

**Folate: Friend or Foe? An investigation into the  
opposing roles of folate in glioma**

**by**

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## Abstract

For individuals diagnosed with a glioma, survival rates have shown little improvement over the last 40 years due to the heterogeneity of tumours and the difficulty of specifically targeting the tumour whilst sparing surrounding healthy tissue. Altered gene methylation is often seen in glioma cells, but methylating agents such as folate, may reverse aberrant methylation. Folate treatment has shown a beneficial effect, reducing risk of certain cancers (colorectal, breast, squamous cell carcinoma) but other studies have shown detrimental results whereby proliferation of cancer increased (mammary, prostate). The aim of this thesis was to investigate the opposing roles of folate in glioma. The glioma cell lines 1321N1, U87 MG and non-cancerous glial SVGp12 cells were used for analysis. Cells were grown in folate deficient, folic or folinic acid supplemented media and compared to standard cell culture media. Cell viability, cell cycle and apoptosis analysis along with methylation status and protein expression of the genes of interest; *PTEN*, *FOLR1*, *RFC*, *PCFT*, and *MTHFR* were analysed to determine differences between cell lines following treatment. The investigation showed that folic and folinic acid behaved differently depending on concentration used and the cell lines treated. Folic acid at 5 µg/ml significantly increased cell viability and protein expression levels in the U87 MG and SVGp12 cell lines, whilst the folinic acid (35 µg/ml) resulted in significant decreased cell viability, increased apoptotic activity and down regulation of the folate transporters in the 1321N1, U87 MG and SVGp12 cell lines. Folate treatment did not significantly alter cell cycle phase. Altered methylation of genes specific for folate metabolism and transport did not explain the cytotoxic effects of folate in cell lines. In conclusion, the work presented here signifies that folinic acid rather than folic acid would be more suitable for glioma treatment. The effect of folinic acid treatment on glioma had not been previously studied, and the knowledge obtained here regarding the effects of folic and folinic acid treatment on folate transporter expression in glioma has advanced understanding.

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## List of abbreviations

5-MTHF	5 methyltetrahydrofolate
5-MeTHF	5,10 methylenetetrahydrofolate
A/O	Antioxidant
BCPC	Brain cancer propagating cells
BSA	Bovine serum albumin
CNS	Central Nervous System
CSCs	Cancer stem cells
CSF	Cerebral spinal fluid
DHFA	Dihydrofolic acid
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNMTs	DNA methyltransferases
ECACC	European Collection of Authenticated Cell Cultures
EMEM	Eagle's minimal essential medium
FA	Folic Acid
FBS	Foetal bovine serum
FDH	Formyltetrahydrofolate dehydrogenase
Fo A	Folinic Acid
FOLR1	Folate receptor 1
GF-AFC	Glycylphenylalanyl-aminofluorocoumarin
GFAP	Glial fibrillary acidic protein
HDAC	Histone deacetylase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HFM	Hereditary folate malabsorption
HLA	Human leukocyte antigen

MDA	Methyl donating agents
MDD	Major depressive disorder
MDS	Myelodysplastic syndrome
MGMT	O <sup>6</sup> -methylguanine DNA methyltransferase
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MS-PCR	Methylation specific polymerase chain reaction
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTRR	Methionine synthase reductase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NEAA	Non-essential amino acid
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate buffered saline
PCFT	Proton coupled folate transporter
PES	Pheazine ethyl sulfite
PFA	Paraformaldehyde
PTEN	Phosphatase and tensin homolog
RFC	Reduced folate carrier
RFU	Raw fluorescence units
SAM	S-adenosyl methionine
SD	Standard deviation
SNPs	Single nucleotide polymorphisms
SSRIs	Selective serotonin reuptake inhibitors
STRs	Short tandem repeats

TAE	Tris-acetate- Ethylenediaminetetraacetic acid
TBST	Tris buffer tween
THF	Tetrahydrofolate
TIC	Tumour Initiating Cells
TMZ	Temozolomide
WHO	World Health Organisation

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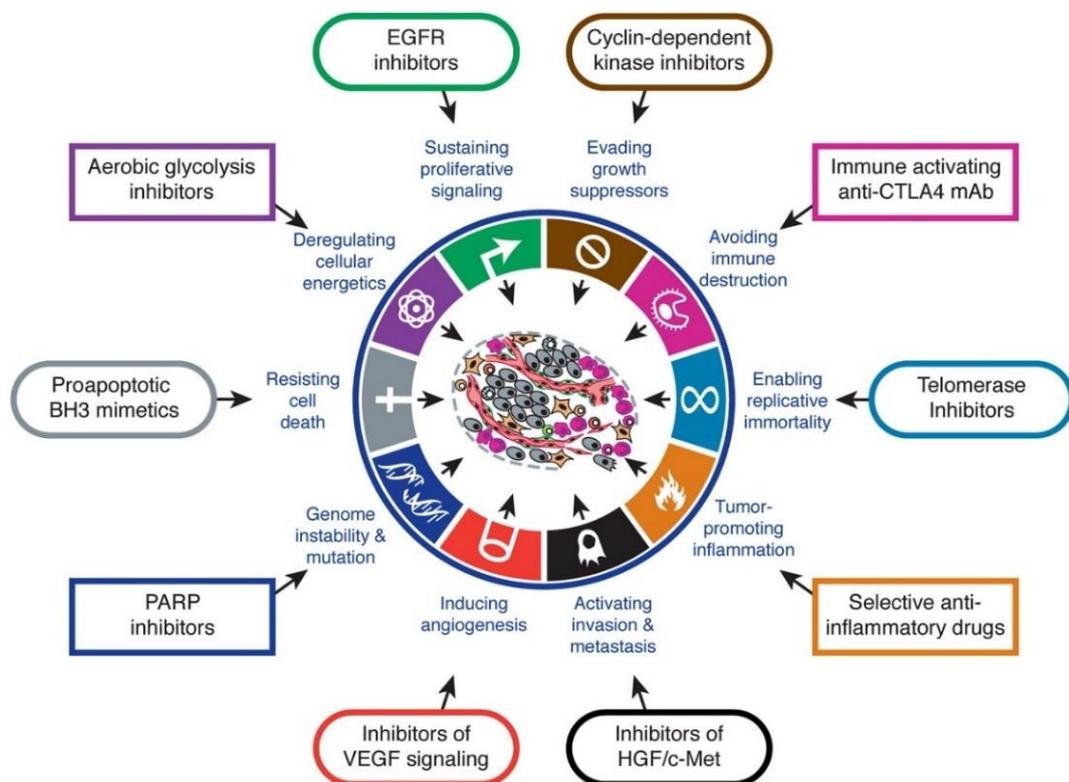
# Chapter 1

# 1 INTRODUCTION

## 1.1 Origin of cancer

Tumours can be defined as a collection or growth of abnormal cells which can result from a multistep and complex acquisition of multiple somatic and/or germline genetic mutations and epigenetic modulation. At the simplest level, the origin of cancer may be viewed as oncogenes with increased function, and tumour suppressor genes with decreased function, leading to tumourigenesis (Vescovi *et al.*, 2006).

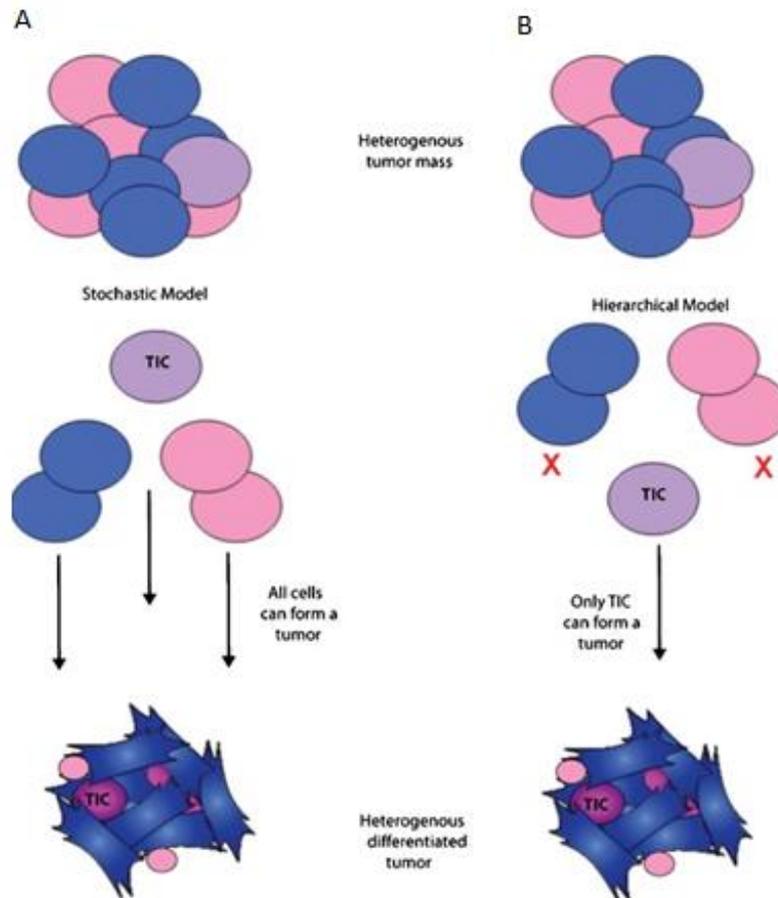
The landmark paper by Hanahan and Weinberg (2011) summarised that malignant growth can occur following manifestation of ten essential cell physiology alterations shown in Figure 1.1 (Hanahan and Weinberg). According to the mathematical model of Frank (2007), 6-7 of these alterations are required in order for a cell to become malignant (Frank, 2007).



**Figure 1.1 The hallmarks of cancer** (Hanahan and Weinberg 2011).

The surrounding textboxes indicate current therapeutic targets that are exploited to counteract malignant growth.

The heterogeneity of tumour phenotype has widely been acknowledged as a major obstacle for treatment of cancer. Two models have been proposed to explain the tumorigenic process: the stochastic and the hierarchical model (Figure 1.2). The stochastic model suggests that the origin of cancer growth is random and all cancer cells have the potential to renew and differentiate, dependent upon an acquisition of a particular set of somatic mutations. For the stochastic theory, tumours are assumed to be biologically homogeneous, but show heterogeneity in either intrinsic (for example levels of transcription factors, signalling pathways) or extrinsic factors (individual risk factors, microenvironment, immune response) which can alter the behaviour of individual cells within the tumour (Dick, 2008). In contrast, the hierarchical model suggests that only a small subset of tumour initiating cells can form a tumour and in that respect the cells are like distorted normal tissues, that are biologically distinct from the rest of the hierarchy (Dick, 2009). This hierarchical model also gives support to the theory of the existence of cancer stem cells (CSCs), where CSCs sit at the top of the hierarchy and differentiate into small subsets of cells capable of either self-renewal or maintenance of tumour bulk (Cabrera *et al.*, 2015; Mack *et al.*, 2016; Vescovi *et al.*, 2006). It is acknowledged that subsets of CSCs with differential cellular and functional hierarchy will require different approaches to treatment (Dirks, 2008a). CSCs are now considered to contribute to the majority of malignant cells (Calabrese *et al.*, 2007); hence contributing further evidence to the hierarchy theory as the most favourable model for tumourigenesis (Tabassum and Polyak, 2015).



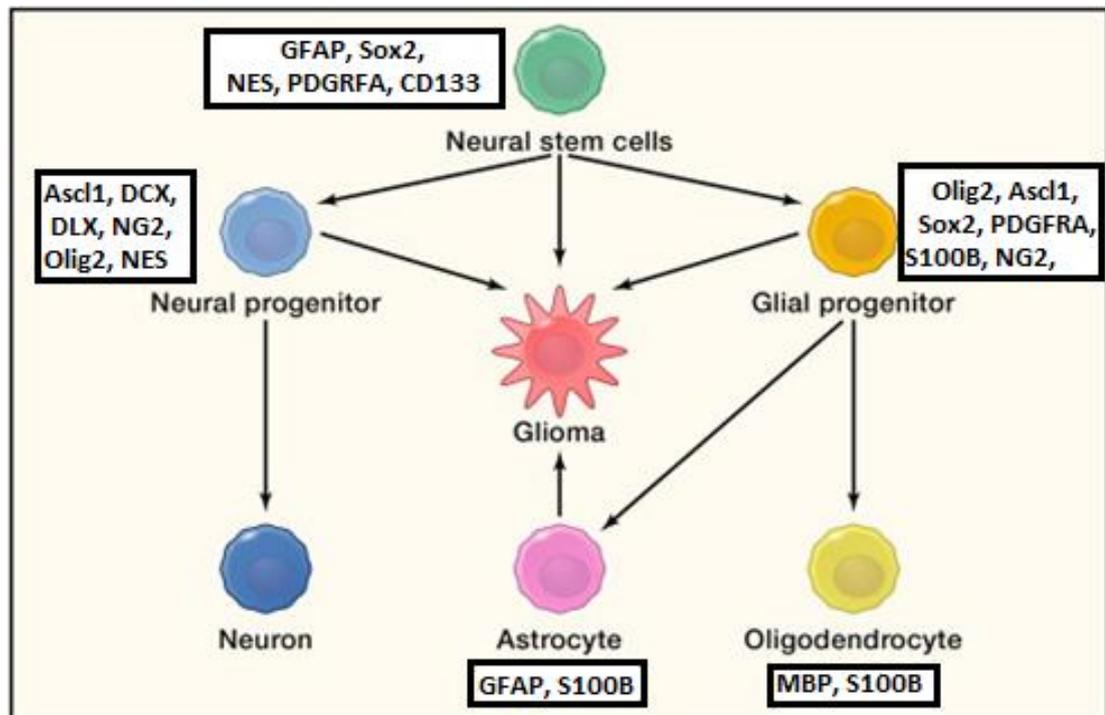
**Figure 1.2 Tumour heterogeneity process**

There are two proposed models for tumour initiation. The stochastic model in which any cell has the potential to differentiate to form a tumour (A) and the hierarchical model which suggests that tumours are formed from tumour initiating cells (B) (Mathews, Cabarcas *et al.* 2011).

One explanation for the origin of cancer is that genetic instability occurs within a stem cell population, or in differentiated cells which have undergone a malignant transformation and have begun to show stem cell like properties (Widschwendter *et al.*, 2007). Tumour progression is then dictated by adaptation to the tumour microenvironment through epigenetic control which then drives selection of genetic variant CSCs most suited to survival, proliferation, invasion and metastasis (Greenhough *et al.*, 2009). The current hypothesis for gliomas is indeed that transformed stem cells may drive cancer progression (Denysenko *et al.*, 2010; Dirks, 2008b; Filbin and Stiles, 2015). Adaptation through epigenetic control is a key research theme that will be explored as part of this thesis.

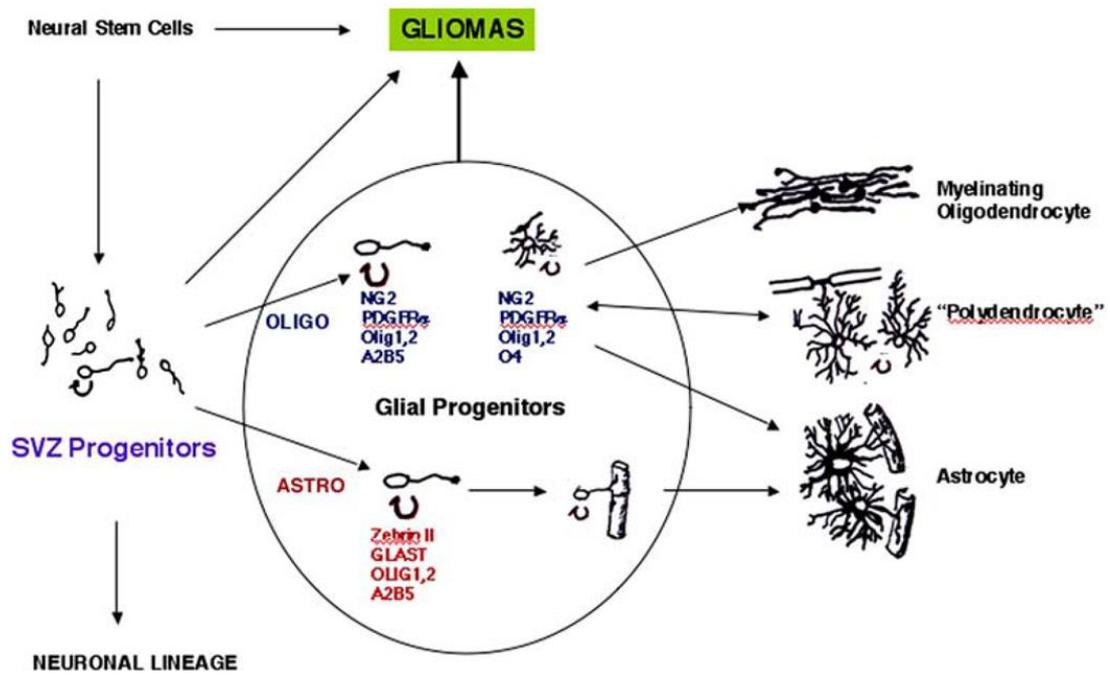
### 1.1.1 Gliomagenesis

Gliomas have been shown to originate from neural stem cells (Noushmehr *et al.*, 2010c), which are cells that have the potential to self renew and can differentiate into neurones, astrocytes and oligodendrocytes, and progenitor cells, that are programmed to differentiate into its target cell (Phillips *et al.*, 2006b)(Figure 1.3). Cells are determined by measurement of the expression of neural and glial markers (Canoll and Goldman, 2008) (Figures 1.3 and 1.4). Several markers have been suggested for the identification of differentiated stem or cancerous cells. There is however controversy surrounding the reliability of each marker (Neradil and Veselska, 2015; Wu and Wu, 2009).



**Figure 1.3 Glioma cell origin**

*Glioma cells can originate from neural stem cells (NSCs) and glial and neural progenitor cells (Chen *et al.*, 2012). Mutations to the neural stem cell, allow for transformation to a malignant glioma. Text in boxes relate to markers for the relative cell type. Glial fibrillary acidic protein (GFAP), Sex determining region Y-box 2 (Sox2), Nestin (NES), Platelet-Derived Growth Factor Receptor Alpha (PDGFR), Prominin-1 (CD133), Achaete-scute homolog 1 (Ascl1), doublecortin (DCX), Distal-less homeobox (DLX), neuron-glia antigen 2(NG2), Oligodendrocyte transcription factor (OLIG2), S100 calcium-binding protein B (S100B), Myelin basic protein (MBP).*

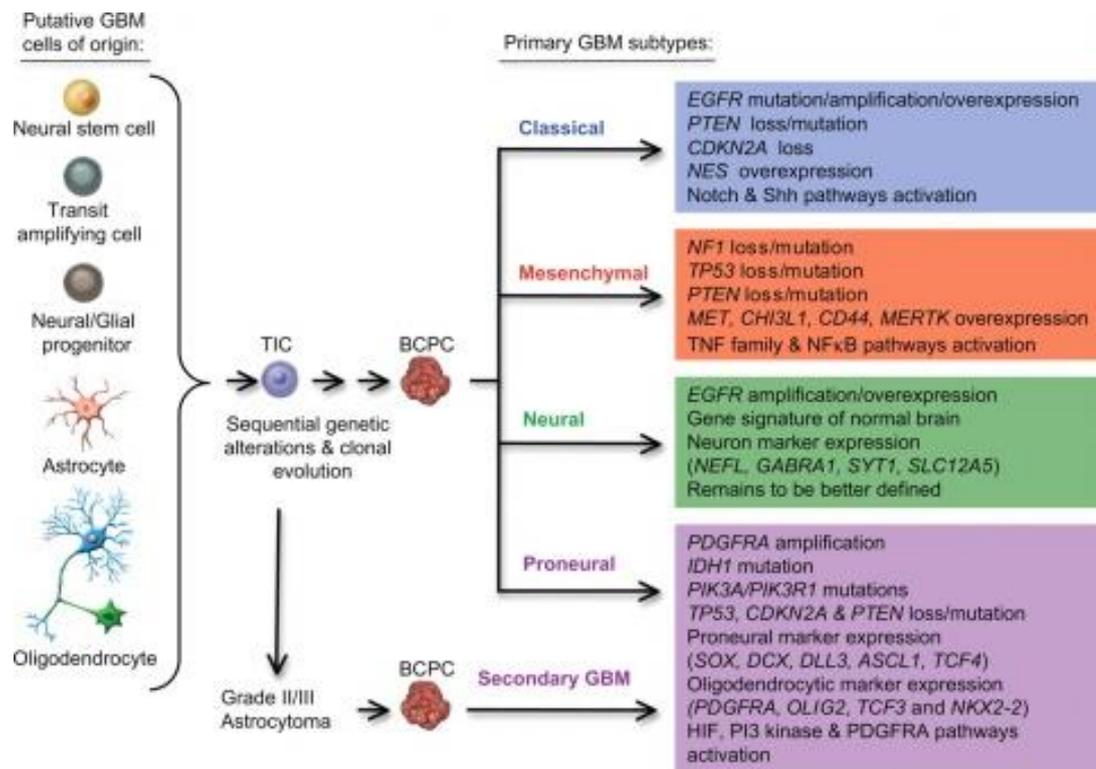


**Figure 1.4** The development of oligodendrocytes and astrocytes from forebrain sub-ventricular zone (SVZ) cells.

Neural stem cells are considered to generate SVZ cells, a foundation of glioma, they generate both neuronal and glial tumours as well as oligodendrocytes and astrocytes (Canoll and Goldman, 2008).

### 1.1.2 Classification of brain tumours

Primary glioma are a *de novo* tumour of the brain or spinal cord (Kyritsis *et al.*, 2010). As can be seen from Figure 1.5 there are many subtypes of glioma, each categorised by distinct molecular markers (Louis *et al.*, 2016b). Glioma that have progressed from lower grade diffuse or anaplastic astrocytomas are identified as being IDH mutants (Louis *et al.*, 2016a; Ohgaki and Kleihues, 2013). All of the historical literature pre-2016 refers to this classification as secondary glioma, hence when reviewing the literature, the term secondary glioblastoma is used, but it should be noted that this classification is now obsolete since the revised World Health Organisation classifications were published in Aug 2016 (Louis *et al.*, 2016a).



**Figure 1.5 Genetic changes in different subtypes of glioma.**

*Tumour Initiating Cells, TIC, accumulate genetic and molecular changes to become brain cancer propagating cells, BCPC, which then differentiate into subtypes of glioma (Van Meir et al., 2010).*

## 1.2 Aetiology

### 1.2.1 Environmental Risk

The reason why glial cells differentiate into cancerous tumour cells is not fully understood. Therapeutic ionising or high dose radiation is the only established extrinsic risk factor, where a notable link has been found in children treated for leukaemia with x-ray irradiation (Bondy et al., 2008; Ohgaki, 2009). The influence of environmental factors such as lead (Anttila et al., 1996; Cocco et al., 1998; Rajaraman et al., 2006; Wong and Harris, 2000), cured meat (Blowers et al., 1997; Boeing et al., 1993; Chen et al., 2002; Giles et al., 1994; Huncharek et al., 2003; Lee et al., 1997), smoking (Blowers et al., 1997; Zheng et al., 2001), electromagnetic fields (Armstrong et al., 1994; Savitz and Loomis, 1995; Theriault et al., 1994; Villeneuve et al., 2002; Wrensch et al., 1999) and head injury (Gurney et al., 1996; Hochberg et al., 1984;

Schlehofer *et al.*, 1992) on risk of tumour development have yielded conflicting and inconclusive results. It is hypothesised that epigenetic modulation in response to extrinsic factors may be the driver behind changes following exposure to any of the aforementioned risks which will be discussed further in Section 1.7.2.

### 1.2.2 Genetic Risk

About 5% of brain tumours are thought to be linked to hereditary genetic factors (Cancer.Net 2012). Research has suggested that gliomas are likely to occur due to the inheritance and/or acquisition of several low risk mutated genes, rather than a few high risk mutations (Liu *et al.*, 2010) (Table 1.1).

**Table 1.1 Inherited predisposition to glioma.** Occurrence due to inherited or acquisition of several low risk mutated genes (Kyritsis, Bondy et al. 2010).

Cancer Syndrome	Altered Gene	Tumours
Li–Fraumeni syndrome	<i>TP53</i>	Sarcoma, breast, brain, leukaemia, adrenocortical carcinoma
Families with patients with multifocal glioma, glioma + second cancer	<i>TP53</i>	Various other tumours
Melanoma–astrocytoma syndrome	<i>P14 (ARF)</i>	Melanoma, astrocytoma
Neurofibromatosis 1	<i>NF1</i>	Glioma, neurofibroma, pheochromocytoma, meningioma, schwannoma
Neurofibromatosis 2	<i>NF2</i>	Bilateral acoustic schwannoma, meningioma, glioma, neurofibroma, ependymoma
Turcot's syndrome (type 1) (hereditary nonpolyposis cancer syndrome)	Mismatch repair genes ( <i>MLH1, MSH2, MSH6, PMS2</i> )	Colorectal carcinoma, glioma
Turcot's syndrome (type 2)	<i>APC</i>	Colorectal carcinoma, primary brain tumour
BRCA syndrome	<i>BRCA-1, BRCA-2</i>	Breast, ovarian, prostatic, pancreatic, glioma

The Online Mendelian Inheritance in Man (OMIM) website which catalogues human genes and genetic disorders, have identified 237 genes which relate to glioma. The Cancer Genome Atlas pilot project analysed 601 genes from 91 tumour-normal pairs, and found that only a small number of genes were regularly presenting with significant mutation (Network, 2008) including *TP53*, and *PTEN* which are involved in signalling pathways, tumour suppression and growth factor receptors (Brennan, 2011).

### **Tumour protein p53 (TP53)**

The *TP53* gene is frequently mutated in both primary and secondary glioblastoma but differences were observed in the location of the mutation where 57% of secondary *TP53* mutations occurred at codons 248 and 273, compared to only 17% for primary glioblastomas (Ohgaki and Kleihues, 2007). The remainder of *TP53* mutations in primary glioblastoma were more evenly dispersed across the different codons (Ohgaki and Kleihues, 2007).

### **Phosphate and tensin homolog protein (PTEN)**

The phosphatase and tensin homolog (PTEN) is a tumour suppressor protein, encoded by the *PTEN* gene that is frequently mutated in a large number of cancers, including breast, prostate, melanoma and endometrium (Milella *et al.*, 2015; Wu *et al.*, 2003). *PTEN* is located at 10q23.3 and is 108,818 base pairs in length, coding for a protein of 403 amino acids, and 47166 Da, and it has 1135 known single nucleotide polymorphisms (SNPs). It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumour suppressor by negatively regulating the AKT/PKB signalling pathway. Whilst *PTEN* inactivation is not required for tumour initiation, the loss of function is a hallmark for progression to a higher grade and is often related to alteration in methylation (Koul, 2008). Epigenetic silencing by *PTEN* promoter methylation has been observed as a mechanism of functional loss in some endometrial cancers (Dillon and Miller, 2014), in a subset of prostate cancer cell lines, and in melanoma, which in turn was linked to progression (Goel *et al.*, 2004). Mutation and deletion of the *PTEN* gene is frequently seen in primary glioblastoma, but is rare in low grade stages of almost all tumour types (Ohgaki and Kleihues, 2013).

Our knowledge of *TP53* and *PTEN* alterations in regards to cancer is well founded, and does not warrant further research, however, *PTEN* will be included as a positive control for methylation and protein expression studies in the present study.

### 1.3 Presentation and diagnosis

Brain tumours are often identified as the presentation of neurological symptoms which can include seizures, nausea, vomiting, headaches, imbalance and hemiparesis (Fomchenko and Holland, 2005), as well as tinnitus, personality change and memory loss. These symptoms can occur over a period of weeks to months due to intracranial pressure, tumour invasion or obstructive hydrocephalus (Ropper *et al.*, 2014). The presence of a glioma will then be confirmed with performance of high resolution magnetic resonance imaging (MRI) scans and histological analysis following biopsy.

Gliomas are the most common intracranial malignant tumour in humans (Ware *et al.*, 2003), and are graded by clinical, histological and molecular appearance as defined by the World Health Organisation (WHO) (Louis *et al.*, 2016b) The WHO histological grading system, as summarised in Table 1.2, aids to predict the biological behaviour of a neoplasm, malignancy, inform treatment choices, and predict prognostic patient outcome (Huse and Holland, 2010; Louis *et al.*, 2007).

**Table 1.2 WHO histological grading system**

Grade	Presentation
Grade I	Low proliferative potential. Potentially cured following surgery.
Grade II	Infiltrative. Often re-occurs. Some progression to higher grade e.g. diffuse astrocytoma progress to anaplastic astrocytomas or glioblastomas.
Grade III	Histological evidence of malignancy. E.g., nuclear atypia, brisk mitotic activity. Treatment is often with adjuvant radiotherapy and/or chemotherapy.
Grade IV	Cytologically malignant. Mitotically active, necrosis prone neoplasms. Rapid pre- and post- operative disease evolution. Fatal Outcome.

Diffuse astrocytomas are characterised by extensive and diffuse infiltration of tumour cells , with no defined tumour edge (Claes *et al.*, 2007). Anaplastic astrocytomas are rare tumours that develop from astrocytes, they are poorly differentiated, and lack specialisation (Smith *et al.*, 2001). Glioblastomas are the most frequent, and most malignant primary brain tumour (Parsons *et al.*, 2008). Oligodendrogliomas originate from two different cell types, oligodendrocytes, and glial cells (Persson *et al.*, 2010). Astrocytic tumours have been graded and separated into subtypes as shown in Table 1.3.

**Table 1.3 Tumours of neuroepithelial tissue** (Louis *et al.*, 2016b).

<b>Diffuse Astrocytic and Oligodendroglial Tumours</b>
Diffuse astrocytoma, IDH-mutant
Gemistocytic astrocytoma, IDH-mutant
Diffuse astrocytoma, IDH-wildtype
Diffuse astrocytoma, NOS
Anaplastic astrocytoma, IDH-mutant
Anaplastic astrocytoma, IDH-wildtype
Anaplastic astrocytoma, NOS
Glioblastoma, IDH-wildtype
Giant cell glioblastoma
Gliosarcoma
Epithelioid glioblastoma
Glioblastoma, IDH-mutant
Glioblastoma, NOS
Diffuse midline glioma, H3 K27M-mutant
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted
Oligodendroglioma, NOS
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted
Anaplastic oligodendroglioma, NOS
<b>Other Astrocytic Tumours</b>
Pilocytic astrocytoma
Pilomyxoid astrocytoma
Subependymal giant cell astrocytoma
Pleomorphic xanthoastrocytoma
Anaplastic pleomorphic xanthoastrocytoma

## 1.4 Incidence and prognosis

Prognosis often depends on several factors, where increased survival has been observed with young age, pilocytic histology, gross total resection, good Karnofsky performance status (how well a person is able to look after themselves, with a score of 100 being no complaints, and no symptoms of diseases, to 0 which is classified as deceased) (Stieber, 2001).

Medulloblastoma is the most frequently occurring malignant brain tumour within pediatric patients (Silvia, 2005), and arises from transformation of progenitor cells of the external granular layer of the cerebellum (Vescovi *et al.*, 2006). The most common low grade (I and II) tumours located within the central hemisphere and observed in adults are: pilocytic; low grade astrocytomas arising from astrocytes; oligodendrogliomas from oligodendrocytes and mixed oligo-astrocytomas (Grier and Batchelor, 2006; Stieber, 2001). The most common is the high grade (IV) glioma, which currently accounts for more than 50% of all gliomas (Adamson *et al.*, 2009). Gliomas are the most frequent brain tumour to affect adults (Huse and Holland, 2010), particularly for those of Caucasian descent who are more frequently affected than those of African or Asian ancestry (Ohgaki and Kleihues, 2005). Although there has been improved overall patient survival over the last 15 years for some cancer patients, most notably colon and kidney (Rachet *et al.*, 2009b); the same has not been observed for those with brain tumours. In 2006, the 1 year survival rate for those with brain cancer was only 35.8% in men, and even lower for women (30.1%). This is only a slightly increased survival rate from 2000, when males had a 30.9% survival rate (Rachet *et al.*, 2009b).

Prognosis is best understood as median survival time, which gives a clearer indication of how long a patient can expect to survive rather than how many people have made it to a specific time point. The median survival rate for patients diagnosed with glioma in 2010 was 15 months (Martinez *et al.*, 2010), but this has since been reported to be 6.1 months (Brodbelt *et al.*, 2015). Recurrence following resection occurs in around 90% of patients, at 2 cm from

the original site (Liang *et al.*, 2009). For individuals with an intracranial glioma, the 5-year survival rate was found to be 4-5% and has remained at this level for the last 40 years (McLendon and Halperin, 2003; Rachet *et al.*, 2009a). Since Grade III and IV tumours were found to be the most common, yet lethal tumours, it was decided to focus solely on these grades in the thesis presented here.

## 1.5 Treatment Options

Gliomas are particularly difficult to treat due to the infiltration of cells amongst normal cells and structures, which often leads to recurrence, and subsequent malignancy (Vescovi *et al.*, 2006). Symptoms and appearance of a primary tumour are very similar to brain metastases, however treatments vary between the two (Campos *et al.*, 2009).

Glioma patients are treated with surgery, depending upon the location of the tumour, followed by radiation. Once the timing, effective dose, and benefit over possible toxicity side effects have been established, chemotherapy is administered, which is most effective for tumours with chromosomal losses (Grier and Batchelor, 2006). Cranial radiotherapy and chemotherapy remains the mainstay of treatment, despite the adverse neurological side effects which can reduce cognitive ability, as well as affecting emotional and physical health (Ehrlich, 2002). In 2005, the Stupp protocol was developed, which used surgery, radiotherapy and the DNA alkylating agent, temozolomide (TMZ). TMZ methylates the N<sup>7</sup> and O<sup>6</sup> positions of guanine and the N<sup>3</sup> position on adenine resulting in a continuous cycle of DNA base mismatch repair (MMR) which eventually leads to strand-breaks and apoptosis of the cancerous cell. The results of additional TMZ treatment with radiotherapy were seen to be positive, with a 37% reduction in risk of death, which was a 2.5 month median survival benefit. Whilst this may not have seemed a great increase, it was a positive step, particularly given that the 2 year survival rate is 27% percent compared to 10% survival rate when compared to radiotherapy alone (Stupp *et al.*, 2005).

An emerging novel glioma treatment is Optune, a skull cap containing electrodes, which works by delivering oscillating electrical fields to the tumour to disrupt the formation of the mitotic spindle during metaphase, which leads to dielectrophoretic movement of charged and polar molecules during anaphase and telophase disrupting normal cytokinesis (Gutin and Wong, 2012). Phase 3 Trials have shown increased progression free survival and overall survival in patients with glioma wearing the Optune cap and receiving TMZ compared to TMZ alone (Stupp *et al.*, 2015). Temozolomide is an alkylating agent that works by delivering a methyl group to purine bases of DNA, resulting in damage to the DNA triggering death of the cell (Jihong *et al.*, 2012).

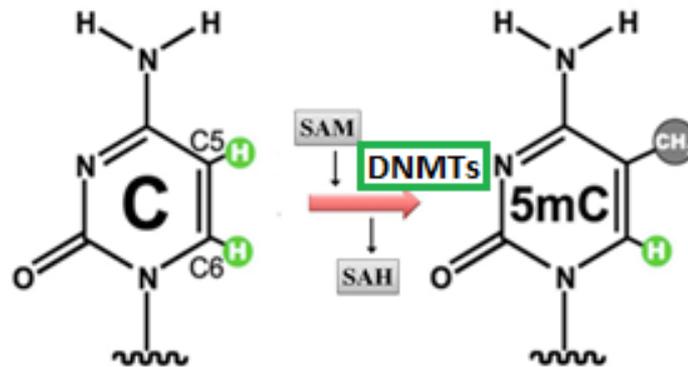
## 1.6 Variability in response to treatment

Glioma often exhibit cells in varying degrees of differentiation within the same tumour, for example the *CDKNA* gene, which encodes for the p16 protein, controlling the cell cycle, may be mutated in one cell, but not in that of another (Kyritsis *et al.*, 2010). The O<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*) gene, which is involved in DNA repair, is silenced in some glioma patients due to methylation of the promotor region (Esteller *et al.*, 1999). Individuals with lower levels of *MGMT* expression, were found to have increased rate of survival, as chemotherapy induced DNA damage was not repaired, and hence treatment was more effective. Methylation has long been seen to affect gene expression, and further research is required to consider the factors which affect methylation status in glioma patients.

## 1.7 DNA Methylation

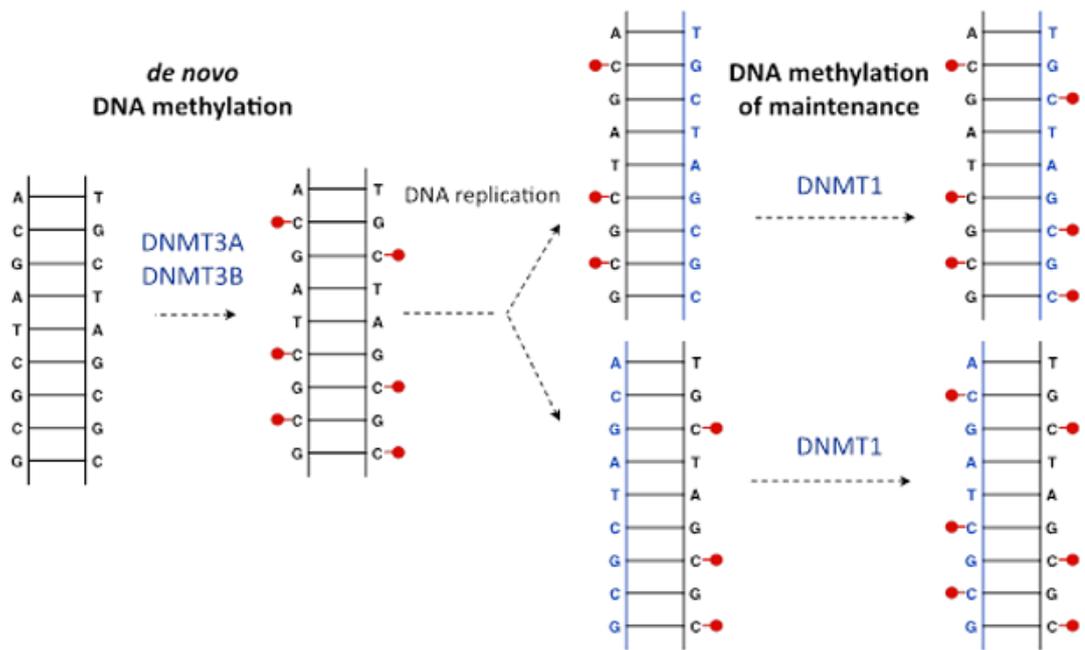
Epigenetic modifications such as DNA methylation and histone modification are important for regulation of genome expression (Kulis and Esteller, 2010). DNA methylation can be described as a switch, which can activate or repress gene transcription as necessary to regulate over or under expressed genes. Gene expression is altered by removal or addition of a methyl group to the C<sup>5</sup> position of the cytosine ring creating an epigenetic alteration (Das

and Singal, 2004). S-adenosyl methionine (SAM) acts as a methyl donor and methylation is regulated by a group of enzymes known as DNA methyltransferases (DNMTs) that catalyse the addition of methyl groups to cytosine residues (Foltz *et al.*, 2009) (Figure 1.6).



**Figure 1.6 Transfer of methyl group.** S-adenosyl methionine (SAM) is used as a methyl donor, which when catalysed by DNA methyltransferases, DNMTs creates S-adenosyl homocysteine (SAH) as the cofactor product (Maresca *et al.*, 2015).

Methylation within the genome does not occur uniformly, but instead occurs within regions, usually at sites known as CpG islands (where a cytosine base is followed by a guanine) which are areas rich in CpG pairing (Hatada *et al.*, 2006). Methylation is usually reversible but the rare occurrence of dense DNA methylation results in the irreversible silencing of gene expression (Abdolmaleky *et al.*, 2004; Baylin, 2005). DNA methylation can occur either *de novo*, or as maintenance methylation in order to preserve methylation status during DNA replication (Das and Singal, 2004) (Figure 1.7).



**Figure 1.7 DNA methylation patterns** (Maresca et al., 2015)

*De novo methylation is present from embryonic development, where methylation patterns are established. Maintenance methylation then occurs to preserve DNA methylation patterns after every DNA replication cycle (Law and Jacobsen, 2010).*

The genes commonly affected by methylation are the ones involved in regulation of the cell cycle, DNA repair, growth signalling and apoptosis. Any subsequent modulation may result in the initiation and progression of tumours (Widschwendter and Jones, 2002).

### 1.7.1 Tumourigenic Effect of Altered Methylation

DNA methylation does not always have a tumourigenic effect, however, tumourigenesis often occurs when normal gene function is altered, either through hyper- or hypomethylation of the gene leading to aberrant gene expression, which can result in tumourigenesis depending on the gene affected (Goodman and Watson, 2002). Indeed, many cancer cells have at least one hypermethylated gene in each major cellular pathway, but the target gene may differ between tumour types (Hervouet *et al.*, 2010; Paz *et al.*, 2003).

Hypomethylation is more commonly seen in solid tumours and can occur both globally throughout the genome, or regionally (Goodman and Watson, 2002). Hypomethylation usually occurs at heterochromatic DNA repeats, and at transcription control sequences (Ehrlich, 2002). Hypomethylation can result in evasion of apoptosis by reactivating antiapoptotic genes (Hervouet *et al.*, 2010). When activation of proto oncogenes is promoted, chromosomal instability often follows both of which can lead to tumourigenesis (Hervouet *et al.*, 2009b). Global hypomethylation has been seen to be present in glioma (Hervouet *et al.*, 2010) with increasing hypomethylation correlated with malignancy of the brain tumour (Ehrlich, 2002).

Hypermethylation occurs at a greater frequency in cancer than hypomethylation, with the most susceptible genes being those involved in cell cycle regulation, DNA repair and apoptosis (Das and Singal, 2004).

The genes which are most likely to be affected will vary depending upon the cancer (Table 1.4). Hypermethylation is a process which occurs to repress proapoptotic genes in order to prevent apoptosis from occurring, which results in uncontrolled cell growth.

**Table 1.4 Frequently methylated genes in different cancers** (Baylin, 2005; Rodriguez *et al.*, 2008)

Gene / Gene Product	Function	Tumour Type
<i>Rb</i>	Cell-cycle regulation	Retinoblastoma
<i>APC</i>	Wnt signal transduction	Colorectal and other cancers
<i>p14/ARF</i>	Cell-cycle regulation	Colorectal cancer
<i>p15/CDKN2B</i>	Cell-cycle regulation	Leukaemias
<i>p16/CDKN2A</i>	Cell-cycle regulation	Various cancers
<i>BRCA1</i>	DNA repair	Breast, ovarian cancer
<i>VHL</i>	Tumour suppressor	Renal cell cancers
<i>hMLH1</i>	DNA mismatch repair	Colorectal, gastric, endometrial cancers
<i>ER-<math>\alpha</math></i>	Estrogen receptor- $\alpha$	Breast, colorectal, other cancers
<i>HRH2</i>	Regulates cell growth and differentiation	Colorectal cancer
<i>CPLX2</i>	Involved in synaptic vesicle exocytosis	Colorectal cancer
<i>SNCB</i>	Regulates SNCA aggregation process	Colorectal cancer
<i>PTEN</i>	Tumour suppressor	Breast, glioma, pancreatic, colorectal cancer
<i>FOLR1</i>	Folate Transporter	Ovarian, Colorectal

Changes to DNA methylation patterns not only affect gene expression but have also been seen to have a resulting effect on histone modifications, as well as altering chromatin structure (Rodriguez *et al.*, 2008), all of which have an effect on transcription (Nguyen *et al.*, 2001).

The location of aberrant methylation is significant. Promoter hypermethylation is often one of the first events seen during tumour progression and is usually observed in genes which

would already be expected to have an increase in regional promoter hypermethylation as a consequence of aging (Baylin *et al.*, 2001). The CpG islands which are found frequently within gene promoters are often methylated by *de novo* methylation, and have been associated with silencing expression of specific tumour suppressor genes during carcinogenesis (Table 1.5) (Hatada *et al.*, 2006). Trithorax and polycom group proteins, which are part of the two main cell-memory systems, are thought to have a role in tumour aberrant gene silencing and promoter DNA methylation (Rodriguez *et al.*, 2008).

**Table 1.5 Genes silenced by promoter CpG island methylation** (Abdolmaleky *et al.*, 2004)

Gene	Protein	Function
<i>CD4</i>	CD4 Antigen	Regulates T-cell activation.
<i>CD8</i>	CD8 Antigen	Involved in T-cell mediated killing.
<i>CDKN2A</i>	Cyclin-dependent kinase inhibitor 2A	Tumour suppressor.
<i>HBB</i>	Haemoglobin beta	Oxygen transport.
<i>IFNG</i>	Interferon, gamma	Immunoregulatory function.
<i>IL4</i>	Interleukin 4	Involved in B-cell activation process.
<i>OXTR</i>	Oxytocin receptor	Oxytocin receptor.
<i>PTEN</i>	Phosphatase and Tensin Homolog	Tumour suppressor (Kang <i>et al.</i> , 2002)

## 1.7.2 Methylation and Environmental Factors

It has long been established that disease is not often the result of genetics alone, but that environmental factors play a role in its aetiology.

### 1.7.2.1 Diet

Folic acid, choline and methionine are classed as methyl donation agents (MDA) which can be obtained through diet, they play such an important role because they interact at the point that homocysteine is converted to methionine (Niculescu *et al.*, 2002). Methyl deficient diets can cause DNA hypomethylation, due to a reduced availability of the methyl groups required resulting in an unmethylated CpG site which results in increased expression of numerous oncogenes (Wainfan and Poirier, 1992).

MDAs are thought to be responsible for directing the flux of one carbon (C<sub>1</sub>) metabolites towards DNA methylation, promoting an increased rate of DNA remethylation (Batra *et al.*, 2010). Aberrant regional hypermethylation, as previously mentioned localises to the CpG islands of a gene which leads to transcriptional silencing (Shukeir *et al.*, 2006b). Given the role DNA methylation has in transforming a normal cell to a malignant phenotype, it was hypothesised that supplementation of the diet with MDAs may be potential used to limit the aggressiveness of glioma, and the methylation status of other genes should be investigated.

### 1.7.2.2 Alcohol

It has already been established that the brain is susceptible to the action of alcohol and potentially to its carcinogenic effects (Boffetta and Hashibe, 2006), yet there have been inconsistent results in studies linking alcohol and glioma. A study by Baglietto *et al.*, (2011) suggested that alcohol consumption increased the risk of glioma, in a dose-response dependent manner, with an increased hazard risk (HR) for every additional 10 g/day of alcohol in a 15-year study. Altered gene expression through alcohol induced changes in

methylation were proposed as a mechanism of action, along with changes related to alcohol metabolism (Baglietto *et al.*, 2011). Alcohol consumption has been seen to inhibit the metabolism and transport of folate, resulting in a folic acid deficiency which in turn affects DNA methylation and increased carcinogenic risk. Increased DNA methylation within the colon has been seen in patients who consume alcohol, so increased levels of folate intake were proposed to have a protective effect in individuals with high alcohol consumption (Mason, 2011). A contradictory study by Hurley *et al.*, (1996) found no association between lifetime alcohol consumption and glioma risk for all levels of alcohol consumption from 0 g/day to >29.7 g/day.

A study by Wani *et al* (2011) showed that rats chronically dosed with 1 g/kg/day ethanol for 3 months resulted in hypomethylation of the reduced folate carrier (RFC), inhibiting transporter function (Wani *et al.*, 2011).

Alcohol consumption also affects mRNA levels of DNMT in the sperm which can therefore alter the paternal genomic imprinting, and possibly lead to an inherited risk of DNA methylation alteration and contribute to neural tube defects (Davis and Uthus, 2004).

### 1.7.2.3 *Smoking*

Studies investigating the associated risk between allergy, as an atopic disease which causes a heightened immune system response and smoking and glioma, showed that there was an inverse risk between allergy history and glioma, with no relation between the risk of glioma and smoking history (Lachance *et al.*, 2011; Linos *et al.*, 2007). This is in contrast to a previous study which showed that in patients with colorectal tumours, current smokers as opposed to those who had never smoked, or were former smokers, were more likely to have an increased propensity for promoter hypermethylation, than those who had previously or never smoked (Marsit *et al.*, 2007). There is evidence to suggest that smoking does have an increased risk

of glioma in people who have the at risk genotype, the T variant of the MTHFR gene (Smith *et al.*, 2008).

#### 1.7.2.4 Other Factors

Azacitidine, a demethylating agent is able to inhibit methylation, and has been shown to reverse epigenetic changes in target genes in myelodysplastic syndrome (MDS) (Baylin, 2005). This again shows that methylation is an epigenetic change that is reversible, so therefore it was hypothesised that a methyl donating agent, such as folate may also be able to alter the methylation status of genes which play a role in glioma initiation and progression. This potential is particularly important given that there has been evidence to suggest that folate intake protects against cancers (Mason, 2011).

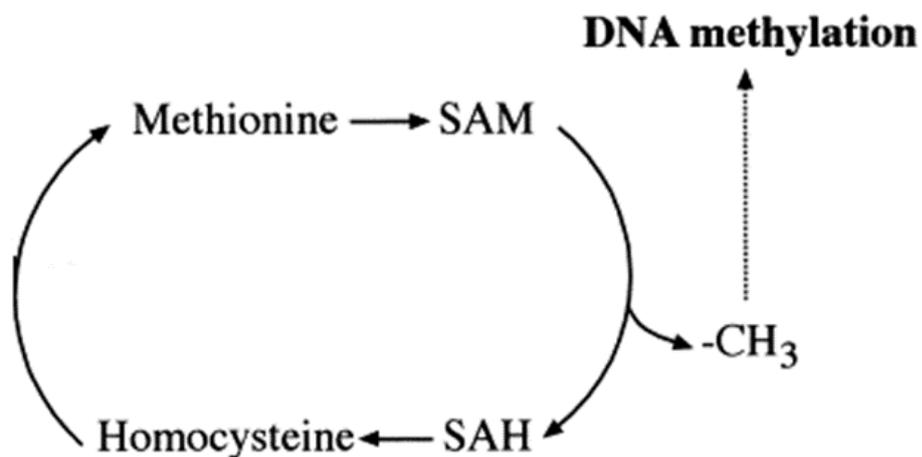
### 1.7.3 Methylation and Neurological Conditions

Alterations to the methylation status of patients with neurological conditions has been observed in individuals with Alzheimer's (West *et al.*, 1995). Rett's syndrome is a neurological condition which occurs due to a mutation within the *MECP2* gene which is involved in methylation (Francke, 2006) and Angelman's syndrome, another neurological condition has also been linked to DNA methylation function (Costello and Plass, 2001), and is the result of a deletion of the 15q11-13 chromosome region (Dittrich *et al.*, 1992). Lupus, an autoimmune disease, has been linked with hypomethylation (Attwood *et al.*, 2002), showing the multifunctional effect DNA methylation has on different genes (Table 1.5). An increased risk of brain cancer in lupus patients has also been seen (Chen *et al.*, 2010). Child abuse not only has well documented psychological effects, but has also been seen to influence cardiovascular disease risk, and has a long term effects on the immune system (Mehta *et al.*, 2013). Post mortems of suicide victims with and without childhood abuse have shown differences in epigenetic status and levels of gene expression, particularly the *NR3C1* gene in the hippocampus which is linked to hypothalamic-pituitary-adrenal (HPA) response

(McGowan *et al.*, 2009). *NR3C1* expression has been seen to be reduced in individuals with a history of childhood abuse, compared to those without, and with increased methylation. Cytosine methylation is incredibly stable, so alterations are unlikely to be as the result of changes in pH in the brain following death (McGowan *et al.*, 2009). These examples serve to show the large and varied role methylation plays.

#### 1.7.4 Methylation and Gliomas

Aberrant DNA methylation has been seen in many studies to be associated with tumour progression (Jones and Baylin, 2007) and glioma in particular (Kim *et al.*, 2006a; Uhlmann *et al.*, 2003b). It is important to understand the underlying factors and sources of variability that determine methylation. S-adenosyl methionine (SAM) is the major methyl donor within the brain (Obeid *et al.*, 2007). Metabolism of SAM yields a methyl group and S-Adenosyl homocysteine (SAH) which rapidly hydrolyses to adenosine and homocysteine, before being recycled back to the precursor methionine by addition of a methyl group (Figure 1.8).



**Figure 1.8 SAM Cycle:**

*S-Adenosyl methionine (SAM) is converted to S-Adenosyl Homocysteine (SAH) resulting in production of a methyl group. SAH is then hydrolysed to adenosine and homocysteine, before being enzymatically converted back to methionine with the addition of a methyl group (Li *et al.*, 2003a).*

### 1.7.5 Methylated Genes in Glioma

Methylated genes have been proposed as prospective cancer biomarkers (Duffy *et al.*, 2009).

A summary of aberrantly methylated genes are often seen in glioma, as is shown in Table 1.6.

**Table 1.6 Methylated Genes in Glioma.**

Gene	Location	Function	Reference
<i>MGMT</i>	10q26.3	Repairs alkylated guanine.	(Etcheverry <i>et al.</i> 2010) (Bady <i>et al.</i> , 2016; Stupp <i>et al.</i> , 2009)
<i>MTHFR</i>	1p36.22	Moves methyl groups in biosynthesis pathway	(Linnebank <i>et al.</i> , 2008)
<i>DNMT1</i> <i>DNMT3A</i> <i>DNMT3B</i>	19p13.2 2p23.3 20q11.21	Involved in de novo methylation	(Zhang <i>et al.</i> , 2011a)
<i>PTEN</i>	10q23.31	Self-renewal and proliferation of stem cells.	(Etcheverry <i>et al.</i> 2010) (Hervouet <i>et al.</i> , 2009b)
<i>RB</i>	13q14.2	Negative regulator of cell cycle.	(Etcheverry <i>et al.</i> , 2010)
<i>CDKN2A</i>	9p21.3	Tumour suppressor.	(Etcheverry <i>et al.</i> , 2010)
<i>TP53</i>	17p13.1	Tumour suppressor.	(Etcheverry <i>et al.</i> 2010) OMIM
<i>RASSF1A</i>	3p21.31	Tumour suppressor.	(Horiguchi <i>et al.</i> , 2003)
<i>SLIT2</i>	4p15.31	Guides cell migration.	(Dallol <i>et al.</i> , 2003)
<i>GLM1</i>	17q21.1, 3p25	Genetic Locus.	OMIM
<i>PEG3</i>	19q13.4	Tumour suppressor.	OMIM
<i>BEX2</i>	Xq22	Tumour suppressor.	OMIM
<i>BEX1</i>	Xq22.1-q22.2	Involved in cell cycle progression and neuronal differentiation.	OMIM
<i>MLH1</i>	3p21.3	DNA damage signalling.	OMIM
<i>L3MBTL</i>	20q12	Regulates chromatin modification.	OMIM
<i>LTB4R</i>	14q11.2-q12	Involved in inflammatory signalling pathways.	OMIM
<i>EBF3</i>	10q26.3	Involved in neurogenesis and may be a tumour suppressor.	OMIM
<i>SLC22A18</i>	11p15.4	Organic cation transporter	(Chu <i>et al.</i> , 2011)
<i>LHX9</i>	1q31.1	Involved in gonadal development	(Vladimirova <i>et al.</i> , 2009)

#### 1.7.5.1 MGMT

O<sup>6</sup>-methylguanine-DNA methyltransferases (MGMT) is an enzyme which repairs DNA adducts after exposure to alkylating agents, it works by removing alkyl groups from the O<sup>6</sup> position of guanine (Yin *et al.*, 2003). Research has shown that when the CpG island in the promoter region of the MGMT gene is methylated, it becomes silenced and prevents transcription from occurring (Hegi *et al.*, 2004), which is often seen in glioma patients (Stupp *et al.*, 2009). Damage to the DNA as a result of chemotherapy prevents any further cell cycle replication from occurring, and further tumour growth. The silencing of the MGMT gene as a result of methylation, and repressed transcription ability means that the tumour is unable to repair the DNA damage (Stupp *et al.*, 2010). This indicates the use of the MGMT gene as a possible therapeutic target.

#### 1.7.5.2 DNA Methyltransferases

DNA methyltransferases (DNMT) are responsible for regulating the status and intensity of methylation within the genome (Zhang *et al.*, 2011b). *DNMT1* is responsible for the maintenance and *de novo* methylation of tumour suppressor genes, whilst *DNMT3A* and *DNMT3B* are only responsible for *de novo* methylation. Genetic alteration of *DNMT1* and *DNMT3B* through methylation can result in *p16*, a tumor suppressor gene, being silenced, which allows for the abnormal growth of cells (Das and Singal, 2004). Decreased expression of *DNMT3A* and increased expression of *DNMT1* and *DNMT3B* have been seen in glioma (Zhang *et al.*, 2011b), suggesting a link between aberrant *DNMT* expression and abnormal DNA methylation and gliomagenesis.

The value of analysing the DNMTs goes further as by looking at their levels of expression, and the methylation status of apoptosis associated genes, a correlation between apoptosis and methylation status was established by (Hervouet *et al.*, 2010). This indicates the role the DNMTs have in maintaining the methylation status of apoptotic genes.

### 1.7.5.3 MTHFR

One carbon metabolism is important to maintain nucleotide synthesis and repair and is involved in the methylation process (Friso and Choi, 2005). It involves two branches of biosynthesis. The first, involves purine and thymidine, whilst the second involves the synthesis of methionine and adenosylmethionine. Methylenetetrahydrofolate reductase (MTHFR) moves methyl groups from the first branch to the second branch of biosynthesis (Das and Singal, 2004). As with most genes, there is more than one polymorphism. The T variant of this gene has been linked to an increased cancer risk, including endometrial, breast and ovarian (Gershoni-Baruch *et al.*, 2000), whilst decreasing the risk of others, such as colorectal cancer (Chen *et al.*, 1996). This polymorphism along with low folate intake also increases the risk of carcinogenesis by impairing DNA methylation and DNA synthesis/repair (Das and Singal, 2004).

Given that methylation status aids in determining patient outcome, this is seen in reference to the *MGMT* promotor, patients with a methylated *MGMT* promoter are almost twice as likely to survive as those with an unmethylated promoter (Hong *et al.*, 2016) (Hegi *et al.*, 2005). Methylation status also holds the potential to inform of the best treatment option, as it determines whether cells will be resistant to temozolomide treatment (Brandes *et al.*, 2008). Modifying methylation status could also be used as a novel therapeutic strategy. One such under researched area to consider would be the use of natural supplements. Following a literature review for causes of changes in methylation status, it was decided to pursue folate; a key methylating agent in the methylation process and biosynthetic pathways all of which are intrinsically linked to cancer progression. Henceforth, two key genes that are altered in glioma and involved in folate metabolism were chosen for further investigation within the scope of this thesis: *PTEN* and *MTHFR*.

## 1.8 Natural Supplements as an adjuvant therapy

Patients have often turned to natural supplements as an adjuvant therapy to help treat cancer, especially since side effects of chemotherapy have such an impact on quality of life and patients often feel disillusioned with conventional treatment in the face of such poor survival statistics (Lovgren *et al.*, 2011; Shorofi, 2011). A summary of the effects of dietary supplementation or deficiency is shown in Table 1.7.

**Table 1.7 Natural Supplements and Cancer.**

Supplement	Presence/Absence	Result	Cancer Type	Author
Vitamin D	Deficiency	Increased Risk	Colon, Breast, Ovarian, Prostate	(R. Zhang & Naughton, 2010), (Zhang & Naughton, 2010), (M. F. Holick, 2008, Lucas <i>et al.</i> , 2010)
Vitamin E	Supplementation	Reduced Risk	Lung	(Yang <i>et al.</i> , 2012)
Selenium	Deficiency	Increased Risk	Malignant Brain Tumour	(El-Yazigi, Al-Saleh, & Al-Mefty, 1984) (Sundaram <i>et al.</i> , 2000) (Norman <i>et al.</i> , 2003)
Folate	Supplement	Decreased Risk	Colorectal	(Martínez <i>et al.</i> , 2012)
Calcium	Supplement	Decreased Risk	Breast	(Chen <i>et al.</i> , 2010)
Calcium	Supplement	Increased	Prostate	(Van Poppel and Tombal, 2011)

## 1.9 Folate

### 1.9.1 Dietary sources of folate

Folate, a natural derivative of vitamin B<sub>9</sub>, is one of several bioactive food components which can modulate DNA methylation, and is obtained naturally from dietary sources including: orange juice, dark green leafy vegetables, dried beans and peas, and peanuts (Davis and Uthus, 2004) (Table 1.8). Mammalian cells do not possess the metabolic enzymes required for folate biosynthesis, so this requirement must be obtained from our diet (Desmoulin *et al.*, 2012).

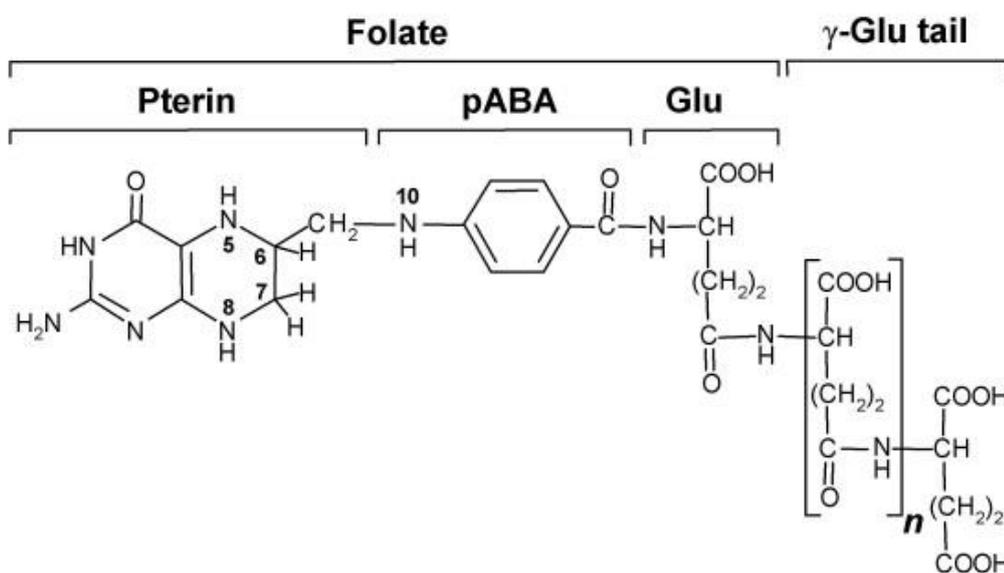
**Table 1.8 Folate Content of Foods** (Rampersaud *et al.*, 2003).

Food	Folate Content (µg per 100g)
Beef liver	212 µg
Lentils, cooked	181 µg
Chickpeas, cooked	172 µg
Beans (black, kidney), cooked	149 µg
Spinach, raw	194 µg
Spinach, cooked	146 µg
Asparagus, cooked	149 µg
Greens (turnip), cooked	118 µg
Orange juice, ready to drink	30 µg
Strawberries, fresh	24 µg
Broccoli, cooked	108 µg
Tomato juice	20 µg
Peanuts, dry roasted	145 µg
Lettuce, romaine	136 µg
Cantaloupe, fresh	21 µg
Lettuce, iceberg	29 µg
Banana	20 µg
Grapefruit	10 µg

Folate enters the body through diet, mostly in the forms; 5-methyl and 10-formyl-tetrahydrofolate. These are polyglutamate forms that cannot cross the biological membranes, so must be converted to monoglutamates by and gamma-glutamyl hydrolase, (GGH). Once these monoglutamates are in the enterocytes, they are converted back into polyglutamates by folypolyglutamate synthetase, (FPGS), which adds glutamate residues (Wani *et al.*, 2011). This maintains a concentration gradient favouring monoglutamate entry into the cell, therefore, a balance of these enzymes is crucial.

### 1.9.2 Forms of Folate

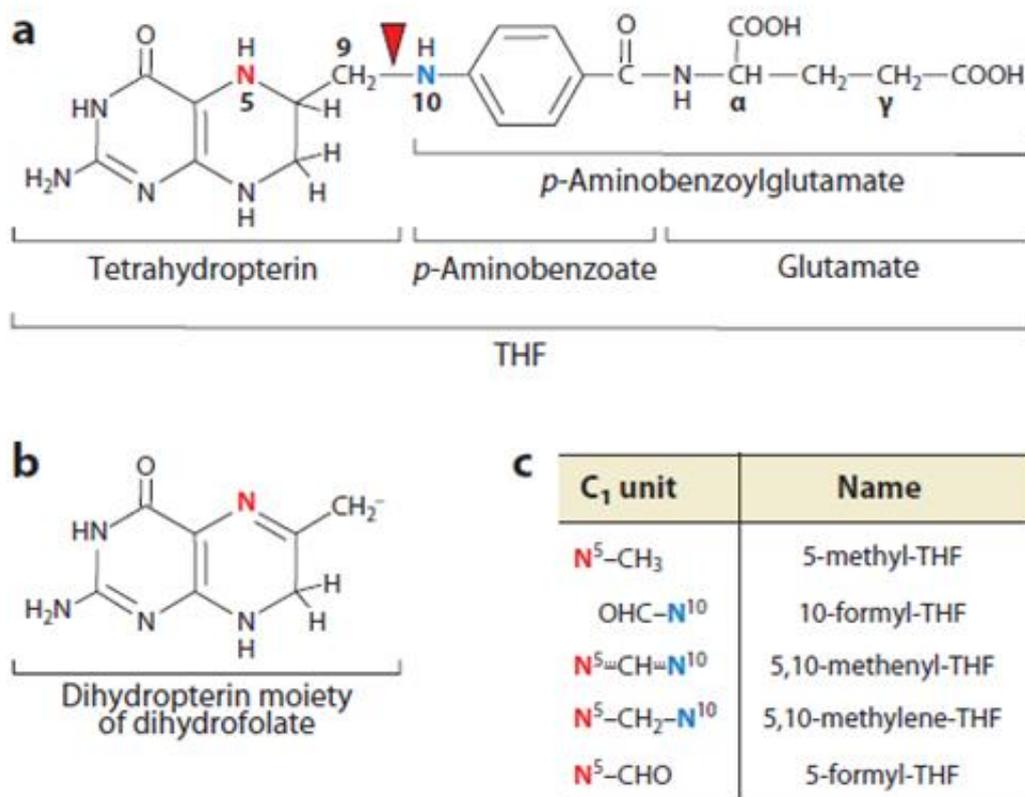
Folate is the generic term for the group of heterocyclic bioactive vitamin compounds that are based on the pteridine ring p-aminobenzoic acid structure conjugated to one or glutamate units (Figure 1.9) (de Crécy-Lagard *et al.*, 2007).



**Figure 1.9** The structure of tetrahydrofolate.

The pterin ring shown exists in tetrahydro form. The ring is fully oxidised in folic acid. Folates usually have a  $\gamma$ -linked polyglutamyl tail of up to about eight residues attached to the first glutamate. One-carbon units (formyl, methyl, etc.) can be coupled to the N5 and/or N10 positions. (de Crécy-Lagard *et al.*, 2007).

The folates differ by variations in oxidation state, one-carbon substitution and polyglutamate chain length (Shane, 2011) (Figure 1.10).



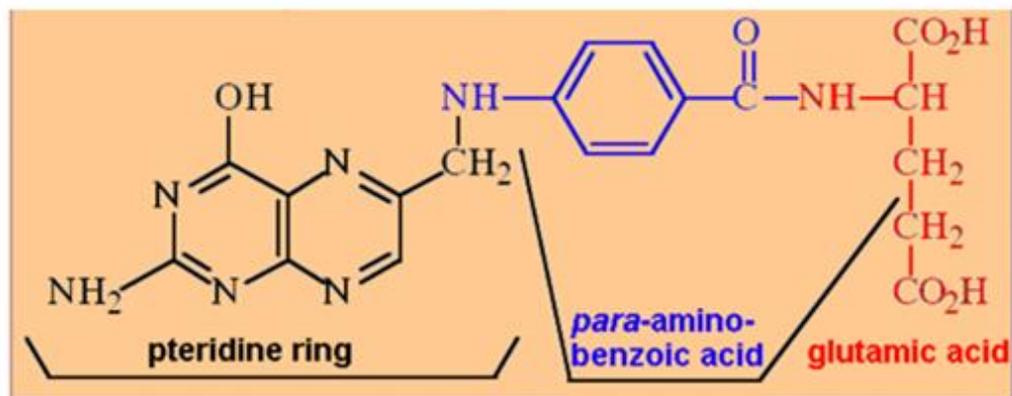
**Figure 1.10 Different forms of folate** (Hanson and Gregory, 2011)

The structure of folates **(a)** chemical structure of tetrahydrofolate (THF), monoglutamyl form. The red arrowhead marks the oxidatively labile C9-N10 bond. A polyglutamyl tail can be attached via the  $\gamma$ -carboxyl group of the glutamate moiety. **(b)** The 7,8-dihydropterin moiety of dihydrofolate. **(c)** The various one-carbon(C<sub>1</sub>) substituents of THF.

### 1.9.2.1 Folic acid

Folic acid is a synthetic oxidised structure that requires activation by multistep enzyme reactions into dihydrofolic acid (DHFA) in the presence of cofactor NADPH, and then further converted to the reduced tetrahydrofolate form (THFA) (the four new hydrogen atoms are shown in green in Figure 1.11), which can then polymerise to form the various polyglutamates found in living organisms. The multistep enzymatic reductions are summarised in Figure 1.12.

