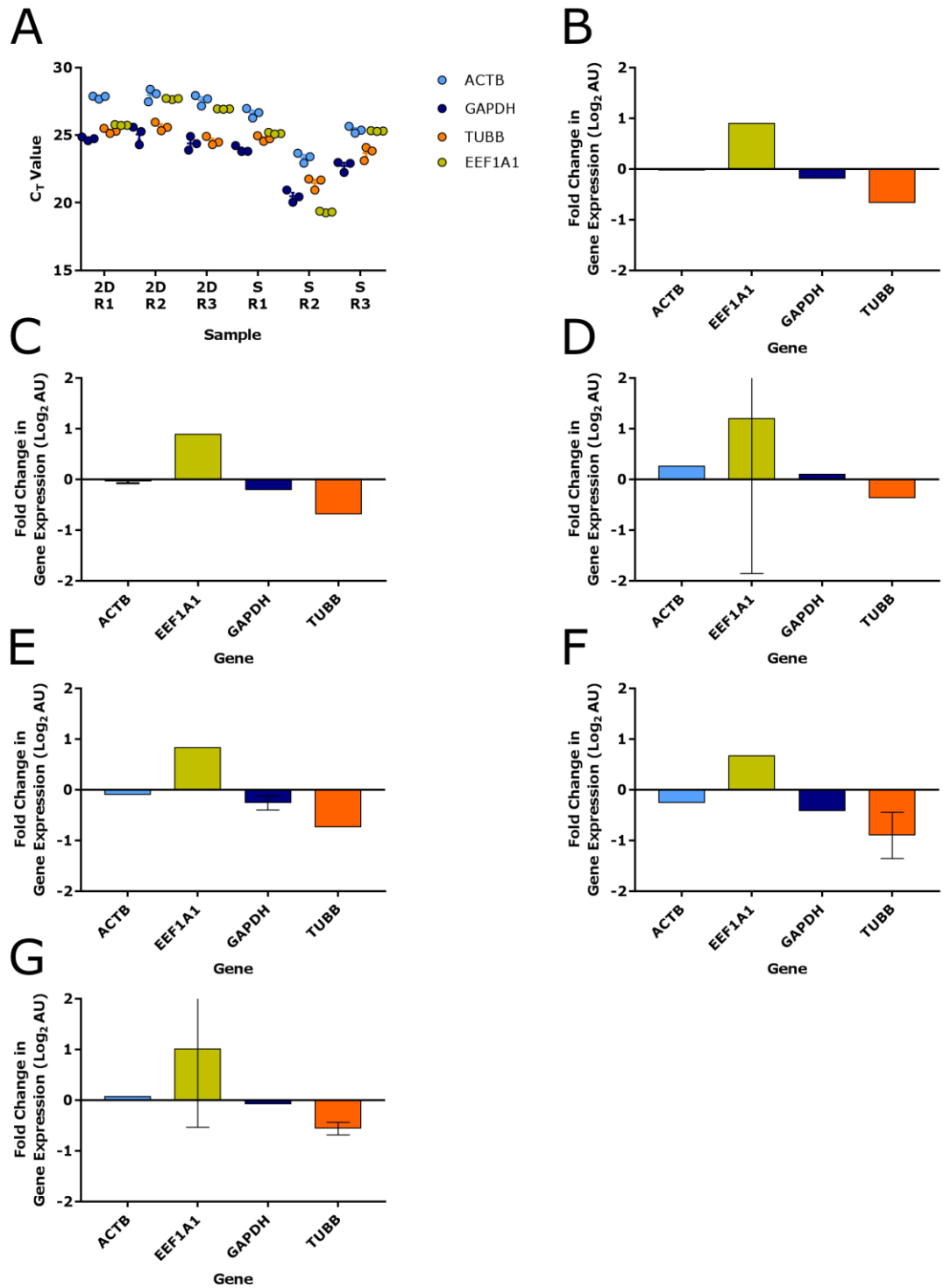


Figure 8.8 – Expression stability of *ACTB*, *EEF1A1*, *GAPDH* and *TUBB*. **A**, threshold breaking cycle (C_T) values for each replicate of 2D and suspension cultured MDA231 cells. **B**, reference gene stability normalised with *ACTB*, *EEF1A1*, *GAPDH* and *TUBB*. **C**, reference gene stability normalised without *ACTB*. **D**, reference gene stability normalised without *EEF1A1*. **E**, reference gene stability normalised without *GAPDH*. **F**, reference gene stability normalised without *TUBB*. **G**, reference gene stability normalised without *EEF1A1* or *TUBB*. All plotted with SEM, (n=3).

8.2.3 Cell Count by Flow Cytometry

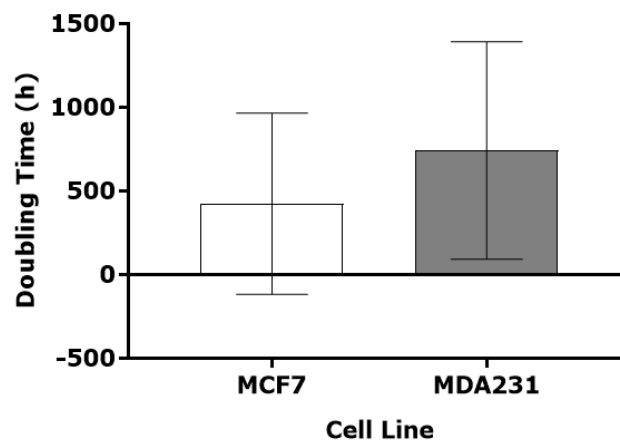


Figure 8.9- Doubling time of MCF7 and MDA231 cells in suspension, calculated by flow cytometry. Cell count measured in this way for the use in growth curves provided highly inconsistent data.

8.3 Statistical Tables

8.3.1 MCF7 and MDA231 Monolayer Growth Rate

Table 8.3 – Post hoc two-stage linear step up statistical table for MCF7 and MDA231 monolayer culture comparing within cell lines.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7				
Low Glucose Serum Free vs. Low Glucose Complete	-20.15	No	0.9406	0.7359
Low Glucose Serum Free vs. High Glucose Serum Free	-12.34	No	0.9406	0.8362
Low Glucose Serum Free vs. High Glucose Complete	11.36	No	0.9406	0.8489
Low Glucose Complete vs. High Glucose Serum Free	7.81	No	0.9406	0.8958
Low Glucose Complete vs. High Glucose Complete	31.51	No	0.9406	0.5988
High Glucose Serum Free vs. High Glucose Complete	23.7	No	0.9406	0.6917
MDA231				
Low Glucose Serum Free vs. Low Glucose Complete	10.99	No	0.9132	0.8539
Low Glucose Serum Free vs. High Glucose Serum Free	61.91	No	0.9132	0.3073
Low Glucose Serum Free vs. High Glucose Complete	20.77	No	0.9132	0.7281
Low Glucose Complete vs. High Glucose Serum Free	50.92	No	0.9132	0.3985
Low Glucose Complete vs. High Glucose Complete	9.783	No	0.9132	0.8697
High Glucose Serum Free vs. High Glucose Complete	-41.14	No	0.9132	0.4935

Two-stage linear step-up procedure.

Table 8.4 – Post hoc two-stage linear step up statistical table for MCF7 and MDA231 monolayer culture comparing within media types.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7 - MDA231				
Low Glucose Serum Free	3.71	No	0.9979	0.9504
Low Glucose	34.85	No	0.9979	0.5611
High Glucose Serum Free	77.96	No	0.8518	0.2028
High Glucose	13.12	No	0.9979	0.8260

Two-stage linear step-up procedure.

Table 8.5 – Post hoc two-stage linear step up statistical table for MCF7 and MDA231 monolayer culture comparing within cell lines, excluding high glucose serum⁻.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	-20.15	No	0.0783	0.0746
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	11.36	No	0.2047	0.2924
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	31.51	Yes	0.0210	0.0100
MDA231				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	10.99	No	0.3799	0.3080
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	20.77	No	0.2114	0.0671
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	9.783	No	0.3799	0.3618

Two-stage linear step-up procedure.

Table 8.6 – Post hoc two-stage linear step up statistical table for MCF7 and MDA231 monolayer culture comparing within media types, excluding high glucose serum free.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7 - MDA231				
Low Glucose Serum ⁻	3.71	No	0.5078	0.7254
Low Glucose Serum ⁺	34.85	Yes	0.0115	0.0055
High Glucose Serum ⁺	13.12	No	0.2392	0.2278

Two-stage linear step-up procedure.

8.3.2 NADH Turnover Assay**Table 8.7 – V_{max} comparison between media and cell types.**

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7:Complete vs. MCF7:Serum Free	0.3779	No	0.0533	0.0380
MCF7:Complete vs. MDA231:Complete	0.7221	Yes	0.0006	0.0002
MCF7:Complete vs. MDA231:Serum Free	0.6276	Yes	0.0018	0.0008
MCF7:Serum Free vs. MDA231:Complete	0.3442	No	0.0609	0.0580
MCF7:Serum Free vs. MDA231:Serum Free	0.2497	No	0.1393	0.1659
MDA231:Complete vs. MDA231:Serum Free	-0.0945	No	0.4181	0.5973

Two-stage linear step-up procedure.

