The Effect of Hypoxia on Protein Phosphatase 2A (PP2A) in MDA-MB-231 Cells,

And, a Pilot Study to Establish a Primary Breast Cancer Cell Model from Fine Needle Aspirations.

By

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Declaration

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Abstract

Background: Cancerous tumours are characterised by areas of insufficient blood supply, with resulting hypoxia and nutrient deprivation. The mechanisms by which cancer cells adapt to these conditions are of interest due to potential clinical relevance. Although immortalised cell lines have been used extensively in cancer research, a primary cancer cell model could potentially shed additional light on cancer biology. Protein phosphatase 2A (PP2A) is a Serine/Threonine protein phosphatase which has been implicated in hypoxia, although contradictory reports regarding its involvement has been published.

Aims:

1. To establish a primary cell culture model using fine needle aspiration (FNA) for cell collection.
2. To investigate the effect of hypoxia on these primary cells.
3. To investigate the effect of hypoxia on PP2A expression, post-translational modification and activity in MDA-MB-231 cells.

We hypothesized that hypoxia would induce a reduction in PP2A activity, thereby favouring the phosphorylation and activation of enzymes associated with survival and proliferation.

Methods: Nine FNA samples were collected from patients at Tygerberg Hospital. Samples were subjected to processing and cultured at 37°C in suitable growth medium containing HEPES and epidermal growth factor. Cells were maintained in culture for 1.5–6 weeks and medium was refreshed initially after 3-4 days, and there after every second day. Although cells appeared to attach and survive initially, numbers dwindled until none were visible. We are therefore as of yet unable to establish a primary cancer cell model. Potential variables which could be of importance in future attempts to culture primary cells, include using a suitable attachment matrix, as well as optimizing the culture medium.

Concerning PP2A in hypoxia in standard cell culture: MDA-MB-231 cells were cultured in a gas mixture of 5%CO_2, 0.5%O_2 for varying lengths of time, in growth medium containing only 1% FBS, where after cells were harvested and the following primary end-points investigated: (1.) The expression, phosphorylation and methylation of the catalytic subunit of PP2A; (2.) PP2A activity; and (3.) cell viability.

Results: Following 72 hours hypoxia, Western blotting showed a decrease in total PP2A (control: 1.00±0.1426 arbitrary units (AU) vs hypoxia: 0.3513±0.07558 AU; n=3; p≤0.05), as well as unexpectedly a decrease in the relative phosphorylation of both PKB (phospho-to-total (p/t) ratio: control: 1.211±0.1820 AU vs hypoxia: 0.2088±0.03583 AU; n=3; p≤0.05) and ERK (control: 1.00±0.1519 AU vs hypoxia: 0.3493±0.05206 AU; n=3; p≤0.05). This led us to investigate shorter durations of hypoxia, specifically in the range between 2 and 8 hours. No significant changes in PP2A expression, post-translational modification or activity were observed, although there was a trend in increasing PP2A activity associated with hypoxia, which was highlighted by a significant increase in cells exposed to 6 hours of chemically stabilized HIF1α (characteristic of hypoxia) (control: 134.7±33.74 AU vs positive control: 193.6±35.31 AU; n=2; p≤0.05). Four and six hours of hypoxia were consistently associated with a significant decrease in ATP.

Discussion and Conclusion: Although we observed an interesting pattern of increased PP2A activity associated with hypoxia, our results failed to show a convincing link. Based on this, as well as literature, further research is needed: It would be interesting to investigate intermediate durations of less strenuous hypoxia (1% O_2 instead of 0.5%). Modulating the activity of PP2A in hypoxia, followed by assessment of cell viability and pro-survival signalling would also contribute value.

Opsomming

Agtergrond: ‘n Tekort aan genoegsame bloedvoorsiening, met gepaardgaande hipoksie en ‘n tekort aan voedingstowwe, is kenmerkend van kankeragtige tumore. Die meganismes ter sprake by die aanpassing van kankerselle by hierdie kondisies is van belang as gevolg van moontlike kliniese toepaslikheid. Alhoewel onsterflike sellyne ekstensief gebruik is in kanker-navorsing, kan ‘n primêre kankersel-model moontlik bykomende inligting aangaande kankerbiologie oplewer. Proteïen fosfatase 2A (PP2A) is ‘n Serien/Threonien proteïen fosfatase wat vantevore betrek is by hipoksie, alhoewel teenstrydige inligting aangaande sy deelname gepubliseer is.

Doelstellings:

1. Om ‘n primêre selkultuur model daar te stel deur selle te gebruik wat deur fynnaaldaspirasie (FNA) versamel is.
2. Om die effek van hipoksie op hierdie primêre selle te ondersoek.
3. Om die invloed van hipoksie op PP2A uitdrukking, post-sintese modifikasie en aktiwiteit in MDA-MB-231 selle te ondersoek.

Ons hipotese was dat hipoksie ‘n afname in PP2A aktiwiteit tot gevolg sou hê om sodoende die fosforilering en aktivering van ensieme wat betrokke is by oorlewing en selvermeerdering te bevoordeel.

Metodes: Nege FNA monsters is versamel by pasiënte in Tygerberg Hospitaal. Hierdie monsters is verwerk en in kultuur geplaas teen 37°C in gepaste groeimedium wat beide HEPES en epidermale groeifaktor bevat het. Selle is in kultuur onderhou vir tussen 1.5 en 6 weke. Medium is inisieel eers vervang na 3-4 dae en daarna elke tweede dag. Alhoewel dit eers voorgekom het of die selle geheg en oorleef het, het die getal selle uiteindelik afgeneem totdat niks sigaar was nie. Ons is dus tot op hede onsuksesvol in ons pogings om ‘n primêre kankersel-model daar te stel. Veranderlikes wat in aggeneem kan word in toekomstige pogings om primêre selle in kultuur te onderhou sluit in die gebruik van ‘n gepaste hegtings-matriks, asook optimalisering van die groeimedium.

Aangaande die effek van hipoksie op PP2A in standaard selkultuur: MDA-MB-231 selle is onderhou in ‘n gasmengsel van 5%CO_2, 0.5%O_2 tesame met groemedium wat slegs 1% FBS bevat het vir wisselende tydsdure. Selle is daarna versamel en die volgende eindpunte

ondersoek: (1.) Die uitdrukking, fosforilering en metilering van die katalitiese subeenheid van PP2A; (2.) PP2A aktiwiteit; en (3.) seloorlewing.

Resultate: Western-kladanalise na 72 uur hipoksie het ‘n afname in totale PP2A (Kontrole: 1.00±0.1426 arbitrêre eenheide (AE) vs hipoksie: 0.3513±0.07558 AE; n=3; p≤0.05), asook ‘n onverwagse afname in die relatiewe fosforilering van beide PKB (fosfo-tot-totaal (p/t) verhouding: kontrole: 1.211±0.1820 AE vs hipoksie: 0.2088±0.03583 AE; n=3; p≤0.05) asook ERK (kontrole: 1.00±0.1519 AE vs hipoksie: 0.3493±0.05206 AE; n=3; p≤0.05) getoon. Hierdie resultate het ons genoop om korter periodes van hipoksie te ondersoek, spesifiek tussen 2 en 8 ure. Geen beduidende verskille in die uitdrukking, post-sintese modifikasie of aktiwiteit van PP2A is egter waargeneem nie, alhoewel PP2A aktiwiteit geneig het om toe te neem in assosiasie met hipoksie. Hierdie is uitgelig deur ‘n beduidende toename in PP2A aktiwiteit in selle blootgestel aan 6 uur van die chemiese stabilisering van HIF1α (wat kenmerkend is van hipoksie) (kontrole: 134.7±33.74 AE vs positiewe kontrole: 193.6±35.31 AE; n=2; p≤0.05). Vier en ses uur van hipoksie was konstant geassosieer met ‘n afname in ATP.

Bespreking en gevolgtrekking: Alhoewel ons ‘n interessante patroon van toenemende PP2A aktiwiteit in assosiasie met hipoksie waargeneem het, het ons resultate geen oortuigende verwantskappe getoon nie. In die lig hiervan, asook die literatuur, word verdere ondersoek vereis: Dit sal interessant wees om intermediêre tydsdure met minder ingrypende hipoksie (1% O_2 instede van 0.5%) te ondersoek. Dit sal ook van waarde wees om die aktiwiteit van PP2A in hipoksie te moduleer, gevolg deur analises van sel-oorlewing en pro-oorlewings seintransduksie.

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List of Abbreviations:

ACSR	: Aids and Cancer Specimen Resource
ANOVA	: Analysis of Variance
Asn	: Asparagine
ATP	: Adenosine Triphosphate
AU	: Arbitrary Units
BAD	: Bcl-2-associated Death Promoter
BSA	: Bovine Serum Albumin
DiFMUP	: 6,8-difluoro-4-methylumbelliferyl phosphate
DMEM	: Dulbecco's Modified Eagle Medium
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylenediaminetetraacetic acid
EGF	: Epidermal Growth Factor
EGTA	: Egtazic acid
EPO	: Erythropoietin
ER	: Estrogen Receptor
ERK	: Extracellular Regulated Kinase
FBS	: Fetal Bovine Serum
FCS	: Fetal Calf Serum
FISH	: Fluorescent *in situ* hybridisation
FIH	: Factors Inhibiting HIF-1
FNA	: Fine Needle Aspirate
HER-2/C-erb	: Human EGF receptor 2
Hg	: Mercury
HRE	: Hypoxia Response Elements
HREC	: Health Research Ethics Committee
IGF2	: Insulin-like Growth Factor 2
IL-1β	: Interleukin-1β
IL-32β	: Interleukin-32β
I2PP2A	: Inhibitor of PP2A
KSR	: Kinase Suppressor of Ras
LDH-A	: Lactate Dehydrogenase – A

LOH	: Loss of Heterozygosity
OA	: Okadaic Acid
MAPK	: Mitogen-Activated Protein Kinase
MEK	: Mitogen-Activated Protein Kinase Kinase
MUP	: 4-methylumbelliferyl
PBS	: Phosphate Buffered Saline
PCR	: Polymerase Chain Reaction
PDK	: Pyruvate Dehydrogenase Kinase
PHD	: Prolyl Hydroxylase
PI3K	: Phospho-inositol 3 Kinase
PKB/Akt	: Protein Kinase B
PP1	: Protein Phosphatase 1
PP2A	: Protein Phosphatase 2A
PR	: Progeterone Receptor
Pro	: Proline
PTEN	: Phosphate and Tensin Homolog
PVDF	: Polyvinylidene Fluoride
RAF	: Rapidly Accelerated Fibrosarcoma
RAS	: Retrovirus-associated DNA Sequences
SD	: Standard Deviation
SDS-PAGE	: Sodium Dodecyl Sulphate – Polyacrylamide Gel
SEM	: Standard Error of the Means
Ser	: Serine
TBS	: Tris Buffered Saline
TGFα	: Transforming Growth Factor α
Thr	: Threonine
TNFα	: Tumour Necrosis Factor α
Tyr	: Tyrosine
VEGF	: Vascular Endothelial Growth Factor

List of Symbols:

%	-	Percentage
°C	-	Degrees Celsius
A	-	Amperes
g	-	Grams
kDa	-	Kilo Dalton
kg	-	Kilogram
L	-	Litre
m	-	Meter
M	-	Molar
mg	-	Milligram
min	-	Minute
ml	-	Millilitre
mM	-	Milimolar
mmHg	-	Pressure (Millimetres of Mercury)
mW	-	Miliwatt
ng	-	Nanogram
nM	-	Nanomolar
v	-	Volume
V	-	Volts
α	-	Alpha
β	-	Beta
µg	-	Microgram
µl	-	Microliter
µM	-	Micromolar

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Chapter 1: Literature Review

1.1 The Burden of Breast Cancer

Statistics concerning the rapidly increasing global incidence of breast cancer sees this unrelenting disease account for 23% per annum, of new cancer cases around the world, and it has been stated to be overall leading cause of cancer related deaths among women worldwide (Shibuya *et al.*, 2002; Parkin *et al.*, 2005). According to *Cancer Research UK*, approximately 1 in 8 women will develop breast cancer within their lifetime (World Cancer Research Fund International, 2017). It is the most common neoplastic disease in women the world over, accounting for a staggering 22.9% of all global cases, and 13.7% of cancer related deaths (Jemal *et al.*, 2011). There are however, variations in incidence, mortality and survival between predominantly Western, industrialised countries and the developing world, with more than three quarters of breast cancer related deaths reported to be occurring in 'third world' developing countries (Hortobagyi *et al.*, 2005). This discordance has been reported to be largely related to the lack of preventative screening methods available leading to diagnosis at a late stage of cancer progression, along with poor access to patient care with below standard treatment (Vorobiof *et al.*, 2001; Walker *et al*., 2004). The highest incidences of the disease have been reported in well developed, urbanised countries and regions, yet it presents a more favourable survival rate in comparison to incidences in the developing world (Hortobagyi *et al.*, 2005; Ferlay *et al.*, 2010). South Africa also follows the global cancer trend, with breast cancer presenting the primary cancer in women (Vorobiof *et al.*, 2001). It has been reported that the incidence of breast cancer in South Africa is far higher in white South African women than compared to black women. However the occurrence of the disease in black South Africans appears to increase among women living in urban areas (Walker *et al*., 2004). African women from traditionally rural areas have been reported to show characteristic protective factors against the development of breast cancer, such as a later onset of menstruation, a younger age at birth of first child usually with prolonged lactation, and increased levels of physical activity in comparison to their non-African counterparts. However, these favourable factors appear to decrease with a direct correlation to the increasing levels of urbanisation (Walker *et al*., 2004).

Predisposition to breast cancer includes genetic factors (such as familial susceptibility), gender, age, exposure to radiation and other diseases of the breast (Turnbull and Rahman, 2008; Cancer Genome Atlas, 2012). It has been predicted that, by the year 2030, the mortality rate of South African women with breast cancer will reach around 56.9% (Ferlay *et al.*, 2010).

The vast majority of malignant human tumours have been found to consist of cancerous epithelial cells along with a multitude of different cell types (such as fibroblasts, immune cells, adipocytes and normal non-cancerous epithelial cells), and of course, the components of the extracellular matrix (McAllister and Weinberg, 2010). Taken together, these components are collectively named the cancer microenvironment, or the stroma. Recent studies have alluded to the fact that all of the different cell types existing in the cancer microenvironment play an important role in the growth, survival and metastasis of the cancer cells within solid tumours (Quail and Joyce, 2013).

The human breast can be described as consisting of mammary gland lobules, lactiferous ducts connecting the lobules, and stroma consisting of connective tissue, adipose tissue and a dense network of blood and lymph vessels (Badowska-Kozakiewicz *et al*., 2015). It has been found that the majority of breast cancers occur within the lactiferous duct tissue, however it is in fact possible for lesions to grow in all breast structures. Cancerous lesions within the lactiferous duct that do not infiltrate the surrounding tissue, have been described as non-invasive ductal carcinomas and account for approximately 20% of all breast cancers. Invasive ductal carcinoma of no special type are the most common form of invasive breast cancer, accounting for up to 80% of all cases (Badowska-Kozakiewicz *et al*., 2015). Chemotherapeutic agents (anthracyclines) used for breast cancer therapy have been found to be effective in treating patients diagnosed with breast cancer, however, they are cytotoxic to a wide assortment of systems in the body (Barrett-Lee *et al.*, 2009). Resistance to cancer treatment therapies has become a prominent issue faced by patients, accounting for over 90% of treatment failures (Coley, 2008), with a vast majority of sufferers experiencing a re-occurrence of the disease with poor prognosis. Thus, there exists a serious requirement for novel strategies to overcome these problems and improve clinical outcomes and treatment.

1.2 Models Used to Study Breast Cancer

1.2.1 Immortalised cell lines

The bulk of our knowledge on breast cancer and breast cancer biology can be attributed to research done utilising breast cancer cell lines to investigate therapies, new therapeutic drugs or hormone treatments, as well as to gain a better insight into the mechanisms underlying tumour initiation and evolution of this relentless disease (Lacroix *et al*., 2004). Whether these cell lines are an accurate and realistic representation of cancerous tumours *in vivo*, and whether the molecular profiles of breast tumours are reflected in cell line models (Holliday and Speirs, 2011) remains a topic of debate among researchers, as it is somewhat unclear as to whether cultured cells are truly representative of *in vivo* conditions *(Correa et al*., 2009). Vargo-Gogola and Rosen described breast cancer as a collection of breast diseases rather than a single disease, having a diverse range of histopathologies along with both genetic and genomic variations and outcomes. They identified a major challenge facing breast cancer research being the availability of experimental models of the disease that accurately represent the multiple varieties of the disease (Vargo-Gogola and Rosen, 2007). Most available lines have been derived from metastatic tumours, aspirates or pleural effusions, representing more aggressive behaviours and phenotypes, and are mostly genetically homogenous, converse to the heterogeneity of the *in vivo* tumour (Burdall *et al.*, 2003; Vázquez *et al.*, 2004). The extent to which a single cell line can mirror the heterogeneity of clinical carcinoma samples has been questioned, as well as if an *in vivo* phenotype can be maintained in culture (Holliday and Speirs, 2011). Complex inter-relationships existing *in vivo* are often lost when cell lines are cultured, with cells frequently found to be sensitive to culture conditions altering the cell's phenotypes and resulting in the inappropriate activation of certain pathways, or altered differentiation (Holliday and Speirs, 2011). Neve *et al.* suggested that no single cell line can be a true representation of the *in vivo* tumour cell population, yet a battery of cell lines could show the heterogeneity observed in primary breast cancer tissue (Neve *et al.*, 2006). Based on this, it could be argued that future research studies should make use of additional methods or strategies to try and mitigate shortcomings of cell lines. Such methods could include injecting cells back into animals, as with xenografts (Kuperwasser *et al.*, 2005; Stuelten *et al.*, 2010), or changing and optimising the culture conditions with the additions of attachment-favouring products such as Matrigel, promoting 3D culturing (Bergstraesser and Weitzman, 1993; Ip and Darcy, 1996). Alternatively, protocols could include other models all together, such as primary cell models (S P Ethier *et al.*, 1993; Speirs *et al.*, 1998; Li *et al.*, 1998).

Even with the metastatic nature of the cells, successful long-term culture has been limited, as it is probable that the cells undergo specific phenotype and genotype alterations in the simplified culture conditions when compared to *in vivo* cells (Burdall *et al.*, 2003; Lacroix *et al*., 2004). Culture conditions used to propagate cell lines create vastly differing environments to the microenvironments within the breast tissue (Vargo-Gogola and Rosen, 2007). It has also been identified that the same cancer cell lines cultured in different laboratories and/or under other conditions can evolve differently, giving rise to divergent sub-populations and preventing inter-laboratory comparisons of data on the same line (Lacroix *et al*., 2004). The metastatic foundation of most breast cancer cell lines also raises doubts as to the relationship of the lines to primary tumours, and it has been argued that most culturing conditions used for the establishing of the cell lines might eliminate some types of cancer cells initially present in the sample (Lacroix *et al*., 2004).

Previous research however, suggests that breast cancer cell lines can likely reflect the features of the cancerous tumours from which they originate (Lacroix *et al*., 2004), and in many instances, remain powerful experimental and investigative tools (Holliday and Speirs, 2011). Experiments performed on cell lines have established important principles, and allowed for the construction of useful hypotheses (Correa *et al*., 2009). A major benefit of using immortalised cell lines, is that they provide a ceaseless source of easily cultured, self-replicating material with little to no contamination by fibroblasts or stromal cells, that can easily be grown and propagated over a long time (Burdall *et al.*, 2003; Lacroix *et al*., 2004; Holliday and Speirs, 2011).

Because of their ease of use, immortalised cancer cell lines will likely continue to be used in research as models for breast cancer. However, research findings suggest that the cell lines available might not be accurate representations of *in vivo* tumour cells, and the phenotype of the primary cells might alter significantly when interacting with surrounding tumour stroma *in vivo* (Lacroix *et al*., 2004). It is important for researchers to understand the shortfalls and to consider all limitations when designing experiments and interpreting experimental data, as relying on a single cell line could generate misleading data (Holliday and Speirs, 2011).

1.2.2 Isolated primary cells

Successful isolation and culture of primary breast cancer cells provides an alternative model to immortalised cell lines, and is potentially of great importance for the establishment of a model of cells originating from an *in vivo* tumour for the advancement of our knowledge and understanding of breast cancer biology. As mentioned above, many investigative studies have made use of commercially available immortalised cell lines, however, recently it has come to light that these cell lines might not dependably display the true characteristics of the original tumour cells (Keller *et al.*, 2010). Burdall *et al.* reported that the aggressive immortalised cell lines derived from tumour metastases, offer a bias toward more rapidly progressive and late stage cancer, rather than low grade and early stage disease. Accordingly, it could be argued that it is clinically more relevant to make use of cells isolated directly from a primary tumour as a more accurate representation of cancer pathophysiology (Burdall *et al.*, 2003).

The establishing and maintenance of isolated cells in primary culture as a more representative and viable *in vitro* model of breast cancer has been a major research target (Speirs *et al.*, 1998). The advantage of this being that cells derived directly from tumour tissue offer a more accurate representation of the *in vivo* tumour, thereby furthering our understanding of cancer pathology, and allowing for characterisation of cell cultures which can be compared to the original tumour (Burdall *et al.*, 2003). It is pertinent for researchers to select a cell culture model that accurately reflects as many of the *in vivo* characteristics of the cells as possible, hence the essential need for a successful isolation and culturing protocol for primary cancer cells (Vu *et al.*, 2015).

Previous studies have been done to identify the optimal conditions for the procurement of primary breast cancer cells (Vu *et al.*, 2015). It has been found that with isolation, many other cell types are often also present, such as connective-tissue fibroblasts, immune cells and vascular endothelial cells (Sung *et al.*, 2007). Slow proliferation rates of isolated primary breast cancer cells often results in an overgrowth of these non-malignant cells, which threaten the successful culture and propagation of tumour cells (Dairkee *et al.*, 1995; Speirs *et al.*, 1998). Many attempts to isolate and culture cancer cells from breast tumour tissue have thus been largely unsuccessful due to this overgrowth of fibroblasts rapidly adapting to the *in vitro* conditions, and proliferating swiftly, outgrowing any epithelial counterparts

(Freshney, 1985; Burdall *et al.*, 2003; Lacroix *et al*., 2004). Amadori *et al*. were only able to report a success rate of 0.7% (Amadori *et al.*, 1993), while Gazdar *et al.* obtained isolated cells from a mere 18 out of 177 cultures (Gazdar *et al.*, 1998). Limitations and further barriers often encountered in the isolation procedure include low availability of human breast tumour tissue, the lack of consensus in literature regarding a successful, reproducible protocol for the isolation, cultivation and characterisation of the cancer cell population (Weigand *et al.*, 2016), as well as obtaining sufficient quantities of cancer cells in culture for molecular and cell biology studies (Speirs *et al.*, 1998). Furthermore, there often also exists a lack of clear identification and morphological distinction between non-malignant and malignant cells in culture (Dairkee *et al.*, 1995).

The poor efficiency of the isolation procedure is often attributed to the technical difficulties associated with the extraction of viable tumour cells from the surrounding stromal tissue (Lacroix *et al*., 2004). However, Vázquez *et al.* were able to derive and characterize three novel hormone responsive cell lines from human breast carcinomas through isolation and culture (Vázquez *et al.*, 2004). Lacroix *et al.* reported that freshly isolated breast cancer cells can be considered to be representative of cancer cells *in vivo* (Lacroix *et al*., 2004). Although more technically challenging to establish, if properly characterized, isolated cells can offer a more clinically relevant model likely to provide more significant data (Burdall *et al.*, 2003). However, it has been suggested that long-term culturing can alter the cancer cell's properties (Lacroix *et al*., 2004). Although the limitations of primary culture exist with the afore-mentioned slow doubling times and poor characterisation due to rapid overgrowth of fibroblasts, when cultured over the short term, the cells appear able to maintain many characteristics associated with breast tumours (Speirs *et al.*, 1998). There is little opportunity for the transformations frequently observed in long-term culture of immortalised cell lines, to take place when primary cells are maintained in culture for only a limited length of time. However, due to the loss of cell-cell interactions, the possibility does exist that the isolated cells may well behave differently under culture conditions in comparison to when forming part of the tissue *in vivo* (Burdall *et al.*, 2003).

1.2.2.1 Primary cell isolation from excised tumour tissue

In literature to date, there does not appear to be any general consensus on a single reproducible and successful method for primary cell isolation and propagation in culture. Many studies describe an isolation protocol requiring the mechanical disruption and enzymatic digestion of the tumour tissue (Burdall *et al.*, 2003), with further differential centrifugation following the addition of collagenase for digestion. This allows for cell separation based on differing sedimentation rates (Emerman and Wilkinson, 1990; Speirs *et al*., 1996), and subsequent propagation of sufficient quantities of cells (Speirs *et al.*, 1998; Loveday *et al.*, 2002). Research published by Dairkee *et al*. describes a 6 hour partial enzymatic digestion with 66% of samples giving rise to tumour epithelial cells presenting with a proliferative capacity (Dairkee *et al.*, 1997). Interestingly, the same research group had previously described an isolation protocol using a custom made sandwich model creating an hypoxic gradient whereby only malignant cells survived (Dairkee *et al.*, 1995), however, further reported use of this technique is limited.

In terms of sample collection, once patient consent has been obtained from breast cancer patients, tumour tissue is removed often by needle biopsy or through mastectomy surgery. When received in the laboratory following clinical and pathological testing, the tumour mass is often vastly reduced (Vázquez *et al.*, 2004). Tissues are generally washed extensively in phosphate buffered saline (PBS) and subjected to disaggregation into fragments to remove any excess adipose and vascular material (Vu *et al.*, 2015; Weigand *et al.*, 2016), followed by the addition of collagenase and/or hyaluronidase for digestion, and overnight incubation on a rotor at 37°C (Speirs *et al.*, 1998; Vázquez *et al.*, 2004; Weigand *et al.*, 2016). This digestion step preferentially disrupts the cancer cell's contact with any stromal cells present, instigating release of single tumour cells while the remaining tumour epithelium remains intact. This allows for differential centrifugation and fractionation in later steps to successfully isolate the cancer cells for culturing (DeRose *et al.*, 2013). In a previous argument by Li *et al*., it had been reported that approaches to the isolation of primary breast tumour cells dependent on the mechanical and/or enzymatic disaggregation and digestion of connective tissue, required large quantities (in excess of several grams worth) of tumour tissue. Furthermore, the cultures that have managed to proliferate using these techniques, are frequently slow-growing with a high occurrence of fibroblast overgrowth along with other breast tissue (Li *et al.*, 1998).

Several studies have proceeded to characterise and plate the resulting fractions following digestion and a filtration steps, in a culture medium of Dulbecco's Modified Eagle medium (DMEM) and HAMS-F12 with low (5%) serum and supplemented with growth factors, insulin and bovine serum albumin (BSA) and HEPES (Soule *et al.*, 1990; Speirs *et al.*, 1998; Vázquez *et al.*, 2004; Ponti *et al.*, 2005; Marsden *et al.*, 2012; Weigand *et al.*, 2016).

However, since the culturing of cells isolated through protease digestion of solid tumours can encourage and increase the risk of overgrowth of any stromal cells and fibroblasts that might be present (Speirs *et al.*, 1998; Loveday *et al.*, 2002), there is a need for selective culture conditions allowing for the propagation of cancer cells only (Hass and Bertram, 2009). Preferential growth of primary cancer cells is stimulated most often in serum-free, or low serum conditions permitting this selection (Emerman and Wilkinson, 1990). However, cells may still require a complex culture medium (including growth factors), and a reduced cell proliferation rate is frequently experienced (Vu *et al.*, 2015). Although short term culture of isolated cancer cells can prove successful, it is still an imperfect model due to the nature of the mechanical and/or enzymatic digestion methods causing disruption to the normal tissue architecture and cell-cell interactions, which would normally occur *in vivo* (Burdall *et al.*, 2003). In a protocol described by Hood *et al.*, these functions and interactions can be maintained when a tissue slice (400 µm thick) from an excised tumour is placed *in vitro* for up to 6 days allowing cells to proliferate along with the adequate diffusion of nutrients from the surrounding culture medium (Hood and Parham, 1998). Further studies have also reported isolation techniques, whereby digestion with collagenase and differential centrifugation are not implemented. Hass *et al.*, van Pham *et al.*, and Vu *et al.* each describe protocols where tumour tissue samples were cut into fragments, washed with PBS and seeded directly onto uncoated culture dishes in serum-free growth medium with appropriate supplementations, and incubated at 37°C (Hass and Bertram, 2009; Van Pham *et al.*, 2012; Vu *et al.*, 2015). This approach led to the subsequent outgrowth of primary derived breast cancer cells spread out from the tumour fragments and forming tumour clusters, which were later separated and placed in a new culture dish to encourage the continual outgrowth of primary cancerous cells (Hass and Bertram, 2009). The success rate of further culture of the isolated primary breast cancer cells is greatly increased when using optimised growth media to enhance tumour cancer cell growth whilst preventing the rapid overgrowth of stromal cells and fibroblasts (Vu *et al.*, 2015).

Following an isolation protocol, many studies make use of characterisation techniques to identify and confirm successful isolation and propagation of primary breast cancer cells. Techniques used include flow cytometry analysis using cancer specific cell surface markers $CD44^{+}/CD24^{-}$ (Van Pham *et al.*, 2012; DeRose *et al.*, 2013; Vu *et al.*, 2015), immunocytochemistry (Weigand *et al.*, 2016), immunostaining, and polymerase chain reaction (PCR) (Ethier *et al.*, 1993). In an effort to improve the success rate of primary breast cancer cultures, researchers have employed a number of approaches such as optimising the dissociation process of tumour tissue (Dairkee *et al.*, 1997), improving the growth medium formulation (Band *et al.*, 1990; Ethier *et al.*, 1993) and simulating an *in vivo* microenvironment, in culture (Dairkee *et al.*, 1995).

1.2.2.2. Primary Cell Isolation through Fine Needle Aspiration (FNA)

Fine needle aspiration (FNA), first reported to be used by Martin and Ellis in 1930 (Martin and Ellis, 1930), is a well-established, safe, minimally invasive, accurate technique to obtain samples from solid breast lumps, and is commonly used in the pathological characterization, evaluation and diagnosis of palpable and non-palpable lesions (Schmitt *et al*., 1995a; Schmitt *et al*., 1995b; Li *et al.*, 1998; Rosa, 2008) . It is usually a fast technique, rarely presenting any complications, and commonly performed after the detection of a mass or lesion (Frable, 1983; Rosa, 2008). It is an inexpensive and relatively painless technique, and is advantageous in that it can be performed at the bedside of patients in outpatient clinics etc. (McLoughlin *et al*., 1978). Commonly, a fine 23-gauge needle attached to a syringe is used to remove cells from the tissue, referred to as a 'pass' (Figure 1.1). This is achieved by moving the needle back and forth through the tumour while pulling back on the syringe plunger to maintain suction, creating a pressure difference to draw cells into the needle (Li *et al.*, 1998). FNA has become an important tool in clinical cytology and is frequently found to complement and supplement histology practices, being used extensively as a tool for obtaining samples from superficial organs of the body such as lymph nodes, breast tissue, thyroid and salivary glands for diagnostic purposes, in developed as well as developing countries (Das, 2003).

The use of frozen sections of cancerous lesions has largely been replaced by FNA which has proven advantageous in providing assessment of hormonal status in patients, as well as many other significant biomarkers (Schmitt (a), Bento and Amendoeira, 1995; Schmitt

(b), Figueiredo and Lacerda, 1995). Two important factors which determine the cellular yield of the FNA are reported to be a) the skill of the individual performing the techniques, and more importantly, b) the cellularity of the malignant lesion. Furthermore, the number of actual viable proliferation competent cells present in the FNA sample, will determine whether propagation in culture will occur (Li *et al.*, 1998). In addition to the detection of neoplasia through morphological analysis, cytological smears of breast cancer FNA samples have been used in independent research, through indirect immuno-localisation, towards outcomes such as the determination of estrogen and progesterone receptors (Skoog *et al.*, 1990), the expression of oncogene p53 (Lavarino *et al.*, 1998) and the expression of the protein, erb-B2, also referred to as HER2 (Troncone *et al.*, 1996).

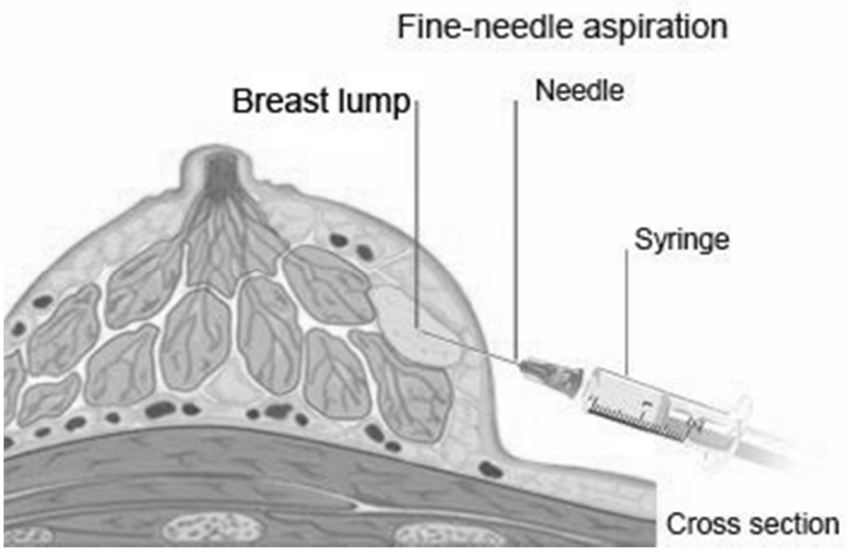


Figure 1.1 Cross section of a fine needle aspiration of a breast lump (Cancer Helpline, 2017).

Since the FNA technique was implemented in the 1930's (Martin and Ellis, 1930), it has been used predominantly as a diagnostics related technique. Aside from the advantages of patient diagnosis and prognosis, it has been proposed that FNA can be used to obtain samples of malignant cells from cancer patients for the purpose of research (Araujo *et al.*, 1999). It was suggested by Hammond *et al.,* that it is possible to harvest tumour cells from small quantities of tumour material present in primary breast cancer FNA samples (Hammond *et al*., 1984). However, there exists in literature, a vast gap pertaining to this research idea, although two particular studies from the 90's allude to the notion that cells obtained through FNA can be cultured and propagated *in vitro* for further research (Li *et al.*,

1998; Araujo *et al.*, 1999). Breast cancer cell lines have in the past been useful in the research of tumorigenicity, hormone responsiveness and drug-resistance as well as other aspects (Möbus *et al.*, 1998). However, the majority of *in vitro* breast cancer research investigations have been carried out on well characterised cell lines, whose phenotype as well as biological behaviour could be different to that of the primary cancer, as the cells are often derived from secondary metastases rather than the primary tumour itself (Amadori *et al.*, 1993; Siwek *et al.*, 1998). Thus, there remains a need for the establishment of a cell model that retains the original tumour characteristics and can offer a better representative model for the study of hormone expression and neoplastic behaviours such as the progression and mechanisms involved in breast cancer (Araujo *et al.*, 1999).

It has been demonstrated, that primary breast cultures can be established from culturing material obtained through FNA (Li *et al.*, 1998; Araujo *et al.*, 1999), although there are several obstacles that interfere with and hinder isolation. Commonly, the presence and overgrowth of fibroblasts in the sample is a primary concern for all isolation techniques, as they are generally the first cell type to adhere to culture plates. This is especially true in the case of FNA sample collection (Gartner *et al.*, 1996). Cleaning fibroblasts out of the sample is often tedious and time consuming (Amadori *et al.*, 1993; Gartner *et al.*, 1996), thus plural or visceral effusions, which have been commonly used to establish the majority of available breast cancer cell lines as cultures, prove far easier to establish (Siwek *et al.*, 1998). Aspirates also frequently contain varying amounts of other contaminating cells, such as red blood cells, lymphocytes and mesenchymal cells (Li *et al.*, 1998).

In a study conducted by Li *et al.*, FNA samples were collected from 25 patients. The samples were retrieved from tumours already excised from patients, prior to the specimen being cut open. A small, 23-gauge fine needle and syringe were used to aspirate the sample through continuous moving of the needle back and forth through the lesion while maintaining suction in the syringe by pulling back on the plunger. The aspirate was expelled into suitable transport medium and the process was repeated several times until a suspension containing visible fragments was obtained. The sample suspension was then centrifuged and the cell pellet re-suspended in suitable MCDB170 growth medium (supplemented with calcium, insulin, epidermal growth factor (EGF), hydrocortisone, bovine pituitary extract and fetal calf serum (FCS)), plated into culture dishes and maintained under standard conditions (37°C in

5% CO_2) (Hammond *et al.*, 1984). Differential trypsinisation was implemented to remove any contaminating fibroblasts only after the initial cell attachment and growth of the culture (Wolman *et al.*, 1985). Cultures were passaged 3-7 times at sub-confluence using routine trypsinisation. It was found that cell proliferation was high during the first three sub-culturing cycles, with duplication time between 48-72 hours. In subsequent passaging, cell numbers appeared to increase more slowly, with a doubling rate of between 10 and 12 days. Through immunofluorescence, loss of heterozygosity (LOH) analysis and fluorescent *in situ* hybridisation (FISH) analysis, the researchers found they were able to expand viable cells from the FNA and obtain pure populations of cancerous epithelial cells presenting the same distinctive characteristics as that of the original tumour tissue, and in all cases, the phenotype of the cultures closely resembled the cancerous lesion from which they were derived.

The objective of a similar study conducted by Araujo *et al.* was “to verify the potential role of FNA cytology in obtaining malignant cells from primary breast cancer, for the establishment of a primary breast cancer cell line” (Araujo *et al.*, 1999). The study was conducted on four patients with the outcome of one successfully obtained primary cell line. FNA samples were collected under sterile conditions using a small, 23-gauge needle attached to a syringe. The material was then centrifuged, and the pellet washed and re-suspended in DMEM medium supplemented with HEPES, FCS, EGF and penicillin/streptomycin. The suspension was seeded into a 25 cm^3 culture flask and cultured under standard conditions (37°C in 5% CO_2). It took approximately one week for the cells to attach to the bottom of the cultures plate and to begin to proliferate. Cells were sub-cultured when a stage of confluence was reached and approximately 15 passages of sub-culturing occurred. It was reported that the cells appeared to reach confluence in more or less 2 weeks with a doubling time between 48-72 hours. Growth kinetics, xenografts and immunocytochemistry analysis showed that the cultured cells established from the FNA sample presented with the same morphological characteristics as that of the original tumour. It was also concluded that only a small percentage of fibroblasts were present in the FNA sample and cancer cells appeared to represent the bulk of the tumour. This suggests that the use of the FNA technique to obtain malignant tumour cells for the purpose of laboratory culturing might be advantageous due to reduced risk for contamination of fibroblasts in culture. However, it is thought that the number of tumour cells present in the original FNA sample could play an important role in the success rate of establishing the primary culture (Araujo *et al.*, 1999).

The use of FNA could prove to be a possible alternative in establishing a viable cell culture from a primary tumour, with no need for enzymes such as trypsin or collagenase for cell detachment from the surrounding stroma, as predominantly free, isolated cells can be obtained (Araujo *et al.*, 1999). When performing an FNA, the bevelled tip of the needle easily shears through the tumour tissue, dislodging the less cohesive tumour cells, which are easily aspirated from the tissue and stromal elements. In this way, a more enriched sample is obtained that contains predominantly malignant cancer epithelium (Li *et al.*, 1998). The afore mentioned studies show that relatively small numbers of cells within the FNA sample can be successfully propagated in culture, and could prove useful in research studies (Li *et al.*, 1998; Araujo *et al.*, 1999). Regarding direct patient care, the existence of a strategy that can lead to an in-depth analysis of an FNA sample culture of a cancerous tumour, could be beneficial in the establishment of more effective patient specific therapeutic interventions that could potentially replace surgery or in the least, function as adjuvant therapy to surgical intervention (Li *et al.*, 1998). However, minimal research has been conducted since these principle studies in the 1990's possibly suggesting that the techniques and culture of primary FNA samples might not be as successful as hoped.

1.3 The Effect of Hypoxia in Cancer

A sufficient oxygen supply to the tissues is essential for the maintenance and proper functioning of cells, and to ensure appropriate energy reserves in the form of ATP (Badowska-Kozakiewicz *et al*., 2015). Hypoxia can be described as the reduction or deficiency of oxygen in tissues, with molecular oxygen levels reaching less than 2%. Moderate hypoxia is defined as tissue oxygen levels of 1.2%, while deep hypoxia or anoxia is defined as below 0.2%. Cells that are unable to adapt to the hypoxic environment and nutrient deprivation that often accompanies hypoxia, will likely undergo apoptosis and/or necrosis (O'Reilly, 1996). For cell survival to continue, cells are required to adapt to these new hypoxic conditions by making a switch to anaerobic metabolism, or to restore the appropriate levels of tissue oxygenation (Badowska-Kozakiewicz *et al*., 2015). Cells assume a range of responses in order to maintain a precise balance between the requirement of oxygen as an energy substrate of oxidative phosphorylation and metabolic reactions, and the intrinsic risk of possible oxidative damage to cellular macromolecules (Zhou *et al.*, 2006; Ruan *et al*., 2009). Aside from the restoration of appropriate tissue oxygenation levels through increased angiogenesis (Strese *et al.*, 2013), other mechanisms of adaptation and compensation include increased levels of lung ventilation, reduced blood viscosity,

decreases in the resistance of peripheral vessel, the redistribution of blood flow, and a switch to anaerobic metabolism, favouring glycolysis, facilitated through an increase in expression of glucose transporters (Semenza, 2003; Badowska-Kozakiewicz *et al*., 2015). With the adaptive shift from respiration to glucose metabolism, a decrease in intracellular pH follows due to the increase in production of lactic acid, leading to a state of acidosis (Zheng *et al.*, 2005; Badowska-Kozakiewicz *et al*., 2015). Acidosis during hypoxia is often thought to contribute to the susceptibility of cells to undergo apoptosis, with metabolically active cells found to be more prone to this demise than their silent counterparts (Zheng *et al.*, 2005). Glycolysis and inhibition of apoptosis are therefore key regulators of cell survival, specifically in cancer cells (Park *et al.*, 2015). In fact, the onset of hypoxia in the context of cancer, can also promote and facilitate cell proliferation through the provocation of adaptive responses, inciting a more aggressive tumour phenotype with often accompanying chemoresistance (Brown and Giaccia, 1998; Semenza, 2003; Zhou *et al.*, 2006). These alterations may in turn contribute to an anti-apoptotic phenotype and tumour progression in the cancer environment, with an increase in invasion and metastasis contributing to an increase in cell survival (Semenza, 2003; Zhou *et al.*, 2006; Strese *et al*., 2013).

Cancerous tumours developing from epithelial tissue are characterised by growth and unrestricted replication with the potential to metastasise and infiltrate surrounding tissue (Vaapil *et al.*, 2012). Regions of hypoxia are characteristic of the cancer microenvironment due to insufficient angiogenesis leading to a decrease in oxygen and nutrient supply, and ultimately to the subsequent stimulation of migration of endothelial cells to the region of tumour growth. This subsequently initiates disorderly angiogenesis which further impedes oxygen supply and contribute to persistently increasing hypoxia (Sorrentino and Carlo, 2009; Filho *et al*., 2010; Heddleston *et al.*, 2010). Bousquet *et al.* proposed that a lack of oxygen to tissues can be seen as a hallmark of cancer, as it appears to be the driving force for malignant tumour progression with proteomic changes favouring cell survival under hypoxic conditions, leading to a more aggressive phenotype (Bousquet *et al.*, 2015). Cells are able to adapt to the limited oxygen by slowing down cellular processes to preserve energy and by altering their metabolism to maximise energy gain (Carrer and Wellen, 2015). Sclerotic lesions developing in breast tissue are frequently poorly oxygenated and it has been hypothesised that hypoxia may play an important role in the cancerous transformation of cells in the hypoxic microenvironment (Vaapil *et al.*, 2012). Growth arrest and/or necrosis occurs in the unvascularised regions when there is an insufficient supply of oxygen

(Tannock, 1968; Evans *et al*., 2001). In a review written by Badoswska-Kozakiewicz *et al.*, it was discussed that due to factors such as poor vascularisation, limited oxygen supply with increased ischaemia, and large distances between cells, cancerous cells appear to be largely susceptible to increasing hypoxia and disease progression (Badowska-Kozakiewicz *et al.*, 2015). Genetic defects in cell cycle checkpoints, as well as in DNA repair mechanisms, have been found to lead to genetic instability in cells, resulting in the progressive acquisition of several genetic, somatic and even epigenetic alterations promoting tumorigenesis (Hanahan and Weinberg, 2000). Hypoxia as a stress on the tumour microenvironment can provoke genetic instability through an increase in gene amplification, chromosomal rearrangement and the induction of intra-chromosomal fragile sites within cells (Coquelle *et al.*, 1998; Bristow and Hill, 2008). It has also been reported that conditions of hypoxia can lead to the suppression of the expression of DNA mismatch repair genes, leading to an increase in the occurrence of mutagenesis (Bindra *et al*., 2007; Huang *et al.*, 2007). These alterations cause cancer cells to adopt functional characteristics that oppose normal homeostasis such as facilitating a switch to a glycolytic metabolism, promoting the ability for unlimited replication potential, inducing irregular angiogenesis, resisting growth inhibitory factors, evading apoptosis, evading attacks from the immune system, promoting proliferation without exogenous growth factors, as well as invasion and metastasis (Hanahan and Weinberg, 2000; Bao *et al.*, 2004) (Figure 1).

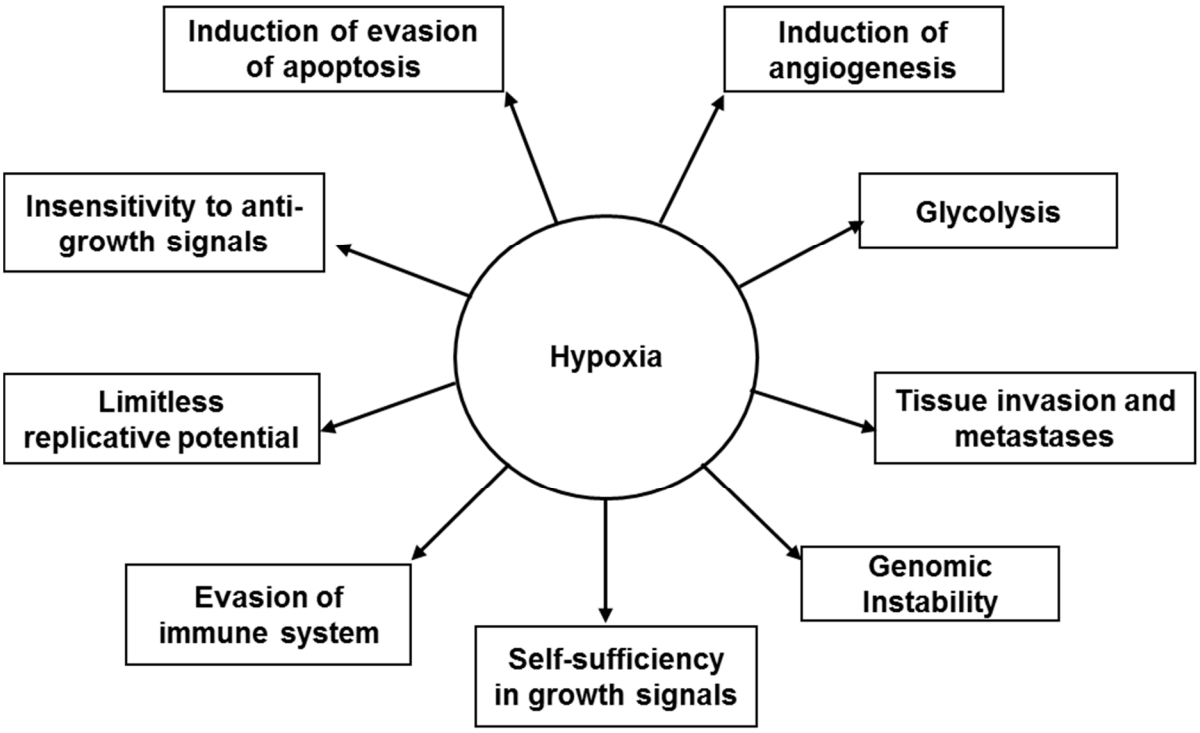


Figure 1.2 ***Adapted from Ruan et al, 2009.*** **Hypoxia is associated with several aspects of tumorigenesis, including promoting a switch to glycolysis, the induction of angiogenesis, evasion of apoptosis, genomic instability, resistance to growth inhibitory factors, evasion of the immune system, unlimited replication potential, invasion and metastasis (Ruan *et al*., 2009).**

1.3.1 Hypoxia and HIF-1α

Conditions of hypoxia have been found to lead to changes in gene expression, induction and suppression of specific genes, with hypoxia-inducible factor 1 (HIF-1) identified as the predominant transcription factor involved in the adaptation of cells to the hypoxic environment and the switch to anaerobic metabolism facilitating cell survival (Brahimi-Horn *et al*., 2007; Badowska-Kozakiewicz *et al*., 2015). This heterodimer is composed of one of three α-subunit isoforms, together with a single HIF-1β subunit, and is activated when the α-subunit is expressed or becomes stabilised under conditions of hypoxia (Kaelin, 2002; Pugh and Ratcliffe, 2003; Semenza, 2004). Kaelin *et al.* have reported that a decrease in the hydroxylation of Pro564/402 and Asn803 occurs with hypoxia, resulting in the stabilisation of HIF-1α and subsequent recruitment of transcriptional cofactors leading to the activation of HIF-1. Association of the β subunit with HIF-1α thus activates HIF-1 and results in the expression of target genes (Kaelin, 2002). The discovery of HIF-1 has led to the identification of the molecular mechanisms involved in the adaptive responses of cells to the

changes in oxygenation of body tissues (Zhou *et al.*, 2006). HIF-1 target genes include erythropoietin (EPO), VEGF, insulin-like growth factor 2 (IGF2) and transforming growth factor α (TGFα), all associated with pro-survival/proliferation, as well as cell death pathways (Semenza, 2003; Greijer and Wall, 2004; Brahimi-Horn *et al*., 2007). It has been reported that the hypoxia induced activation of HIF-1 plays a critical role in responses such as proliferation, angiogenesis, immunosurveilance, tumour invasion and survival (Ruan *et al*., 2009). However, it plays an important further role in the maintenance of oxygen homeostasis (Kaelin and Ratcliffe, 2008), and as such is essential in systemic homeostasis (Badowska-Kozakiewicz *et al*., 2015). The exact role of HIF-1 in cell adaptation and/or death is uncertain, since its activation has been shown to favour both pro-survival as well as pro-death pathways. Thus, there exists a paradox between the induction of both pro-survival and pro-apoptotic genes as a consequence of HIF-1 activation under hypoxic conditions (Zhou *et al.*, 2006). A balance between these activators and inhibitors is necessary for the regulation of either adaptation or apoptosis of cells in the growing tumour (Strese *et al.*, 2013).