## A - Folic Acid (oxidised)

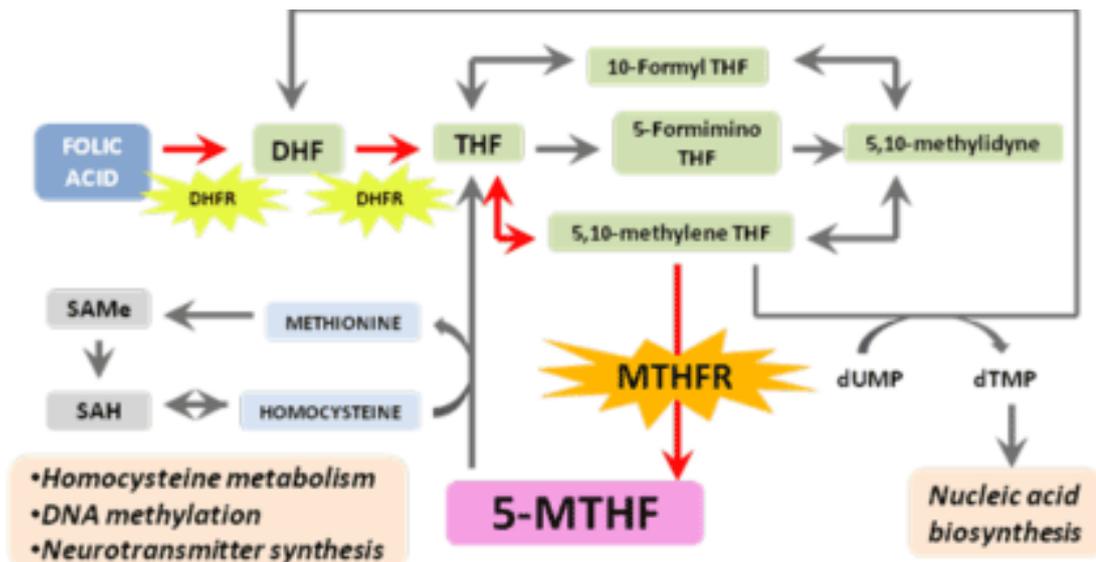


## B - Tetrahydrofolate (reduced)



**Figure 1.11 Oxidation site comparison** (Taylor and May, 2008)

Comparison between (A) folic acid and (B) tetrahydrofolate in terms of sites of oxidation at the 5, 6, 7 and 8 position on the pteridine ring.

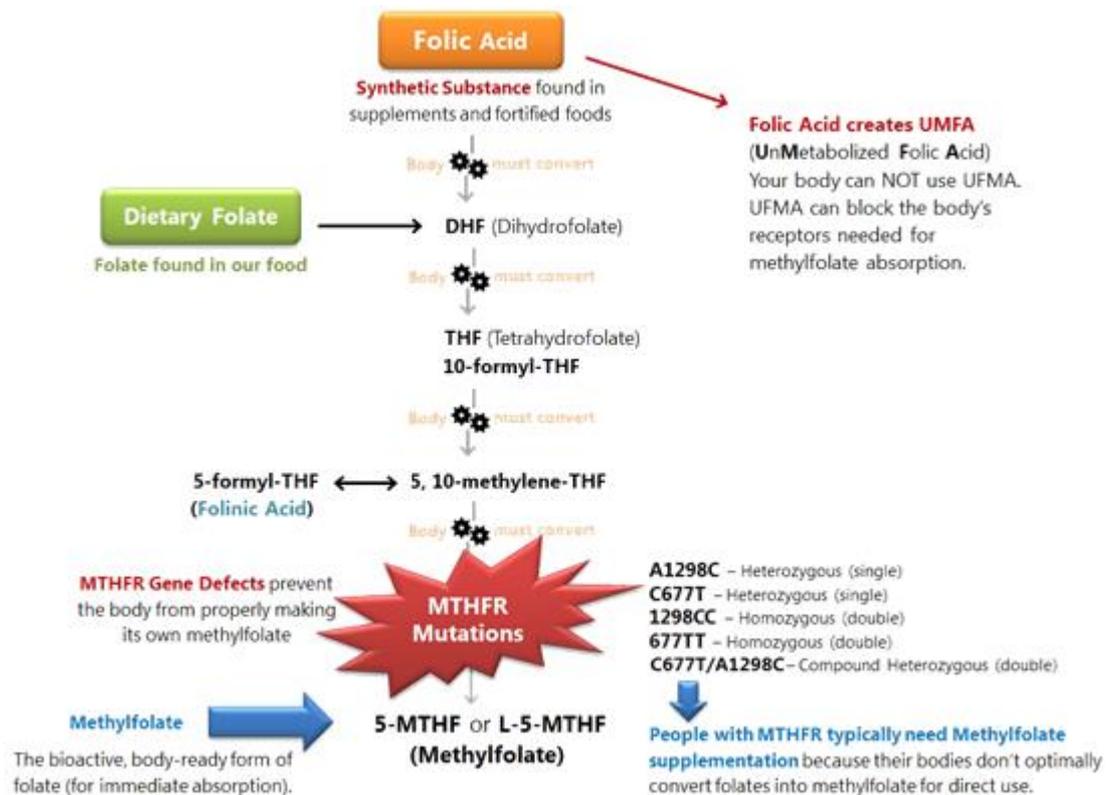


**Figure 1.12** Multienzymatic reduction of folic acid to activated methyl donor form (Heim, 2016).

Folic acid is an oxidised form of folate that must be enzymatically reduced to function as a methyl donor. It is first converted to dihydrofolate (DHF) by dihydrofolate reductase (DHFR) before being converted again to tetrahydrofolate (THF). THF is converted to 5,10-methylene THF by serine hydroxymethyltransferase. 5,10-methylene THF is then converted to 5-methyltetrahydrofolate (5-MTHF) by 5,10-methylene tetrahydrofolate reductase (MTHFR).

### 1.9.2.2 Folinic Acid

Folinic acid, also known as 5-formyl tetrahydrofolate, is a reduced metabolite of folic acid that is rapidly converted to 5, 10 methyl tetrahydrofolate and exhibits activity equivalent to folic acid. Folinic acid can be administered in calcium or sodium salt forms, known by the name leucovorin. Folinic acid, 5-formyl tetrahydrofolate, is a preferable supplement to folic acid because it is absorbed into the body as a reduced metabolite, arriving at the folate metabolism pathway as 5,10 methylenetetrahydrofolate (5-MeTHF). 5-MeTHF is found in the serum at a concentration range of 206 – 1110 nmol/L (Sobczyk *et al.*, 2014).

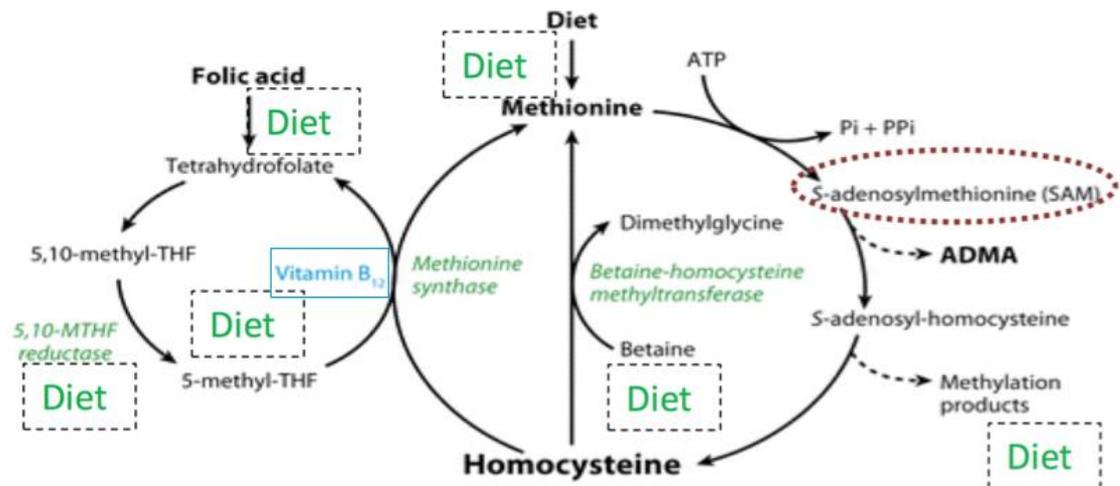


**Figure 1.13 Conversion of folic acid and folinic acid to 5-MTHF (Rawlins, 2015).**

Folic acid is converted to dihydrofolate (DHF) by dihydrofolate reductase (DHFA) before being converted again to tetrahydrofolate (THF). THF is then converted to 5,10-methylene THF by serine hydroxymethyltransferase. Folinic Acid in the form 5-formyl THF is catalysed by 5-formyl-THF cyclo-ligase to 5,10-methylene THF. 5,10-methylene THF is then converted to the biologically active 5-methyltetrahydrofolate (5-MTHF) by 5,10-methylene tetrahydrofolate reductase (MTHFR).

Inter-individual variability exists in the enzymatic steps required to activate folic acid, due to different MTHFR mutations (Castro *et al.*, 2004). A lack of MTHFR would be exposed by folic acid supplementation because MTHFR is required to convert it to the biologically active form 5-MTHF, and a build-up of folic acid would be toxic. Folate that entered the body naturally as methylfolate would not need to be converted by MTHFR so mutations in this gene would not be noted (Scaglione and Panzavolta, 2014). This can be bypassed following direct administration of the reduced metabolite folinic acid, therefore folinic acid is proposed to be a more reliable treatment in terms of predicting bioactivity and reducing the potential of creating a folate trap (James *et al.*, 2004). A folate trap occurs when there is a deficiency in

vitamin B<sub>12</sub>. When MTHFR catalyses the conversion of 5,10-methylene-THF into 5-methyl-THF it is irreversible, and can only be used in remethylation dependent upon the presence of vitamin B<sub>12</sub>, so when there is a decreased level of vitamin B<sub>12</sub>, levels of 5-methyl-THF will still continue to rise but will be unable to release methyl groups, so vitamin B<sub>12</sub> and folate are both required for methylation to continue (Figure 1.14).



**Figure 1.14 Folate and methylation cycle**

Vitamin B<sub>12</sub> is required for the conversion of 5-methyl-THF to tetrahydrofolate (THF), without it 5-methyl-THF levels will increase, but methyl groups will be unable to be released (Maron and Loscalzo, 2009).

### 1.9.3 Functional role of folate

Folate is essential for carrying methyl groups within the cell, and can act as either a donor or receiver of one-carbon groups in enzyme catalysed reactions (Li *et al.*, 2003a). Folate also plays an important role in nucleotide synthesis, metabolism and growth within mammalian cells through maintenance of base sequence integrity of genomic DNA (Choi and Mason, 2002; Li *et al.*, 2003b; Wani *et al.*, 2011). The role folate plays in nucleotide synthesis, suggests that a deficiency would affect cells that divide most rapidly (Ulrich and Potter, 2006).

## 1.9.4 Folate and Disease

### 1.9.4.1 Folate and Neurological Conditions

Although folate deficiency is now known to be associated with an increased risk of cancer, in the breast, colon and lung, due to reduced DNA stability (Zhang *et al.*, 2015), it was first linked to megaloblastic anaemia in the 1930s (Johnston, 2009). Folate also has a role in foetal brain development, as well as affecting the brain in other conditions. Prenatal folate availability is linked to brain development, where deficiency is associated with neural tube defects and is the second most common type of serious birth defect (Yi *et al.*, 2011). Neural tube defects are a congenital malformation where the neural tubes fail to close during embryogenesis (Pitkin, 2007). They occur at a decreased frequency in women taking folic acid before and during pregnancy (Opladen *et al.*, 2010), reducing the risk in offspring by 50-75% (Kirkbride *et al.*, 2012). Folic acid supplementation however does not prevent hydrocephalus, a condition when there is an imbalance in the production and drainage of CSF, resulting in an accumulation of fluid in the brain, subsequently causing increased cranial pressure which can result in major neurological conditions (Cains *et al.*, 2009). Folic acid may in fact increase occurrence, folinic acid and THF however, reduces risk and serves to improve foetal brain development, highlighting the difference between forms of folate, hypothesised to be due to an altered balance of folate metabolites (Cains *et al.*, 2009).

Folate also affects the adult brain. Low folate levels have been associated with cognitive decline and some forms of Alzheimer's disease in elderly patients (D'Anci and Rosenberg, 2004). There is also an association between low folate and depression, particularly major depressive disorder (MDD) due to the reduced response to selective serotonin reuptake inhibitors (SSRIs) (Alpert *et al.*, 2002). Decreased levels of folate as the result of phenytoin or barbiturates, have also been linked to mental alterations including depression, apathy, and cognitive decline (Edward, 2006). Schizophrenia is another disorder likely to be due partially

as a result of epigenetic influences. Rates of occurrence are not 100 % in monozygomatic twins, which suggests the influence of epigenetic mechanisms (Kirkbride *et al.*, 2012). One of the genes involved is *MTHFR*, which is also involved in one carbon metabolism. Studies performed showed that schizophrenic patients present with epigenetic dysregulation of the brain, and examination of the frontal cortices showed that between these patients and the control subjects there was a difference in the DNA methylation pattern for genes involved in neurotransmission and brain development (Auta *et al.*, 2013). This supports the hypothesis that epigenetic alterations affect brain function through changes to the mRNA and protein abundance in specific neuronal or glial cell type (Kirkbride *et al.*, 2012).

#### 1.9.4.2 Folate and Cancer

Folate levels have also been linked to cancer initiation and progression. A strong link between a low level of folate intake and colorectal cancer has been well established (Wang *et al.*, 2012). It was also seen that increased intake of folate, supplementation with 1 mg/day, before the formation of neoplasms in colorectal cancer, compared to those who did not receive additional folate, reduced the risk of cancer (Ulrich and Potter, 2007). Due to the role folate plays in the synthesis of nucleotides, it can be hypothesised that an abundant availability of folate would aid in the proliferation of rapidly dividing cells, as hyperproliferation can result in the growth of malignant neoplasms (Mason, 2011). The higher rate of proliferation within neoplastic cells means that cells require a larger level of folate in order to maintain the increased level of thymidine synthesis, which occurs due to the increased rate of DNA synthesis.

#### 1.9.5 Implications of Folate Status

Intake of folate from diet tends to be at a low level, around 200-400 µg/day, despite the recommend daily allowance of folate for adults being 400 µg a day (Chan *et al.*, 2013).

### 1.9.5.1 Folate Deficiency

Folate deficiency can result from a reduced dietary intake or as the result of genetic causes which can affect folate uptake and/or metabolism (De Haan, 2010). Hereditary folate malabsorption, (HFM) is an autosomal recessive disorder (Shin *et al.*, 2011) which occurs when there is a mutation within the proton coupled folate transporter (*PCFT*) gene. It is most frequently treated with leucovorin, racemic 5-formylTHF, also known as folinic acid. Folic acid is not advised as it binds so tightly to the folate receptors, it can create competition for the transport of 5-methylTHF across the blood-choroid plexus-CSF barrier (Shin *et al.*, 2011). Competition between folic acid and endogenous 5-methylTHF could prevent 5-methylTHF from entering the cell, so 5-methylTHF would be the recommended supplement. Therefore, reduced levels may reduce methyl availability; a requirement for normal cell function. Ensuing methyl deficiency may increase with tumourigenesis. One aim of this thesis is to gain a greater understanding of the role the different folate transporters have in glioma.

Folate deficiency tends to result in global hypomethylation or promoter DNA hypermethylation (Kim, 2009). A deficiency in folate means a reduced availability of methyl moieties required to synthesise SAM, resulting in passive loss of DNA methylation; global hypomethylation (Salbaum, 2012). The mechanism of action behind the observed gene hypermethylation is not fully understood but is believed to be as a result of increased DNMT activity seen during folate deficient conditions (Neumann, 2007). Chronic deficiency can result in an increase in both *TP53* and genome wide methylation (Das and Singal, 2004) as it has been seen that diets which are deficient in folate have been associated with a decreased expression of tumour suppression genes (Li *et al.*, 2003a). Folate deficient diets have also been reported to increase carcinogenic risk due to alteration in the methylation status of apoptotic genes (Li *et al.*, 2003a). It is not only folate which appears to influence our risk, diets deficient in zinc, which cause the histone deacetylase, (HDAC) inhibitors to become

inactive, results in HDAC binding to methylated cytosines which leads to global hypomethylation (Singh *et al.*, 2003).

#### 1.9.5.2 Folate Application

Folic acid is frequently added to food which has caused controversy given the increasing number of foods being fortified with folic acid, due to concerns raised around uncertainties of the long-term effects of raised oxidised folic acid levels (Crider *et al.*, 2011). Addition of folic acid is thought to exacerbate the neurological symptoms caused by a deficiency in vitamin B<sub>12</sub>, a condition commonly observed in the elderly which can occur as the result of a poor diet, medication, stomach or intestine conditions or having pernicious anaemias (Morris *et al.*, 2007). High folate levels, caused by an increase of folate supplemented food within the diet when combined with low levels of B<sub>12</sub>, has been seen in studies to produce a higher risk of cognitive impairment. This folate trap is due to high levels of folate inhibiting 5-MTHF formation which decreases synthesis of methionine, which is linked to cognitive function (Miller, 2003; Smith *et al.*, 2008). The folate trap increases the levels of homocysteine which increases cellular toxicity and results in an autoimmune response (Perla-Kaján *et al.*, 2007).

Following folate application, genes can be up or down regulated in response, the genes which showed most significant change are those involved in cell cycle, cell differentiation, cell division, and transport. The form of folate also has a role in which genes will be affected, natural folates play a key role in biosynthetic processes, whilst antifolates have a role in cancer chemotherapy (Zhao *et al.*, 2005). Antifolates, such as methotrexate (MTX) work by targeting the metabolism of folate (Gonen and Assaraf, 2012a), inhibiting the enzyme dihydrofolate reductase (DHFR) that is involved in tetrahydrofolate synthesis, therefore causing cells to die (Weinstein *et al.*, 1971).

Although folic acid supplementation has been seen to decrease the risk of carcinogenesis (Clarke *et al.*, 2010), it also has the potential, if administered in too high a dose, to actually

increase the risk of cancer (Mason *et al.*, 2007; Neuhouser *et al.*, 2011)(Table 1.9-1.10). Animal studies have also shown that the dose and timing of folate intervention are critical (Kim, 2004a). Indeed, the application of folate before neoplastic foci formation has been shown to suppress development and progression of tumours, however if folate is administered after these neoplastic foci have begun to form, then it can actually serve to enhance the development and progression of preneoplastic cell growth (Smith *et al.*, 2008). This could also be due to polymorphisms within the gene which code for enzymes relating to one-carbon metabolism. This genotype can then turn the protective effect of folate into one which is harmful (Smith *et al.*, 2008).

Doses of folate which are too high, above the 100 nM range in blood plasma can result in inhibition of folate dependant enzymes such as methionine synthase, methylenetetrahydrofolate reductase, and dihydrofolate reductase (Nijhout *et al.*, 2004; Tactacan *et al.*, 2010).

Current methods of treatment of cancer often involve macromolecular drugs, such as protein toxins; momordin, and pseudonomous exotoxin, drug encapsulating liposomes such as doxorubicin. These macromolecular drugs have a high molecular weight which can result in permeability problems at the tumour membrane which limits their ability to work as chemotherapeutic drugs. Folic acid would make a good ligand for macromolecular drugs too large to pass the membrane on their own, and is currently being developed as it could aid in the selective targeting and delivery of macromolecular drugs into tumour cells due to the high levels of folate receptor expression in cancer cells, and the specificity of macromolecular drugs to target cancer cells for a prolonged period (Lu and Low, 2002). The advantage of folic acid as a ligand is its high binding affinity, low immunogenicity, ease of modification, small size, stability during storage, compatibility with a variety of organic and aqueous solvents low cost, and is readily available.

**Table 1.9 Altered Folate Status in Disease (Detrimental).**

**Detrimental Effect**

Model	Species	Condition	Dose	Folate Status and End Point	Author
<i>In vivo</i>	Rat	Mammary Cancer	13-130 $\mu$ M	Deficiency – Suppresses Supplementation – Initiates and Promotes	(Baggott <i>et al.</i> , 1992)
<i>In vivo</i>	Rat	Mammary Cancer	0, 2 or 8 mg/kg	Deficiency – Suppresses Supplementation – No significant effect	(Kotsopoulos <i>et al.</i> , 2003)
<i>In vivo</i>	Human	Leukaemia	pteroyldiglutamic acid 100-300 mg pteroylaspartic acid 40 mg	Supplementation – Increases progression	(Farber <i>et al.</i> , 1947)
<i>In vivo</i>	Human	Prostate Cancer	1 mg	Supplementation – Increases risk	(Figueiredo <i>et al.</i> , 2009)

**Table 1.10 Altered Folate Status in Disease (Protective).**

**Protective Effect**

Model	Species	Cancer Type	Dose	Folate Status and End Point	Author
<i>In vivo</i>	Mouse	Colorectal Cancer	8 mg/kg	Supplementation – Suppresses ileal polyps and colonic aberrant crypt foci	(Song <i>et al.</i> , 2000)
<i>In vivo</i>	Human	Neural Tube Defects	100-1000 µg	Supplementation – Decreases occurrence	(Kim, 2004b; Safdar <i>et al.</i> , 2007)
<i>In vivo</i>	Human	Spina Bifida	1.0 ppm	Supplementation – Decrease occurrence	(Arth <i>et al.</i> , 2016)
<i>In vivo</i>	Human	Post Menopausal Breast Cancer	Median values µg daily 160, 204, 237, 287, 582	Supplementation – Decreased Occurrence	(Ericson <i>et al.</i> , 2007)
<i>In vivo</i>	Human	Squamous cell carcinoma in Men (Heavy Smokers)	400 µg	Supplementation – Protective Effect	(Bandera <i>et al.</i> , 1997)
<i>In vivo</i>	Human	Bronchial Metaplasia	10 mg 10-20 mg	Supplementation – Reversal of bronchial metaplasia	(Heimburger <i>et al.</i> , 1988; Saito <i>et al.</i> , 1994)
<i>In vivo</i>	Human	Cervical Dysoplasia	100µg/ 1,000 k cal	Supplementation – Reduces risk	(Buckley <i>et al.</i> , 1992; VanEenwyk <i>et al.</i> , 1992)

To date, extensive studies have been reviewed on the role of methylation in tumour initiation and progression, including purported protective effects of folate in prophylactic cancer therapy as well as potentially stimulatory growth effects in cancer (Feinberg *et al.*, 2016; Sharma *et al.*, 2010). A key link that has been poorly researched is the key role of folate, a methylating agent, on the progression of glioma. This gap in current research knowledge formed the key emphasis and direction of this thesis.

## 1.10 Folate Transport

Folates are highly polar hydrophilic anionic molecules, only capable of traveling through biological membranes by specialised transporters (Hamid *et al.*, 2009). Dietary folate is initially taken up in the gut before it reaches the liver where it is then metabolised to 5-methyltetrahydrofolate (5-MTHF) before it is distributed by the bloodstream. Cells then uptake the 5-MTHF using the folate transporters PCFT, RFC, and FOLR1 (Steinfeld *et al.*, 2009a). The folate receptor 1 (FOLR1) (also known as folate receptor alpha) is particularly notable for its high binding affinity for circulating 5-MTHF (Ramaekers *et al.*, 2002).

Normal brain development and function depend on the active transport of folates across the blood-brain barrier which is transported into the brain by the folate receptor 1 in the choroid plexus (Smith *et al.*, 2008). Folic supplementation around conception has long been recognised as reducing the incidence of congenital malformation, as it is crucial for placental and foetal development. Folinic acid therapy has been used to treat patients with a defect in the folate receptor alpha, by reducing clinical symptoms which included motor dysfunction, developmental regression and epileptic seizures (Steinfeld *et al.*, 2009c).

### 1.10.1 Folate Receptor 1

The folate receptor 1 (FOLR1) is the gene that codes for the folate receptor alpha (FR $\alpha$ ) protein, it is the most studied of the folate transporters. It is predominately found on polarised epithelial cells, and active macrophages. It binds preferentially to oxidised folates, through the process of receptor mediated endocytosis (Table 1.11).

**Table 1.11 FOLR1 Genomics**

<b>Location</b>	11q13.3-q14.1
<b>Length</b>	6,766 base pairs
<b>Protein</b>	257 amino acids, 29819 Da
<b>SNPs</b>	124

The FR $\alpha$  is present in low levels on normal epithelia but is seen to be elevated 2-fold in malignant tissues originating from the epithelia, including the brain (Sega and Low, 2008a). The degree of this over expression in the FR $\alpha$  is found to be correlated to a histologic grade of the tumour and, the advanced stage of the cancer. This indicates the need for elevated levels of folate in rapidly growing tumours (Lu and Low, 2002). This suggests that malignant cells can successfully compete for folate even when levels are low. In fact, enhanced delivery has already been seen both *in vitro* and *in vivo* of tumour cells which are FR positive. High expression levels of FR $\alpha$  may be because of the requirements of the cell to increase DNA synthesis and growth (Basal *et al.*, 2009).

*FOLR1* is also of interest due to its polymorphic variability in expression (Table 1.12) (Kotsopoulos *et al.*, 2010). The current agents that are presently used to target the folate receptor are; farletuzumab, vintafolide and IMG853 (Vergote and Leamon, 2015). These drugs are currently being tested on ovarian cancer, which has the most increased levels of expression (Lutz, 2015; Vergote and Leamon, 2015).

FOLR1 is not only involved in the cellular uptake of folic acid, but disruption to its expression cause birth defects, including abnormal heart development and neural tube defects. FR $\alpha$  acts as a transcription factor for pluripotency genes including Oct4, Lin28 and Sox2 in neural crest cells (Mohanty, 2017). It has been suggested that FR $\alpha$  increases the expression of these genes as a way to derive pluripotent stem cells from neural crest stem cells. This is important because it aids understanding of the functions of FR $\alpha$  at an early developmental time point, and helps explain why folic acid supplementation prior to pregnancy obviates NTDs (Mohanty, 2016).

**Table 1.12 FOLR1 Expression Levels**

Transporter	Expression Level	Cell Line / Cancer Type	Ref
FOLR1	Decreased	U87MG	EMBL-EL
FOLR1 and 3	Increased	ovarian carcinoma	(Yuan <i>et al.</i> , 2009)
FOLR1 and 3	Increased	epithelial ovarian	(Kotsopoulos <i>et al.</i> , 2010).

### 1.10.2 Folate Receptor 2

Folate Receptor 2 (*FOLR2*) is the gene that codes for the folate receptor beta protein (FR $\beta$ ). The protein encoded by this gene is a member of the folate receptor (*FOLR*) family, and these genes exist in a cluster on chromosome 11, it is most often expressed by mesenchymal cells (Opladen *et al.*, 2010). Members of this gene family have a high affinity for folic acid and for several reduced folic acid derivatives, and they mediate delivery of 5-MTHF to the interior of cells. This protein has a 68% and 79% sequence homology with the FOLR1 and FOLR3 proteins, respectively (Wallis *et al.*, 2009). Although this protein was originally thought to be specific to placenta, it can also exist in other tissues, and it may play a role in the transport of methotrexate in synovial macrophages in rheumatoid arthritis patients (Puig-Kröger *et al.*, 2009). Multiple transcript variants that encode the same protein have been found for this gene (Table 1.13).

**Table 1.13 FOLR2 Genomics**

<b>Location</b>	11q13.3-q13.5
<b>Length</b>	5,350 base pairs
<b>Protein</b>	255 amino acids, 29280 Da
<b>SNPs</b>	108

### 1.10.3 Folate Receptor 3

The Folate Receptor 3 (*FOLR3*) codes for folate receptor gamma (FR $\gamma$ ). This gene is another member of the folate receptor (*FOLR*) family. This gene includes two polymorphic protein variants; the shorter one has two base deletion in the coding sequence, resulting in a truncated polypeptide. Both protein products are constitutively secreted in hematopoietic tissues and are potential serum markers for certain hematopoietic malignancies. The longer protein has a 71% and 79% sequence homology with the FR $\alpha$  and FR $\beta$  proteins (GeneCards, 2016) (Table 1.14).

**Table 1.14 FOLR3 Genomics**

<b>Location</b>	11q13
<b>Length</b>	25,022 base pairs
<b>Protein</b>	243 amino acids, 27638 Da
<b>SNPs</b>	101

From the four isoforms of the Folate Receptor;  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ . FR $\alpha$  was chosen for further study in this thesis, because it is expressed mostly in epithelial cells such as the choroid plexus (Gonen and Assaraf, 2012b). FR $\beta$  is expressed in the placenta, colon, thymus, and spleen (Elnakat and Ratnam, 2004). FR $\gamma$  is not involved in cellular uptake (Dosio *et al.*, 2010) and FR $\delta$  is difficult to detect in human tissue, and may be a splice variant or pseudogene (Spiegelstein *et al.*, 2000)

#### 1.10.4 Reduced Folate Carrier

The reduced folate carrier (RFC) is a glycoprotein found ubiquitously distributed in virtually all cells and it is the primary pathway for the uptake of physiological folates. It has multiple transmembrane domains, with a greater affinity for reduced folates than folic acid, and works as a bi-directional anion exchange mechanism to transport 5-MTHF (Mauritz *et al.*, 2008) (Table 1.15).

**Table 1.15 RFC Genomics**

<b>Location</b>	21q22.3
<b>Length</b>	50,840 base pairs
<b>Protein</b>	591 amino acids, 64868 Da
<b>SNPs</b>	15

#### 1.10.5 Proton Coupled Folate Transporter

The proton-coupled folate transporter (PCFT), has optimal activity at a low pH. It is essential for folate absorption within the intestines, and is the predominant folate transporter in the central nervous system. PCFT is highly expressed within the small intestine, but is also expressed in other organs such as the brain (Steinfeld *et al.*, 2009a). Loss of function of the PCFT is associated with hereditary folate malabsorption (Zhao *et al.*, 2009) (Table 1.16).

**Table 1.16 PCFT Genomics**

<b>Location</b>	17q11.2
<b>Length</b>	12,556 base pairs
<b>Protein</b>	459 amino acids, 49771 Da
<b>SNPs</b>	16

The FOLR proteins play a key role in the assimilation, distribution and retention of folates derived from food.

Understanding the factors that affect folate transport, can aid our understanding of folate transport in the CSF-blood-brain barrier. Given that there is an overexpression of the FR $\alpha$  in tumour cells compared to non-cancerous cells, FR $\alpha$  make an ideal therapeutic target as it functions in the transport of folate in tumours, but not normal tissue (Jackman *et al.*, 2011). Specifically targeting the folate receptor in human epithelial cancer cells grown in mice has already begun (Zwicke *et al.*, 2012). The RFC, along with other intracellular targets are not tumour specific, making the use of antifolate drugs as a possible cancer treatment difficult. One study already shows potential, delivering paclitaxel to FR-positive tumours in order to enhance the effects of chemotherapy whilst reducing the negative side effects (Wang *et al.*, 2009).

The three main transporters as discussed are FR $\alpha$ , which uptakes folate by the process of endocytosis. The RFC, which takes up folate in a bi-directional manner, and the PCFT which works best at low pH and is predominantly found in the intestines. The folate transporters have different effects on tumour growth, overexpression of FR $\alpha$  is seen in ovarian cancer, which may promote tumour growth. Whilst reduced expression of the RFC correlates with cancer progression in colorectal cancer (Siu *et al.*, 2012). The folate transporters function in different ways, one study recently showed that when RFC expression was eliminated, methotrexate, an antifolate used in chemotherapy, could still be transported with no change in influx, in rat small intestinal cells (IEC-6), and HeLa cells (Zhao *et al.*, 2005), and it could therefore be hypothesised that if expression in one is lost, the others could continue transporting folate. However, there have been many studies that show loss of function of the *FOLR1* gene impairs cerebral folate transport leading to a deficiency, and resulting in developmental regression, epilepsy and leukodystrophy (Steinfeld *et al.*, 2009c). Mutations to the *PCFT* resulting in loss of function have also been seen to result in hereditary folate

malabsorption (HFM) (Shin *et al.*, 2011). Other factors that also have an effect are the transport capacity and kinetics of the folate transporters which are also affected by extracellular folate concentrations (Steinfeld *et al.*, 2009c).

The folate transporters which will be studied are the *FOLR1* due to its variability in expression between cancerous and non-cancerous tissue. *RFC* because it has a greater affinity for reduced folates, such as folinic acid and *PCFT* because it is highly expressed within the brain.

### 1.11 Folate Metabolism

Folate metabolism also plays a role in causing folate deficiency, the metabolic and subcellular route of the internalised folates is determined by the type of folate-transporting proteins involved. and might result in transcytosis of monoglutamyl folate or subcellular accumulation of polyglutamyl folate metabolites that cannot cross cell membranes, again resulting in deficiency (Fowler, 1998). Folate is required for one carbon metabolism, a prerequisite for DNA synthesis, and is crucial to the formation of the main methylating agent S-adenosylmethionine (SAM) as described in Section 1.10 (Bailey and Gregory, 1999). Folates are composed of three chemical compounds, a pteridine ring, p-aminobenzoic acid, PABA, and a glutamate residue and can be reduced or oxidised (Gonen and Assaraf, 2012a). The enzymes central to folate metabolism are 5,10-methylenetetrahydrofolate reductase (MTHFR) methionine synthase (MTR) and methionine synthase reductase (MTRR) (Bethke *et al.*, 2008), and formyltetrahydrofolate dehydrogenase (*FDH*).

A study by Raemaekers (2002), identified a new metabolic disorder which presented with low 5-MTHF in the cerebrospinal fluid, and responded to folinic acid supplements. The cause for this disturbed folate transfer across the blood-brain barrier was unknown (Raemaekers *et al.*, 2002) but it is possible that it could have been due to an inherited disorder (Table 1.17).

**Table 1.17 Inherited disorders of folate transport and metabolism (Steinfeld et al., 2009b).**

<b>Definite</b>	Methylene-H4 folate reductase deficiency
	Functional methyltetrahydrofolate (methyl-H4 folate):
	Homocysteine methyltransferase (methionine synthase) deficiency caused by cobalamin E (cblE) or G(cblG) disease
	Glutamate formiminotransferase deficiency
	Hereditary folate malabsorption
<b>Putative</b>	Dihydrofolate reductase deficiency
	Methenyl-H4 folate cyclohydrolase deficiency
	Cellular uptake defects
	Primary methyl-H4 folate:homocysteine methyltransferase deficiency

### 1.11.1 MTHFR

One carbon metabolism is important to maintain nucleotide synthesis and repair, and is involved in the methylation process (Friso and Choi, 2005). It involves two branches of bio synthesis. The first, involves purine and thymidine, whilst the second involves the synthesis of methionine and adenosylmethionine. Methylenetetrahydrofolate reductase (MTHFR) is the enzyme that is involved in moving methyl groups from the first branch to the second (Das and Singal, 2004). MTHFR is also responsible for converting 5,10-methylene-THF to 5-MTHF (Figure 1.12 and 1.13). There is more than one polymorphism for this gene, the T variant has been linked to an increased risk of some cancers, including endometrial, breast and ovarian (Gershoni-Baruch *et al.*, 2000), whilst decreasing the risk of others, such as colorectal cancer (Chen *et al.*, 1996). This polymorphism along with low folate intake also increases the risk of carcinogenesis by impairing DNA methylation and DNA synthesis/repair (Das and Singal, 2004) (Table 1.18).

**Table 1.18 MTHFR Genomics**

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<b>Location</b>	1p36.22
<b>Length</b>	21,198 base pairs
<b>Protein</b>	656 amino acids, 74597 Da
<b>SNPs</b>	74

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Rapidly proliferating cancer cells are susceptible to disruption of folate metabolism due to a significant requirement of folate pools for DNA/RNA synthesis for rapid division. The metabolism of folate also plays a role in the potential to develop a glioma because as with most genes, polymorphisms also exist for the folate metabolism genes. Bethke *et al.* (2008) looked at the following genotypes; *MTHFR* C677T and A1298C, *MTRR* A2756G and *MTR* A66G which were taken from primary brain tumour patients. The differences in polymorphic variants differed between ethnicities, particularly *MTHFR* A1298C and C6677T which could explain the differences in susceptibility to gliomas observed between ethnic groups (Bethke *et al.*, 2008) as these two genes along with *MTRR* A66G were seen to be associated with primary brain tumour in the form of glioma and meningioma. The association between *MTRR* 2756G and glioblastoma was also seen in an earlier study (Semmler *et al.*, 2006).

### 1.11.2 ALDH1L1

Formyltetrahydrofolate dehydrogenase (*FDH*) is also known as ALDH1L1, a folate metabolic enzyme. It catalyses the conversion of 10-formyltetrahydrofolate to tetrahydrofolate. The protein expression levels are highest in the liver, followed by the brain, it is also relatively high in the heart, kidney, blood, plasma and platelets (Anguera *et al.*, 2006). Its loss of function is associated with decreased apoptosis, increased cell motility and cancer progression (Anthony, 2007).

ALDH1L1 is involved in two reactions of *de novo* purine biosynthesis; removal of excess pools of folate activated one-carbon units, and repossession of cellular folate in the form of tetrahydrofolate and inhibition of purine synthesis. Without purine synthesis, there would be a depletion of intracellular 10-formyltetrahydrofolate followed by the loss of *de novo* purine biosynthesis. This loss diminishes DNA/RNA availability, leading to induction of apoptotic cell death, and down regulation in tumours may enhance proliferation (Krupenko *et al.*, 2015).

*FDH*, although central to folate metabolism is also functionally related to *FR $\alpha$* , involved in transporting 5-MTHF to different areas of the brain (Naz *et al.*, 2016) (Table 1.19)

**Table 1.19 ALDH1L1 Genomics**

<b>Location</b>	3q21.3
<b>Length</b>	94,434 base pairs
<b>Protein</b>	902 amino acids, 98829 Da
<b>SNPs</b>	3

### 1.11.3 Chosen Genes of Interest

There were many different genes that could have been chosen for further analysis in this thesis given the complexity of the methylation cycle, and the differing methylation agents available, however, it was decided to focus on folate given the opposing roles it plays in tumourgenesis, and in particular to focus on the transporters, due to their potential for chemotherapeutic drug delivery. *FOLR1* is of particular interest given its variability in expression between cancerous and non-cancerous tissue. *RFC* was chosen because it has a greater affinity for reduced folates, such as folinic acid, so was important for a study comparing forms of folate treatment. *PCFT* was selected because it has been seen to be highly expressed within the brain. The *PTEN* gene was chosen as a positive control within the

study as there was already literature published regarding its relation to glioma and methylation. *MTHFR* was chosen as the folate metabolism gene of interest over *ALDH1L1* because *ALDH1L1* is functionally related to *FOLR1*. *MTHFR* was also chosen due to its opposing role in cancer outcome.

## 1.12 Study Aims

The background literature review revealed that patients with high grade (III/ IV) have a bleak prognosis and survival rates have shown virtually no improvement for 40-years, therefore the thesis here focused on this condition. Advances in the theories of origin of cancer have highlighted that aberration in methylation may be a driver for neoplasia progression. Although this has been researched in other tumours, notably colorectal, breast and lung cancer, there is a paucity of information on the role of methylation in brain cancer. Folate supplementation has long been used for a variety of conditions as a methylating agent, but again the effects in glioma are contradictory and data are sparse. In particular, lack of understanding of the role of folate transport and metabolism in brain tumours was apparent. To contribute to the current gaps in knowledge identified and to help improve patient outcome for this dire condition, ***the main aim of the thesis was to investigate the opposing roles of folate in glioma.***

The specific aims were:

1. To authenticate the glioma (U87 MG and 1321N1) and glial (SVGp12) cells lines to be used in experiments, and to establish growth behaviour, in particular, reference to cell doubling times and to optimise cell viability assays.
2. To assess the effect the different forms of folic and folinic acid had on the viability of glioma and glial cells with respect to dose and duration of exposure

3. To determine the potential mechanism of changes in cell viability following folate treatment.

4. To assess the effect the different forms of folic and folinic acid had on the methylation status of the chosen genes of interest: *FR1*, *RFC*, *PCFT*, *PTEN* and *MTHFR*.

5. To establish if folate deficiency, folic or folinic acid treatment altered the expression of the proteins of interest; *PTEN*, *FOLR1*, *RFC*, *PCFT* and *MTHFR*.

The objectives of the project were to undertake steps to achieve the aims of the thesis. A series of experiments were performed on cell lines as all research needs to be proven at *in vitro* level before moving on to clinical applications.

If results demonstrate efficacy of folic or folinic acid, in terms of therapeutic potential, it could become a beneficial dietary supplement which could be easily implemented into clinical trials given that it is already so well characterised.

## CHAPTER 2

## 2 MATERIALS AND METHODS

### 2.1 Tissue Culture

#### 2.1.1 Cell Lines

The immortalised human cell lines used for analysis were 1321N1, representative of a malignant astrocytoma, sub clone of grade IV U118MG, U87MG representative of an astrocytoma grade IV/glioblastoma, and SVGp12, a non-cancerous foetal astrocyte. These cell lines were all obtained from the European Collection of Cell Cultures (ECACC) in Salisbury, UK. University ethical approval was obtained for the use of biohazards, risk assessments and COSHH forms were completed for all work carried out.

#### 2.1.2 Media and Supplements

Folate free media and its additional supplements were purchased from Sigma Aldrich, Dorset, UK and from Lonza, Nottingham, UK. Phosphate buffered saline, PBS, ethanol, and all plastic ware were ordered from Fisher Scientific, Leicestershire, UK. A summary of all tissue culture consumables and suppliers is given in Table 2.1.

**Table 2.1 Tissue Culture Reagents and Suppliers**

<b>Reagent</b>	<b>Abbr, Storage Temperature</b>	<b>Supplier</b>
Eagle's minimal essential medium (EMEM)	EMEM, 2-8 °C	Sigma, Dorset UK
Dulbecco's modified eagle's medium (DMEM)	DMEM, 2-8 °C	Sigma, Dorset UK
Dulbecco's modified eagle's medium (DMEM) (Folate Free)	DMEM, 2-8 °C	Sigma, Dorset UK
Sodium bicarbonate	Room Temperature	Sigma, Dorset UK
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)	HEPES, Room Temperature	Sigma, Dorset UK
Foetal bovine serum Cat no:10082139	FBS, -20 °C	Gibco, Paisley, UK
L-glutamine	-20 °C	Lonza, Slough, UK
Sodium pyruvate	2-8 °C	Lonza, Slough, UK
Non-essential amino acid	NEAA, 2-8 °C	Lonza, Slough, UK
Phosphate buffered saline (PBS) 0.1M pH7.41	PBS, Room Temperature	Thermo Fisher Scientific, Leicestershire, UK

The 1321N1 cell line was maintained in DMEM, supplemented with 10 % v/v, 10 % FBS, 5.96 g/ L HEPES, 3.7 g/ L sodium bicarbonate and 2 mM L-glutamine as recommended by the cell line supplier ECACC. The U87MG and SVGP12 cell lines were maintained in EMEM supplemented with 10 % FBS, 5.96g/L HEPES, 3.7g/ L Sodium Bicarbonate, 2 mM L-glutamine,

1mM sodium pyruvate, and 1 % v/v NEAA as recommended by the cell line provider ECACC. The cell lines were cultured at 37°C in a humidified incubator supplemented with 5 % CO<sub>2</sub>, in T75 flasks.

### 2.1.3 Cryopreserving and Thawing Cells

Cells were defrosted as required from liquid nitrogen storage by rapidly thawing in a water bath set at 37 °C. Growth media was then changed within 12-24 hours. Confluent cells that required cryopreservation were pelleted by centrifugation, and resuspended in freezing media which consisted of the respective supplemented media and 10 % dimethyl sulphoxide (DMSO). Cell suspensions were transferred to cryotubes and placed inside a freezing container (Mr Frosty™) (Nalgene, UK) containing isopropanol for a slow freeze for 24 hours at -80 °C, the cryovials were then transferred to a liquid nitrogen dewer and stored at a temperature of -190 °C.

### 2.1.4 Passaging Cells

Cells were grown to 75 % confluency in a flask before passage. Subculturing of the cells took place in a laminar flow hood using aseptic techniques. Complete media, 0.25 mM trypsin and 0.1 M PBS at pH 7.4 were pre-warmed to 37 °C in a water bath. Media from the flask was aspirated, and cells were washed with the PBS, which was aspirated and then trypsin was added to the flask. The flask was incubated with the trypsin for 5 minutes at 37 °C. To check the cells has detached they were examined under an inverted light microscope at 10 X magnification (Leica DMIL, Milton Keynes, UK). Following successful detachment, an equal volume of media to trypsin was added to the flask to prevent any more trypsinisation from occurring. This cell suspension was mixed in the flask and then added to a 15 ml tube for centrifugation at 179 x g for 5 minutes at room temperature in a benchtop centrifuge (ALC, Buckinghamshire, UK). Supernatant was aspirated, and the cell pellet was resuspended in 5 ml of fresh media, and then 20 µl was reserved to perform a cell count so that cells could be

seeded at  $5 \times 10^4$  cells per ml in a T75 flask. The flask was returned to the incubator at  $37^\circ\text{C}$  and 5 %  $\text{CO}_2$ .

## 2.2 Cell Counting

One hundred microlitres of cell suspension was added to a sterile 1.5 ml tube, and an equal volume of 100  $\mu\text{l}$  of 0.4 % trypan blue and mixed by pipetting up and down. Ten microlitres of this suspension was added to a newbauer haemocytometer and cells were counted under the inverted light microscope at 10 X magnification (Leica DMIL, Milton Keynes, UK). Viable cells appeared as clear round cells, whereas non-viable cells had taken up the dye and appeared blue. The number of viable cells in each chamber were counted, and an average of the five chambers counted was calculated. This number was then corrected to allow for dilution factors and multiplied by the haemocytometer factor  $\times 10^4$  to give a number of viable cells per 1ml cell suspension. Cells were seeded at  $1 \times 10^6$  per mL in T75 flasks, or at a density of 500 cells per well in a 96 well plate or as detailed in Chapter 3, when PrestoBlue optimisation was performed.

## 2.3 Growth Curves

Cell growth was calculated for 1321N1, U87MG and SVGp12 cells by seeding at an initial density of 20,000 cells per mL into a 24 well tissue culture plate and kept in an incubator at  $37^\circ\text{C}$  and 5 %  $\text{CO}_2$ . The cells were counted daily by using a haemocytometer, as described in Section 2.2 and the experiment was performed in triplicate.

## 2.4 Immunostaining of cells

### 2.4.1 Fixation of cells

Cells were seeded onto a 0.16 mm thick coverslip in a 24 well plate at a density of  $1 \times 10^4$  per ml. When they had reached 50% confluence, the media was removed, and the

coverslips were washed with PBS three times. The cells were then fixed onto the coverslip using 4 % paraformaldehyde (PFA) for 15 minutes at room temperature, before being washed again with PBS three times.

#### 2.4.2 Fluorescent Immunostaining

The cells were fixed on the coverslips were washed three times with PBS and then incubated with 0.1 M glycine for 10 minutes in order to quench background fluorescence. Cells were then washed with PBS five times. Cells were incubated with 0.1 % triton X-100 for 10 minutes at room temperature in order to make them permeable, followed by five washes in PBS. The non-specific binding was then blocked by incubating with 10 % goat serum for 1 hour, and then washed three times with PBS. Cells were incubated in the required antibody, as detailed in Table 2.2, for 1 hour at room temperature and then washed in PBS three times. The cells were then incubated with a 1:1000 dilution of the secondary antibody Alexa Fluor® 488F9ab' 2 fragment of goat anti-rabbit IgG (LifeTechnologies, UK) for 30 minutes in the dark and then washed with PBS three times before they were mounted on a microscope slide using VECTASTAIN mounting media (Vector Laboratories, Peterborough, UK). The slides were incubated in the dark for 24 hours before they were analysed to prevent photo exposure of fluorophores. To prevent the cells from drying out overnight, the corners of the coverslip were painted with clear nail varnish.

**Table 2.2 Rabbit polyclonal primary antibodies and dilutions used for immunostaining**

Primary antibody	Antibody dilutions in 0.1 M PBS, pH 7.4	Manufacturer
GFAP	1 : 300	Abcam, Cambridge, UK
HLA	1 : 250	Santacruz, Middlesex, UK
Isotype Control	1 : 100	Santacruz, Middlesex, UK

## 2.5 Treatment

### 2.5.1 Reagents

Cells were treated using various supplements: folic acid, folinic acid and antioxidant and vehicle controls (Sigma, Dorset, UK) (Table 2.3 and 2.4). Stock solutions of: 1 M of NaOH in distilled water, 40µg/ml folic acid solution in NaOH as recommended (Sigma, Dorset, UK), and 40µg/ml folinic acid in DNA and RNA Free Water were created. The vehicle did not exceed 0.08 % in the final reaction volume.

Folate free media was prepared by a 1 in 10 dilution of DMEM without folic acid (Sigma, Dorset, UK) in autoclaved distilled water. To this 26.19 mM of sodium bicarbonate (Fisher Scientific, Leicestershire, UK) and 24.99 mM of HEPES (Sigma, Dorset, UK) was added under sterile conditions.

Cells were grown in standard cell culture media EMEM or DMEM which contained 4 µg/ mL folic acid, and from here onwards will be referred to simply as standard cell culture media as detailed in Section 2.1. Cells were treated with an additional 40 µg/ mL folic acid or 40 µg/ mL folinic acid, added to standard cell culture media, which was defined as high dose, or an additional 4 µg/ mL folic acid or 4 µg/ mL folinic acid, added to standard cell culture media and this was defined as a low dose.

Treatments used are detailed in the Appendix 1.

## 2.6 Cell Viability Analysis

In order to assess the viability of cells they were defrosted from liquid nitrogen storage, and seeded into T75 flasks along with 20 ml of standard media. Cells were then grown until confluent and a cell count was performed by using a haemocytometer as described in Section 2.2. Cells were then seeded into a black walled clear bottom 96 well plate (Fisher Scientific, Leicestershire, UK), to reduce cross talk between the wells in the fluorescence assay. Cells were seeded at a density of 500 cells per well and 100  $\mu$ L of normal growth media was added. The following day, the media was aspirated, and replaced with fresh control or treatment media as detailed in Section 2.4. Cells were returned to the incubator for 24 hours. The next day 10  $\mu$ L of PrestoBlue (Fisher Scientific, Leicestershire, UK) was added to each well containing cells and 6 wells containing only media which was required to calculate background fluorescence. The plate was returned to a 37 °C incubator for 1 hour and then fluorescence was read using a Tecan GENios Pro plate reader, (Tecan, Theale, UK) at an excitation wavelength 535 nm and an emission wavelength 615 nm.

PrestoBlue is a live cell assay, so following readings, the PrestoBlue was removed from the wells, and replaced with fresh media. The plate was then returned to the incubator at 37°C until the next time point. Fluorescence levels are measurable as the result of conversion of resazurin to resorufin, and correlate to the total level of metabolic activity within the cell, producing quantitative data. Light is absorbed by the PrestoBlue dye molecules which become excited to a higher energy level, then as energy is lost through collisions, they return to their ground state. As they return, energy is emitted as light at longer wavelengths, due to the energy lost. Treatment levels were compared to the control, which was the cells grown in standard cell culture media, or those grown in folate deficient conditions dependent upon the comparison analysis being performed. The average fluorescence values of the no cell

control wells were subtracted from the treated cells well to give a value, which was then plotted.

## 2.7 Cell Cycle Analysis

Cells were treated as described in Section 2.4, and then cells were trypsinised and transferred to a sterile centrifuge tube, followed by centrifugation with a benchtop centrifuge (ALC, Buckinghamshire, UK) at 224 x g for 5 minutes at room temperature. The supernatant was then removed and the cells resuspended in 1 ml of PBS, and the cells are centrifuged again at 224 x g for 5 minutes at room temperature. The supernatant was then removed and the pellet agitated to resuspend in the remaining media. The cells were then fixed by adding 2 ml of ice cold 70 % ethanol drop by drop, whilst vortexing and then stored at -20 °C for 24 hours. Following thawing, samples were then centrifuged at 224 x g for 5 minutes at room temperature and the supernatant was removed. One millilitre of ice cold PBS was then added and the cells were centrifuged at 224 x g for 5 minutes at room temperature, and the supernatant removed again. The pellet was resuspended in 1 ml of PBS containing 50 µg/ ml propidium iodide and 100 µg/ ml RNase and then cells were incubated at 37 °C for 30 minutes, then stored at 4 °C in the dark.

Cells were analysed using the FACS-Aria flow cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA). Cells were passed through a nozzle in order to separate them into single cells. A 15 mW argon laser with a fixed wavelength of 488 nm was used to excite the propidium iodide which had stained the double stranded DNA in treated cells. As the propidium iodide lost energy it emitted red fluorescence which was measured at 615nm. As the cells passed through the laser, light was scattered forward and sideways, and detected then converted to an electric signal and amplified, where voltage is relative to the number of photons. In order to exclude any debris, or clumps of non-single cells, samples were gated using forward *verses* side scatter. The resulting histogram was set with gates that were adjusted so that the G<sub>0</sub>/G<sub>1</sub>

peak appeared at channel 50. Subsequent gates were set up to show the remaining cell cycle phases, S and G<sub>2</sub>/M.

Cells were treated for 7 days and cell cycle analysis was assessed on Days 2 and 7 using a flow cytometer following staining of the cells with propidium iodide (PI). Histograms were produced indicating the mean fluorescence intensity at each stage of cell cycle; where P2 was representative of G<sub>0</sub>-G<sub>1</sub>, P3 was representative of S Phase and P4 was representative of G<sub>2</sub>-M. A 488nm laser was used to excite the cells, and red fluorescence was measured at 615nm, and it was ensured that a minimum of 10,000 events were analysed. Samples were gated using forward scatter versus side scatter in order to exclude any cell debris or clumps of cells, this was then termed the “live gate”. The G<sub>0</sub>/G<sub>1</sub> peak was then adjusted to appear around channel 50, and software attached to the FACS determined how many cells were in each phase of the cell cycle.

## 2.8 Apoptosis Analysis

Cells were seeded in white walled 96 well plates (Fisher Scientific, Leicestershire, UK) at the same density used for the cell viability assay (Section 2.5) (500 cells/ well), and left for 24 hours before treatment. Treatment was performed for the required time, as detailed in Chapter 5. The extent of apoptosis was assessed using the Caspase-Glo 3/7 Assay (Promega, Hampshire, UK) by measuring the activity level of the executioner caspases; caspase 3 and 7 which play a key role in apoptosis discussed in more detail in Chapter 5. The caspase-Glo 3/7 buffer and substrate were first equilibrated to room temperature before the buffer was transferred to the substrate bottle, the reagents were mixed by gentle inversion. One hundred microlitres of caspase-Glo 3/7 reagent was added to each well containing cells, and control wells containing only media to account for background luminescence. The wells were then mixed by using a plate shaker set to 300-500 rpm for 30 seconds. The plate was left to

incubate at room temperature for 1 hour. Luminescence was then measured using the Tecan GENios Pro plate reader, (Tecan, Theale, UK).

## 2.9 DNA Extraction for methylation studies

### 2.9.1 Puregene DNA Extraction

For the Genra Puregene DNA extraction method (Qiagen, Manchester, UK), cells were scraped in the flask into PBS, and then centrifuged to form a pellet before the supernatant was removed, leaving around 20  $\mu$ l to allow for re-suspension. One hundred and fifty microliters of cell lysis solution was added before the sample was vortexed. Proteinase K was then added (7.5  $\mu$ l) and the samples vortexed and incubated at 56°C for 30 minutes with further vortexing for 30 seconds every 10 minutes to allow for improved cell lysis. Fifty microlitres of protein precipitation solution was then added and the samples were vortexed again for 30 seconds, then put on ice for 5 minutes to cool. The samples were centrifuged with a benchtop centrifuge (ALC, Buckinghamshire, UK) for 3 minutes at room temperature at 13,000 x g, and replaced on ice to allow visualisation of the white protein pellet. One hundred and fifty microliters of isopropanol was placed into new tubes, and the supernatant was added, gently inverted and incubated at -20 °C for 20 minutes. The samples were centrifuged at 13,000 x g for 5 minutes at room temperature, and the supernatant was discarded. One hundred and fifty microlitres of ethanol was added and the pellet resuspended by inversion, followed by centrifugation for 1 minute at 13,000 x g at room temperature. The resulting supernatant was discarded and the tubes were inverted and the pellet was allowed to air dry for 30 minutes and then re-suspended in 20  $\mu$ l of DNA hydration solution. The samples were incubated at 65 °C for 1 hour to allow the DNA to dissolve and then left at room temperature overnight.

### 2.9.2 QIAamp DNA Extraction

Cells were seeded in 6 well plates at a density of 10,000 cells per well, and grown until confluent then subject to treatment as detailed in Section 2.5. DNA was extracted using the QIAamp DNA mini kit (Qiagen, Manchester, UK). Cells were first trypsinised and placed into a 1.5 ml tube and centrifuged with a benchtop centrifuge (ALC, Buckinghamshire, UK) at 300 x g for 5 minutes at room temperature. The supernatant was removed and cells were re-suspended in 200 µl of PBS. Twenty microlitres of proteinase K was added and samples were incubated for 10 minutes at 56 °C at room temperature, followed by centrifugation at 300 x g for 5 min at room temperature. Two hundred microlitres of 100 % ethanol was added, and mixed by vortexing, and centrifuged again briefly for 30 seconds at 1,000 x g at room temperature to remove any drops. This solution was added to a QIAamp mini spin column, and centrifuged at 6,000 x g for 1 minute at room temperature. The QIAamp column was placed in a clean collection tube and 500 µl of wash buffer AW1 wash buffer was added, and the tube was centrifuged for 1 minute at 6,000 x g at room temperature. The column was again placed in a clean collection tube and then 500 µl of wash buffer AW2 was added and the tube was centrifuged at 20,000 x g for 3 minutes at room temperature. The spin column was then placed in another clean tube and 200 µl of elution buffer AE was added and the tube was incubated at room temperature for 1 minute before being centrifuged at 6,000 x g for 1 minute at room temperature. DNA quantity and quality was measured using the Nanadrop 2000 spectrophotometer at a wavelength of 260nm (Thermo Scientific, Leicestershire, UK). Extracted DNA was stored at -20°C for short term storage, and at -80°C for longer storage of more than a month.

## 2.10 Bisulphite Treatment for detection of methylated DNA

The deamination of cytosine to uracil by treatment with bisulfite allows 5-methylcytosine (5mc) to be distinguished from cytosine (Grunau *et al.*, 2001) because 5mC does not react with the bisulfite and will remain as cytosine.

DNA was mixed with a solution containing urea and sodium meta bisulphite, and then incubated at 50 °C which results in the breakage of the DNA to form fragmented single strands (Ehrich *et al.*, 2007).

The DNA was bisulphite converted using the EZ DNA Methylation-Gold Kit (Cambridge Bioscience, Cambridgeshire, UK). The CT conversion reagent was prepared by adding 900 µl of RNA free water, 300 µl of M-dilution buffer and 50 µl of M-dissolving buffer to a tube of the CT conversion reagent. The solution was then mixed at room temperature by vortexing for 10 minutes. The M-wash buffer was prepared by adding 24 ml of 100 % ethanol to 6 ml of wash buffer. One hundred and thirty microliters of the CT conversion reagent was added to 20 µl of the DNA sample extracted using the method in Section 2.8. The sample was mixed by pipetting the solution up and down, then centrifuging for 30 seconds at room temperature at 1,000 x g to collect the liquid to the bottom of the tube. The tube was then placed in a thermal cycler under the following conditions; 98 °C for 10 minutes, and then 64 °C for 2.5 hours. Six hundred microlitres of M-binding buffer was added to a Zymo-Spin IC Column, and the column was then placed into a collection tube. The heated DNA sample was placed into the IC Column and inverted several times. The column was then centrifuged at 12,000 x g for 30 seconds at room temperature before the collection was discarded. One hundred microlitres of M-wash buffer was added to the column and centrifuged again at 12,000 x g at room temperature for 30 seconds. Two hundred microliters of M-desulphonation buffer was added to the column and left to stand at room temperature for 20 minutes, before centrifugation for 30 seconds at 12,000 x g at room temperature. Two hundred microlitres

of M-wash buffer was added to the column and centrifuged for 30 seconds at room temperature at 12,000 x g. Another 200 µl of M-wash buffer was added and centrifuged again for 30 seconds at 12,000 x g at room temperature. The column was paced into a clean micro centrifuge tube and 10 µl of M-elution buffer was added directly to the column. The column was then spun for 30 seconds at 12,000 x g at room temperature. Eluted DNA was stored at -20°C for short term storage, and at -80°C for longer storage for over a month.

## 2.11 Methylation Specific PCR

### 2.11.1 Primer Design

The promoter region of the chosen genes, summarised in Table 2.3, was used to design the primers by calculating 2000 bp upstream of the gene.

**Table 2.3 Location of Genes of Interest**

Gene	Location	Ensembl version
PTEN	10q23.3	ENSG00000171862
FOLR1	11q13.3-q14.1	ENSG00000110195
RFC (SLC19A1)	21q22.3	ENSG00000173638
PCFT (SLC46A1)	17q11.2	ENSG00000076351
MTHFR	1p36.22	ENSG00000177000

In order to avoid PCR bias towards unmethylated sequences, which occurs in most PCR amplifications (Wojdacz *et al.*, 2008) the primers designed were required to be of a length of 18-22 base pairs with a GC content of 40-60%, producing a product below 600 base pairs with a difference in melting temperature between the two primers  $\pm 3$  °C.

The MethPrimer Program (Li and Dahiya, 2002) was used to design methylation specific primers using the above requirements to create a methylated sense strand, a methylated antisense strand, an unmethylated sense strand, and an unmethylated antisense strand.

Compatibility of the unmethylated sense strand was checked to ensure it was aligned with the promoter region of the gene using the BioEdit program (Hall, 1999). The GC / AT rule, applied in Table 2.4, calculates the primer annealing temperature using the formula shown in Equation 2.1 where A, C, G and T represent the number of adenine, cytosine, guanine and thymine bases respectively in the primer sequence concerned.

**Equation 2.1 GC/AT Rule**

$$T = 2^{\circ}(A + T) + 4^{\circ}(G + C)$$

**Table 2.4 DNA Methylation Primers**

Gene	Primer Sequence	Annealing Temperature				Expected amplicon size (bp)	Extension time (amplicon dependant - 25bp/s)
		NCBI Blast	Invitrogen Custom Primers	GC/AT Rule	Experimental Temperature		
PTEN METHYLATED	SENSE 5' GTT TTT TGG GTT TTT GAA ATT TAA C '3 UNSENSE 5' ACA ACC TAC TAT TAT ATC GCC AAC G '3	53.78 58.85	64.00 71.00	62.00 70.00	56.00 56.00	168	6.7 seconds
PTEN UNMETHYLATED	SENSE 5' TTT TTG GGT TTT TGA AAT TTA ATG '3 UNSENSE 5' CAA CCT ACT AAT ATA TCA CCA ACA TA '3	52.13 54.48	62.00 68.00	58.00 68.00	56.00 56.00	165	6.6 seconds
FOLR1 METHYLATED	SENSE 5' GTT GTA GAA AGG ATA TTT GAG TTG C '3 UNSENSE 5' ACA AAT AAA AAA AAT AAA ATC CGA A '3	56.74 51.49	70.00 61.00	70.00 58.00	56.00 56.00	236	9.4 seconds
FOLR1 UNMETHYLATED	SENSE 5' GTT GTA GAA AGG ATA TTT GAG TTG TTG '3 UNSENSE 5' ACA AAT AAA AAA AAT AAA ATC CAA A '3	57.39 49.84	70.00 59.00	72.00 56.00	56.00 56.00	241	9.6 seconds
RFC METHYLATED	SENSE 5' AGG GAA AAC GTA TAA GGT TTC GTC '3 UNSENSE 5' GTC AAC ATC CTA AAA TAC CAC TCG '3	59.07 57.65	69.00 70.00	66.00 68.00	56.00 56.00	127	5.1 seconds
RFC UNMETHYLATED	SENSE 5' AGG AAA ATG TAT AAG GTT TTG TTG A '3 UNSENSE 5' CAT CAA CAT CCT AAA ATA CCA CTC A '3	55.40 57.14	66.00 69.00	64.00 68.00	56.00 56.00	123	4.9 seconds
PCFT METHYLATED	SENSE 5' TTT GTA GAG TTA TCG GGA GAT TAA C '3 UNSENSE 5' CGC GAA TAC ACC TAA CTA AAC GTA '3	56.45 58.97	69.00 70.00	68.00 68.00	56.00 56.00	100	4 seconds
PCFT UNMETHYLATED	SENSE 5' TTG TAG AGT TAT TGG GAG ATT AAT GT '3 UNSENSE 5' CCA CAA ATA CAC CTA ACT AAA CAT A '3	56.20 55.10	68.00 68.00	68.00 66.00	56.00 56.00	101	4 seconds
MTHFR METHYLATED	SENSE 5' TAG ATT TAG GTA CGT GAA GTA GGG TAG AC '3 UNSENSE 5' GAA AAA CTA ATA AAA AAC CGA CGA A '3	61.29 55.39	75.00 66.00	82.00 64.00	56.00 56.00	181	7.2 seconds
MTHFR UNMETHYLATED	SENSE 5' TTT AGG TAT GTG AAG TAG GGT AGA TGT '3 UNSENSE 5' CAA AAA ACT AAT AAA AAA CCA ACA AA '3	59.16 53.20	72.00 63.00	74.00 62.00	56.00 56.00	178	7.1 seconds

### 2.11.2 MS-PCR Master Mix

The Thermo Scientific 2X ReddyMix PCR Master Mix with 1.5mM MgCl<sub>2</sub> (Thermo Scientific, Leicestershire, UK) was used to create a MS-PCR Master Mix. Following thawing, the Master Mix was vortexed and then centrifuged with a benchtop centrifuge (ALC, Buckinghamshire, UK) briefly for 30 seconds at 1,000 x g at room temperature. A Master Mix was created as shown in Table 2.5.

**Table 2.5 MS-PCR Master Mix**

Reagent	Volume	Final Concentration
2x ReddyMix PCR Master Mix	12.5 µl	1X
Forward Primer 10 µM	1.25 µl	0.5 µM
Reverse Primer 10 µM	1.25 µl	0.5 µM
Template DNA, bisulphite treated	0.5-10 µl	0.5-125ng
Water, RNA Free	Up to 25 µl	

The mix was gently vortexed, then spun down before being added to the Veriti thermal cycler (Thermo Fisher Scientific, Leicestershire, UK).

### 2.11.3 PCR Conditions

The MS-PCR was performed under the conditions summarised in Table 2.6.

**Table 2.6 PCR Conditions for MS-PCR**

Step	Temperature, °C	Time	Number of Cycles
Initial Denaturation	95	2 mins	1
Denaturation	95	25 secs	35
Annealing	As Optimised	35 secs	
Extension	72	65secs	
Final Extension	72	5 mins	1

#### 2.11.4 Gel Visualisation

Following the MS-PCR as described in Section 2.11.3, samples were loaded into a 2 % agarose Gel. The agarose gel was made using 1g of agarose powder (Fisher Scientific, Leicestershire, UK) in 50ml of 1X Tris-acetate- Ethylenediaminetetraacetic acid, TAE Buffer, consisting of 40 mM Tris, 20 mM Acetate and 1 mM EDTA to a pH of 8.6. This was heated in a microwave until the agarose was fully dissolved. The solution was then allowed to cool down to around 60 °C before 5 µl of SafeView (NBS Biologicals, Cambridgeshire, UK) was added. The gel was then allowed to set with a small tooth comb at room temperature. The gel was then transferred to a gel electrophoresis tank (Embi Tec Run One Electrophoresis Cell, San Diego) filled with 1X TAE buffer. Ten microlitres of each sample was loaded in each well whilst 5 µl of 100 bp ladder, (exACT Gene 100bp PCR DNA Ladder, Fisher BioReagents, Leicestershire, UK) was added to each end wells. The gel was run at 100 V for 20 minutes and then transferred to a gel visualiser Bio Rad Gel Doc XR+ with Image Lab Software, (Hemel Hempstead, Hertfordshire, UK), and the fluorescence emission of SafeView was detected at 515 nm.

#### 2.12 Protein Extraction

Cells were grown in T75 flasks, and upon confluency protein was extracted using Radioimmunoprecipitation assay buffer, RIPA buffer (Sigma Aldrich, Dorset, UK), and protease and phosphatase inhibitors. Media was aspirated from the flask, and cells were washed twice with 5 ml of PBS, and then the PBS was aspirated. One millilitre of RIPA Buffer was added and the flasks were stored on ice for 5 minutes. A cell scraper was used to detach cells from the flask, and the lysate was transferred to a microcentrifuge tube and stored on ice. The lysate was centrifuged with a benchtop centrifuge (ALC, Buckinghamshire, UK) at 8,000 x g for 10 minutes at 4°C to pellet cell debris. The supernatant was then transferred to a clean microcentrifuge tube on ice and a Bradford assay performed to assess protein quantity before western blot analysis.

### 2.13 Bradford Coomassie Assay

The coomassie (Bradford) protein assay (Thermo Scientific, Leicestershire, UK) was used to determine the concentration of protein extracted. Bovine serum albumin (BSA) protein standards were diluted in RIPA lysis buffer in a range from 0 – 2000 µg/ ml. Five microlitres of extracted protein samples were added to the plate and mixed with 250 µl of Bradford reagent so they could be measured against the known BSA standards. Absorbance was measured at 595nm on a Tecan GENios Pro plate reader (Tecan, Theale, UK). The absorbance of the BSA standards were plotted against the known concentrations of BSA, and a line of best fit was added using linear regression. Using the equation of the line of best fit, concentration of the extracted protein was calculated from the absorbance and then adjusted for the dilution factor. All protein samples were diluted in dH<sub>2</sub>O to 2 mg/ mL and stored at -20°C until western blotting analysis.

### 2.14 Western Blotting

The 2 mg/ ml protein samples and both ladders; a prestained molecular weight ladder (New England Biolabs, UK) and a western blotting ladder (Invitrogen, Paisley, UK) were defrosted. The protein samples were then mixed with 2 x Laemmli buffer at a ratio of 1:1 and then were briefly vortexed before being placed in a heat block (Techne, Staffordshire, UK) for 5 minutes at 90 °C. Samples are then briefly vortexed again before loading into the gel.

The gel consisted of a 4 % stacking gel and a 10 % resolving gel which was poured into a vertical plate. The gels were added to an electrophoresis tank and filled with 1.5 M tris glycine buffer pH 7.4. The samples were loaded into the wells at a concentration of 10µg and a ladder was loaded in to the two end ladder wells. The gel was run for 2 hours at a constant voltage of 200 V and then transferred onto a nitrocellulose membrane using the wet method. This consisted of a 0.2 µm nitrocellulose membrane (Biorad, UK), Whatmann filter paper and sponges creating a sandwich with the gel in the middle which was then placed back in the

transfer tank filled with transfer buffer, ensuring proteins would travel in the correct direction from gel to the nitrocellulose membrane. The transfer took 1 hour at 90V in an ice bath.