Table 8.8– k_m comparison between media and cell types.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7:Complete vs. MCF7:Serum Free	-93.4	No	0.2950	0.1010
MCF7:Complete vs. MDA231:Complete	34.59	No	0.6797	0.5394
MCF7:Complete vs. MDA231:Serum Free	-9.64	No	0.9072	0.8640
MCF7:Serum Free vs. MDA231:Complete	128	No	0.1646	0.0261
MCF7:Serum Free vs. MDA231:Serum Free	83.76	No	0.2950	0.1405
MDA231:Complete vs. MDA231:Serum Free	-44.23	No	0.6797	0.4331

Two-stage linear step-up procedure.

Table 8.9 – Kinetic model comparison between media and cell types.

Comparison of Fits	MCF7 Serum ⁺	MCF7 Serum [−]	MDA231 Serum ⁺	MDA231 Serum [−]
Null hypothesis	Michaelis-Menten	Michaelis-Menten	Michaelis-Menten	Michaelis-Menten
Alternative hypothesis	Allosteric sigmoidal	Allosteric sigmoidal	Allosteric sigmoidal	Allosteric sigmoidal
P value	0.7308	0.8018	0.5435	0.6256
Conclusion (alpha = 0.05)	Do not reject null hypothesis	Do not reject null hypothesis	Do not reject null hypothesis	Do not reject null hypothesis
Preferred model	Michaelis-Menten	Michaelis-Menten	Michaelis-Menten	Michaelis-Menten
F (DFn, DFd)	0.1191 (1, 87)	0.06338 (1, 87)	0.372 (1, 87)	0.2398 (1, 87)
Michaelis-Menten				
Best-fit values				
Vmax	1.179	0.8011	0.4569	0.5514
Km	75.1	168.5	40.51	84.74
Std. Error				
Vmax	0.2047	0.1126	0.04182	0.0833
Km	39.52	55.95	13.51	37.43
95% CI (profile likelihood)				
Vmax	0.851 to 1.727	0.6226 to 1.105	0.3825 to 0.5476	0.4167 to 0.7626
Km	25.83 to 212.3	89.59 to 337.6	20.49 to 75.28	35.64 to 201.3
Goodness of Fit				
Degrees of Freedom	88	88	88	88
R square	0.2068	0.5196	0.3081	0.2869
Absolute Sum of Squares	30.99	3.015	2.336	4.457
Sy.x	0.5934	0.1851	0.1629	0.225
Constraints				
Km	Km > 0	Km > 0	Km > 0	Km > 0

Appendix

Allosteric sigmoidal				
Best-fit values				
Vmax	1.085	0.7487	0.4283	0.4983
h	1.22	1.089	1.271	1.262
Khalf	62.14	144	35.91	67.29
Kprime	154.2	223.7	94.62	202.7
Std. Error				
Vmax	0.2782	0.2067	0.05344	0.1055
h	0.6999	0.3624	0.5094	0.5868
Khalf	38.65	91.2	11.31	33.76
Kprime	369.1	275.9	155.1	416.4
95% CI (profile likelihood)				
Vmax	0.7694 to +infinity	0.5354 to +infinity	0.3541 to 0.8114	0.3714 to +infinity
h	??? to 3.603	??? to 1.987	0.4531 to 2.543	??? to 3.054
Khalf	24.99 to +infinity	68.92 to +infinity	20.48 to 319	32.38 to +infinity
Goodness of Fit				
Degrees of Freedom	87	87	87	87
R square	0.2079	0.52	0.3111	0.2888
Absolute Sum of Squares	30.95	3.013	2.326	4.445
Sy.x	0.5964	0.1861	0.1635	0.226
Constraints				
h	h > 0	h > 0	h > 0	h > 0
Khalf	Khalf > 0	Khalf > 0	Khalf > 0	Khalf > 0

Extra sum of squares F-test

8.3.3 Cell Morphology of Migratory MCF7 and MDA231

Table 8.10 – Nuclei diameter comparison of migrating cells.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7 0 vs. MCF7 16	-0.3777	No	0.4696	0.8395
MCF7 0 vs. MDA231 0	-0.631	No	0.4696	0.6403
MCF7 0 vs. MDA231 16	-15.54	Yes	<0.0001	<0.0001
MCF7 16 vs. MDA231 0	-0.2533	No	0.4696	0.8944
MCF7 16 vs. MDA231 16	-15.16	Yes	<0.0001	<0.0001
MDA231 0 vs. MDA231 16	-14.91	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure.

Table 8.11 – Cell diameter comparison of migrating cells.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7 0 vs. MCF7 16	-10.49	No	0.0579	0.1655
MCF7 0 vs. MDA231 0	-22.2	Yes	<0.0001	<0.0001
MCF7 0 vs. MDA231 16	-76.97	Yes	<0.0001	<0.0001
MCF7 16 vs. MDA231 0	-11.71	No	0.0549	0.1307
MCF7 16 vs. MDA231 16	-66.48	Yes	<0.0001	<0.0001
MDA231 0 vs. MDA231 16	-54.77	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure.

Table 8.12 – Cell diameter : nuclear diameter ratio comparison.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7 0 vs. MCF7 16	-0.7927	Yes	0.0005	0.0018
MCF7 0 vs. MDA231 0	-1.668	Yes	<0.0001	<0.0001
MCF7 0 vs. MDA231 16	-1.508	Yes	<0.0001	<0.0001
MCF7 16 vs. MDA231 0	-0.8748	Yes	0.0003	0.0008
MCF7 16 vs. MDA231 16	-0.7151	Yes	0.0032	0.0150
MDA231 0 vs. MDA231 16	0.1596	No	0.0868	0.4960

Two-stage linear step-up procedure.

8.3.4 Suspension Culture Viability

Table 8.13 – Viability comparison between culture conditions at each time point.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D vs. 2D _{Mim}	2.467	No	>0.9999	0.7018
2D vs. 2D _T	1.5	No	>0.9999	0.8158
2D vs. 2D _{PTX}	-0.03333	No	>0.9999	0.9959
2D vs. S	2	No	>0.9999	0.7562
2D vs. S _{Mim}	-1.633	No	>0.9999	0.7998
2D vs. S _T	3.7	No	>0.9999	0.5661
2D vs. S _{PTX}	-0.9667	No	>0.9999	0.8807
2D _{Mim} vs. 2D _T	-0.9667	No	>0.9999	0.8807
2D _{Mim} vs. 2D _{PTX}	-2.5	No	>0.9999	0.6980
2D _{Mim} vs. S	-0.4667	No	>0.9999	0.9422
2D _{Mim} vs. S _{Mim}	-4.1	No	>0.9999	0.5251
2D _{Mim} vs. S _T	1.233	No	>0.9999	0.8481
2D _{Mim} vs. S _{PTX}	-3.433	No	>0.9999	0.5944
2D _T vs. 2D _{PTX}	-1.533	No	>0.9999	0.8118
2D _T vs. S	0.5	No	>0.9999	0.9381
2D _T vs. S _{Mim}	-3.133	No	>0.9999	0.6269
2D _T vs. S _T	2.2	No	>0.9999	0.7327
2D _T vs. S _{PTX}	-2.467	No	>0.9999	0.7018
2D _{PTX} vs. S	2.033	No	>0.9999	0.7522
2D _{PTX} vs. S _{Mim}	-1.6	No	>0.9999	0.8038
2D _{PTX} vs. S _T	3.733	No	>0.9999	0.5627
2D _{PTX} vs. S _{PTX}	-0.9333	No	>0.9999	0.8847
S vs. S _{Mim}	-3.633	No	>0.9999	0.5731
S vs. S _T	1.7	No	>0.9999	0.7918
S vs. S _{PTX}	-2.967	No	>0.9999	0.6453
S _{Mim} vs. S _T	5.333	No	>0.9999	0.4091
S _{Mim} vs. S _{PTX}	0.6667	No	>0.9999	0.9175
S _T vs. S _{PTX}	-4.667	No	>0.9999	0.4697
48				
2D vs. 2D _{Mim}	-11.33	No	0.4384	0.0831
2D vs. 2D _T	-9.8	No	0.5566	0.1325
2D vs. 2D _{PTX}	-11.1	No	0.4384	0.0895
2D vs. S	-17.93	No	0.1187	0.0073
2D vs. S _{Mim}	-16.53	No	0.1268	0.0129
2D vs. S _T	-17.7	No	0.1187	0.0081
2D vs. S _{PTX}	-15.03	No	0.1696	0.0231
2D _{Mim} vs. 2D _T	1.533	No	0.9682	0.8118
2D _{Mim} vs. 2D _{PTX}	0.2333	No	>0.9999	0.9711
2D _{Mim} vs. S	-6.6	No	0.6828	0.3079
2D _{Mim} vs. S _{Mim}	-5.2	No	0.7278	0.4208
2D _{Mim} vs. S _T	-6.367	No	0.6828	0.3251
2D _{Mim} vs. S _{PTX}	-3.7	No	0.8760	0.5661
2D _T vs. 2D _{PTX}	-1.3	No	0.9682	0.8400
2D _T vs. S	-8.133	No	0.6828	0.2102
2D _T vs. S _{Mim}	-6.733	No	0.6828	0.2983
2D _T vs. S _T	-7.9	No	0.6828	0.2234
2D _T vs. S _{PTX}	-5.233	No	0.7278	0.4179
2D _{PTX} vs. S	-6.833	No	0.6828	0.2913