It has also been suggested that secondary stressors, such as the progressively more acidic environment alongside genetic alterations, may play a role in the mechanism of action of HIF-1, mediating the susceptibility of cells to either adaptation and malignant progression, or alternatively to progress through hypoxia-induced cell death (Zhou *et al.*, 2006). Only phosphorylated HIF-1α is able to bind to HIF-1β under hypoxic conditions, while dephosphorylated HIF-1α preferentially binds to p53 to bring about pro-apoptotic responses (Suzuki *et al*., 2001). Thus the phosphorylation status of HIF-1α during hypoxia is key in determining whether apoptosis or proliferation is promoted (Semenza, 2003).

In a normoxic environment, HIF-1α is typically unstable, difficult to detect, and earmarked for degradation by hydroxylation catalysed by prolyl hydroxylase (PHD) proteins, or, ‘oxygen sensors’ (Kaluz *et al*., 2008). Changes in the oxygen supply to the cells affects the enzyme’s activity, thus functioning as an oxygen sensor system (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). Under conditions of hypoxia, PHD’s and factors-inhibiting-HIF-1 (FIH) are inactivated due to the absence of oxygen. HIF-1α is stabilised, and translocates to the cell nucleus where it forms a heterodimer with the HIF-1β subunit, and interacts with cofactors for binding to hypoxia-response elements (HRE) of promoter genes, fundamental in mediating systemic responses to hypoxia (Bertout *et al*., 2008; Chandel and Simon, 2008; Simon and Keith,

2008). Initial increases in levels of HIF-1α might be responsible for cell adaptation to hypoxia, with cancer cells favouring angiogenesis stimulation by vascular endothelial growth factor (VEGF) (Hicklin and Ellis, 2004), the inhibition of apoptosis (Zhou *et al.*, 2006), adaptation to the increasing extracellular acidic pH (Cardone and Casavola, 2005), and the upregulation of proliferative proteins (Bogenrieder and Herlyn, 2003). An over expression of HIF-1 has commonly been associated with cancerous lesions with mean oxygen levels in tumour tissue presenting at approximately 10 mmHg while healthy, oxygenated tissue levels are around 63 mmHg (Cancer Genome Atlas, 2012). This reduced partial pressure of oxygen has been linked to the increase in expression of HIF-1 particularly, for example, in the pre-invasive high microvascular density stages of breast cancer, and may suggest a possible role of HIF-1 in cancer progression (Edge and Compton, 2010; Cancer Genome Atlas, 2012). It has been postulated that the overexpression of HIF-1 is linked to an increased risk of tumour invasion and metastasis with poor patient prognosis (Ruan *et al*., 2009). HIF-1 induced angiogenesis has been implicated in tumour growth and metastases due to the associated increases in lactate production (from increased glycolysis) and overexpression of VEGF, leading to poor patient prognosis (Bos *et al.*, 2003; Edge and Compton, 2010). A number of reports have thus supported the idea that hypoxia plays a predominantly pro-survival role in tumour tissue, with HIF-1α particularly exerting an anti-apoptotic response (Zhang *et al.*, 2004). Resistance to apoptosis induced by hypoxia has been linked to the development of chemo- and radiation resistance with an increase in invasive and metastasis potential (Hockel and Vaupel, 2001). Mechanistically, several factors have been reported to promote tumour metastases, induced by hypoxia, all of which are either directly or indirectly influenced by HIF-1 transcription regulators (Le *et al*., 2004; Chan and Giaccia, 2007; Vooijs *et al.*, 2008; Lunt *et al*., 2009). Taking all the effects of HIF-1 activation into account, it is perhaps not surprising that an increase in HIF-1α expression has been associated with an increase in patient mortality (Zhou *et al.*, 2006).

1.3.2 Metabolic Adaptations

It is well known that normal, non-cancerous cells rely predominantly on mitochondrial oxidative phosphorylation as an efficient method of ATP production for metabolic processes, rather than glycolysis (Zheng, 2012). In contrast to this, under hypoxic conditions, cancer cells have been reported to preferentially switch their glucose metabolism from oxidative phosphorylation to oxygen independent glycolysis as a primary mechanism of ATP production, whereby components of the glycolytic pathway become up-regulated (Harris,

2002; Semenza, 2003; Brahimi-Horn *et al*., 2007; Denko, 2008). This adaptive shift to anaerobic metabolism is often regarded as an important hallmark of cancer cells under hypoxic conditions (Gatenby and Gillies, 2008). In 1926 Otto Warburg first described this inefficient pathway for the production of energy in cancer cells, which has been termed, the 'Warburg effect'. In his research, Warburg hypothesised that the cancer cell's altered metabolism could be due to defective mitochondria, in turn affecting the cell's ability to oxidize glucose to CO_2 efficiently (Warburg *et al*., 1927). However, it has since been reported that the ability of cancer cell mitochondria to carry out oxidative phosphorylation is not defective. Instead, mitochondrial metabolism undergoes a reprogramming in order to better cope with the synthesis of macromolecules (Ward and Thompson, 2012). Recently, Lee *et al.* suggested that the cancer cells might have the inclination to preferentially produce energy through oxidative phosphorylation rather than increased glycolysis, but this is largely dependent on the surrounding conditions of the microenvironment (Lee and Yoon, 2015). This theory has been termed the 'reverse Warburg effect', and proposes that the oxygen deprived cancer cells stimulate an increase in aerobic glycolysis, with a subsequent increase in lactate production, in surrounding stromal cells which is then converted to pyruvate and utilized by the oxidative phosphorylation pathway in cancer cells. Thus, the stromal cells seem to 'feed' the cancer cells with pyruvate, thereby preventing the development of an acidic tumour microenvironment (Lee and Yoon, 2015). This concept advocates that both the surrounding stromal cells, as well as the cancer cells might have an influential role on the energy metabolism of each other, thus influencing cancer growth and progression. The understanding of the proposed mechanisms still remains unclear, however, Fiaschi *et al.* have reported that an increased production of ROS within the cancer cell environment in addition to the direct increase in aerobic glycolysis, can lead to the activation of HIF-1α and NFKβ, another important transcription factor (Fiaschi and Chiarugi, 2012), along with subsequent induction of oxidative stress and autophagy in the surrounding stromal cells and microenvironment (Pavlides *et al.*, 2012). Taken together, these alterations contribute and ultimately lead to an increase in the production of metabolic substrates, such as lactate and ketones, which are shifted into cancer cells and utilized for their progression and survival (Anastasiou *et al.*, 2011; Ros *et al.*, 2012).

1.3.3 Cellular Signalling and Inflammation

Cancer cells have also been found to be able to modify growth-inhibitory signalling events. Phosphatase and tensin homolog (PTEN), a tumour suppressor known to inhibit the phospho-inositol 3 kinase (PI3K) – protein kinase B (PKB) pathway which regulates cell survival and growth, is reported to be mutated, or even deleted in some forms of cancer, leading to the promotion of tumour growth and proliferation through HIF-mediated responses (Zundel *et al.*, 2000; Harris, 2002). Related to this, several researchers have reported that the PI3K/PKB survival pathway is activated under conditions of hypoxia, thus promoting cell survival and proliferation in various cancer types (Song *et al.*, 2005; Furuta *et al.*, 2008; Wai and Kuo, 2008; Zeng *et al.*, 2008; Walsh *et al.*, 2009).

Another potential role player in this setting is interleukin-32β (IL-32β), a pro-inflammatory cytokine, which has been associated with hypoxia. It has been shown that conditions of hypoxia play a role in its activation and over-expression in cancer. In this regard, hypoxia induced ROS has been shown to enhance IL-32β production (Park *et al.*, 2013). In turn, IL-32β enhances the migration and invasive potential of numerous tumour types, including breast cancer (Nishida *et al.*, 2009; Nold-Petry *et al*., 2009; Sorrentino and Carlo, 2009; Tsai *et al.*, 2014). Park *et al.* have reported that hypoxia-induced IL-32β also plays a role in the stimulation of glycolysis in hypoxia, mediated through the activation of lactate dehydrogenase (LDH), as well as the stimulation and activation of Src through the protection of its phosphorylation. This suggests that a hypoxia-ROS-IL-32β – Src-glycolysis interlinking pathway might be associated with the molecular regulation of cancer cell metabolism (Park *et al.*, 2013; Park *et al.*, 2015).

1.3.4 Hypoxia Summary

A review by Zhou *et al.* concluded that conditions of hypoxia appear to influence a wide range of molecular pathways to promote and facilitate adaptation of cells, apoptosis and chemoresistance. The responses of cells to hypoxia are determined through both pro- and anti-apoptotic factors, along with proliferative signals, a shifting in microenvironmental pH, as well as clonal selection. It has further been suggested that apoptosis may be initiated in conditions of severe and prolonged hypoxia/anoxia due to the presence of secondary stressors, such as acidosis, leading to the onset of apoptotic pathways. The development of chemoresistance and possibly clonal selection may occur in response to the opposing

signals delivered by pro-survival and pro-death pathways, leading to the selection of cells displaying a genetic or epigenetic growth advantage (Zhou *et al.*, 2006). Vaapil *et al.* further concluded that conditions of hypoxia lead to a less differentiated phenotype in cells, and impairs cellular differentiation of non-malignant mammary epithelial cells by 'trapping' the cells in an undifferentiated, proliferative state increasing the risk of genetic alterations and mutations (Vaapil *et al.*, 2012).

Although hypoxia may prove toxic to both normal and cancerous cells, cancer cells appear to be able to undergo alterations permitting their adaptation and increasing their ability for survival and proliferation (Harris, 2002; Powis and Kirkpatrick, 2004). They are able to break the balance between pro- and anti-apoptotic factors, and continue to survive under adverse conditions such as hypoxic stress (Semenza, 2002; Bao *et al.*, 2004; Blagosklonny, 2004). For critical understanding of cancer development and progression, it is important to understand the molecular mechanisms involved in the induction of cell death as well as cellular adaptations to such signals.

1.4 Protein Phosphatase 2A (PP2A) in Cancer

The transfer of phosphates between substrates is catalysed by the action of phosphatase and kinase enzymes. While the transfer of phosphate from ATP or GTP to a protein substrate is catalysed by protein kinases, protein phosphatases catalyse the transfer of the phosphate group from a phosphorylated protein to a water molecule (Cheng *et al.*, 2011). Phosphorylation and dephosphorylation of proteins within the cell, are fundamental mechanisms employed for signal transduction and numerous other cellular functions, where the action of protein phosphatases and kinases encompass a broad substrate specificity *in vitro*, while performing specific and defined functions *in vivo* (Sontag, 2001). Disturbances of the action of kinases and phosphatases within the cell due to either genetic and/or chemical factors, can thus yield a range of disease phenotypes, including that of cancer. Dysregulated phosphate-mediated signalling could lead to the deregulation of cell growth and adhesion, as well as gene expression, all of which are hallmarks of a transformed cellular phenotype, and could lead to progressive invasiveness and an increased cell motility in endothelial cells (Gabel *et al.*, 1999; Sontag, 2001; Wang *et al.*, 2004). Ruvolo *et al.* described the presence of pathway dysregulation in the cell as being much like a 'switch', whereby the activation of certain mutations can cause signalling to be stuck in the "On

position" leading to continual stimulation of proliferation and survival pathways, while alternatively the inactivation of certain phosphatases, such as PP2A, could be viewed as a defective "Off switch" again leading to the continued activation of these pro-survival pathways (Ruvolo, 2016). Abnormal activation or regulation of signal transduction pathways can thus play a role in transforming normal cells into malignant cells, promoting cell survival properties and leading to cellular resistance to potential drug therapies (Ruvolo, 2016).

The ubiquitously expressed PP2A is one of the main serine/threonine (Ser/Thr) phosphatases in mammalian cells involved in the regulation of nearly all cellular activities including signal transduction, cell proliferation, apoptosis, cell division, survival, growth and development (Eichhorn *et al*., 2009; Danilo *et al*., 2013; Seshacharyulu *et al.*, 2013). PP2A therefore plays an important role in the maintenance of cellular homeostasis. It has been found to be responsible for over 90% of all Ser/Thr activity within the cell, accounting for up to 1% of all cellular proteins (Eichhorn *et al*., 2009) and responsible for reversing the action of kinases in most major signalling pathways (Schönthal, 1998; Janssens and Goris, 2001; Sontag, 2001).

1.4.1 Structure and Regulation of PP2A

Structurally, PP2A is a multifarious hetero-trimer holoenzyme, composed of an active catalytic core dimer where the catalytic C subunit (PP2A-C) associates with the scaffold A subunit (PP2A-A, also known as PT65), with a regulatory B subunit binding to the AC core. Each subunit exists not as stable monomers, but rather in multiple isoforms, allowing for a structurally diverse enzyme (Mumby, 2007; Seshacharyulu *et al.*, 2013; Kiely and Kiely, 2015). Two isoforms (α and β) of the scaffold and catalytic subunits exist, whereas an ever-growing number of regulatory B subunits are continually being discovered without being functionally redundant (Shu *et al.*, 1997; Zhao *et al.*, 1997; Sontag, 2001).

Each of these subunits is responsible for different roles within the cell. The 36 kDa C subunit is the enzymatically active component and together with the A subunit, is responsible for dephosphorylation events (Eichhorn *et al.*, 2009). The 60 kDa A subunit appears to function predominantly as a scaffold protein, assembling the different subunits into a single holoenzyme (Wong *et al.*, 2010; Ruvolo, 2016). Thirdly, B subunits act as target modules,

providing distinct functional substrate specificity. These subunits control cellular localisation whilst displaying differential affinities for the core enzymes, competing for binding to the core and differentially replacing or exchanging one another in the PP2A complex (Sontag, 2001). The catalytic activity of PP2A is thus modulated through the binding of a regulatory B subunit to the AC core, the extent of which varies according to the type of subunit present, as well as the substrate available (Sontag, 2001). The association of the catalytic core with a wide variety of regulatory B subunits gives rise to the formation of different heterotrimeric PP2A holoenzyme complexes with diverse specificities (Eichhorn *et al*., 2009). It has been found that endogenous PP2A activity is modulated by the expression of regulatory B subunits (Garcia *et al*., 2000), as the B subunits influences the sub-cellular localization, as well as substrate selection of PP2A (Ruvolo, 2016). The over expression of mutant scaffold A subunits binding to catalytic C subunits but not to B, may lead to the dysregulation of intracellular PP2A (Ruediger *et al.*, 1997). Thus, the regulation of PP2A is significantly affected by any changes occurring in the ratio of endogenous core enzymes to holoenzymes (Ruediger *et al.*, 1997; Garcia *et al*., 2000).

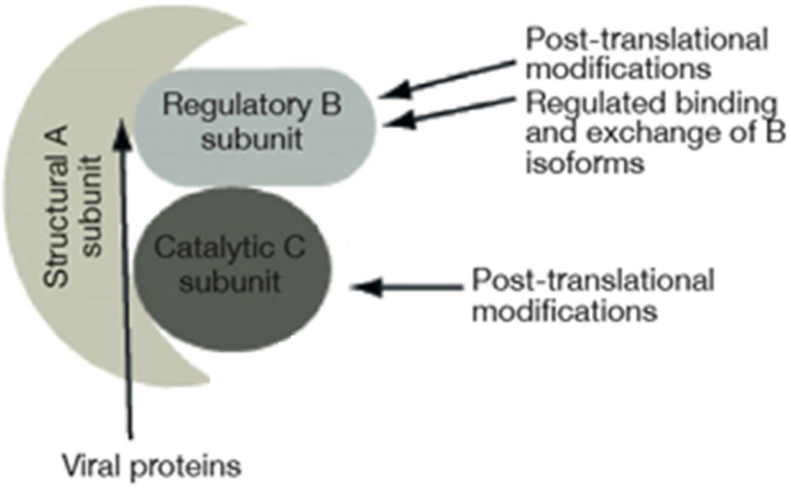


Figure 1.3 ***Adapted from Martin et al, 2010*****. PP2A structure and mechanisms of regulation (Martin, Kettmann and Dequiedt, 2010)**

Post-translational modifications, such as phosphorylation or methylation, of proteins can influence the structure, charge and enzymatic activity of proteins to a great extent, thereby promoting differently modified proteins that display different activities (Nolan *et al.*, 1964). Inappropriate regulation, or dysregulation, of reversible phosphorylation can therefore have a substantial effect on the responses of the cell to its surrounding environment (Eichhorn *et*

al., 2009). Modifications of the catalytic core subunits of the PP2A enzyme complex, influences the binding affinity of regulatory B subunits, as well as the activity of the catalytic subunit itself, thereby modulating enzyme function (Janssens *et al*., 2008; Janssens and Rebollo, 2012).

It has been argued that the phosphorylation of PP2A on both its Thr^{304} and Tyr^{307} residues shows an association with the inactivation of the enzyme, while reactivation occurs through dephosphorylation, including auto-dephosphorylation (Guo and Damuni, 1993; Brautigan, 1995). In response to growth factors, PP2A can become transiently phosphorylated and thus, inactivated (Brautigan, 1995). PP2A actively dephosphorylates many kinases within the cell, and because resting levels of the enzyme's activity is generally relatively high, it can be argued that the inactivation of PP2A might be a requirement for the activation of extra-cellular signal-related cascades (Sontag, 2001). Furthermore, Sontag *et al.* have also suggested that post-translational methylation of the catalytic C subunit greatly influences the ability of the regulatory B subunit to interact with the AC core, thus controlling the specificity of PP2A (Sontag, 2001).

The activity and specificity of this heterotrimeric enzyme are highly regulated, and a growing amount of research points to PP2A as a tumour suppressor (Eichhorn *et al*., 2009). The role of methylation of PP2A remains poorly described in the context of cancer. In a study conducted by Du *et al*. it was reported that reversible methylation of the catalytic subunit of PP2A, regulates the enzymes activity. They further suggested that high glucose levels lead to significant increases in methylation of PP2A and that therapies inhibiting methylation also lead to inhibition of PP2A activity (Du *et al*., 2010). Another report by Israël *et al*. relating to Alzheimers disease, showed that a failure of PP2A methylation leads to the hyperphosphorylation of Tau protein and the formation of tangles in the brain (Israël and Schwartz, 2011). Methylation of PP2A and its regulation have remained elusive in literature and thus we aimed to investigate the effect of this post-translational modification of PP2A.

1.4.2 PP2A in Cancer

The connection between PP2A and tumorigenesis has been strengthened through the discovery of mutations existing in genes encoding the scaffold A subunit of the enzyme in human breast carcinomas, however, neoplastic progression and cell invasiveness may also be promoted through mutations occurring in certain regulatory B subunits (Sontag, 2001). Interestingly, Ratnasinghe e*t al.* found that the expression and activity of PP2A appears to be decreased in breast cancer cells presenting with drug-resistance (Ratnasinghe *et al*., 1998). PP2A was first implicated as a tumour suppressor based on experiments conducted with the selective PP2A inhibitor, okadaic acid (OA), leading to tumour promotion (Bialojan and Takai, 1988). In later research, Schontal and colleagues showed that when mice were treated with OA, tumorous skin lesions developed due to the activation of several cancer promoting pathways (Schönthal, 2001). Dohoney *et al.* and Li and co-workers have also suggested that the direct dephosphorylation of the known tumour suppressor p53 at Ser^{37} and Thr^{55} by PP2A, results in an increased stabilisation of p53 together with a subsequent increase in apoptosis following DNA damage (Dohoney *et al.*, 2004; Li *et al.*, 2007).

1.4.2.1 The Effects of PP2A in Signalling

Further data reviewed by Manning *et al.* have shown, that under normal cellular conditions, PP2A is found to actively inhibit the activity of PKB (also known as Akt), a Ser/Thr kinase, and known oncogene which has been implicated as a principal role player in a number of tumour promoting signalling pathways (Manning and Cantley, 2007). Under normal cellular conditions, PKB is tightly regulated by phosphorylation in order to regulate pro-survival pathways and control growth. However, this regulation has been reported to become de-regulated in human cancers (Bellacosa *et al.*, 1995; Carpten *et al.*, 2007; Kuo *et al.*, 2008).

Abnormal RAS proteins have been found to facilitate uncontrolled cell division leading to the development of cancerous tumours (Mumby, 2007; Sablina *et al.*, 2007). PP2A is one of the intracellular regulators involved in the modulation of the activity of the RAS-RAF1-MAPK pathway (Ory *et al.*, 2003; Kao *et al.*, 2004; Adams *et al.*, 2005). PP2A has been found to selectively target several kinases in this pathway, suggesting that the regulation of RAS-RAF-MEK-ERK signalling by PP2A may be dependent on the context (Eichhorn *et al*., 2009). Downstream activation of the MAPK pathway correlates with dephosphorylation of RAF-1 by PP2A, however, inhibition of B subunits or phosphorylation of PP2A has been found to

also result in the phosphorylation of ERK, downstream from RAF (Sontag *et al.*, 1993; Sonoda *et al.*, 1997). Supporting studies suggest that, while PP2A positively stimulates MAPK signalling at RAF1, there also exists a negative regulatory role for PP2A in this pathway (Wassarman *et al.*, 1996). It has been found to inhibit several proteins in the RAF-MEK-ERK pathway, including RAF, and the direct dephosphorylation of ERK by specific B subunits of PP2A leads to the inhibition of ERK activity (Eichhorn *et al*., 2009). The deregulation of PP2A in either the RAS-RAF-MEK-ERK pathways therefore appears to play a crucial role in the phenotypic cellular transformation to an oncogenic state (Eichhorn *et al*., 2009).

It is therefore clear that the deregulation of PP2A greatly contributes to cancer progression through the stabilisation of proto-oncogenes and destabilisation of tumour suppressors (i.e. p53), through the activation of protein kinases involved in mitogenic signalling (MAPK and PKB), and through the loss of signalling pathways focussing on promoting apoptosis (Eichhorn *et al*., 2009). The deregulation of PP2A in a number of signalling pathways can therefore lead to the promotion of tumorigenesis (Eichhorn *et al*., 2009).

Endogenous tumorigenic inhibitors of PP2A, such as ‘endogenous inhibitor of PP2A (I2PP2A)’ and ‘cancerous inhibitor of PP2A (CIP2A)’ (also known as SET), have been found to play important roles in the survival of cancer cells and the development of drug resistance (Perrotti and Neviani, 2006; Danilo *et al*., 2013; Khanna *et al*., 2013). These inhibitors bind the phosphatase enzyme, diminishing its catalytic activity and leading to the promotion of malignant cell transformation through the suppression of PP2A (Ruvolo, 2016). Khanna *et al.* have reported elevated levels of CIP2A in several cancers (Khanna *et al*., 2013), while others have demonstrated an association of CIP2A with a poor prognosis for solid tumours (Dong *et al.*, 2011; Khanna *et al*., 2013; Barragán *et al.*, 2015). It can be postulated that inhibitors of PP2A play an important role in mediating drug resistance in cancer cells, with CIP2A specifically promoting a resistance to doxorubicin, a known and popular therapeutic drug (Choi *et al.*, 2011). The overexpression of I2PP2A has been found to increase the activation of ERK, thus supporting the notion of the antagonistic role of PP2A in the MAPK pathway (Al-Murrani *et al*., 1999; Härmälä-Braskén *et al.*, 2003). High levels of this inhibitor observed in several human malignancies thus points to a conceivable role for the deregulation of PP2A by I2PP2A in cancer (Fornerod *et al.*, 1995; Carlson *et al.*, 1998). Therapeutic strategies aiming to suppress these inhibitors and re-activate PP2A are

continually being researched and developed to enhance possible future cancer therapies (Ruvolo, 2016).

In this regard, sphingolipid second messenger molecules such as ceramide, have been associated with signalling leading to decreased proliferation and decreased survival, through the inactivation of I2PP2A and CI2PA/SET (Mukhopadhyay *et al.*, 2009). This implies that ceramide-mediated apoptosis could possibly involve the reactivation of PP2A in some cancer types (Ruvolo, 2016). Evidence to support this exists whereby FTY-720, a sphingosine analogue has been shown to suppress CIP2A/SET and induce cell death through the re-activation of PP2A (Perrotti and Neviani, 2013; Cristóbal *et al.*, 2014; Ciccone *et al*., 2015).

Promotion of wide scale activation of cellular kinases through the suppression of PP2A can be achieved through targeting of the catalytic core (Ruvolo, 2016), and the loss or deregulation of specific PP2A holoenzymes can thus be argued to be a critical factor in cell transformation and cancer development (Eichhorn *et al*., 2009). Targeting PP2A through drug interventions can potentially show therapeutic benefits in some malignancies, though depending on the cellular context, the different PP2A isoforms can act as either tumour suppressors or promotors. These reported findings suggest the necessity for the strict regulation of PP2A activity, as dysregulated reduced activity leads to the promotion of cellular transformation, even though a minimal level of PP2A activity is necessary for normal cell survival (Ruvolo, 2016).

1.5 Aims and Hypothesis

The majority of research conducted has been performed on established, homogenous cancerous cell lines which do not provide an accurate representation of the *in vivo* tumour. With ethical clearance, we aimed to set up a cell culture model, for culturing primary breast cancer cells isolated through fine needle aspiration (FNA) from patients presenting with breast cancer at Tygerberg Hospital. Although it is clinically more relevant to use primary cultures of cancerous cells, limitations do exist. Cancerous cells present slow population doubling times with a finite lifespan, along with the possible contamination by fibroblasts and normal epithelial cells due to their heterogenic nature. The loss of cell-cell interactions in

culture may also lead to a change in behaviour of cells when compared to their responses as part of a tissue/organ (Burdall *et al.*, 2003). As drug therapies are predominantly directed against primary tumours, it is still more relevant to establish a primary cell culture representative of the *in vivo* tumour (Burdall *et al.*, 2003). A primary cell culture model will thus provide a useful tool for future research with a more accurate representation of cancer cells within the human body and characteristics comparable with the original tumour. In this study, we intended to build on the limited number of reports concerning primary cell isolation and culture by attempting to replicate these studies in the context of FNA.

In the second half of the study, we aimed to investigate the effect that hypoxia has on PP2A within an immortalised breast cancer cell line, MDA-MB-231. As discussed above, malignant tumours are characterised by having areas of insufficient blood supply leading to hypoxia and nutrient deprivation to which cancer cells have been found to adapt (Vaapil *et al.*, 2012; Carrer and Wellen, 2015). A potential role player in this adaptation is the major Ser/Thr phosphatase, PP2A, involved in the regulation of most cellular activities and therefore playing an important role in the maintenance of homeostasis (Eichhorn, Creyghton and Bernards, 2009; Danilo Perrotti and Neviani, 2013; Seshacharyulu *et al.*, 2013). It has been implicated as a tumour suppressor in several forms of cancer, and its activity status and dysregulation have been linked to tumorigenesis (Eichhorn, Creyghton and Bernards, 2009). This study will therefore focus on determining the effect of chronic hypoxia and serum starvation on PP2A in cancer cells. The results generated will contribute to delineating the role of PP2A in this context, which could be of great interest as PP2A is a potentially drug-able target.

1.5.1 Aims

1. To establish a primary cancer cell culture model using FNA as a method of cell sample collection.

2. Once established, we aimed to investigate the effect of hypoxia on the primary cells.

3. We also aimed to investigate the effect of hypoxia on protein phosphatase 2A (PP2A) expression, post translational modification and activity in a breast cancer cell line.

1.5.2 Objectives

1. To set up a pilot study for the isolation, culture and proliferation of cancer cells from fine needle aspirates obtained from apparent primary breast cancer lesions in patients presenting at the FNA clinic at Tygerberg Hospital.

2. Compare frozen FNA samples with established primary cultures under normoxic conditions in order to determine the effect of *in vitro* culturing on the cancer cells. This will entail the comparison of each primary culture with its original FNA sample.

3. Investigate the effect of hypoxia on the expression, post translational modification and activity of PP2A in an immortalised breast cancer cell line, MDA-MB-231. This will be achieved through Western blotting for the expression, phosphorylation and methylation of the catalytic subunit of PP2A (PP2A-C). PP2A activity will be measured through the incubation of immunoprecipitated PP2A with a fluorescent phosphatase substrate.

4. Determine the sensitivity of both the primary, as well as the commercial breast cancer cell line to hypoxia through the measurement of ATP present in cells following hypoxia. A CellTitre Glo Cell viability assay kit will be used in this determination.

1.5.3 Hypothesis

Research has shown that cancer cells are able to adapt and survive under conditions of hypoxia and several studies have shown that the inhibition of PP2A, has been implicated in carcinogenesis and drug resistance, as well as in hypoxia (Ratnasinghe *et al*., 1998; Eichhorn *et al*., 2009).

We therefore hypothesised that hypoxia would induce a decrease in PP2A activity, favouring the phosphorylation and activation of enzymes associated with survival and proliferation.

Chapter 2: Methods and Techniques

2.1 Isolation and Culture of Primary Breast Cancer Cells

With ethical clearance and approval from the Human Research Ethics Committee of Stellenbosch University, a pilot study with the aim of isolating and culturing cells from breast cancer carcinomas was conducted in collaboration with the Department of Pathology at Tygerberg Hospital. The inclusion criteria for the patients were: female patients below the age of 50 years, presenting with undiagnosed breast carcinomas, and who were radiation and/or chemotherapy naïve. The exclusion criteria included patients receiving and responding to cancer treatment, and patients older than 50. Patients were recruited from the Fine Needle Aspirate (FNA) clinic at Tygerberg Hospital. Samples were collected by trained nurses with the consent of the patient. This collection was the 3rd or 4th pass performed on the patients with initial passes used for hospital processing and pathological diagnosis. The FNA was expelled directly into 3 ml room temperature growth medium, consisting of Dulbeccos Modified Eagle Medium (DMEM) (Lonza, BE12-604F), supplemented with 10% fetal bovine serum (FBS) (Sigma F6178), 2 mM HEPES, 1 µg/ml Epidermal Growth Factor (EGF), and 1% Penicillin/Streptomycin solution (Sigma, P4333). Samples were maintained at room temperature and transported by foot (~15 minutes) back to the Medical Physiology laboratories of Stellenbosch University, adjacent to the hospital, where they were centrifuged at room temperature for 5 minutes at 500xg. The pellet of the first sample received was re-suspended in 5 ml of 'TAC' buffer (DeRose *et al.*, 2013) containing 170 mM Tris (pH 7.4) and 150 mM Ammonium Chloride (pH 7.4), in order to lyse red blood cells present. The sample was incubated for 3 minutes in a water bath at 37°C, followed by centrifugation for 5 minutes at 500xg at room temperature. These steps were repeated, as many red blood cells remained in the pellet after the initial iteration. The pellet obtained was then re-suspended in 1 ml of warmed growth medium and seeded into one well of a 24 well plate, and incubated at 37°C in a humidified atmosphere with 5% CO_2. On all samples received, growth medium was initially refreshed after ~4 days, to allow the cancer cells adequate time to attach to the surface of the culture plate. Thereafter, medium was refreshed every second day, with warmed phosphate buffered saline (PBS) used for washing steps. Sample 1 was left for the initial 4 days to allow for attachment, but unfortunately no cells appeared to attach to the culture plate after the first growth medium change.

Therefore, the second sample received from the FNA clinic was centrifuged as above and re-suspended directly into 1 ml warmed growth medium, seeded onto a 24 well plate and incubated. No attempt was made to lyse the red blood cells present in this sample. Initially, the presence of the red blood cells made visualisation of the cell population impossible, but after 3 days, growth medium was refreshed and from there onwards, cells were more clearly visible. Cells attached well and appeared to grow in number and size but never quite reached confluency over a 6 week period.

The third and fourth samples collected were again centrifuged for 5 minutes at 500xg at room temperature. The pellet was re-suspended in 1 ml warmed growth medium and again seeded into wells on a 24 well plate and incubated. Then, after one hour, medium containing all unattached cells was removed from the wells and transferred to a clean, adjacent well on the plate. Fresh medium was added to the first well. This was repeated on the second well after a further hour. The rationale behind this was that fibroblasts attach fairly quickly to culture plates, and will hopefully remain in the first two wells so that the third well might contain cancer cells only. Gartner *et al*. observed that fibroblasts are generally the first cell type to adhere to culture plates (Gartner *et al.*, 1996). Plates were incubated at 37°C in a humidified atmosphere with 5% O_2 and 5% CO_2. Cells were maintained in culture for four weeks. However, cells did not appear to grow prolifically, and the numbers of cells dwindled away until nothing was left.

For samples five to nine, we reverted back to the protocol used on sample two, whereby samples were centrifuged immediately upon reception in the laboratory, followed by re-suspension in 1 ml warmed growth medium, seeding onto 24 well plates and incubation as before, at 37°C in a humidified atmosphere under 95% O_2 and 5% CO_2. In some instances, some of the samples presented a gelatinous supernatant after centrifugation. This was collected and re-centrifuged, and the pellet generated was re-suspended in 1 ml growth medium and seeded onto the 24 well plate as well. In some cases, a sort of ‘tissue clump’ was visible floating in the supernatant. This was removed and disaggregated in a separate 1 ml of growth medium and seeded separately onto the 24 well plate.

2.2 Culturing Immortalised Cell Lines

A commonly investigated triple negative human breast cancer cells line, MDA-MB-231 (PTEN wild type), was obtained through colleagues in the department of Physiological Sciences at Stellenbosch University. This cell line has been characterised by the lack of expression of estrogen receptors (ER), progesterone receptors (PR), as well as human epidermal growth factor receptor 2 (HER2) receptors, hence the term 'triple negative' (Holliday and Speirs, 2011), and has been shown to be rich in the expression of cell surface markers associated with mammary cancer stem cells such as $CD44^+$ and $CD24^-$ (Prat *et al.*, 2009). These cells were seeded onto 100 mm cell culture plates, and maintained in a proliferative state through culture in high glucose (4.5 g/L) DMEM (Lonza, BE12-604F) supplemented with 10% FBS (Sigma F6178) and 1% penicillin/streptomycin solution (Sigma, P4333), at 37°C in a humidified atmosphere containing 95% O_2 and 5% CO_2. The use of adhesion factor to coat culture plates prior to culturing was not necessary as cells adhered well to the surface of the dishes.

Culture medium was refreshed every 2-3 days and warmed phosphate buffered saline (PBS) used for washing steps. Cells were passaged at 70-80% confluency using a combination of trypsinisation (plates incubated at 37°C for 4 minutes in 0.25% trypsin), and cell scraping to lift cells from the base of the culture plates. Cell-trypsin suspensions were quenched in culture medium, centrifuged at 423xg for 4 minutes at 4°C, and re-seeded onto fresh culture plates. Cultures were maintained in this manner until enough plates were obtained for experimental protocols to proceed.

2.3 Exposure of Cells to Hypoxia

Once a sufficient number of plates of ~80% confluent cells were obtained, plates were washed with warmed PBS and exposed to varying lengths of hypoxia. Control plates received standard growth medium and were kept under standard culturing conditions (described above), while hypoxic experimental plates received growth medium with reduced serum (1% FBS), and were exposed to 4 minutes of hypoxic gas flow (0.5% O_2, 5% CO_2 and 95% N_2) (Afrox, 802732-RC-A) to displace O_2, and sealed within an air-tight hypoxic chamber for the intended duration of hypoxia (Figure 2.1). Cobalt Chloride ($CoCl_2$) (Sigma, C8661), a known chemical inducer of hypoxic-inducible factor (HIF-1), which is associated with hypoxia (Wu and Yotnda, 2011), was used as a positive control for the purposes of

Western Blotting and phosphatase assay experiments. 200 mM $CoCl_2$ was added to positive control plates in standard growth medium, and all plates were then incubated at 37°C in a humidified atmosphere for the desired length of time.

Hypoxia was initially measured over 72 hours, which was found to be too potent. As such, it was decided that varying time points should be investigated, namely 24, 48 and 72 hours. This was further revised to 2 hours, 4 hours, 6 hours and 8 hours.

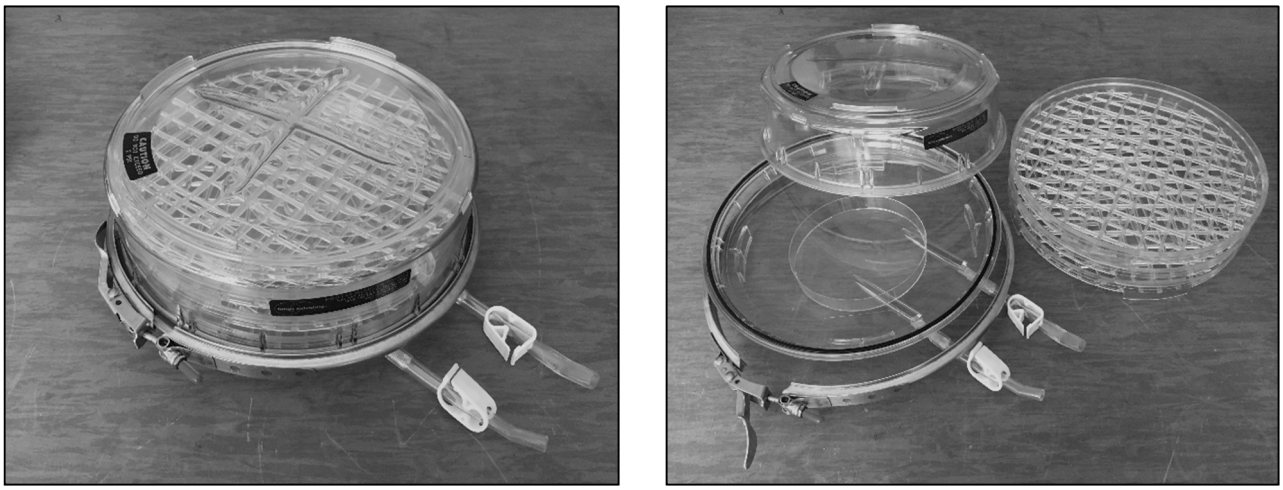


Figure 2.1 Billups-Rothenberg Inc. Modular Incubation Chamber (Hypoxic chamber) with rubber 'O' ring and metal sleeve for an air-tight seal. Culture plates are placed on the grid-trays and sealed inside the chamber, receiving hypoxic gas flow through the inlet valve.

2.4 Western Blotting

2.4.1 Lysate Preparation

Following incubation, culture plates were placed directly on ice and cells were collected from the plates through scraping and centrifugation (423xg for 4 minutes at 4°C) to obtain cell pellets used for lysate preparation (on ice). Cell pellets were re-suspended in 300 µl ice cold lysis buffer (Table 2.1), with 0.5 mm zirconium beads and placed in a bullet blender homogeniser (Next Advance Bullet Blender) at 4°C for three cycles of 1 minute on speed 4, with 5 minutes rest between each cycle, for mechanical disruption. Samples were kept on ice for ~20minutes followed by re-centrifugation at 12074xg for 20 minutes at 4°C. Supernatants were removed, transferred to clean Eppendorf tubes and kept on ice. Protein concentrations were determined using the Bradford assay method (Bradford, 1976), and

sample lysates were prepared accordingly in Laemmli Sample buffer, boiled for 4.5 minutes and stored at -80°C until needed.

Table 2.1: Components of the Lysis buffer used for the preparation of lysate samples for Western blotting. Buffer made up to 10ml with distilled water.

Component	Concentration
Tris + EGTA	20 mM 1 mM
EDTA	10 mM
NaCl	150 mM
β-glycerophosphate	0,93 mM
Tetra sodium pirophosphate	3.76 mM
Na_3VO_4	1 mM
Leupeptin	0,01 µg/ml
Aprotinin	0,01 µg/ml
1%-Triton-X	1%
PMSF	0,3 mM

2.4.2 Protein Separation and Detection

Proteins were separated through SDS-page electrophoresis using BioRad Criterion pre-cast 4-20% gradient gels (Bio-rad, 5678095), and were resolved according to molecular weight. ~30 µg of protein per sample was loaded into the gel along with a pre-stained molecular weight marker. Gels were run for 10 minutes at 100V, 200A, followed by 50 minutes at 200V, 200A. Thereafter, proteins were transferred to an inert polyvinyl fluoride (PVDF) membrane via either a semi-wet TurboBlot system (7 minutes at 2.5A constant; up to 25V), or a wet tank-transfer system (35 minutes at 200V, 200mA). Membranes were briefly placed in methanol to fix proteins, allowed to air dry for 20 minutes, and then placed in 5% fat free milk (made in TBS-Tween) for at least an hour. The fat free milk acts as a blocking agent to minimise non-specific binding of antibodies. Membranes were then washed with Tris-buffered saline (TBS)-0.1% Tween 20 solution with 4 quick hand washes, followed by three cycles of 10 minutes each. Thereafter, they were incubated at 4°C in primary antibody specific for the protein of interest (Table 2.2). Membranes were then re-washed in TBS-Tween as before and further incubated for an hour at room temperature in an appropriate secondary antibody specific for the primary antibody used. A further washing step was performed followed by exposure of the membranes for protein detection using either a

ChemiDoc system in association with stain free technology (Biorad, 2017), or exposed and developed in a traditional dark room. Visualisation of the protein of interest was made possible through the chemiluminescence interaction between the Bio Rad Clarity™ ECL detection reagent (Bio-rad, 170-5061) and the anti-rabbit/mouse horseradish peroxidase conjugated secondary antibodies used.

Bio Rad's stain free imaging technology in association with imagers enabled with stain free detection capabilities, such as the ChemiDoc system, makes use of a trademarked tri-halo compound to enhance protein fluorescence of tryptophan amino acids within proteins, upon exposure to ultra-violet light. The proteins fluoresce directly within the gel upon photoactivation, allowing for immediate visualisation and imaging of proteins present on the gel and membrane at any stage during electrophoresis and blotting. Normalisation of the proteins can therefore be performed by measuring the total protein directly on the membrane, or 'transfer image', to obtain the total density of each lane. Specialised software then interprets the data in three dimensions and the total background is subtracted from the sum of the density of all the bands in each lane. In this way, total protein normalisation eliminates the need for immunodetection of housekeeping proteins for normalisation, along with staining with Ponceau, Coommassie or other dyes. It produces a much stronger signal, improves precision and reliability of Western blotting data, and stripping and re-probing steps can be circumvented, thus saving time (Bio Rad, 2017; Gilda and Gomes, 2013; Gürtler *et al.*, 2013).

Accordingly, bands generated using the ChemiDoc were analysed using Image Lab™ Software (Bio-Rad). Normalisation of 'transfer images' and exposed membranes using stain free technology, was used for the standardisation of protein loading. Normalisation of blots was expressed as pixel values which were then expressed relative to control. The resulting values were expressed as arbitrary units (AU) and used for final statistical analysis. Phospho-to-total and methyl-to-total ratios were calculated from pixel values of phosphorylation and methylation, divided by total pixel values and expressed relative to control, as arbitrary units. GraphPad Prism6 was used to conduct T-tests and 1-way Analysis of Variance (ANOVA) with Dunnett and Bonferroni post-hoc tests. Data was expressed as means ± SEM (standard error of the mean), with $p \leq 0.05$ considered statistically significant.

Table 2.2: Primary and secondary antibodies used for Western blotting

Primary Antibodies Used	Supplier	Code	Secondary Antibody
Total PP2A-C	Cell Signalling	20385	Anti-Rabbit Conjugate
Phosphorylated PP2A-C	R&D Systems	AF3980	Anti-Rabbit Conjugate
Methylated PP2A-C	Santa Cruz	Sc81603	Anti-Mouse Conjugate
Total PKB	Cell Signalling	9272S	Anti-Rabbit Conjugate
Phosphorylated PKB	Cell Signalling	4060S	Anti-Rabbit Conjugate
Total ERK	Cell Signalling	9102S	Anti-Rabbit Conjugate
Phosphorylated ERK	Cell Signalling	9101S	Anti-Rabbit Conjugate
HIF-1α	Cell Signalling	14179S	Anti-Rabbit Conjugate

Secondary Antibodies Used	Supplier	Code
Anti-Rabbit Conjugate	Cell Signalling	7074S
Anti-Mouse Conjugate	Cell Signalling	7076S

2.5 Protein Phosphatase Assay

2.5.1 Lysate Preparation

Following hypoxic incubation as previously described, sample lysates were prepared in the same way as reported for Western blotting above, although the components of the lysis buffer differed (Table 2.3). Protein concentrations were determined using the Bradford assay method (Bradford, 1976), and sample lysates made up to a protein content of 400 µg/µl.

Table 2.3: Components of the Lysis buffer used in the preparation of lysate samples for the protein phosphatase assay.

Component	Concentration
Na_3VO_4	1 mM
NaCl	150 mM
HEPES	50 mM
Glycerol	5 %
Leupeptin	0,01 µg/ml
Aprotinin	0,01 µg/ml
β-mercaptoethanol	0.1 %
Benzamidine	1,3 mM
EDTA	0,01 µM
PMSF	10 mM

2.5.2 Antibody Incubation

10 µl PP2A-C antibody (Santa-Cruz, Sc-6110) was added to each sample. Samples were then incubated at 4°C, overnight in a rotating wheel.

2.5.3 Bead Slurry Preparation

An agarose bead slurry was prepared for a volume of 50 µl slurry needed per sample i.e. 1.2 ml for 24 samples. 400 µl Agarose bead (Sigma, P2545) was added to each of three clean Eppendorf tubes, together with 1 ml lysis buffer (Table 2.3), and centrifuged at 4°C at 12000xg for 45 seconds. The supernatant was then discarded and bead pellet re-suspended in 500 µl lysis buffer. This washing process was repeated three times where after slurries were left overnight at 4°C, ready to be used for immunoprecipitation.

2.5.4 Immunoprecipitation

Prepared bead slurries were gently vortexed to re-suspend all beads. Beads were all transferred to one single Eppendorf tube to ensure a consistent bead suspension, and the total volume of the tube was corrected to 1.2 ml with lysis buffer. 50 µl of well suspended bead slurry was added to each sample, which were then incubated for four hours at 4°C under continual agitation using a rotating wheel. Following incubation, samples were washed as before using prepared assay buffer (Table 2.4). The final bead pellet was re-suspended in a volume of 150 µl assay buffer and assayed for PP2A activity in a 96 well plate using a DiFMUP assay.

Table 2.4: Components of the assay buffer used during activity measurement of PP2A.

Component	Concentration
HEPES + $MgCl_2$	50 mM 5 mM
β-mercaptoethanol	0.02 %
BSA	1,5 µM

2.5.5 PP2A Activity Measurement: the DiFMUP Assay

An EnzCheck® phosphatase assay kit was purchased from Molecular Probes (E12020). This kit is designed for use in a 96-well micro-plate for the continuous fluorescent assay of phosphatases with neutral, alkaline or moderately acidic pH optima. The substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) is a fluorinated relative of 4-methylumbelliferfyl (MUP) and is ideal for the continuous assay of phosphatases. The kit also contains 6,8-difluoror-7-hydroxy-4-methyl-coumarin, a reference standard for the generation of a standard curve. In this assay, DiFMUP is hydrolysed by any and all phosphatases to a product, DiFMU. Therefore, by measuring the increase in DiFMU associated fluorescence, one can estimate the phosphatase activity in the samples. In order to determine the activity of specific phosphatases, one has to either make use of specific phosphatase inhibitors, or enzyme purification (as with immunoprecipitation).