The membrane was then blocked in blocking buffer consisting of 1 x fish gelatine from Sigma Aldrich (Dorset, UK) for 1 hour at room temperature on a rocker set to 40 rpm. The blocking buffer was then decanted, and the membrane was washed with washing buffer (1M tris buffer, pH 7.4, 0.1% Tween20) (TBST) three times for 10 minutes each time at room temperature. The membrane was then incubated with primary antibody at the chosen dilutions as detailed in Table 2.7. This was incubated overnight at 4°C on the rocker set to 40 rpm. The antibody was then decanted, and the membrane was washed with TBST for 10 minutes on a shaker and repeated 3 times. The secondary antibody conjugated to horseradish peroxidase was then added at a concentration of 1:10000 for 1 hour at room temperature on the rocker. The secondary antibody was then decanted and the membrane was washed with TBST for 10 minutes, and repeated 3 times.

The membrane was then removed and covered with Amersham ECL prime Western blotting chemiluminescent detection reagent (GE Healthcare Life Sciences, Buckinghamshire, UK) at a ratio of 1:1 buffer to luminol. The membrane was left for 5 minutes before being transferred to the XPS Chemi Doc (Biorad, UK) where it was visualised and analysed, using densitometry.

**Table 2.7 Concentration of Rabbit Polyclonal Primary Western Blot Antibodies**

Common Name	Gene Name	AbCam Ref	Size	Dilution
RFC	<i>SLC19A1</i>	Ab62302	66kDa	1:1000
PCFT	<i>SLC46A1</i>	Ab25134	50-55kDa	1:1000
FOLR1	<i>FOLR1</i>	Ab137347	30kDa	1:500
MTHFR	<i>MTHFR</i>	Ab125707	75kDa	1:500
PTEN	<i>PTEN</i>	Ab32199	47kDa	1:500

Protein expression levels were assessed by performing densitometry following Western immunoblot analysis. To minimise loading variation, a Bradford assay was performed and equal concentrations of protein were loaded into each well. In addition, the loading control  $\beta$ -actin was probed for and results were normalised relative to  $\beta$ -actin expression. Cells grown in folate free or folic or folinic acid treatment were then compared as a percentage of the control sample, which was cells grown in standard cell culture media.

## 2.15 Statistical Analysis

Data were expressed as mean values  $\pm$  standard deviation (mean  $\pm$  SD) with  $n = 3$  of triplicate experiments. Statistical analysis was carried out in IBM SPSS version 23 and GraphPad Prism 6 using the students t-test, one way ANOVA, with Tukey's or Dunnett's post-test. Any probability values,  $p < 0.05$  were considered statistically significant (\*), any values,  $p < 0.01$  were considered very statistically significant (\*\*).

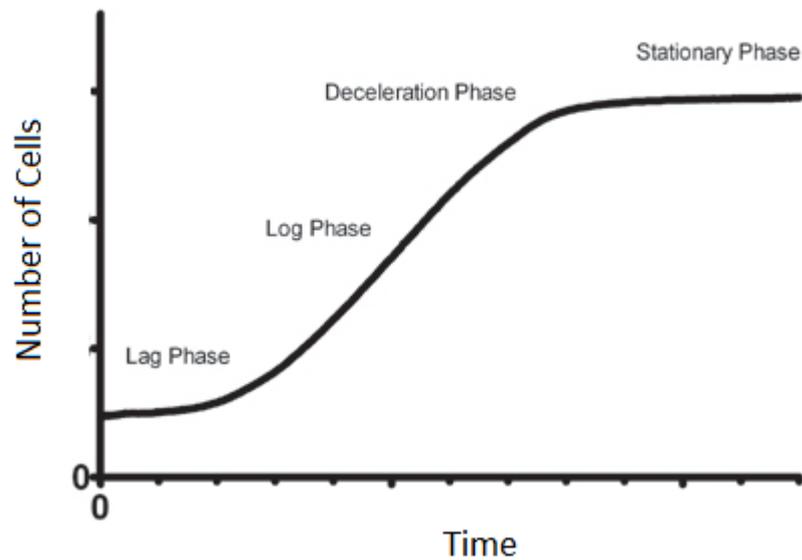
## CHAPTER 3

### 3 CYTOLOGICAL EVALUATION AND ASSAY OPTIMISATION

#### 3.1 Introduction

Cell culture is a crucial model for the study of effects of drug treatments on cells. Genetic changes often seen within the original tumour are also present within the cell lines (Capes-Davis *et al.*, 2010). Whilst use of immortalised cell lines results in greater inter-experimental consistency and therefore improved reproducibility of results compared to variation between primary cell cultures, there is the potential for these cell lines to be unreliable. Cell lines have the potential to be mis-identified, as was seen when the HeLa cervical carcinoma cells were found in many other cultured cell lines (Skloot, 2011). Regrettably, this was not a singular example (Røslund *et al.*, 2009; Torsvik *et al.*, 2010). Cell lines have also been seen to be contaminated with mycoplasma or have phenotypically drifted from the primary source (Geraghty *et al.*, 2014), therefore characterisation is vital, and mycoplasma contamination should be periodically monitored (Kaplan and Hukku, 1998).

In order to perform downstream assays in cultured cells, cells need to be within the exponential phase of growth. As shown in Figure 3.1, cells begin in an initial lag phase, where they do not divide but are adjusting to the conditions within the flask or plate, adhering to the plate and establishing cell-cell contact and communication. The next stage is the log phase where the cells are rapidly proliferating and growing exponentially, and it is at this stage where cells are most viable, so is the point at which any assays should be performed. Following this, the cells will enter a plateau phase where rate of proliferation decreases, and cells become most vulnerable to external factors. If cells are grown past this phase they will begin to decline, and cell death will occur as part of the cell cycle (Phillips and Clayton, 1997).

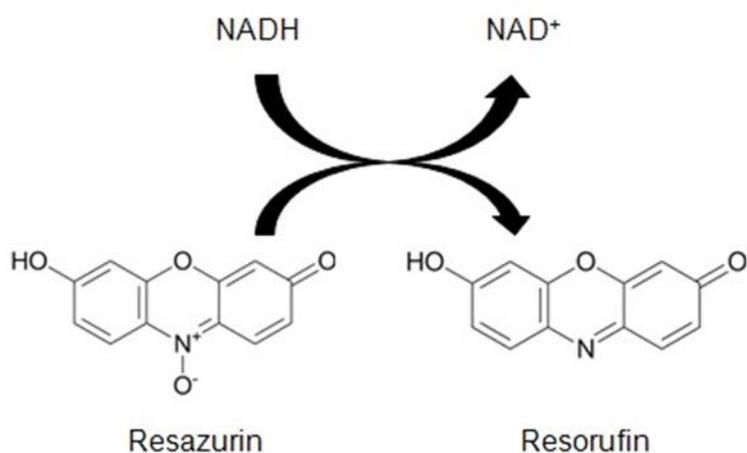


**Figure 3.1 Stages of cell growth (Held, 2010)**

*Cells begin the Lag phase before progressing to the Log phase, also known as exponential growth phase. This phase was of most interest as cells from this phase were analysed. Cells then begin to decelerate before entering the stationary phase.*

Cells grow at different rates dependent upon the cell type and conditions they are exposed to so it is important that growth curves are performed in order to determine the doubling time of the cells.

Accurate prediction of the viability of cancerous cells following *in vitro* drug treatment is a critical part of screening for efficacy during drug development. Cell viability assays have the ability to show the number of live cells through the use of a biological event that occurs in living cell, but not following cell death (Riss *et al.*, 2004). In many commercially available viability kits for metabolic function (PrestoBlue assay, Alamar blue assay), resazurin is used as a redox dye that assesses the ability of viable cells to reduce the dye to a fluorescent product, whereby in a reducing environment within the cell cytosol, where oxygen is removed, is associated with cell viability (Figure 3.2).

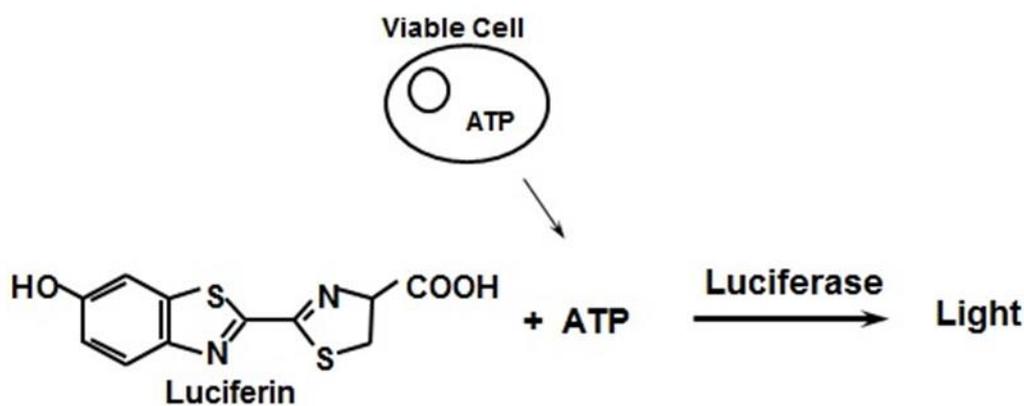


**Figure 3.2 The reduction of resazurin to resorufin.**

*The PrestoBlue assay, used to assess cell viability, utilises the metabolic activity of viable cells to reduce resazurin to resorufin by removal of oxygen (Riss et al., 2004).*

In eukaryotic cells, most catabolic redox reactions occur in the cytoplasm and mitochondria (Rawson, et al., 2014). There are several assays available for use to assess cell viability. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was the first homogenous cell viability assay, which measures nicotinamide adenine dinucleotide (NADH) carrying electrons from one reaction to another. When cells die they lose their ability to convert MTT into formazan, a colour formation which gives an indication of viable cells, giving an indication of mitochondrial activity. NADH works by reducing the formazan (MTT) reagent. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2Htetrazolium (MTS) assay works in a similar fashion, except it is negatively charged, rather than the positively charged MTT. It works with an intermediate electron acceptor phenazine ethyl sulfate (PES) that can transfer electrons from the cytoplasm or plasma membrane to facilitate the reduction of the tetrazolium into the coloured formazan product (Riss et al., 2004). The glycyphenylalanyl-aminofluorocoumarin (GF- AFC) assay works by detecting protease activity, which is limited to viable cells, the peptide substrate, Gly-Phe-AFC, enters intact cells, where it is cleaved by cytoplasmic amino peptidase activity removing the glycine

and phenylalanine amino acids to release AFC to generate a fluorescent signal proportional to the number of living cells. The live-cell protease becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. Another assay that utilises the loss of membrane activity, is the ATP assay. As shown in Figure 3.3, when cells lose membrane integrity, they lose the ability to synthesise ATP and any remaining ATP in the cytoplasm is depleted by endogenous ATPases. ATP detection reagent contains detergent to lyse cells, ATPase inhibitors then stabilise the ATP that is released from the cells. Luciferin, the stable form of luciferase, is used as the substrate to catalyse the reaction that generates lights to measure ATP.



**Figure 3.3 ATP assay mechanism.**

*Luciferin is used as a substrate, which when combined with ATP released from live cells, is catalysed to produce light which can be measured (Riss et al., 2004).*

The PrestoBlue assay was chosen over other assays available to assess the number of living cells present following treatment, compared to untreated cells because it is more sensitive than the tetrazolin assay (Xu, 2015), and can be used for repeat measurements for time course experiments, whereas other viability assays, such as ATP are terminal due to toxicity of assay components.

Cell viability assays will establish whether the rate of proliferation has increased, however a decrease in cell viability does not necessarily mean a cytotoxic response, further assays need to be performed to check for apoptosis, cell quiescence or even sub-optimal culture conditions. Cell cycle analysis can show whether the cells are in cell cycle arrest, or whether they are in the apoptotic phase or if they have become necrotic (Elmore, 2007). For this reason, further apoptosis assays and cell cycle analysis were undertaken in Chapter 5 following folate treatment. It is also important when to optimise all assays for the particular cell line and growth conditions utilised. PrestoBlue is a cell viability assay that uses a resazurin-based cell viability reagent, whereby only viable cells have the ability to reduce the dye, hence proving a quantitative measure of cell proliferation. The compound in PrestoBlue is blue in colour with little fluorescence, which becomes highly fluorescent and changes to pink-purple in colour when reduced by viable cells. Cell viability assays can be used to indicate cell growth, because as cells continue to divide and multiply, there will be more viable cells to observe. Previous studies using PrestoBlue all date from 2011, where the studies published using PrestoBlue have analysed different aspects of cell biology, including plasma membrane integrity (Reisetter *et al.*, 2011), cell viability (Deepe and Buesing, 2011; Gillaux *et al.*, 2011) and cytotoxicity (Broekman *et al.*, 2011). PrestoBlue has been used on a range of cell types, including the normal Chinese hamster ovary cell line (CHO) mouse skin melanoma (B16F0) and human squamous cell carcinoma (COLO16) (Istivan *et al.*, 2011).

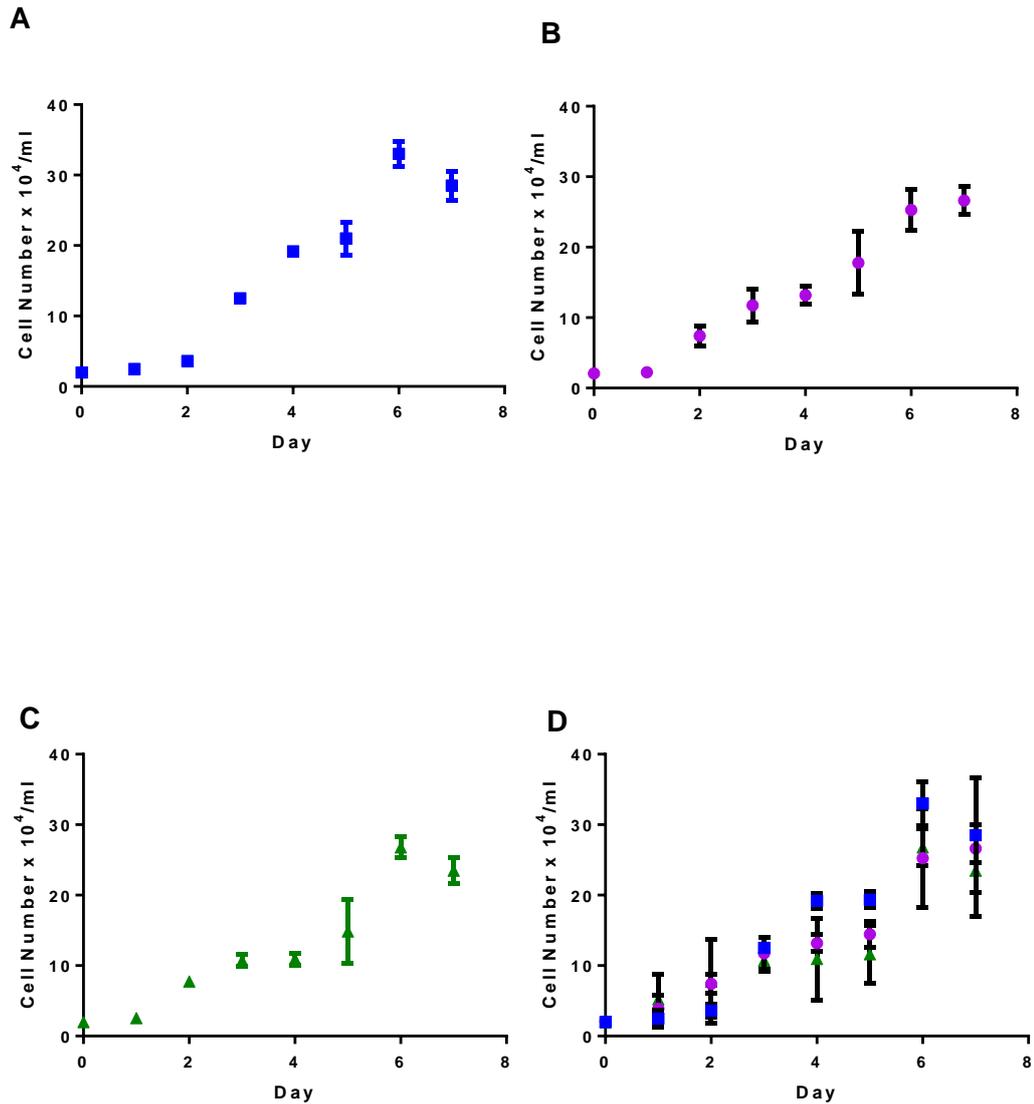
### 3.1.1 Aim of Chapter

The aim of this chapter was to authenticate the cells lines used in experiments, and to establish growth behaviour, in particular reference to cell doubling times. This chapter also aimed to validate the conditions of assays being used in future chapters.

## 3.2 Results

### 3.2.1 Standard Cell Culture Media Growth Curves

As shown in Figure 3.4, the U87MG cells displayed exponential growth between days 2 and 5, whilst the 1321N1 cell line showed exponential growth between days 2 and 6. The SVGp12 cell line showed exponential growth between days 2 and 5. The doubling time for the 1321N1 cell line was fastest with a time of 20.2 hours compared to 36.3 hours for the U87MG cells and 50.6 hours for the SVGp12 cells (Table 3.1).

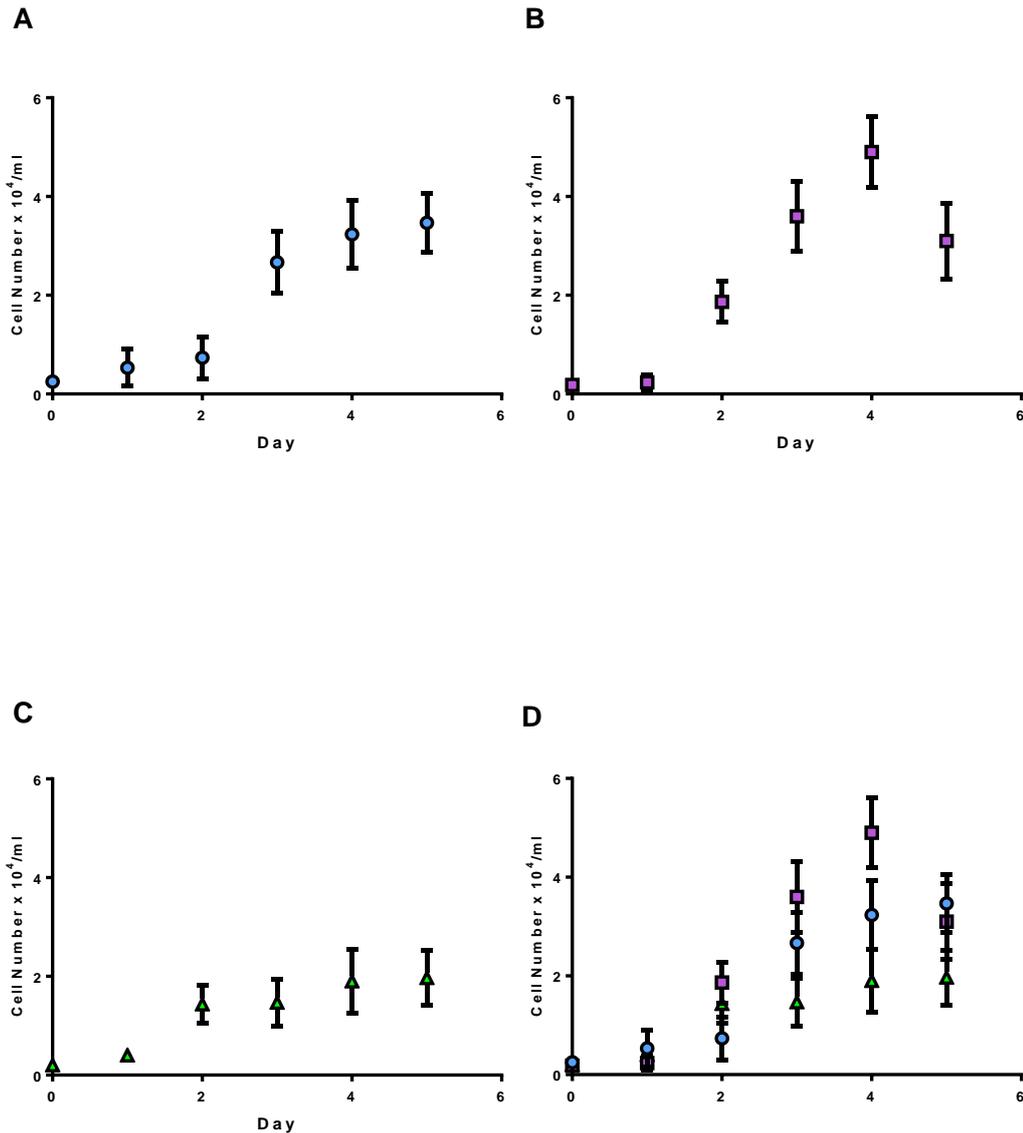


**Figure 3.4 Cell growth curves over seven days in standard cell culture media.**

Standard cell culture media contains 4µg/ml folic acid. **A)** Cell growth curve for 1321N1, Grade II Glioma Cell Line **B)** Cell growth curve for U87MG, Grade IV Glioma Cell Line **C)** Cell growth curve for SVGp12, Non-Cancerous Foetal Glial Cell Line **D)** Cell growth curve for all Cell Lines. Graphs show the log and stationary phases. The data points are means of three independent replicates (n=3), error bars represent ± standard deviation.

### 3.2.2 Folate Free Media Growth Curves

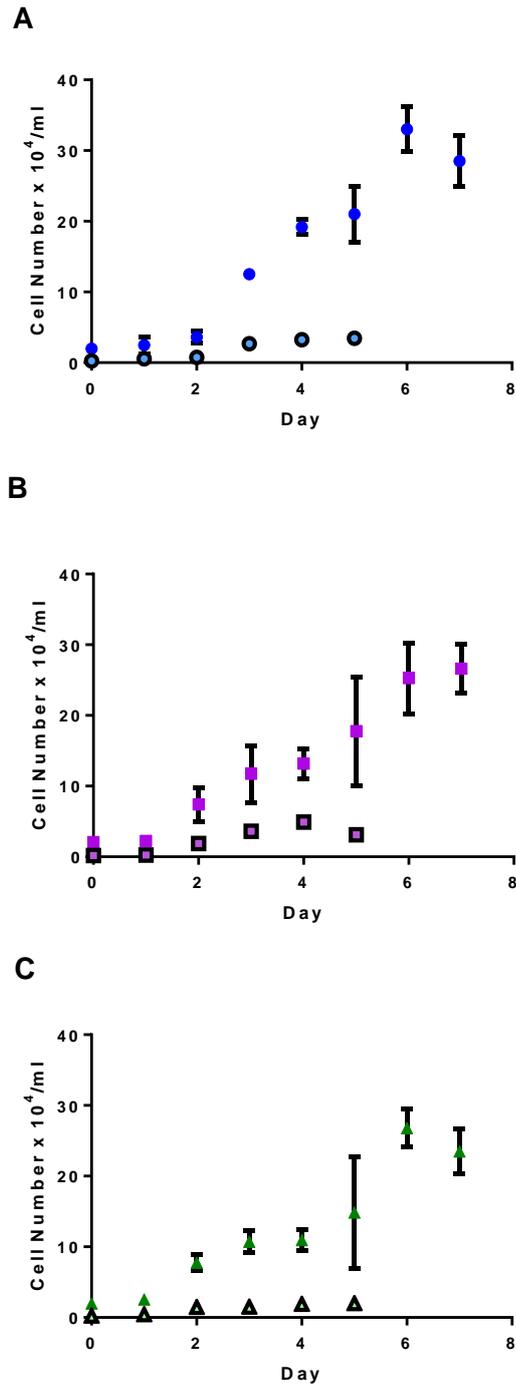
Cells lines were also grown without folate in the media as shown in Figure 3.5. The 1321N1 cell line showed exponential growth between Days 2 - 4. The U87MG cell line also showed exponential growth between Days 2 - 4. The SVGp12 cell line showed exponential growth between Days 1 - 3. The doubling time for the 1321N1 cell line was fastest with a time of 41.03 hours compared to 45.24 hours for the U87MG cells and 54.47 hours for the SVGp12 cells (Table 3.1).



**Figure 3.5 Cell growth curves over seven days in folate free media.**

*Folate free media contains 0 $\mu$ g/ml folic acid. **A)** Cell growth curve for 1321N1, Grade II Glioma Cell Line **B)** Cell growth curve for U87MG, Grade IV Glioma Cell Line **C)** Cell growth curve for SVGp12, Non-Cancerous Foetal Glial Cell Line **D)** Cell growth curve for all Cell Lines. Graphs show the lag and stationary phases. The data points are means of three independent replicates ( $n=3$ ), error bars represent  $\pm$  standard deviation.*

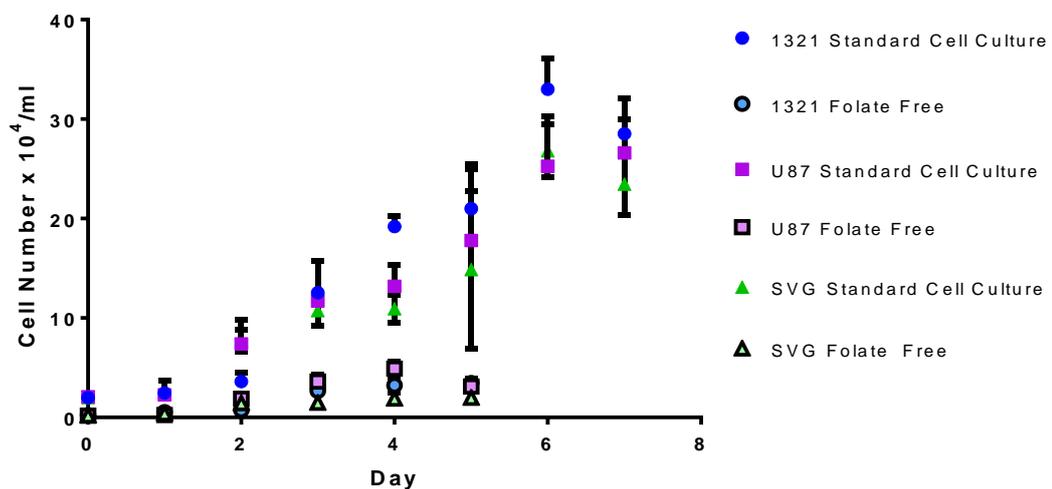
When comparing the same cell line grown with and without folate in the media, it was seen that the absence of folate significantly decreases the rate of cell growth in all cell lines (Figure 3.6), this was assessed by performing a t-test to compare the mean doubling times of folate deficient cells to those grown in standard cell culture media.



**Figure 3.6 Cell growth curves over seven days comparing standard and folate free media.**

Standard cell culture media contains 4µg/ml folic acid. Folate free media contains 0µg/ml folic acid. **A)** Cell growth curve for 1321N1, Grade II Glioma Cell Line grown in standard cell culture media and folate free media **B)** Cell growth curve for U87MG, Grade IV Glioma Cell Line grown in standard cell culture media and folate free media **C)** Cell growth curve for SVGP12, Non-Cancerous Foetal Glial Cell Line grown in standard cell culture media and folate free media. Graphs show the log and stationary phases. The data points are means of three independent replicates (n=3), error bars represent ± standard deviation. A Student's t-test was performed to assess significance between cells grown in standard cell culture media and folate free media.

As can be seen from both Figure 3.7 and Table 3.1 cells grown in folate deficient media grow much more slowly than cells grown in standard cell culture media. The cell doubling time for 1321N1 cells grown in standard cell culture media is 20.16 hours, half the time of cells grown in folate free media which have a doubling time of 41.03 hours. The U87MG cells grown in Standard Cell Culture Media have a doubling time of 36.3 hours, this is faster than the cells grown in folate free media which have a doubling time of 45.24 hours. The SVGp12 cells grown in standard cell culture media have a doubling time of 50.56 hours which is again faster than the cells grown in folate free media which have a doubling time of 54.47 hours.



**Figure 3.7 Cell number comparison between folate free and standard cell culture media grown cells.**

Cells were grown over seven days in standard cell culture media containing  $4\mu\text{g/ml}$  folic acid and folate free media containing  $0\mu\text{g/ml}$  folic acid. Cells grown in folate free media grew more slowly than those grown in standard cell culture media in all three cell lines. A Student's *t*-test was performed to assess significance between cells grown in standard cell culture media and folate free media.

The following calculation was used to calculate cell doubling time from two points chosen in the exponential phase of growth.

**Equation 1 Cell Doubling Time Equation (Roth, 2006).**

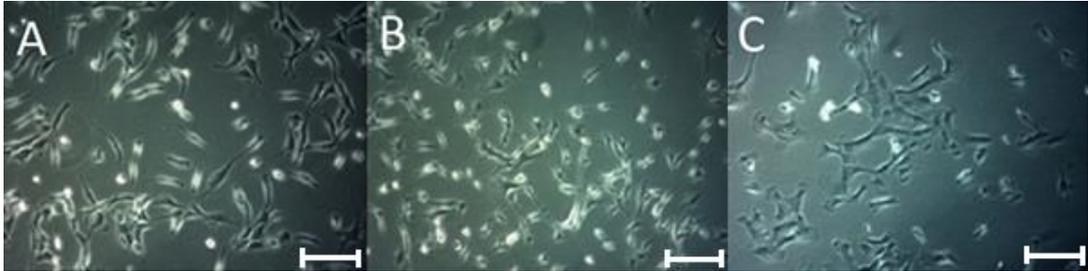
$$\text{DoublingTime} = \frac{\text{duration} * \log(2)}{\log(\text{FinalConcentration}) - \log(\text{InitialConcentration})}$$

Where log is the logarithm to base 10. Units used were hours.

**Table 3.1 Cell Doubling Times**

Cell Line	Treatment	Doubling Time (Hours)	Standard Deviation (+/-)	Significance (t-test)
1321N1	Folate Free	41.03	0.695	$p < 0.01$
1321N1	Standard Cell Culture Media	20.16	4.619	$p < 0.01$
U87	Folate Free	45.24	0.767	$p < 0.01$
U87	Standard Cell Culture Media	36.30	7.671	$p < 0.01$
SVGP12	Folate Free	54.47	0.642	$p < 0.01$
SVGP12	Standard Cell Culture Media	50.56	8.637	$p < 0.01$

The cells were also visualised under a light microscope at 10X magnification. Cells were elongated in appearance with star like projections visible, characteristics typically seen in astrocytes (Figure 3.8).

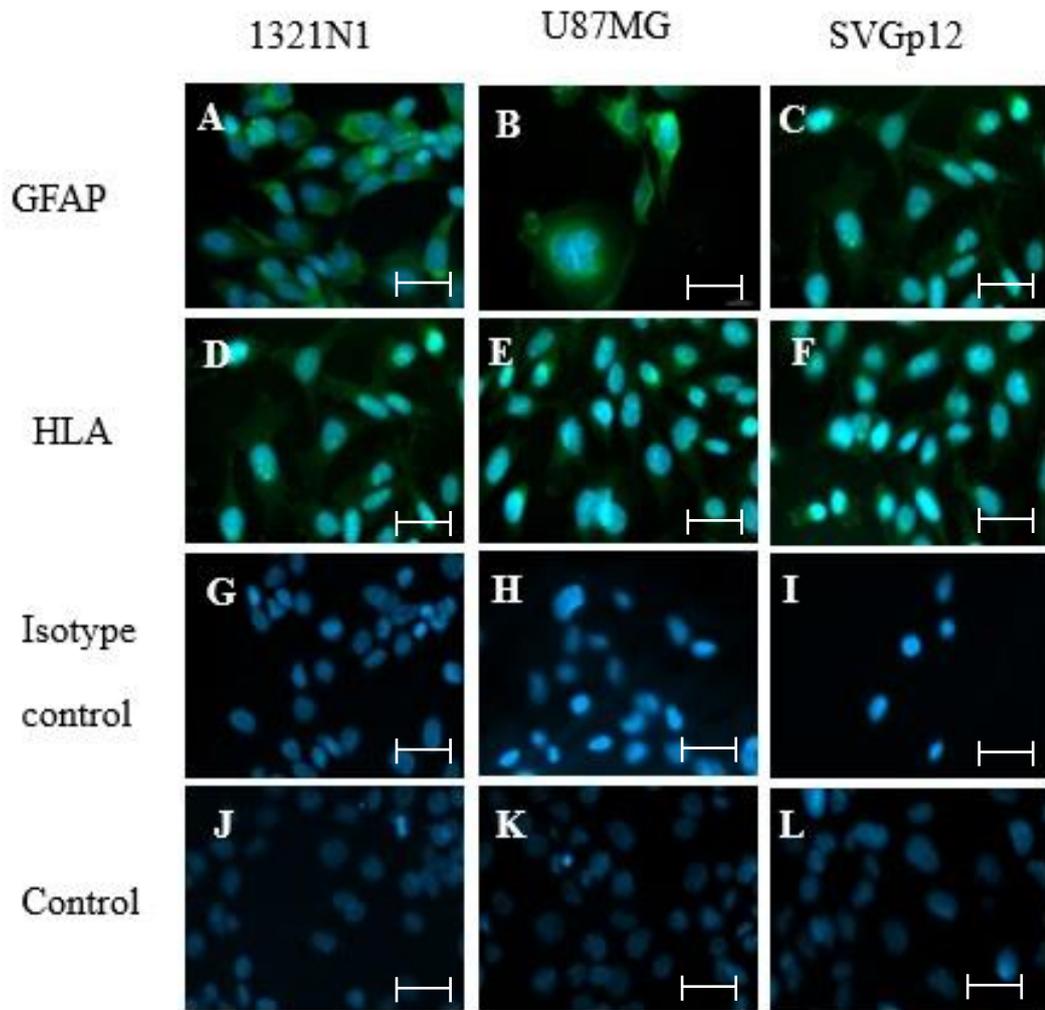


**Figure 3.8** *Microscope visualisation of cells.*

*Cells were grown for 5 days, in standard cell culture media and then observed under a light microscope at 10X magnification. A) 1321N1 cells B) U87MG cells C) SVGp12 cells. The scale bar represents 20 µm.*

### 3.2.3 Characterisation of Cell Lines by Immunocytochemistry

In order to ensure the cell lines were as reported, the 1321N1, U87MG, and SVGp12 cell lines were characterised to ensure they were of both glial and human origins. Human leukocyte antigen (HLA) and glial fibrillary acidic protein (GFAP) were used for confirmation. All three cell lines expressed both HLA and GFAP, as shown for the merged images in Figure 3.9. Green HLA and GFAP staining was seen to be cytoplasmic and the nucleus was stained blue by DAPI. An example of the single channel images is shown in Appendix 3, where it was confirmed the staining was not nuclear. A negative control was used in the form of an isotype, because it has all the non-specific characteristics of the antibodies used for characterisation, but does not have any specificity for the target cell.

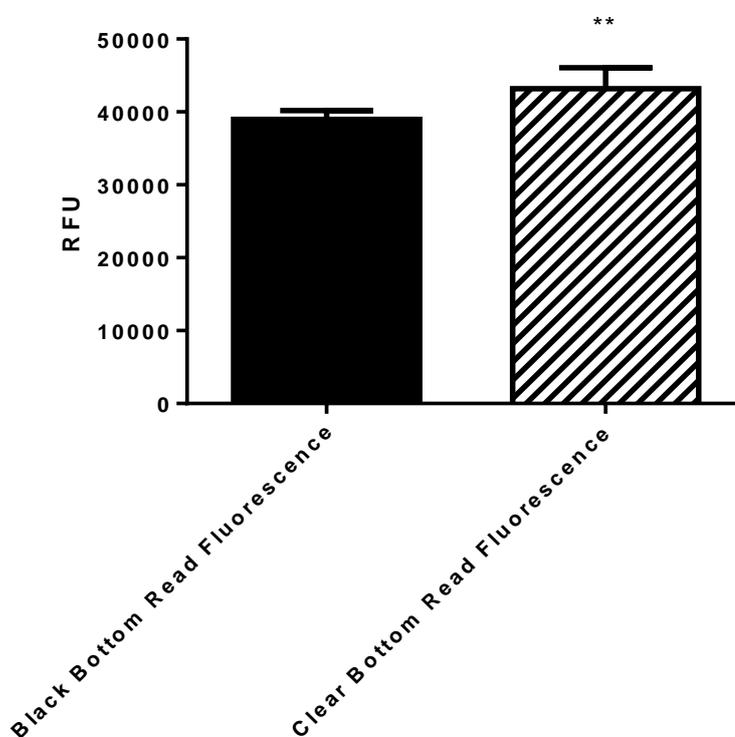


**Figure 3.9 Immunofluorescent Staining of Cell Lines.**

Cells were grown for 5 days in standard cell culture media. **A-C** represent staining with GFAP (green) and counterstained with DAPI (nuclear staining). **D-F** represent staining with HLA (green) counterstained with DAPI (nuclear staining). **G-I** represents the Isotype Control which was rabbit IgG primary antibody. **J-L** represents a no antibody control. The scale bar represents 50  $\mu\text{m}$

### 3.2.4 Plate Optimisation

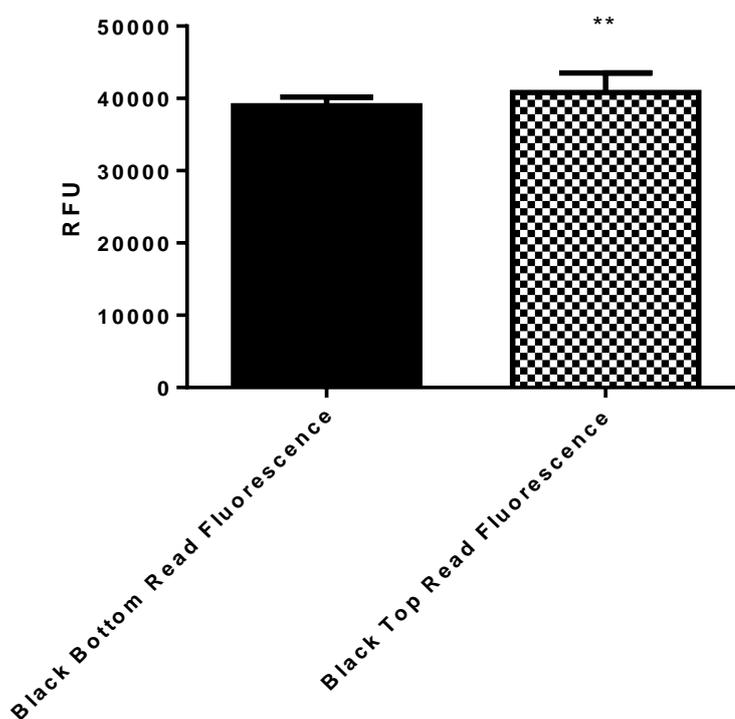
To determine whether black walled 96-plates were required for the fluorescence measurements in the PrestoBlue assay, the mean raw fluorescence units (RFU) of a standard solution of cell culture media was compared to conventional clear 96-well plates. A significantly higher RFU value was measured in the clear 96-well plates compared to the black walled 96-well plates (Figure 3.10), indicating cross talk, therefore black wall plates were chosen.



**Figure 3.10 Plate colour optimisation.**

*Cells were seeded into black and clear 96 well plates with standard cell culture media containing 4µg/ml folic acid and PrestoBlue (1:10). Raw fluorescence units were then measured following sixty minutes incubation. A significantly higher level of variance is seen in the clear plate. The data points are means of twenty replicates (n=20), from one experiment, error bars represent ± standard deviation. A Student's t-test was performed to assess significance.*

A significantly higher RFU value was measured when measure from the top, rather than the bottom of the plate (Figure 3.11). Due to the significant difference seen between the two samples using a t-test it was not necessary to perform a Bland Altman's repeatability coefficient test.



**Figure 3.11 Plate reading optimisation.**

*Cells were seeded into black 96 well plates with standard cell culture media containing 4µg/ml folic acid, and PrestoBlue (1:10). Raw fluorescence units were then measured following sixty minutes incubation from both the top and bottom. A significantly higher level of variance is seen in the plate read from the top. The data points are means of twenty replicates (n=20), from one experiment, error bars represent ± standard deviation. A Student's t-test was performed to assess significance.*

Given the results seen, cell viability assays using the PrestoBlue assay will be measured in a black plate using bottom read fluorescence to gain optimal accuracy.

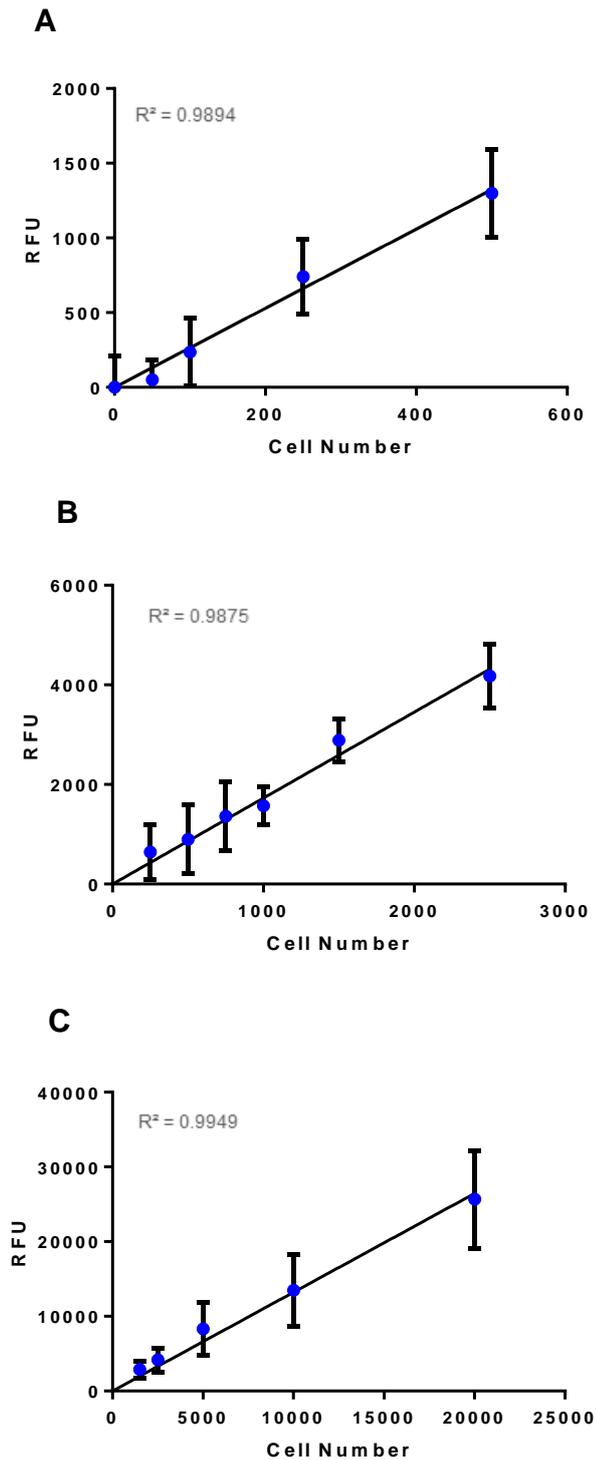
### 3.2.5 PrestoBlue Assay Optimisation

It was essential that all cell lines were tested for linearity, as seen in Sections 3.2.1 and 3.2.2, each cell line showed different growth kinetics, so theoretically different proliferation kinetics would also expect to be seen. The fastest growing 1321N1 cell line, would be more likely to lose linearity sooner compared to the slowest growing SVGp12 cells. So it was important to ensure that the linearity of proliferation measures for all three cell lines were tested.

The recommended incubation time of cells with PrestoBlue is 10 minutes to 2 hours (although up to 24 hours can be used). The sensitivity of the assay was reported to be as low as 12 cells per well but assay optimisation of incubation time and cell number was crucial to ensure linearity.

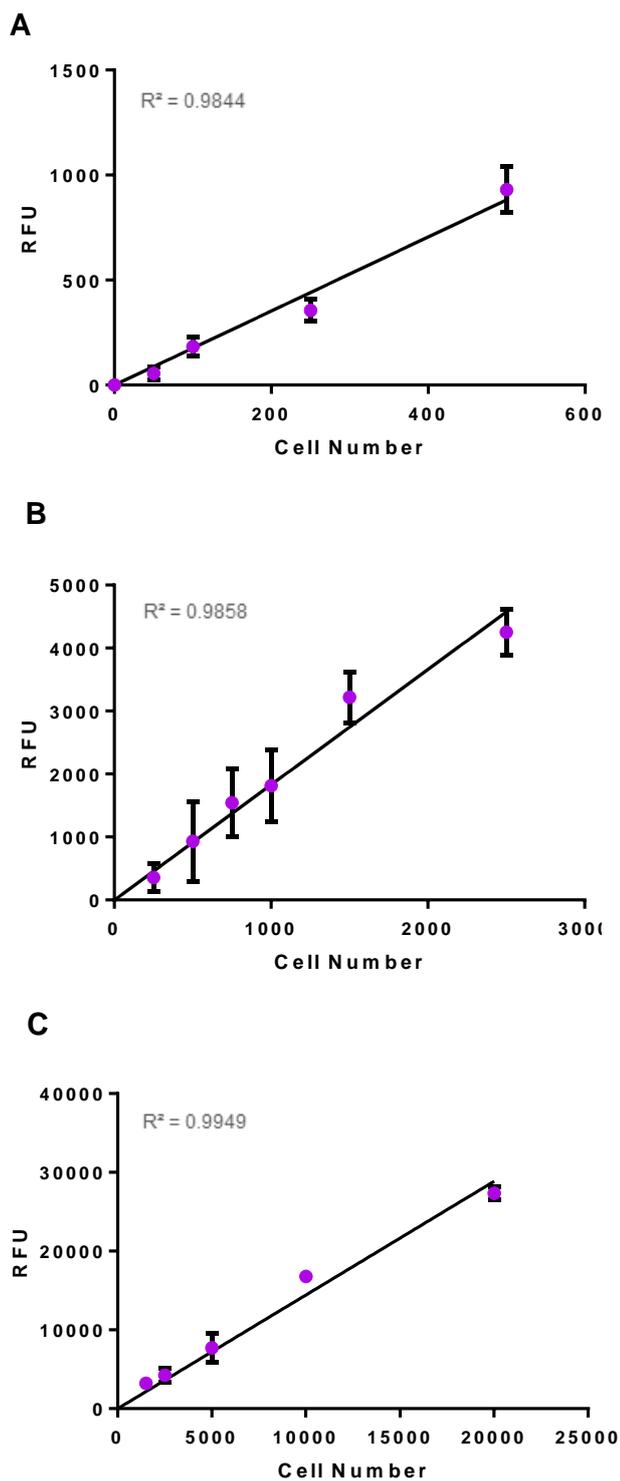
Cells were incubated with PrestoBlue for 60 minutes, as this was the midway point of recommended incubation times. The lowest range of cell numbers analysed were those typically chosen for seeding density: 50, 100, 250 and 500. The highest range of cell number analysed were those that might be expected following 7 days of treatment based on cell doubling numbers: 1500, 2500, 5000, 10,000 and 20,000. A mid-point in cell number were then chosen that would be typically observed following 5 days of treatment, when most assays were performed. This was predicted based on cell doubling numbers from kinetic experiments to assess linearity at: 250, 500, 750, 1000, 1500 and 2500. Based on these assumptions the range of cell numbers 50 – 20,000 were incubated in PrestoBlue at time points from 10– 1440 minutes (24 hours) as detailed in the appendix 2. The optimisation was performed for all three cell lines; 1321N1, U87MG and SVGp12. The aim was to confirm the incubation time, and appropriate cell number to be analysed, ensuring the fluorescence measurements remained linear, considering cells would be grown for up to 7 days with viability being assessed every other day.

Linearity was seen across the range of cell numbers 50 – 20,000 in all three cell lines 1321N1, U87MG and SVGp12 when cells were incubated with PrestoBlue for 60 minutes (Figures 3.12-3.14).



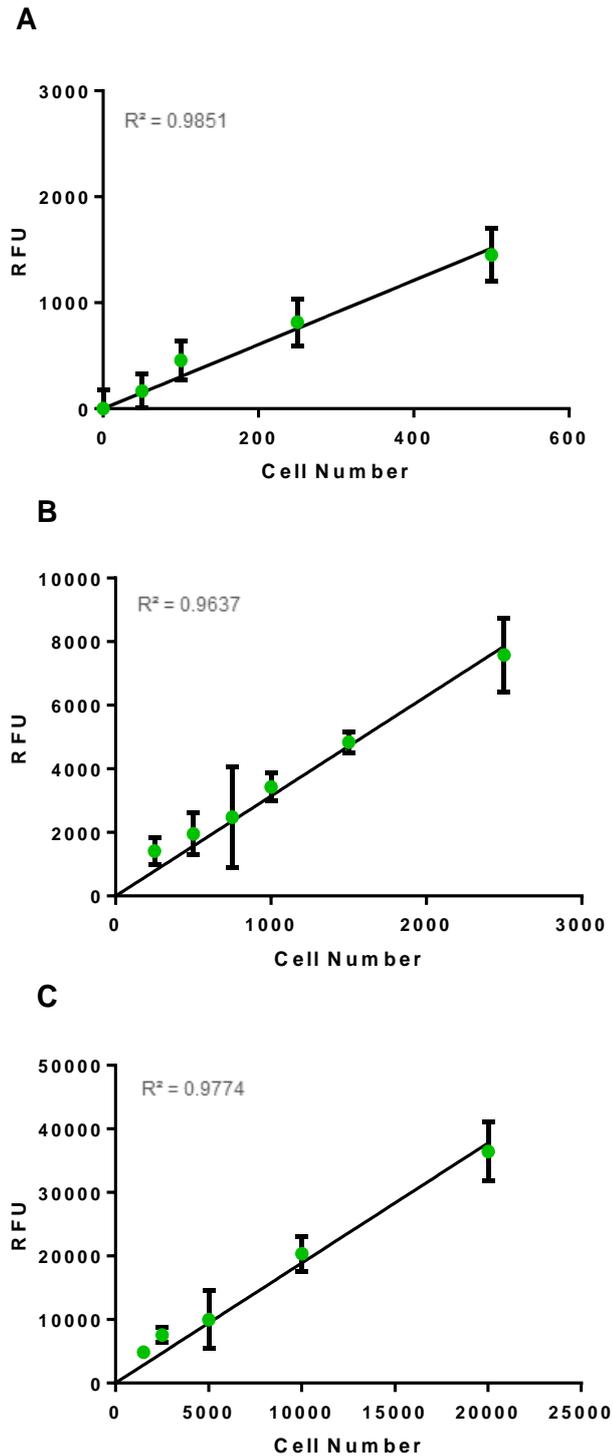
**Figure 3.12 PrestoBlue Linearity across 0 – 20,000 1321N1 Cells.**

1321N1 cells were seeded into 96 well plates with standard cell culture media containing 4µg/ml folic acid, and PrestoBlue (1:10). Raw fluorescence units were then measured following sixty minutes' incubation. **(A)** 50 – 500 cells. **(B)** 250 – 2500 cells. **(C)** 1500 – 20,00 cells. The data show that there was linearity across the range of cells 0 – 20,000. The data points are means of four replicates (n=4), from three independent experiments, error bars represent ± standard deviation.



**Figure 3.13 PrestoBlue Linearity across 0 – 20,000 U87MG Cells**

*U87MG cells were seeded into 96 well plates with standard cell culture media containing 4 $\mu$ g/ml folic acid, and PrestoBlue (1:10). Raw fluorescence units were then measured following sixty minutes' incubation. (A) 50 – 500 cells. (B) 250 – 2500 cells. (C) 1500 – 20,00 cells. The data show that there was linearity across the range of cells 0 – 20,000. The data points are means of four replicates (n=4), from three independent experiments, error bars represent  $\pm$  standard deviation.*

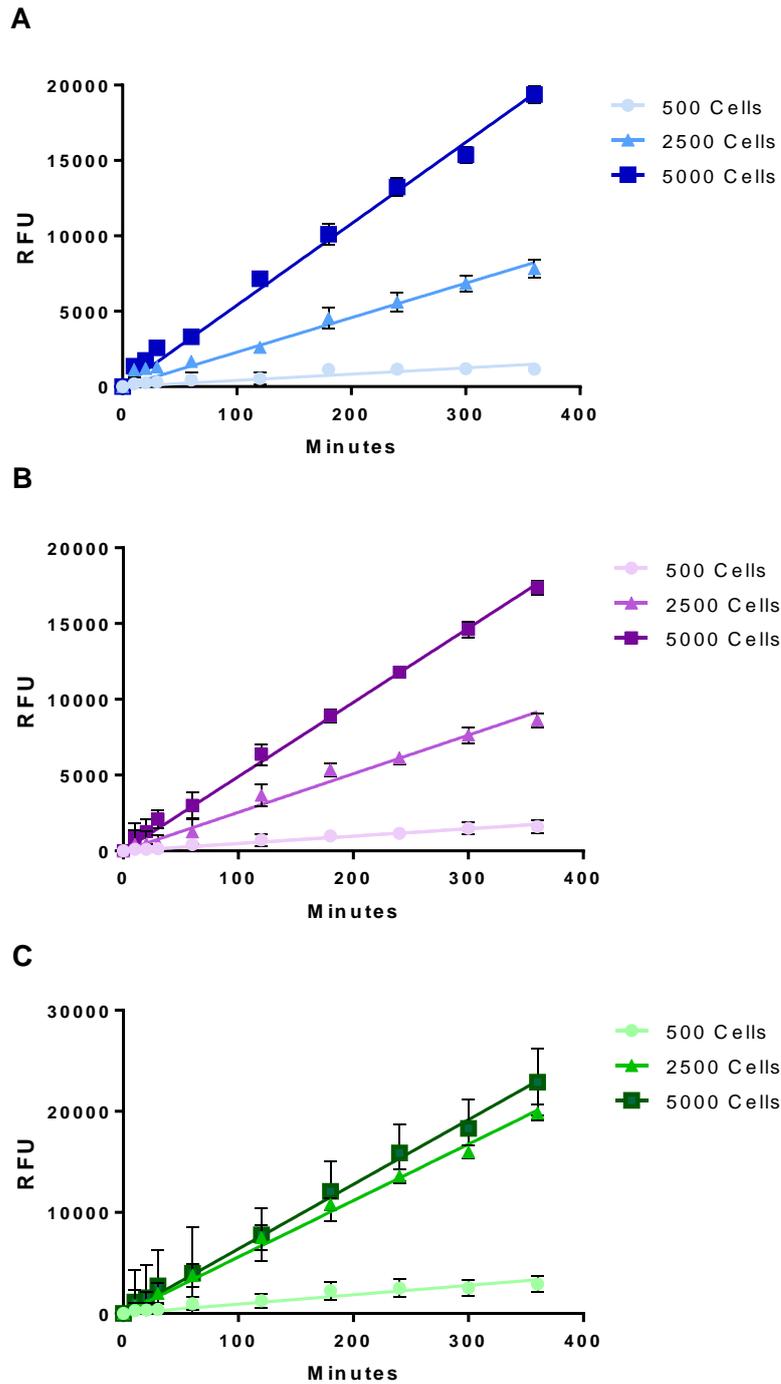


**Figure 3.14 PrestoBlue Linearity across 0 – 20,000 SVGp12 Cells**

SVGp12 cells were seeded into 96 well plates with standard cell culture media containing  $4\mu\text{g/ml}$  folic acid, and PrestoBlue (1:10). Raw fluorescence units were then measured following sixty minutes' incubation. **(A)** 50 – 500 cells. **(B)** 250 – 2500 cells. **(C)** 1500 – 20,00 cells. The data show that there was linearity across the range of cells 0 – 20,000. The data points are means of four replicates ( $n=4$ ), from three independent experiments, error bars represent  $\pm$  standard deviation.

After 5 days of treatment, when the majority of assay were performed, 120 hours will have passed, allowing the cell population to have doubled 3 times. With a seeding density of 500 cells this would equating to an approximate cell number on Day 5 of 4000 cells, so it was important to establish linearity in this range. Three cell number densities were chosen; 500, 2500 and 5000 cells. The cells were then incubated in PrestoBlue over a range of times; 10 minutes to 360 minutes.

Linearity was seen for the cells seeded at 500, 2500 and 5000 when they were incubated with PrestoBlue for a range of time from 10 to 360 minutes (Figure 3.15). This supports the use of PrestoBlue as an assay to be indicate cell viability over a time period of 7 days where cell numbers will vary from intial seeding density to days analysed to the final time point.



**Figure 3.15 Linearity of PrestoBlue over 0 -360 minutes' incubation at 500, 2500 and 5000 cells**

Cells were seeded into 96 well plates with standard cell culture media containing 4µg/ml folic acid, and PrestoBlue (1:10). Raw fluorescence units were then measured following incubation at 10, 20, 30, 60, 120, 180, 240, 300 and 360 minutes. **(A)** 1321N1 cells at a seeding density of 50, 2500 and 5000 cells. **(B)** U87MG cells at a seeding density of 50, 2500 and 5000 cells. **(C)** SVGp12 cells at a seeding density of 50, 2500 and 5000 cells. The data show that there was linearity across the range of cells 0 – 20,000 at the different incubation times. The data points are means of four replicates (n=4), from three independent experiments, error bars represent ± standard deviation.

### 3.3 Discussion

The primary aim of this chapter was to authenticate the cell lines being used. In order to ensure that the cells being used for analysis were as described, they were authenticated by testing for human and glial markers. The results showed that all three cells line 1321N1, U87MG and SVGp12 were all of human origin as they tested positive for the human leukocyte antigen (HLA) marker, and were all of glial origin as they all tested positive for the glial fibrillary acidic protein (GFAP). GFAP, a member of the cytoskeletal protein family, was chosen because it is widely expressed in astroglial cells, and has already been used in several published studies to confirm cells are of glial origin (Gomes *et al.*, 1999; Ignatova *et al.*, 2002; Jung *et al.*, 2007). Whilst GFAP is also a marker for neural stem cells, these cells have not been used within the lab, therefore GFAP was used as a marker to confirm there had been no cross contamination with other non-neural cells. HLA is a molecule involved in the regulation of immune response, and is the human version of the major histocompatibility complex which rejects foreign organs (Choo, 2007). It is specific to humans, and is often used to assist cross contamination checks between cell lines (Braun *et al.*, 2000).

The preferred option for authenticating cell lines would be to use short tandem repeat (STR) DNA profiling. STR analysis has the ability to identify cell lines by being able to differentiate between individuals of the same species (Almeida *et al.*, 2016) due to their polymorphic nature. Each STR consists of between 2 to 9 base pairs, but the number of repeats will vary greatly between individuals. The National DNA database use 17 loci in order to discriminate between individuals (Almeida *et al.*, 2016).

The use of cell lines for scientific research is not always looked upon favourably due to the propensity for phenotypic drift. Immortalised cell lines do however, have many advantages compared to primary cell culture, including low cost, a short doubling time, less serum requirement and reduced variation between experiments (Masters, 2000). Cancerous cell

lines only show a snapshot of a tumour at a given time. Whilst data from a cell line will only give you a picture of one stage of the tumour, it also means that results are more reproducible. Assays performed at different time points using primary tissue which differentiates over time could have variable results, whilst informative, if a certain time point in the tumour stage is being focused on this would not be beneficial.

It has been argued that using an immortalised cell line is not the same as using primary tissue, but studies in breast cell lines and tumours (Wistuba *et al.*, 1998) as well as in lung cell lines and tumours (Wistuba *et al.*, 1999) show there are similar genotypic and phenotypic properties between the cell lines and corresponding tumour. Similar gene expression has also been seen in another study which looked at 60 different human cancer cell lines and their tissue equivalent (Ross *et al.*, 2000). Cell lines have also been seen to respond to drug treatments in a similar way to their comparable primary tissue (Walker *et al.*, 1987). Cell lines do have a draw back in that they tend to represent only high grade tumours, this is because the high grade tumours tend to harbour the mutations needed to create a cell line.

There are also issues raised regarding quality control when using cell lines, but these can easily be addressed. Cross contamination with other cell lines is unfortunately an issue which can occur on a regular basis (Dirks *et al.*, 1999; Gartler, 1967). Many cell lines are actually HeLa cells (Nelson-Rees *et al.*, 1981; Nelson-Rees *et al.*, 1974), the first human cell line created (Lucey *et al.*, 2009). This potential pitfall can be avoided if cell line authentication techniques such as immunohistochemistry or DNA fingerprinting using short tandem repeat PCR (ST-PCR) based genotyping are used to allow cell lines to be identified.

Another problem is microbial contamination of cell lines in the form of mycoplasma, which affects gene expression and cell behaviour, to the point that studies performed with mycoplasma infected cell lines are invalid (Nikfarjam and Farzaneh, 2012). It again can be

easily checked for by regularly screening and testing cell lines. Cells were regularly tested for mycoplasmas within the lab which were all found to be free from mycoplasmas.

The use of cell lines is particularly useful in this project as alterations to methylation of a gene are usually first detected in cell lines, in lung cancer (Virmani *et al.*, 1998) and breast carcinomas (Shivapurkar *et al.*, 1999).

This chapter also aimed to establish the growth behaviour of the cells being used for analysis. Establishing the growth curves of cell was important given that this project will focus largely on cell viability over time, and this information will be critical in aiding initial seeding density so that cells can continue to grow undisturbed in a well without the need to passage. The average doubling time for all cells was 41 hours, if cells were grown for 7 days (168 hours) this would allow for the population to double 4 times. This knowledge informed the decision to seed cells at a number of 500 cells per well in a 96 well plate, so that after having been treated for 7 days, the final number would approximately be 8000 cells, which is sufficiently low enough that the cells did not need to be passaged. This was important because every time the cells were passaged it allowed the potential for greater genetic variability because cell lines have a tendency to produce variations when they are continuously grown and frequently passaged. To counter this, passage numbers were kept to a minimum, and cells ideally not used above 10 passages (Masters and Stacey, 2007). It is also important to know that any changes seen in viability, apoptosis or cell death are as the result of treatment, and not because the cells have entered the plateau phase, the phase before cell death occurs.

Establishing linearity and fully optimising an assay is important to ensure that any results from future assays are reliable, and easily reproducible, and should be the start of any investigation (Dawson *et al.*, 2010). Linearity is important to ensure that the samples being tested are in the limits of the analytic measurement range (Jhang *et al.*, 2004), and allows any deterioration in the reagent to be observed. The results confirmed that there was linearity

across the range of cell numbers to be used when cells were incubated with PrestoBlue for 60 minutes. It is important to ensure that consistency is kept across all cell lines to provide an accurate comparison, and so a time of 60 minutes was identified to give the best linearity across all three cells line.

To summarise, it can be seen that this chapter has fulfilled its aim to confirm that the cells used were of both human and glial origin. The information obtained regarding cell growth patterns and doubling times was then used to ensure that when cells were analysed in future experiments they would be in the exponential phase of cell growth, rather than the lag or plateau phase. The doubling times also allowed for more precise assay optimisation by providing expected cell numbers after 7 days, and assisting with choosing initial cell seeding density. The assay optimisation showed that the PrestoBlue assay which was to be used to determine the viability of cells following treatment was linear across the expected range of cell number which supports the reliability of future results.

# CHAPTER 4

## 4 ASSESSING THE EFFECT OF FOLATE TREATMENT ON CELL VIABILITY IN GLIOMA CELL LINES

### 4.1 Introduction

Neoplastic progression can be defined as deregulated cell proliferation and suppressed cell death (Evan and Vousden, 2001). One reason for this aberration is due to altered methylation, an epigenetic change that can affect the expression of genes without altering the primary DNA sequence (Suzuki and Bird, 2008). If the genes affected are involved in cell proliferation and apoptosis this may result in an increased carcinogenic risk (Jackson-Grusby *et al.*, 2001).

Folate is the predominant methyl donor, which is required to be in the form of THF to enter the folate pool. Following conversion of folic acid, or the naturally occurring 5-MethylTHF into THF; THF is used in DNA methylation, and DNA synthesis (Crider *et al.*, 2012). The methylation properties of THF and its potential to reverse aberrant methylation, a hallmark seen in glioma, has meant that folate has been considered as a potential treatment (Noushmehr *et al.*, 2010a). It has already been seen both *in vitro* and *in vivo*, in prostate cancer, that DNA methylating agents such as the methyl donor S-adenosylmethionine (SAM) or methyl DNA-binding domain protein 2 antisense oligonucleotide (MBD2-AS) may reduce cancer cell growth (Shukeir *et al.*, 2006a). Folate supplementation has also been considered as a potential prophylactic therapy given that low folate status, defined as 160 µg/ L, was associated with increased cancer risk in colorectal cancer patients (Giovannucci, 2002; Lamprecht and Lipkin, 2003). It has also been observed that folate supplementation has the potential to increase cancer progression in an *in vivo* human study of colon cancer of cell proliferation (Kim, 2003).

Following a literature search, only one previous study was found by Hervouet *et al.*, (2009), where effects of folate treatment on cell proliferation were investigated on *in vitro* glioma cell lines U373. It was found that cells treated for 7 days with 4 and 40 µg/ml folate with and without an antioxidant at 20 µmol/L, exhibited reduced cell proliferation, the form of folate used however, was not specified (Hervouet *et al.*, 2009b). It was apparent from the reports in the literature that studies into the effects of folate in glioma were sparse and contradictory, however due to the success of folate supplementation in other cancers, it was felt this was a novel and under researched area to explore further in the thesis here.

For this investigation here, both folic and folinic acid were considered as possible rescue agents due to the differences in the way the two forms are metabolised. Folic acid was used as a rescue agent because in its oxidised form it is more stable than the reduced naturally occurring folate (O'Leary and Sheehy, 2001). It was also considered because of its role in cancer prevention as discussed in Chapter 1. Folinic acid was also considered as a rescue agent because fewer enzymatic conversions are required for it to reach the biologically active form, compared to folic acid (Greenberg *et al.*, 2011). Adjuvant folinic acid supplementation alongside fluorouracil and oxaliplatin treatment has been recommended as a colorectal cancer treatment by NICE (Wade *et al.*, 2015), yet the effects in glioma have not been investigated. The dosing range chosen for this study here was guided by the only other reported *in vitro* study in glioma cell lines of concentrations 4 and 40 µg/ml (Hervouet *et al.*, 2009).

Cell viability assays such as PrestoBlue are a useful tool to measure levels of cell proliferation because counting the number of viable cells this can give an indication of the rate of proliferation, and can assess the response of cells following treatment (Boncler *et al.*, 2014).

Cells were treated daily because, although folic acid is stable, the half-life of folinic acid is around 6 hours (Torres *et al.*, 2015). A study by Moussa (2015) measured cell viability as soon

as 24 hours, following folate treatment at a concentration of 2, 20, and 100 nM in human placental choriocarcinoma JEG-3 cells (Moussa *et al.*, 2015), whilst another study performed proliferation assays on days 1, 2 and 3 in the oral cancer cell lines CAL27, SCC15, and SCC25 (Moody *et al.*, 2012). A more recent study treated human liver adenocarcinoma cells; SK-HEP-1, and PLC/PRF/5 cells for 3 days, as this was a sufficient time period to observe changes in protein expression (Price *et al.*, 2015), which will be assessed here in Chapter 7. Seven days was chosen as the optimum duration to treat the cells with folate following on from the protocol by Hervouet *et al.*, (2009) and growth kinetic analysis performed in Chapter 3, which indicated that cells were in the active growth phase for up to 7-days.

#### 4.1.1 Aim of Chapter

The aim of this chapter was to assess the effect the different forms of folic and folinic acid had on the viability of glioma cells.

#### 4.1.2 Hypothesis

The controversies surrounding the benefits of folic acid supplementation in the literature, alongside the purported risks of stimulating of cell growth in cancerous cells following folic acid supplementation has meant there is a clear knowledge gap in ascertaining whether folate derivatives are suitable as an adjuvant or prophylactic therapy in glioma patients. The following hypotheses were formulated:

##### ***Hypothesis One***

Folic acid and folinic acid would cause an increase in cell growth, in a dose dependent manner, hence this would be detected as an increase in cell viability.

### ***Hypothesis Two***

A dose-dependent increase in cell growth would be more pronounced in the cancerous cell lines U87MG and 1321N1 compared to the non-cancerous control cell line SVGp12, due to the greater metabolic demands of the cancerous cells.

### ***Hypothesis Three***

Folinic acid would have greater bioactivity compared to folic acid due to the fewer enzymatic steps required for conversion to the active metabolite 5-MTHF.

#### **4.1.3 Objectives**

To test the hypotheses and address the overall aim the following steps were formulated:

**Objective 1.** To examine whether daily dosing with folic or folinic acid over 7 days affects cell viability in cell lines compared to standard cell culture conditions.

**Objective 2.** To examine whether anti-oxidants are required to be added to cultures for removal of ROS, associated with cancer microenvironment and high levels of folate following treatment.

**Objective 3.** To examine whether there is a time-dependent effect of changes in cell viability following daily folate dosing.

**Objective 4.** To examine whether there is a dose-response effect on cell viability in all cell lines following treatment with folic and folinic acid.

**Objective 5.** To examine whether there is an effect on cell viability following growth in folate deficient media in all cell lines

**Objective 6.** To examine whether there is a difference in effect on cell viability, comparing folic acid verses folinic acid.

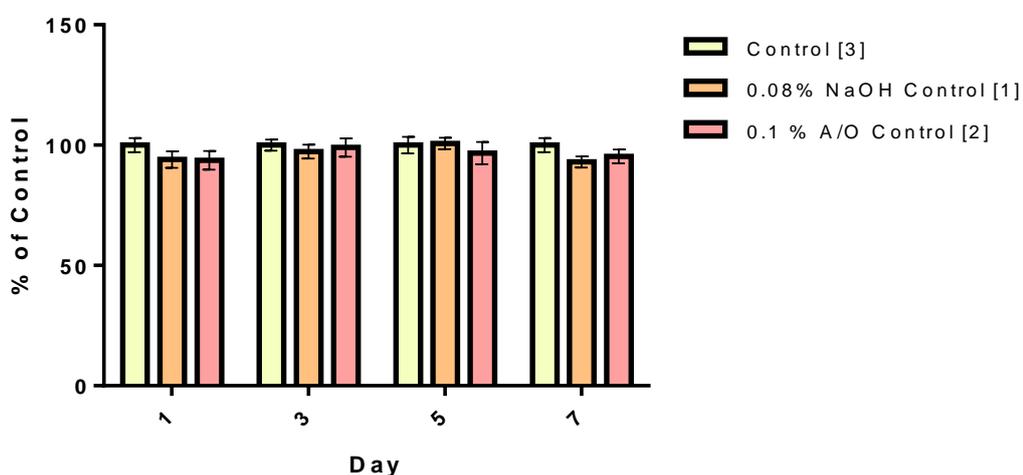
**Objective 7.** To examine whether there is a difference in response following folic or folinic acid treatment, comparing cancerous verses non-cancerous cell lines.