Appendix

	Mean Diff.	Discovery?	q value	Individual P Value
48				
2D _{PTX} vs. S _{Mim}	-5.433	No	0.7278	0.4004
2D _{PTX} vs. S _T	-6.6	No	0.6828	0.3079
2D _{PTX} vs. S _{PTX}	-3.933	No	0.8760	0.5420
S vs. S _{Mim}	1.4	No	0.9682	0.8279
S vs. S _T	0.2333	No	>0.9999	0.9711
S vs. S _{PTX}	2.9	No	0.9506	0.6527
S _{Mim} vs. S _T	-1.167	No	0.9682	0.8562
S _{Mim} vs. S _{PTX}	1.5	No	0.9682	0.8158
S _T vs. S _{PTX}	2.667	No	0.9506	0.6790
96				
2D vs. 2D _{Mim}	2.9	No	0.7151	0.6527
2D vs. 2D _T	4.367	No	0.6282	0.4986
2D vs. 2D _{PTX}	17.13	No	0.0513	0.0102
2D vs. S	-5.6	No	0.5725	0.3862
2D vs. S _{Mim}	-6.4	No	0.5676	0.3226
2D vs. S _T	-5.9	No	0.5694	0.3615
2D vs. S _{PTX}	-1.833	No	0.8147	0.7759
2D _{Mim} vs. 2D _T	1.467	No	0.8264	0.8198
2D _{Mim} vs. 2D _{PTX}	14.23	No	0.1302	0.0310
2D _{Mim} vs. S	-8.5	No	0.3697	0.1907
2D _{Mim} vs. S _{Mim}	-9.3	No	0.3504	0.1530
2D _{Mim} vs. S _T	-8.8	No	0.3692	0.1758
2D _{Mim} vs. S _{PTX}	-4.733	No	0.6282	0.4634
2D _T vs. 2D _{PTX}	12.77	No	0.1869	0.0519
2D _T vs. S	-9.967	No	0.3180	0.1262
2D _T vs. S _{Mim}	-10.77	No	0.3125	0.0992
2D _T vs. S _T	-10.27	No	0.3180	0.1155
2D _T vs. S _{PTX}	-6.2	No	0.5676	0.3378
2D _{PTX} vs. S	-22.73	Yes	0.0073	0.0009
2D _{PTX} vs. S _{Mim}	-23.53	Yes	0.0073	0.0006
2D _{PTX} vs. S _T	-23.03	Yes	0.0073	0.0008
2D _{PTX} vs. S _{PTX}	-18.97	Yes	0.0299	0.0047
S vs. S _{Mim}	-0.8	No	0.8665	0.9011
S vs. S _T	-0.3	No	0.8665	0.9628
S vs. S _{PTX}	3.767	No	0.6405	0.5592
S _{Mim} vs. S _T	0.5	No	0.8665	0.9381
S _{Mim} vs. S _{PTX}	4.567	No	0.6282	0.4792
S _T vs. S _{PTX}	4.067	No	0.6341	0.5284

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension; Mim, mimosine; T, thymidine; PTX, paclitaxel

8.3.5 MDA231 Protein Expression in Suspension

Table 8.14 – Relative abundance of proteins of interest normalised to β -actin.

	Mean Diff.	Discovery?	q value	Individual P Value
MCF7 _{2D} - MCF7 _S				
INCENP	1.69	No	0.2728	0.1457
AURKB	0.3181	No	0.6467	0.8212
Borealin	1.58	No	0.2728	0.1732
pH3	4.187	Yes	0.0020	0.0006
XIAP	0.6426	No	0.4953	0.5765
Survivin	0.7801	No	0.4707	0.4981
Ki67	4.767	Yes	0.0006	0.0001
MDA231 _{2D} - MDA231 _S				
INCENP	4.198	Yes	0.0021	0.0020
AURKB	5.823	Yes	0.0008	0.0006
Borealin	8.04	Yes	<0.0001	<0.0001
pH3	2.113	No	0.0707	0.1010
XIAP	-0.01358	No	0.4627	0.9914
Survivin	3.964	Yes	0.0028	0.0033
Ki67	8.391	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure.

8.3.6 Dimethylenastron Treatment of MDA231 Suspension Culture

Table 8.15 – 2n DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D _{DMA} vs. Suspension	-2.067	No	0.6523	0.6212
2D _{DMA} vs. Suspension _{DMA}	2.6	No	0.6523	0.5347
Suspension vs. Suspension _{DMA}	4.667	No	0.6523	0.2685
24				
2D _{DMA} vs. Suspension	-25.97	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	-5.867	No	0.0583	0.1667
Suspension vs. Suspension _{DMA}	20.1	Yes	<0.0001	<0.0001
48				
2D _{DMA} vs. Suspension	-48.33	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	-25.73	Yes	<0.0001	<0.0001
Suspension vs. Suspension _{DMA}	22.6	Yes	<0.0001	<0.0001
72				
2D _{DMA} vs. Suspension	-47.33	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	-18.27	Yes	0.0001	0.0001
Suspension vs. Suspension _{DMA}	29.07	Yes	<0.0001	<0.0001
96				
2D _{DMA} vs. Suspension	-52.47	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	-17.77	Yes	0.0002	0.0002
Suspension vs. Suspension _{DMA}	34.7	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure. DMA, dimethylenastron.

Table 8.16 – S-phase DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D _{DMA} vs. Suspension	-0.5333	No	0.9402	0.6744
2D _{DMA} vs. Suspension _{DMA}	-0.3667	No	0.9402	0.7725
Suspension vs. Suspension _{DMA}	0.1667	No	0.9402	0.8954
24				
2D _{DMA} vs. Suspension	0.1667	No	0.9402	0.8954
2D _{DMA} vs. Suspension _{DMA}	2.8	No	0.0704	0.0336
Suspension vs. Suspension _{DMA}	2.633	No	0.0704	0.0447
48				
2D _{DMA} vs. Suspension	-2.233	No	0.2701	0.0857
2D _{DMA} vs. Suspension _{DMA}	-0.6667	No	0.6297	0.5997
Suspension vs. Suspension _{DMA}	1.567	No	0.3500	0.2223
72				
2D _{DMA} vs. Suspension	-1.133	No	0.4384	0.3744
2D _{DMA} vs. Suspension _{DMA}	1.033	No	0.4384	0.4175
Suspension vs. Suspension _{DMA}	2.167	No	0.2994	0.0950
96				
2D _{DMA} vs. Suspension	1.133	No	0.3931	0.3744
2D _{DMA} vs. Suspension _{DMA}	3.167	No	0.0545	0.0173
Suspension vs. Suspension _{DMA}	2.033	No	0.1830	0.1162

Two-stage linear step-up procedure. DMA, dimethylenastron.

Table 8.17 – 4n DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D _{DMA} vs. Suspension	0.8667	No	0.8868	0.8445
2D _{DMA} vs. Suspension _{DMA}	-1.4	No	0.8868	0.7516
Suspension vs. Suspension _{DMA}	-2.267	No	0.8868	0.6087
24				
2D _{DMA} vs. Suspension	30.8	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	6.367	No	0.0548	0.1566
Suspension vs. Suspension _{DMA}	-24.43	Yes	<0.0001	<0.0001
48				
2D _{DMA} vs. Suspension	48.67	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	25.77	Yes	<0.0001	<0.0001
Suspension vs. Suspension _{DMA}	-22.9	Yes	<0.0001	<0.0001
72				
2D _{DMA} vs. Suspension	40.67	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	9.033	Yes	0.0168	0.0480
Suspension vs. Suspension _{DMA}	-31.63	Yes	<0.0001	<0.0001
96				
2D _{DMA} vs. Suspension	36.53	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	1.4	No	0.2630	0.7516
Suspension vs. Suspension _{DMA}	-35.13	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure. DMA, dimethylenastron.

Table 8.18 – >4n DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D _{DMA} vs. Suspension	1.733	No	0.5472	0.3475
2D _{DMA} vs. Suspension _{DMA}	-0.8333	No	0.6821	0.6496
Suspension vs. Suspension _{DMA}	-2.567	No	0.5287	0.1678
24				
2D _{DMA} vs. Suspension	-5	Yes	0.0208	0.0099
2D _{DMA} vs. Suspension _{DMA}	-3.3	No	0.0831	0.0792
Suspension vs. Suspension _{DMA}	1.7	No	0.2497	0.3567
48				
2D _{DMA} vs. Suspension	1.9	No	0.7662	0.3038
2D _{DMA} vs. Suspension _{DMA}	0.6333	No	0.7662	0.7297
Suspension vs. Suspension _{DMA}	-1.267	No	0.7662	0.4908
72				
2D _{DMA} vs. Suspension	7.8	Yes	<0.0001	0.0002
2D _{DMA} vs. Suspension _{DMA}	8.2	Yes	<0.0001	<0.0001
Suspension vs. Suspension _{DMA}	0.4	No	0.2895	0.8272
96				
2D _{DMA} vs. Suspension	14.8	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	13.2	Yes	<0.0001	<0.0001
Suspension vs. Suspension _{DMA}	-1.6	No	0.1348	0.3853

Two-stage linear step-up procedure. DMA, dimethylenastron.