50 µl of each immune precipitate sample was added, in duplicate, to wells of a 96-well micro-plate, along with 50 µl 200 µM DiFMUP working solution. Blanks were set up to contain 50 µl assay buffer (Table 2.4) with 50 µl DiFMUP, and a standard curve was prepared in duplicate through serial dilution of 6, 8-difluoror-7-hydroxy-4-methyl-coumarin (1.562-100µM) (Table 2.5). The micro-pate was prepared under limited lighting to prevent, or at least minimise, degradation of the DiFMUP substrate, and fluorescence of the samples was measured every 5 minutes for a period of approximately two hours at a wavelength of 355/450 nm, using a BMG Labtech FLUOstar Omega Plate reader at 37°C. Data generated was analysed with the aid of the Biostatistics Unit, Centre for Evidence-based Health Care at Stellenbosch University. Repeated measures ANOVA's were performed with post-hoc Mann Whitney tests, and data presented as means ± SEM with $p \leq 0.05$ considered statistically significant.

2.5.6 Confirmation of Immunoprecipitation

To confirm that immunoprecipitation of PP2A had occurred, Western blotting was carried out as described above. 50 µl sample buffer (Laemmli buffer + Lysis buffer) was added to 50 µl of immunoprecipitate sample. These were submitted to Western blotting alongside non-immunoprecipitated samples which were collected from the original lysates prior to immunoprecipitation. This was done to provide a comparison between immunoprecipitated and non-immunoprecipitated samples. Samples were run in 12% gels and transferred for 1

hour as described above. Membranes were probed for PP2A (cell signalling, 20385S) as well as PP1 (cell signalling, 25825), and were visualised using the Bio Rad Clarity™ ECL detection reagent and ChemiDoc System.

2.6 Cell Viability Assay

A CellTitre-Glo® luminescent cell viability assay kit was purchased from Promega (G755A). This kit is designed for use with multi-well plates, involving the addition of the CellTitre-Glo reagent directly to cells in culture. In this ATP activity assay, the generation of a stable "glow-type" luminescent signal is brought about through the properties of a thermos-stable luciferase, with the simultaneous inhibition of the release of endogenous enzymes from cell lysis, such as ATPase's, preventing any interference with accurate ATP measurement. Traditionally, firefly luciferase is used in these assays, however a unique and more stable mutant form of luciferase developed by Promega, selects for favourable characteristics to improve the performance of the ATP assay. The homogenous assay format involves the addition of the CellTiter-Glo® Reagent directly to cell line culture, resulting in cell lysis and the subsequent generation of a luminescent signal that is directly proportional to the amount of ATP present. Measurements of ATP thus provide an indication of viable and metabolically active cells present (Kangas et al., 1984; Gerhardt *et al.*, 1991; Crouch *et al.*, 1993; Promega, 2015)

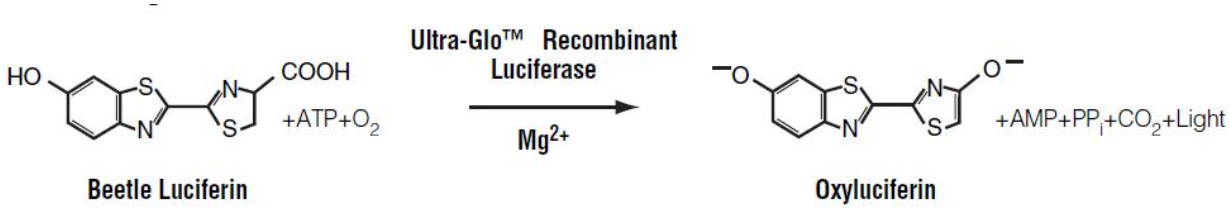


Figure 2.2 The luciferase reaction where luciferase catalyses the mono-oxygenation of luciferin in the presence of Mg^{2+}, ATP and molecular oxygen (Promega, 2015).

MDA-MB-231 cells were grown under standard culturing conditions as previously mentioned. Once 70-80% confluent, cells were divided in a ratio of 1:3 and seeded into four wells of two 96-well plates (control vs hypoxia). The control plate received two extra wells of cells for the purpose of a positive control, which entailed the incubation of the cells with distilled water for 2 hours. This positive control was not added to the hypoxic plate, as the hypoxic chamber could not be opened until the end of the desired incubation period. Cells

on the hypoxic plates received growth medium with reduced serum (1% FBS), and the plates were exposed to 4 minutes of hypoxic gas flow (0.5% O_2, 5% CO_2 and 95% N_2) in a hypoxic chamber where after it was sealed with the hypoxic gas mixture inside (as described previously). Control, positive control and hypoxic (in the hypoxic chamber) 96 well plates were placed in an incubator at 37°C in a humidified atmosphere for the desired length of time. Varying time points were again investigated with hypoxic durations of 2 hours, 4 hours, 6 hours and 8 hours.

Following incubation, an ATP standard curve was prepared in duplicate on the control plate just prior to the addition of the CellTitre-Glo® reagent. A medium (2 µM) and a high (10 µM) concentration of the standard curve was added in duplicate to the hypoxic plate in order to be used as reference samples to facilitate comparison between the two plates. 10 µM ATP was prepared in standard growth medium (described previously), and serial dilutions were prepared on the 96 well plates (1 µM to 10 µM). 100 µl of the CellTitre-Glo® reagent was added to each well, where after the plates were exposed to vigorous shaking for two minutes followed by 10 minutes incubation at room temperature. Following this, a BMG Labtech FLUOstar Omega Plate reader was used determine the luminescence generated from each well. Data generated was analysed using GraphPad Prism6. Student T-tests were conducted and data was presented as means ± SEM with $p \leq 0.05$ considered statistically significant.

Table 2.5: 96 Well micro-titre plate layout used in the phosphatase assays. Rows A and B: Wells 1-7 represent standard curve concentrations (µM) prepared through serial dilutions; wells 9 and 12 represent blanks with assay buffer (AB). Rows D, E, G and H: DiFMUP substrate added to immunoprecipitate samples. C = control, +C = positive control, H = hypoxia.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.562	3.125	6.25	125	25	50	100		50ul AB + DiFMUP			100µl AB
B	1.562	3.125	6.25	125	25	50	100		50ul AB + DiFMUP			100µl AB
C												
D	50ul C1 (2h)	50ul C2 (2h)	50ul +C1 (2h)	50ul +C2 (2h)	50ul H1 (2h)	50ul H2 (2h)	50ul C3 (4h)	50ul C4 (4h)	50ul +C3 (4h)	50ul +C4 (4h)	50ul H3 (4h)	50ul H4 (4h)
E	50ul C1 (2h)	50ul C2 (2h)	50ul +C1 (2h)	50ul +C2 (2h)	50ul H1 (2h)	50ul H2 (2h)	50ul C3 (4h)	50ul C4 (4h)	50ul +C3 (4h)	50ul +C4 (4h)	50ul H3 (4h)	50ul H4 (4h)
F												
G	50ul C5 (4h)	50ul C6 (4h)	50ul +C5 (4h)	50ul +C6 (4h)	50ul H5 (4h)	50ul H6 (4h)	50ul C7 (6h)	50ul C8 (6h)	50ul +C7 (6h)	50ul +C8 (6h)	50ul H7 (6h)	50ul H8 (6h)
H	50ul C5 (4h)	50ul C6 (4h)	50ul +C5 (4h)	50ul +C6 (4h)	50ul H5 (4h)	50ul H6 (4h)	50ul C7 (6h)	50ul C8 (6h)	50ul +C7 (6h)	50ul +C8 (6h)	50ul H7 (6h)	50ul H8 (6h)

Chapter 3: Isolation and Culture of Primary Breast Cancer Cells: A Pilot Study

3.1 Introduction

Immortalised cell lines have been widely used in cancer studies for their ease of culturing self-replicating cells with minimal contamination from other cells and extended culturing capacity (Burdall *et al.*, 2003; Lacroix *et al*., 2004; Holliday and Speirs, 2011). However, whether these cells can provide a true representation of cancer under *in vivo* conditions remains a topic of debate among researchers (Correa, Marshall and Burnier, 2009; Holliday and Speirs, 2011). Thus the establishment and use of successfully isolated and propagated primary cell cultures holds great allure as a research model more able to provide a closer representation of the *in vivo* tumour and microenvironment, as cells would be derived directly from tumour tissue (Speirs *et al.*, 1998; Keller *et al.*, 2010). Primary cell isolation and culture however, is not without its challenges. Studies have found a high prevalence of contamination from other cell types, such as fibroblasts, immune cells and connective tissue, that frequently overgrow cancer cells (Dairkee *et al.*, 1995; Speirs *et al.*, 1998; Sung *et al.*, 2007). Further challenges include a lack of consensus on a successful, reproducible isolation and culture protocol, acquiring sufficient quantities of cancer cells for molecular studies, and a lack of clear identification and morphological distinction between cancerous and non-cancerous cells in culture (Dairkee *et al.*, 1995; Speirs *et al.*, 1998; Weigand *et al.*, 2016). In previous studies, primary breast cancer cells have been isolated from highly metastatic pleural effusions, or from excised tumour tissue requiring mechanical disaggregation or enzymatic digestion for the detachment of cells from surrounding breast tissue. This has been found to encourage and increase the risk of overgrowth of contaminating cells (Siwek *et al.*, 1998; Speirs *et al.*, 1998; Loveday *et al.*, 2002). The use of FNA as a means of primary cell sample collection for the purpose of *in vitro* culturing, has been proposed as an alternative, minimally invasive protocol able to collect tumour samples at an earlier stage, prior to metastasis and can be performed at the bedside of patients with a decreased risk of overgrowth of contaminating cells (McLoughlin *et al*., 1978; Li *et al.*, 1998; Araujo *et al.*, 1999; Rosa, 2008). However, literature does not report on much work conducted on this proposal.

The aim of this pilot study was therefore to establish a successful isolation and primary cell culture model from samples collected by FNA from patients presenting with abnormal breast lumps or lesions. FNA is a well-established technique that is minimally invasive and safe, and is routinely used for the collection of tissue samples for the purposes of diagnosis and characterization of palpable and non-palpable lesions, commonly of the breast, lymph nodes, thyroid and salivary glands (Schmitt *et al*., 1995a; Schmitt *et al*., 1995b; Li *et al.*, 1998; Rosa, 2008). In this technique, a fine, 23-gauge needle attached to a syringe is used to remove cells from the tissue, referred to as a 'pass'. This is achieved by moving the needle back and forth through the tumour while pulling back on the syringe plunger to maintain suction (Li *et al.*, 1998). However, very few cases have been documented where this technique has been used for the collection of samples for the purpose of cellular propagation in culture for research. Two particular studies from the late 90's suggest that cells obtained through FNA can be cultured *in vitro* for further research (Li *et al.*, 1998; Araujo *et al.*, 1999). However, there is no consistent and definitive protocol for this approach and there still remains a need to establish a reliable primary cell model that retains the characteristics of the original *in vivo* tumour, offering a better representation of the progression and mechanisms involved in breast cancer (Araujo *et al.*, 1999).

In 1998, Li and colleagues developed a protocol whereby multiple FNA 'passes' were collected from each of 25 cancerous breast tumours already excised from patients until visible cell pellets were present. The samples then underwent centrifugation and tripsinisation to remove possible contaminating fibroblasts (Li *et al.*, 1998). It was found that cell proliferation was initially rapid but doubling time slowed down after the first three sub-culturing cycles. However, viable cancerous cells were obtained from the FNA samples presenting with characteristics and phenotype closely matching those of the original tumour (Li *et al.*, 1998). A second study conducted the following year by Araujo *et al.* similarly aimed to substantiate the role of FNA sample collection as a means to obtain primary cancer cells with the purpose of establishing a primary breast cancer cell line *in vitro*. This study was conducted on four patients, with only one primary cell line successfully cultured. These FNA samples were collected as described in chapter 1, centrifuged and the pellets re-suspended in suitable medium and seeded directly onto culture plates. It was reported that cells attached to the bottom of the flasks within one week and that approximately 15 cycles of sub-culturing occurred producing cells with similar characteristics and morphology as the

original tumour, and only a small percentage of contaminating fibroblasts was present (Araujo *et al.*, 1999).

These two studies both provided definitive success in their aim of culturing primary breast cancer cells from FNA samples, however, after 1999 no other reports were found of the usage of the FNA technique as a means of sample collection for the propagation and successful culture of primary breast cancer cells for research purposes. This led us to the decision to investigate this technique further and to examine whether in fact this technique does indeed have merit as a sample collection method for successful culture and propagation of primary cells *in vitro,* and if a successful primary cell model could be established.

Unfortunately, due to time restraints and an unanticipated low number of suitable patients, only nine FNA samples were collected and maintained in culture. Of these nine samples, only one sample appeared to actually proliferate and progress through a sub-culture cycle, however, it was uncertain whether the proliferating cells were in fact cancer cells or contaminating fibroblasts as cells did not sufficiently increase in number to allow for characterisation through Flow Cytometry before dying off. We were not able to establish a successful culture from any of the samples collected and no definitive model has yet been established.

3.2 Methods, Materials and Results

Due to the nature of this pilot study, this section will combine the methods and the results. As we were attempting to set up an isolation and culture technique, the protocol was adjusted based on results obtained with each sample, therefore it is in the interest of flow and understanding to merge the information. An overview schematic of the different sample processing is shown in Figure 3.7, however, indicating no obvious emerging patterns with the changes in protocols.

With ethical clearance from the Human Research Ethics Committee (HREC) of Stellenbosch University, the pilot study was intended as a collaboration with the AIDS and Cancer Specimen Resource (ACSR) Biobank in conjunction with the department of Anatomical Pathology at Tygerberg Hospital, Cape Town, South Africa. The ACSR biobank nurses approached patients, visiting the Tygerberg Hospital FNA clinic who met the inclusion criteria, for their consent to donate a single FNA pass for the purpose of this research study. For this purpose the nurses made use of standard informed patient consent forms, approved by HREC (Addendum A). No samples were obtained without prior consent from each individual patient, and patients were only permitted to sign consent once it was determined that they fully understood and accepted the purpose of the study and their anticipated role. The inclusion criteria for suitable patients included females below the age of 50, who were chemo-and radiation therapy naïve. The study set out to obtain 15 FNA samples collected by trained nurses and then transported to the laboratories in the Division of Medical Physiology, Stellenbosch University, to optimise the protocol for successful isolation, propagation and culturing of cancerous cells present in the FNA samples. The protocol decided on was a combination of those described in previous FNA studies (Li *et al.*, 1998; Araujo *et al.*, 1999), as well as from studies on pleural effusions (Siwek *et al.*, 1998) as a starting point. In all cases, the FNA samples were injected into 3ml suitable DMEM growth medium containing FBS, penicillin/streptomycin, HEPES and EGF, and transported to the laboratory for handling.

Table 3.1 shows the age of the patients, the approximate number of days before the first growth medium change to allow cells to attach to the culture plate, the length of time the cells were maintained in culture, if a passage of the cells was possible and the official pathology diagnosis of each sample received.

Table 3.1 Collected FNA Sample Specifics

Patient	Age of Patient	Days before 1st medium change	Length of time maintained in Culture	Passage	Pathology Diagnosis
1	37	~4	NA	NA	Breast Carcinoma: Duct Carcinoma. ER+ PR+ C-erb-
2	37	~3	6 weeks	1	Breast Carcinoma: Duct Carcinoma. ER+ PR- C-erb-
3	42	~3	3 weeks	NA	Breast Carcinoma. ER+ PR+ C-erb-
4	44	~3	2 weeks	NA	Breast Carcinoma: Duct Carcinoma. ER+ PR+ C-erb 2+ FISH requested
5	31	~4	3,5 weeks	NA	Breast Carcinoma: Duct Carcinoma.
6	32	~3	1,5 weeks	NA	Breast Carcinoma ER- PR- C-erb-
7	36	~4	2 weeks	NA	Breast Carcinoma. ER- PR- C-erb+
8	43	~4	2 weeks	NA	Breast Carcinoma: Duct Carcinoma. ER+ PR+ C-erb-
9	48	~3	3 weeks	NA	Breast Carcinoma: Duct Carcinoma.

Upon return to the laboratories in the Division of Medical Physiology, the first sample received from patient 1 was centrifuged for 5 minutes at 500xg at room temperature, where after the pellet was re-suspended in a lysis buffer, containing 170 mM Tris (pH 7.4) and 150 mM ammonium chloride (pH 7.4) (DeRose *et al.*, 2013), intended to lyse red blood cells. The sample was incubated for 3 minutes in a water bath at 37°C followed by re-centrifugation as before. This lysing step was repeated a second time before the cell pellet was re-suspended in 1ml warmed growth medium and seeded into a well on a 24 well plate incubated at 37°C in a humidified atmosphere. Growth medium was refreshed after 3 days to allow time for cancer cells to attach to the culture plate. However, no cells were visible in culture after the medium change and we believe there were no cells left after the isolation procedure, to attach to the culture plate as the lysis buffer appeared to be too potent for the cells. No images were able to be taken as no cells were present in the well.

Based on this result, the second FNA sample received was centrifuged as before, and merely re-suspended into suitable growth medium and allowed to incubate for the cells to attach. What appeared to be a ‘clump of tissue’ was observed to be floating in the supernatant after centrifugation. This was removed, manually dissociated with repeated suction through a pipette tip, re-suspended separately in growth medium and seeded onto

the culture plate. After the first week in culture, cells appeared to have attached in both wells, however cells from the 'tissue clump' appeared more rounded and defined. Cells from the original sample suspension showed many small, round cells forming clusters and only a few larger, more rounded cells (Figure 3.1a). Over the course of approximately six weeks, only cells from the 'tissue clump' suspension appeared to grow in number and size, although the morphology of visible cells seemed to change (Figure 3.1b). After ~4 weeks, these cells were sub-cultured from one well on a 24 well plate, to a single 3.5 mm culture plate. However, the cells appeared branched and dendritic (Figure 3.2) thus leading to a suspicion of fibroblast contamination. Numbers of cells increased extremely slowly and began to dwindle off again before confluence was reached. After approximately 6-7 weeks cells were no longer present in culture.

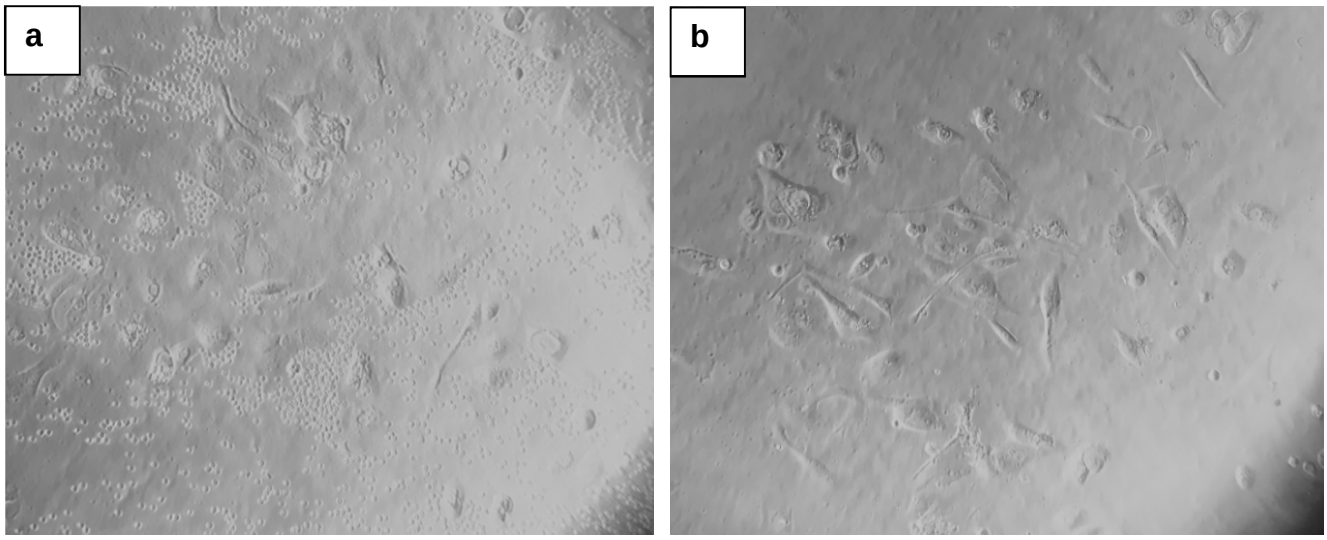


Figure 3.1 FNA sample 2 at ~1 week in culture: a) cells from sample pellet appearing rounded with a few spindle shaped cells and many smaller red blood cells; b) cells from disaggregated and re-suspended 'tissue clump' appearing rounded and clustered together. Images taken with a 12 megapixel iPhone 5S camera through the lens of a microscope (Zeiss, 476100-9901). Magnification = 100x.

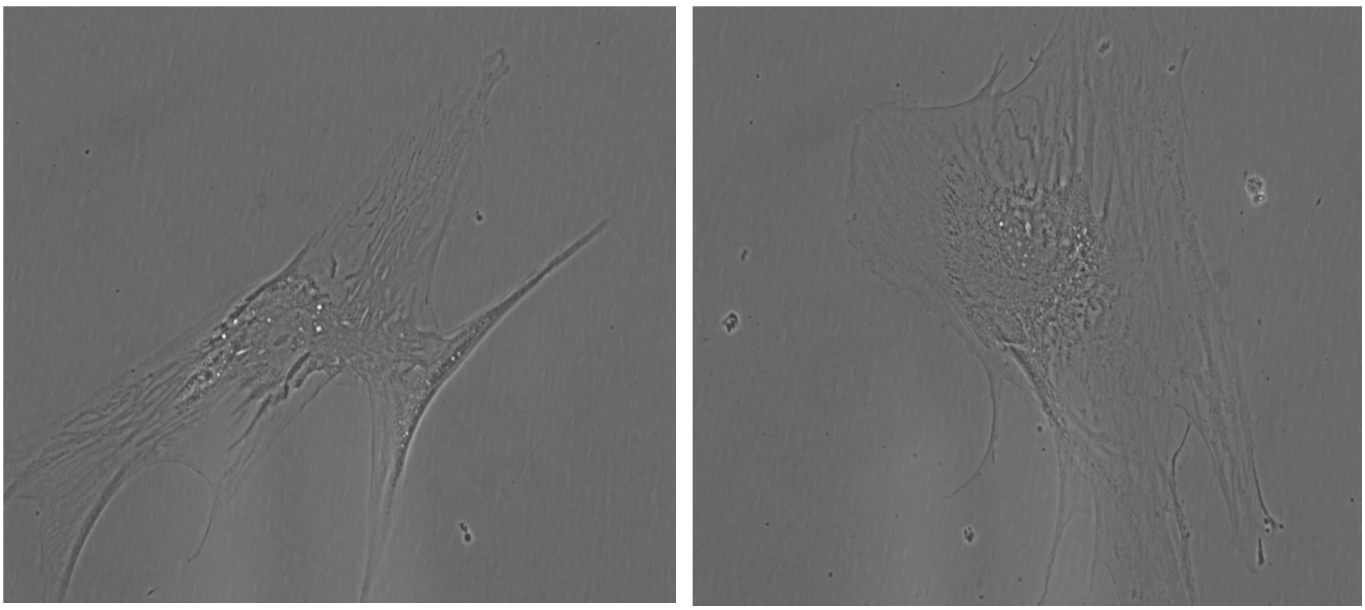


Figure 3.2 FNA sample 2 'tissue clump' grown in culture after ~6 weeks. Long, spindly branches are visible with cells showing a flat and spread morphology. A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

Because cell growth had been exceptionally slow and there was the suspicion of contaminating cells, it was decided to adjust the protocol for samples 3 and 4. These samples too presented with a similar 'clump' of tissue suspended in the supernatant following centrifugation. This was again removed, disaggregated and re-suspended in growth medium, separate to the cell pellet. However, the samples were then subjected to a 'differential' seeding of cells into wells, followed by removal of medium and transferral to a new, clean well after one and two hours respectively, with the hope that fast attaching fibroblasts would attach to the initial wells within the first two hours leaving the third well expectantly free of fibroblast contamination, this based on reports by Gartner *et al.* (Gartner *et al.*, 1996). The cultures were maintained for 2-3 weeks but cells from neither the original pellet resuspension, nor the 'tissue clump' appeared to proliferate or grow in size. Cells in the original pellet re-suspension were more rounded and appeared to clump together forming clusters (Figure 3.3). No 'branched' or 'dendritic' looking cells were observed as with sample 2, however, cells were very sparsely seeded with large distances between clusters, or sections of clusters.

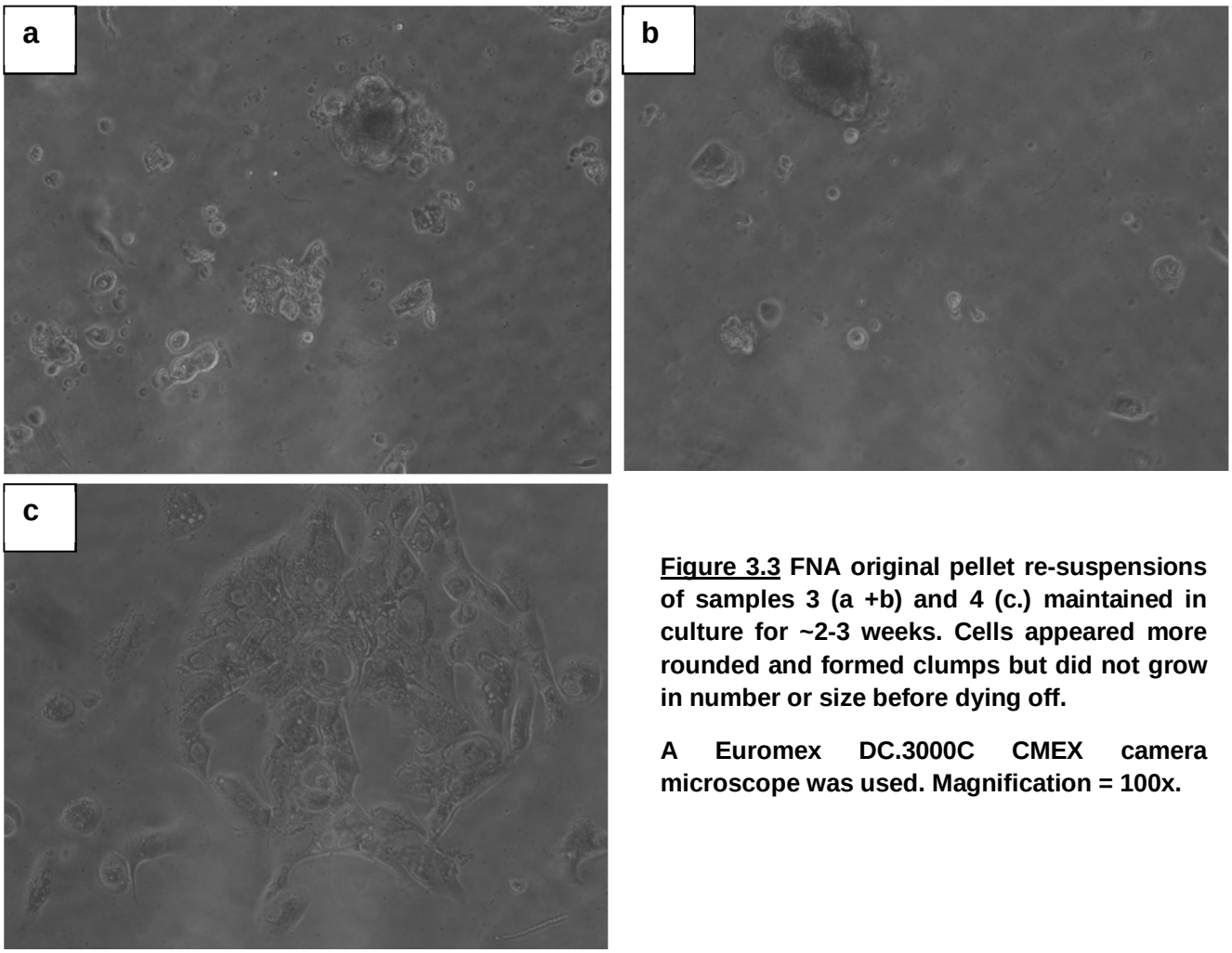


Figure 3.3 FNA original pellet re-suspensions of samples 3 (a +b) and 4 (c.) maintained in culture for ~2-3 weeks. Cells appeared more rounded and formed clumps but did not grow in number or size before dying off.

A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

While samples 3 and 4 remained in culture with no growth, it was decided to revert back to seeding the cells directly into one well for the next samples in the hopes of an increased yield of cells, with possible sub-culture cycles as seen with sample 2. Samples 5 to 9 were treated similarly to sample 2, whereby FNA samples received were centrifuged and the pellet re-suspended in growth medium and immediately seeded into wells of 24 well plates for culture. Following centrifugation, samples 6 and 7 presented a thick, gelatinous supernatant which was removed and re-centrifuged before both pellets from each sample were seeded as before. Cultures were maintained for ~1.5-3 weeks but no growth in number or size was recorded and no sub-culturing was possible.

Sample 5 again presented cells of a more ‘spindly’, flat, ‘dendritic’ appearance (Figure 3.4) as seen in sample 2 (Figure 3.2). Initially, cells appeared to attach well to the culture plate and an increase in cell numbers was observed, albeit slowly and reaching a plateau. However, by 3.5 weeks, cells were no longer visible in culture.

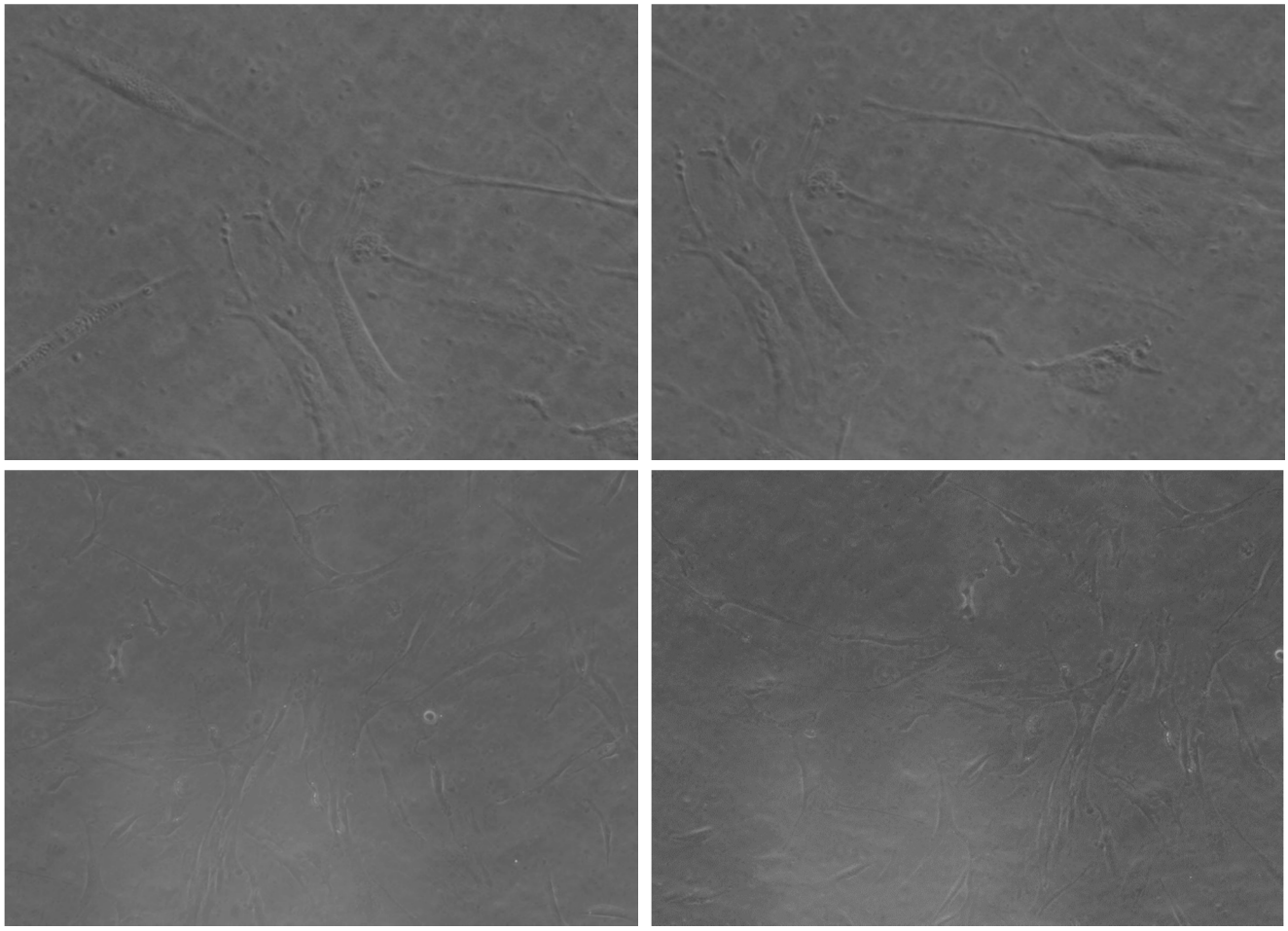


Figure 3.4 Sample 5 maintained in culture for ~3.5 weeks. Cells again appeared branched and dendritic as seen in sample 2 but did not appear to proliferate. A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

Samples 6 and 8 did not fare well at all (Figure 3.5). The protocol did not change, yet following the first growth medium change after 3 days, many red blood cells were still present in the culture wells. With subsequent medium changes, no cells appeared to have actually attached to the culture dish and the samples were discarded after 1.5-2 weeks. We speculate that it is possible we did not obtain enough tumour material in the FNA sample received to allow attachment and propagation of cells.

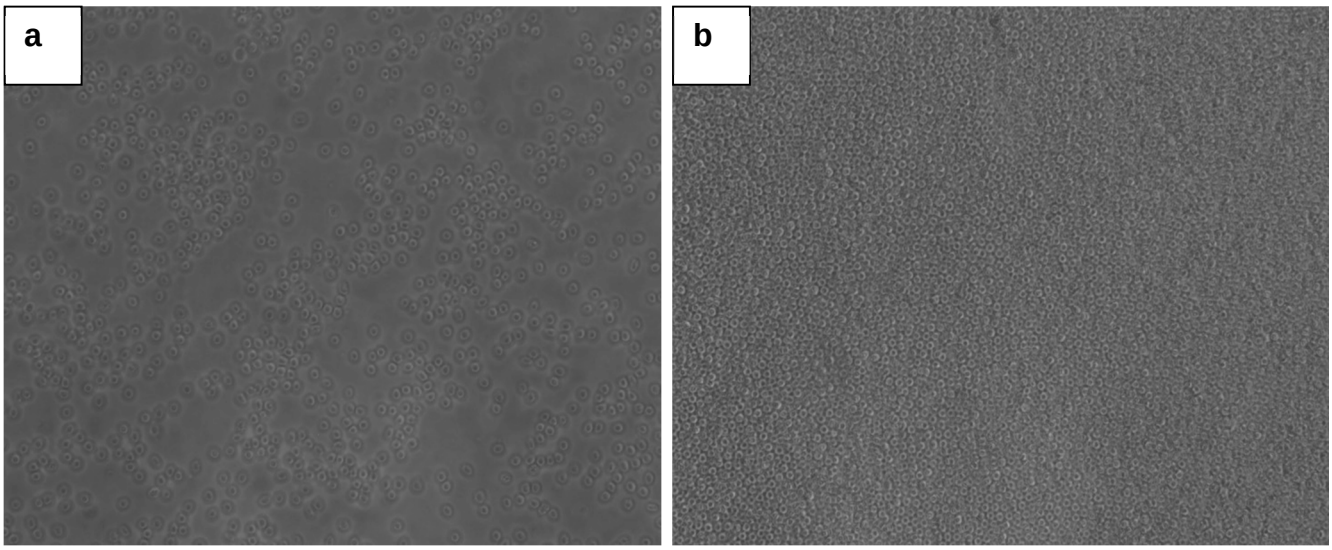


Figure 3.5 Many red blood cells initially visible in sample 6 (a) and 8 (b) following the first growth medium change after the first three days of culture. Thereafter, no cells appeared to have attached. A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

Again, samples 7 and 9 were treated with the same protocol. Following the initial growth medium changes after 3-4 days, a few larger, round cells had appeared to attach to the base of the culture plates (Figure 3.6). Cells were very few in number with large distances between them. The cultures were maintained with regular growth medium changes for 2-3 weeks but the cells did not appear to multiply in number or grow in size before eventually disappearing from culture.

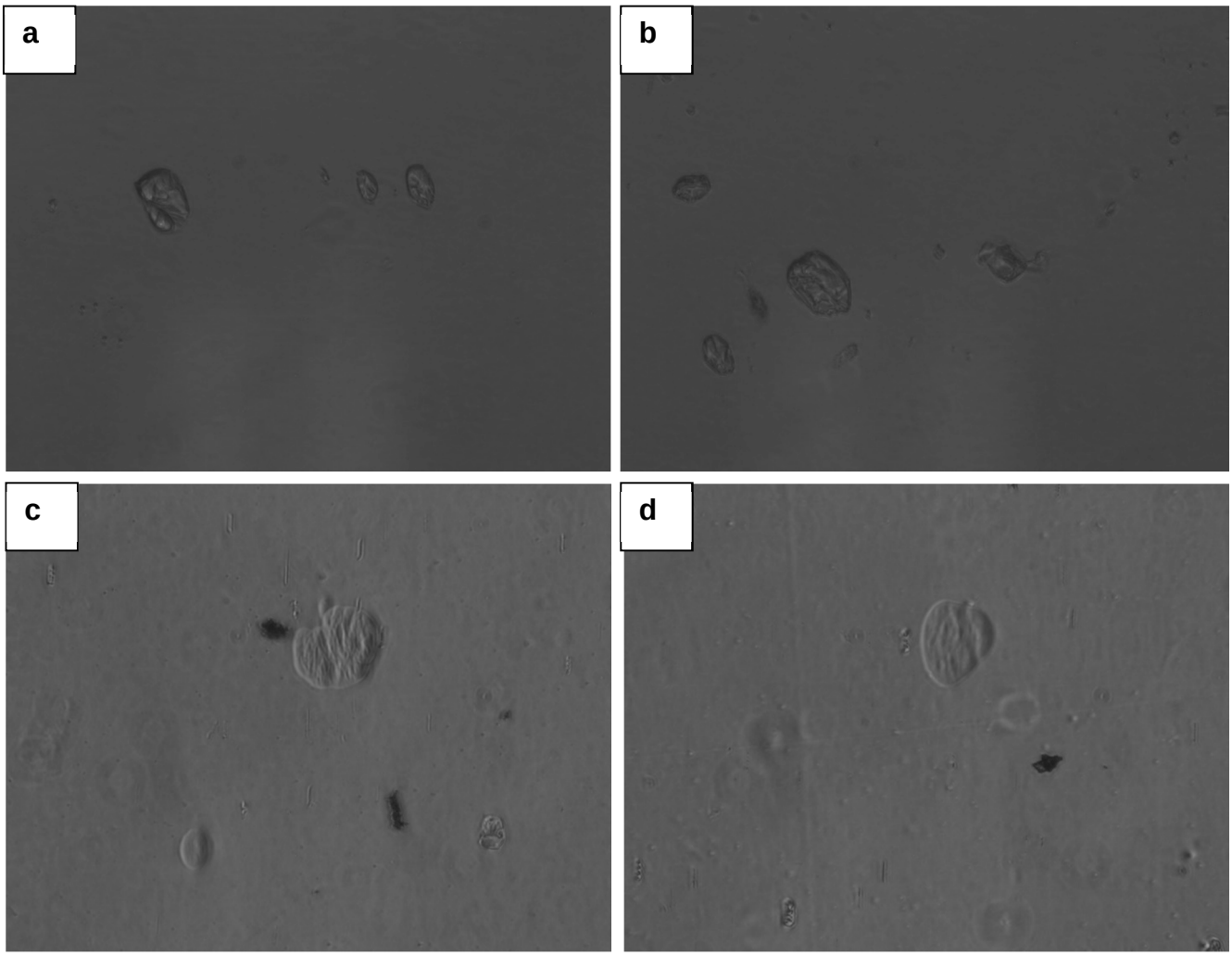


Figure 3.6 A few, rounded cells from sample 7 (a + b) and sample 9 (c + d) appeared in culture for up to 2 weeks but there were no signs of growth in number or size over this duration. Cells eventually disappeared and were no longer visible on the culture plate. A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

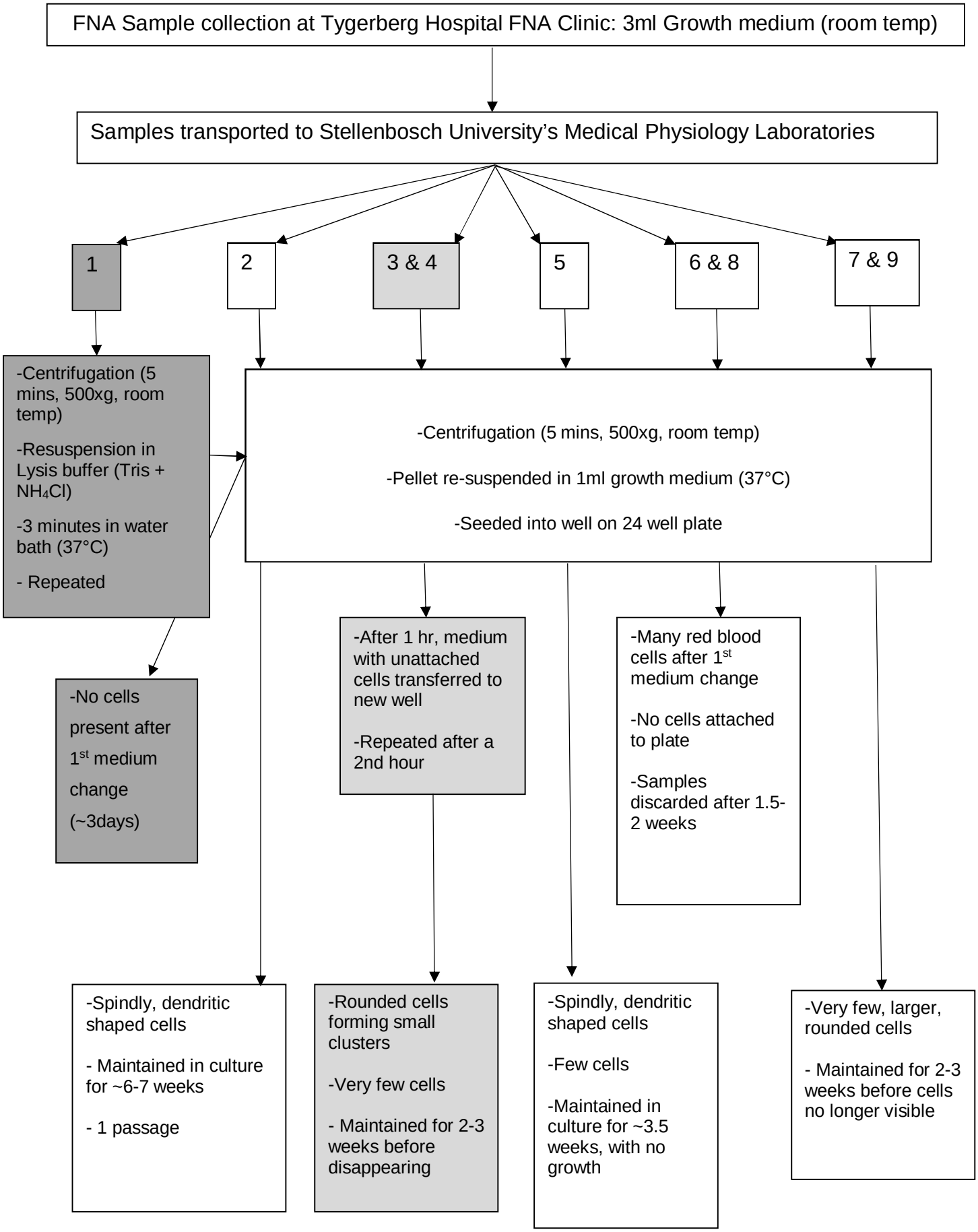


Figure 3.7 Outline schematic of FNA sample processing

3.3 Discussion

Nine women consented to donating a breast lump FNA sample to this research study for the setup of a primary cell culture *in vitro* model. These women ranged in age from 31 years to 48 years old, having had no previous treatment or therapy for the lumps present in their breasts. The samples were collected, transported back to the laboratory at Stellenbosch University and seeded into culture dishes where we left cells on average, 3.6 days to attach to the culture plates. The length of time the cells remained in culture ranged from approximately two to six weeks, until no more cells were visible under a microscope. Of the nine samples collected, Sample 2 appeared to survive the longest and even progressed through a single sub-culture cycle, while in the other samples, cells were visible but no real growth or proliferation was observed contrary to expectations as set out by previous literature.

It is a disappointment that only 9 out of the intended 15 samples were collected for this Pilot study, however a conclusion can be drawn from the culture of the 9 samples that we did in fact receive. As described in literature, the presence of contaminating fibroblasts in any primary sample is frequently a major stumbling block in the culture of these samples (Amadori *et al.*, 1993; Gartner *et al.*, 1996; Z Li *et al.*, 1998). It had been our intention to characterise the cultured cell populations using Flow Cytometry to identify known, distinct breast cancer cell surface markers $CD44^{Hi}$ and $CD24^{Low}$ (Al-Hajj *et al.*, 2003) and confirm whether the cultured populations were indeed cancerous. However, unfortunately due to such low numbers of cells in each culture, we were not able to perform characterisation analyses to confirm the cell types. We were therefore unable to definitively report whether the few cells we had seen in culture, were in fact cancerous breast epithelial cells or rather contaminating cells such as fibroblasts.

Upon initial seeding and imaging of the samples, many presumably red blood cells were present in the culture plate wells, discernible by their distinguishing rounded and concave shape as seen in figure 3.5. These cells were washed away with each growth medium change to leave attached cells in the wells. The cells present in figures 3.2 and 3.4 (samples 2 and 5 respectively) appear to have a similar morphology of spindly, almost dendritic shape, also appearing flatter and more spread out. In comparison, cells pictured in figures 3.3 (samples 3 and 4) and figure 3.6 (samples 7 and 9), appear more rounded and forming

clumps. The differences in morphology of the cells is quite clear and we speculate whether the cells surviving in samples 2 and 5 might well be fibroblasts or pericytes described by Rønnov-Jessen and colleagues (Rønnov-Jessen *et al.*, 1995), as a similar morphology can be observed. Interestingly, smaller, rounded clusters of cells were visible in samples 3 and 4, more in keeping with the description of cancer cells as reported by Li *et al.* and Araujo *et al.* (Li *et al.*, 1998; Araujo *et al.*, 1999). These samples were subjected to centrifugation followed by differential seeding over two hours (see chapter 2). Again, we were unable to characterise these cells due to such few numbers and lack of proliferation in culture. Samples 7 and 9 were quite unsuccessful with very few cells visible on the surface of the culture plates. These cells appeared more rounded but disappeared relatively quickly with no proliferation observed at all. From the official pathology diagnoses received for each patient sample (Table 3.1), we were unable to draw any conclusions regarding possible trends with the associated cultures. However, patient 2 was diagnosed as having an estrogen receptor positive (ER+), progesterone receptor negative (PR-) and C-erb (also known as HER—2) receptor negative, ductal carcinoma. This hormone profile was different from the other 8 patients, and cells from this sample were cultured for the longest time, progressing through a sub-culturing cycle. As stated before however, we cannot know if these were in fact cancer cells or not, as no characterisation was possible. Further investigations are necessary to be able to draw conclusions from the pathological hormone profiles of the samples.

In the study conducted by Li and colleagues on their 25 samples, it was reported that almost half of their cases showed vigorous growth, proliferating through 3-7 sub-culture cycles with a rapid initial doubling time every 48-72 hours which then slowed to every 10-12 days after the third passage. Of the remaining unsuccessful samples, 10 of the 25 showed no cellular attachment or growth and in three cases, although small clusters of cells were initially observed, no further appreciable growth was observed. Their explanation for these unsuccessful cultures of samples alluded to the possibility of an insufficient number of viable and/or proliferation-competent cells present in the FNA sample (Li *et al.*, 1998). It is quite possible that we were also unable to attain sufficient amounts of viable epithelial cancerous cells in the samples.

Successful primary cultures have also been established through FNA sample collection from other cancerous lesions, such as thyroid tumours and uveal melanomas. Thyroid FNA samples were initially cultured in RPMI 1640 growth medium supplemented with gentamicin, penicillin, glutamine and 20% fetal calf serum (FCS) for 2 weeks, followed by trypsinisation and then propagation in DMEM medium supplemented with penicillin/streptomycin, FCS and glutamine for 4-5 weeks, obtaining sufficiently high numbers of cells for experimental purposes (Antonelli *et al.*, 2008). Uveal melanoma FNA samples were cultured in RPMI 1640 supplemented with 5% FBS, penicillin/streptomycin and fungizone, grown to a minimum of 1 million cells for 2 weeks, thus maintaining a stable cell line (Correa, Marshall and Burnier, 2009). In both of these cases, successful primary cultures have been established from samples collected via the FNA technique confirming that the technique can be successful in these instances.