## 4.2 Results

Cells were treated as defined in Section 2.5.1.

### 4.2.1 Negative Controls

Folic acid was resuspended in the solvent sodium hydroxide (NaOH). In addition, some cells were also supplemented with 1  $\mu\text{l}$ / mL of commercially available antioxidant diluted in cell culture media, to reduce the number of free radicals formed by redox reactions, that could go on to damage cellular DNA (Halliwell, 2006). To ensure any effect seen on cell viability was as the result of folate supplementation, not the addition of solvent NaOH or antioxidant three negative controls were prepared. Cells were treated with vehicle controls: [1] 0.08 % NaOH v/v in standard cell culture media; [2] 0.1 % (v/v) antioxidant in standard cell culture media [2] or [3] cells grown in standard cell culture medium for relative comparison of changes in cell viability. The results shown in Figure 4.1 indicated no significance in cell viability between cells grown in 0.08 % NaOH or 0.1 % antioxidant relative to cells grown in standard media.



**Figure 4.1 Determination of the effect of vehicle controls on cell viability,**

*The PrestoBlue assay was used to assess the effect of daily treatment on cell viability with vehicle controls [1] 0.08 % (v/v) NaOH and [2] 0.1 % (v/v) antioxidant (A/O) both diluted in standard cell culture media, relative to cells grown in standard media [3]. No significant effect was observed.*

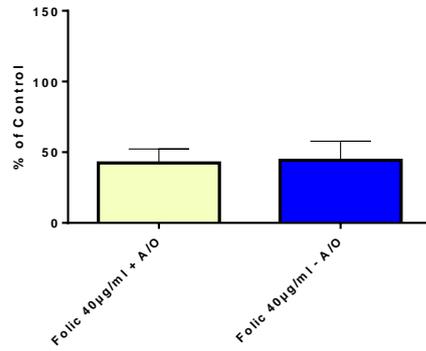
*The data points are the means of three replicates, from three independent experiments, and the error bars represent ( $\pm$ SD). Statistical analysis was the ANOVA with Tukey's post-hoc test.*

#### 4.2.2 The effect of combining antioxidant cocktail with folic acid or folinic acid

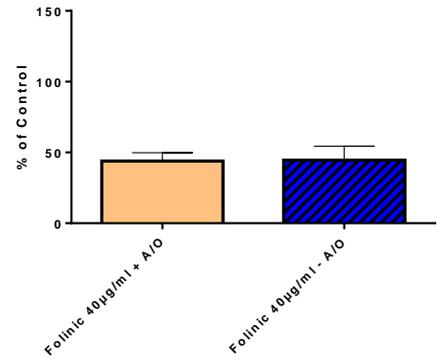
To determine whether an antioxidant was required to stabilise folic acid in the presence of light and to prevent the degradation of folate in liquid, an antioxidant was included for comparison (Monajjemzadeh *et al.*, 2014; Tansey and Schneller, 1955). At the end of seven days of treatment, cell viability was calculated relative to standard cell culture and compared as shown in Figure 4.2. The only significant difference observed between the folic and folinic acid treatments with antioxidant compared to without, was for the SVGp12 cell line with folic acid treatment, therefore from this point onwards extra antioxidant supplementation was excluded.

### 1321N1

A

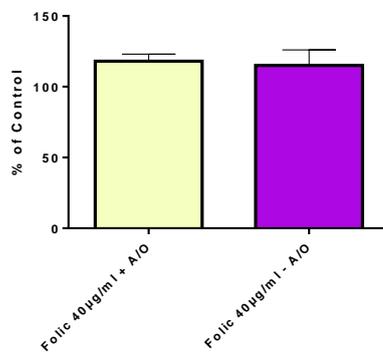


B

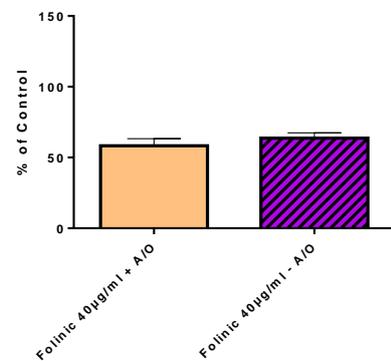


### U87MG

C

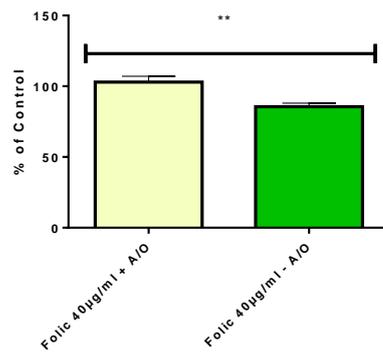


D

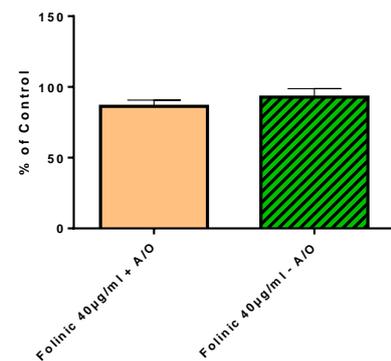


### SVGp12

E



F



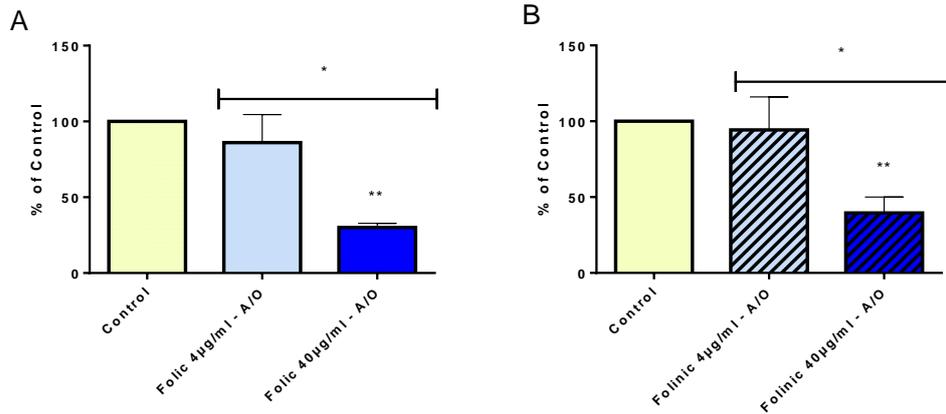
**Figure 4.2 Comparison of cell viability following folic acid and folic acid treatment with and without supplementary antioxidant.**

Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 folic acid **B)** 131N1 folic acid **C)** U87MG folic acid **D)** U87MG folic acid **E)** SVGp12 folic acid **F)** SVGp12 folic acid. There was generally no significant difference in cell viability following additional use of an antioxidant. The data points are means of three replicates (n=3), error bars represent  $\pm$  standard deviation. Statistical analysis was the Student's t- test.

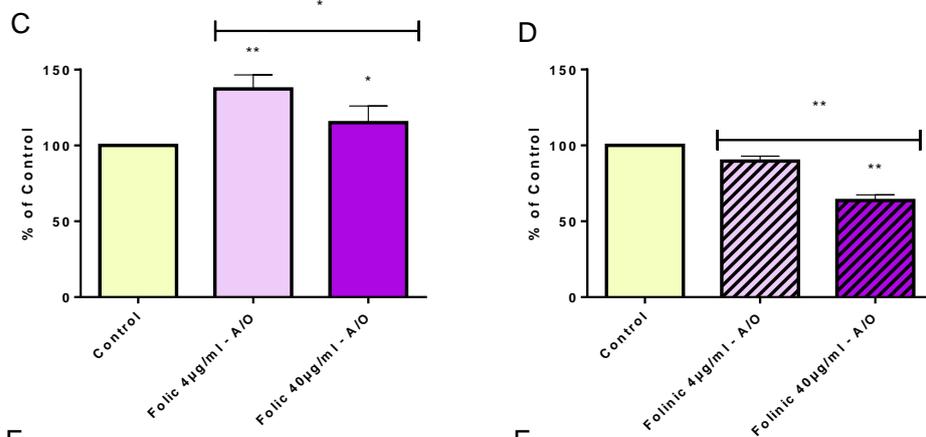
#### 4.2.3 Preliminary comparison of change in cell viability following folic or folinic acid treatment relative to standard cell culture

Preliminary assessments were first performed to ascertain whether folate treatment affected cell viability, before studies with increased replicates were undertaken. A preliminary experiment was conducted to determine whether changes in cell viability were observed following treatment with folic or folinic acid. Cell viability at the end of seven days of daily treatment with either low dose (4 µg/ mL) folic or folinic acid or high dose (40 µg/ mL) folic or folinic acid was compared to standard cell culture media. The results in Figure 4.3 revealed a significant decrease in cell viability between the high dose and low dose for all treatments with the exception of the SVGp12 cell line where no significant change was observed. A significant increase in cell viability was seen for the U87MG cells treated with both a low ( $p < 0.01$ ) and high ( $p < 0.05$ ) dose of folic acid.

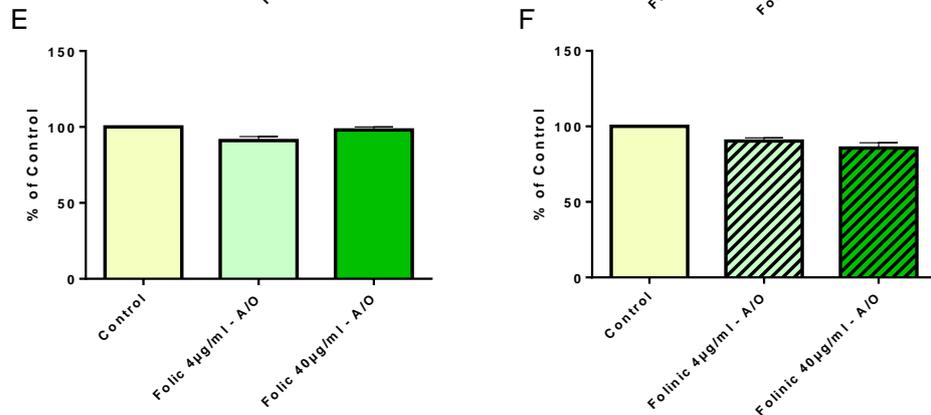
1321N1



U87MG



SVGp12



**Figure 4.3 Comparison of cell viability following daily high and low dose folic and folinic acid treatment**

Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 folic acid **B)** 131N1 folinic acid **C)** U87MG folic acid **D)** U87MG folinic acid **E)** SVGp12 folic acid **F)** SVGp12 folinic acid. The data show that there was a significant decrease in cell viability following treatment with a high concentration of folic and folinic acid compared to the lower concentration of folic and folinic acid respectively in the 1321N1 and U87MG cell lines. There was no significant difference observed in the SVGp12 cell line. The data points are means of three replicates (n=3), in three independent experiment, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

#### 4.2.4 Preliminary assessment of the time-dependent effect of changes in cell viability following folic acid treatment

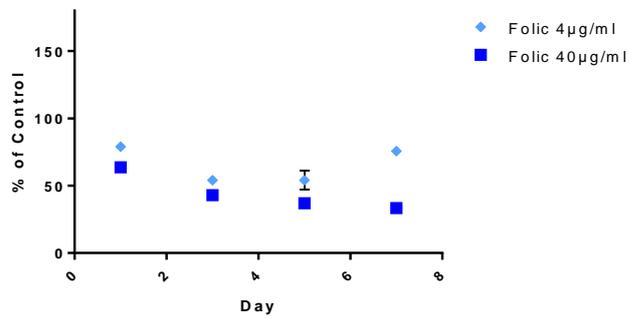
As can be seen in Figure 4.4A, folic acid supplementation at 4 µg/ml on 1321N1 cells continued to reduce cell viability significantly from Day 1 to Day 5 ( $p<0.05$ ). There is then a significant recovery in cell viability on Day 7. The 1321N1 cells treated with the higher level of folic acid supplementation at 40 µg/ml, continued to show a significant reduction in cell viability from Day 1 to Day 7 ( $p<0.01$ ).

In Figure 4.4B U87 MG cells were treated with folic acid supplementation at 4 µg/ml. The cells showed an initial significant increase in viability ( $p<0.01$ ) which then significantly decreased on Day 3 ( $p<0.01$ ). The U87 MG cells that were treated with the higher level of folic acid supplementation at 40 µg/ml, showed a significant reduction in cell viability from Day 1 to Day 3 ( $p<0.01$ ).

In Figure 4.4C SVGP12 cells were treated with folic acid supplementation at 4 µg/ml and at 40 µg/ml showed no significant alteration to cell viability over the 7 days.

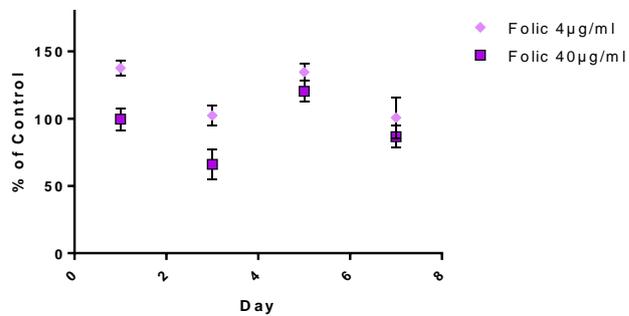
### 1321N1

A



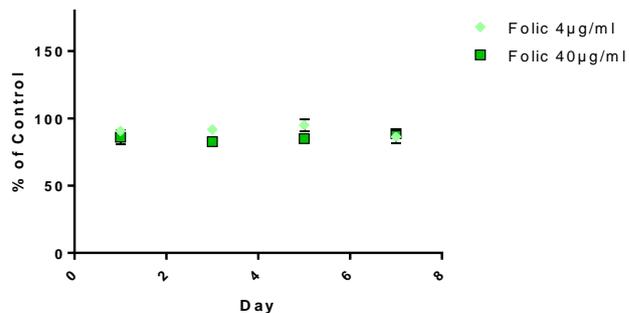
### U87MG

B



### SVGp12

C



**Figure 4.4 Comparison of effects of high and low folic acid treatment on cell viability over time.**

Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4 µg/ml folic acid. **A)** 1321N1 cells treated with folic acid at 4 µg/ml and 40 µg/ml **B)** U87MG cells treated with folic acid at 4 µg/ml and 40 µg/ml **C)** SVGp12 cells treated with folic acid at 4 µg/ml and 40 µg/ml. The data show that there was a decrease in cell viability following treatment over time in the 1321N1 cell line. There was a mixed response in cell viability following treatment in the U87MG cell line. There was no significant difference in cell viability following treatment over time in the SVGp12 cell line. The data points are means of three replicates (n=3), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test and students t-test.

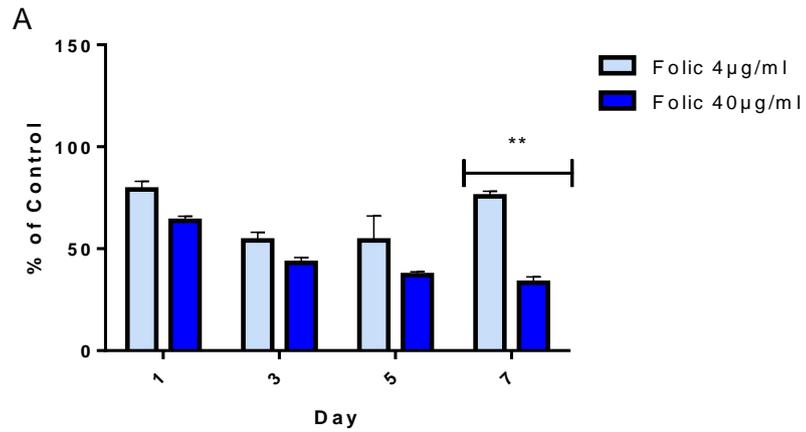
#### 4.2.5 Preliminary Assessment of folic acid concentration effect at set time points

Comparing the effect of low versus high folic acid supplementation in the 1321N1 cell line, the higher concentration on had a significant effect on reducing cell viability on Day 7 ( $p<0.01$ ) as can be seen in Figure 4.5A.

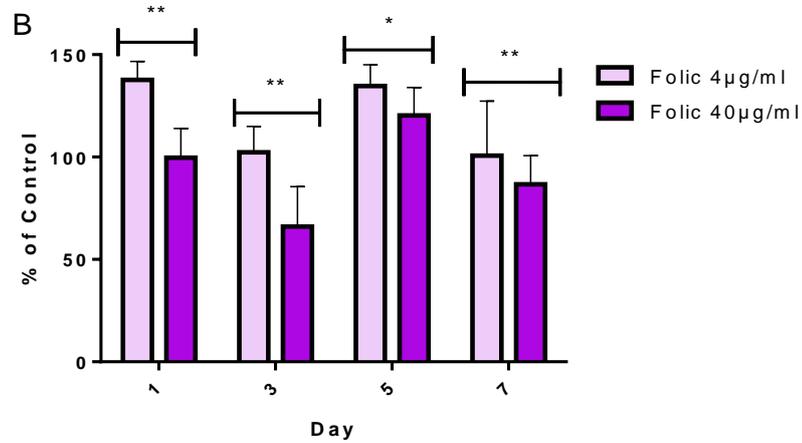
As can be seen in Figure 4.5B in the U87MG cells, when the effect of low versus high folic acid supplementation was compared, the higher concentration of folic acid supplementation had a significantly greater effect on reducing cell viability on Days 1 ( $p<0.01$ ), 3 ( $p<0.01$ ), 5 ( $p<0.05$ ) and 7 ( $p<0.01$ ).

In Figure 4.5C when comparing the effect of low versus high folic acid supplementation on SVGp12 cells, the higher concentration of folic acid supplementation had a significantly greater effect reducing cell viability compared to the lower concentration on Day 5 ( $p<0.05$ ).

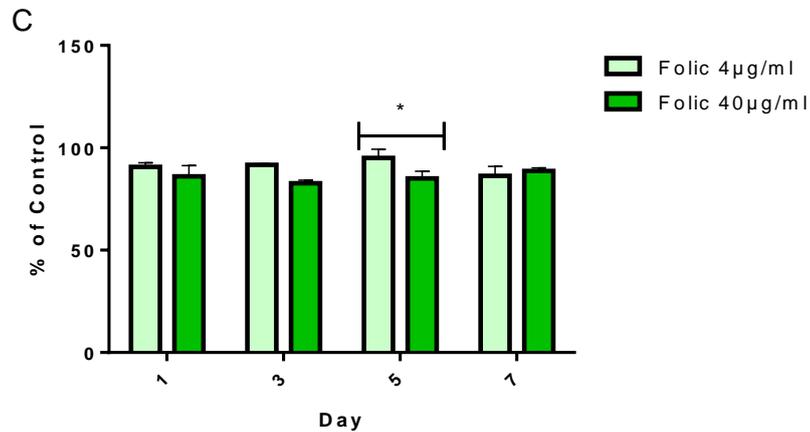
### 1321N1



### U87MG



### SVGp12



**Figure 4.5 Comparison of effects of folic acid at 4 µg/ml and 40 µg/ml on cell viability.**

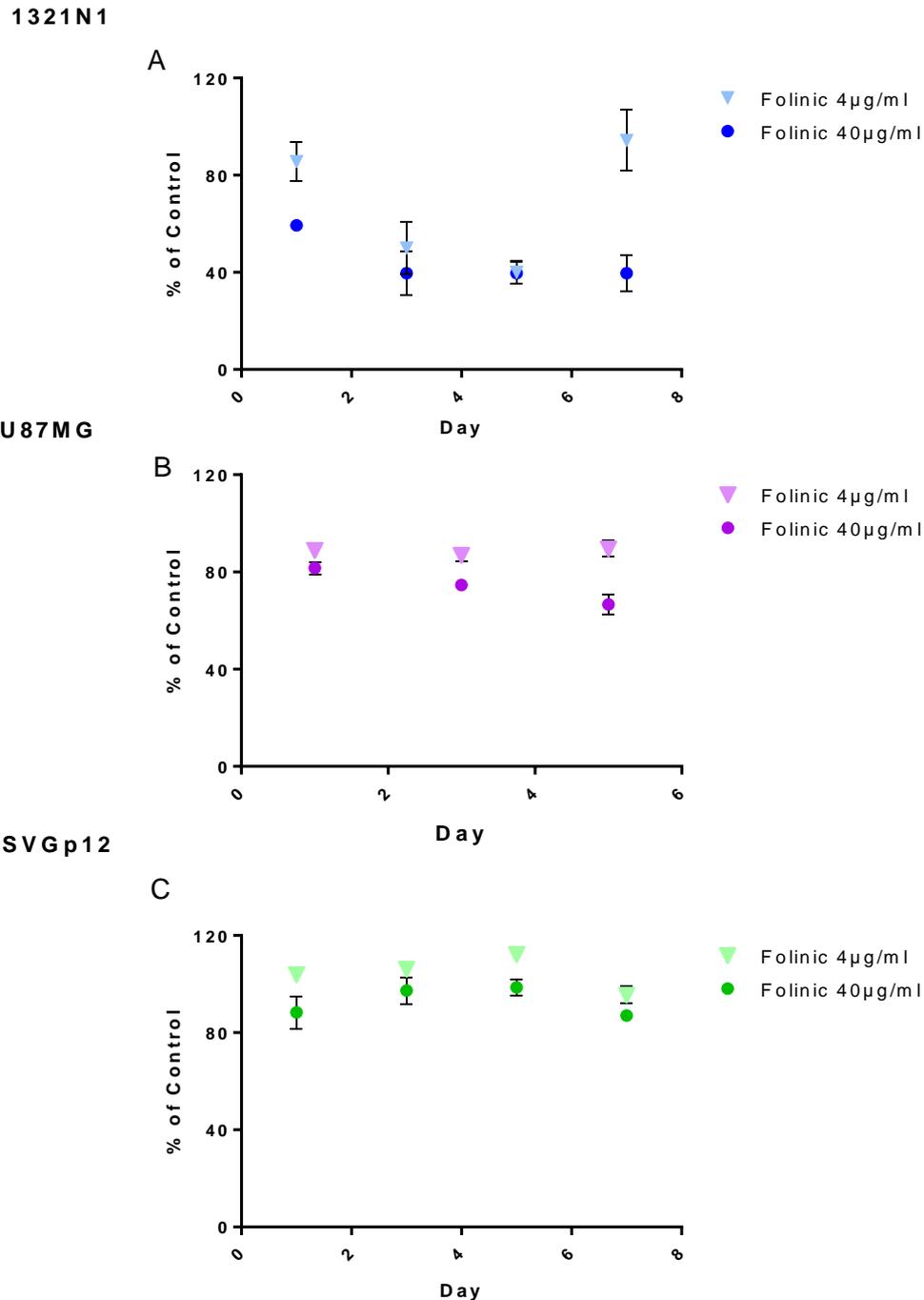
Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4 µg/ml folic acid. **A)** 1321N1 cells treated with folic acid at 4 µg/ml and 40 µg/ml **B)** U87MG cells treated with folic acid at 4 µg/ml and 40 µg/ml **C)** SVGp12 cells treated with folic acid at 4 µg/ml and 40 µg/ml. The data show that there was a significant effect on cell viability following increased concentration of folic acid in the U87MG cells. The data points are means of three replicates (n=3), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test and Student's t-test.

#### 4.2.6 Preliminary assessment of the time-dependent effect of changes in cell viability following folinic acid treatment

In Figure 4.6A folinic acid supplementation at 4 µg/ml on 1321N1 cells significantly reduced cell viability on all days ( $p < 0.01$ ). This began with an initial significant reduction in cell viability from Day 1 – 3 ( $p < 0.01$ ). The 1321N1 cells treated with the higher level of folinic acid supplementation at 40 µg/ml, also showed a significant reduction in cell viability between day 1 and day 3 ( $p < 0.01$ ).

In Figure 4.6B folinic acid supplementation at 4 µg/ml on U87MG cells did not have a significant reduction in cell viability from day 1 – 5. The U87MG cells treated with the higher level of folinic acid supplementation at 40 µg/ml, showed a significant reduction in cell viability from day 1-5 ( $p < 0.05$ ).

In Figure 4.6C SVGp12 cells were treated with folinic acid supplementation at 4 µg/ml and 40 µg/ml showed no significant difference in cell viability across the 7 days.



**Figure 4.6 Comparison of effects of high and low folic acid treatment on cell viability over time.**

Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic acid at 4µg/ml and 40µg/ml **B)** U87MG cells treated with folic acid at 4µg/ml and 40µg/ml **C)** SVGP12 cells treated with folic acid at 4µg/ml and 40µg/ml. The data show that there was a decrease in cell viability following treatment over time in the 1321N1 cell line. There was a mixed response in cell viability following treatment in the U87MG cell line. There was no significant difference in cell viability following treatment over time in the SVGP12 cell line. The data points are means of three replicates (n=3), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test and Students t-test.

#### 4.2.7 Preliminary Assessment of folinic concentration affect at set time dependant points

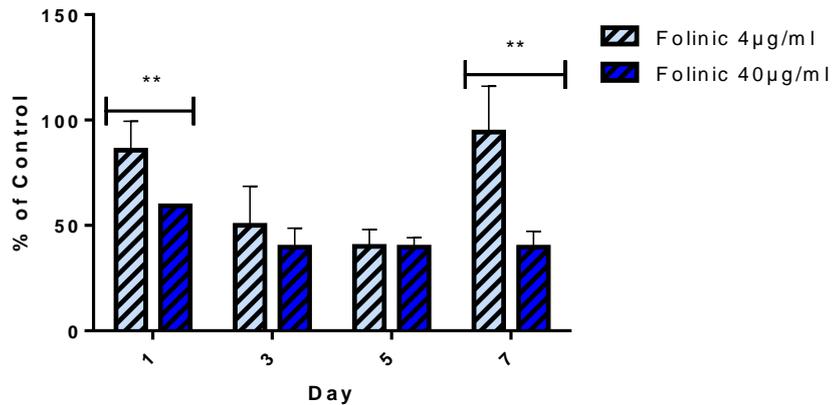
Comparing the effect of low versus high folinic acid supplementation in 1321N1 cells (Figure 4.7A), the higher concentration had a greater effect on reducing cell viability with significance on Day 1 and Day 7 ( $p < 0.01$ ).

In the U87MG cells when comparing the effect of low versus high folic acid supplementation (Figure 4.7A), the higher concentration a significant effect on reducing cell viability was seen on Day 3 ( $p < 0.05$ ) and day 5 ( $p < 0.01$ ).

Comparing the effect of low versus high folinic acid supplementation on SVGp12 cells (Figure 4.7A), the higher concentration of folinic acid supplementation had a very slight effect of decreasing cell viability compared to the lower concentration of folinic acid supplementation, with significance seen on Days 1 and 5 ( $p < 0.01$ ).

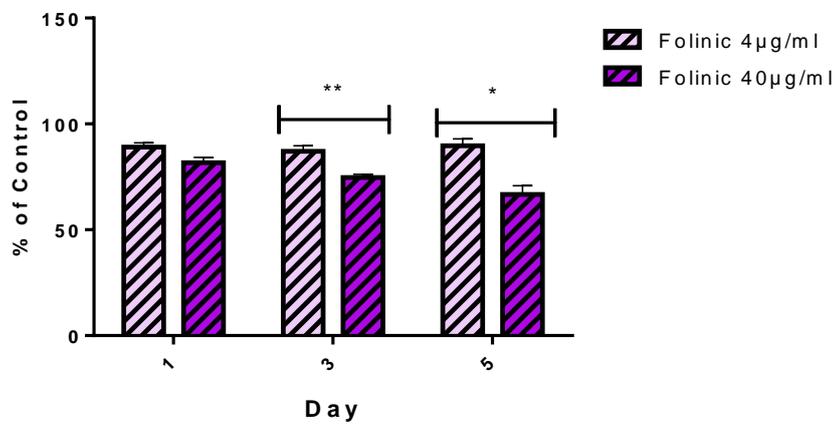
1321N1

A



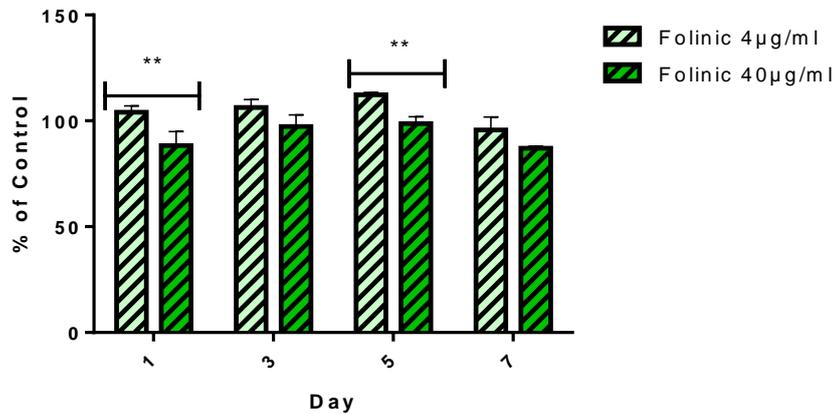
U87MG

B



SVGp12

C



**Figure 4.7 Comparison of effects of folicinic acid at 4µg/ml and 40µg/ml on cell viability.**

Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folicinic acid. **A)** 1321N1 cells treated with folicinic acid at 4µg/ml and 40µg/ml **B)** U87MG cells treated with folicinic acid at 4µg/ml and 40µg/ml **C)** SVGp12 cells treated with folicinic acid at 4µg/ml and 40µg/ml. The data show that there was a significant effect on cell viability following increased concentration of folicinic acid in all cell lines. The data points are means of three replicates (n=3), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test and Students t-test.

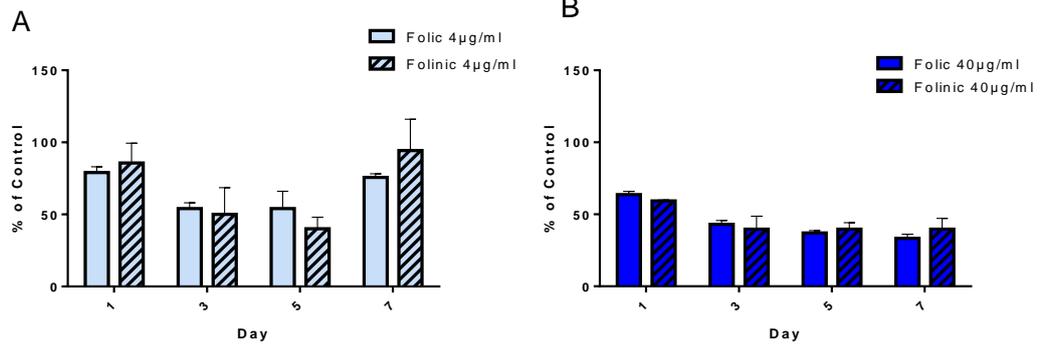
#### 4.2.8 Preliminary assay comparing the effect of folic acid treatment against folinic acid treatment at defined concentrations and set time points.

There was no significant difference in cell viability between treatment types at a concentration of 4 µg/ml or 40 µg/ml in the 1321N1 cells at any of the time points across the 7 days.

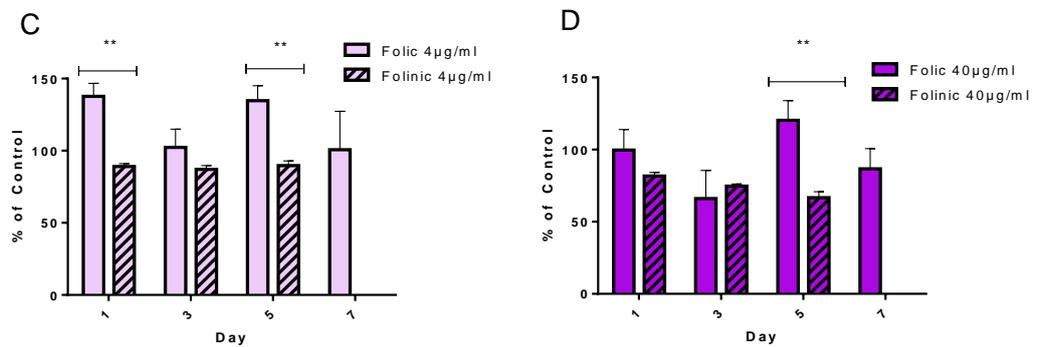
The U87MG cells treated with low concentrations of 4 µg/ml supplementation showed a significant decrease in cell viability for folinic acid compared to folic acid between treatments on Days 1 and 5 ( $p < 0.01$ ). The U87MG cells that were treated at a higher level of supplementation showed a significant decrease in cell viability for folinic acid compared to folic acid between treatments on Day 5 ( $p < 0.01$ ).

The SVGp12 cells treated with low concentrations of 4 µg/ml supplementation showed a significant increase in cell viability for folinic acid compared to folic acid between treatments on Days 1 ( $p < 0.05$ ), and 5 and 7 ( $p < 0.01$ ). The SVGp12 cells that were treated at a higher level of supplementation showed a significant increase in cell viability for folinic acid compared to folic acid between treatments on Days 3 - 5 ( $p < 0.05$ ).

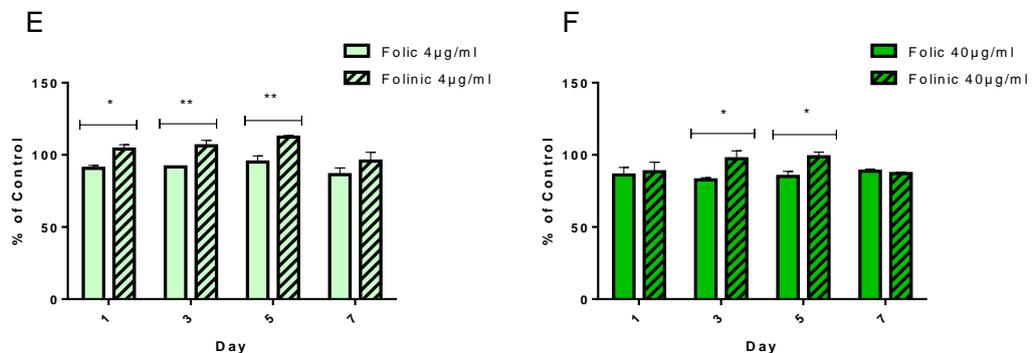
### 1321N1



### U87MG



### SVGp12



#### Figure 4.8 Comparison of effects of folic acid and folinic acid on cell viability

Cells were treated daily for 7 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic and folinic acid at 4µg/ml **B)** 1321N1 cells treated with folic and folinic acid at 40µg/ml **C)** U87MG cells treated with folic and folinic acid at 4µg/ml **D)** U87MG cells treated with folic and folinic acid at 40µg/ml **E)** SVGp12 cells treated with folic and folinic acid at 4µg/ml **F)** SVGp12 cells treated with folic and folinic acid at 40µg/ml. The data show that there was a significant difference on cell viability between treatment type in the U87MG and SVGp12 cell lines. The data points are means of three replicates (n=3), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the Students t-test.

#### 4.2.9 Extended Cell Viability Study

Following on from the preliminary time course experiment, a time dependent effect on cell viability following treatment was observed, with most significant changes occurring on Day 5. Full investigations were carried out again, but with a sample number of 12 rather than 3 to increase the reliability of the results seen.

Low level folic acid supplementation of 4  $\mu\text{g/ml}$  in 1321N1 cells had no significant decrease on cell viability compared to the control cells, however, the high level of folic acid supplementation 40  $\mu\text{g/ml}$  did with a significant decrease in cell viability Figure 4.9A ( $p < 0.01$ ). The difference between the two concentrations was also significant ( $p < 0.05$ ).

Low level 4  $\mu\text{g/ml}$  folic acid supplementation of 1321N1 cells caused a significant reduction in cell viability compared to the control cells ( $p < 0.01$ ). The high level of folic acid supplementation 40  $\mu\text{g/ml}$  also had a significant decrease in cell viability ( $p < 0.01$ ). The difference between the two concentrations however, was not significant.

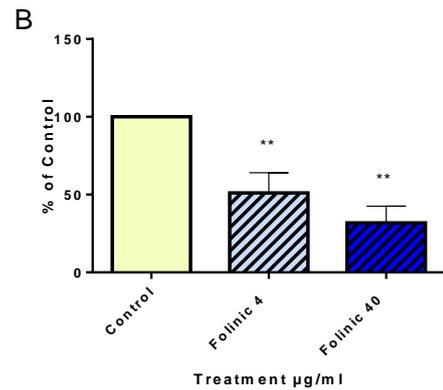
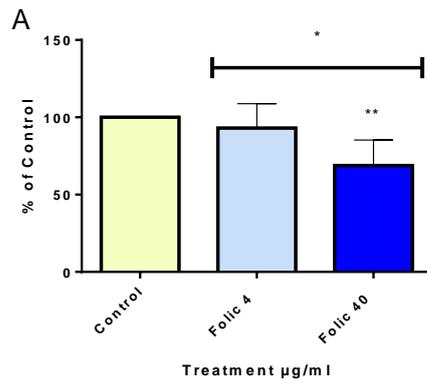
U87 MG cells treated with low and high level folic acid supplementation 4  $\mu\text{g/ml}$  and 40  $\mu\text{g/ml}$  had no significant decrease in cell viability compared to the control cells. The difference between the two concentrations was also not significant.

Low level folic acid supplementation 4  $\mu\text{g/ml}$  of U87MG cells caused no significant reduction in cell viability compared to the control cells. The high level of folic acid supplementation 40  $\mu\text{g/ml}$  did however cause a significant decrease in cell viability compared to the standard cell culture control ( $p < 0.01$ ), but the difference between the two concentrations however, was not significant.

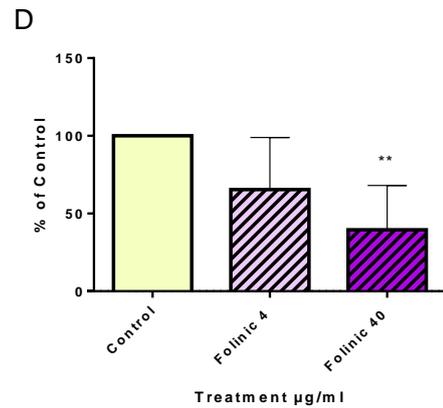
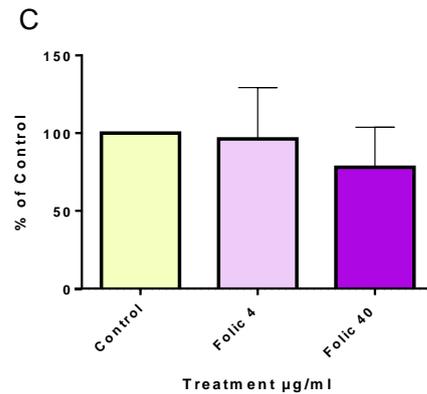
SVGp12 cells treated with low and high level folic acid supplementation 4  $\mu\text{g/ml}$  and 40  $\mu\text{g/ml}$  had no significant decrease in cell viability compared to the control cells. The difference between the two concentrations was also not significant.

Low level folic acid supplementation 4 µg/ml of SVGp12 cells caused no significant decrease in cell viability compared to the control cells, however, the high level of folic acid supplementation 40 µg/ml caused a significant decrease in cell viability ( $p<0.01$ ). The difference between the two concentrations however, was not significant.

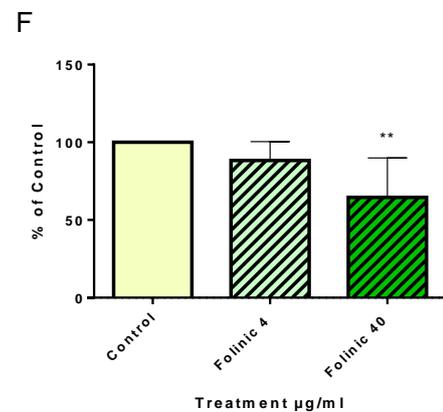
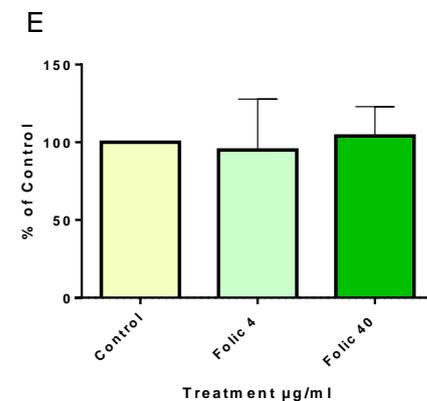
### 1321N1



### U87MG



### SVGp12

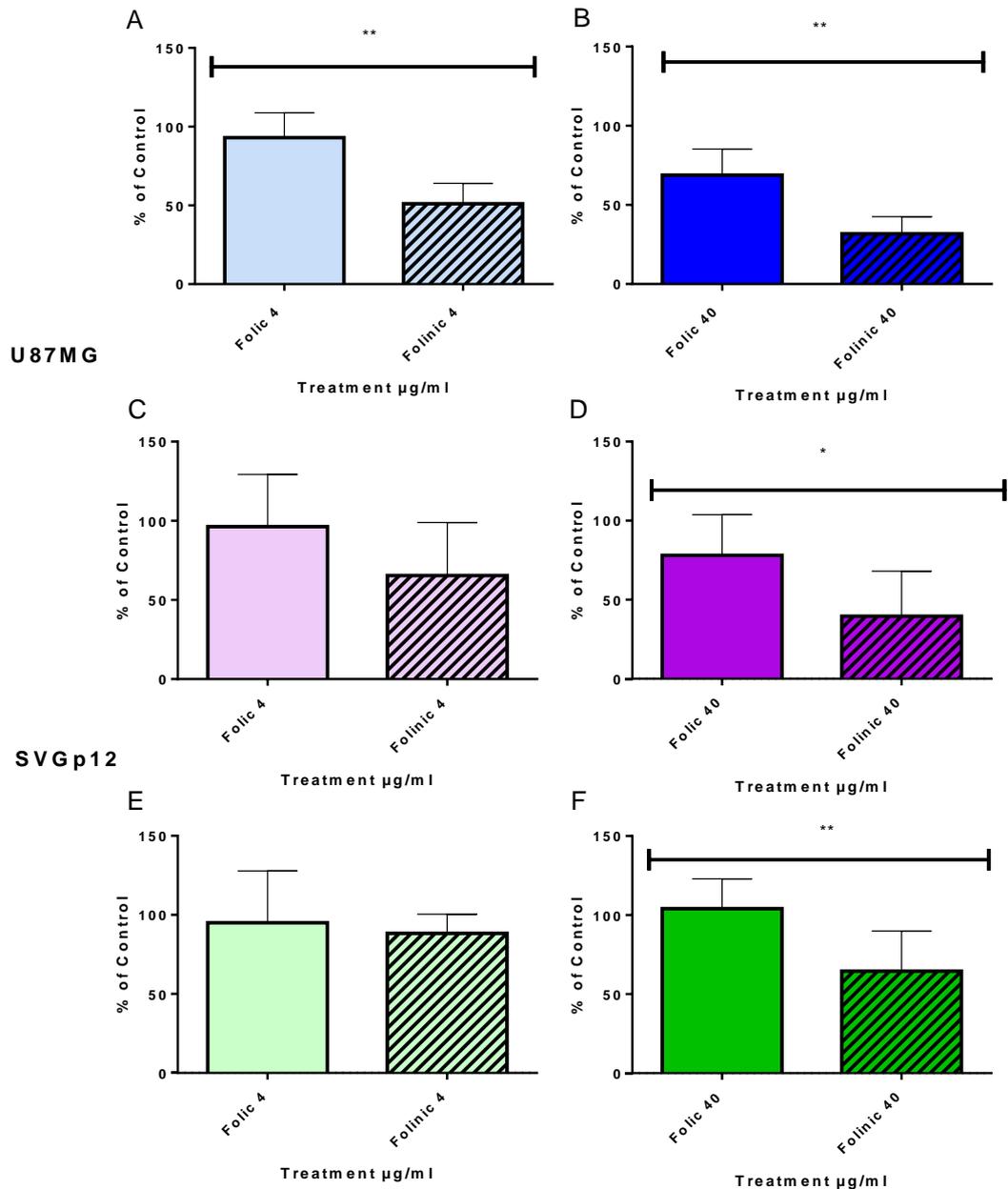


**Figure 4.9 Comparison of high and low doses of folic and folinic acid on cell viability.** Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic acid at 4µg/ml and 40µg/ml **B)** 1321N1 cells treated with folinic acid at 4µg/ml and 40µg/ml **C)** U87MG cells treated with folic acid at 4µg/ml and 40µg/ml **D)** U87MG cells treated with folinic acid at 4µg/ml and 40µg/ml **E)** SVGp12 cells treated with folic acid at 4µg/ml and 40µg/ml **F)** SVGp12 cells treated with folinic acid at 4µg/ml and 40µg/ml. The data show that there was a significant difference in cell viability in the 1321N1 cells treated with an increased concentration of folic acid. The data points are means of twelve replicates (n=12), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

#### 4.2.10 Folate treatment comparison

It was observed that there was a significant decrease in cell viability seen when comparing folinic acid to folic acid ( $p < 0.01$ ) in the 1321N1 cell line, for both low and high concentrations as seen in Figure 4.10A and B. The U87MG cells however, showed no significant difference between folic acid versus folinic acid for both high and low treatments (Figure 4.10C and D). The SVGp12 cells also showed no significant difference between folic and folinic acid treatment at a low concentration; 4  $\mu\text{g}/\text{ml}$  (Figure 4.10E). There was however, a significant decrease in cell viability following folinic acid supplementation compared to folic acid at the high concentration ( $p < 0.01$ ), as can be seen in Figure 4.10F.

1321N1

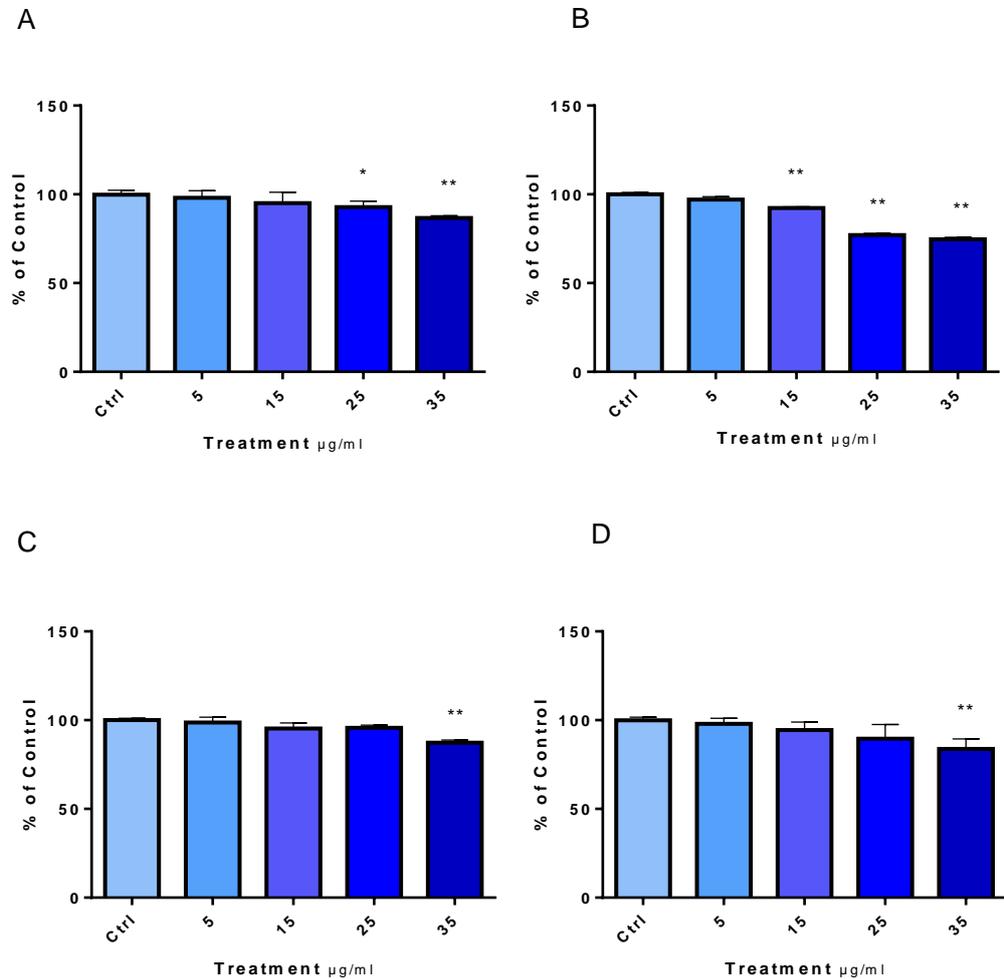


**Figure 4.10 Folate treatment comparison on cell viability.** Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic and folinic acid at 4µg/ml **B)** 1321N1 cells treated with folic and folinic acid at 40µg/ml **C)** U87MG cells treated with folic and folinic acid at 4µg/ml **D)** U87MG cells treated with folic and folinic acid at 40µg/ml **E)** SVGP12 cells treated with folic and folinic acid at 4µg/ml **F)** SVGP12 cells treated with folic and folinic acid at 40µg/ml. The data show that there was a significant difference in cell viability across all cell lines when compared between the folic and folinic acid at 40µg/ml. The data points are means of twelve replicates (n=12), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the Students t-test.

#### 4.2.11 Dose response experiment comparing folic acid supplementation to standard cell culture media

To further explore the apparent dose dependent changes observed in cell viability following folate treatment in the preliminary experiments, a dose-response experiment was conducted at the end of 5 days of daily dosing to confirm if a relationship existed. The experiment was conducted in triplicate and repeated on three separate occasions. Individual experiments are shown in Figures 4.11A-C and Figure 4.11 D shown the mean of all three experiments. Overall, the viability of 1321N1 cells was significantly reduced ( $p < 0.01$ ) compared to the standard cell culture control when the concentration of folic acid was 35  $\mu\text{g}/\text{ml}$  (Figure 4.11 A-D).

## 1321N1

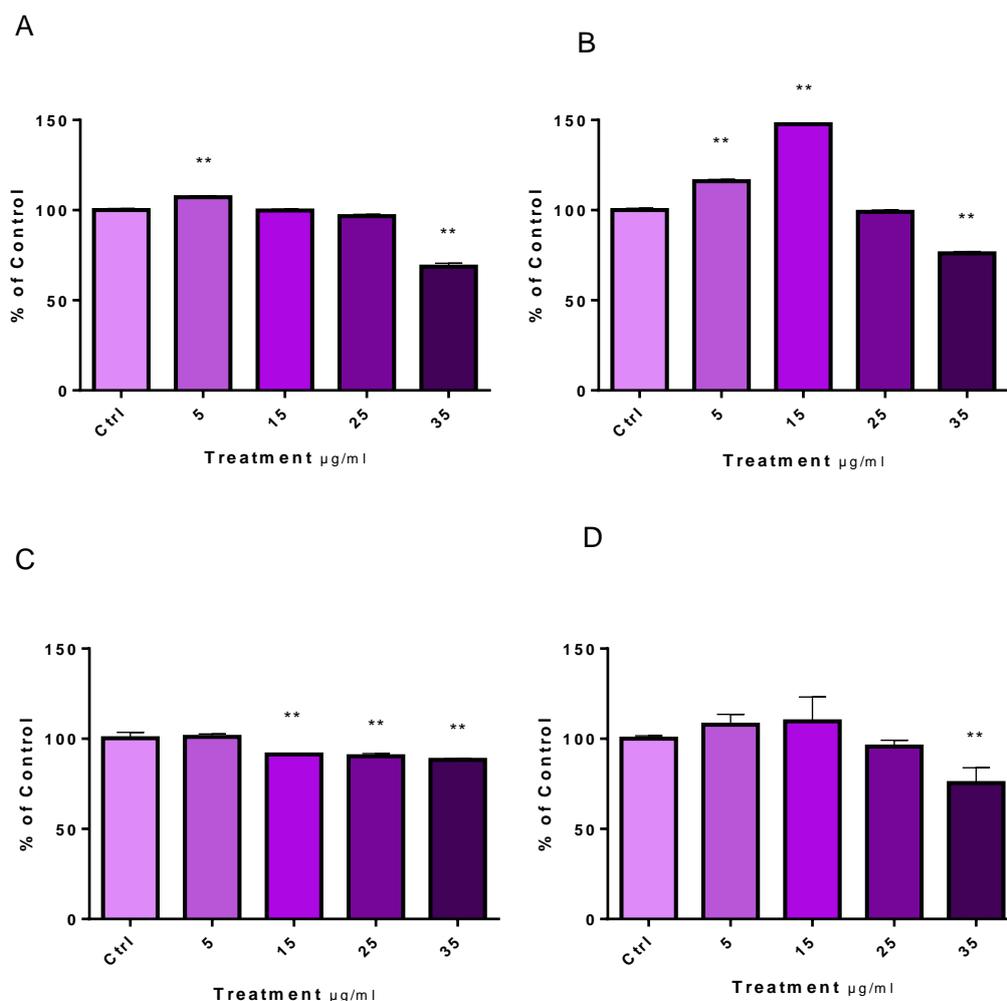


**Figure 4.11 Dose response effect of folic acid on cell viability in 1321N1 cells.**

Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 Plate A **B)** 1321N1 Plate B **C)** 1321N1 Plate C **D)** 1321N1 Mean of Plates A, B and C. The data show that there was a significant difference in cell viability for cells treated with folic acid at 35µg/ml. The data points are means of nine replicates (n=9), of one independent experiment, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

U87 MG cells supplemented with additional folic acid showed mixed results when looking at each individual experiment. When the mean of all three experiments was analysed, only the addition of 35 µg/ml significantly reduced the viability of cells (Figure 4.12D).

### U87MG

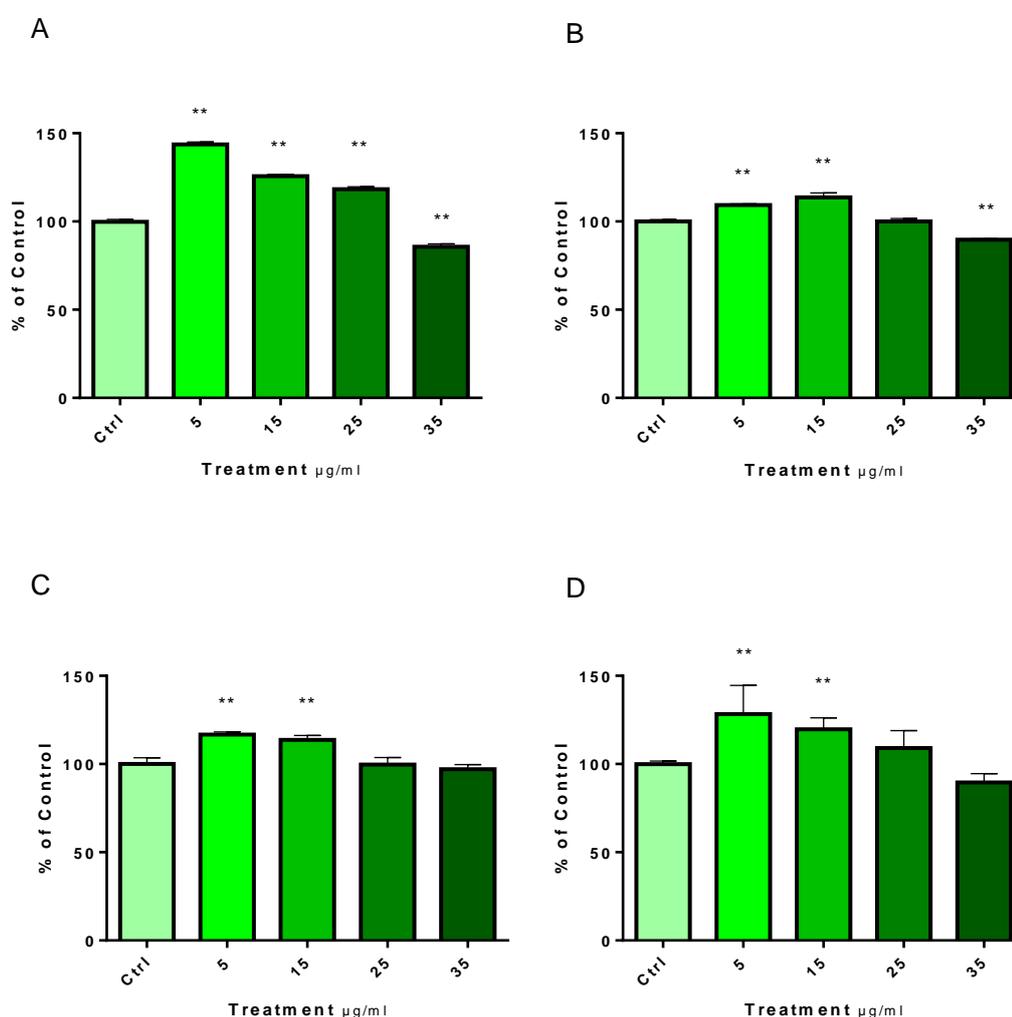


**Figure 4.12 Dose response effect of folic acid on cell viability in U87MG cells**

Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** U87MG Plate A **B)** U87MG Plate B **C)** U87MG Plate C **D)** U87MG Mean of Plates A, B and C. The data show that there was a significant difference in cell viability for cells treated with folic acid at 35µg/ml. The data points are means of nine replicates (n=9), of one independent experiment, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

SVGp12 cells that were supplemented with additional folic acid at a concentration of 5 – 15  $\mu\text{g/ml}$  significantly increased in cell viability compared to standard cell culture conditions (Figure 4.13A-D). In two of the three individual experiments 35  $\mu\text{g/ml}$  folic acid significantly decreased cell viability (Figure 4.13A-B), but when the mean percentage values were calculated from all three experiments, there was no significant decrease observed (Figure 4.13D).

### SVGp12



**Figure 4.13 Dose response effect of folic acid on cell viability in SVGp12 cells.** Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4  $\mu\text{g/ml}$  folic acid. **A)** SVGp12 Plate A **B)** SVGp12 Plate B **C)** SVGp12 Plate C **D)** SVGp12 Mean of Plates A, B and C. The data show that there was a significant difference in cell viability for cells treated with folic acid at 5  $\mu\text{g/ml}$  and 15  $\mu\text{g/ml}$ . The data points are means of nine replicates ( $n=9$ ), of one independent experiment, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

#### 4.2.12 Comparing the effect of folic acid treatment on cancerous versus non-cancerous cells

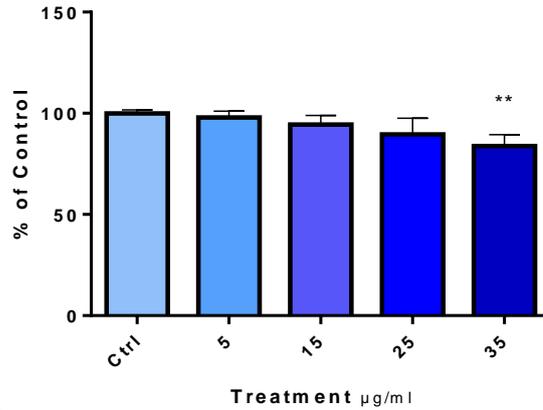
When cells were treated with a low dose of folic acid 5 -15  $\mu\text{g/ml}$  only the non-cancerous SVGp12 cells showed a significant increase in cell viability (Figure 4.14C). When cells were treated with a higher supplementation of folic acid of 35  $\mu\text{g/ml}$  only the cancerous cells showed a significant decrease in cell viability (Figure 4.14A-B).

There was a significant difference in the effect of folic acid treatment at 35  $\mu\text{g/ml}$  seen between non-cancerous and high grade cancerous cells. This difference was also significant when comparing the effects of treatment on the grade of tumour (Figure 4.15A).

When cells were treated with folic acid at 5  $\mu\text{g/ml}$  there was a significant difference observed between the non-cancerous cells and both low and high grade cancerous cells (Figure 4.15B).

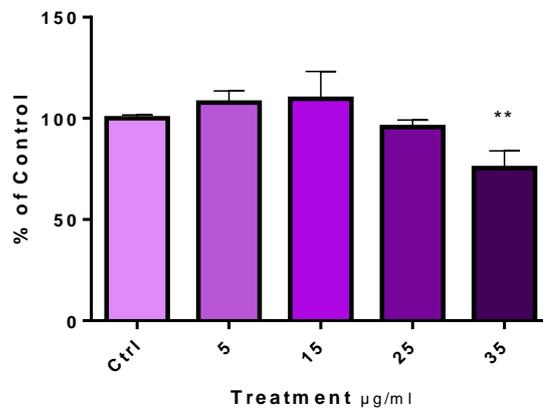
1321N1

A



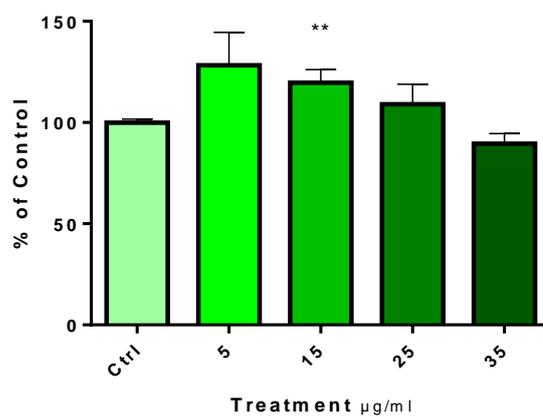
U87MG

B

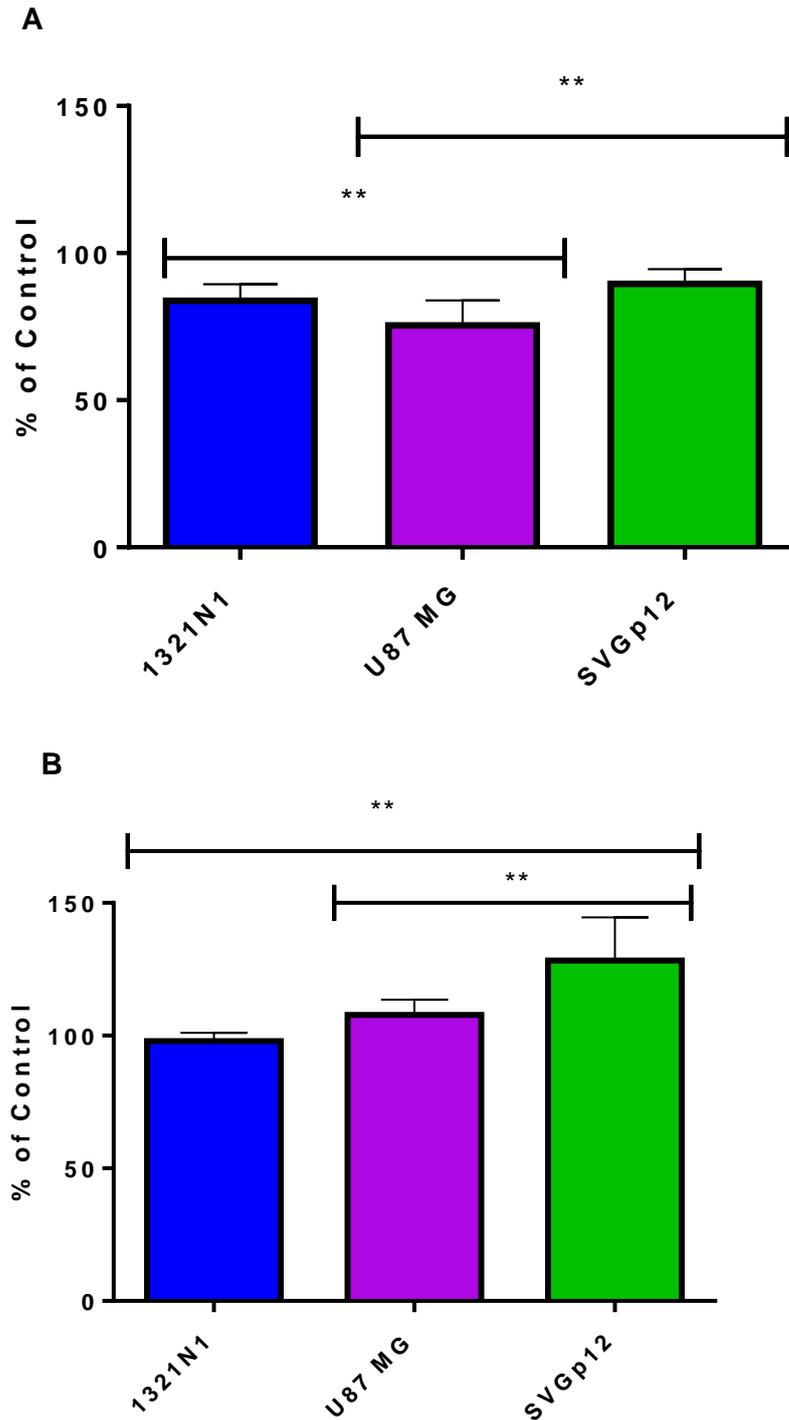


SVGp12

C



**Figure 4.14 Folic acid effects on cell viability.** Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells **B)** U87MG cells **C)** SVGp12 cells. The data show that there was a significant difference in cell viability for 1321N1 and U87MG cells treated with folic acid at 35µg/ml and SVGp12 cells treated with folic acid at 5µg/ml and 15µg/ml. The data points are means of nine replicates (n=9), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

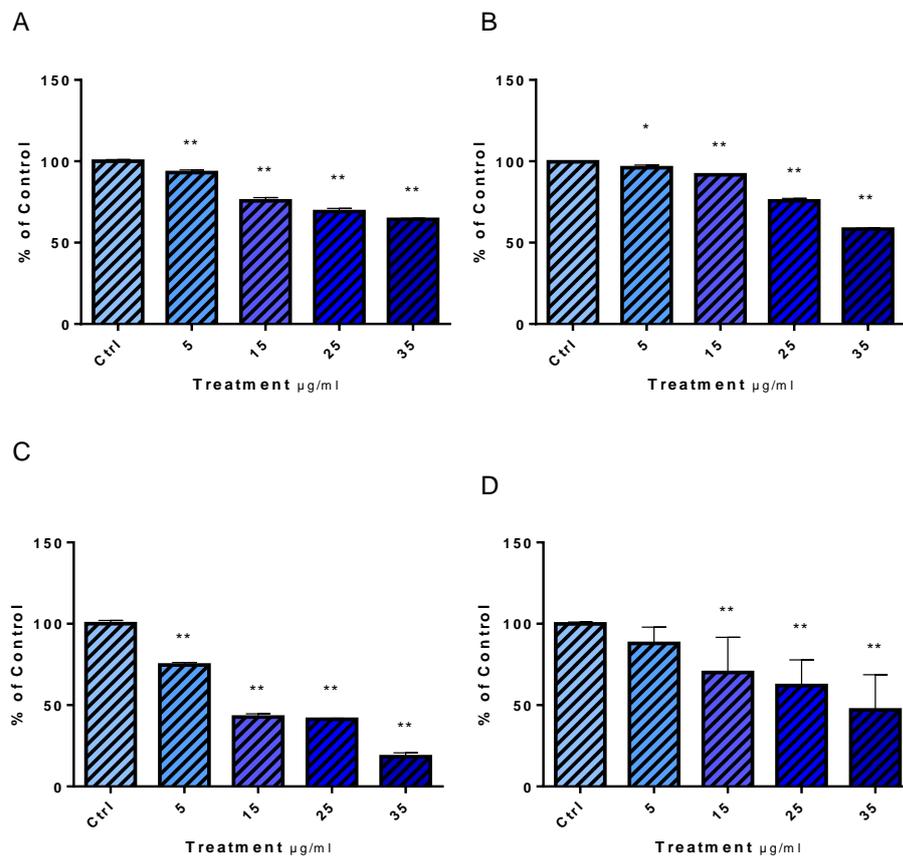


**Figure 4.15 Folic acid effects on cell viability between cancerous and non-cancerous cells**  
 Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** Cells treated with folic acid at 35 µg/ml **B)** Cells treated with folic acid at 5 µg/ml. The data show that there was a significant difference in cell viability between non-cancerous, and high grade cancerous cells at both a low (4µg/ml) and high (40µg/ml) dose of folic acid. The data points are means of nine replicates (n=9), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

#### 4.2.13 Dose response experiment comparing folic acid supplementation to standard cell culture media

A dose response experiment was also conducted for folic acid. 1321N1 cells that were supplemented with additional folic acid all significantly reduced the viability of cells, in a concentration dependent manner in each individual experiment (figure 4.16A-C). When the mean of these three experiments was compared, only folic acid in the supplement range 15-35  $\mu\text{g/ml}$  had a significant decrease on cell viability compared to standard cell culture media (figure 4.16D).