Table 8.19 – $\geq 4n$ DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D _{DMA} vs. Suspension	2.6	No	0.6537	0.5669
2D _{DMA} vs. Suspension _{DMA}	-2.233	No	0.6537	0.6226
Suspension vs. Suspension _{DMA}	-4.833	No	0.6537	0.2903
24				
2D _{DMA} vs. Suspension	25.8	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	3.067	No	0.1750	0.4999
Suspension vs. Suspension _{DMA}	-22.73	Yes	<0.0001	<0.0001
48				
2D _{DMA} vs. Suspension	50.57	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	26.4	Yes	<0.0001	<0.0001
Suspension vs. Suspension _{DMA}	-24.17	Yes	<0.0001	<0.0001
72				
2D _{DMA} vs. Suspension	48.47	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	17.23	Yes	0.0006	0.0006
Suspension vs. Suspension _{DMA}	-31.23	Yes	<0.0001	<0.0001
96				
2D _{DMA} vs. Suspension	51.33	Yes	<0.0001	<0.0001
2D _{DMA} vs. Suspension _{DMA}	14.6	Yes	0.0030	0.0028
Suspension vs. Suspension _{DMA}	-36.73	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure. DMA, dimethylenastron.

8.3.7 Mimosine, Thymidine and Paclitaxel Treatment of MDA231 Suspension Culture

Table 8.20 – 2n DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D vs. 2DMim	-0.7	No	0.9745	0.8618
2D vs. 2DT	-6.1	No	0.3933	0.1338
2D vs. 2DPTX	-0.7333	No	0.9745	0.8553
2D vs. S	-9.667	No	0.2778	0.0195
2D vs. SMim	-8.067	No	0.2778	0.0493
2D vs. ST	-7.767	No	0.2778	0.0580
2D vs. SPTX	-4	No	0.6317	0.3223
2DMim vs. 2DT	-5.4	No	0.4206	0.1833
2DMim vs. 2DPTX	-0.03333	No	>0.9999	0.9934
2DMim vs. S	-8.967	No	0.2778	0.0296
2DMim vs. SMim	-7.367	No	0.2778	0.0717
2DMim vs. ST	-7.067	No	0.2778	0.0836
2DMim vs. SPTX	-3.3	No	0.6470	0.4134
2DT vs. 2DPTX	5.367	No	0.4206	0.1860
2DT vs. S	-3.567	No	0.6470	0.3770
2DT vs. SMim	-1.967	No	0.8464	0.6252
2DT vs. ST	-1.667	No	0.8464	0.6787
2DT vs. SPTX	2.1	No	0.8464	0.6020
2DPTX vs. S	-8.933	No	0.2778	0.0302
2DPTX vs. SMim	-7.333	No	0.2778	0.0729
2DPTX vs. ST	-7.033	No	0.2778	0.0850
2DPTX vs. SPTX	-3.267	No	0.6470	0.4181
S vs. SMim	1.6	No	0.8464	0.6909
S vs. ST	1.9	No	0.8464	0.6369
S vs. SPTX	5.667	No	0.4206	0.1630
SMim vs. ST	0.3	No	>0.9999	0.9405
SMim vs. SPTX	4.067	No	0.6317	0.3144
ST vs. SPTX	3.767	No	0.6450	0.3510
48				
2D vs. 2DMim	4.9	No	0.4162	0.2265
2D vs. 2DT	5.067	No	0.4162	0.2113
2D vs. 2DPTX	4.9	No	0.4162	0.2265
2D vs. S	-3.3	No	0.6032	0.4134
2D vs. SMim	-3	No	0.6032	0.4569
2D vs. ST	-0.1	No	>0.9999	0.9802
2D vs. SPTX	8.067	No	0.1588	0.0493
2DMim vs. 2DT	0.1667	No	>0.9999	0.9669
2DMim vs. 2DPTX	0	No	>0.9999	>0.9999
2DMim vs. S	-8.2	No	0.1588	0.0458
2DMim vs. SMim	-7.9	No	0.1588	0.0540
2DMim vs. ST	-5	No	0.4162	0.2173
2DMim vs. SPTX	3.167	No	0.6032	0.4324
2DT vs. 2DPTX	-0.1667	No	>0.9999	0.9669
2DT vs. S	-8.367	No	0.1588	0.0418
2DT vs. SMim	-8.067	No	0.1588	0.0493
2DT vs. ST	-5.167	No	0.4162	0.2026
2DT vs. SPTX	3	No	0.6032	0.4569

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	Mean Diff.	Discovery?	q value	Individual P Value
2DPTX vs. SMim	-7.9	No	0.1588	0.0540
2DPTX vs. ST	-5	No	0.4162	0.2173
2DPTX vs. SPTX	3.167	No	0.6032	0.4324
S vs. SMim	0.3	No	>0.9999	0.9405
S vs. ST	3.2	No	0.6032	0.4276
S vs. SPTX	11.37	No	0.1178	0.0066
SMim vs. ST	2.9	No	0.6032	0.4719
SMim vs. SPTX	11.07	No	0.1178	0.0080
ST vs. SPTX	8.167	No	0.1588	0.0467
96				
2D vs. 2DMim	5.3	No	0.1481	0.1914
2D vs. 2DT	16.53	Yes	0.0010	0.0001
2D vs. 2DPTX	17.23	Yes	0.0010	<0.0001
2D vs. S	5.033	No	0.1575	0.2143
2D vs. SMim	1.533	No	0.3975	0.7031
2D vs. ST	14.7	Yes	0.0018	0.0006
2D vs. SPTX	11.23	Yes	0.0096	0.0072
2DMim vs. 2DT	11.23	Yes	0.0096	0.0072
2DMim vs. 2DPTX	11.93	Yes	0.0082	0.0045
2DMim vs. S	-0.2667	No	0.4972	0.9471
2DMim vs. SMim	-3.767	No	0.2457	0.3510
2DMim vs. ST	9.4	Yes	0.0241	0.0229
2DMim vs. SPTX	5.933	No	0.1249	0.1445
2DT vs. 2DPTX	0.7	No	0.4692	0.8618
2DT vs. S	-11.5	Yes	0.0096	0.0060
2DT vs. SMim	-15	Yes	0.0017	0.0005
2DT vs. ST	-1.833	No	0.3815	0.6487
2DT vs. SPTX	-5.3	No	0.1481	0.1914
2DPTX vs. S	-12.2	Yes	0.0078	0.0037
2DPTX vs. SMim	-15.7	Yes	0.0014	0.0003
2DPTX vs. ST	-2.533	No	0.3243	0.5295
2DPTX vs. SPTX	-6	No	0.1249	0.1401
S vs. SMim	-3.5	No	0.2495	0.3859
S vs. ST	9.667	Yes	0.0221	0.0195
S vs. SPTX	6.2	No	0.1249	0.1277
SMim vs. ST	13.17	Yes	0.0046	0.0019
SMim vs. SPTX	9.7	Yes	0.0221	0.0191
ST vs. SPTX	-3.467	No	0.2495	0.3904

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension; Mim, mimosine; T, thymidine; PTX, paclitaxel