Unfortunately, we were unable to match these results as all of our nine FNA samples failed to proliferate successfully *in vitro*, thus a primary breast cancer culture model was not established. We can only speculate at this point as to why this might be. As mentioned previously, it is possible that not enough viable cancerous material was present in each FNA. The growth medium we used was a combination of that stipulated in the two breast FNA articles from the 90's, containing HEPES and epidermal growth factor as well as penicillin and FBS in a high glucose DMEM. For future samples it might be worth including insulin or other growth factors into the growth medium to promote growth of the cancer cells, or perhaps making use of RPMI 1640 growth medium as used by others in other tissues (Antonelli *et al.*, 2008; Correa *et al*., 2009). Temperature fluctuations could possibly also play a role in the success of the culture. As the FNA samples are removed directly from the body which is at a temperature of approximately 37°C, having the growth medium and centrifugation steps at room temperature (~22°C) might influence the viability of the cells in the sample. Since maintaining cells at 37°C would logistically be very difficult, cooling cells to 4°C and keeping them on ice could possibly prove a better option for increasing cell viability. Future investigations could include a comparison of cold with room temperature handling of samples. A further possible amendment to future studies would be to consider 3D culturing with the addition of a suitable attachment matrix such as collagen or Matrigel® (Corning®), a solubilized basement membrane hydrogel, to coat the base of the culture plate (Bergstraesser and Weitzman, 1993; Ip and Darcy, 1996; Burdall *et al.*, 2003). This is a variation in the protocol that we would have like to test, but unfortunately time did not allow

for it. Moving forward, for future investigations we would also like to include samples collected from solid tumour masses i.e. tissue slices from mastectomy surgeries, to possibly increase the number of viable cancerous cells in the sample and to optimise culturing conditions. Once this is established, we'd like to return to examining FNA sample collection.

To conclude, although two studies from the 90's have successfully shown that breast cancer samples collected through FNA can successfully be maintained and propagated in culture, and despite the fact that FNA sample collection has been used in other cancer research studies, for the establishment of successful primary cell lines, we were unable to definitively culture and propagate any of the nine breast cancer FNA samples we collected. Due to this, we were also unable to complete our second aim of investigating the effect of hypoxia on an established primary cell culture. Furthermore, we were not able to complete the objectives originally set out to compare frozen FNA samples with established primary cell cultures under normoxic conditions to determine the effect of *in vitro* culturing on primary cells, or to establish the sensitivity of the primary cells to hypoxia through the measurement of ATP present in cells.

There is room for manipulation and improvement of the protocol used and with time, we would have liked to experiment with further amendments in order to achieve all of the original aims and objectives. However, this pilot study remains inconclusive with no successful primary cell culture model established.

Chapter 4: The Effect of Hypoxia on PP2A Activity, Expression and Post-translational Modification

4.1 Introduction

Signal transduction within cells is a process whereby components (typically receptors or enzymes) are required to initiate signalling, as well as inhibit the cascade when necessary. While previous research provides a wide and comprehensive understanding of a range of kinases associated with cellular function, much about the 'brakes' of signal cascades, the phosphatases, is yet to be understood (Haesen *et al.*, 2014). It is speculated that phosphatases are less studied in comparison to kinases, as they are more challenging to work with (Ruvolo, 2016). Protein phosphatase 2A (PP2A) is a ubiquitously expressed protein Serine/Threonine phosphatase, initially described as a tumour suppressor, that is involved in the regulation of almost all cellular activities within the body, including signal transduction, cell proliferation, cell division, growth and development, apoptosis, and cell survival. It is therefore a key role-player in the maintenance of cellular homeostasis (Eichhorn *et al.*, 2009; Perrotti and Neviani, 2013; Seshacharyulu *et al.*, 2013). Previous studies have alluded to the fact that dysregulation, or abnormal inactivation of this phosphatase can lead to the transformation of cells with the development and progression of cancerous phenotypes, promoting cell survival (Gabel *et al.*, 1999; Sontag, 2001; Ruvolo, 2016).

As discussed in chapter 1, conditions of decreased tissue oxygen, or hypoxia, have been identified as hallmark characteristics of most solid-tumour cancers, and is a state to which cancer cells are seen to adapt through different mechanisms of survival, promoting and facilitating cell proliferation, and provoking more aggressive tumour phenotypes (Semenza, 2003; Zhou *et al.*, 2006). In a review by Zhou *et al.*, it was concluded that conditions of hypoxia appear to influence a wide range of molecular pathways to promote and facilitate adaptation of cells, apoptosis and chemoresistance. The responses of cells to hypoxia are determined through both pro- and anti-apoptotic factors, along with proliferative signals, a shift in microenvironmental pH, as well as clonal selection. They further suggested that apoptosis may be initiated in conditions of severe and prolonged hypoxia/anoxia due to the presence of secondary stressors such as acidosis leading to the onset of apoptotic pathways. The development of chemoresistance and possibly clonal selection may occur in

response to the opposing signals delivered by pro-survival and pro-death pathways, leading to the selection of cells displaying a genetic or epigenetic growth advantage (Zhou *et al.*, 2006). Hypoxia thus plays some role in whether proteins involved in signalling pathways become activated or inactivated, and the cascade of effects this has within the cell. It is of importance to this study how the phosphatase PP2A specifically is affected by hypoxic conditions.

Currently in literature there exists some degree of controversy surrounding the actual effect that hypoxia has on PP2A and its state of activation. Some studies have provided evidence that PP2A becomes activated under conditions of hypoxia, inhibiting cell growth and leading to arrest of the cell cycle in the G1/S phase allowing for prolonged survival of cells (Bando *et al.*, 2003; Yung and Tolkovsky, 2003; Hofstetter *et al.*, 2012). Other studies offer opposing evidence that PP2A is phosphorylated, becoming inactive during hypoxia, promoting activation of pro-survival/proliferation signal cascades and tumour formation (Mumby, 2007; Lin *et al.*, 2012). In a review by Mumby *et al*. it was described that mechanisms involved in PP2A inhibition include the expression of SV40 small tumour antigen, the upregulation of the cancerous inhibitor of PP2A (CIP2A) and/or mutations leading to the inactivation of the scaffold subunit of PP2A. These all contribute to enhancing phosphorylation and activity of tumorigenic proteins, including PKB (Mumby, 2007). Chen *et al.* further reported, that suppression of the endogenous A scaffold subunit of PP2A promotes the activation of PKB, thus suggesting that the activation of the PI3K/PKB pathway contributes to cancerous cell transformation, triggered by the suppression of PP2A activity (Chen *et al.*, 2005). When considering these opposing findings, one must however, take into account variations in the different research set ups, such as cancer types investigated, locations within the body, whether primary cells or immortalised cell lines were used, and the influence that various other signalling pathways may have on whether cells survive or progress through apoptosis. Regulation of the function of PP2A appears to be quite complex, involving post translational modifications of the subunits which make up PP2A, which in turn influences the activity and binding affinities of the catalytic and the B subunits, thereby modulating the function of the enzyme (Janssens *et al*., 2008; Janssens and Rebollo, 2012).

We therefore set out to identify the effect of hypoxia on PP2A within the immortalised triple negative breast cancer cell line, MDA-MB-231. This cancerous line has been widely used in

breast cancer studies (Bando *et al.*, 2003; Kang *et al.*, 2003; Ameri *et al.*, 2010; Xie *et al.*, 2016), and we anticipated that it would be a good breast cancer cell model to use for the purpose of our investigations. Our aim was to investigate the effect of hypoxia on PP2A activity and post-translational modification in the MDA-MB-231 cell line, with the objective of making use of Western blotting and phosphatase activity assays to accomplish this aim. ATP cell viability assays were performed to determine the effect of hypoxia on the cells. We hypothesised that hypoxia would lead to a decrease in PP2A activity, thereby favouring the phosphorylation and activation of pro-survival and proliferation associated enzymes.

4.2 Methods and Materials

4.2.1 Culturing immortalised cell lines

The MDA-MB-231 triple negative breast cancer cell line (PTEN wild type), obtained from colleagues, was maintained in culture under standard conditions (37°C, 5% CO_2) in DMEM growth medium supplemented with FBS and penicillin/streptomycin. Cells were passaged at ~80% confluence through a combination of trypsinisation and cell scraping, and growth medium was refreshed every second day, as described in chapter 2.

4.2.2 Exposure of immortalised cell lines to hypoxia

Once a suitable number of cell culture plates was obtained, hypoxic experiments were conducted over various time durations. Initially, 72 hours exposure to hypoxia was carried out but this length of time appeared to be too potent for the cells (cell numbers dwindled drastically) and was revised to investigating a time course of 24, 48 and 72 hours. The latter three time points were only tested once (n=1). The hypoxic duration was further revised to 2 hours, 4 hours, 6 hours and 8 hours. Time exposure experiments for the initial 72 hours and the 2, 4, 6 and 8 hours were carried out on three separate days for each time point (n=3). Cells were used between passages 47-52. For the purposes of Western blotting and the phosphatase activity assays, control plates received standard growth medium (supplemented with 10% FBS), and positive control plates received further supplementation with 200mM $CoCl_2$, a known inducer of HIF-1 (Wu and Yotnda, 2011). Hypoxic experimental plates received growth medium supplemented with 1% FBS and were subjected to 4 minutes of hypoxic gas flow (described in chapter 2) in a sealed hypoxic chamber, followed by incubation in the chamber at 37°C in a humidified atmosphere for the varying time durations.

For the ATP cell viability assays, MDA-MB-231 cells were seeded and grown in two 96 well plates for each time point, namely one control plate and one hypoxic plate. Again, once ~80% confluency, hypoxic experiments were carried out as described in chapter 2. Following hypoxic incubation, cells were harvested and the following primary end-points investigated: (1.) The expression, phosphorylation and methylation of the catalytic subunit of PP2A via Western blotting; (2.) PP2A activity; and (3.) cell viability.

4.3 Western Botting

4.3.1 Method Overview (described in detail in Chapter 2)

Following incubation of cells for the different time points, cell lysates were prepared for the purpose of Western blotting as described in chapter 2. Protein determination of each sample was calculated using the Bradford assay method (Bradford, 1976) and samples were prepared accordingly in Leammlie sample buffer. Between 10-30µg of protein was calculated for each sample. Equal amounts of protein were loaded onto gels and used for each experimental run.

Proteins within the samples were separated according to molecular weight using SDS-page electrophoresis on a pre-cast gradient gel (4-20%) as described in chapter 2. Proteins were transferred to an inert PVDF membrane followed by fixing of the proteins to the membrane, in methanol. 5% fat free milk was used for blocking of the membrane for at least one hour to minimise nonspecific binding of antibodies. Membranes were washed three times and were incubated at 4°C overnight, in the primary antibody of interest. For the initial 72 hour hypoxia experiments, membranes were probed for phosphorylated catalytic subunit of PP2A (PP2A-C) (R&D Systems, AF3989), methylated PP2A-C (Santa Cruz, Sc81603) and total PP2A-C (cell signalling, 20385), as well as total PKB/Akt (Cell Signalling, 9272S), phosphorylated PKB/Akt (Cell Signalling, 4060S), total ERK (Cell Signalling, 9102S) and phosphorylated ERK (Cell Signalling, 9101S). The revised time points of 24, 48 and 72 hours were probed for PKB/Akt (total and phosphorylated), HIF-1α (Cell Signalling, 14179S) and ERK (total and phosphorylated). Blotting for HIF-1α was introduced at this point to confirm that hypoxia had in fact been stimulated, since it is known that stabilisation and increase levels of HIF-1α has been associated with conditions of hypoxia (Kaelin, 2002; Semenza,

2004). The final revised time points of 2, 4, 6 and 8 hours were probed for PP2A (methylated, phosphorylated and total) as well as HIF-1α.

Following overnight incubation, membranes were again washed as previously described in Chapter 2, and incubated at room temperature for an hour, in relevant secondary antibody. All antibodies were incubated in anti-rabbit conjugate secondary antibody (Cell Signalling, 7074S), barring methylated-PP2A, which was incubated in anti-mouse conjugate secondary antibody (Cell Signalling, 7076S). Again, membranes were washed as before, and protein bands were detected using a ChemiDoc system in association with stain free technology (Biorad, 2017) through the chemiluminescence interaction between the Bio Rad Clarity™ ECL detection reagent (Bio-rad, 170-5061) and the anti-rabbit/mouse horseradish peroxidase conjugated secondary antibody used.

Detectable bands were analysed using Image Lab™ Software (Bio-Rad), and normalisation of 'transfer images' and exposed membranes using stain free technology (see chapter 2) for the standardisation of protein loading. Paired t-tests were conducted for data generated from the initial 72 hours hypoxic experiments. One way ANOVA's with Bonferroni and Dunnett post hoc tests were conducted for data generated from the revised time-course hypoxic experiments. Analysis was done using GraphPad Prism 6, and data expressed relative to control (as described in the text), as means ± SEM.

4.3.2. Western Blotting Results

Based on microscope observations, the initial hypoxic incubation period of 72 hours seemed to be too potent for the MDA-MB-231 cells as they appeared to be dying and unable to withstand such a length of hypoxic onslaught. Cell numbers appeared to have visibly and notably decreased in number following hypoxia, with the morphology of the cells appearing shrunken and small. Lysate samples were still prepared and run through SDS-page protein separation and detection techniques. PP2A-C (total, phosphorylated and methylated) was probed for, as the main protein of interesting in the experiment, however we also looked at PKB/Akt and ERK (total and phosphorylated) in order to identify if the cell's survival pathways had in fact been affected. Further, HIF-1α was probed for as a confirmation that hypoxia had been simulated. These graphs provide a representation of what has occurred following 72 hours hypoxia (Figure 4.1-4.5).

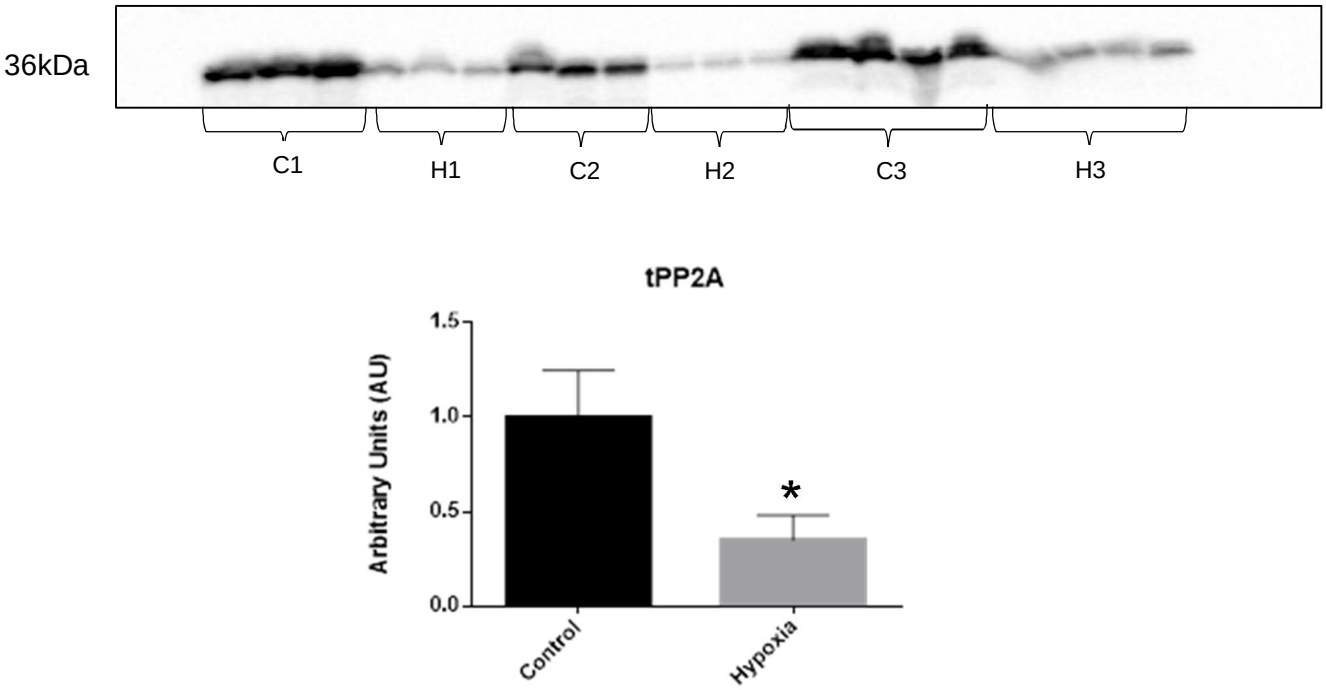


Figure 4.1 **Membrane showing total-PP2A bands and corresponding bar graph indicating control vs hypoxia with a significant decrease in tPP2A in the 72 hour hypoxic samples. C = Control; H = Hypoxia. * = p ≤ 0.05**

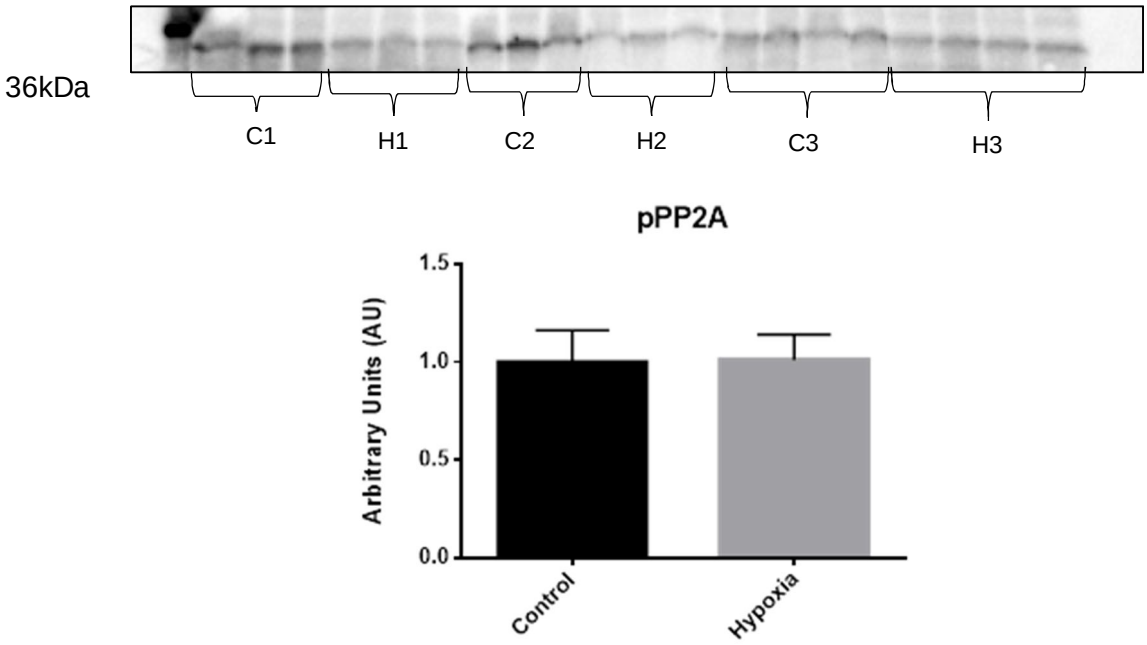


Figure 4.2 **Membrane showing phosphorylated-PP2A bands after 72 hours of hypoxia, and corresponding bar graph indicating control vs hypoxia with no significant difference between the two. C = Control; H = Hypoxia.**

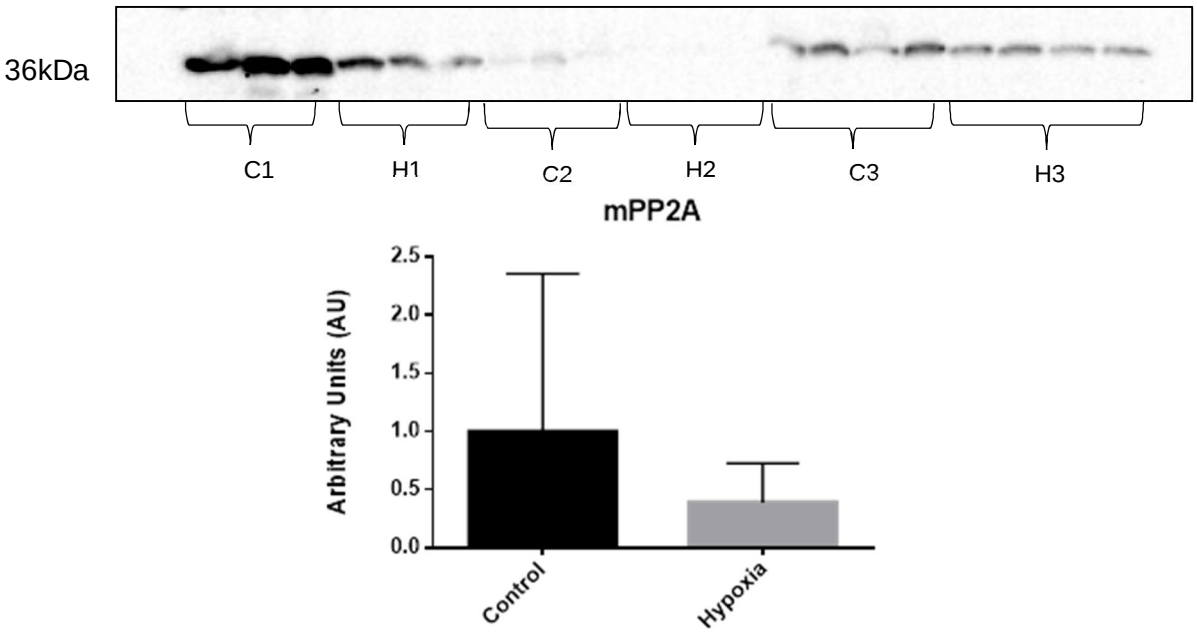


Figure 4.3 Western blot membrane of methylated-PP2A bands after 72 hour incubations with corresponding bar graph indicating control vs hypoxia, showing no significant difference. C = Control; H = Hypoxia

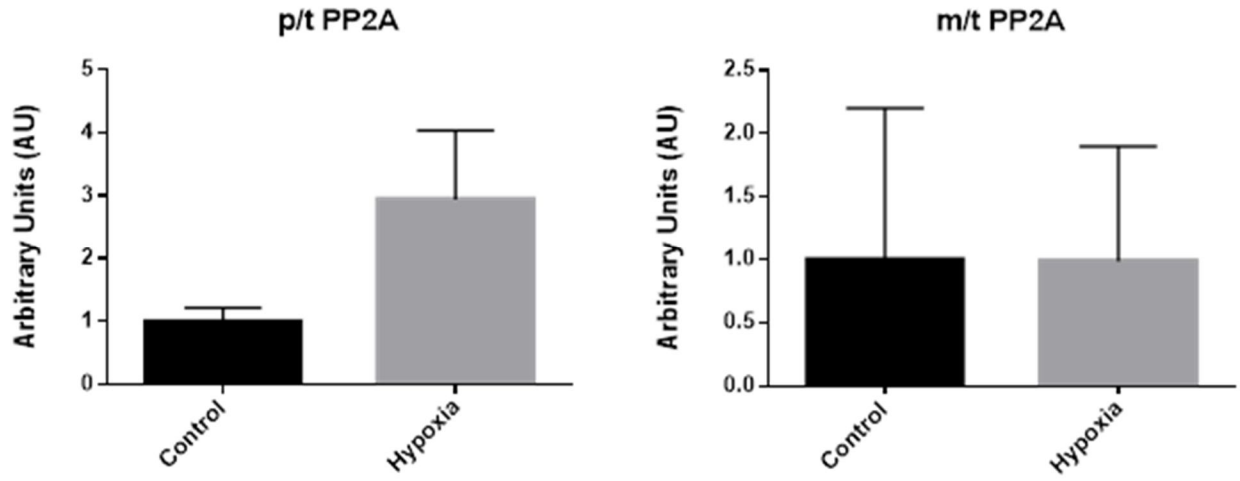


Figure 4.4 Phospho-to-total and methyl-to-total ratios for control vs hypoxia conditions after 72 hours incubation. Neither ratio showed any significant difference, relative to control.

A decrease in total PP2A (tPP2A) expression with hypoxia was significant following 72 hours hypoxia (Control: 1.00 ± 0.1426 AU vs hypoxia: 0.3513 ± 0.07558 AU; n=3; $p \leq 0.05$) (Figure 4.1). However, blots analysed for the phosphorylation and methylation of PP2A (figures 4.2

- 4.3) showed no significant changes with hypoxia relative to control. Furthermore, the phospho-to-total (p=0.0615) and methyl-to-total (p=0.9650) ratios further showed no changes following hypoxia.

HIF-1α as well as survival proteins PKB and ERK were blotted for next to investigate whether hypoxia had in fact been induced, and what its impact was on the phosphorylation status of PKB and ERK following 72 hours hypoxia.

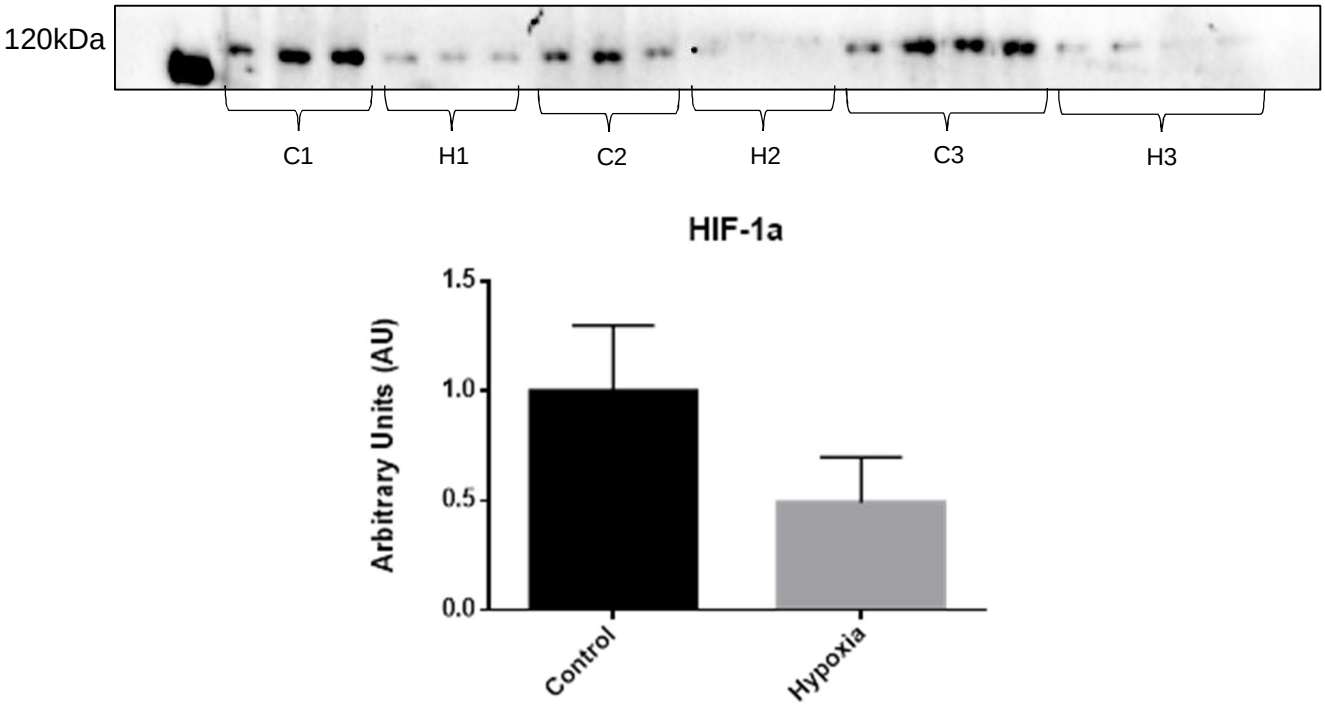


Figure 4.5 Membrane showing HIF-1α bands and corresponding bar graph indicating control vs hypoxia with no significant difference, although an interestingly, and unexpected decrease in HIF-1α during hypoxia is observed.

From visualising the membrane in Figure 4.5, one would expect to have seen a significant decrease in HIF-1α which was not observed using a T-test analysis. However, from literature, one would expect an increase in HIF-1 α. As can be seen from the patchy appearance of the blots, it could be that the binding of one of the antibodies was too weak or unreliable to generate consistent results. The resulting variations in the data could explain the lack of significance.

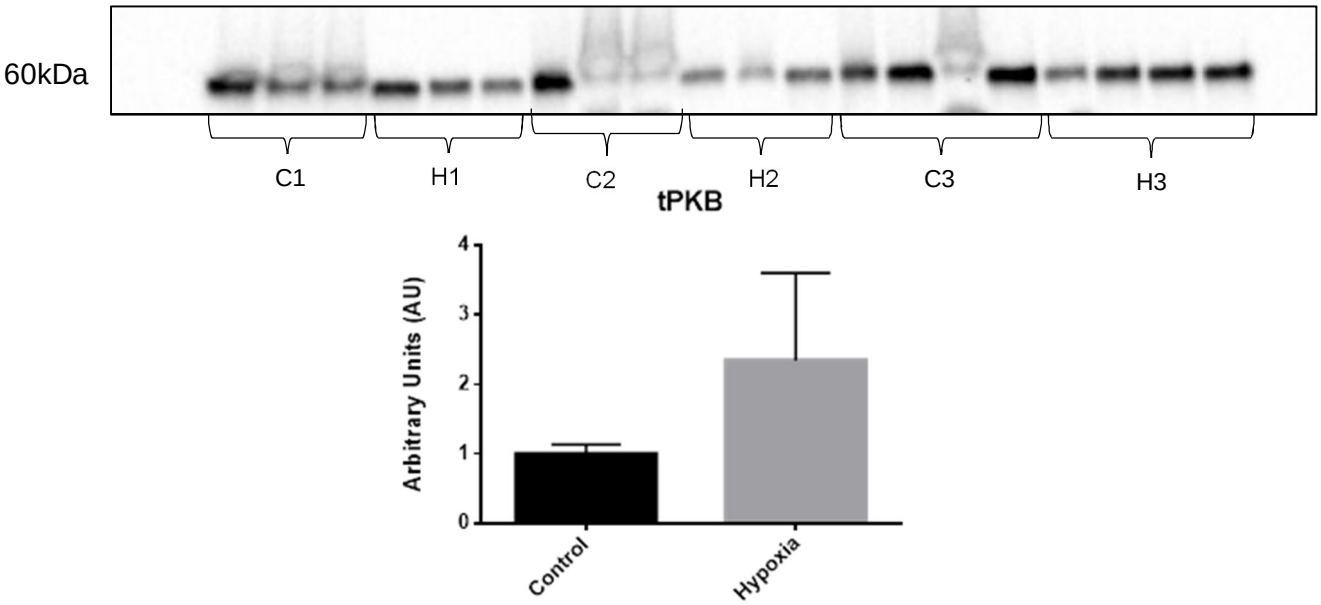


Figure 4.6 **Membrane showing total-PKB bands and corresponding bar graph, indicating control vs hypoxia with no significant increase during hypoxia. C = Control; H = Hypoxia.**

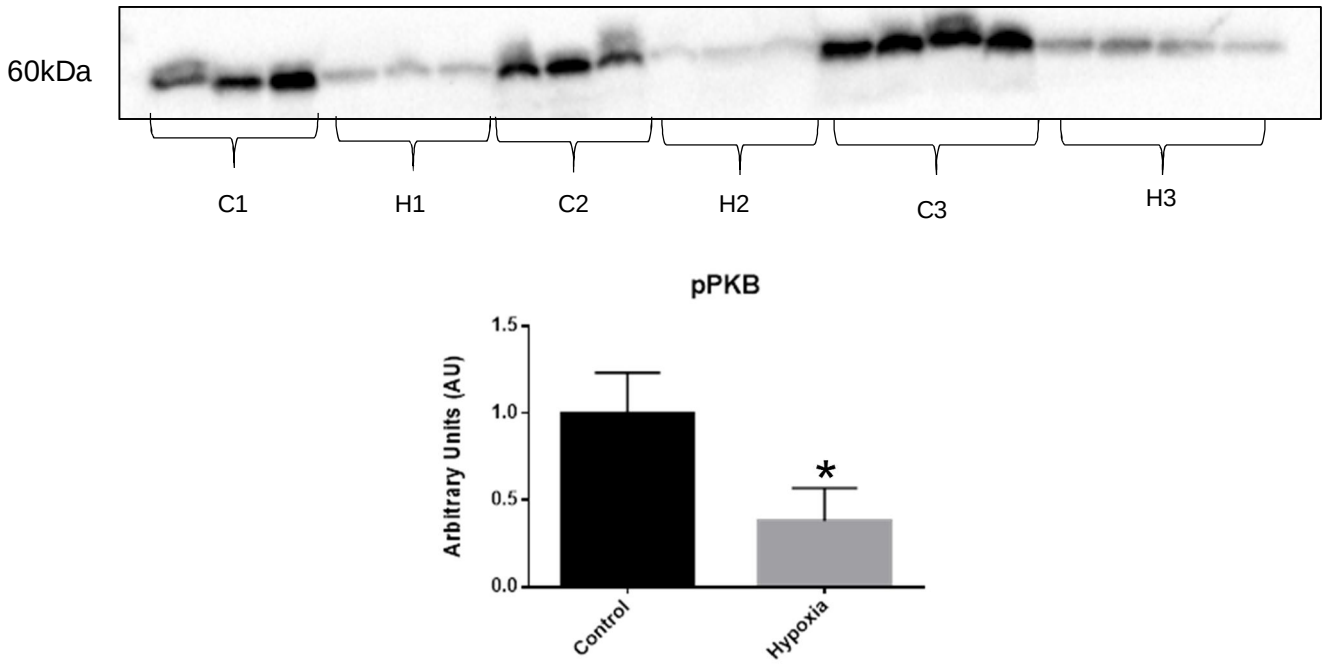


Figure 4.7 **Western blot and corresponding bar graph of phosphorylated PKB, showing a significant decrease with hypoxia. C = Control; H = Hypoxia. * = p≤0.05**

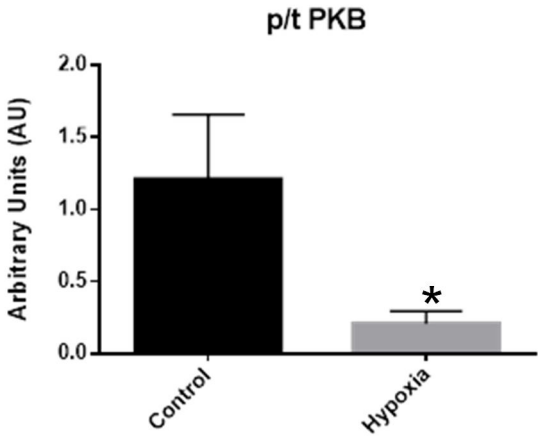


Figure 4.8 Phospho-to-total ratio of PKB showing a significant decrease with 72 hours hypoxia
*** = p≤0.05**

Blotting for PKB showed no significant changes in total PKB (tPKB) following 72 hours hypoxia (Figure 4.6), however, phosphorylation of PKB (pPKB) significantly decreased with hypoxia relative to control (control: 1.00 ± 0.1347 AU vs hypoxia: 0.3812 ± 0.1089 AU; n=3; p≤0.05) (Figure 4.7). Furthermore, the ratio of phosphorylation to total PKB (p/t PKB) following hypoxia was found to decrease significantly relative to control (control: 1.211 ± 0.1820 AU vs hypoxia: 0.2088 ± 0.03583 AU; n=3; p≤0.05) (Figure 4.8).

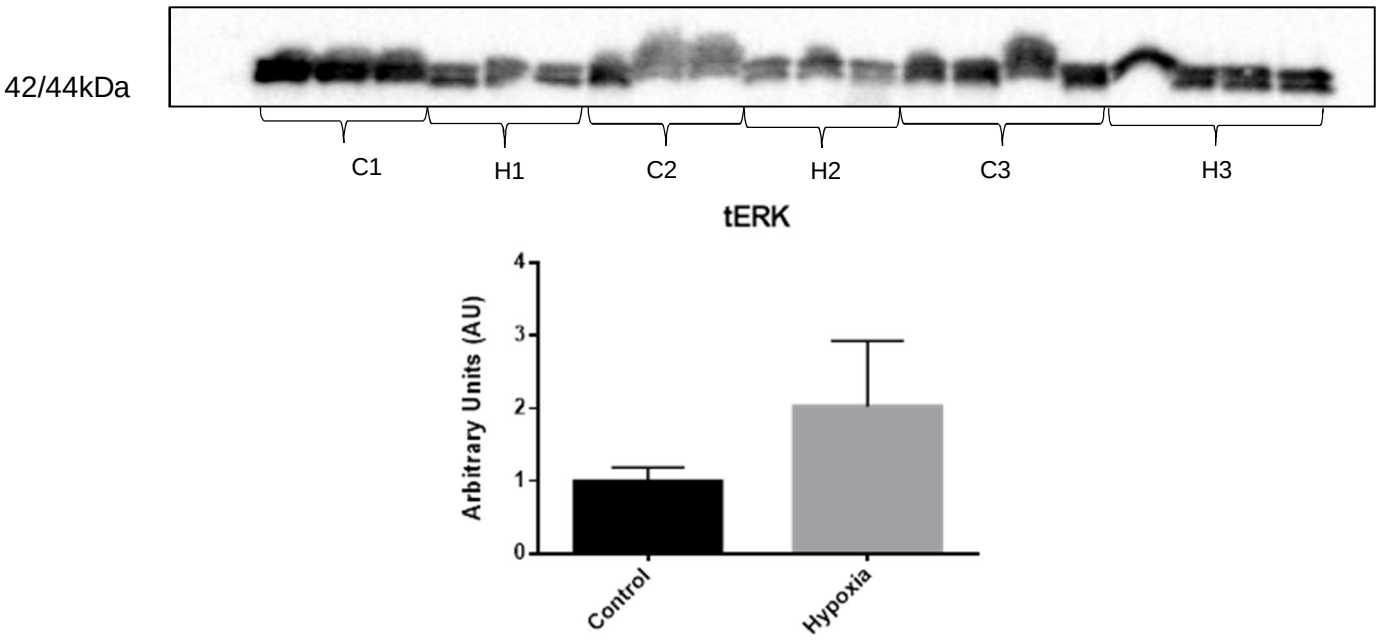


Figure 4.9 Membrane showing total-ERK bands at 42kDa and 44kDa, and corresponding bar graph indicating control vs hypoxia. No significant difference was observed. C = Control; H = Hypoxia. Data for ERK 42 and ERK 44 were combined in this analysis due to difficulties experienced in distinguishing between individual bands.

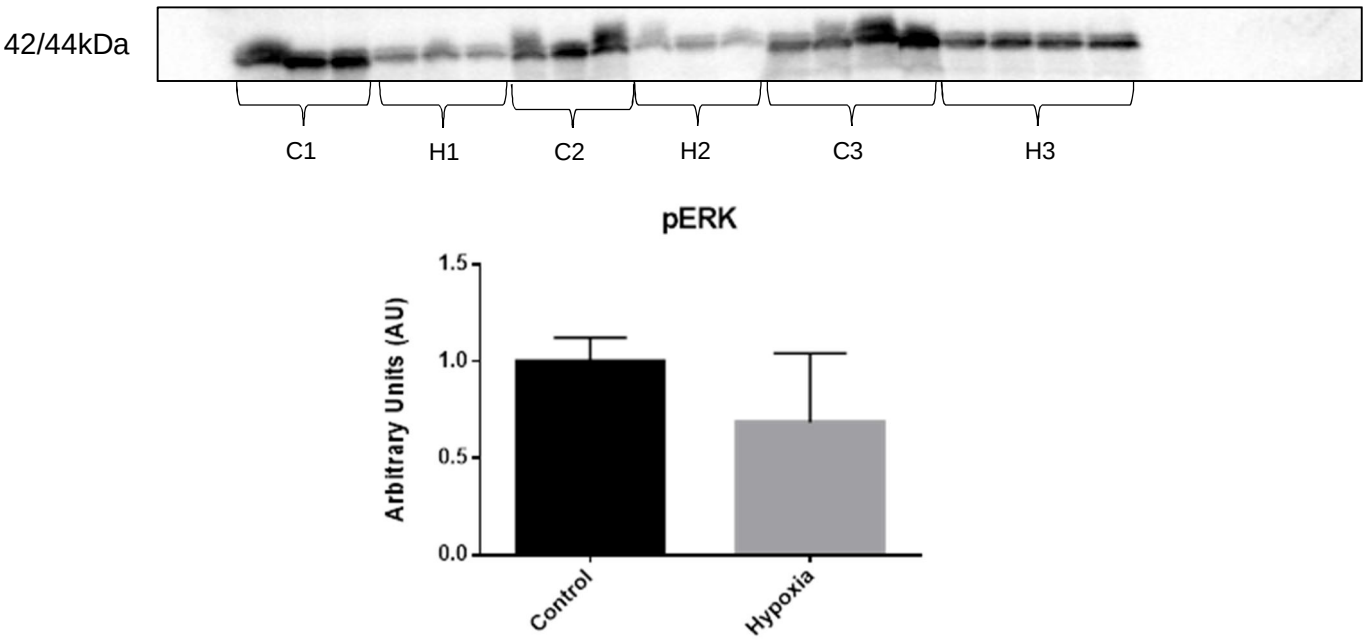


Figure 4.10 **Membrane showing phospho-ERK bands 42kDa and 44kDa, and corresponding bar graph indicating control vs hypoxia. No Significant changes were observed between control and hypoxic samples. C = Control; H = Hypoxia.**

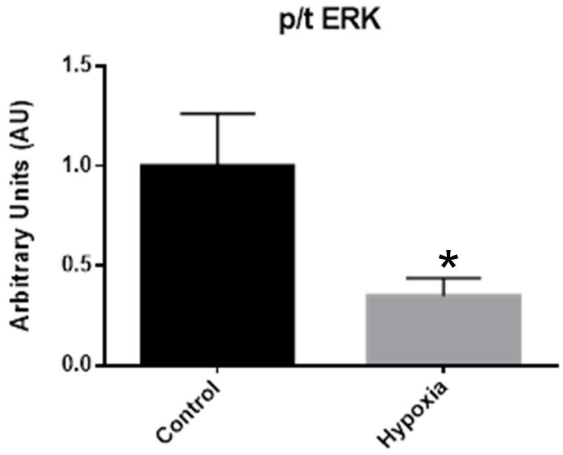


Figure 4.11 **Phospho-to-total ratios for control vs hypoxia conditions. A significant decrease in the p/t ratio was found with hypoxia compared to control following72 hours hypoxia. * = p≤0.05**

Blots probing for ERK showed no significant changes in expression of total ERK (tERK) after 72 hours hypoxia relative to control (Figure 4.9), and similarly, no changes were observed in phosphorylated ERK (pERK) relative to control either (Figure 4.10). However, a significant decrease in the phospho-to-total ratio was measured following hypoxia relative to control (control: 1.00 ± 0.1519 AU vs hypoxia: 0.3493 ± 0.05206 AU; n=3; p≤0.05), indicating that with hypoxia, a decrease in phosphorylation of ERK relative to the amount to total protein

was measured, in comparison to control (Figure 4.11). Our data therefore indicated that 72 hours of hypoxia was associated with a reduction in the activation of the pro-survival pathways mediated by PKB and ERK.

Following Western blotting of samples exposed to 72hours incubation, it was decided from the data obtained to revise the incubation period to a time course to include varying durations of incubation, namely 24hours, 48hours and 72hours. These were conducted only once (n = 1) before further revision. In this time course experiment, antibodies probed for included HIF-1α (to confirm hypoxia), PKB and ERK (both total and phosphorylated) in order to assess the possible effects of the incubation on the survival pathways of the cells. Blotting for PP2A was not included with these revised hypoxic durations as it was our intention to examine the effects of these hypoxic time points on the survival pathways, with the aim of selecting a time point for the investigation of PP2A.

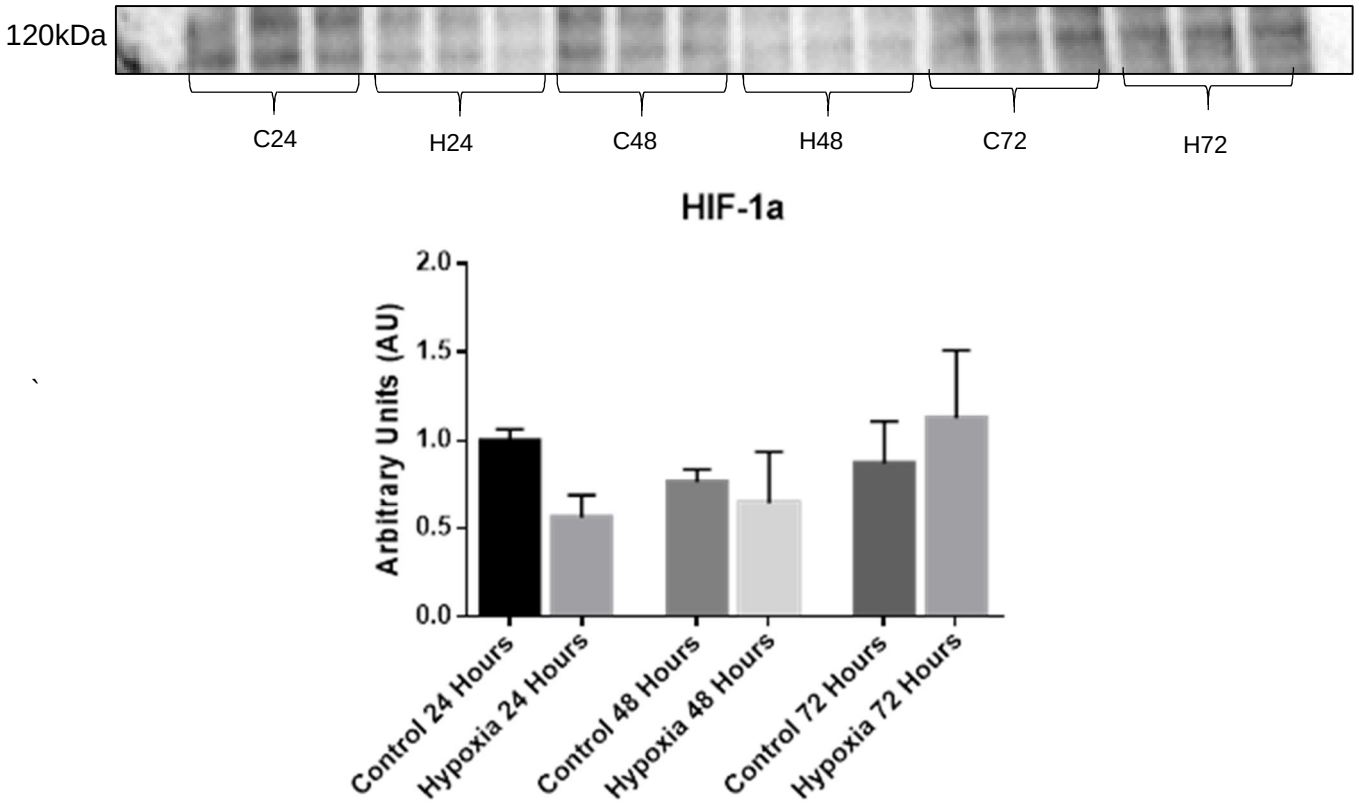


Figure 4.12 Membrane and corresponding bar graph of HIF-1α normalised to 24 hour control, showing no significant differences between control and hypoxia following 24 and 48 hours. C = Control; H = Hypoxia. * = p≤0.05

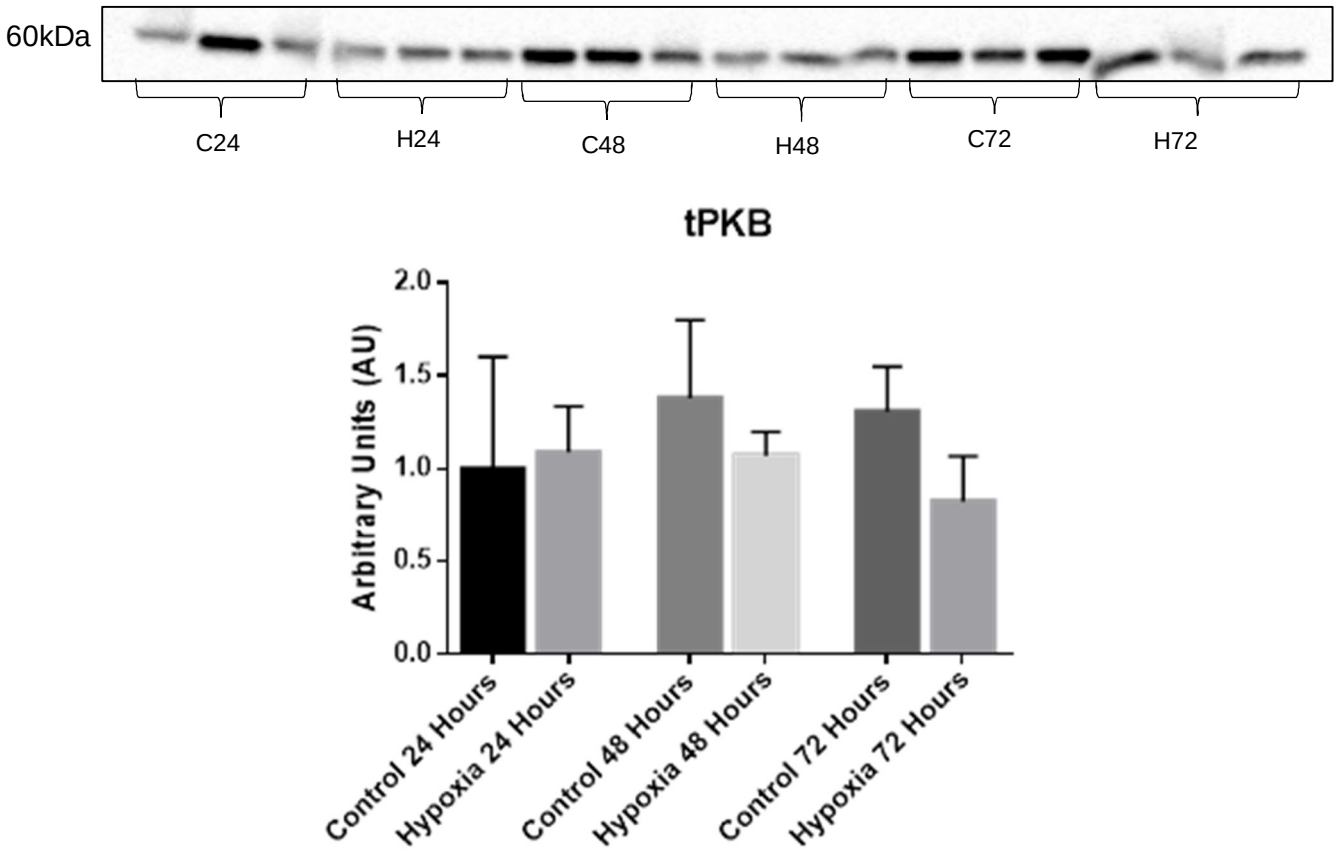


Figure 4.13 Western blotting membrane and corresponding bar graph of total-PKB, normalised to 24 hour control. No significant difference detected between control and hypoxia for any of the three time points measured. C = Control; H = Hypoxia.