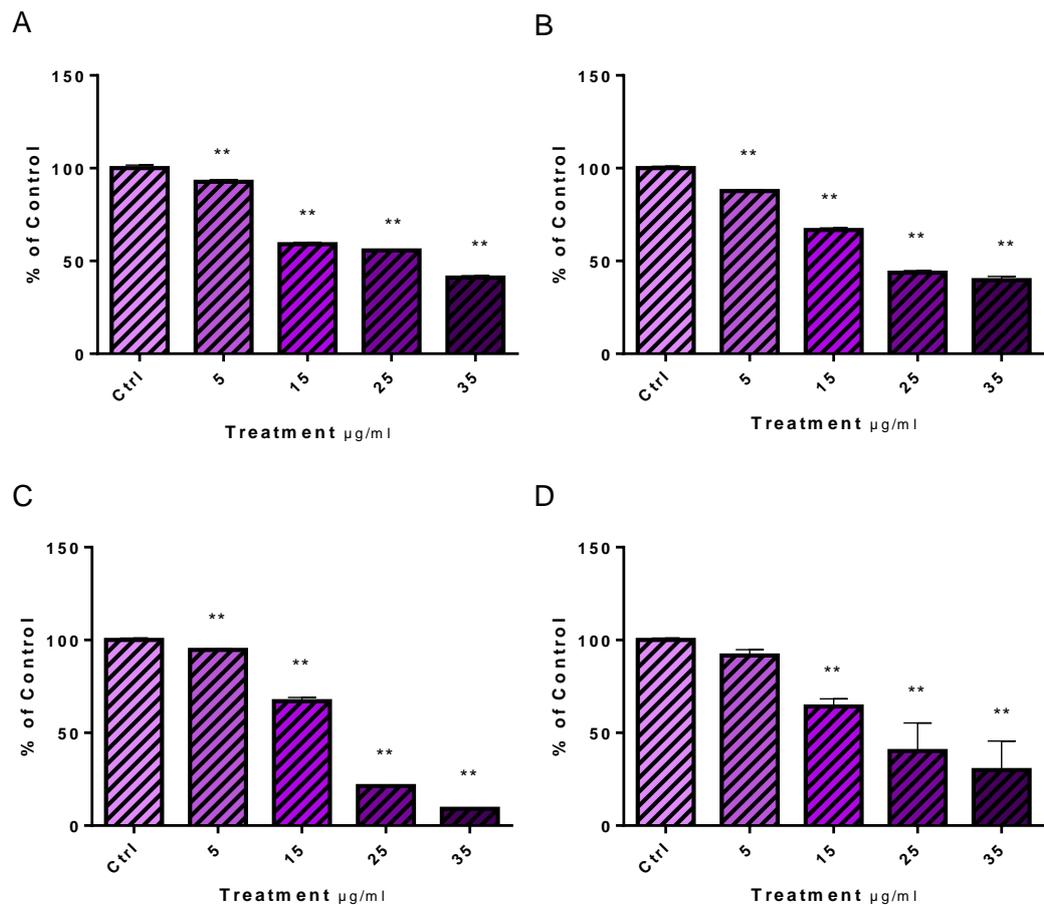
##### 1321N1



**Figure 4.16 Folic acid effect on cell viability of 1321N1 cells.** Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4 $\mu\text{g/ml}$  folic acid. **A)** 1321N1 Plate A **B)** 1321N1 Plate B **C)** 1321N1 Plate C **D)** 1321N1 Mean of Plates A, B and C. The data show that there was a significant difference in cell viability for cells treated with folic acid at 15 $\mu\text{g/ml}$ , 25 $\mu\text{g/ml}$  and 35 $\mu\text{g/ml}$ . The data points are means of nine replicates ( $n=9$ ), of one independent experiment, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

U87 MG cells that were supplemented with additional folic acid at a concentration of 15 – 35  $\mu\text{g}/\text{ml}$  significantly reduced the viability of cells, in a concentration dependent manner when compared to standard cell culture media control (Figure 4.17D).

### U87MG

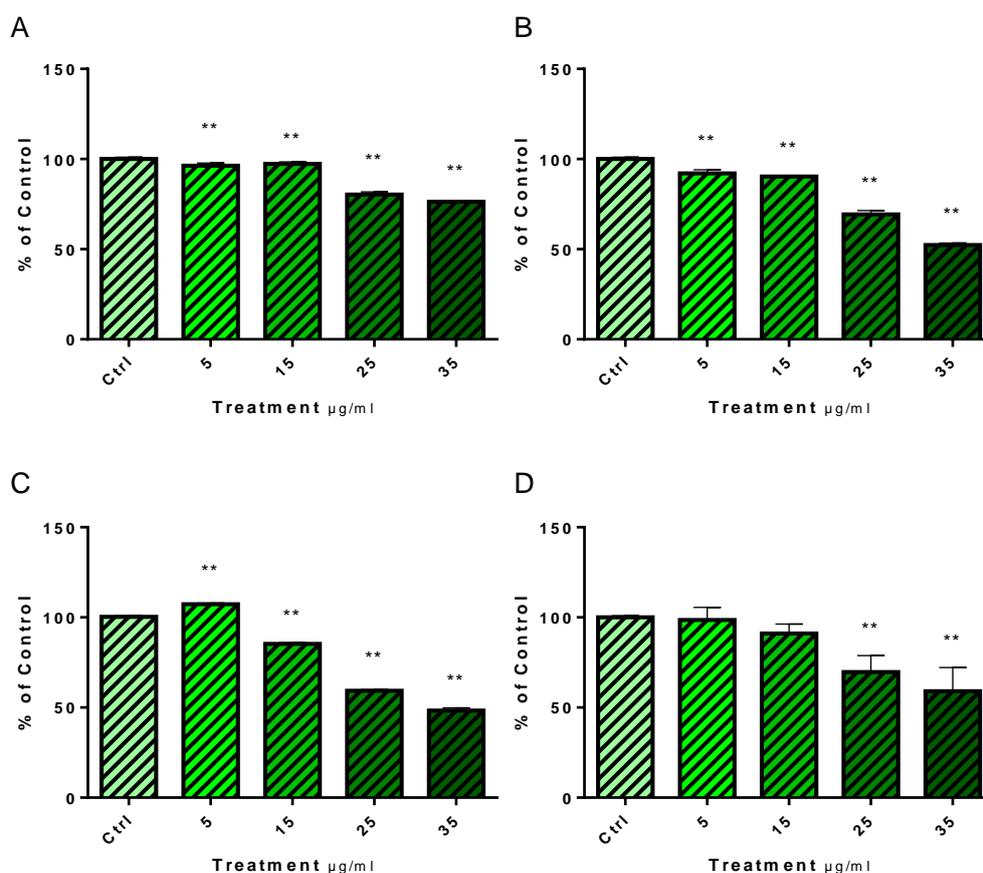


**Figure 4.17 Folic acid effect on cell viability of U87MG cells.**

Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing  $4\mu\text{g}/\text{ml}$  folic acid. **A)** U87MG Plate A **B)** U87MG Plate B **C)** U87MG Plate C **D)** U87MG Mean of Plates A, B and C. The data show that there was a significant difference in cell viability for cells treated with folic acid at  $15\mu\text{g}/\text{ml}$ ,  $25\mu\text{g}/\text{ml}$  and  $35\mu\text{g}/\text{ml}$ . The data points are means of nine replicates ( $n=9$ ), of one independent experiment, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

SVGp12 cells that were supplemented with additional folic acid all significantly decreased in cell viability when analysed on separate plates (Figures 4.18A-C). When the means were combined, only the additional supplements of 25 – 35  $\mu\text{g}/\text{ml}$  caused a significant decrease in cell viability (Figure 4.18D). All comparisons were made to the control standard cell culture media.

### SVGp12



**Figure 4.18 Folic acid effect on cell viability of SVGp12 cells.**

Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4 $\mu\text{g}/\text{ml}$  folic acid. **A)** SVGp12 Plate A **B)** SVGp12 Plate B **C)** SVGp12 Plate C **D)** SVGp12 Mean of Plates A, B and C. The data show that there was a significant difference in cell viability for cells treated with folic acid at 25 $\mu\text{g}/\text{ml}$  and 35 $\mu\text{g}/\text{ml}$ . The data points are means of nine replicates ( $n=9$ ), of one independent experiment, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.

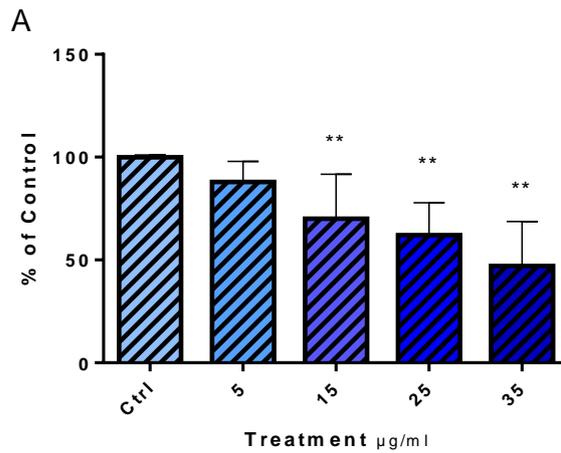
#### 4.2.14 Comparing the effect of folinic acid treatment on cancerous versus non-cancerous cells

The cancerous 1321N1 and U87MG cells that were treated with additional folinic acid at a concentration of 15 – 35  $\mu\text{g}/\text{ml}$  significantly decreased in cell viability (Figure 4.19A-B). The concentration had to increase to 25 – 35  $\mu\text{g}/\text{ml}$  before the non-cancerous SVGp12 cells showed a significant decrease in cell viability (Figure 4.19C).

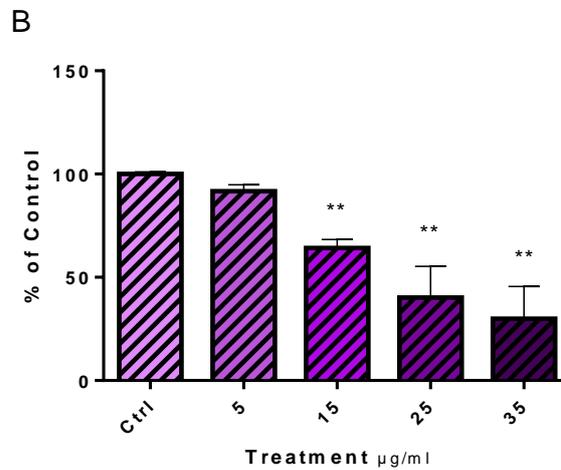
There was a significant difference in the effect of folinic acid treatment at 35  $\mu\text{g}/\text{ml}$  seen between non-cancerous and high grade cancerous cells. (Figure 4.20A).

When cells were treated with folic acid at 5  $\mu\text{g}/\text{ml}$  there was a significant difference observed between the non-cancerous cells and both low and high grade cancerous cells (Figure 4.20B).

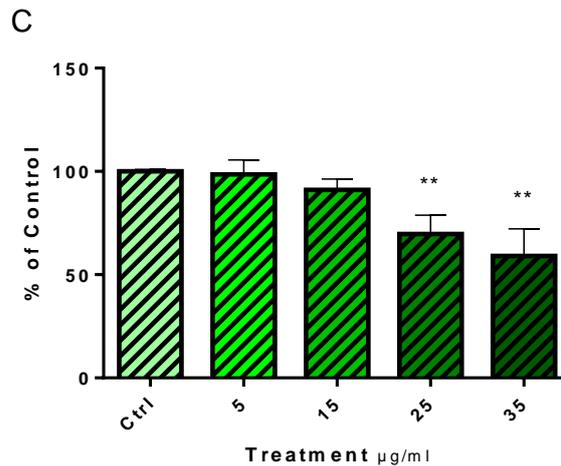
### 1321N1



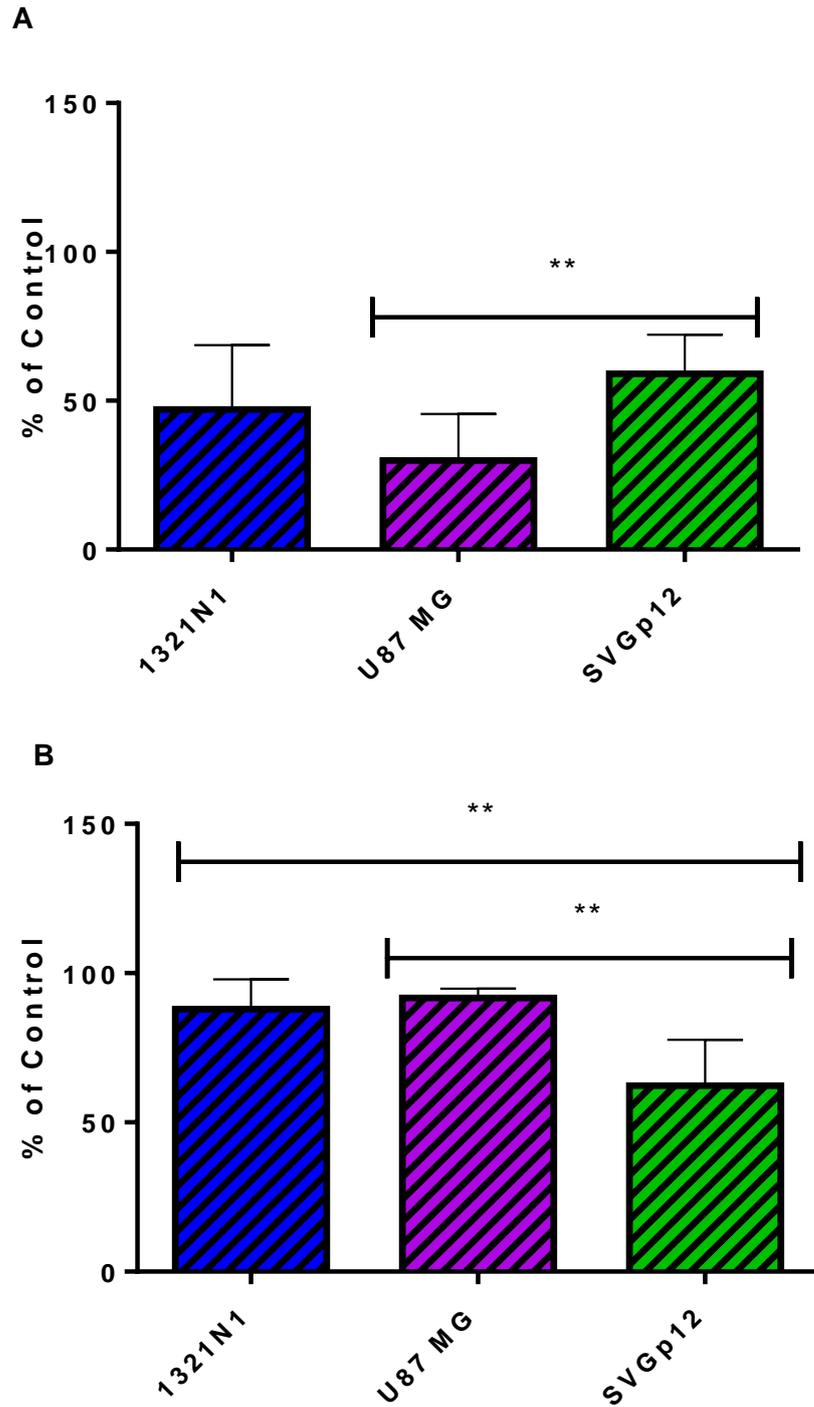
### U87MG



### SVGp12

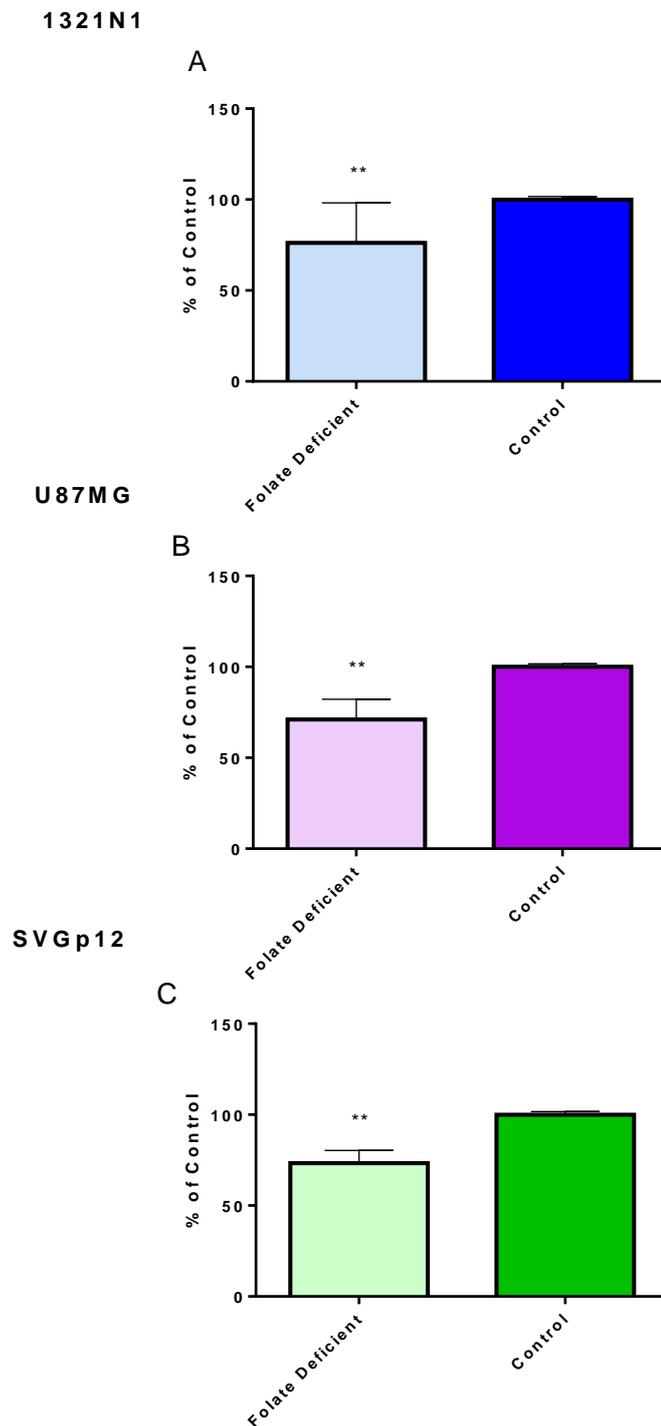


**Figure 4.19 Folinic acid effects on cell viability.** Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells **B)** U87MG cells **C)** SVGp12 cells. The data show that there was a significant difference in cell viability for all cell lines treated with folinic acid at 25µg/ml and 35µg/ml. The data points are means of nine replicates (n=9), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Dunnett's post-hoc test.



**Figure 4.20 Folinic acid effects on cell viability between cancerous and non-cancerous cells**  
 Cells were treated daily for 5 days, and cell viability measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** Cells treated with folic acid at 35 µg/ml **B)** Cells treated with folic acid at 5 µg/ml. The data show that there was a significant difference in cell viability between non-cancerous, and high grade cancerous cells at both a low (4µg/ml) and high (40µg/ml) dose of folic acid. The data points are means of nine replicates (n=9), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

**4.2.15 Effect of cells grown in folate free media relative to standard cell culture media**  
A further experiment was conducted to determine the difference in cell viability of all cell lines grown in folate free media compared to standard cell culture media for 5 days. For all cell lines, cell viability was significantly lower in the cells grown in folate free media compared to the control which was standard cell culture condition (Figure 4.21)



**Figure 4.21 Effect of folate deficient media on cell viability.** Cells were grown in folate free media containing folic acid at  $0\mu\text{g/ml}$  and standard cell culture media containing folic acid at  $4\mu\text{g/ml}$  for 5 days. Cell viability was measured using the PrestoBlue assay. Cells were then analysed as a percentage of the control cells grown in standard cell culture media. **A)** 1321N1 cells **B)** U87MG cells **C)** SVGp12 cells. The data show that there was a significant decrease in cell viability in all cell lines grown in folate free media. The data points are means of six replicates ( $n=6$ ), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the Student's t-test.

### 4.3 Discussion

The main aim of this chapter was to assess the effect of folic and folinic acid on the cell viability of glioma cells, and non-cancerous glial cells. The preliminary data found that vehicle solvents and antioxidants had no significant effect on cell viability and that significant difference in cell viability for the majority of cell lines and treatment groups had occurred by day 5 of daily treatment.

It was hypothesised that both folic and folinic acid supplementation would cause an increase in cell growth compared to cells grown in standard cell culture media, and that this effect would occur in a dose dependant manner given the role folate plays in DNA synthesis which assists cell growth. This hypothesis was not proven.

In the preliminary experiment where effects of high and low doses of folic and acid were compared, only the high concentration of folic acid supplementation caused a significant decrease in cell viability in the 1321N1 relative to the control, although a significant decrease in cell viability was observed between the low and high folic acid treatment groups. In the enhanced study with increased repeats this was only seen for the 1321N1 cells, which shows greater variability between experiments.

The results were more consistent for cells treated with folinic acid. The results showed that there was a decrease in cell viability following treatment with folinic acid at 35 µg/ml compared to standard cell culture conditions in all three cell lines in the enhanced study, which was also seen in the dose response experiment for all three cell lines.

This observed reduction in cell viability was supported by the literature which shows that folate supplementation in the form of folic and folinic acid reduced risk of developing cancer (Table 1.10). The most notable was the inverse relationship between folate supplementation and risk of colorectal cancer development (Ferraroni *et al.*, 1994; Freudenheim *et al.*, 1991;

Meyer and White, 1993). An inverse relationship has also been seen between pre conception folate supplementation and childhood brain tumours (Milne *et al.*, 2012).

More specifically with reference to glioma, the results seen here that folinic acid supplementation significantly decreases cell viability of cancerous glioma cell lines, 1321N1 and U87MG, is corroborated by a paper which showed that folate supplementation also decreased cell proliferation of U373 glioma cells (Hervouet *et al.*, 2009a). The paper however, did not specify what was meant by folate supplementation, whether it was folic acid, folinic acid or a combination of the two, only that the concentration of treatment was 4 and 40 µg/mL, as used in this study (Hervouet *et al.*, 2009a). Proliferation was measured using the Cell titer blue cell viability assay (Promega), which also measure the conversion of resazurin to resorufin, as utilised by the PrestoBlue assay.

Cells grown in folate free media showed a reduction in proliferation compared to those cells grown in standard cell culture, for the both the non-cancerous and cancerous cells. This finding has also been seen in the literature in embryonic multipotent neuroepithelial progenitor cells (Kruman *et al.*, 2005), human T lymphocytes (Courtemanche *et al.*, 2004b), NIE115 neuroblastoma cells (Battaglia-Hsu *et al.*, 2009). This effect is because folate deficiency induces apoptosis, blocks the cell cycle, and increases cell death (Huang *et al.*, 1999).

The second hypothesis formulated was that a greater effect would be seen in the cell viability following folate treatment in the cancerous cells compared to the non-cancerous cells due to the increased metabolic demand of cancer cells. This hypothesis was proven.

The dose response experiment shows that folic acid supplementation at 35 µg/ml significantly decreased the viability of the cancerous 1321N1 and U87MG compared to those in standard cell culture media, whilst the non-cancerous SVGp12 showed no significant decrease in cell viability at this concentration. At the lower concentration treatment 5 – 15

$\mu\text{g/ml}$  SVGp12 cells significantly increased in cell viability whilst no difference is observed in the cancerous cells. This suggests that high concentration folic acid treatment may reduce glioma growth, whilst not affecting non-cancerous cells. These findings also imply that low dose folic acid supplementation at  $5 \mu\text{g/ml}$  could be further investigated as a potential recovery treatment following chemotherapy, whereby the repair of healthy glial cells could be enhanced without encouraging cancerous cell growth. This is in line with the hypothesis that expected folate treatment to increase cell proliferation, but it has been found this was only for non-cancerous cells.

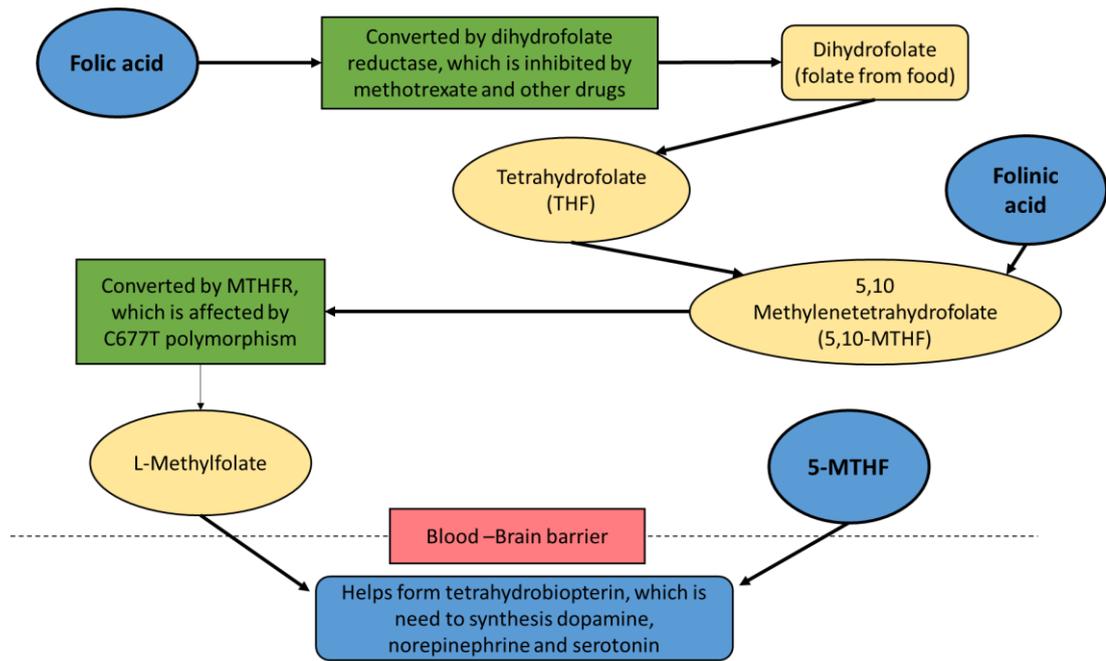
All three cell lines showed significantly decreased cell viability following folic acid supplementation at a concentration of  $35 \mu\text{g/ml}$ , although at a mid-concentration point of  $15 \mu\text{g/ml}$  only the cancerous cells showed a significant decrease in cell viability.

Cancer cells have an increased metabolic demand in comparison to non-cancerous cells due to rapid proliferation (Cairns *et al.*, 2011). Increased cell proliferation requires an increase in methyl donors for one carbon metabolism, DNA synthesis and DNA methylation, in the form of 5-methyl tetrahydrofolate, 5-MTHF (Blom and Smulders, 2011) which could suggest that cancerous cells have a higher uptake of methyl status altering folate, as demonstrated here by higher proliferation in the cancer cell lines at the lower concentration of  $15 \mu\text{g/ml}$ , compared to the non-cancerous cell line.

The final hypothesis was that folic acid would have a greater effect on bioactivity compared to folic acid due to the fewer enzymatic steps required for conversion to the active metabolite 5-MTHF. Folic acid treatment caused a significant reduction in viability in 1321N1, U87MG and SVGp12 cells compared to the same concentration of folic acid where a significant decrease was only observed in the 1321N1 cell line. The effects of folic acid also produced more consistent and reliable data. This hypothesis was proven.

Differences were observed between the different forms of folate in the different cell lines. folic acid had a significantly greater effect at reducing the cell viability of 1321N1 cells than folic acid when cells were treated at a lower concentration of 4 µg/ml. This significant difference was seen in all three cells lines; 1321N1, U87MG and SVGp12 when folate supplementation was increased to 40 µg/ml. Both folic and folic acid treatments were chosen to assess cell viability effect, given the biological and metabolic differences between the two. Folic acid enters the methylation pathway in an oxidised state, and is more stable, and for this reason tends to be used more often as a supplement, however folic acid must later be reduced in the body to dihydrofolate, and then tetrahydrofolate (Wright *et al.*, 2007). Folic acid enters in a reduced state as 5-MTHF and therefore can enter directly into the methylation pathway. Folic acid binds tightly to the folate receptors, creating competition with 5-methylTHF for transport across the blood-choroid plexus CSF barrier (Shin *et al.*, 2011), and has the potential to interfere with the metabolism and transport of natural folate (Smith *et al.*, 2008), thereby causing a reduction in the available physiological form required; 5-MTHF.

Folic and folic acid are also metabolised in different ways, folic acid requires more enzymatic steps than folic acid before it can cross the blood brain barrier (Figure 4.22). The first step is to be converted to dihydrofolate by dihydrofolate reductase which can be inhibited by methotrexate a common chemotherapeutic agent (Tian and Cronstein, 2007).



**Figure 4.22 The different metabolic pathways of folic and folinic acid.**

*Folic acid must be converted four times before it reaches the form of L-methylfolate to cross the blood brain barrier, this is twice as many steps as folinic acid.*

Folinic acid requires fewer enzymatically steps for it to become biologically active than folic acid which may explain why folinic acid had a greater effect on reducing cell viability. The biologically active form 5-MTHF is more effective than FA as it can donate a methyl group for methionine synthesis and maintenance methylation of DNA (Wang, 2003).

In summary, high dose folinic acid had a greater effect at reducing cell viability, compared to folic acid, but folic acid had a more cell specific response at the low dose. In order to understand the mechanism of the reduction in cell viability, cell cycle and apoptosis analysis will be performed. Methylation status will also be assessed to ascertain whether treatment is altering any methylation patterns which may be affecting gene expression in those involved in folate transport. In order to understand the specific nature of folic acid western blot analysis will be performed to ascertain folate transporter expression levels.

Treatment will continue for 5 days for all further investigations, because the literature as detailed in Section 4.1, corroborates this is sufficient time for downstream assays to assess alterations including change in methylation status, and altered protein level to have occurred.

# CHAPTER 5

## 5 CELL CYCLE AND APOPTOSIS

### 5.1 Introduction

In Chapter 4, cell viability was significantly reduced in the U87MG and 1321N1 cell lines following treatment with high dose folate, particularly in the form of folic acid. The next step was to investigate the cause of the observed decrease in cell viability. The reduction of resazurin, an oxidation–reduction indicator, can provide a marker of metabolically active cells based on viable cell numbers. Resazurin reduction assays do not however indicate the mechanism of reduction in viable cells with active metabolism, which could be due to necrosis, apoptosis or cell cycle arrest (Riss *et al.*, 2015).

#### 5.1.1 Cell Cycle

The point at which a eukaryotic cell decides whether to begin mitosis, is influenced by extracellular growth factors that signal cell proliferation, rather than availability of nutrients (Blagosklonny and Pardee, 2002). The M Phase is where mitosis and cytokinesis occur, it takes around an hour to complete and during this stage the daughter chromosomes separate and the cell divides by cytokinesis. The interphase which prepares the cell for mitosis, takes about 23 hours. During this stage, cell growth and DNA synthesis occurs and most cells in a population are in interphase. As shown in Figure 5.1, there are several phases that occur during Interphase: G<sub>1</sub>, S and G<sub>2</sub> phase (Cooper, 2000). The G<sub>1</sub> phase prepares the cell to enter the S phase and during this time the cell is metabolically active and continues to grow. In the S Phase DNA replication occurs, finally in the G<sub>2</sub> phase the cell grows and proteins are synthesised.

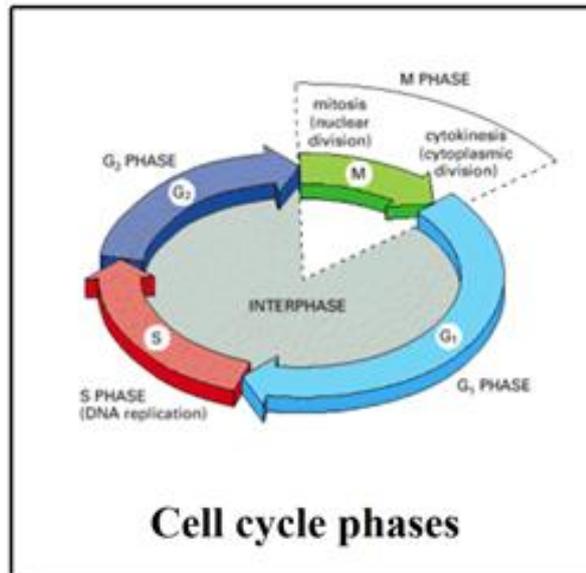
There are many checkpoints a cell must go through during the cell cycle (Sherr and McCormick, 2002). The G<sub>1</sub> checkpoint is the predominant point at which cells will be checked before entering cell cycle arrest on either a temporary or permanent basis (Bartek *et al.*,

1997). If the cell passes this restriction point, it is then committed to the rest of cycle, even if conditions later change. If the cell cycle stops at the restriction point it will enter  $G_0$ . Cells can remain in this stage for long periods without proliferating, they are still metabolically active, and therefore the Prestoblue assay will indicate that the cells are still viable, although they have ceased growth, and reduced rates of protein synthesis. When calculating cell viability relative to the control group using the Prestoblue assay; cells grown in standard culture conditions will continue to proliferate over time and therefore comparison to a group of cells that may have arrested will appear to decrease in viability. Cells remain in  $G_0$  unless called upon by growth factors. Any arrest at  $G_1$  is regulated by the *TP53* tumour suppressor gene; a checkpoint gene found to be frequently mutated in most cancers (Bargonetti and Manfredi, 2002; Nyberg *et al.*, 2002).

The S Phase checkpoint, slows down DNA synthesis if required (Kastan and Bartek, 2004). The  $G_2/M$  checkpoint prevents any cells that have acquired DNA damage in the  $G_2$  phase from initiating mitosis (Xu *et al.*, 2002).

If any damage to the DNA occurs, for example from reactive oxygen species, cell cycle arrest will occur at  $G_1$  or  $G_2$  (Blagosklonny and Pardee, 2002). These arrests are often temporary to allow cellular damage to be repaired as it allows the availability of essential growth factors to be restored. Apoptosis however may subsequently occur if the damage cannot be repaired.

A loss of these restriction points distinguishes between cancerous and normal cells. An aberration at the  $G_1$  checkpoint is a particularly common in cancerous cells.



**Figure 5.1 Cell Cycle Phases**

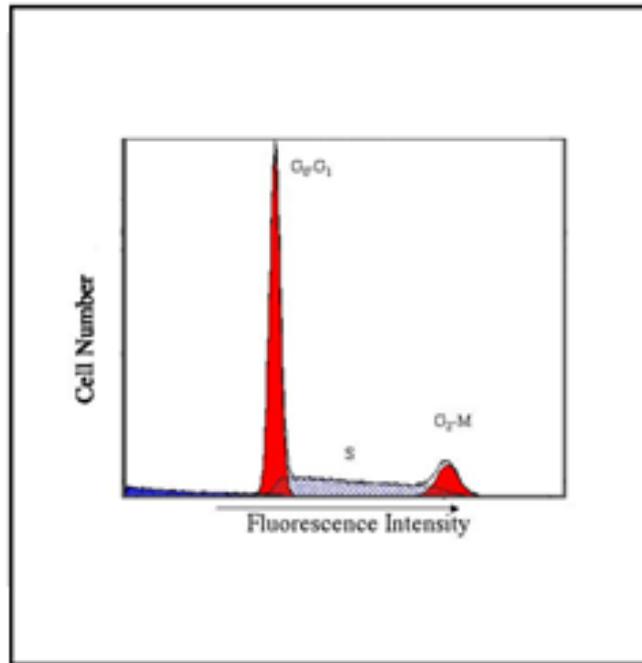
There are four phase in the cell cycle, the M phase, where mitosis and cytokinesis occur. The G<sub>1</sub> phase, the location of the first checkpoint, and when the cell prepares to enter the S phase. The S phase is when DNA replication occurs. Finally the cell enters the G<sub>2</sub> phase where the cell grows and the proteins synthesise (Tabll and Ismail, 2012).

### 5.1.2 Folic Acid and Cell Cycle

The primary role of folic acid is the generation of single carbon groups which are transferred to various compounds. Folic acid can be found in the diet as foylpolylglutamate and is then split into monoglutamates which are absorbed by the proximal jejunum (Bohnsack and Hirschi, 2004). Folate is crucial to a diet as deficiency results in a reduction in proliferation because cells lacking folate will accumulate in the S phase, as a result of nucleotide imbalance and slow DNA synthesis (Koury *et al.*, 2000b). Folate deficiency will also induce apoptosis, block the cell cycle block, and increase cell death (Huang *et al.*, 1999). When folate is re-introduced, a reversal occurs and proliferation is restored (Courtemanche *et al.*, 2004a). In a study involving HepG2 cells, representing liver cancer, folate deficiency induced apoptosis. Preceding this apoptosis there was a drop in folate level, as the result of a cell cycle block. Folate supplementation was able to rescue apoptotic cells and normalise growth (Huang *et al.*, 1999).

### 5.1.3 Determining Cell Cycle Stage

In order to determine the stage of cell cycle, the  $G_1$ , S, and  $G_2$  phases must be identified through biochemical criteria. The S phase is able to incorporate radioactive thymidine which is exclusive to DNA synthesis.  $G_1$  cells are diploid, containing two of each chromosome, referred to as  $2n$ , whilst cells in  $G_2$  have a DNA content known as  $4n$  (Mao *et al.*, 2012). Cellular DNA content can be determined by the incubation of cells with a fluorescent dye that binds to DNA. Fluorescence intensity is then analysed in a flow cytometer, or fluorescence activated cell sorter. The most commonly used stains are propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) (Pozarowski and Darzynkiewicz, 2004). PI is a fluorescent dye, that binds to DNA stoichiometrically, the more DNA, the greater the fluorescence. This stained solution is then passed through the flow cytometer which uses a laser to pick up the emitted fluorescence signal, which is converted into an electronic pulse whose amplitude is proportional to the amount of DNA present (Nunez, 2001). An example of these measurements is shown in Figure 5.2 for  $G_1$  diploid cells. Apoptotic cells stained with PI show a broad hypodiploid peak that will be distinguishable from the narrow peak of diploid cells in the red fluorescence channel (Riccardi and Nicoletti, 2006).



**Figure 5.2 A typical DNA histogram**

*A typical DNA histogram should produce a diploid profile, when the  $G_0/G_1$  peak is equal that of a known reference, and the S and  $G_2/M$  phases are relatively low. Profiles are the result of flow cytometry analysis (Tabll and Ismail, 2012.)*

#### 5.1.4 Apoptosis

The Prestoblue assay is not capable of distinguishing the difference between the two modes of cell death, apoptosis and necrosis, or whether cells are in cell cycle arrest. Necrosis does not involve a regular DNA degradation pattern, but begins with the loss of cell membrane integrity, the cytoplasm and mitochondria swell and then the cell and internal organelles lyse (Kroemer *et al.*, 1998). Necrosis often affects groups of adjacent cells.

Apoptosis however, is a regulated event, characterised by a change in cell nuclear morphology involving chromatin condensation, pyknosis, irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis and karyorrhexis, fragmentation of the nucleus and only individual cells are affected (Kroemer *et al.*, 1998).

Apoptotic cells fragment DNA into around 200 bp fragments, and then these cells are broken into many apoptotic bodies following apoptosis, which are phagocytosed by nearby cells (King and Cidlowski, 1998). Apoptotic stimuli have the ability to arrest growth of a cell or induce cell death. These stimuli can include the G<sub>1</sub> checkpoint regulators; TP53, pRb, and E2F. Growth arrest in order to suppress apoptosis is induced by pRb, whilst TP53 and E2F would induce apoptosis (King and Cidlowski, 1998)

Wildtype TP53 has the ability to cause cells to arrest or undergo apoptosis as the result of DNA damage. Whether arrest or apoptosis occurs can be influenced by several factors, response is often influenced by the specific cell type due to its cellular content and which growth factors are present. Exogenous factors such as radiation, result in genetic changes such as DNA damage (Amundson *et al.*, 1998). The extent of the DNA damage will also affect whether a cell will enter arrest or undergo apoptosis (Vermeulen *et al.*, 2003).

Apoptosis was first described in 1972, as programmed cell death (Kerr *et al.*, 1972), it consists of three major components; the BCL-2 proteins, the caspases, and the Apaf-1/CED-4 protein that relays the signal from the BCL-2 protein to the caspase protein (Adams and Cory, 1998). There are over 14 different caspases (Thornberry and Lazebnik, 1998), caspase 1 and 11 relate to cytokine processing (Wang *et al.*, 1998). Caspases 2, 3, 6, 7, 8,9 and 10 all play a role in the initiation and process of apoptosis (Budihardjo *et al.*, 1999). There are two main routes for the caspase activating cascade that commences apoptosis; initiation from the cell surface death receptor, and secondly from changes in the integrity of the mitochondria (Budihardjo *et al.*, 1999). These are both regulated by different proteins upstream which result in the cleavage of an initiator caspase; caspase 9 in the cell surface death receptor model, and caspase 8 in the mitochondrial model. Dependent upon the caspase 8 or 9, they will then cleave the executioner caspases (Slee *et al.*, 2001); caspases 3, 6 and 7 to activate them (Budihardjo *et al.*, 1999).

Measuring levels of caspase can be achieved through a variety of methods. These include; immunoblotting, measuring enzyme activity by the cleavage of synthetic substrates, as well as through the use of caspase inhibitors and affinity labelling (Köhler *et al.*, 2002). Apoptosis will be measured by two methods in this chapter, through the use of PI staining and flow cytometry, and through measuring caspase activity levels.

The assay used to measure levels of apoptosis was the Caspase-Glo 3/7 assay from Promega, which measures the level of caspase 3/7 activity. Caspase 3 is a useful indicator of level of apoptosis given that it is found in almost all mammalian cells, and because it is the primary executioner caspase (Slee *et al.*, 2001). Caspase 7 is also measured as it has been seen to have a similar sequence to caspase 3 (Lakhani *et al.*, 2006). Caspase 3 is the effector caspase most frequently involved in neuronal apoptosis (Jaraskog *et al.*, 2004). In gliomas, caspases are activated in the order caspase 8, then caspase 3 and 7 (Burguillos *et al.*, 2011; Shen *et al.*, 2016).

It was decided that Days 2 and 7 would be analysed, because the initial cell viability assay showed most significant alteration began to occur around Day 5. Choosing Day 2 and 7 allowed a snapshot before and after this time point to detect whether any potential differences occurred before or after this observed change in cell viability.

### 5.1.5 Aim of Chapter

The aim of this chapter was to determine if folate treatment, which was seen to reduce the viability of cells, was doing so as the result of cell cycle arrest, or through increased levels of apoptosis, compared to cells grown in standard cell culture media.

### 5.1.6 Hypothesis

The previous chapter showed that cells grown in folate free conditions led to a reduction in cell viability, but this was not observed following additional folate treatment. It was therefore hypothesised that:

#### **Hypothesis One**

The observed reduction in cell viability following supplementation with high dose folate was due to cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> checkpoint.

#### **Hypothesis Two**

Due to the reduced viability of cells seen in Chapter 4, it is expected that there will be a higher level of apoptosis in cells treated with high dose (40 µg/ml) folic acid.

### 5.1.7 Objectives

**Objective 1** To ascertain the cell cycle phase profile for each cell line for cells grown in standard cell culture media at set time points of Day 2 and Day 7 to ascertain a normal profile for each cell line.

**Objective 2** To examine whether low or high dose folic or folic acid treatment has an effect on cell cycle phase compared to the cells grown in standard cell culture media at set time points on Day 2 and 7.

**Objective 3** To examine whether treatment with folic or folic acid at low or high dose have an effect on levels of apoptosis measured by caspase activity at a set time point of Day 5 to compare with cell viability experiments.

**Objective 4** To examine whether low and high dose of folic or folic acid treatment affects caspase activity compared to the cells grown in standard cell culture media at a set time points of Day 5.

**Objective 5** To examine whether there is a difference in levels of caspase activity between cancerous and non-cancerous cells.

**Objective 6** To establish whether a short term 24 hour study or longer 5 day study best shows changes to apoptotic activity.

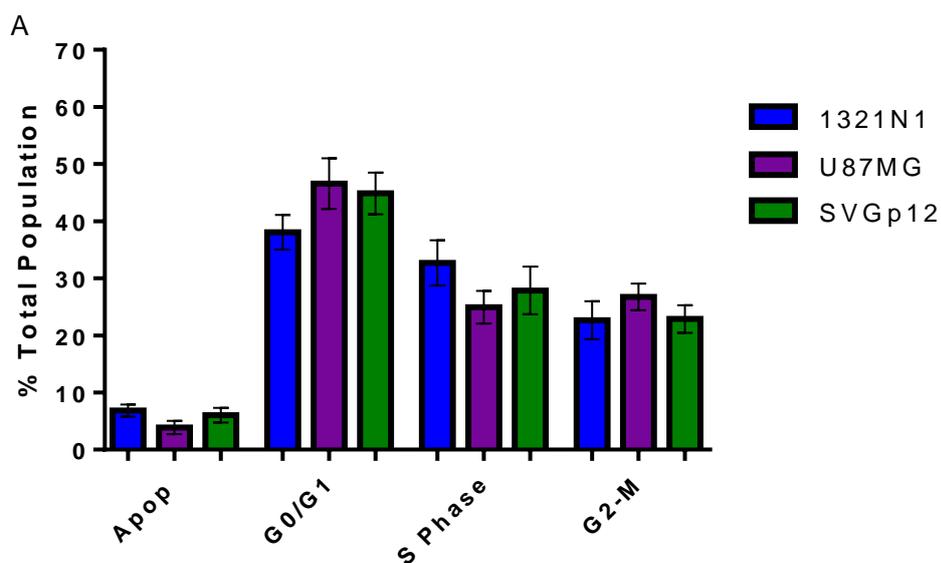
## 5.2 Results

Cells were treated and analysed using flow cytometry as summarised in Section 2.7.

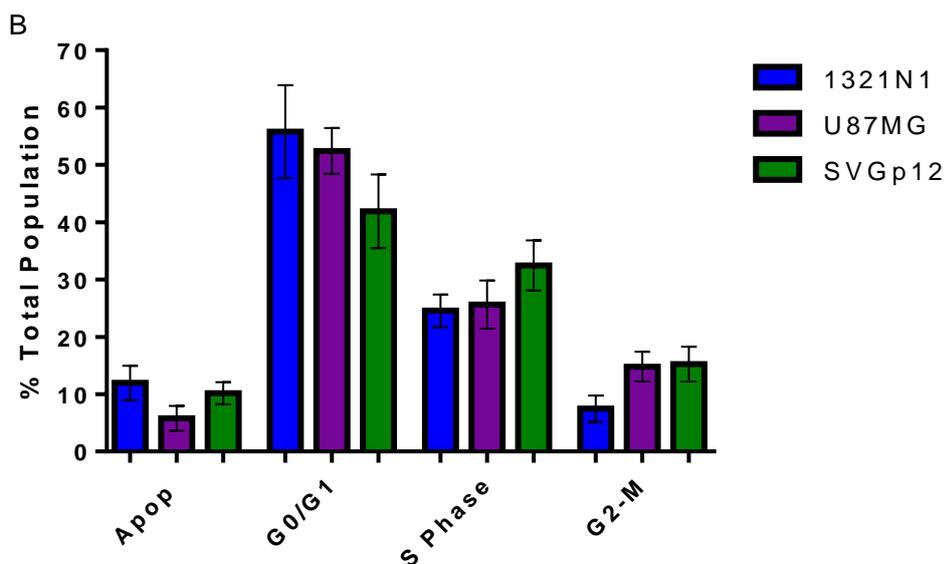
### 5.2.1 A comparison of cell cycle phase between cell lines grown in standard cell culture media at set time points

Cells were analysed by comparing the percentage of cell population in each stage of the cell cycle by ANOVA with Tukey's post hoc test. Initially, the cell lines were compared on both Day 2 and Day 7, and showed no significant difference in any cell cycle stage on either day between the cell lines (Figure 5.3). During standard cell culture conditions, the largest percentage of cells were in the  $G_0/G_1$  phase. This knowledge of a normal cell cycle phase profile for each cell line will allow comparisons to be made in each cell line following treatment.

## Day 2



## Day 7



### Figure 5.3 Comparison of Cell Cycle Phase between Cell Lines

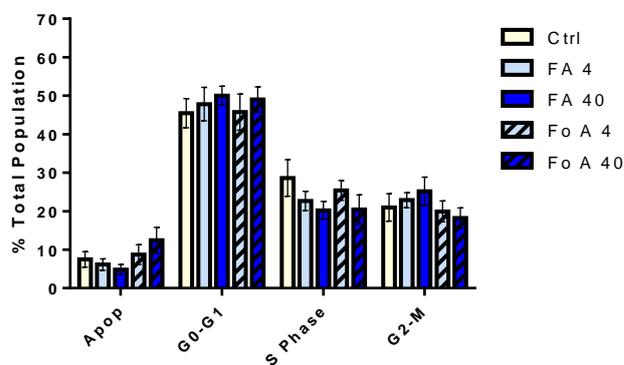
Cells were grown in standard cell culture media containing 4 $\mu$ g/ml folic acid. The percentage of sub G<sub>1</sub> apoptotic, G<sub>1</sub>, S and M phase populations were determined by propidium iodide (PI) staining and flow cytometry, A 488nm laser was used to excite the cells, and red fluorescence was measured at 615nm, a minimum of 10,000 events were analysed. Samples were gated using forward scatter versus side scatter in order to exclude any cell debris or clumps of cells. The G<sub>0</sub>/G<sub>1</sub> peak was then adjusted to appear around channel 50, and software attached to the FACS determined how many cells were in each phase of the cell cycle. **(A)** Cell cycle profile on Day 2 **(B)** Cell cycle profile on Day 7. Results are displayed as a percentage of the total population. ANOVA with Tukey's post hoc test was performed.

### 5.2.2 A comparison of folic acid and folinic acid treatment on cell cycle phase

The effect different treatments had on cell cycle phase was also analysed, by comparing each cell cycle phase between each treatment within each cell line. Apart from a significant increase of 10 % percentage of SVGp12 cells at G<sub>0</sub>-G<sub>1</sub> phase between the cells grown in standard cell culture media (control) and cells treated with folic acid at 4 µg/ml on Day 7 (Figure 5.5), there was no significant difference seen on either Day 2 (Figure 5.4) or Day 7 (Figure 5.5).

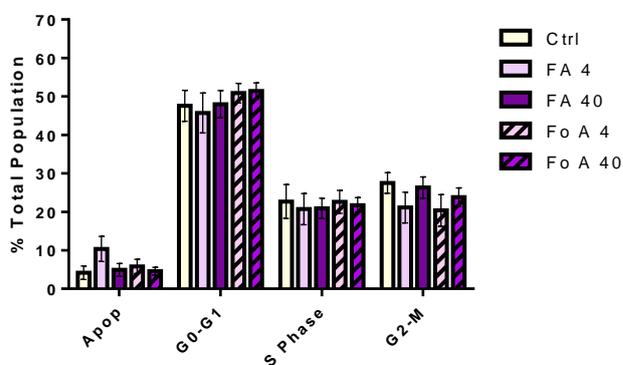
1321N1

A



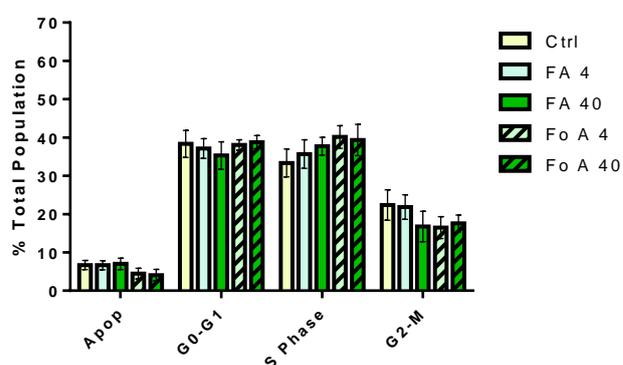
U87MG

B



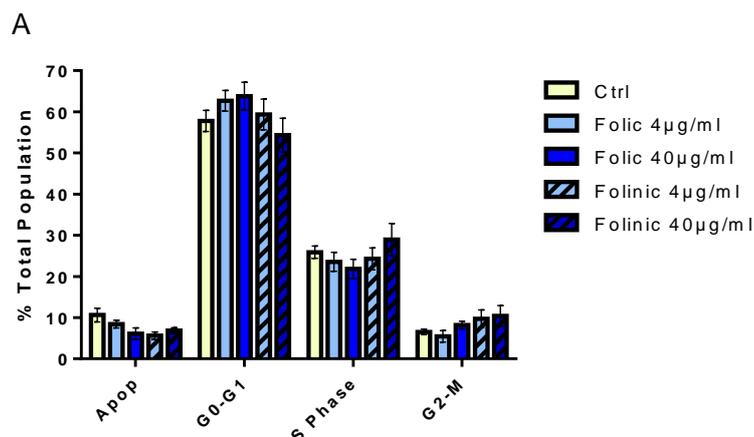
SVGp12

C

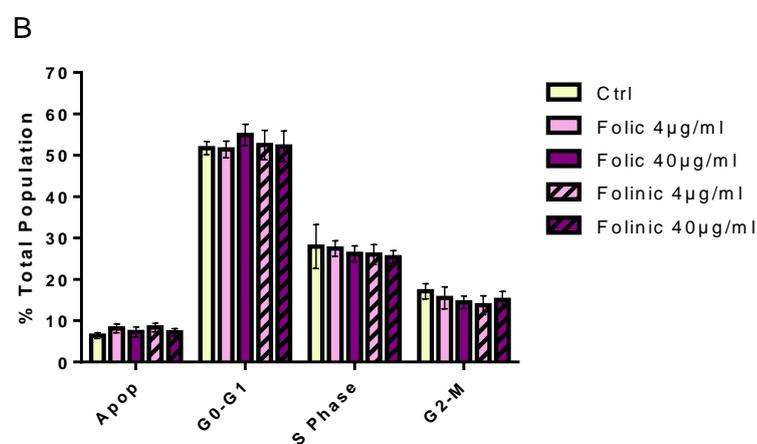


**Figure 5.4 Comparison of cell cycle phase by treatment on Day 2.** Cells were grown in standard cell culture media containing 4 $\mu$ g/ml folic acid. The percentage of sub G<sub>1</sub> apoptotic, G<sub>1</sub>, S and M phase populations were determined by propidium iodide (PI) staining and flow cytometry, A 488nm laser was used to excite the cells, and red fluorescence was measured at 615nm, a minimum of 10,000 events were analysed. Samples were gated using forward scatter versus side scatter in order to exclude any cell debris or clumps of cells. The G<sub>0</sub>/G<sub>1</sub> peak was then adjusted to appear around channel 50, and software attached to the FACS determined how many cells were in each phase of the cell cycle. **(A)** 1321N1 cells **(B)** U87MG cells **(C)** SVGp12 cells. Results are displayed as a percentage of the total population. ANOVA with Tukey's post hoc test was performed.

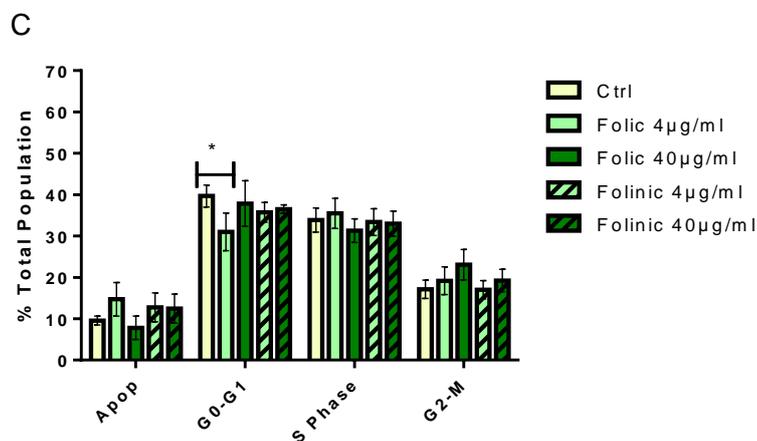
1321N1



U87MG



SVGp12



**Figure 5.5 Comparison of cell cycle phase by treatment on Day 7.** Cells were grown in standard cell culture media containing 4µg/ml folic acid. The percentage of sub G<sub>1</sub> apoptotic, G<sub>1</sub>, S and M phase populations were determined by propidium iodide (PI) staining and flow cytometry, A 488nm laser was used to excite the cells, and red fluorescence was measured at 615nm, a minimum of 10,000 events were analysed. Samples were gated using forward scatter versus side scatter in order to exclude any cell debris or clumps of cells. The G<sub>0</sub>/G<sub>1</sub> peak was then adjusted to appear around channel 50, and software attached to the FACS determined how many cells were in each phase of the cell cycle. **(A)** 1321N1 cells **(B)** U87MG cells **(C)** SVGp12 cells. Results are displayed as a percentage of the total population. ANOVA with Tukey's post hoc test was performed.

### 5.2.3 Measure of apoptosis in response to treatment with low or high dose of folic acid following five days treatment

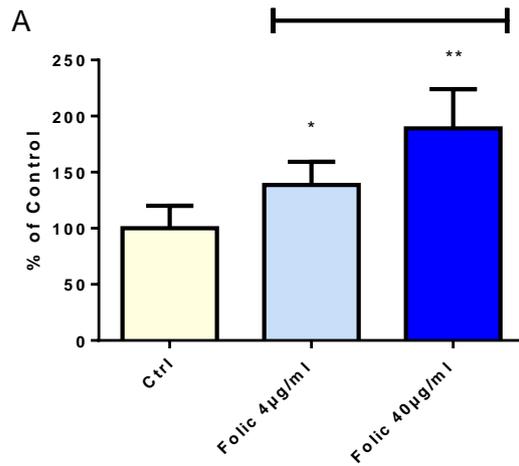
Given that there was no alteration to cell cycle observed in response to increasing dose of folate treatment, which was unexpected, caspase activity analysis was performed following 5 days of daily folate treatment. The advantage of using a caspase assay to detect levels of apoptosis is that cells can be assayed undisturbed within the well of a plate, without needing to be detached and treated with propidium iodide as required for flow cytometry which may affect the cells.

The 1321N1 cells had a significant increase in apoptosis following treatment with folic acid at a concentration of 4  $\mu\text{g/ml}$  and 40  $\mu\text{g/ml}$  compared to standard cell culture conditions and between the low and high doses (Figure 5.6A).

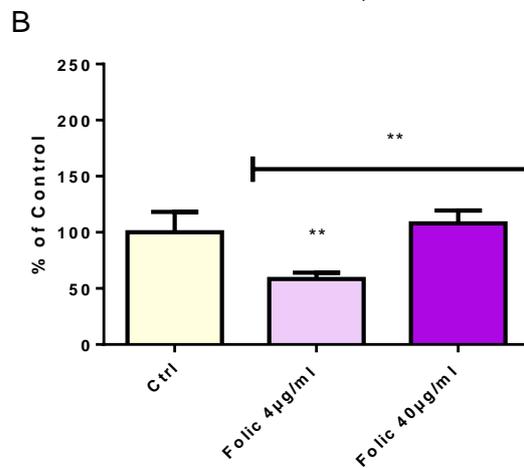
The U87MG cells treated with 4  $\mu\text{g/ml}$  of folic acid showed a significant decrease in level of caspase 3/7 activity compared to the control cells, whilst those treated with 40  $\mu\text{g/ml}$  of folic acid showed no significant difference. There was a significant increase in caspase 3/7 activity seen between low and high levels of folic acid supplementation (Figure 5.6B).

The SVGp12 cells treated with 4  $\mu\text{g/ml}$  of folic acid showed a similar response to the U87MG cells with a significant decrease in caspase 3/7 activity but no significant difference when cells were treated with 40  $\mu\text{g/ml}$  folic acid. There was a significant increase in caspase 3/7 activity seen between low and high levels of folic acid supplementation (Figure 5.6C).

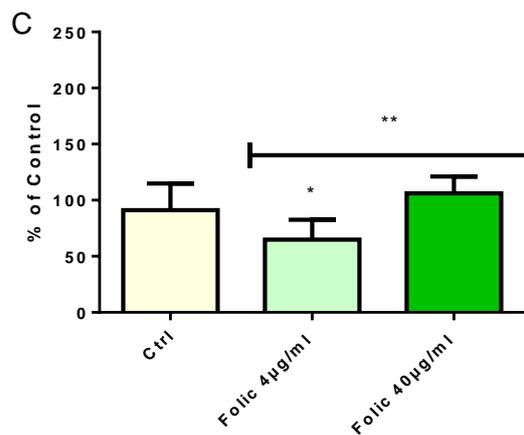
1321N1



U87MG



SVGp12



**Figure 5.6 Caspase 3/7 activity following folic acid treatment.** Cells were treated daily for 5 days, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic acid at 4µg/ml and 40µg/ml **B)** U87MG cells treated with folic acid at 4µg/ml and 40µg/ml **C)** SVGp12 cells treated with folic acid at 4µg/ml and 40µg/ml. The data show that there was a significant increase in caspase 3/7 activity in all cell lines treated with an increased concentration of folic acid. The data points are means of three replicates (n=12), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

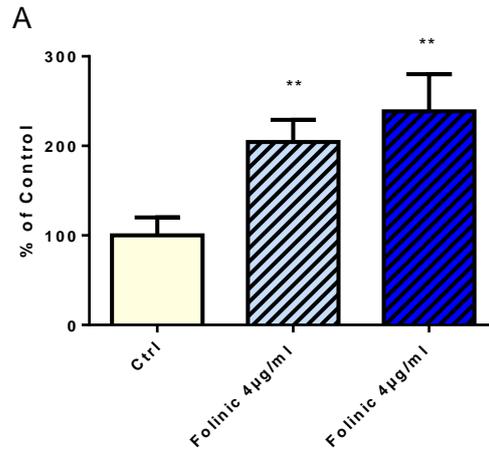
#### 5.2.4 Measure of apoptosis in response to treatment with low or high dose of folic acid following five days treatment

The 1321N1 cells had a significant increase in apoptosis following treatment with folic acid at both 4 and 40 µg/ml (Figure 5.7A). This significant difference however was not seen between the low and high level of folic acid supplementation.

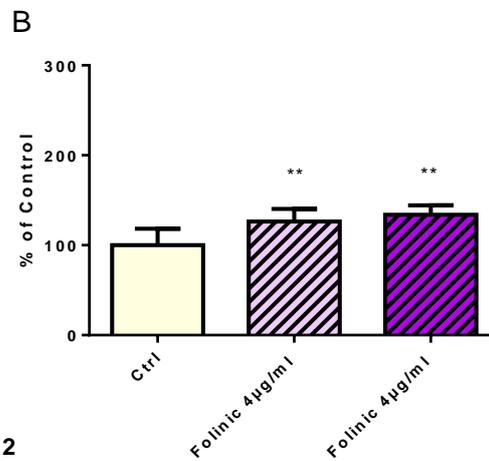
U87MG cells treated with both concentrations of folic acid, showed a significant increase in caspase 3/7 activity in comparison to the control cells (Figure 5.7B). This significant difference was not seen between low and high level of folic acid supplementation.

SVGp12 cells again behaved similarly to the U87MG cells with a significant increase in level of caspase activity when treated with folic acid at both 4 and 40 µg/ml (Figure 5.7C). This significant difference however was not seen between supplementation with low and high levels of folic acid.

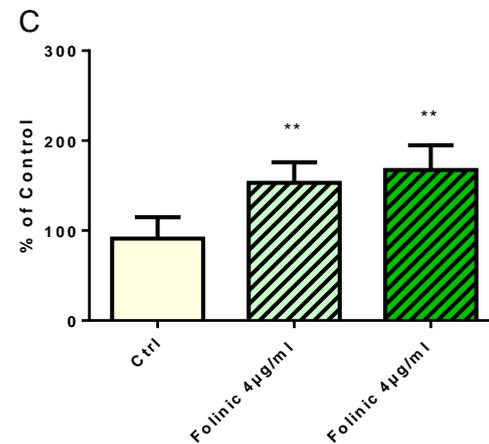
### 1321N1



### U87MG



### SVGp12



**Figure 5.7 Caspase 3/7 activity following folicinic acid treatment.** Cells were treated daily for 5 days, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folicinic acid. **A)** 1321N1 cells treated with folicinic acid at 4µg/ml and 40µg/ml **B)** U87MG cells treated with folicinic acid at 4µg/ml and 40µg/ml **C)** SVGp12 cells treated with folicinic acid at 4µg/ml and 40µg/ml. The data show that there was a significant increase in caspase 3/7 activity in all cell lines treated with low (4µg/ml) and high (40µg/ml) doses of folicinic acid compared to the control cells. The data points are means of three replicates (n=3), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

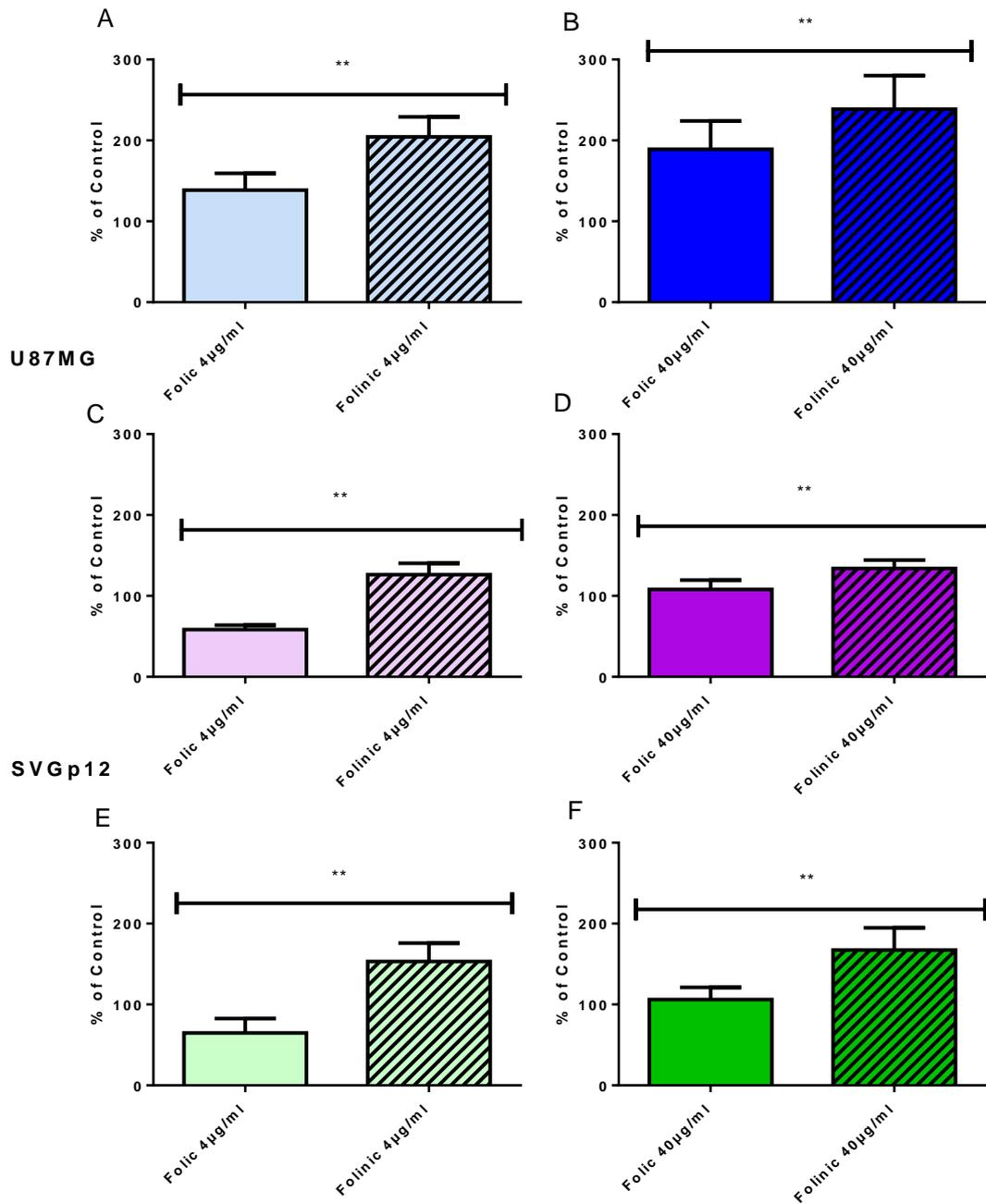
### 5.2.5 Measure of apoptosis in response to treatment comparing low or high dose of folic and folinic acid following five days treatment

Comparing between forms of folate, there was a significant increase in caspase 3/7 activity in 1321 cells treated with folinic acid compared to folic acid at 4  $\mu\text{g/ml}$ . This significant increase was also seen between folic and folinic acid at 40  $\mu\text{g/ml}$  (Figure 5.8A).

There was a significant increase in caspase activity when cells were treated with folinic acid compared to folic acid at 4  $\mu\text{g/ml}$ . This significant increase was also seen for cells treated with folinic acid compared to folic acid at 40  $\mu\text{g/ml}$  (Figure 5.8B).

A significant increase in caspase activity was observed when cells were treated with folinic acid compared to folic acid at 4  $\mu\text{g/ml}$ . A similar trend was observed for cells treated with folinic acid compared to folic acid at 40  $\mu\text{g/ml}$  (Figure 5.8C).

1321N1



**Figure 5.8 Comparison of folic acid versus folinic acid treatment on caspase 3/7 activity.**

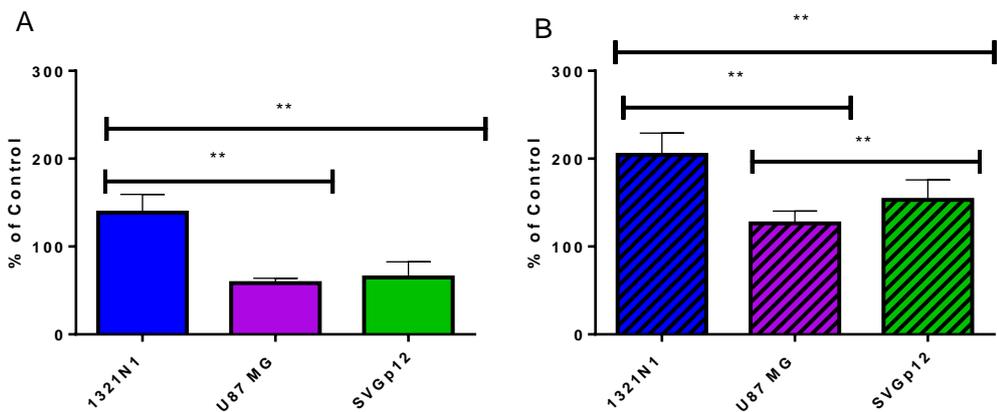
Cells were treated daily for 5 days, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic and folinic acid at 4µg/ml **B)** 1321N1 cells treated with folic and folinic acid at 40µg/ml **C)** U87MG cells treated with folic and folinic acid at 4µg/ml **D)** U87MG cells treated with folic and folinic acid at 40µg/ml **E)** SVGp12 cells treated with folic and folinic acid at 4µg/ml **F)** SVGp12 cells treated with folic and folinic acid at 40µg/ml. The data show that there was a significant difference in caspase 3/7 activity across all cell lines when compared between the folic and folinic acid at both 4µg/ml and 40µg/ml. The data points are means of three replicates (n=3), of three independent experiments, error bars represent ± standard deviation. Statistical analysis was the Students t-test.