Table 8.21 – S-phase DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D vs. 2DMim	0.3667	No	>0.9999	0.8745
2D vs. 2DT	0.06667	No	>0.9999	0.9771
2D vs. 2DPTX	-0.9	No	>0.9999	0.6983
2D vs. S	-0.5667	No	>0.9999	0.8071
2D vs. SMim	-2	No	>0.9999	0.3906
2D vs. ST	0.5	No	>0.9999	0.8294
2D vs. SPTX	-1.267	No	>0.9999	0.5857
2DMim vs. 2DT	-0.3	No	>0.9999	0.8971
2DMim vs. 2DPTX	-1.267	No	>0.9999	0.5857
2DMim vs. S	-0.9333	No	>0.9999	0.6878
2DMim vs. SMim	-2.367	No	>0.9999	0.3104
2DMim vs. ST	0.1333	No	>0.9999	0.9542
2DMim vs. SPTX	-1.633	No	>0.9999	0.4826
2DT vs. 2DPTX	-0.9667	No	>0.9999	0.6772
2DT vs. S	-0.6333	No	>0.9999	0.7850
2DT vs. SMim	-2.067	No	>0.9999	0.3751
2DT vs. ST	0.4333	No	>0.9999	0.8519
2DT vs. SPTX	-1.333	No	>0.9999	0.5662
2DPTX vs. S	0.3333	No	>0.9999	0.8858
2DPTX vs. SMim	-1.1	No	>0.9999	0.6359
2DPTX vs. ST	1.4	No	>0.9999	0.5470
2DPTX vs. SPTX	-0.3667	No	>0.9999	0.8745
S vs. SMim	-1.433	No	>0.9999	0.5376
S vs. ST	1.067	No	>0.9999	0.6461
S vs. SPTX	-0.7	No	>0.9999	0.7630
SMim vs. ST	2.5	No	>0.9999	0.2842
SMim vs. SPTX	0.7333	No	>0.9999	0.7521
ST vs. SPTX	-1.767	No	>0.9999	0.4478
48				
2D vs. 2DMim	-0.8667	No	0.5955	0.7090
2D vs. 2DT	-10.13	Yes	0.0003	<0.0001
2D vs. 2DPTX	-3.033	No	0.2926	0.1951
2D vs. S	1.8	No	0.4194	0.4393
2D vs. SMim	3.667	No	0.1918	0.1187
2D vs. ST	1.4	No	0.4787	0.5470
2D vs. SPTX	-0.1667	No	0.7071	0.9427
2DMim vs. 2DT	-9.267	Yes	0.0007	0.0002
2DMim vs. 2DPTX	-2.167	No	0.3897	0.3526
2DMim vs. S	2.667	No	0.3330	0.2537
2DMim vs. SMim	4.533	No	0.1159	0.0553
2DMim vs. ST	2.267	No	0.3862	0.3310
2DMim vs. SPTX	0.7	No	0.6163	0.7630
2DT vs. 2DPTX	7.1	Yes	0.0104	0.0035
2DT vs. S	11.93	Yes	<0.0001	<0.0001
2DT vs. SMim	13.8	Yes	<0.0001	<0.0001
2DT vs. ST	11.53	Yes	<0.0001	<0.0001
2DT vs. SPTX	9.967	Yes	0.0003	<0.0001
2DPTX vs. SMim	4.833	No	0.0970	0.0416
2DPTX vs. ST	6.7	Yes	0.0146	0.0056
2DPTX vs. SPTX	4.433	No	0.1159	0.0607

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	Mean Diff.	Discovery?	q value	Individual P Value
S vs. SPTX	1.867	No	0.4194	0.4227
SMim vs. ST	-0.4	No	0.6713	0.8632
SMim vs. SPTX	-1.967	No	0.4184	0.3984
ST vs. SPTX	-2.267	No	0.3862	0.3310
96				
2D vs. 2DMim	-1.133	No	0.7067	0.6257
2D vs. 2DT	-5.333	No	0.1323	0.0252
2D vs. 2DPTX	-4.633	No	0.1889	0.0504
2D vs. S	1.767	No	0.5343	0.4478
2D vs. SMim	2.467	No	0.4104	0.2906
2D vs. ST	-1.867	No	0.5284	0.4227
2D vs. SPTX	-2.2	No	0.4532	0.3453
2DMim vs. 2DT	-4.2	No	0.2190	0.0751
2DMim vs. 2DPTX	-3.5	No	0.2619	0.1360
2DMim vs. S	2.9	No	0.3529	0.2151
2DMim vs. SMim	3.6	No	0.2619	0.1254
2DMim vs. ST	-0.7333	No	0.7418	0.7521
2DMim vs. SPTX	-1.067	No	0.7067	0.6461
2DT vs. 2DPTX	0.7	No	0.7418	0.7630
2DT vs. S	7.1	Yes	0.0303	0.0035
2DT vs. SMim	7.8	Yes	0.0303	0.0015
2DT vs. ST	3.467	No	0.2619	0.1397
2DT vs. SPTX	3.133	No	0.3167	0.1810
2DPTX vs. S	6.4	No	0.0518	0.0079
2DPTX vs. SMim	7.1	Yes	0.0303	0.0035
2DPTX vs. ST	2.767	No	0.3653	0.2366
2DPTX vs. SPTX	2.433	No	0.4104	0.2971
S vs. SMim	0.7	No	0.7418	0.7630
S vs. ST	-3.633	No	0.2619	0.1220
S vs. SPTX	-3.967	No	0.2419	0.0922
SMim vs. ST	-4.333	No	0.2184	0.0666
SMim vs. SPTX	-4.667	No	0.1889	0.0488
ST vs. SPTX	-0.3333	No	0.8304	0.8858

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension; Mim, mimosine; T, thymidine; PTX, paclitaxel

Table 8.22 – 4n DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D vs. 2DMim	-0.4667	No	0.9898	0.8416
2D vs. 2DT	3.033	No	0.9074	0.1978
2D vs. 2DPTX	1.967	No	0.9074	0.4014
2D vs. S	1.9	No	0.9074	0.4174
2D vs. SMim	1.2	No	0.9074	0.6078
2D vs. ST	0.06667	No	>0.9999	0.9772
2D vs. SPTX	3.133	No	0.9074	0.1837
2DMim vs. 2DT	3.5	No	0.9074	0.1384
2DMim vs. 2DPTX	2.433	No	0.9074	0.3001
2DMim vs. S	2.367	No	0.9074	0.3134
2DMim vs. SMim	1.667	No	0.9074	0.4766
2DMim vs. ST	0.5333	No	0.9898	0.8194
2DMim vs. SPTX	3.6	No	0.9074	0.1278
2DT vs. 2DPTX	-1.067	No	0.9074	0.6482
2DT vs. S	-1.133	No	0.9074	0.6278
2DT vs. SMim	-1.833	No	0.9074	0.4339
2DT vs. ST	-2.967	No	0.9074	0.2077
2DT vs. SPTX	0.1	No	>0.9999	0.9658
2DPTX vs. S	-0.06667	No	>0.9999	0.9772
2DPTX vs. SMim	-0.7667	No	0.9772	0.7428
2DPTX vs. ST	-1.9	No	0.9074	0.4174
2DPTX vs. SPTX	1.167	No	0.9074	0.6178
S vs. SMim	-0.7	No	0.9772	0.7645
S vs. ST	-1.833	No	0.9074	0.4339
S vs. SPTX	1.233	No	0.9074	0.5979
SMim vs. ST	-1.133	No	0.9074	0.6278
SMim vs. SPTX	1.933	No	0.9074	0.4094
ST vs. SPTX	3.067	No	0.9074	0.1930
48				
2D vs. 2DMim	0.2667	No	>0.9999	0.9091
2D vs. 2DT	3.3	No	0.6562	0.1619
2D vs. 2DPTX	-0.06667	No	>0.9999	0.9772
2D vs. S	2.967	No	0.6562	0.2077
2D vs. SMim	1.8	No	0.7641	0.4422
2D vs. ST	-2.167	No	0.7262	0.3556
2D vs. SPTX	0.1	No	>0.9999	0.9658
2DMim vs. 2DT	3.033	No	0.6562	0.1978
2DMim vs. 2DPTX	-0.3333	No	>0.9999	0.8865
2DMim vs. S	2.7	No	0.6705	0.2509
2DMim vs. SMim	1.533	No	0.7666	0.5124
2DMim vs. ST	-2.433	No	0.7262	0.3001
2DMim vs. SPTX	-0.1667	No	>0.9999	0.9431
2DT vs. 2DPTX	-3.367	No	0.6562	0.1538
2DT vs. S	-0.3333	No	>0.9999	0.8865
2DT vs. SMim	-1.5	No	0.7666	0.5215
2DT vs. ST	-5.467	No	0.4692	0.0227
2DT vs. SPTX	-3.2	No	0.6562	0.1747
2DPTX vs. SMim	3.033	No	0.6562	0.1978
2DPTX vs. ST	1.867	No	0.7641	0.4256
2DPTX vs. SPTX	-2.1	No	0.7262	0.3705

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	Mean Diff.	Discovery?	q value	Individual P Value
S vs. ST	-1.167	No	0.8649	0.6178
S vs. SPTX	-5.133	No	0.4692	0.0319
SMim vs. ST	-2.867	No	0.6562	0.2232
SMim vs. SPTX	-3.967	No	0.6562	0.0942
ST vs. SPTX	-1.7	No	0.7641	0.4678
96				
2D vs. 2DMim	2.4	No	0.4407	0.3067
2D vs. 2DT	0.5	No	0.8397	0.8305
2D vs. 2DPTX	3.5	No	0.3149	0.1384
2D vs. S	-0.2667	No	0.8864	0.9091
2D vs. SMim	-1.733	No	0.5698	0.4592
2D vs. ST	-6.1	No	0.0631	0.0116
2D vs. SPTX	-2.833	No	0.3899	0.2285
2DMim vs. 2DT	-1.9	No	0.5427	0.4174
2DMim vs. 2DPTX	1.1	No	0.6967	0.6380
2DMim vs. S	-2.667	No	0.4122	0.2567
2DMim vs. SMim	-4.133	No	0.2225	0.0815
2DMim vs. ST	-8.5	Yes	0.0086	0.0006
2DMim vs. SPTX	-5.233	No	0.0985	0.0289
2DT vs. 2DPTX	3	No	0.3690	0.2027
2DT vs. S	-0.7667	No	0.7799	0.7428
2DT vs. SMim	-2.233	No	0.4657	0.3412
2DT vs. ST	-6.6	No	0.0598	0.0066
2DT vs. SPTX	-3.333	No	0.3239	0.1578
2DPTX vs. S	-3.767	No	0.2766	0.1115
2DPTX vs. SMim	-5.233	No	0.0985	0.0289
2DPTX vs. ST	-9.6	Yes	0.0039	0.0001
2DPTX vs. SPTX	-6.333	No	0.0608	0.0089
S vs. SMim	-1.467	No	0.6300	0.5308
S vs. ST	-5.833	No	0.0703	0.0155
S vs. SPTX	-2.567	No	0.4166	0.2747
SMim vs. ST	-4.367	No	0.2008	0.0662
SMim vs. SPTX	-1.1	No	0.6967	0.6380
ST vs. SPTX	3.267	No	0.3239	0.1661