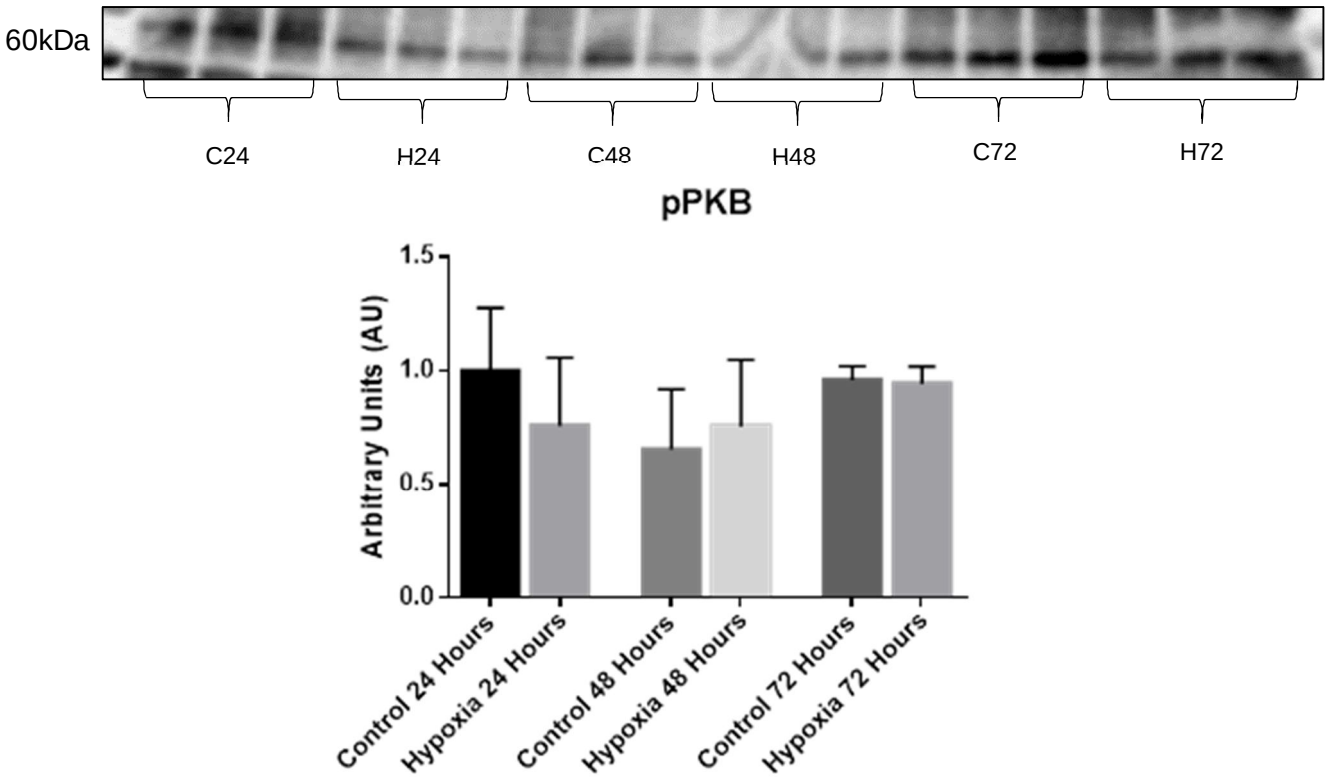


Figure 4.14 Exposed membrane and corresponding bar graph of phosphorylated PKB, normalised to 24 hour control. No significant difference observed. C = Control; H = Hypoxia.

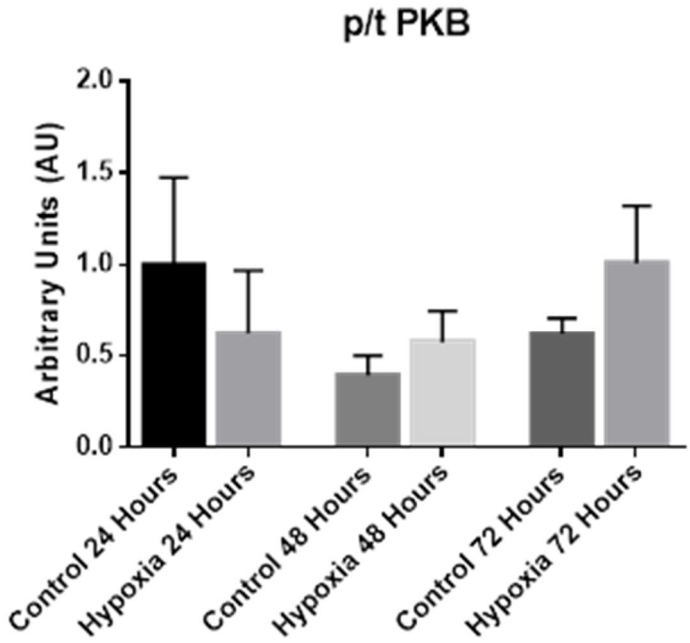


Figure 4.15 Phospho-to-total ratio of PKB, normalised to 24 hour control. No significant differences found between control and hypoxia exposed samples for each of the three time points measured.

Following the analysis of the blots from the revised incubation times, it was observed that HIF-1α appeared initially to follow a downward trend at 24 and 48 hours (Figure 4.12). Although these changes were not statistically significant in comparison to control, it is still interesting to observe, especially since based on reports in literature, we expected to observe an increase in stability and expression of HIF-1α with hypoxia. An increase was seen following 72 hours, however this was still not significant. Similarly, analysis of the expression and phosphorylation of PKB showed no significant changes after hypoxic exposure relative to control (Figures 4.13 and 4.14). The ratio of phospho-to-total PKB further remained unchanged in all of the hypoxic time durations (Figure 4.15).

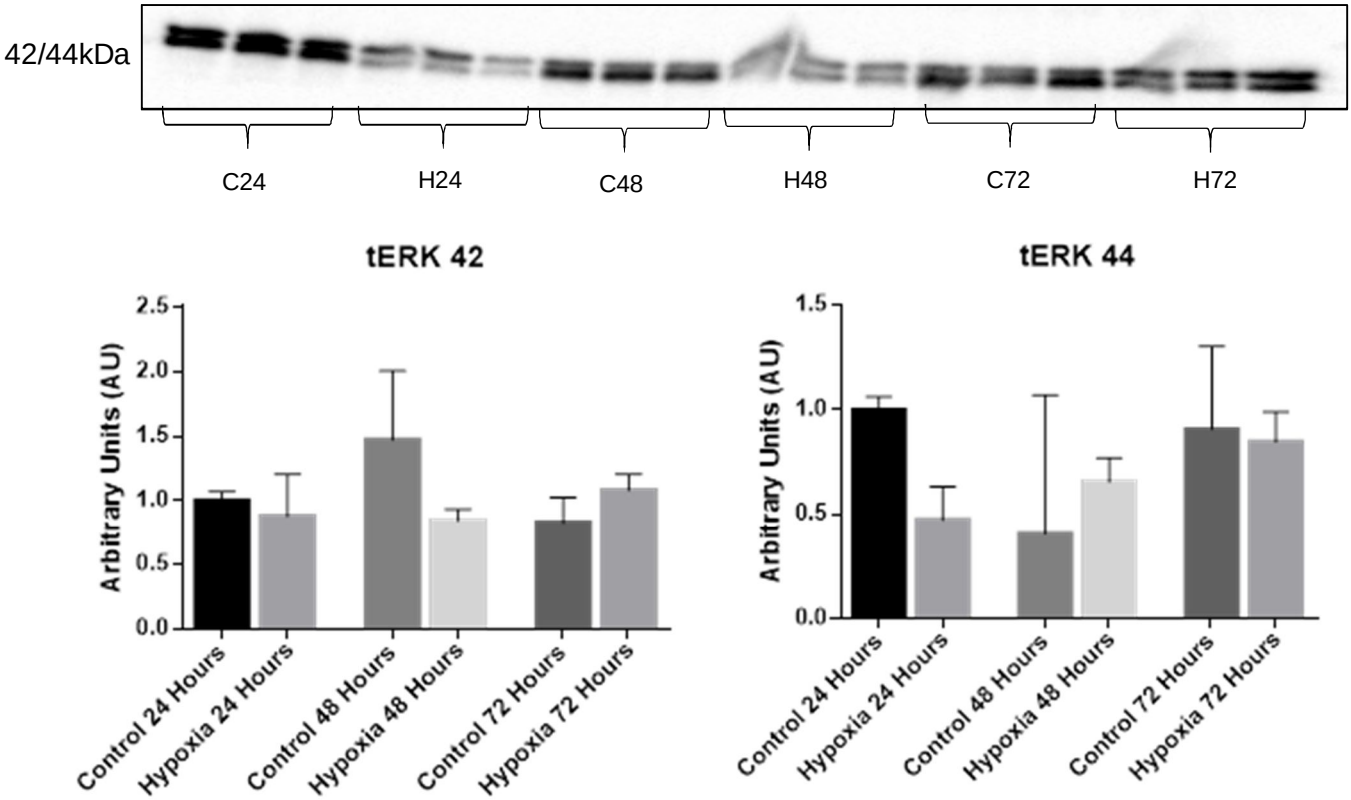


Figure 4.16 Exposed membrane with corresponding bar graphs for total ERK 42 and 44 respectively. Data was normalised to 24 hour control. C = Control; H = Hypoxia.

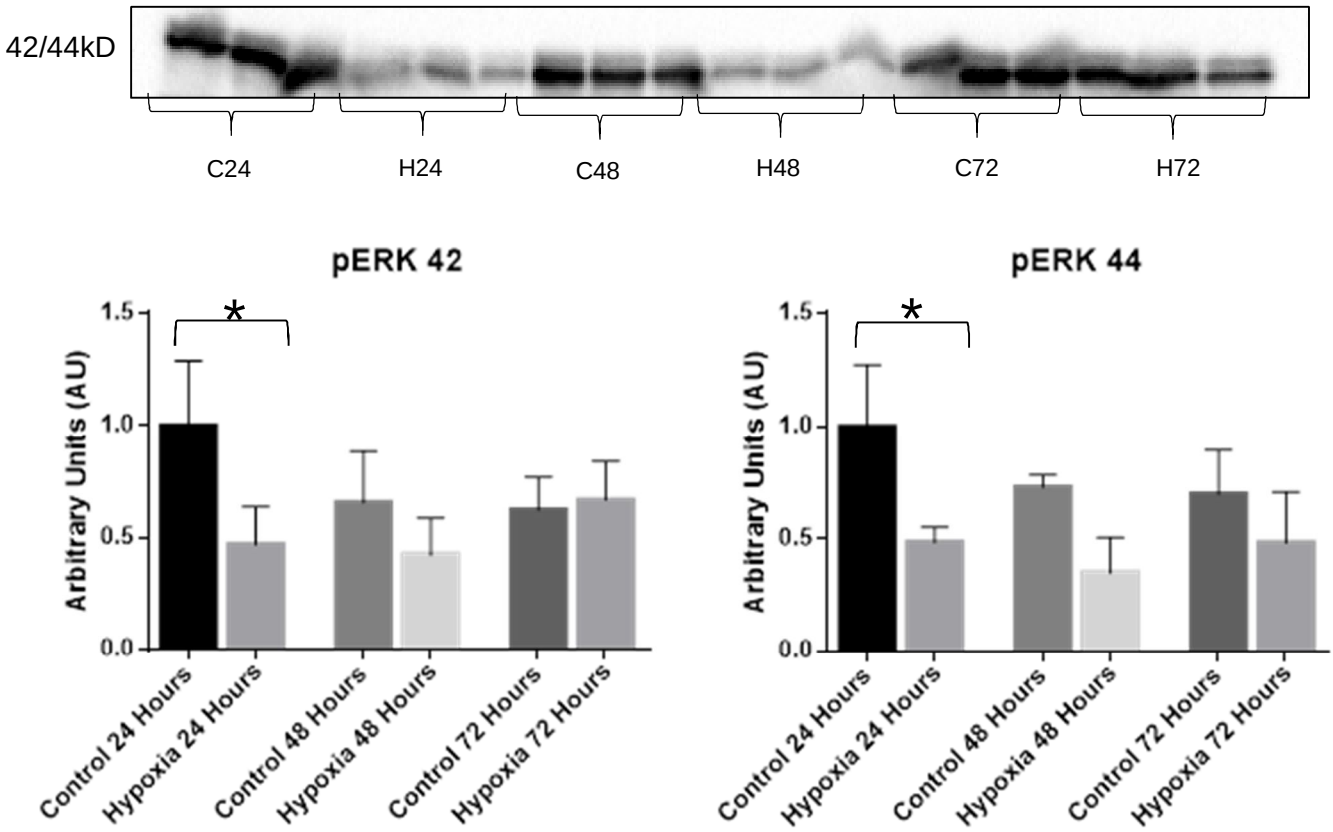


Figure 4.17 Western blot membrane with corresponding bar graphs of phosphorylated ERK 42 and ERK 44, normalised to 24 hour control. A significant decrease in pERK was observed in 24 hours hypoxia compared to 24 hours control for both pERK 42 and pERK 44 respectively. C = Control; H = Hypoxia. * = p≤0.05

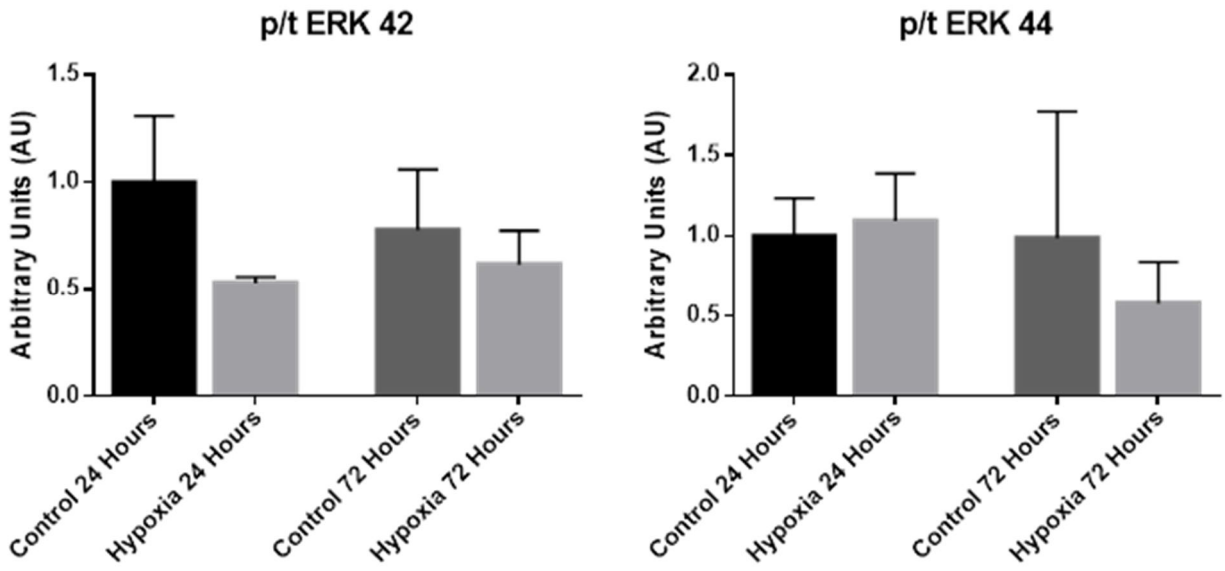


Figure 4.18 Phospho-to-total ratio of ERK 42 and 44 respectively, normalised to 24 hour control. No significant difference found between control and hypoxia for the different time points examined.

No changes in the expression of tERK were observed following any of the hypoxic time points (Figure 4.16), however, significant decreases in the phosphorylation of both pERK 42 (control: 1.00 ± 0.1660 AU vs hypoxia: 0.4696 ± 0.09681 AU; n=3; $p \leq 0.05$) and pERK 44 (control: 1.00 ± 0.1586 AU vs hypoxia: 0.4849 ± 0.03826 AU; n=3; $p \leq 0.05$) was observed following 24 hours hypoxia relative to 24 hour controls (Figure 4.17). For the analysis of the ratio of phospho-to-total ERK, data for 48 hours hypoxia was omitted from the graphs due to technical challenges with an anomaly in the gel, or the way in which the samples ran. This caused to too much variation in the data, leading to skewed and unrepresentative graphs. No significant changes in this ratio were observed following any of the hypoxic time points (Figure 4.18).

These results prompted further revision of the hypoxic incubation times to include shorter durations. The lack of significant changes in PKB expression and phosphorylation along with the unanticipated HIF-1α trends led us to wonder whether the durations of hypoxic incubation were still too potent for the MDA-MB-231 cell. The significant decrease in phosphorylation ERK 42 and 44 following 24 hours hypoxia relative to control further suggested the possibility of shorter hypoxia times may be favourable. Moving forward with the experiments, the further revised hypoxic incubation times were thus implemented (2, 4, 6 and 8 hours) and results plotted accordingly. Representative blots are shown in Figures

4.19- 4.24. Because the results obtained for HIF-1α thus far did not match expectations as reported in literature, such that HIF-1α expression and activity is increased with hypoxia (Kaelin, 2002; Semenza, 2004), $CoCl_2$ was introduced in these experiments as a positive control for hypoxia (refer to chapter 2). It has been described in literature as a chemical inducer of the transcription factor, HIF-1α (Wu and Yotnda, 2011) thus all pathways and proteins downstream from HIF-1α that become activated during hypoxia, will also become activated with treatment with 200 mM $CoCl_2$. Blots were normalised to 2 hour control and stain free technology was a control for loading. Each of these time points was repeated in triplicate on separate days (n=3) and the data averaged and subjected to 1-way ANOVA.

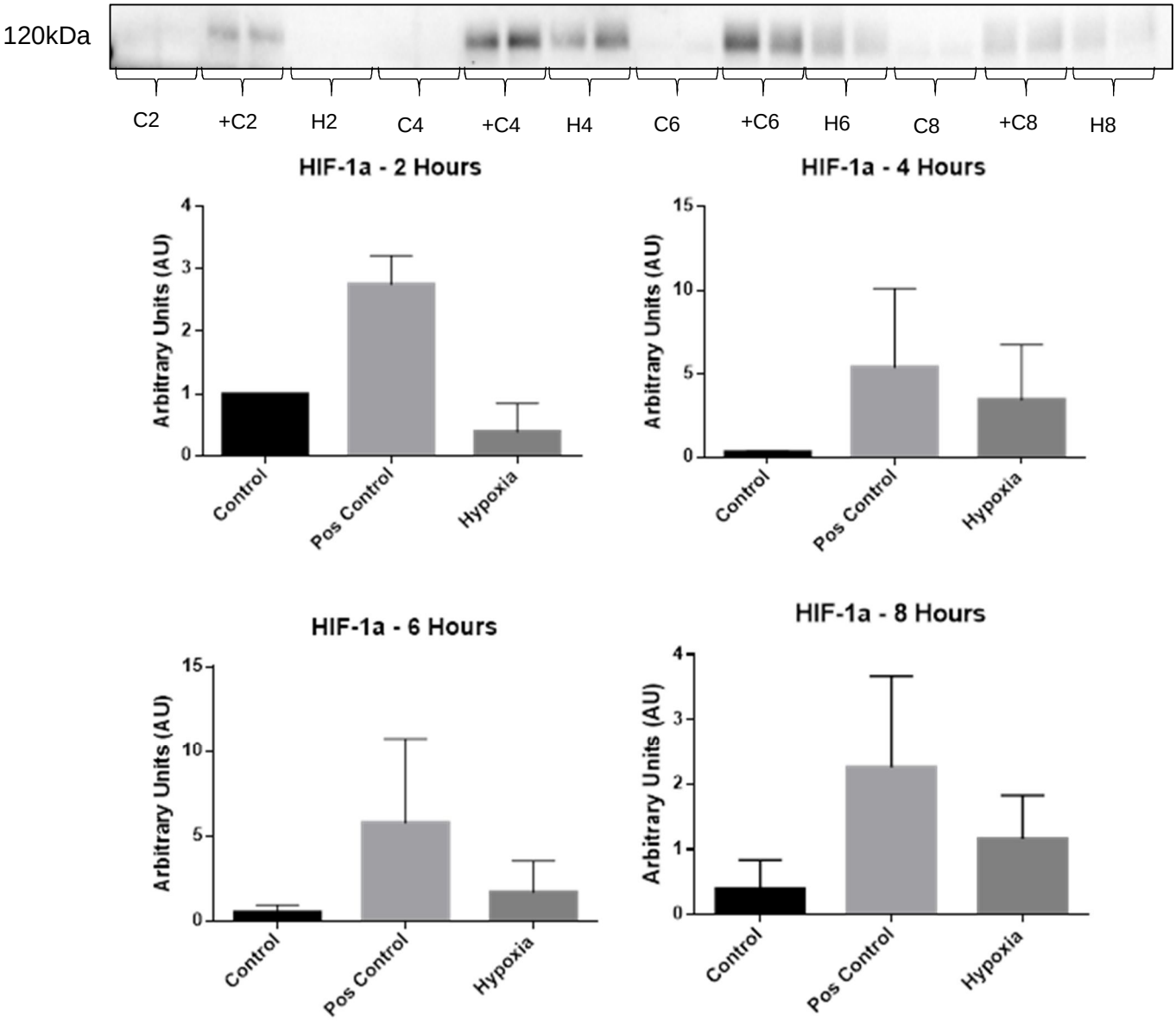


Figure 4.19 Individual graphs of HIF-1α with corresponding Western blot. Data was normalised to 2 hour control and no significant changes were observed despite the visual trend of increased HIF-1α expression in positive control samples relative to control. C = Control; +C = Positive Control; H = Hypoxia. Numbers indicate time point measured, e.g. C2 = 2 hr control

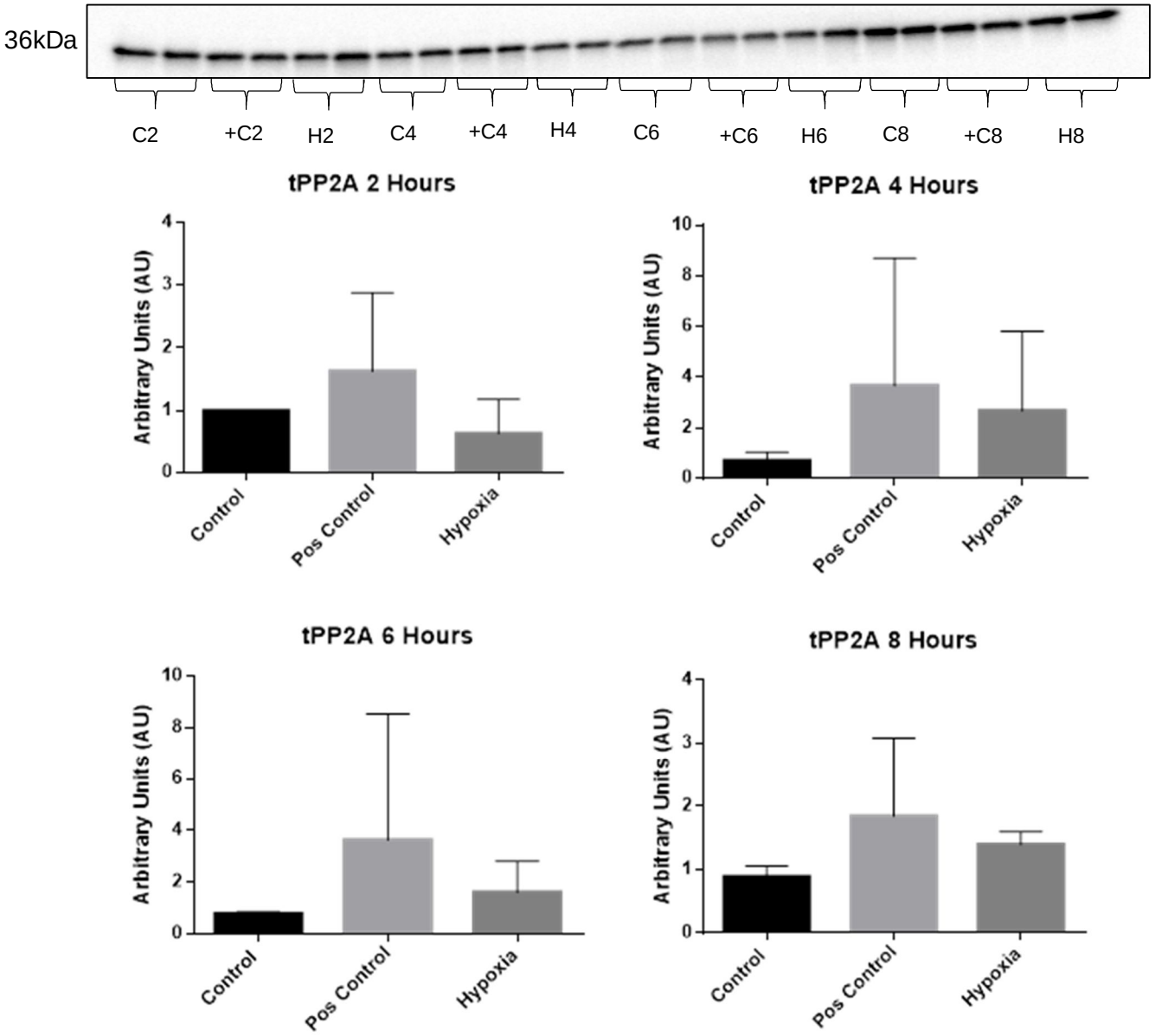


Figure 4.20 **Total PP2A protein expression across all four time points measured, normalised to 2 hour control. Despite a visual increase in hypoxia relative to control, no statistical differences were observed for PP2A expression. C = Control; +C = Positive Control; H = Hypoxia. Numbers indicate time point measured**

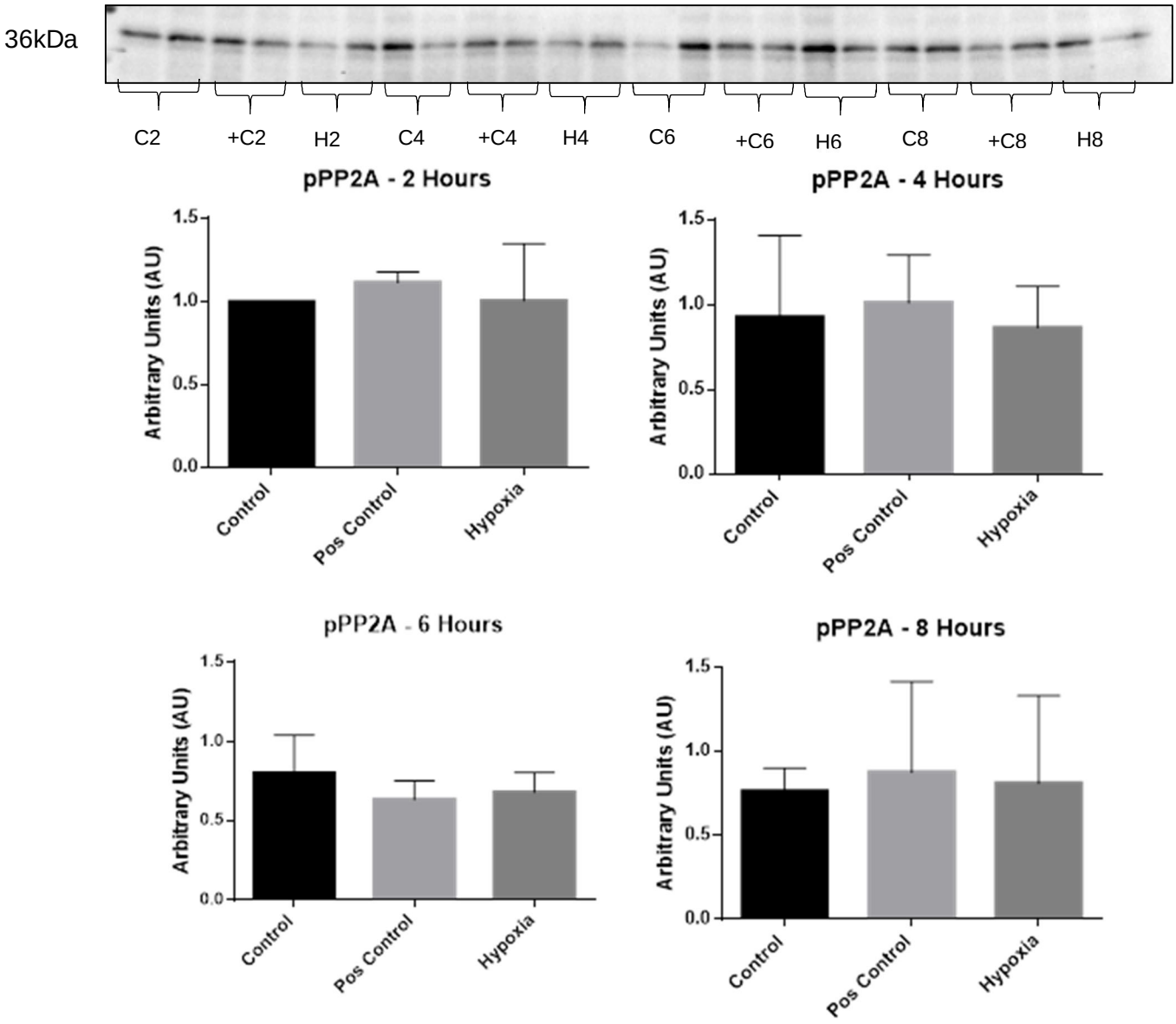


Figure 4.21 **Phosphorylated PP2A showed no statistically significant changes relative to control over any of the four time points. Data was normalised relative to 2 hour control. C = Control; +C = Positive Control; H = Hypoxia. Numbers indicate time point measured**

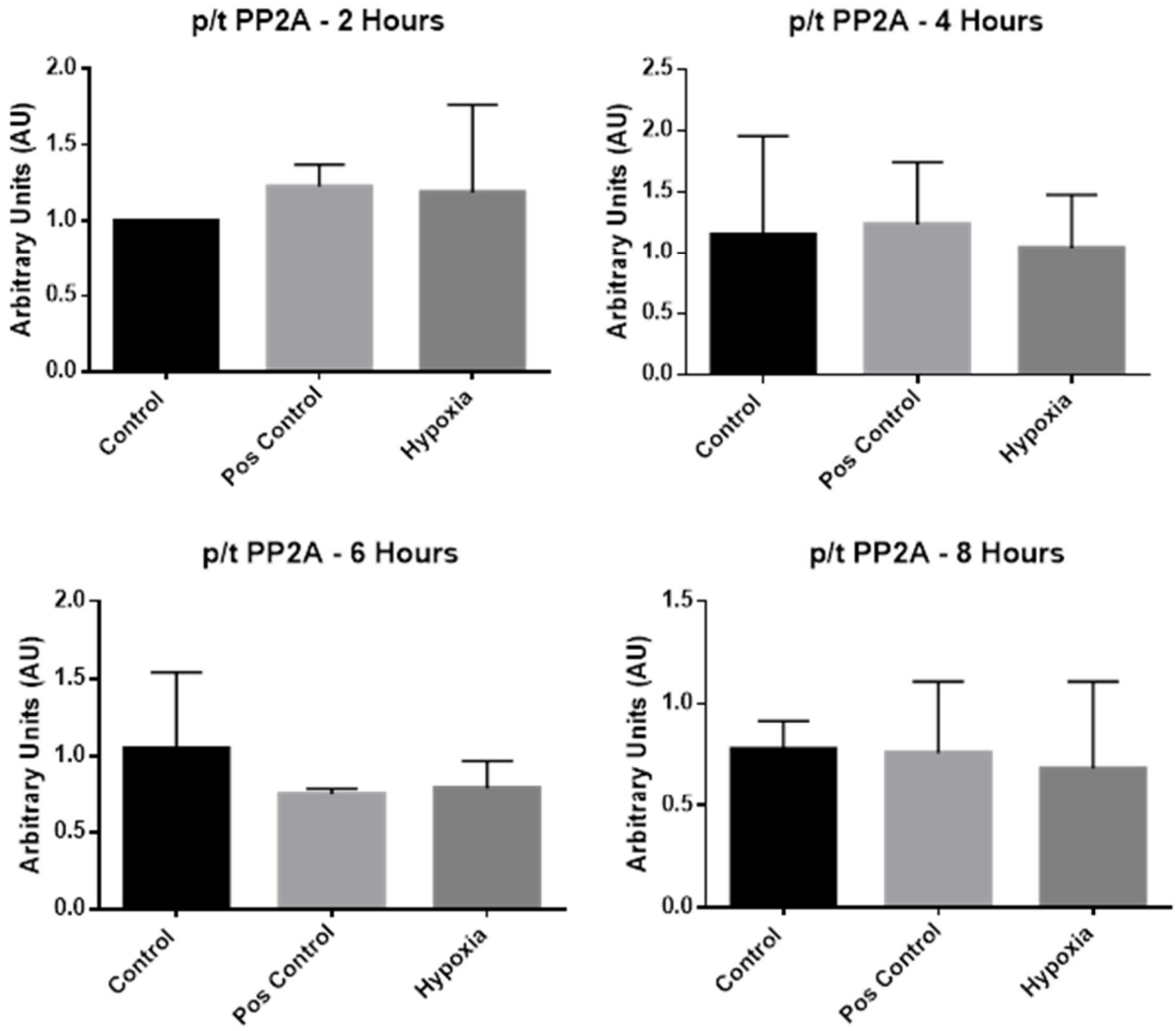


Figure 4.22 Phospho-to-total ratio of PP2A expression across all four time points, expressed relative to 2 hour control.

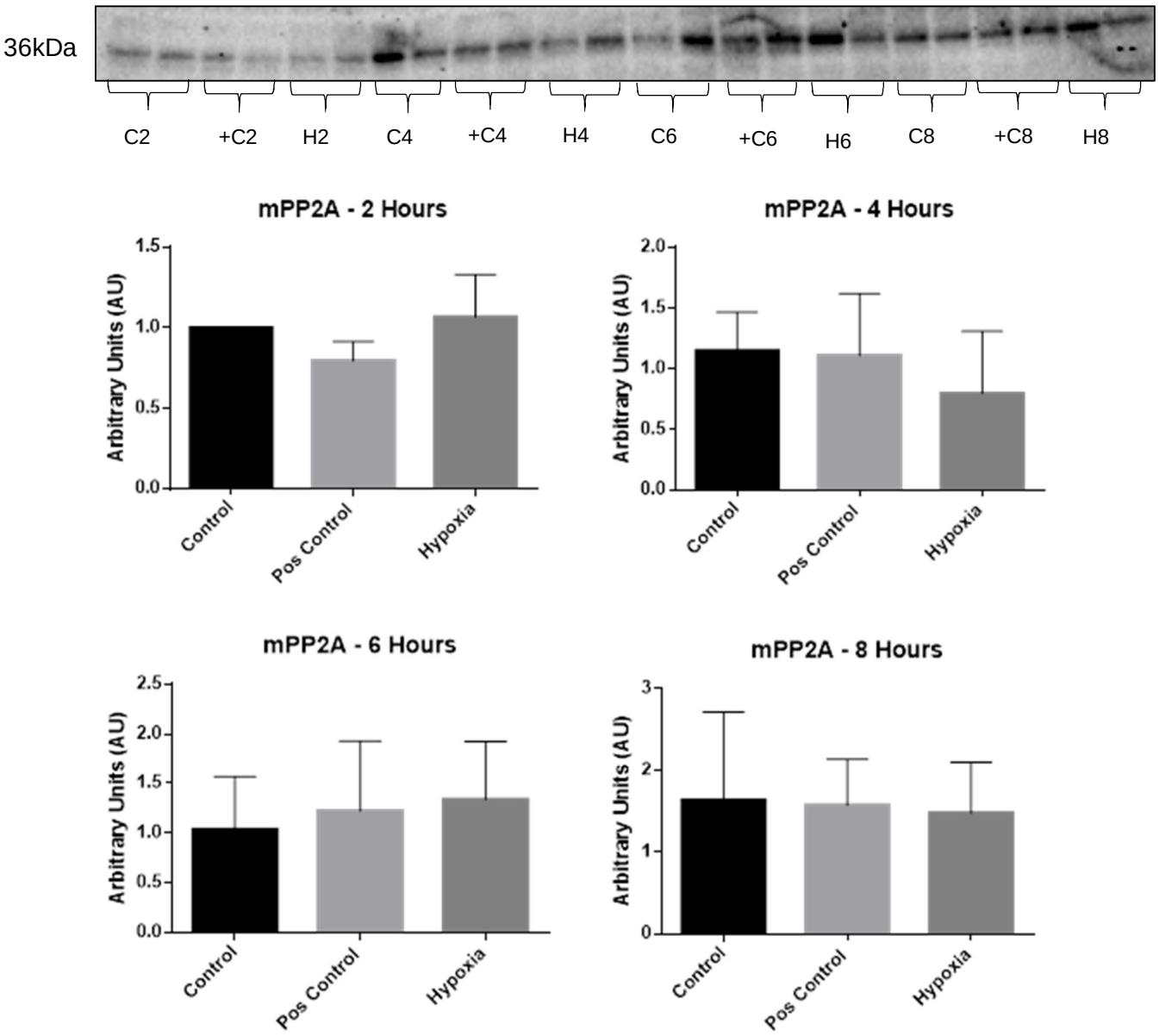


Figure 4.23 Methylated PP2A expression showed no significant changes with hypoxia relative to control. Data was normalised to 2 hour control. C = Control; +C = Positive Control; H = Hypoxia. Numbers indicate time point measured

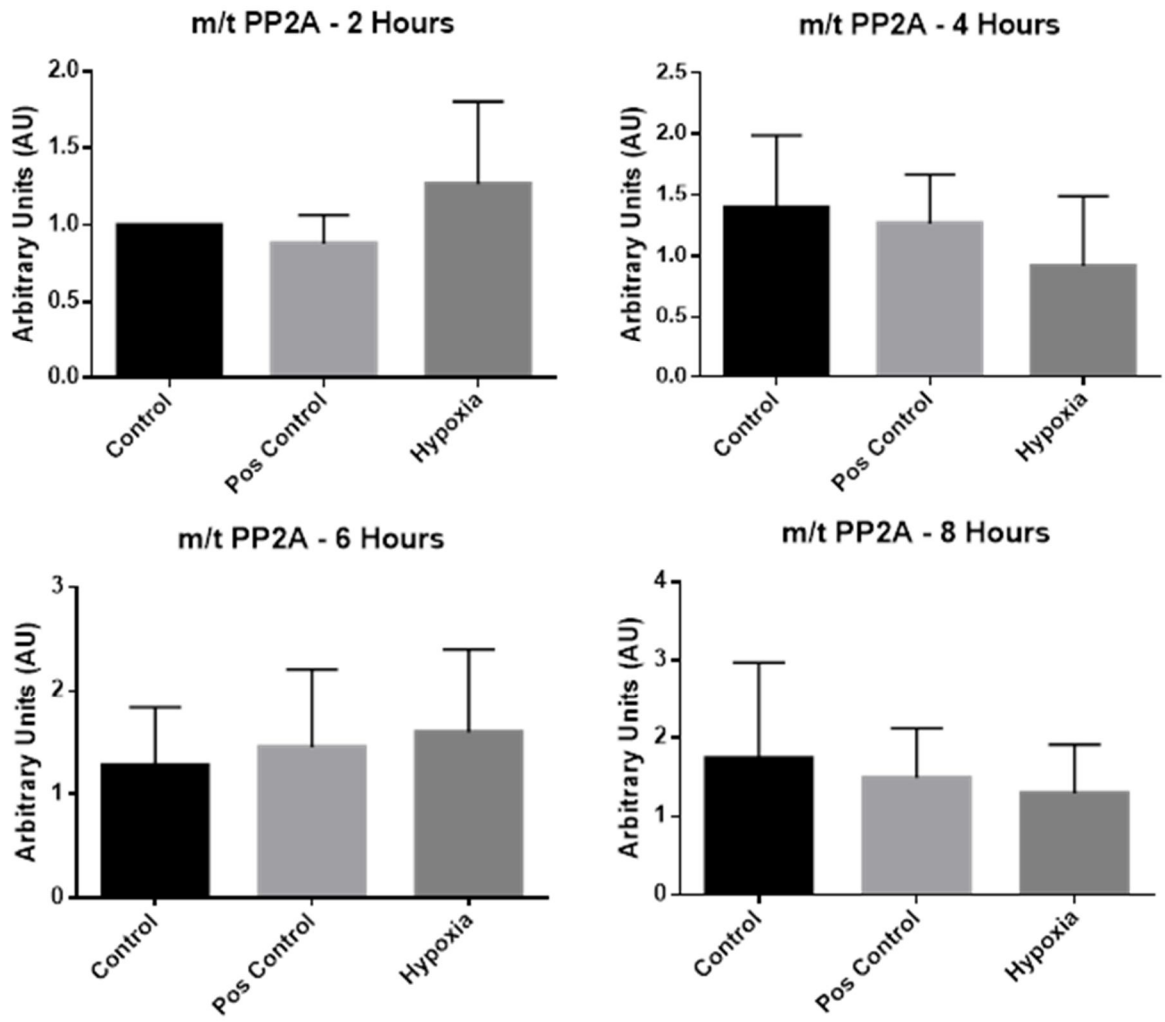


Figure 4.24 Methyl-to-total ratio of PP2A expression, normalised to positive control. No significant changes were observed for any of the time points measured.

Following hypoxic incubation for 2, 4, 6 and 8 hours, Western blotting analysis indicated no significant changes in the levels of HIF-1α relative to control (Figure 4.19). Similarly, the expression, phosphorylation or methylation of PP2A remained unchanged following hypoxia relative to control, as did the ratios of phospho-to-total and methyl-to-total PP2A (Figures 4.20-4.24). This suggests that hypoxia does not appear to have an effect on PP2A expression of post-translational modification.

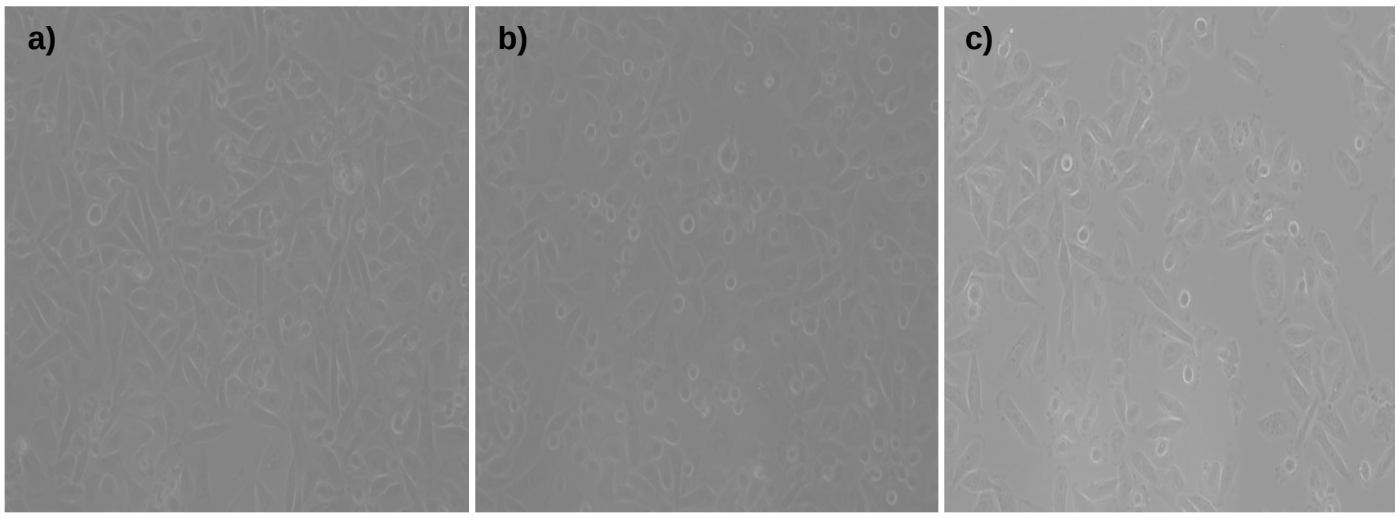


Figure 4.25 **Representative images of the MDA-MB-231 cells after 2 hours incubation: a) control cells (10% FBS), b) positive control (10% FBS + 200mM $CoCl_2$), and c) hypoxic cells (1% FBS + 4mins hypoxic gas). A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.**

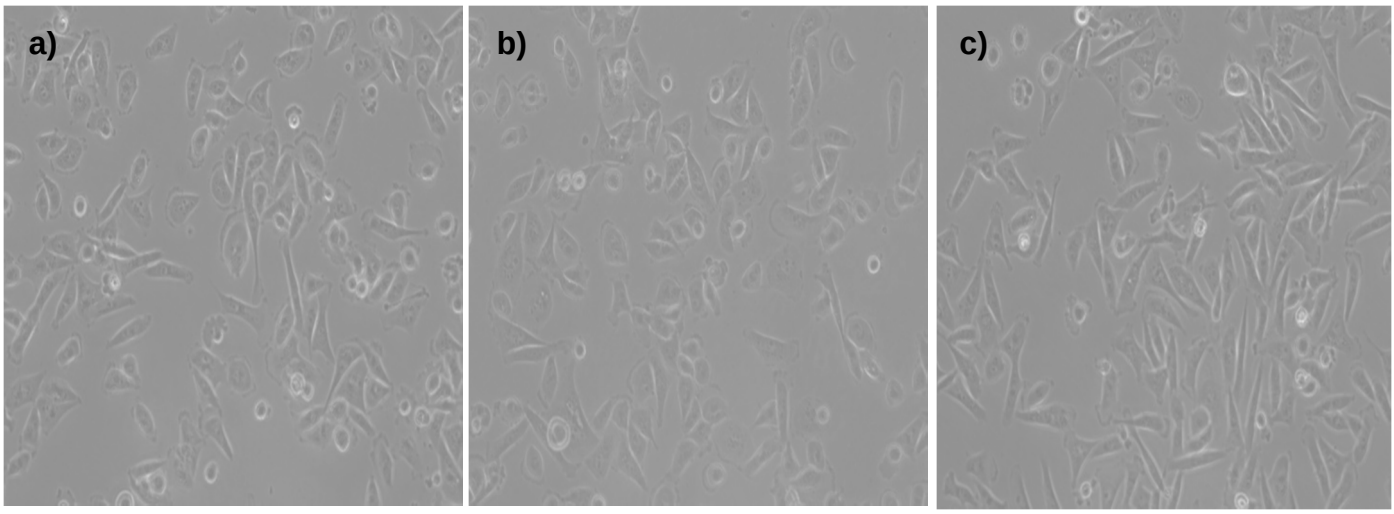


Figure 4.26 **Representative images of the MDA-MB-231 cells after 4 hours incubation: a) control cells (10% FBS), b) positive control (10% FBS + 200mM $CoCl_2$), and c) hypoxic cells (1% FBS + 4mins hypoxic gas). A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.**

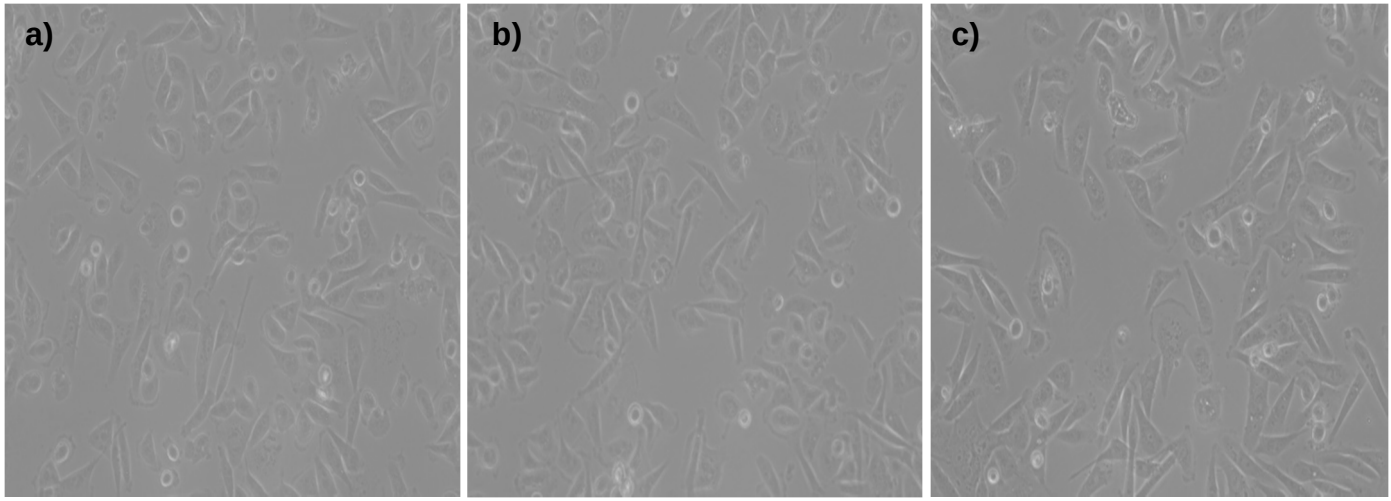


Figure 4.27 Representative images of the MDA-MB-231 cells after 6 hours incubation: a) control cells (10% FBS), b) positive control (10% FBS + 200mM $CoCl_2$), and c) hypoxic cells (1% FBS + 4mins hypoxic gas). A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

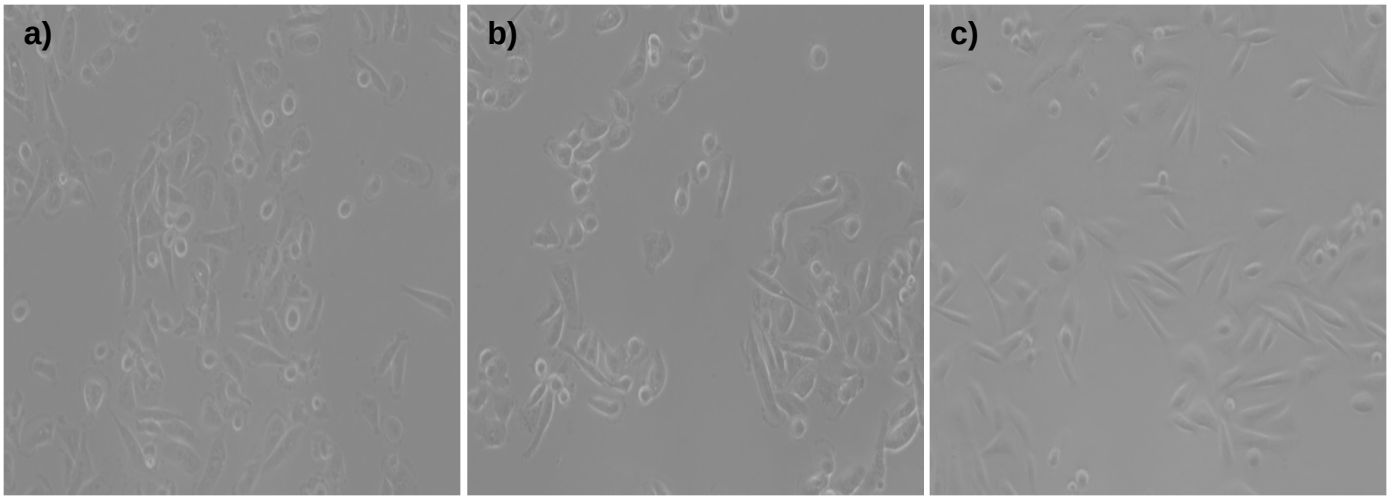


Figure 4.28 Representative images of the MDA-MB-231 cells after 8 hours incubation: a) control cells (10% FBS), b) positive control (10% FBS + 200mM $CoCl_2$), and c) hypoxic cells (1% FBS + 4mins hypoxic gas). A Euromex DC.3000C CMEX camera microscope was used. Magnification = 100x.