### 5.2.6 Comparing the effect of folic and folinic acid treatment on cancerous versus non-cancerous cells

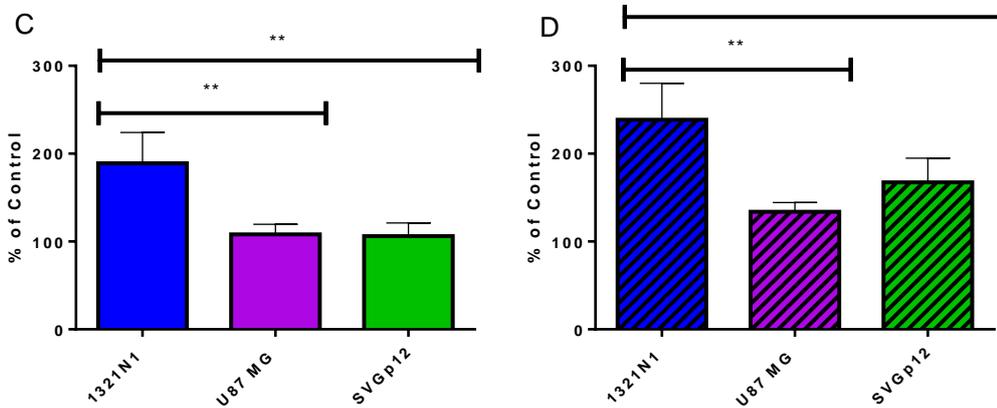
Following treatment with folic acid at 35 µg/ml there was a significant difference in apoptotic activity between the non-cancerous SVGp12 cells and the low grade cancerous 1321N1 cells (Figure 5.9A). There was also a significant difference between the grades of cancerous cells. When the same concentration of folinic acid was used to treat the cells there was a significant difference in levels of apoptosis between the non-cancerous SVGp12 and low grade 1321N1 and high grade U87 MG cells. There was also a significant difference seen between the grades of cancerous cells (Figure 5.9B).

When the cells were treated with a lower concentration of folic acid at 5 µg/ml a similar result was observed, as was seen when the cells were treated with the higher concentration. There was a significant difference in apoptotic activity between the non-cancerous SVGp12 cells and the low grade cancerous 1321N1 cells (Figure 5.9C). There was also a significant difference between the grades of cancerous cells, 1321N1 and U87 MG. When the cells were treated with folinic acid at 5 µg/ml there was a significant difference observed in apoptotic activity between the non-cancerous SVGp12 cells and the low grade 1321N1 cells. There was also a significant difference seen between the two grades of cancerous cells (Figure 5.9D).

35  $\mu\text{g}/\text{ml}$



5  $\mu\text{g}/\text{ml}$



**Figure 5.9 Folic and folinic acid effects on caspase 3/7 activity between cancerous and non-cancerous cells**

Cells were treated daily for 5 days, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4  $\mu\text{g}/\text{ml}$  folic acid. **A)** Cells treated with folic acid at 35  $\mu\text{g}/\text{ml}$  **B)** Cells treated with folic acid at 5  $\mu\text{g}/\text{ml}$  **C)** Cells treated with folinic acid at 35  $\mu\text{g}/\text{ml}$  **D)** Cells treated with folinic acid at 35  $\mu\text{g}/\text{ml}$ . The data show that there was a significant difference in caspase 3/7 activity between non-cancerous, and low grade cancerous cells at both a low (4  $\mu\text{g}/\text{ml}$ ) and high (40  $\mu\text{g}/\text{ml}$ ) dose of folic and folinic acid. The data points are means of nine replicates (n=9), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Tukey's post-hoc test.

### 5.2.7 Caspase 3/7 Activity over 24 Hours

It was important to analyse caspase 3/7 activity over 24 hours following treatment to ascertain whether treatment was causing an initial apoptotic effect because it can take as little as 2-3 hours from the initiation of caspase to completion of the apoptotic cascade (Elmore, 2007).

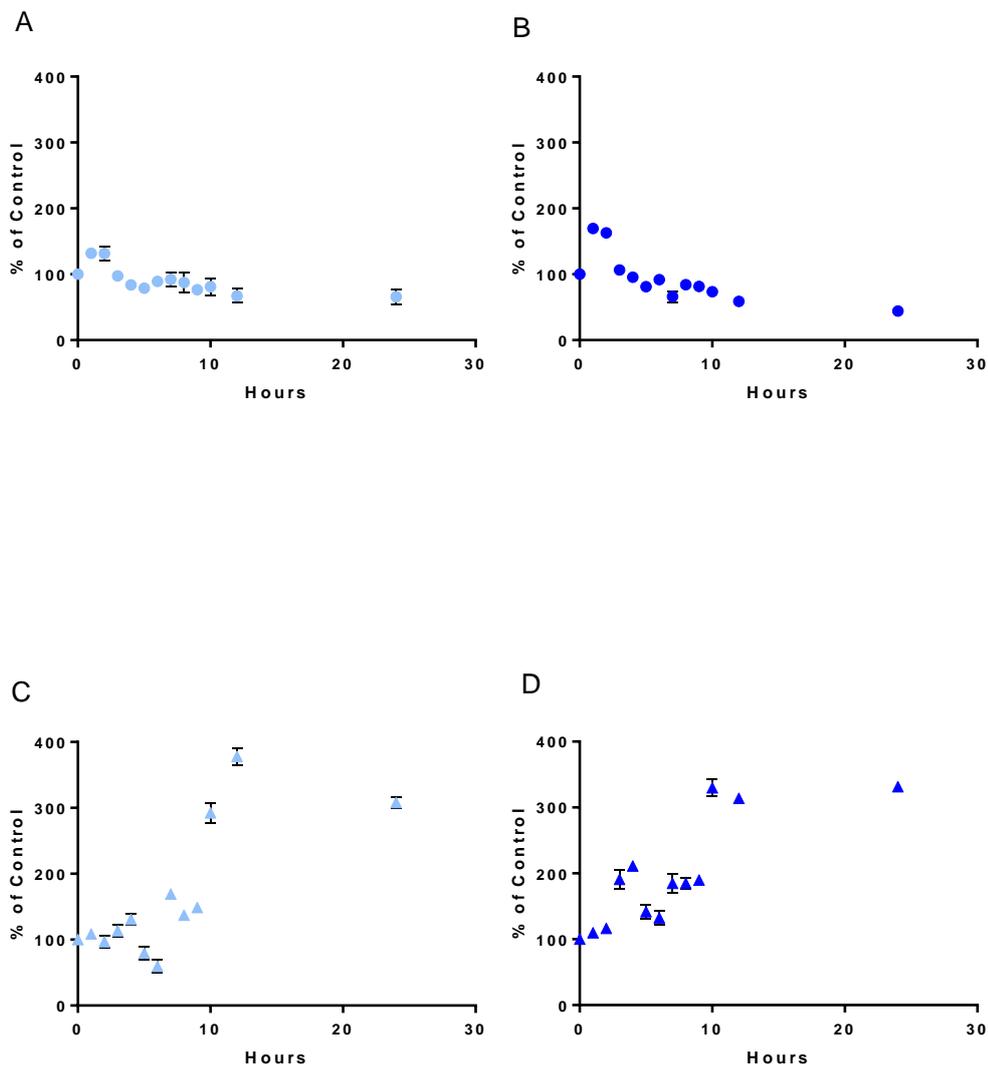
When caspase activity in the treated cells was compared to the untreated control cells, it was seen that 1321N1 cells treated with folic acid at a concentration of 4 µg/ml initially showed a greater level of caspase 3/7 activity for the first 2 hours, then slowly decreased to a level that was lower than the control cells (Figure 5.10A).

A similar response was seen for the 1321N1 cells treated with a high level of folic acid supplementation (40 µg/ml). There was a greater level of caspase activity for the first 2 hours, but again this decreased lower than the control cells (Figure 5.10B). 1321N1 cells treated with folinic acid showed a much greater level of caspase 3/7 activity.

The low dose of folinic acid showed a similar level of caspase activity for the first 4 hours before it decreased to a level lower than the control cells and then greatly increased to almost 400 % of what was seen in the control cells after 12 hours (Figure 5.10C).

The cells treated with the high level of folinic acid maintained a level of caspase activity higher than that of the control cells, peaking after 11 hours at a level around 320 % more than the control cells (Figure 5.10D).

## 1321N1



**Figure 5.10 Caspase 3/7 activity of 1321N1 cells following folic and folinic acid treatment.**

Cells were treated for 24 hours, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 cells treated with folic acid at 4µg/ml **B)** 1321N1 cells treated with folic acid at 40µg/ml **C)** 1321N1 cells treated with folinic acid at 4µg/ml **D)** 1321N1 cells treated with folinic acid at 4µg/ml. The data points are means of three replicates (n=3), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnet's post-hoc test.

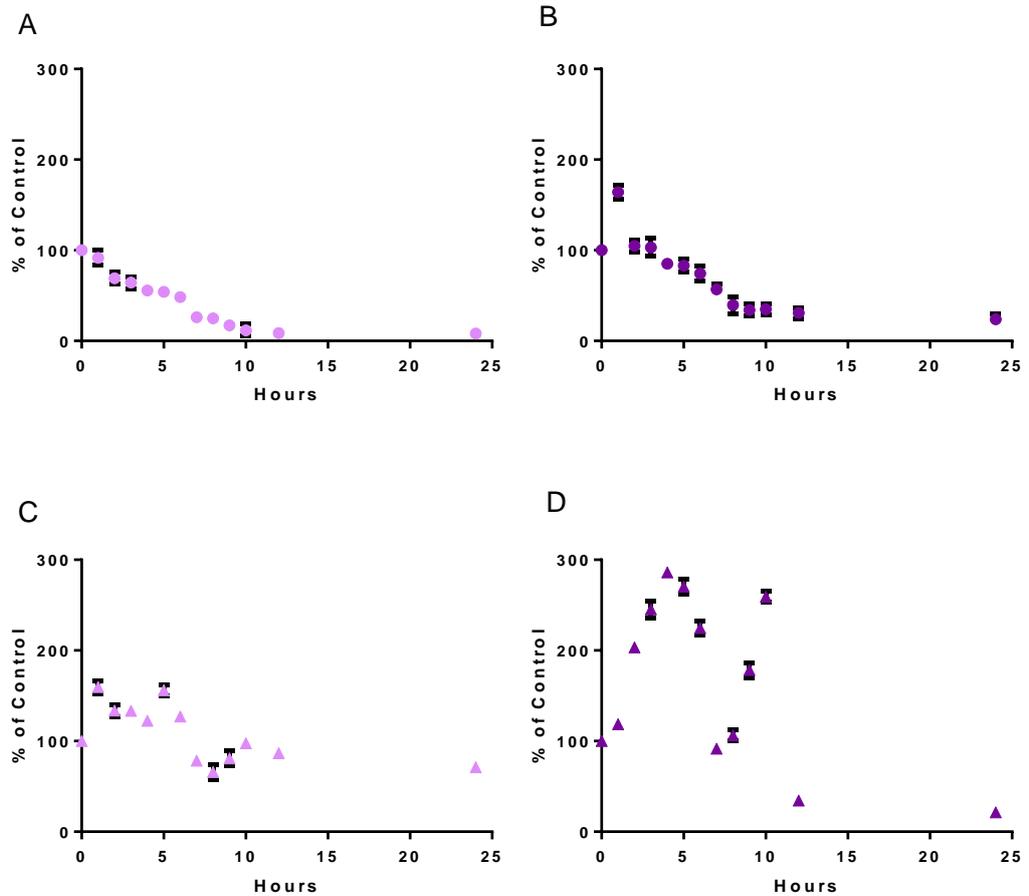
Caspase activity in the treated cells was calculated as a percentage relative to the untreated control cells in standard cell culture conditions. It was observed that U87MG cells treated with folic acid at a concentration of 4 µg/ml showed a lower level of caspase 3/7 activity than the control cells over the 24 hours, which gradually decreased from 1 hour to 24 hours after supplementation (Figure 5.11A).

A similar response was seen for the U87MG cells treated with the high level of folic acid supplementation (40 µg/ml). Although an initial peak in caspase 3/7 activity was seen at the first hour; caspase activity reached a comparable level to the control cells for the next 2 hours before decreasing in activity at a level lower than the control (Figure 5.11B).

U87MG cells treated with low dose folinic acid (4 µg/ml) showed a much greater level of caspase 3/7 activity for the first 6 hours before it decreased to a level lower than the control cells (Figure 5.11C).

The cells treated with the high level of folinic acid (40 µg/ml) maintained a level of caspase activity higher than that of the control cells, with levels peaking 5 hours after supplementation before levels decreased, and then peaked again 11 hours after supplementation at a level around 270 % more than the control cells (Figure 5.11D).

## U87MG



**Figure 5.11 Caspase 3/7 activity of U87MG cells following folic and folinic acid treatment.**

Cells were treated for 24 hours, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing  $4\mu\text{g/ml}$  folic acid. **A)** U87MG cells treated with folic acid at  $4\mu\text{g/ml}$  **B)** U87MG cells treated with folic acid at  $40\mu\text{g/ml}$  **C)** U87MG cells treated with folinic acid at  $4\mu\text{g/ml}$  **D)** U87MG cells treated with folinic acid at  $4\mu\text{g/ml}$ . The data points are means of three replicates ( $n=3$ ), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnet's post-hoc test.

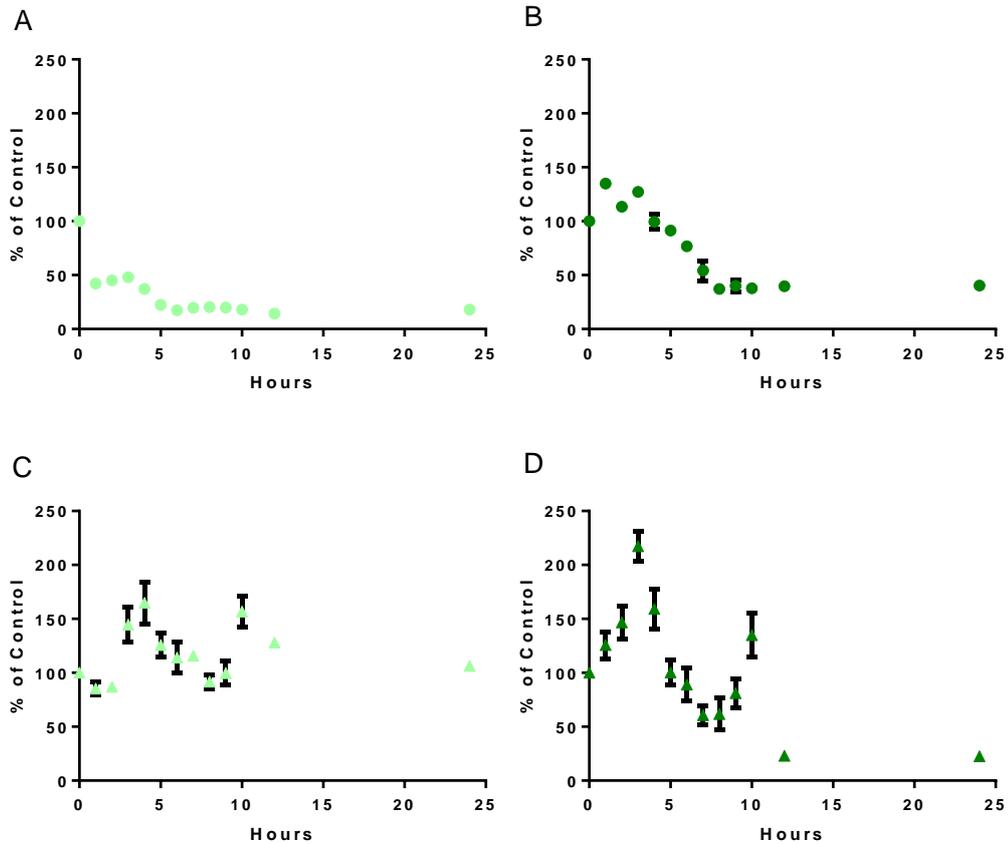
When caspase activity in the treated cells was compared to percentage of caspase activity in the untreated control cells, it was seen that SVGp12 cells treated with folic acid at a concentration of 4 µg/ml showed a much lower level of caspase 3/7 activity than the control cells over the 24 hours (Figure 5.12A).

A similar response was seen for the SVGp12 cells treated with the high level of folic acid supplementation (40 µg/ml). Although an initial peak in caspase 3/7 activity was seen for the first three hours, it then reached a comparable level to the control cells for the next hour before decreasing in activity to a level lower than the control (Figure 5.12B).

SVGp12 cells treated with 4 µg/ml folic acid showed a much greater level of caspase 3/7 activity for the first 4 hours before it decreased to a level similar to that of the control cells before peaking again in activity at 10 hours following supplementation (Figure 5.12C).

The cells treated with the high level of folic acid (40 µg/ml) maintained a level of caspase activity much higher than that of the control cells, with levels peaking 3 hours after supplementation before decreasing, and then peaking again 11 hours after supplementation at a level around 150 % more than the control cells; a lower peak than the initial one seen (Figure 5.12D).

## SVGp12



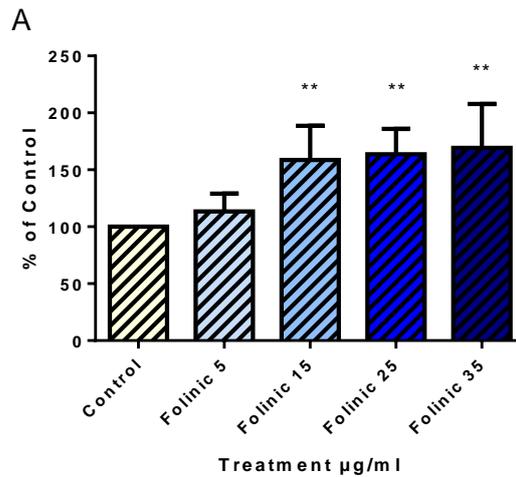
**Figure 5.12 Caspase 3/7 activity of SVGp12 cells following folic and folinic acid treatment.**

Cells were treated for 24 hours, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing  $4\mu\text{g/ml}$  folic acid. **A)** SVGp12 cells treated with folic acid at  $4\mu\text{g/ml}$  **B)** SVGp12 cells treated with folic acid at  $40\mu\text{g/ml}$  **C)** SVGp12 cells treated with folinic acid at  $4\mu\text{g/ml}$  **D)** SVGp12 cells treated with folinic acid at  $4\mu\text{g/ml}$ . The data points are means of three replicates ( $n=3$ ), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnet's post-hoc test.

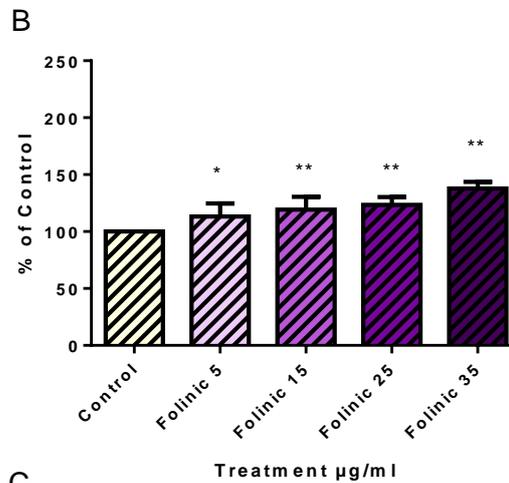
### 5.2.8 Dose-response of folic acid and caspase 3/7 activity

Given that most difference in apoptosis activity was seen in folic acid treated cells, a concentration range of folic acid treatments was prepared and cells were treated for 5 Days, and then levels of caspase 3/7 activity were recorded. 1321N1 cells treated with folic acid show a significant increase in caspase activity when treated with concentration of 15, 25, and 35  $\mu\text{g/ml}$  (Figure 5.13A). A significant increase in caspase activity was seen for U87MG cells when treated with all concentrations of folic acid; 5,15, 25, and 35  $\mu\text{g/ml}$  (Figure 5.13B). SVGp12 cells also showed a significant increase in levels of caspase 3/7 when treated with all concentrations of folic acid; 5,15, 25, and 35  $\mu\text{g/ml}$  (Figure 5.13C). All statistical comparisons were relative to caspase activity in cells subject to standard cell culture conditions (control).

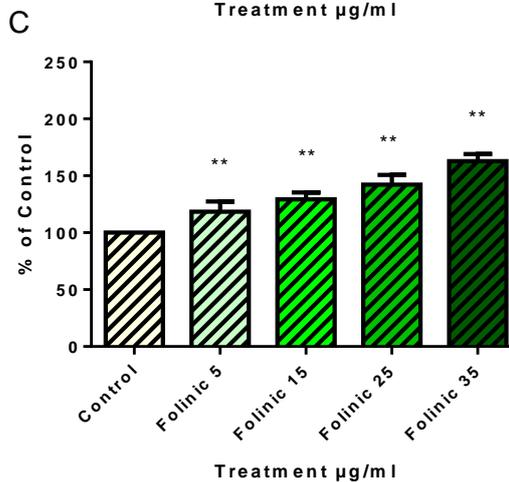
1321N1



U87MG



SVGp12



**Figure 5.13 Caspase 3/7 activity over 24 Hours following folinic acid treatment.** Cells were treated for 24 hours, and caspase 3/7 activity was measured using the Caspase 3/7 Glo assay. Cells were then analysed as a percentage of the control cells which were grown in standard cell culture media containing 4µg/ml folic acid. **A)** 1321N1 caspase 3/7 activity **B)** U87MG caspase 3/7 activity **C)** SVGp121321N1 caspase 3/7 activity. The data points are means of three replicates (n=3), of three independent experiments, error bars represent  $\pm$  standard deviation. Statistical analysis was the ANOVA with Dunnet's post-hoc test.

### 5.3 Discussion

In Chapter 4, it was seen that cells treated with both folic (with the exception of SVGp12) and folinic acid showed a significant decrease in cell viability compared to the untreated control cells. The main aim in this chapter was to ascertain the mechanisms behind this altered cell viability. The reason for this difference in cell viability in the treated cells was hypothesised as being the result of cell cycle arrest. Viability may have appeared to have decreased in treated cells compared to those grown in standard cell culture media, because the cells were no longer proliferating, so it would appear that viable cell numbers had decreased. The second hypothesis was that the reduction in viability was due to increased levels of apoptosis. Cell cycle arrest was considered first due to the varied effect folate supplementation had on cell viability, which differed between the three cell lines (Chapter 4). The flow cytometry results here in Chapter 5 showed that there was no significant difference observed in regards to the number of cells in each cell cycle phase between the cell lines; 1321N1, U87MG and SVGp12. It was also seen that treatment had no effect on the number of cells in each phase in any of the cell lines on either Day 2 or Day 7. This is supported by a previous study which also showed there was no difference in cell cycle phase of human breast tissue in individuals with different plasma folate concentrations (Llanos, Dumitrescu *et al.* 2015). Cell cycle analysis only shows a snapshot of the cells in each phase, given that there was no difference observed between the treated cells and control cells on either Day 2 or Day 7. This would indicate that treatment is not having an effect on factors which could alter cell cycle progression, such as DNA damage, a lack of growth factors and nutrients (Foster *et al.*, 2010). The high level of SVGp12 cells in the S-phase of the cell cycle after both 2 and 7 days of treatment was of particular interest. An increased number of cells in the S-phase will occur when there is DNA damage to the cells which requires repair and increases the length of time spent in the S-phase. (Borel *et al.*, 2002). If the cells cannot be repaired they will remain in

the S-phase. The results seen indicate that treatment is resulting in DNA damage occurring to the SVGp12 cells. The increased number of cells in the S-phase would also explain why the SVGp12s cells were the cell line with the slowest growth kinetics as seen in Chapter 3. Folate metabolism maintains *de novo* synthesis of purines which supports cell proliferation. Impairment can lead to misincorporation of uracil into DNA, resulting in strand breaks, causing the cells to arrest in the S-phase which reduces the rate of cell proliferation. This supports the results seen in Chapter 4 which also showed a reduction in cell proliferation of the SVGp12 cells, and would suggest the mechanism of action was apoptosis activity.

The effect of folate deficiency on cell cycle regulation had hoped to be answered in this chapter, however this was not achieved, as there were no cells growing in folate free conditions when flow cytometry analysis was performed, therefore the first hypothesis was not proven. It would be expected however that cells grown in folate free conditions would suffer cell cycle arrest as indicated by the reduction in cell viability in Chapter 4, which is supported by evidence from the literature (Mason and Choi, 2000).

From previous investigations undertaken by Koury *et al.*, (2000), reduced proliferation following folate deficiency in haematopoietic cells in megaloblastic anaemia was the result of cell cycle arrest, predominantly due to extended S Phase (Koury *et al.*, 2000a). An increased percentage population of cells in the S-Phase following folate depletion was also seen in HepG2 cells and linked to increased apoptosis (Koury and Horne, 1994). Subsequent incubation with a folate supplement was able to reverse this reduction in DNA synthesis (Huang *et al.*, 1999; Koury *et al.*, 2000a), and cells no longer initiated apoptosis. This rescue by folate supplementation can be seen as soon as 6 hours later, although it took 24 hours before cells were at a level to those that had not been grown in folate deficient media (Courtemanche *et al.*, 2004a).

Although there was no alteration to cell cycle following folate treatment, or even an increase in levels of apoptosis from the flow cytometry data, increased apoptosis was still considered, because the unobserved changes may have been due to the method of detection. Using flow cytometry and PI staining to establish levels of apoptosis is not always reliable, for multiple reasons. Cells undergoing apoptosis do not always show DNA fragmentation so may be missed from analysis, and within the hypodiploid peak itself there may also be nuclear fragments and chromosome clumps (Riccardi and Nicoletti, 2006). If G<sub>2</sub>/M or late S phase cells undergo apoptosis the loss of DNA from these cells mean that they will not show in the sub G<sub>1</sub> peak, but instead will be indistinguishable from the G<sub>1</sub>/early S phase (Wlodkovic *et al.*, 2009). Flow cytometry data also only gives a snapshot of cellular activity at a particular moment.

Given the knowledge that cells that spend a prolonged period in the S-Phase then enter the apoptotic pathway, it was then hypothesised that as no alteration to the cell cycle profile was seen, the extended S-Phase may have been missed, and the decrease in cell viability seen in Chapter 4 may still have been the result of increased apoptotic activity.

The results showed that high levels of folic acid supplementation resulted in an increased level of apoptosis compared to the control cells across all three cell lines; 1321N1, U87MG and SVGp12. This correlates with the results seen in Chapter 4, that treatment resulted in decreased levels of proliferation. This increase in levels of apoptosis was also seen in human epithelial cells which observed a relationship between increased folic acid levels in the serum and increased apoptotic activity, however this was not linear (Ghaffari *et al.*, 2016) but this non-linear relationship was also seen in this study (Figure 5.9).

The increase in apoptotic activity following folate treatment has also been seen in the liver of rats fed 25 mg/kg folic acid twice a week (Marsillach *et al.*, 2008). Folic acid supplementation has also been seen to decrease the rate of cell growth of cancerous COL-

205 cells, a human colorectal adenocarcinoma cell line (Kuo *et al.*, 2015), by activating the TP53 signalling pathway, a protein that is regularly linked to apoptosis and cancer. Another study showed that folate supplementation alters apoptosis following an investigation in patients with a gastric lesion. Individuals were given folic acid at a concentration of 10 mg, three times a day for 3 months. This not only served to increase the level of folate in the gastric mucosa, but also increased the level of TP53 expression and the level of epithelial apoptosis occurring (Cao *et al.*, 2005).

The journey from gastrointestinal epithelial tissue to carcinoma occurred with an increasing level of apoptosis inhibition. High levels of TP53 protein were induced by folic acid supplementation, and overexpression of this *TP53* tumour suppressor gene could cause cell cycle arrest and ultimately initiate apoptosis as was seen. Folic acid supplementation does not just alter apoptotic activity in cancerous cells but also significantly decreases apoptosis in neural stem cells (Jia *et al.*, 2008). This is similar to the results observed in this study which demonstrated both the cancerous and non-cancerous cells were affected by folate treatment causing an increase in apoptotic activity. This again involves the TP53 gene, which regulates the expression of p53 in the nucleus, which is known to be frequently mutated in cancerous cells (Bartek *et al.*, 1990; Sherr, 1996).

The U87MG and SVGp12 cells that were treated with a lower dose of Folic acid (4 µg/ml) showed a significant decrease in level of apoptosis compared to the control cells. The cells when treated with the higher dose, 40 µg/ml showed a significant increase in caspase activity (Figure 5.9). This has also been seen in the literature which showed that when extravillous trophoblast cells were supplemented with folic acid there was an increase in apoptosis and reduced proliferation when concentrations were in the micro molar ( $10^{-6}$ ) range, however when concentration were reduced to nano molar ( $10^{-9}$ ) range, there was a reverse effect, apoptosis decreased and proliferation increased (Williams *et al.*, 2011). Folic acid has a

molecular weight of 441.4 g/mol which means that the low dose had a molarity of 9.1  $\mu$ M whilst the high dose was 90.6  $\mu$ M. Folinic acid has a molecular weight of 473.4 g/mol which calculated to a low and high dose concentration of 8.4  $\mu$ M and 84.5  $\mu$ M respectively, so an increase in apoptosis would expect to be seen, which was observed.

The levels of caspase activity were different between the three cell lines; 1321N1, U87MG and, SVGp12 (Figure 5.9), which is to be expected given the difference in cell type. This supports the results shown in Chapter 4, which showed that cell viability was altered amongst the different cell lines. The results seen, correlate with other studies that have shown that the effect of folate deficiency on gene expression in genes involved in apoptosis is not only highly cell specific, but the resulting changes seen in expression vary in level between cell lines (Novakovic *et al.*, 2006). Temozolomide, a known chemotherapeutic therapy for glioblastoma, aids patient survival by increasing initiation of apoptosis, observed as an increase in caspase 3 activity (Shi *et al.*, 2010). Glioma progression is as the result of increased cell proliferation, which can be as the result of microglia which promote cell growth and invasion in gliomas (Hambardzumyan *et al.*, 2016), which are frequently found at the edge of the tumour, apoptosis does still occur. This imbalance between cell proliferation and level of apoptotic activity is grade dependent, with the greatest imbalance seen at the higher grades. Studies have shown that there is less caspase 3 activity in the higher grade tumours (Kobayashi *et al.*, 2007). This could indicate why folate treatment had a smaller effect on the levels of caspase 3/7 recorded in the high grade U87MG cell line than the lower grade 1321N1 cell line. If there was an initial lower level of caspase activity occurring, it would be expected that this lower base level would be observed following treatment. Studies have shown that in general, apoptosis is inhibited in glioblastomas (Tirapelli *et al.*, 2010), but it should be noted that the cell death that does occur in malignant glioma is as the result of apoptotic activity rather than necrosis (Kleihues *et al.*, 2002).

When cells were treated with folic acid there was an increased level of apoptosis across all three cell lines when treated with both low and high levels in a dose dependant manner. This dose dependant relationship was seen in colon cancer cells which showed increased apoptotic activity following treatment, this time with folic acid at a concentration of 0, 10, 50 and 100 mg/ml (Attias *et al.*, 2006).

When the cells were grown in folate free conditions, apoptosis also increased, which was also seen in another study involving HeG2 cells (Abdel Nour *et al.*, 2007). Folate deficiency results in an increased accumulation of DNA damage in the neurons which leads to an increased level of apoptosis (Kruman *et al.*, 2002).

Although apoptosis levels were measured after Day 5, a 24 hour reading was also undertaken to see what effect supplementation would initially have, given that the half-life of folic acid is around 2 hours (Loew *et al.*, 1987), whilst the half-life for reduced folates such as folic acid is 6.2 hours (Drugs.com, 2016). As previously mentioned a rescue by folate supplementation can be seen as soon as 6 hours later, although it took 24 hours before cells were at a level to those that had not been grown in folate deficient media (Courtemanche *et al.*, 2004a). This difference in half-life between folic and folic acid may also explain the significant difference in apoptosis levels seen between the two forms.

It should be noted that there is a disadvantage to using caspase as an indicator of apoptotic activity. There have been studies that showed that caspase 3 and 7 can be activated by caspase 8 and not produce an apoptotic effect (Burguillos *et al.*, 2011; Shen *et al.*, 2016). Another way to measure levels of apoptosis in the future could be through western blotting, using an anti-PARP antibody, which is an early indicator of apoptosis (Attias *et al.*, 2006). Another method would be to stain cells with monoclonal antibodies to detect single stranded DNA, which is an indicator of early apoptosis (Frankfurt, 2004). Caspase 3/7 activity however

was used to assess level of apoptotic activity as it is the most frequently used method within the literature.

In summary, this chapter aimed to ascertain whether the reduction in cell viability observed in Chapter 4, was as the result of cell cycle arrest, or increased apoptotic activity. The results in this chapter conclude that this alteration was as the result of increased levels of apoptosis. In regards to the first hypothesis that cells treated with folate would present with an increased number of cells in the G<sub>1</sub> phase due to cell cycle arrest, this was disproven as no significant difference was observed in cell cycle profile following treatment.

In terms of hypothesis two, that there would be a higher level of apoptosis for all treatments, but particularly for cells treated with folic acid, this was proven. All three cell lines treated with both folic and folic acid showed increased levels of apoptotic activity, which was significantly greater following folic acid treatment when the two forms were compared. This increase in apoptotic activity was expected due to the decreased in cell proliferation seen in Chapter 4 following treatment with folic and folic acid.

Progression through the phases of the cell cycle is regulated by different cyclin-dependent kinase (CDK) complexes, whose expression can be altered by changes to methylation of the promoter, which can lead to cancer (Llanos *et al.*, 2015). This is of particular interest given that folate is a methylating agent and has been seen to alter the methylation status of other genes (Chapter 1.9). It was then hypothesised that folate treatment may therefore be able to modify methylation status of the promoter regions of different genes, this area will be discussed in more detail in Chapter 6.

# CHAPTER 6

## 6 METHYLATION STATUS ANALYSIS OF GLIOMA CELLS FOLLOWING FOLATE TREATMENT.

### 6.1 Introduction

The results from Chapters 4 and 5 showed a change in cell viability and apoptosis following folate treatment, which may be due to altered gene expression. Gene expression is controlled in different ways, including, but not limited to; RNA transcription and DNA methylation (Phillips and Goodman, 2008) so, methylation status of the folate transporters and metabolising enzymes may explain the differing response to folic or folinic acid treatment measured by changes in cell viability and apoptosis previously reported in Chapter 4 and 5. DNA methylation is the predominant epigenetic alteration that occurs within human DNA, whereby a methyl group is added to the 5' position of a cytosine or guanine nucleotide (Thon *et al.*, 2013). DNA methylation has both direct and indirect effects; directly by repressing transcription, via inhibition of specific transcription factors binding, and indirectly by recruiting methyl CpG binding proteins (Robertson, 2005). CpG islands are a C (cytosine) base followed immediately by a G (guanine) base (CpG). DNA methylation is associated with transcriptional silencing, and works by locking genes in to an 'off' position (Phillips and Goodman, 2008). In normal cells DNA methylation occurs in repetitive genomic regions: satellite DNA, long interspersed transposable elements (LINES) and short interspersed transposable elements, (SINES) (Robertson, 2005). These selected nucleotides tend to be found in 'CpG islands' which occur in the promoter region of more than 50% of all human genes.

CpG islands are small stretches of nucleotide bases, 300-3000 bp in length, and tend to be non-methylated within the coding region of gene, so gene expression can occur, and methylated in the non-coding regions. The first study to show that methylation had an effect on gene expression was by McGhee and Ginder (1979) who looked at chicken beta-globin

locus cells (McGhee and Ginder, 1979). They found a correlation between DNA methylation patterns and the subsequent activity of individual genes. This study was followed up by another research group in 1980 (Jones and Taylor, 1980) who noted that inhibition of DNA methylation induced formation of muscle cells.

The link between DNA methylation and cancer was first seen in 1983 (Gama-Sosa *et al.*, 1983), when it was observed that cancer cells presented with more hypomethylation than the matched non-cancerous cells which subsequently resulted in genomic instability, a hallmark of cancerous cells (Feinberg and Tycko, 2004). There are many cancers which are affected by the altered methylation of specific genes as summarised in Table 6.1.

**Table 6.1 Cancers affected by methylated genes.**

Gene	Abb	Cancer	Effect	Reference
Melanoma Antigen	MAGE	Testis Cancer	Frequently demethylated and re-expressed in cancer	(De Smet <i>et al.</i> , 1999)
S100 calcium binding protein A4	S100A4	Colon Cancer	Demethylation and increased expression in cancer	(Nakamura and Takenaga, 1998)
Serine Protease Inhibitor Gene	SERPIN5	Gastric Cancer	Demethylation and increased expression in cancer	(Akiyama <i>et al.</i> , 2003)
Putative Oncogene $\gamma$ -synuclein	SNCG	Breast and Ovarian Cancer	Demethylation and increased expression in cancer	(Gupta <i>et al.</i> , 2003)

DNA methylation information is a useful therapeutic tool, as patient response to different therapies can vary dependent upon the methylation status of affected genes (Thon *et al.*, 2013). DNA methylation also has an effect on genomic imprinting, an epigenetic modification

of a specific parental chromosome in the gamete or zygote that leads to a differential expression of the two alleles of a gene in the somatic cells of the offspring (Feinberg *et al.*, 2002).

There have already been genetic markers identified to aid glioma characterisation, the isocitrate dehydrogenase (*IDH*) gene was one of the first molecular alterations identified to occur in glioma formation, and is used to differentiate between *IDH* wildtype and *IDH* mutant which were previously referred to as primary and secondary glioblastomas respectively (Louis *et al.*, 2016a; Thon *et al.*, 2013). Much has already been done to research the methylation status of the DNA repair enzyme, O<sup>6</sup>-methylguanine–DNA methyltransferase (*MGMT*) which works by removing alkyl adducts from the O<sup>6</sup> position of guanine, which if allowed to remain can result in base mispairing, double stranded breaks which ultimately lead to unplanned apoptosis (Zhang *et al.*, 2013). The *MGMT* promoter region is frequently methylated in glioblastomas, 35-45% in grade III and IV tumours, and 80% in grade II tumours (Thon *et al.*, 2012). Survival of patients with a methylated *MGMT* promoter was almost double those with an unmethylated promoter (Hegi *et al.*, 2005). Individuals with a hypermethylated *MGMT* gene correlated with an improved survival outcome (Esteller, 2002; Rivera *et al.*, 2010). This increased survival was the result of the effects of alkylating chemotherapeutic drug temozolomide (TMZ), which alkylates the N<sup>7</sup> or O<sup>6</sup> positions of guanine residues, damaging the DNA of cells, and ultimately forcing the tumour cells into apoptosis. Patients with a methylated *MGMT* promoter have an inactivated gene, which means that the tumours cells are not able to repair the damage caused by TMZ, and therefore the chemotherapeutic treatment is more effective (Hegi *et al.*, 2005).

Given that there has already been so much work analysing the methylation status of the *MGMT* gene, it was not chosen for this study. Other genes which have also seen to be aberrantly methylated in glioblastoma include; *CDKN2A*, *PTEN* and *TP53* (Amatya *et al.*, 2005;

Bello and Rey, 2006; Costello *et al.*, 1996; Nakamura *et al.*, 2001; Watanabe *et al.*, 2001), so *PTEN* was chosen as a positive control in the study here.

The promoter region of the gene was chosen for analysis here, given that there are so many studies which show the presence of promoter-associated CpG island hypermethylation in human glioma (Kim *et al.*, 2006b; Martinez *et al.*, 2009; Martinez *et al.*, 2007; Nagarajan and Costello, 2009; Stone *et al.*, 2004; Tepel *et al.*, 2008; Uhlmann *et al.*, 2003a). Promoter CpG island hypermethylation tends to result in transcriptional silencing of the gene, and is a potential useful biomarker (Noushmehr, Weisenberger *et al.* 2010). The genes were chosen as discussed in Section 1.11.3.

### 6.1.1 Aim of Chapter

The aim of this chapter was to assess the effect the different forms of folic and folinic acid had on the methylation status of the chosen genes of interest: *FR1*, *RFC*, *PCFT*, *PTEN* and *MTHFR*.

### 6.1.2 Hypothesis

Methylation status is known to have an effect on gene expression, and can be a key marker of difference between not only cancerous and non-cancerous tissues, but also between grades of tumour. Given the role folate has in the methylation cycle, it was hypothesised that:

#### ***Hypothesis One***

Cells treated with folic or folinic acid can alter the methylation status compared to cells grown in standard cell culture media

### ***Hypothesis Two***

The methylation response to folic and folinic acid treatment will differ depending on the cell line because of the documented altered methylation patterns between cancer and non-cancerous cell and between low and high grade glioma (Uhlmann, Rohde *et al.* 2003).

### ***Hypothesis Three***

Methylation status of folate transporters and metabolising enzymes may explain the differing response to folic or folinic acid treatment measured by changes in cell viability and apoptosis previously reported in Chapter 4 and 5.

#### **6.1.3 Objectives**

**Objective 1.** Examine whether daily dosing with folic or folinic acid affects methylation status of genes of interest after 5 days in cell lines compared to standard cell culture conditions.

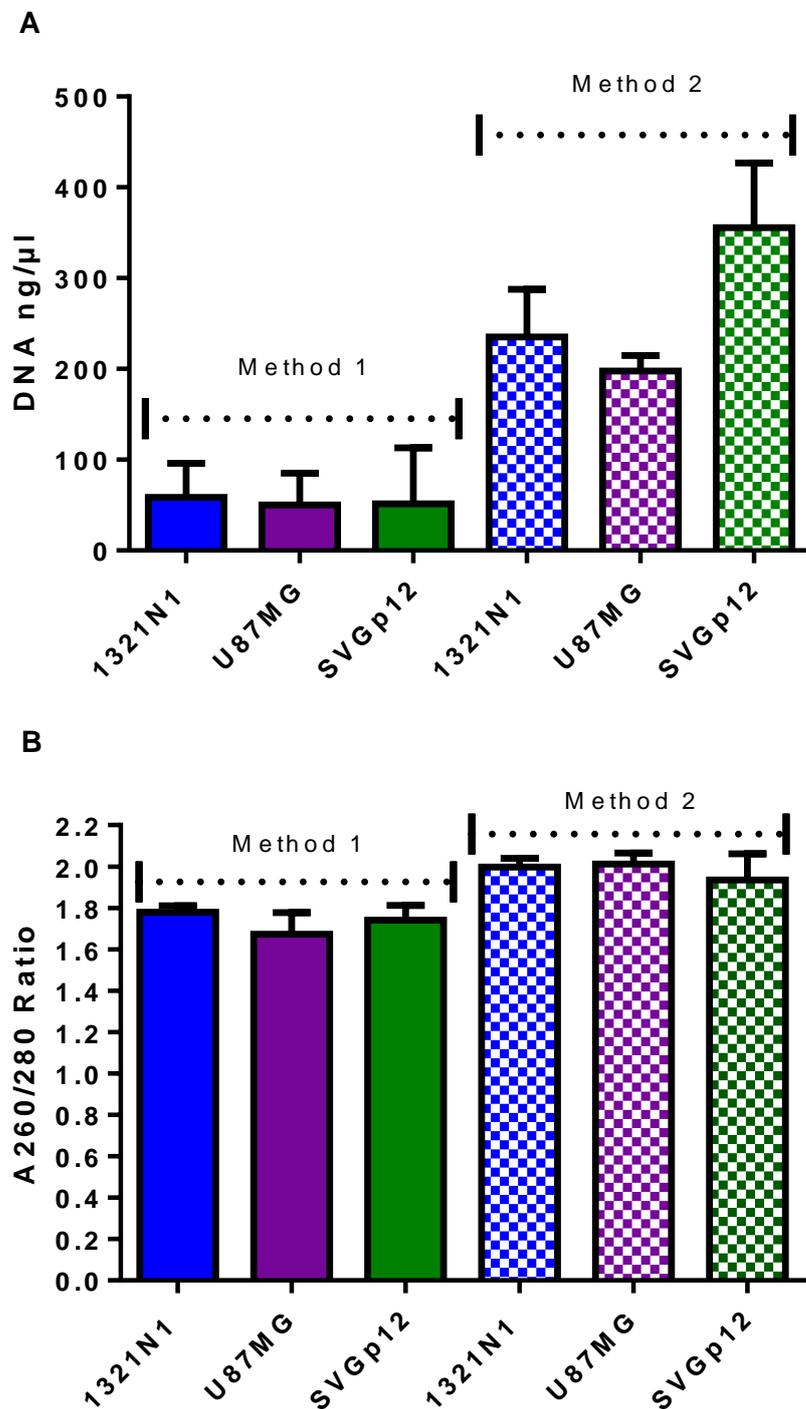
**Objective 2.** Examine whether methylation status is different between cancerous and non-cancerous cells.

**Objective 3.** Examine whether methylation status is different between different grades of tumour.

## 6.2 Results

Two different methods were chosen to extract genomic DNA from cultured cells for use to analyse methylation status of the genes of interest because it was essential the purest DNA was used for MS-PCR. The first method was Puregene DNA Extraction, described in Section 2.8.1. The second method used was the QIAmp DNA Extraction method detailed in Section 2.8.2. The ideal purity should have an absorbance ratio 260 nm / 280 nm (A260/280) of around 1.8 – 2.0. The first method, was not as efficient at extracting DNA as the second method, as indicated by a mean 17-fold higher extraction (Figure 6.1A). The DNA extracted using the first method, also did not meet the required mean A260/280 ratio threshold of 1.8, so the second method was continued for all future DNA extractions (Figure 6.1B).

## 6.2.1 DNA Extraction Optimisation



**Figure 6.1 Comparison of DNA extraction efficiency and purity.**

DNA was extracted using two different DNA extraction kits; Genra Puregene (Qiagen) and QIAmp DNA mini kit (Qiagen) to assess. **A)** DNA Quantity **B)** DNA Purity. A value of 1.8-2.0 is considered pure DNA. The data points are means of nine replicates ( $n=9$ ), from one independent experiment. Error bars represent  $\pm$  standard deviation.

### 6.2.2 Primer Control Results

Each primer was tested against complementary DNA, and uncomplementary DNA to ensure that any bands seen were specific to the methylation status. For all gels shown in Figure 6.2, the primers specific for methylated genes only amplified methylated DNA and the primers specific for unmethylated DNA only amplified unmethylated DNA.

The *PTEN* gene was expected to produce a band with length 168 bp in the methylated sample and 165 bp in the unmethylated sample. The band observed in Lanes 1-3 confirm that the methylated primers were specific for the methylated *PTEN* promoter (Figure 6.2A). The empty lanes in 4-6 show that the primers were specific for methylation and did not pick up the unmethylated *PTEN* promoter. The band observed in Lanes 7-9 confirm that the unmethylated primers were specific for the unmethylated *PTEN* promoter (Figure 6.2A). The empty lanes in 10-12 show that the primers were specific for unmethylated promoter and did not pick up the methylated *PTEN* promoter.

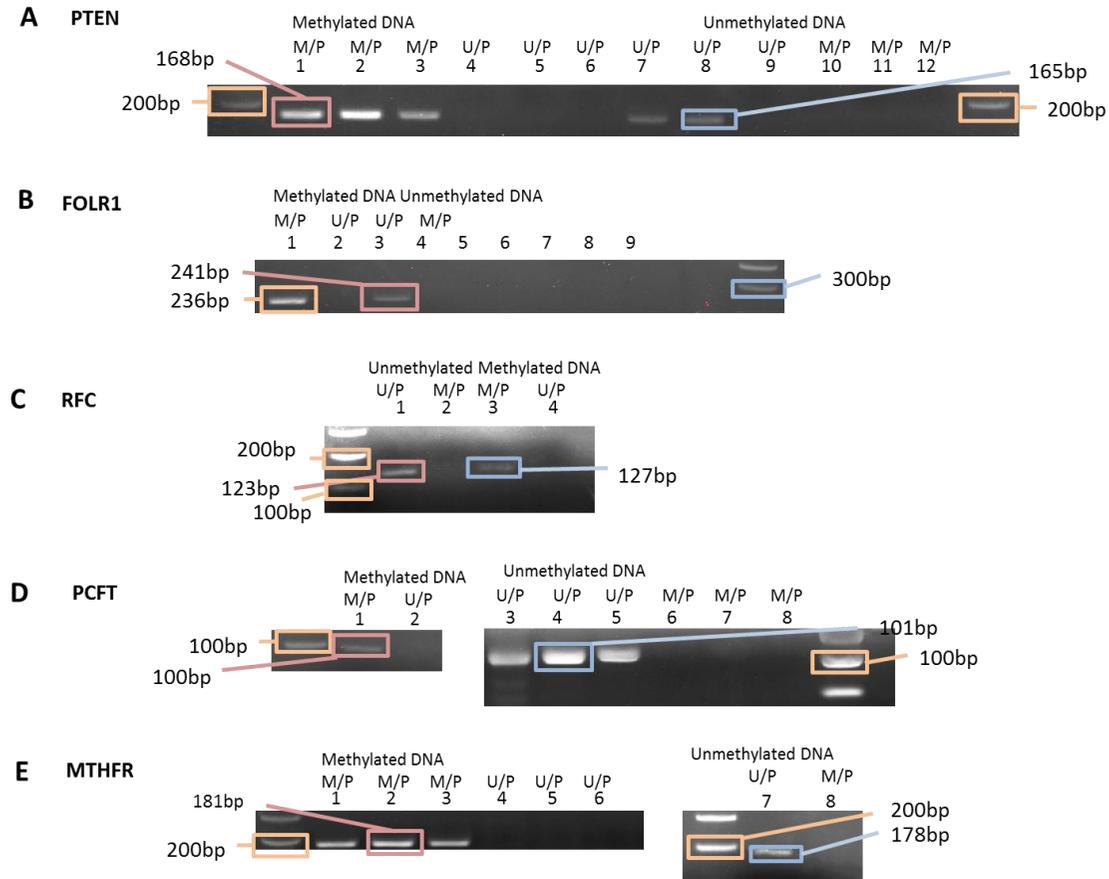
The *FOLR1* gene was expected to produce a band with length 236 bp in the methylated sample and 241 bp in the unmethylated sample. The band observed in Lane 1 confirms that the methylated primers were specific for the methylated *FOLR1* promoter (Figure 6.2B). The empty Lane 2 shows that the primers were specific for methylation and did not pick up the unmethylated *FOLR1* promoter. The band observed in Lane 3 confirms that the unmethylated primers were specific for the unmethylated *FOLR1* promoter (Figure 6.2B). The empty lane in 4 shows that the primers were specific for unmethylated promoter and did not pick up the methylated *FOLR1* promoter. The empty lanes 5 – 9 were blank wells.

The *RFC* gene was expected to produce a band with length 127 bp in the methylated sample and 123 bp in the unmethylated sample. The band observed in Lane 1 confirms that the methylated primers were specific for the methylated *RFC* promoter (Figure 6.2C). The empty

Lane 2 shows that the primers were specific for methylation and did not pick up the unmethylated *RFC* promoter. The band observed in Lane 3 confirms that the unmethylated primers were specific for the unmethylated *RFC* promoter (Figure 6.2C). The empty lane in 4 shows that the primers were specific for unmethylated promoter and did not pick up the methylated *RFC* promoter.

The *PCFT* gene was expected to produce a band with length 100 bp in the methylated sample and 101 bp in the unmethylated sample. The band observed in Lane 1 confirms that the methylated primers were specific for the methylated *PCFT* promoter (Figure 6.2D). The empty Lane 2 shows that the primers were specific for methylation and did not pick up the unmethylated *PCFT* promoter. The band observed in Lanes 3-5 confirm that the unmethylated primers were specific for the unmethylated *PCFT* promoter (Figure 6.2D). The empty Lanes in 6-8 show that the primers were specific for unmethylated promoter and did not pick up the methylated *PCFT* promoter.

The *MTHFR* gene was expected to produce a band with length 181 bp in the methylated sample and 178 bp in the unmethylated sample. The bands observed in Lanes 1-3 confirm that the methylated primers were specific for the methylated *MTHFR* promoter (Figure 6.2E). The empty lanes 4-6 show that the primers were specific for methylation and did not pick up the unmethylated *MTHFR* promoter. The band observed in Lane 7 confirms that the unmethylated primers were specific for the unmethylated *MTHFR* promoter (Figure 6.2E). The empty lane in 8 shows that the primers were specific for unmethylated promoter and did not pick up the methylated *MTHFR* promoter.



**Figure 6.2 Primer quality control testing.**

Methylated and unmethylated primers were tested against methylated and unmethylated control human DNA (Qiagen) to ensure specificity. **A) PTEN B) FOLR1 C) RFC D) PCFT E) MTHFR.** Where M/P means methylated primers and U/P means unmethylated primers.

A ladder was run to ensure that the band observed matched the expected PCR product size.

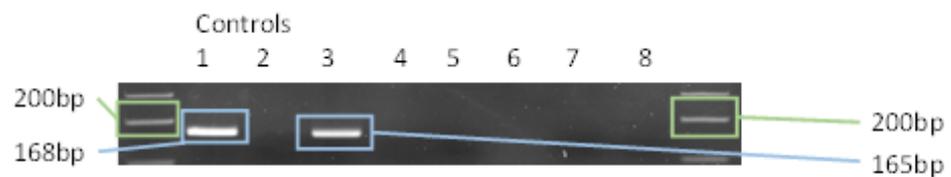
All PCR product gave bands at the expected size as detailed in Table 6.2

**Table 6.2 Expected product sizes**

<b>Gene</b>	<b>Methylated Band Size</b>	<b>Unmethylated Band Size</b>
FOLR1	236	241
PCFT	100	101
RFC	127	123
MTHFR	181	178
PTEN	168	165

### 6.2.3 Quality Control Results

To determine that MS-PCR was producing the expected results, a quality control gel was run for every MS-PCR, using the PTEN primer as a positive control. Controls were performed using the Qiagen Epi Controls, to ensure specificity of primers amplifying methylated, and unmethylated DNA and to check for bisulphite conversion success by including unbisulphite treated DNA. As can be seen in Figure 6.3, no non-specific bands were observed when methylated primers were incubated to control unmethylated DNA (Lane 2) and *vice versa* (Lane 4) and no contaminating bands were observed in the template and enzyme free controls (Lane 5 and 6). The absence of bisulphite conversion prevented any MS-PCR product formation (Lanes 7 and Lanes 8).



1. Methylated Primers with Control Methylated DNA
2. Methylated Primers with Control Unmethylated DNA
3. UnMethylated Primers with Control Unmethylated DNA
4. UnMethylated Primers with Control Methylated DNA
5. No Template DNA Control
6. No Enzyme Control
7. Unbisulfite treated DNA with UnMethylated Primers
8. Unbisulphite treated DNA with Methylated Primers

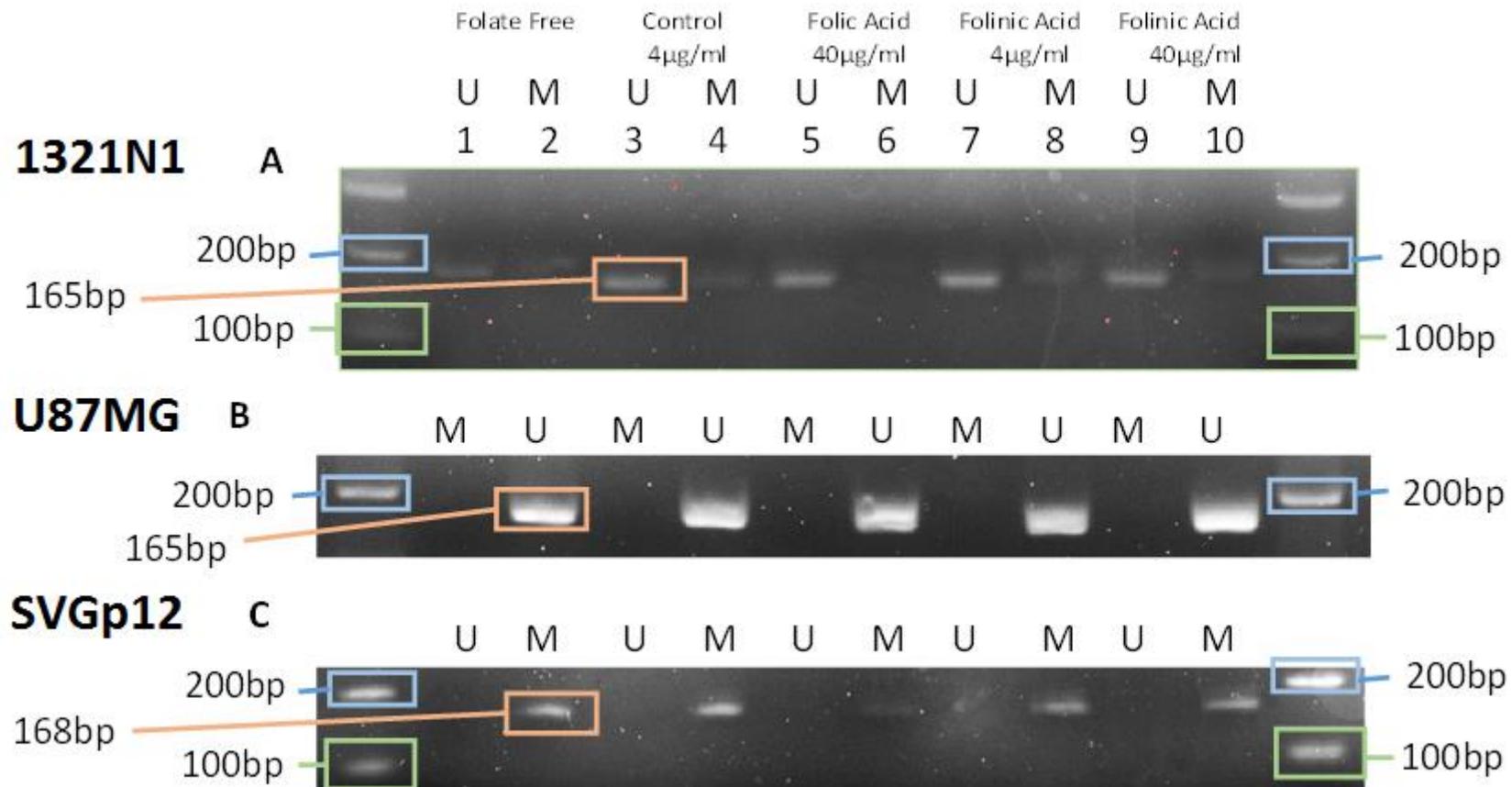
**Figure 6.3 MS-PCR Control Gel**

To ensure the specificity of primers (1-4), contaminate free samples (5-6), and successful bisulphite conversion (7-8), control samples were run for every PCR performed. Primers were used with Qiagen Epi Control DNA. PCR products were then visualised on an agarose gel.

## 6.2.4 Methylation status of cells treated with folic or folinic acid compared to cells grown in standard cell culture media

### 6.2.4.1 Phosphate and tensin homolog protein (PTEN)

For the 1321N1 cell line (Figure 6.4A) in all samples amplification of the *PTEN* promotor, was achieved with both primers, specific for methylated DNA and for unmethylated DNA, and the expected lengths 168 bp and 165 bp respectively, and therefore these samples were classified as hemimethylated as shown by a faint band in both the methylated (Lanes 2, 4, 6, 8, 10) and unmethylated lanes (Lanes 1, 3, 5, 7, 9)). The unmethylated PTEN appeared stronger in treated cells (Lanes 4, 6, 8 and 10) compared to the folate free conditions (Lane 2). There did not appear to be a difference in methylated PTEN between any of the treatment groups. The higher grade glioma, U87MG showed different methylation patterns with complete methylation of the *PTEN* promoter and no difference in methylation between the treatment groups (Figure 6.4B). The SVGp12 cell line representing the non-cancerous cells showed complete methylation of PTEN and little difference between treatment groups.



**Figure 6.4 Methylation status of the PTEN gene.**

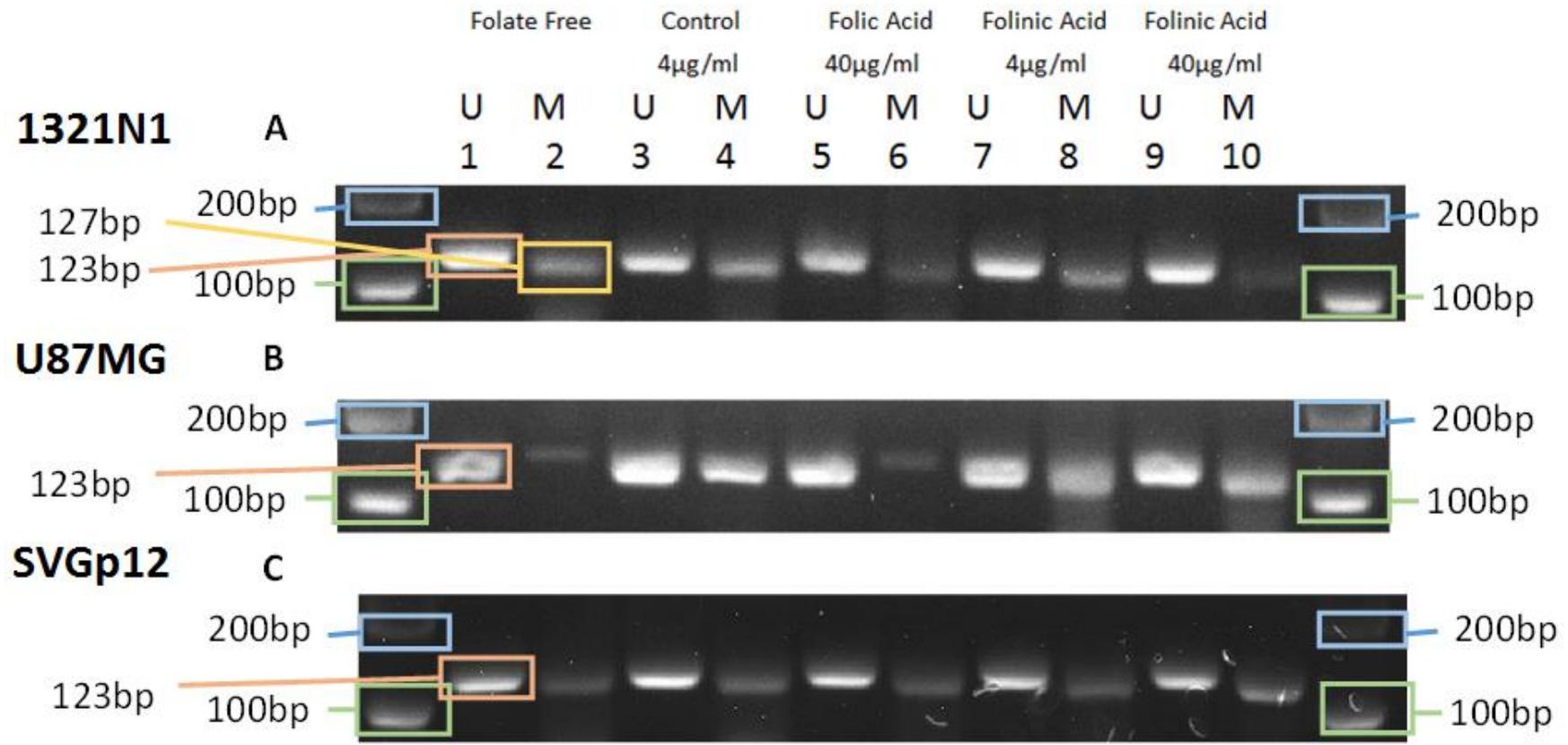
Cells were treated for 5 days before genomic DNA was extracted using the QIAmp DNA mini kit, and then bisulphite converted using the EZ DNA Methylation-Gold Kit. Methylated and unmethylated PTEN primers were tested against the DNA obtained from treated cells of the (A) 1321N1, (B) U87MG and (C) SVGp12 cell lines. Three independent experiments were performed showing the same findings.

#### 6.2.4.2 *Reduced Folate Carrier*

The 1321N1 cell line, showed hemimethylation of the *RFC* promoter (Figure 6.5A), although there is some discrepancy in the length of PCR product for the methylated primer, which alters between the gels, in some presenting with a shorter length than the unmethylated sample (Figure 6.5A and C), in another appearing at a longer length (Figure 6.5B). Following treatment of 1321N1 cells at higher concentrations of both folic and folinic acid, had a higher ratio of unmethylation than methylation of the *RFC* promoter was observed (Figure 6.5A Lanes 5 and 9).

The high grade, U87MG cell line grown in folate free media showed a greater level of unmethylation in the *RFC* promoter region compared to U87MG cells grown in standard cell culture media (Figure 6.5B Lane 1). A similar response was observed for the U87MG cells treated with folic acid at 40 µg/ml (Figure 6.5B Lane 5 and 6). A more even balance was seen for hemimethylation in Lanes 7 and 8, and 9 and 10, for the U87MG cells treated with low and high concentrations of folinic acid.

The SVGp12 cell line showed hemi methylation of the *RFC* promoter in the control cells, those grown in folate free conditions, and those treated with folic and folinic acid (Figure 6.5C). There did not appear to be any difference between treatment groups.

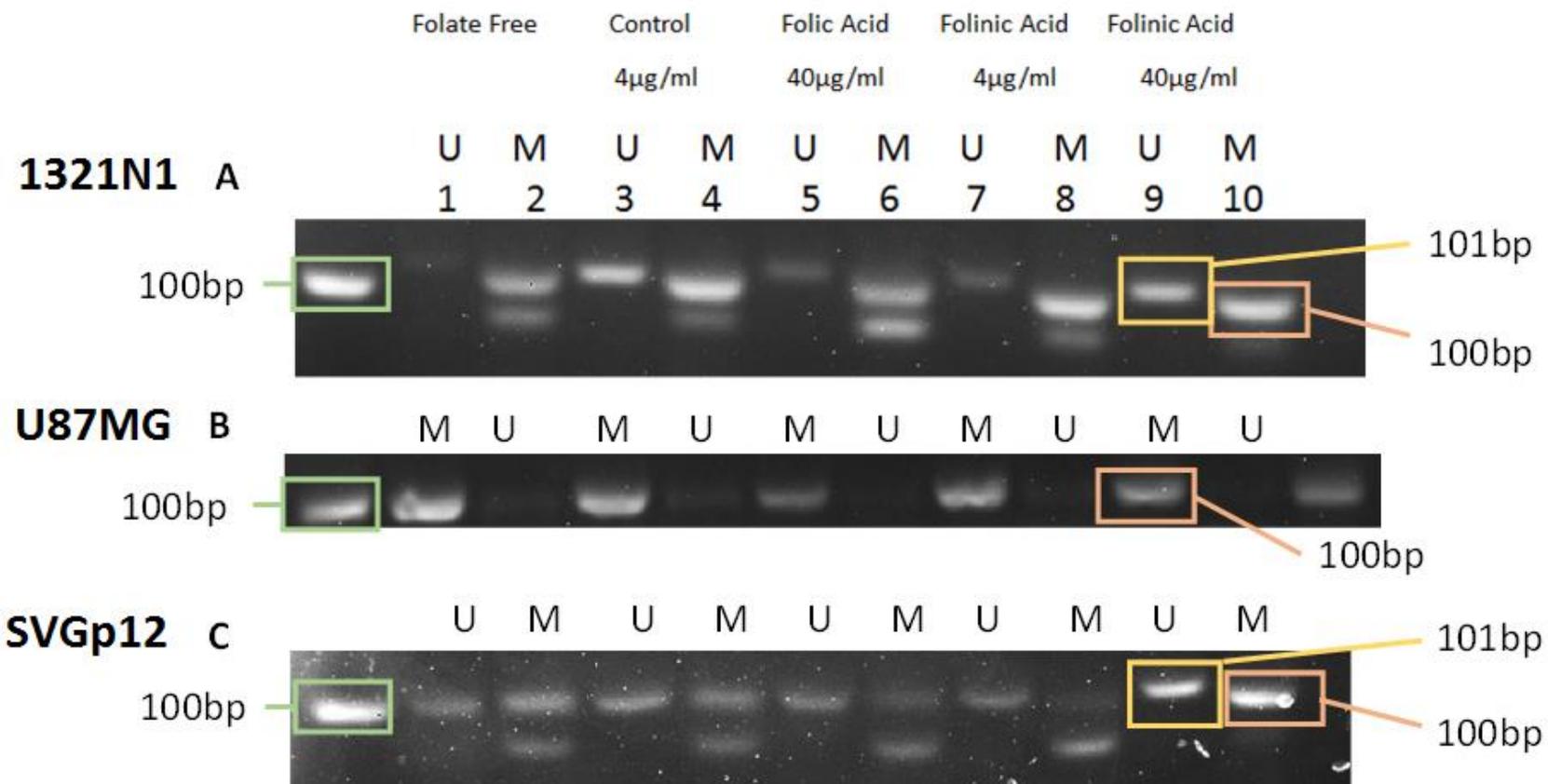


**Figure 6.5 Methylation status of the RFC gene.**

Cells were treated for 5 days before genomic DNA was extracted using the QIAmp DNA mini kit, and then bisulphite converted using the EZ DNA Methylation-Gold Kit. Methylated and unmethylated RFC primers were tested against the DNA obtained from treated cells of the (A) 1321N1, (B) U87MG and (C) SVGp12 cell lines. Three independent experiments were performed showing the same findings.

### 6.2.4.3 Proton Coupled Folate Transporter

The 1321N1 cell line showed a general trend for hemimethylation of the *PCFT* promoter (Figure 6.6A Lanes 3-10), with a double band observed in the methylated lanes (Lanes 2, 4, 6, 8, 10) The cells grown in folate free media showed methylation (Lane 2). The high grade, U87MG cell line showed that there was a trend towards greater methylation of the *PCFT* promoter in the cells grown in folate free conditions which was comparable to the control cells (Lanes 1 and 2, and 3 and 4). The SVGp12 cell line showed hemimethylation of the *PCFT* promoter, this hemimethylation was unaffected by treatment (Figure 6.6C Lanes 1-10).