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension; Mim, mimosine; T, thymidine; PTX, paclitaxel

Table 8.23 – >4n DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D vs. 2DMim	0.8	No	0.9825	0.8689
2D vs. 2DT	3	No	0.9280	0.5366
2D vs. 2DPTX	-0.3333	No	0.9924	0.9452
2D vs. S	8.333	No	0.5210	0.0902
2D vs. SMim	8.867	No	0.5210	0.0720
2D vs. ST	7.2	No	0.5210	0.1418
2D vs. SPTX	2.133	No	0.9702	0.6600
2DMim vs. 2DT	2.2	No	0.9702	0.6501
2DMim vs. 2DPTX	-1.133	No	0.9825	0.8151
2DMim vs. S	7.533	No	0.5210	0.1246
2DMim vs. SMim	8.067	No	0.5210	0.1007
2DMim vs. ST	6.4	No	0.5466	0.1905
2DMim vs. SPTX	1.333	No	0.9825	0.7832
2DT vs. 2DPTX	-3.333	No	0.9050	0.4925
2DT vs. S	5.333	No	0.6197	0.2740
2DT vs. SMim	5.867	No	0.5622	0.2295
2DT vs. ST	4.2	No	0.7602	0.3879
2DT vs. SPTX	-0.8667	No	0.9825	0.8581
2DPTX vs. S	8.667	No	0.5210	0.0784
2DPTX vs. SMim	9.2	No	0.5210	0.0623
2DPTX vs. ST	7.533	No	0.5210	0.1246
2DPTX vs. SPTX	2.467	No	0.9702	0.6112
S vs. SMim	0.5333	No	0.9924	0.9124
S vs. ST	-1.133	No	0.9825	0.8151
S vs. SPTX	-6.2	No	0.5466	0.2045
SMim vs. ST	-1.667	No	0.9825	0.7310
SMim vs. SPTX	-6.733	No	0.5466	0.1688
ST vs. SPTX	-5.067	No	0.6267	0.2984
48				
2D vs. 2DMim	-4.3	No	0.9563	0.3768
2D vs. 2DT	1.767	No	0.9563	0.7156
2D vs. 2DPTX	-1.8	No	0.9563	0.7105
2D vs. S	-1.467	No	0.9697	0.7622
2D vs. SMim	-2.467	No	0.9563	0.6112
2D vs. ST	0.8667	No	0.9697	0.8581
2D vs. SPTX	-8	No	0.9563	0.1035
2DMim vs. 2DT	6.067	No	0.9563	0.2142
2DMim vs. 2DPTX	2.5	No	0.9563	0.6064
2DMim vs. S	2.833	No	0.9563	0.5594
2DMim vs. SMim	1.833	No	0.9563	0.7053
2DMim vs. ST	5.167	No	0.9563	0.2891
2DMim vs. SPTX	-3.7	No	0.9563	0.4464
2DT vs. 2DPTX	-3.567	No	0.9563	0.4629
2DT vs. S	-3.233	No	0.9563	0.5055
2DT vs. SMim	-4.233	No	0.9563	0.3841
2DT vs. ST	-0.9	No	0.9697	0.8527
2DT vs. SPTX	-9.767	No	0.9563	0.0483
2DPTX vs. SMim	0.3333	No	0.9924	0.9452
2DPTX vs. ST	-0.6667	No	0.9697	0.8906
2DPTX vs. SPTX	2.667	No	0.9563	0.5826

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	Mean Diff.	Discovery?	q value	Individual P Value
S vs. ST	-1	No	0.9697	0.8365
S vs. SPTX	2.333	No	0.9563	0.6305
SMim vs. ST	-6.533	No	0.9563	0.1816
SMim vs. SPTX	3.333	No	0.9563	0.4925
ST vs. SPTX	-5.533	No	0.9563	0.2566
96				
2D vs. 2DMim	-6.567	No	0.4680	0.1794
2D vs. 2DT	-11.7	No	0.1796	0.0190
2D vs. 2DPTX	-16.1	Yes	0.0461	0.0016
2D vs. S	-6.533	No	0.4680	0.1816
2D vs. SMim	-2.267	No	0.8251	0.6403
2D vs. ST	-6.733	No	0.4680	0.1688
2D vs. SPTX	-6.2	No	0.4831	0.2045
2DMim vs. 2DT	-5.133	No	0.5393	0.2922
2DMim vs. 2DPTX	-9.533	No	0.2050	0.0537
2DMim vs. S	0.03333	No	>0.9999	0.9945
2DMim vs. SMim	4.3	No	0.5393	0.3768
2DMim vs. ST	-0.1667	No	>0.9999	0.9726
2DMim vs. SPTX	0.3667	No	>0.9999	0.9397
2DT vs. 2DPTX	-4.4	No	0.5393	0.3659
2DT vs. S	5.167	No	0.5393	0.2891
2DT vs. SMim	9.433	No	0.2050	0.0562
2DT vs. ST	4.967	No	0.5393	0.3080
2DT vs. SPTX	5.5	No	0.5393	0.2595
2DPTX vs. S	9.567	No	0.2050	0.0529
2DPTX vs. SMim	13.83	No	0.0863	0.0061
2DPTX vs. ST	9.367	No	0.2050	0.0578
2DPTX vs. SPTX	9.9	No	0.2050	0.0454
S vs. SMim	4.267	No	0.5393	0.3804
S vs. ST	-0.2	No	>0.9999	0.9671
S vs. SPTX	0.3333	No	>0.9999	0.9452
SMim vs. ST	-4.467	No	0.5393	0.3587
SMim vs. SPTX	-3.933	No	0.5650	0.4185
ST vs. SPTX	0.5333	No	>0.9999	0.9124

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension; Mim, mimosine; T, thymidine; PTX, paclitaxel

Table 8.24 – $\geq 4n$ DNA content analysis of MDA231 cells in different culture conditions.

	Mean Diff.	Discovery?	q value	Individual P Value
0				
2D vs. 2DMim	0.3333	No	>0.9999	0.9470
2D vs. 2DT	6.033	No	0.6671	0.2320
2D vs. 2DPTX	1.633	No	0.9474	0.7446
2D vs. S	10.23	No	0.4166	0.0455
2D vs. SMim	10.07	No	0.4166	0.0490
2D vs. ST	7.267	No	0.6270	0.1514
2D vs. SPTX	5.267	No	0.6671	0.2959
2DMim vs. 2DT	5.7	No	0.6671	0.2584
2DMim vs. 2DPTX	1.3	No	0.9474	0.7953
2DMim vs. S	9.9	No	0.4166	0.0527
2DMim vs. SMim	9.733	No	0.4166	0.0567
2DMim vs. ST	6.933	No	0.6270	0.1706
2DMim vs. SPTX	4.933	No	0.6671	0.3272
2DT vs. 2DPTX	-4.4	No	0.6899	0.3817
2DT vs. S	4.2	No	0.6899	0.4036
2DT vs. SMim	4.033	No	0.6899	0.4224
2DT vs. ST	1.233	No	0.9474	0.8056
2DT vs. SPTX	-0.7667	No	0.9933	0.8784
2DPTX vs. S	8.6	No	0.4759	0.0909
2DPTX vs. SMim	8.433	No	0.4759	0.0971
2DPTX vs. ST	5.633	No	0.6671	0.2640
2DPTX vs. SPTX	3.633	No	0.7266	0.4695
S vs. SMim	-0.1667	No	>0.9999	0.9735
S vs. ST	-2.967	No	0.8076	0.5545
S vs. SPTX	-4.967	No	0.6671	0.3240
SMim vs. ST	-2.8	No	0.8076	0.5769
SMim vs. SPTX	-4.8	No	0.6671	0.3403
ST vs. SPTX	-2	No	0.9221	0.6900
48				
2D vs. 2DMim	-4.033	No	0.8692	0.4224
2D vs. 2DT	5.067	No	0.7704	0.3145
2D vs. 2DPTX	-1.867	No	0.9484	0.7097
2D vs. S	1.5	No	0.9535	0.7647
2D vs. SMim	-0.6667	No	0.9555	0.8942
2D vs. ST	-1.3	No	0.9535	0.7953
2D vs. SPTX	-7.9	No	0.7282	0.1195
2DMim vs. 2DT	9.1	No	0.7262	0.0741
2DMim vs. 2DPTX	2.167	No	0.9320	0.6657
2DMim vs. S	5.533	No	0.7282	0.2724
2DMim vs. SMim	3.367	No	0.8692	0.5026
2DMim vs. ST	2.733	No	0.9067	0.5859
2DMim vs. SPTX	-3.867	No	0.8692	0.4417
2DT vs. 2DPTX	-6.933	No	0.7282	0.1706
2DT vs. S	-3.567	No	0.8692	0.4777
2DT vs. SMim	-5.733	No	0.7282	0.2557
2DT vs. ST	-6.367	No	0.7282	0.2076
2DT vs. SPTX	-12.97	No	0.3616	0.0123
2DPTX vs. SMim	3.367	No	0.8692	0.5026
2DPTX vs. ST	1.2	No	0.9535	0.8108
2DPTX vs. SPTX	0.5667	No	0.9555	0.9100