There appears to be no obvious visible difference in number or morphology of cells following hypoxic incubation and the addition of $CoCl_2$ relative to control plates.

4.4 Phosphatase Activity Assay

4.4.1 Method Overview (described in detail in Chapter 2)

Following incubation of cells for the different time points, cell lysates were prepared for the purpose of the phosphatase activity assays as described in detail in chapter 2. Protein determination of each sample was calculated using the Bradford assay method (Bradford, 1976) and samples were prepared to a protein concentration of 400µg/µl.

The PP2A catalytic subunit was then isolated from these lysates using immunoprecipitation. Samples were incubated in a rotating wheel overnight at 4°C with PP2A-C antibody. Following this, 50µl of previously prepared agarose bead slurry was added to each sample with a further four hour incubation at 4°C in a rotating wheel. Samples were then washed and the bead-antibody pellet re-suspended in assay buffer (Table 2.4) and used to prepare 96 well plates for the activity assay. An EnzCheck® phosphatase assay kit (Molecular Probes, E12020) was used for the detection of fluorescent product, correlating to the amount of phosphatase activity present and samples were measured every 5 minutes over a period of approximately 2 hours using a BMG Labtech FLUOstar Omega Plate reader.

Data was analysed with the help of a biostatistician at the Stellenbosch University Biostatistics Unit, Centre for Evidence-based Health Care. Phosphatase assay experiments were repeated for each time point (2, 4, 6 and 8 hours respectively), 3 times over, on separate days (n=3). The rate of change for each group at each time point was measured as well as the time-by-group effect. A repeated measures ANOVA was conducted, however no significant differences were observed. Western blotting (as previously described) was carried out to confirm immunoprecipitation of PP2A in the samples, whereby non-immunoprecipitated cell lysates were blotted against immunoprecipitate samples and membranes were probed for both total PP2A protein and protein phosphatase 1 (PP1). This was done to confirm that the immunoprecipitation was successful and per implication that the phosphatase activity measured was indeed primarily due to PP2A.

4.4.2 Phosphatase Activity Assay Results

The degree of DiFMUP hydrolysis within the immunoprecipitated samples was measured over approximately 2 hours. The fluorescent signal detected thus provided an indication of the amount of phosphatase activity within each sample. Experiments were repeated three times for each hypoxic incubation period (2, 4, 6 and 8 hours) and averages used to plot graphs for the signal generation over time.

Western blotting of immunoprecipitated samples was conducted to observe whether PP2A had in fact been successfully precipitated in the samples. Figure 4.29 shows the presence of PP2A in immunoprecipitated samples as well as present in non-immunoprecipitated samples prepared prior to immunoprecipitation. Blotting for PP1 was also carried out as a means to observe whether the activity measured in the assay was not contaminated by other major Ser/Thr phosphatases, such as PP1, suggesting that what was measured in the assay, was in fact, PP2A (Figure 4.30). Thus, successful immunoprecipitation was confirmed by the presence of PP2A but not PP1 in immunoprecipitate samples relative to non-immunoprecipitated samples.

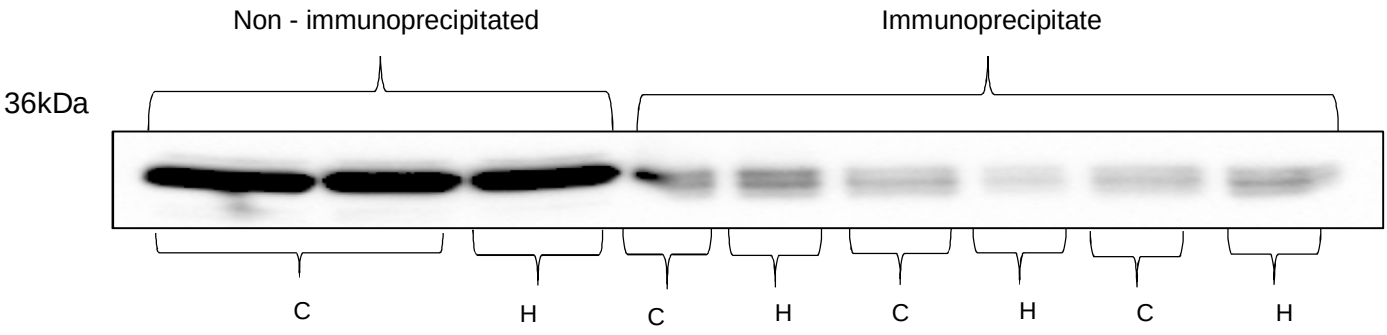


Figure 4.29 Representative Western blot of PP2A, confirming the immunoprecipitation of PP2A in samples used for phosphatase assay. Clearly visible bands present at 36kDa in immunoprecipitation samples. C = control samples, H = Hypoxic samples.

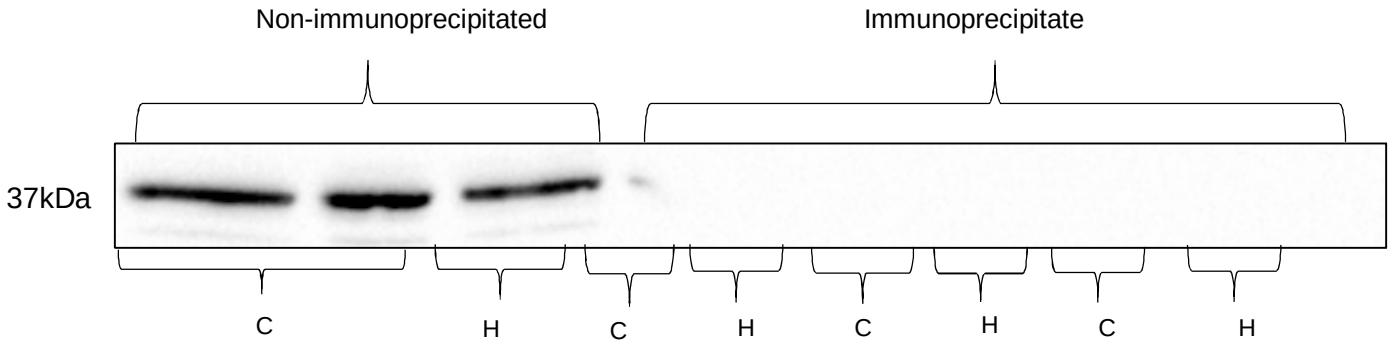
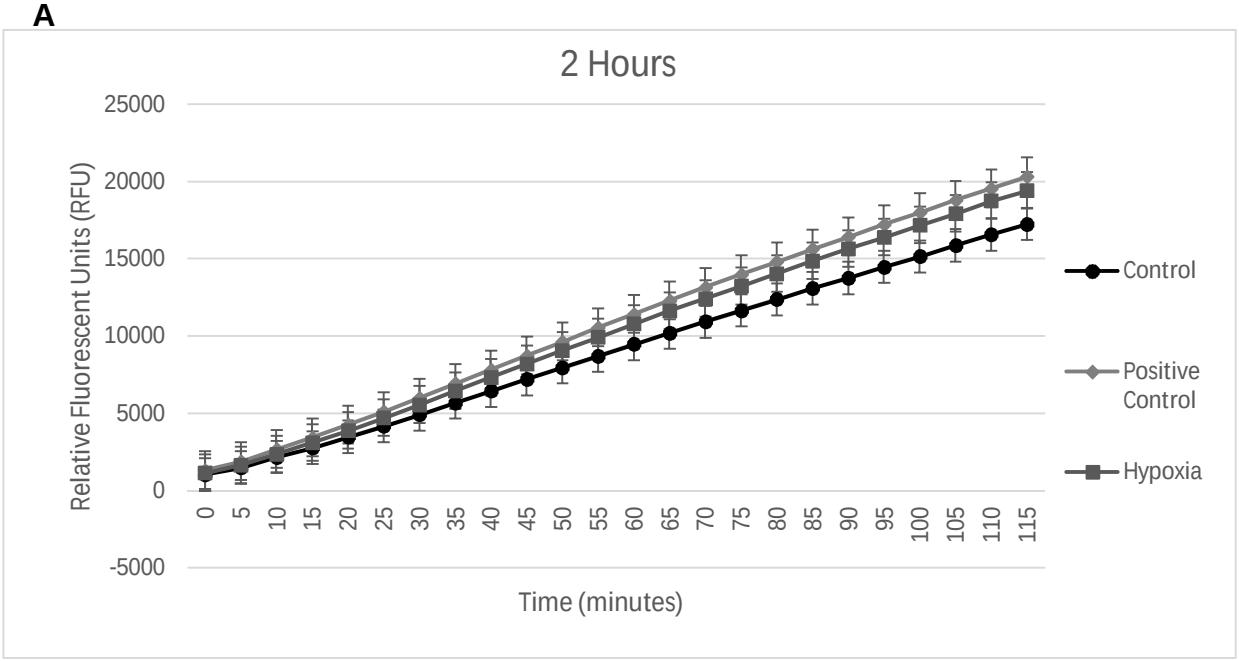


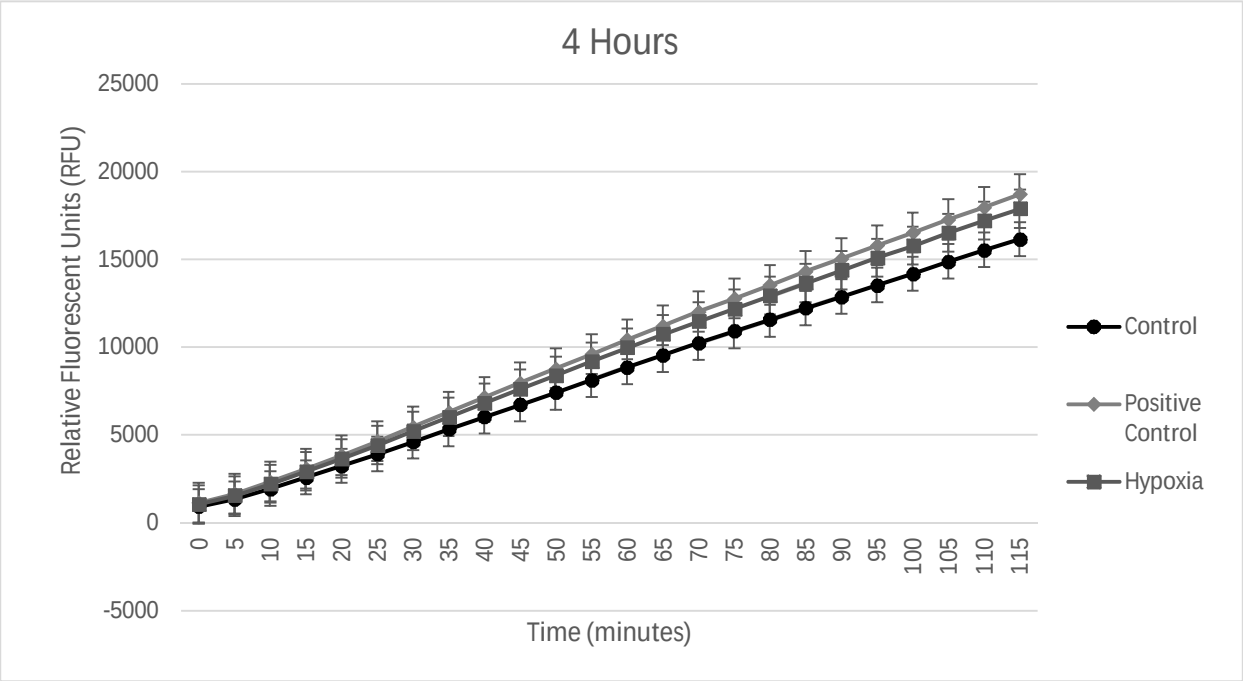
Figure 4.30 Representative Western blot showing the presence of PP1 in the non-immunoprecipitated samples but not in the immunoprecipitatied samples. C = control samples, H = Hypoxic samples.

From the graphs in Figure 4.31, one can see that the signal generation over time, for each group and for each hypoxic incubation time, remained statistically constant with no significant differences found relative to control.

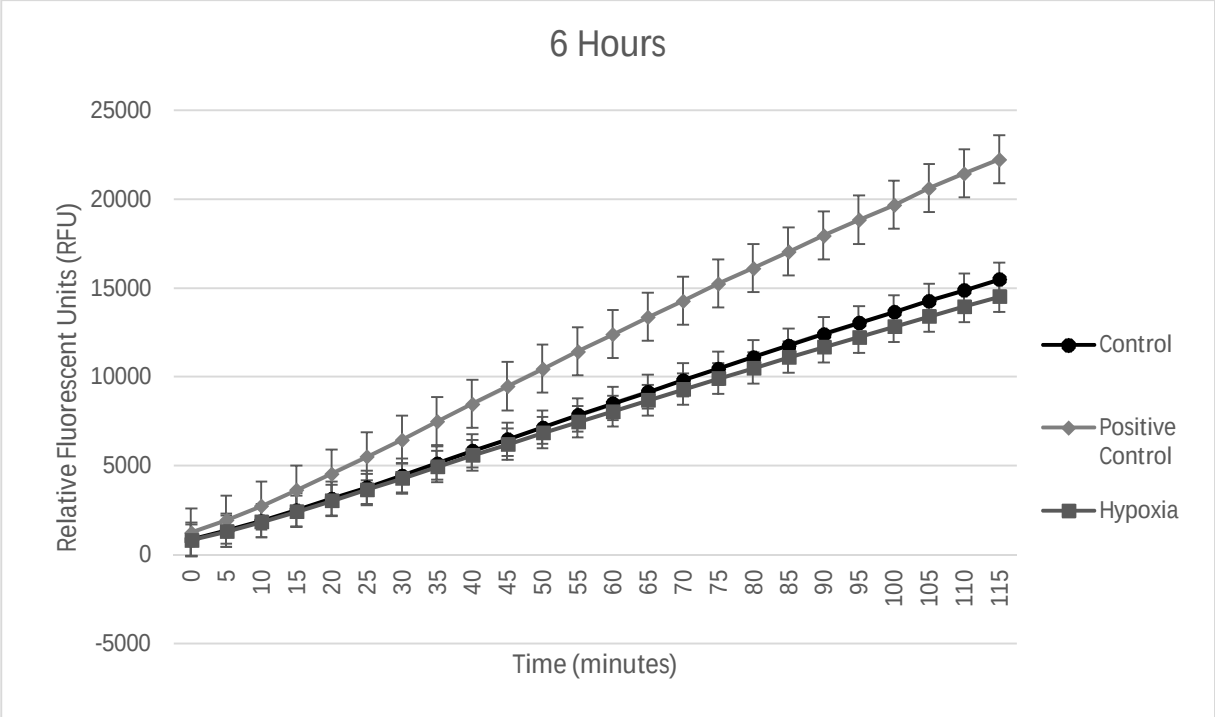
A



B



C



D

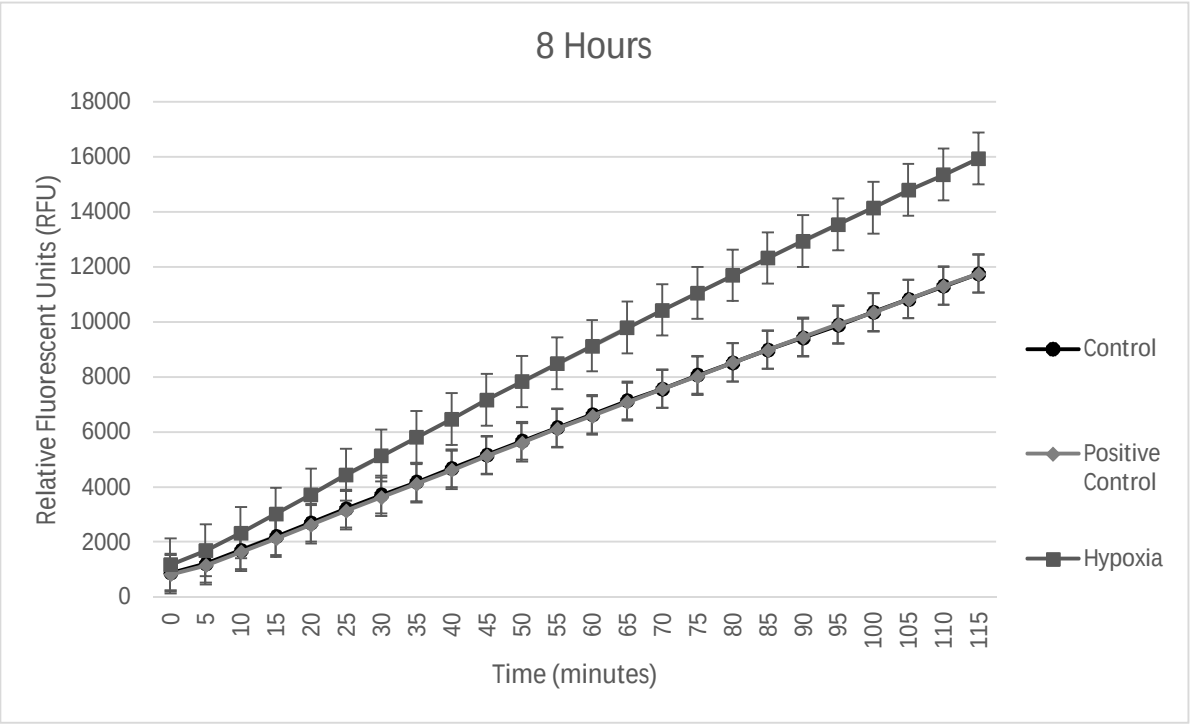


Figure 4.31 Average signal generation over time. Samples exposed to A) 2 hours hypoxia, B) 4 hours hypoxia, C) 6 hours hypoxia and D) 8 hours hypoxia respectively. (n=3). The distribution of absolute change is the same across the groups. ANOVA's and Mann-Whitney post hoc tests confirmed no significant differences.

The average velocity of the reactions was then calculated as the absolute change in signal generation over 115 minutes and expressed as fluorescent units (FU) per minute (Figure 4.32). This was further analysed for each hypoxic incubation time point. 1-way ANOVA's were conducted with Dunnett post hoc tests. The velocity of the reactions of hypoxic samples as well as positive control samples showed no significant changes from that of control for the 2, 4 and 8 hour hypoxic times, however a significant increase was observed in the positive control group following 6 hours hypoxia, relative to control (control: 134.7 ± 33.74 AU vs positive control: 193.6 ± 35.31 AU; n=2; $p \leq 0.05$). However, for this time point, only 2 experimental sets of data were used (n=2) due to the third repeated experiment providing too little protein in the sample to enable immunoprecipitation experiments. Interestingly, following 8 hour hypoxia, there appears to be a visual increased rate of change in hypoxic samples relative to control, contrary to the trend observed in the previous three time points. However, this was not statistically significant (control: 102.2 ± 0.7696 AU vs hypoxia: 138.6 ± 50.82 AU; n = 2; p = 0.6018). Data for this time point was also constricted to two sets of experimental repeats as too little protein was present in cells of the third

repetition to allow for immunoprecipitation and assay. Thus, it is possible that with an n of 3, significant differences may have been observed after 8 hours hypoxia.

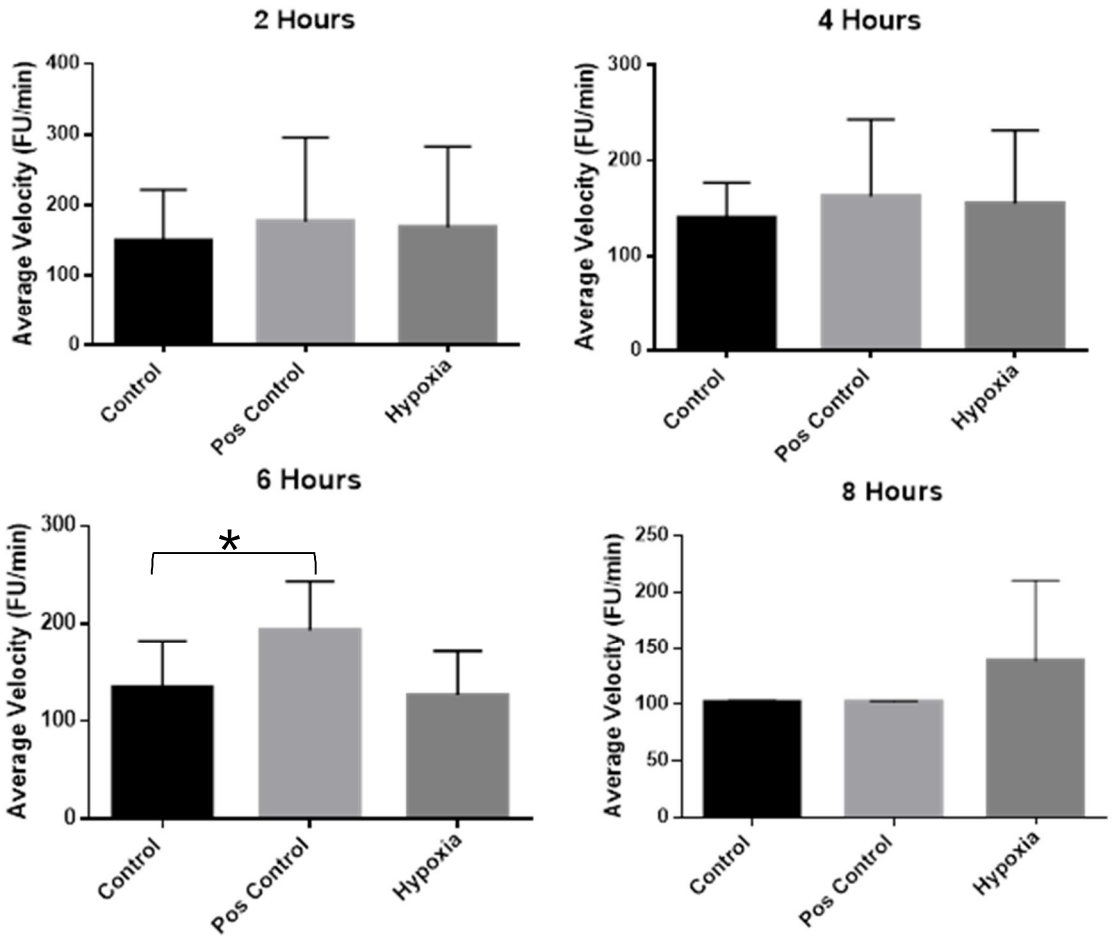


Figure 4.32 Average velocity (change in signal generation over time) for each time point measured. A significant change was only observed at 6 hours for positive control relative to control. * = p≤0.05

With the aid of the Biostatistics Unit, Centre for Evidence-based Health Care at Stellenbosch University, repeated measures ANOVA's were conducted to determine the effect of time on each group. However, insufficient residual degrees of freedom were detected and thus no statistical conclusions could be drawn from the graphs (Figure 4.33 and 4.34). Because of this, data was pooled to possibly provide an indication of whether hypoxia in general, had any effect on the activity of PP2A. The slopes of the graphs of hypoxia vs control (Figure 4.35) and positive control vs control (Figure 4.36) were compared; however, no statistical significance was found, indicating that regardless of the time intervention, hypoxia did not appear to have any effect in the rate of change of activity of PP2A over time.

Figure 4.33 and 3.34 are representative comparisons of signal generation of the four different hypoxic incubation durations conducted. The trend is thought-provoking and could prompt the inclusion of longer hypoxic incubation periods, possibly 12 and/or 16 hours, for future experiments. Furthermore, an increase in n-number would be beneficial as well as possibly a comparison between the three different runs conducted on one single plate. This however, would be logistically challenging and would cost some experimental compromise.

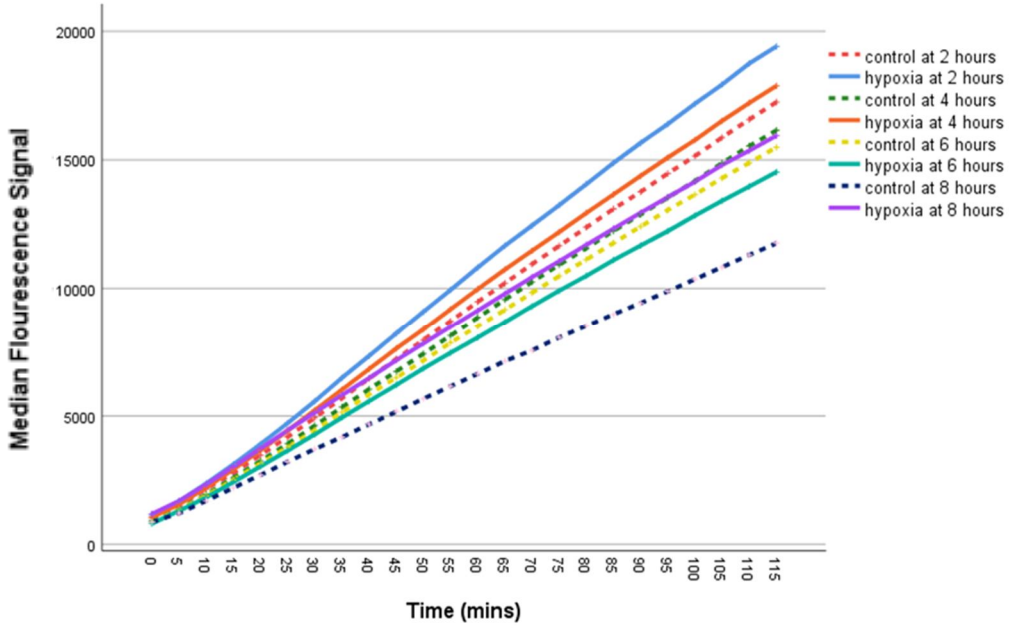


Figure 4.33 A representative comparison of the signal generation of the different hypoxic incubation times with corresponding controls. Fluorescence appears to increase over time.

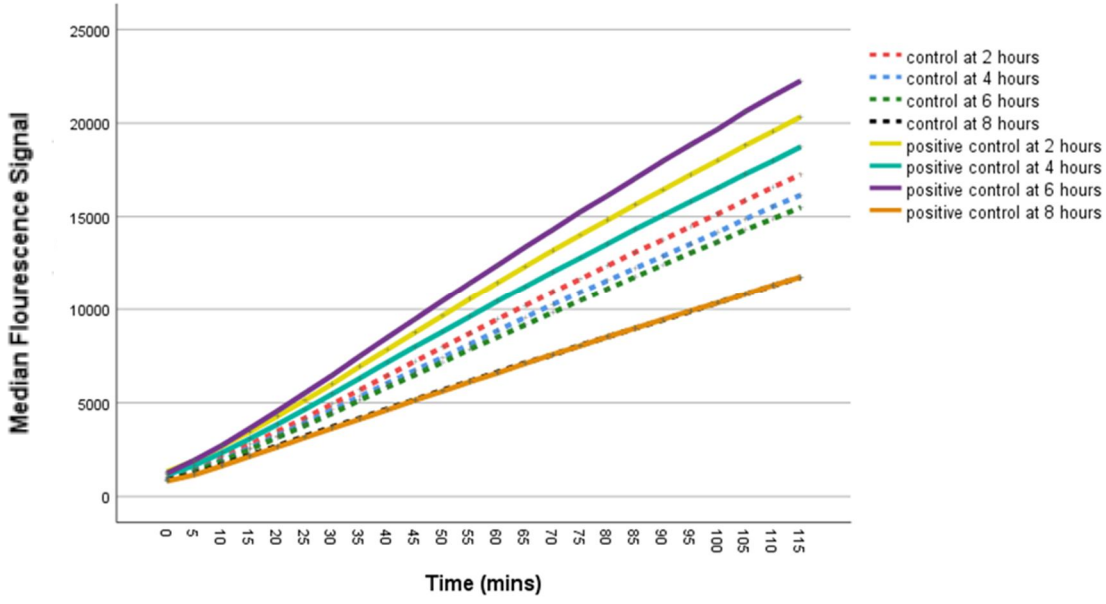


Figure 4.34 A representative comparison of the signal generation of the different incubation times for control and positive control samples.

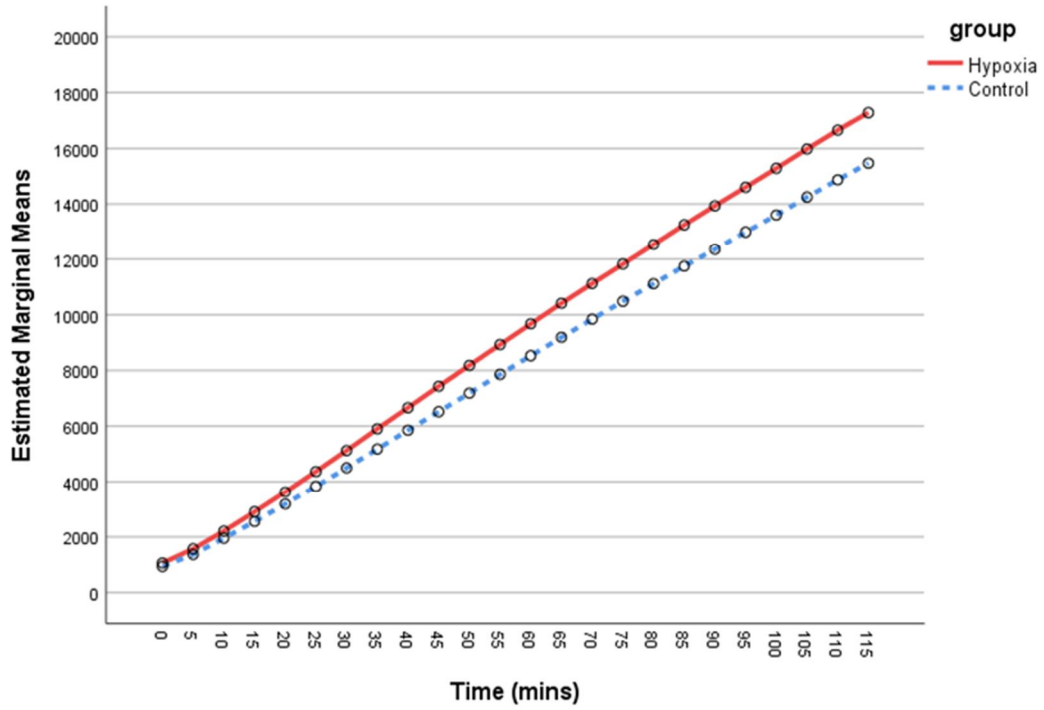


Figure 4.35 A repeated measures ANOVA conducted for control groups vs hypoxia groups showed no significant differences between the groups over time. p=0.586

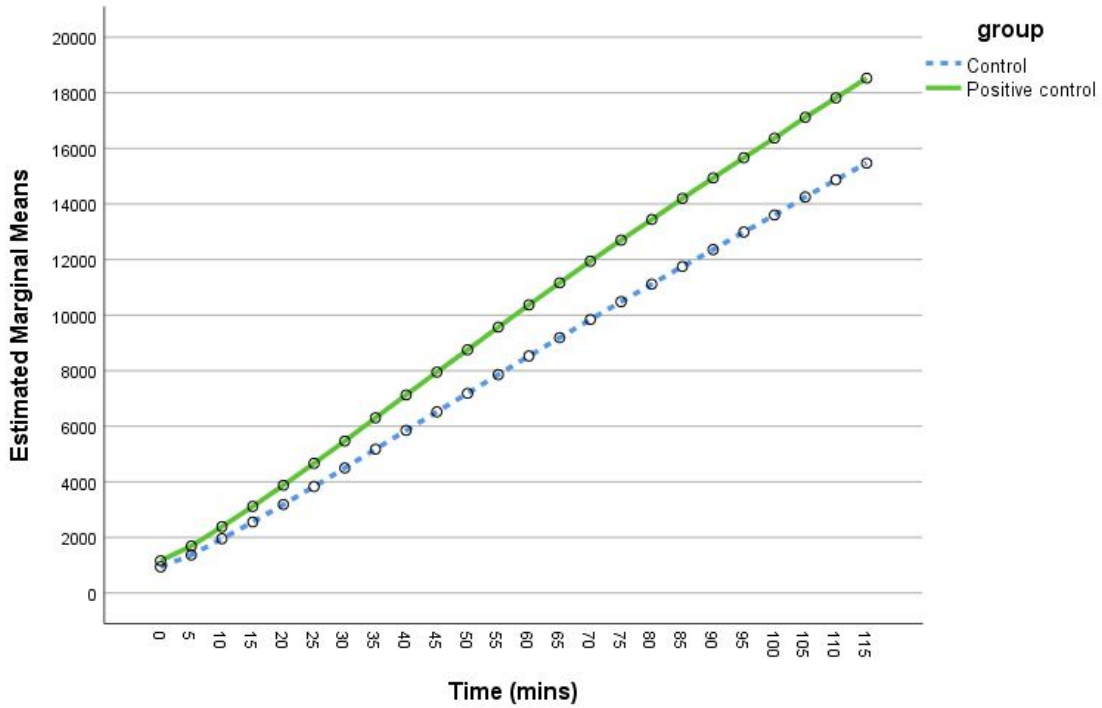


Figure 4.36 A repeated measures ANOVA conducted for control groups vs positive control groups also showed no significant differences between the groups over time. p=0.381

From the data generated and the statistics performed, we saw that there appeared to be no association of phosphatase activity with an increase in hypoxic exposure relative to controls. The rate of change of controls, positive controls and hypoxic groups showed no differences and the effect of time within each group also confirmed that no significant changes occur within each group over time. We are thus compelled to accept the null hypothesis that hypoxia does not have an effect on PP2A activity.

4.5 ATP Cell Viability Assay

4.5.1 Method Overview (described in detail in Chapter 2)

MDA-MB-231 breast cancer cells were seeded and grown in 96 well plates under standard conditions to a confluency of ~80% whereupon plates were exposed to varying lengths of hypoxic incubations as described in detail in chapter 2. Control cells were seeded onto separate 96 well plates and incubated at 37°C for the corresponding time points measured. A CellTitre-Glo® luminescent cell viability assay kit (Promega G755A) was used to measure the amount of ATP present in the wells, providing an indication of the degree of metabolic activity of the cells in each well. An increase in luminescent signal correlates to an increased level of ATP production indicating increased viability of the cells.

100µl of the CellTitre-Glo® reagent was added to each well by hand and a BMG Labtech FLUOstar Omega Plate reader was used determine the luminescence generated from each well. Data generated was analysed using paired T-Tests on GraphPad Prism6 and presented as means ± SEM with $p \leq 0.05$ considered statistically significant.

4.5.2 ATP Cell Viability Assay Results

Figure 4.37 shows a representative control plate standard curve of ATP signal generation measured, and confirming the linearity of the measurements in this assay. We did not however make use of the standard curve to calculate ATP levels as our primary concern was the relative change, relative to control. The luminescent signal measured alone is sufficient in this regard.

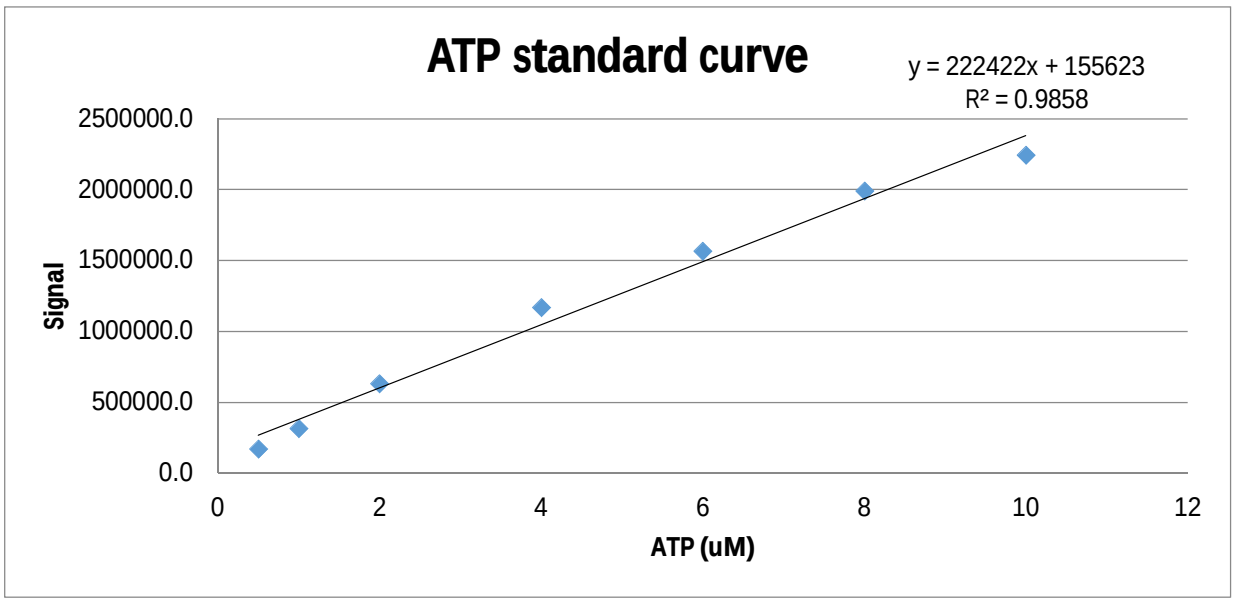


Figure 4.37 Representative ATP standard curve indicating the linear measurement of ATP.

Data collected from three separate runs of each time point (n=3) were analysed as averages of each run. Paired T-tests indicated that no significant changes in ATP levels were present in the hypoxic samples relative to control (Figure 4.38). However, when each repeated run for each time point for was analysed independently, an interesting visual trend of decreased ATP following 4 and 6 hour hypoxia relative to control were detected (Figures 4.39 and 4.40). Although no statistical conclusions can be drawn from this observation, it is still pertinent to note the degree of repeatability present. Four wells of cells per time point per run were measured for both hypoxic and control plates. These individual analyses provide clearly visible trends in the data which is lost due to too much variability when the data is pooled and expressed as averages for each time point.

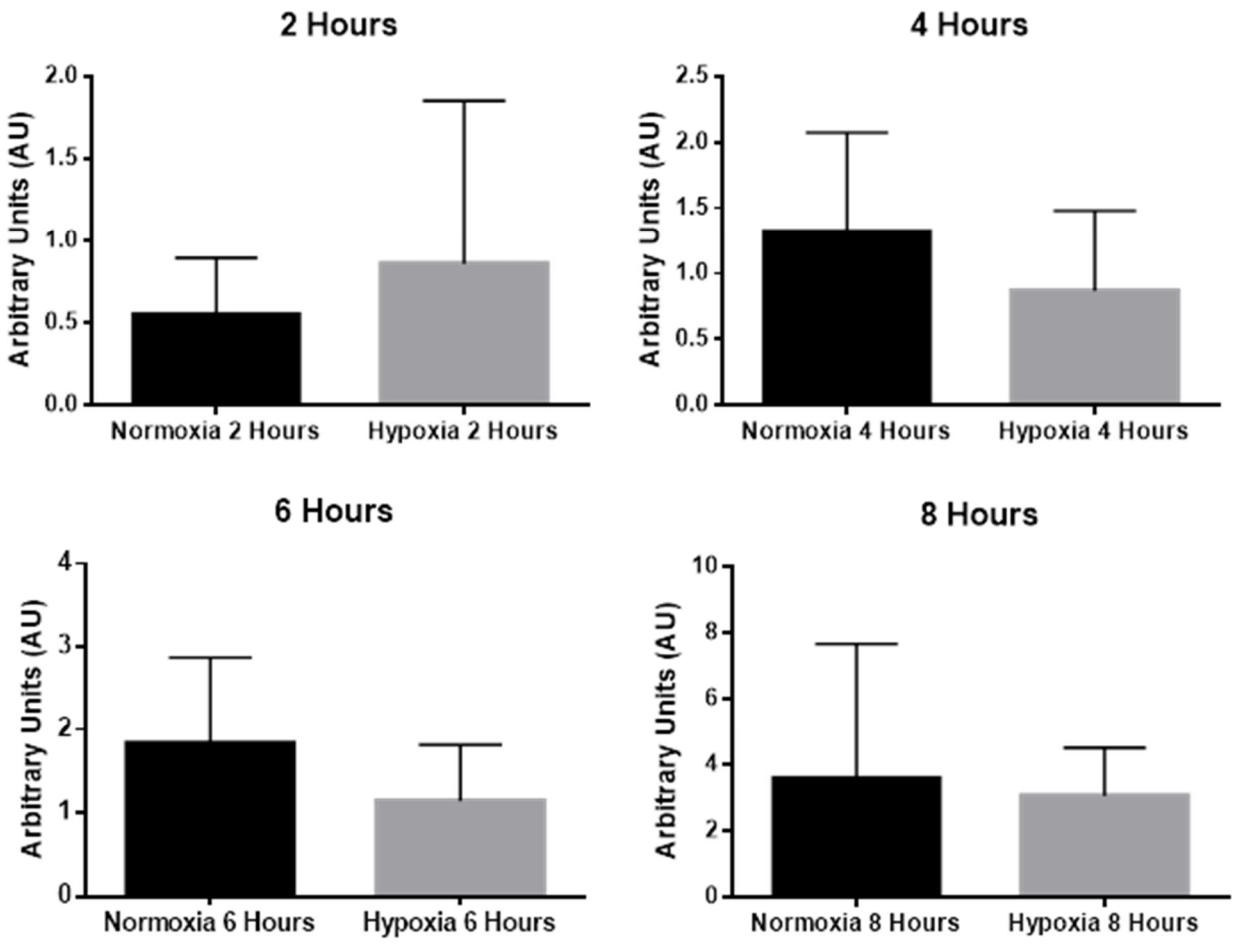


Figure 4.38 Paired T-tests indicated no significant changes in ATP levels for hypoxia samples relative to control for each time point measured. (n=3).

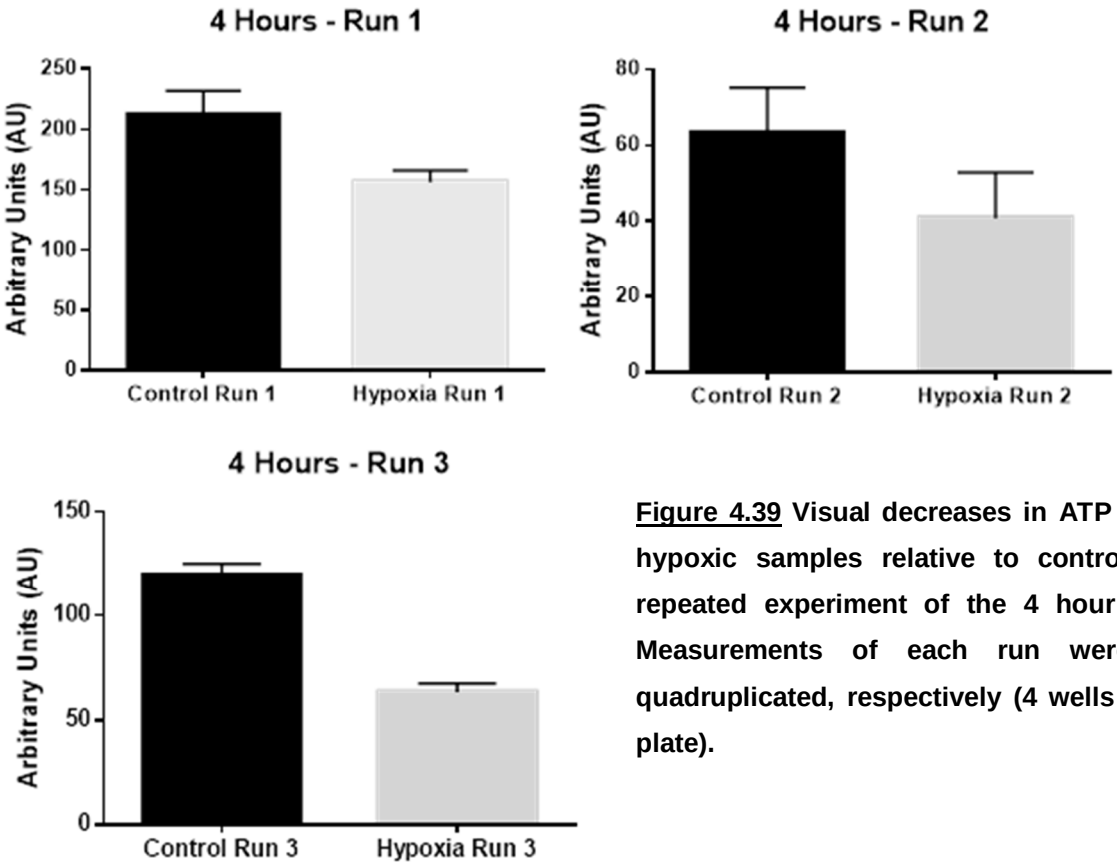


Figure 4.39 Visual decreases in ATP observed in hypoxic samples relative to controls for each repeated experiment of the 4 hour time point. Measurements of each run were done in quadruplicated, respectively (4 wells of cells per plate).