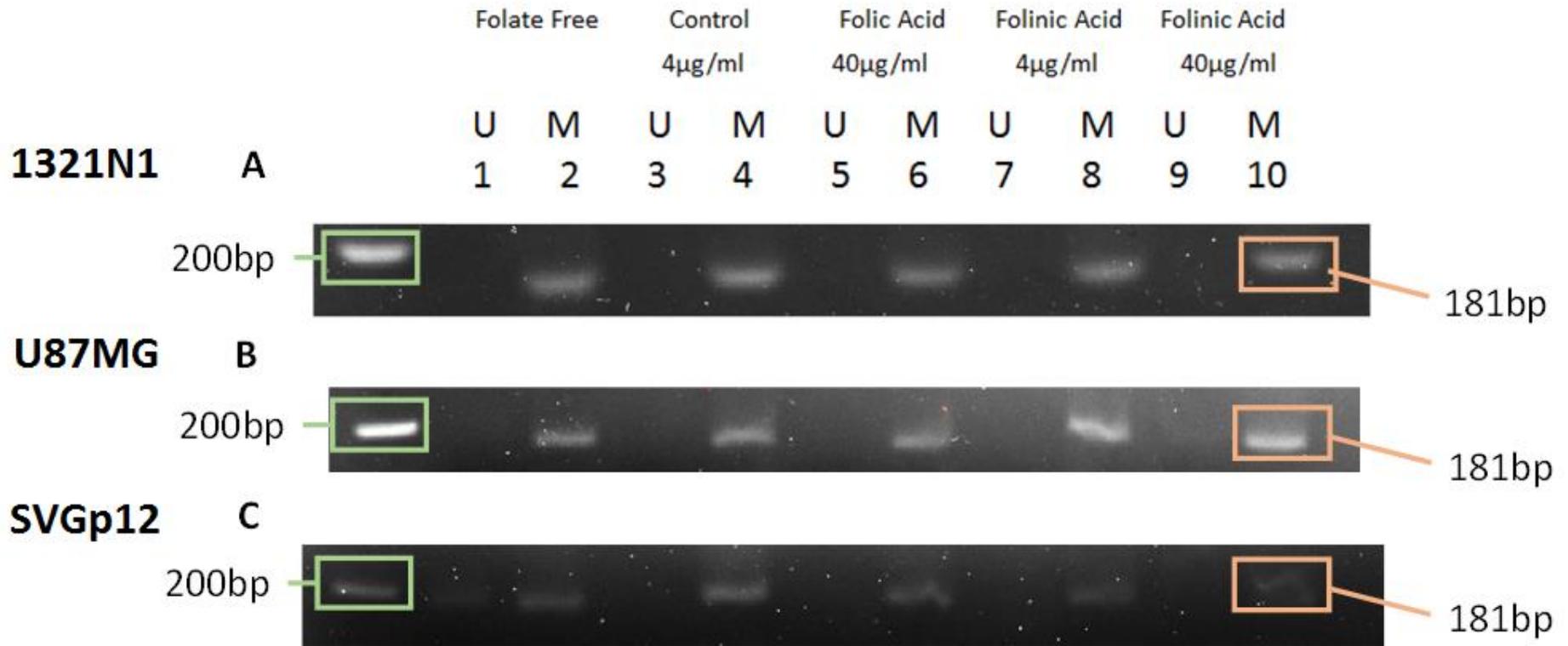


**Figure 6.6 Methylation status of the PCFT gene.**

Cells were treated for 5 days before genomic DNA was extracted using the QIAmp DNA mini kit, and then bisulphite converted using the EZ DNA Methylation-Gold Kit. Methylated and unmethylated PCFT primers were tested against the DNA obtained from treated cells of the (A) 1321N1, (B) U87MG and (C) SVGp12 cell lines. Three independent experiments were performed showing the same findings.

#### 6.2.4.4 *Methyltetrahydrofolatereductase*

In all samples amplification of the *MTHFR*, was achieved with primers specific for methylated DNA. There was no difference in *MTHFR* methylation seen between any of the treatments in any of the cells lines, 1321N1 (Figure 6.7A), U87MG (Figure 6.7B) or SVGp12 (Figure 6.7C), however, slight hemimethylation was seen in the *MTHFR* promotor in the SVGp12 cell line when the cells were grown in folate free media, although this is with more of a balance towards unmethylation (Lane 1).

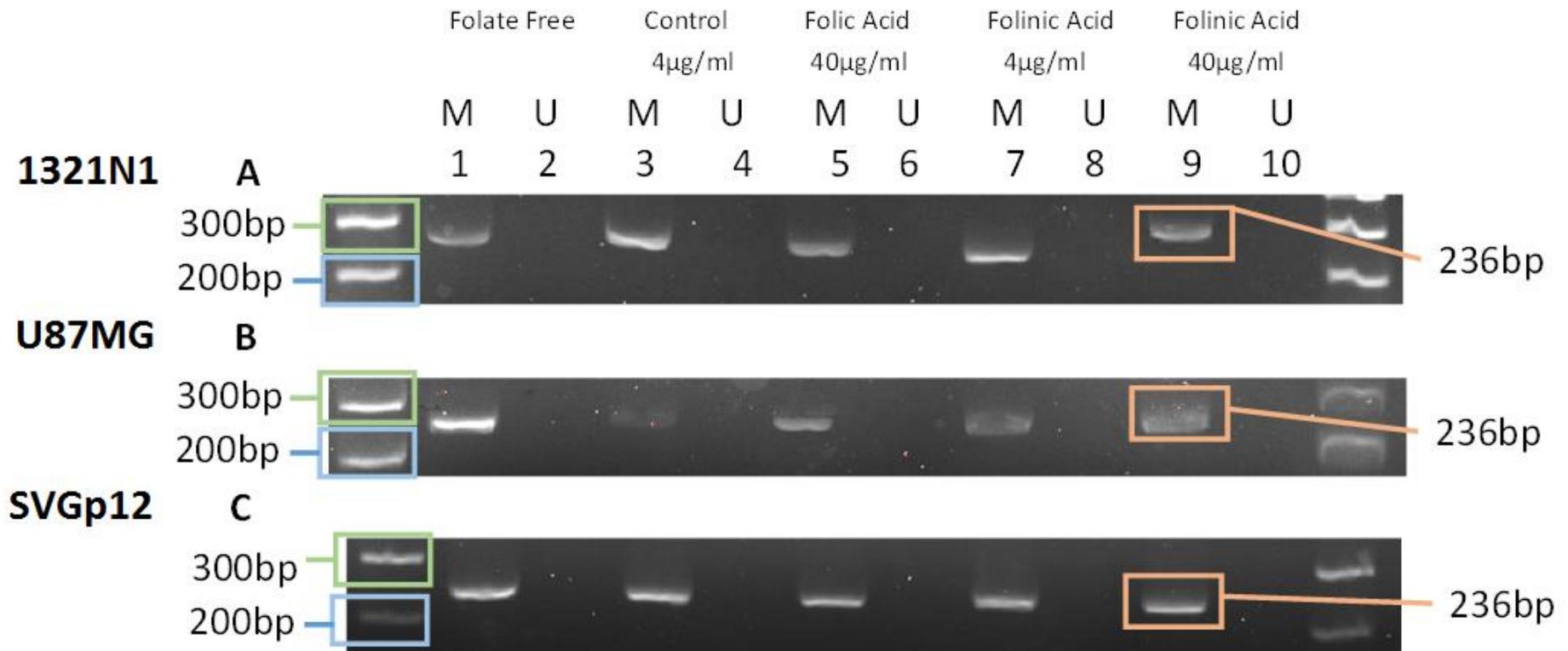


**Figure 6.7 Methylation status of the MTHFR gene.**

Cells were treated for 5 days before genomic DNA was extracted using the QIAmp DNA mini kit, and then bisulphite converted using the EZ DNA Methylation-Gold Kit. Methylated and unmethylated MTHFR primers were tested against the DNA obtained from treated cells of the (A) 1321N1, (B) U87MG and (C) SVGp12 cell lines. Three independent experiments were performed showing the same findings.

#### 6.2.4.5 *Folate Receptor 1*

In all samples amplification of the *FOLR1* was achieved with primers, specific for methylated DNA. There was no difference seen between the effect of folate supplementation on altering methylation status in any of the cells lines, 1321N1 (Figure 6.8A), U87MG (Figure 6.8B) or SVGp12 (Figure 6.8C).



**Figure 6.8 Methylation status of the FOLR1 gene.**

Cells were treated for 5 days before genomic DNA was extracted using the QIAmp DNA mini kit, and then bisulphite converted using the EZ DNA Methylation-Gold Kit. Methylated and unmethylated FOLR1 primers were tested against the DNA obtained from treated cells of the (A) 1321N1, (B) U87MG and (C) SVGp12 cell lines. Three independent experiments were performed showing the same findings.

### 6.2.5 Comparison of methylation status between non-cancerous cells and different grades of glioma

When comparing the methylation status of the genes of interest between the cell lines grown in standard cell culture media, the only genes for which there were a difference were *PTEN* and *PCFT*. The *RFC*, *FOLR1* and *MTHFR* showed no difference in methylation status between the cancerous and non-cancerous cells, or between the grade of tumours (Figures 6.5, 6.7 and 6.8).

The *PTEN* gene was methylated in the non-cancerous cells, then became hemi methylated in the lower grade cancerous cells, and then unmethylated in the higher grade cancer cells, showing an altered methylation pattern from non-cancerous to cancerous and through the grades (Figure 6.4).

The *PCFT* gene showed hemi methylation in both the non-cancerous and low grade cancerous cells, but the higher grade cells showed a methylated *PCFT* promoter in the cells grown in standard cell culture media (Figure 6.6).

### 6.3 Discussion

The main aim of this chapter was to establish what affect folate, a methylating agent, would have on altering methylation status of the genes of interest.

It was hypothesised that supplementation would alter the methylation status of the gene, in comparison to the cells grown in standard cell culture media, however, no notable differences were observed in methylation status following treatment with either folic or folinic acid, or those grown in folate deficient media, and the hypothesis was not proven.

This was unexpected from what has been seen in the literature, as altered methylation of the *RFC* in human breast cancer MDA-MB-231 and MCF-7 cells was possible by treatment with 5-aza-2'-deoxycytidine, 5-aza-CdR, another demethylating agent (Worm *et al.*, 2001). Folate supplementation to the human colonic adenocarcinoma cell line, SW620, grown in folate deficient conditions had been seen to alter methylation status, by inducing hypomethylation (Wasson *et al.*, 2006).

No difference in methylation following treatment may have been observed because although a change in viability and apoptosis levels were seen in Chapter 4, it is still unclear whether a change to methylation occurs first which results in transcriptional silencing of a gene, or the silencing of the gene results in an altered methylation status (Jones, 2012).

The second hypothesis was that a difference in methylation status would be seen between the non-cancerous and cancerous cell lines, and between the different grades of tumour. This hypothesis was partly proven for *PTEN* and *PCFT* which showed altered methylation in the non-cancerous cells, and unmethylated in the high grade glioma for *PTEN* and hemimethylation in the non-cancerous and low grade glioma cells compared to methylated in the high grade glioma cells for *PCFT*.

The non-cancerous cell line, SVGp12 showed complete methylation of the *PTEN* promoter, whilst 1321N1 (grade 3/4) showed hemimethylation and the high grade glioma, U87MG

showed complete unmethylation. These results have also been seen in a study in patients with glioma where patients with a low grade glioma also had *PTEN* promoter methylation, whilst the *de novo* glioma tended to have an unmethylated *PTEN* promoter (Wiencke *et al.*, 2007). These replicated results suggested *PTEN* promoter methylation status could be used to assist tumour grading and confirmed the MS-PCR was working correctly.

A slight difference in the *PCFT* was observed altering from hemi methylated in the non-cancerous and low grade glioma cells to methylated in the high grade glioma cells. The *PCFT* gene plays a key role in transporting folates into the central nervous system (Diop-Bove *et al.*, 2009). It was found to be methylated in the high grade glioma cell line, U87MG, but only hemimethylated in the grade 3/4 1321N1 cell line and non-cancerous cell line SVGp12. This correlates with other research that showed higher levels of methylation of the *PCFT* gene in the colorectal cancer tissue, at CpG sites 9-12 and 14 which are all located in the body of the gene (Farkas *et al.*, 2015b). It was also seen in the methylated lane of the *PCFT* gene, that there was a double band. It is possible that this additional band was as the result of non-specific binding, usually in the form of primer dimers, or the presence of a pseudo gene, but given the similar length of the band seen, it may be representative of heterozygosity at a particular locus, which has been seen in the *PCFT* gene (Zhao *et al.*, 2011).

The results showed that *FOLR1* was methylated across all cell lines and following all treatments. This was potentially unexpected given that there is known to be increased *FOLR1* expression levels between cancerous and non-cancerous cells, which will be discussed further in Chapter 7. It has been previously hypothesised that that an increase in *FOLR1* expression was due to hypomethylation of the gene (Elwood *et al.*, 1997), although the results have been inconclusive (Kelemen, 2006).

No difference in *FOLR1* methylation across cell lines in this study, may have been because only one area of the promoter was analysed. The methylation status of the *FOLR1* promoter

changes dependent upon which CpG island was investigated. In CRC CpG sites 1 and 2, located at the translation start site, were less methylated in CRC than the non-cancerous control samples. The CpG 14 island was downstream of the gene and was more methylated in the CRC than the normal tissue (Farkas *et al.*, 2015b).

The reduced folate carrier (*RFC*) a transmembrane bidirectional anion channel, showed hemimethylation across all three cell lines, with no notable differences between the non-cancerous and cancerous cell lines. This does not correlate with observations seen in CRC where there was a greater level of methylation in the *RFC* in CRC tissue at CpG sites 3-5 and 11-13, which are sites all within the promoter region (Farkas *et al.*, 2015b). Heavy promoter methylation was also seen in the MDA-MB-231 breast cancer cell line (Moscow *et al.*, 1997; Worm *et al.*, 2001). The variability in methylation status that has been recorded in the *FOLR1* has been seen in the *RFC*. Promoter hypermethylation was only seen in 42% of the ovarian cancer samples (Siu *et al.*, 2012). The diversity of results in terms of methylation status was also seen in primary central nervous system lymphomas samples, where only 30% of samples were methylated, and even fewer at 8 % in the diffuse large B-cell lymphomas samples. The *RFC* methylated band appeared at different length within different PCR gels, this is likely due to the different polymorphisms of the gene between the cell lines (De Marco *et al.*, 2003).

The *MTHFR* gene of which there are 24 different polymorphisms (Sibani *et al.*, 2000), has been seen to be hypermethylated in human lung cancer tissue compared to matched non-cancerous control tissue (Vaissière *et al.*, 2009). The *MTHFR* C677T was associated with global hypomethylation and *MGMT* demethylation (Shelnutt *et al.*, 2004) which occurred when there was low levels of folate (Liu *et al.*, 2013).

There are many difficulties associated with assessing methylation status of genes within gliomas, first of all there have been few studies looking at methylation patterns of the promoter region in varying genes in glioblastoma (Farkas *et al.*, 2015a; Lai *et al.*, 2014), and

it is difficult to determine whether the observations seen are patient or tumour specific, so the results presented here for glioma cell lines are highly novel. The Cancer Genome Atlas Project (TCGA) was established to provide a source of information regarding tumours including; methylation patterns, gene mutations, copy number and expression levels (Noushmehr *et al.*, 2010b). Another problem often encountered is availability of matched healthy tissue samples to compare to cancerous brain tissue, as it recommended to leave as much healthy brain tissue intact as possible during resection. Often instead unmatched samples have often been used for comparison. Methylation patterns also vary dependent upon the particular region of the brain, and by age of the patient (Verhaak, Hoadley *et al.* 2010, Hernandez, Nalls *et al.* 2011). So global methylation change would not make an ideal biomarker as alterations to methylation are very gene specific and it would not be practical to investigate all genes. The use of cell lines in this project, makes the results more reproducible and aims to give a basis of understanding.

Another problem reported as the result of variation in methylation patterns seen, was that variation can occur within the tumour itself. One recent study showed that there was greater variation seen in methylation status of glioma samples compared to control brain samples. Glioma tumour tissue showed 1389 CpG sites were hypomethylated compared to the control samples, whilst 475 CpG sites were hypermethylated. This difference seen was affected by the grade of tumour. Grade one had a higher degree of hypomethylation. The difference seen between the number of hyper methylated tumour genes compared to the control samples may be because the normal samples tended to already be hypermethylated at the CpG sites (Lai, Chen *et al.* 2014).

In summary, in the aim of this chapter was to assess the effect the different forms of folic and folinic acid had on the methylation status of the chosen genes of interest: *FR1*, *RFC*, *PCFT*, *PTEN* and *MTHFR*. In terms of the first hypothesis that treatment would alter methylation status compared to those grown in standard cell culture media, there was no difference

observed, so this hypothesis was disproven. It was also hypothesised that a difference in methylation status would be observed between cancerous and non-cancerous cells, and between the different tumour grades. This difference was only observed in the *PTEN* and *PCFT* genes, which could make these two genes potential biomarkers to aid tumour grading. It was also hoped that this methylation study would allow comparisons to be made between the methylation stats of the folate transporters and metabolising enzymes and the findings regarding viability and apoptosis in Chapters 4 and 5, but this was not possible, given that treatment had no alteration on methylation of the genes analysed.

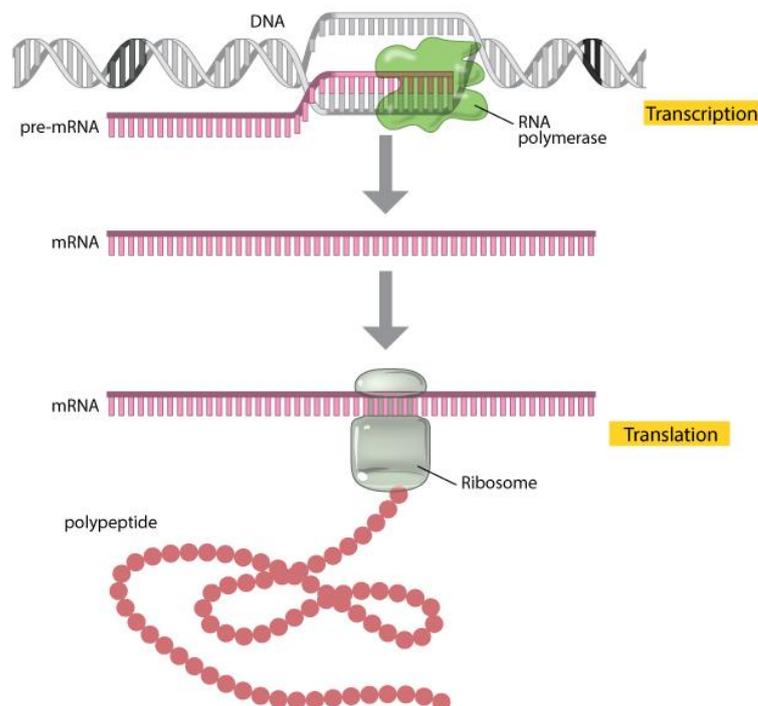
The semi quantifiable results of methylation status seen would benefit from future work, such as pyrosequencing to determine more quantitatively whether there was a difference seen in methylation status that was not observable though MS-PCR, however, given the many references which suggest a link between methylation status and protein expression, and to aid further understanding of the mechanisms behind the altered cell viability and apoptotic levels following treatment, protein expression levels will be analysed.

# CHAPTER 7

## 7 PROTEIN EXPRESSION LEVELS

### 7.1 Introduction

Although Chapter 6 revealed no difference in the methylation status of genes of interest RFC, FOLR1 and MTHFR following treatment with folic and folinic acid, regardless of cell type or grade of tumour, it was decided to investigate other mechanisms of variability that may explain cell viability and apoptosis differences following treatment. There are different ways in which gene expression can be assessed, either through the analysis of mRNA expression levels, or through the expression of the corresponding protein levels. DNA is copied to mRNA through transcription, and this mRNA is then translated into a protein molecule (Figure 7.1) (Clancy and Brown, 2008). Every hour a mammalian cell will produce two copies of a particular mRNA, compared to dozens of copies of the corresponding protein (Vogel and Marcotte, 2012).



**Figure 7.1 DNA to Protein.**

DNA is transcribed into mRNA, which is then translated into a protein, made up of many amino acids (Clancy and Brown, 2008).

It has been observed from various studies that there is no clear correlation between gene and protein expression level, some are highly correlated, whilst others are not; particularly in cancer tissue. (Kosti *et al.*, 2016; Koussounadis *et al.*, 2015). A recent study utilising the work of the Human Proteome Map Project (HPM) and the Genotype-Tissue Expression (GTEx) project contradicts this and showed that most genes have a positive gene-protein expression correlation, although this study did not include brain cells (Kosti *et al.*, 2016). Transcript information is only part of the picture, and can only partially predict protein levels as errors can occur in the transcription between mRNA to protein (Gout *et al.*, 2013). In the Chapter here, protein expression was chosen as a measurable end-point rather than using mRNA levels to determine gene expression because protein is still detectable even when mRNA expression is not, and protein expression is more closely related to corresponding activity.

### 7.1.1 Aim of Chapter

The aim of this chapter was to establish if folate deficiency, folic or folinic acid treatment altered the expression of the proteins of interest; PTEN, FOLR1, RFC, PCFT and MTHFR, compared to the cells grown in standard cell culture media.

### 7.1.2 Hypotheses

Despite much research regarding the role of folate in affecting protein expression of the folate transporters FOLR1, PCFT and RFC, in colorectal cancer, little has been undertaken in glioma, this is a clear gap in knowledge that needs to be researched, given the benefits of folate supplementation reducing colorectal cancer risk as has already been discussed in Section 1.9.

#### ***Hypothesis One***

Folate deficiency is expected to cause an upregulation in protein expression of the folate transporters in all three cell lines, as the cells try to compensate for restricted folate.

### ***Hypothesis Two***

Folic and folinic acid supplementation are both expected to increase the expression of the proteins of interest compared to the cells grown in standard cell culture media.

### ***Hypothesis Three***

An increase in protein expression of the chosen proteins is expected between the cell lines as cancer cells have an increased metabolic demand.

### ***Hypothesis Four***

Altered methylation status relative to cell type will be measurable by a corresponding change in protein expression.

## **7.1.3 Objectives**

### **Objective 1**

Measure levels of protein expression for cells grown in folate deficient conditions, and compare to those grown in standard cell culture media.

### **Objective 2**

Measure levels of protein expression for cells treated with folic or folinic acid supplementation and compare to those grown in standard cell culture media.

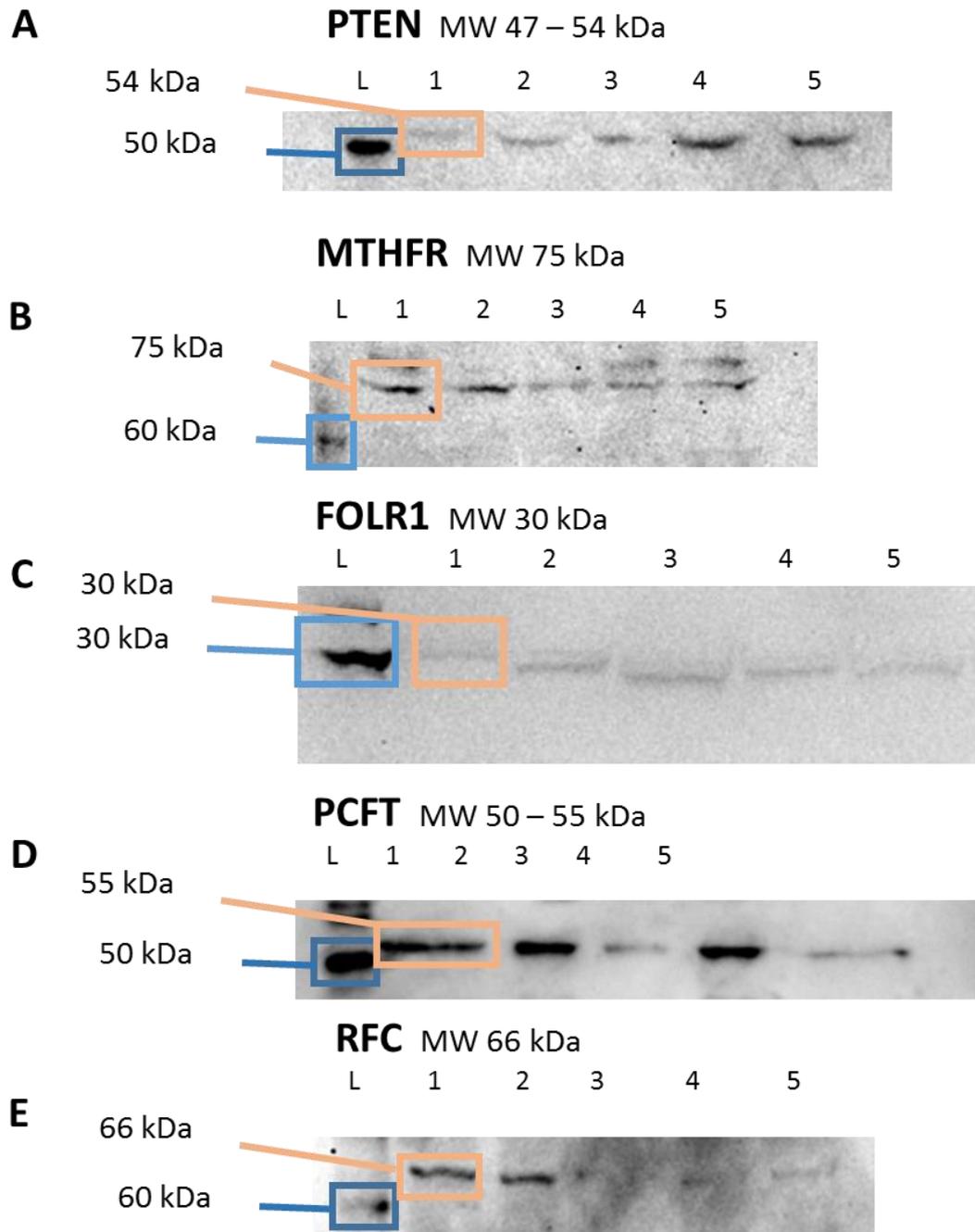
### **Objective 3**

Measure whether there is a difference in protein expression levels between the different cell lines.

## 7.2 Results

### 7.2.1 Antibody Validation

The PTEN protein was expected to produce a band of 47 – 54 kDa which was visible in Figure 7.2A. The MTHFR protein should have a weight of 75 kDa, this was again seen in Figure 7.2B. The FOLR1 protein had an expected weight of 30 kDa which was seen in Figure 7.2C. The PCFT protein had an expected weight of 50-55 kDa which was seen in Figure 7.2D. The RFC protein had an expected weight of 60 kDa which was seen in Figure 7.2E. All the primers used were validated with rat liver lysate, as the liver is known to have high expression levels of the folate transporters (Visentin *et al.*, 2014).



**Figure 7.2 Western immunoblot - antibody validation.**

Protein extracted from rat liver lysate using RIPA buffer, underwent western blotting. **A)** PTEN had a molecular weight 47-54 kDa **B)** MTHFR had a molecular weight 75 kDa **C)** FOLR1 had a molecular weight 30 kDa **D)** PCFT had a molecular weight 50-55 kDa **E)** RFC had a molecular weight 66 kDa. Bands produced were of the expected weight, showing specificity.

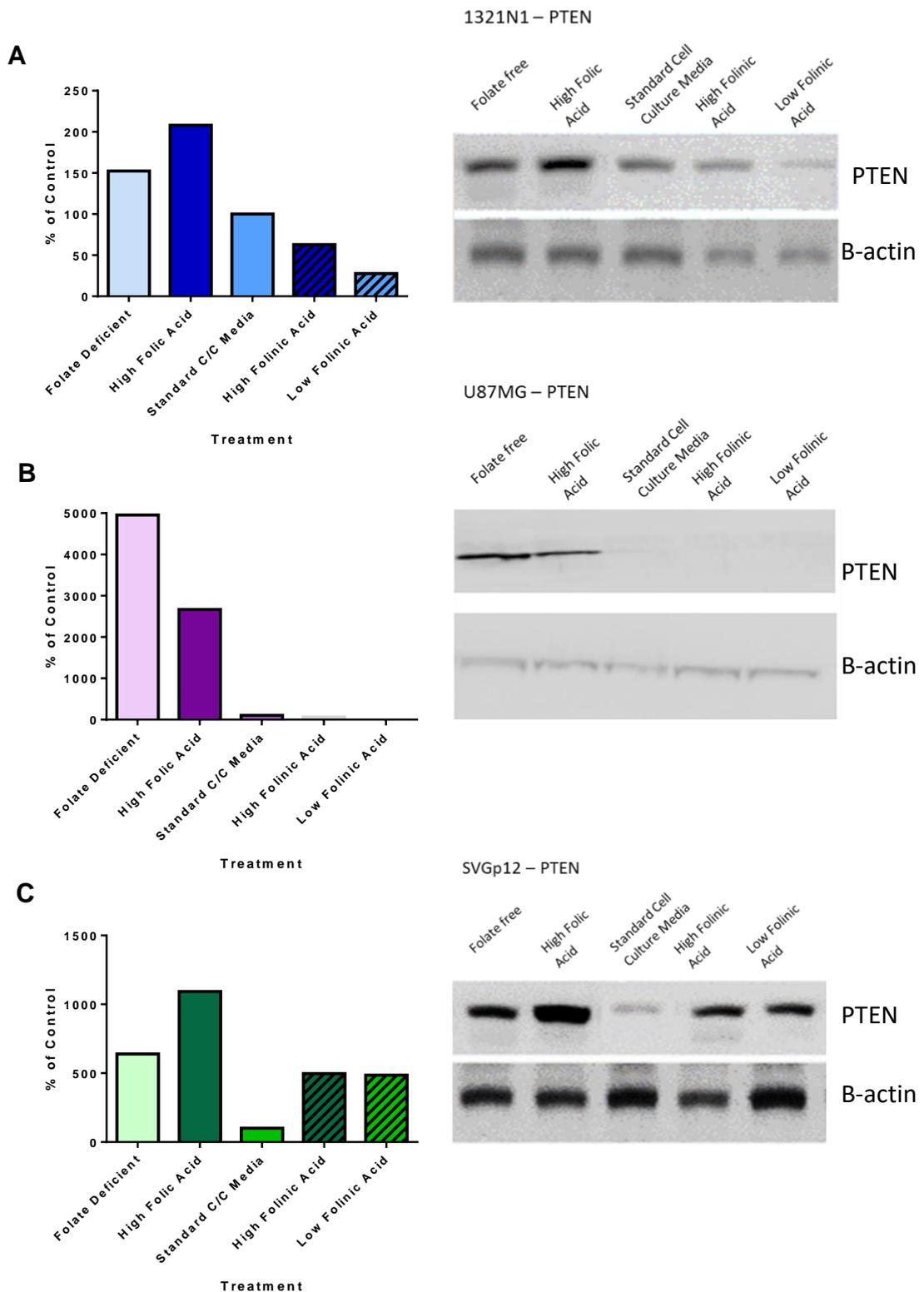
## 7.2.2 Protein expression following 5 day folate treatment

### 7.2.2.1 *Phosphate and tensin homolog protein (PTEN)*

The results show that the 1321N1 cells express PTEN at a higher level in cells treated with high folic acid at 40 µg/ml and a lower expression in folate free media compared to cells grown in standard cell culture media (Figure 7.3A). The cells that were grown in folic acid expressed PTEN at a lower level than those grown in standard cell culture media. There was also an increase seen between cells grown with low (4 µg/ml) and high (40 µg/ml) concentrations of folic acid (Figure 7.3A).

The U87 MG cells grown without folate, and those grown with the high concentration of folic acid expressed PTEN at a higher level than those grown in standard cell culture media. As, was seen in the 1321N1 cells, those treated with folic acid at both concentrations showed PTEN at a lower expression level (Figure 7.3B). There was no band visible for the cells treated with folic acid at the low and high concentrations.

All SVGp12 cells treated with high concentration of folic acid, folate deficient media, and folic acid, expressed PTEN at a higher level than those grown in standard cell culture media (Figure 7.3C).



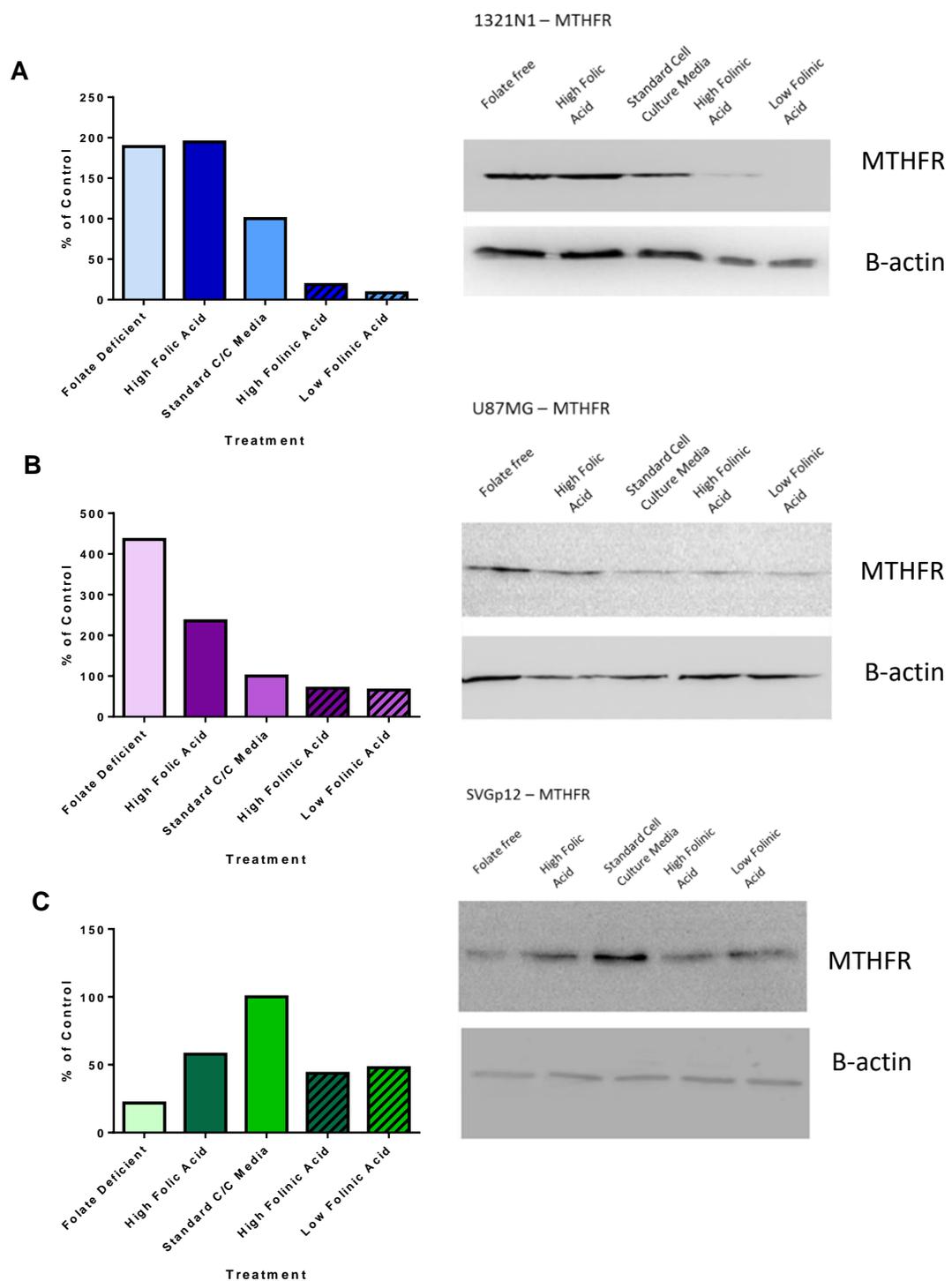
**Figure 7.3 PTEN protein expression.** Cells were treated daily for 5 days with folate free media (0 $\mu$ g/ml), standard cell culture media containing 4 $\mu$ g/ml folic acid, high (40 $\mu$ g/ml) folic acid, folinic acid at low (4 $\mu$ g/ml) and high 40 $\mu$ g/ml) concentration, before undergoing protein extraction and western blotting. Protein levels of PTEN (54kDa), were determined by densitometry analysis relative to the beta-actin loading control for (A) 1321N1, (B) U87MG, and (C) SVGp12 cell lines. The data is from one independent experiment.

### 7.2.2.2 *Methyltetrahydrofolatereductase*

The results showed that the 1321N1 cells expressed MTHFR at a higher level in cells treated with folic acid at 40  $\mu\text{g/ml}$  and those grown in folate free media, compared to those grown in standard cell culture media (Figure 7.4A). The cells that were grown in folic acid expressed MTHFR at a lower level than those grown in standard cell culture media. There was also a decrease seen between those grown with low (4  $\mu\text{g/ml}$ ) and high (40  $\mu\text{g/ml}$ ) concentrations of folic acid.

The U87MG cells showed a similar response, the cells grown without folate, and those grown with the high concentration of folic acid expressed MTHFR at a higher level than those grown in standard cell culture media (Figure 7.3B). Again, as was seen in the 1321N1 cells, those treated with folic acid at both concentrations showed MTHFR at a lower expression level. There was however no difference seen between those grown with low (4  $\mu\text{g/ml}$ ) and high (40  $\mu\text{g/ml}$ ) concentrations of folic acid.

The SVGp12 cells responded in a different manner to the cancerous cells, (1321N1 and U87MG). SVGp12 cells treated with a high concentration of folic acid, or folate deficient media expressed lower MTHFR than those grown in standard cell culture media (Figure 7.3C). SVGp12 cells treated with folic acid at both concentrations however showed a similar pattern to the cancerous cells as MTHFR was expressed at a lower level than those grown in standard cell culture media. There was no difference seen between those grown with low (4  $\mu\text{g/ml}$ ) and high (40  $\mu\text{g/ml}$ ) of folic acid.



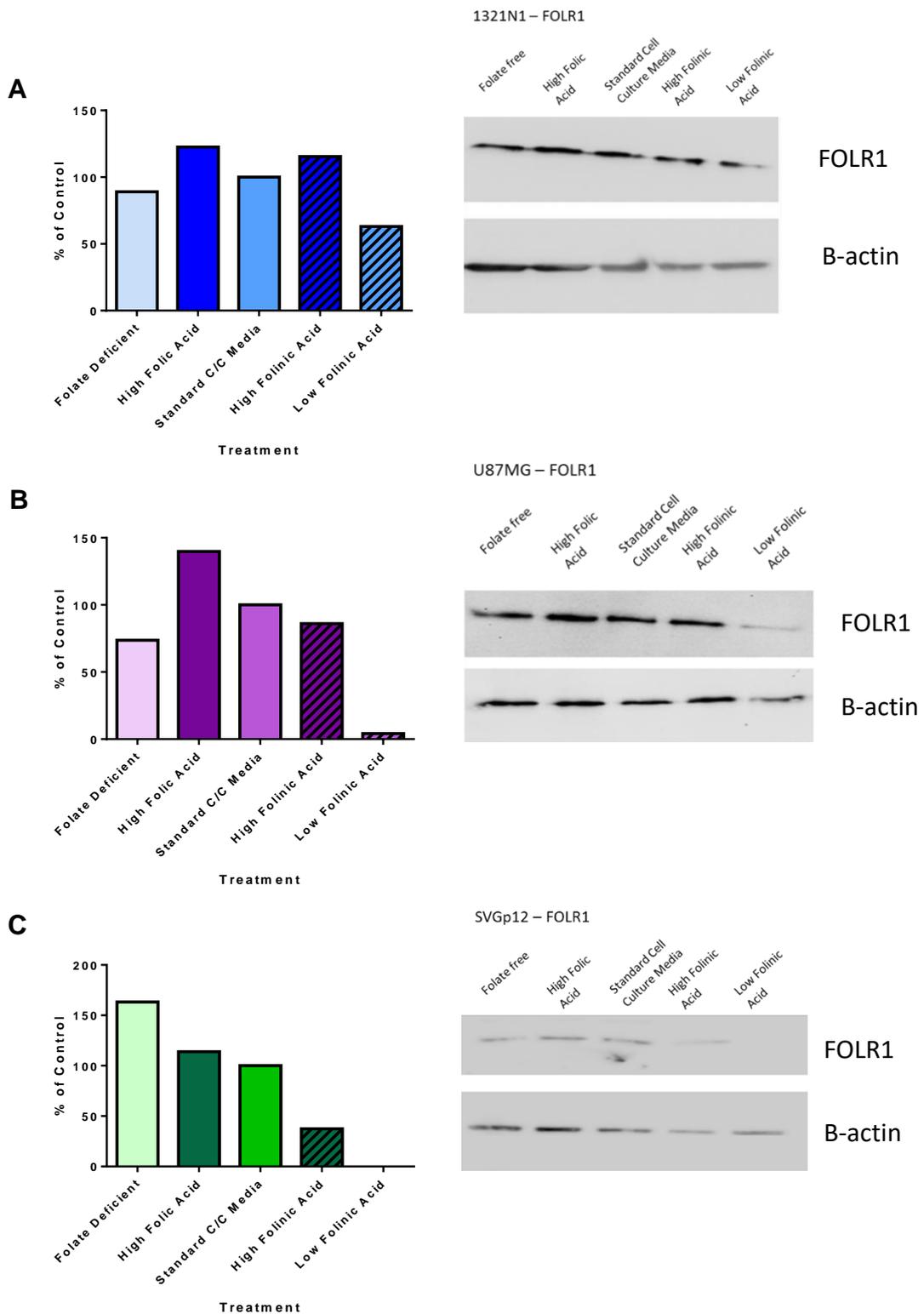
**Figure 7.4 MTHFR protein expression.** Cells were treated daily for 5 days with folate free media ( $0\mu\text{g/ml}$ ), standard cell culture media containing  $4\mu\text{g/ml}$  folic acid, high ( $40\mu\text{g/ml}$ ) folic acid, folinic acid at low ( $4\mu\text{g/ml}$ ) and high ( $40\mu\text{g/ml}$ ) concentration, before undergoing protein extraction and western blotting. Protein levels of MTHFR (75kDa), were determined by densitometry analysis relative to the beta-actin loading control for **(A)** 1321N1, **(B)** U87MG, and **(C)** SVGp12 cell lines. The data is from one independent experiment.

### 7.2.2.3 *Folate Receptor 1*

The results showed that 1321N1 cells expressed FOLR1 at a higher level following treatment with folic acid at 40 µg/ml, and also following treatment with folinic acid at the higher supplementation of 40 µg/ml (Figure 7.5A). Cells grown without folate, and those grown with a low concentration of folinic acid showed a decrease in FOLR1 expression compared to those grown in standard cell culture media. There was also an increase between cells grown with low (4 µg/ml) and high (40 µg/ml) concentrations of folinic acid.

The U87MG cells showed a similar response to the 1321N1 cells; the cells grown with folic acid at 40 µg/ml showed increased expression of FOLR1 compared to the cells grown in standard cell culture media (Figure 7.5B). The cells grown in folate free media and those treated with folinic acid at both concentrations 4 µg/ml and 40 µg/ml, showed FOLR1 expression at a lower expression than was seen in the cells grown in standard cell culture media. There was also an increase seen between cells grown with low (4 µg/ml) and high (40 µg/ml) concentrations of folinic acid.

SVGp12 cells responded in a different manner to the cancerous cells (1321N1 and U87MG). Cells treated with a high concentration of folic acid, and those grown in folate deficient media, all expressed FOLR1 at a lower level than those grown in standard cell culture media (Figure 7.5C). Cells treated with folinic acid at both concentrations however showed a similar pattern to the cancerous cells as FOLR1 was expressed at a lower level than those grown in standard cell culture media. There was no difference seen between those grown in low (4 µg/ml) and high (40 µg/ml) concentrations of folinic acid.



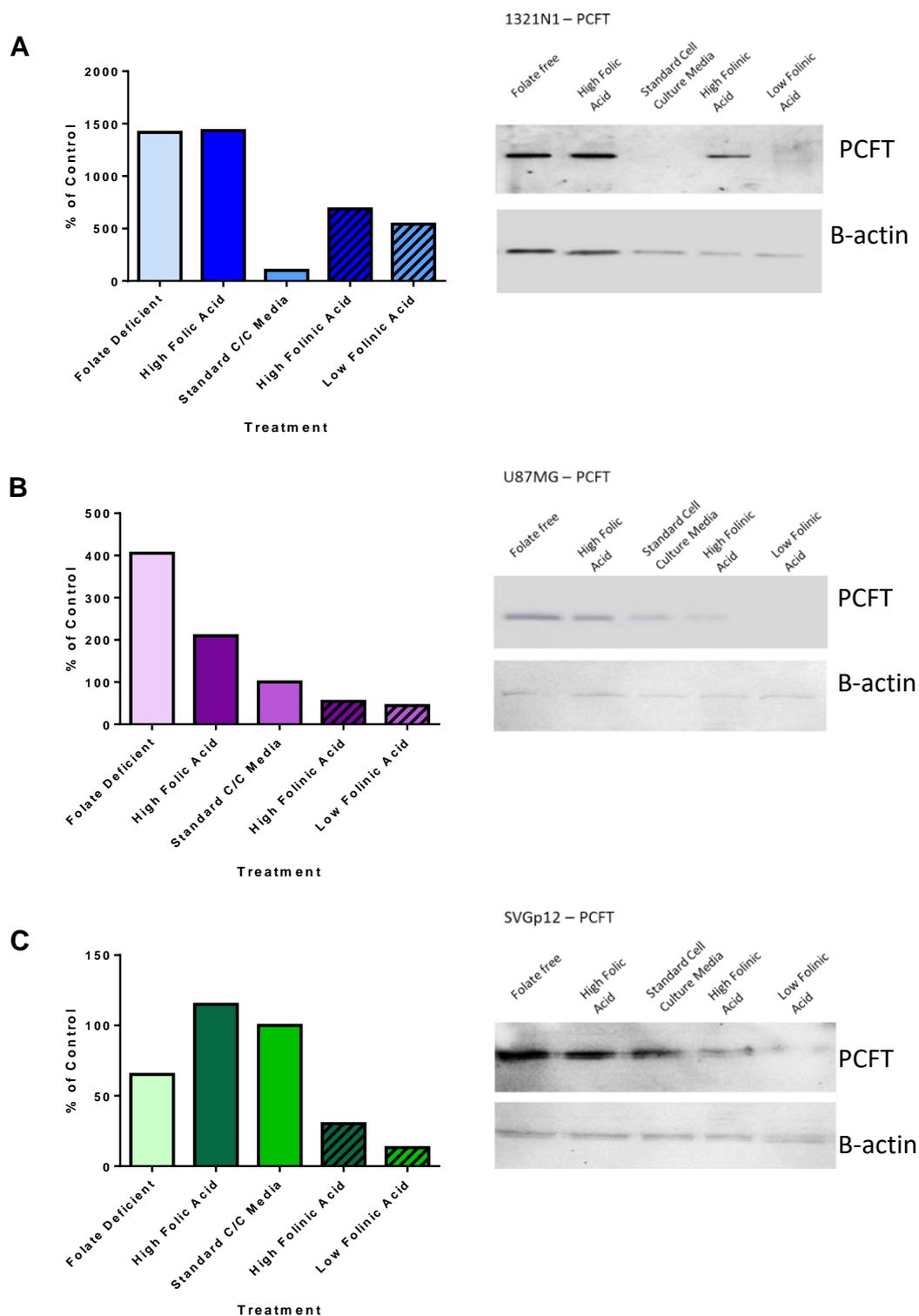
**Figure 7.5 FOLR1 protein expression.** Cells were treated daily for 5 days with folate free media (0µg/ml), standard cell culture media containing 4µg/ml folic acid, high (40µg/ml) folic acid, folinic acid at low (4µg/ml) and high (40µg/ml) concentration, before undergoing protein extraction and western blotting. Protein levels of FOLR1 (30kDa), were determined by densitometry analysis relative to the beta-actin loading control for **(A)** 1321N1, **(B)** U87MG, and **(C)** SVGp12 cell lines. The data is from one independent experiment.

#### 7.2.2.4 *Proton Coupled Folate Transporter*

The 1321N1 cells that were grown with a high concentration of folic acid and cells grown without folate had an increased expression of PCFT (Figure 7.6A). The cells treated with folic acid also had an increase in PCFT expression, and an increase was also seen between the low and high concentrations.

The U87 MG cells treated with high folic acid and cells grown without folate had an increased expression, which was also seen in the 1321N1 cells (Figure 7.6B). The cells treated with folic acid at both concentrations had a decreased level of expression compared to the cells grown in standard cell culture media. There was also an increase seen between the low and high concentrations.

The SVGp12 cells grown with high folic acid had an increased level of expression of PCFT compared to the cells grown in standard cell culture media. The cells grown without folate, and those treated with folic acid had a decreased level of expression; showing a similar response to the U87MG cells (Figure 7.6C). An increase was seen again between the low and high concentration of folic acid.



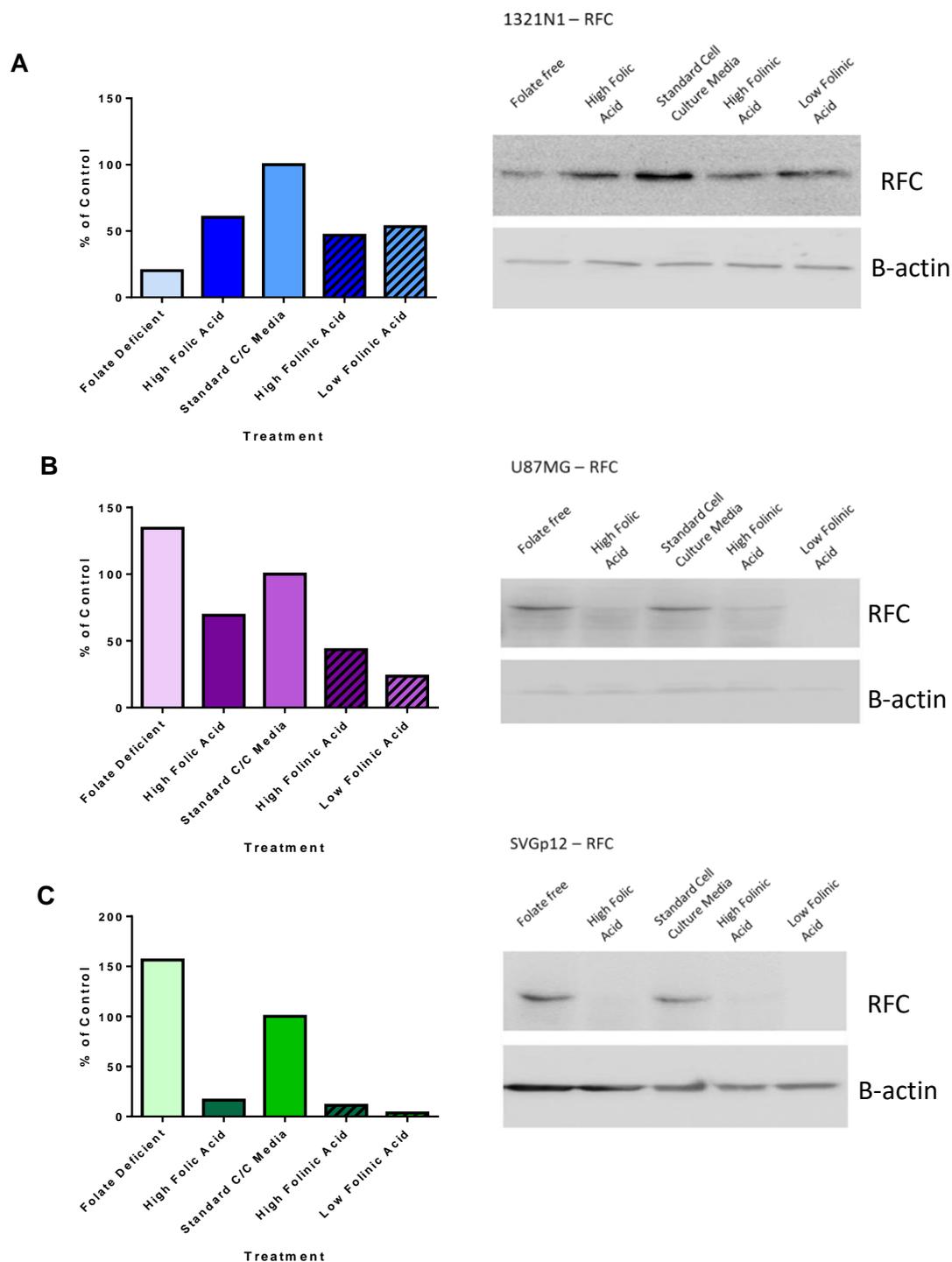
**Figure 7.6 PCFT protein expression.** Cells were treated daily for 5 days with folate free media ( $0\mu\text{g/ml}$ ), standard cell culture media containing  $4\mu\text{g/ml}$  folic acid, high ( $40\mu\text{g/ml}$ ) folic acid, folinic acid at low ( $4\mu\text{g/ml}$ ) and high ( $40\mu\text{g/ml}$ ) concentration, before undergoing protein extraction and western blotting. Protein levels of PCFT ( $55\text{kDa}$ ), were determined by densitometry analysis relative to the beta-actin loading control for **(A)** 1321N1, **(B)** U87MG, and **(C)** SVGp12 cell lines. The data is from one independent experiment.

#### 7.2.2.5 *Reduced Folate Carrier*

The 1321N1 cells had a decreased level of expression when grown in folate deficient media compared to the cells grown in standard cell culture media. The cells treated with folic acid at both a low and high concentration also had a decreased level of expression compared to the cells grown in standard cell culture media (Figure 7.7A), although it did appear that the band for low folic acid was not present. An increase was seen between the low and high concentration of folic acid.

The U87MG cells grown in folate deficient media had a higher level of RFC expression than cells grown in standard cell culture media (Figure 7.7B). The cells treated with a high concentration of folic acid and both concentrations of folic acid had a decreased level of expression in comparison to the control. An increase was seen between the low and high concentration of folic acid.

The SVGp12 cells treated with the high concentration of folic acid and both concentrations of folic acid showed decreased expression compared to the cells grown in standard cell culture media (Figure 7.7C). The cells grown in folate free media showed an increase in expression.



**Figure 7.7 RFC protein expression.** Cells were treated daily for 5 days with folate free media ( $0\mu\text{g/ml}$ ), standard cell culture media containing  $4\mu\text{g/ml}$  folic acid, high ( $40\mu\text{g/ml}$ ) folic acid, folinic acid at low ( $4\mu\text{g/ml}$ ) and high ( $40\mu\text{g/ml}$ ) concentration, before undergoing protein extraction and western blotting. Protein levels of RFC ( $66\text{kDa}$ ), were determined by densitometry analysis relative to the beta-actin loading control for **(A)** 1321N1, **(B)** U87MG, and **(C)** SVGp12 cell lines. The data is from one independent experiment.

In Table 7.1 a summary of the overall trends in protein expression is given for each protein. In general, folinic acid treatment caused a decrease in protein expression of all proteins of interest and both folate deficient and high folic acid treatment caused an increase in expression in each protein. Some of these changes appeared to be dependent on cell type. The U87MG cells show all proteins analysed were down regulated following folinic treatment with both a low (4 µg/ml) and high dose (40 µg/ml). This was also seen in the SVGp12 cells except when grown in folate deficient conditions. The 1321N1 and U87 MG cells when grown in folic acid at 40 µg/ml resulted in increased expression of the proteins being analysed except RFC.

**Table 7.1 Summary of Protein Expression – Treatment Comparison**

*Following 5 days of daily treatment cells were compared to those grown in standard cell culture media containing 4µg/ml of folic acid.*

**Folate Deficient**

Gene	1321N1	U87MG	SVGp12
PTEN	↑	↑	↑
MTHFR	↑	↑	↓
FOLR1	↓	↓	↑
PCFT	↑	↑	↓
RFC	↓	↑	↑

**Low Folinic**

Gene	1321N1	U87MG	SVGp12
PTEN	↓	↓	↑
MTHFR	↓	↓	↓
FOLR1	↓	↓	↓
PCFT	↑	↓	↓
RFC	↓	↓	↓

**High Folic**

Gene	1321N1	U87MG	SVGp12
PTEN	↑	↑	↑
MTHFR	↑	↑	↓
FOLR1	↑	↑	↑
PCFT	↑	↑	↑
RFC	↓	↓	↓

**High Folinic**

Gene	1321N1	U87MG	SVGp12
PTEN	↓	↓	↑
MTHFR	↓	↓	↓
FOLR1	↑	↓	↓
PCFT	↑	↓	↓
RFC	↓	↓	↓

### 7.2.3 Protein expression comparison between cancerous versus non – cancerous cells

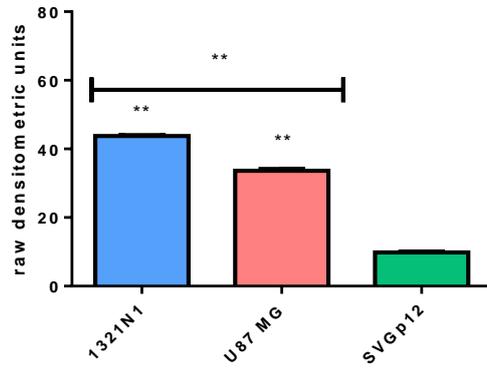
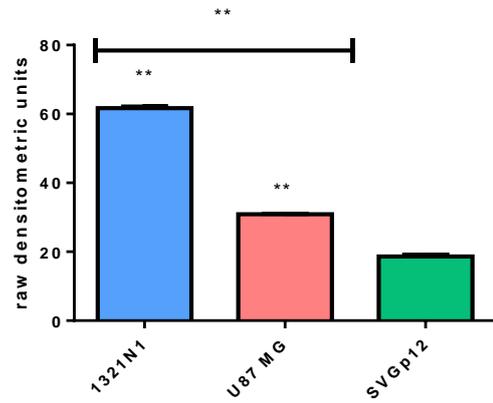
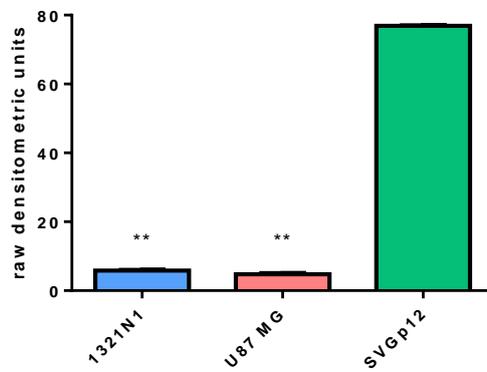
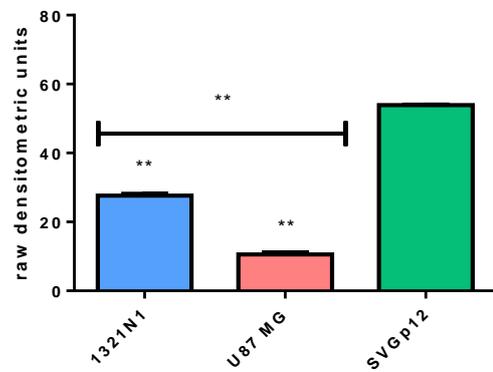
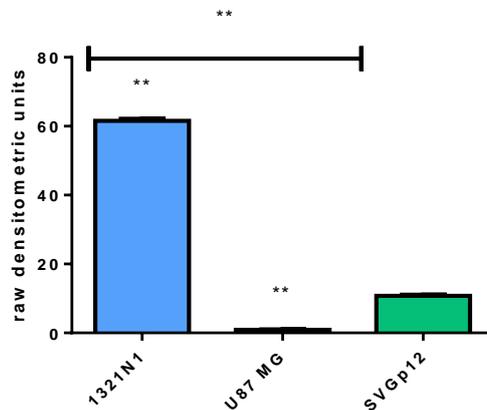
The 1321N1 and U87 MG cells showed a significantly higher level of FOLR1 expression compared to the non-cancerous control SVGp12 cells (Figure 7.7A). There was also a significant difference between the 1321N1 and U87 MG cells.

The 1321N1 and U87 MG cells also showed a significantly higher level of RFC expression compared to the non-cancerous control SVGp12 cells, and there was also a significant higher expression in 1321N1 compared to U87 MG cells (Figure 7.7B).

There was a significantly decreased level of PCFT expression in the cancerous cells 1321N1 and U87 MG cells compared to the non-cancerous cells (Figure 7.7C).

There was a significantly decreased level of MTHFR expression in the cancerous cells compared to the non-cancerous cells, and this level of expression was significantly lower in the high grade U87 MG cells compared to the lower grade 1321N1 cells (Figure 7.7D).

The 1321N1 cells showed a significantly greater level of PTEN expression compared to the non-cancerous SVGp12 cells (Figure 7.7E). The high grade U87MG cells however, showed a significantly lower level of expression.

**A - FOLR1****B - RFC****C - PCFT****D - MTHFR****E - PTEN**

**Figure 7.7 Protein expression levels in cells grown in standard cell culture media.**

Cells were grown in standard cell culture media for 5 days before undergoing protein extraction and western blotting and probing for the proteins of interest **A)** FOLR1 (30kDa) **B)** RFC (66kDa) **C)** PCFT (55kDa) **D)** MTHFR (75kDa) **E)** PTEN (54kDa). The data is from one independent experiment.

In Table 7.2 a summary of the overall trends in protein expression is given for each protein. In general, folinic acid treatment caused a decrease in protein expression of all proteins of interest and both folate deficient and high folic acid treatment caused an increase in expression in each protein. Some of these changes appeared to be dependent on cell type. The PCFT protein was upregulated in the 1321N1 cell line for all treatments whereas U87MG and SVGp12 showed down regulation following folinic acid treatment. The PTEN protein was also cell type specific, showing a general trend of up regulation in the SVGp12 cells, and down regulation following folinic acid treatment in the 1321N1 and U87MG cells. The MTHFR protein was down regulated in the SVGp12 cells following all treatments and down regulated following folinic acid treatment in the 1321N1 and U87MG cells. The RFC protein was down regulated in the 1321N1 cells, and generally also down regulated in the U87 MG and SVGp12 cells, except when grown in folate deficient conditions.

**Table 6.2 Summary of Protein Expression – Gene Comparison**

Following 5 days of daily treatment cells were compared to those grown in standard cell culture media containing 4µg/ml of folic acid.

**PTEN**

Treatment	1321N1	U87MG	SVGp12
Folate Deficient	↑	↑	↑
High Folic	↑	↑	↓
Low Folinic	↓	↓	↑
High Folinic	↓	↓	↑

**PCFT**

Treatment	1321N1	U87MG	SVGp12
Folate Deficient	↑	↑	↓
High Folic	↑	↑	↑
Low Folinic	↑	↓	↓
High Folinic	↑	↓	↓

**MTHFR**

Treatment	1321N1	U87MG	SVGp12
Folate Deficient	↑	↑	↓
High Folic	↑	↑	↓
Low Folinic	↓	↓	↓
High Folinic	↓	↓	↓

**RFC**

Treatment	1321N1	U87MG	SVGp12
Folate Deficient	↓	↑	↑
High Folic	↓	↓	↓
Low Folinic	↓	↓	↓
High Folinic	↓	↓	↓

**FOLR1**

Treatment	1321N1	U87MG	SVGp12
Folate Deficient	↓	↓	↑
High Folic	↑	↑	↑
Low Folinic	↓	↓	↓
High Folinic	↑	↓	↓

### 7.3 Discussion

The aim of this chapter was to establish the effect of folate on protein expression levels. This was conducted by investigating cells grown in folate deficient, folic or folinic acid supplemented media in non-cancerous and cancerous cells representing different grades of glioma.

It was hypothesised that cells grown in folate deficient conditions would have an increased level of folate transporter expression in order to maximise possible folate uptake. The results showed that there was an increased level of FOLR1 expression in the SVGp12 cells grown in folate free media compared to standard cell culture media, but this was not seen in the cancerous cells, partially proving the first hypothesis.

Increased protein expression of RFC, PCFT, and FR $\alpha$  following folate deficient growth has also been seen in HeLa cells, in response to increased transcription (Zhao *et al.*, 2008). Other possible explanations which may contribute to the altered expression include changes to regulatory mechanisms of transcription and translation including; binding of transcription factors, histone modifications and post transcriptional and post translation modifications (Thakur and Kaur, 2015). In a study human of colon cancer cell lines, C26-A, C26-10, C26-G and WiDr grown in folate deficient media, RFC expression was down regulated which correlates with the results seen in the 1321N1 cells (Backus *et al.*, 2000).

Another paper also showed increased expression of the RFC and PCFT, in the intestinal brush border membrane cells when grown in folate deficient conditions. This was suggested as being the result of an increased number, or activity of the folate transporters, rather than an increased affinity of folate for the transporters (Visentin *et al.*, 2014). The increased mRNA expression also seen along with the increased protein expression indicates that transcriptional and translational regulatory mechanisms are involved (Wani *et al.*, 2012b).

A study by Liu *et al.*, (2015) showed RFC expression increased in the intestines of mice fed a folate free diet compared to those fed a diet of 2mg/kg folic acid (Liu *et al.*, 2005). The PCFT also increases in expression in mice intestinal cells when treated with folate deficient conditions compared to those fed a standard folate diet of 2 mg/kg folic acid (Qiu *et al.*, 2007).

The second hypothesis was that cells grown in folic acid and folinic acid supplemented media would have altered protein expression levels compared to cells grown in standard cell culture conditions. Folic acid at a concentration of 40 µg/ml generally caused an increased level of expression compared to cells grown in standard cell culture media. Folinic Acid supplementation at both a low (4 µg/ml) and high (40 µg/ml) concentrations generally caused a decrease in protein expression level across all three cell lines. This hypothesis was proven for proteins FOLR1, MTHFR, and RFC.

The results observed in this chapter correlate with this evidence in the literature that cells treated with high levels of folic acid decreased protein expression, as detailed below. Caco-2 cells representative of intestinal epithelia cells showed decreased protein levels of RFC when treated with excessive levels of folic acid which was at a concentration of 100 µmol/L, compared to those grown in supplemented concentration of 25 µmol/L which was used as standard folic acid levels (Ashokkumar *et al.*, 2007).

Previous studies have shown that FOLR1 is regulated by the concentration of folate in cell culture medium (Matsue *et al.*, 1992). Human nasopharyngeal carcinoma (KB) cells cultured in media containing low levels of 5-methyltetrahydrofolate, at a concentration of 2-10 nM, showed higher expression of folate transport protein compared to the cells grown in the higher concentration of folic acid at 2300 nM (Kane *et al.*, 1988)

The third hypothesis that protein expression of metabolising enzymes and transporters would be higher in cancerous cells to cope with increased metabolic demands was only proven for one protein. The cancerous cells showed increased expression of the PCFT compared to the cells grown in standard cell culture media. The RFC protein increased in expression level in the U87 MG but also for the non-cancerous SVGp12 cells too. The FOLR1 increased in expression in the SVGp12 cells but did not for the cancerous cells.

There was a great difference in protein expression levels between the cancerous and non-cancerous cells, FOLR1 and RFC was higher in expression level, whilst PCFT and MTHFR were down regulated in comparison to the non-cancerous cells. PTEN behaved differently, it was up regulated in the 1321N1 cell line, and down regulated in the U87 MG cell line. There was also a significant difference seen in expression levels of FOLR1, RFC, MTHFR and PTEN between the different grades of glioma. Folate is transported across the cell membrane using the folate transporters; RFC, PCFT and folate receptors  $\alpha$ ,  $\beta$ , and  $\gamma$  with expression levels varying between the cell types (Farkas *et al.*, 2015b).

FOLR1 is more highly expressed in epithelial tumours than the comparative non-cancerous tissue (Evans *et al.*, 2008; Parker *et al.*, 2005; Toffoli *et al.*, 1997; Weitman *et al.*, 1994), and folate receptor one has been seen to be more highly expressed in glioblastoma tissue than that of normal brain tissue (Wu *et al.*, 2014), which was also seen in this study. This overexpression increases folate uptake, and therefore providing a growth advantage to tumorous tissues, (Necela *et al.*, 2015). It has been hypothesised that this could be due to the modulation of folate uptake from the serum (Kane *et al.*, 1988).

The final hypothesis stated at the beginning of the chapter was that differences in protein expression levels between the cell lines would be reflected by altered methylation status measured in Chapter 6. Comparing the two genes that showed altered methylation status between the cell lines; PTEN and PCFT, there was no correlation to protein expression so this

hypothesis was not proved. This absence of correlation has also been observed between the methylation status of the *MGMT* gene and its protein in human glioblastoma cells, (Uno *et al.*, 2011).

Maintenance of folate metabolism is probably dependent on the intricate balance of all metabolic enzymes and transporters within the folate pathway, and likewise changes in expression should be considered holistically rather than in isolation (Sharp *et al.*, 2004). For example, the altered expression levels of the folate transporters may not be only due to the availability of folate, but may also relate to the extent of metabolism (Sharp *et al.*, 2004). For example, there are less enzymatic steps required for folinic acid metabolism, therefore intracellular levels of 5-MTHF are easier to maintain and in turn this was reflected here by a lower expression of RFC following folinic acid treatment. Conversely, during folic acid treatment there are more metabolic steps, therefore it may be harder to maintain intracellular levels of 5-MTHF and in turn FOLR1 expression is increased. This would imply that intermediate metabolites, such as 5-MTHF, are critical sensors for cellular folate status and may play a key role in regulation of expression of transporters. This theory has been supported by Naz *et al.*, who found FDH, a key folate metabolic enzyme in particular is functionally related to FR $\alpha$ , and assists in transporting 5-MTHF to different areas of the brain (Naz *et al.*, 2016). The differences seen between the non-cancerous and cancerous cells could again be due to folate metabolism issues. Cancer cells proliferate rapidly, and therefore are more susceptible to folate metabolism disruption, as this could result in limited folate availability for DNA synthesis, a mechanism essential for cell division. Future work would hope to focus on linking cellular folate status, folate metabolism and regulation of expression of folate enzymes and transporters to gain a clearer understanding.