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	Mean Diff.	Discovery?	q value	Individual P Value
S vs. ST	-2.167	No	0.9320	0.6657
S vs. SPTX	-2.8	No	0.9067	0.5769
SMim vs. ST	-9.4	No	0.7262	0.0654
SMim vs. SPTX	-0.6333	No	0.9555	0.8994
ST vs. SPTX	-7.233	No	0.7282	0.1532
96				
2D vs. 2DMim	-4.167	No	0.6956	0.4073
2D vs. 2DT	-11.2	No	0.2868	0.0293
2D vs. 2DPTX	-12.6	No	0.2178	0.0148
2D vs. S	-6.8	No	0.4779	0.1788
2D vs. SMim	-4	No	0.6956	0.4262
2D vs. ST	-12.83	No	0.2178	0.0132
2D vs. SPTX	-9.033	No	0.3569	0.0762
2DMim vs. 2DT	-7.033	No	0.4779	0.1646
2DMim vs. 2DPTX	-8.433	No	0.3569	0.0971
2DMim vs. S	-2.633	No	0.8014	0.5997
2DMim vs. SMim	0.1667	No	>0.9999	0.9735
2DMim vs. ST	-8.667	No	0.3569	0.0885
2DMim vs. SPTX	-4.867	No	0.6541	0.3337
2DT vs. 2DPTX	-1.4	No	0.8820	0.7800
2DT vs. S	4.4	No	0.6956	0.3817
2DT vs. SMim	7.2	No	0.4779	0.1551
2DT vs. ST	-1.633	No	0.8756	0.7446
2DT vs. SPTX	2.167	No	0.8155	0.6657
2DPTX vs. S	5.8	No	0.5660	0.2503
2DPTX vs. SMim	8.6	No	0.3569	0.0909
2DPTX vs. ST	-0.2333	No	>0.9999	0.9629
2DPTX vs. SPTX	3.567	No	0.7022	0.4777
S vs. SMim	2.8	No	0.8014	0.5769
S vs. ST	-6.033	No	0.5660	0.2320
S vs. SPTX	-2.233	No	0.8155	0.6561
SMim vs. ST	-8.833	No	0.3569	0.0827
SMim vs. SPTX	-5.033	No	0.6541	0.3176
ST vs. SPTX	3.8	No	0.6956	0.4495

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension; Mim, mimosine; T, thymidine; PTX, paclitaxel.

8.3.8 Mitochondrial Polarisation in Suspension

Table 8.25 – TMRE:MitoTracker ratios of MDA231 cells cultured in 2D and Suspension.

	Mean Diff.	Discovery?	q value	Individual P Value
Mean Fluorescence				
MDA231 _{2D} - MDA231 _S				
MitoTracker	28.98	No	0.4250	0.8094
TMRE	-375.8	Yes	0.0023	0.0022
Minimum Fluorescence				
MDA231 _{2D} - MDA231 _S				
MitoTracker	19.03	No	0.4069	0.7751
TMRE	-161.5	Yes	0.0175	0.0167
Maximum Fluorescence				
MDA231 _{2D} - MDA231 _S				
MitoTracker	-78.77	No	0.4111	0.7831
TMRE	-3096	Yes	<0.0001	<0.0001
Ratio				
MDA231 _{2D} - MDA231 _S				
Mean	-0.976	Yes	0.0104	0.0197
Min	-0.4629	No	0.0931	0.2659
Max	-3.504	Yes	<0.0001	<0.0001

Two-stage linear step-up procedure. 2D, Monolayer; S, Suspension.

8.3.9 MRC5 and MDA231 Co-culture Monolayer Growth Rate

Table 8.26 – Doubling time of MRC5 and MDA231 cells in monolayer co-culture compared between media types.

	Mean Diff.	Discovery?	q value	Individual P Value
MRC5^{GFP}				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	53.69	No	0.2781	0.0883
Low Glucose Serum ⁻ vs. High Glucose Serum ⁻	31.18	No	0.4981	0.3162
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	71.92	No	0.1536	0.0244
Low Glucose Serum ⁺ vs. High Glucose Serum ⁻	-22.51	No	0.5839	0.4679
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	18.23	No	0.5839	0.5561
High Glucose Serum ⁻ vs. High Glucose Serum ⁺	40.74	No	0.4038	0.1923
75:25				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	-7.147	No	0.8981	0.8172
Low Glucose Serum ⁻ vs. High Glucose Serum ⁻	15.43	No	0.8981	0.6181
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	21.07	No	0.8981	0.4967
Low Glucose Serum ⁺ vs. High Glucose Serum ⁻	22.58	No	0.8981	0.4665
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	28.22	No	0.8981	0.3638
High Glucose Serum ⁻ vs. High Glucose Serum ⁺	5.637	No	0.8981	0.8553
50:50				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	0.08333	No	>0.9999	0.9978
Low Glucose Serum ⁻ vs. High Glucose Serum ⁻	26.62	No	0.6187	0.3914
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	29.55	No	0.6187	0.3419
Low Glucose Serum ⁺ vs. High Glucose Serum ⁻	26.53	No	0.6187	0.3928
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	29.46	No	0.6187	0.3432
High Glucose Serum ⁻ vs. High Glucose Serum ⁺	2.93	No	>0.9999	0.9245
25:75				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	8.047	No	0.8344	0.7947
Low Glucose Serum ⁻ vs. High Glucose Serum ⁻	23.09	No	0.8344	0.4567
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	31.16	No	0.8344	0.3164
Low Glucose Serum ⁺ vs. High Glucose Serum ⁻	15.04	No	0.8344	0.6270
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	23.12	No	0.8344	0.4561

	Mean Diff.	Discovery?	q value	Individual P Value
High Glucose Serum ⁻ vs. High Glucose Serum ⁺	8.077	No	0.8344	0.7939
MDA231				
Low Glucose Serum ⁻ vs. Low Glucose Serum ⁺	165.1	Yes	<0.0001	<0.0001
Low Glucose Serum ⁻ vs. High Glucose Serum ⁻	90.63	Yes	0.0031	0.0119
Low Glucose Serum ⁻ vs. High Glucose Serum ⁺	195.3	Yes	<0.0001	<0.0001
Low Glucose Serum ⁺ vs. High Glucose Serum ⁻	-74.43	Yes	0.0042	0.0201
Low Glucose Serum ⁺ vs. High Glucose Serum ⁺	30.21	No	0.0580	0.3312
High Glucose Serum ⁻ vs. High Glucose Serum ⁺	104.6	Yes	0.0005	0.0015

Two-stage linear step-up procedure.

Table 8.27 – Doubling time of MRC5 and MDA231 cells in monolayer co-culture compared between cell lines.

	Mean Diff.	Discovery?	q value	Individual P Value
Low Glucose Serum ⁻				
MRC5 ^{GFP} vs. 75:25 MRC5 ^{GFP} :MDA231	67.86	Yes	0.0416	0.0330
MRC5 ^{GFP} vs. 50:50 MRC5 ^{GFP} :MDA231	51.18	No	0.0985	0.1036
MRC5 ^{GFP} vs. 25:75 MRC5 ^{GFP} :MDA231	50.3	No	0.0985	0.1095
MRC5 ^{GFP} vs. MDA231	-113.2	Yes	0.0033	0.0021
75:25 MRC5 ^{GFP} :MDA231 vs. 50:50 MRC5 ^{GFP} :MDA231	-16.69	No	0.4130	0.5899
75:25 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	-17.57	No	0.4130	0.5706
75:25 MRC5 ^{GFP} :MDA231 vs. MDA231	-181.1	Yes	<0.0001	<0.0001
50:50 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	-0.88	No	0.6157	0.9773
50:50 MRC5 ^{GFP} :MDA231 vs. MDA231	-164.4	Yes	<0.0001	<0.0001
25:75 MRC5 ^{GFP} :MDA231 vs. MDA231	-163.5	Yes	<0.0001	<0.0001
Low Glucose Serum ⁺				
MRC5 ^{GFP} vs. 75:25 MRC5 ^{GFP} :MDA231	7.03	No	>0.9999	0.8201
MRC5 ^{GFP} vs. 50:50 MRC5 ^{GFP} :MDA231	-2.427	No	>0.9999	0.9374
MRC5 ^{GFP} vs. 25:75 MRC5 ^{GFP} :MDA231	4.657	No	>0.9999	0.8802
MRC5 ^{GFP} vs. MDA231	-1.857	No	>0.9999	0.9521
75:25 MRC5 ^{GFP} :MDA231 vs. 50:50 MRC5 ^{GFP} :MDA231	-9.457	No	>0.9999	0.7597
75:25 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	-2.373	No	>0.9999	0.9388
	Mean Diff.	Discovery?	q value	Individual