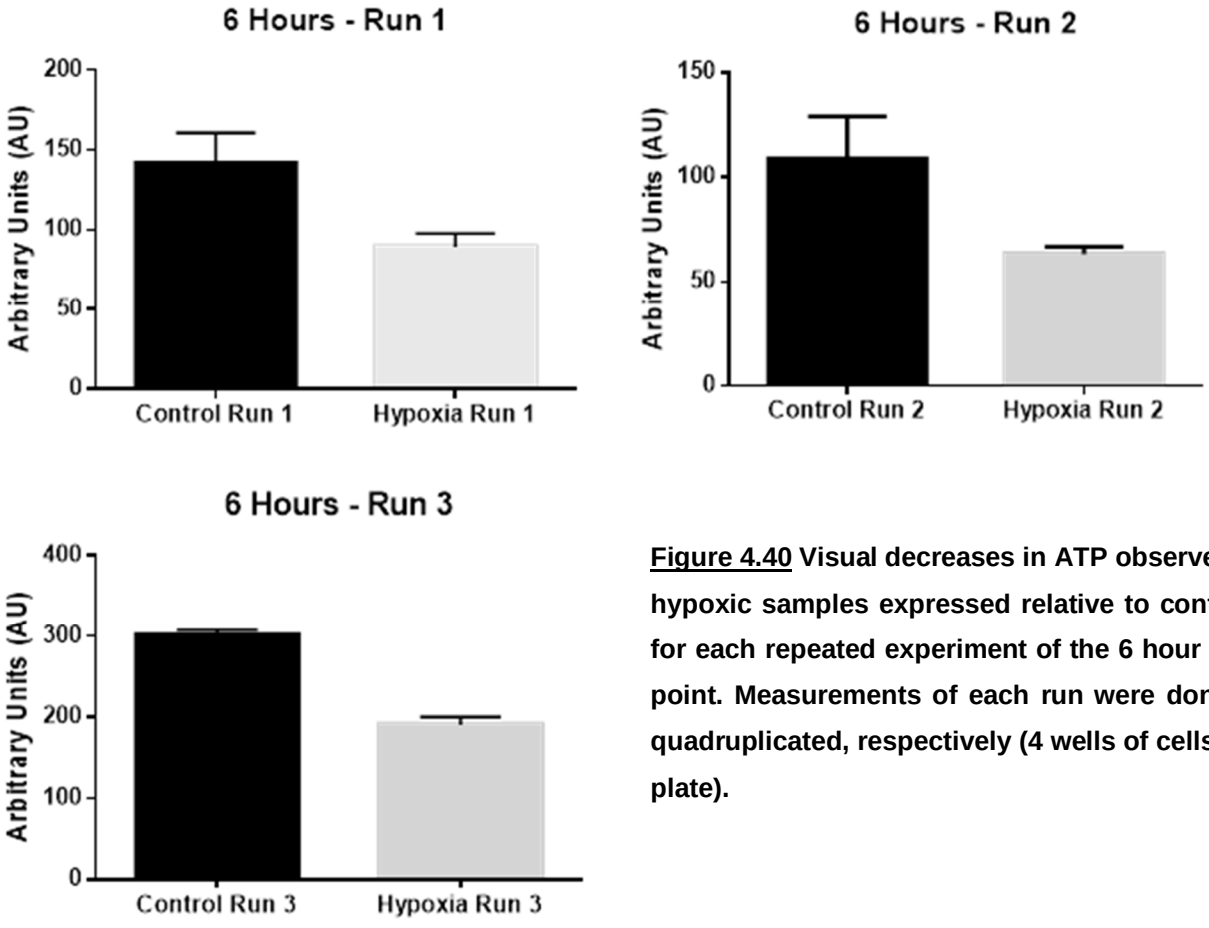


Figure 4.40 Visual decreases in ATP observed in hypoxic samples expressed relative to controls for each repeated experiment of the 6 hour time point. Measurements of each run were done in quadruplicated, respectively (4 wells of cells per plate).

It is thus important to note that consistent results were only observed following 4 and 6 hours hypoxia. This indicates that the data was more robust at these time points. To investigate the variability seen following 2 and 8 hours hypoxia, we would need to increase the n-number of the experiment.

Although the above analyses of the individual runs showed notable and repeated visual changes, when pooled together, the variation in the data lead to a finding of no statistical significance as seen in Figure 4.38.

4.6 Discussion

Previous reports in literature have shown that cancer cells are able to adapt and survive in conditions of hypoxia, with several studies suggesting that the inhibition of PP2A has been implicated in carcinogenesis and drug resistance, as well as in hypoxia (Ratnasinghe, Phang and Yeh, 1998). This led to the formulation of our hypothesis that hypoxia would induce a decrease in PP2A activity, thereby favouring the phosphorylation and activation of enzymes associated with survival and proliferation.

In a study conducted by Lin *et al*. involving tumour initiating or stem cell (TIC's), it was reported that PP2A activity is suppressed in these TIC's in a variety of cancers. Their experiments made use of TIC's cultured in 1% oxygen for 2, 4 and 6 days. Analysis of apoptosis assays indicated a large degree of cell death accompanying hypoxia, while phosphatase activity assays and Western blotting for PP2A found a decrease in PP2A activity with an increase in PP2A phosphorylation following hypoxia. These results combined with their results from cell migration and invasion assays, PCR, and flow cytometry analyses, led to the conclusion that suppression of PP2A leads to independent cell growth and tumour formation, essential factors for tumour initiation. In essence, PP2A suppression may promote tumour initiation (Lin *et al.*, 2012). In an earlier study conducted by Chen *et al*., mutations in the Aα subunit of PP2A were introduced to immortalised cells, which led to defects in its binding to the other PP2A subunits, thereby impairing PP2A activity. Suppression of the expression of this subunit favoured the activation of the PKB survival pathway promoting tumour formation. Immunoprecipitation with phosphatase activity assays, apoptosis analysis and proliferation assays showed that over-expression of Aα

mutations and suppression of PP2A phosphatase activity lead to enhanced tumour formation. The same researchers also investigated PKB phosphorylation in these cells and found increased levels of pPKB in the cells expressing the Aα mutations, however, increased levels of pPKB were not detected in wild type PP2A cells. The results from their experiments further identified that 50% suppression of endogenous PP2A lead to cancerous cell transformation, while further suppression of PP2A lead to cell cycle arrest and apoptosis (Chen *et al.*, 2005).

4.6.1 Western Blotting

At the onset of this research project, it was decided to investigate a hypoxic incubation period of 72 hours based on previous studies that had conducted similar hypoxic experiments, some ranging from 6 hours incubation (Prior *et al.*, 2014; Xie *et al.*, 2016), to 18 hours (J. Park *et al.*, 2015), 24 hours (Nagelkerke *et al.*, 2013), 48 hours (Cronin *et al*., 2010) and 72 hours (Ren *et al.*, 2013; Bousquet *et al.*, 2015). Some studies even went so far as to incubate cells for between 9-21 days of hypoxic exposure (Coyle, Izzo and Chu, 2009; Vaapil *et al.*, 2012). Thus we anticipated that after 72 hours of hypoxia, we would be able to measure some degree of the effect of hypoxia on PP2A. We made use of a hypoxic gas mixture of 0.5% O_2, 5% CO_2 and the balance, N_2, and cells were incubated at 37°C in a humidified atmosphere. However, after the 72 hour incubation period, cultures had decreased in cell numbers, with shrunken, smaller morphologies. Western blotting analysis for total PP2A (tPP2A) showed a significant decrease, while blots for phosphorylated PP2A (pPP2A) and methylated PP2A (mPP2A) showed no significant changes following hypoxia relative to control (Figure 4.1 – 4.4). The ratios p/t PP2A and m/t PP2A further showed no significance. We also blotted for HIF-1α as it is a known and predominant transcription factor involved in cellular adaptation to hypoxia (Brahimi-Horn *et al*., 2007; Badowska-Kozakiewicz *et al*., 2015). From the analysis we saw an unexpected decrease in HIF-1α in hypoxia relative to control (Figure 4.5). Although this decrease was not statistically significant, it was an interesting observation as reportedly, HIF-1α is stabilised, activated and elevated under conditions of hypoxia (Bertout *et al*., 2008; Chandel and Simon, 2008; Ruan *et al*., 2009). Variations in the data, temperature fluctuations as in the case of HIF-1α, or sub-optimal Western blotting and antibody binding may have had an influence over these results.

Pro-survival pathways such as that leading to the activation of PKB, have been reported to be activated in many types of cancers through either amplification of associated genes, or through mutations of components of the signalling pathways, disrupting normal signalling and favouring cell proliferation and survival (Nicholson and Anderson, 2002; Yung and Tolkovsky, 2003; Song *et al*., 2005; Furuta *et al.*, 2008). Lawlor and Alessi described PKB as a critical role player in cancer progression, through the stimulation of cell proliferation along with the inhibition of proteins that mediate apoptosis (Lawlor MA, 2001). Under normal cellular conditions, PKB is tightly regulated in order to inhibit pro-survival pathways and retard growth, however, in human cancers, this regulation has been reported to become de-regulated (Bellacosa *et al.*, 1995; Carpten *et al.*, 2007; Kuo *et al.*, 2008). In its activated, phosphorylated state, PKB directly phosphorylates transcription factors controlling expression of pro-and anti-apoptotic genes, leading to the negative regulation of factors promoting death gene expression, as well as positively regulating factors that induce survival genes. This promotes the development of angiogenesis, neovascularization and evasion of apoptosis, ultimately promoting tumour progression (Nicholson and Anderson, 2002).

The ubiquitously expressed phosphatase, PP2A, has been found to actively dephosphorylate and inhibit the activity of PKB for regulation of cell survival pathways (Manning and Cantley, 2007). However, regulation of PP2A in hypoxia has been shown to be abnormal, or dysregulated, contributing to cancer progression through the transformation of cells and the upregulation of phosphorylated PKB and ERK alike (Eichhorn *et al*., 2009; Ruvolo, 2016). We therefore decided to further blot for total PKB (tPKB), phosphorylated PKB (pPKB), total ERK (tERK) and phosphorylated ERK (pERK) after 72 hours hypoxia to gain a better indication of the effect of hypoxia on whether the MDA-MB-231 cells were adapting to hypoxia, or were in fact dying. When analysed, the results showed a significant decrease in pPKB with hypoxia relative to control (Figure 4.7), as well as significant decreases in the phospho-to-total ratios of both PKB and ERK with hypoxia relative to control (Figures 4.8 and 4.11 respectively). This indicates that there is less phosphorylated (active) PKB and ERK in comparison to the amount of total protein present during hypoxia, and suggests that these survival proteins are possibly being inactivated through dephosphorylation. This result was unexpected as we anticipated to find an increase in activation of PKB and ERK correlating with an increased inactivation of PP2A with hypoxia. This, along with the fact that cell numbers appeared to be decreasing after 72 hours of hypoxia, called into question whether the MDA-MB-231 breast cancer cells were in fact able

to adapt and survive under such a long and potent duration of hypoxia (0.5% O_2 with 1% FSB).

In a study conducted by Xie *et al.* also on MDA-MB-231 cells, they made use of hypoxic incubation times of 2, 6, 12, 24 and 48 hours and cells were exposed to 1% O_2. They measured the effect of hypoxia on proliferation and found that growth curves of control vs hypoxia overlapped at 2, 6, 12, and 24 hours, but separated at 48 hours however, changes were not significant. Cell apoptosis was also measured with results indicating that a significant reduction of apoptotic cells was indicated after 48 hours hypoxia relative to normoxic counterparts. The same group also looked at the expression of stem cell markers, which was significantly increased after 48 hours hypoxia, as well as colony formation which also indicated increased rates at 48 hours of hypoxia. These results obtained by this research group all confirm the notion that hypoxia alters metabolic pathways and promotes cell survival with the inhibition of apoptosis (Xie *et al.*, 2016). Although this study did not investigate signalling pathways as with this current study, the cell line used was the same and the hypoxic incubation times appeared to induce cellular changes within the cells.

Our 72 hour data prompted the revision of our incubation periods to include 24 hours as well as 48 hours exposure to hypoxia in the experimental protocol. However, Western blotting for PKB and ERK along with HIF-1α saw no significant changes in phosphorylation states other than a decrease in pERK 42/44 after 24 hours hypoxia relative to 24 hour control (Figure 4.17). Total PKB and ERK levels showed no significant changes over the 24, 48 and 72 hour experiments relative to control, with the phospho-to-total ratios similarly unchanged. HIF-1α again showed a decreasing trend following 24 and 48 hours hypoxia relative to control, however, an increase following 72 hours hypoxia was observed. This data however, was not statistically significant, although it was surprising and unexpected (Figure 4.12). Upon exposure to oxygen, HIF-1α is typically unstable, difficult to detect and rapidly degraded by hydroxylation (Kaluz *et al*., 2008). We speculate that we may have in fact lost most of the stable HIF-1α through degradation when cells were exposed to re-oxygenation upon opening of the hypoxic chamber. Care was taken to handle cells quickly and all experimental steps following incubation were carried out on ice to slow cellular metabolism and thereby slow the breakdown of this protein. However, the results shows a visual decrease after 24 and 48 hours hypoxia, which opposes the significant increases that were

expected based on literature (Zhou *et al.*, 2006; Bertout *et al*., 2008; Chandel and Simon, 2008; Ruan *et al*., 2009). The hypoxic gas mixture used for the experiments contained only 0.5% O_2, and the hypoxic chamber remained sealed with an air-tight rubber 'o'-ring and metal sleeve (Figure 2.1), thus we are certain that our stimulation of hypoxic conditions was sufficient, and the results we have seen are due to reasons relating to cellular signalling or perhaps technical issues concerning the various steps of the lysate protocol and/or lysate handling and storage. Thus it is possible that we were not able to accurately measure an accumulation of the protein due to re-exposure of the cells to oxygen upon removal from the hypoxic chamber. However, for future studies and peace of mind, measuring the presence of oxygen within the hypoxic chamber using either a specialized probe or dye, would indeed be a very relevant and useful step in the protocol. This would also confirm whether the chamber was sealed correctly and if the gas flow time was sufficient.

The revised time experiments (24, 48 and 72 hours) were only conducted once each (n=1) and are thus seen as purely observational data without statistical power. The trends show a loss of cells with hypoxia, as well as a decrease in activation (phosphorylation) of PKB and ERK at 72 and 24 hours respectively, which does not support the notion that these MDA-MB-231 breast cancer cells are able to display resistance to hypoxia. And further revision of hypoxic incubation times was considered.

In a recent study by Smith *et al.*, the uptake of F-Fluorodeoxyglucose (FDG), a widely used PET tracer, was measured in breast cancer cells mediated via HIF-1α. FDG enters cells via glucose transporters (GLUT) which are frequently seen to be upregulated in cancer cells (Smith *et al*., 2013). Low oxygen levels further enhance FDG uptake in cultured breast cancer cells, thus in order to measure the uptake, hypoxia was stimulated for 1, 2, 4, 8, 16 and 24 hours respectively. Their results showed that treatment with hypoxia rapidly induced HIF-1 proteins, peaking at between 1- 4 hours with levels increasing ~10 fold in MDA-MB-231 cells. Increased FDG uptake was detected after a mere 1 hour hypoxic incubation and reached a maximum after 4 hours, thereafter decreasing to base line by 16 hours. The rapid increase and peak between 1-8 hours hypoxia is consistent with a similar study by Burgman *et al.* in 2001, also on breast cancer cells (Burgman *et al.*, 2001). These results further suggest that shorter periods of hypoxic incubation appear to have a significant effect on some cellular signalling pathways, including that of HIF-1.

Taking into account that these studies made use of hypoxic incubation times far shorter than those previously used in this study, we decided to further re-adjust the project protocol to include 2, 4, 6 and 8 hours of hypoxia. These experiments were repeated three times for each time point (n=3). As these time points were decided on to focus on the aim of the project, namely PP2A, we did not continue blotting for PKB and ERK. Western blotting for tPP2A, pPP2A and mPP2A (Figure 4.20-4.24) showed no significant changes with hypoxia relative to control. Blots for HIF-1α (Figure 4.19) also showed no significant changes, however, the trend appears to now show an increase in the expression of HIF- α with hypoxia at 4, 6 and 8 hours, relative to control. Although not statistical, this trend is closer to what was initially expected. Furthermore, the positive control for HIF-1α follows an increasing trend with an increase in hypoxic incubation time, reaching a maximum after 4 hours. This does somewhat cast some doubt on the efficacy of our hypoxic intervention, although large variations do exists in the data. This could be contributed to the high instability of HIF-1α and the technical difficulties experienced in working with the enzyme due to rapid degradation upon re-oxygenation following hypoxic incubation.

Figures 4.25-4.29 show images of MDA-MB-231 cells in culture under control (normoxia), positive control (supplementation with 200 µM $CoCl_2$) and hypoxic (0.5% O_2) conditions, for each time point. It can be seen that not much variation exists between number and morphology of cells for each condition. Experiments were conducted when confluency was estimated to be 70-80%, thus an amount of discrepancy may exist between plates of different experiments/time points due to human error on different days. Xie *et al.* noted that hypoxia could inhibit the growth of MDA-MB-231 cells very slightly, but this inhibition is not significant relative to control cells cultured under normoxia (Xie *et al.*, 2016).

From the analyses conducted above, there proved to be a surprising and unexpected lack of significant changes in PP2A, and unexpected decreases in phosphorylation of pro-survival proteins PKB and ERK following 72 and 24 hours hypoxia respectively. There also appears to be no link or evidence suggesting an association between PP2A and the duration of hypoxia in the MDA-MB-231 breast cancer cell line, at least in our hands.

4.6.2 Phosphatase Activity Assay

Results from the phosphatase assays (n=3) seemed to correlate to and confirm the results obtained from the Western blotting analysis of PP2A, that hypoxia did not show any significant effect on PP2A activity following 2, 4, 6 and 8 hours hypoxic incubation. Fluorescence was measured every 5 minutes over a period of 2 hours for both hypoxic and control cells for each of the four incubation times. One way ANOVA analyses showed that no significant changes were observed within each group (incubation time) for control vs hypoxia, thus no rate of change differences were observed. Phosphatase activity during hypoxia did not significantly increase over time, relative to control (Figure 4.29). The average velocity for each time point was also analysed and the only significant change observed was seen in positive control samples relative to control after 6 hours (Figure 4.30). The trends however, observed in Figure 4.31 (D), Figure 4.32, Figure 4.33 and Figure 4.34, indicated an increase in PP2A as hypoxia continues. This could suggest further investigations are needed with increased n-numbers, increased durations of hypoxia, or attempting a different approach to measuring PP2A activity.

Western blots were performed with whole cell lysates alongside immunoprecipitated samples used in the phosphatase assay, probing for PP2A and PP1 respectively. Figure 4.29 shows the representative blot for PP2A with clearly visible bands across the blot at 36 kDa. This confirms the presence of PP2A in both the non-immunoprecipitated and the immunoprecipitated samples, indicating that the protocol had been successful. Blotting for PP1 as shown in Figure 4.30 served to prove that other phosphatases were not present in the immunoprecipitate and that the activity measured in the assay was not contaminated by other major Ser/Thr phosphatases. This suggests that the lack of change in PP2A activity between control and hypoxia is not due to errors in the protocol but more likely that in fact hypoxia is not influencing activation of the enzyme. However, as a significant decrease in PP2A activity during hypoxia was also not observed, it appears that hypoxia is not leading to the phosphorylation and inactivation of PP2A either. PP2A remains unaffected, converse to our original hypothesis. It is difficult to speculate as to why this is. A phosphatase activity assay conducted on glioblastoma multiforme-derived tumour stem-like cells by Hofstetter *et al.*, showed that at 80% confluency, cells exposed to 6 hours of hypoxia (1% O_2) or 200 µM $CoCl_2$, exhibited significantly marked increases in PP2A activity (Hofstetter *et al.*, 2012). We expected to observe some change in activity levels of PP2A, but instead it appears to remain similar to control conditions.

4.6.3 ATP Cell Viability Assay

Paired T-tests of the results obtained from the ATP cell viability assay indicated that no changes in ATP levels were present following any of the hypoxic time points. However, a degree of variability existed within the data so it was decided to analyse each repeated run of the experiments individually to possibly find a trend. Notable decreases in ATP were consistently detected in hypoxic samples for each of the three repeated experiments of 4 hours and 6 hours incubation respectively (Figures 4.39 and 4.40). The individual analyses pertaining to 4 hours and 6 hours hypoxia, provide a clearly visible trend in the sets of data which is lost due to too much variability when the data is pooled and expressed as averages for each time point (Figure 4.38).The fact that the same trends were measured on each of these individual runs for 4 hours and 6 hours, alludes to the robustness and the repeatability of the observations. This decrease in ATP at 4 hours and 6 hours, suggests that at these time points, cells are less metabolically active than their control counterparts, or that possibly there are less viable cells present. Yung *et al.* found in their ATP analyses following oxygen and glucose deprivation that after the first 4 hours cells still contained approximately 50% of their initial ATP, however after 6 hours, only 8.5% of initial ATP levels remained. Interestingly, this loss in ATP was accompanied by a loss of phosphorylation of kinases ERK and PKB, however these decreases were concluded to be due to glucose deprivation rather than the lack of oxygen (Yung and Tolkovsky, 2003). From this we could speculate that as hypoxia progresses, the activity of the kinases decreases. From our results we observed no changes in PP2A, thus if the interaction between phosphatases and kinases is viewed as a 'balancing act', then even though PP2A activity remained unchanged following hypoxia, it would appear that the 'balance' has shifted in favour of PP2A activity by virtue of a reduction in kinase activity. In our experimental protocol, cells were cultured in high glucose DMEM medium, which does not then explain the decreases in ATP levels in accordance with Yung's results. The decrease in ATP could possibly be contributed to the Warburg effect where the adaptive shift from oxidative phosphorylation to use inefficient glycolysis, produces less ATP (Warburg *et al*., 1927). In a more recent study involving PP2A and hypoxia, it was found that PP2A mediates the decrease, or loss, of metabolic activity along with ATP production during hypoxia. Their conclusion was that hypoxia induced PP2A activity leading to cell cycle arrest and decreased ATP consumption, promoting tumour cell survival (Hofstetter *et al.*, 2012).

4.6.4 Summary

Although all of these results do not tie in with our initial hypothesis which states that we anticipated a decrease in PP2A activity, favouring the activation of pro-survival pathways, it is interesting to note that controversy over the role of PP2A in cancer exists in literature, and there are research groups who report opposing results and propose different conclusions as to its role during hypoxia. In an article published in 2012 by Hofstetter and colleagues, evidence suggests that hypoxia in actual fact may induce PP2A, rather than inhibit it, leading to cell survival through G1/S phase arrest of the cell cycle. Their research was conducted on glioblastoma multiforme-derived tumour stem-like cells, and hypoxia was stimulated with 1% oxygen or the use of 200 µM $CoCl_2$. Results showed that PP2A activity was positively correlated with increased HIF-1α expression, and that its activity was induced after 6 hours of hypoxia, leading to the mediation of G1/S phase growth inhibition and a reduction in the consumption of ATP. This hypoxia-induced PP2A activity therefore was seen to decrease the cells' metabolic activity by limiting proliferation and enhancing survival under severe hypoxic conditions. Experiments were conducted with okadaic acid (OA), a specific inhibitor of PP2A, where it was shown that PP2A inhibition prevented this cell cycle arrest and allowed cells to continue to proliferate, depleting intracellular ATP levels and eventually progressing though apoptosis or necrosis (Hofstetter *et al.*, 2012). In 2015, Carrer and Wellen proposed that cells are able to adapt to hypoxia, or limited oxygen supply, by slowing down cellular processes thereby preserving energy by altering their metabolism to maximise energy gain (Carrer and Wellen, 2015). Studies prior to these have also alluded to the notion that growth arrest and/or necrosis occurs in unvascularised regions where an insufficient supply of oxygen is present (Tannock, 1968; Evans *et al*., 2001). These reports call into question whether our expectation of 'adaptation to hypoxia' was correct. We expected that cells would thrive with an increase in cell survival signalling, but perhaps adaptation could also include sections of the cell population dying off, while the remaining population slows down and only later emerges as resistant, more aggressive cells.

Another earlier study by Yung and Tolkovsky in 2003 on astrocytes, had similar conclusions that in fact PP2A becomes activated during oxygen and glucose deprivation, thereby promoting the dephosphorylation and inactivation of survival proteins PKB and ERK. Their research showed that erasure of ERK and PKB phosphorylation, thereby inactivating these enzymes, is mediated through the activation of OA-sensitive phosphatases such as PP2A. This is manifested between 6 and 8 hours from the onset of oxygen deprivation, with a

parallel decrease in ATP. However, it was determined that it was the lack of glucose rather than the lack of oxygen that promoted the dephosphorylation of ERK (Yung and Tolkovsky, 2003).

These studies mentioned were conducted on a variety of tumours rather than solely on the breast so one could argue that the cellular signalling could substantially vary. However, the fact remains that there are conflicting reports as to the effect hypoxia has on PP2A and subsequently which signalling pathways become activated or inactivated. La Croix *et al*. also reported that the same cancer cell lines cultured in different laboratories and/or under several conditions can evolve differently, thus giving rise to divergent sub-populations and preventing inter-laboratory comparisons of data on the same cell line (Lacroix *et al*., 2004)

From the Western blotting and phosphatase activity assay experiments, we have seen that PP2A activity did not appear to increase, nor did it decrease. The ATP results suggest that hypoxia is however affecting the cells, specifically at 4 and 6 hours, as ATP levels are decreased relative to control. These decreases however, are thus unlikely to be connected to PP2A activity, and it is more likely that some other signalling pathway within the cells has been affected rather than the PP2A. It is possible that there are contributing variables to our protocol that could be influencing the results, such as age of cells, passage number, robustness of these particular cells, genetic mutations inherent to this line or a number of other factors.

In essence, we are investigating an *in vivo* problem using an *in vitro* cell line. Our central premise was that these MDA-MB-231 cells would adapt to the hypoxic conditions and continue to proliferate. Although the ATP assay that we conducted was a cell viability assay, with more time we would have liked to perform additional Propidium Iodide/ Annexin V staining to distinguish between apoptosis and necrosis. From this we would have been able to conclude whether cells were still very much present, just less metabolically active and producing less ATP, or in fact whether cell numbers had decreased due to cell death. The protein yields measured through Bradford assays (Bradford, 1976) following hypoxia for the purposes of Western blotting and the phosphatase assay, showed very little variation between time points. This suggests that cells exposed to hypoxia were still present in culture

with similar protein yields relative to control cells. This could therefore propose that cells have not been lost to apoptosis or necrosis, but have rather gone into cell cycle arrest with decreased cellular metabolic demands. As mentioned, though this would need to be confirmed through further experimental assessment. Expanding on the hypoxic incubation periods to perhaps include 12, 16 and 32 hours might aid in further investigations, as well as making use of multiple cell lines for comparisons. The ATP data show that after 4 and 6 hours of hypoxia, these cancer cells appear to already be producing less ATP, yet Western blotting data shows that although some degree of cell loss was experienced, there was a population of cells continuing to survive for up to 72 hours in hypoxia. This could indicate that a portion of the cells have adapted to the hypoxic conditions. From all the results obtained, there appears to be no link or evidence of association between PP2A and duration of hypoxia in this breast cancer cell line, and our initial hypothesis regarding the effects on PP2A was incorrect.

Chapter 5: Conclusion

5.1 Establishing Primary Breast Cancer Cell Cultures

Establishing successful primary cell cultures from breast cancer cells obtained through fine needle aspiration (FNA), retaining the characteristics of the original *in vivo* tumour, is a research goal that if achieved, could have numerous benefits for breast cancer research and clinical treatment. Advantages potentially include specific drug targeting and resistance management, the development of patient-specific therapies, as well as contributing to a broader understanding of the cellular functioning of cancer cells. In our pilot study we had hoped to be able to set up and institute such a primary cell model with the intention of comparing cultured cells to their frozen original un-cultured counterparts, and performing further hypoxic experiments on the cells. Apart from two prominent studies conducted in the late 90's, not much work has been published referring to the successful set up of a primary cell model in this way, but based on these reports, we aimed to confirm and improve on these successful cell cultures.

Our initial pilot study protocol included samples from 15 patients to set up the culturing technique. Unfortunately, we were only able to collect 9 FNA samples of the intended 15 due to unforeseen waiting periods and time constraints. However, even with 9 primary samples, we were unable to successfully propagate cells in culture. Proliferating cells were observed in one of the cultures, however cells did not survive long, nor did the population of cells expand sufficiently for us to confirm whether they were in fact cancerous epithelial cells, or merely contaminating cells such as fibroblasts or pericytes (Rønnov-Jessen *et al.*, 1995).

5.1.1 Limitations and Future Directions

Limitations of this study include the protocol design and the availability of patients. At the onset, we expected to receive patient samples on a regular and frequent basis, however, in reality, often weeks went by where no suitable patients visited the FNA clinic. We were therefore not able to collect all 15 samples. A second obstacle we did not anticipate was the length of time taken to obtain ethical clearance to begin sample collection. In this 2 year study, patient samples were only collected in the second half of the second year due to waiting periods connected to ethical approval, as well as the setting up of research

collaborations with the Department of Anatomical Pathology at Tygerberg Hospital. This meant that we did not have enough time to continue collection of samples before conclusion of the study.

Regarding the actual protocol and handling of the samples, a further limitation was that samples were taken directly out of the body at 37°C, but our transport medium and conditions in the laboratory were at 22°C due to technical limitations. This change in temperature may or may not have affected the cells in the samples. In future, we would like to find a way of maintaining a constant 37°C at all times. However, keeping samples on ice and maintaining them at a low temperature could be an alternative approach, and could possibly serve to enhance the viability of the collected cells in the sample. Both of these temperature changes should be investigated to identify the optimal temperature range for successful, viable isolation.

Contaminating cells within the FNA samples is also a limitation faced. Samples included many red blood cells which were difficult to remove without lysing cancer cells present. They also made visualisation of cultures impossible in the initial few days after seeding, due to the intense opaque red colouration of the growth medium. Thus we were unable to identify if cells had attached to culture plates and had to make estimations based on literature. Contaminating fibroblasts, mesenchymal cells and lymphocytes are a well-documented limitation in primary cell cultures, with their over growth commonly overwhelming and out numbering growth of cancer cells (Amadori *et al.*, 1993; Gartner *et al.*, 1996; Zheng Li *et al.*, 1998). However, this was not something that was struggled with in this study. In the majority of the cases, no cells appeared to grow for very long while in other cases, nothing grew at all. This then alludes to non-optimal culturing conditions, or it is possible that we did not receive enough cancerous material in the FNA samples we acquired. Li *et al*. also reported that the number of actual viable proliferation competent cells present in the FNA samples will determine whether propagation in culture will occur (Li *et al.*, 1998).

For futures studies, it would be interesting to incorporate the use of a suitable attachment matrix such as collagen or a Matrigel® (Corning®) coating to the culture plates to determine if this could aid attachment of cancerous epithelial cells. Manipulations of the growth medium could also be implemented, possibly to include different growth factors such as estrogen and/or insulin, as well as varying percentages of FBS content. The use of RPMI 1640 growth medium instead of high glucose DMEM could also be tested. Furthermore, investigations including cells harvested from excised cancerous lesions obtained through surgery could prove beneficial in ensuring a greater number of cancerous cells are harvested per sample.

If and when a primary model is established, we would like to continue our characterisation of the model by comparing primary cultured cells with their corresponding uncultured original sample as set out in our initial aims and objectives. In addition, we would like to assess the effects of hypoxia on these cells. We would like to even go so far as to investigate the specific protein profiles of these cultures to get a better idea of notable proteins that may or may not be up or down regulated in conjunction with culturing *per se*. Proteomic analysis could be used to identify adaptations in the cancer cells' protein content due to *in vitro* culturing, as well as associated long term hypoxic culturing. Furthermore, identification and localisation of multiple proteins within samples, as well as determining the sensitivity of the cancer cells toward traditional therapies could be investigated. Introducing drug targeting and drug resistance studies to primary cultures would be a further advancement in the research which could add considerable value to the field of breast cancer.

5.2 The Effect of Hypoxia on PP2A

The effect that hypoxia has on PP2A in cancerous cells has led to much debate and controversy among researchers as different studies have concluded with quite different results. Several studies have proposed that PP2A becomes activated under conditions of hypoxia, inhibiting cell growth and leading to arrest of the cell cycle in the G1/S phase leading to prolonged survival of cells (Bando *et al.*, 2003; Yung and Tolkovsky, 2003; Hofstetter *et al.*, 2012). Other studies propose that PP2A is phosphorylated and inactivated during hypoxia, promoting survival, proliferation and tumour formation (Mumby, 2007; Lin *et al.*, 2012). The exact mechanisms involved are still poorly understood and the influence of other signalling pathways leads one to appreciate that cellular signal transductions do not

occur as stand-alone processes. There are many contributing pathways involved, sometimes bringing about unexpected effects.

In this study, we hypothesised that hypoxia would induce a decrease in PP2A activity through an increase in its phosphorylation, bringing about the promotion of phosphorylation of pro-survival pathways. Our data suggests however, that PP2A activity was neither increased nor decreased. We observed a reduction in ATP at 4 hours and 6 hours that could indicate either a loss of cells, or that while cells are surviving, they may have reduced metabolic activity and are showing some degree of adaptation to hypoxia. However, further research is thus necessary to confirm this.

From these results we are forced to reject our initial hypothesis and conclude that in our MDA-MB-231 breast cancer cell cultures, hypoxia does not seem to have an effect on PP2A and there appears no link or association between the two, contrary to those previously stated in literature.

5.2.1 Limitations and Future Directions

Limitations of this part of the study could be contributed to the setup of hypoxia. Oxygen levels were decreased to a mere 0.5% and it is possible that this severity of reduced oxygen could be too potent for this particular cell line. Hofstetter *et al.* suggested that in instances of severe hypoxia, cancer cells arrest in G1/S phase of the cell cycle (Hofstetter *et al.*, 2012). Therefore, it is possible that the intensity of hypoxia in our experiments could have caused cells to enter cell cycle arrest which could be associated with the lack of any further PP2A-associated responses to hypoxia.

Cell robustness, age, passage number and associated possible genetic mutations of the cell line could all play roles in these hypoxic experiments. We are trying to investigate an *in vivo* phenomenon, making use of an *in vitro* cell line, as such, for future experiments it would be advantageous to investigate a range of different breast cancer cell lines for a better, more descriptive comparison of results. It could therefore be investigated whether results observed are the same in some, or none of the other cell types. This comparison would be of importance as the *in vivo* tumour microenvironment is largely heterogenous, thus if one

is able to observe repeated results in multiple cell lines, it would be a good indication of the conditions existing within the tumour cells.

Furthermore, we would like to increase our hypoxic incubation times to include 12, 16 and 36 hours of exposure. It is possible that the time points we measured may have been too short (2, 4, 6 and 8 hours), or alternatively, too long (48 and 72 hours). A wider variety of incubation times might give a better representation of the effect of hypoxia on cell signalling. It is interesting to note that the 72 hour data of the first revised time course experiments, did not mirror the data obtained from the initial 72 hour experiments. It is possible that we were too hasty in looking for an ideal time point. For further studies, it would be pertinent to conducted further experiments across these time points. Regarding whether cells were surviving with a lowered metabolic demand, it would be preferential to include analyses using Propidium Iodide/Annexin V staining to confirm cell death within samples as the ATP assay that we conducted was merely a measure of cell viability, and not apoptosis as well. Obtaining new cells of a younger age having gone through fewer sub-culturing cycles, could also be advantageous as well as increasing the replication number of the experiments for robustness and reproducibility.

This study has contributed further to the confusion surrounding the role of PP2A in hypoxia, however, some thought provoking trends were observed suggesting that extended future work is necessary.

References

Adams, D. G. *et al.* (2005) 'Positive Regulation of Raf1-MEK1/2-ERK1/2 Signaling by Protein Serine/Threonine Phosphatase 2A Holoenzymes', *Journal of Biological Chemistry*, 280(52), pp. 42644–42654

Al-Hajj, M. *et al.* (2003) 'Prospective identification of tumorigenic breast cancer cells', *Cell Biology*. Department of Internal Medicine, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA., 100(7), pp. 3983–3988.

Al-Murrani, S. W., Woodgett, J. R. and Damuni, Z. (1999) 'Expression of I2PP2A, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity.', *The Biochemical Journal*, 341 (Pt 2), pp. 293–8.

Amadori, D. *et al.* (1993) 'Establishment and characterization of a new cell line from primary human breast carcinoma', *Breast Cancer Research and Treatment*. Springer, 28(3), pp. 251–260.

Ameri, K. *et al.* (2010) 'Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype', *British Journal of Cancer*, 102, pp. 561–569.

Anastasiou, D. *et al.* (2011) 'Inhibition of Pyruvate Kinase M2 by Reactive Oxygen Species Contributes to Cellular Antioxidant Responses', *Science*, 334(6060), pp. 1278–1283.

Antonelli, A. *et al.* (2008) 'Primary cell cultures from anaplastic thyroid cancer obtained by fine-needle aspiration used for chemosensitivity tests', *Clinical Endocrinology*, 69(1), pp. 148–152.

Araujo, R. W. *et al.* (1999) 'Fine needle aspiration as a tool to establish primary human breast cancer cultures in vitro', *Acta Cytologica*. Karger Publishers, 43(6), pp. 985–990.

Badowska-Kozakiewicz, A. M., Budzik, M. P. and Przybylski, J. (2015) 'Hypoxia in breast cancer Characteristics of the hypoxia-inducible factor', *Polish Journal of Pathology*, 66(4), pp. 337–346.

Band, V. *et al.* (1990) 'Tumor Progression in Four Mammary Epithelial Cell Lines Derived from the Same Patient', *Cancer Research*, 50(22).

Bando, H. *et al.* (2003) 'Genes commonly upregulated by hypoxia in human breast cancer cells MCF-7 and MDA-MB-231', *Biomedicine and Pharmacotherapy*. Elsevier Masson, 57(8), pp. 333–340.

Bao, S. *et al.* (2004) 'Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway', *Cancer Cell*. Cell Press, 5(4), pp. 329–339.

Barragán, E. *et al.* (2015) 'CIP2A high expression is a poor prognostic factor in normal karyotype acute myeloid leukemia', *Haematologica*, 100(5).

Barrett-Lee, P. J. *et al.* (2009) 'Expert opinion on the use of anthracyclines in patients with advanced breast cancer at cardiac risk', *Annals of Oncology : Official Journal of the European Society for Medical Oncology,* 20(5), pp. 816–827.

Bellacosa, A. *et al.* (1995) 'Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas.', *International Journal of Cancer*, 64(4), pp. 280–5.

Bergstraesser, L. M. and Weitzman, S. A. (1993) 'Culture of normal and malignant primary human mammary epithelial cells in a physiological manner simulates in vivo growth patterns and allows discrimination of cell type.', *Cancer Research*, 53(11), pp. 2644–54.

Bertout, J. A., Patel, S. A. and Simon, M. C. (2008) 'The impact of O2 availability on human cancer.', *Nature Reviews. Cancer*. Howard Hughes Medical Institute, 8(12), pp. 967–75.

Bialojan, C. and Takai, A. (1988) 'Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics.', *The Biochemical Journal,* 256(1), pp. 283–90.

Bindra, R. S., Crosby, M. E. and Glazer, P. M. (2007) 'Regulation of DNA repair in hypoxic cancer cells', *Cancer and Metastasis Reviews*, 26(2), pp. 249–260.

Bio Rad (2017) *Stain-Free Imaging Technology*. Available at: http://www.bio-rad.com/en-za/applications-technologies/stain-free-imaging-technology (Accessed: 22 November 2017).

Biorad (2017) *ChemiDoc MP System | Life Science Research | Bio-Rad*. Available at: http://www.bio-rad.com/en-za/product/chemidoc-mp-imaging-system (Accessed: 22 June 2017).

Blagosklonny, M. V (2004) 'Prospective strategies to enforce selectively cell death in cancer cells', *Oncogene*, 23, pp. 2967–2975.

Bogenrieder, T. and Herlyn, M. (2003) 'Axis of evil: molecular mechanisms of cancer metastasis, *Oncogene*, 22, pp. 6524-6536.

Bos, R. *et al.* (2003) 'Levels of hypoxia-inducible factor-1α independently predict prognosis in patients with lymph node negative breast carcinoma', *Cancer*, 97, pp. 1573-1581.

Bousquet, P. A. *et al.* (2015) 'Hypoxia strongly affects mitochondrial ribosomal proteins and translocases, as shown by quantitative proteomics of HeLa cells', *International Journal of Proteomics*. Hindawi Publishing Corporation, 2015, pp. 1–9.

Bradford, M. (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry*, 72, 248-254.

Brahimi-Horn, M. C., Chiche, J. and Pouysségur, J. (2007) 'Hypoxia signalling controls metabolic demand', *Current Opinion in Cell Biology*. Elsevier Current Trends, pp. 223–229.

Brautigan, D. L. (1995) 'Flicking the switches: phosphorylation of serine/threonine protein phosphatases', *Seminars in Cancer Biology*, 6(4), pp. 211–217.

Bristow, R. G. and Hill, R. P. (2008) 'Hypoxia and metabolism: Hypoxia, DNA repair and genetic instability', *Nature Reviews Cancer*. Nature Publishing Group, 8(3), pp. 180–192.

Brown, J. and Giaccia, A. (1998) 'The unique physiology of solid tumors: opportunities (and problems) for cancer therapy', *Cancer research*, 58, pp. 1408-1416.

Burdall, S. E. *et al.* (2003) 'Breast cancer cell lines: friend or foe?', *Breast Cancer Research*. Current Science Ltd, 5(2), p. 89.

Burgman, P. *et al.* (2001) 'Hypoxia-Induced increase in FDG uptake in MCF7 cells.', *Journal of Nuclear Medicine*, 42(1), pp. 170–5.

Cancer Genome Atlas (2012) 'Comprehensive molecular portraits of human breast tumors', *Nature*, 490(7418), pp. 61–70.

Cancer Helpline (2017) *Diagnosis Cancer,* Available at: http://www.cancerhelpline.in/diagnose-cancer (Accessed: 29 November 2017).

Cardone, R. and Casavola, V. (2005) 'The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis', *Nature Reviews. Cancer*, 5, pp. 786-795

Carlson, S. G. *et al.* (1998) 'Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms' tumor.', *Journal of the American Society of Nephrology,* 9(10), pp. 1873–80.

Carpten, J. D. *et al.* (2007) 'A transforming mutation in the pleckstrin homology domain of AKT1 in cancer', *Nature*, 448(7152), pp. 439–444.

Carrer, A. and Wellen, K. E. (2015) 'Metabolism and epigenetics: A link cancer cells exploit', *Current Opinion in Biotechnology*. Elsevier Current Trends, pp. 23–29.

Chan, D. A. and Giaccia, A. J. (2007) 'Hypoxia, gene expression, and metastasis', *Cancer and Metastasis Reviews*. Kluwer Academic Publishers-Plenum Publishers, 26(2), pp. 333–339.

Chandel, N. S. and Simon, M. C. (2008) 'Hypoxia-inducible factor: roles in development, physiology, and disease', *Cell Death and Differentiation*. Nature Publishing Group, 15(4), pp. 619–620.

Chen, W. *et al.* (2005) 'Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity.', *Cancer Research*. American Association for Cancer Research, 65(18), pp. 8183–92.

Cheng, H.-C. *et al.* (2011) 'Regulation and function of protein kinases and phosphatases.', *Enzyme Research*. Hindawi, p. 794089.

Choi, Y. *et al.* (2011) 'Increase in CIP2A expression is associated with doxorubicin resistance', *FEBS Letters,* 5, pp 755-760.

Ciccone, M., Calin, G. A. G. and Perrotti, D. (2015) 'From the Biology of PP2A to the PADs for Therapy of Hematologic Malignancies', *Frontiers in Oncology*, 5, pp. 1–10.

Coley, H. M. (2008) 'Mechanisms and strategies to overcome chemotherapy resistance in metastatic breast cancer', *Cancer Treatment Reviews*. Elsevier, 34(4), pp. 378–390.

Coquelle, A *et al.* (1998) 'A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs.', *Molecular Cell*. Cell Press, 2(2), pp. 259–265.

Correa, Z. M. S., Marshall, J. and Burnier, M. N. (2009) 'Fine needle aspiration biopsy to reestablish cell culture in an animal model of uveal melanoma', *Arquivos Brasileiros de Oftalmologia*, 72(3), pp. 515–518.

Coyle, C. H., Izzo, N. J. and Chu, C. R. (2009) 'Sustained hypoxia enhances chondrocyte matrix synthesis', *Journal of Orthopaedic Research*, 27(6), pp. 793–799.

Cristóbal, I. *et al.* (2014) 'PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential', *Molecular Cancer*, 13(4), pp. 938-947

Cronin, P. A., Wang, J. H. and Redmond, H. P. (2010) 'Hypoxia increases the metastatic ability of breast cancer cells via upregulation of CXCR4', *BMC Cancer*, 10(1), p. 225.

Crouch, S. P. M. *et al.* (1993) 'The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity', *Journal of Immunological Methods*, 160, pp. 81–88.

Dairkee, S. H. *et al.* (1995) 'Selective cell culture of primary breast carcinoma', *Cancer research*, 55(12), pp. 2516–2519.

Dairkee, S. H., Paulo, E. C., *et al.* (1997) 'Partial enzymatic degradation of stroma allows enrichment and expansion of primary breast tumor cells', *Cancer Research*, 57(8), pp. 1590–1596.

Das, D. K. (2003) 'Fine-needle aspiration cytology: Its origin, development, and present status with special reference to a developing country, India', *Diagnostic Cytopathology*. 28(6), pp. 345–351.

Denko, N. C. (2008) 'Hypoxia, HIF1 and glucose metabolism in the solid tumour', *Nature Reviews. Cancer*. Nature Publishing Group, 8(9), p. 705.

DeRose, Y. S. *et al.* (2013) 'Patient-Derived Models of Human Breast Cancer: Protocols for In Vitro and In Vivo Applications in Tumor Biology and Translational Medicine', *Current Protocols in Pharmacology*. Wiley Online Library, p. 14.23. 1-14.23. 43.

Dohoney, K. M. *et al.* (2004) 'Phosphorylation of p53 at serine 37 is important for transcriptional activity and regulation in response to DNA damage', *Oncogene*, 23(1), pp. 49–57.

Dong, Q. *et al.* (2011) 'CIP2A is overexpressed in non-small cell lung cancer and correlates with poor prognosis', *Annals of surgical Oncology*, 18(3), pp. 857-865.

Du, Y., Kowluru, A. and Kern, T. S. (2010) 'PP2A contributes to endothelial death in high glucose: inhibition by benfotiamine.', *American Journal of Physiology.* 299(6), pp. R1610-7.

Edge, S. and Compton, C. (2010) 'The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM', *Annals of Surgical Oncology*, 17(6), pp. 1471-1474.

Eichhorn, P. J. A., Creyghton, M. P. and Bernards, R. (2009) 'Protein phosphatase 2A regulatory subunits and cancer', *Biochimica et Biophysica Acta - Reviews on Cancer*. Elsevier B.V., 1795(1), pp. 1–15.

Emerman, J. T. and Wilkinson, D. A. (1990) 'Routine culturing of normal, dysplastic and malignant human mammary epithelial cells from small tissue samples', *In vitro Cellular & Developmental Biology*. Springer, 26(12), pp. 1186–1194.

Ethier, S. P. *et al.* (1993) 'Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media', *Cancer Research*, 53(3), pp. 627–635.

Evans, S. M. *et al.* (2001) 'Hypoxic Heterogeneity in Human Tumors', *American Journal of Clinical Oncology*, 24(5), pp. 467–472.

Ferlay, J. *et al.* (2010) 'Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008', *International Journal of Cancer*. Wiley Online Library, 127(12), pp. 2893–2917.

Fiaschi, T. and Chiarugi, P. (2012) 'Oxidative Stress, Tumor Microenvironment, and Metabolic Reprogramming: A Diabolic Liaison', *International Journal of Cell Biology*. Hindawi, 2012, pp. 1–8.

Filho, A. L., Lopes, J. and Schmitt, F. (2010) 'Angiogenesis and breast cancer', *Journal of Oncology*, 2010, pp. 1782-1790.

Fornerod, M. *et al.* (1995) 'Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements.' *Oncogene*, 10(9), pp. 1739–48.

Frable, W. J. (1983) 'Fine-needle aspiration biopsy: a review', *Human Pathology*, 14(1) pp. 9–28.

Freshney, R. I. (1985) 'Induction of differentiation in neoplastic cells', *Anticancer Research*, 5(1), pp. 111–130.

Furuta, E. *et al.* (2008) 'Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1', *Cancer Research*, 68(4), pp. 1003-1011.

Gabel, S. *et al.* (1999) 'Protein phosphatases 1 and 2A maintain endothelial cells in a resting state, limiting the motility that is needed for the morphogenic process of angiogenesis', *Otolaryngology - Head and Neck Surgery*, 121(4), pp. 463–468.

Garcia, A., Cereghini, S. and Sontag, E. (2000) 'Protein phosphatase 2A and phosphatidylinositol 3-kinase regulate the activity of Sp1-responsive promoters', *Journal of Biological Chemistry*, 275(13), pp. 9385–9389.

Gartner, F. *et al.* (1996) 'Establishment and characterization of two cell lines derived from human diffuse gastric carcinomas xenografted in nude mice', *Virchows Archiv*. Springer-Verlag, 428(2), pp. 91–98.

Gatenby, R. A. and Gillies, R. J. (2008) 'A microenvironmental model of carcinogenesis', *Nature Reviews: Cancer*, 8(1), pp. 56-61.

Gazdar, A. F. *et al.* (1998) 'Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer', *International Journal of Cancer*, 78, pp. 766–774.

Gerhardt, R. *et al.* (1991) 'Characterization of in vitro chemosensitivity of perioperative human ovarian malignancies by adenosine triphosphate chemosensitivity assay', *American Journal of Obstetrics and Gynecology*. Mosby, 165(2), pp. 245–255.

Gilda, J. E. and Gomes, A. V (2013) 'Stain-Free total protein staining is a superior loading control to Î²-actin for Western blots', *Analytical Biochemistry*, 440, pp. 186–188.

Greijer, A. and Wall, E. Van der (2004) 'The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis', *Journal of Clinical Pathology*, 57(10), pp. 1009-1014.

Guo, H. and Damuni, Z. (1993) 'Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A.', *Proceedings of the National Academy of Sciences of the United States of America*, 90(6), pp. 2500–2504.

Gürtler, A. *et al.* (2013) 'Stain-Free technology as a normalization tool in Western blot analysis'. *Analytical Biochemistry*, 433(2), pp. 105-111.

Haesen, D. *et al.* (2014) 'The Basic Biology of PP2A in Hematologic Cells and Malignancies.', *Frontiers in Oncology*, 4, p. 347.

Hammond, S. L., Ham, R. G. and Stampfer, M. R. (1984) 'Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract', *Proceedings of the National Academy of Sciences*, 81(17), pp. 5435–5439.

Hanahan, D. and Weinberg, R. A. (2000) 'The hallmarks of cancer.' *Cell*, 100(1), pp. 57–70.

Härmälä-Braskén, A.-S. *et al.* (2003) 'Type-2A protein phosphatase activity is required to maintain death receptor responsiveness', *Oncogene*. Nature Publishing Group, 22(48), pp. 7677–7686.

Harris, A. L. (2002) 'Hypoxia – A key regulatory factor in tumour growth', *Nature Reviews Cancer*, 2(1), pp. 38–47.

Hass, R. and Bertram, C. (2009) 'Characterization of human breast cancer epithelial cells (HBCEC) derived from long term cultured biopsies', *Journal of Experimental & Clinical Cancer Research*. BioMed Central, 28(1), p. 1.

Heddleston, J. *et al.* (2010) 'Hypoxia inducible factors in cancer stem cells', *British journal of Cancer,* 120, pp. 789-795.

Hicklin, D. and Ellis, L. (2005) 'Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis', *Journal of Clinical Oncology*, 23(5), pp. 1011-1027.

Hockel, M. and Vaupel, P. (2001) 'Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects', *Journal of the National Cancer Institute*, 93(4), pp. 266-276.

Hofstetter, C. P. *et al.* (2012) 'Protein Phosphatase 2A Mediates Dormancy of Glioblastoma Multiforme-Derived Tumor Stem-Like Cells during Hypoxia', *PLoS ONE*, 7(1), p. e30059.

Holliday, D. L. and Speirs, V. (2011) 'Choosing the right cell line for breast cancer research', *Breast Cancer Research*. BioMed Central, 13(4), p. 1.

Hood, C. J. and Parham, D. M. (1998) 'A simple method of tumour culture', *Pathology-Research and Practice*. Elsevier, 194(3), pp. 177–181.

Hortobagyi, G. N. *et al.* (2005) 'The global breast cancer burden: variations in epidemiology and survival', *Clinical Breast Cancer*. Elsevier, 6(5), pp. 391–401.

Huang, L. E. *et al.* (2007) 'Hypoxia-induced genetic instability—a calculated mechanism underlying tumor progression', *Journal of Molecular Medicine*. Springer-Verlag, 85(2), pp. 139–148.

Ip, M. M. and Darcy, K. M. (1996) 'Three-dimensional mammary primary culture model systems', *Journal of Mammary Gland Biology and Neoplasia*. Kluwer Academic Publishers-Plenum Publishers, 1(1), pp. 91–110.

Israël, M. and Schwartz, L. (2011) 'The metabolic advantage of tumor cells', *Molecular Cancer, 10(1) p. 70.*

Ivan, M. *et al.* (2001) 'HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing', *Science*, 292(5516), pp. 464-468.

Jaakkola, P. *et al.* (2001) 'Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation', *Science*, 292(5516), pp. 468-472.

Janssens, V. and Goris, J. (2001) 'Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.' *The Biochemical Journal*, 353(Pt 3), pp. 417–39.

Janssens, V., Longin, S. and Goris, J. (2008) 'PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail)', *Trends in Biochemical Sciences*, 33(3), pp. 113–121.

Janssens, V. and Rebollo, A. (2012) 'The Role and Therapeutic Potential of Ser / Thr Phosphatase PP2A in Apoptotic Signalling Networks in Human Cancer Cells', *Current molecular medicine*, 12, pp. 1–19.

Jemal, A. *et al.* (2011) 'Global cancer statistics', *CA: a Cancer Journal for Clinicians*. Wiley Online Library, 61(2), pp. 69–90.

Kaelin, W. (2002) 'How oxygen makes its presence felt', *Genes & Development*, 16(12), pp 1441-1445.

Kaelin, W. G. and Ratcliffe, P. J. (2008) 'Oxygen Sensing by Metazoans: The Central Role of the HIF Hydroxylase Pathway', *Molecular Cell*. Cell Press, 30(4), pp. 393–402.

Kaluz, S., Kaluzová, M. and Stanbridge, E. J. (2008) 'Does inhibition of degradation of hypoxia-inducible factor (HIF) α always lead to activation of HIF? Lessons learnt from the effect of proteasomal inhibition on HIF activity', *Journal of Cellular Biochemistry*.,104(2), pp. 536–544.

Kang, Y. *et al.* (2003) 'A multigenic program mediating breast cancer metastasis to bone', *Cancer Cell*, 3(6), pp. 537–549.

Kangas, L., Grönroos, M. and Nieminen, A. L. (1984) 'Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro.' *Medical biology*, 62(6), pp. 338–43.

Kao, G. *et al.* (2004) 'C. elegans SUR-6/PR55 cooperates with LET-92/protein phosphatase 2A and promotes Raf activity independently of inhibitory Akt phosphorylation sites', *Development*, 131(4), pp. 755–765.

Keller, P. J. *et al.* (2010) 'Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines', *Breast Cancer Research*. BioMed Central, 12(5), p. 1.

Khanna, A., Pimanda, J. and Westermarck, J. (2013) 'Cancerous inhibitor of protein phosphatase 2A, an emerging human oncoprotein and a potential cancer therapy target', *Cancer research*, 73(22), pp. 6548-6553.

Kiely, M. and Kiely, P. (2015) 'PP2A: The Wolf in Sheep's Clothing?' *Cancers*. Multidisciplinary Digital Publishing Institute, 7(2), pp. 648–669. .

Kuo, Y.-C. *et al.* (2008) 'Regulation of Phosphorylation of Thr-308 of Akt, Cell Proliferation, and Survival by the B55α Regulatory Subunit Targeting of the Protein Phosphatase 2A Holoenzyme to Akt', *Journal of Biological Chemistry*, 283(4), pp. 1882–1892.

Kuperwasser, C. *et al.* (2005) 'A mouse model of human breast cancer metastasis to human bone.' *Cancer research*, 65(14), pp. 6130–8.

Lacroix, M., Leclercq, G. and Vi, M. (2004) 'Relevance of breast cancer cell lines as models for breast tumours: an update', *Breast Cancer Research and Treatment*. Springer, 83(3), pp. 249–289.

Lavarino, C. *et al.* (1998) 'Detection of TP53 mutation, loss of heterozygosity and DNA content in fine-needle aspirates of breast carcinoma.' *British journal of Cancer*. Nature Publishing Group, 77(1), pp. 125–30.

Lawlor, M. A. and Alessi, D. R. (2001) 'PKB/Akt: A key mediator of cell proliferation, survival and insulin responses?' *Journal of Cell Science*, 114(16), pp. 2903-2910.

Le, Q.-T., Denko, N. C. and Giaccia, A. J. (2004) 'Hypoxic gene expression and metastasis', *Cancer and Metastasis Reviews*. Kluwer Academic Publishers, 23(3/4), pp. 293–310.

Lee, M. and Yoon, J.-H. (2015) 'Metabolic interplay between glycolysis and mitochondrial oxidation: The reverse Warburg effect and its therapeutic implication.' *World journal of biological chemistry*. Baishideng Publishing Group Inc, 6(3), pp. 148–61.

Li, H.-H. *et al.* (2007) 'A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55.', *The EMBO journal*, 26(2), pp. 402–411.

Li, Z. *et al.* (1998) 'Propagation of genetically altered tumour cells derived from fine-needle aspirates of primary breast carcinoma.' *Cancer Research*, 58(415), pp. 5271–5274.

Loveday, R. L. *et al.* (2002) 'Intracellular flow cytometric analysis of primary cultured breast tumor cells', *Cancer Investigation*. Taylor & Francis, 20(3), pp. 340–347.

Lunt, S. J., Chaudary, N. and Hill, R. P. (2009) 'The tumor microenvironment and metastatic disease', *Clinical & Experimental Metastasis*. Springer Netherlands, 26(1), pp. 19–34.

Manning, B. D. and Cantley, L. C. (2007) 'AKT/PKB Signaling: Navigating Downstream', *Cell*, 129(7), pp. 1261–1274.

Marsden, C. G. *et al.* (2012) 'A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies', *BMC cancer*. BioMed Central, 12(1), p. 1.

Martin, H. E. and Ellis, E. B. (1930) 'Biopsy by needle puncture and aspiration.' *Annals of Surgery*. 92(2), pp. 169–181.

Martin, M., Kettmann, R. and Dequiedt, F. (2010) 'Recent insights into Protein Phosphatase 2A structure and regulation: the reason why PP2A is no longer considred as a lazy passive housekeeping enzyme.', *Biotechnol. Agron. Soc. Environ*, 14(1), pp. 243–252.

McAllister, S. S. and Weinberg, R. A. (2010) 'Tumor-host interactions: a far-reaching relationship.' *Journal of Clinical Oncology ,* 28(26), pp. 4022–4028.

McLoughlin, M. J., Ho, C. S. and Tao, L. C. (1978) 'Percutaneous needle aspiration biopsy.' *Canadian Medical Association journal*. Canadian Medical Association, 119(11), pp. 1324–8.

Möbus, V. J. *et al.* (1998) 'Differential characteristics of two new tumorigenic cell lines of human breast carcinoma origin', *International Journal of Cancer*, 77(3), pp. 415–423.

Mukhopadhyay, A. *et al.* (2009) 'Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling', *The FASEB Journal*, 23(3), pp. 751-763.

Mumby, M. (2007) 'PP2A: Unveiling a reluctant tumor suppressor', *Cell*, 130(1), pp. 21–24.

Nagelkerke, A. *et al.* (2013) 'Hypoxia stimulates migration of breast cancer cells via the PERK/ATF4/LAMP3-arm of the unfolded protein response', *Breast Cancer Research*. BioMed Central Ltd, 15(1), p. R2.

Neve, R. M. *et al.* (2006) 'A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes', *Cancer Cell*, 10(6), pp. 515–527.

Nicholson, K. and Anderson, N. (2002) 'The protein kinase B/Akt signalling pathway in human malignancy', *Cellular Signalling*. Pergamon, 14(5), pp. 381–395.

Nishida, A. *et al.* (2009) 'Interleukin-32 expression in the pancreas', *Journal of Biological Chemistry*, 284(26), pp. 17868-17876.

Nolan, C. *et al.* (1964) 'Further Studies on the Site Phosphorylated in the Phosphorylase b to a Reaction', *Biochemistry*, 3, pp. 542–551.

Nold-Petry, C., Nold, M. and Zepp, J. (2009) 'IL-32–dependent effects of IL-1β on endothelial cell functions', *Proceedings of the National Academy of Sciences of the United States of America,* 106(10), pp. 3883-3888.

O'Reilly, M. *et al.* (1996) 'Angiostatin induces and sustains dormancy of human primary tumors in mice', *Nature Medicine*, 2(6), pp. 689-692.

Ory, S. *et al.* (2003) 'Protein phosphatase 2A positively regulates Ras signalling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites.' *Current biology : CB*, 13(16), pp. 1356–64.
Park, J. *et al.* (2013) 'Interleukin-32β stimulates migration of MDA-MB-231 and MCF-7cells via the VEGF-STAT3 signaling pathway', *Cellular Oncology*, 36(6), pp. 493-503.

Park, J. S. *et al.* (2015) 'Hypoxia-induced IL-32β increases glycolysis in breast cancer cells', *Cancer Letters*. Elsevier, 356(2), pp. 800–808.

Parkin, D. M. *et al.* (2005) 'Global cancer statistics, 2002', *CA: a Cancer Journal for Clinicians*. Wiley Online Library, 55(2), pp. 74–108.

Pavlides, S. *et al.* (2012) 'Warburg Meets Autophagy: Cancer-Associated Fibroblasts Accelerate Tumor Growth and Metastasis *via* Oxidative Stress, Mitophagy, and Aerobic Glycolysis', *Antioxidants & Redox Signaling*. Mary Ann Liebert, Inc. 16(11), pp. 1264–1284.

Perrotti, D. and Neviani, P. (2006) 'ReSETting PP2A tumour suppressor activity in blast crisis and imatinib-resistant chronic myelogenous leukaemia', *British Journal of Cancer*, 95(7), pp. 775-781.

Perrotti, D. and Neviani, P. (2013) 'Protein phosphatase 2A: A target for anticancer therapy', *The Lancet Oncology*. Elsevier Ltd, 14(6), pp. e229–e238.

Ponti, D. *et al.* (2005) 'Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties', *Cancer Research*, 65(13), pp. 5506–5511.

Powis, G. and Kirkpatrick, L. (2004) 'Hypoxia inducible factor-1α as a cancer drug target', *Molecular Cancer Therapeutics*, 3(5), pp. 647-654.

Prat, A. *et al.* (2009) 'Mammary development meets cancer genomics', *Nature Medicine*. BioMed Central, 15(8), pp. 842–844.

Prior, S. *et al.* (2014) 'Mitochondrial respiratory function induces endogenous hypoxia', *PloS one*. Public Library of Science, 9(2), p. e88911.

Promega (2015) 'CellTiter-Glo ® Luminescent Cell Viability Assay Instructions for Use of Products G7570, G7571, G7572 and G7573', pp. 608–277. Available at: https://worldwide.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-glo-luminescent-cell-viability-assay-protocol.pdf (Accessed: 22 November 2017).

Pugh, C. and Ratcliffe, P. (2003) 'Regulation of angiogenesis by hypoxia: role of the HIF system', *Nature medicine*, 9(6), pp. 677-684.

Quail, D. F. and Joyce, J. A. (2013) 'Microenvironmental regulation of tumor progression and metastasis', *Nature Medicine*. Nature Research, 19(11), pp. 1423–1437.

Ratnasinghe, D., Phang, J. M. and Yeh, G. C. (1998) 'Differential expression and acivity of phosphatases and protein kinases in adriamycin sensitive and resistant human breast cancer MCF-7 cells.' *International Journal of Oncology*, 13(1), pp. 79-163.

Rdnnov-Jessen, L. *et al.* (1995) 'The Origin of the Myofibroblasts in Breast Cancer: Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells.' Journal of *Clinical. Investigation*, 95(2), pp. 859–873.

Ren, Y. *et al.* (2013) 'Hypoxia modulates A431 cellular pathways association to tumor radioresistance and enhanced migration revealed by comprehensive proteomic and functional studies', *Molecular & Cellular Proteomics*., 12(2), pp. 485–498.

Ros, S. *et al.* (2012) 'Functional metabolic screen identifies 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 4 as an important regulator of prostate cancer cell survival', *Cancer Discovery*, 2(4), pp. 328-343.
Rosa, M. (2008) 'Fine-Needle Aspiration Biopsy : A Historical Overview', *Diagnostic Cytopathology*, 36(11), pp. 773–775.

Ruan, K., Song, G. and Ouyang, G. (2009) 'Role of hypoxia in the hallmarks of human cancer', *Journal of Cellular Biochemistry*, 107(6), pp. 1053–1062.

Ruediger, R. *et al.* (1997) 'Increasing the ratio of PP2A core enzyme to holoenzyme inhibits Tat-stimulated HIV-1 transcription and virus production', *Virology*, 238(2), pp. 432-443.

Ruvolo, P. P. (2016) 'The broken "Off" switch in cancer signaling: PP2A as a regulator of tumorigenesis, drug resistance, and immune surveillance', *BBA Clinical*, pp. 87–99.

Sablina, A. A. *et al.* (2007) 'The Tumor Suppressor PP2A Aβ Regulates the RalA GTPase', *Cell*, 129(5), pp. 969–982.

Schmitt, F. C., Bento, M. J. and Amendoeira, I. (1995a) 'Estimation of estrogen receptor content in fine-needle aspirates from breast cancer using the monoclonal antibody 1d5 and microwave oven processing: Correlation with paraffin embedded and frozen sections determinations', *Diagnostic Cytopathology*, 13(4), pp. 347–351.

Schmitt, F. C., Figueiredo, P. and Lacerda, M. (1995b) 'Expression of c-erb B-2 protein and DNA ploidy in breast carcinogenesis', *Archives of Pathology and Laboratory Medicine*, 119(9), pp. 815–820.

Schönthal, A. H. (1998) 'Role of PP2A in intracellular signal transduction pathways.' *Frontiers in Bioscience,* 3(6), pp. D1262-73.

Schönthal, A. H. (2001) 'Role of serine/threonine protein phosphatase 2A in cancer.' *Cancer letters*, 170(1), pp. 1–13.

Semenza, G. (2002) 'HIF-1 and tumor progression: Pathophysiology and therapeutics', *Trends in Molecular Medicine*. Elsevier Current Trends, 8(4 SUPPL.), pp. S62–S67.

Semenza, G. (2003) 'Targeting HIF-1 for cancer therapy', *Nature Reviews Cancer*, 3(10), pp. 721–732.

Semenza, G. (2004) 'Hydroxylation of HIF-1: oxygen sensing at the molecular level', *Physiology*, 19(4), pp. 167-182.

Seshacharyulu, P. *et al.* (2013) 'Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer', *Cancer Letters*. Elsevier Ireland Ltd, 335(1), pp. 9–18.

Shibuya, K. *et al.* (2002) 'Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000', *BMC Cancer*. BioMed Central, 2(1), p. 1.

Shu, Y. *et al.* (1997) 'Molecular genetic analysis of Rts1p, a B' regulatory subunit of Saccharomyces cerevisiae protein phosphatase 2A.' *Molecular and Cellular Biology*. American Society for Microbiology, 17(6), pp. 3242–53.

Simon, M. C. and Keith, B. (2008) 'The role of oxygen availability in embryonic development and stem cell function.' *Nature reviews. Molecular cell biology*. Howard Hughes Medical Institute, 9(4), pp. 285–96.

Siwek, B. *et al.* (1998) 'Establishment and characterization of three new breast-cancer cell lines', *International Journal of Cancer*, 76(5), pp. 677–683.

Skoog, L. *et al.* (1990) 'Immunocytochemical analysis of receptors for estrogen and progesterone in fine-needle aspirates from human breast carcinomas', *Diagnostic Cytopathology*, 6(2), pp. 95–98.

Smith, T. A. D., Zanda, M. and Fleming, I. N. (2013) 'Hypoxia stimulates 18F-Fluorodeoxyglucose uptake in breast cancer cells via Hypoxia inducible Factor-1 and AMP-activated protein kinase', *Nuclear Medicine and Biology*. Elsevier Inc., 40(6), pp. 858–864.

Song, G., Ouyang, G. and Bao, S. (2005) 'The activation of Akt/PKB signaling pathway and cell survival', *Journal of Cellular and Molecular Medicine*. Blackwell Publishing Ltd, 9(1), pp. 59–71.

Sonoda, Y. *et al.* (1997) 'Stimulation of interleukin-8 production by okadaic acid and vanadate in a human promyelocyte cell line, an HL-60 subline. Possible role of mitogen-activated protein kinase on the okadaic acid-induced NF-kappaB activation.' *The Journal of Biological Chemistry*, 272(24), pp. 15366–72. d

Sontag, E. *et al.* (1993) 'The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation', *Cell*. Cell Press, 75(5), pp. 887–897.

Sontag, E. (2001) 'Protein phosphatase 2A: the Trojan horse of cellular signaling.' *Cellular Signalling*, 13(1), pp. 7–16.

Sorrentino, C. and Carlo, E. Di (2009) 'Expression of IL-32 in human lung cancer is related to the histotype and metastatic phenotype', *Journal of Respiratory and Critical Care,* 180(8), pp. 769-779.

Soule, H. D. *et al.* (1990) 'Isolation and Characterization of a Spontaneously Immortalized Isolation and Characterization of a Spontaneously Immortalized Human Breast', *Cancer*, pp. 6075–6086.

Speirs, V. *et al.* (1998) 'Short-term primary culture of epithelial cells derived from human breast tumours', *British Journal of Cancer*, 78(11), pp. 1421–1429.

Speirs, V., White, M. C. and Green, A. R. (1996) 'Collagenase III: a superior enzyme for complete disaggregation and improved viability of normal and malignant human breast tissue', *In Vitro Cellular & Developmental Biology-Animal*. Springer, 32(2), pp. 72–74.

Strese, S. *et al.* (2013) 'Effects of hypoxia on human cancer cell line chemosensitivity', *BMC Cancer*. BioMed Central, 13(1), p. 1.

Stuelten, C. H. *et al.* (2010) 'Transient Tumor-Fibroblast Interactions Increase Tumor Cell Malignancy by a TGF-β Mediated Mechanism in a Mouse Xenograft Model of Breast Cancer', *PLoS ONE*, 5(3), p. e9832.

Sung, S. Y. *et al.* (2007) 'Tumor microenvironment promotes cancer progression, metastasis, and therapeutic resistance', *Current Problems in Cancer*, 31(2), pp. 36–100.

Suzuki, H., Tomida, A. and Tsuruo, T. (2001) 'Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia.' *Oncogene*, 20(41), pp. 5779–88.

Tannock, I. (1968) 'The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour.' *British Journal of Cancer*, 22(2), pp. 258-273.

Troncone, G. *et al.* (1996) 'c-erbB-2 expression in FNAB smears and matched surgical specimens of breast cancer', *Diagnostic Cytopathology*, 14(2), pp. 135–139.

Tsai, C. *et al.* (2014) 'Interleukin-32 increases human gastric cancer cell invasion associated with tumor progression and metastasis', *Clinical Cancer Research*, 20(9), pp. 2276-2288.
Turnbull, C. and Rahman, N. (2008) 'Genetic Predisposition to Breast Cancer: Past, Present, and Future', *Annual Review of Genomics and Human Genetics*, 9(1), pp. 321–345.

Vaapil, M. *et al.* (2012) 'Hypoxic conditions induce a cancer-like phenotype in human breast epithelial cells', *PloS one*. Public Library of Science, 7(9), p. e46543.

Van Pham, P. *et al.* (2012) 'Isolation of breast cancer stem cells by single-cell sorting.' *Biomedial Tissue Culture*, pp. 59-72.

Vargo-Gogola, T. and Rosen, J. M. (2007) 'Modelling breast cancer: one size does not fit all', *Nature Reviews Cancer*. Nature Publishing Group, 7(9), pp. 659–672.

Vázquez, S. M. *et al.* (2004) 'Three novel hormone-responsive cell lines derived from primary human breast carcinomas: Functional characterization', *Journal of Cellular Physiology*, 199(3), pp. 460–469.

Vooijs, M. *et al.* (2008) 'Hypoxic regulation of metastasis via hypoxia-inducible factors', *Current Molecular Medicine*, 8(1), pp. 60-67.

Vorobiof, D. A. *et al.* (2001) 'Breast cancer incidence in South Africa', *Journal of clinical Oncology,* 19(18 Suppl), pp. 125S–127S.

Vu, B. T. *et al.* (2015) 'Optimization of culture medium for the isolation and propagation of human breast cancer cells from primary tumour biopsies', *Biomedical Research and Therapy*, 2(2), pp. 207–219.

Wai, P. Y. and Kuo, P. C. (2008) 'Osteopontin: regulation in tumor metastases.', *Cancer and Metastasis Reviews*. Springer US, 27(1), pp. 103–118.

Walker, A. R., Adam, F. I. and Walker, B. F. (2004) 'Breast cancer in black African women: a changing situation', *Journal of the Royal Society for the Promotion of Health*, 124(2), pp. 81–85.

Walsh, S. *et al.* (2009) 'Hypoxia increases normal prostate epithelial cell resistance to receptor-mediated apoptosis *via* AKT activation', *International Journal of Cancer*, 124(8), pp. 1871–1878.

Wang, Z. *et al.* (2004) 'Mutational Analysis of the Tyrosine Phosphatome in Colorectal Cancers', *Science*, 304(5674), pp. 1164–1166.

Warburg, O., Wind, F. and Negelein, E. (1927) 'The metabolism of tumors in the body', *The Journal of General Physiology*. The Rockefeller University Press, 8(6), p. 519.

Ward, P. S. and Thompson, C. B. (2012) 'Metabolic Reprogramming: A cancer hallmark even Warburg did not anticipate', *Cancer Cell*. Cell Press, pp. 297–308.

Wassarman, D. A. *et al.* (1996) 'Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in Drosophila.' *Genes & Development*. Cold Spring Harbor Laboratory Press, 10(3), pp. 272–8.

Weigand, A. *et al.* (2016) 'Selective isolation and characterization of primary cells from normal breast and tumors reveal plasticity of adipose derived stem cells', *Breast Cancer Research*. BioMed Central, 18(1), p. 1.

Wolman, S. R. *et al.* (1985) 'Growth of diploid cells from breast cancers', *Cancer Genetics and Cytogenetics*. Elsevier, 16(1), pp. 49–64.

Wong, L. L. *et al.* (2010) 'Silencing of the PP2A catalytic subunit causes HER-2/neu positive breast cancer cells to undergo apoptosis', *Experimental Cell Research*. Elsevier Inc., 316(20), pp. 3387–3396.

World Cancer Research Fund International (2017) *Breast cancer statistics*, *2017*. Available at: http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/breast-cancer-statistics (Accessed: 16 September 2017).

Wu, D. and Yotnda, P. (2011) 'Induction and Testing of Hypoxia in Cell Culture', *Journal of Visualized Experiments*, (54), pp. 4–7.

Xie, J. *et al.* (2016) 'Hypoxia regulates stemness of breast cancer MDA-MB-231 cells', *Medical Oncology*. Springer US, 33(5), p. 42.

Yung, H. W. and Tolkovsky, A. M. (2003) 'Erasure of kinase phosphorylation in astrocytes during oxygen-glucose deprivation is controlled by ATP levels and activation of phosphatases', *Journal of Neurochemistry*. Blackwell Science Ltd, 86(5), pp. 1281–1288.

Zeng, R. *et al.* (2008) 'Biliverdin Reductase Mediates Hypoxia-Induced EMT via PI3-Kinase and Akt', *Journal of the American Society of Nephrology*, 19(2), pp. 380–387.

Zhang, X. *et al.* (2004) 'Enhancement of hypoxia-induced tumor cell death in vitro and radiation therapy in vivo by use of small interfering RNA targeted to hypoxia-inducible factor-1α', *Cancer Research*, 64(22), pp. 8139-8142.

Zhao, Y. *et al.* (1997) 'Saccharomyces cerevisiae homologs of mammalian B and B' subunits of protein phosphatase 2A direct the enzyme to distinct cellular functions.', *The Journal of Biological Chemistry*, 272(13), pp. 8256–62.

Zheng, J. (2012) 'Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation.' *Oncology Letters*, 4(6), pp. 1151-1157.

Zheng, M. *et al.* (2005) 'Intracellular acidosis-activated p38 MAPK signaling and its essential role in cardiomyocyte hypoxic injury.' *The FASEB Journal,* 19(1), pp. 109–111.

Zhou, J. *et al.* (2006) 'Tumor hypoxia and cancer progression', *Cancer letters*. Elsevier, 237(1), pp. 10–21.

Zundel, W. *et al.* (2000) 'Loss of PTEN facilitates HIF-1-mediated gene expression.' *Genes & Development*. Cold Spring Harbor Laboratory Press, 14(4), pp. 391–6. 1.

Addendum A:

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITILE OF RESEARCH PROJECTS:

1. Proteomic investigation into the effect of hypoxic culturing on primary breast carcinoma cells.

REFERENCE NUMBER: S16/03/048

PRINCIPAL INVESTIGATORS:
Dr Derick van Vuuren
Miss Victoria Patten

ADDRESS:
Division of Medical Physiology, FISAN building, Tygerberg Campus, Francie van Zyl drive, Stellenbosch University, Bellville.

CONTACT NUMBER:
Dr Derick van Vuuren – 021 9389390

Dear patient

You are being invited to take part in our research study on breast cancer, and we would appreciate if you could take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions you may have about any part of this project that you may not fully understand as it is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. Should you so decline, this will in no way affect you negatively by any means. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

*This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research*

What is the study all about and how can you help?

- The study will be conducted at the department of Medical Physiology of Stellenbosch University, Tygerberg campus, and we are hoping to recruit approximately 50 participants.
- During your visit to the FNA clinic at Tygerberg Hospital, **we would like to ask your permission to take 1 to 2 extra fine needle aspirates (FNA)** when the nurses perform the routine diagnostic FNA procedures, to use in our research. We will use this aspirate to grow more cancer cells (if you do have cancer) that can be used in further research to assess variations in the proteins present within the cells, differences in molecular signalling as well as cell death processes.
- **For mastectomy patients**, when the cancer tumour is removed from your breast during surgery, a biopsy (small piece of the tumour) is routinely taken by the surgeon to send to the pathologist for classification. **For our study, we would like to ask your permission take a small piece of this tumour (already removed from you) to use in our research.** We will use this tumour tissue to grow more cancer cells that can be used in further research to assess variations in the proteins present within the cells, differences in molecular signalling as well as cell death processes.
- Cancer cells are able to adapt and thrive in hypoxic (reduced oxygen levels) conditions in the body. We would like to set up and characterise a primary cell culture model to investigate the effect of hypoxia on culturing cells and provide a useful tool for future research with a more accurate representation of cancer cells within the human body and characteristics comparable with the original tumour

Why have you been invited to participate?

- To make this study clinically significant, we need as many volunteers as possible to take part. We would like to ask your participation because either you have come to the FNA clinic with an undiagnosed lump in your breast, OR you have been diagnosed with breast cancer.

What will your responsibilities be?

- You will have no responsibilities during this project.

Will you benefit from taking part in this research?

- You will not benefit directly from this study, but future patients will benefit if this study is successful.

Are there any risks involved in your taking part in this research?

- The FNA will incur absolute minimal discomfort and risk.
- Mastectomy patients: Taking part in this study will not induce any further risk apart from the usual risk factors involved in the mastectomy surgery.

Do you have to take part?

- It is entirely your decision to take part in this research study or not, and it is completely acceptable if you decide to withdraw at any point during the study. The usual standard of care will apply to all patients.

Who will have access to your medical records?

- For us to make sense of our results, we will need to know how your cancer (if applicable) has been treated until now, and also what the diagnostic findings of the biopsy will be. All the information collected will be treated as confidential and protected. If it is used in a publication or thesis, the identity of the participant will remain anonymous. Only the researchers (Dr Derick van Vuuren, Professor A-M Engelbrecht and Ms V Patten) will have access to the information.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

- The risk to patients receiving FNA is minimal. Taking part in this study will not induce any further risk outside of usual risk factors involved in FNA procedures.
- For Mastectomy patients: Taking part in this study will not induce any further risk apart from the usual risk factors involved in surgery. No injury can occur as a direct result of taking part in this study.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study. There will be no costs involved for you, if you do take part.

Is there anything else that you should know or do?

- Should you have any further queries about the study, or encounter any problems, please feel free to contact **Dr Derick van Vuuren** at tel 021-938 9390.
- You can contact the **Health Research Ethics Committee** at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …… agree to take part in a research study entitled “Proteomic investigation into the effect of hypoxic culturing on primary breast carcinoma cells”.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ……. on (*date*) …….. 2017.

.....	.....
Signature of participant	**Signature of witness**

Declaration by investigator

I *(name)* ……. declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....……. On (*date*) ……..…….. 2017.

.....	.....
Signature of investigator	**Signature of witness**

Declaration by interpreter

I *(name)* …….. declare that:

- I assisted the investigator (*name*) …….. to explain the information in this document to (*name of participant*) …….. using the language medium of Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…….. on (*date*) ……...……2017

....

Signature of interpreter **Signature of witness**

Addendum B

DEELNEMERINLIGTINGSBLAD EN -TOESTEMMINGSVORM

TITEL VAN DIE NAVORSINGSPROJEK:

Ondersoek na die effek van langtermyn hipoksiese blootstelling op die proteoom van primêre mammakarsinoom selle.

VERWYSINGSNOMMER: S16/03/048

HOOFNAVORSER:
Dr Derick van Vuuren
Mej Victoria Patten

ADRES:
Afdeling van Geneeskundige Fisiologie, FISAN gebou, Tygerberg kampus, Stellenbosch Universiteit, Francie van Zjil Rylaan, Bellville.

KONTAKNOMMER:
Dr Derick van Vuuren – 021 938 9390

Beste Pasiënt

U word versoek om deel te neem aan ’n navorsingsprojek wat handel oor borskanker. Lees asseblief hierdie inligtingsblad op u tyd deur, aangesien die detail van die navorsingsprojek hierin verduidelik word. Indien daar enige deel van die navorsingsprojek is wat u nie ten volle verstaan nie, is u welkom om die navorsingspersoneel of dokter daaroor uit te vra. Dit is baie belangrik dat u ten volle moet verstaan wat die navorsingsprojek behels en hoe u daarby betrokke kan wees. U deelname is **volkome vrywillig** en dit staan u vry om deelname te weier. U sal op geen wyse hoegenaamd negatief beïnvloed word indien u sou weier om deel te neem nie. U mag ook te eniger tyd aan die navorsingsprojek onttrek, selfs al het u ingestem om deel te neem.

Hierdie navorsingsprojek is deur die Gesondheidsnavorsingsetiekkomitee (GNEK) van die Universiteit Stellenbosch goedgekeur en sal uitgevoer word volgens die etiese riglyne en beginsels van die Internasionale Verklaring van Helsinki en die Etiese Riglyne vir Navorsing van die Mediese Navorsingsraad (MNR).

Wat behels hierdie navorsingsprojek en hoe kan u deelneem?

- Die studie self gaan plaasvind by die departement van Geneeskundige Fisiologie van die Universiteit van Stellenbosch, op die Tygerberg kampus. Ons hoop om nagenoeg 50 deelnemers aan die projek te werf.
- **Ons wil graag u toestemming vra om 1 of 2 ekstra fynnaald aspirate (FNA) by u te versamel** tydens die roetine diagnostiese FNA prosedures, waaraan u blootgestel gaan word tydens u besoek aan die FNA kliniek by Tygerberg hospitaal. Ons gaan hierdie aspiraat gebruik om meer kankerselle (indien u kanker het) te groei, wat ons dan sal kan gebruik in verdere navorsing aangaande verskille in die proteïene teenwoordig in die selle, verskille in hoe die selle aanpas by hul omgewing en ook seldood prosesse.
- **Vir mastektomie pasiënte**, nadat die kankergewas chirurgies verwyder is vanuit u bors word die hele monster roetineweg deur die chirurg na ‘n patoloog gestuur vir evaluasie en klassifikasie. **Vir ons studie wil ons graag u toestemming vra om ’n 2-3 mm dik snit van die gewas (wat reeds uit u bors verwyder is) te gebruik in ons studie.** Ons gaan hierdie tumorweefsel gebruik om kankerselle in die laboratorium te groei, wat dan weer gebruik sal word vir verdere navorsing aangaande verskille in die proteïene teenwoordig in die selle, verskille in hoe die selle aanpas by hul omgewing en ook seldood prosesse.
- Die weerstand van kankerselle teen anti-kanker medikasies speel ’n belangrike rol in die oorlewing van tumorselle en beperk dus die effektiewe gebruik van hierdie medikasies. Ons stel belang in hoe kankerselle se metaboliese prosesse aanpas om uiteindelik by te dra tot hul weerstandigheid. Die uiteindelike doel is om hierdie prosesse te manipuleer sodat borskanker meer effektief behandel kan word.

Waarom is u genooi om deel te neem?

- Om hierdie studie klinies betekenisvol te maak het ons soveel as moontlik vrywilligers nodig om aan die studie deel te neem. Ons wil graag u deelname versoek omdat u óf die FNA kliniek besoek met ‘n ongediagnoseerde knop in u bors, óf u is gediagnoseer met borskanker.

Wat sal u verantwoordelikhede wees?

- U sal geen verantwoordelikhede tydens hierdie studie hê nie.

Sal u voordeel trek deur deel te neem aan hierdie navorsingsprojek?

- U sal nie direk voordeel trek deur u deelname aan hierdie studie nie, maar toekomstige pasiënte mag dalk wel bevoordeel word indien die studie suksesvol is.

Is daar enige risiko's verbonde aan u deelname aan hierdie navorsingsprojek?

- Die FNA gaan gepaard met minimale ongemak en risiko.
- Vir mastektomie pasiënte: Deelname aan hierdie studie sal u nie blootstel aan enige addisionele risiko nie, buiten vir die gewone risikofaktore wat betrokke is by mastektomie chirurgie.

Watter alternatiewe is daar indien u nie instem om deel te neem nie?

- U deelname aan hierdie studie is geheel en al u eie keuse. Dit is heeltemal aanvaarbaar indien u ter enige tyd tydens die studie besluit om u deelname te onttrek. Die gewone standaard van behandeling sal van toepassing wees vir alle pasiënte, ongeag deelname aan hierdie studie, al dan nie.

Wie sal toegang hê tot u mediese rekords?

- Om sin te maak van ons resultate sal ons moet weet hoe u kanker (indien van toepassing) tot op hede behandel is, asook die diagnostiese bevindings van die biopsie. Al die inligting wat ingesamel gaan word sal as konfidensieel hanteer word en sal ook beskerm word. Indien dit gebruik word in 'n publikasie of tesis, sal die identiteit van die deelnemer anoniem bly. Slegs die navorsers (Dr Derick van Vuuren, Prof A-M Engelbrecht en Me Patten) sal toegang tot hierdie inligting hê.

Wat sal gebeur in die onwaarskynlike geval van 'n besering wat mag voorkom as gevolg van u deelname aan hierdie navorsingsprojek?

- Die risiko vir pasiënte op wie FNA uitgevoer gaan word is minimaal. Deelname aan hierdie studie gaan geen verdere risiko, buiten die gewone risikofaktore verwant aan FNA, tot gevolg hê nie.
- Vir mastektomie pasiënte: Deelname aan hierdie projek gaan geen verdere risiko tot gevolg hê nie, buiten vir die standaard risikofaktore wat betrokke is by chirurgie. Geen besering kan plaasvind as 'n direkte gevolg van deelname aan hierdie projek nie.

Sal u betaal word vir deelname aan die navorsingsprojek en is daar enige koste verbonde aan deelname?

Nee, u sal nie betaal word vir deelname aan die navorsingsprojek nie. U sal geen addisionele kostes aangaan indien u wel besluit om deel te neem aan die projek nie.

Is daar enigiets anders wat u moet weet of doen?

- U kan **Dr Derick van Vuuren** kontak by tel **021 938 9390** indien u enige verdere vrae het of enige probleme ondervind.
- U kan die **Gesondheidsnavorsingsetiek administrasie** kontak by 021-938 9207 indien u enige bekommernis of klagte het wat nie bevredigend deur u studiedokter hanteer is nie.
- U sal 'n afskrif van hierdie inligtings- en toestemmingsvorm ontvang vir u eie rekords.

Verklaring deur deelnemer

Met die ondertekening van hierdie dokument onderneem ek, ……....……..……..……., om deel te neem aan 'n navorsingsprojek getiteld "*Ondersoek na die effek van langtermyn hipoksiese blootstelling op die proteoom van primêre mammakarsinoom selle."*

Ek verklaar dat:

- Ek hierdie inligtings- en toestemmingsvorm gelees het of aan my laat voorlees het en dat dit in ’n taal geskryf is waarin ek vaardig en waarmee ek gemaklik is.
- Ek die geleentheid gehad het om vrae te stel en dat al my vrae bevredigend beantwoord is.
- Ek verstaan dat deelname aan hierdie navorsingsprojek **vrywillig** is en dat daar geen druk op my geplaas is om deel te neem nie.
- Ek te eniger tyd aan die navorsingsprojek mag onttrek en dat ek nie op enige wyse deur my ontrekking benadeel sal word nie.
- Ek gevra mag word om van die navorsingsprojek te onttrek voordat dit afgehandel is indien die studiedokter of navorser van oordeel is dat dit in my beste belang is, of indien ek nie die ooreengekome navorsingsplan volg nie.

Geteken te *(plek)* .....…….. op *(datum)* ……..…….. 2017.

.....	.....
Handtekening van deelnemer	**Handtekening van getuie**

Verklaring deur navorser

Ek *(naam)* ……..…… verklaar dat:

- Ek die inligting in hierdie dokument verduidelik het aan …….……
- Ek hom/haar aangemoedig het om vrae te vra en voldoende tyd geneem het om dit te beantwoord.
- Ek tevrede is dat hy/sy al die aspekte van die navorsingsprojek soos hierbo bespreek, voldoende verstaan.
- Ek 'n tolk gebruik het/nie 'n tolk gebruik het nie. (*Indien 'n tolk gebruik is, moet die tolk die onderstaande verklaring teken.)*

Geteken te *(plek*) .....…….. op *(datum*) ……..…….. 2017.

....

Handtekening van navorser **Handtekening van getuie**

Verklaring deur tolk

Ek *(naam)* ……..……. verklaar dat:

- Ek die navorser *(naam*) ……..…… bygestaan het om die inligting in hierdie dokument in Afrikaans/Xhosa aan *(naam van deelnemer)* ……..... te verduidelik.
- Ons hom/haar aangemoedig het om vrae te vra en voldoende tyd geneem het om dit te beantwoord.
- Ek ’n feitelik korrekte weergawe oorgedra het van wat aan my vertel is.
- Ek tevrede is dat die deelnemer die inhoud van hierdie dokument ten volle verstaan en dat al sy/haar vrae bevredigend beantwoord is.

Geteken te *(plek*) .....…….. op *(datum*) ……..……..... 2017.

.....	.....
Handtekening van tolk	**Handtekening van getuie**

Addendum C

Phosphatase Assay Statistical Analysis Models

```
      name:  <unnamed>
       log:  C:\Users\tonyae\Documents\Stellenbosch\Consulting\Victoria Patten\6
> feb 2018.log
  log type:  text
 opened on:   6 Feb 2018, 10:03:44

. use "C:\Users\tonyae\Documents\Stellenbosch\Consulting\Victoria Patten\victoria
>  long data.dta"

. encode group, generate(group_n)

. codebook group_n

-------------------------------------------------------------------------------
group_n                                                         (unlabeled)
-------------------------------------------------------------------------------

                 type:  numeric (long)
                label:  group_n

                range:  [1,3]                units:  1
        unique values:  3                    missing .:  0/864

           tabulation:  Freq.   Numeric  Label
                        288         1  Control
                        288         2  Hypoxia
                        288         3  Pos Control
```

Multiple linear regression analysis using PP@A as the dependent variable and incubation time, measurement time, and group (hypox vs control) as independent variables and adjusting for the repeated measures over measurement time using clustered robust standard errors.
Hypoxia vs control
model 1
no interaction term between time and group.
only time is significant (p<0.001)meaning there was on average a 684 unit increase in PP2A with every 5 minute increase in measurement time. Incubation time did not affect PP2A nor did group.

```
. regress PP2A i.Incubation  time  i.group_n if group_n<=2, vce(cluster id)

Linear regression                               Number of obs     =        480
                                                F(5, 19)          =      29.87
                                                Prob > F          =     0.0000
                                                R-squared         =     0.6196
                                                Root MSE          =     3815.5
```

```
                                (Std. Err. adjusted for 20 clusters in id)
------------------------------------------------------------------------------
             |               Robust
       PP2A |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  Incubation |
           4 |  -691.9705   2208.681    -0.31   0.757    -5314.794    3930.853
           6 |  -1735.205   2138.998    -0.81   0.427    -6212.178    2741.769
           8 |  -2136.194   2241.688    -0.95   0.353    -6828.102    2555.713
             |
        time |   684.3865   68.34555    10.01   0.000     541.3376    827.4354
             |
     group_n |
     Hypoxia |   1047.307   1469.445     0.71   0.485    -2028.277    4122.892
       _cons |    567.72   1423.577     0.40   0.694    -2411.861    3547.301
------------------------------------------------------------------------------
```

. gen timegroup = time* group_n
model 2
Interaction term between time and group.
only time is significant (p=0.006)meaning there was on average a 569 unit increase in PP2A with every 5 minute increase in measurement time. Incubation time did not affect PP2A nor did group. THere was no interaction between time and group, therefore final model is model 1.

```
. regress PP2A i.Incubation  time  i.group_n timegroup if group_n<=2, vce(cluster
>  id)

Linear regression                               Number of obs     =        480
                                                F(6, 19)          =      25.82
                                                Prob > F          =     0.0000
                                                R-squared         =     0.6214
                                                Root MSE          =     3810.2

                                (Std. Err. adjusted for 20 clusters in id)
------------------------------------------------------------------------------
             |               Robust
       PP2A |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  Incubation |
           4 |  -691.9705   2211.015    -0.31   0.758    -5319.678    3935.737
           6 |  -1735.205   2141.258    -0.81   0.428    -6216.908    2746.499
           8 |  -2136.194   2244.057    -0.95   0.353    -6833.059    2560.671
             |
        time |   569.5443    184.582     3.09   0.006     183.2097     955.879
             |
     group_n |
     Hypoxia |   90.28922   492.4886     0.18   0.856    -940.5012     1121.08
   timegroup |   76.56145   135.6891     0.56   0.579    -207.4392    360.5621
       _cons |   1046.229   1414.797     0.74   0.469    -1914.975    4007.433
------------------------------------------------------------------------------
```

control vs pos control

model 1
No interaction term
only time is significant (p<0.001)meaning there was on average a 748 unit increase in PP2A with every 5 minute increase in measurement time. Incubation time did not affect PP2A nor did group.

```
. regress PP2A i.Incubation  time  i.group_n  if group_n>1, vce(cluster id)

Linear regression                               Number of obs     =        480
                                                F(5, 19)          =      21.35
                                                Prob > F          =     0.0000
                                                R-squared         =     0.5540
                                                Root MSE          =     4786.5

                                   (Std. Err. adjusted for 20 clusters in id)
------------------------------------------------------------------------------
             |               Robust
        PP2A |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  Incubation |
           4 |  -867.8307   2831.739    -0.31   0.763    -6794.728    5059.067
           6 |   -813.888   2715.079    -0.30   0.768    -6496.614    4868.838
           8 |  -3056.063   2757.494    -1.11   0.282    -8827.563    2715.438
             |
        time |   748.4501   83.06968     9.01   0.000     574.5833     922.317
             |
     group_n |
Pos Control  |   658.4974     1866.9     0.35   0.728    -3248.969    4565.964
       _cons |      866.7   1941.428     0.45   0.660    -3196.755    4930.155
------------------------------------------------------------------------------
```

Model 2
Interaction term added
No significant interaction between time and group, therefore revert to model 1.

```
. regress PP2A i.Incubation  time  i.group_n timegroup if group_n>1, vce(cluster
> id)

Linear regression                               Number of obs     =        480
                                                F(6, 19)          =      18.95
                                                Prob > F          =     0.0000
                                                R-squared         =     0.5547
                                                Root MSE          =     4788.2

                                   (Std. Err. adjusted for 20 clusters in id)
------------------------------------------------------------------------------
             |               Robust
        PP2A |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  Incubation |
           4 |  -867.8307   2834.731    -0.31   0.763     -6800.99    5065.329
           6 |   -813.888   2717.948    -0.30   0.768    -6502.618    4874.842
           8 |  -3056.063   2760.407    -1.11   0.282    -8833.661    2721.536
```

```
             |
        time |   619.5355   419.5653     1.48   0.156    -258.6248    1497.696
             |
     group_n |
Pos Control  |   13.92409     579.36     0.02   0.981     -1198.69    1226.538
   timegroup |   51.56586   165.8883     0.31   0.759    -295.6423     398.774
       _cons |   1188.987   1744.681     0.68   0.504    -2462.673    4840.646
------------------------------------------------------------------------------

. log close
      name:  <unnamed>
       log:  C:\Users\tonyae\Documents\Stellenbosch\Consulting\Victoria Patten\6
> feb 2018.log
  log type:  text
 closed on:   6 Feb 2018, 10:13:06
```