In summary, it was seen that folate deficiency does cause an increased expression level of the folate transporters, PCFT in the cancerous cells, FOLR1 in the non-cancerous cells, and the RFC in the non-cancerous and high grade cancerous cells, required to increase the uptake

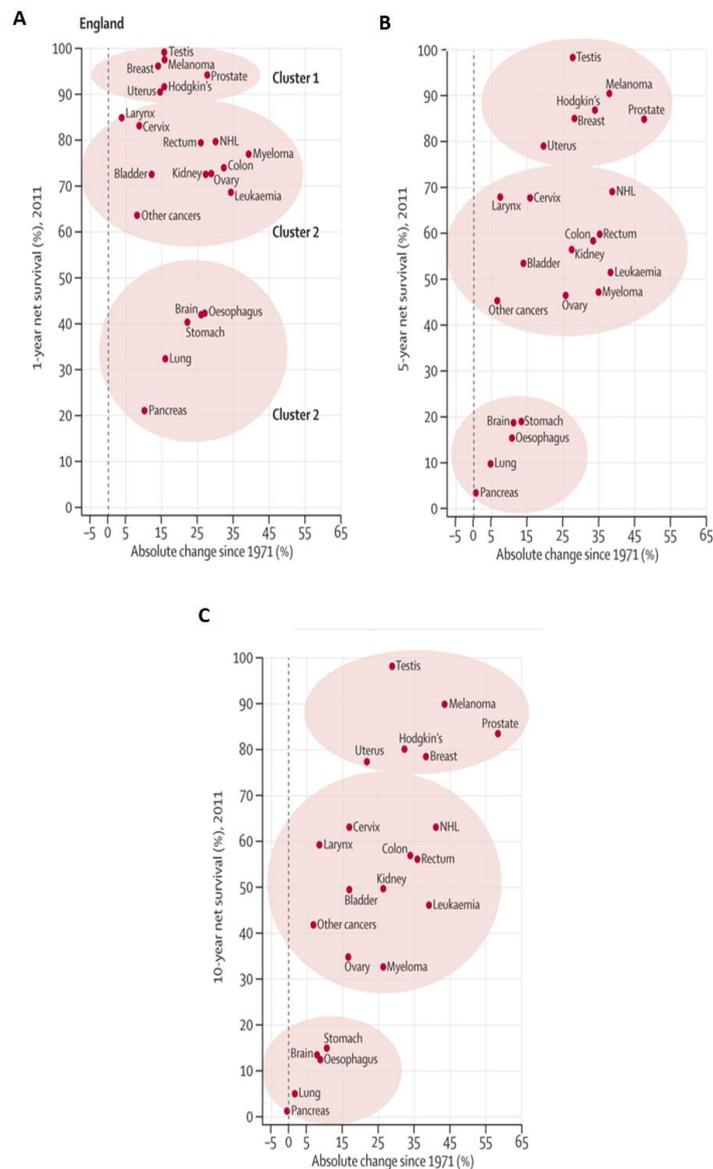
of folate into the cell. In particular, it was observed that folic acid supplementation increased protein expression of PTEN, FOLR1, PCFT, and MTHFR, whilst folinic acid appeared to down regulate protein expression levels of PTEN, MTHFR, FOLR1, PCFT, and RFC. This could be a potential treatment target for restoration or exploitations genes that have been seen to be up or down regulated in cancerous cells compared to their non-cancerous counterpart. There was also a difference in expression levels for all genes which was seen between cancerous and non-cancerous cells, and between grades of tumour, this difference could be used as a potential biomarker to identify cancerous tissues, or to assist in grading of a tumour.

# Chapter 8

## 8 DISCUSSION

### 8.1 General Discussion

The survival rates of patients with high grade glioma still remain low, with little alteration over the last 10 years, despite the advances in life expectancy for many other cancer patients (Figure 8.1) (Quaresma *et al.*).



**Figure 8.1** Change in cancer survival rates;

The change in cancer survival rates after (A) 1 Year, (B) 5 Years and (C) 10 Years as of 2011. Glioma is represented as 'Brain' (Quaresma *et al.*, 2015).

This continued poor survival rate is in part due to the lack of funding for research agreed by parliament, with brain tumours receiving only 3.3% of site specific cancer research funding (UK Parliament, 2015). The large genetic variation seen between tumours is also a hindrance, making a 'blanket' treatment for all patients ineffective, due to the heterogeneous molecular nature (Burger and Kleihues, 1989; Phillips *et al.*, 2006a). The invasive nature of brain tumours, and their close proximity to healthy tissue, means that surgery often leaves behind tumorous cell which chemotherapy is administered to target, but there is often difficulty with these drugs being effluxed at the blood brain barrier (Mrugala, 2013).

Folate is a naturally occurring vitamin that can cross the blood brain barrier, through different folate transporters, such as folate receptor alpha, which is one of the transporters expressed at higher levels in cancerous cells compared to non-cancerous cells (Cheung *et al.*, 2016; Zhao *et al.*, 2009). Folinic acid, although not as abundant as the naturally occurring 5-MTHF, is also found naturally, and is found in the reduced state, whilst folic acid the oxidised form is more stable and found synthetically in food. The different redox states mean that folic and folinic acid have a different affinity for different transporters, and this difference combined with the different expression levels of the folate transporters may play a key role in glioma treatment.

Folate was also considered as a treatment option due to its antioxidant properties, which are frequently published as being beneficial to health (Joshi *et al.*, 2001; Van Duyn and Pivonka, 2000). The body, as a by-product of respiration, produces free radicals called reactive oxygen species (ROS). which are mutagenic and can potentially result in cancer (Shi *et al.*, 2012). Antioxidants such as folic acid, accept electrons from the free radicals and reduce the risk of developing cancer (Fiedor and Burda, 2014). Antioxidants have also been seen to increase the risk of cancer, a study showed that smokers who were given beta carotene supplements were 16% more likely to develop lung cancer than smokers who did not receive a supplement (The Alpha-Tocopherol, 1994). Another study of smokers given beta-carotene and vitamin A

had a 28% increase in lung cancer occurrence, and 17% increase in death (Omenn *et al.*, 1996). It is also inaccurate to assume that free radicals are only damaging, without them we would have difficulty fighting many bacterial infections, as they stimulate immune response (Pham-Huy *et al.*, 2008).

Antioxidants can be obtained through diet, and it seems that diet is a crucial factor in not only brain tumour risk, but ways to slow progression, as improved survival by up to 5 years has been observed in brain tumour patients on the energy restricted ketone diet (Schwartz *et al.*, 2015). These studies however, have had limited patient numbers (19-30). A ketone diet has been suggested as a treatment due to the different energy requirements between normal and tumorous cells. Normal cells use glucose and ketones for energy, whilst tumour cells are more dependent upon glucose, so glucose restricted, high ketone diets theoretically mean that tumour cells will not have the energy source required for proliferation whilst normal cells still will (Schwartz *et al.*, 2015). A ketone diet has also been seen to reduce ROS production in the brain; cancer cells have increased ROS levels (Stafford *et al.*, 2010). It has been seen that the ketone diet significantly slowed down tumour growth, reduced ROS production associated with tumour growth, altered expression of genes involved in ROS production in the tumour; glutathione peroxidase 7 (Gpx7), peroxiredoxin 4 (Prdx4) and Cox2 (Stafford *et al.*, 2010)

The key role of folate in DNA synthesis, as a methyl donor, and cell growth has meant that folate has opposing roles and can both protect against developing neoplasia, as well as promoting cancerous growth (Tables 1.9 and 1.10) (Wani *et al.*, 2012a).

The aim of this thesis was to assess the effect of folate treatment in gliomas In particular, to focus on the effect of folate treatment on cell viability in both cancerous and non-cancerous cells, and to understand the mechanism behind any altered cell viability.

The thesis began by first optimising any experimental procedures that were to be performed to ensure reproducibility and confidence in the results seen. Characterisation of the cells used was also essential to ensure that the results seen were relevant to glioma. This was of huge importance given a recent study that came out at the end of this project which highlighted that many U87 MG cells, although still of glial origin, are not from the patient sources originally detailed (Allen *et al.*, 2016). Initial growth curves comparing cells grown in folate free media were significantly slower growing compared to standard cell culture.

Cell viability following folate treatment was first analysed because if folate supplementation had no significant effect on viability, there would have been little reason to continue the investigation. Treatment with folic acid showed mixed results, but there was a significant decrease in cell viability in the 1321N1 cells treated at 35  $\mu\text{g}/\text{mL}$  compared to standard cell culture media after 5 days of daily treatment. Treatment with folinic acid however showed more consistent results, with high level treatment (35  $\mu\text{g}/\text{ml}$ ) significantly reducing the cell viability in all three cells lines. This was supported in the literature which showed the cell viability of villous trophoblast BeWo cell line reduced following treatment with 2  $\text{mg}/\text{ml}$  folic acid (Ahmed *et al.*, 2016).

Given this significant difference in viability seen it was necessary to understand the mechanisms behind it, whether the perceived reduction in cell viability was as the result of cell cycle arrest, pausing further proliferation, or whether the cells were undergoing increased apoptosis.

Cell cycle analysis showed no difference in the percentage of each cell cycle phase following treatment in any of the cell lines, there was also no significant difference in levels of apoptosis which was unexpected. Unfortunately, no positive control was used in the flow cytometry analysis, to ensure that any apoptotic activity was being recorded. In future work a positive control for apoptosis should be incorporated, such as a set of cells treated with camptothecin

a topoisomerase inhibitor that interferes with the essential function of topoisomerase in DNA replication (Liu *et al.*, 2000). A more sensitive test for apoptotic activity was decided upon by analysing levels of caspase 3/7 activity which did show a significant difference.

Folic Acid has been shown here to be a possible treatment for cancerous cells given its ability to reduce cell proliferation by increasing levels of apoptosis. Folic acid is advantageous because it has been shown in other studies that apoptosis can be reversed by increasing the availability of 5-MTHF when needed, to prevent any ongoing damage to the non-cancerous cells (Akoglu *et al.*, 2004). This affect was seen in a report that showed Caco2 cells that were treated with folic acid at a concentration of 4µg/ml underwent cell cycle arrest, with an accumulation of cells within the S phase resulting in cell death (Akoglu *et al.*, 2004), and noted that the supplementation with folic acid causes cells to accumulate in the S phase. with the majority of studies have only reported the use of folic acid as a supplementation, the effects of other forms of folate, such as folinic acid on cell growth have not been investigated which makes the work done here novel. (Duthie, 2001),

A better measure of cell cycle phase would have been the use of bromodeoxyuridine (BrdU) incorporation rather than propidium iodide staining. BrdU is incorporated into the cells at S Phase, and these cells can then be identified at different time points when stained for at later time points (Welschinger and Bendall, 2015).

Following on from the cell viability and apoptosis studies, the methylation status of the genes of interest were analysed. In this study no notable changes in methylation status were observed between the cell lines, or following treatment with either folic or folinic acid. This may be because there was no difference in the genes chosen. A review of folate treatment and methylation studies, showed there was no linear relationship between folate supplementation and methylation status (Crider *et al.*, 2012). Another explanation is that the wrong coding sequence of the gene was being analysed, or it may have been due to the

method of analysis, as MS-PCR is only a semi-quantitative method, and may not have been sensitive enough to observe any alterations.

The protein expression levels showed that folate deficiency does cause an increase in expression level of the folate transporters; the PCFT in the cancerous cells 1321N1 and U87MG and FOLR1 in the non-cancerous SVGp12 cells. The RFC is upregulated in the U87 MG and SVGp12 cells, in order to increase the uptake of folate into the cell. This finding has also been seen in the literature (Thakur *et al.*, 2016; Wani *et al.*, 2012b) Supplementation with folic acid at 40 µg/ml caused an increase in protein expression for PTEN, MTHFR, FOLR1 and PCFT, whilst folinic acid at both 4 and 40 µg/ml appeared to down regulate protein expression levels of the PTEN, MTHFR, FOLR1, PCFT and RFC proteins.

The differences in protein expression observed in the treated cells compared to the cells grown in standard cell culture media led to a potential treatment option for genes that have been seen to be up or down regulated in cancerous cells compared to their non-cancerous counterpart. For example, folic acid at a concentration of 40 µg/ml could be used to increase expression of genes that are downregulated such as PTEN, MTHFR, FOLR1 and PCFT, whilst folinic acid at 4 or 40 µg/ml could reduce expression of proteins that are over expressed, such as FOLR1, a known protein that is overexpressed in cancerous tissue (Evans *et al.*, 2008).

The difference in protein expression levels of PTEN, FOLR1, RFC, PCFT, and MTHFR observed between cancerous and non-cancerous cells, and between grades of tumour, could be used as a potential biomarker to identify cancerous tissues, or to assist in grading of a tumour, although extensive validation studies would be required to confirm this.

## 8.2 Limitations

Due to resource constraints the only way of establishing methylation status was through methylation specific PCR, however, this technique is only suitable when candidate genes are known and have been chosen, which limits the genes that can be analysed. Primers were

designed for specific regions of the genes involved and will therefore only assess alteration to methylation status at that particular region. It is possible that different codons within the gene may show different results.

Profiling the whole genome would allow for greater information regarding the epigenetic changes that occur during tumourgenesis (Bujko *et al.*, 2014; Calvo *et al.*, 2014). A technique most suitable for this would be Line 1 pyrosequencing which allows for high throughput analysis, followed by whole genome bisulphite sequencing which will allow for specific genes and regions that are differently methylated to be identified (Kurdyukov and Bullock, 2016). Bisulphite sequencing works by comparing bisulphite treated DNA to non-treated control DNA, it is still however a costly technique (Ziller *et al.*, 2015).

A chronic dosing study would have been interesting to observe the long term effects of folate supplementation given that fortified folate supplementation in food industry has become so widespread, and individuals are exposed to a higher level of folate than previously seen. A chronic study would allow assessment of the observed benefits of reducing neural tube defects in infants versus any possible increase in tumourgenesis occurrence (Joubert *et al.*, 2016; Mortensen *et al.*, 2015).

It would also have been interesting to perform a study analysing the combined effects of folic and folinic acid supplementation, as they have been seen to have differing effects in both this study. In addition, looking at the effect of folic and folinic acid supplementation in reducing the side effects of methotrexate treatment in rheumatoid arthritis patients (Morgan *et al.*, 2004).

It would have also have been ideal to have performed analysis on primary tissue to observe what effect occurred following folate treatment on cells with mixed origin as well as *in vivo* studies as treatment was shown to have differing effects on the different cells lines.

### 8.3 Future Work

The recommended course of treating glioma through resection followed by radiotherapy plus concurrent temozolomide application is unlikely to change (Weller *et al.*, 2014). This is an invasive procedure which has detrimental psychological and physical effects on patients (Henriksson *et al.*, 2011; Rooney and Grant, 2010). Tumours that are slow growing may remain undetected for years until the mass grows to a size big enough to produce symptoms (Pouratian and Schiff, 2010), however if detected early enough, perhaps targeted chemotherapy may reduce progression of glioma growth and the need for resection. The chemotherapeutic drugs used to treat glioma are currently carmustine wafers which stop division in the cancer cell, and temozolomide, an alkylating agent which delivers a methyl group to purine bases of DNA (O<sub>6</sub>-guanine; N<sub>7</sub>-guanine and N<sub>3</sub>-adenine), resulting in DNA cross-linkages which inhibits DNA and cellular replication (Wesolowski *et al.*, 2010). Carmustine wafers however need to be placed at the tumour site, and temozolomide is not cancer cell specific.

Following on from the literature and evidence seen in Chapter 7, that there is an increased expression of the FOLR1 in glioma tissue compared to that of non-cancerous origin (Kobel *et al.*, 2014; Necela *et al.*, 2015). The folate receptor works via a process termed receptor-mediated endocytosis (Low and Kularatne, 2009). The overexpression of the FOLR1 in cancer cells has been utilised by using folic acid as a drug targeting ligand. Following intracellular delivery, the drug is released and remains near to the nucleus of the cell whilst the receptor is recycled back to the cell surface (Yang *et al.*, 2006). This has already been successfully trialled in humans with encouraging results. A folate conjugate was created <sup>111</sup>In-DTPA-folate, that was seen to localise to cancer cells in the kidneys, without creating a toxic effect due to its rapid clearance from the kidneys (Sega and Low, 2008b).

There are however limitations, the cancer would need to have a high expression of folate receptor alpha, so more work would be needed to characterise levels of folate receptor between tumour grades and the attached drug needs to be membrane permeable (Low and Kularatne, 2009). To establish whether cells increased the levels of FOLR1 in order to find the required folate, proteomic analysis would be performed to ascertain the levels of protein.

Another area of future research would be to measure folate levels both outside and inside of the cells, to establish whether the altered protein expression levels of the folate transporters were having an effect on the level of folate being transported into the cell.

#### 8.4 Conclusion

So, in conclusion, is folate a friend or foe? It would appear that it is both. The results observed in this study indicate that folic acid has the potential to reduce glioma growth, by increasing levels of apoptosis, and therefore slowing the rate of growth of tumours. Given this clinical potential, folic acid, and the more naturally abundant form of folate, 5-MTHF, should be further researched as a potential therapeutic tool or adjuvant therapy.

Folic acid however appears to increase the rate of cancerous growth, the rate of cell proliferation increased following treatment. The different responses seen are due in part to the different expression levels of the folate transporters. Higher protein expression levels of the FOLR1, a transporter with a high affinity for folic acid, were seen in the cancerous cells. This increased expression theoretically would allowed for increased intracellular levels of oxidised folate, required for the increased metabolic demands of the cancer cells to be met. The thesis findings suggest that folic acid supplementation should be discontinued, and instead the advice should be to use a naturally occurring form of folate, either as folic acid or 5-MTHF.

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## 10 APPENDICES

1. *Cell Culture Treatments for 1321N1 Cell Line* where: NaOH represents the solvent required to dissolve folic acid and A/O represents antioxidant supplementation

	Folate Free Media	NaOH Control	Antioxidant (A/O) Control	Folic Acid 4µg/ml with A/O	Folic Acid 4µg/ml without A/O	Folic Acid 40µg/ml with A/O	Folic Acid 40µg/ml without a A/O	Folinic Acid 4µg/ml with A/O	Folinic Acid 4µg/ml without A/O	Folinic Acid 40µg/ml with A/O	Folinic Acid 40µg/ml without A/O
<b>FF Media</b>	44.00ml	44.00ml	43.95ml	43.95ml	44.00ml	43.95ml	44.00ml	43.95ml	44.00ml	43.95ml	44.00ml
<b>FBS</b>	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml
<b>L Glut</b>	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
<b>Pen Strep</b>	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
<b>NaOH</b>	-	40µl	-	-	-	-	-	-	-	-	-
<b>Antioxidant</b>	-	-	0.05ml	0.05ml	-	0.05ml	-	0.05ml	-	0.05ml	-
<b>Folic Acid in NaOH</b>	-	-	-	4µl	4µl	40µl	40µl	-	-	-	-
<b>Folinic Acid</b>	-	-	-	-	-	-	-	2µl	2µl	20µl	20µl
<b>TOTAL Volume</b>	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml

**2. Cell Culture Treatments for U87MG and SVGp12 Cell Lines** where: NaOH represents the solvent required to dissolve folic acid and A/O represents antioxidant supplementation

	Folate Free Media	NaOH Control	Antioxidant (A/O) Control	Folic Acid 4µg/ml with A/O	Folic Acid 4µg/ml without A/O	Folic Acid 40µg/ml with A/O	Folic Acid 40µg/ml without A/O	Folinic Acid 4µg/ml with A/O	Folinic Acid 4µg/ml without A/O	Folinic Acid 40µg/ml with A/O	Folinic Acid 40µg/ml without A/O
<b>FF Media</b>	43.00ml	43.00ml	42.50ml	42.50ml	43.00ml	42.50ml	43.00ml	42.50ml	43.00ml	42.50ml	43.00ml
<b>FBS</b>	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml
<b>L Glut</b>	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
<b>Pen Strep</b>	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
<b>NEAA</b>	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
<b>Na Pyr</b>	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
<b>NaOH</b>	-	40µl	-	-	-	-	-	-	-	-	-
<b>Antioxidant</b>	-	-	0.05ml	0.05ml	-	0.05ml	-	0.05ml	-	0.05ml	-
<b>Folic Acid in NaOH</b>	-	-	-	4µl	4µl	40µl	40µl	-	-	-	-
<b>Folinic Acid</b>	-	-	-	-	-	-	-	2µl	2µl	20µl	20µl
<b>TOTAL Volume</b>	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml	50ml

### 3. Western blotting recipes

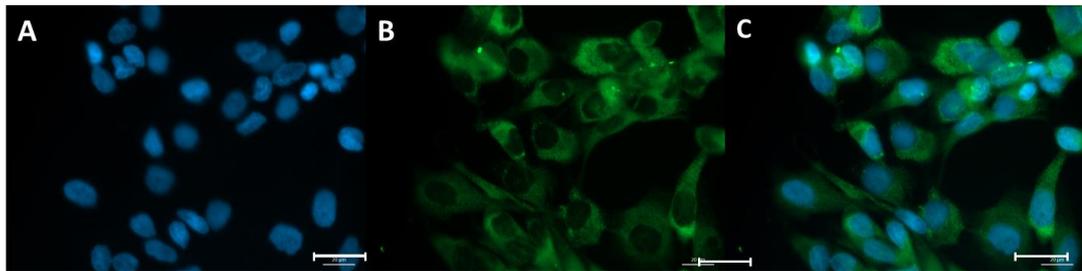
a) Recipe for casting resolving and stacking gel for SDS-PAGE.

Materials	10% Resolving Gel (for two gels)	4% Stacking Gel (for two gels)
dH <sub>2</sub> O	4.7 mL	4.5 mL
1.5M Tris Buffer (pH 8.8)	2.6 mL	-
1M Tris Buffer (pH 6.8)	-	0.76 mL
10% SDS	100 µl	60 µl
40% Acrylamide	2.5 mL	0.6 mL
10% APS (freshly made only)	100 µl	60 µl
TEMED (added prior to pouring gel)	10 µl	6 µl
Total Volume	10 mL	6 mL

b) Buffer Recipes

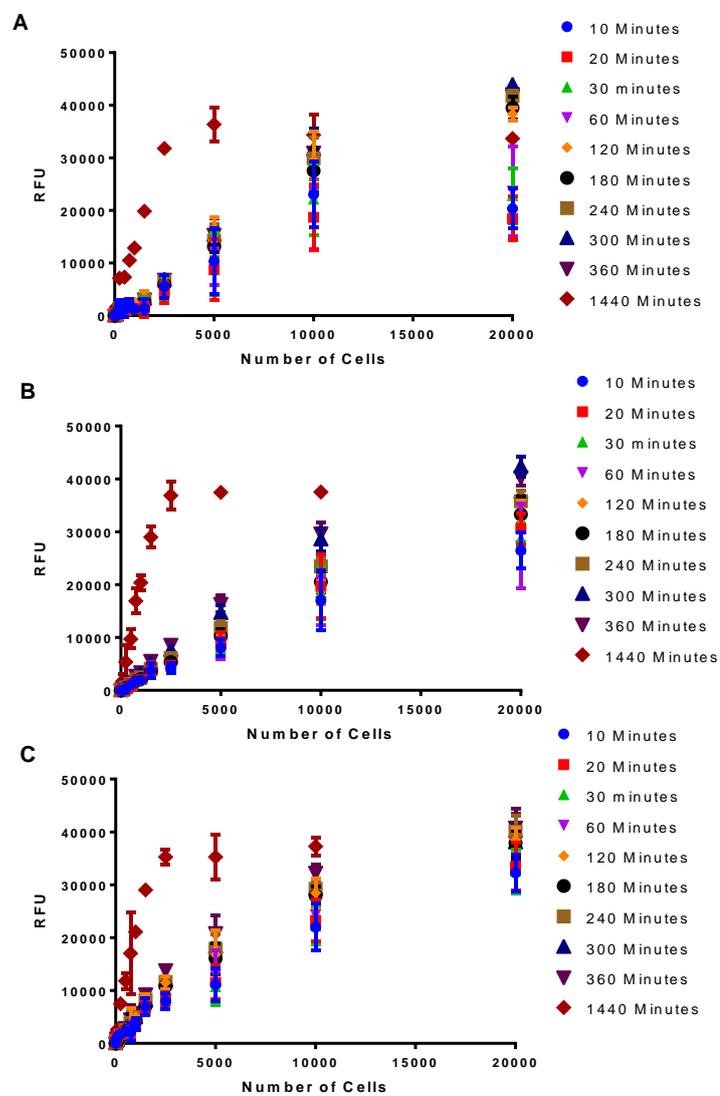
Buffer	Recipe
Sample loading buffer	<ul style="list-style-type: none"> <li>• 4% SDS</li> <li>• 10% 2-mercaptoethanol</li> <li>• 20% glycerol</li> <li>• 0.004% bromophenol blue 0.125 M Tris HCl</li> </ul>
Blocking Buffer	<ul style="list-style-type: none"> <li>• 5 ml 10 x Fish Gelatine</li> <li>• 45 ml PBS</li> </ul>
Electrophoresis buffer (10X)	<ul style="list-style-type: none"> <li>• 303 g Trisbase (FW 121.1) 1440 g glycine (FW 75.07)</li> <li>• 100 g SDS</li> </ul>
Transfer buffer (10X)	<ul style="list-style-type: none"> <li>• 303 g Trisbase</li> <li>• 1440 g glycine</li> </ul>
Wash buffer (10X)	<ul style="list-style-type: none"> <li>• 24.23 g Trizma HCl</li> <li>• 80.06 g NaCl</li> <li>• 800 ml distilled water</li> <li>• pH to 7.6</li> </ul>
Stripping buffer	<ul style="list-style-type: none"> <li>• 15 g glycine</li> <li>• 1 g SDS</li> <li>• 10 ml Tween 20</li> <li>• 800ml with ultrapure water Adjust pH to 2.2</li> </ul>

#### 4. Immunochemistry Images



1321N1 cells were grown for 5 days in standard cell culture media. **A** represent staining with DAPI (nuclear staining). **B** represents staining with GFAP (green) **C** represents the merged images. Where the scale bar represents 50  $\mu\text{m}$ .

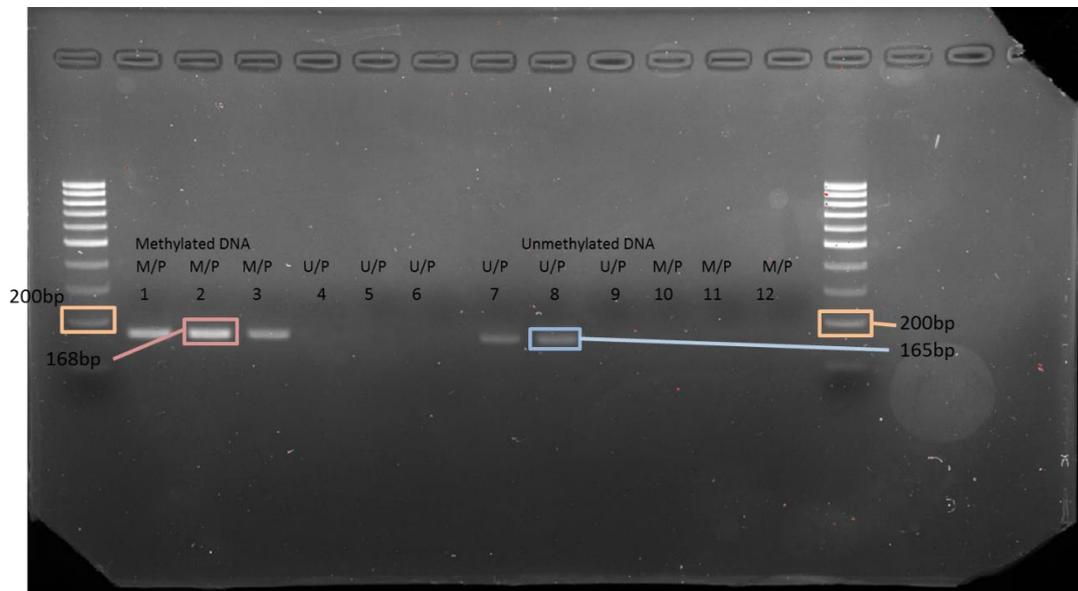
#### 5. PrestoBlue Optimisation Graphs



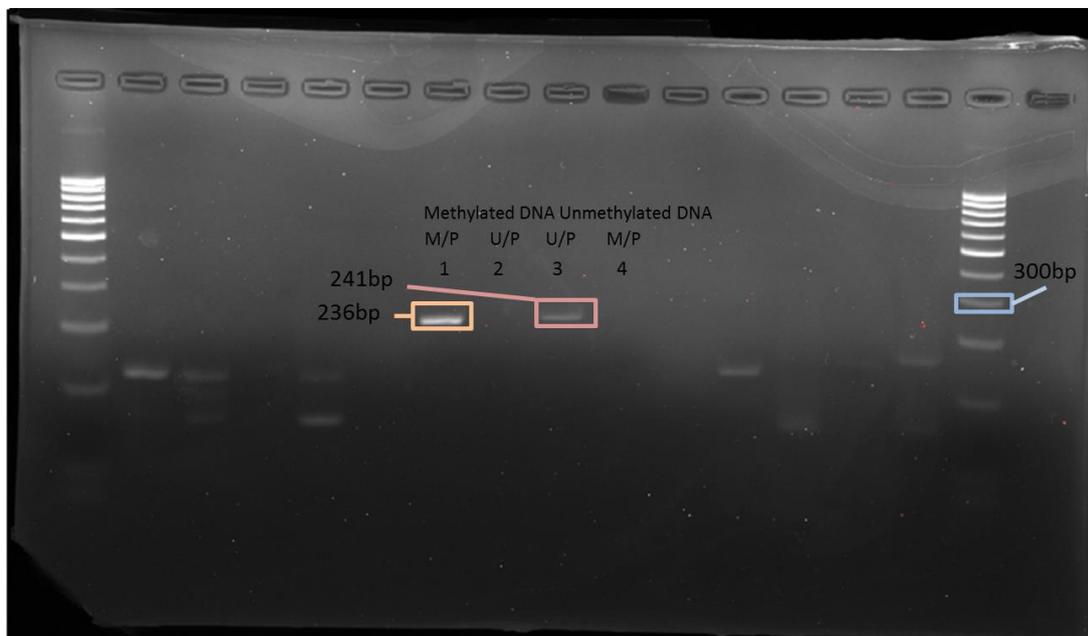
Where **A**) 1321N1 **B**) U87MG **C**) SVGp12

## 5. Whole MSPCR Gels

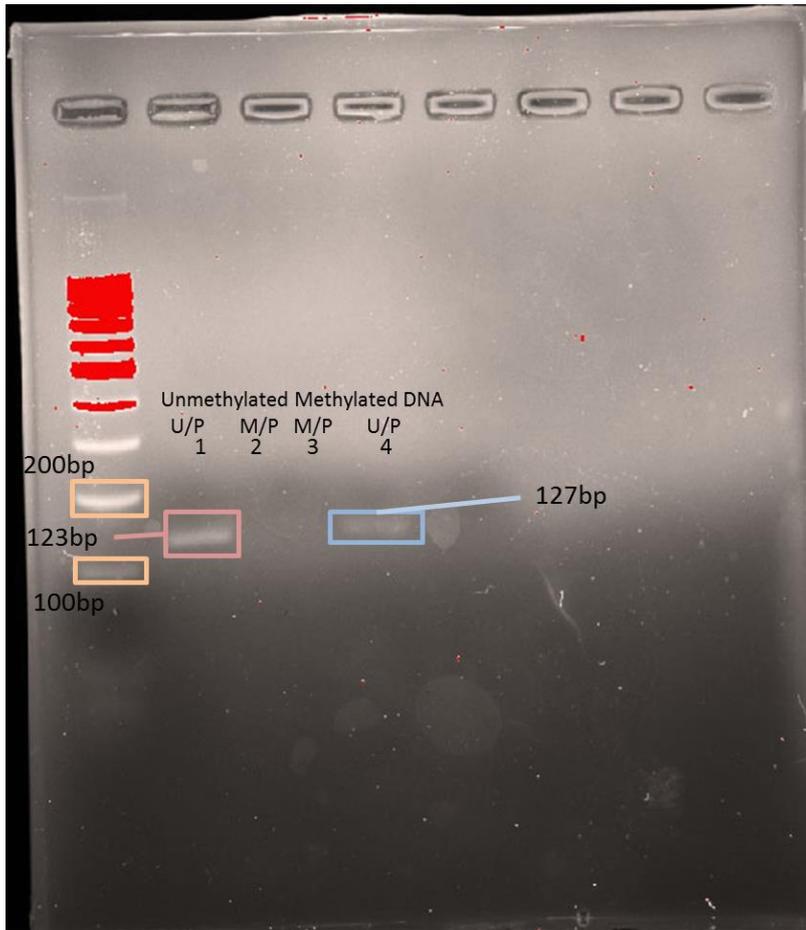
**PTEN Primer Optimisation;** where M/P is methylated primer and U/P is unmethylated primer.



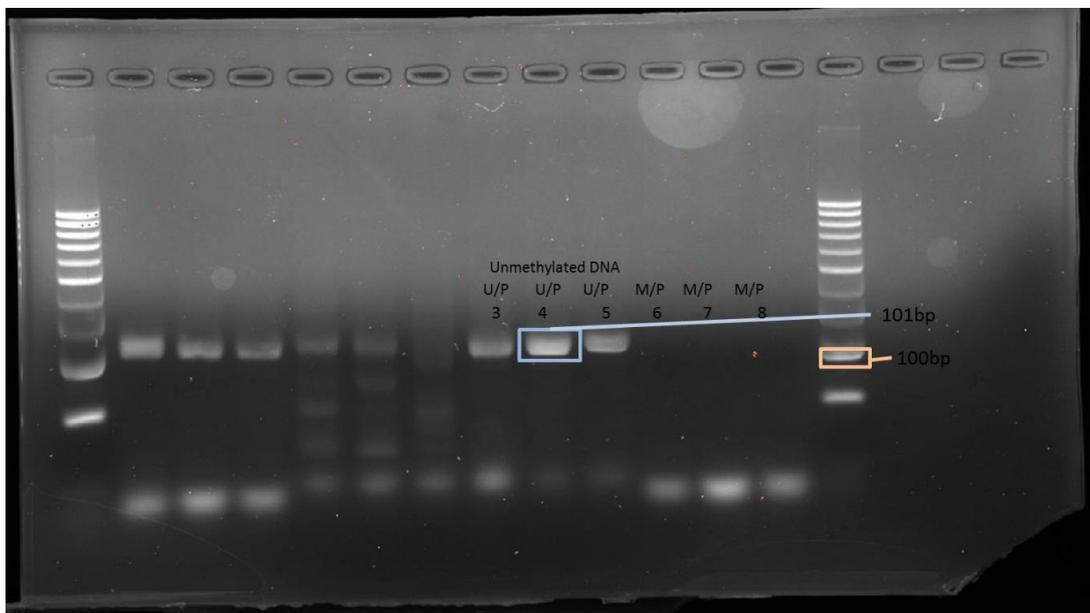
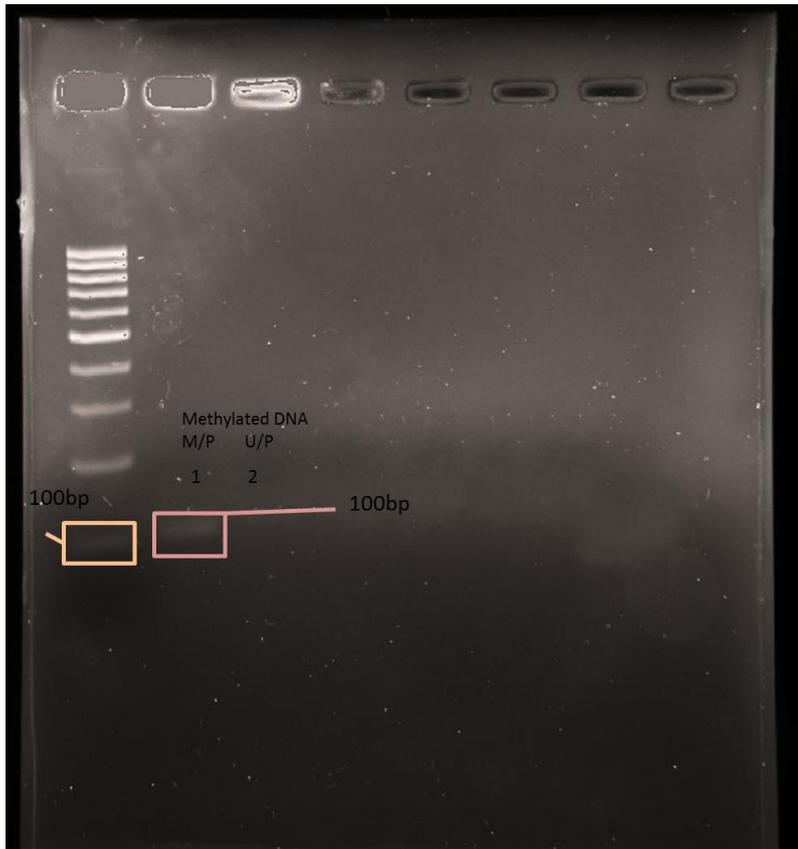
**FOLR1 Primer Optimisation;** where M/P is methylated primer and U/P is unmethylated primer.



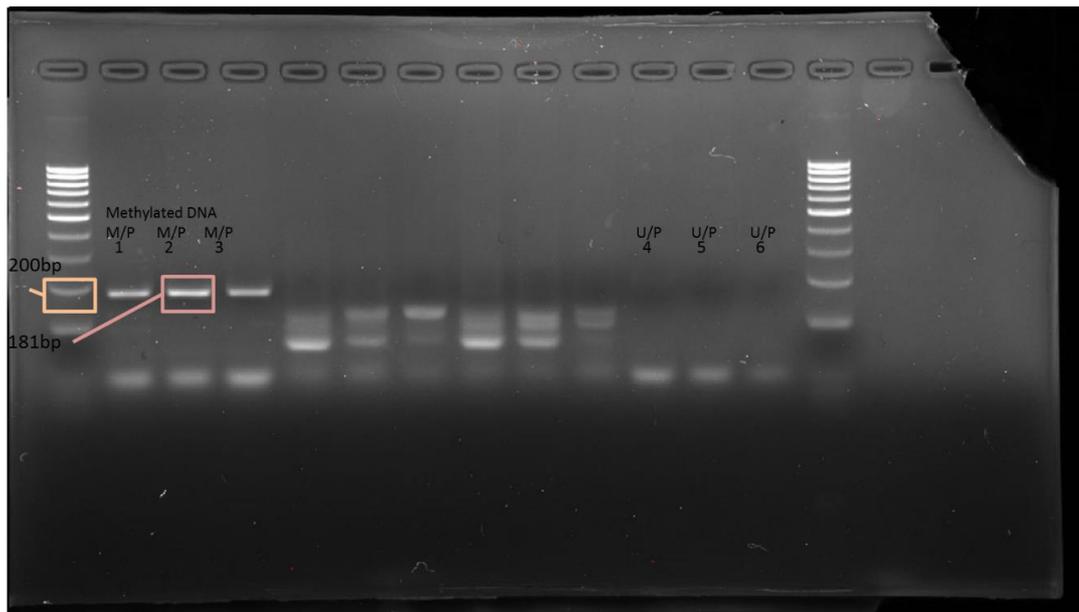
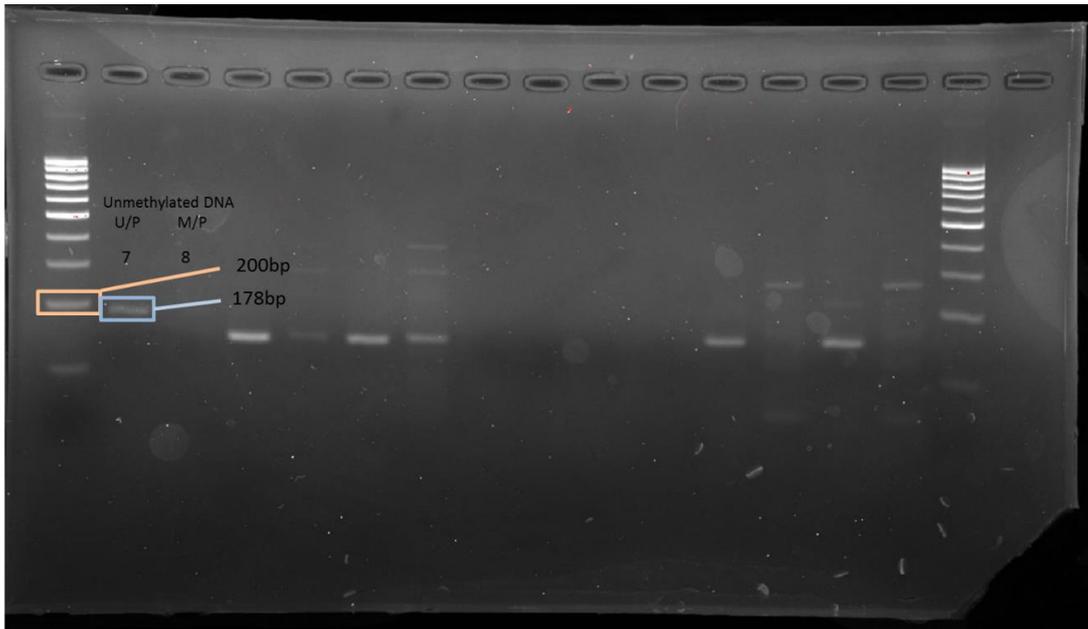
**RFC Primer Optimisation;** where M/P is methylated primer and U/P is unmethylated primer.



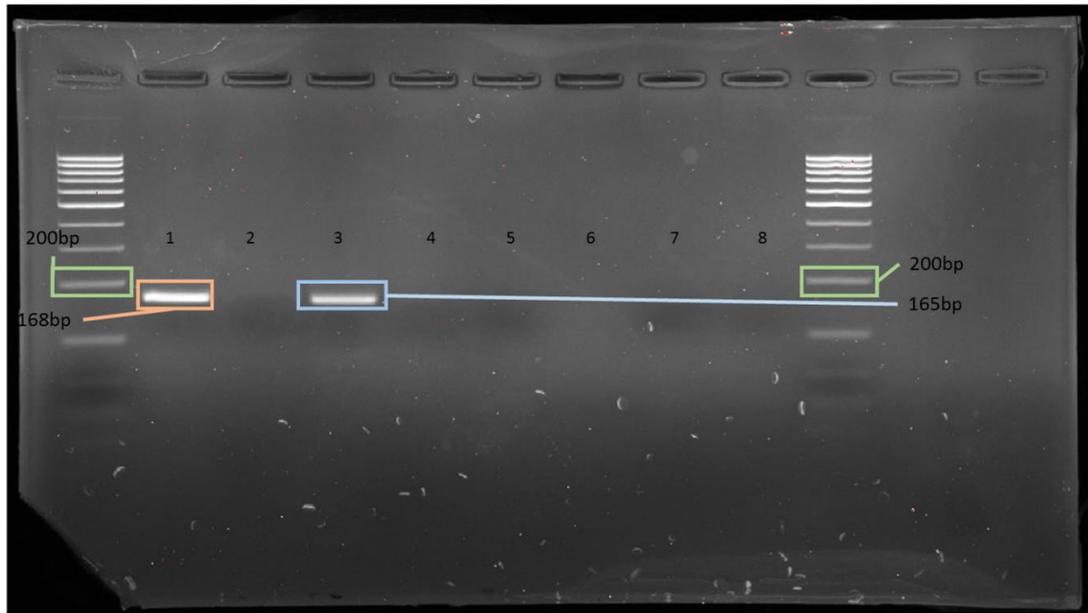
**PCFT Primer Optimisation;** where M/P is methylated primer and U/P is unmethylated primer.



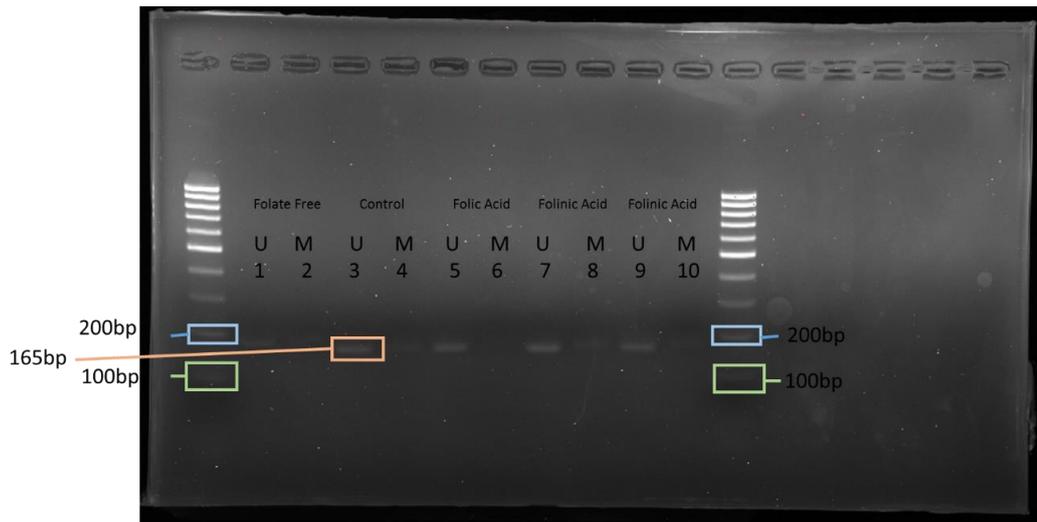
**MTHFR Primer Optimisation;** where M/P is methylated primer and U/P is unmethylated primer.



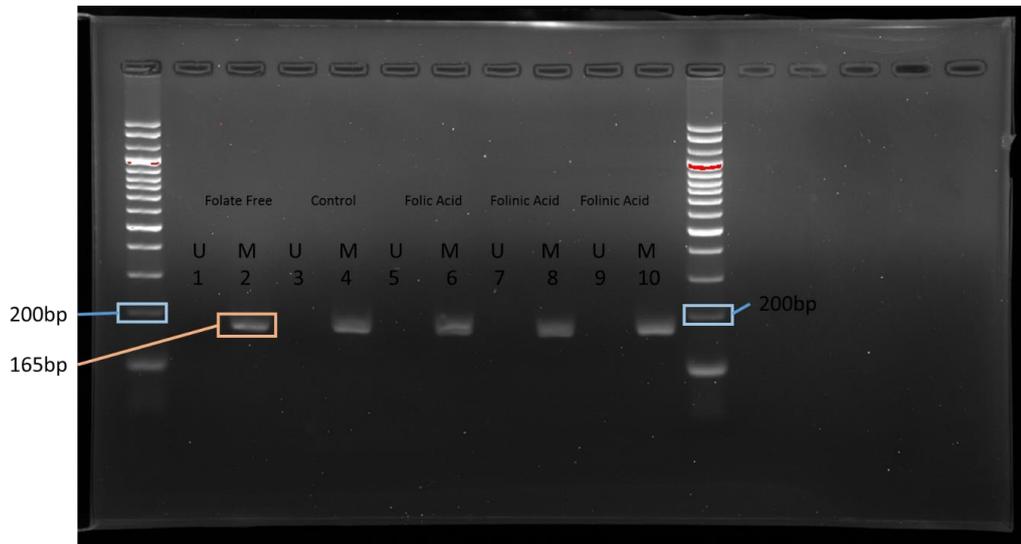
MSPCR Control Gel



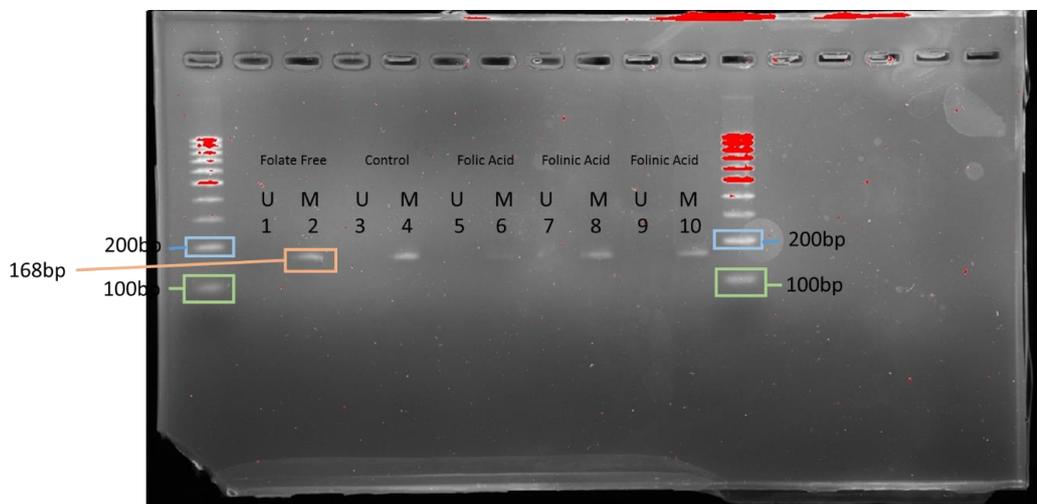
**PTEN Samples - 1321**



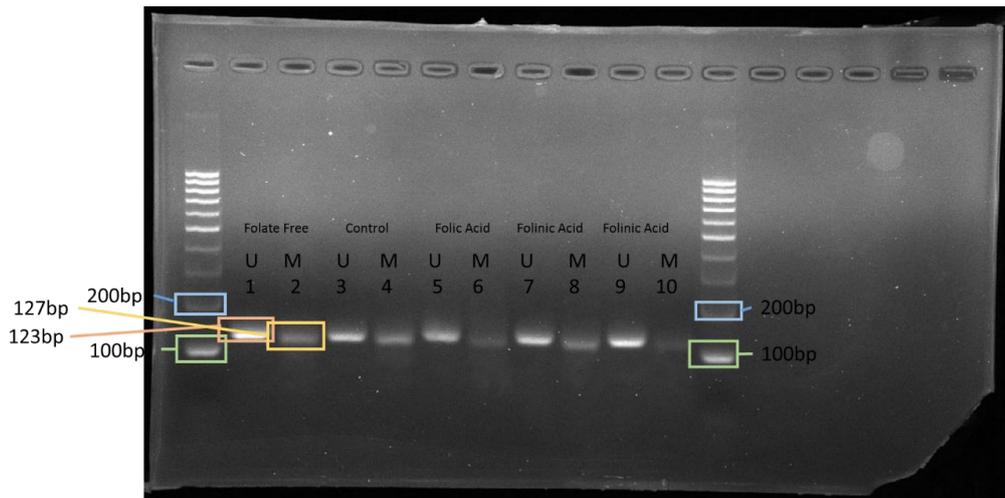
**U87**



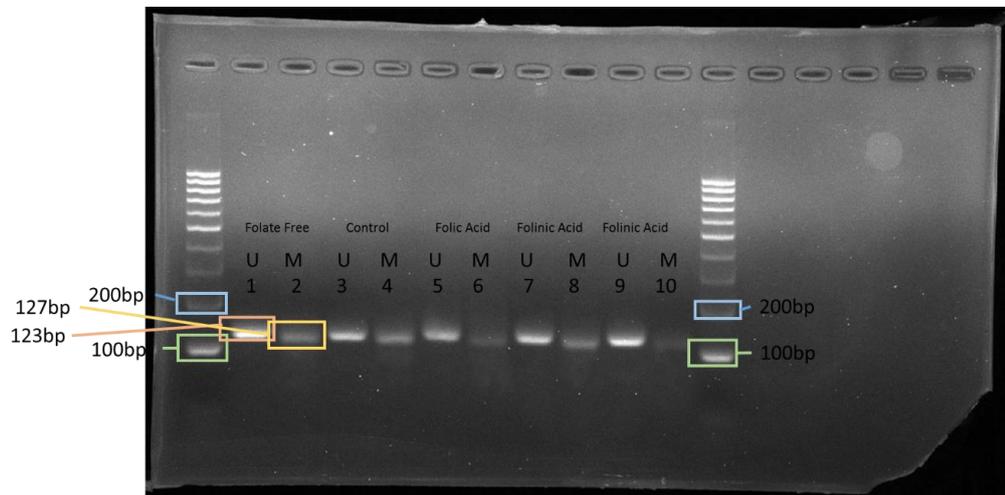
**SVG**



### RFC Samples - 1321



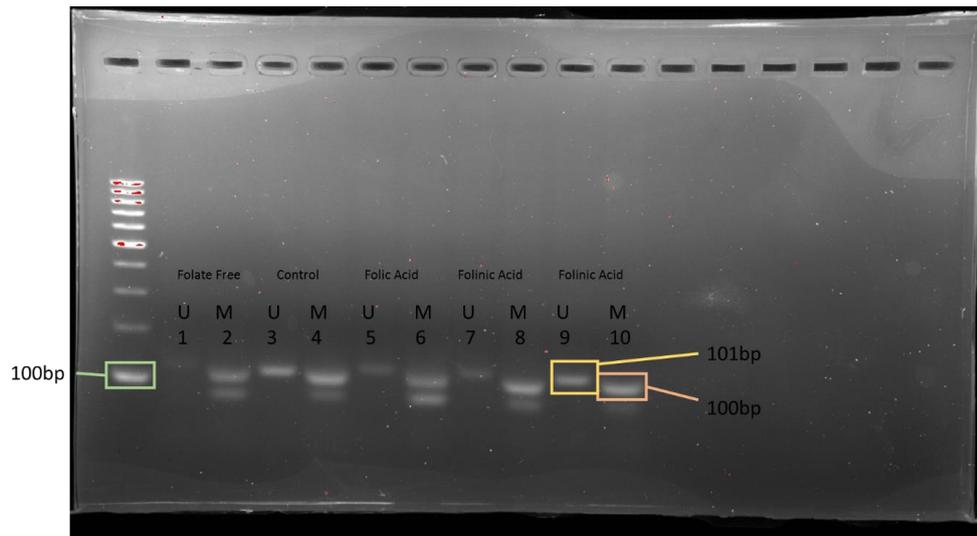
### U87



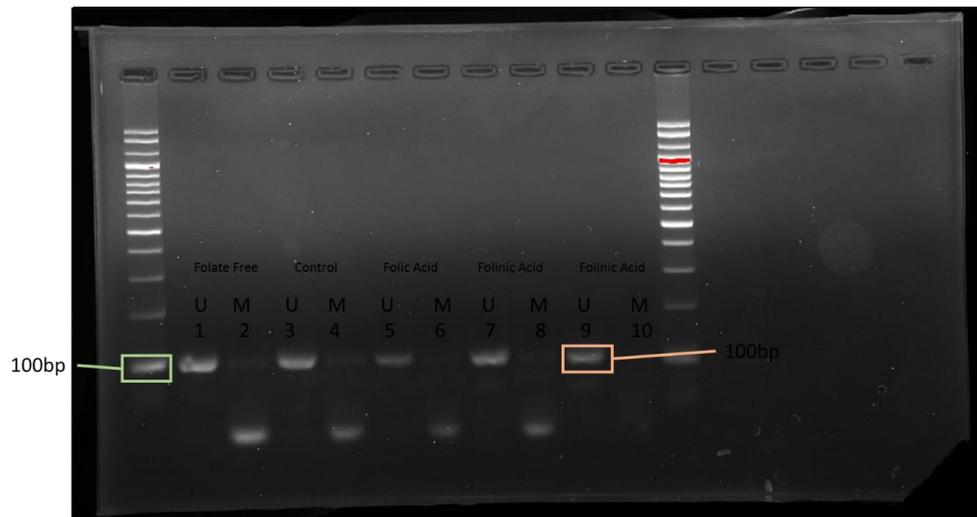
### SVG



PCFT Samples - 1321



U87



SVG

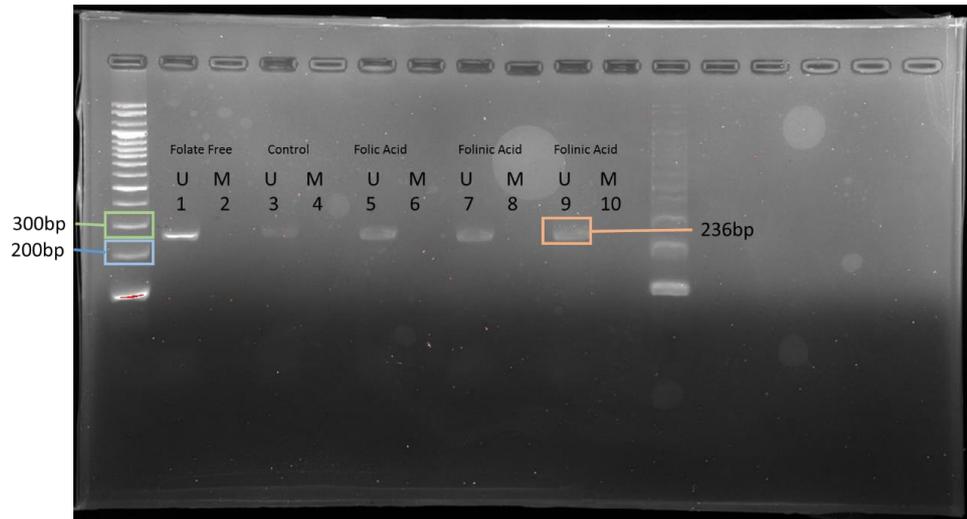




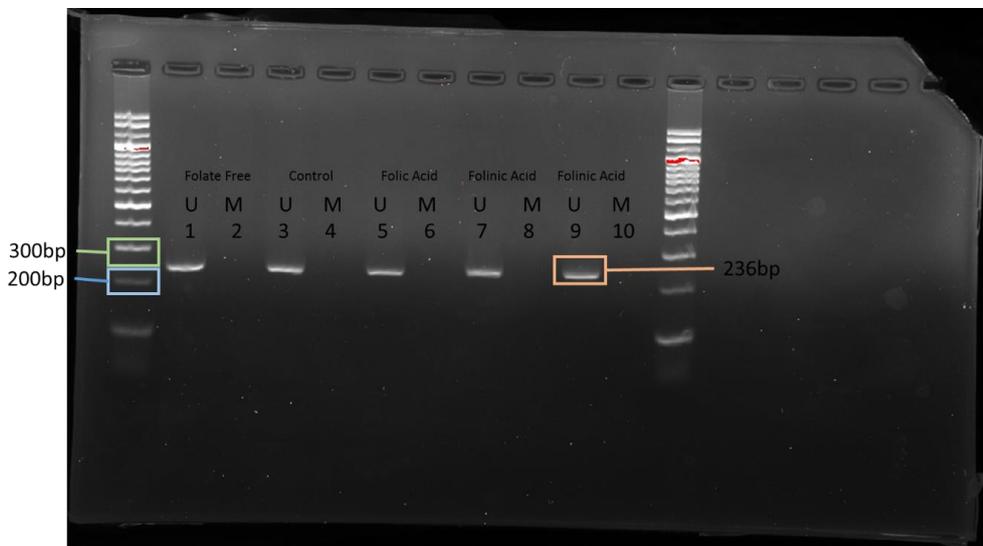
### FOLR1 Samples - 1321



### U87



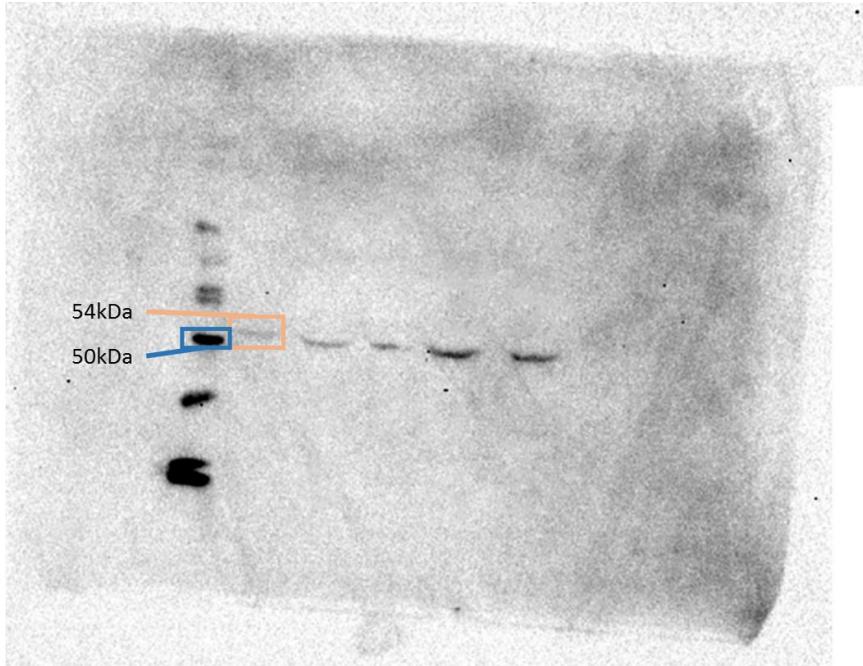
### SVG



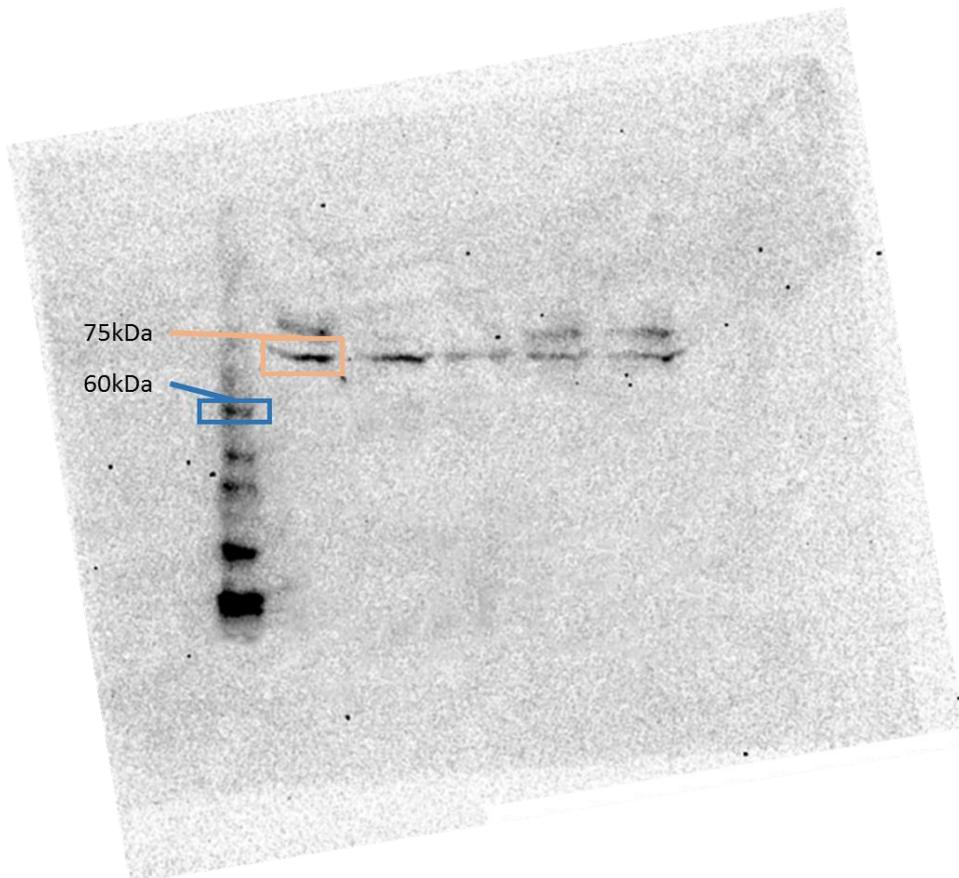
## 6. Full Western Gels

### Appendix Western Immunoblotting Antibody Validation

#### PTEN (54 kDa)



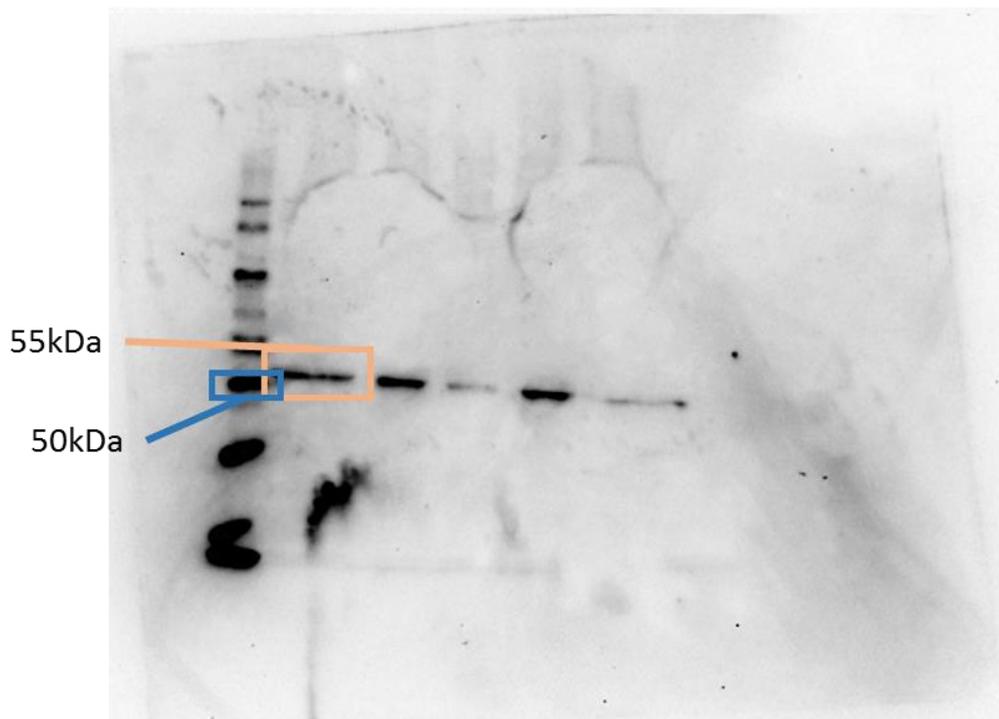
#### MTHFR (75 kDa)



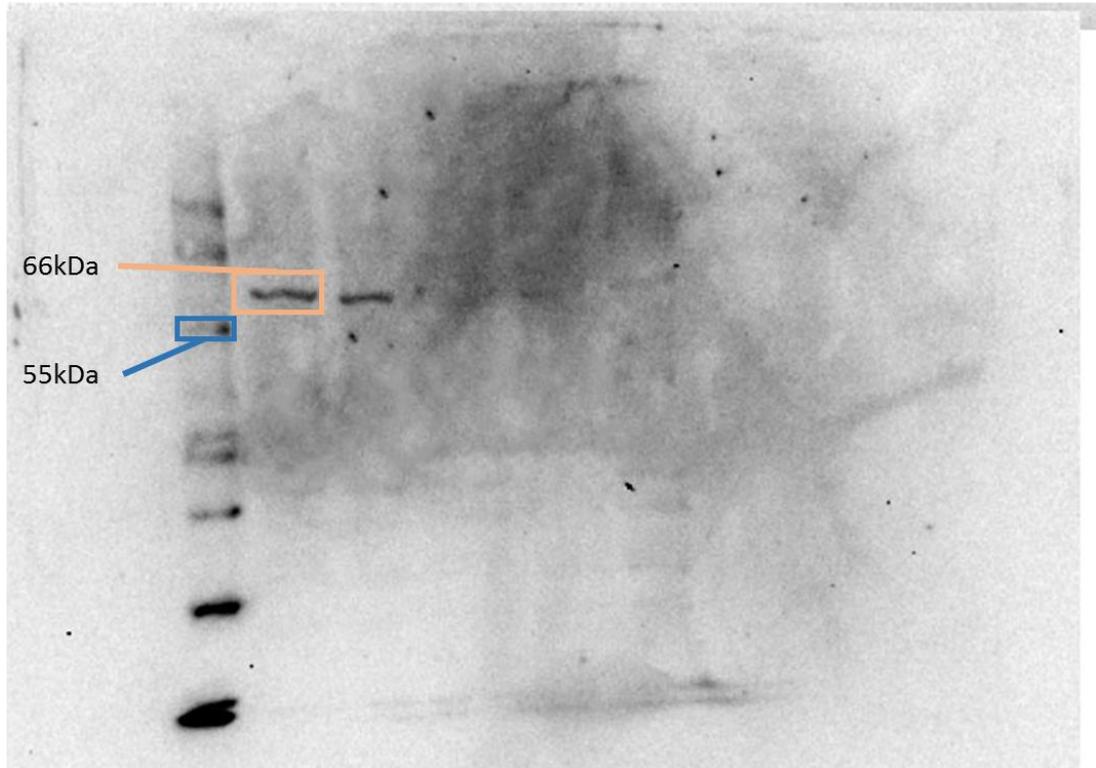
**FOLR1 (30 kDa)**



**PFCT (55 kDa)**

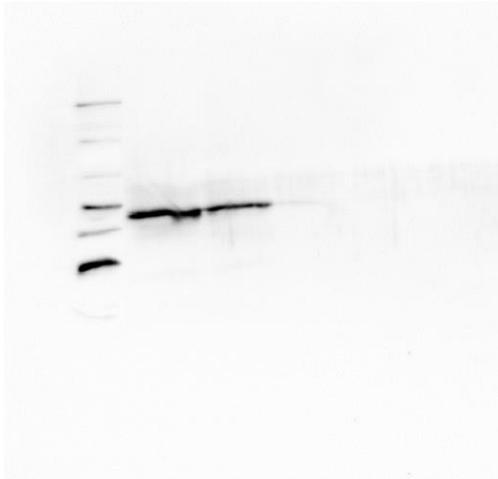


**RFC (66 kDa)**

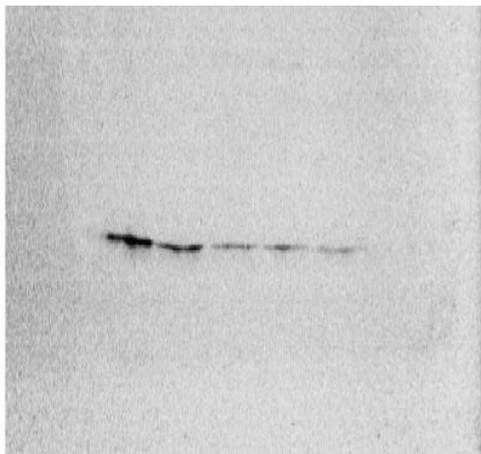


**Sample Gels for Human Cell lysate**

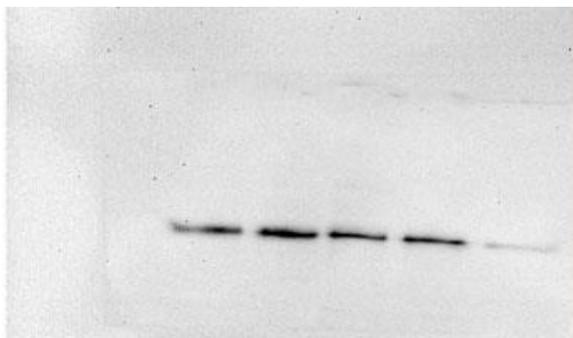
**PTEN**



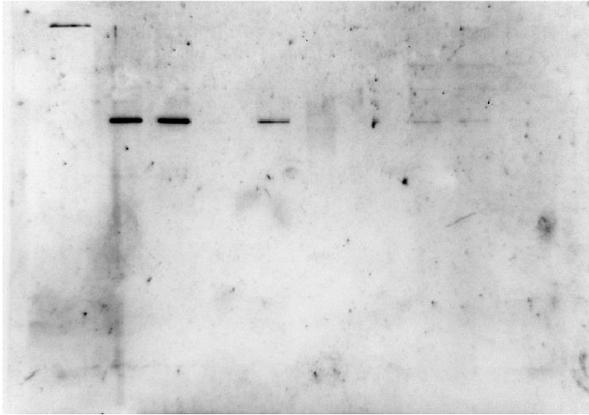
**MTHFR**



**FOLR1**



**PCFT**



**RFC**