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				P Value
75:25 MRC5 ^{GFP} :MDA231 vs. MDA231	-8.887	No	>0.9999	0.7738
50:50 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	7.083	No	>0.9999	0.8188
50:50 MRC5 ^{GFP} :MDA231 vs. MDA231	0.57	No	>0.9999	0.9853
25:75 MRC5 ^{GFP} :MDA231 vs. MDA231	-6.513	No	>0.9999	0.8331
High Glucose Serum ⁻				
MRC5 ^{GFP} vs. 75:25 MRC5 ^{GFP} :MDA231	52.12	No	0.1435	0.0976
MRC5 ^{GFP} vs. 50:50 MRC5 ^{GFP} :MDA231	46.62	No	0.1679	0.1371
MRC5 ^{GFP} vs. 25:75 MRC5 ^{GFP} :MDA231	42.21	No	0.1860	0.1771
MRC5 ^{GFP} vs. MDA231	-53.78	No	0.1435	0.0878
75:25 MRC5 ^{GFP} :MDA231 vs. 50:50 MRC5 ^{GFP} :MDA231	-5.503	No	0.6516	0.8587
75:25 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	-9.913	No	0.6516	0.7485
75:25 MRC5 ^{GFP} :MDA231 vs. MDA231	-105.9	Yes	0.0082	0.0014
50:50 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	-4.41	No	0.6516	0.8865
50:50 MRC5 ^{GFP} :MDA231 vs. MDA231	-100.4	Yes	0.0082	0.0023
25:75 MRC5 ^{GFP} :MDA231 vs. MDA231	-95.98	Yes	0.0082	0.0033
High Glucose Serum ⁺				
MRC5 ^{GFP} vs. 75:25 MRC5 ^{GFP} :MDA231	17.01	No	>0.9999	0.5827
MRC5 ^{GFP} vs. 50:50 MRC5 ^{GFP} :MDA231	8.803	No	>0.9999	0.7759
MRC5 ^{GFP} vs. 25:75 MRC5 ^{GFP} :MDA231	9.54	No	>0.9999	0.7577
MRC5 ^{GFP} vs. MDA231	10.12	No	>0.9999	0.7434
75:25 MRC5 ^{GFP} :MDA231 vs. 50:50 MRC5 ^{GFP} :MDA231	-8.21	No	>0.9999	0.7906
75:25 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	-7.473	No	>0.9999	0.8090
75:25 MRC5 ^{GFP} :MDA231 vs. MDA231	-6.89	No	>0.9999	0.8236
50:50 MRC5 ^{GFP} :MDA231 vs. 25:75 MRC5 ^{GFP} :MDA231	0.7367	No	>0.9999	0.9810
50:50 MRC5 ^{GFP} :MDA231 vs. MDA231	1.32	No	>0.9999	0.9659
25:75 MRC5 ^{GFP} :MDA231 vs. MDA231	0.5833	No	>0.9999	0.9849

Two-stage linear step-up procedure.

8.3.10 MRC5 and MDA231 Suspension Co-culture Protein Expression

Table 8.28 – MDA231 protein expression in 2D and suspension.

	Mean Diff.	Discovery?	q value	Individual P Value
MDA231 _{2D} - MDA231 _s				
Actin	0.003405	No	>0.9999	0.9943
Survivin	0.84	No	0.2272	0.1082

Two-stage linear step-up procedure.

Table 8.29 – MRC5^{GFP} protein expression in 2D and suspension.

	Mean Diff.	Discovery?	q value	Individual P Value
MRC5 ^{GFP} _{2D} - MRC5 ^{GFP} _s				
Actin	0.02167	No	>0.9999	0.9508
GFP	-0.4282	No	0.9172	0.2912
Survivin	-0.01238	No	>0.9999	0.9770

Two-stage linear step-up procedure.

Table 8.30 – Co-culture protein expression in 2D and suspension.

	Mean Diff.	Discovery?	q value	Individual P Value
Co-culture _{2D} - Co-cultures				
Actin	-0.003368	No	>0.9999	0.9926
GFP	-0.8225	No	0.2009	0.0638
Survivin	-0.1712	No	>0.9999	0.6409

Two-stage linear step-up procedure.

8.3.11 MRC5 and MDA231 Suspension Co-culture Survivin Staining

Table 8.31 – Viability comparison between culture conditions at each time point.

P value	0.1078
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=1.751 df=10.99
How big is the difference?	
Mean ± SEM of column A	1.066 ± 0.3302, n=5
Mean ± SEM of column B	2.044 ± 0.4505, n=8
Difference between means	0.9779 ± 0.5586
95% confidence interval	-0.2517 to 2.207
R squared (eta squared)	0.218
F test to compare variances	
F, DFn, Dfd	2.979, 7, 4
P value	0.3082
P value summary	ns
Significantly different (P < 0.05)?	No

Unpaired t test with Welch's correction.

8.3.12 MRC5 and MDA231 Suspension Co-Culture Collagen I and Laminin Staining

Table 8.32 – Area fraction of collagen I and laminin.

	Mean Diff.	Discovery?	q value	Individual P Value
Collagen I				
Control vs. MRC5 ^{GFP}	3.266	Yes	0.0129	0.0139
Control vs. Coculture	3.048	Yes	0.0129	0.0245
MRC5 ^{GFP} vs. Coculture	-0.2183	No	0.2407	0.6876
Laminin				
Control vs. MRC5 ^{GFP}	5.651	Yes	<0.0001	<0.0001
Control vs. Coculture	5.297	Yes	0.0001	0.0002
MRC5 ^{GFP} vs. Coculture	-0.3536	No	0.1804	0.5156

Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

Table 8.33 – Fluorescence intensity of collagen I and laminin.

	Mean Diff.	Discovery?	q value	Individual P Value
Collagen I				
Control vs. MRC5 ^{GFP}	-5047	No	0.0963	0.0611
Control vs. Coculture	-2666	No	0.3444	0.3280
MRC5 ^{GFP} vs. Coculture	2380	No	0.0963	0.0381
Laminin				
Control vs. MRC5 ^{GFP}	2663	No	0.6082	0.3159
Control vs. Coculture	2359	No	0.6082	0.3862
MRC5 ^{GFP} vs. Coculture	-303.6	No	0.8254	0.7861

Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

8.3.13 MRC5 and MDA231 Suspension Co-culture E-cadherin and Cortactin Staining

Table 8.34 – Area fraction of E-cadherin and cortactin.

	Mean Diff.	Discovery?	q value	Individual P Value
MRC5 ^{GFP} - Coculture				
E-cadherin	-0.01428	No	0.5149	0.9807
CTTN	-1.984	Yes	0.0067	0.0064

Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

Table 8.35 – Fluorescence intensity of E-cadherin and cortactin.

	Mean Diff.	Discovery?	q value	Individual P Value
MRC5 ^{GFP} - Coculture				
E-cadherin	-1618	No	0.1937	0.1845
CTTN	1976	No	0.1937	0.1123

Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

8.3.14 GAG Stain Intensity of Native vs. Decellularised Rat lung

Table 8.36 –PAS-D staining of native and decellularised rat lung column statistics.

	Native Lung ECM	DCM Lung ECM
W	0.9658	0.9238
P value	0.6446	0.4660
Passed normality test (alpha=0.01)?	Yes	Yes
P value summary	ns	ns

Shapiro-Wilk normality test

Table 8.37 – PAS-D stain intensity comparison of native and decellularised rat lung.

P value	0.0451
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=3.146 df=3.304
How big is the difference?	
Mean ± SEM of column A	132.6 ± 7.208, n=3
Mean ± SEM of column B	159.1 ± 4.389, n=3
Difference between means	26.55 ± 8.439
95% confidence interval	1.035 to 52.06
R squared (eta squared)	0.7497
F test to compare variances	
F, DFn, Dfd	2.697, 2, 2
P value	0.5409
P value summary	ns
Significantly different (P < 0.05)?	No

Unpaired t test with Welch's correction

8.3.15 pH3 and β-actin Immunoblotting of MDA231 cells Grown in rDLM

Table 8.38 – Immunoblot of MDA231 in monolayer and rDLM culture.

Appendix

	Mean Diff.	Discovery?	q value	Individual P Value
β -actin				
2D vs. 3D	0.6173	No	0.1172	0.3350
2D vs. N	5.411	Yes	<0.0001	<0.0001
3D vs. N	4.793	Yes	<0.0001	<0.0001
pH3				
2D vs. 3D	7.712	Yes	<0.0001	<0.0001
2D vs. N	6.069	Yes	<0.0001	<0.0001
3D vs. N	-1.643	Yes	0.0185	0.0176
Laminin				
2D vs. 3D	-4.372	Yes	<0.0001	<0.0001
2D vs. N	-3.984	Yes	<0.0001	<0.0001
3D vs. N	0.3879	No	0.2044	0.5841

Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli